Original Article

The Effects of Nitric Oxide on Blood Coagulation Process

Armand R[1], Sadeghi Hashjin G[2*], Rasouli A[2], Asghari M[3]

1. Department of Biology, Faculty of Basic Sciences, Behbahan Khatam Alanbia University of Technology,Behbahan, Iran
2. Department of Comparative Biosciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
3. Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

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Abstract

Background & Objective: Nitric oxide (NO) in body can be made of amino acid L-arginine nitric oxide synthase (NOS). Donor drugs that release NO in the body are exogenous sources for this free radical. According to available sources, NO and its precursor (L-Arginine) can affect blood coagulation process. This work investigates the effect of NO on blood coagulation process *in vitro* using bovine blood as a model.

Materials & Methods: Blood samples were taken from 5 apparently healthy adult Holstein cows and were separately exposed to ethylene diamine tetra acetic acid (EDTA) anticoagulation materials and sodium citrate. Then, the blood containing anticoagulation substance was incubated for 30 min at temperature of 37°C with 10, 100 and 1000 µM of donors of NO, including sodium nitroprusside (SNP), nitroglycerine (GTN), isosorbide dinitrate (ISDN). In addition, 1, 10 and 100 mM of substances affecting NOS (either L-Arginine, L-NAME or L-Arginine + L-NAME) were applied in relevant groups. After incubation, following general tests of coagulation were adopted: prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (FIB), hemoglobin (Hb), platelet count (PLT), red blood cell (RBC) count, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), hematocrit (HCT), white blood cells (WBC) count and methemoglobin (MetHb).

Results: Obtained results indicate that among all relevant works, combination of L-Arginine and L-NAME in applied concentrations can cause significant reduction of red blood cells and hemoglobin concentration ($p<0.05$). Despite some changes in other parameters, they did not reach the level of significance.

Conclusion: In general, it could be mentioned that endogenous or exogenous NO in the model applied in this study may not have a decisive impact on blood coagulation process. However, the non-specific effects observed in the L-Arginine + L-NAME group on the number of red blood cells and hemoglobin concentration need further studies.

Keywords: Nitric oxide, Complete Blood Count (CBC), In Vitro

Introduction

NO is one of the 10 tiny molecules in the environment weighing about 30 daltons. The molecule has a half-life of about 4 minutes in presence of oxygen, although its stability reduces in biologic systems and reaches about 2-30 sec. Then, it changes into nitrite and nitrate in combination with oxygen and water (1). NO in body can be made of amino acid of L-Arginine under the effect of enzymes named nitric oxide synthase (NOS). L-Arginine is a semi-essential amino acid, which is essential for natural life of human and animals. Along with its nutritional role, the substance is also a precursor of nitric oxide. Construction analogues of this material such as L-NAME (L-NG-Nitroarginine methyl ester) act as false substrate; meaning that they would be combined with nitric oxide and inhibit the production of NO through competing with L-Arginine and taking its position (2,3). Intravenous infusion of L-NAME (an eNOS

***Corresponding Author: Sadeghi Hashjin Goudarz**, Department of Comparative Biosciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
Email: gsadeghi@ut.ac.ir
https://orcid.org/0000-0002-3513-4530

inhibitor) in rats caused a reduction of RBC elongation index (4).

NO is an important signaling molecule that acts in many tissues to regulate different physiological and pathological processes (5), and is a key molecule involved in the control of blood pressure, blood flow, and brain activity, both before and during a brain attack (stroke, either due to a blockage or rupture of an artery in the brain (6)). Also a free radical is found at higher than normal concentrations within inflammatory multiple sclerosis (MS) and lesions (7,8). NO and NOS are inducing factors in the progression of Alzheimer's disease (AD) (9). Knockdown of iNOS and nitric oxide synthase (eNOS) significantly inhibited colorectal cancer cell growth. In other words, NOS inhibitors inhibited colorectal cancer cell growth and migration, associated with suppression of angiogenesis pathway. The combined use of NOS inhibitors with 5-fluorouracil showed enhanced inhibition of cell proliferation and migration (10).

Drugs that produce nitric oxide or control its production may be beneficial in acute stroke 6. NOaa neurotransmitters are released from nerve ending and are considered as factors in non-adrenergic-non-cholinergic (NANC) neural system (2,3). Endothelium-derived NO is a potent in vitro inhibitor of platelet adhesion and aggregation (11). Platelets express the endothelial form of the eNOS and generate NO. Platelet-derived NO plays an important role in the regulation of platelet aggregation and secretion. Changes in the activity of platelet eNOS are responsible for the abnormal platelet activation encountered in different pathological situations (e.g. hypertension and diabetes) (12). In the blood vessel wall, NO is produced mainly from l-arginine by the enzyme endothelial eNOS but it can also be released non-enzymatically from S-nitrosothiols or from nitrate/nitrite13. Recent literature indicates that endothelial NO synthase (eNOS) can modulate cancer-related events (angiogenesis, apoptosis, cell cycle, invasion, and metastasis) (7). Goldstein et al (2012) evaluated effects of inhaled NO on hemostasis in the presence of heparin using aspirin as a positive control. The results showed that inhaled NO, when administered with heparin, exhibited no significant additive effects on ACT, PT, APTT, bleeding time or platelet aggregation (14).

Evidence indicates that through inhibiting blood coagulation process, the substance may be effective to inhibit coagulation disorders related to cardiovascular diseases. Moreover, it indicated that donors of NO inhibit operations of platelets and as a result, their positive role can be considered to inhibit vascular diseases. In addition to the mentioned effects by NO and its donors, it indicated that the substance can also affect coagulation process, since its precursor (L-Arginine) provides condition for controlling the activation of hemostasis in human blood in vitro (15). Animal studies suggest that NO is important in basal cerebral blood flow (CBF) regulation and that it may mediate the vasodilatory response to carbon dioxide (16).

Materials & Methods

Five apparently healthy female cows were examined in Karaj teaching hospital. 100 cc of blood was drawn from jugular vein, 50 cc of which was mixed with citrate anticoagulant, and 50 cc of EDTA with anticoagulant, and transferred to blood test tubes. Tubes containing L-NAME, L-Arginin and L-NAME were used in dilutions of 1, 10, and 100 mM Sodium SNP, GTN, and ISDN were in dilutions of 10, 100, and 1000 µM. 0.25 cc of the dilutions were mixed with 2.25 cc of blood and incubated at 37 ° C for 30 min. After 30 min, the tubes containing the citrate anticoagulant were centrifuged at 2500 rpm for 10 min, and then the plasma was separated to Measure PT, APTT, Fib. From tubes containing EDTA anticoagulant; Hb, PLT, RBC, McH, MCV, MCHC, Hct, WBC were measured, and dilutions were prepared for examination of platelet morphology. Met Hemoglobin levels were then measured by Evelyn and Malloy method.

Results

A) Effects of Cow Blood with NOS effects including: L-ARG, L-NAME, ARG/L-NAME on the following indices (Data on changes in the following indices and factors compared with control group Avg± Average error of mean are provided)**:**

PT; in combination (ARG/ L-NAME) was slightly increased at all 1, 10 and 100 mM concentrations compared to control groups, indicating partial inhibition of coagulation. The compound (ARG / L-NAME) at 100 mM concentration had the best effect on increasing PT time (Table 1).

APTT; in the compound (ARG/ L-NAME) was partially decreased at all 1, 10 and 100 mM concentrations compared to the control group, indicating that this compound partially accelerated the coagulation. L-NAME increased the anticoagulant effect at 10 mM concentration and increased APTT time (Table 1).

Fib; in combination (ARG/ L-NAME) at all concentrations of 1, 10 and 100 mM decreased slightly in comparison to the control group, indicating partially accelerated coagulation. L-NAME increased the anticoagulant effect at 100 mM concentration and increased the time of Fib (Table 1).

Hb; in combination (ARG/ L-NAME) was significantly decreased at 1, 10, and 100 mM (P <0.05) compared to controls. L-Arginine also decreased hemoglobin at all three concentrations and L-NAME at all three concentrations (Table 1).

PLT; in combination (ARG/ L-NAME) was partially decreased at all 1, 10, and 100 mM concentrations compared to controls. Which indicates a decrease in platelet activity. The combination (ARG/ L-NAME) and L-NAME decreased platelets at all three concentrations (Table 1).

RBC; in combination (ARG/ L-NAME) at all concentrations of 100, 10 and 1 mM decreased significantly (P <0.05) compared to the control group (Table 1).

Table 1. Effects Of NOS Modulators On PT, APTT, FIB, HB, PLT and RBC In Blood

Blood parameters	NOS Modulators	plasma concentration per mM		
		1	10	100
PT	L-Arginine	20.32±0.80	16.30±4.22	21.42±0.45
	L-NAME	19.65±1.11	20.68±1.02	21.46±0.92
	L-Arginine+L-NAME	20.96±0.80	22.38±1.58	23.90±1.46
APTT	L-Arginine	34.00±1.50	33.80±1.98	35.75±1.28
	L-NAME	33.40±1.35	36.80±1.78	33.40±1.72
	L-Arginine+L-NAME	33.80±1.29	32.20±1.19	33.40±1.52
FIB	L-Arginine	227.80±10.98	229.60±9.07	218.80±7.50
	L-NAME	238.00±8.02	227.20±8.41	245.00±44.07
	L-Arginine+L-NAME	212.20±15.30	202.80±8.29	198.20±9.88
HB	L-Arginine	8.72±0.64	8.74±0.63	8.72±0.55
(*P<0.05)	L-NAME	8.64±0.64	8.62±0.61	8.46±0.56
	*L-Arginine+L-NAME	7.82±0.56	7.74±0.57	7.80±0.54
PLT	L-Arginine	280.80±44.20	283.60±45.21	281.20±43.16
	L-NAME	276.00±39.88	277.00±42.64	274.00±43.37
	L-Arginine+L-NAME	265.20±42.04	229.00±44.40	239.00±47.49
RBC	L-Arginine	5.85±0.22	5.89±0.19	5.85±0.21
(*P<0.05)	L-NAME	5.72±0.24	5.73±0.23	5.76±0.22
	*L-Arginine+L-NAME	5.35±0.19	5.25±0.18	5.32±0.20

B) Effects of cow blood with NO donors (SNPs), SNP GTN, ISDN on the following indices (Data on changes in the following indices and factors compared with control group Avg± Average error of mean Provided)**:**

PT; in the ISDN compound at all concentrations of 100, 10, and 1 mM was slightly increased compared to the control group, indicating partial inhibition of coagulation. ISDN at a concentration of 10 µM increased PT and had the greatest effect on preventing coagulation (Table 2).

APTT; in the ISDN compound at all concentrations of 100, 10 and 1 mM decreased slightly in comparison to the control group, indicating that it partially accelerated the coagulation. SNP at 1000µM increased APTT and had the greatest effect on preventing the coagulation process (Table 2).

Fib; in GTN in all three concentrations of 1, 10 and 100 mM decreased slightly in comparison to the control group and in SNP compound increased in 1000 and 10 concentrations and decreased in 100 concentration. And 10 and 100 µM were increased compared to the control group, indicating partially inhibition of coagulation. ISDN at a concentration of 1000µM increased Fib and had the greatest effect on preventing the coagulation process (Table 2).

Hb; was slightly decreased in GTN, ISDN, SNP compounds compared to controls. ISDN had the greatest effect on hemoglobin depletion at concentration 10 M (Table 2).

PLT; in the GTN and SNP compounds had a slightly increased coagulation inhibition compared to the control group. GTN at 1000µM had the greatest effect on preventing coagulation and reducing platelets (Table 2).

RBC; in the ISDN compound was slightly increased compared to the control group and the GTN and sodium SNP compounds decreased. (Table 2).

Table 2. Effects Of NO Donors On PT, APTT, FIB, HB, PLT and RBC In Blood

Blood parameters	NO donors	plasma concentration (µM)		
		10	100	1000
	SNP	20.16±0.70	19.33±1.55	19.75±1.28
PT	GTN	20.48±0.95	18.54±1.74	18.78±1.74
	ISDN	20.46±0.64	19.93±1.25	20.04±0.94
	SNP	34.40±1.79	34.40±1.79	35.00±2.21
APTT	GTN	33.80±1.82	33.60±1.89	34.20±1.71
	ISDN	34.00±1.77	32.50±1.45	33.80±1.60
	SNP	232.80±22.96	219.00±11.68	233.60±42.70
FIB	GTN	225.00±9.35	225.80±17.38	218.80±8.29
	ISDN	239.00±18.82	196.00±14.94	251.60±19.72
	SNP	8.66±0.64	8.60±0.65	8.62±0.63
HB	GTN	8.54±0.61	8.56±0.65	8.54±0.64
	ISDN	8.44±0.61	8.54±0.58	8.60±0.56
	SNP	293.20±43.98	285.80±44.65	283.00±44.45
PLT	GTN	286.20±41.49	285.80±47.75	269.80±35.04
	ISDN	292.80±45.57	286.20±46.64	278.20±45.35
	SNP	5.80±0.23	5.72±0.24	5.76±0.20
RBC	GTN	5.78±0.22	5.76±0.22	5.79±0.24
	ISDN	5.87±0.22	5.84±0.17	5.85±0.20

Discussion

According to obtained results from the study and findings of relevant works, combination of L-Arginine and L-NAME in used concentrations could result in significant decline of number of RBCs on one hand and Hb on the other hand. Despite to existence of some changes in other parameters, except for the mentioned items, other items were not significant statistically. In general, it could be found that NO with internal origin (androgen) or external (exogenous) in applied model can have no significant effect on coagulation process. However, observed non-special effects in compound of L-Arginine and L-NAME on number of RBCs and Hb rate may have important outcomes and need further studies.

The results of Stief et al (2001) studies show that Arginine in concentrations of 5-100 mM inhibited the WBCT, PT, APTT, IVBT-CT, and WBA. Arginine (50 mM) resulted in a twofold prolongation of WBCT, PT, or IVBT-CT (the anti-epinephrine action is superior to the anti-ADP action), a four-fold prolongation of APTT or a 60% inhibition of WBA (17). Findings confirm that L-Arg suppresses platelet aggregation in whole blood. Other authors also demonstrated that this effect is associated with enhanced NOS (18). Kucukatay et al (2003) in a study on the effects of NO on red blood cell deformability have found that both NOS [No-nitro-L-arginine methyl ester (L-NAME) and S methylisothiourea] inhibitors significantly reduced RBC deformability above a threshold concentration, whereas the NO donors SNP and DETA-NONOate increased deformability at optimal concentrations. These results thus indicate the importance of NO as a determinant of RBC mechanical behavior and suggest its regulatory role for normal RBC deformability (19). The results of Ryszard Korbut et al. (1993) showed that the deformability of RBC was increased by NO donors, such as sydnonimine (SIN-1) or sodium nitroprusside, and reduced by the NOS inhibitor, L-NAME. Our results showed that RBC in the ISDN compound was slightly increased compared to the control group and the GTN and SNP compounds decreased (20). This is close to the research by Ryszard Korbut et al. Stief et al (2001) found that stimulation of producing NO androgen can result in the reduction of concentration of platelets and reduction of PT and APTT, which indicate enforcement effect of NO on inhibition of blood coagulation (17), Which is in line with our results.

Conclusions

In general, it could be mentioned that NO with internal origin (androgen) or external origin (exogenous) in the model applied in this study can have a decisive impact on blood coagulation process. However, unspecialized effects observed in compound of L-Arginine and L-NAME can have significant effects on the number of red blood cells and hemoglobin concentration and needs further studies.

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Conflict of Interests

The authors declare no conflicts of interest.

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مقاله پژوهشی

بررسی تاثیر نیتریک اکسید بر فرایند انعقاد خون

رحام آرمند[۱]، گودرز صادقی هشجین[۲]*، علی رسولی[۲]، مژگان اصغری[۳]

۱. گروه زیست شناسی، دانشکده علوم پایه، دانشگاه صنعتی خاتم الانبیاء(ص) بهبهان، بهبهان، ایران
۲. گروه علوم زیستی مقایسه ای، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران
۳. دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران

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چکیده

زمینه و هدف: نیتریک اکساید (NO) در بدن از اسید آمینه L-Arginine و تحت تأثیر آنزیم نیتریک اکسید سنتاز (NOS) ساخته می شود. داروهای دهنده نیتریک اکساید (NO donors) در بدن از خود نیتریک اکساید آزاد میکنند. بر اساس منابع موجود، این ماده و پیش ساز آن (L-Arginine) در فرایند انعقاد خون نیز مؤثر است. از آنجا که مطالعات نشان میدهد استفاده از ضد انعقادهایی مثل سیترات سدیم در شرایط خارج بدنی به صورت کامل از انعقاد خون ممانعت نمی کند، این مطالعه تأثیر NO را بر روند انعقاد خون در شرایط آزمایشگاهی بررسی کرده است .

مواد و روش ها: بدین منظور نمونه های خون از ۵ رأس گاو ماده بالغ هولشتاین به ظاهر سالم اخذ و به صورت مجزا با مواد ضد انعقاد اتیلن دی آمین ترا استیک (EDTA) و سیترات سدیم مجاور گردیدند. سپس خون حاوی ماده ضد انعقاد به مدت ۳۰ دقیقه در دمای ۳۷ درجه سانتیگراد با غلظتهای ۱۰ ، ۱۰۰ و ۱۰۰۰ میکرومولار از مواد آزاد کننده نیتریک اکساید(NO donors) شامل نیتروپروساید سدیم (SNP)، نیتروگلیسیرین (GTN)، ایزوسورباید دی نیترات (ISDN)، و با غلظتهای ۱ ، ۱۰ و ۱۰۰ میکرومولار از مواد مؤثر بر نیتریک اکساید سنتاز شامل L-Arginine، L-NAME و ترکیب L-NAME+L-Arginine انکوبه شد. و آزمایش های کلی جهت بررسی انعقاد خون از جمله زمان پروترومبین (PT)، زمان ترومبوپلاستین نسبی فعال (APTT)، فیبرینوژن (FIB)، میزان هموگلوبین (Hb)، پلاکت ها (PLT)، گلبول قرمز خون (RBC)، هموگلوبین گلوبولی (McH)، میانگین حجم گلوبولی (MCV)، میانگین غلظت هموگلوبین گلوبولی(MCHC) ، هماتوکریت (HCT)، گلبول های سفید(WBC) و مت هموگلوبین (Met Hb) انجام شد.

نتایج: نشان داده است که ترکیب (L-NAME + L-Arginine) در غلظت های استفاده شده موجب کاهش معنی دار در تعداد گلبول های قرمز و میزان هموگلوبین شده است ($P<0.05$). علیرغم وجود برخی تغییرات در سایر پارامترها، سایر موارد از نظر آماری معنی دار نبودند.

نتیجه گیری: در مجموع میتوان نتیجه گرفت که نیتریک اکساید با منشأ داخلی (آندوژن) یا خارجی (اگزوژن) در مدل مورد استفاده در این پژوهش تأثیر قطعی بر روند انعقاد خون ندارد. لیکن، اثرات غیراختصاصی مشاهده شده در ترکیب L-Arginine با L-NAME بر تعداد گلبولهای قرمز و میزان هموگلوبین نیازمند مطالعات بیشتری میباشد.

کلمات کلیدی: نیتریک اکساید ، شمارش کامل خون (CBC) ، شرایط آزمایشگاهی

***نویسنده مسئول: گودرز صادقی هشجین،** گروه علوم زیستی مقایسه ای، دانشکده دامپزشکی، دانشگاه تهران، تهران، ایران.
Email: gsadeghi@ut.ac.ir
https://orcid.org/0000-0002-3513-4530