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In vitro effect of sodium nitrite on platelet aggregation in human platelet rich plasma – Preliminary report

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Abstract. – OBJECTIVE: The role of nitrates and nitric oxide on platelet functions has obtained an increasing attention with respect to their potential effects on cardiovascular disorders. In this study we aimed to analyze the effect of sodium nitrite on platelet functions in human platelets.

PATIENTS AND METHODS: This *in vitro* study was designed to show the effect of sodium nitrite on platelet functions in seven healthy volunteers. Blood samples were centrifuged to prepare platelet rich plasma and platelet poor plasma. Platelet rich plasma was diluted with the platelet poor plasma to have a final count of 300,000 ± 25,000 platelets. Platelet rich plasma was incubated with six different increasing doses (from 10 µM to 5 mM) of sodium nitrite for 1 hour at 37°C. Then stimulating agents including collagen (3 µg ml^{-1}), adenosine diphosphate (10 µM), and epinephrine (10 µM) were added to the cuvette. Changes in light transmission were observed for 10 minutes. In addition spontaneous aggregation were performed in control group with all aggregating agents separately.

RESULTS: Effect of sodium nitrite on agonist-induced platelet aggregation depends on the concentration of sodium nitrite. Compared with control group, agonist-induced platelet aggregations were significantly suppressed by sodium nitrite at the concentration of 5, 1.0 and 0.5 mM.

CONCLUSIONS: Our results suggested that sodium nitrite has inhibitory effects *in vitro* on platelet aggregation in a dose-dependent manner.

Key Words:
Sodium nitrite, Platelet aggregation, Nitric oxide, *In vitro*.

Introduction

Platelets are non-nucleated blood cells, and play an initial role in coagulation pathway by adhesing the injured endothelium and aggregating with other platelets. For balancing procoagulant and anticoagulant state, platelet activities is controlled through various mechanism and molecules. Adenosine di-phosphate (ADP) is one of these stimulating molecules, which predominantly show its effect via G-protein coupled receptors. Collagen, which is one of the major components of the vascular wall, is another major stimulating molecule[1]. Epinephrine is another agonistic molecule, which predominantly affects with sensitizing the platelets to other activating agents[2].

Nitric oxide (NO), which is well known as the bioactive form of nitrates, is considered as one of the important inhibitors of platelet functions. NO is mainly generated from L-arginine by enzymatic reaction of nitric oxide synthase. Beside this endogenous source, another alternative pathway for NO production involves serial reductive pathways of nitrates[3,4].

There are some studies demonstrating the inhibitory effects of nitrates on platelet functions in the literature[1,4-6]. Most of these papers are focused on NO rather than nitrates. However, there is limited information about the effects of sodium nitrite. Furthermore, there is also a lack of information about the concentration depended activities of testing molecule over platelet functions. In this study we aimed to analyze *in vitro* effect of sodium nitrite on platelet functions at wide range of concentrations.

Patients and Methods

The study was performed in accordance with the Helsinki Declaration. Blood samples were

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obtained from seven healthy volunteers following informed consent and local ethics committee approval.

Study Population

All subjects were required to discontinue aspirin or other non-steroidal anti-inflammatory agents at least 10 days. They also required not consuming garlic or Chinese food within last 2 days, and no alcohol for last 24 hours. Patients with medications, which have effects on platelet functions, were excluded. Furthermore, patients with history of abnormal platelet dysfunction, thrombosis, abnormal bleeding, active neoplasia or active inflammatory disorders were also excluded.

Blood Collection

Venous blood was collected under light tourniquet through 19 gauge needles into vacutainers. Four tubes of blood collection were performed in the morning after a light breakfast. All the samples were provided of testing within 2-4 h of collection.

Blood Sample Preparation

Platelets were counted with an automated cell counter device (ABX Pentra DX 120, Horiba, France). Platelet aggregation was measured with a platelet lumiaggregometer (Model 560CA, Chrono-log Corporation, Havertown, PA, USA) according to the manufacturer's instructions. Platelet-rich plasma (PRP) was separated from 3.2% citrate-anticoagulated blood as previously described[7,8]. The remaining blood was centrifuged within the next 10 minutes at 1250 g for preparing platelet-poor plasma (PPP). PRP calibration was made with PPP. Leukocyte or red blood cell contamination was controlled with an automated cell counter device (ABX Pentra DX 120, Horiba, France). Platelet counts was adjusted to 300,000 ± 25,000 platelets L^{-9} for each PRP samples. After preparation of samples, the aggregation procedure was performed in computerized aggregometer.

Study Protocol

PRP was incubated with increasing doses (from 10 mM to 5 mM) of sodium nitrite for 1 hour at 37°C. Then stimulating agents (Chronopar reagents, Havertown, PA, USA) including collagen (3 g ml^{-1}), ADP (10 M), and epinephrine (10 M) were added to the cuvette. Changes in light transmission were observed for 10 minutes. Maximal amplitudes of the aggregation curves were used for quantitative analysis. In addition spontaneous aggregation were performed in control group with all aggregating agents separately.

Statistical Analysis

SPSS 15.0 (SPSS Inc. Software, Chicago, IL, USA) statistical software was used for statistical analyses. The data are shown as mean ± SEM of independent measurements. Aggregation data presented as mean ± SD. Two tailed, Mann-Whitney U test was used consistently throughout the study. *p* values less than 0.05 were considered significant.

Results

Effect of sodium nitrite on agonist-induced platelet aggregation depends on the concentration of sodium nitrite. Compared with control group, agonist-induced platelet aggregations were significantly suppressed by sodium nitrite at the concentration of 5, 1.0 and 0.5 mM. Results of the study were summarized and illustrated in Table I and Figure I, respectively.

Discussion

Since first demonstration of the antiplatelet effects of nitrates in 1991, antiaggregan effects of these molecules are obtained an increasing attention with respect to their potential benefits in cardiovascular diseases[5,9]. Researchers were focused on the antiplatelet mechanisms of nitrates to find how these molecules influence platelet dependent hemostasis. Several studies[4,6,10,11] declared that this inhibitory effect occurs through nitric oxide, which is an active form of nitrite, and nitrates. Physiologic nitric oxide generation is mainly depended on two mechanisms. First bioconversion of L-arginine to nitric oxide by endothelial nitric oxide synthases; and second, reduction of exogenous nitrates to nitric oxide[6,11,12]. Most of the studies revealed that this reduction occurs in pathophysiologic conditions. Actually, intrinsic biological activity of nitrite is limited at physiological states. Reduction of nitrite to nitric oxide is dependent to deoxygenation or low oxygen levels and it mainly occurs in hypoxic states like chronic tissue ischemia or ischemia reperfusion[1,4,6,10]. However, there is lack of evidence

Table I. Effects of sodium nitrite on human platelet aggregation, *in vitro*. The platelet aggregation was induced by addition of collagen (3 µg ml^{-1}), ADP (10 µM), epinephrine (10 µM), respectively.

Stimulating agent			Collagen (3 µg ml^{-1})	ADP (10 µM)	Epinephrine (10 µM)
	Control group		77.85 ± 10.38	62.14 ± 6.44	77.00 ± 14.60
Study Group (sodium nitrite)	5 mM	Agg.	13.40 ± 7.40	12.80 ± 6.97	12.40 ± 2.88
		p-value	< 0.001	< 0.001	< 0.001
	1 mM	Agg.	17.40 ± 8.64	11.66 ± 6.59	14.60 ± 5.85
		p-value	< 0.001	< 0.001	< 0.001
	0.5 mM	Agg.	28.28 ± 11.87	36.85 ± 16.65	20.71 ± 10.71
		p-value	0.012	0.007	0.001
	0.1 mM	Agg.	59.42 ± 18.72	45.85 ± 7.92	41.00 ± 22.39
		p-value	0.238	0.615	0.073
	0.05 mM	Agg.	70.28 ± 13.76	50.57 ± 4.31	57.85 ± 10.25
		p-value	0.404	0.095	0.571
	0.01 mM	Agg.	74.57 ± 6.94	54.28 ± 8.69	66.71 ± 6.80
		p-value	0.232	0.502	0.313

about the antiplatelet effects of sodium nitrite under physiological conditions[13].

Srihirun et al[10] reported that nitrite anions inhibited platelet aggregation in the presence of deoxygenated erythrocytes, and declared that nitrite anions show its inhibitory effects with only deoxygenated state through reduction of nitrite to nitric oxide. Many other studies[1,5,6] concordant with Srihirun et al[10], and they all declared the hypoxic state is the main factor for the conversion of nitrites to nitric oxide.

Park et al[4] claimed that, sodium nitrite significantly inhibit platelet aggregation at deoxygenated state, while it has no effect at physiologic concentrations (0.1 mM, 1 mM, 10 mM) on PRP stimulating with 1 mm arachidonic acid and 2 mm ADP. Similar to Park et al[4], Srihirun et al[10] also reported that sodium nitrite has no effect on PRP (stimulated with 8 mm ADP and 2.5 mg/ml collagen), at 0.01 mM, 0.1 mM, 1 mM, 10 mM and 100 mM concentrations. They also observed an inhibitory effect of sodium nitrite at 0.1 mM, 1 mM and 10 mM concentration on erythrocyte enriched PRP (stimulated with ADP and collagen at same concentrations).

Different from these studies, we added sodium nitrite to PRP with stimulating agents, without any erythrocyte and or deoxygenated state. However, we observed a significant inhibition of platelet aggregation with several concentrations of stimulating agents. From this point, our study is different from previous studies.

This difference is originating from our study protocol. We use six different concentrations of sodium nitrite (5 mM, 1 mM, 0.5 mM, 0.1 mM, 0.05 mM, 0.01 mM) and we found a statistically significant inhibitory effect at 5 mM, 1 mM and 0.5 mM concentrations. Previous papers were all studied with restricted-ranged concentrations of sodium nitrite, which may directly affect the results.

We consider that there may be another explanation for this discordance. All investigators declared that nitrite anions have inhibitory effect on platelets through nitric oxide. We hypothesized that, there may be another pathway from platelet aggregation to nitrite ions itself, and therefore exogenous nitrite uptake could inhibit platelet functions directly or mediated. This could be important and worth for future investigations.

Conclusions

Our findings suggested that sodium nitrite inhibits platelet aggregation in physiologic conditions *in vitro*, although the exact mechanism is not known. Although the well-known mechanism is based on nitric oxide pathway, there may be another pathway affects the platelet directly, which may be not discovered yet. There should be further molecular studies to clarify this issue. We conclude that sodium nitrite could be used at defined concentrations as a platelet inhibitor in the treatment of cardiovascular disorders, after extensive further investigations in the future.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

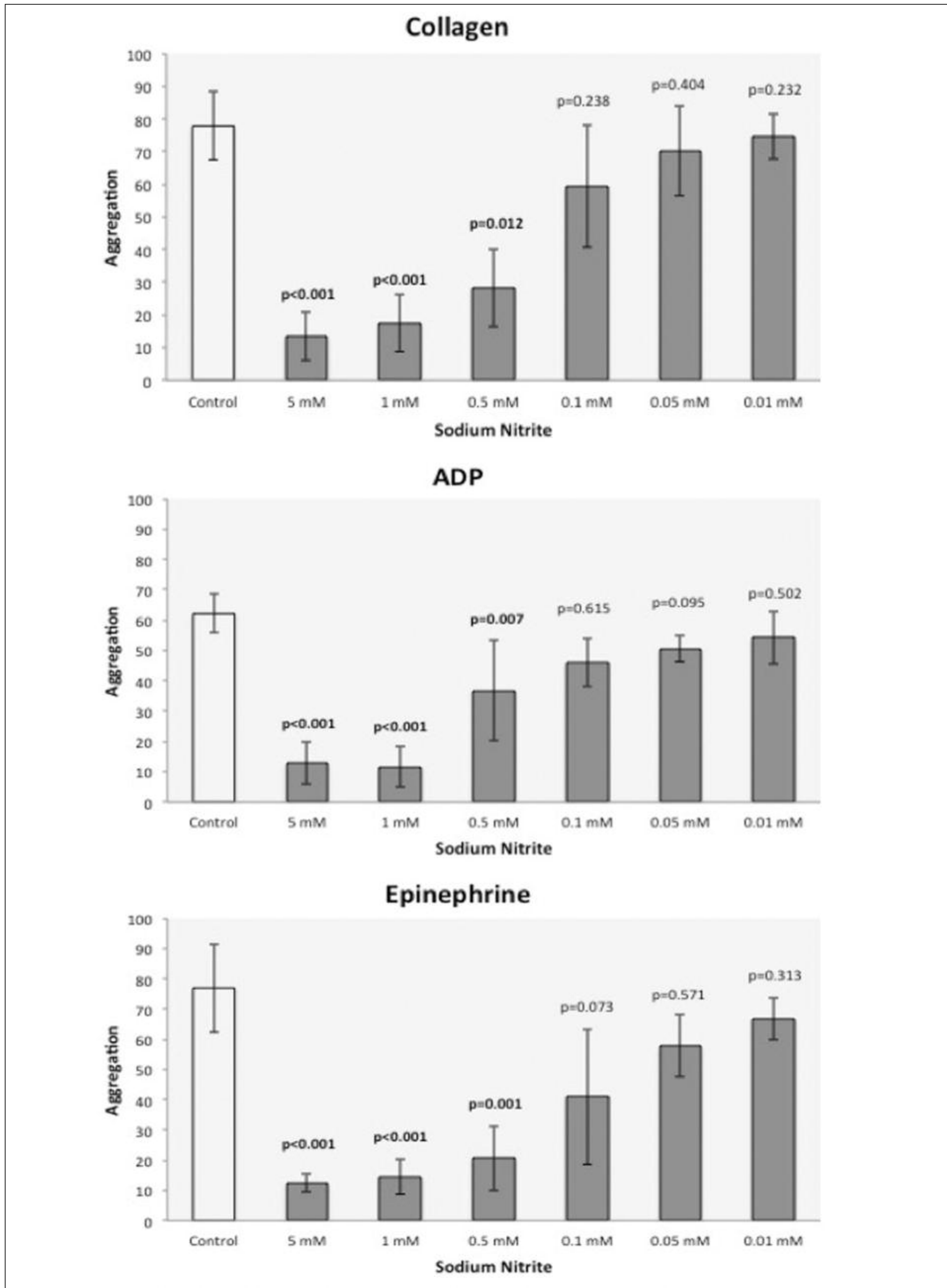

Figure 1. Comparison of maximal aggregation according to control group.

References

1) Akrawinthawong K, Park JW, Piknova B, Sibmooh N, Fucharoen S, Schechter AN. A flow cytometric analysis of the inhibition of platelet reactivity due to nitrite reduction by deoxygenated erythrocytes. PLoS One 2014; 9: e92435.
2) Kose EA, Nevruz O, Honca M, Yildirim V. *In vitro* effect of dexmedetomidine on platelet aggregation. Braz J Anesthesiol 2013; 63: 415-418.
3) Doganci S, Yildirim V, Bolcal C, Korkusuz P, Gumusel B, Demirkilic U, Aydin A. Sodium nitrite and cardioprotective effect in pig regional myocardial ischemia-reperfusion injury model. Adv Clin Exp Med 2012; 21: 713-726.
4) Park JW, Piknova B, Nghiem K, Lozier JN, Schechter AN. Inhibitory effect of nitrite on coagulation processes demonstrated by thrombelastography. Nitric Oxide 2014; 40: 45-51.
5) Park JW, Piknova B, Huang PL, Noguchi CT, Schechter AN. Effect of blood nitrite and nitrate levels on murine platelet function. PLoS One 2013; 8: e55699.
6) Dautov RF, Stafford I, Liu S, Cullen H, Madhani M, Chirkov YY, Horowitz JD. Hypoxic potentiation of nitrite effects in human vessels and platelets. Nitric Oxide 2014; 40: 36-44.
7) Ural AU, Yilmaz MI, Avcu F, Yalcin A. Treatment with cerivastatin in primary mixed hyperlipidemia induces changes in platelet aggregation and coagulation system components. Int J Hematol 2002; 76: 279-283.
8) Doganci S, Yildirim V, Ozgur G, Yildirim M, Ertas Z, Cetin T, Gumusel B, Avcu F. *In vitro* effect of intermedin/adrenomedullin 2 on platelet aggregation in human. Gulhane Med J 2015; 57: 41-43.
9) Laustiola KE, Vuorinen P, Porsti I, Metsa-Ketela T, Manninen V, Vapaatalo H. Exogenous GTP enhances the effects of sodium nitrite on cyclic GMP accumulation, vascular smooth muscle relaxation and platelet aggregation. Pharmacol Toxicol 1991; 68: 60-63.
10) Srihirun S, Sriwantana T, Unchern S, Kittikool D, Noulsri E, Pattanapanyasat K, Fucharoen S, Piknova B, Schechter AN, Sibmooh N. Platelet inhibition by nitrite is dependent on erythrocytes and deoxygenation. PLoS One 2012; 7: e30380.
11) Jablecka A, Bogdanski P, Balcer N, Cieslewicz A, Skoluda A, Musialik K. The effect of oral L-arginine supplementation on fasting glucose, HbA1c, nitric oxide and total antioxidant status in diabetic patients with atherosclerotic peripheral arterial disease of lower extremities. Eur Rev Med Pharmacol Sci 2012; 16: 342-350.
12) Son Y, Lee JH, Cheong YK, Jung HC, Jeong SO, Park SH, Pae HO. Piceatannol, a natural hydroxylated analog of resveratrol, promotes nitric oxide release through phosphorylation of endothelial nitric oxide synthase in human endothelial cells. Eur Rev Med Pharmacol Sci 2015; 19: 3125-3132.
13) Yildirim V, Doganci S, Yesildal F, Kaya E, Ince ME, Ozkan G, Gumusel B, Avcu F, Ozgurtas T. Sodium nitrite provides angiogenic and proliferative effects in vivo and *in vitro*. Med Sci Monit Basic Res 2015; 21: 41-46.