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Nattokinase migliora il flusso sanguigno inibendo l'aggregazione piastrinica e la formazione di trombi

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Astratto

Gli effetti della nattokinase sull'aggregazione piastrinica *in vitro* e sulla trombosi *in vivo* sono stati studiati rispetto all'aspirina. Il plasma ricco di piastrine di coniglio è stato incubato con nattokinase e induttori di aggregazione collagene e trombina, ed è stato analizzato il tasso di aggregazione piastrinica. Nattokinase ha inibito significativamente le aggregazioni piastriniche indotte sia dal collagene che dalla trombina. Nattokinase ha anche ridotto la formazione di trombossano B_2 dalle piastrine attivate dal collagene in modo dipendente dalla concentrazione. I ratti sono stati somministrati per via orale con nattokinase per 1 settimana e le loro arterie carotidi sono state esposte. La trombosi arteriosa è stata indotta applicando il 35% di $FeCl_3$carta da filtro imbevuta per 10 minuti e il flusso sanguigno è stato monitorato con una sonda laser Doppler. Nattokinase ha ritardato l'occlusione arteriosa indotta da $FeCl_3$ in modo dose-dipendente, raddoppiando il tempo di occlusione a 160 mg/kg. Inoltre, una dose elevata (500 mg/kg) di nattokinase ha impedito completamente l'occlusione, come ottenuto con l'aspirina (30 mg/kg). I risultati indicano che la nattokinasi estratta dalla soia fermentata inibisce l'aggregazione piastrinica bloccando la formazione di trombossano e quindi ritarda la trombosi in seguito a lesione ossidativa della parete arteriosa. Pertanto, si suggerisce che nattokinase potrebbe essere un buon candidato senza effetti negativi per il miglioramento del flusso sanguigno.

Parole chiave: Aggregazione piastrinica, trombossano B_2 , trombosi, nattokinasi

La trombosi dovuta all'occlusione dell'arteria embolica è una delle principali cause di malattie cardiovascolari compreso l'ictus (angina). Sebbene le piastrine siano importanti nell'omeostasi fisiologica, la loro aggregazione gioca un ruolo cruciale nella formazione del trombo [1]. Cioè, in caso di lesione arteriosa, i ligandi adesivi come il collagene e i fattori di von Willebrand

(vWF) nonché gli agonisti solubili tra cui l'adenosina difosfato (ADP) e la trombina vengono esposti o generati. Tali fattori di coagulazione facilitano l'adesione delle piastrine alle pareti arteriose danneggiate, l'attivazione e l'aggregazione [2].

Il collagene supporta il legame delle piastrine alla regione danneggiata delle arterie attraverso i recettori della superficie piastrinica come la glucoproteina VI e l'integrina α2β1 [3]. Il legame al collagene induce l'attivazione piastrinica attraverso la trasduzione del segnale mediata dalla tirosina chinasi, e quindi le piastrine stimolate aderiscono alle pareti arteriose, che dipende dal rilascio di agonisti ADP e prostaglandina H2/trombossano A2 (TXA2) dai granuli piastrinici., 5]. TXA_2, un induttore di vasocostrizione e aggregazione piastrinica, svolge un ruolo chiave nell'omeostasi delle arterie. Pertanto, TXA_2è considerato un mediatore eziologico nel progresso dell'aterosclerosi e dell'ischemia miocardica [6]. Il TXA_2 è generato dall'acido arachidonico durante la reazione di ossidazione catalizzata dalla cicloossigenasi (COX) e dal trombossano sintasi, e quindi rapidamente ossidato in un trombossano B_2 inattivo stabile (TXB_2) [7]. Pertanto, la concentrazione ematica di TXB_2 dopo la coagulazione del sangue è un marcatore specifico per la valutazione dell'attività della COX-1 [8].

Poiché i metalli di transizione facilitano la formazione di radicali ossidativi, inducendo lesioni cellulari e tissutali, nonché danni alle cellule endoteliali che portano alla trombosi, l'applicazione di cloruro ferrico ($FeCl_3$) alle arterie è stata utilizzata come modello per valutare l'efficacia degli stimolatori del flusso sanguigno antitrombotico [9]. Nattokinase, un enzima trombolitico ricco di 'Natto', alimento tradizionale orientale, è prodotto da *Bacillus subtilis* Natto durante la fermentazione dei semi di soia [10 - 12]. È stato riportato che la nattokinase ha dissolto i trombi *in vitro* e ha prevenuto la trombosi della coda di ratto indotta da carragenina *in vivo* [13]. Nel presente studio, abbiamo dimostrato l'attività antitrombotica della nattokinasi in un modello di trombosi dell'arteria carotide indotta da $FeCl_3$, oltre ai suoi effetti sulla formazione di TXB_2 e sull'aggregazione piastrinica come meccanismi di azione.

Il Bacillus subtilis Natto è stato coltivato in un terreno (pH 7,0) composto da 66% di polvere di soia, 30% di amido di mais, 3% di olio di soia e 1% di carbonato di calcio a 35°C per 24 ore. Il mezzo di coltura comprendente le cellule è stato filtrato attraverso un filtro Celite 505 da 2 um (Celite Co., Lompoc, CA, USA) per rimuovere i solidi e la vitamina K2 , seguito da una filtrazione da 0,2 um. Il filtrato (30% finale) è stato miscelato con maltodestrina indigeribile più estratto di soia (7:3) (Daesang Co., Gunsan, Corea), e quindi essiccato a spruzzo. La nattokinase NSK-SD preparata è stata conservata a 4°C fino al momento dell'uso, sciolta in acqua purificata e somministrata ai ratti in un volume di 1 mL/kg.

Six-month-old male New Zealand white rabbits (body weight 2.0 kg) and 7-week-old male Sprague-Dawley rats (body weight 200-220 g) were from Daehan-Biolink (Eumseong, Korea), and subjected to the experiment after 1-week acclimation to the laboratory environment. The animals were housed in each cage with free access to feed and water under constant environmental conditions (22±2°C; 40-70% relative humidity; 12-hour light-dark cycle; 150-300 lux brightness). All the animal experiments were conducted according to the Standard Operation Procedures, and approved by the Institutional Animal Care and Use Committee of Chungbuk National University, Korea.

Blood sample was collected from the ear artery of rabbits directly into anti-coagulant citrate dextrose solution containing 0.8% citric acid, 2.2% trisodium citrate, and 2% dextrose. Platelet-rich plasma (PRP) was obtained by centrifugation at 230 *g* for 10 min. Platelets were sedimen-

ted by centrifugation of the PRP at 800 g for 15 min and washed with a HEPES buffer (pH 6.5) [9,14]. The washed platelets were resuspended (4×10^8 cells/mL) in the HEPES buffer (pH 7.4).

Platelet aggregation was measured with an aggregometer (Chrono-Log Co., Harbertown, CA, USA) according to the turbidimetric method of Born [15] as previous described [14]. In brief, the washed platelet suspension was preincubated with nattokinase (400 μg/mL) or aspirin (200 μg/mL) as a reference control at 37°C in the aggregometer under stirring at 1,000 rpm. After 3-min preincubation, platelet aggregation was induced by adding collagen (2.5 μg/mL) or thrombin (0.1 U/mL). The extent of aggregation was expressed as a percentage of the vehicle control value stimulated with collagen or thrombin alone.

TXB_2 released from platelets was assessed using a kit according to the manufacturer's instructions. In brief, washed rabbit platelets (4×10^8 cells/mL) were preincubated with nattokinase (1-1,000 μg/mL) or aspirin (100 μM) as a reference control at 37°C for 3 min in an aggregometer, and aggregation was induced by adding collagen (10 μg/mL). The reaction was stopped by 5 mM indomethacin and 2 mM EGTA, centrifuged at 1,200 rom for 2 min, and analyzed for the concentration of TXB_2 by enzyme-linked immunosorbent assay.

Rats (n=8/group) were orally administered with nattokinase (50, 160, or 500 mg/kg) or aspirin (30 mg/kg) for 1 week. Forty min after the final administration, the animals were anesthesized by intramuscular injection of Zoletil® (1 mL/kg). Under constant maintenance of body temperature (36-37°C) using a heating pad, the right carotid artery of rats were exposed, and dissected away from the vagus nerve and surrounding tissues. Aortic blood flow rate was monitored with a laser Doppler flowmeter (AD Instruments, Colorado Springs, CO, USA). At the time point of 1 hour after the final administration, arterial thrombosis was induced by wrapping the artery with a Whatman No. 1 filter paper (3 mm in diameter) saturated with 35% $FeCl_3$ solution near (5 mm anterior to) the flowmeter probe for 10 min. The blood flow was monitored for 90 min. A part of the animals (n=3/group) were sacrificed at the time point of 50 min from the application of $FeCl_3$, and the arteries were cut to observe the thrombus in the artery.

The results are presented as means±standard deviation. The significance of differences of all results was analyzed by one-way analysis of variance followed by the Dunnett's multiple-range test correction. Statistical significance was set a priority at $P<0.05$.

Nattokinase (400 μg/mL) significantly inhibited platelet aggregation induced by collagen (2.5 μg/mL) by approximately 40% (Figure 1), although the effect of nattokinase was inferior to that of aspirin (200 μg/mL). Nattokinase also markedly suppressed the platelet aggregation induced by thrombin (0.1 U/mL), exhibiting an effect comparable to that of aspirin.

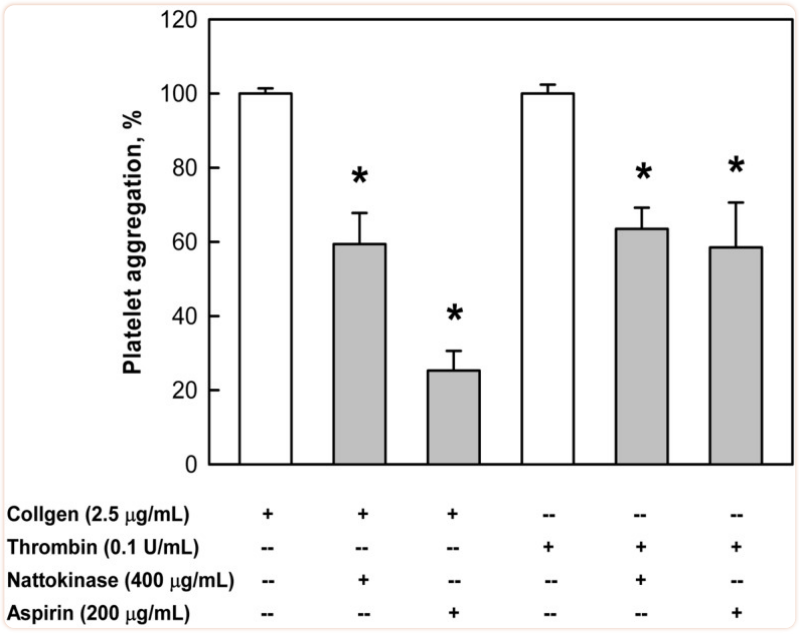

Figure 1

Inhibition by nattokinase (400 μg/mL) or aspirin (200 μg/mL) of platelet aggregation induced by collagen (2.5 μg/mL) or thrombin (0.1 U/mL). *Significantly different from vehicle controls (collagen or thrombin alone).

During collagen-induced platelet aggregation, TXB_2 formation was inhibited by nattokinase in a concentration-dependent manner; by 20%, 32.4%, and 37.5% at 10, 100, 1,000 μg/mL, respectively (Figure 2). Notably, the effect of a high dose (1,000 μg/mL) was similar to that (35.8%) of aspirin (100 μM).

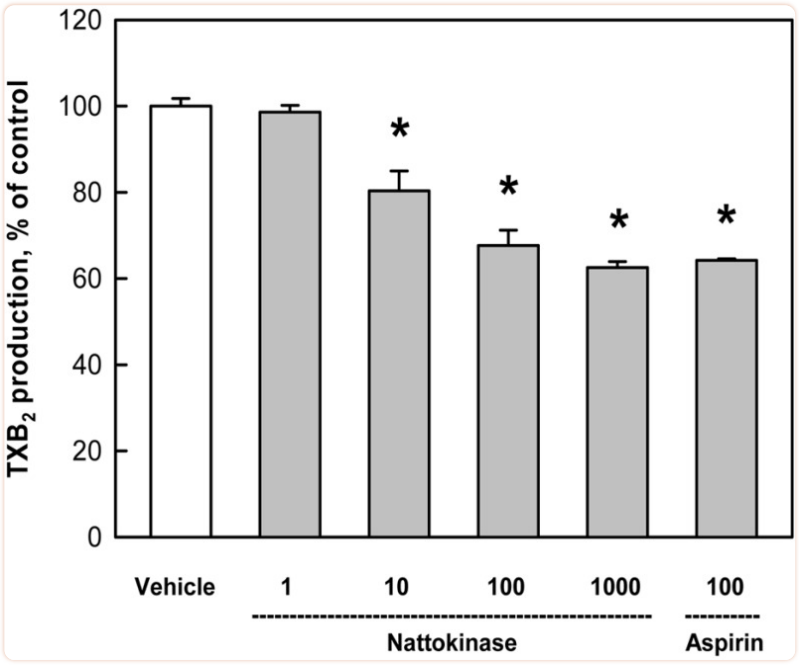

Figure 2

Inhibition by nattokinase (1-1,000 μg/mL) or aspirin (100 μM) of thromboxane B_2 production from rabbit platelets induced by collagen (2.5 μg/mL). *Significantly different from vehicle control.

Ten-min application of 35% $FeCl_3$ to the external surface of carotid artery caused gradual decrease in the blood flow that near-fully ceased in 40 min (Figure 3). However, 1-week oral treatment with nattokinase inhibited the thrombus formation in a dose-dependent manner, in which the blood flow was maintained longer than 90 min in the rats treated with a high dose (500 mg/kg) of nattokinse or with aspirin (30 mg/kg).

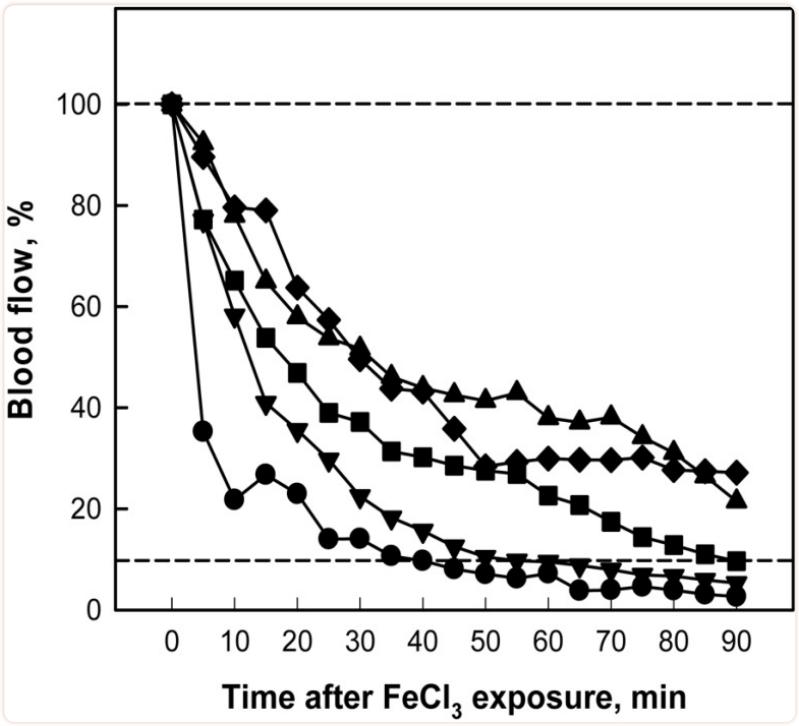

Figure 3

Time-course of carotid arterial blood flow following $FeCl_3$ application outside the arterial wall. Nattokinase and aspirin were orally administered for 1 week prior to $FeCl_3$ exposure. Lower dot line indicates a practical cessation of blood flow. ●, vehicle; ▾, nattokinase 50 mg/kg; ▪, nattokinase 160 mg/kg; ♦, nattokinase 500 mg/kg; ▴, aspirin 30 mg/kg.

The mean occlusion time in the vehicle control group was calculated to be 38.5 min, based on the time point when the blood flow dropped to 10% (practical cessation) of initial flow rate (Figure 4). In comparison, treatment with 50 and 160 mg/kg of nattokinase extended the occlusion time to 55.0 and 90.1 min, respectively. In addition, a high dose (500mg/kg) of nattokinase prolonged the occlusion time longer than 120 min, as achieved with aspirin (30 mg/kg).

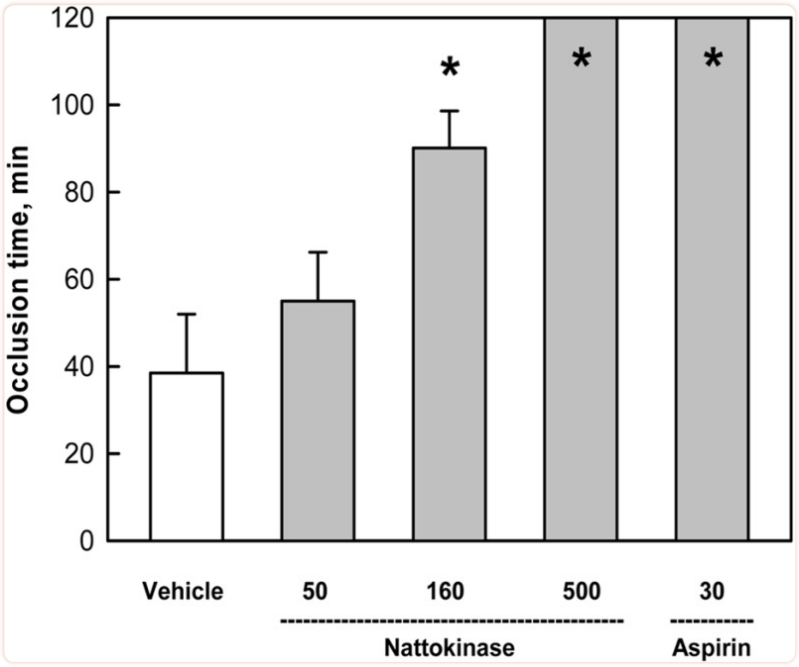

Figure 4

Time to occlusion of carotid arteries after application of $FeCl_3$ to outside the arterial wall. Nattokinase (50-500 mg/kg) and aspirin (30 mg/kg) were orally administered for 1 week prior to $FeCl_3$ exposure. *Significantly different from vehicle control.

As dissected 50 min after the application of $FeCl_3$, the arteries were entirely plugged with thrombi in the vehicle control group (Figure 5). However, in the animals treated with nattokinase or aspirin, the thrombi were small and loose, without fully obstructing the arterial lumens.

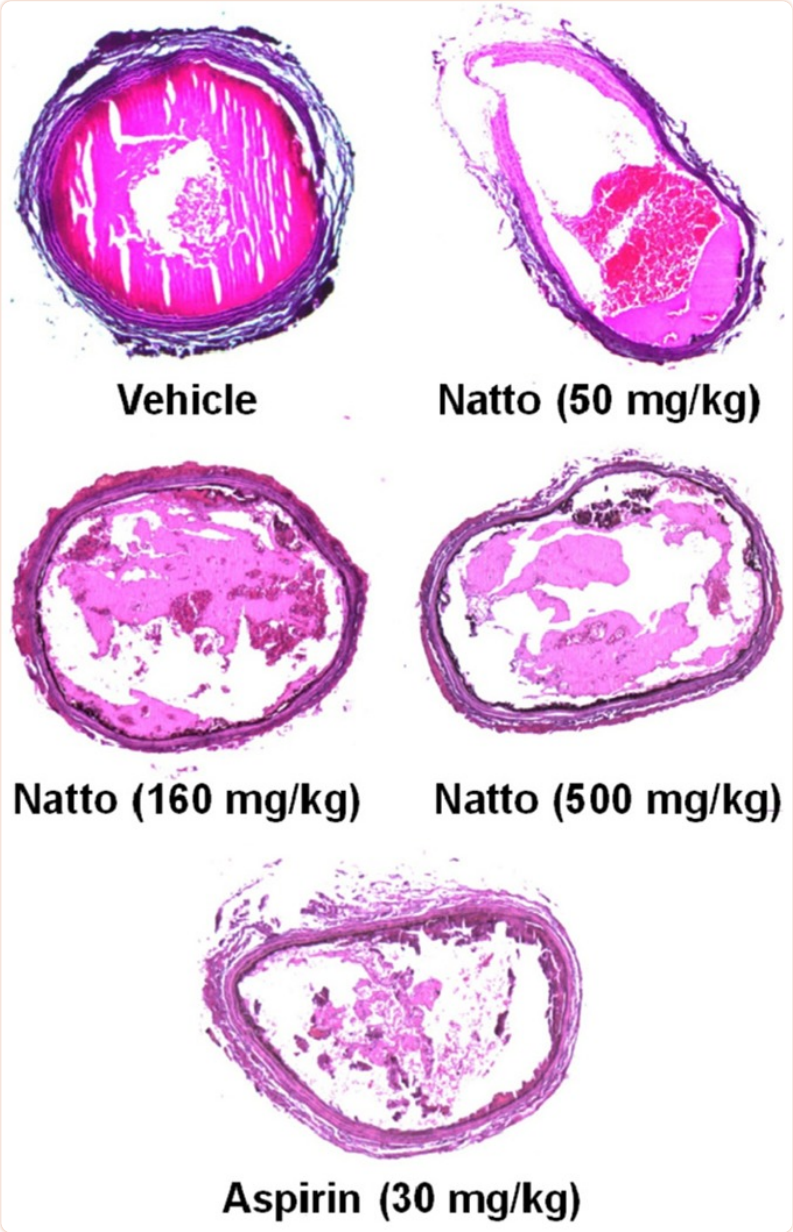

Figure 5

Representative findings of arterial thrombi produced by $FeCl_3$ application outside the arterial wall. Nattokinase (50-500 mg/kg) and aspirin (30 mg/kg) were orally administered for 1 week prior to $FeCl_3$ exposure.

Oral administration of nattokinase from *Bacillus subtilis* Natto substantially inhibited both the collagen- and thrombin-induced platelet aggregations. The results mean that nattokinase not only inhibits blood clotting triggered by thrombin, but also blocks TXA_2-mediated adhesion of platelets to the injured vessel walls as shown in the collagen-induced TXB_2 formation [3-5]. Therefore, it is inferred that the effects of nattokinase are similar to those of aspirin, a well-known blood flow enhancer exerting its effect via both mechanisms.

$FeCl_3$ brings about oxidative endothelial injury and exposure of subendothelial extracellular matrix. Then platelets interact with collagen and vWF in the matrix via their respective platelet membrane receptors, leading to platelet adhesion. Activated platelets undergo calcium mobilization and the release of ADP and TXA_2 to further accelerate recruitment and aggregation of

platelets for thrombus formation [16]. Based on the *in vitro* results, *in vivo* anti-thrombotic efficacy of nattokinase was anticipated. Indeed, oral administration of nattokinse delayed the occlusion time in a $FeCl_3$-induced artery thrombosis model. Notably, the effects of crude nattokinase at high doses were comparable to those of aspirin (30 mg/kg), a purified drug, in which nattokinase doubled the occlusion time and especially fully prevented occlusion at 160 and 500 mg/kg, respectively.

It was also reported that subcutaneous injection of nattokinase substantially reduced the tail vein infarction in a carrageenan-induced tail thrombosis model [13]. The result indicates that nattokinase also attenuates inflammation-induced vascular thrombosis, in addition to the effects in the oxidative injury-mediated thrombosis in the present study. It is of interest to note that oral administration of nattokinase enhanced fibrinolytic activity and the production of tissue-type plasminogen activator in the plasma [11,17]. The findings represent possibilities of both the prevention and treatment of ischemic diseases via inhibition of thrombus formation and facilitation of fibrinolysis, since nattokinase dissolves the fibrin clot [18,19]. However, it is unclear whether a part of nattokinase proteins is absorbed in the gastrointestinal tracts without digestion or ingredients other than nattokinase activating intrinsic fibrinolytic machinery exist in the compound.

It is well known that non-steroidal anti-inflammatory drugs including aspirin can induce gastric ulcers and bleeding at high doses [20]. Thus, there is a need for an effective improvement of blood flow without risk of adverse effects, and natural products should fulfill this requirement. In the present study, the nattokinase extracted from fermented soybeans displayed excellent anti-platelet aggregation and anti-thrombotic activities *in vitro* and *in vivo*. Although additional exact action mechanisms remain to be clarified, it is suggested that nattokinase could be a good health functional food for the improvement of blood flow.

Acknowledgments

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References

1. Majid A, Delanty N, Kantor J. Antiplatelet agents for secondary prevention of ischemic stroke. *Ann Pharmacother.* 2001;35(10):1241–1247. [PubMed] [Google Scholar]

2. Jackson SP, Nesbitt WS, Kulkarni S. Signaling events underlying thrombus formation. *J Thromb Haemost.* 2003;1(7):1602–1612. [PubMed] [Google Scholar]

3. Farndale RW, Sixma JJ, Barnes MJ, de Groot PG. The role of collagen in thrombosis and hemostasis. *J Thromb Haemost.* 2004;2(4):561–573. [PubMed] [Google Scholar]

4. Konno C, Oshima Y, Hikino H. Morusinol, isoprenoid flavone from Morus root barks. *Planta Med.* 1977;32(2):118–124. [PubMed] [Google Scholar]

5. Cowan DH. Platelet adherence to collagen: role of prostaglandin-thromboxane synthesis. *Br J Haematol.* 1981;49(3):425–434. [PubMed] [Google Scholar]

6. Dogné JM, Hanson J, de Leval X, Pratico D, Pace-Asciak CR, Drion P, Pirotte B, Ruan KH. From the design to the clinical application of thromboxane modulators. *Curr Pharm Des.* 2006;12(8):903–923. [PubMed] [Google Scholar]

7. Arita H, Nakano T, Hanasaki K. Thromboxane A2: its generation and role in platelet activation. *Prog Lipid Res.* 1989;28(4):273–301. [PubMed] [Google Scholar]

8. Gilmer JF, Murphy MA, Shannon JA, Breen CG, Ryder SA, Clancy JM. Single oral dose study of two isosorbide-based aspirin prodrugs in the dog. *J Pharm Pharmacol.* 2003;55(10):1351–1357. [PubMed] [Google Scholar]

9. Lee JJ, Yang H, Yoo YM, Hong SS, Lee D, Lee HJ, Lee HJ, Myung CS, Choi KC, Jeung EB. Morusinol extracted from Morus alba inhibits arterial thrombosis and modulates platelet activation for the treatment of cardiovascular disease. *J Atheroscler Thromb.* 2012;19(6):516–522. [PubMed] [Google Scholar]

10. Sumi H, Hamada H, Tsushima H, Mihara H, Muraki H. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. *Experientia.* 1987;43(10):1110–1111. [PubMed] [Google Scholar]

11. Sumi H, Hamada H, Nakanishi K, Hiratani H. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol.* 1990;84(3):139–143. [PubMed] [Google Scholar]

12. Ko JH, Yan JP, Zhu L, Qi YP. Identification of two novel fibrinolytic enzymes from Bacillus subtilis QK02. *Comp Biochem Physiol C Toxicol Pharmacol.* 2004;137(1):65–74. [PubMed] [Google Scholar]

13. Kamiya S, Hagimori M, Ogasawara M, Arakawa M. In vivo evaluation method of the effect of nattokinase on carrageenan-induced tail thrombosis in a rat model. *Acta Haematol.* 2010;124(4):218–224. [PubMed] [Google Scholar]

14. Lee JJ, Jin YR, Yu JY, Munkhtsetseg T, Park ES, Lim Y, Kim TJ, Pyo MY, Hong JT, Yoo HS, Kim Y, Yun YP. Antithrombotic and antiplatelet activities of fenofibrate, a lipid-lowering drug. *Atherosclerosis.* 2009;206(2):375–382. [PubMed] [Google Scholar]

15. Born GV, Cross MJ. The aggregation of blood platelets. *J Physiol.* 1963;168:178–195. [PMC free article] [PubMed] [Google Scholar]

16. Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest.* 2005;115(12):3355–3362. [PMC free article] [PubMed] [Google Scholar]

17. Fujita M, Hong K, Ito Y, Fujii R, Kariya K, Nishimuro S. Thrombolytic effect of nattokinase on a chemically induced thrombosis model in rat. *Biol Pharm Bull.* 1995;18(10):1387–1391. [PubMed] [Google Scholar]

18. Suzuki Y, Kondo K, Matsumoto Y, Zhao BQ, Otsuguro K, Maeda T, Tsukamoto Y, Urano T, Umemura K. Dietary supplementation of fermented soybean, natto, suppresses intimal thickening and modulates the lysis of mural thrombi after endothelial injury in rat femoral artery. *Life Sci.* 2003;73(10):1289–1298. [PubMed] [Google Scholar]

19. Urano T, Ihara H, Umemura K, Suzuki Y, Oike M, Akita S, Tsukamoto Y, Suzuki I, Takada A. The profibrinolytic enzyme subtilisin NAT purified from Bacillus subtilis Cleaves and inactivates plasminogen activator inhibitor type 1. *J Biol Chem.* 2001;276(27):24690–24696. [PubMed] [Google Scholar]

20. Rao ChV, Ojha SK, Radhakrishnan K, Govindarajan R, Rastogi S, Mehrotra S, Pushpangadan P. Antiulcer activity of Utleria salicifolia rhizome extract. *J Ethnopharmacol.* 2004;91(2-3):243–249. [PubMed] [Google Scholar]