Comparative Study of Nitric Oxide Scavenging Effect in Several Herbal Extracts

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In this study, we investigated in vitro nitric oxide (NO) scavenging effect on nine herbal extracts using an NO donor S-nitroso-N-acetylpenicillamine (SNAP) compared with vitamin C. All herbal extracts effectively reduced the generation of NO radicals in a dose-dependant manner and over 65% in 10 h. Especially, *Ephedrae herba*, *Carthami flos* and *Lonicerae flos* declined over 75% of NO scavenging effect, suggested that the herbal extracts are the powerful free radical scavengers and may be effective in clinical applications.

Key words : nitric oxide, S-nitroso-N-acetylpenicillamine, Griess reagent, antioxidant

Introduction

Oxygen free radicals have been implicated as causative agents in traumatic and ischemic brain injury and brain edema. Nitric oxide (NO) is produced in various cell types and tissues by nitric oxide synthase from the substrate L-arginine. While this free radical is an important signaling molecule, it has also been implicated as neurotoxin, for example, as a causative agent in cerebral ischemia. As a result of this cytotoxic action, NO and its oxidized metabolite may aggravate brain damage[1]. Kimura et al.[2] showed that the L-arginine-NO synthase pathway was activated during reperfusion after focal cerebral ischemia, indicating that interaction between NO and superoxide during early reperfusion is likely. Simultaneous production of NO and superoxide in the ischemic brain might produce highly cytotoxic peroxynitrite[3]. In specific neuronal pathways, NO functions alternatively as a neurotransmitter or a second messenger. Recently, it has been reported that NO is a key molecule in migraine and other vascular headaches[4,5].

Free radicals, including NO, have been closely associated with the pathogenesis of Parkinson's disease. In Parkinson's disease, the level of NO has been reported to increase in the brain[6-8]. Moreover, NO has been implicated in the pathogenesis of Alzheimer's disease[9,10]. Previous studies have demonstrated that oxygen free radical scavengers might be useful in the treatment of brain edema and brain injury. However, there are only a limited number of oxygen free radical scavengers with demonstrable clinical efficacy.

The aim of this study is to investigate the effects of herbal extracts on NO radical scavenging in vitro using a S-nitroso-N-acetylpenicillamine (SNAP) generating NO system.

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Materials and methods

1. Meterials

Sodium phosphate monobasic, sodium phosphate dibasic, sodim chloride, sulphanilamide, naphthyletylenediamine dihydrochloride and H_3PO_4 were purchased from Sigma Korea. S-nitroso-N-acetylpenicillamine (SNAP) was prepared by Yoo et al.[11].

2. Herbal extracts

To obtain the aqueous extract, 100 g of dried herbal was added to 100 ㎖ of the boiled-distilled water and extraction was performed by heating at 80℃ for 2 h. Then the extracts was filtered and freezing dried (Biocryos, Seoul).

3. Assay of NO radical savenging activity

NO generated from SNAP was measured by the Griess reagent. 1 mM of SNAP was prepared by dissolving the powder in phosphate buffered saline (PBS) pH 7.4. The reaction mixture (1 ㎖) containing 1 mM SNAP and PBS was incubated at 37C. Samples (100 ㎕) of the incubation were

removed and diluted with 100 ㎕ of Griess reagent (1% sulphanilamide and 0.1% naphthyletylenediamine dihydrochloride in 2% H_3PO_4). The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylene -diamine was read at 540 nm, and referred to the absorbance of standard solutions of sodium nitrite treated in the same way with Griess reagent. The plot between the concentration of nitrite and incubation time exhibited the best incubation time for nitrite production from SNAP.

4. Effect of the herbal extracts on NO radical scavenging activity

Various concentrations of the herbal extracts and SNAP (1 mM, final concentration) in PBS in a final volume of 1 ㎖ were incubated at 37C. A control experiment without the herbal extracts but with the equivalent amount of vehicles was conducted in an identical manner of control. After incubation, 100 ㎕ samples of reaction mixtures containing nitrite were removed and diluted with 100 ㎕ of Griess reagent. Vitamin C was used as a reference standard. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. The absorbances of their chromophores at measured wavelengths were in a concentration-dependent manner. Addition of varied concentrations of these compounds into the reaction mixture affected an increase in total absorbance upon treatment with Griess reagent.

5. Statistical analysis

Data was presented as % means of at least three separate experiments. Comparison between two values was analyzed using Student's t-test.

Results

SNAP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce a nitrite ion that can be estimated using Griess reagent. The incubation time for SNAP to generate a maximum concentration of nitrite ion is 24 h. Incubation of solutions of SNAP in PBS at 37℃ from 1 h to 24 h resulted in linear time-dependent nitrite production (Fig. 1). In this study, vitamin C was used as the reference NO radical scavengers (Table 1). The compound that possesses NO scavenging activity inhibited nitrite formation by competing with oxygen to react with NO. But this didn't lead to the reduction of nitrite concentration in the assay media, the other data showed that 0.2 ㎎/㎖, 2.0 ㎎/㎖, 3.5 ㎎/㎖ of vitamin C scavenged approximately 50%, 78%, and 95% of NO radical. Therefore vitamin C is an extreme antioxidant.

All the herbal extracts scavenged NO radicals in a dose-dependent manner (Table 1). The final concentration (1.0 ㎎/㎖) of Caesalpinia Sappan was 74.40% for 5 h and 68. 45% for 10 h, Linderae Radix was 65. 9% and 64.9%, Teucrium veronicoides was 66.15% and 60.01%, Citri reticulatae viride pericarpium was 68.41% and 72.81, Psoraleae fructus was 70.35% and 70.46%, Amomi fructus was 71.21% and 72.75%, Ephedrae herba was 56.20% and 78.39%, Carthami flos was 54.05% and 82.03% and Lonicerae flos was 58.38% and 78.02%. All the herbal extracts scavenged over 65% in 10 h. Especially, Ephedrae herba, Carthami flos and Lonicerae flos declined over 75% of NO scavenging effect.

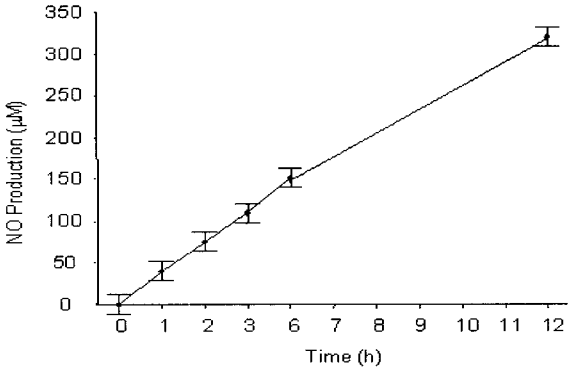

Fig. 1. Typical nitric oxide producing curve according to time. NO generated from SNAP (1 mM) was measured by the Griess reagent. These data were repeated for three experiments.

Discussion

In acute brain injury, oxygen free radicals are thought to cause oxidative damage to a wide variety of molecular and cellular targets, including lipids, proteins, DNA, membrane structures, and mitochondria. Oxygen free radicals of potential importance in cerebral ischemia include superoxide and hydroxyl radicals. The hydroxyl radical is a highly reactive free radical and excessive production of this species can induce lipid peroxidation. Recently, we investigated the scavenging activity of Caesalpinia Sappan towards reactive oxygen free radicals using the scavenging assay of DPPH radical and the protection assay against bovine serum albumin12. In this study, it was demonstrated that the herbal extracts could scavenge NO radical. NO is a small, highly reactive molecule that passes readily through neuronal membranes and has a half-life on the order of milliseconds to seconds. NO can also be neurotoxic due to its radical properties and NO toxicity derives from its reaction with superoxide to form peroxynitrite. Peroxynitrite is a powerful oxidizing agent that, when

Table 1. In Vitro NO scavenging Effect of traditional medicine

Herb	Time	Concentration (mg/ml)	NO Production (%)a	NO Scavenging effect (%))b
Caesalpinia sappan	5h	0.00	100.00	0.00
		0.01	122.17	-22.17
		0.10	60.48	39.52
		0.25	45.10	54.90
		0.50	33.03	66.97
		1.00	25.60	74.40
	10h	0.00	100.00	0.00
		0.01	134.48	-34.48
		0.10	45.40	54.60
		0.25	39.84	60.16
		0.50	33.37	66.63
		1.00	31.55	68.45
Linderae radix	5h	0.00	100.00	0.00
		0.01	63.83	36.17
		0.10	35.80	64.20
		0.25	29.84	70.16
		0.50	33.45	66.55
		1.00	34.18	65.82
	10h	0.00	100.00	0.00
		0.01	52.27	47.73
		0.10	34.72	65.28
		0.25	34.85	65.15
		0.50	34.85	65.15
		1.00	35.10	64.90
Teucrium veronicoides	5h	0.00	100.00	0.00
		0.01	55.64	44.36
		0.10	37.35	62.65
		0.25	32.68	67.32
		0.50	36.58	63.42
		1.00	33.85	66.15
	10h	0.00	100.00	0.00
		0.01	41.19	58.81
		0.10	31.27	68.73
		0.25	28.35	71.65
		0.50	27.89	72.11
		1.00	39.99	60.01
Citri reticulatae viride pericarpium	5h	0.00	100.00	0.00
		0.01	58.17	41.83
		0.10	44.23	55.77
		0.25	35.95	64.05
		0.50	33.33	66.67
		1.00	31.59	68.41
	10h	0.00	100.00	0.00
		0.01	42.40	57.60
		0.10	33.25	66.75
		0.25	29.25	70.75
		0.50	28.48	71.52
		1.00	27.19	72.81
Psoraleae frutus	5h	0.00	100.00	0.00
		0.01	74.22	25.78
		0.10	36.10	63.90
		0.25	32.23	67.77
		0.50	23.76	76.24
		1.00	29.65	70.35
	10h	0.00	100.00	0.00
		0.01	40.41	59.59
		0.10	31.46	68.54
		0.25	29.41	70.59
		0.50	24.55	75.45
		1.00	29.54	70.46
Amomi fructus	5h	0.00	100.00	0.00
		0.01	45.49	54.51
		0.10	42.42	57.58
		0.25	37.14	62.86
		0.50	29.45	70.55
		1.00	28.79	71.21
	10h	0.00	100.00	0.00
		0.01	37.85	62.15
		0.10	36.82	63.18
		0.25	33.73	66.27
		0.50	28.87	71.13
		1.00	27.25	72.75
Ephedrae herba	5h	0.00	100.00	0.00
		0.01	48.21	51.79
		0.10	36.91	63.09
		0.25	38.84	61.16
		0.50	42.42	57.38
		1.00	43.80	56.20
	10h	0.00	100.00	0.00
		0.01	66.70	33.30
		0.10	26.31	73.69
		0.25	22.76	77.24
		0.50	20.64	79.36
		1.00	21.61	78.39
Carthami flos	5h	0.00	100.00	0.00
		0.01	49.19	50.81
		0.10	41.62	58.38
		0.25	38.92	61.08
		0.50	45.14	54.86
		1.00	45.95	54.05
	10h	0.00	100.00	0.00
		0.01	45.64	54.36
		0.10	22.33	77.67
		0.25	19.31	80.69
		0.50	19.31	80.69
		1.00	17.97	82.03
Lonicerae flos	5h	0.00	100.00	0.00
		0.01	58.38	41.62
		0.10	47.03	52.97
		0.25	44.05	55.95
		0.50	42.70	57.30
		1.00	41.62	58.38
	10h	0.00	100.00	0.00
		0.01	52.22	47.78
		0.10	32.03	67.97
		0.25	26.25	73.75
		0.50	21.53	78.47
		1.00	21.98	78.02
Vitamin C	5h	0.00	100.00	0.00
		0.01	108.31	-8.31
		0.10	108.00	-8.00
		0.25	119.08	-19.08
		0.50	130.77	-30.77
		1.00	150.77	-50.77
	10h	0.00	100.00	0.00
		0.01	112.80	-12.80
		0.10	138.40	-38.40
		0.25	139.76	-39.76
		0.50	152.41	-52.41
		1.00	167.92	-67.92

protonated, decays to form the highly reactive hydroxyl radical. This indicates that scavenging NO may constitute an approach to reducing hydroxyl radical mediated damage. In

the present study, an in vitro NO assay involving NO radical scavenging analysis was employed and was found to effective for the detection of NO. Using this method, we have demonstrated a direct, dose-dependent scavenging activity of the herbal extracts towards NO. The data obtained suggest the herbal extracts are the powerful free radical scavengers and may be effective in clinical applications. Furthermore, the scavenging effects of the herbal extracts towards NO observed in vitro constitute a novel mechanism by which this drug may exert its protective effect against brain edema and brain injury.

In conclusion, we investigated in vitro NO scavenging effect on nine herbal extracts using a NO donor SNAP compared with vitamin C. All herbal extracts effectively reduced the generation of NO radicals in a dose-dependant manner and over 65% in 10 h. Especially, Ephedrae herba, Carthami flos and Lonicerae flos declined over 75% of NO scavenging effect, suggested that the herbal extracts are the powerful free radical scavengers and may be effective in clinical applications.

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