

Journal of Medicinal Food: <http://mc.manuscriptcentral.com/medicinalfood>

Effects of silibinin against prothrombin kringle-2-induced neurotoxicity in the nigrostriatal dopaminergic system *in vivo*

Journal:	<i>Journal of Medicinal Food</i>
Manuscript ID	JMF-2018-0078
Manuscript Type:	Original Article
Date Submitted by the Author:	29-May-2018
Complete List of Authors:	Leem, Eunju; Kyungpook National University, School of Life Sciences, BK21 plus KNU Creative BioResearch Group, Institute of Life Science & Biotechnology Oh, Yong-Seok ; Daegu-Gyeongbuk Institute of Science and Technology Graduate School, Department of Brain-Cognitive Science Shin, Won-Ho; Korea Institute of Toxicology, Predictive Model Research Center, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology Jeong, Jae Yeong; Korea Institute of Toxicology, Predictive Model Research Center, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology; Kyung Hee University School of Medicine, Department of Biochemisry and Molecular Biology, Department of Neuroscience Graduate School Jin, Byung Kwan; Kyung Hee University School of Medicine, Department of Biochemisry and Molecular Biology, Department of Neuroscience Graduate School Shin, Minsang; School of Medicine, Kyungpook National University, Department of Microbiology Kim, Dong Woon; Chungnam National University School of Medicine, Department of Anatomy, Brain Research Institute Jang, Jin-Hyeok; Daegu-Gyeongbuk Institute of Science and Technology Graduate School, Department of Brain-Cognitive Science Kim, Hyung-Jun; Korea Brain Research Institute, Department of Neural Development and Disease Moon, Gyeongjoon; Kyungpook National University, School of Life Sciences, BK21 plus KNU Creative BioResearch Group, Institute of Life Science & Biotechnology Kim, Sang Ryong; Kyungpook National University, School of Life Sciences, BK21 plus KNU Creative BioResearch Group, Institute of Life Science & Biotechnology; Brain Science and Engineering Institute, Kyungpook National University
Keyword:	neurodegeneration
Manuscript Keywords (Search Terms):	Microglia, Nigrostriatal dopaminergic system, Prothrombin kringle-2, Silibinin

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

SCHOLARONE™
Manuscripts

For Peer Review

Effects of silibinin against prothrombin kringle-2-induced neurotoxicity in the nigrostriatal dopaminergic system *in vivo*

Running Title: Silibinin against pKr-2-induced neuroinflammation

Eunju Leem,^{1,*} Yong-Seok Oh,^{2,*} Won-Ho Shin,^{3,*} Jae Yeong Jeong,^{3,4} Byung Kwan Jin,⁴ Minsang Shin,⁵ Dong Woon Kim,⁶ Jin-Hyeok Jang,² Hyung-Jun Kim,⁷ Gyeong Joon Moon,^{1,#} and Sang Ryong Kim^{1,8,#}

¹ School of Life Sciences, BK21 plus KNU Creative BioResearch Group, Institute of Life Science & Biotechnology, Kyungpook National University, Daegu 41566, Republic of Korea.

² Department of Brain-Cognitive Science, Daegu-Gyeongbuk Institute of Science and Technology, Daegu 42988, Republic of Korea.

³ Predictive Model Research Center, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Daejeon 34114, Republic of Korea.

⁴ Department of Biochemistry and Molecular Biology, Department of Neuroscience Graduate School, School of Medicine, Kyung Hee University, Seoul 02447, Republic of Korea.

⁵ Department of Microbiology, School of Medicine, Kyungpook National University, Daegu 41944, Republic of Korea.

⁶ Department of Anatomy, Brain Research Institute, Chungnam National University School of Medicine, Daejeon 34134, Republic of Korea.

⁷ Department of Neural Development and Disease, Korea Brain Research Institute, Daegu 41062, Republic of Korea.

⁸ Brain Science and Engineering Institute, Kyungpook National University, Daegu 41944, Republic of Korea.

*These authors contributed equally to this work.

#Correspondence: Dr. Sang Ryong Kim and Dr. Gyung Joon Moon

Tel.: +82-53-950-7362, Fax: +82-53-943-2762

E-mail addresses: srk75@knu.ac.kr (S.R. Kim) and neobios7@gmail.com (G.J. Moon)

Abstract

Neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease, can be caused by numerous factors, however, neurotoxic inflammation by activated microglia is a common feature in such diseases, suggesting that the control of microglial activation is useful to protect neurons in the adult brain. Recently, we reported that upregulation of prothrombin kringle-2 (pKr-2), a domain of prothrombin that is generated by active thrombin, could induce the death of nigral dopaminergic (DA) neurons through microglial activation in the adult brain. Here, we show that silibinin, a flavonoid found in milk thistle, can suppress the production of neurotoxic inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β), following pKr-2 treatment in the substantia nigra of the mouse brain. Moreover, as demonstrated by immunohistochemical staining, reversed-phase high-performance liquid chromatography (HPLC) analysis, and open-field behavioral tests, silibinin treatment protects the nigrostriatal DA system from pKr-2-induced neurotoxicity *in vivo*. Thus, we conclude that silibinin may be a beneficial natural compound offering anti-inflammatory effects against pKr-2-induced neurotoxicity, resulting in protection of the nigrostriatal DA system, which can be applied in PD.

Key Words

Microglia · Neurodegeneration · Nigrostriatal dopaminergic system · Prothrombin kringle-2 · Silibinin

INTRODUCTION

Parkinson’s disease (PD) is characterized by a progressive loss of nigral dopaminergic (DA) neurons and a reduction in striatal dopamine levels.¹⁻⁶ Although the main etiology of PD remains controversial, accumulated evidence suggests that neuroinflammatory processes are crucial for the initiation and progression of PD, and the control of neurotoxic inflammation is considered as one of the therapeutic strategies against PD.^{7,8} We recently reported that the upregulation of prothrombin kringle-2 (pKr-2), a domain of prothrombin that is generated by active thrombin, could induce microglial activation, resulting in overexpression of neurotoxic inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), and degeneration of the nigrostriatal DA projection in the adult brain, although pKr-2 itself was not directly toxic to neurons.^{1,2} Moreover, our previous observations showed that pKr-2 levels were upregulated in the brains of patients with PD,⁹⁻¹³ suggesting that limiting pKr-2-induced microglial activation might be an effective therapeutic strategy for protecting DA neurons in the adult brain.

Silibinin, a polyphenolic flavonoid and the major biologically active compound of milk thistle, has been shown to have neuroprotective effects in models of ischemia and Alzheimer’s disease (AD).⁹⁻¹³ In addition, treatment with silibinin attenuated the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, resulting in protection of DA neurons against 1-methyl-4-phenylpyridinium (MPP⁺)-induced neurotoxicity in the substantia nigra (SN) of the adult rat brain.⁹ This suggests that its administration has the potential to inhibit neurotoxic inflammation in the nigrostriatal DA system *in vivo*. In the present study, therefore, we examined whether silibinin treatment could attenuate pKr-2-induced microglial activation and prevent neurodegeneration in the nigrostriatal DA system following pKr-2 treatment in the SN of the mouse brain.

MATERIALS AND METHODS

Materials

The following materials, excluding silibinin and pKr-2, were purchased from commercial companies: rabbit anti-tyrosine hydroxylase (TH; Pel-Freez, Brown Deer, WI), rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1; Wako Pure Chemical Industries, Osaka, Japan), mouse anti-TNF- α (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-IL-1 β (Santa Cruz Biotechnology), mouse anti- β -actin (Santa Cruz Biotechnology), rabbit anti- β -actin (Cell Signaling, Beverly, MA), biotinylated anti-rabbit IgG (Vector laboratories, Burlingame, CA), Texas Red-conjugated anti-mouse IgG (Vector Laboratories), fluorescein isothiocyanate-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Bar Harbor, ME), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Enzo Life Sciences, Farmingdale, NY), and HRP-conjugated anti-mouse IgG (Thermo Fisher Scientific, Rockford, IL).

Institutional review of animal protocols

The male C57BL/6 mice (8-10 weeks old, 23–25 g) used in this study were obtained from Daehan Biolink (Eumseong, Republic of Korea). The mice were housed in a controlled environment and were provided with food and water *ad libitum*. All surgical experiments were performed in accordance with approved animal protocols and guidelines established by the Animal Care Committee of Kyungpook National University (No. KNU 2016-42).

Silibinin administration

Silibinin [(2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl]chroman-4-one; C₂₅H₂₂O₁₀] was obtained from Sigma-Aldrich (St. Louis, MO; Fig. 1A). To examine the effects of silibinin against pKr-2-induced neurotoxic inflammatory responses in the nigrostriatal DA system, mice received intraperitoneal injection of silibinin (50, 100, or 200 mg/kg/day), dissolved in 0.9% saline containing 0.25% carboxymethylcellulose (Sigma-Aldrich), starting 1 day before intranigral injection of pKr-2 and continued daily until 1 day or 6 days post-injection. The brains were harvested for each analysis at the indicated time points (Fig. 1A).

Intranigral pKr-2 injection

As previously described, with some modifications,¹⁴ mice were anesthetized with chloral hydrate solution and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) with a mouse adapter. Each animal received a unilateral injection of pKr-2 alone [Haematologic Technologies, Inc., Essex Junction, VT; 24 µg in 2 µl phosphate-buffered saline (PBS); Sigma-Aldrich] into the right SN (AP: -3.2 mm; ML: -1.3 mm; DV: -4.0 mm, relative to bregma) at a rate of 0.5 µl/min.¹⁵ After the injection, the needle was left in place for an additional 5 min and then slowly retracted. The animals were sacrificed and analyzed at the indicated time points for each experiment (Fig. 1A).

Open-field test

The open-field test was performed as described previously, with some modifications.¹⁶ Briefly, a week after the pKr-2 injection (Fig. 1A), mice were placed individually in the right side of a test chamber (40 × 40 × 40 cm) enclosed with white acrylic walls. After a 1-min

adaptation period, animal behaviors were recorded for 5 min using a video camera. Changes in locomotor activity were analyzed offline with video-tracking software (SMART, Panlab, Barcelona, Spain) and displayed as graphs depicting the total distance traveled (in cm) and velocity (in cm/sec). The test chamber was cleaned between trials with 70% ethyl alcohol. To minimize stress levels, the tests were performed under low-illumination conditions.

Immunohistochemical staining procedures

Immunohistochemistry was performed as previously described,^{17,18} with some modifications for the present study. The animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After post-fixation and cryoprotection using a 30% sucrose solution, 30- μ m-thick brain sections were processed for immunohistochemical staining. Briefly, sections were rinsed in 0.1 M PBS and incubated with primary antibodies at 4 °C for 2 days. Tissues were rinsed with PBS containing 0.5% bovine serum albumin, incubated at room temperature with appropriate biotinylated secondary antibodies, and then processed with an avidin-biotin complex kit (Vector Laboratories). The signal was detected by incubating sections in 0.5 mg/ml 3,3'-diaminobenzidine (Sigma-Aldrich) in 0.1 M PB containing 0.003% H₂O₂. The primary antibodies were rabbit anti-TH or rabbit anti-Iba1. Immunostained SN and striatum tissue samples were mounted on gelatin-coated slides. Samples were counterstained with 0.5% cresyl violet (Sigma-Aldrich) and then analyzed with a bright-field microscope (Axio Imager, Carl Zeiss, Göttingen, Germany).

Brain sections for double-immunofluorescence staining were rinsed and incubated for 2 days with the following pairs of antibodies: rabbit anti-Iba1 and mouse anti-TNF- α . After 2 days, sections were rinsed and incubated with fluorescence-conjugated secondary antibodies

for 1 h, and then washed and mounted with antifade reagent (Invitrogen, Camarillo, CA). The stained sections were analyzed under a fluorescence microscope (Axio Imager).

Stereological estimation

Both hemispheres of each mouse brain were analyzed to determine the number of TH-positive neurons as previously described, with some modifications.¹⁹ Briefly, the entire SN from every section was identified as the region of interest, and the total number of TH-positive neurons was counted using the optical fractionator's method with the Olympus Computer-Assisted Stereological Toolbox system version 2.1.4 (Olympus, Ballerup, Denmark). The number of cells in the SN was quantitatively expressed as a percentage of that in the contralateral control side.

Quantitative determination of striatal TH immunostaining

The whole striatum was identified as previously described.¹⁹ Briefly, the density of striatal TH-positive fibers was measured using the Multi Gauge version 3.0 (Fujifilm, Tokyo, Japan). To control for variations in background illumination, the density of the corpus callosum was subtracted from the density of the striatum for each section. TH-positive fiber innervation of the striatum was expressed quantitatively as the optical density on the ipsilateral lesion side as a percentage of the optical density on the contralateral control side.

Western blot analysis

The SN tissues were prepared from the animals at the indicated time points after injection of

pKr-2, as previously described.^{14,17,18} Briefly, animal SN tissues were removed and sliced using a brain matrix (Roboz Surgical Instrument Co., Gaithersburg, MD), and the tissues were homogenized and centrifuged at 4 °C for 15 min at 14,000 g. The supernatant was transferred to a fresh tube, and the concentration was determined using a DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA). The samples were boiled at 100 °C for 5 min before gel loading, and equal amounts of protein were loaded into each lane with the loading buffer. Proteins were separated using gel electrophoresis (Bio-Rad Laboratories) and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). The membranes were then incubated overnight at 4 °C with specific primary antibodies: rabbit anti-TH (1:2000; Pel-Freez), rabbit anti-Iba1 (1:2000; Wako Pure Chemical Industries), mouse anti-TNF- α (1:500; Santa Cruz Biotechnology), rabbit anti-IL-1 β (1:500; Santa Cruz Biotechnology), and mouse anti- β -actin (1:1000; Santa Cruz Biotechnology). After washing with Tris-buffered saline with Tween-20, the membranes were incubated with the appropriate secondary antibodies (Invitrogen) for 1 h at room temperature, and the blots were finally developed using enhanced chemiluminescence (ECL) western blot detection reagents (GE Healthcare Life Sciences, Marlborough, MA). The signal was analyzed using an LAS-500 image analyzer (GE Healthcare Life Sciences) and Multi Gauge version 3.0 (Fuji Photo Film).

Dopamine and its metabolites in the striatum

The measurements of striatal dopamine and its metabolites using reversed-phase high-performance liquid chromatography (HPLC) with an electrochemical detector were performed as previously described.¹⁴ Briefly, animal striatum tissues were removed and sliced using a brain matrix. The brain tissues were homogenized and centrifuged, and then the

supernatants were injected into an autosampler at 4 °C (Waters 717 plus autosampler) and eluted through a Sunfire C18 column (4.6 × 100 mm × 5 µm; Waters Corporation, Milford, MA) with a mobile phase for catecholamine analysis (Chromosystems, Munich, Germany). The peaks of dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were analyzed using an ESA Coulochem III electrochemical detector and integrated using a commercially available software program (ChemStation software, Agilent Technologies, Santa Clara, CA). All samples were normalized for protein content as spectrophotometrically determined using the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA).

Statistical analysis

All values are expressed as the mean ± standard error of the mean. Multiple comparisons among groups were performed with one-way analysis of variance (ANOVA) followed by Tukey’s *post hoc* tests. All statistical analyses were performed using Sigma Stat software (Systat Software, San Leandro, CA).

RESULTS

Protection of DA neurons by silibinin treatment against pKr-2-induced neurotoxicity

To examine the protective effects of silibinin on the nigrostriatal DA projections against pKr-2-induced neurotoxicity, we unilaterally injected pKr-2 into the SN of the mouse brain at 1 day after intraperitoneal administration of silibinin (50, 100, or 200 mg/kg/day; Fig. 1A). The brains were harvested at 7 days after pKr-2 treatment, and then the immunostaining for TH, indicating DA neurons, was performed on coronal sections including the SN or striatum. The mice treated with pKr-2 alone showed an apparent loss of TH-positive neurons and fibers in the SN and striatum, respectively (Fig. 1B). However, silibinin treatment prevented the loss in both SN and striatum of pKr-2-treated mice (Fig. 1B). As the quantitative results show, there was a significant degeneration of TH-positive neurons and fibers following pKr-2 treatment (Fig. 1C, D; $^{**}p < 0.001$ vs. control), and silibinin treatment at 200 mg/kg significantly attenuated the loss of both TH-positive neurons and fibers compared to pKr-2 alone (Fig. 1C, D; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. pKr-2 alone). Silibinin treatment at 50 or 100 mg/kg also showed protective patterns in both the SN and striatum against pKr-2 toxicity, but the effects were not significant (Fig. 1C).

Preservation of dopamine and its metabolites by silibinin treatment in the striatum of pKr-2-treated mice

In addition to the histological protection, we further investigated the levels of dopamine and its metabolites, DOPAC and HVA, in the striatum using reversed-phase HPLC with an electrochemical detector. Similar to our previous report,¹⁴ mice treated with pKr-2 showed a significant reduction in the levels of dopamine, DOPAC, and HVA in the striatum, by 57%

($**p < 0.01$), 39% ($*p < 0.05$), and 34% ($**p < 0.01$), respectively, compared to control mice (Fig. 2A). In contrast, the levels of dopamine, DOPAC, and HVA in the striatum of silibinin-treated mice were preserved by 85% ($^{##}p < 0.01$), 102% ($^{#}p < 0.05$), and 108% ($^{###}p < 0.01$), respectively, compared to control mice (Fig. 2A). There were no significant changes in the basal levels of dopamine and its metabolites in the striatum of silibinin-treated mice (Fig. 2B). To examine whether the protected dopaminergic system following silibinin treatment could contribute to the preservation of locomotor activity from pKr-2-induced neurodegeneration, we measured the alteration of mouse behaviors using the open-field test *in vivo*. Consistent with the HPLC results, our observations revealed that treatment with pKr-2 alone induced a significant hypo-locomotor activity compared to the moving distance per time of control mice (Fig. 2C, D; $**p < 0.01$). However, treatment with 200 mg/kg of silibinin significantly improved locomotor activity compared to the pKr-2-treated mice (Fig. 2C, D; $^{#}p < 0.05$).

Anti-inflammatory effects of silibinin in the SN treated with pKr-2

To examine whether silibinin treatment could inhibit microglial activation and reduce the production of activated microglia-derived pro-inflammatory cytokines, indicating a protective mechanism of silibinin against pKr-2-induced neurotoxicity, we performed the double-immunofluorescence staining for microglia and pro-inflammatory cytokines, such as TNF- α , and IL-1 β , and western blotting for the levels of those proteins, respectively, 1 day after injection of pKr-2 into the SN (Fig. 3). Immunofluorescence staining showed that silibinin treatment did not induce any change in basal TNF- α (Fig. 3A). However, silibinin administration attenuated the increase in the expression of TNF- α as well as in the number of activated microglia following pKr-2 treatment (Fig. 3A). The alteration of IL-1 β expression was similar to that in TNF- α levels following silibinin or pKr-2 injection (data not shown).

Consistent with the immunofluorescence staining results, western blotting showed that the levels of TNF- α , IL-1 β , and Iba1 were significantly increased in the pKr-2-treated mice compared with control mice (Fig. 3B; * $p < 0.05$, ** $p < 0.01$). However, silibinin treatment significantly reduced the expression of TNF- α , IL-1 β , and Iba1 compared with pKr-2 treated mice (Fig. 3B; # $p < 0.05$, ## $p < 0.01$). No significant changes in TH levels were observed in any group at 1 day after injection of pKr-2 into the SN, suggesting that pKr-2 induced the occurrence of microglial activation before lesion of DA neurons in the pKr-2-treated SN *in vivo*.¹⁴

DISCUSSION

Prothrombin, synthesized mainly in the liver and then secreted into the bloodstream, is cleaved to produce fragment 1-2 (kringle regions) and thrombin during activation, and activated thrombin, a serine protease that converts soluble fibrinogen into insoluble fibrin for blood coagulation, can cleave pKr-1 and -2.^{1,14} In addition to the accumulation of prothrombin and thrombin, which is suspected to occur due to blood–brain barrier leakage, in both PD and AD brains,^{1,14} we recently reported that a significant increase in pKr-2 expression was also observed in the postmortem brain of patients with PD,^{1,14} suggesting that the relationship between pKr-2 upregulation and the pathogenesis of PD might be clinically relevant.

Activated microglia-induced neuroinflammation has been considered a major contributor to progressive neuronal loss in neurodegenerative diseases, such as PD and AD.^{1,14} Recently, we reported that upregulation of pKr-2, which has been proposed as an endogenous molecule for microglia activation with no direct neurotoxicity, induced the progressive degeneration of the nigrostriatal DA system through the overexpression of activated microglia-derived inflammatory cytokines such as TNF- α and IL-1 β *in vivo*. These findings suggest that the control of pKr-2-induced microglial activation may be helpful to reduce the loss of DA neurons under neurotoxic inflammation in the adult brain, and that the development and utilization of a potential bio-molecule inhibiting pKr-2-induced neurotoxic events can be useful to prevent neurodegeneration in the adult brain, which could be applied in PD.

Flavonoids are one of the largest families of phenolic compounds and are widely found in foods and beverages of plant origin.¹³ In general, the bioavailability of flavonoids is low due to poor absorption, rapid excretion, and the extensive biotransformation and conjugation that occurs during their absorption from the intestine, in the liver, and in cells.¹³ However, despite

their low bioavailability, extensive studies have reported that many flavonoids can provide numerous health benefits, including a reduction in the risk of PD, due to their biologic activity including anti-oxidative and anti-inflammatory properties.¹³ These reports show the intense interest in the potential of flavonoids to modulate neuronal function and prevent neurodegeneration from neurotoxic conditions in the adult brain.

Silibinin, a flavonoid derived from milk thistle seeds, can protect neurons against age-dependent neurodegenerative diseases^{10,11,20} and ischemic stroke,¹³ and its administration has been shown to induce anti-inflammatory activity through inhibiting microglial activation, resulting in the reduction of pro-inflammatory cytokines, in various models of inflammation *in vivo* and *in vitro*.²¹⁻²³ We also reported recently that silibinin treatment attenuated the expression of pro-inflammatory cytokines such as TNF- α and IL-1 β , resulting in neuroprotective effects in an MPP⁺-treated animal model of PD and a kainic acid-induced animal model of epileptic seizure,^{9,24} suggesting that it might have the potential to inhibit neurotoxic inflammation in the nigrostriatal DA system *in vivo*.

In the present study, we examined whether silibinin treatment could protect the nigrostriatal DA system from neurotoxicity induced by pKr-2 treatment in the SN of the mouse brain, and whether its administration could attenuate the pKr-2-induced microglial activation. Our results show that silibinin treatment significantly reduced the degeneration of the nigrostriatal DA projections following injection of pKr-2 (Fig. 1), suggesting the potential of silibinin as a protective molecule against pKr-2-induced neurotoxicity. Consistent with the preservation of the nigrostriatal DA projections, silibinin treatment apparently preserved striatal dopamine and its metabolites as well as locomotor function against pKr-2-induced neurotoxicity (Fig. 2). Moreover, our observations show that silibinin treatment could attenuate pKr-2-induced microglial activation in the SN, resulting in the down-regulation of TNF- α and IL-1 β (Fig. 3).

1
2
3
4 Altogether, our results show that silibinin could inhibit neurotoxic inflammatory responses
5
6 elicited by pKr-2-induced microglial activation, consequently resulting in the protection of
7
8 the nigrostriatal DA projections *in vivo* (Fig. 4). Therefore, we conclude that silibinin is a
9
10 natural product that is beneficial in the protection of the nigrostriatal DA system from
11
12 neurotoxic conditions induced by pKr-2 upregulation, and that its neuroprotective effects
13
14 through anti-inflammatory activity may be usefully applied to prevent neurodegeneration in
15
16 PD.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ACKNOWLEDGMENTS

This study was supported by grants from the National Research Foundation of Korea (NRF-2014R1A1A2056508 and 2017R1A2B4002675), the Korea Health Technology R&D Project of the Ministry of Health & Welfare (HI14C1135), and the KBRI Research Program of the Ministry of Science, ICT & Future Planning (18-BR-02-06) funded by the Korean government.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

REFERENCES

1. Kim SR, Chung ES, Bok E, *et al.*: Prothrombin kringle-2 induces death of mesencephalic dopaminergic neurons in vivo and in vitro via microglial activation. *J Neurosci Res.* 2010;88(7):1537-1548.

2. Leem E, Jeong KH, Won SY, Shin WH, Kim SR: Prothrombin Kringle-2: A Potential Inflammatory Pathogen in the Parkinsonian Dopaminergic System. *Exp Neurobiol.* 2016;25(4):147-155.

3. Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K: Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res.* 2004;318(1):121-134.

4. Jankovic J: Parkinson's disease: clinical features and diagnosis. *J Neurol Neurosurg Psychiatry.* 2008;79(4):368-376.

5. Antony PM, Diederich NJ, Kruger R, Balling R: The hallmarks of Parkinson's disease. *FEBS J.* 2013;280(23):5981-5993.

6. Saiki S, Sato S, Hattori N: Molecular pathogenesis of Parkinson's disease: update. *J Neurol Neurosurg Psychiatry.* 2012;83(4):430-436.

7. Dheen ST, Kaur C, Ling EA: Microglial activation and its implications in the brain diseases. *Curr Med Chem.* 2007;14(11):1189-1197.

8. Wang Q, Liu Y, Zhou J: Neuroinflammation in Parkinson's disease and its potential as therapeutic target. *Transl Neurodegener.* 2015;4:19.

9. Jung UJ, Jeon MT, Choi MS, Kim SR: Silibinin attenuates MPP(+)-induced neurotoxicity in the substantia nigra in vivo. *J Med Food.* 2014;17(5):599-605.

10. Lee Y, Park HR, Chun HJ, Lee J: Silibinin prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease via mitochondrial stabilization. *J Neurosci Res.* 2015;93(5):755-765.

11. Lu P, Mamiya T, Lu LL, *et al.*: Silibinin prevents amyloid beta peptide-induced memory impairment and oxidative stress in mice. *Br J Pharmacol.* 2009;157(7):1270-1277.
12. Marrazzo G, Bosco P, La Delia F, *et al.*: Neuroprotective effect of silibinin in diabetic mice. *Neurosci Lett.* 2011;504(3):252-256.
13. Wang M, Li YJ, Ding Y, *et al.*: Silibinin Prevents Autophagic Cell Death upon Oxidative Stress in Cortical Neurons and Cerebral Ischemia-Reperfusion Injury. *Mol Neurobiol.* 2016;53(2):932-943.
14. Shin WH, Jeon MT, Leem E, *et al.*: Induction of microglial toll-like receptor 4 by prothrombin kringle-2: a potential pathogenic mechanism in Parkinson's disease. *Sci Rep.* 2015;5:14764.
15. Paxinos G, Franklin KBJ: *The mouse brain in stereotaxic coordinates.* (Compact 2nd ed.) Elsevier Academic Press, Boston, MA, USA, 2004.
16. Kim BW, Jeong KH, Kim JH, *et al.*: Pathogenic Upregulation of Glial Lipocalin-2 in the Parkinsonian Dopaminergic System. *J Neurosci.* 2016;36(20):5608-5622.
17. Kim SR, Kareva T, Yarygina O, Kholodilov N, Burke RE: AAV transduction of dopamine neurons with constitutively active Rheb protects from neurodegeneration and mediates axon regrowth. *Mol Ther.* 2012;20(2):275-286.
18. Nam JH, Leem E, Jeon MT, *et al.*: Induction of GDNF and BDNF by hRheb(S16H) transduction of SNpc neurons: neuroprotective mechanisms of hRheb(S16H) in a model of Parkinson's disease. *Mol Neurobiol.* 2015;51(2):487-499.
19. Kim HD, Jeong KH, Jung UJ, Kim SR: Naringin treatment induces neuroprotective effects in a mouse model of Parkinson's disease in vivo, but not enough to restore the lesioned dopaminergic system. *J Nutr Biochem.* 2016;28:140-146.
20. Song X, Zhou B, Cui L, *et al.*: Silibinin ameliorates Abeta25-35-induced memory deficits in rats by modulating autophagy and attenuating neuroinflammation as well as

oxidative stress. *Neurochem Res.* 2017;42(4):1073-1083.

21. Fernandes V, Sharma D, Kalia K, Tiwari V: Neuroprotective Effects of Silibinin: An In-silico and In-vitro Study. *Int J Neurosci.* 2018:1-26.

22. Wang MJ, Lin WW, Chen HL, *et al.*: Silymarin protects dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activation. *Eur J Neurosci.* 2002;16(11):2103-2112.

23. Song J-H, Kim K-J, Lee B-Y: Silibinin Suppresses Mediators of Inflammation through the Inhibition of TLR4-TAK1 Pathway in LPS-induced RAW264.7 Cells. *Journal of Food and Nutrition Research.* 2016;4(8):515-521.

24. Kim S, Jung UJ, Oh YS, *et al.*: Beneficial Effects of Silibinin Against Kainic Acid-induced Neurotoxicity in the Hippocampus in vivo. *Exp Neurobiol.* 2017;26(5):266-277.

FIGURE LEGENDS

Fig. 1. Preservation of the nigrostriatal DA projections by silibinin treatment against pKr-2-induced neurotoxicity.

(A) Experimental schematic for effects of silibinin against pKr-2-induced neurotoxicity in the nigrostriatal DA system. Mice received a daily intraperitoneal injection of silibinin (50, 100, or 200 mg/kg/day), starting 1 day before pKr-2 treatment into the SN, and were sacrificed at the indicated time points according to the experimental purpose. (B) Representative coronal sections of the SN and the striatum (STR) stained with anti-TH at 7 days after pKr-2 treatment. Scale bar, 200 μ m in the left panels (SN) and 1,000 μ m in the right panels (STR), respectively. (C) The number of TH-positive neurons in the SN. $^{**}p < 0.001$ and $^{##}p < 0.01$ vs. CON and pKr-2 alone, respectively (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). (D) The optical densities of the striatal TH-positive fibers. $^{**}p < 0.01$ and $^{#}p < 0.05$ vs. CON and pKr-2 alone, respectively (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). CON, control.

Fig. 2. Effects of silibinin on the functional protection of the nigrostriatal DA system *in vivo*.

(A, B) The levels of striatal dopamine and its metabolites, which were measured with HPLC, were quantitatively expressed as a percentage of control. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. CON; $^{#}p < 0.05$ and $^{##}p < 0.01$ vs. pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). (C, D) Locomotor activity was measured with the open-field test. $^{**}p < 0.01$ and $^{#}p < 0.05$ vs. CON and pKr-2 alone, respectively (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). CON, control.

Fig. 3. Anti-inflammatory effects of silibinin in the pKr-2-treated SN.

(A) Representative double immunofluorescence staining for anti-Iba1 (green) and anti-TNF- α (red) in the SN of mice at 1 day after pKr-2 treatment. Scale bar, 20 μ m. (B) Western blotting to measure the levels of TH, TNF- α , IL-1 β , and Iba1 in the SN. * p < 0.05 and ** p < 0.01 vs. CON; # p < 0.05 and ### p < 0.01 vs. pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test; n = 4 for each group). CON, control; SNpc, Substantia nigra pars compacta; SNr, Substantia nigra reticulate.

Fig. 4. Schematic representation of silibinin against pKr-2-induced neurotoxic inflammation.

The increase in prothrombin kringle-2 (pKr-2), an endogenous microglial activator, induces microglial activation, resulting in neurodegeneration through inflammatory responses such as the production of pro-inflammatory cytokines, in the nigrostriatal DA system of adult brain (upper schematic). However, its neurotoxic inflammation can be significantly inhibited by silibinin administration, consequently resulting in the protection of the nigrostriatal DA projections *in vivo* (lower schematic). Thus, these observations suggest that silibinin is a beneficial natural compound offering anti-inflammatory effects against pKr-2-induced neurotoxicity in the nigrostriatal DA system.

Fig. 1

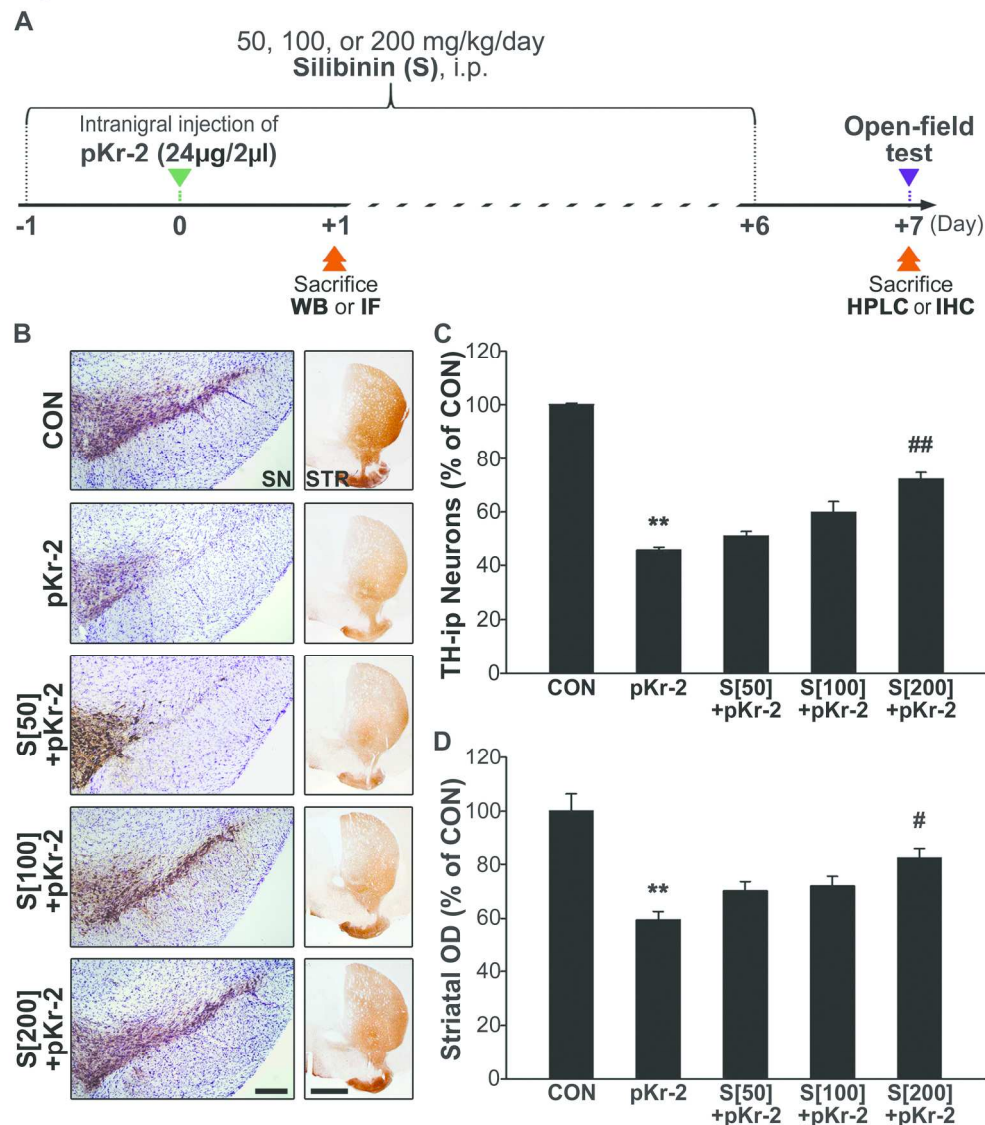


Fig. 1. Preservation of the nigrostriatal DA projections by silibinin treatment against pKr-2-induced neurotoxicity.

(A) Experimental schematic for effects of silibinin against pKr-2-induced neurotoxicity in the nigrostriatal DA system. Mice received a daily intraperitoneal injection of silibinin (50, 100, or 200 mg/kg/day), starting 1 day before pKr-2 treatment into the SN, and were sacrificed at the indicated time points according to the experimental purpose. (B) Representative coronal sections of the SN and the striatum (STR) stained with anti-TH at 7 days after pKr-2 treatment. Scale bar, 200 μ m in the left panels (SN) and 1,000 μ m in the right panels (STR), respectively. (C) The number of TH-positive neurons in the SN. ** $p < 0.001$ and ## $p < 0.01$ vs. CON and pKr-2 alone, respectively (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). (D) The optical densities of the striatal TH-positive fibers. ** $p < 0.01$ and # $p < 0.05$ vs. CON and pKr-2 alone, respectively (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). CON, control.

189x225mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

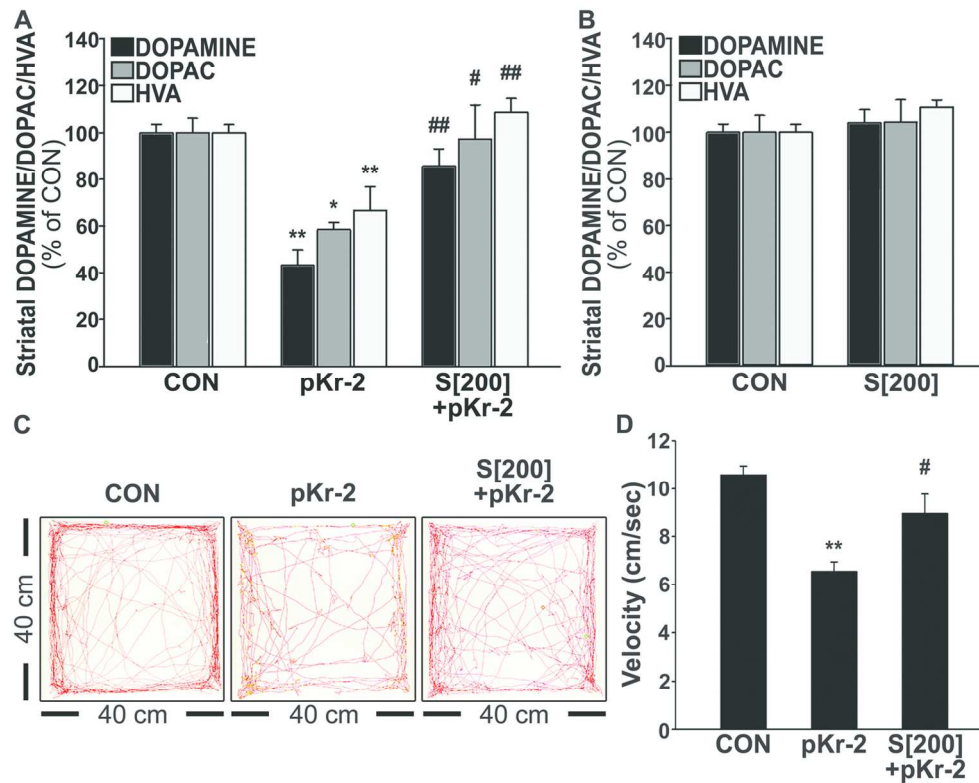
Fig. 2

Fig. 2. Effects of silibinin on the functional protection of the nigrostriatal DA system in vivo. **(A, B)** The levels of striatal dopamine and its metabolites, which were measured with HPLC, were quantitatively expressed as a percentage of control. * $p < 0.05$ and ** $p < 0.01$ vs. CON; # $p < 0.05$ and ## $p < 0.01$ vs. pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). **(C, D)** Locomotor activity was measured with the open-field test. ** $p < 0.01$ and # $p < 0.05$ vs. CON and pKr-2 alone, respectively (one-way ANOVA with Tukey's *post hoc* test; $n = 4$ for each group). CON, control.

138x114mm (300 x 300 DPI)

Fig. 3

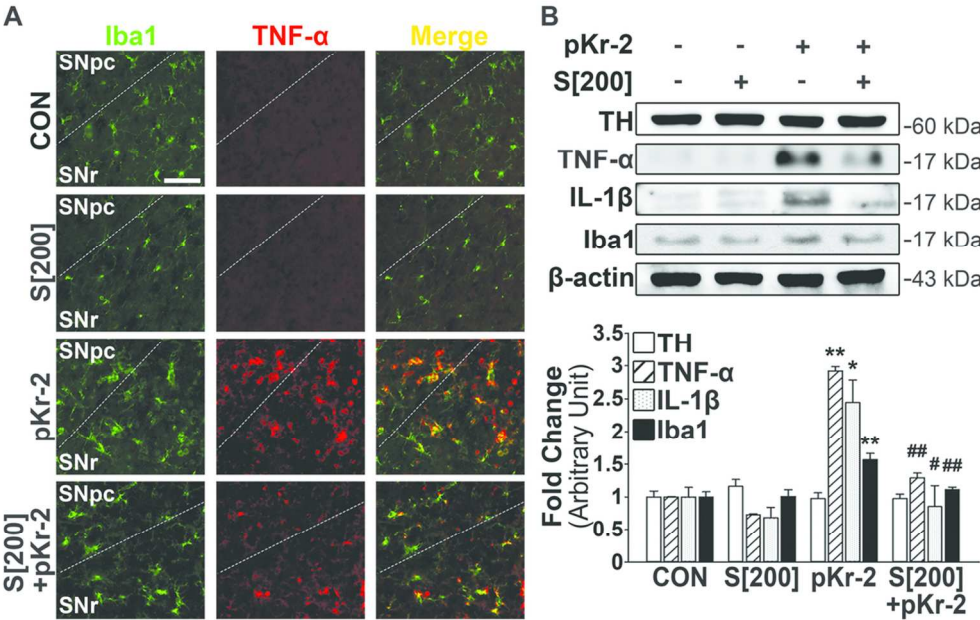


Fig. 3. Anti-inflammatory effects of silibinin in the pKr-2-treated SN. **(A)** Representative double immunofluorescence staining for anti-Iba1 (green) and anti-TNF-α (red) in the SN of mice at 1 day after pKr-2 treatment. Scale bar, 20 μm. **(B)** Western blotting to measure the levels of TH, TNF-α, IL-1β, and Iba1 in the SN. **p* < 0.05 and ***p* < 0.01 vs. CON; #*p* < 0.05 and ##*p* < 0.01 vs. pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test; *n* = 4 for each group). CON, control; SNpc, Substantia nigra pars compacta; SNr, Substantia nigra reticulata.

106x70mm (300 x 300 DPI)

Fig. 4

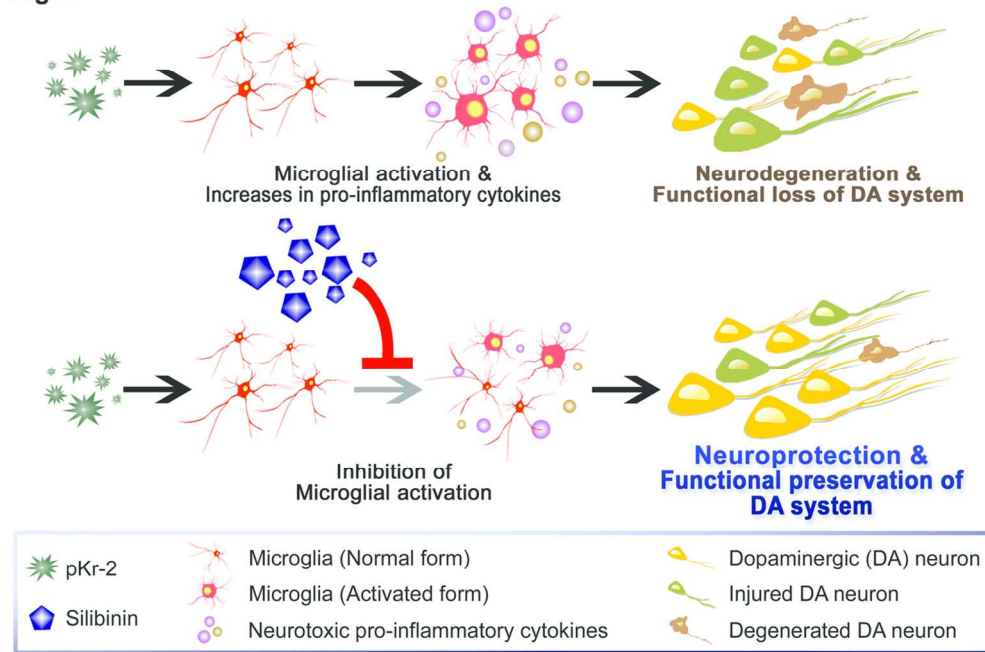


Fig. 4. Schematic representation of silibinin against pKr-2-induced neurotoxic inflammation.

The increase in prothrombin kringle-2 (pKr-2), an endogenous microglial activator, induces microglial activation, resulting in neurodegeneration through inflammatory responses such as the production of pro-inflammatory cytokines, in the nigrostriatal DA system of adult brain (upper schematic). However, its neurotoxic inflammation can be significantly inhibited by silibinin administration, consequently resulting in the protection of the nigrostriatal DA projections *in vivo* (lower schematic). Thus, these observations suggest that silibinin is a beneficial natural compound offering anti-inflammatory effects against pKr-2-induced neurotoxicity in the nigrostriatal DA system.

115x80mm (300 x 300 DPI)