Review

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Scientific evidence for therapeutic effects of Chinese prescription Kangen-karyu from pre-clinical animal experiments

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Summary **Chinese prescription Kangen-karyu, comprised of six crude drugs, has received much attention due to its numerous biological activities. The present study reports therapeutic evidence for Kangen-karyu from pre-clinical animal experiments related to human diseases. Kangen-karyu showed beneficial effects on type 1 diabetes and related complications through the suppression of protein expression related to advanced glycation endproducts and oxidative stress. Kangen-karyu reduced oxidative stress *via* the regulation of dyslipidemia, and also exerted a renoprotective effect mainly through its antioxidant properties during the development of diabetic nephropathy in type 2 diabetes. In addition, Kangen-karyu showed neuroprotective effects by attenuating the spatial memory impairment and neuronal death induced by diabetes. Kangen-karyu counteracted oxidative stress and ameliorated tissue damage possibly associated with aging. These findings provide scientific evidence to explain the efficacy of Kangen-karyu based on its underlying therapeutic effects.**

Keywords: Kangen-karyu, diabetes, diabetic nephropathy, cognitive deficit, dementia, aging

1. Introduction

Traditional Chinese medicine has received much attention as a source of novel therapeutic agents due to their multiple beneficial effects and absence of toxic and/or side effects (*1*). Therapy in traditional Chinese medicine is aimed to correct maladjustments and restore the self-regulatory ability of the body (*2*). For example, dermatologic disease can be successfully cured with traditional Chinese medicine by improving "Ki" stagnation in the spleen, lung, and kidney. "Ki" is an intrinsic energy to maintain human health as well as to cure sickness (*3*). In addition, in traditional Chinese medicine, apparently distinct diseases (according to modern diagnostics) can share a common pattern and be treated with the same formula (*2*). Consequently, traditional Chinese medicine influences changes at multi-system and multi-organ levels. However, there has been virtually no attempt to logically analyze multi-target strategies in traditional Chinese medicine.

Kangen-karyu (Guan-Yuan-Ke-Li in Chinese), one of our major interests among traditional Chinese medicine agents, has been developed in Japan by the modification of herbal constituents of Kan-shin No. 2 (Guan-xin No. 2 in Chinese) (*4*). Kan-shin No. 2 was originally created following traditional Chinese medicine practice to cure blood stagnation, and it has been used to treat thrombosis, myocardial infarction, and cerebral infarction in China (*5*). Kangen-karyu consists of six herbs (Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma) (Figure 1), and has been clinically used as a treatment for cardiovascular diseases such as angina pectoris and cerebrovascular diseases (*6*). A typical high-performance liquid chromatogram of Kangen-karyu is given in Figure 2; lithospermic acid B, lithospermic acid, and rosmarinic acid derived from Salviae Miltiorrhizae Radix, and

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Figure 1. Crude drugs of Kangen-karyu.

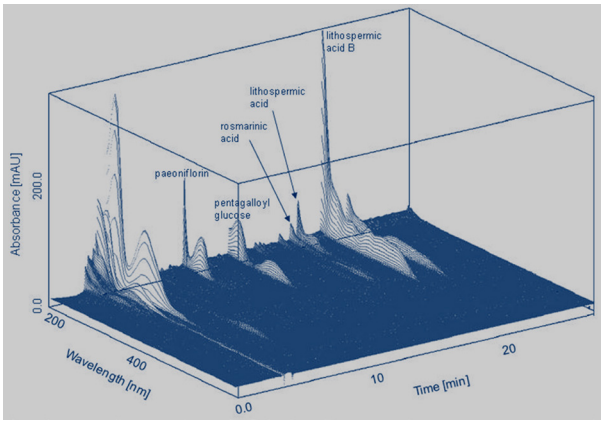

Figure 2. Three-dimentional HPLC of Kangen-karyu showing its major compounds.

paeoniflorin and pentagalloyl glucose derived from Paeoniae Radix are also detected. In this review, we have summarized the therapeutic evidence for Kangen-karyu from pre-clinical animal experiments related to human diseases. These pre-clinical experimental results provide scientific evidence that may explain the efficacy of traditional Chinese medicine at multi-organ levels and may also help to identify the common mechanism underlying therapeutic effects against distinct diseases.

2. Dysmetabolic syndrome in type 1 diabetes

Diabetes induced by streptozotocin (STZ) in animal models is associated with type 1, characterized by a loss of β cells of islets of Langerhans in the pancreas, leading to insulin deficiency. STZ-treated rats showed markedly increased serum glucose, triglyceride (TG), and total cholesterol levels. The elevated serum TG level was significantly reduced by oral administration of Kangen-karyu (50, 100, or 200 mg/kg body weight/day for 20 days) in a dose-dependent manner, whereas serum levels of glucose and total cholesterol were mildly affected. These results suggest that Kangen-karyu can prevent diabetic pathological conditions induced by hyperglycemia and hyperlipidemia in diabetes (*7*). In addition, Kangen-karyu dose-dependently lowered expression levels of N^{ε}-(carboxymethyl)lysine, one of the major components of advanced glycation endproducts (AGEs) closely associated with pathogenesis of diabetes and liver cirrhosis (*8,9*), and a receptor for AGEs, as well as the expression levels of nuclear factor-kappa B (NF-κB), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) associated with oxidative stress (*10*). Especially, thiobarbituric acid (TBA)-reactive substance levels in both serum and hepatic tissue and COX-2 expression increased by STZ were recovered by Kangen-karyu (200 mg/kg body weight) to normal levels. Collectively, Kangen-karyu showed beneficial effects on type 1 diabetes and related complications such as atherosclerosis, liver disease, as well as cardiovascular diseases (*7*).

3. Dysmetabolic syndrome in type 2 diabetes

Patients with type 2 diabetes often exhibit dyslipidemia and an increase of the TG content in the liver and skeletal muscle (*11,12*). In contrast to patients with insulin-deficient diabetes (type 1) who are in hypoleptinemic states, patients with type 2 diabetes often show increased adiposity and elevated leptin levels (*13-17*). We investigated the effects of Kangen-karyu on abnormal lipid metabolism in type 2 diabetic C57BLKS/J *db/db* mice (*18*). Male *db/db* mice were divided into 3 orally administered groups: vehicle (control), Kangen-karyu 100, or 200 mg/kg body weight/day. Age-matched non-diabetic *m/m* mice were used as the normal group. Serum TG and total cholesterol levels in *db/db* mice were increased compared with those of *m/m* mice. However, the administration of Kangen-karyu reduced hyperlipidemia in *db/db* mice through a decline in the serum levels of TG and total cholesterol. In addition, the markedly elevated serum TBA-reactive substance levels in *db/db* mice were significantly reduced by Kangen-karyu administration at a dose of 200 mg/kg body weight. The hepatic TG and total cholesterol levels of *db/db* mice were markedly higher than those of *m/m* mice, but these elevated lipid levels were significantly reduced by 200 mg/kg Kangen-karyu administration. Also, oil red O staining showed that the increased lipid deposition level in the liver of *db/db* control mice was improved by Kangen-karyu administration, as shown in Figure 3. Expression of sterol regulatory element-binding protein-1 in the liver of *db/db* mice was significantly down-regulated by the administration of Kangen-karyu at a dose of 200 mg/kg body weight. Kangen-karyu caused a slight elevation in the expression of peroxisome proliferator-activated receptor α in the liver of *db/db* mice. These results suggest that the administration of Kangen-karyu can improve liver dysfunction caused by abnormal lipid metabolism and oxidative stress in type 2 diabetic mice.

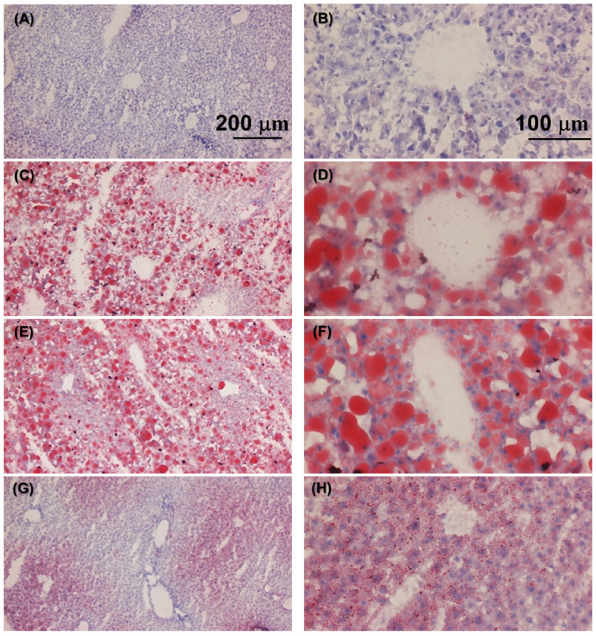

Figure 3. Oil red O staining of the liver. (A and B) Non-diabetic *m/m* mice; **(C and D)** vehicle-treated *db/db* mice; **(E and F)** Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; **(G and H)** Kangen-karyu 200 mg/kg body weight-treated *db/db* mice. Figures were taken from Yamabe *et al*. (*18*).

4. Diabetic nephropathy in type 2 diabetes

Diabetic nephropathy is one of the serious complications in patients with either type 1 or 2 diabetes mellitus. Multiple factors are involved in the pathogenesis of diabetic nephropathy, such as hyperglycemia, hypertension, hyperlipidemia, and oxidative stress (*19*). Hyperglycemia generates reactive oxygen species (ROS), which contribute to apoptosis in podocytes and mesangial and tubular cells. In fact, several researchers have demonstrated that ROS generation induced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the mitochondrial electron-transport chain is an early event in the development of diabetic renal disease (*20,21*). In addition, oxidative stress, in turn, activates different processes involving protein kinase C, NF-κB, cytokines, and others (*19*). Diabetic nephropathy also includes several important pathophysiological developments such as albuminuria, mesangial matrix expansion, glomerulosclerosis, glomerular and tubular hypertrophy and apoptosis, and extracellular matrix gene expression, as well as macrophage accumulation and activation (*22*). The present study was conducted to examine whether Kangen-karyu has an ameliorative effect on diabetes-induced alterations in the kidney of type 2 diabetic *db/db* mice. Kangen-karyu (100 or 200 mg/kg body weight) was administered for 18 days to *db/db* mice, and its effect was compared with vehicle-treated *db/db* and *m/m* mice. The administration of Kangen-karyu decreased the elevated serum glucose concentration in *db/db* mice, and reduced the increased oxidative biomarkers including the generation of ROS and lipid peroxidation in the serum and kidney. Increased serum creatinine and urea nitrogen levels, which reflect renal dysfunction, and renal structural changes, representing glomerular enlargement, in *db/db* mice were significantly lowered by Kangen-karyu administration. The *db/db* mice exhibited up-regulation of NADPH oxidase subunits, NF-κB, COX-2, and iNOS levels in the kidney; however, Kangen-karyu treatment significantly reduced those expressions. Moreover, augmented expression of apoptosis-related proteins, cytochrome *c* and Bax, were down-regulated by Kangen-karyu administration (*23*). Thus, these results provide important evidence that Kangen-karyu exhibited a pleiotropic effect on several oxidative stress-related parameters and exerted a renoprotective effect on the development of diabetic nephropathy in type 2 diabetic *db/db* mice (Figure 4).

5. Cognitive dysfunction in type 2 diabetes

Cognitive deficits such as Alzheimer's disease and vascular dementia involve a variety of risk factors such as aging, vascular disorders, and diabetes. Several lines of evidence suggest an association between cognitive deficits such as Alzheimer's disease and diabetes, and demonstrate that diabetes increases the risk of developing Alzheimer's disease several fold (*24*). About 80% of Alzheimer's disease patients appear to be diabetic or to have abnormal blood glucose levels and defects in insulin signaling that are associated with accumulation of the neurofibrillary tangles and senile plaques of Alzheimer's disease (*25*). Similar learning and memory deficits have been reported using *db/db* mice, an animal model of type 2 diabetes (*26*). This animal strain exhibits not only hyperglycemia and hyperinsulinemia but also impaired hippocampus-dependent cognitive performance and long-term potentiation. These deficits have been reported to become evident in adulthood at 10 weeks of age and over. We investigated the effect of Kangen-karyu on the water maze performance and expression levels of brain-derived neurotrophic factor (BDNF) and central cholinergic marker proteins such as choline acetyltransferase (ChAT) and muscarinic receptor subtypes (M_1, M_3, and M_5 receptors) of an animal model of *db/db* mice with a diabetic insult to clarify if Kangen-karyu can be used as an anti-dementia drug effective for diabetes-related cognitive deficits. Therefore, seven-week-old *db/db* (Y-*db/db*) mice received daily administration of Kangen-karyu for 12 weeks. At 18 weeks of age (O-*db/db*), the animals were given the water maze test. Compared with age-matched control strain mice (O-*m/m*), vehicle-treated O-*db/db* mice showed impaired learning and memory performance. Kangen-karyu (100 or 200 mg/kg body weight per day) ameliorated the performance of O-*db/db* mice without affecting their serum glucose level. O-*db/db* mice had lower levels of BDNF mRNA and its protein in the brain than O-*m/m* mice. Expression levels of central

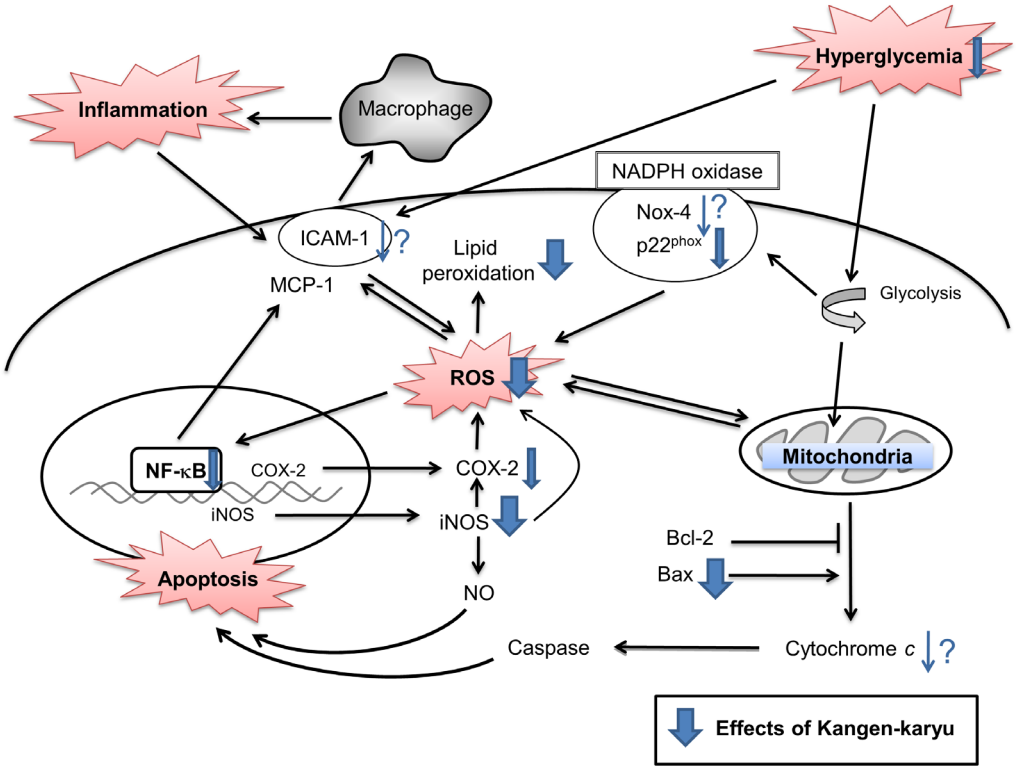

Figure 4. Possible mechanisms for the renoprotective effects of Kangen-karyu. Kangen-karyu moderated hyperglycemia and effectively attenuated oxidative stress including ROS and lipid peroxidation. Furthermore, Kangen-karyu suppressed the protein expression of $p22^{phox}$, one of the subunits of NADPH oxidase, NF-κB-targeting proinflammatory iNOS and COX-2, and pro-apoptotic Bax. Figure was taken from Park *et al*. (*23*).

cholinergic marker proteins in the hippocampus and the number of cholinergic cells in the medial septum and basal forebrain were also significantly lower in O-*db/db* than in O-*m/m* mice, whereas no significant differences in the expression levels of these factors and the cell number were found between Y-*m/m* and Y-*db/db* mice. Kangen-karyu treatment significantly reversed the down-regulated levels of cholinergic markers, the ChAT-positive cell number and BDNF expression, in *db/db* mice (*27*) (Figure 5). These findings suggest that Kangen-karyu prevents diabetes-induced cognitive deficits by attenuating the dysfunction of the central cholinergic system.

6. Reno-protective effect in aged rats

Many theories have been proposed to explain the aging process including the free radical theory (*28*), oxidative stress hypothesis of aging (*29*), the mitochondrial theory (*30*), and the molecular inflammation hypothesis (*31*). These are all specific to a particular cause of physiological changes occurring with aging. This study examined whether Kangen-karyu has a reno-protective effect on the age-related oxidative stress and inflammatory response through the phosphoinositide 3-kinase (PI3K)/Akt or mitogen-activated protein kinase (MAPK) pathways in aged rats. Administration of Kangen-karyu caused a slight decrease in the serum glucose level and a significant decrease in the serum insulin level in old rats. The increased levels of serum renal functional (urea nitrogen) and oxidative parameters were significantly reduced by Kangen-karyu. The old rats showed increased renal damage associated with expression of the PI3K/Akt, MAPK pathway-derived pro-inflammatory transcription factors (NF-κB and activator protein-1), and pro-inflammatory genes (COX-2, iNOS, and tumor necrosis factor-α). However, these unfavorable outcomes were reversed by Kangen-karyu administration in old rats. Kangen-karyu treatment of old rats improved the overall renal function, such as serum urea nitrogen and morphological characteristics. In addition, the old rats exhibited a dysregulation of protein expression related to insulin resistance, oxidative stress, and inflammation in the kidney, but Kangen-karyu administration significantly reduced expression of inflammatory proteins through the PI3K/Akt pathway (*32*) (Figure 6). These results provide important evidence that Kangen-karyu has a pleiotropic effect on the PI3K/Akt and MAPK pathways, showing reno-protective effects against development of inflammation in old rats.

7. Anti-dementia effect in senescence-accelerated mice prone (SAMP8)

The brain is one of the most sensitive tissues to oxidative stress because of its high content of oxidized substrates such as polyunsaturated fatty acids and neurotransmitters. Nevertheless, ROS are constantly

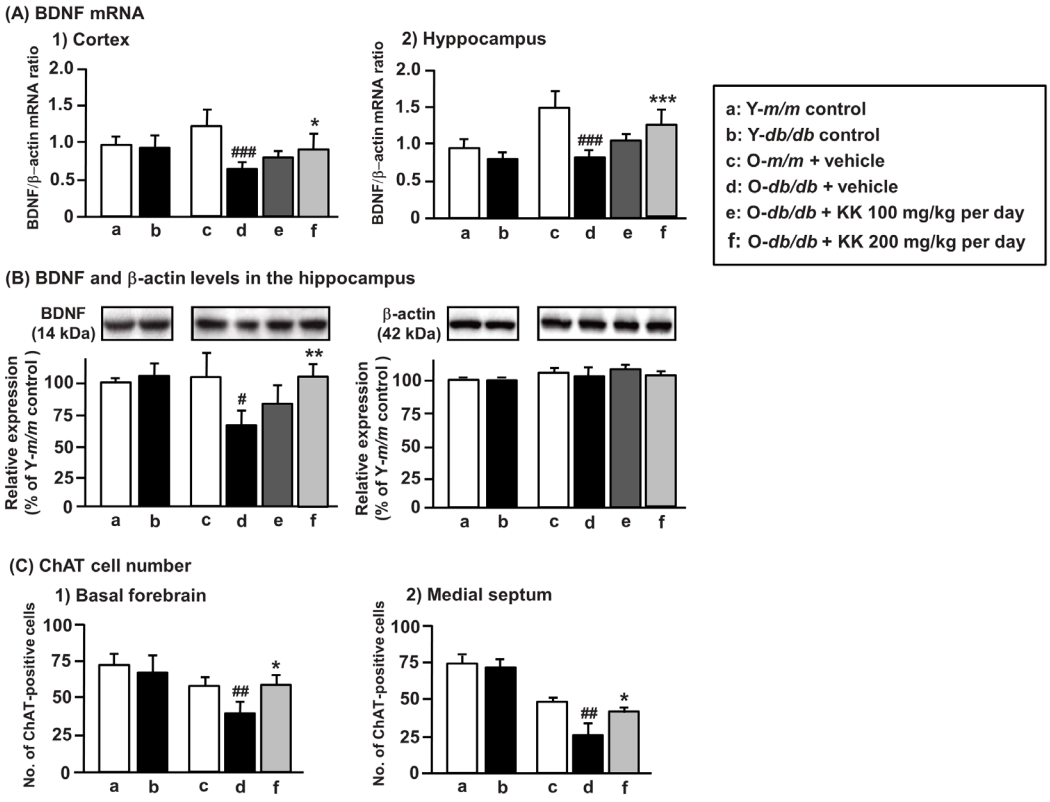

Figure 5. BDNF expression and ChAT cell number in the brain of *db/db* mice. (A) BDNF mRNA; **(B)** BDNF protein; **(C)** ChAT-positive cell number. Values are expressed as the mean ± SD of 4-5 mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs*. vehicle-treated O-*m/m* mice (*t*-test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. vehicle-treated O-*db/db* mice (one-way ANOVA). Figures were taken from Zhao *et al*. (*27*) and partially modified.

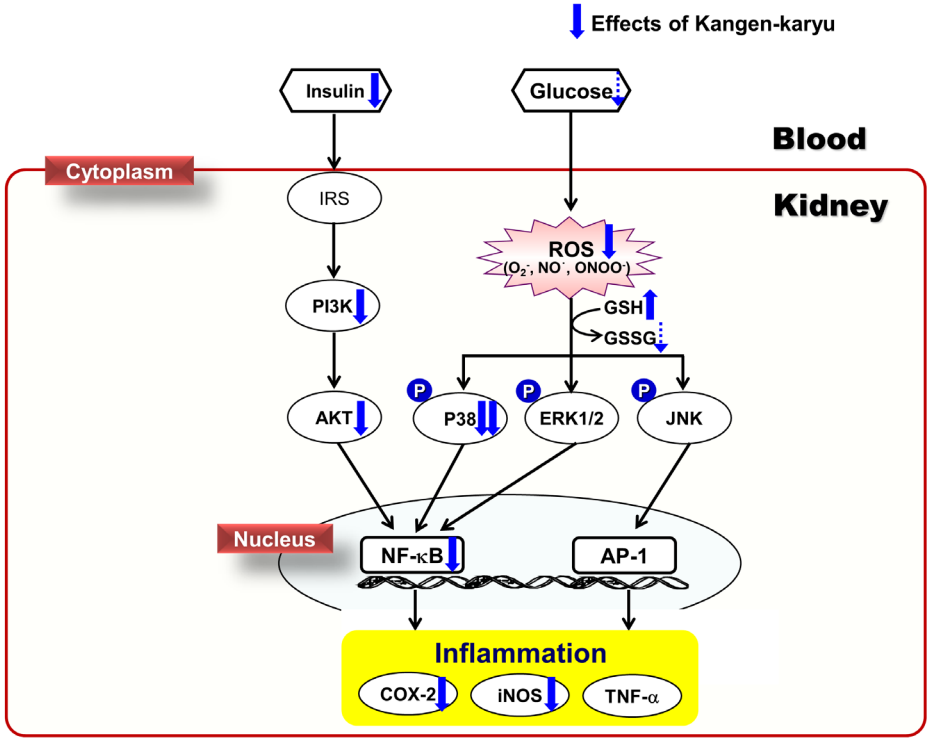

Figure 6. Possible mechanisms of Kangen-karyu in the kidney of old rats. The administration of Kangen-karyu caused a slight decrease in the serum glucose level and a significant decrease in the serum insulin level in the old rats. The increased oxidative parameters were reduced by Kangen-karyu. The old rats exhibited a dysregulation of the protein expression related to insulin resistance, oxidative stress, and inflammation in the kidney, but Kangen-karyu administration significantly reduced the expression of the inflammatory proteins through the PI3K/Akt pathway. Figure was taken from Park *et al*. (*32*).

produced through its high consumption of oxygen for energy metabolism and also the metabolism of neurotransmitter molecules (*33*). Thus, oxidative stress-induced neuronal damage and cell death play a critical role in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease (*34,35*). The nucleus basalis magnocellularis-lesioned rat is considered to be a model of the cholinergic dysfunction observed in the cerebral cortices of patients with Alzheimer's disease. The cholinergic markers, acetylcholine release and ChAT activity, were decreased in the cerebral cortex of the nucleus basalis magnocellularis-lesioned rat. Orally administered Kangen-karyu (125 mg/rat, twice a day for 2 days) following nucleus basalis magnocellularis-lesioning (injection of ibotenic acid) significantly preserved the cholinergic markers. These results suggest that Kangen-karyu preserves the activity of cholinergic neurons in the cerebral cortex following nucleus basalis magnocellularis-lesioning (*36*). The anti-dementia effect of Kangen-karyu on aging-induced

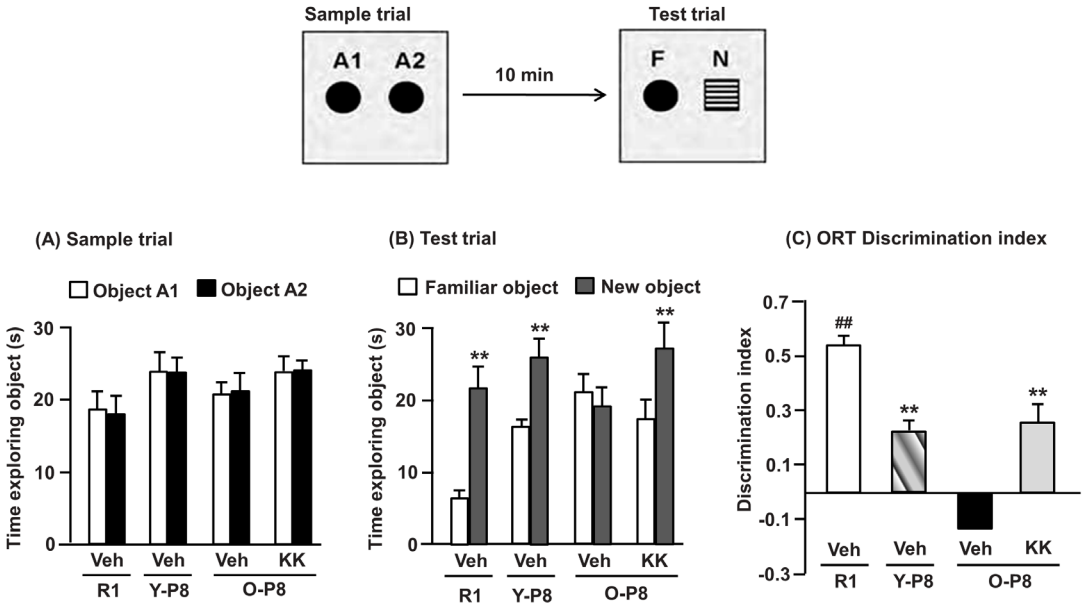

Figure 7. Effects of Kangen-karyu on aging-induced cognitive deficits using object recognition test (ORT). Values are expressed as the mean ± SEM of 5-8 mice. **(B)** $^{**}p < 0.01$ *vs*. time spent exploring a familiar object (paired t-test). **(C)** $^{\#\#}p < 0.01$ *vs*. vehicle-treated O-P8 group (*t*-test). $^{**}p < 0.01$ *vs*. vehicle-treated O-P8 group (Student-Newman-Keuls test). Figures were taken from Zhao *et al*. (*36*).

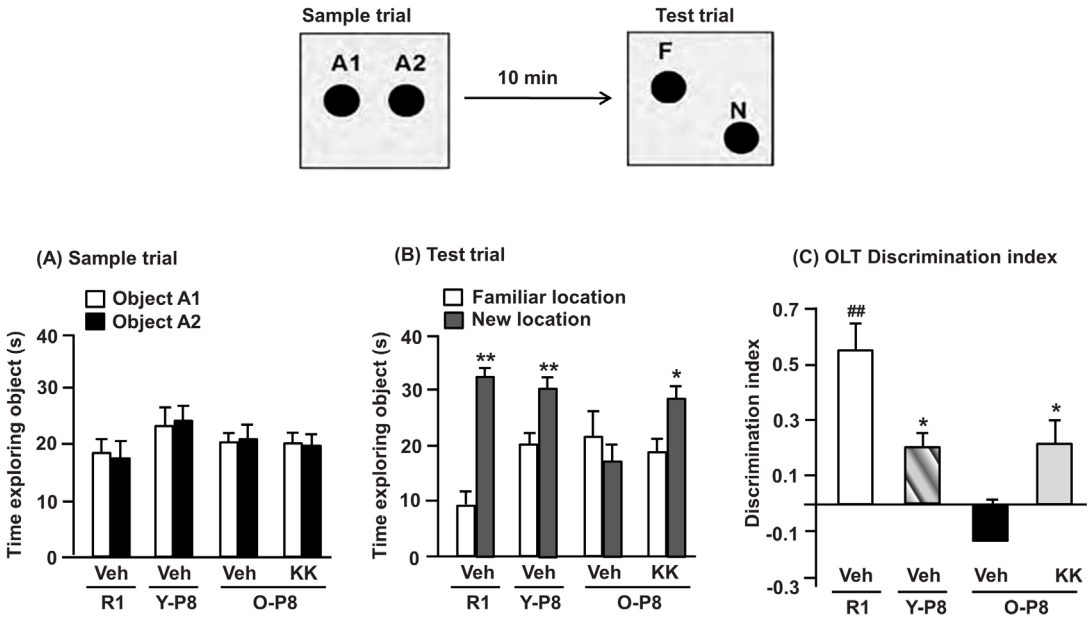

Figure 8. Effects of Kangen-karyu on aging-induced cognitive deficits using object location test (OLT). Values are expressed as the mean ± SEM of 5-8 mice. **(B)** $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. respective time exploring the object placed in a familiar location (paired *t*-test). **(C)** $^{\#\#}p < 0.01$ *vs*. vehicle-treated O-P8 group (*t*-test). $^{*}p < 0.05$ *vs*. vehicle-treated O-P8 group (Student-Newman-Keuls test). Figures were taken from Zhao *et al*. (*36*).

cognitive deficits and its mechanism was also examined in SAMP8. Twenty-week-old SAMP8 (older SAMP8) were used as an animal model of aging, and an age-matched senescence-resistant inbred strain (SAMR1) and 8-week-old SAMP8 (young SAMP8) were used as controls. Older SAMP8 received an oral administration of Kangen-karyu daily (100 mg/kg body weight) or water vehicle for 22 days. Compared with the controls, older SAMP8 exhibited cognitive deficits in object recognition and object location tests; however, Kangen-karyu improved the deficits caused by aging, as shown in Figures 7 and 8. The levels of biochemical factors related to neuro-plasticity and learning and memory, *i.e*., phosphorylated forms of *N*-methyl-D-aspartate receptor 1, Ca^{2+}/calmodulin-dependent protein kinase II, and cAMP-responsive element-binding protein, and brain-derived neurotrophic factor, were significantly decreased in older SAMP8 compared with those in the control animals, but Kangen-karyu normalized the levels of these factors. Moreover, mRNA and protein levels of vascular endothelial growth factor (VEGF) and its receptor type 2 in the cerebral cortices of older SAMP8 were down-regulated by aging, but these levels were reversed by Kangen-karyu administration (*36*). These findings suggest that the normalization of neuro-plasticity-related neuronal signaling and VEGF systems in the brain may be one of the mechanisms underlying the ameliorative effects of Kangen-karyu on cognitive deficits in older SAMP8.

8. Drug interaction

Extensive studies on the interactions between modern drugs and herbal medicines have been conducted, and predictable adverse effects must be avoided (*37*). Warfarin and ticlopidine hydrochloride have long been used as anticoagulants to prevent thrombosis and embolism (*38-40*). Patients taking these agents are monitored by measuring the prothrombin time to achieve the desired anticoagulant effect and minimize the risk of bleeding (*41*). In the studies of Makino *et al*. (*4,42*), pharmacological interactions between Kangen-karyu and warfarin or ticlopidine hydrochloride were assessed by measuring tail-bleeding time using normal mice. Warfarin or ticlopidine hydrochloride alone significantly prolonged tail-bleeding time, which was further prolonged, significantly, by the combination of Kangen-karyu at a dose that did not cause pharmacokinetic interactions with warfarin or ticlopidine hydrochloride. Therefore, in the combined therapy using Kangen-karyu and warfarin or ticlopidine hydrochloride to prevent thrombosis, a synergistic action of these drugs (that is, the effect of an anti-coagulant and anti-platelet) was expected. In the synergistic effect between Kangen-karyu and wafarin or ticlopidine hydrochloride, the anti-thrombotic effect would be augmented, although adverse effects such as a tendency toward hemorrhage might occur. Physicians are expected to consider the value of combined therapy and regulate the dosage of both medicines to prevent such adverse effects.

9. Conclusion and perspective

In this review, we investigated the multi-target therapeutic effects of traditional Chinese medicine on several human diseases using pre-clinical animal experiments. First, Kangen-karyu showed favorable effects on hypertriglyceridemia, AGE formation, and oxidative stress in STZ-treated rats, suggesting beneficial effects on type 1 diabetes, diabetic hepatopathy, and liver diseases. Second, Kangen-karyu may improve oxidative stress *via* regulation of dyslipidemia in the *db/db* type 2 diabetic mice model, and it also exhibited a pleiotropic effect on several oxidative stress-related parameters and exerted a renoprotective effect on development of diabetic nephropathy in *db/db* mice. Furthermore, type 2 diabetic *db/db* mice exhibited severe cognitive deficits and degeneration of the basal forebrain cholinergic complexes; however, Kangen-Karyu attenuated diabetes-related cognitive deficits and cholinergic dysfunction. Third, Kangen-karyu counteracted oxidative stress and ameliorated tissue damage possibly associated with aging. Finally, Kangen-karyu exhibited neuroprotective effects by preventing spatial memory impairment and neuronal death induced by aging. Taken together, therapeutic effects of Kangen-karyu at multi-system and multi-organ levels are closely related to maintenance of the self-regulatory ability in the body. Therefore, use of Kangen-karyu as a multi-target agent is warranted, when a predictable drug interaction with anticoagulants exists.

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