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Review

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Silkworm fungal infection model for identification of virulence genes in pathogenic fungus and screening of novel antifungal drugs

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Summary **The silkworm infection model has the potential to replace conventional animal models for evaluation of the efficacy and toxicity of investigational antifungal agents. Silkworms are relatively inexpensive, can be simply grown in large numbers and can be easily infected with pathogenic fungi, including mutant strains. Antifungal agents can then be injected into the silkworm either *via* the hemolymph to mimic intravenous administration or directly into the gut for oral administration, and their antifungal effect can be evaluated. Common features regarding the mechanisms of pharmacokinetics between the silkworm and mammals result in consistent therapeutic effectiveness of antifungal agents. ASP2397, a promising new antifungal agent, was discovered using the silkworm model. The conclusion is that silkworms can be a more ethical and less expensive alternative to standard animal models, particularly for the identification and testing of new antifungal agents.**

Keywords: Silkworm, fungal infection model, antifungal agents, virulence factors, drug development

1. Introduction

Pathogenic fungi can cause serious deep mycosis, such as pneumonia, in humans. Patients with weakened immune functions, such as those suffering from leukemia or acquired immune deficiency syndrome (AIDS), or those under treatment with immunosuppressive therapy, are predisposed to fungal infections. There are four classes of therapeutic agents for deep mycosis: polyenes, azoles, echinocandins, and fluoropyrimidines. There are limitations in the practical application of these agents in the clinic due to their known adverse effects and antifungal activity. Therefore, the development of novel antifungal agents for deep mycosis is desired.

Animal models mimicking infectious diseases in human are used to understand virulence of pathogenic microorganisms and to evaluate therapeutic effects of drug candidates. Mice and rats have been used as fungal infection models (*1*). Sacrificing a large number of mammals for infection experiments incurs not only a high cost but can also raise ethical issues with respect to animal welfare. To solve these problems, we are proposing use of the silkworm as an animal model of infectious diseases (*2-5*). The silkworm is an insect whose breeding method has been well developed during a long history of sericulture. The cost needed to breed silkworms is relatively low, and the use of insects like silkworms avoids ethical concerns that arise when vertebrate animals are used for research. Therefore, we can easily conduct experiments using a large number of silkworms. Moreover, studies using silkworms are relatively straightforward because they move very slowly and their size is larger as compared to other invertebrate animal models such as the fruit fly or nematode. By using syringes, one can inject accurate volumes of samples containing pathogens or candidate agents into the body fluid of silkworms. Furthermore, injection of the samples into the hemolymph or the gut can be distinguished in silkworms. The former corresponds to intravenous injection in humans, and the latter to oral administration.

The silkworm infection model of *Staphylococcus aureus* has already been shown to be useful for

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screening mutant strains with low virulence (*3,4*). The mutant strains of *S. aureus* that had low killing ability against silkworms also showed low virulence against mice. Therefore, the silkworm infection model may be useful for understanding common mechanisms of bacterial virulence between silkworms and mice, and possibly humans.

The silkworm shares common mechanisms of drug metabolism with mammals. Namely, chemicals incorporated in the silkworm body are modified by hydroxylation of the first phase reactions by cytochrome P450s followed by the second phase reactions of conjugation to highly water-soluble substances to be excreted. We demonstrated the process by using 7-ethoxycoumarin, which is generally used as a model compound to study drug metabolism in mammals (*6*). The ED_{50} values, *i.e.*, the amount of reagent needed for therapeutic effects on half the population of animals, which indicates the therapeutic effect of antibiotics, in the infection model of silkworms, were consistent with the values in mammals (*7*). The LD_{50} values, *i.e.*, the amount of reagent needed for killing half the population of animals, which indicates toxicity of chemicals, were also consistent between silkworms and mammals (*8*). Therefore, the silkworm infection model can be used to evaluate both the therapeutic effects and toxicity of candidate chemicals under consideration as anti-infective agents. By using the *S. aureus* infection model of silkworms, we recently discovered a novel antibiotic, lysocin E (*9*) suggesting the usefulness of silkworms in the discovery of novel antibiotics.

The above-mentioned results suggest that the silkworm may be appropriate for screening genes responsible for virulence and novel therapeutic agents against other pathogenic microorganisms. In this review, we describe recent progress in the study of silkworm infection models for pathogenic fungi.

2. Silkworm fungal infection model

Silkworms die within a few days after injection with *Aspergillus fumigatus*, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, or *Cryptococcus neoformans* (*7,10-13*). Heat treatment (121°C, 15 min) of the fungi eliminates the killing ability of *C. albicans* or *C. neoformans* (*12,14*). Killing of the silkworms by *C. neoformans* infection is greatly influenced by temperatures. At 37°C, *C. neoformans* grows in the body of silkworms resulting in a killing effect, whereas at 27°C, the fungus does not kill silkworms. The capsule thickness and the overall size of the cell of *C. neoformans* increase in the silkworm hemolymph at 37°C, where pathogenicity of *C. neoformans* is exhibited, whereas apparent morphological changes are not observed at 27°C (*12*). The capsule has been shown to be necessary for pathogenicity of *C. neoformans* against mammals. Taken together, these findings suggest that fungal infection models of the silkworm are useful to understand the mechanisms of pathogenicity of fungi against mammals, including humans.

2.1. *Identification of fungal genes responsible for pathogenicity to silkworms*

To understand the molecular mechanism of fungal virulence, genetic approaches may provide useful information. To achieve this, we have attempted to isolate mutant strains the lack pathogenicity against silkworms. Silkworms are of an appropriate size for injection by syringes with needles, such that accurate volumes of sample can be injected into the hemolymph of silkworms (Figure 1). Pathogenicity of mutants can be demonstrated quantitatively by determination of the ED_{50}, *i.e.*, the number of cells needed to kill 50% of the population of silkworms. Using this information, one can identify genes responsible for the virulence of pathogens. So far, more than 10 mutants of *C. albicans*, *C. glabrata*, or *C. neoformans* were found to have low pathogenicity against silkworms (*15*).

Calcineurin complex CMP1 (also called CNA1), a serine/threonine protein kinase of *C. albicans*, and protein kinases SIT4 and YVH1 have been reported to be required for virulence against mammalian animals (*16-19*). Injection experiments of mutants whose genes encode these protein kinases were artificially disrupted and showed reduced pathogenicities compared to the wild strain (*10*). Pathogenicity against the silkworm of a disrupted strain of the PTC1 gene encoding another protein kinase also was shown to be decreased (*10*). Pathogenicity of the disrupted mutant of the PTC1 gene identified by using the silkworm model has also

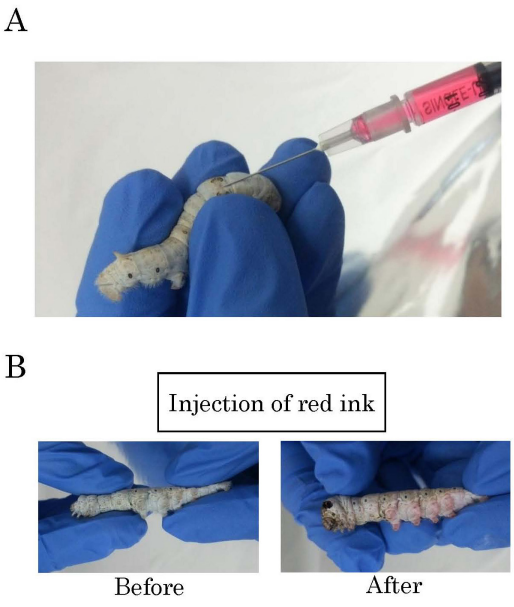

Figure 1. Injection method of liquid into silkworm. (A) Injection of sample into silkworm hemolymph using Syringe. **(B)** Color of silkworm legs (left) change to red (right).

been reported to be decreased in mice (*10*). The results that genes which are pathogenic against mice are also pathogenic against silkworms suggest that the silkworm model of infection by *C. albicans* is useful for screening virulence factors.

We reported that silkworms ingesting a high glucose diet showed the symptoms corresponding to diabetes in mammals (*20,21*). We screened *C. glabrata* mutant strains that have low pathogenicity against the diabetic model of silkworms (*11*). As a result, we found that the *cyb2* gene was needed for pathogenicity against diabetic silkworms (Figure 2). Mutants of the *hap2* and *hap5* genes in which the RNA level of the *cyb2* gene is low also showed low pathogenicity against diabetic silkworms. The *cyb2* gene encodes a protein that has 65% homology with lactate dehydrogenase in *Saccharomyces cerevisiae*, which was found to be an adaptation factor for survival in the intestine. A deficient strain of the *cyb2* gene in infection of the gastrointestinal tract using a diabetic murine model showed decreased adaptation in the mouse cecum (*11*).

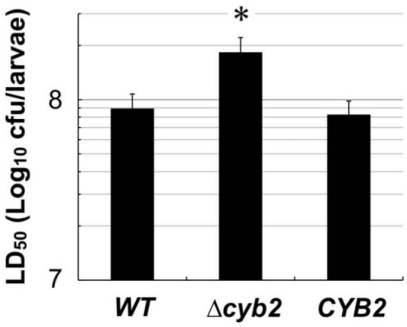

Figure 2. The virulence of *C. glabrata* Δ*cyb2* strain is attenuated in infection model of silkworm. $N = 10$. Asterisks: $p = 0.05$ with Student *t*-test *vs*. *WT* or *CYB2*. *WT*; wild type strain, Δ*cyb2*: deletant strain, *CYB2*: revertant strain. Ueno K e*t al*., 2011.

These results suggest that the diabetic silkworm/fungal infection model is useful for screening genes needed for virulence of fungi against diabetes patients.

We found that strains deficient in the *can*, *gpa1*, or *pka1* genes, which are required for virulence in *C. neoformans* against mammals, also showed low pathogenicity against silkworms (*12*). The product of the *can* gene is considered to contribute to the pathogenicity in mammals *via* the calcineurin signaling pathway (*22*). The product of the *gpa1* gene, an α-subunit of G-protein, was shown to contribute to the capsule formation (*23*). Pka1 is a protein kinase that functions downstream of Gpa1, and is known to contribute to the capsular formation (*24*). These results suggest that the fungal infection model of silkworms contributes to understanding the virulence mechanism in *C. neoformans* on a molecular level.

2.2. *Evaluation of therapeutic effects of antifungal drugs and screening of novel antifungal drugs using a silkworm fungal infection model*

We evaluated the therapeutic effects in silkworms of antifungal agents that currently are used for clinical purposes (*7,12*). Killing effects of silkworms by *C. albicans* or *C. tropicalis* infection were abolished by the administration of sufficient amount of amphotericin B or fluconazole. The ED_{50} values were consistent with those in the mouse infection models (Table 1). Injection of amphotericin B, flucytosine, ketoconazole, and fluconazole into the hemolymph showed therapeutic effects against *C. neoformans* infection of the silkworm (Table 2). On the other hand, injection of amphotericin B into the midgut of the silkworm did not show a therapeutic effect, which suggests that it may not be absorbed in the intestinal tract (Table 2). This finding

Table 1. ED_{50} of antifungal agents in a silkworm-infection model with *C. tropicalis* or *C. albicans*. (Hamamoto H *et al.*, 2004)

Antifungal agents	True fungus	ED_{50} in silkworm (μg/g of larva)	MIC (μg/mL)	ED_{50} /MIC ratio in	
				Silkworm	Mouse
Amphotericin B	*C. tropicalis*	1.8	3.2	0.6	0.2
	C. albicans	4.1	1.6	2.6	1.3
Fluconazole	*C. tropicalis*	1.8	1.6	1.1	7.4
	C. albicans	1.8	0.4	4.5	8.6

Table 2. Therapeutic effects of antifungal agents on silkworm infection by *C. neoformans*. (Matsumoto Y *et al*., 2012)

Antifungal agents	MIC (μg/mL^{-1})	ED_{50} (μg of antifungal agent g^{-1} of larva) of drug administrated by i.h. or i.m.	
		i.h.	i.m.
Amphotericin B	4 ± 2	14 ± 10	> 250
Flucytosine	21 ± 7	6 ± 1	9 ± 7
Fluconazole	7 ± 6	2 ± 1	9 ± 3
Ketoconazole	0.1 ± 0.1	19 ± 2	14 ± 10
Micafungin	> 100	> 125	N.D.

i.h., intra hemolymph. i.m., intra midgut. N.D., not determined.

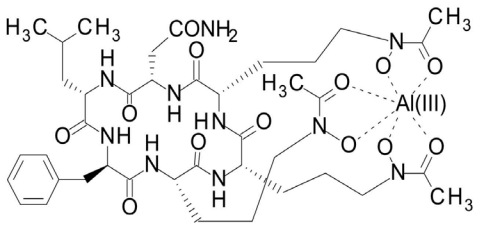

Figure 3. Structure of ASP2397. Nakamura I *et al*., 2016.

is in keeping with the fact that Amphotericin B is not absorbed in the intestinal tract in mammals, and therefore does not show a therapeutic effect when administered orally. We previously reported common features between silkworms and mammals regarding absorption capacity of various chemicals in the intestine (*25*). We therefore propose that the silkworm fungal infection model may be useful as an alternative method to assess the intestinal absorption of antifungal drugs.

2.3. *Discovery of a novel therapeutic agent against Aspergillosis infection using the silkworm fungal infection model*

We recently reported the discovery of a novel therapeutic agent using an *Aspergillus fumigatus* infection model of silkworms (*13*). *A. fumigatus* killed silkworms 2 days after injection. Amphotericin B and voriconazole showed therapeutic effects in the model. Screening of natural products derived from fungal species allowed us to identify ASP2397 (Figure 3), which showed a therapeutic effect against *A. fumigatus* infection in silkworms. This compound was also therapeutically effective in a mouse infection model of *A. fumigatus*. Initially, *in vitro* antifungal activity was used as an indicator for purification of compounds from a crude extract of a culture supernatant of the fungi that produced the therapeutically effective antifungal. However, a purified fraction which exhibited antifungal activity did not show a therapeutic effect in the silkworm infection model. Therefore, we conducted further purification by monitoring the therapeutic effect in the silkworm model instead of antifungal activity in test tubes. Eventually, ASP2397 was purified and identified as a therapeutically effective compound. These results suggest that therapeutically effective antifungal drugs can be purified by monitoring the therapeutic effect in the silkworm infection model. ASP2397 is expected to demonstrate safety and effectiveness in nonclinical studies and human clinical trials.

3. Conclusion

The silkworm infection model is a promising new approach for the identification of new antifungal agents. This model was used to identify the promising new antifungal agent, ASP2397.

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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.

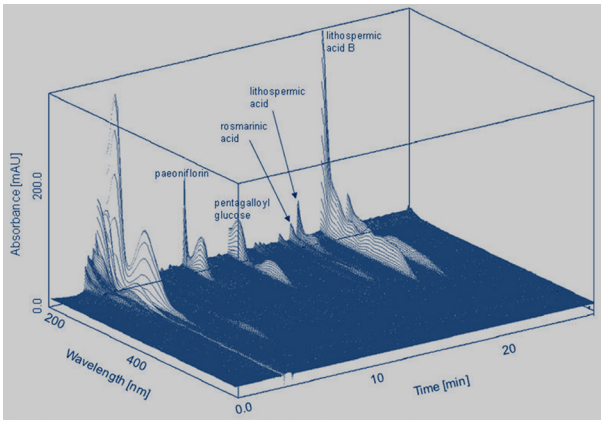

Figure 2. Three-dimentional HPLC of Kangen-karyu showing its major compounds.

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.

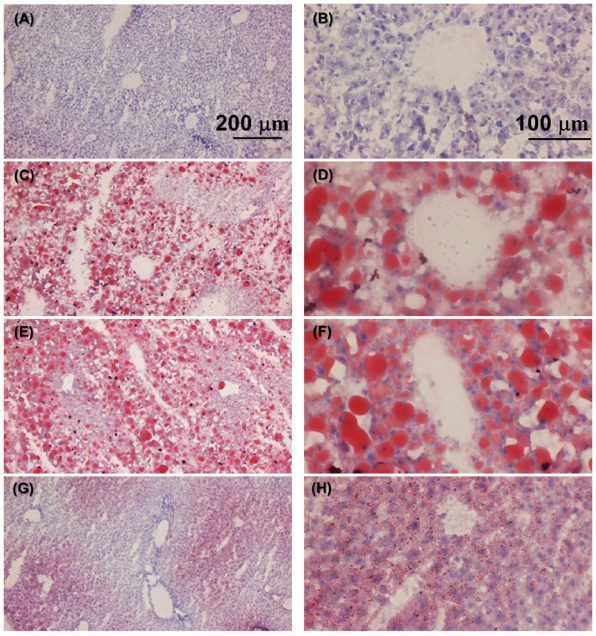

Figure 3. Oil red O staining of the liver. (A and B) Non-diabetic *m/m* mice; **(C and D)** vehicle-treated *db/db* mice; **(E and F)** Kangen-karyu 100 mg/kg body weight-treated *db/db* mice; **(G and H)** Kangen-karyu 200 mg/kg body weight-treated *db/db* mice. Figures were taken from Yamabe *et al*.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

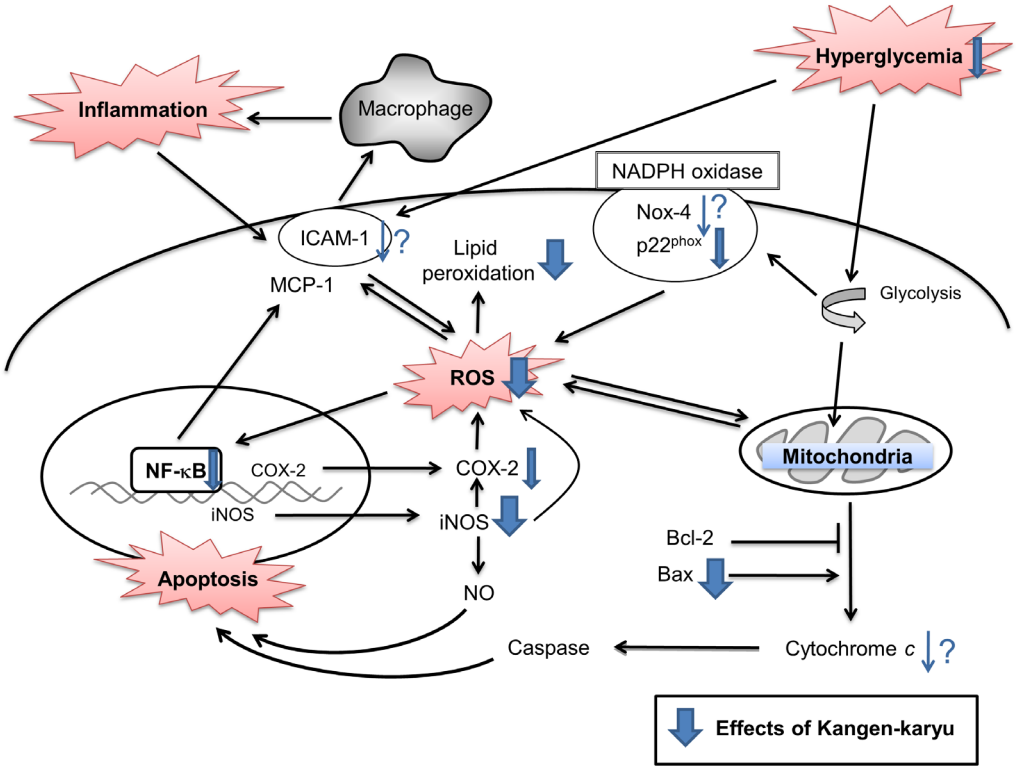

Figure 4. Possible mechanisms for the renoprotective effects of Kangen-karyu. Kangen-karyu moderated hyperglycemia and effectively attenuated oxidative stress including ROS and lipid peroxidation. Furthermore, Kangen-karyu suppressed the protein expression of $p22^{phox}$, one of the subunits of NADPH 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

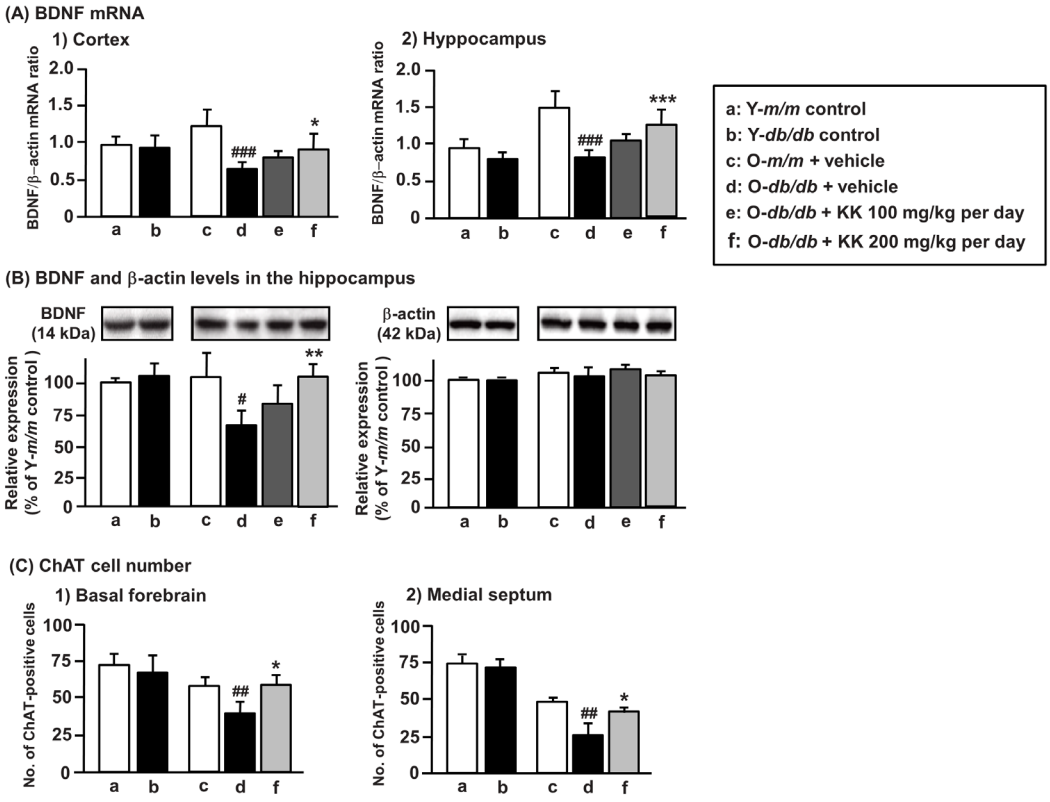

Figure 5. BDNF expression and ChAT cell number in the brain of *db/db* mice. (A) BDNF mRNA; **(B)** BDNF protein; **(C)** ChAT-positive cell number. Values are expressed as the mean ± SD of 4-5 mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs*. vehicle-treated O-*m/m* mice (*t*-test).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

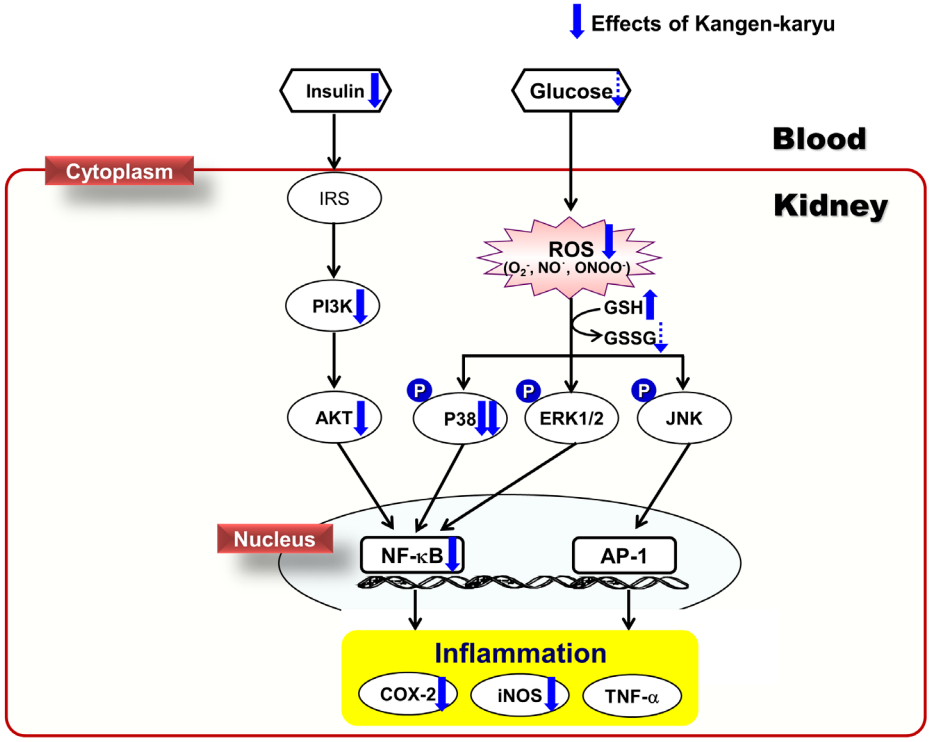

Figure 6. Possible mechanisms of Kangen-karyu in the kidney of old rats. The administration of Kangen-karyu caused a slight decrease in the serum glucose level and a significant decrease in the serum insulin level in the old rats. The increased oxidative parameters were reduced by Kangen-karyu. The old 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

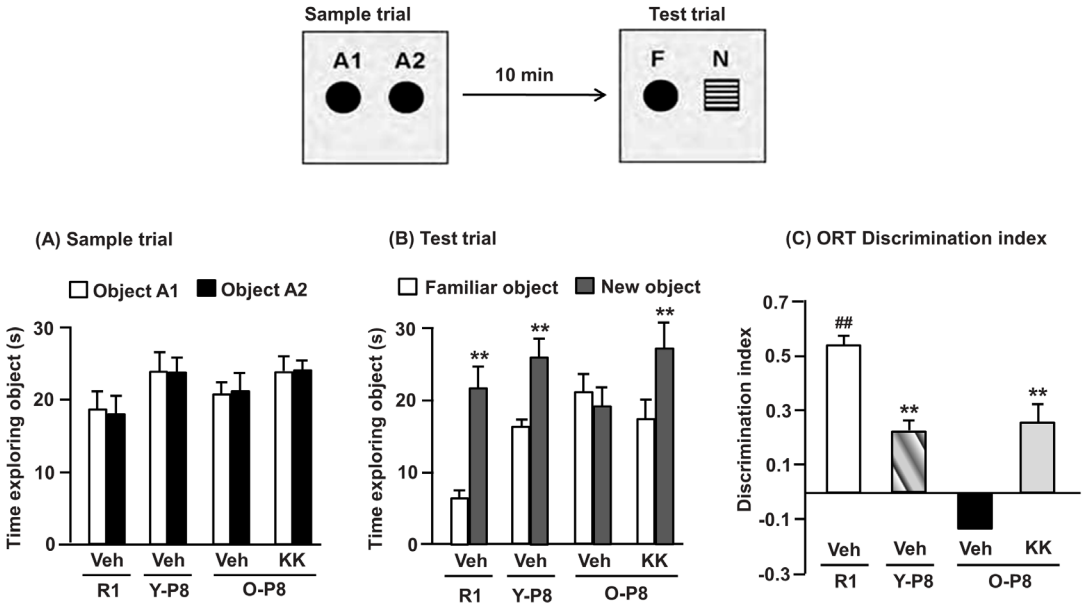

Figure 7. Effects of Kangen-karyu on aging-induced cognitive deficits using object recognition test (ORT). Values are expressed as the mean ± SEM of 5-8 mice. **(B)** $^{**}p < 0.01$ *vs*. time spent exploring a familiar object (paired t-test). **(C)** $^{\#\#}p < 0.01$ *vs*. vehicle-treated O-P8 group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

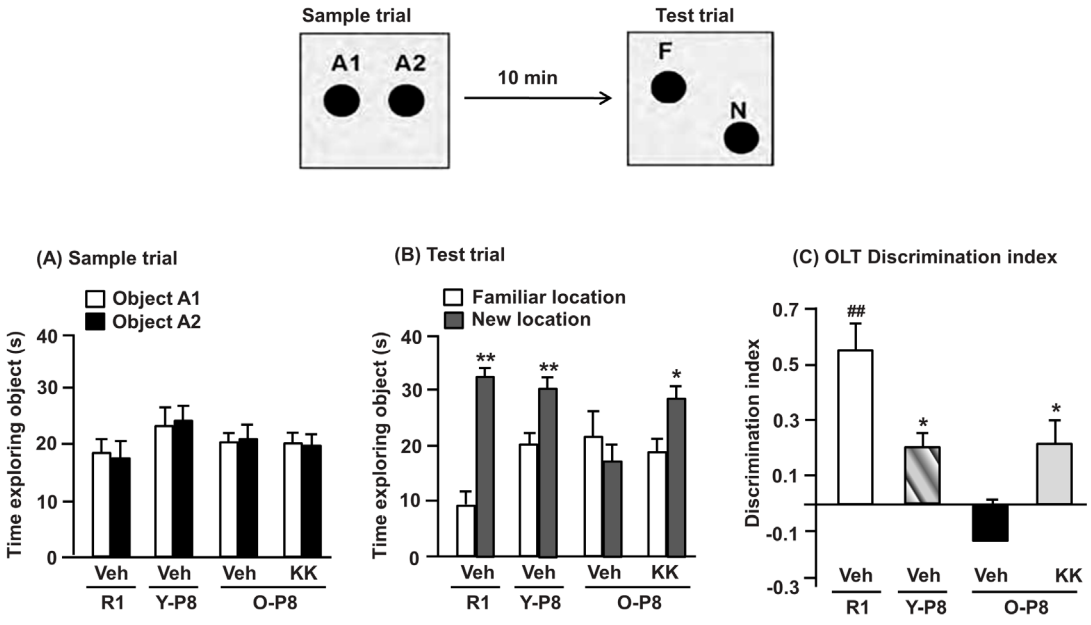

Figure 8. Effects of Kangen-karyu on aging-induced cognitive deficits using object location test (OLT). Values are expressed as the mean ± SEM of 5-8 mice. **(B)** $^{*}p < 0.05$, $^{**}p < 0.01$ *vs*. respective time exploring the object placed in a familiar location (paired *t*-test). **(C)** $^{\#\#}p < 0.01$ *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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Mini-Review

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The exercise paradox may be solved by measuring the overall thrombotic state using native blood

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Summary **While exercise is widely believed to prevent atherothrombotic diseases, it occasionally causes sudden death. This exercise paradox may be due to the inadequate testing of the thrombotic and thrombolytic status. A recently developed shear-induced thrombosis/endogenous fibrinolysis test performed with non-anticoagulated blood samples allows the assessment of the thrombotic state of an individual both at rest and after exercise. This sensitive and physiologically relevant test may help to solve the aforementioned exercise paradox.**

Keywords: Exercise, platelet reactivity, platelet aggregation, coagulation, fibrinolysis, non-anticoagulated blood

1. Introduction

Prevention of atherothrombotic diseases such as coronary artery disease and stroke is an important social issue. Evidence from epidemiological and clinical studies suggests that regular exercise is an efficient way to prevent such diseases. However, the benefit of exercise is still a subject of debate that is referred to as the "exercise paradox" or the "double-edged sword in exercise" (*1-5*).

Findings from studies that have sought to explain that paradox are a subject of debate (*6-10*). The intensity of exercise of endurance training may be responsible for this paradox according to the American College of Sports Medicine (Table 1) (*11*). Inconsistent evidence might be partly due to differences between the laboratory tests used to study the thrombotic status. Hemostasis tests in common use are performed on anticoagulated blood (*12-14*). Anticoagulants interfere with the mechanism of hemostasis and render the obtained results unphysiological. To overcome this limitation, some tests (shear-induced platelet-rich thrombosis and thrombolysis tests) use non-anticoagulated whole blood (*15-20*). The aim of the present review was to compare the effects of short-term and long-term exercise on the thrombotic state. These effects were measured with two types of tests using either anticoagulated or non-anticoagulated blood in order to understand the exercise paradox.

2. Measurement of the effects of acute and long-term exercise on the thrombotic/fibrinolytic state using anticoagulated blood

2.1. *Effect of acute exercise on platelet function*

Only a few studies have examined the effect of low-intensity exercise (< 49% maximal oxygen consumption, $\%\dot{V}O_{2max}$) on platelet reactivity. However, the effect of moderate-intensity exercise (50-74% $\dot{V}O_{2max}$) and heavy-intensity or strenuous exercise (> 75% $\dot{V}O_{2max}$) on platelet reactivity have been extensively studied. Studies using conventional tests have measured agonist-induced platelet aggregation, release of markers of platelet activation, such as β-thromboglobulin, platelet factor 4, and P-selectin, and an increase in metabolites such as thromboxane B_2 after moderate-intensity exercise (*21-24*). Those studies failed to yield conclusive results regarding the effect of moderate-intensity exercise on platelet reactivity.

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Table 1. Classification of the intensity of an endurance exercise

Classification of intensity	$\%\dot{V}O_{2max}$	%HRmax	RPE scale
Very light	< 30	< 35	< 10
Light	30-49	35-59	10-11
Moderate	50-74	60-79	12-13
Heavy	75-84	80-89	14-16
Very heavy	> 85	> 90	> 16

$\dot{V}O_{2max}$, maximal volume of oxygen consumed per minute; HR, heart rate; RPE, Borg rating of perceived exertion. (Modified from Mahler DA, Froelicher VF, Miller NH, York TD. General principles of exercise prescription. In: ACSM's Guidelines for Exercise Testing and Prescription, 5th ed., 1995).

Heavy-intensity exercise (> $75\%\dot{V}O_{2max}$) seems to enhance platelet reactivity.

2.2. *Effect of acute exercise on coagulation and fibrinolytic activity*

Moderate-intensity exercise is known to increase fibrinolytic activity without raising levels of coagulation activation markers, while heavy-intensity exercise concurrently activates both the fibrinolytic and coagulation system (*25*). Fibrinolytic activity is enhanced by exercise and the extent of that enhancement mainly depends on the intensity of exercise (*7,10,25-27*). In healthy volunteers and patients with peripheral arterial disease, coronary artery disease, metabolic syndrome, or hypertension, moderate-intensity exercise consistently increased tissue plasminogen activator (t-PA) activity (*28-33*) but not plasminogen activator inhibitor-1 (PAI-1) activity (*31,34*). Heavy-intensity exercise seems to enhance fibrinolytic activity along with coagulation (*25,35-37*).

2.3. *Effect of long-term exercise on coagulation and fibrinolytic activity*

Antithrombotic effects of regular or long-term exercise have been measured in healthy volunteers and patients of various ages. Studies have found that long-term exercise inhibited thrombin generation, reduced platelet reactivity and fibrin formation, and increased fibrinolytic activity (*28,38-41*). These results suggest that long-term exercise may help to prevent sudden death.

Because of individual variation, the thrombotic state of individuals needs to be measured before and during long-term exercise training. Since the thrombotic state is governed by a balance of and overall interaction between coagulation, fibrinolysis, platelet reactivity, and flow, the overall thrombotic state needs to be measured in individuals.

3. Effects of exercise on the thrombotic state assessed using non-anticoagulated blood

A wide variety of tests are used to measure the individual components of the hemostasis system, but their results are difficult to interpret. An overall test of hemostasis is greatly needed to assess the actual thrombotic state of an individual (*12-14*). Kovacs and her colleagues have focused on creating tests that can simultaneously measure platelet reactivity, coagulation, and endogenous thrombolytic (fibrinolytic) activity using one blood sample. Such a test, named the Global Thrombosis Test (GTT), is now commercially available. This test induces platelet-rich thrombus formation in non-anticoagulated (native) blood solely by shear forces, as opposed to conventional tests that use chemical agonists. Further, this test detects the spontaneous lysis of formed autologous thrombi in the same blood sample (*15-20*).

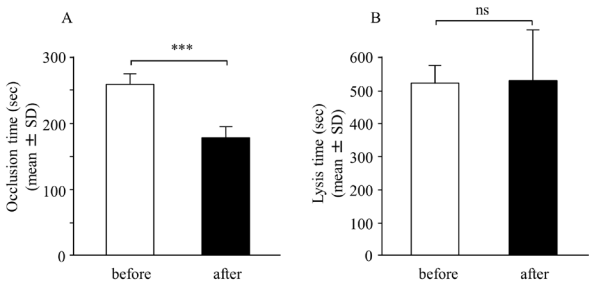

Figure 1. Effects of acute exercise on platelet reactivity (A) and fibrinolytic activity (B) as measured with a thrombotic status analyser. ***$p < 0.001$, ns: not significant. (Modified from Ikarugi *et al*. Thromb Res. 1997; 85:351-356).

3.1. *Effect of acute exercise*

In a study by the current authors, results from a thrombotic status analyser indicated that low-intensity exercise (50% $\dot{V}O_{2max}$, 40 min) does not affect platelet reactivity (*42*). In contrast, moderate-intensity exercise (60% $\dot{V}O_{2max}$, 20 min) significantly increased platelet reactivity but did not affect fibrinolytic activity (Figure 1) (*43*).

In another study by the current authors, results from a haemostatometer verified that the effects of exercise at different levels of intensity on the thrombotic status depended on the individual's anaerobic threshold (AT), platelet reactivity, and coagulation not only during exercise but also during the recovery period after exercise (*44*). A significant increase in platelet reactivity (H1) and coagulation (CT1) was observed immediately and

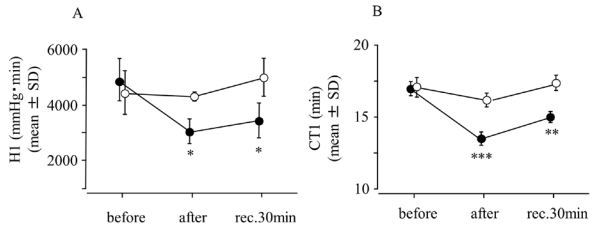

Figure 2. Effects of acute exercise on platelet reactivity (A: H1) and coagulation (B: CT1) as measured with a haemostatometer. Effects of low-intensity exercise (Ex-VT 90%; approximately 55%$\dot{V}O_{2max}$, 30 min) and high-intensity exercise (Ex-VT 130%; approximately 80%$\dot{V}O_{2max}$, 30 min) on platelet reactivity and coagulation; ○, Ex-VT 90%; ●, Ex-VT 130%; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. before. (Modified from Ikarugi *et al*. Pathophysiol Haemost and Thromb. 2003; 33:127-133).

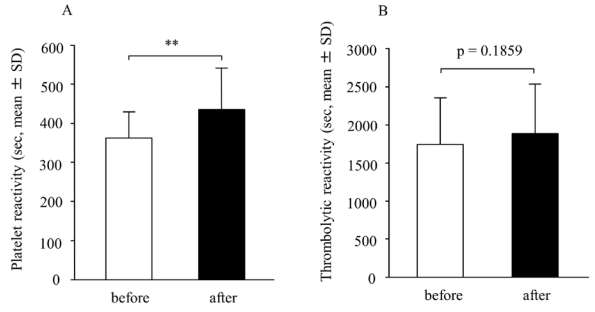

Figure 3. Effects of three months' exercise on platelet reactivity (A: OT) and endogenous fibrinolysis (B: LT) as measured with the Global Thrombosis Test. Effects in n = 30 patients with metabolic syndrome; **$p < 0.01$. (Modified from Baba Y. Master's thesis, Kobe Gakuin University, 2012).

30 minutes after heavy-intensity exercise (corresponding to approximately 80% $\dot{V}O_{2max}$), but such changes were not observed after moderate-intensity exercise (corresponding to approximately 55%$\dot{V}O_{2max}$) (Figure 2).

3.2. *Effect of long-term exercise*

Research used the GTT to measure the thrombotic state in patients with metabolic syndrome before and after three months' exercise (*45*). Results of that research are shown in Figure 3. A thrombotic state indicated by hyper-platelet reactivity before long-term exercise abated after exercise, but long-term exercise did not appear to affect endogenous fibrinolytic activity.

The thrombotic state was measured in healthy volunteers and patients under various conditions. The shear-induced thrombosis and thrombolysis tests were performed on non-anticoagulated blood (haemostatometer, thrombotic status analyser, and GTT). The thrombotic state was influenced by age (*46*), smoking (*46-48*), gender (*47*), and race (*49*). These findings corroborate the results of clinical studies indicating that physical exercise or sports helped to prevent a prothrombotic state and increased an individual's quality of life (*3,38,41,50*).

In conclusion, using the GTT and a non-anticoagulated blood sample to measure the thrombotic state at rest and immediately after exercise may help to solve the exercise paradox.

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Mini-Review

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Short-lived non-coding transcripts (SLiTs): Clues to regulatory long non-coding RNA

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Summary **Whole transcriptome analyses have revealed a large number of novel long non-coding RNAs (lncRNAs). Although the importance of lncRNAs has been documented in previous reports, the biological and physiological functions of lncRNAs remain largely unknown. The role of lncRNAs seems an elusive problem. Here, I propose a clue to the identification of regulatory lncRNAs. The key point is RNA half-life. RNAs with a long half-life ($t_{1/2}$ > 4 h) contain a significant proportion of ncRNAs, as well as mRNAs involved in housekeeping functions, whereas RNAs with a short half-life ($t_{1/2}$ < 4 h) include known regulatory ncRNAs and regulatory mRNAs. This novel class of ncRNAs with a short half-life can be categorized as Short-Lived non-coding Transcripts (SLiTs). I consider that SLiTs are likely to be rich in functionally uncharacterized regulatory RNAs. This review describes recent progress in research into SLiTs.**

Keywords: Non-coding RNA, RNA degradation, RNA decay, RNA-Seq, BRIC-Seq

1. Introduction

Recent transcriptome analyses have revealed thousands of intergenic, intronic, and cis-antisense long non-coding RNAs (lncRNAs) that are expressed from mammalian genomes. LncRNAs are defined as RNA molecules greater than 200 nucleotides in length that do not contain any apparent protein-coding potential (*1-4*). The majority of lncRNAs are transcribed by RNA polymerase II (Pol II), as evidenced by Pol II occupancy, 5' caps, histone modifications associated with Pol II transcriptional elongation, and polyadenylation (*5*). Although the importance of lncRNAs to processes such as transcriptional regulation, organization of nuclear structure, and post-transcriptional processing has been documented in previous reports (*2,6,7*), the biological and physiological functions of a great many lncRNAs remain largely unknown. Which lncRNA molecules should we target? Here, I present a clue to the selection of lncRNAs.

In 2012, two independent research groups reported that ncRNA half-lives vary over a wide range, and that they are comparable to those of mRNAs in mice and humans (*8,9*). Moreover, a genome-wide approach for determining RNA stability, which is called 5'-bromo-uridine (BrU) immunoprecipitation chase-deep assay (BRIC), or BRIC through deep sequencing (BRIC-Seq) (*9-11*), revealed that ncRNAs with short half-lives (RNA half-life $t_{1/2}$ < 4 h) included known regulatory ncRNAs, such as HOX transcript antisense RNA (HOTAIR), antisense noncoding RNA in the inhibitors of CDK4 locus (ANRIL)/CDKN2B antisense RNA 1 (CDKN2B-AS1), and growth arrest specific 5 (GAS5). BRIC-Seq has revealed 785 lncRNAs with short half-lives, termed Short-Lived non-coding Transcripts (SLiTs) (*9*), and I have selected 26 lncRNAs that are short-lived ($t_{1/2}$ < 4 h) in HeLa-Tet-off cells (Table 1), longer than 200 nt, and fulfill the established criteria for lncRNA classification. In this review, we will describe SLiTs identified in studies to date.

2. MIR4435-2HG_v1 and v2

Yang *et al*. reported that MIR4435-2 host gene (MIR4435-2HG), also known as AK001796, is up-

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Table 1. The 26 short-lived long ncRNAs that were investigated in this study

Name	Another name	Accession No.	Length (nt)	$t_{1/2}$*
MIR4435-2HG_v2	LINC0541471_v2	NR_024373	557	2.3
LOC100216545	KMT2E-AS1	NR_024586	3,615	2.3
FLJ43663	LINC-PINT	NR_015431	2,964	2.4
ANRIL	CDKN2B-AS1	NR_003529	3,857	2.4
FAM222A-AS1	-	NR_026661	1,178	2.4
LINC00473_v1	-	NR_026860	1,832	2.4
MIR22HG	-	NR_028502	2,699	2.4
LINC00473_v2	-	NR_026861	1,123	2.4
LINC00152	CYTOR	NR_024204	828	2.4
HOTAIR	-	NR_003716	2,337	2.5
MIR4435-2HG_v1	LINC0541471_v1	NR_015395	809	2.5
TTN-AS1	-	NR_038271	2,033	2.6
GAS5	-	NR_002578	651	2.6
SNHG15	-	NR_003697	837	2.6
LINC00662	-	NR_027301	2,085	2.7
HCG18	-	NR_024052	6,814	2.9
LOC728431	LINC01137	NR_038842	997	3.0
TUG1	-	NR_002323	7,115	3.2
LOC550112	UBA6-AS1	NR_015439	2,301	3.2
NEAT1_v1	-	NR_028272	3,756	3.3
LINC00667	-	NR_015389	3,979	3.4
GABPB-AS1	-	NR_024490	4,139	3.4
LINC01184	FLJ33630	NR_015360	2,977	3.5
ZFP91-CNTF	-	NR_024091	3,544	3.6
NEAT1_v2	-	NR_131012	22,743	3.7
IDI2-AS1	-	NR_024628	1,107	3.7

*These values are taken from a previous report (Tani 2012)

regulated and acts as an oncogene in lung cancer tissues and cell lines (*12*). In MIR4435-2HG knockdown experiments, cell proliferation and growth were reduced in lung cancer cells and tumorigenesis was slowed, and cell-cycle arrest was observed with increased numbers of cells in G0/G1. Moreover, they also found that MIR4435-2HG is downregulated in resveratrol-treated lung cancer cells (*12*).

3. ANRIL/CDKN2B-AS1

Two independent groups reported that ANRIL, also known as CDKN2B-AS1, associates with and recruits polycomb repression complex (PRC)-1 and PRC-2 on the INK4 locus to repress the transcription of p15 and p16 (*13,14*). Knockdown of ANRIL increases p15 and p16 expression, causing inhibition of cell proliferation and cellular senescence in human fibroblasts (13,14). Higher levels of ANRIL are regarded as a risk factor in several types of human cancers, including hepatocellular carcinoma (*15*), lung cancer (*16*), ovarian cancer (*17*), gastric cancer (18), bladder cancer (*19*), and colorectal cancer (*20*). ANRIL also regulates a large number of genes related to gene expression, cell proliferation, cell adhesion, and apoptosis (*21*), suggesting that ANRIL is involved in various cellular processes.

4. LINC00473_v1 and v2

Reitmair *et al*. reported that expression of long intergenic non-protein coding RNA 473 (LINC00473)_v1, also known as C6orf176, is cyclic adenosine monophosphate (cAMP)-mediated (*22*). cAMP mediates diverse cellular signals, including prostaglandin E2-mediated intraocular pressure-lowering activity in human ocular ciliary smooth muscle cells. Knockdown of LINC00473_v1 shows modulation of several cAMP-responsive genes. LINC00473_v1 is a potential biomarker and/or therapeutic target in the context of diseases linked to deregulated cAMP signaling.

5. LINC00152 / CYTOR

Recently, reports concerning long intergenic non-protein coding RNA 152 (LINC00152), also known as cytoskeleton regulator RNA (CYTOR), have been increasing. First, LINC00152 expression is increased in gastric cancer tissue (*23*). The expression level of LINC00152 in gastric carcinoma is significantly increased compared with matched normal tissue and normal mucosa from healthy controls. LINC00152 also acts as a novel biomarker in predicting diagnosis of hepatocellular carcinoma (*24*). Moreover, LINC00152 acts as an oncogene, because knockdown of LINC00152 inhibits cell proliferation and colony formation, promotes cell cycle arrest at G1 phase, triggers late apoptosis, reduces the epithelial to mesenchymal transition program, and suppresses cell migration and invasion (*25*). LINC00152 directly binds with epidermal growth factor receptor (EGFR)

which causes activation of Phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) signaling (*26*). LINC00152 is involved in the oncogenesis of hepatocellular carcinoma by activating the mechanistic target of the rapamycin (mTOR) signaling pathway (*27*).

6. HOTAIR

The most thoroughly studied representative of the modulation chromatin state of lncRNAs is HOTAIR (*28*). HOTAIR is transcribed within the homeobox C (HOXC) locus, and interacts with the catalytic subunit of PRC2 and enhancer of zeste homolog 2 (EZH2) (*29,30*). Knockdown of HOTAIR causes a reduction in PRC2 occupancy, a local decrease in H3K27 trimethylation, and the activation of genes within the homeobox D (HOXC) HOXD locus on chromosome 2 (*29*). HOTAIR acts as a molecular scaffold to connect PRC2 and lysine (K)-specific demethylase 1A (LSD1) complexes (*30*). While the 5' end of HOTAIR is required for interaction with PRC2, its 3' end has been shown to interact with the H3K4-demethylase LSD1 *in vitro*. This interaction results in the physical bridging of a small subfraction of PRC2 and LSD1-containing repressive complexes, with HOTAIR knockdown causing loss of either or both complexes at a subset of target genes (*30*). Recently, RNA-chromatin immunoprecipitation (ChIP) and chromatin Isolation by RNA Purfication (ChIRP) reveals that HOTAIR is regulated by estrogens and able to control estrogen receptors (ERs) function by interacting with estrogen receptor (ER)α/ERβ, and HOTAIR is present on pS2, human telomerase reverse transcriptase (hTERT) and HOTAIR promoters at the estrogen response elements (ERE)/endothelial nitric oxide synthase (eNOS) peaks (*31*).

7. GAS5

GAS5 was originally isolated from a screen for potential tumor suppressor genes expressed at high levels during growth arrest (*32*). The human GAS5 gene is a multiple small nucleolar RNA (snoRNA) host gene that encodes 10 box C/D snoRNAs within 11 introns, and has been classified as a member of the 5'-terminal oligopyrimidine tract (5' TOP) gene family, characterized by an upstream oligopyrimidine tract sequence (*33*). GAS5 transcript abundance is increased during growth arrest induced by either serum starvation or treatment with translation inhibitors (*34*). GAS5 functions as a starvation- or growth arrest-linked riborepressor for the glucocorticoid receptor (GR) by binding to the DNA-binding domain of the GR, acting as a decoy glucocorticoid response element (GRE), thus competing with DNA GREs for binding to the GR (*35*). The degradation pathway of GAS5 regulates GAS5 function, which modulates the apoptosis-related genes cellular inhibitor of apoptosis protein 1 (cIAP2) and serum/glucocorticoid regulated kinase 1 (SGK1) (*36*).

8. TUG1

Taurine up-regulated 1 (TUG1) causes growth-control genes to relocate from the repressive environment of Polycomb bodies, where they interact with co-repressor complexes, to the gene activation milieu of the interchromatin granules, by selectively interacting with methylated and unmethylated polycomb 2 proteins present on growth-control gene promoters (*37,38*). TUG1 is upregulated by p53 upon DNA damage in p53 wild-type, but not p53 mutant cells. TUG1 also serves as a diagnostic biomarker and therapy target for hepatocellular carcinoma and promotes cell growth and apoptosis by epigenetic silencing of kruppel like factor 2 (KLF2) (*39*).

9. NEAT1_v1 and v2

Nuclear paraspeckle assembly transcript 1 (NEAT1) has been found to localize specifically to paraspeckles where it forms an essential structural component (*40-42*). Recently, two independent research groups reported that the NEAT1 - splicing factor proline and glutamine rich (SFPQ) interaction plays roles in both repression and activation of genes, which likely depend on the context of the promoter sequence or interplay with other transcriptional factors (*43-44*). Hirose *et al*. reported the role of NEAT1 in transcriptional regulation through sequestering of SFPQ from the RNA-specific adenosine deaminase B2 (ADARB2) gene in response to proteasome inhibition (*43*). Imamura *et al*. reported that NEAT1 expression is induced by infection with the influenza virus or herpes simplex virus. This upregulation of NEAT1 results in relocation of SFPQ, a NEAT1- binding paraspeckle protein and repressor of interleukin 8 (IL8) transcription, from the IL8 promoter to the paraspeckles, leading to transcriptional activation of IL8 (*44*).

10. Other lncRNAs

The functions of other lncRNAs remain to be elucidated; however, we have found that lncRNAs highly and rapidly respond to chemical stresses as follows. In HeLa Tet-off cells, six SLiTs [MIR22 host gene (MIR22HG), GABPB1 antisense RNA 1 (GABPB1-AS1), LINC00152, IDI2 antisense RNA 1 (IDI2-AS1), small nucleolar RNA host gene 15 (SNHG15), and LINC01184) respond to chemical stressors (cisplatin, cycloheximide, or mercury II chloride) (*45*). In human-induced pluripotent stem cells (hiPSCs), six novel lncRNAs (ANRIL, MIR22HG, GABPB1-AS1, LINC01184, LINC00152, and LINC0541471_v2) respond to chemical stressors (cycloheximide, hydrogen peroxide, cadmium, or arsenic) (*46*).

11. Concluding remarks

Although the biological and physiological functions of some SLiTs have been documented, those of others remain unknown. We have found unknown SLiTs that respond strongly to chemical stresses; thus, we can use these SLiTs as surrogate indicators of chemical stress responses in human cells. I believe that the relationships of the unknown SLiTs in this review and RNA-binding proteins will be determined in the future.

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Original Article

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Lactic acid bacteria of the *Leuconostoc* genus with high innate immunity-stimulating activity

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Summary **We screened lactic acid bacteria that exhibited high innate immunity-stimulating activity by monitoring muscle contraction activity in silkworms. Heat-treated fractions of lactic acid bacteria, *Leuconostoc carnosum* #7-2, *Leuconostoc gelidum* #4-2, and *Leuconostoc mesenteroides* 8/11-3, had high (250-460 units/mg) innate immunity-stimulating activity. These lactic acid bacteria proliferated in milk to concentrations of 1 × 10^6 colony forming unit/mL. The present findings suggest that the silkworm muscle contraction assay is useful for screening lactic acid bacteria with high innate immunity-stimulating activity, and that the assay can be used for the production of fermented foods made from milk.**

Keywords: Lactic acid bacteria, *Leuconostoc* sp., silkworm, innate immunity

1. Introduction

Lactic acid bacteria produce lactic acid and are used for the production of various fermented foods, such as yogurt and kimchi (Korean pickles). Lactic acid bacteria are considered effective for the treatment of diarrhea and regulation of immune responses (*1-3*). An established method to efficiently isolate lactic acid bacteria that are beneficial for human health would be useful for the development of foods containing functional lactic acid bacteria.

"Innate immunity-stimulating activity" is now recognized as an important function of lactic acid bacteria. Innate immunity, which does not involve antibodies, is the front line defense system in all animals and plants. In mammals, various stimuli promote the secretion of cytokines from immunocompetent cells such as macrophages, and induce the transmission of signals to other immunocompetent cells, followed by the production of specific antibodies that eliminate pathogens. In invertebrate animals, such as insects, which do not possess acquired immunity, elimination of pathogens relies solely on innate immunity. The innate immune systems in insects and mammals have many common features. For example, cells called hemocytes ingest invasive foreign pathogens as well as macrophages in mammals (*4*). Moreover, Toll-like receptors, which are related to the innate immune response in mammals, have high homology with Toll receptors, which function in innate immune responses in *Drosophila melanogaster* (*5*). We previously reported that stimulation of the innate immune system activates a cytokine called paralytic peptide in silkworms, resulting in muscle contraction. This means that muscle contraction is coupled with the activation of innate immunity in silkworms. We established a simple method for measuring innate immunity-stimulating activity in various samples using this system (*6*). We demonstrated by monitoring muscle contraction ability in mouse macrophages that purified polysaccharides from green tea stimulate the production of cytokines (*7*). Using this system, we also demonstrated that certain species of lactic acid bacteria exhibit relatively high innate immunity-stimulating activity (*8*). Here we describe that three strains of lactic acid bacteria of the genus *Leuconostoc* isolated from kimchi and rice bran

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have high innate immunity-stimulating activity.

2. Materials and Methods

2.1. *Isolation and identification of lactic acid bacteria*

Samples obtained from kimchi and rice bran were streaked on deMan, Rogosa and Sharpe (MRS) agar (Becton, Dickinson and Company, MD, USA) plates containing 0.5% calcium carbonate (Wako Pure Chemical Industries, Osaka, Japan). After incubation at 30°C under anaerobic conditions, bacterial colonies with transparent halos were isolated and further characterized. The bacterial colonies were cultured in 15 mL of MRS broth (Becton, Dickinson and Company) under anaerobic conditions at 30°C. Bacterial pellets were collected by centrifugation, suspended into 0.9% NaCl (Wako Pure Chemical Industries), mixed well with the same volume of 80% glycerol (Wako Pure Chemical Industries), and stored at −80°C. The bacterial 16S rDNA was amplified by colony polymerase chain reaction. A homology search was carried out on a database using BLAST. Bacterial species exhibiting the highest sequence homology were identified.

2.2. *Characterization of bacteria*

Gram staining was performed using Gram color (Merck, Kenilworth, NJ, USA). Bacteria were fixed by passing slides through the flame of a Bunsen burner. For scanning electron microscopy (SEM), bacterial cells were pre-fixed with 2.5% glutaraldehyde (POLYSCIENCES, INC., Warrington, PA, USA) in 0.1 M cacodylate (TAAB, England) buffer (pH 7.2), post-fixed with 1% osmium tetroxide (Merck) in the same buffer, and freeze-dried in t-butyl alcohol (Wako Pure Chemical Industries). The samples were examined with field-emission SEM (JSM- 7500F, JEOL, Japan). The carbohydrate metabolism of each strain was determined using an Api 50 CH system (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan) by the following procedure. The lactic acid bacterial colonies were suspended and adjusted to match a McFarland turbidity standard of 2. One hundred-fifty microliters of the bacterial suspension was added to the Api plate, which was incubated at 30°C for 48 h, and then carbohydrate metabolism was determined based on the color change. The catalase activity of each strain was determined by suspending the bacterial colonies in 3% hydrogen peroxide (Wako Pure Chemical Industries). Other enzyme activities were determined using an Api Zym system (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan).

2.3. *Measurement of innate immunity-stimulating activity of lactic acid bacteria using silkworm muscle specimens*

IHC was performed on 4-μm sections that had been e Innate immunity-stimulating activities of lactic acid bacteria were determined by measuring silkworm muscle contraction (*6*). Lactic acid bacterial suspensions cultured in 100 mL of MRS were autoclaved at 121°C for 20 min, and the cells were collected by centrifugation at 8,000 rpm at 4°C. After washing with 50 ml of 0.9% NaCl, the pellet was suspended in 1 mL of 0.9% NaCl. Fifty microliters of diluted suspension was injected into silkworm muscle specimens, and the length of the silkworm muscle specimens was measured 10 min after the injection. One unit of activity was defined as a sample dose that decreased the length of silkworm muscle specimens by 15% (*6*). Dry weights of the samples were determined after evaporation to calculate the specific activity.

2.4. *Growth test of lactic acid bacteria in milk*

Glycerol stocks (1 μL) of lactic acid bacteria were added to 50 mL of milk (Meiji Co., Ltd., Japan), and cultured under anaerobic conditions at 30°C for 1 day. The number of viable cells in the milk was determined after spreading diluted samples on MRS agar plates and anaerobically culturing them at 30°C for 20 h.

3. Results

3.1. *Isolation of lactic acid bacteria with high innate immunity-stimulating activity*

We isolated lactic acid bacteria from kimchi and rice bran, popular fermented foods in Korea and Japan, on MRS agar containing 0.5% calcium carbonate. Colonies of lactic acid bacteria were identified by transparent halos, which indicate the production of lactic acid. Isolated lactic acid bacteria were cultured in MRS following by heat treatment at 121°C for 15 min.

Muscle contraction was measured to evaluate the innate immunity-stimulating activity (*6*). Each bacterial suspension was injected into silkworm muscle specimens. The activity of most of the lactic acid bacteria was much lower than 50 units/mg, but three lactic acid bacteria, #7-2, #4-2, and 8/11-3, exhibited activities higher than 100 units/mg (Table 1).

3.2. *Characterization of the isolated lactic acid bacteria*

Species of the three strains of lactic acid bacteria, #7-2, #4-2, and 8/11-3, that exhibited high innate immunity-stimulating activity in the silkworm muscle contraction assay were determined by sequencing their 16S rDNA. The results indicated that #7-2, #4-2, and 8/11-3 were *Leuconostoc carnosum*, *Leuconostoc gelidum*, and *Leuconostoc mesenteroides*, respectively. We recognized that the genus of all three strains with high innate immunity-stimulating activities were *Leuconostoc*

Table 1. Species and innate immunity-stimulating activity of three bacterial strains isolated from fermented foods

Strains	Origins	Species	Identity (%)	Relative activity[a] (units/mg)
#7-2	Kimchi	*Leuconostoc carnosum*	99	460 ± 240 (n = 3)
#4-2	Rice bran	*Leuconostoc gelidum*	99	250 ± 140 (n = 3)
8/11-3	Kimchi	*Leuconostoc mesenteroides*	99	250 ± 190 (n = 4)

One unit of activity was defined as the activity required to decrease the length of silkworm muscle specimens by 15% (*6*). Values were mean ± standard deviation.

Table 2. Carbohydrate metabolism of the three lactic acid bacteria strains isolated from fermented food

Items	#7-2	#4-2	8/11-3
Control	–	–	–
Glycerol	–	–	–
Erythritol	–	–	–
D-Arabinose	–	–	–
L-Arabinose	–	+	+
D-Ribose	+	+	+
D-Xylose	–	+	+
L-Xylose	–	–	–
D-Adonitol	–	–	–
Methyl-β-D-Xylopyranoside	–	–	–
D-Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Solbose	–	–	–
L-Rhamnose	–	–	–
Dulcitol	–	–	–
Inositol	–	–	–
D-Mannitol	–	+	+
D-Sorbitol	–	–	+
Methyl-α-D-Mannopyranoside	–	–	–
Methyl-α-D-Glucopyranoside	+	+	+
N-Acetyl Glucosamide	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
Esculin ferric citrate	+	+	+
Salicin	+	+	+
D-Cellobiose	+	+	+
D-Maltose	+	+	+
D-Lactose	–	–	+
D-Melibiose	+	+	+
D-Sucrose	+	+	+
D-Trehalose	+	+	+
Inulin	–	–	–
D-Melezitose	+	+	+
D-Raffinose	+	–	+
Starch	+	+	–
Glycogen	–	–	–
Xylitol	–	–	–
Gentiobiose	+	+	+
D-Turanose	+	+	+
D-Lyxose	–	–	–
D-Tagatose	+	+	+
D-Fucose	–	–	–
L-Fucose	–	–	–
D-Arabitol	–	–	–
L-Arabitol	–	–	–
Gluconate	+	+	+
2 Keto Gluconate	–	+	–
5 Keto Gluconate	–	–	–

The carbohydrate metabolism capacity was determined by Api 50 CH system. Lactic acid bacteria on agar plates was suspended in suspension medium, and the bacterial density was adjusted to a McFarland turbidity standard of 2. One hundred-fifty microliters of the bacterial suspension was added to an Api plate and incubated at 30°C for 48 h. The metabolism capacity for each carbohydrate was determined based on the color change.

Table 3. Enzymatic activities of three lactic acid bacteria strains isolated from fermented foods

Items	#7-2	#4-2	#8/11-3
Catalase	–	–	–
Alkaline phosphatase	+	–	+
Esterase (C4)	+	+	+
Esterase lipase (C8)	+	+	+
Lipase (C14)	–	–	–
Leucine aminopeptidase	+	+	+
Valine aminopeptidase	–	–	–
Cysteine aminopeptidase	–	–	–
Trypsin	–	–	–
Chymotrypsin	–	+	–
Acid phosphatase	+	+	+
Phosphoamidase	+	+	+
α-Galactosidase	–	–	+
β-Galactosidase	–	–	+
β-Glucuronidase	–	–	–
α-Glucosidase	+	+	+
β-Glucosidase	–	+	+
β-Glucosaminidase	–	–	–
α-Mannosidase	–	–	–
α-Fucosidase	–	–	–

The catalase activity of each strain was determined by suspending the bacterial colony in 3% hydrogen peroxide. Other enzyme activities were determined by Api Zym system. Lactic acid bacteria were suspended in suspension medium, and adjusted to a McFarland turbidity standard of 5 or 6. Sixty-five microliters of the bacterial suspension was added to an Api plate and incubated at 37°C for 4.5 h. After incubation, enzymatic activities were determined based on the color change.

(Table 1). Moreover, we examined the bacteria for Gram staining and in an electron micrograph. We also performed a carbohydrate metabolism test and an enzymatic activity test of these lactic acid bacteria (Tables 2 and 3, Figures 1 and 2). All three strains were Gram-positive coccal bacteria (Figure 1). SEM confirmed that these lactic acid bacteria had a coccal shape and were arranged in pairs or short chains (Figure 2). Results of the carbohydrate metabolism test were consistent with the notion that these bacteria belong to the *Leuconostoc* genus. All three strains exhibited esterase (C4), esterase lipase (C8), leucine aminopeptidase, acid phosphatase, phosphoamidase, and α-glucosidase activities. In addition, #7-2 and 8/11-3 exhibited alkaline phosphatase activity, #4-2 exhibited chymotrypsin activity, 8/11-3 exhibited α-galactosidase and β-galactosidase activities, and #4-2 and 8/11-3 exhibited β-glucosidase activity.

3.3. *Growth of lactic acid bacteria in milk*

Plant-origin lactic acid bacteria are generally considered

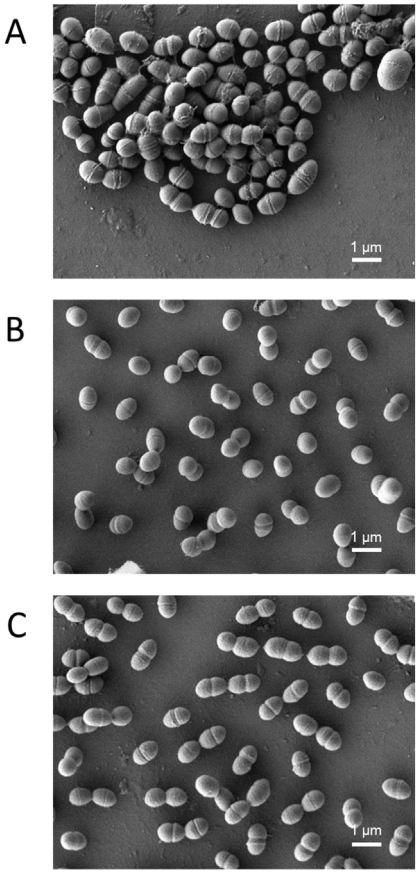

Figure 2. Scanning electron micrographs of lactic acid bacteria. Bacteria are shown at 10,000× magnification. **(A)** *Leuconostoc carnosum* #7-2, **(B)** *Leuconostoc gelidum* #4-2, (C) *Leuconostoc mesenteroides* 8/11-03.

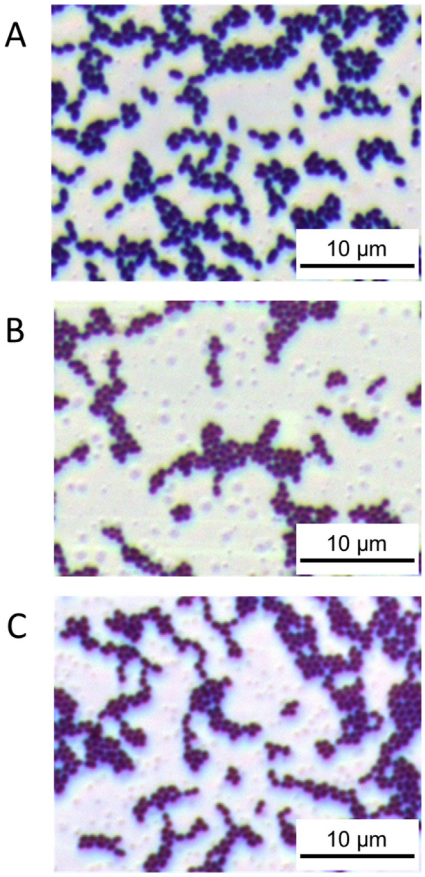

Figure 1. Gram stain of lactic acid bacteria. After Gram staining, bacteria cells were observed under an optical microscope at 1,000× magnification. **(A)** *Leuconostoc carnosum* #7-2, **(B)** *Leuconostoc gelidum* #4-2, **(C)** *Leuconostoc mesenteroides* 8/11-03.

Table 4. Growth of isolated lactic acid bacteria in milk

Strains	Origins	Species	Number of viable cells (before culture)	Number of viable cells (after culture)
#7-2	Kimchi	*Leuconostoc carnosum*	9.0×10^5 (cells/mL)	3.4×10^6 (cells/mL)
#4-2	Rice bran	*Leuconostoc gelidum*	$< 1.0 \times 10^3$ (cells/mL)	1.0×10^7 (cells/mL)
8/11-3	Kimchi	*Leuconostoc mesenteroides*	4.6×10^4 (cells/mL)	1.0×10^8 (cells/mL)

Glycerol stocks of lactic acid bacteria were added to 50 mL milk (Meiji Co., Ltd.) using a 1-μL disposable loop, and anaerobically cultured at 30°C for 1 day. The number of viable cells in the milk was calculated by counting the number of colonies that formed on MRS agar plates spread with 100 μL of diluted milk and anaerobically cultured at 30°C.

to be inappropriate for manufacturing yogurt as they lack the ability to grow in milk. Some plant-origin lactic acid bacteria, such as *Lactococcus lactis*, however, can grow in milk (*8,9*). We examined whether the above-mentioned lactic acid bacterial strains could grow in milk. Lactic acid bacteria were cultivated in milk at 30°C for 24 h. Diluted samples were spread on agar plates, and the number of colonies was counted. The numbers of all three bacterial strains increased to more than 1.0×10^6 colony forming units (cfu)/mL (Table 4).

4. Discussion

In this paper, we describe three strains of lactic acid bacteria of the *Leuconostoc* genus, isolated from kimchi and rice bran, that exhibited high innate immunity-stimulating activities as assessed by monitoring silkworm muscle contraction. Lactic acid bacteria of the *Leuconostoc* genus are used conventionally for manufacturing fermented foods, such as sauerkraut and kefir, which means that humans have experience eating these lactic acid bacteria. Recently, much attention has been focused on the use of lactic acid bacteria for maintaining good health. Establishment of methods for identifying functional lactic acid bacteria, however, are difficult. Our proposed method for measuring innate immunity-stimulating activity by silkworm muscle contraction is considered to be applicable for the

discovery of lactic acid bacteria that have high innate immunity-stimulating activity.

Plant-origin lactic acid bacteria are considered suitable for manufacturing kimchi and fermented rice bran, but not for the production of yogurt because they generally do not grow in milk. The three strains of lactic acid bacteria described in this paper did not solidify milk, but #4-2 and 8/11-3 strains grew in milk and reached a concentration greater than 1×10^7 cfu/mL. Fermented milk containing a minimum 8.0% milk solids-not-fat content should contain more than 1×10^7 cfu/mL of lactic acid bacteria according to the Ministry of Health, Labor and Welfare in Japan (*10*). Therefore, these lactic acid bacteria are considered useful for manufacturing fermented beverages made from milk.

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Decreased sugar concentration in vegetable and fruit juices by growth of functional lactic acid bacteria

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Summary ***Leuconostoc carnosum* #7-2, *L. gelidum* #4-2, and *L. mesenteroides* 8/11-3, which were isolated from fermented plant foods, are lactic acid bacteria. We previously reported that these bacteria are functional lactic acid bacteria whose innate immunity-stimulating activities are high based on a silkworm muscle contraction assay. The concentrations of these three lactic acid bacteria increased to more than 1×10^6 colony forming units (cfu)/mL in various vegetable and fruit juices when the pH values were appropriately adjusted. As the bacteria grew in the vegetable and fruit juices, the pH decreased and the concentrations of total sugars and glucose also decreased. These findings suggest that these functional lactic acid bacteria can be used to produce vegetable and fruit juices with reduced sugar levels, which is expected to be beneficial for human health.**

Keywords: Juice, lactic acid bacteria, sugar concentration

1. Introduction

Vegetable and fruit juices are commonly ingested beverages. These juices contain abundant sugars, including sucrose, glucose, and fructose, and their ingestion may lead to excessive intake of carbohydrates. Reducing the sugar levels in vegetable and fruit juices may help to decrease the risk of lifestyle-related diseases such as diabetes and obesity resulting from excessive carbohydrate ingestion. Unlike the methods that are used to decrease the sugar content of carbonated drinks, sports drinks, and coffee drinks, methods that effectively remove sugars from vegetable and fruit juices without affecting the taste of the juice are limited.

Lactic acid bacteria are used to ferment foods, such as yogurt and pickles. We have studied functional lactic acid bacteria with innate immunity-stimulating activity and postprandial hyperglycemia inhibitory activity (*1,2*). The use of functional lactic acid bacteria for manufacturing fermented food is thought to contribute to maintaining human health. We demonstrated that stimulating the silkworm immune system induces the production of a cytokine called paralytic peptide, whose pharmacologic activities include muscle contraction (*3*). Lactic acid bacteria strains *Leuconostoc carnosum* #7-2, *L. gelidum* #4-2, and *L. mesenteroides* 8/11-3, which were previously isolated from kimchi and rice bran (fermented plant foods) have high innate immunity-stimulating activities as determined using the silkworm muscle contraction assay.

In this article, we describe that sugar concentrations in juices can be reduced by these lactic acid bacteria. Vegetable and fruit juices, in which functional lactic acid bacteria grow, are expected to be useful for promoting health.

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2. Materials and Methods

2.1. *Lactic acid bacteria*

The lactic acid bacteria used in this study are listed in Table 1.

2.2. *Growth of lactic acid bacteria in vegetable and fruit juices*

Lactic acid bacteria were precultured anaerobically

in deMan, Rogosa and Sharpe (MRS) liquid media (Becton, Dickinson and Company, MD, USA) at 30°C. Vegetable and fruit juices (50 mL) were added to 50 µL to 500 µL of precultured lactic acid bacteria, and further cultured at 30°C for 24 to 94 h. The number of viable cells was calculated by counting the number of colonies that formed on MRS agar plates spread with diluted samples and anaerobically cultured at 30°C. The pH of the culture was maintained between 6 and 7 by adding a 10N NaOH solution.

Glycerol stocks of lactic acid bacteria were prepared and stored at -80°C. Specifically, a single colony was isolated from MRS agar (Becton, Dickinson and Company) containing 0.5% calcium carbonate (Wako Pure Chemical Industries, Osaka, Japan) that was spread with lactic acid bacteria and anaerobically cultured at 30°C. The bacterial colony was picked up and inoculated in 15 mL of MRS media, and anaerobically cultured at 30°C. Bacterial pellets were collected after centrifugation. The pellet was suspended into 2 mL of 0.9% NaCl (Wako Pure Chemical Industries), and mixed well with same volume of 80% glycerol (Wako Pure Chemical Industries). The glycerol stocks were stored at -80°C.

Table 1. Lactic acid bacteria used in this study

Strains	Origins	Species
#7-2	Kimchi	*Leuconostoc carnosum*
#4-2	Rice bran	*Leuconostoc gelidum*
8/11-3	Kimchi	*Leuconostoc mesenteroides*

2.3. *Determination of sugar concentrations in vegetable and fruit juices*

Total sugar concentration in the juices was determined by the phenol-sulfuric acid method according to the following procedure: Each juice sample (100 µL) was centrifuged at 8,000 rpm for 10 min. Phenol (5%; Wako Pure Chemical Industries) was added to the sample and vortexed vigorously for 5 s. Sulfuric acid (500 µL; Wako Pure Chemical Industries) was added to the sample and vortexed until it produced heat. After incubation at room temperature for 20 min, the OD_{490} was measured. Glucose concentration was determined using an ACCU-CHEK strip F (Roche Diagnostics K.K., Tokyo, Japan).

3. Results

3.1. *Growth of lactic acid bacteria in vegetable and fruit juices*

We previously reported three strains of lactic acid bacteria, *L. carnosum* #7-2, *L. gelidum* #4-2, and *L. mesenteroides* 8/11-3, with high innate immunity-stimulation activities. These lactic acid bacteria grow in milk. In the present study, we examined whether those bacterial strains could grow in vegetable or fruit juice. The concentration of *L. mesenteroides* 8/11-3 increased to more than 1.0×10^6 colony forming units (cfu)/mL after culture for 70 h in broccoli, mixed vegetable, and apple juices (Table 2). Moreover, the concentration of this strain increased to 1.0×10^6 cfu/mL after culture for 94 h in bitter melon and mandarin orange juices

Table 2. Growth of lactic acid bacteria in vegetable and fruit juices without pH adjustment

Strains	Juices (Before neutralization)	Bacterial concentration in juice (cfu/mL)	
		After 70 h	After 94 h
L. carnosum #7-2	Bitter melon	$< 1.0 \times 10^3$	N.D.
	Broccoli	$< 1.0 \times 10^3$	N.D.
	Mixed vegetable	$< 1.0 \times 10^3$	N.D.
	Apple	$< 1.0 \times 10^3$	N.D.
	Mandarin orange	$< 1.0 \times 10^3$	N.D.
	Kiwi fruit	$< 1.0 \times 10^3$	N.D.
L. gelidum #4-2	Bitter melon	$< 1.0 \times 10^3$	N.D.
	Broccoli	1.0×10^5	1.5×10^4
	Mixed vegetable	1.0×10^4	1.7×10^4
	Apple	$< 1.0 \times 10^3$	N.D.
	Mandarin orange	$< 1.0 \times 10^3$	N.D.
	Kiwi fruit	$< 1.0 \times 10^3$	N.D.
L. mesenteroides 8/11-3	Bitter melon	1.0×10^5	1.0×10^6
	Broccoli	1.0×10^6	1.4×10^7
	Mixed vegetable	1.0×10^6	4.6×10^7
	Apple	1.0×10^6	1.2×10^7
	Mandarin orange	1.0×10^3	1.0×10^6
	Kiwi fruit	$< 1.0 \times 10^3$	N.D.

Vegetable and fruit juices were autoclaved at 121°C for 20 min. Lactic acid bacteria were precultured anaerobically in MRS liquid media at 30°C. Aliquots (10 mL) of vegetable and fruit juices with 50 µL of the preculture was further cultured at 30°C. (N.D., not determined).

Table 3. Growth of lactic acid bacteria in vegetable and fruit juices with pH adjustment

Strains	Juices (After neutralization)	Bacterial concentration in juice (cfu/mL)	
		After 24 h	After 48 h
L. carnosum #7-2	Kiwi fruit	1.0×10^2	N.D.
	Apple ①	1.0×10^4	N.D.
	Orange ①	N.D.	1.4×10^8
	Grapefruit	N.D.	1.4×10^7
L. gelidum #4-2	Kiwi fruit	N.D.	1.4×10^5
	Apple ①	N.D.	6.7×10^6
	Orange ①	N.D.	1.1×10^8
	Grapefruit	N.D.	2.1×10^8
L. mesenteroides 8/11-3	Kiwi fruit	N.D.	4.7×10^6
	Apple ①	N.D.	2.3×10^8
	Orange ①	N.D.	1.1×10^9
	Grapefruit	N.D.	1.1×10^8

Adjustment of fruit juice pH was conducted by adding 10N NaOH solution. Neutralized juices into which preculture was added was cultured at 30°C. One hundred grams of kiwi fruit were cut, and 500 ml of water was added and crushed by a juicer and neutralized. Apple ① (Tokyo Meiraku Co Ltd), orange ①, and grapefruit juices were neutralized before use. These fruit juices were autoclaved at 121°C for 20 min. (N.D., not determined).

Table 4. Change of sugar concentration and pH in fruit juices with lactic acid bacterial culture

Strains	Juices (After neutralization)	Culture time (h)	Concentration of total sugar (mg/mL)		Concentration of glucose (mg/mL)		pH of juices	
			Without bacteria	With bacteria	Without bacteria	With bacteria	Without bacteria	With bacteria
L. carnosum #7-2	Kiwi fruit	48	15	N.D.	19	N.D.	7	N.D.
	Apple ①	48	79	N.D.	2.9	N.D.	6	N.D.
	Apple ②	72	72	67	N.D.	N.D.	7	4.5
	Orange ①	48	72	45	2.8	<0.1	6	N.D.
	Orange ②	72	69	49	N.D.	N.D.	7	4
	Orange ③	24	95	75	N.D.	N.D.	7	6
	Orange ④	48	95	65	N.D.	N.D.	7	4
	Orange ⑤ (slow juicer)	24	93	77	N.D.	N.D.	7	5
	Orange ⑥ (slow juicer)	48	93	68	N.D.	N.D.	6	4
	Grapefruit	48	65	44	2.4	N.D.	6	N.D.
L. gelidum #4-2	Kiwi fruit	48	15	8	19	1.9	7	N.D.
	Apple ①	48	79	N.D.	2.9	N.D.	6	N.D.
	Apple ②	72	72	64	N.D.	N.D.	7	4
	Orange ①	48	72	50	2.8	< 0.1	6	N.D.
	Orange ②	72	69	60	N.D.	N.D.	7	4
	Orange ③	24	95	62	N.D.	N.D.	7	4
	Orange ④	48	95	68	N.D.	N.D.	7	4
	Orange ⑤ (slow juicer)	24	93	70	N.D.	N.D.	7	5
	Orange ⑥ (slow juicer)	48	93	75	N.D.	N.D.	6	4
	Grapefruit	48	65	46	2.4	< 0.1	6	N.D.
L. mesenteroides 8/11-3	Kiwi fruit	48	15	2	19	1.4	7	N.D.
	Apple ①	48	79	59	2.9	4.3	6	N.D.
	Apple ②	72	72	62	N.D.	N.D.	7	4
	Orange ①	48	72	34	2.8	1.7	6	N.D.
	Orange ②	72	69	39	N.D.	N.D.	7	3
	Orange ③	24	95	49	N.D.	N.D.	7	3
	Orange ④	48	95	47	N.D.	N.D.	7	3
	Orange ⑤ (slow juicer)	24	93	73	N.D.	N.D.	7	3
	Orange ⑥ (slow juicer)	48	93	54	N.D.	N.D.	6	3
	Orange ⑦ (slow juicer)	48	98	55	N.D.	N.D.	6	3
	Grapefruit	48	65	46	2.4	<0.1	6	N.D.

Neutralized juices into which preculture were added was cultured at 30°C for 2-3 days. Apple juice ①, ②, orange juice ①, ②, ③, and ④ were neutralized and autoclaved before use. Orange ⑤, ⑥, and ⑦(US) were prepared by slow juicer. Oranges (⑤, ⑥: from Australia, ⑦: from US) were crushed by slow juicer, and neutralized. These juices were autoclaved before use. (N.D., not determined).

(Table 2). The concentration of *L. mesenteroides* 8/11-3 after culture for 70 h in kiwi fruit juice was less than 1.0×10^3 cfu/mL (Table 2). The concentrations of *L. carnosum* #7-2 and *L. gelidum* #4-2 after culture for 70 h in bitter melon, broccoli, mixed vegetable, apple, mandarin orange, and kiwi fruit juices were lower than 1.0×10^5 cfu/mL (Table 2).

Neutralizing tomato juice with sodium hydrogen carbonate facilitates the growth of lactic acid bacteria (*4*). Therefore, we examined whether these lactic acid bacteria could grow in neutralized fruit juices. The concentrations of all three strains were increased to 1.4×10^7 cfu/mL after 48 h in orange and grapefruit juices (Table 3). Moreover, the concentrations of *L. gelidum* #4-2 and *L. mesenteroides* 8/11-3 in neutralized kiwi fruit and apple juices increased to 1.4×10^5 cfu/mL after 48 h (Table 3). The pH of apple and orange juices decreased after the growth of lactic acid bacteria (Table 4).

3.2. *Decreased sugar concentration after growth of lactic acid bacteria in vegetable and fruit juices*

Bacterial growth consumes sugars in culture media. We determined the total sugar and glucose concentrations in neutralized juices in which the three strains of lactic acid bacteria were cultured. The concentration of total sugar decreased under all conditions in which bacteria grew (Table 4). In particular, the total sugar content of kiwi fruit juice in which *L. mesenteroides* 8/11-3 grew decreased to 13% that of the original level. Glucose concentrations of juices in which *L. mesenteroides* 8/11-3 grew, except apple juice, decreased (Table 4). In particular, the glucose concentration decreased to below the detection limit in orange and grapefruit juices in which *L. carnosum* #7-2 and *L. gelidum* #4-2 grew, and in kiwi fruit and grapefruit juices in which *L. mesenteroides* 8/11-3 grew. These results indicate that growth of these three strains of lactic acid bacteria, *L. carnosum* #7-2, *L. gelidum* #4-2, and *L. mesenteroides* 8/11-3, decreased the concentration of total sugars and glucose in fruit juices.

4. Discussion

In this paper, we demonstrated that the growth of *L. carnosum* #7-2, *L. gelidum* #4-2, and *L. mesenteroides* 8/11-3 decreased sugar concentrations in vegetable and fruit juices. These three strains are functional lactic acid bacteria with high innate immune system-stimulating activity, as determined in silkworms by a muscle contraction assay. Vegetable and fruit juices are generally acidic, and bacteria generally do not grow well in these juices. We showed that neutralizing the juices with sodium hydroxide provides the appropriate conditions for growing the three strains of lactic acid bacteria. Besides a low pH, insufficient nutrition or the presence of bactericidal substances inhibit bacterial growth. To solve these problems, it is necessary to identify such factors in other juice samples.

Excessive intake of sucrose, glucose, and fructose from fruit juices is thought to contribute to the development of diabetes and obesity (*5-8*). We propose here that fermenting juices using lactic acid bacteria may solve this problem. The lactic acid bacterium *L. mesenteroides* 8/11-03 strain grew in bitter melon and broccoli juices. Therefore, this lactic acid bacterium may reduce sugars not only in fruit juices, but also in vegetable juices. Lactic acid bacteria decrease the sugar content of vegetable juices, fruit juices, and mixed juices (*4,9-11*). In addition, the lactic acid bacteria *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* decrease the glucose and fructose concentrations of a juice made from rice flour (*12*). To our knowledge, there are no reports that "functional lactic acid bacteria" decrease the sugar content of juices. The presence of the functional lactic acid bacteria may confer additional beneficial effects to juices for maintaining human health.

The Ministry of Health, Labor, and Welfare in Japan reports that fermented milk containing a minimum of 8.0% milk solids-not-fat content should contain at least 1×10^7 cfu/mL of lactic acid bacteria (*13*). In this study, we demonstrated that the concentrations of the three strains of lactic acid bacteria increased to 1×10^7 cfu/mL in vegetable and fruit juices after the pH was neutralized. Lactic acid bacteria of the *Leuconostoc* genus have long been used for manufacturing fermented foods, indicating that humans have experience ingesting those lactic acid bacteria. Therefore, we propose here the manufacture of vegetable and fruit juices containing these lactic acid bacteria as fermented foods that may be beneficial to human health.

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Ability of community pharmacists to promote self-care and self-medication by local residents [I]: Improvements in bone mineral density

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Summary **This study was conducted in order to establish a health management method for the elderly in a community through follow-ups of bone mineral density (BMD) measurement results over a 1-year period based on BMD measurements performed by pharmacists and a guidance program. Regarding BMD measurement results, the percent young adult mean (%YAM: mean BMD in healthy persons of the same sex aged between 51 and 82 years old) significantly increased in Period I, during which the intervention by pharmacists was performed (6 months after the start of measurements), but significantly decreased in Period II, during which this intervention was not performed (between 7 and 12 months after the start of measurements). Based on these results, lifestyle improvements were effective in Period I regardless of sex or age; however, it may be important to maintain an improved diet and subject motivation in the future. The results of this study suggest that community pharmacists play an important role in community medicine through positive intervention for local residents' health support.**

Keywords: Bone mineral density, nutrition, exercise, health support pharmacy, health expectancy

1. Introduction

The number of patients with osteoporosis in Japan, which is a rapidly aging society, has increased annually, reaching approximately 13,000,000. Health expenditure for osteoporosis is estimated to be approximately one trillion yen (*1,2*). According to a survey conducted by the Ministry of Health, Labour and Welfare in 2013, fall-related fractures were found to be the third most common reason (14.6%) for a state requiring support/nursing (*3*). Vertebral body/hip fractures were frequent in patients with osteoporosis, raising an important issue for the current medical system to prevent sequelae, such as fracture-related reductions in viability and long-term restrictions in daily living.

According to the World Health Organization (WHO), osteoporosis is a disease that is characterized by a low bone mass and the microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequential increase in the risk of fractures (*4*). Regarding the risk of fractures, assessments using bone mineral density (BMD) measurements are recommended by the "Japanese 2015 Guidelines for Prevention and Treatment of Osteoporosis", which were edited by the Japan Osteoporosis Society, Japanese Society for Bone and Mineral Research, and Japan Osteoporosis Foundation (*1*). In these guidelines, BMD of < 70% young adult mean (%YAM) has been established as a diagnostic criterion for osteoporosis; however, routine health check-ups do not currently involve BMD measurements. Furthermore, special qualifications and measurement-place settings are not necessary; therefore, positive activities, such as the addition of BMD to measurement items and holding measurement sessions for local residents, may be important in the future.

Regarding BMD measurements, informing each subject of their measurement results alone does not lead to healthy bone maintenance. A previous study reported that effective exercise/diet guidance for individuals based on BMD measurement results contributed to the

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prevention of osteoporosis (*5*), suggesting future trends in community medicine.

The Ministry of Health, Labour and Welfare recommended that pharmacists, as a "health support pharmacy", provide advice regarding nutrition and lifestyle to individuals in order to prolong national health expectancy (*6*). However, few studies have reported outcomes of BMD education for the elderly by pharmacists. In the present study, we examined changes in BMD with the presence or absence of diet-improving guidance by pharmacists involving local residents.

2. Materials and Methods

2.1. *Study design and subjects*

This study was conducted on the campus of Meiji Pharmaceutical University (Kiyose City, Tokyo, Japan) between December 2013 and December 2014. Subjects were 50 middle-aged or elderly residents in the neighborhood of the university (9 males, 41 females), with a mean age of 69 years (range: 51 to 82 years) (Table 1). Subjects were recruited for BMD measurements through advertising or posters. We excluded individuals who had cardiac attacks or stroke within 6 months, those with heart diseases (angina pectoris, heart failure, and severe arrhythmia), those with serious complications related to diabetes, those with markedly high blood pressure, those with chronic obstructive pulmonary disease, those with acute arthralgia, arthritis, low back pain, or neuralgia, those with acute inflammation such as pneumonia and hepatitis, those who had been instructed to restrict exercise by physicians, and those who had undergone surgery for cataracts or glaucoma within 6 months. The protocol of this study was approved by the Ethics Review Board of Meiji Pharmaceutical University. Written informed consent was obtained from all subjects.

BMD measurements were performed before the intervention by pharmacists (1st session) and 1.5, 3, and 6 months after the start of the intervention (2nd, 3rd, and 4th sessions), with the intervention period (6 months) being Period I. BMD was measured again at 12 months (5th session), with the non-intervention period (6 months) being Period II. In 40 subjects, the intervention by pharmacists and BMD measurements (1st to 4th sessions) were completed in Period I. At the completion of Period II, the 5th session of measurements was conducted in 23 subjects.

Table 1. Subject characteristics at baseline

Number of patients	*n* = 50
Mean age	69 ± 8.6* (51-82)
Men	9
Women	41
Thyroid disease	1
Diabetes	0
Prostatomegaly	1
Asthma	2
Gynopathy	0
High blood pressure	4
Epilepsy	0
Osteoporosis	1
Thrombosis / embolism	0
Digestive disorder	1
Alcohol habit (every day)	3
Smoking habit	0
Previous smoker	3

*mean ± SD

2.2. *BMD measurements*

BMD was measured using the ultrasound bone densitometer Model CM-200 (Furuno Electric Co., Ltd., Hyogo Prefecture, Japan). Using the ultrasonic pulsed penetration method, jelly for ultrasonography was applied to the right calcaneus, with the right knee of each subject being flexed at 90 degrees, and measurements were then conducted. Measurement items consisted of the speed of sound (SOS), T-score, Z-score, %YAM, and %AGE. After height, abdominal circumference, and body weight had been measured using the high-precision organization composition DF-851 (Yamato Scale Co., Ltd., Hyogo Prefecture, Japan), pharmacists performed lifestyle guidance based on the measurement results.

2.3. *Intervention by pharmacists*

In the intervention by pharmacists, each subject was given advice related to diet, sun exposure, and exercise (Table 2). Regarding diet, pharmacists explained that calcium, vitamin D, and vitamin K were essential for bone formation. Foods containing these nutrients and ingestion methods were explained in detail. Each subject was also instructed to adequately expose his/her body to sunlight because exposure to ultraviolet rays

Table 2. Contents of the calendar for recording

Items	Points
Scores regarding diet	
Ingestion of 3 nutrients	4
Ingestion of 2 nutrients	3
Ingestion of 1 nutrient	2
No intention to ingest each nutrient	1
No record (blank column)	0
Scores regarding sun exposure	
Exposure to the sun for 1 hour or more	4
Exposure to the sun for 30 minutes to 1 hour	3
Exposure to the sun for 30 minutes or less	2
No sun exposure	1
No record (blank column)	0
Scores regarding exercise	
Pilates was performed in addition to other types of exercise	4
Pilates was performed	3
Exercise other than Pilates was performed	2
No exercise	1
No record (blank column)	0

from sunlight leads to the synthesis of vitamin D in the skin (*7*). Adequate walking or low-intensity exercise (Pilates) was recommended for exercise. Each subject was instructed to perform exercise involving Pilates in order to improve articular stability and muscular mobility through trainers' guidance 3 times a week for 6 months after the start of the intervention. A calendar was delivered to record diet, sun exposure, and exercise every day in Period I, but not in Period II.

2.4. *Data analysis*

The paired *t*-test was performed in order to analyze the group consisting of subjects who participated in this study in Period I (1^{st} to 4^{th} sessions of measurements: 40 subjects) and the group consisting of subjects who participated during Periods I and II (1^{st} to 5^{th} sessions of measurements: 23 subjects). A p-value < 0.05 was regarded as significant.

3. Results

3.1. *Subject information*

Subject information is presented in Table 1. Fifty subjects initially participated in the 1^{st} session of measurements. Of these, 40 participated in all measurement sessions (1^{st} to 4^{th} sessions) in Period I. BMD was measured in these subjects (54 to 85 years, mean: 69 ± 8.6 years) using %YAM as an index according to the diagnostic criteria prepared by the Japan Osteoporosis Society. Age-plotted results are shown in Figure 1. Mean BMD was 67.1%YAM.

3.2. *Presence or absence of the intervention by pharmacists*

Serial changes (●) in %YAM (n = 40) in the presence of the intervention by pharmacists in Period I and those (○) in %YAM (n = 23) in its absence during Periods I and II are shown in Figure 2. Mean %YAM values in the presence of the intervention 3 and 6 months after the start of measurements were 70.8 and 74.5%YAM, respectively, and were significantly higher than that at the start of measurements (67%YAM) ($p < 0.05^{*}$ and $p < 0.01^{**}$, respectively). However, the intervention by pharmacists increased %YAM by 7.5% in Period I, whereas a 4.3% decrease was observed after the completion of Period II (74.5 → 70.2%YAM) ($p < 0.01^{**}$).

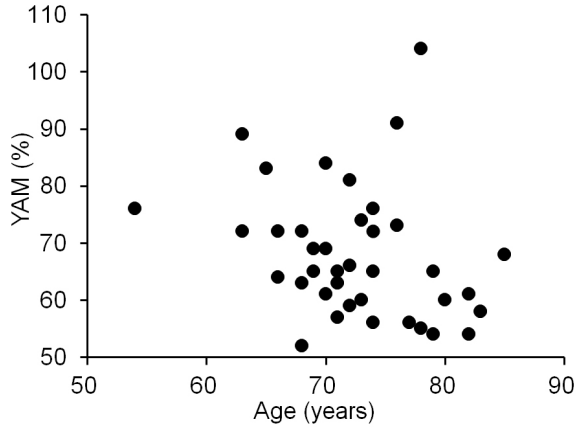

Figure 1. Relationship between YAM (%) and age before the intervention. (n = 40)

3.3. *Effects of the dietary intervention*

Serial changes in %YAM (n = 40) in the presence of the dietary intervention by pharmacists in Period I are shown in Figure 3. Group A (●) showed a score of ≥ 3

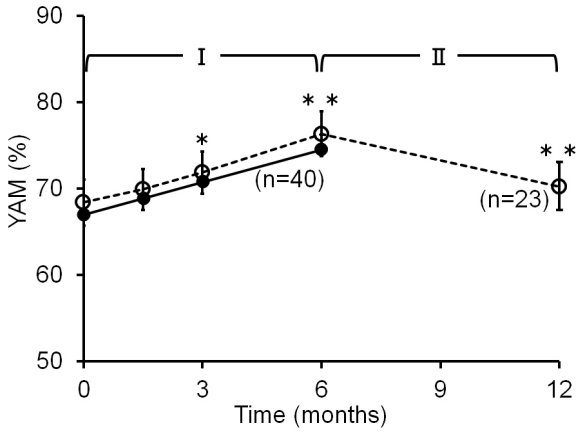

Figure 2. Effects of the pharmacist intervention on YAM (%). Solid line (●): 6 months (n = 40), Dotted line (○): 12 months (n = 23), paired t-test: $p < 0.05^{*}$, $p < 0.01^{**}$, With (I) and without (II) the pharmacist intervention.

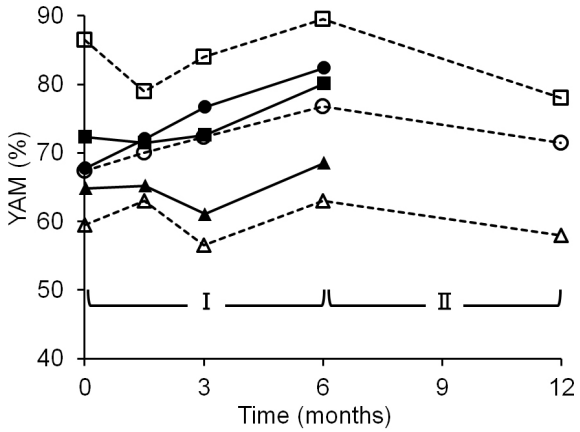

Figure 3. Effects of various food habit points on YAM (%). Solid line: 6 months (n = 40); Group A (●): Food habit points are more than 3. (n = 30), Group B (■): Food habit points are less than 3 and more than 2. (n = 6), Group C (▲): Food habit points are less than 2 and more than 0. (n = 4). Dotted line: 12 months (n = 23); Group A (○): Food habit points are more than 3. (n = 19), Group B (□): Food habit points are less than 3 and more than 2. (n = 2), Group C (Δ): Food habit points are less than 2 and more than 1. (n = 2). With (I) and without (II) pharmacist intervention.

points. In Group B (■), scores ranged between 2 and 2.9 points. In Group C (▲), scores ranged between 0 and 1.9 points. The "diet" score was calculated based on the calendar recorded by each subject every day through the 1st to 4th sessions of measurements in Period I. As shown in Figure 3 (solid lines), %YAM increased linearly until the 4th session of measurements (6 months after the start of measurements) in Group A (●) (n = 30). In Groups B (■) (n = 6) and C (▲) (n = 4), %YAM slightly increased from the 3rd session of measurements (3 months after the start of measurements). %YAM (n = 23) in Periods I and II are shown as dotted lines in Figure 3. In Group A (○) (n = 19), %YAM increased linearly until the 4th session of measurements in Period I. In Groups B (□) (n = 2) and C (Δ) (n = 2), a decrease was observed in the 2nd or 3rd session of measurements, whereas an increase was noted in the 4th session of measurements. However, all groups showed a decrease in the 5th session of measurements in Period II. The values obtained in Groups B (□) and C (Δ) returned to those at the start of measurements.

3.4. *Effects of the sun exposure intervention*

Serial changes in %YAM (n = 40) in the presence of the sun exposure intervention in Period I are shown in Figure 4. As indicated by the solid lines, %YAM increased linearly until the 4th session of measurements in Groups A (●) (n = 11), B (■) (n = 27), and C (▲) (n = 2). %YAM (n = 23) in Periods I and II are shown as dotted lines in Figure 4. In Groups A (○) (n = 9), B (□) (n = 13), and C (Δ) (n = 1), %YAM increased linearly until the 4th session of measurements, whereas a decrease was observed in the 5th session of measurements. The value in Group B (□) returned to that at the start of measurements.

3.5. *Effects of the exercise intervention*

Serial changes in %YAM (n = 40) in the presence of the exercise intervention in Period I are shown in Figure 5. As indicated by the solid lines, %YAM increased linearly until the 4th session of measurements in Group A (●) (n = 30). In Groups B (■) (n = 6) and C (▲) (n = 4), an increase was observed in the 4th session. %YAM (n = 23) in Periods I and II are shown as dotted lines in Figure 5. In Groups A (○) (n = 4), B (□) (n = 17), and C (Δ) (n = 2), a slight decrease was noted in the 2nd or 3rd session of measurements, whereas an increase occurred in the 4th session. However, %YAM subsequently decreased until the 5th session, similar to that observed for the dietary and sun exposure interventions.

3.6. *Comprehensive effects of the intervention*

Serial changes in %YAM (n = 40) in Period I with respect to the total score of "diet", "sun exposure", and "exercise" are shown in Figure 6. As indicated by the solid lines, %YAM increased linearly until the 4th session of measurements in Groups A (●) (n = 16) and B (■) (n = 23). In Group C (▲) (n = 1), a decrease was observed in the 3rd session (3 months after the start of measurements), whereas a slight increase was noted in the 4th session. %YAM (n = 23) in Periods I and II are shown as dotted lines in Figure 6. In Group A (○) (n = 11), a decrease occurred in the 3rd session of

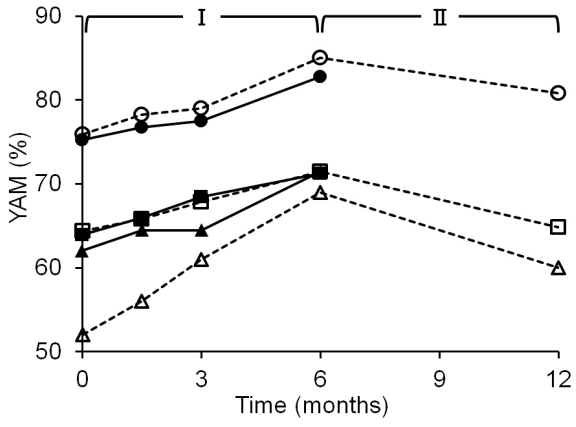

Figure 4. Effects of various sun exposure habit points on YAM (%). Solid line: 6 months (n = 40); Group A (●): Sun exposure habit points are more than 3. (n = 11), Group B (■): Sun exposure habit points are less than 3 and more than 2. (n = 27), Group C (▲): Sun exposure habit points are less than 2 and more than 0. (n = 2). Dotted line: 12 months (n = 23); Group A (○): Sun exposure habit points are more than 3. (n = 9), Group B (□): Sun exposure habit points are less than 3 and more than 2. (n = 13), Group C (Δ) Sun exposure habit points are less than 2 and more than 1. (n = 1). With (I) and without (II) pharmacist intervention.

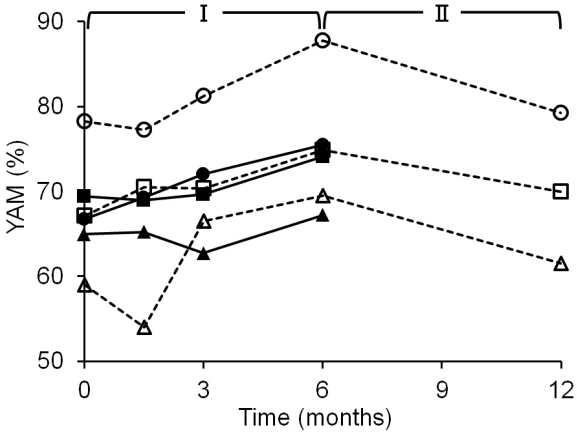

Figure 5. Effects of various fitness habit points on YAM (%). Solid line: 6 months (n = 40); Group A (●): Fitness habit points are more than 3. (n = 30), Group B (■): Fitness habit points are less than 3 and more than 2. (n = 6), Group C (▲): Fitness habit points are less than 2 and more than 0. (n = 4). Dotted line: 12 months (n = 23); Group A (○): Fitness habit points are more than 3. (n = 4), Group B (□): Fitness habit points are less than 3 and more than 2. (n = 17), Group C (Δ): Fitness habit points are less than 2 and more than 1. (n = 2). With (I) and without (II) pharmacist intervention.

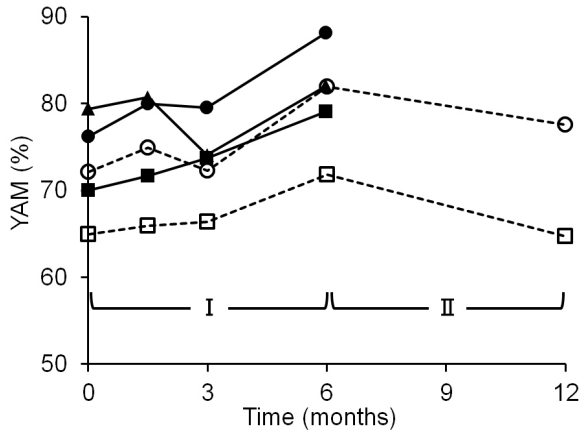

Figure 6. Effects of overall habit points on YAM (%). Solid line: 6 months (n = 40); Group A (●): Overall habit points are more than 3. (n = 16), Group B (■): Overall habit points are less than 3 and more than 2. (n = 23), Group C (▲): Overall habit points are less than 2 and more than 0. (n = 1). Dotted line: 12 months (n = 23); Group A (○): Overall habit points are more than 3. (n = 11), Group B (□): Overall habit points are less than 3 and more than 2. (n = 12). With (I) and without (II) pharmacist intervention.

measurements, whereas an increase was noted in the 4th session. In Group B (□) (n = 12), %YAM serially increased until the 4th session of measurements. No subject was assigned to Group C (Δ). In all groups, a decrease was observed in the 5th session (12 months after the start of measurements). The value in Group B (□) (n = 13) returned to that at the start of measurements.

4. Discussion

Regarding the effects of diet, the balanced ingestion of calcium (*8,9*)/vitamin D (*10*)/vitamin K (*11*) is recommended by the "Japanese 2015 Guidelines for Prevention and Treatment of Osteoporosis" (*1*), and several studies have reported that it increases BMD, thereby preventing fractures (*12-14*). In the intervention performed in the present study, a leaflet was delivered to visually promote understanding, and guidance through detailed explanations was conducted. Subjects were instructed to ingest milk products, soybean products, small fish, and seaweed as foods containing high levels of calcium, fish (particularly bluefish), salmon, dried mushrooms, and wood ear mushrooms as foods containing high levels of vitamin D, and fermented soybeans, vegetables (green vegetables), liver, and cheese as foods containing high levels of vitamin K. They were also instructed to avoid excessive consumption of foods containing high levels of phosphorus (processed foods and some soft drinks), salt, foods containing high levels of caffeine (coffee and tea), and alcohol (*15*).

As shown in Figure 3, BMD increased linearly in Group A (●) in which 3 nutrients (calcium, vitamin D, and vitamin K) were consciously ingested after pharmacists recommended foods that promote an increase in BMD. However, no increase was observed in BMD until the 3rd session of measurements (3 months after the start of measurements) in Group B (■) or C (▲) without this intervention. This result suggests the importance of intentional nutrient ingestion. In addition, in Groups A (○) and B (□), a decrease was observed in BMD after the completion of Period II (in the 5th session of measurements). However, in Group B (□), the value was lower than at the start of measurements, whereas there was a gradual rebound in Group A (○). This result reflects subjects in Group A (○) complying with pharmacists' instructions, indicating that it is important for pharmacists to maintain subject motivation at a high level.

Regarding the effects of sun exposure, vitamin D is ingested from meals or synthesized in the skin through exposure to ultraviolet rays. Therefore, adequate sun exposure is recommended in addition to a balanced diet (*7,10*).

As shown in Figure 4, %YAM increased linearly in Groups A (sun exposure for 30 minutes or more/day), B (sun exposure for 15 to 30 minutes/day), and C (sun exposure for 0 to 15 minutes/day) following pharmacists' recommendations. On the other hand, a decrease was observed in %YAM in each group after the completion of Period II (in the 5th session of measurements). In Group A (○), there was a gradual rebound, similar to that observed with the dietary intervention. The necessity of adequate exposure to sunlight for approximately 15 minutes/day is emphasized by the guidelines (*1*). The results obtained in the present study suggest the importance of consciously going outdoors, even for a short time, during the daytime.

Regarding the effects of exercise, a larger number of clinical studies have compared the influence of exercise interventions on BMD in healthy adults than in patients with osteoporosis. A previous study reported that aerobic exercise or walking increased lumbar vertebral and proximal femoral BMD (*16*). Another study investigating the prevention of decreases in BMD indicated that high-intensity loading on bones, such as weight training, prevented a decrease in %YAM (*17*). However, it may not be realistic to recommend weight training for subjects (particularly the elderly) in community medicine. In the present study, the mean age of subjects was 69 years, and mean BMD at the start of measurements was 67.1%; therefore, aerobic exercise was not conducted, and low-intensity Pilates involving stretching was performed 3 times a week for 6 months in Period I.

As shown in Figure 5, %YAM increased linearly in Group A (other types of exercise were conducted in addition to Pilates at a high frequency) after the exercise intervention by pharmacists. However, it slowly increased in Groups B (other types of

exercise were conducted in addition to Pilates at a low frequency) and C (absence of exercise). Since BMD is measured at the calcaneus, calcaneal loading related to the continuation of exercise based on a fixed program may have been reflected by an increase in BMD in Group A (●). On the other hand, the value in Group A (○) after the completion of Period II (in the 5th session of measurements) was similar to that at the start of measurements, as demonstrated for diet and sun exposure, suggesting that a rebound phenomenon occurs in a relatively short period, in contrast to the results obtained for diet and sun exposure.

Regarding comprehensive effects, subjects were classified into Groups A to C based on the total score of "diet", "sun exposure", and "exercise" as comprehensive parameters (Figure 6). As shown in Figure 6 (solid lines), an increase was observed in BMD in Groups A (●) and B (■), whereas a similar value to that before the start of the intervention was noted in Group C (▲); in subjects complying with the guidance of pharmacists, the effects achieved were reflected by numerical data, and a marked difference was observed between these subjects and those who did not comply with the guidance provided. As indicated by the dotted lines in Figure 6, the rebound phenomenon slowly decreased, even after completion of the intervention period in Group A (○), and final BMD was higher than that before the intervention. However, in Group B (□), it was similar to that at the start of the intervention. A comparison of subject backgrounds between Groups A (n = 11) and B (n = 12) revealed that mean ages, 71.5 and 73 years, respectively, were similar. Therefore, the intervention was more effective in the group in which the "guidance program for increasing BMD" was consciously conducted. However, the results obtained also showed that BMD decreased when consciousness for lifestyle improvements was reduced.

In the future, it may be important for a home pharmacy or pharmacist to positively and continuously perform health consultations/management for local residents in order for each pharmacy to function as a "health support pharmacy". If the importance of self-medication is recognized by individual residents under pharmacists' guidance, it may lead to activation of the entire area; therefore, pharmacists need to accomplish this goal.

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Original Article

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Olive and ginkgo extracts as potential cataract therapy with differential inhibitory activity on aldose reductase

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Summary **Aldose reductase (AR) has been the leading target in the treatment of diabetic cataract. Although numerous synthetic AR inhibitors (ARI) have been identified, their adverse side effects currently preclude their use. Olive leaf extract (OLE) as well as ginkgo leaf extract (GLE) are natural supplements that have wide therapeutic indices and a plethora of salutary effects during diabetes that so far untested on sugar cataract progression. As such, the present study sought to evaluate the AR-inhibiting properties of OLE and GLE using the isolated enzyme from rabbit lens. Biochemical analyses revealed that both OLE and GLE inhibited rabbit lens AR activity in a concentration-dependent manner with half maximal inhibitory concentration (IC_{50}) 65 µg/mL and 72.5 µg/mL, respectively. Interestingly, the results of kinetic studies exhibited a differential pattern of inhibition by these two extracts. While an non-competitive inhibition of AR was promoted by OLE recognized by significant decrease in the apparent maximum velocity (V_{max}) (0.12 ± 0.009677 µM/min *versus* 0.278 ± 0.0013677 µM/min) without significant change in Michaelis constant (Km), the GLE showed a competitive pattern of inhibition characterized by significant increase in apparent Km (4.4 ± 0.0068 µM), without change in V_{max} value. It would appear that these classes of natural extracts represent effective and safe therapeutic options that hold the great promise for treatment not only diabetic cataract, but also other ocular diseases characterized by uncontrolled AR activity.**

Keywords: Rabbit lenses, polyol pathway, aldose reductase, NADPH, enzyme inhibiting, cataract, olive extract, ginkgo extract

1. Introduction

Diabetes, the silent killer, is the biggest national health threat due to its deadly and costly complications despite appropriate therapeutic measures. Among the most common secondary diabetic complications is cataract, the opacity of the lens that produces painless gradual loss of vision (*1*). Diabetic cataract is usually acquired during persistence hyperglycemia through increasing the glucose level greatly in eye lens where glucose entry is independent of insulin. This excess glucose is metabolized *via* an accessory pathway known as the polyol pathway. Activation of this pathway leads to the accumulation of the osmolyte sorbitol in eye lens resulting in osmotic swelling and subsequent hydropic lens fibers that degenerate to form sugar cataracts (*2*). Aldose reductase (AR) is the rate-limiting enzyme of the polyol pathway that catalyzes a NADPH-dependent reduction of a glucose to sorbitol and is found abundantly in eye lens (*3*). Nevertheless, this enzyme has a low affinity for glucose, and little substrate is processed under physiological conditions but its activity is more pronounced with chronic hyperglycemia (*4*). As such, pharmacologic regulation of AR activity is a rational approach to modulate early pathological pathways associated with cataract genesis long before the occurrence of vision loss among diabetics. This view has been strengthened recently by the finding

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that the administration of AR inhibitors at the onset of diabetes prevents diabetic cataract formation in experimental animals (*5*).

While a number of structurally diverse compounds inhibit AR significantly, many of these compounds possess poor pharmacokinetic properties or side-effects not associated with the specific inhibition of AR (*6*). These side effects range from severe allergic reactions with sorbinil (*7*), impaired kidney function with zenarestat (*8*), to alteration of liver function with tolrestat (*9*). These problems reflect a need for the development of new, more effective and safe drug which may rise the therapeutic benefits for diabetic patients. As there is still no safe AR inhibitor (ARI), there is a renewed interest in recent times to use plant-based medicine in the area of health care owing to its efficient, safe, and economic features.

Several plant species have been used for prevention or managing diabetes by the native Americans, Chinese, Africans, South Americans, and Asian Indians. However, a limited number of medicinal plant species have been studied and validated for their hypoglycemic properties using laboratory diabetic animal models and in clinical studies using human subjects. Among the antidiabetic plants recommended by traditional practitioners are olive and ginkgo on which the retrieved research has focused to utilizing them as alternatives to current antidiabetic therapies. However, they have not been investigated for their beneficial effects on diabetes cataract.

There are several studies have been conducted on the efficacy of the olive leaf extract (OLE). These studies proved that OLE has a powerful effect on lowering blood glucose level (*10*). Similarly, ginkgo (*Ginkgo biloba*) is one of the oldest living tree species and its leaves are among the most extensively studied herbs in use today. In Europe and the United States, ginkgo supplements are among the best-selling herbal medications. Interestingly, Kudolo and his colleagues provided evidence for lowering hyperglycemia among diabetic patients treated by *Ginkgo biloba* (*11-12*). However, no thoroughly study to date has investigated the independent and combined effects of these extract on polyol pathway and its subsequent sugar cataract progression. Therefore, this study sought to pursue the protective effect of both OLE and GLE on AR activity and to gain insight into the inhibition mechanism involved therein.

2. Materials and Methods

2.1. *Materials*

DL-glyceraldehyde (GA), lithium sulfate, reduced nicotinamide adenine dinucleotide phosphate (NADPH) and Dimethylsulfoxide (DMSO), were obtained from Sigma Chemical Company (Sigma-Aldrich, St Louis, MO, USA). All other chemicals and solvents were of analytical grade and were obtained from local company (Al-Gomheria Co., Mansoura, Egypt).

2.2. *Extracts preparation*

Total extract of olive leaf was obtained by macerating dried leaves with 70% methanol at 25°C for 48 hours, the extract was evaporated under vacuum at 45°C; then the dried powder was dissolved in DMSO to prepare the stock solution of 1 mg/mL. Extract of Ginkgo leaves was ready obtained as dried powder from (Pharco-Pharm Company, Cairo, Egypt) and then dissolved in DMSO to prepare the stock solution of 1 mg/mL.

2.3. *Preparation of crude AR from rabbit lenses*

Following the method of Pottinger, 1967, with some modifications (*13*), twenty rabbits of 2 months old and average weight of 1.5 kg were purchased from local Cuniculture (Mansoura, Egypt). Animal care and protocols were in accordance with and approved by Institutional Animal Ethics Committee. Immediately after rabbits were killed by decapitation, the eyes were removed and placed in a dish of isotonic saline. Lenses were dissected by posterior approach and homogenized in 10 volumes of 100 mM potassium phosphate buffer pH 6.2. The homogenate was centrifuged at 15,000× g for 30 min at 4°C and the resulting supernatant was used as the source of AR and stored in -20°C until used.

2.4. *Determination of anti-AR activity in vitro*

The reaction mixture was prepared at (25 ± 1°C), with a total volume of 2.3 mL cuvette, containing Na-K phosphate buffer (pH 6.8), 0.05 mM NADPH, 0.02 M LiSO4, enzyme preparation equivalent to 0.8 g protein, and 0.01 M GA as a substrate with or without plant extracts. The reaction was initiated by addition of NADPH and continued by 10 min. The change in the absorbance (Abs) at λ_{max}, 340 nm due to NADPH oxidation was followed in a Bio-lab spectrophotometer (Biolab Scientific Ltd, Ontario, Canada). A negative control (Neg. Ctrl) was prepared using DMSO (the solvent of extracts) in phosphate buffer (pH 6.8). Various concentrations of inhibitors were added to the assay mixture and incubated for 5-10 min before initiating the reaction by NADPH as described above. At the end, the inhibitory activity of the extracts was calculated using the following formula:

$$\% \text{ ARI} = \frac{\Delta\text{Abs. (Neg. Ctrl.)} - \Delta\text{Abs. (Extract)}}{\Delta\text{Abs. (Neg. Ctrl.)}}$$

The percent of inhibition with test compounds was calculated considering the AR activity in the absence of inhibitor was 100%. The concentration of each test sample giving 50% inhibition (IC_{50}) was then estimated.

2.5. *Kinetic parameters*

K_m and V_{max} of rabbit lens AR were determined with varying concentrations of glyceraldehyde in the absence and presence of OLE or GLE. K_m and V_{max} were estimated by Lineweaver-Burk double reciprocal plots. Inhibition constant (Ki) for each extract was determined using the following formulas:

$$\text{Ki for competitive inhibitor} = \frac{\text{Km [I]}}{(\text{Km}_{app}\text{-Km})}$$

$$\text{Ki for non-competitive inhibitor} = \frac{\text{Vmax}_{app}\text{ [I]}}{\text{Vmax- Vmax}_{app}}$$

2.6. *Data analysis*

The results were expressed as mean ± SD. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by the post hoc test (Fisher's PLSD) when indicated. Significance was defined as $p < 0.05$.

3. Results

3.1. *Validation of the analytical method used*

AR which is found primarily in the eye lens is the key relay in the polyol pathway that is initiating sugar cataract formation. Rodent lens is known to have the highest AR activity compared to other species (*14*). Therefore, we have assessed the inhibitory potential of OLE and GLE against AR isolated from the eye lens of rabbits. In order to evaluate the activity of the isolated enzyme, we have successfully validated an analytical method by the adaptation of the procedure given by Hayman and Kinoshita with some modifications (*15*). Firstly, the validation of this method was based on the consumption of the co-factor, reduced NADPH, in the AR catalyzed-NADPH-dependent conversion of glucose to sorbitol. Under the used analytical conditions, enzymatic curves were done by monitoring the absorbance change of NADPH to NADP+ using absorbance spectroscopy. The starting spectrum was characteristic of NADPH, which absorbs light at both λ_{max}, 260 and 340 nm, whereas NADP+ only absorbs light at λ_{max}, 260 not 340 nm (Figure 1A). Moreover, linear and concentration-dependent dose-response trends of the absorbance were seen at 340 using the values obtained from the standards NADPH (Figure 1B). This dose-response curve was then used to calculate the concentration of the consumed NADPH during AR activity and hence the velocity of the enzymatic reaction which was monitored by measuring the λ_{max} 340 nm absorbance decrease as a function of time (Figure 1C).

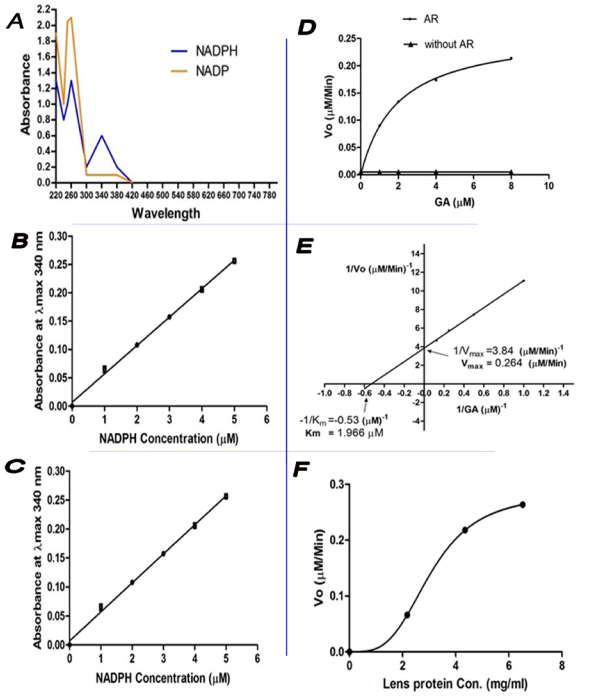

Figure 1. Validation of the analytical method used. (A) Absorbance spectra of NADPH and NADP. **(B)** Dose response at λ_{max}, 340 nm, for different NADPH concentrations. **(C)** Time resolved absorbance response of NADPH at λ_{max}, 340 nm obtained for 1, 2, 4, 8 mM of DL-glyceraldehyde (GA) as a substrate in aldose reductase (AR) catalyzed reactions. **(D)** Hyperbolic curve of AR-catalyzed NADPH-dependent reactions in which the reaction velocity (Vo) is dependent on GA concentration. **(E)** Lineweaver-Burk plot of the AR catalyzed reaction with variable substrate concentrations of GA (0.05-8 µM) for determination of Vmax, the reaction velocity at saturated substrate concentration, and the Michaelis constant, K_m. **(F)** AR activity at different concentrations of lens protein measured by the rate of NADPH oxidation at λ_{max} 340 nm.

Secondly, the initial velocity (Vo) of AR-catalyzed reaction was dependent on GA concentration; this relationship is typically hyperbolic, with a linear increase at lower concentrations until the reaction approaches saturation, at which point further increases in substrate will not increase reaction velocity (Figure 1D). Two important parameters are typically calculated using kinetic assay data: V_{max} (0.264 ± 0.0137 µM/min), the reaction velocity at saturated substrate concentration, and the Michaelis constant, K_m (1.996 µM), which is a measure of the affinity of the enzyme for the substrate. These parameters are obtained through a double-reciprocal plot of velocity against GA concentration for the linear portion of the original curve (Figure 1E).

To further validate the activity of the isolated AR, we have measured Vo at various enzyme concentrations, using a substrate concentration that is well above the K_m (Figure 1F). As shown in Figure 1F, increasing the added enzyme concentration to 6 mg/ml lens protein caused a progressive increase in the rate of NADPH consumption and thereby the velocity of the reaction.

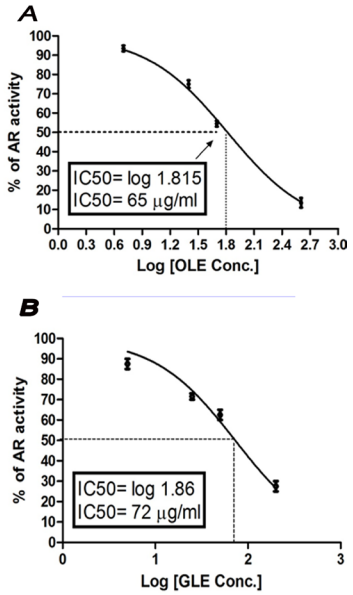

Figure 2. Representative graph for inhibiting rabbit lens aldose reductase activity by (A) olive leaf extract (OLE) and (B) ginkgo leaf extract (GLE) and the IC_{50} of each extract.

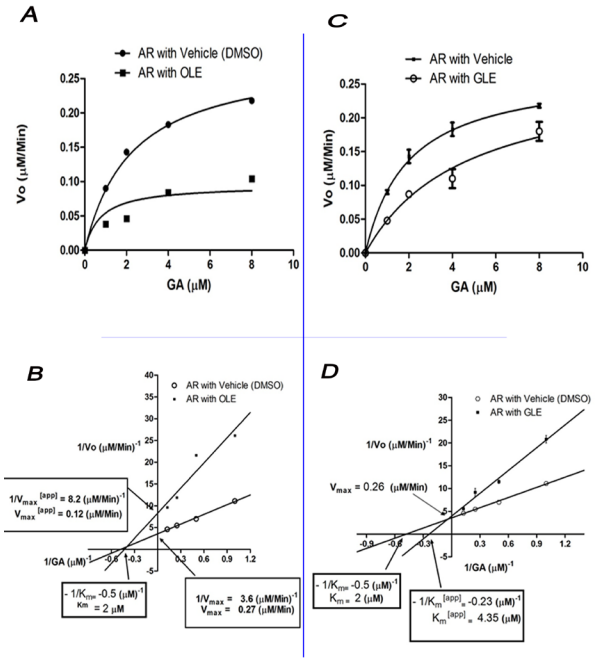

Figure 3. Kinetic data for determination the type of inhibiting aldose reductase by either OLE or GLE. (A) and **(B)** Representative hyperbolic and double-reciprocal plots, respectively, for the inhibitory effect of OLE on rabbit lens AR in the presence or absence of OLE (65 µg/mL) in three independent experiments (uncompetitive type of inhibition). **(C)** and **(D)** Representative hyperbolic and double-reciprocal plots, respectively, for the inhibitory effect of GLE on rabbit lens AR in the presence or absence of GLE (72.5 µg/mL) in three independent experiments (competitive type of inhibition).

3.2. *Olive leaf and ginkgo leaf extracts mitigated lens AR activity in a dose dependent and differential manner*

Given the primary role played by AR in the initiation of the entire sequence of cataractous change during diabetes and the fact that GLE and OLE exhibited a plethora of benefits in experimental diabetes (*16*), we aimed to investigate whether GLE and OLE, may also be effective in attenuation of AR activity. To address this point, the ability of both extracts to reduce AR activity was determined using the enzyme pre-incubated with indicated concentrations of OLE or GLE for half an hour before the addition of the substrate (GA). As shown in Figure 2A and B, extracts of both olive leaf and ginkgo inhibited rabbit lens AR activity in a concentration-dependent manner with IC_{50} values 65 µg/mL and 72.5 µg/mL, respectively.

In light of olive leaf and ginkgo extracts' anti-AR effect, interest in their mechanisms of inhibition has been expanded to explore the type of inhibition. Consequently, kinetic studies were performed and Lineweaver-Burk plots were constructed. Interestingly, the results obtained from enzyme kinetic studies exhibited a differential pattern of inhibition by these two extracts. As shown in Figures 3A and 3B, an non-competitive inhibition of AR was promoted by OLE which was recognized by its characteristic effect on the V_{max} (0.278 ± 0.0013677 µM/min) that was decreased significantly in the apparent maximum velocity (56%; 0.12 ± 0.009677 µM/min) while K_m (2 ± 0.0017 µM) did not differ as compared with the substrate GA (1.996 ± 0.0012 × 10^{-3} µM). On the other hand, GLE showed a competitive inhibition with an increased (220%) apparent K_m for GA (4.4 ± 0.0068 µM), while V_{max} remained unchanged (Figures 3C and 3D).

Next, from the aforementioned differential AR inhibitory properties of both OLE and GLE, a renewed interest in the combination anti-AR therapies has been stimulated. To underscore this point, the capacity of both extract to mitigate AR activity was evaluated individually as well as in combination therapy. As shown in Figure 4A, the combination ratio of 3:1, GLE:OLE was much more effective than either GLE or OLE alone in inhibiting AR. This finding was supported by the kinetic data in that showed a mixed type of inhibition at the ratio used because it influences both K_m and V_{max} (Figures 4B and 4C).

4. Discussion

Biochemical studies have shown that activation of AR in the eye lens is an early event that occurs in response to diabetes prior to mature cataract formation resulting in the accumulation of high concentrations of polyols. These polyols lead to excessive hydration, loss of membrane permeability, and leakage of free amino acids, glutathione, myoinositol, and others. The sequelae is a hyper-osmotic associated oxidative insult that is postulated to be the primary cause for the development of diabetic cataract (*17*). Thus, by targeting AR, the onset and progression of sugar cataract can be delayed or even

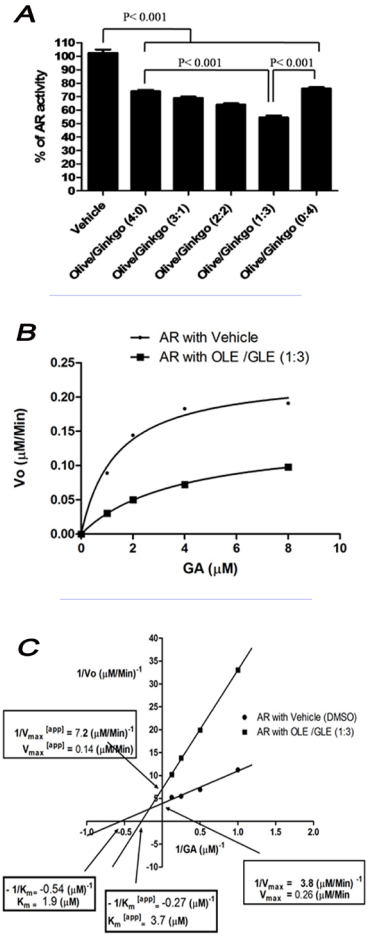

Figure 4. Representative graph for inhibiting rabbit lens aldose reductase activity by (A) different combination ratios between OLE and GLE. (B) and (C) Representative hyperbolic and double-reciprocal plots, respectively, for the inhibitory effect of the combination between OLE and GLE in 1:3 ratio on rabbit lens AR in the presence or absence of this combination in three independent experiments (mixed type of inhibition).

prevented. Unfortunately, AR inhibitors have fallen short. The problem is that AR inhibitors are very effective at preventing cataracts but they have been eliminated from any eye disease-related clinical trials because of adverse side effects (*6*). When diabetic patients were treated with the AR inhibitor sorbinil, enhancement of the nerve conduction velocity was observed (*18*), but generally, the effect had shown to be modest with major adverse reaction of hypersensitivity similar to that seen with other hydantoins. Even though no clinically important adverse reaction was observed with ponalrestat, the subsequent AR inhibitor of a different structure, it failed to provide beneficial effects in randomized controlled study (*19*). Tolrestat, another class of inhibitors, showed increased serum levels of alanine aminotransferase or aspartate aminotransferase as side effect (*20*). Because of the inability to demonstrate efficacy in the multicenter double blind studies, the clinical development of tolrestat was eventually withdrawn.

In the present study, we put forward a new evidence for introducing Olive and Ginkgo leaf extracts as natural AR inhibitors. These natural extracts have wide therapeutic index with very low toxicity (*21-23*) and their use have shown a plethora of salutary effects in different animal models of experimental diabetes.

Chronic treatment with OLE inhibited the high glucose-induced neural damage and suppressed diabetes-induced thermal hyperalgesia observed in diabetic rat (*24*). Additionally, the chronic treatment with GLE improved the vascular function in diabetic nephrotic patients (*25*). Recently, OLE has been reported for its potential hypoglycemic activity following chronic systemic administration in overweight middle-aged men who are at risk of developing the metabolic syndrome as well as in experimental animals (*10,26*). Likewise, GLE has been shown to possess anti-hyperglycemic activity in streptozotocin (STZ)-induced diabetic rats (*27*). These anti-diabetic effects are mediated *via* preservation of insulin positive β-cells and restoration of the glucose metabolic enzyme activities (*28, 29*).

In our *in vitro* studies, GLE showed a remarkable inhibition of AR suggesting the presence of potential enzyme inhibiting compound(s) in the extract. To find the mechanism of inhibition, we have formulated double reciprocal plot from the kinetics data and the results indicate the competitive mode of inhibition by GLE with a Ki value of 62.5 μM. On the other hand, OLE inhibited the AR activity non-competitively with a Ki value of 114.36 μM. In other words, although AR inhibition by GLE can be overcome by adding higher concentrations of substrate, no amount of substrate can overcome AR inhibition by OLE. Moreover, their combination was much more effective than either GLE or OLE alone, a finding that was supported by the kinetic data.

Collectively, the experiments in this study provide new insights into the mechanisms of anti-cataraceous effects of both GLE and OLE by attenuating AR activity in a differential manner. Consequently, it would appear that these classes of natural extract represent effective, safe and well tolerated therapeutic options that hold the best hope for treatment lens diseases characterized by uncontrolled NADPH-dependent AR activity.

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Case Report

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Rivaroxaban-induced chest wall spontaneous expanding hematoma

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Summary **Rivaroxaban is an oral direct Factor Xa inhibitor approved in the European Union and the United Sates for the single-drug treatment of several thromboembolic diseases in adults. It has been evaluated in large phase III clinical trials and has been found to have similar efficacy and safety with standard therapy. Herein, is described a very rare case of a rivaroxaban-induced spontaneous expanding chest wall hematoma, that required surgical intervention, in a breast cancer patient. Use of the Naranjo adverse drug reaction probability scale indicated a probable relationship (score of 7) between the patient's development of hematoma and treatment with rivaroxaban. Physicians should be cautious when prescribing rivaroxaban in groups of patients associated with increased bleeding risk such as patients with impaired renal or hepatic function, hypertension, coronary heart disease, heart failure, patients with certain types of cancers and patients receiving concomitant medications which may alter the pharmacokinetic or pharmacodymamic parameters of rivaroxaban. Anticoagulant treatment should be tailored to each individual patient weighing the bleeding risk against the risk of recurrent thrombosis.**

Keywords: Rivaroxaban, hematoma, anticoagulation, bleeding, spontaneous

1. Introduction

Rivaroxaban is an oral direct Factor Xa inhibitor that has been approved for the prevention of venous thromboembolism in patients undergoing elective hip or knee replacement surgery, for stroke prevention in patients with nonvalvular atrial fibrilation and for the treatment and secondary prevention of recurrent deep vein thrombosis and pulmonary embolism (*1,2*). Bleeding is the most common complication of anticoagulant therapy (*3*). Patients with cancer have an increased risk of venous thromboembolism compared to patients without cancer and they are at increased risk of bleeding during anticoagulant therapy (*4,5*). Herein is described a case of a rivaroxaban induced spontaneous expanding chest wall hematoma that required surgical intervention, in a breast cancer patient.

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2. Case Report

A 67-year-old Caucasian female presented with a 24-hour history of acute chest wall pain associated with a palpable mass and extensive bruising in a previous left mastectomy site. She had undergone a left modified radical mastectomy five months prior to the current admission for a pT3N3aM0 invasive ductal breast carcinoma, after neoadjuvant chemotherapy. Chemotherapy consisted of four cycles of fluorouracil, epirubicin and cyclophosphamide (FEC) followed by four cycles of docetaxel. One month prior to surgery she developed left lower extremity deep vein thrombosis for which she was treated with rivaroxaban 15 mg twice daily for three weeks, followed by 20 mg daily. Preoperatively a retrievable inferior vena cava filter was inserted. Postoperatively, she received adjuvant radiotherapy and hormonal therapy with letrozole, an aromatase inhibitor.

She was unable to recall any trauma, whereas she did not have any history of bleeding manifestations and there was no family history of bleeding disorders. In adittion, she had no history of any chronic illness and she was not taking any medications apart from letrozole 2.5 mg daily.

Physical examination revealed a left chest wall

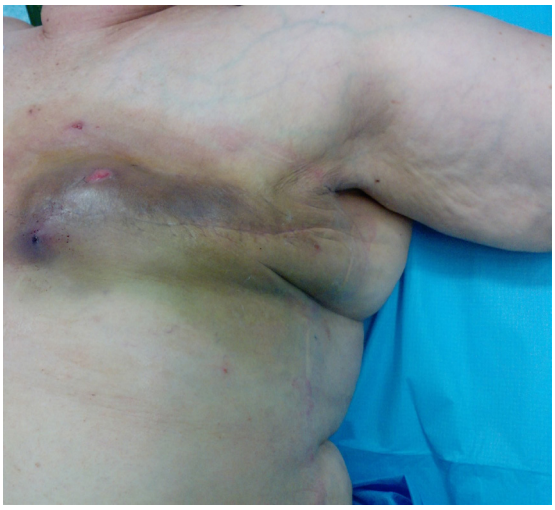

Figure 1. Photo showing signs of chest wall hematoma in a previous left mastectomy site. Signs of skin ischemia are also noted.

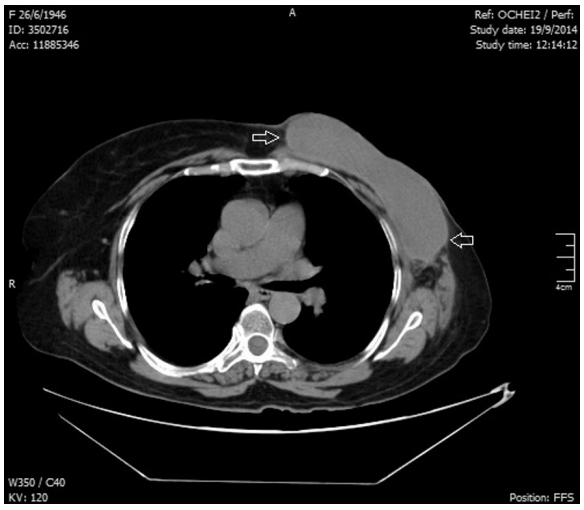

Figure 2. Contrast enhanced CT scan of the chest demonstrating a large left chest wall hematoma measuring 17 × 8 × 5 cm (arrows).

painful swelling associated with extensive ecchymosis (Figure 1). A complete blood count revealed a hematocrit of 32.4%, a white blood cell count of 5.600 × 10^3 mL, a hemoglobin level of 10.5 × 10^3 dL, and a platelet count of 200 × 10^3 mL. Coagulation studies revealed: international normalized ratio (INR): 0.95 (range 0.85-1.15) and activated partial thromboplastin time (APTT): 28.2s (range 26-38). Liver function tests and renal function tests were within normal limits. Blood Urea: 38.7 mg/dl (range 20.0-50.0) and serum creatinine: 1.00 mg/dl (range 0.20-1.50).

A contrast enhanced computed tomography (CT) scan of the chest revealed a large cutaneous hematoma measuring 17 × 8 × 5 cm in the previous left mastectomy site (Figure 2). There were no signs of local or regional recurrence of breast cancer. Rivaroxaban was discontinued and a local compression of the hematoma was applied. Despite compression, the hematoma continued to expand and signs of skin ischemia were noted and we therefore decided to proceed with hematoma evacuation and exploration. During surgery a large amount of blood clots was evacuated and a thorough irrigation of the large cavity was performed. A thorough inspection ruled out local recurrence from breast cancer. No bleeding vessel was found but a diffuse oozing was noted. The incision was closed after a drain was placed and compression was applied. The patient had an uncomplicated postoperative course without any signs of recurrent bleeding. During hospital stay a thorough investigation for haemorrhagic disorder was negative. She was discharged three days later after her anticoagulant therapy was modified. She is well without any signs of recurrent bleeding 23 months after the hematoma formation.

3. Discussion

Rivaroxaban has gained approval for the treatment and prevention of venous thromboembolism in adults. It has been evaluated in large phase III trials and has demonstrated non inferior or superior efficacy with a similar safety profile to current treatment standards (*2,3,6*). It has certain advantages over traditional agents.

Rivaroxaban has predictable pharmacokinetic and pharmacodynamic parameters thus allowing a fixed dose without the need of routine coagulation monitoring. It additionally has low potential for drug and food interactions. It has a rapid onset of action, reaches maximal plasma concentration 2-4 hours after administration and has high bioavailability (*2,6*). After administration, two thirds of the drug is metabolized to inactive metabolites in liver *via* cytochrome P450 (CYP) 3A4/A5 and CYP2J2, half of which is excreted through the kidneys and other half is excreted *via* the fecal route. The other one-third is excreted unchanged by the kidneys (*1*). Rivaroxaban, however, is contraindicated in patients with severe renal impairment and in patients with hepatic disease associated with coagulopathy and clinically relevant bleeding risk (*1*).

The acute deep vein thrombosis (DVT) randomized study enrolled 3449 patients and compared the efficacy and safety of rivaroxaban with standard treatment with enoxaparin and a vitamin K antagonist in patients with acute symptomatic DVT (*6*). The authors suggested that oral rivaroxaban at 15 mg twice daily for three weeks followed by 20 mg daily may provide an effective single drug approach to the initial and continued treatment of venous thrombosis (*6*). The proportion however, of patients with active cancer at the time of enrolment was 7% for both groups thus indicating that more data are needed in this subgroup (*6*).

A prespecified pooled analysis of the EINSTEIN DVT and EINSTEIN pulmonary embolism (PE) studies involving 8,252 patients suggested that simple drug therapy with rivaroxaban resulted in similar efficacy to standard therapy and was associated with a significantly

lower rate of major bleeding (*3*). The reduction was mainly seen in fatal and non fatal critical site bleeding such as intracranial and retroperitoneal bleeding. In fragile patients the incidence of major bleeding was reduced from 4.5% with standard treatment to 1.3% with rivaroxaban therapy, while in cancer patients the incidence of both bleeding and recurrent DVT tended to be lower in the rivaroxaban group (*3*).

Bleeding complications are frequent in patients treated with rivaroxaban but mainly consist of minor bleeding events (*7*). In the large prospective noninterventional oral anticoagulation registry of daily care patients (Dresden NOAC registry) with 1,775 rivaroxaban patients enrolled, major bleeding represented only 6% of the bleeding events. Sixty per cent of these cases managed conservatively with tamponade compression and red blood cell transfusions, while in the remaining 40% of major bleeding events a surgical or interventional treatment and rarely procoagulant treatment with prothrombin complex concentrates was required (*7*).

In a pharmacovigilance study of 27,467 patients taking rivaroxaban 496 major bleeding events occurred indicating an incidence of 2.86 per 100 person years (*8*). Major bleeding affected more frequently elder patients with hypertension, coronary heart disease, heart failure and renal disease. The most common site of bleeding was the gastrointestinal tract (88.5%) followed by intracranial bleeding (7.5%). Fourteen patients died indicating a fatal bleeding incidence of 0.08 per 100 person years (*8*).

The treatment of choice for cancer associated venous thromboembolism (VTE) is generally accepted to be at least 6 months of low molecular weight heparin (LMWH) (*4,9*). The effectiveness and safety of rivaroxaban is similar for venous thromboembolism patients with and without active malignancy, although borderline higher rates of major bleeding and non major clinically relevant bleeding have been reported in patients with cancer (*10*). In a retrospective review of 237 active cancer patients treated with rivaroxaban the authors reported that the recurrence and major bleeding events were low despite the fact that a half of the patients had metastatic disease (*9*).

Currently, there are no specific reversal agents for rivaroxaban. In addition, no prospective randomized clinical trials for patients presenting with acute bleeding have been conducted (*1*).

Discontinuation of rivaroxaban 20-30 hours before an elective surgery is sufficient to minimize the bleeding risk. In cases of severe bleeding discountinuation of rivaroxaban along with compression or appropriate surgical or interventional treatment are necesseary, while for life threatening bleeding the use of prothrombin complex concetrate is needed (*1*). In a recent study andexanet alfa, a recombinant modified human factor Xa decoy protein, reduced the anti-factor Xa activity by 92% in a series of 27 healthy older rivaroxaban treated participants within minutes after administration, without any side effects. The authors however, did not present any data on the efficacy and safety of andexanet in patients requiring urgent reversal of factor Xa inhibitor due to a bleeding or emergency surgery (*11*).

Patients with cancer receiving anticoagulant therapy have a higher bleeding risk than patients without cancer (*5,12,13*). Overall, 241 (5.1%) out of 4,709 patients with active cancer enrolled in the RIETE multicenter prospective registry, developed a major bleeding event which in most cases occurred during the first three months after the initiation of the anticoagulant therapy. The most common sites of bleeding were the gastrointestinal tract (49%), genitourinary tract (18%), and the brain (11%). One third of the patients who developed major bleeding died (*12*).

The bleeding in a cancer patient may present either as a localized bleeding diathesis as a result of tumor invasion or as a generalized hemorrhagic tendency (*13*). Apart from the known bleeding risk factors such as age and impaired renal function, cancer patients, particularly those under anticoagulation, may have specific risk factors that further influence bleeding. These factors include certain types of solid tumors such as gastric, neck or lung cancer, thrombocytopenia, platelet dysfunction, prior surgeries, metastatic disease, myelosuppressive chemotherapy and use of vascular endothelial growth factor (VEGF) receptor tyrosine-kinase inhibitors (*13*). Since there is no reported score assessing the risk of bleeding, an individual approach to assess the bleeding risk is essential when anticoagulant treatment is initiated especially in certain cancer patients, those with renal impairment, hypertension, coronary heart disease, heart failure and the very elderly (*8,12,13*).

Our patient had no history of any chronic disease and was not receiving any medication other than letrozole 2.5 mg daily. She had completed chemotherapy and radiotherapy seven and five months ago respectively. All laboratory values were within the normal range. There is no reported interaction between rivaroxaban and aromatase inhibitors. The use of rivaroxaban is however recent and all potential drug interactions may have not yet been reported. Based on the adverse drug reaction probability scale described by Naranjo *et al*. (*14*), a score of 7 indicated that the treatment with rivaroxaban was the probable cause for the development of the spontaneous chest wall hematoma in our patient.

In conclusion, physicians should be cautious when prescribing rivaroxaban in certain groups of patients associated with increased bleeding risk, such as patients with renal or hepatic impairment, hypertension, coronary heart disease, heart failure, patients with certain types of cancer and patients receiving concomitant medications which may alter the pharmacokinetic and pharmacodynamic parameters of rivaroxaban. Anticoagulant treatment should be tailored to each

individual patient weighing the bleeding risk against the risk of recurrent thrombosis.

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Case Report

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Cocoon carcinomatosa: An unusual cause of intestinal obstruction

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Summary **Abdominal cocoon, also termed sclerosing encapsulating peritonitis, is an uncommon entity associated with formation of a fibro-collagenous membrane around intestinal loops resulting in intestinal obstruction. Most reported cases are idiopathic, but many other causes have been implicated in cocoon formation, including use of continuous ambulatory peritoneal dialysis, peritoneal tuberculosis, and connective tissue disease. However, peritoneal carcinomatosis is a rarely reported entity that causes this condition. Reported here are two cases of abdominal cocoon secondary to peritoneal carcinomatosis. Both patients presented with intestinal obstruction; one underwent surgery but the other refused surgery.**

Keywords: Peritoneum, peritonitis, intestinal obstruction, abdominal pain, sclerosis

1. Introduction

Abdominal cocoon, also referred to as sclerosing encapsulating peritonitis (SEP), is related to small bowel encasement by a cocoon like fibro-collagenous sac resulting in intestinal obstruction. It can be either idiopathic or secondary. The pathogenesis of abdominal cocoon is theorized to be related to an increase in the release of fibrogenic cytokines resulting in deposition of fibrin-like material on the peritoneum (*1*). Abdominal cocoon may present with intestinal obstruction or with nonspecific symptoms like nausea, vomiting, and malnutrition. Ultrasound or computed tomography can be used for diagnosis, and scans will reveal a thick membrane-like covering around loops of the small intestine (*1,2*). Surgical intervention with resection of the membrane and adhesiolysis is the usual treatment. Peritoneal carcinomatosis occurs due to peritoneal seeding by various neoplastic diseases. In very rare cases, such peritoneal seeding can lead to formation of a membranous cocoon surrounding the bowel that causes intestinal obstruction. Such cocoon formation in peritoneal carcinomatosis is a rarely reported entity. Reported here are two cases of cocoon formation resulting from peritoneal carcinomatosis.

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2. Case Report

2.1. *Case 1*

An 18-year-old male presented with gradually increasing abdominal pain of one month's duration that was generalized and colicky in nature. The patient had features of acute intestinal obstruction (recurrent vomiting, abdominal distension, and obstipation) for 3 days before he was seen. The patient was started on anti-tubercular therapy at another facility based on a low serum-ascites albumin gradient (SAAG) and an adenosine deaminase (ADA) level of 48 U/L in ascitic fluid. On examination, the abdomen was tender and distended with the presence of nodules on palpation. An abdominal x-ray revealed dilated bowel loops with multiple air-fluid levels, and a contrast-enhanced computed tomography (CECT) scan of the abdomen revealed the presence of gross ascites with nodular peritoneal thickening, omental caking, and scalloping of the liver (Figure 1). The patient underwent an urgent exploratory laparotomy that revealed the presence of multiple nodular deposits over the peritoneum with adherent small bowel and a surrounding membranous "cocoon". The cocoon was excised and a loop ileostomy was created around 30 cm proximal to the IC junction. Histopathological examination of biopsy samples from omental deposits and the peritoneal cocoon revealed the

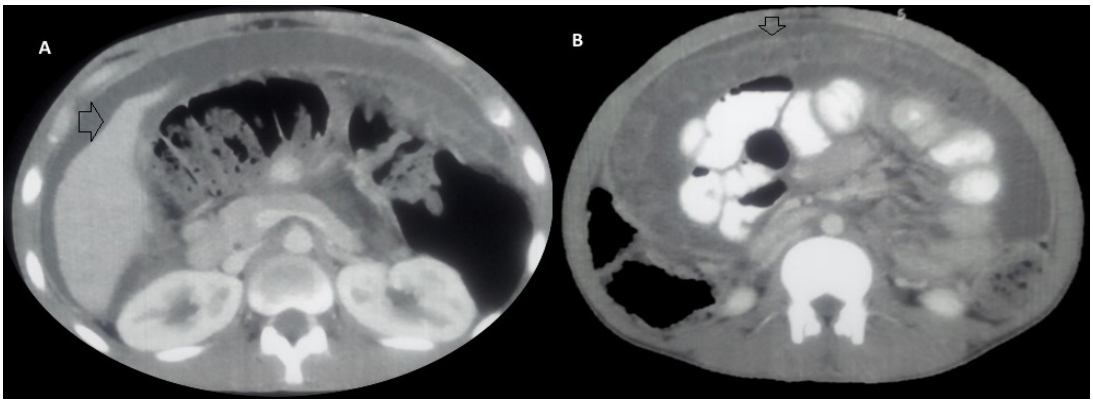

Figure 1. CT scan showing ascites, liver scalloping (A), and clumping of bowel loops with membrane (B). The arrow indicates liver scalloping **(A)** and a membrane surrounding the bowel loops **(B)**. CT, computed tomography.

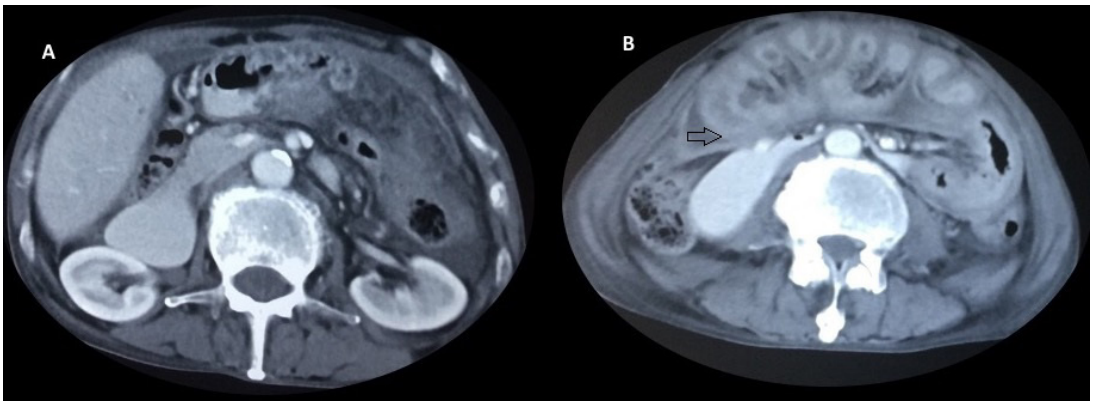

Figure 2. CT scan showing ascites (A) and clumped bowel loops encased by a membrane (B). The arrow indicates clumped bowel loops encased by a membrane **(B)**. CT, computed tomography.

presence of metastatic mucinous adenocarcinoma. The patient was advised to follow up for treatment of the primary lesion and discharged.

2.2. *Case 2*

A 70-year-old male was evaluated for gradually increasing abdominal pain of two months' duration that was generalized and colicky in nature. The patient had a history of recurrent vomiting, abdominal distension, and constipation for 15-20 days. On examination, a vague lump approximately 22 × 20 cm in size was palpable in the periumbilical area. The lump was firm, non-tender, and non-mobile in nature with surface nodularity. On ultrasonography, the bowel loops appeared clumped in the periumbilical area with a surrounding sac-like structure. Contrast-enhanced CT of the abdomen revealed the presence of peritoneal thickening with clumped small bowel loops surrounded by a membrane "cocoon". Analysis of ascitic fluid revealed the presence of a low SAAG and ADA in ascitic fluid was 13 U/L. On further evaluation, analysis of the ascitic fluid revealed the presence of metastatic adenocarcinoma. The patient was counselled for the need of a further workup and various treatment options, but the patient left treatment against medical advice.

3. Discussion

First described by Foo *et al*. in 1978, SEP is an uncommon entity (*2*). The term encompasses three components: "sclerosing", which refers to the growth of dense collagenous fibrotic tissue, "encapsulating", which refers to small bowel encasement and restricted motility due to this fibrotic process, and "peritonitis", which refers to the inflammatory changes in the peritoneal membrane, though these may not be universal (*1,2*). SEP may be idiopathic or secondary to chronic ambulatory peritoneal dialysis, prior abdominal surgery, beta blocker therapy, post-liver transplantation, abdominal tuberculosis, ventriculoperitoneal shunts, and peritoneovenous shunts (*1-5*). Patients may be entirely asymptomatic or have non-specific symptoms like nausea, anorexia, and diffuse abdominal pain or they can present with stark features of intestinal obstruction.

Plain films of the abdomen may reveal dilated small bowel loops due to obstruction and these loops may be restricted to one location due to encapsulation (*6*).

Barium studies may reveal a serpentine or concertina-like configuration of dilated small bowel loops fixed in a U-fashioned cluster often termed a "cauliflower" sign (Figure 2B). On CT, a fibrous membrane surrounding the bowel loops is usually seen. Other signs include fixation of intestinal loops, mural thickening of the bowel wall, ascites, and lymphadenopathy (*7*). On CT, peritoneal carcinomatosis can appear with thickening, nodularity, and enhancement of the peritoneum and diffuse tumor infiltration of the mesentery producing a pleated or stellate pattern. Omental caking is another key CT feature of peritoneal carcinomatosis due to replacement of the omental fat by the tumor and fibrosis. In rare cases, such seeding with fibrosis can lead to the formation of a thick membrane (cocoon) surrounding the bowel, resulting in obstructive symptoms. Peritoneal nodules can also appear hyperechoic with acoustic shadowing from psammomatous calcification (*8*).

There are only a few case reports of cocoon secondary to peritoneal carcinomatosis in the literature. Various malignancies like ovarian and colorectal carcinoma have been found to present with cocoon formation (*9,10*). In one diagnosed case of colorectal carcinoma with peritoneal carcinomatosis, cocoon formation occurred after chemotherapy and was treated surgically (*10*). Interestingly, abdominal cocoon formation in cases of metastatic gastroenteropancreatic neuroendocrine tumors (NET) has been reported in late stages and is associated with poor surgical outcomes and universal mortality. In fact, a NET-related cocoon has been reported to be a type 4 cocoon that completely encases the entire contents of the abdomen. Surgical outcomes are poor and therefore surgery is not recommended (*11*). In conclusion, abdominal cocoon may be caused by peritoneal carcinomatosis and it represents a late feature of that condition with limited therapeutic options.

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