ORIGINAL ARTICLE

Effects of the Aqueous Extract of the Chinese Medicine Danggui-Shaoyao-San on Rat Pineal Melatonin Synthesis

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Abstract

OBJECTIVES: The purpose of this study is to investigate if the aqueous extract of the Chinese medicineDanggui-Shaoyao-San (DSS) can increase the plasma level of melatonin and enhance the function of the pineal gland of naturally aged rats.
METHODS: The rats were treated with DSS at doses of 3ml or same volume of distilled water by oral administration at 11 p.m. for three weeks. The plasma level of melatonin were measured by radioimmunoassay. The function of pineal gland were measured through three parameters: pineal beta adrenergic receptor binding investigated by [3H]DHA binding; pineal expression of NAT mRNA detected by real-time RT-PCR; phosphorylation of CREB (P-CREB) and total level of CREB (T-CREB) measured by western blot analysis.
RESULTS: DSS significantly increased melatonin level at night after oral administration for 3 weeks. By measurement of pineal [3H]DHA binding, it was found DSS improved the β-adrenergic receptors binding in pineals. The stimulatory effect of DSS on the expression of NAT mRNA in the old rat pineal gland has been demonstrated in this study. Western blot analysis showed that DSS significantly increased phosphorylation of CREB.
CONCLUSIONS: Our results indicate that a downstream pathway for DSS induction of melatonin synthesis in the rat pineal gland acts via cyclic AMP-dependent cascade and transcription mechanism.

Abbreviations:

DSS	– Danggui-Shaoyao-San
[3H]DHA	– 3H-Dihydroalprenolo
AD	– Alzheimer's disease
NAT	– N-acetyltransferase
CREB cAMP	– responsive element binding protein
P-CREB	– phosphorylation of CREB
T-CREB	– total level of CREB
Aβ	– β-amyloid protein
SCN	– suprachiasmatic nucleus

INTRODUCTION

Danggui-Shaoyao-San (DSS), a famous traditional Chinese complex prescription, first recorded in 'JinKui-YaoLue' (early in the third century A.D.), consisting of six Chinese herbs (Table 1), has been widely used for amenorrhea, infertility and menopausal syndrome in China and Japan (Hagino, 1994). It has been reported that DSS significantly reduces the amyloid beta25-35-induced neuronal death (Egashira *et al.* 2005) and has free radical scavenging properties (Stefek & Benes, 1994). Clinical application of DSS to postmenopausal women with the Alzheimer type of dementia improves the cognitive functions and sleep disturbance (Fukushima, 1994; Mizushima, 1989).

The pineal gland exhibits a diurnal secretory rhythm of its hormone, melatonin, which is synchronized by N-acetyltransferase (NAT) within a rhythm (Klein & Weller, 1970). Pineal activity is paced by the suprachiasmatic nucleus of hypothalamus (Moore & Klein, 1974) via the sympathetic neurons which synapse in the superior cervical ganglion (Kappers, 1960). NE turnovers in these neurons, and thus pineal beta receptor stimulation, is within a 24-hr rhythm (Brownstein & Axelrod, 1974).Besides playing an important role as a transmitter of photoperiodic information, melatonin shows the antioxidant (Reiter *et al.* 2005), oncostatic (Vijayalaxmi *et al.* 2002), anti-aging (Reiter *et al.* 2002) and immunomodulatory (Carrillo-Vico *et al.* 2005)properties, as well as many other physiological functions (Sallinen *et al.* 2005). As aging advances, the nocturnal production of melatonin decreases in animals of various species, including humans (Waldhauser *et al.* 1998; Mahlberg *et al.* 2008). Previous study claimed that the age-related suppression of melatonin synthesis may even be greater in individuals who died of Alzheimer's disease (Skene *et al.* 1990). Many researchers believe changing melatonin levels might be an important component of aging and senescence (Reiter, 1995; Waldhauser *et al.* 1998). However, the mechanisms that underlie reducing lipid peroxidation and improving sleep disturbance by DDS are not clear. The aim of this study was to investigate whether DSS rejuvenated the function of aged rat pineal, including the plasma melatonin level, pineal beta adrenergic receptor binding, and mRNA expression of NAT.

MATERIALS AND METHODS

DSS preparation

DSS consists of six medicinal plants as shown in Table 1. Six herbs were purchased from Guangzhou Medicinal Materials Company, Guangdong Province, China, and identified by the Department of Chinese Compound Prescription of Guangzhou University of Chinese Medicine. Aqueous extract of DSS was prepared as following procedure. In brief, six medicinal materials were mixed in proportion and were macerated for 1 h with eight times (v/w) distilled water, and then decocted for 2 h, after which the filtrate was collected and the residue was decocted again for 1.5 h with six times (v/w) distilled water. The filtrates were mixed, condensed to 1g crude drug per ml and stored in 4°C. The drug was prepared one time every two days.

Animals

Sprague-Dawley rats (20-month-old, 450–500g body wt; 3-month-old, 200–250 body wt) were obtained from Center of Experimental Animal, Sun Yat-sen University. The experiment began after a 1-week preliminary period. The animal care conditions were at room temperature of 23±1°C, humidity of 55~65%, and lights-on at 6 a.m. and lights-off at 6 p.m. All animals were treated in accordance with the Guidelines for Animal Care and Use published by the Ministry of Science and Technology of P.R. China. The aged rats were divided randomly into two groups and treated with DSS at doses of 3ml (DSS group) or same volume of distilled water (aged control group) by oral administration at 11 p.m. for three weeks. The young rats were also given with distilled water orally at the same time (young control group).

Tissue samples were obtained in the 21st day. Blood samples were drawn through tail veins of every group animals at 11:30 a.m. and 11:30 p.m. At night blood were drawn in dim red light. The aged rats were sacrificed by decapitation and pineals were rapidly removed in the presence of a dim red light at 11:30p.m.–12:00p.m.

Melatonin radioimmunoassay

The melatonin level of plasma was determined with commercially available radioimmunologic assay kit (Labor Diagnostika Nord GmbH & Co. KG.). Data analysis was performed with a standard curve and half logarithmic plot of melatonin concentration versus the relationship of measured radioactivity to total activity. Melatonin concentration is measured in pg/ml.

Measurement of pineal [3H]DHA binding.

Pineal beta adrenergic receptor binding was investigated by the method of Greenberg and Weiss (Greenberg and Weiss 1978). Pineals were respectively homogenized in 750 μl of 50 mM Tris buffer, pH 8.0, containing 3 mM Mgcl. Aliquots of 250μl were incubated with various concentrations of [3H]DHA (New England

Table 1. Recipe of Danggui-shaoyao-san (DSS) formulation

Components	Ratio
1. Dang Gui (*Angelica sinensis* (Oliv.) Diels., root)	3
2. Bai Shao (*Paenonia lactiflora* Pall., root)	3
3. Chuan Xiong (*Ligusticum chuanxiong* Hort., rhizome)	2
4. Bai Shu (*Astractylodes macrocephala* Koidz., root and rhizome)	4
5. Fu Ling (Poria cocos (Schw.) Wolf., fungus nucleus)	4
6. Ze Xie (*Alisma orientale* (Sam.) Juzep., rhizome)	4

Table 2. The plasma concentration of melatonin (pg/ml, *n*=10)

	Day	Night	N-D
Young Control	8.4±0.43	39.7±2.63	31.3±2.92
Aged Control	7.3±0.28	11.4±0.53**	4.1±0.61**
DSS	8.4±0.53	27.2±1.91☆☆**	18.8±2.34☆☆**

In the daytime the concentration of melatonin had no significant difference among the three groups, but during the dark period, the increase in the old rats was much less than in the young animal. After 3-week DSS treatment, the melatonin had a rise but didn't reach the young rats' level. n-d means concentration of melatonin at night subtracts daytime concentration. The symbol (**) denotes significance between old rats and young rats at $P < 0.01$. The symbol (☆☆) denotes significance between old control and DSS-treated rats at $P< 0.01$.

Nuclear; 44.9 Ci/mmol) at 37°C for 10min in the presence or absence of 7μM dL-propranolol. Reactions were terminated by rapid dilution of the samples with cold Tris-Mgcl buffer, followed by rapid vacuum filtration through Whatman GF/C glass fiber filters. The filters were rapidly washed continually with 10 ml of cold buffer, put in 80°C for 30min to dry. Then the filters were placed in dimethyl benzene containing 3.5% PO and 0.5% POPOP and counted for radioactivity. Specific binding was defined as the difference between total binding and nonspecific binding obtained in the presence of propranolol. Specific binding was expressed in femtomoles of [3H]DHA bound per pineal.

Western blot analysis

Two pineal glands were homogenized in 50μl of protein extraction buffer [50 mmol/li Tris (pH 8), 150 mmol/l NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 mmol/l EDTA, 10 mmol/l β-glycerophosphate, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 2 μg/ml leupeptin, 0.1 mmol/l PMSF] for determination of protein content by Western blot. (Spessert *et al*. 2000) Approximately 15 μg of protein extract was separated by 10% SDS/PAGE. The gel was transblotted onto a PVDF membrane; blocked with 10% milk powder in TBST (50 mM Tris, pH 7.4, containing 150 mM NaCl and 0.05% Tween) four hours, and incubated in primary antibody (polyclonal antibodies against pCREB and total CREB: 1:1000 in TBS containing 5% skim-milk,) for 20 h in 4°C. After 3 washes with TBST, it was treated with horseradish peroxidase-labeled secondary antibody (1:2000 in TBS containing 5% skim-milk) for 2h.in room temperature. After 3 washes with TBST for 30 min, chemiluminescent substrate was added. The chemiluminescence of the membrane was exposed to X-ray film. Membranes were normalized with a polyclonal Ab against β-actin protein. Because qualitative analysis of blots can overestimate the magnitude of results, semiquantitative scan densitometry of blots was done with a Scan LKB (Amersham Pharmacia).

Real-time fluorescent quantitative RT-PCR

Total RNA was prepared from pineal glands using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Isolated total RNA was quantified using a spectrophotometer (Amersham Biosciences Ultraspec 3100 Pro). Aliquots of total RNA (1 μg) were reverse-transcribed using random primers and Superscript II-Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA equivalent to 20 ng of total RNA was subjected to real-time PCR analysis (ABI7000; Appliedbiosystem, USA) following standard protocols. PCR Primers (Invitrogen) and Taqman probes for AANAT were designed by Primer Express 2.0 software (Appliedbiosystem, USA). The primer and probe for AANAT (Table 2) were commercially synthesized by DaAn Gene Co. Ltd. of Sun Yat-sen University (China). Each reaction (25 μl) contained 2.5 μl of reaction buffer (10×), 6 mM of MgCl2, 0.2 mM of dNTP, 0.6 mM of each primer, 0.25 μl of SureStar TaqDNA Polymerase, and 2 μl of cDNA dilutions. The cycling condition consisted of one cycle at 93°C for 3 min and 40 two-segment cycles (93°C for 30 s and 55°C for 45 s). In each run a negative control (distilled water) was included. Briefly, 10-fold serial dilutions of control cDNA were amplified by the ABI7000 PCR machine (Appliedbiosystem, USA). Ct Value (initial amplification cycle) of each standard dilution was plotted against standard cDNA copy numbers. On the basis of the standard curves for each gene, the sample cDNA

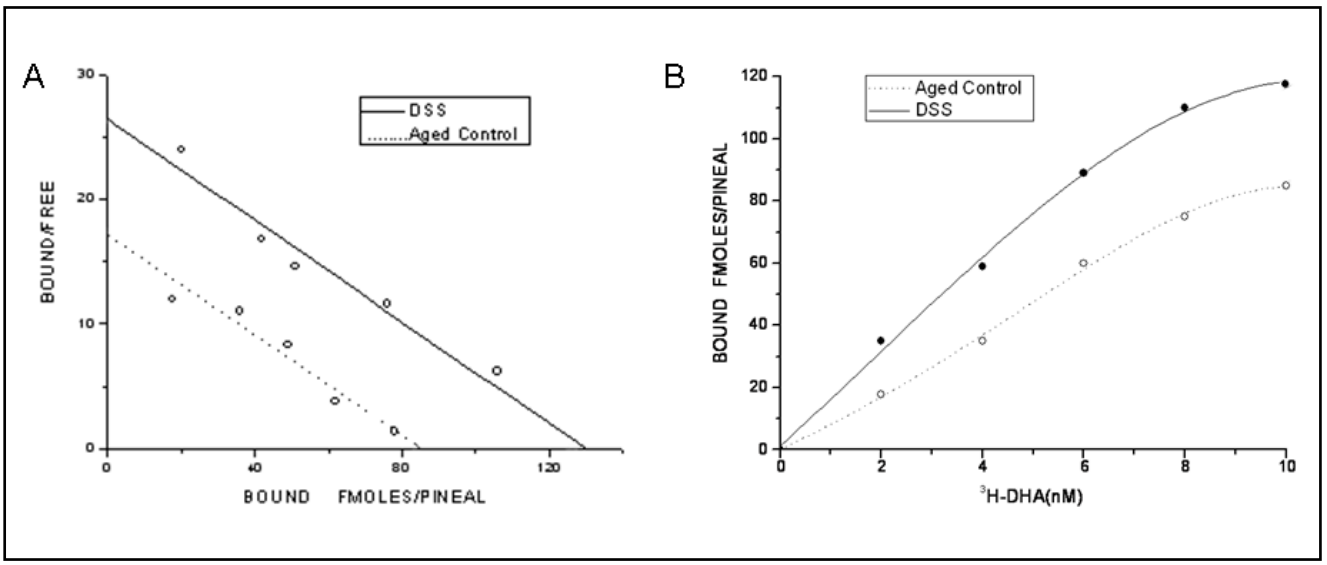

Fig. 1. Effect of DSS treatment (3 weeks) on [3H]DHA binding in the rat pineal. Each point represents the mean of five experiments (n=10). A, Scatchard plot. The Bmax values were 126.25±13.57 and 84.52±11.28 fmol/pineal and Kd values were 4.84±0.42 and 4.73±0.56 nM for old control and DSS groups, respectively. B, saturation curve for [3H]DHA binding.

copy number was calculated according to the sample Ct value. Standard curves and PCR results were analyzed using ABI7000 software (Appliedbiosystem, USA). The target gene primers were: NAT (f): 5'-GCG CGA AGC CTT TAT CTC AGT-3'; NAT (r): 5'-GAG GAA GTG CCG GAT CTC ATC-3. The fluorescent Probe was 5'-FAM-TCG GGT ACC TGC CCC CTC C-TAMRA-3'.

Statistical analyses

The results were reported as the mean ± S.E.M. Statistical analysis was performed by using the one-way or two-way ANOVA test followed by Student's t-test. P value of less than 0.05 was considered significant.

RESULTS

Effects of DSS treatment on melatonin release from aged rats' pineal glands at night

The plasma melatonin at night decreased with aging, but in the old rats the daytime concentration of melatonin was not significantly lower than that of young animals (Table 2). There was no significant difference between the two old groups in daytime. By oral administration of DSS for 3 weeks melatonin at night had a rise in the old group (p< 0.01, compared to the age-matched control), but did not increase to the level of young rats (p< 0.01).

Effect of DSS oral administration on pineal [3H]-DHA binding of aged rats

The effect of 3 weeks of treatment with DSS on pineal [3H]DHA binding is presented in Figure 1. The data represent averages of five separate experiments each consisting of three pooled animals. Both saturation curves and scatchard analyses indicate a DSS-induced increase in the number of pineal [3H]DHA specific binding sites (Bmax). The kinetic parameters obtained by averaging the binding constants obtained in the five separate experiments indicate a significant increase (p< .035, two tailed t test) in the number of [3H]DHA binding sites without a change in binding affinity (Kd).

Effect of DSS treatment on pineal expression of NAT mRNA detected by real-time RT-PCR

After amplification using Lightcycler fluorescent PCR system, the results showed that mRNA level of NAT at night of aged pineals increased significantly after 3-week DSS treatment (Fig 2, p< 0.05).

Effects of DSS treatment on phosphorylation of CREB (P-CREB) and total level of CREB (T-CREB)

In order to clarify the possible mechanisms that DSS-enhanced expression of NAT mRNA, we have investigated whether DSS exerts its effect by enhancing phosphorylation of cyclic AMP response element-binding protein (CREB), which acts as a transcription factor to induce expression of NAT mRNA in the present study. The results showed that DSS significantly increased P-CREB (p< 0.05) but did not change T-CREB levels in rat pineal glands (Fig. 3).

DISCUSSION

Norepinephnine released from sympathetic nerve endings in the pineal gland regulates the synthesis of the pineal hormone, melatonin (Axelrod, 1974). Norepinephnine binding with β-adrenergic receptors stimulates pineal adenylate cyclase activity and increases cyclic AMP levels in the gland (Axelrod, 1974). Norepinephnine, as well as dibutyryl cyclic AMP, induce and acti-

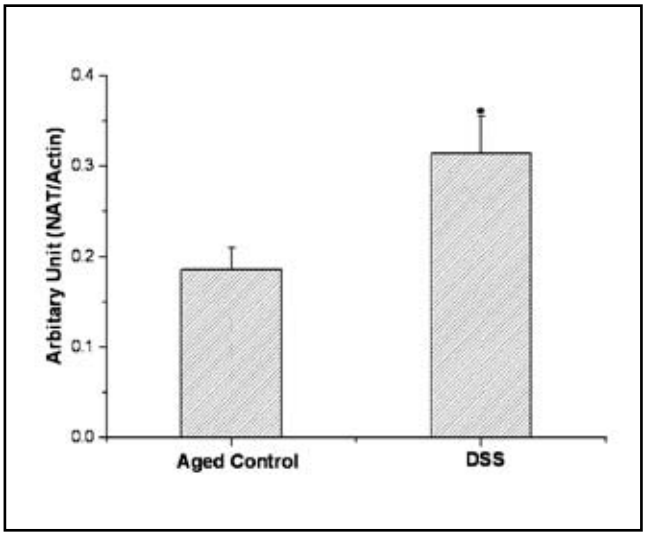

Fig 2. By using the real-time RT-PCR method, the mRNA expression level of NAT gene was measured in aged pineals (n=10). The symbol (*) denotes significance between old control and DSS treated rats at p<0.05.

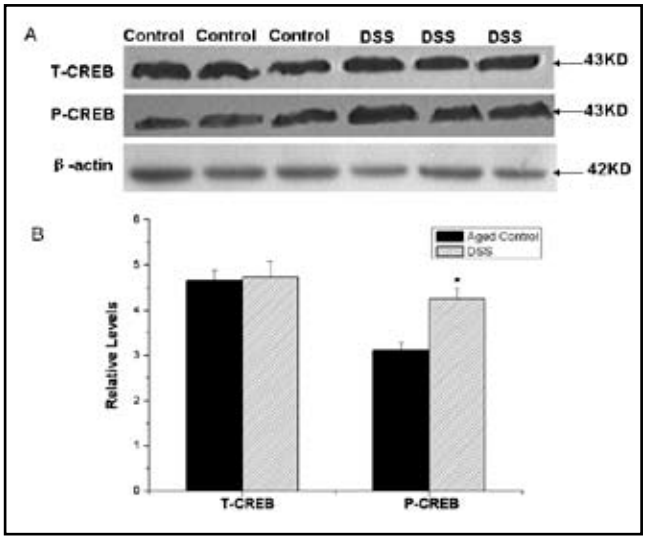

Fig. 3. Effects of DSS on phosphorylation of CREB (P-CREB) and total level of CREB (T-CREB) in rat pineal glands assessed by Western blot analysis (n=10). The P-CREB and T-CREB bands were quantified by densitometry and the changes were represented in graph. Results are expressed as mean±S.E.M. of five independent experiments. The asterisk symbol (*) denotes significance between old control and DSS-treated rats (p < 0.05).

vate NAT, the enzyme that catalyzes the rate-limiting step in melatonin synthesis, thus increasing melatonin production (Spessert *et al.* 2000). Norepinephrine controls NAT transcription through a cyclic AMP-dependent cycle by activating protein kinase A and this leads to phosphorylation of CREB (Spessert *et al.* 2000). The promoter region of the rat NAT gene contains one cyclic AMP-responsive element (CRE)-like sequence, an inverted CCAAT box and activating protein-1 (AP-1) (Chetsawang & Govitrapong, 2005).These events cause the phosphorylation of the DNA-binding protein, CREB (Chetsawang & Govitrapong, 2005). The effect of cyclic AMP on NAT occurs via the activation of NAT gene transcription through a CRE–CCAAT complex (Chetsawang & Govitrapong, 2005). Our results showed that oral administration of DSS for three weeks (3ml/day) significantly increased melatonin production at night, β-adrenergic receptors binding in pineals and NAT mRNA level of pineals in old rats. It has been shown that the increase in NAT mRNA expression is followed by an increase in NAT activity (Klein *et al.* 1997). Several lines of evidence indicate that NAT enzyme activity closely relates to NAT protein levels, which is usually regulated by transcription and translation mechanisms (Zatz *et al.* 2000). Our results showed that DSS significantly increased P-CREB but did not change T-CREB levels in old rat pineal glands. Oral administration of DSS for 3 months increased the contents of NE, DA and 5-HT to modulate metabolism of monoamine neurotransmitters changed by aging on aged mice (Kou *et al.* 2005). Although it has reported that two herbs (Danggui and Fuling) contain melatonin (Chen *et al.* 2003), we have measured that the level of melatonin in the aqueous extract of DSS is very low. Our results indicate that at least one downstream messenger pathway for DSS activating adrenergic system on the induction of melatonin synthesis in the rat pineal gland acts via cyclic AMP-dependent cascade and transcription mechanism. Oral administration of DSS increases the plasma melatonin level partly through enhancing the function of the pineal gland.

Numerous studies have shown that melatonin production decreases with age in humans. Reduced concentrations have been observed in plasma melatonin (Iguichi *et al.* 1982; Waldhauser *et al.* 1998)and urinary 6-hydroxymelatonin (Young *et al.* 1988). The major urinary metabolite of melatonin, 6-sulphatoxymelatonin has also been shown to be reduced with age (Kennaway *et al.* 1999). We found that the plasma melatonin of the old rats decreased apparently than that of the young rats at night and the melatonin rhythm had been disrupted in the old rats. However, the pineal gland does not degenerate (Pardo *et al.* 1990); even in very old subjects the pineal parenchyma is still histologically active (Arieti, 1954). But the central clock SCN shows age-related degenerative alterations. The circadian rhythm of melatonin levels is regulated by the SCN, the clock of the brain. Circadian and circannual rhythmicity of neuropeptide synthesizing neurons of the human SCN, such as vasopressin, are reduced with aging (Hofman, 2000). One report claimed that the age-related suppression of melatonin synthesis may even be greater in individuals who died of Alzheimer's disease (Skene *et al.* 1990). Impairment of melatonin secretion is not only related to age but also to severity of mental impairment (Magri *et al.* 1997). Like the naturally aging subjects, the AD patients' pineals have no apparently degeneration (Wu *et al.* 2003). No evidence has been observed in this

structure of neurofibrillary tangles, the accumulation of neurofilaments, tau, hyperphosphorylated tau or β-amyloid deposition in pinealocytes (Pardo *et al.* 1990). But pretangles (Ohashi *et al.* 1999) and tangles were found (Stopa *et al.* 1999) in the SCN of AD patients indicating that the SCN is affected by AD. In AD there was a marked decrease in the number of vasopressinexpressing neurons in the SCN which occurred at an earlier age and was more dramatic than in non-AD patients (Swaab *et al.* 1987). We propose that these degenerative changes in the SCN most probably result in a disrupted melatonin synthesis, and may underlie the common circadian rhythm disorders in AD and aging persons. The pineal glands of AD and naturally aging subjects have no apparently dysfunction. DSS maybe affect on the pineal glands directly and increase melatonin secretion, because it's difficult to improve the SCN that has been a specific lesion for AD or aging in 3 weeks by using DSS. The close relationship between nocturnal increase of endogenous melatonin, and the timing of human sleep and the sleep-promoting effects of exogenous melatonin prompted many investigators to suggest that melatonin is involved in the physiological regulation of sleep. The function of DSS to improve sleep disturbance of AD patients (Fukushima, 1994; Mizushima, 1989) maybe correlates with increasing melatonin level.

Melatonin is now known to be a multi-faceted free radical scavenger and antioxidant. It detoxifies a variety of free radicals and reactive oxygen intermediates, including the hydroxyl radical, peroxynitrite anion, singlet oxygen and nitric oxide (Tan *et al.* 2000). Melatonin also crosses all morphophysiological barriers, e.g., the blood-brain barrier and, placenta, and distributes throughout the cell; these features increase the efficacy of melatonin as an antioxidant (Reiter, 1995). Skaper *et al*. suggested that melatonin possesses broad-spectrum free radical scavenging and antioxidant activities, and prevents kainic acid-induced neuronal lesions, glutathione depletion and reactive oxygen species-mediated apoptotic nerve cell death (Skaper *et al.* 1999). DSS has also been reported to have free radical scavenging properties (Stefek & Benes, 1994). It increased the activity of superoxide dismutase activity and scavenged 1,1-diphenyl-2-picyrylhydrazyl radicals, superoxide, and hydroxyl radicals (Ueda *et al.* 1996). It has been reported that DSS significantly reduced the amyloid beta25-35-induced neuronal death (Egashira *et al.* 2005). Clinical application of DSS to postmenopausal women with dementia of the Alzheimer type has been shown to enhance their cognitive functions and to improve their sleep disturbance (Fukushima, 1994; Mizushima, 1989). The composition of DSS is not very clear and we don't know whether DSS can cross blood-brain barrier. The neuroprotective action of DSS may be linked with improvement of pineal and melatonin secretion.

In conclusion, the plasma melatonin level decreased with aging. Oral administration of DSS can improve the pineal function and increase melatonin level by activating adrenergic system via cyclic AMP-dependent cascade and transcription mechanism. The action of DSS to improve sleep disturbance and protect nervous system may be related to enhance of melatonin level.

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