Supplimentary Information

Memory-improving effect of formulation-MSS by activation of hippocampal MAPK/ERK signaling pathway in rats

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1) Supplementary Materials and Methods

Preparation of MSS

MSS was made from the three materials mixture comprising maesil concentrate (MS, *Prunus mume Sieb. et Zucc*) with disodium succinate (SS) and Span80 (Sp) (3.6:4.6:1 ratio, respectively). MS was prepared from Maesil fruits before full maturity were purchased at the first week of June. 10 kg of maesil fruits which have no defect were washed clean with service water, dried, and then ground on a steel sheet or in a mixer, thus making maesil juice. The maesil juice concentrate was obtained by heating with slow stirring at 50 °C in a stainless container. The concentration process was performed until the green juice became a sticky dark brown liquid, thus preparing about 200 g of a MS having a water content of 14 ± 3%. The prepared concentrate was placed in a sterilized bottle in a hot state, then cooled and stored at 4 °C in refrigerator until being taken out of the bottle. For the standardization of MS, each of the contents of SA and fumaric acid in MS was quantified with gas chromatograph by using standards. Each dehydrated 1~1.5 mg of MS was added in 100 μℓ of N, O-bis (trimethylsilyl) acetamide (Aldrich Chemical Company), and the mixtures was allowed to react in an oven at 60 °C for 60 minutes. 900 μℓ of CHCl3 (HPLC grade) was added to each of the reaction materials, and the resulting mixtures were used as analytical samples. 1 μℓ of each of the trimethylsilylated sample solutions was collected and analyzed with GC (GC-14B, Shimadzu, Japan). As a detector, FID (flame ionization detector) was used, and as a column, DB-5HP (30m X 0.32 mm ID X 0.1 μm film thickness; J&W, Folsom, CA, USA) was used. The oven temperature was elevated from 100 °C at a rate of 15°C/min and maintained at a final temperature of 320 °C for 5 minutes. The injector port and the detector port were all maintained at a temperature of 300 °C. Each of the tests was repeated three times and the test results were analyzed. As a result, the regression equation of fumaric acid was y=420921x – 5921.2 (r²=0.9995), and the regression
equation of SA was $y=265256x + 22047$ ($r^2=0.9981$). As a result of substitution into each of the regression equation, the MS had a fumaric acid content of 8% (2.55 mg/31.6 mg of MS), but no SA was detected in the MS. Disodium succinate (SS, sodium succinate dibasic hexahydrate) and Span80 (Sorbitan monooleate, Sp) were purchased from Sigma and Aldrich, respectively.

Animals

Adult male Sprague Dawley rats (6 weeks old) purchased from Core tech., Central Animal Research Facility, Korea, were used after a week adjustment (180 ~ 200 g) in the new environment. Rats were housed three or four per cage in polypropylene cages (22.5 x 35.5 x 15 cm) in a temperature (23±2 °C), humidity (50–55%) and light-controlled (12-h light/dark cycle) environment, with food and water ad libitum. The experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No.80-23). All efforts were made to minimize both the suffering and the number of animals used. Each animal was randomly assigned to each group by using SAS (v. 9.1) program and then, randomly classified into control and test groups. Each group consisting of 7 animals administered daily for 3 weeks. The test materials were orally administered as 1ml volume by using jondae at 10:40 a.m. every day. The control group was administered only distilled water. Morris water maze test began at the 1st day of the 3rd week of administration. For tail suspension test (TST) mice (ICR, male, 4 weeks old, 25g~30g b.w) were used and each group consisting of 10 animals administered daily for 1 or 3 weeks (Experiments were independently repeated two times. See details below “Examination of Anti-depressive effect”).

MWM test

The Morris water maze, consisted of a large circular black pool 180 cm diameter, 50 cm height, was filled with water to the depth of 30 cm at $23\pm1^\circ C$, and then, illuminated by sparse in direct light. In the
pool, a submerged platform (round, 10 cm diameter, 2 cm below surface) was hidden on a fixed location in a center of one quadrant of the pool. The rat could climb on the platform to escape from the necessity of swimming. During a series of trials the rat was trained to locate the platform. The animals were given 4 acquisition trials per day on 5 consecutive days. A different starting position was used on each trial. Four points, equally spaced along the circumference of the pool, were arbitrarily assigned as: N, E, S and W, on this basis, the pool area was divided into 4 quadrants. These points served as the starting positions at which the rat was lowered gently into the water, with its head facing the wall of the water maze. The rat was given a maximum of 90 s to find the hidden platform and was allowed to stay on it for 10 s and then placed for 20 s in a holding cage before the next trial began, resulting in an inter-trial interval of 35s. Rats that failed to locate the platform were guided and put onto it by the experimenter. At the end of the session, the rat was dried with a towel before being returned to its home cage. On each trial, the time and distance needed to reach the platform was measured, and automatically registered on a video computer system Ethovision v3.1. In addition, the pool was subdivided in different zones for analysis of the search pattern of the rats. The zone of the pool was subdivided in four equal quadrants, one of which contained the platform in the center. The time spent in the quadrant of the pool containing the hidden platform was registered and also expressed as the total time or distance spent in the quadrant zone of the pool. After 4 or 5 days of the final learning training, a working memory test called as probe trial was performed without platform at 24 hr later. The working memory was measured by retention time or distance in each quadrant without platform for 60s and then continually measured for more 30s of short-term spatial memory task (total 90s). However, the hidden escape platform was put in a different location (opposite site of the previous location) and one starting position was employed in order to obtained data of the short term working memory test by measuring the continued 3 more trials. In the short-term working memory test, data analysis was focused upon the savings in escape performance between the first and subsequent trials. Authors expressed the short-term spatial memory as a ‘short-term working memory index’. These values,
more detailed, were obtained by the subtraction of the escape latencies between the first and the average of subsequent 2nd–4th trials. For effective measurement of the savings-in-escape performance (Buresová et al., 1985), 90s after the start of swimming, the platform hidden 20cm below the water surface was automatically ascended to 2cm by a mechanic system operated by air pressure pump. Since the escape latency often inhibited by the unwanted touch at the first trial when rats were unconsciously passed the submerged platform during the given 90s swimming. The 1st trial data for 60s swimming were used as probe trial data.

**Examination of Anti-depressive effect**

An anti-depressant effect was tested using a tail suspension test (TST), which is most generally used for the screening of anti-depressant drugs (Steru, 1985). To analyze the antidepressant effect, ICR mice (male, 4 weeks of old, 20 ± 5 g) were divided into groups with each group consisting of 10 animals. The MS or MS containing mixtures were administered to the animal groups by varying doses (whole experiments were independently repeated two times), followed by TST. Specifically, the test groups were administered orally with several dosages of MS or several formulations including MS and the control group was administered with distilled water at the same time (p.m. 2:00) daily for 1 or 3 weeks. At 1 hour after administration at the last day (7th or 21st day) 1-2 cm of the tail end of each mouse was suspended to the corner of a 80-cm–height horizontal plane using a strongly adhesive tape, and then recorded on a video recorder for 5 minutes. Based on a time of 4 min other than the first 1 min, the immobility time of the test animals was measured by using the video tape recording. In TST, anti-depressive effect by decreasing the immobility time was measured by suspending the tail of test animals and measuring each of the agitation time and immobility time of the animals during the test period. As positive control compound, imipramine (Im, 10mg/kg b.w. see Fig. 3) was intraperitoneally injected once a day for one week (Nomura et al. 1991).
Analysis for the changes of signal transduction molecules in brain

In case of test animals, which showed a significant difference in a probe test, the brain hippocampus was isolated and then, the mRNA expression levels of memory and learning-related signaling substances e.g., CREB, NMDAR (NR1, NR2A, NR2B), ERK1/2 and TrkB were examined comparatively with the control group in order to analyze the improvement of learning and memory function of the composition-administered group with regard to molecular signaling mechanisms. Rat hippocampal total RNA was extracted using the Mini RNA Isolation II (ZYMO RESEARCH) according to the manufacturer’s protocol. cDNAs were synthesized from total RNA (5 µg) with random hexamer as the primer using the TaqMan reverse transcription reagents (Applied Biosystems) according to the manufacturer’s instructions. The resulting cDNAs were then subjected to real-time PCR as follows. The expression level of mRNA was evaluated using quantitative real-time PCR based on specific sets of primers. Primer pair of each gene for real-time PCR was designed by using Primer3 software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to standardize the values. Each reaction consisted of 1X SYBR Green PCR Master Mix (Applied Biosystems), 50 ng cDNA in a total volume of 20 µl and 50 nM specific primer sets (5’ ACC TGC TGG ACC GGA TGT T 3’ and 5’ GGG TGA GCC AGT GCT TCC T 3’ forward and reverse primers for ERK1, 5’ TGC TTT CTC TCC CGC ACA A 3’ and 5’ GGT TTT GAG TCA GCG TTT GG 3’ forward and reverse primers for ERK2, 5’ GAA ACA GCT TCT TCT GGC TCA GCG TTT GG 3’ forward and reverse primers for CREB, 5’ AAT TGT GGA TTC CGG CTT AAA GT 3’ and 5’ CCG CAG GTT GCC GTT CT 3’ forward and reverse primers for TrkB, 5’ AGC GGG TAA ACA ACA GCA ACA 3’ and 5’ TCC GCT TGG CCA CTG AGT 3’ forward and reverse primers for NR1, 5’ TGC TTT CCT CGA ACC CTT CA 3’ and 5’ CTG AGA CGA TGA TGG CCA GCA TCA 3’ forward and reverse primers for NR2A, 5’ GTG AGA GAT GGA ATT GCC ATC A 3’ and 5’ GCC TCA GGG ATG AAA CTG
TGT T forward and reverse primers for NR2B, 5′ TGC CAA GTA TGA TGA CAT CAA GAA G 3′ and 5′ AGC CCA GGA TGC CCT TTA GT 3′ forward and reverse primers for GAPDH) using the following parameters on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems): 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Data were collected and recorded by ABI PRISM 7900 SDS Software (Applied Biosystems) and expressed as a function of threshold cycle (CT). Relative quantitative gene expression was calculated with the 2^{-\Delta\Delta CT} method (Livak and Schmittgen, 2001).

Analysis for phosphorylation of ERK1/2 and CREB in brain

The brain hippocampal tissues from each rat were rapidly removed and homogenized in lysis buffer composed of 150 mM sodium chloride (Sigma), 1 % Nonidet P-40 (Fluka), 0.5 % sodium deoxycholic acid (Sigma), 0.1 % sodium dodecyl sulfate (Amersham Biosciences), 50 mM Tris-HCl (pH 7.5, Sigma), protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail (Sigma). The homogenate was centrifuged at 13,000 rpm for 10 min at 4 °C. Protein concentrations in the supernatant were determined using a protein assay kit (Bio-Rad), with bovine serum albumin as the standard. Equivalent amounts of protein for each sample were resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinilidene difluoride (PVDF) membranes (Amersham Bioscience). After blocking the non-specific sites with tris-buffered saline (TBS) containing 0.5% Tween 20 and 5% skim milk (Difco Laboratories) for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4 °C. After washing in TBS with 0.5% Tween 20, the membranes were incubated with the horseradish-peroxidase-conjugated IgG secondary antibody (1:1000, dilution in TBS with 0.5% Tween20 for 1h) (Santa Cruz). Membranes were then washed three times with TBS with 0.5% Tween 20 and developed with chemiluminescence kit (Amersham Bioscience) according to the conditions recommended by the manufacturer’s instructions. The primary antibodies used were pERK1/2 (phosphorylated ERK1/2), ERK1/2 (1:1000, Santa Cruz); pCREB (phosphorylated CREB), CREB (1:500,
Upstate); β-actin (1:1000, Sigma). The films were scanned and densitometric analysis of the bands was performed using the software "Quantity One"(Bio-Rad). Quantitation was performed with reference to the invariant cytoskeletal protein, β-actin and expressed additionally as a percentage of control.

Statistics

We assessed two parameters of spatial performance in parallel: escape latency and search efficiency. A group mean was calculated from the median escape latencies of each animal’s performance per session. In addition to recording the escape latency, search efficiency was calculated as a retention time (or moving distance) in correct quadrant trials. Statistical analysis of the differences in both escape latencies and search efficiencies between control and test components-treated animals on successive days of testing were performed using two-way analysis of variance (ANOVA) with repeated measures (the two factors being group and session, matched by subjects). Bonferroni posttests were performed to assess daily differences. For TST, real-time PCR analysis and western blot analysis, Newman-Keuls Multiple Comparison test or Student’s t-test was performed to evaluate the significance of the differences. All data were obtained by the two-tailed analysis. The p values of less than 0.05 were considered to be statistically significant.

2) Supplementary Discussion

Effect of MS as a health supplement

The ethanol extract or fruit-juice concentrate of maesil contains compounds which markedly improved the fluidity of human blood (Chuda et al., 1999), intestinal availability of nutritionally pharmacologically or physiologically active compounds that undergoes intestinal metabolism by
the SGLT1 (Na+/glucose cotransporter)-mediated absorption (Mizuma et al., 2005). Among compounds of maesil, it was reported that Benzyl β-D-glucopyranoside (BG) and chlorogenic acid (CA) may participate in rat blood pressure level and may contribute to relieving the tension in Menopausal rats caused by ether stress (Ina et al., 2004). Maesil has been known as one of health supplement foods in Korea and Japan and receives attention as a food material for the prevention of cardiovascular diseases, such as thrombosis, a reduction in neutral lipid, and blood pressure lowering associated with the inhibition of ACE.

**Hippocampus extraction time**

The expression of mRNA-related signal transduction molecules was found to be dependent upon hippocampus extraction timing after behavioral tests. It also was dependent upon which side of the brain hippocampus was selected. While processing of spatial scenes involves the parahippocampus, the right hippocampus appears particularly involved in memory for locations within an environment, with the left hippocampus more involved in context-dependent episodic or autobiographical memory (Burgess et al., 2002). On the basis of our preliminary experiments, we took out the hippocampus from brain at 40min. after finishing final relearning followed by using the right hippocampus for mRNA level and by using the left side for the analysis of protein levels such pERK and pCREB as compared to their control.

**Analysis of the signal transduction molecules**

Alterations in the expression of BDNF and specific glutamate receptor subtypes such NR2B, may underlie the exercise ability to enhance neurogenesis and reduce the threshold for LTP in the dentate gyrus of hippocampus (J. Farmer et al., 2004). Since the MWM tests were enforced at the same experimental condition in both control and test groups, the increase of TrkB and NR2B mRNA should be caused by MSS treatment. Manipulations that reduce BDNF expression in hippocampus can also reduce NMDA receptor subunit expression (Roceri et al., 2002). However, our results showed that the mRNA
level of BDNF was significantly reduced as compared with the control values (data not shown) although the both mRNA of NR2B and TrkB increased in hippocampus. The discordance of BDNF and TrkB mRNA expression in the experiment was not well understood at present, although synaptic activity can have a profound effect on BDNF expression, the signal cascades underlying activity-dependent BDNF expression are not seems to be identified. Recent report showed that the phosphorylated CREB binds to BDNF promoter and up-regulates the expression of BDNF in amygdala which helps the consolidation of fear memory (Li-Chin Ou and Po-Wu Gean, 2007). The phosphorylated protein levels of ERK and CREB are generally considered as more final advanced steps than those of NR2B and TrkB in memory improving cascades. Phosphorylation of CREB protein induces memory-related gene expression in the cell nucleus. Although many kinases can participate in the synaptic activity, MAPK-signalling pathways play dominant roles in activation of CREB (Tully T et al., 2003). So, we assayed the protein levels of ERK and CREB ahead of NR2B and TrkB. The MAPK activation of brain function in vivo may be due to cellular stress (Katya Belelovsky et al., 2007). Our results could not be obtained without stress conditions by the consecutive Morris water maze test itself. However, the memory enhancing effect of MSS were clearly discriminated under the same behavioral test conditions.

**Serum analysis and acute toxicity of MSS**

When glucose, GOT, GPT and cholesterol in the serum were analyzed, there were no significant differences between MSS-treated and control groups. Also, no significant changes in bodyweight and diet quantity were observed during the 3 weeks of administration. Single oral administration of 4 and 8 g/kg b.w. of MSS, also, showed no death for 2 weeks and no specific differences in autopsy.
3) Supplementary Table 1

Effect of SA$_{100}$ and formulation-MS$_{180}$SA$_{100}$Sp$_{100}$ administration on memory function in MWM test.

Sole administration of SA$_{100}$ did not change the learning or working memory as shown in table. Co-administration (SA$_{100}$Sp$_{100}$) enhanced the ability to learning as compared to SA alone in normal rats (Supplementary-Fig. 1). However, the administration of MS$_{180}$SA$_{100}$Sp$_{100}$ increased the swimming velocity, thus the distance moved in correct quadrant was significantly increased as compared to the control values. It suggests that MS should be related with sensory motor activity.

| Formulations | Treatment (3 weeks) | Escape time in the 4th day of learning | Working memory (probe test) in correct quadrant | Short term working memory index (sec) |
|--------------|---------------------|--------------------------------------|-----------------------------------------------|-------------------------------------|
|              |                     | Retention time (sec) | Moved distance (cm) | Velocity (cm/sec) |                               |
| SA$_{100}$   | control             | 16.7±3.6               | 23.7±1.6             | 313.2±45.3      | 12.6±1.2                     | 39.0±15.9                           |
|              | test                | 13.3±3.1               | 20.7±3.0             | 304.5±61.7      | 13.7±2.7                     | 50.2±6.4                            |
| MS$_{180}$SA$_{100}$Sp$_{100}$ | control | 17.3±2.4               | 22.1±4.8             | 288.6±74.2      | 11.3±3.1                     | 24.1±9.7                            |
|              | test                | 19.6±4.0               | 23.1±1.1             | 445.5±30.8*     | 19.5±1.4*                    | 50.5±18.1*                          |

Each data represents the mean ± SEM (n = 7), $^a$: escape latency (sec), $^*$, p < 0.05 vs control.

$^b$: Short term working memory index was calculated by the differences between escape latency of the 1st trial and the average escape latency of the 2nd–4th trials (See supplementary work, MWM test in Materials and Methods).
4) Supplementary figures legends 1 through 3

(Supplementary Fig. 1)

Effect of spatial memory and learning on 3 week-treatment of SA100Sp100-formulation in MWM test

Values are expressed as the mean number of escape latencies ± S.E.M. Co-administration of SA and Sp (SA100Sp100) enhanced the ability to learning as compared to administration of SA alone in normal rats. The analysis of two-way ANOVA for 4 days of learning (left figure) was significant (p=0.0008, F:12.51 vs control) and the 4th day was significant in Bonferroni posttests (*, p<0.05 vs control) although the working memory in correct quadrant of SA100Sp100 group was not so significant (p=0.089, right figure). The working memory in probe test was expressed as distance moved within the correct quadrant. SA100Sp100: succinic acid 100mg/kg b.w. and span80 (sorbitan monooleate) 100mg/kg b.w. mixture were orally administered every day for 3 weeks.
Antidepressant effect on 3 week-treatment of MS in tail suspension test of mice.

Values are expressed as the mean values ± S.E.M. *: p < 0.05; **: p < 0.01; ***: p<0.001 vs control in Newman-Keuls Multiple Comparison test. Maesil concentrate (MS) was dissolved in distilled water and then orally administered to ICR mice for 3 weeks as a daily dosage of 90, 180, 360, 720mg/kg b.w., respectively (n = 30 ~ 40/group). The each administration of MS showed significant antidepressant effect dose-dependently at MS$_{90}$, MS$_{180}$ and maintained the level to some extent at MS$_{360}$, MS$_{720}$ in TST.
Effect of spatial memory and learning on 3 week-treatment of MS180SA100 in MWM test

Values are expressed as the mean escape latencies ± S.E.M. of 4 trials /session/day for 4 days of learning in Morris water maze test. MS180SA100 without addition of Span80 did not show any significant differences in both working memory and short term working memory to newly moved platform as compared to control values in the water maze test. MS180: maesil extract 180 mg/kg b.w.; SA100: succinic acid 100mg/kg

(Supplementary-Fig. 3)
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