Original Article

The Stimulation of GV20 with Laser Produces Neuroprotective and Cognitive Enhancing Effects by Improving Cholinergic Function, Oxidative Stress Status and Apoptosis in Hippocampus

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Background: The cognitive impairment in menopause with obesity is rising in accompany with the increased prevalence of menopause and obesity. Laser application can improve oxidative stress status and neurodegeneration in hippocampus together with memory enhancement. Therefore, the beneficial effect of laser stimulation at GV20, one of the acupoints commonly used for treating dementia, on memory impairment and neurodegeneration in menopause with obesity has been considered.

Objective: To elucidate this issue, the present study aimed to determine the neuroprotective and memory enhancing effects of GV20 stimulation with red laser on in animal model of menopause with obesity.

Materials and Methods: Female Wistar rats were exposed to the bilateral ovariectomy (OVX) and fed with high fat diet for 16 weeks. Then, OVX rats which fed with diet were exposed to a 10 minute-stimulation with red laser once daily for 14 days. Spatial memory was assessed every 7 days. At the end of study period, acetylcholinesterase activity (AChE), an oxidative stress status and apoptosis were measured.

Results: It was found that GV20 stimulation enhanced spatial memory and neurons densities in CA1, CA2, CA3 and Dentate gyrus of hippocampus. The decreased AChE, MDA and Bax+ cells density together with the increase in SOD, CAT, GPx and Bcl-2+ cells in hippocampus were also observed.

Conclusion: Taken all together, the neuroprotective and cognitive enhancing effects of GV20 stimulation by red laser might occur via the improvements of cholinergic, oxidative stress status and apoptosis.

Keywords: GV20 stimulation, Memory enhancement, Neuroprotective effect, Red laser

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Over the recent decades, the proportion of women who are menopausal in the general population is continually rising in accompany with the increasing longevity. Several studies have demonstrated the association between the menopausal transition is associated with weight gain[1,2]. It has been found that the increased body mass index (BMI) and upper body fat distribution (indicated by waist-to-hip ratio) are associated with later age at natural menopause[3].

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Obesity can lead to many medical disorders, such as diabetes, heart disease, hypertension, and cancer[4]. Obesity and overweight are usually related to poorer cognition across lifespan[5]. More recently, obesity and its associated comorbid conditions have been identified as significant risk factors for the development of AD[6]. The probable mechanisms associated with this condition are associated with structural changes, including atrophy, in the brain during ageing[7]. In addition, obesity also decreased Bcl-2 expression but increased Bax and Caspase-3 expressions in hippocampus. Moreover, oxidative stress status in hippocampus also increased in obese rats[8].

Acupuncture has been long term used for treating various disorders, including menopause related symptoms[9], obesity[10] and memory impairment[11,12] for a long time since ancient period. During the recent decade, it has been found

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that the stimulation of acupoints by using other tools such as laser beam has gained much attention due to the effective and non-invasive approach. Laser acupuncture can improve waist hip ratio and cholesterol in obese condition[13], cognitive function[14-18]. GV20 has been used as the potential acupoint for treating vascular dementia[19] and memory impairment[20]. Based on aforementioned information, the authors hypothesized that the stimulation of GV20 could improve memory in experimental menopause with obesity. Since no data were available, the present study was set up to investigate the effect of the stimulation of GV20 acupoint with red laser on memory and brain oxidative stress status together with apoptosis in animal model of menopause induced by bilateral ovariectomy which was fed with a high fat diet.

Materials and Methods

Animals

Adult female Wistar rats, 8 weeks old (180 to 220 g), were obtained from National Animal Center, Salaya, Nakhon Pathom. The animals were housed in group of 5 per cage in standard metal cages at 22±2°C on 12: 12 h light: dark cycle. All animals were given freely access to food and water. The experiments were performed to minimize animal suffering and the experimental protocols was approved by the Institutional Animal Care and Use Committee Khon Kaen University, Thailand (AEMDKKU 001/2559).

Surgery, habituation, and estrous cycle determination

Female rats were anesthetized using an intraperitoneal injection of thiopental sodium (40 mg/kg), then an incision was made in the midline of the abdomen and the bilateral ovaries were removed. To assure that the ovariectomy is successfully performed and the level of estrogen decreased, the cells lining in the vagina of the rats were monitored for seven consecutive days and the exploration was started on the third day after the operation. The ovariectomized rats with the estrogen deprivation should have vaginal cells appearance as that in diestrus[21].

Induction of obesity

High dietary fat intake is a major risk factor for development of cardiovascular and metabolic dysfunction including obesity, cardiomyopathy and hypertension[22,23].

Excess consumption of egg especially its yolk has been implicated in hyperlipidemia[24,25]. The high fat diet contained 12.5 g of cholesterol per 1 kg of diet. In addition, freshly cooked yolk from eggs 625 g was also used for preparing this formula. The eggs were cooked for 15 minutes, cooled, and the yolks were separated and homogenized. Normal diet was ground and mixed with 200 g of condensed milk and egg yolk. The obtained mixture was homogenized and then diet was made and dried at 40°C to a constant weight.

Experimental groups and protocol

All animals were randomly divided into 4 groups as follows: Group 1: Naive intact, Group 2: OVX + HFD; OVX rats which were fed with a high fat diet (HFD), Group 3: OVX rats + HFD + Sham laser acupuncture at GV20; OVX rats which were fed with a high fat diet and received sham laser acupuncture at GV20, Group 4: OVX rats + HFD + laser acupoint at GV20; OVX rats which were fed with a high fat diet and received laser acupuncture at GV20.

After two weeks of surgical procedure, rats were fed with high fat diet for 16 weeks to induce obesity. The animals in group 2 to 4 were fed with high fat diet. The high-fat diet was modified from the diet that induces obesity in which 27.58% of total energy was derived from fat. After 16 weeks of the feeding period, rats which showed percent change of body weight more than 25 percent higher than the control group were selected for the laser treatment study.

Laser acupuncture

Fifteen minutes before laser acupuncture was applied, all rats were anesthetized with thiopental sodium at dose of 40 mg/kg BW (intraperitoneal injection) to reduce stress.

Laser acupuncture was performed by laser stimulation using the Weberneedle® Compact (Lauenforde, Germany). The laser needle used in this study emits a cold red laser (658 nm/50 mW). The treatment of laser acupuncture or at GV20 (Baihui) acupoint was performed 10 minutes each time, once daily for 2 weeks. During sham laser acupuncture procedure, the laser needles were placed on GV20 but the laser turned off. Acupoint GV20 is located on the top of the head at the intersection of middle sagittal line and the connection of two ear apexes[26].

Assessment of spatial memory by Morris water maze test

A circular pool with a diameter of 160 cm was filled with water to cover the platform. The fixed cues were placed in the room. After the acclimatization to the testing room and learn the given cues, the rat was allowed to the pool and learn to find a platform within 60 seconds and remained on the platform for 5 minutes. During the test session, time that the rats took to find the platform was regarded as escape latency. The test was performed after 24 h later in the same protocol but the platform was removed. The time that rats swam in the quadrant on which the platform was placed before was regarded as retention time.

Assessment of oxidative stress status and acetylcholine activity in brains

At the end of the study, all experimental animals were euthanized with an intraperitoneal injection of thiopental sodium (60 mg/kg). Under deep anesthesia, the rats were perfused transcardially with 0.9% NaCl solution before collecting brain samples. Hippocampus was isolated and homogenate with 50 volume of 0.1 M phosphate buffer saline. Then, the homogenate was used for the determination of the acetylcholinesterase (AChE) activity and oxidative status including malonaldehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione

peroxidase (GPx). The protein concentration in brain homogenate was determined by using a Thermo Scientific NanoDrop 2,000 c spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA), and measured the optical density at the wavelength of 280 nm.

AChE activity was determined by using Ellman's method with slightly modifications[27]. The mixture of 20 µl of sample solution, 200 µl of 0.1 mM sodium phosphate buffer (pH 8.0) and 10 µl of 0.2 M DTNB (5, 5'-dithio-bis-(2-nitrobenzoic acid)) (Sigma-Aldrich, USA) were mixed and incubated at room temperature for 5 minutes. Then, DTNB was added to react with 15 mM acetylcholine thiochloride (ACTI) (Sigma-Aldrich, USA) (10 µl). After the incubation for 5 minutes, the yellow color was developed and measured at 412 nm by using spectrophotometer.The activity of AChE was calculated according to the equation below and expressed as nmol/min.mg protein.

AChE activity = (ΔA/1.36x104) x 1/ (20/230) C

(ΔA = the difference of absorbance/minute, C = protein concentration of brain homogenate).

The determination of MDA level, briefly as following, 100 µl of sample solution, 100 µl of 8.1% sodium dodecyl sulphate (SDS) (Sigma-Aldrich, USA) and 375 µl of 20% acetic acid (Sigma-Aldrich, USA), and 150 µl of distilled water (DW) were mixed. Then, 375 µl of 0.8% of thiobarbituric acid (TBA) (Sigma-Aldrich, USA) was added to the mixture, shaken, and warmed for 30 min in a boiling water bath. After cooling to the room temperature, 500 µl of water and 2.5 ml of the mixture of n-butanol and pyridine at the ratio of 15: 1 were added, mixed together, and centrifuged at 4,000 rpm for 10 minutes. The supernatant was retrieved and determined at an absorbance of 532 nm by spectrophotometer. MDA level was expressed as nmol/mg protein[28].

SOD activity was measured by inhibition of the formazan. The reaction mixture contained the following solutions: 57 mM phosphate buffer solution (KH_2PO_4) (Sigma-Aldrich, USA), 0.1 mM EDTA (Sigma-Aldrich, USA), 10 mM cytochrome C (Sigma-Aldrich, USA) solution,50 µM of xanthine (Sigma-Aldrich, USA) solution at the volume of 200 µl and 20 µl of xanthine oxidase (0.90 mU/ml) (Sigma-Aldrich, USA) solution. Then 20 µl of tissue sample was added to the reaction mixture. The mixture absorbance was measured at 415 nm using microplate reader. SOD enzyme (Sigma-Aldrich, USA) activities at the concentrations of 0 to 25 units/ml were used as standard and the results were expressed as units/mg protein[29].

CAT activity was determined by mixing the reaction solution which contained 50 µl of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0) (BDH Chemicals Ltd, UK), 25 µl of H_2SO_4 (Sigma-Aldrich, USA), 150 µl of 5 mM $KMnO_4$ (Sigma-Aldrich, USA) and 10 µl of brain homogenate. Then, the changes in absorbance of the reaction solution was read at 490 nm and expressed as units/mg protein[30].

GPx activity was detected by the method Rotruck, 1973 [31]. The volume of the reaction mixture containing 10 µl of 1 mM dithiothreitol (DTT) (Sigma-Aldrich, USA) in 6.67 mM potassium phosphate buffer (pH 7), 100 µl of 1 mM sodium azide (Sigma-Aldrich, USA) in 6.67 mM potassium phosphate buffer (pH 7), 10 µl of 50 mM glutathione (Sigma-Aldrich, USA) solution and 100 µl of 30% hydrogen peroxide (BDH Chemicals Ltd, UK) and sample 20 µl were mixed. Then, the mixed solution was shaken for 5 minutes before adding 10 µl of DTNB (5,5-dithiobis-2-nitrobenzoic acid) (Sigma-Aldrich, USA). The absorbance at 412 nm was recorded against the blank using a spectrophotometer. The activity of GPx in tissues was expressed as units/mg protein using a spectrophotometer.

Immunohistochemical staining of brains

The brains were removed and placed in 4% paraformaldehyde overnight. Then, the tissues were fixed in 30% sucrose as a cryoprotectant for 72 hours at 4°C. The brains were then frozen and sectioned (20 µm) with a cryostat, and consecutive sections were transferred into 24 well plates with 0.1 M phosphate buffered saline (pH 7.4). The sections were boiled using microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for 10 minutes and cooled to room temperature. After washing with PBS 3 times (5 minutes/time), the sections were incubated in 0.3% H_2O_2 (1 ml of 30% H_2O_2 in 100 ml of deionized water) to block endogenous peroxidase for 20 minutes and then washed using the same method as above. The sections were blocked in a mixture of 0.3% Triton X-100 (Fluka Chemika, Buchs, Switzerland), 1% (w/v) bovine serum album (BSA) and 10% normal goat serum for 20 minutes at room temperature. After rinsing 3 times with PBS, the sections were incubated with primary antibody to Bcl-2 (1: 500, Abcam, Cambridge, MA, USA) or Bax (1: 500, Abcam, Cambridge, MA, USA) at 4°C overnight. After washing in PBS, primary antibodies were detected with the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse commercial kit (Dako, Glostrup, Denmark) by incubation at room temperature for 30 minutes. The sections were rinsed with PBS and incubated for 5 minutes with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, USA). Positive staining was recognized as a brown color. The negative controls were performed by substitution of primary antibodies with PBS and no immunoreactive neuron was detected. The sections were mounted on gelatin-coated slides and counterstained with cresyl violet and dehydrated with graded alcohols, cleared with xylene and mounted with DPX mountant and observed under a light microscope. Immunoreactive neurons were characterized by brown granules in the cytoplasm. Counts were performed in three adjacent fields and expressed as the mean number of positive cells/255 µm[32,33].

Statistical analysis

Data were presented as mean ± standard error of mean (SEM). The statistical analysis of the experiment was carried out using IBM SPSS Statistic (version 21). The analysis was performed using one-way analysis of variance (ANOVA), following by *post hoc* test. A probability levels less than 0.05 were accepted as significance.

Results

Effect on memory changes

Table 1 demonstrated that at 7 and 14 days of study, OVX rats which were fed with a high fat diet (HFD) significantly increased escape latency (p-value <0.05; compared to naive intact rats). However, no changes in retention time were observed. Sham laser failed to produce the significant changes on escape latency in OVX which fed with HFD. GV20 stimulation with red laser significantly enhanced retention time of OVX rats which fed with HFD diet both at 7 and 14 days of treatment (p-value <0.05 and 0.01 respectively; compared to OVX rats + HFD diet). In addition, it also decreased escape latency in OVX rats with HFD diet (p-value <0.05; compared to OVX rats with HFD diet).

Changes of oxidative stress status and AChE

OVX rats which fed with HFD diet showed the increased AChE and MDA but decreased SOD, CAT and GPx activities (p-value <0.01, 0.01, 0.01, 0.01 and 0.05 respectively; compared to naive intact rats). Sham GV20 failed to modify the mentioned parameters. However, GV20 stimulation with red laser significantly decreased AChE and MDA but increased SOD, CAT and GPx activity (p-value <0.01 all; compared to OVX rats +HFD diet) as shown in Table 2.

Apoptotic change

Sham laser acupuncture failed to modify the neurons densities in hippocampus (Figure 1). However, GV20 significantly enhanced neurons densities in hippocampus (p-value <0.05 all; compared to OVX rats + HFD diet). In addition to the neuron density in the mentioned area, the densities of neurons stained with Bax+ (apoptotic inducing protein) and Bcl-2+ (apoptotic suppression protein) in hippocampus were also investigated in order to assess the effect of GV20 on apoptosis. Sham GV20 laser acupuncture failed to induce the significant changes on Bax+ and Bcl-2+ neurons in all areas of hippocampus as shown in Figure 1 and 2. Interestingly, GV20 laser acupuncture also significantly decreased Bax+ cells density but increased Bcl-2+ cells in all areas mentioned earlier.

Discussion

The present study clearly demonstrated that the stimulation of GV20 acupoint simultaneously enhanced memory, decreased MDA level, AChE activity, and Bax+cell but increased SOD, CAT, and GPx enzymes, densities of the survival neurons and Bcl-2+ cells in the hippocampus.

The current data showed that OVX rats with obesity showed the reduction in SOD, CAT and GPx activities giving rise to the elevation of oxidative stress which in turn enhanced the attack of polyunsaturated fatty acid (PUFA) and oxidative giving rise to the elevation of malondialdehyde (MDA) level. The elevation of MDA level observed in this study was in agreement with the previous studies which showed that obesity decreased total antioxidant capacity[34,35]. The elevation of oxidative stress in hippocampus is reported to induce memory impairment [36]. Based on aforementioned information, the cognitive enhancing effect induced by the

Table 1. The effect of laser acupuncture at GV20 acupoints on spatial memory

Treatment groups	7 days		14 days	
	Escape latency (s)	Retention time (s)	Escape latency (s)	Retention time (s)
Naive intact	5.54±0.609	21.65±1.86	5.40±0.57	21.65±1.58
OVX + HFD	10.38±1.46*	18.06±1.86	11.83±3.58*	23.42±2.29
OVX + HFD + sham GV20	10.25±0.52*	26.58±0.93^{aa}	10.42±1.58*	27.00±2.18
OVX + HFD + GV20	6.88±0.97	27.42±0.77^{a}	5.20±0.72^{a}	36.10±1.63**,^{aa}

Data are presented as mean ± SEM (n = 6/group). *,**p<0.05, 0.01; compared to naive intact rats and $^{a,aa}p$<0.05, 0.01; compared to OVX + HFD

Table 2. The effect of laser acupuncture at GV20 on acetylcholine esterase (AChE) and oxidative stress status in hippocampus

Treatment groups	AChE activity (nmol/mg protein)	MDA level (nmol/mg protein)	SOD activity (U/mg. Protein)	CAT activity (U/mg. protein)	GPxactivity (U/mg protein)
Naive intact	0.89±0.03407	0.069±0.002	32.783±1.82	37.222±2.30	4.8984□0.41
OVX + HFD	0.53±0.021**	0.262±0.007**	9.51±0.491**	4.78±0.472**	2.34±0.260*
OVX + HFD + sham GV20	0.51±0.101**	0.261±0.007**	9.36±0.450**	4.13±0.477**	1.61±0.424*
OVX + HFD + GV20	0.34±0.039**,^{aa}	0.031±0.004**,^{aa,bb}	14.77±1.052**,^{aa}	11.44±1.240**,^{aa}	6.06±0.656^{aa,bb}

Data are presented as mean ± SEM (n = 6/group). *,**p<0.05, 0.01; compared to naive rats, ^{aa}p<0.01; compared to OVX + HFD and ^{bb}p<0.01; compared to OVX + HFD + sham GV20

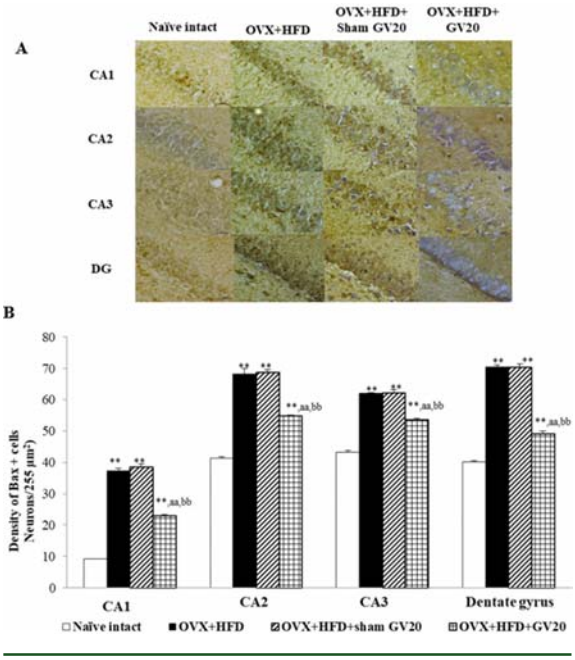

Figure 1. The effect of laser acupuncture at GV20 on density of Bax + neurons in hippocampus. (A) Light microscope of coronal sections of in CA1, CA2, CA3 and dentate gyrus of hippocampus were stained by immunohistochemistry at 40X magnification. (B) Density of Bax + neurons in the CA1, CA2, CA3 and dentate gyrus of the hippocampus. Data are presented as mean ± SEM (n = 6/group). *,**p<0.05, 0.01 respectively; compared to naive intact, $^{a,aa}p$<0.05, 0.01 respectively; compared to OVX + HFD and $^{b,bb}p$<0.05, 0.01 respectively; compared to OVX + HFD + sham GV20.

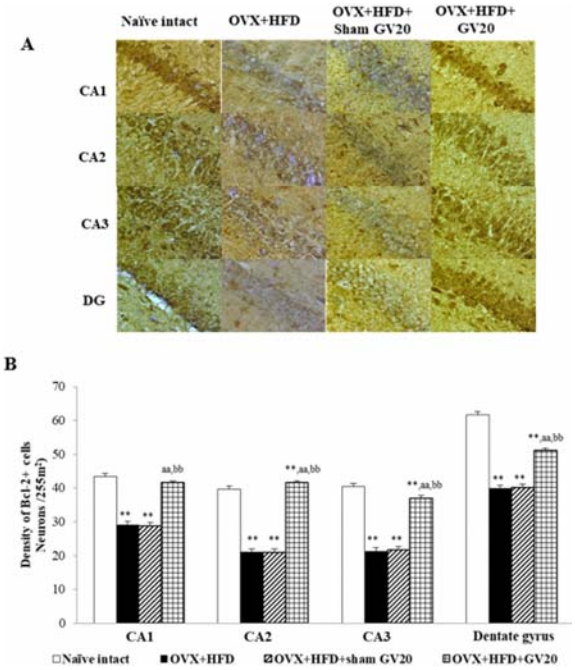

Figure 2. The effect of laser acupuncture at GV20 on density of Bcl-2 + neurons in hippocampus. (A) Light microscope of coronal sections of in CA1, CA2, CA3 and dentate gyrus of hippocampus were stained by immunohistochemistry at 40x magnification. (B) Density of Bcl-2 +neurons in the CA1, CA2, CA3 and dentate gyrus of the hippocampus. Data are presented as mean ± SEM (n = 6/group). *,**p<0.05, 0.01 respectively; compared to naive intact, $^{a,aa}p$<0.05, 0.01 respectively; compared to OVX + HFD and $^{b,bb}p$<0.05, 0.01 respectively; compared to OVX + HFD + sham GV20.

stimulation of GV20 acupoint observed in this study might occur partly the improvement of oxidative stress status in both areas just mentioned.

It has been well known that hippocampus contributes the crucial role on cognitive function, especially working memory[37]. In addition to oxidative stress status mentioned earlier, cholinergic system is also essential for memory performance[38]. The suppression of AChE in hippocampus can also improve memory in various conditions[14-18,39]. In addition, apoptosis in hippocampus also plays the essential roles on memory performance[40,41]. It has been revealed that Bcl-2 family proteins play the pivotal role on apoptosis. Bcl-2 protein is regarded as antiapoptotic protein whereas Bax protein is regarded as a proapoptotic protein. Therefore, the reduction in Bax+ cells but elevation in Bcl-2+ cells densities indicated the reduction of apoptosis and neurodegeneration in the hippocampus. Based on these pieces of information, both the suppression of AChE which in turn enhanced the cholinergic system in the hippocampus together with the reduction of apoptosis induced by the elevation of Bcl-2+ but decreased Bax +cells giving rise to the increase in survival neurons in an area just mentioned also contributes the role on the memory enhancing effect of GV20 stimulation.

The present study is the first study to demonstrate the cognitive enhancing effect and neuroprotective effect of GV20 stimulation. The possible underlying mechanism may occur partly via the decreased oxidative stress status by decreasing MDA but increasing SOD, CAT and GPx activities in hippocampus. The increase in cholinergic function and apoptosis also play the roles.

What is already known on this topic?

Acupuncture has been long term used for treating various disorders, including menopause related symptoms, obesity and memory impairment. GV20 is one of the acupoints commonly used for treating dementia.

What this study adds?

The stimulation of GV20 with laser produces neuroprotective and cognitive enhancing effects by improving cholinergic function, oxidative stress status and apoptosis in hippocampus.

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Potential conflicts of interest

The authors declare no conflict of interest.

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