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Effect of sildenafil citrate on brain central fatigue after exhaustive swimming exercise in rats

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Sildenafil citrate, a potent and selective inhibitor of phosphodiesterase type-5, is used clinically to treat erectile dysfunction and pulmonary arterial hypertension. We investigated the effect of sildenafil citrate on brain central fatigue through serotonin (5-hydroxytryptamine, 5-HT) synthesis after exhaustive swimming exercise in rats. The rats in the sildenafil citrate-treated groups received sildenafil citrate orally once a day for 14 consecutive days at respective dosage. On the 14 days after starting experiment, each animal was submitted to swimming test with intensity equivalent to overload. The exhaustion was defined as a state in which coordinated movements did not return to the water surface for breathing within 10 sec. Immunohistochemistry for 5-HT, tryptophan hydroxylase (TPH), and western blot for serotonergic type 1A (5-HT1$_A$) receptor and 5-HT transporter (5-HTT) were performed. Exhaustive swimming exercise increased 5-HT and TPH expressions in the dorsal raphe and sildenafil citrate suppressed 5-HT and TPH expressions in the exhaustive swimming exercise rats. Exhaustive swimming exercise increased 5-HT1$_A$ receptor and 5-HTT expressions in the dorsal raphe and sildenafil citrate suppressed 5-HT1$_A$ receptor and 5-HTT expressions in the exhaustive swimming exercise rats. The significant suppressing effect appeared in the 20-mg/kg sildenafil citrate. Sildenafil citrate might be proposed as a potential ergogenic aid through anticentral fatigue.

Keywords: Sildenafil citrate, Central fatigue, Exhaustive exercise, Serotonin

INTRODUCTION

Central fatigue is associated with regulation of serotonergic neurotransmitter system, such as serotonin (5-hydroxytryptamine, 5-HT), tryptophan hydroxylase (TPH), 5-HT receptor, and 5-HT transporter (5-HTT) (Cordeiro et al., 2017). 5-HT is an important neurotransmitter, which acts as a biochemical messenger and regulator of the brain function (Kim et al., 2017). Increase in the concentration of 5-HT in the brain during prolonged exercise impairs central nervous system functions and thus bring about a deterioration in exercise performance (Newsholme et al., 1992). 5-HT is modulated by many factors involved in the intrinsic regulation of central neurotransmission, which include TPH and serotonergic type 1A (5-HT1$_A$) receptor (Foley et al., 2006). TPH catalyzes the rate-limiting step of serotonin biosynthesis in the dorsal raphe (Rind et al., 2000). 5-HT1$_A$ receptor negatively regulates the activity of 5-HT neurons and is expressed in presynaptic autoreceptor on raphe neurons (Albert and Lemonde, 2004).

Sildenafil citrate, a potent and selective inhibitor of phosphodiesterase type-5, is used clinically to treat erectile dysfunction and pulmonary arterial hypertension (Mostafa, 2008). Sildenafil citrate increases local concentration of cyclic guanosine monophosphate, thus causing vasodilatation by relaxation of arterial wall smooth muscle fibers, especially in the corpus cavernosum and lungs (Harrold et al., 2000). Some studies have proposed the application of sildenafil citrate on several diseases, including myocardial in-

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farction, heart failure, and stroke (Guazzi et al., 2011; Kukreja et al., 2005). In addition, sildenafil citrate has been shown to improve exercise capacity in subjects with cardiopulmonary diseases (Kloner, 2004) and in healthy subjects in hypoxia (Hsu et al., 2006).

As sildenafil citrate is an effective pulmonary vasodilator (Schermuly et al., 2004), sildenafil citrate is a potent ergogenic aid during exercise performed under hypoxic conditions. However, anti-fatigue effect of sildenafil citrate is the tendency to focus on only peripheral fatigue in hypoxic conditions. Therefore, we investigated effects of sildenafil citrate administration on brain central fatigue after exhaustive swimming exercise in rats.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (8 weeks old) were used in this experiment. The experimental procedure was performed in accordance with the animal care guidelines of the National Institutes of Health and the Korean Academy of Medical Sciences. The rats were randomly divided into four groups (n = 10 in each group): the control group, the exhaustive exercise group, the exhaustive exercise and 20-mg/kg sildenafil citrate-treated group, and the exhaustive exercise and 40-mg/kg sildenafil citrate-treated group. The rats in the sildenafil citrate-treated groups received sildenafil citrate (Sigma Chemical Co., St. Louis, MO, USA) orally once a day for 14 consecutive days at respective dosage. The rats in the control and exercise groups received normal saline orally once a day for 14 consecutive days.

Exhaustive swimming exercise

On the 14 days after starting experiment, each animal was submitted to exhaustive swimming test with intensity equivalent to overload (a metal ring weight attached to the animal's torso) of 10% of the body weight (Ding et al., 2009). The exhaustion was defined as a state in which coordinated movements did not return to the water surface for breathing within 10 sec.

Tissue preparation

After the exhaustive swimming exercise test, the rats were deeply anesthetized with Zoletil 50 anesthesia (40 mg/kg, intraperitoneally; Vibac Laboratories, Carros, France). After complete anesthesia, the rats were transcardially perfused with 0.05 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.5 M sodium phosphate buffer at pH 7.4. The brain was removed, postfixed in the same fixative overnight, and transferred to a 30% sucrose solution for cryoprotection. Serial 40-μm-thick coronal sections were cut with a freezing microtome (Leica, Nussloch, Germany).

Immunohistochemistry for 5-HT and TPH

Immunohistochemistry for 5-HT and TPH was performed, according to the previously described method (Shin et al., 2017). An average of eight sections was selected in each brain region spanning from Bregma -7.30 to -8.00 mm. The sections were incubated in PBS for 10 min and they were next washed three times with PBS. The sections were then incubated in 1% H_2O_2 for 30 min, and then they were incubated overnight with rabbit anti-5-HT antibody (Oncogene Research Product, Cambridge, UK) at a dilution of 1:500 or with mouse anti-TPH antibody (Oncogene Research Product) at a dilution of 1:500. The sections were incubated for 1 hr with biotinylated anti-rabbit secondary antibody or with anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA), and they were subsequently incubated with avidin–biotin–peroxidase complex (Vector Laboratories) for 1 hr at room temperature. Immunoreactivity was visualized by incubating the sections in a solution consisting of 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris-buffer (pH, 7.6) for approximately 3 min. The sections were finally mounted on gelatin-coated glass slides. The slides were air-dried overnight at room temperature, and the coverslips were mounted using Permount (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Western blot analysis

Western analysis was performed, according to the previously described method (Shin et al., 2017). Dorsal raphe tissues were dissected. Sample tissues were stored at -70°C until analysis. The tissues were lysed in ice-cold lysate buffer containing 50 mM HEPES (pH, 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 2-μg/mL leupeptin, 1-μg/mL pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride, after which the mixture was incubated for 30 min at 4°C. The protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein of 30 μg was separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto a nitrocellulose membrane (Whatman, Clifton, NJ, USA). Mouse anti-β actin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-5-$HT1_A$ receptor antibody (1:2,000; Abcam, Cambridge, UK) and rabbit anti-5-

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HTT (1:2,000; Abcam) were used as a primary antibody. A horseradish peroxidase-conjugated anti-mouse secondary antibody was used for β-actin, and an anti-rabbit secondary antibody was used for 5-HT1$_A$ receptor and 5-HTT. Band detection was performed using the enhanced chemiluminescence detection system (Santa Cruz Biotechnology). The bands were quantified using an Image-Pro Plus computer-assisted image analysis system (Media Cybernetics Inc., Bethesda, MD, USA).

Statistical analysis

Differences among the groups were evaluated using IBM SPSS Statistics ver. 23.0 (IBM Co., Armonk, NY, USA) by the one-way analysis of variance followed by Duncan *post hoc* test. All values are expressed as the mean±standard error of the mean. Statistically

Table 1. Effect of sildenafil citrate on exhaustive swimming time in rats

Exhaustive exercise group	Exhaustive exercise and 20 mg sildenafil citrate-treated group	Exhaustive exercise and 40 mg sildenafil citrate-treated group
18.85±0.69 min	26.39±4.75 min*	20.89±0.90 min

The results are presented as the mean±standard error of the mean.
*P<0.05 compared to the exhaustive exercise group.

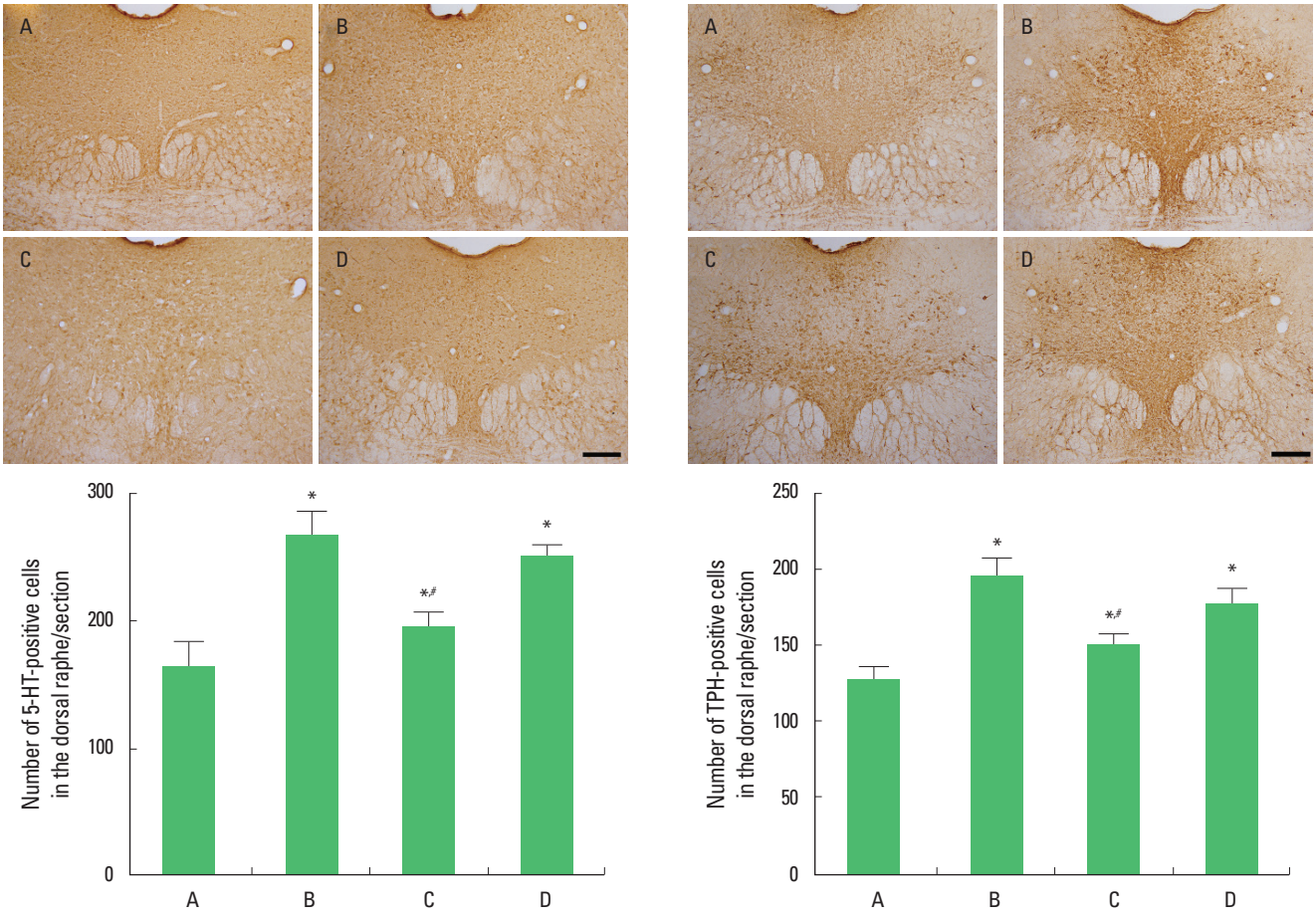

Fig. 1. Effects of sildenafil citrate on 5-hydroxytryptamine (5-HT). Upper panel: Photomicrographs showing 5-HT expressions in the dorsal raphe. Lower panel: The results are presented as the mean±standard error of the mean. The scale bar represents 250 μm. A, control group; B, exhaustive exercise group; C, exhaustive exercise and 20-mg/kg sildenafil treatment group; D, exhaustive exercise and 40-mg/kg sildenafil treatment group. *P<0.05 compared to the control group. #P<0.05 compared to the exhaustive exercise group.

Fig. 2. Effects of sildenafil citrate on tryptophan hydroxylase (TPH). Upper panel: Photomicrographs showing TPH expressions in the dorsal raphe. Lower panel: The results are presented as the mean±standard error of the mean. The scale bar represents 250 μm. A, control group; B, exhaustive exercise group; C, exhaustive exercise and 20-mg/kg sildenafil treatment group; D, exhaustive exercise and 40-mg/kg sildenafil treatment group. *P<0.05 compared to the control group. #P<0.05 compared to the exhaustive exercise group.

significant differences were established at $P < 0.05$.

RESULTS

Effect of sildenafil citrate on exhaustive swimming time

Exhaustive swimming time is presented in Table 1. Exhaustive swimming time was increased in the exhaustive exercise and sildenafil citrate-treated groups than exhaustive swimming exercise group. The significant increasing effect on exhaustive swimming time appeared in the 20-mg/kg sildenafil citrate ($P < 0.05$).

Effect of sildenafil citrate on 5-HT expression in the dorsal raphe

Photomicrographs of 5-HT-positive cells in the dorsal raphe are presented in Fig. 1. Exhaustive swimming exercise increased 5-HT expression in the dorsal raphe ($P < 0.05$) and sildenafil citrate suppressed 5-HT expression in the exhaustive swimming exercise rats ($P < 0.05$). The significant suppressing effect appeared in the 20-mg/kg sildenafil citrate.

Effect of sildenafil citrate on TPH expression in the dorsal raphe

Photomicrographs of TPH-positive cells in the dorsal raphe are presented Fig. 2. Exhaustive swimming exercise increased TPH expression in the dorsal raphe ($P < 0.05$) and sildenafil citrate suppressed TPH expression in the exhaustive swimming exercise rats ($P < 0.05$). The significant suppressing effect appeared in the 20-mg/kg sildenafil citrate.

Effect of treadmill exercise on 5-HT1$_A$ receptor and 5-HTT expression in the dorsal raphe

The 5-HT1$_A$ receptor and 5-HTT expressions are shown in Fig. 3. Exhaustive swimming exercise increased 5-HT1$_A$ receptor and 5-HTT expressions in the dorsal raphe ($P < 0.05$) and sildenafil citrate suppressed 5-HT1$_A$ receptor and 5-HTT expressions in the exhaustive swimming exercise rats ($P < 0.05$). The significant suppressing effect appeared in the 20-mg/kg sildenafil citrate.

DISCUSSION

Sildenafil citrate decreases pulmonary vascular resistance and increases VO_{2max} in hypoxic normal subjects (Ghofrani et al., 2004; Richalet et al., 2005). Galiè et al. (2005) reported that sildenafil citrate improved exercise capacity and pulmonary hemodynamic in patients with pulmonary arterial hypertension. Moreover, chronic sildenafil citrate treatment increased dopamine and

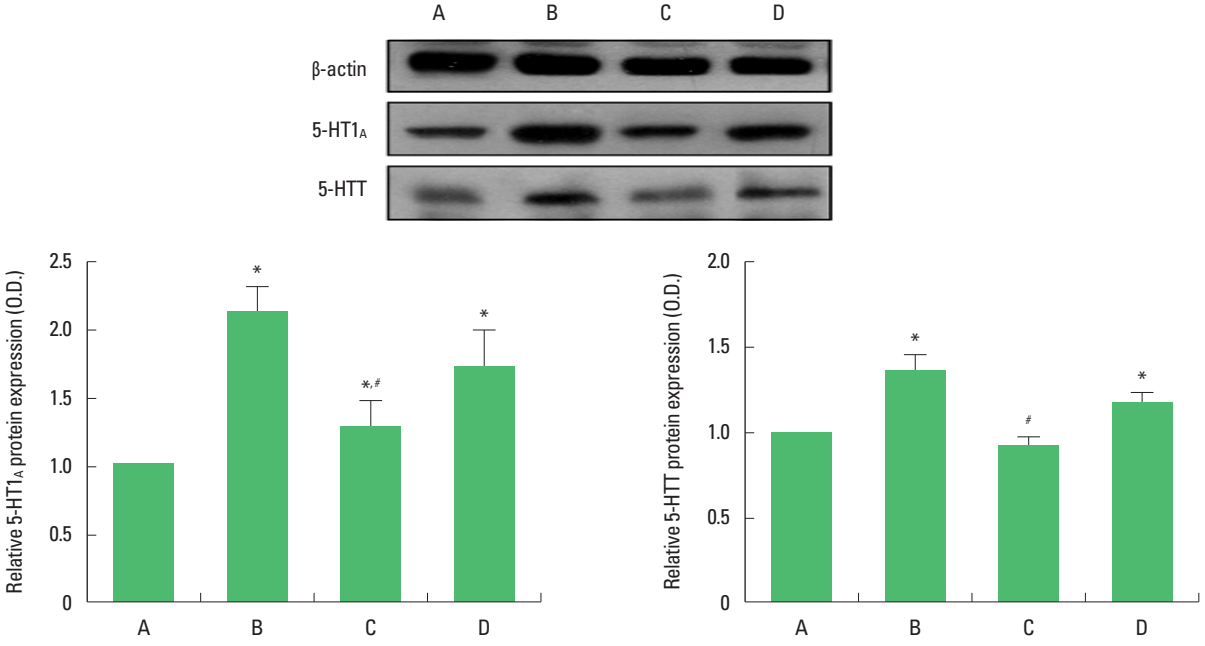

Fig. 3. Effects of sildenafil citrate on 5-hydroxytryptamine-1A (5-HT1$_A$) receptor and 5-hydroxytryptamine transporter (5-HTT) expressions in the dorsal raphe. Upper panel: The expressions of 5-HT1$_A$ and 5-HTT. Lower panel: The results are presented as the mean ± standard error of the mean. A, control group; B, exhaustive exercise group; C, exhaustive exercise and 20-mg/kg sildenafil treatment group; D, exhaustive exercise and 40-mg/kg sildenafil treatment group. *$P < 0.05$ compared to the control group. #$P < 0.05$ compared to the exhaustive exercise group.

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5-HT turnover rate in the medial preoptic area and nucleus accumben in the normal male rats (Kyratsas et al., 2013). In the present study, treatment of 20-mg sildenafil citrate prolonged exhaustive swimming time.

Central fatigue has been proposed the implication of various neurotransmitters such as serotonin, norepinephrine, and dopamine (Newsholme et al., 1992). Among these, serotonin is known to play the most important role in the pathogenesis of central fatigue (Kim et al., 2017). Increment in the 5-HT concentration in the brain is associated with mental fatigue (Davis et al., 2000). Increased concentration of 5-HT leads to the onset of fatigue (Soares et al., 2007), while decreased 5-HT concentration could delay the time to fatigue (Seo et al., 2011). In addition, exhaustive exercise increases 5-HT concentrations and then causes decrement of endurance performance (Caperuto et al., 2009). Inhibition of 5-HT production in the brain could increase endurance exercise performance (Seo et al., 2011). The synthesis of 5-HT is modulated by the hydroxylation of the amino acid tryptophan, which is the rate-limiting step catalyzed by TPH (Carkaci-Salli et al., 2006). Reduction in TPH expression decreases in 5-HT synthesis (Park et al., 2019). In the present study, treadmill exercise increased 5-HT synthesis and TPH expression in the dorsal raphe and 20-mg sildenafil citrate suppressed the exhaustive exercise-induced increase of 5-HT synthesis and TPH expression in the dorsal raphe.

Treadmill exercise upregulates the levels of 5-HT and 5-HT1$_A$ receptor in rats with permanent middle cerebral artery occlusion (Lan et al., 2014). 5-HT1$_A$ receptor expression in the dorsal raphe was reduced by olfactory bulbectomy and treadmill exercise increased 5-HT1$_A$ receptor expression in the olfactory bulbectomized rats (Shin et al., 2017). Exhaustive exercise increased 5-HT1$_A$ receptor and 5-HTT and colostrum serum treatment suppressed exhaustive exercise-induced 5-HT1$_A$ receptor and 5-HTT expression in the dorsal raphe (Kim et al., 2017). Primary pulmonary hypertension is caused by increased expression of the 5-HTT (Eddahibi et al., 2001). Degree of central fatigue depends on the function of the serotonin system (Kim et al., 2017; Maluchenko et al., 2009). In the present study, treadmill exercise increased 5-HT1$_A$ receptor and 5-HTT expression in the dorsal raphe and 20-mg sildenafil citrate suppressed the exhaustive exercise-induced increase of 5-HT1$_A$ receptor and 5-HTT expressions in the dorsal raphe.

These results may support sildenafil citrate as an ergogenic aid to improve exercise performance. Sildenafil citrate might be proposed as a potential ergogenic aid through anticentral fatigue.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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