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KEYWORDS: cognitive, discrimination, reinforce, operant, spinach

INTRODUCTION

Spinacia oleracea Linn. (spinach; SP) is a green leafy vegetable in the Amaranthaceae family. It is an annual plant that grows in low temperature place. In Thailand, it is usually grown in the north part. SP has a high nutritional value and is a rich source of vitamins, especially vitamin B9 or folate. Raw spinach is found 194 µg/100g of folate containing (<http://en.wikipedia.org/wiki/Spinach>). Folate or folic acid is an essential vitamin, which is necessary for various body functions, particularly brain function (Sittig *et al.*, 2012). It has been suggested to consume 400 µg/day of folic acid for adolescents and adults (http://en.wikipedia.org/wiki/Folic_acid). The procedure of conditional discrimination has been used to study learning, memory, and perception (Saunders and Spradlin, 1989). Operant conditioning technique, in particular, is a type of learning in which the likelihood of a behavior is increased or decreased through rewards and/or punishments. This method deals with cognitive thought process. Discrimination between conditions occurs when a subject learns to respond to only one stimulus and inhibit the response to all other stimuli (Rees *et al.* 1985). Although a subject receives many different stimuli, but is only given reinforcement for responding to only one of those stimuli, it learns to discriminate between the conditions. Therefore, the purpose of this study was to determine whether SP could improve learning and memory impairment induced by scopolamine via operant conditioning procedure, which might lead to cognitive improvement in rats.

MATERIALS AND METHODS

Materials

Twenty four male Wistar rats were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom. Age of the rats at the beginning of study was 2 months old. All procedures and animal care were approved by Institutional Animal Care and Use Committee of Thailand Institute of Scientific and Technological Research.

SP was prepared in a dried powder form and was controlled the dried process to have approximately 800-1,000 µg/100g of folate content. It was disposed in distilled water (DW) to make water supplementation with concentrations of 6 (SP6) and 12 (SP12) mg/ml. Folic acid (FO), a standard drug for learning and memory, was dissolved in DW and prepared in water supplement form at a concentration of 0.02 mg/ml. Scopolamine, an acetylcholinesterase, was prepared in 0.9% normal saline solution. It was used to induce an impairment of learning and memory in rats. A milk mixture (tap water : Carnation® sweetened condensed milk was 3:1) was used as a reward for the operant task (McKerchar *et al.* 2005).

Operant conditioning system (Lafayette Instrument Company, USA) with 4 operant rat chambers were used to determine operant behavior.

Methods

Rats were trained the operant behavior in operant conditioning chambers 5 days a week in a 30-min session for approximately 4 months. The programmed contingencies were identical for each session consisting of 3 cycles with 10 min each. Each 10 min contained 4 steps. At the first 2 min, the rats pressed the right lever (an active lever) with response being reinforced, and milk was delivered under the light over the right lever (the right light) was on. When the right light was gone, a press on the right lever was no any consequences for 2 min. Next, the right light was programmed to flash for 4 min with milk delivery under pressing an active lever. At the last 2 min, the right light was off again with no response under any presses on the right lever. The condition of pressing an active lever with lighting of the right light for the first 2 min was scheduled as a fixed ratio (FR). In this schedule, rats were reinforced under

FR10 (every 10 lever presses for 1 milk reward (RE)). The condition of pressing an active lever with flashing of the right light for 4 min was a variable ratio (VR) schedule, which rats were trained to response the VR 2 or 3 (randomly 2 or 3 lever presses for 1 reward). Pressing on an inactive lever (the left one) was specified as a wrong lever (WR) with no any consequences. This manner usually occurs when learning and memory are impaired since the rats have never been trained to press that inactive lever.

Rats were randomly divided into four groups. They were orally given for 21 days with water supplementation (as regular water supply) of 0.02 mg/ml FO, 6 and 12 mg/ml SP, and distilled water (DW; as a control group). Amount of water supplement intake of each group was recorded and computed the approximate dosage of each supplementation receiving per day. On the last day of the experiment (day 21), rats were injected (i.p.) with 0.5 mg/kg scopolamine. Thirty minutes later, rats were exposed to a 30-min session of the operant acquisition procedure. The operant behaviors of each rat were recorded by ABET II software. For each schedule (FR and VR), rat's behaviors were computed as the following; the number of active lever presses, the number of inactive lever presses, and the number of reward (RE). All data were expressed as mean \pm standard error. Statistical analysis for comparing treatment effects of each group of rats was done by one-way ANOVA. Comparisons among groups were conducted using the Tukey post-hoc analysis. Student's t-test techniques were also used. Statistical significance was defined as $p < 0.05$.

RESULTS

Amount of water supplement intake and approximately equivalent dose of each group were shown in Table 1. FO consumption at the concentration of 0.02 mg/ml was 22 ml/day/rat. An equivalent dose of FO was approximately 0.5 mg/kg/day. Intakes of both SP6 and SP12 were 23 ml/day/rat, which were computed to approximately 140 and 280 mg/kg/day equivalent doses, respectively. DW was also consumed 23 ml/day/rat.

The number of active lever presses, the number of rewards, and the number of inactive lever presses were shown in Figure 1. The SP12 group, in responding to FR condition, showed the highest numbers of lever presses in all 3 cycles. A statistical difference was found in cycle 1 ($p = 0.021$). Corresponding to lever pressing, the number of rewards of SP12 (REFRSP12) was significantly higher than others ($p = 0.029$). For VR condition, SP12 also showed the highest numbers of lever presses in all 3 cycles. This group of rat exhibited statistical differences in cycle 1 and 2 ($p = 0.002$ and 0.001 , respectively). As well as the number of active lever presses, SP12 revealed remarkably highest numbers of rewards in both cycle 1 and 2 with $p = 0.004$ and 0.002 , respectively. However, there were no any differences in responding to FR and VR schedules of FO group comparing with the control. The numbers of wrong lever presses were found in all groups of rats, but there were no any significant differences among groups. Interestingly, the lowest numbers of wrong lever presses were found in the SP12 group.

Table 1 Amount of water supplement intake and approximately equivalent doses of FO and SP comparing with amount of DW intake (in average)

Group	Amount of water supplement intake (ml/day/rat)	Approximately equivalent dose (mg/kg/day)
DW	23	-
FO	22	0.5
SP6	23	140
SP12	23	280

DISCUSSION

Cognitive impairment could be produced by 0.5 mg/kg scopolamine injection since the number of inactive lever pressing, which usually exhibited under the influence of learning and memory impairment, was found in all groups of rats. SP, particularly 280 mg/kg dose, showed evidence to reverse the learning and memory impairment induced by scopolamine as expressed the high number of active lever presses and low number of inactive lever presses. FO exhibited low number of active lever presses and number of rewards. Possibly, the given dose of FO in this experiment was quite lower than expectation. The SP12 rats, equally 280 mg/kg dose, initially discriminated stimulus between conditions better than the SP6 rats (approximately 140 mg/kg dose) did. Discrimination between conditions resulted in learning and memory enhancing (Rees *et al.* 1985). Therefore, the data demonstrate that SP at a dose of 280 mg/kg probably affect learning and memory-related cholinergic transmission; however, it is necessary to confirm the acetyl cholinesterase levels in the brain and serum in the further study.

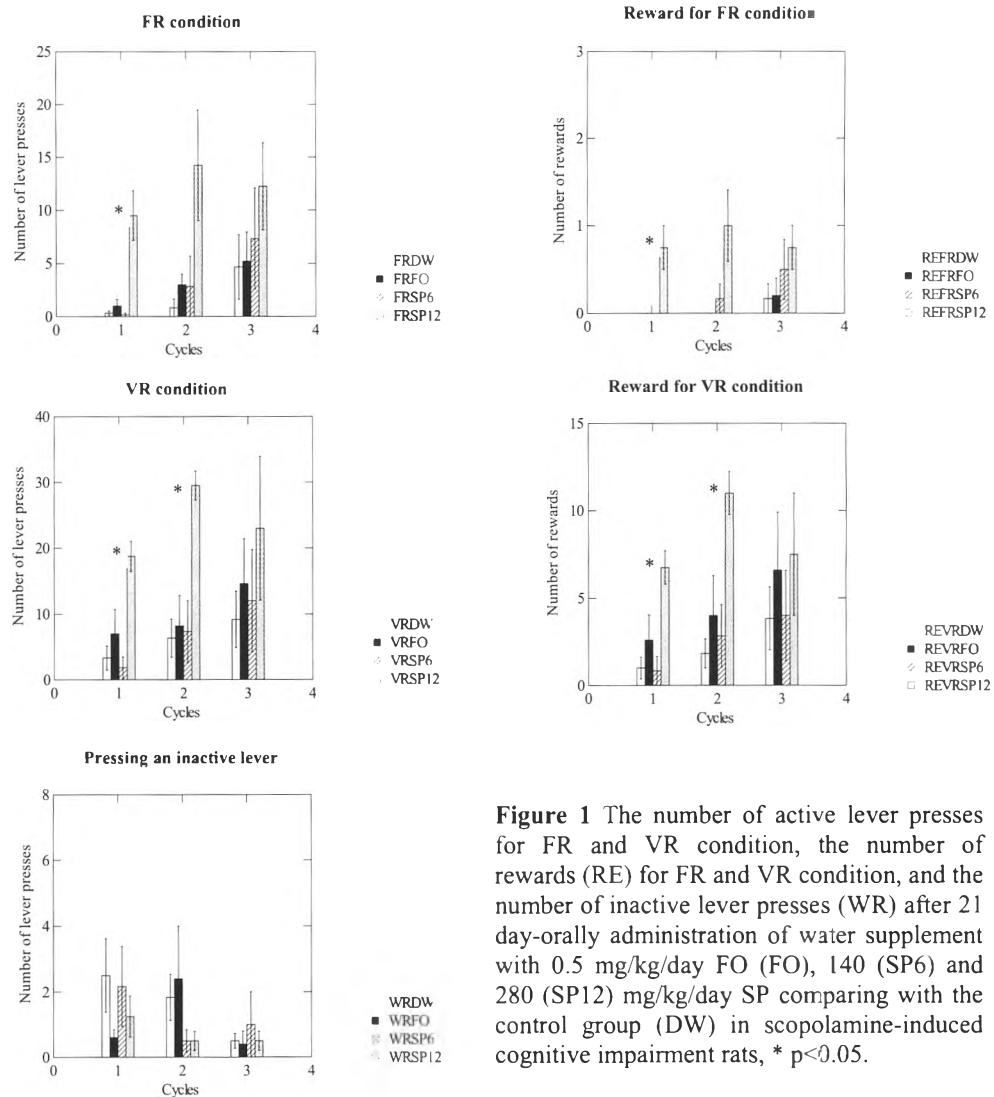


Figure 1 The number of active lever presses for FR and VR condition, the number of rewards (RE) for FR and VR condition, and the number of inactive lever presses (WR) after 21 day-orally administration of water supplement with 0.5 mg/kg/day FO (FO), 140 (SP6) and 280 (SP12) mg/kg/day SP comparing with the control group (DW) in scopolamine-induced cognitive impairment rats, * p<0.05.

CONCLUSION

As an indication to diminish the effect of scopolamine-induced learning and memory impairment via operant conditioning procedure was found in SP12 rats, it suggested that 21 day-consumption of SP supplements at 280 mg/kg/day possibly aid the promotion of brain functions, especially learning and memory, involved cholinergic pathway.

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http://en.wikipedia.org/wiki/Folic_acid

<http://en.wikipedia.org/wiki/Spinach>