



## The effects of *Anethum graveolens* essence on scopolamine-induced memory impairment in mice

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### Abstract

Since *Anethum graveolens* (Dill) has phytoestrogenic compounds and it is proven that estrogens exert beneficial effects on cognition; the aim of this study was to understand if this plant can improve memory performance. Male Balb/c mice weighing 25-30 g were used in this study and memory was assessed by the novel object recognition task. In this method, the difference in the exploration time between a familiar object and a novel object is taken as an index of memory performance (recognition index, RI). Scopolamine significantly reduced memory index (RI = -15.5% ± 3.0). Dill essence (100 mg/kg, ip) prevented the harmful effects of scopolamine on memory (RI = 40% ± 5.5), thus RI did not differ with control animals (RI = 50% ± 5.8). In addition, 17-β estradiol also prevented memory impairment in animals (0.2 mg/kg, ip; RI = 35.8% ± 6.5). Nevertheless, the beneficial effects of dill essence were antagonized by prior injection of tamoxifen (1 mg/kg, ip; RI = -30% ± 7.8). Although phytoestrogens are not steroids, the beneficial effect of dill on memory, at least in part, may have been achieved by estrogenic receptors present in the brain. Thus dill essence could be promising in improving memory and cognition, mainly in postmenopausal women.

**Keywords:** *Anethum graveolens*; Dill; Estrogen; Memory; Phytoestrogen; Tamoxifen

### INTRODUCTION

Memory is controlled via ample receptors, neurotransmitters, and hormones in the brain. Previously the profound effect of glucocorticoids on memory performances was revealed (1). Experiments showed that not only the glucocorticoid receptors, but also mineralocorticoid receptors play an important role in recognition memory impairment following morphine withdrawal (2). Estrogen is also a steroid that regardless of its obvious effects on females' reproductive functions shows beneficial effects on cognition. In the present study effects of, *Anethum graveolens*, on memory is going to be considered in the novel object recognition task.

Estrogens are required for the normal sexual maturation and growth in females. Gonadal steroids show beneficial effects on normal cognitive performance. In aging men and women circulating levels of these

hormones have been positively correlated with cognitive function (3,4). Evidently postmenopausal replacement of ovarian steroids protects against declines in verbal memory in normal healthy women, while decreasing the risk of Alzheimer's disease (5). Researchers have shown that estrogen enhances learning and memory in rats when administered a few days or weeks after ovariectomy (6,7).

Estrogens have numerous effects on the brain, both in adulthood and during development (8). These actions of estrogen are mediated by two distinct estrogen receptors, ER alpha (ERα) and ER beta (ERβ) (9). Studies of their tissue distribution indicate that ERα has high expression in uterus, testis, ovary and kidney, while ERβ is expressed mainly in uterus, testis, ovary, bone, lung and brain. In brain, ERα plays a critical role in regulating reproductive neuroendocrine function and behavior. Although both

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receptors are expressed by neurons in the arcuate nucleus and hippocampus, ER $\beta$  is more prevalent in the hippocampus (8).

*A. graveolens* (Dill) is a plant species cultivated worldwide for the flavoring and curative properties in digestive disturbances, flatulence and gastro-intestinal spasms, urinary infections, insomnia, galactogenic hyposecretion (10). The major components of dill are flavonoids, phenolic compounds and essential oil (11). This herb also consist kaempferol, trans-anethole and limonene which show phytoestrogenic properties (12).

Phytoestrogens are polyphenolic non-steroidal plant compounds with estrogen-like biological activity. Based on their chemical structure, phytoestrogens can be classified into four main groups, i.e., isoflavonoids, flavonoids, stilbenes, and lignans (13). Our former findings revealed the beneficial effect of phytoestrogen containing plant *Foeniculum vulgare* on memory performance (14). Since dill contains phitoestrogens, its estrogenic properties have been previously determined (15). Thus the aim of the present study was to evaluate if this herb could have beneficial effects on cognition. In order to elucidate the underlying possible mechanism of this effect, tamoxifen, as a selective estrogen receptor modulator was used.

## MATERIALS AND METHODS

### *Animals*

Male Balb/c mice weighing 25-30 g were housed in cages of six at  $21 \pm 2$  °C in a 12-h light-dark cycle with the lights on at day time 6 AM-6 PM. Tap water and standard food pellets were available *ad libitum*. Tests were performed only after the mice had acclimated to the above environment for at least 2 days. In order to minimize circadian rhythm influence, all experiments were conducted between 08:00 and 13:00 h, in a special noise-free room with controlled illumination. Minimum of six mice were used for each treatment group. Experiment was comprised of 9 groups: 1) saline only, 2) dill essence only, 3) scopolamine only, 4) dill and scopolamine, 5) estradiol only, 6) estradiol and scopolamine, 7) tamoxifen only, 8) tamoxifen plus dill plus

scopolamine, 9) dimethyl sulfoxide (DMSO) plus dill plus scopolamine. All animal procedures were approved by the Ethics Committee of Isfahan University of Medical Science and performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals.

### *Novel object recognition task (NORT)*

This experiment was performed as described previously by Ennaceur and Delacour (1988) with some modification (16). Briefly, square wooden open-field (35 × 35 × 40 cm) with the inside painted in dark black and a white floor, provided locally, was used. It was placed in a dark room with a uniform dim light toward the apparatus. At the first day, animals were submitted to a habituation session in the open field and allowed to freely explore the arena in the presence of two objects for at least 15 min. On experimental day, animals were submitted to two trials spaced by an intertrial interval (20 min). During the first trial (acquisition trial, T1), animals were placed in the arena containing two identical objects for an amount of time necessary to explore the objects for 20 s. Any mouse not exploring the objects for 20 s within the 12 min period was excluded from experiments. Exploration is defined as the animal directing the nose within 2 cm of the object while looking at, sniffing, or touching it. For the second trial (test trial, T2), one of the objects presented in the first trial was replaced by new object, animals were placed back in the arena for 5 min and total time spent in exploration of each object was determined. Animals' behavior was recorded by a webcam mounted on top of the apparatus and analyzed later.

### *Drug therapy*

Memory impairment was induced by scopolamine (Iran Exir; 0.5 mg/kg, sc, single dose) injected right after T1. Dill essence (DILSAN 2 % essence, Barij Essence Iran; 100 mg/kg, ip) was administered 15 min before T1. Estradiol valerate (Abureyhan Iran; 0.2 mg/kg, ip) was injected 15 min before T1. Tamoxifen (Iran hormone; 1 mg/kg, ip) was injected 30 min before T1. Tamoxifen was

dispersed in DMSO before being diluted with normal saline. Control animals received normal saline (Irandar, Iran) or DMSO (Merck, Germany) solution relatively. All injections were adjusted for 10 ml/kg mice body weight.

### **Estradiol assay**

Plasma estradiol level was measured in animals treated with dill. At the end of the procedure mice were decapitated and blood sample were collected and the harvested serum samples were stored at -17 °C. Analysis was performed by enzyme immunoassay kit (Estradiol test system, Monobind Inc, USA). This ELISA is based on the competitive reaction between estradiol and the enzyme labeled estradiol for the antibody binding sites on the magnetic particles. The assay was performed according to the manufacturers' protocols. Very briefly appropriate serum, control or specimen was pipetted in the wells. Estradiol biotin reagent was added to all wells. The microplate was swirled, covered and incubated for 30 min at room temperature. Ensuing estradiol enzyme reagent was added and swirled, and incubated for another 90 min. After discarding the substances from the microplate wash buffer was added and decanted for 3 times. Substrate solution was added and incubated for 20 min. After addition of the stop solution, absorbance was recorded at 450 nm.

### **Data processing and statistical analysis**

The following parameters were measured:

time required to achieve 20 s of object exploration on T1 (duration of T1), time spent in active exploration of the familiar (F) or novel (N) object on T2. Recognition memory was evaluated using a recognition index (RI) calculated for each animal using the formula  $(N-F/N+F) \times 100$  corresponding to the difference between the time exploring the novel and the familiar objects, corrected for total time exploring both objects (1,17). Positive values indicate a good discrimination performance, while negative values or those around zero indicate very poor discrimination capacity.

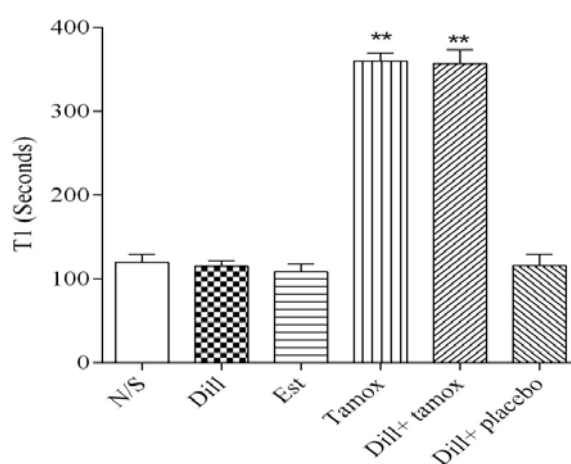
### **Statistical analysis**

Results were expressed as group mean  $\pm$  SEM. All results were analyzed by a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests, *P* values less than 0.05 were considered significant. The software used for data analyzing and drawing graphs was the GraphPad Prizm 5.

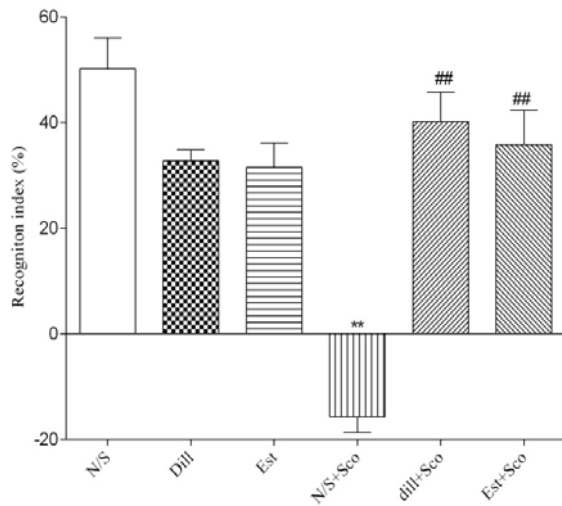
## **RESULTS**

### **Effect of drugs on acquisition time (T1)**

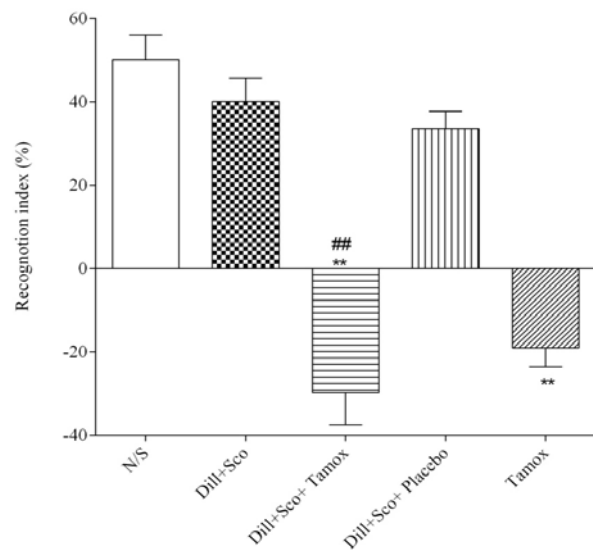
As it is shown in Fig. 1 the mean acquisition time for animals treated by dill or estradiol does not differ from control animals (120 s  $\pm$  9.4). But this amount of time was considerably higher for tamoxifen treated groups (approximately  $\times$  3).



**Fig. 1.** Effect of different treatments on duration of T1 (time required to achieve 20 s of object exploration in the first trial) in mice ( $n = 6$ ). Results are expressed as group mean  $\pm$  SEM and analyzed by ANOVA followed by Tukey's multiple comparison tests ( $n = 6$ ). \*\* $P < 0.001$  compared to the control group. N/S; stands for normal saline, Est; estradiol, Tamox; tamoxifen, and placebo is DMSO (1/9, v/v).



**Fig. 2.** The effect of dill and estradiol (Est) each alone (ip, 15 min before T1) or together with scopolamine (Sco; sc, right after T1) on memory performance. Memory was assessed by recognition index  $RI = (N-F/N+F) \times 100$ , in the novel object recognition task. Results are expressed as group mean  $\pm$  SEM and analyzed by ANOVA followed by Tukey's multiple comparison tests ( $n = 6$ ).  $**P < 0.001$  compared with control values that received only saline (N/S, blank bar).  $###P < 0.001$  compared with the memory impaired group (N/S+Sco).



**Fig. 3.** The effect of tamoxifen (Tamox) alone (ip, 30 min before T1) or together with schopolamin (Sco; sc, right after T1) and dill on memory performance. Memory is expressed as recognition index  $RI = (N-F/N+F) \times 100$ , in the novel object recognition task. Results are expressed as group mean  $\pm$  SEM and analyzed by ANOVA followed by Tukey's multiple comparison tests ( $n = 6$ ).  $**P < 0.001$  compared with control values, that received only saline (N/S, blank bar).  $###P < 0.001$  compared with placebo group.

**Table1.** Time spent exploring the objects in the test trial (T2) by different animal groups.

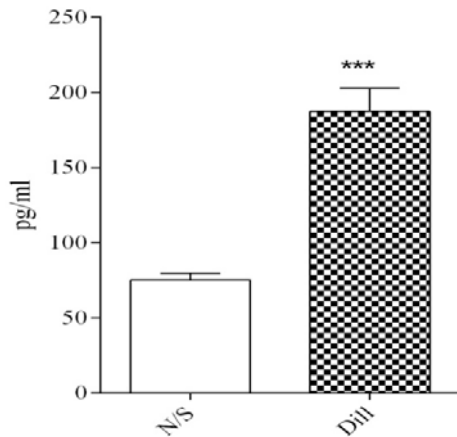
Animal group	New object	Familiar object
Control	41.8 $\pm$ 8.2*	15 $\pm$ 6.2
Scopolamine	12.6 $\pm$ 7.8	17.1 $\pm$ 6.4
Dill	22.6 $\pm$ 10.6*	11.3 $\pm$ 5.6
Dill+Scopolamine	19.6 $\pm$ 8.1*	8.3 $\pm$ 5.6
Estradiol+Scopolamine	19 $\pm$ 9.1*	9.8 $\pm$ 7.5
Tamoxifen+Dill+Scopolamine	2.3 $\pm$ 1.5	3.6 $\pm$ 1.6
Placebo+Dill+Scopolamine	24.1 $\pm$ 7.1*	11.8 $\pm$ 3.3
Tamoxifen	0.33 $\pm$ 1.7	3.8 $\pm$ 0.7

\* $P < 0.05$  comparing times spent exploring new and familiar objects in each group of animals.

**Effect of drugs on memory performance (RI)**

Fig. 2 shows that dill or estradiol ( $RI = 32.7\% \pm 2.1$ ,  $RI = 31.5\% \pm 4.6$  respectively) did not affect memory performance compared with the control group, ( $RI = 50.1\% \pm 5.9$ ). In other words these animals spent more time recognizing the new object in the open field (Table 1). One sample t test presented significant difference from zero; this indicates normal discrimination between the novel and the old objects. As it was expected scopolamine impaired memory performance,

which was presented by the very low memory index ( $RI = -15.6\% \pm 3$ ). While treatment groups receiving either dill or estradiol prior to memory impairment showed normal memory performances ( $RI = 40\% \pm 5.6$  and  $RI = 36\% \pm 6.6$  respectively). These data showed significant memory improvement compared with scopolamine group. Fig. 3 shows the effect of tamoxifen on memory performance. Tamoxifen alone impaired memory performance ( $RI = -19\% \pm 4.6$ ), that is, animals spent more time exploring the old object in the open field (Table 1).



**Fig. 4.** Estradiol concentration in mice serum following dill administration. Dill or normal saline was injected ip 15 min prior to T1 and samples were collected after the memory test (T2). Analysis was performed by enzyme immune assay kit. Values are group mean  $\pm$  SEM, and compared by Student's t-test.  $N = 6$ . \*\*\* $P < 0.0001$  compared to saline group values.

Adding tamoxifen to the dill+scopolamine treatment also deteriorated memory performance (RI =  $-29.6\% \pm 7.8$ ). While this effect was not seen in the placebo treated animals (RI =  $33.6\% \pm 4.1$ ).

#### **Serum estradiol**

Serum estradiol level raised significantly following dill administration ( $187.3 \text{ pg/ml} \pm 15.2$ ) in animals compared to control animals ( $P < 0.007$ ) (Fig. 4).

### **DISCUSSION**

These results indicate that dill essence restored memory performance in animals concomitantly receiving scopolamine. While this effect was similar to estradiol treated animals, on the other hand tamoxifen reversed dill beneficial effects. Thus somehow the beneficial effects of dill essence by estrogen receptors on memory performance could be assumed.

The object recognition task allows a rapid evaluation of memory performance in mice. Animal experiments generally use emotionally affecting learning tasks, in contrast to studies of memory in human subjects. Nonetheless in this method no rewarding or aversive

stimulation is used during training, so the learning occurs under conditions of relatively low stress or arousal (17).

In order to evaluate the validity of this memory paradigm, we examined the effect of scopolamine on the performance of mice in the object recognition task (data not shown). In agreement with the results of other studies on the object recognition task (14,18), a single injection of  $0.5 \text{ mg/kg}$  scopolamine administered after T1 caused amnesia in mice.

Aromatase is the enzyme which converts androgen to estrogen. Aromatase is expressed in the brain, bone and adipose tissue in addition to the gonads (19). Therefore, estrogen can be produced locally in the brain as estrogen receptors are also expressed in the brain (20). Studies of the relationship between endogenous estrogen and testosterone levels and cognition in men are conflicting (21), nevertheless this is not a matter of debate here. Because estrogen receptors are present in male and female brain, male mice were used in this experiment.

The results of the first trial explained that tamoxifen treated animals required more time for acquisition. They needed more time in order to explore the objects in the open field. Many studies have investigated the effects of estrogen on animal's ability to learn and remember (22). Tamoxifen is a selective estrogen receptor modulator that has estrogen antagonistic effects in some tissues and is used for the treatment and prevention of breast cancer. Hippocampus has a profound effect on learning and memory performance. It has been shown that estrogen is critical for hippocampus development and tamoxifen can antagonize these effects (23). Eberling and colleagues report that women taking tamoxifen had lower frontal lobe glucose metabolism and smaller hippocampal volumes than women taking unopposed estrogen (24). Our experiments also supported this somehow by longer time required by the animals for learning at the first trial.

Apart from the well known dill traditional uses it is a galactagogue that is known to increase the flow of milk in nursing mothers and will be taken by the baby in the milk to help prevent colic (11). Dill can be used as a

regulatory agent of the menstrual cycle (11). *A. graveolens* extract both in low dose and high dose increased estrous cycle duration and progesterone concentration and induced infertility (15). This indicates that the estrogenic property of dill is high enough to suppress the normal estrous cycle. Estrogen has effects on sexual differentiation and reproduction, but can also alter brain structure and function with effects on mood, cognition, and emotional behavior (25). Since dill BARIJ essence is abundant and it can readily be prepared by all individuals it was used in this experiments. Dill essence or estradiol both showed beneficial effects on memory performance. This was evaluated by the values which were significantly higher than zero, which presents good discrimination between the novel and the old object. They prevented the harmful effects of scopolamine on memory function. A number of rodent studies that specifically looked at the effects of estrogen on the hippocampus have shown evidence of neuroprotection. For example, estrogen increases the density of dendritic spines and synapses in the CA1 region (26) and regulates the cyclic breakdown of excitatory synapses on dendritic spines (27). The exact mechanisms by which estrogen enhances cognitive performance are unknown, but probably they include actions on specific brain structures and neurotransmitters. The brain regions that provide cholinergic innervations for the hippocampal have high estrogen concentration (28) and are neurochemically sensitive to estrogen as seen by changes in synthesis, turnover, and release of acetylcholine. On the basis of our experiments, the anatomical sites of interaction between dill essence and scopolamine induced memory deficit is not clear. Considering the role of acetylcholine in memory processing and also the estrogen receptors, it is likely that the systemic manipulations in the present experiments affected memory, at least in part, through increasing acetylcholine concentration thus preventing the harmful effects of scopolamine on memory performance.

Tamoxifen was also used to understand the possible actions of dill essence on estrogenic receptors. Using tamoxifen and dill essence

concomitantly could not protect against the harmful effects of scopolamine on memory performance. Dill essence contains flavonoids, a subclass of phytoestrogens that may be responsible for the positive effects on memory performance. Nevertheless, on the basis of our experiment, the exact component that could exert the beneficial effects on memory performance and cognition could not be deduced. Previously we obtained similar results by experimenting *Foeniculum vulgare* (fennel), another herb containing phytoestrogens (14). Although phytoestrogens are polyphenolic non-steroidal structure, their effects are partly performed by ERs. Thus dill essence beneficial effect on memory performance was antagonized by tamoxifen. Evidently fennel beneficial effects on anxiety were also reported to be antagonized by tamoxifen (29).

Serum estradiol level increased dramatically in dill essence treatment animals indicating the profound amount of phytoestrogens presented in the essence. This is also in support of the assumption that the effects of dill essence on preventing memory damage is because of the rise in the serum estradiol capacity.

## CONCLUSION

Dill essence proved to be useful in preventing scopolamine-induced memory deficit. This effect was parallel with the effects of estradiol on memory. Since these protective effects were antagonized by tamoxifen thus ER maybe involved. Although we did not measure acetylcholine concentration but increasing levels of acetylcholine was assumed to be the cause of dill beneficial effects. It is suggested that the flavonoids and phenolic components of *A. graveolens* to be considered separately on memory performances. Accordingly this herb proves promising to be used as a remedy for middle age individuals to prevent cognition impairment induced by estrogen deficiency and aging.

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