

Effect of Isoflavone on Memory in Rat Model of Surgically Induced Menopause; Possible Role of Glutamate and Gamma Amino Butyric Acid

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Abstract:

Background: Menopause is a biological process that can cause various troublesome symptoms such as hot flashes and emotional changes but can also affect cognitive functions. Soy isoflavones (SIF) are natural compounds that gained popularity as an alternative treatment for menopausal symptoms.

Aim: The aim of this work was to investigate the effect of isoflavone treatment on memory in ovariectomized female albino rats. **Method:** 32 female albino rats were divided into 4 groups consisting of 8 rats in each group; sham, sham+isoflavone, bilateral Ovariectomy (OVX), OVX + isoflavone. After isoflavone treatment, the animals underwent the novel object recognition test (NORT), and their performance was evaluated at the end of the experiment. Gamma amino butyric acid (GABA) and glutamate were measured in hippocampal tissues and hormonal level of follicle stimulating hormone (FSH) & 17 β -estradiol (E2) were measured in serum

Results: Animals with ovariectomy showed significant increase ($P < 0.05$) in GABA and decreased glutamate in parallel with a decrease in NORT performance and decreased level of (E2). However, in the isoflavone treated group, there was a significant decrease ($P < 0.05$) in GABA and increase in glutamate observed while there was improvement in NORT performances and an increase in E2 level. **Conclusion:** These results suggest that isoflavone could improve menopause associated cognitive impairment through modulating brain level of GABA and glutamate therefore; isoflavone could be an effective tool to combat this undesirable feature of the natural menopause.

Key words: Ovariectomy, Isoflavone, Cognition, GABA, Glutamate.

Abbreviations: SIF; soy isoflavone, OVX; Ovariectomy, NORT; novel object recognition test, GABA; Gamma amino butyric acid, FSH; Follicle Stimulating Hormone, E2; 17 β -estradiol.

Introduction:

Menopause is a permanent endpoint of the menstrual cycle that occurs naturally or by induction of surgical procedure, chemotherapy, or radiation (1). Natural menopause is recognized to have occurred after 12 consecutive months of amenorrhea (2). Surgically induced menopause would follow bilateral ovariectomy, or sometimes unilateral ovariectomy (3).

Hormonal changes are prominent with menopause, with increased serum follicle stimulating hormone (FSH) and decreased serum estradiol (E2) (4). Estrogen deficiency following menopause has been associated with cognitive decline (5).

It is widely accepted that compromised memory reported in menopausal women may emerge mainly from declines in steroid hormones levels, particularly estradiol (6). Many women complain of changes in their cognitive function during the menopause transition (7).

As a result of the increased side effects of hormone replacement therapy (HRT) and the increased risk of breast cancer, stroke, and thromboembolism (8), alternative options for hormone therapy are increasing and many products containing soy phytoestrogens have

recently been used in woman to alleviate menopause symptoms(9). SIF have gained popularity as an alternative treatment for menopausal symptoms for people who cannot or are unwilling to take HRT (10).

The hippocampus is crucially involved in learning and memory (11). It is implicated in all aspects of the declarative memory, the semantic memory, and the episodic memory (12).

Glutamate acts as the main excitatory neurotransmitter in the CNS and is a proximal regulator of cognitive domains such as learning and memory (13). On the other hand, gamma amino butyric acid (GABA) is the most abundant and widely distributed inhibitory neurotransmitter in the CNS. GABA receptors are putative sites for ovarian hormone effects (13).

A certain balance of neuronal transmission between the major excitatory neurotransmitter glutamate and the inhibitory substance GABA is required to maintain normal functions of brain, including learning and memory (14).The decrease of glutamate or increase of GABA (i.e., reduction of the ratio of (Glutamate/GABA) can lead to impairment of learning and memory (15).

This study was designed to demonstrate the effect of soy isoflavone on short term recognition memory in surgically induced menopause in rats and to explore the possible role of glutamate and GABA in this process.

Material and methods:

Chemical used:

Commercially available isoflavones tablets were obtained from MEPACO pharmaceutical company. Each one tablet contains 50 mg of soy isoflavone.

Animals:

This prospective experimental study was conducted on 32 adult female albino wistar rats, 6-8 weeks old, weighing between 180 and 250 g. They were obtained from the Experimental Animal Unit of Moshtohor faculty of Agriculture. In physiology department, the animals were acclimatized to the laboratory conditions for 10 days prior to the initiation of the experiment, with free access to water and diet. They were placed at room temperature (25° c) with a 12:12-h light/dark cycle. The study period lasted for 120 days from March 2020 to June 2020. Experimental rats were under complete healthy conditions all over the experiment and under care of a professional technician and a qualified researcher. All procedures were approved by ethical committee

of Benha faculty of medicine. No rats were died throughout the experiment. At the end of the study the rats were incinerated at Benha university hospital incinerator.

Experimental design:

I. Rats were randomly assigned to two groups each group contains 16 rats as follows: Sham-operated and Ovariectomy (OVX). Rats in OVX groups underwent bilateral ovariectomy operation. Surgery was performed under anesthesia induced by intra-peritoneal (I.P) injection of 40 mg/kg of thiopental sodium. A longitudinal incision (0.5-1cm) was made in the midline area of lower abdomen (**16**) and the ovaries were then located and a silk thread (5-0) was tightly tied around the oviduct, including the ovarian blood vessels. The oviduct was sectioned, and the ovaries removed in both sides, taking good care in leaving the knot intact. Finally, the skin and the muscular wall were then sutured with a silk thread (4-0). In sham-operated rats, same surgical procedure was performed, but ovaries were just palpated, not removed. After OVX, rats were given 1.75 mg/kg amoxicillin once daily (I.M) for 3 days to protect the rats against infection (**17**).

II. For assurance of menopause blood samples (Whole-blood samples about 2 ml)

were collected from retroorbital vein at day 30 after ovariectomy. Blood was then centrifuged, and sera were taken for estimation of serum hormonal profile including FSH and E2 using enzyme-linked immunosorbent assay (ELISA) technique (3). After assurance of induced menopause, we started the experiment.

III. The rats included in this study were further subdivided into 4 main groups:

- **Group I :(sham group):** sham-operated rats without removal of the ovary.
- **Group II :(sham group+ isoflavone):** sham operated rats received isoflavone by oral gavage at a dose of 100 mg/kg/day for 90 days (18).
- **Group III :(bilateral ovariectomy group):** The animals in this group had bilateral ovariectomy operation.
- **Group IV: (bilateral ovariectomy group + isoflavone):** The animals in this group had OVX and isoflavone treatment.

IV. After 90 days of isoflavone treatment, we evaluated recognition memory by the novel object recognition test (NORT) in all the studied groups.

(NORT) is a behavioral assay of memory that relies on rodent's innate exploratory behavior without externally applied rules or reinforcement (19). It is based on the innate preference of the rodent to explore the novel object rather than the familiar one (20).

NORT has habituation, sample, and choice phases. Experiments were carried out in an isolated chamber. *Habituation Phase:* Rats were habituated properly to the open box for 5 min each day for 5 days. Each rat was allowed to explore the box to be familiar with it (20). *Sample phase:* 24 hours after the last habituation session, the rats were trained for recognition of the two identical objects placed in the test arena for 3 minutes (21). Their behavior was recorded with a video camera. After the sample phase, the objects and the open field were cleaned with 70% alcohol to minimize olfactory cues (22). *Choice phase:* 15 minutes after the sample phase, rats were again placed for 3 min in the testing box (21). Animals were observed for 3 min in a box containing a familiar object and a novel object, which was different in shape and color, and their behavior was recorded with a video camera. *Evaluation:* Results were assigned by comparing the time spent with the familiar and novel objects.

V. We recorded the following measures:

- **e1:** the total time spent exploring the two identical objects in the sample phase **(21)**.
- **e2:** the total time spent exploring the two identical objects in the choice phase **(21)**.
- **d1: the discrimination index:** (time spent for novel object minus time spent for familiar object) **(21)**.
- **d2: the discrimination ratio:** (novel - familiar/novel + familiar). This ratio makes it possible to adjust for any differences in the total amount of exploration time **(21)**.
- **Recognition index:** This is the time spent exploring the novel object divided by the total time. This means all values will fall between 0 and 1. It is often multiplied by 100 = novel/ (novel+ familial) * 100 **(19)**.

VI. Specimen preparation:

All rats were decapitated after the last behavioral test and brains were immediately excised. Hippocampi were quickly dissected and stored at -80 °C until analysis was performed to determine the level of GABA and glutamate by ELISA.

Statistical analysis:

The data were analyzed using the program: Statistical package for social science (SPSS) version 23.0 (SPSS Inc., Chicago, IL, USA, 2000). In the statistical comparison between the different groups, the significance of difference was tested using one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) test for comparison between every two groups. The p-values < 0.05 were considered statistically significant.

Results:

Table (1): The mean of serum FSH and E2 in sham and ovariectomized groups 30 days after ovariectomy.

	Sham	OVX	P value
FSH (mIU/mL)	3.65 ± 0.62	12.78 ± 1.23 *	<0.05
E2 (pg/mL)	62.4 ± 3.37	23.98± 3.96 *	<0.05

Data are represented as Mean ± SD n=16

P value < 0.05 was considered statistically significant. - P value >0.05 was considered statistically insignificant.

* P < 0.05 vs. sham Group.

From table (1):

Ovariectomy resulted in a significant increase (**P < 0.05**) in FSH level and a significant decrease (**P < 0.01**) in E2 level when compared with that of sham group.

Table (2): The mean of FSH, E2, GABA and Glutamate in different experimental groups 90 days after isoflavone treatment.

	group I (sham)	group II (sham + isoflavone)	Group III (ovariectomy group)	Group IV (ovariectomy + isoflavone)	P value
FSH mIU/mL	3.88 ± 0.80	4.02 ± 0.92	14.81 ± 2.8 * +	9.22 ± 2.05 * + #	<0.05
E2 pg/mL	59.02 ± 3.9	59.92 ± 5.96	14.81 ± 2.8 * +	9.22 ± 2.05 * + #	<0.05
GABA pg/mg tissue	110.86 ± 7.7	107.8 ± 6.8	181.2 ± 10.89 * +	134.13 ± 11.6 * + #	<0.05
Glutamate nmol/mg tissue	9.98 ± 1.45	8.92 ± 1.37	4.9 ± 0.78 * +	7.54 ± 1.25 * + #	<0.05

group; group IV: ovariectomy + isoflavone (100 mg/kg for 90 days). P < 0.05 is significant tested by one-way analysis of variance (ANOVA) and post hoc multiple comparison LSD method.

* P < 0.05 vs. Group I; + P < 0.05 vs. Group II; # P < 0.05 vs. Group III.

From table 2:

- There was a non-significant change (**P > 0.05**) in FSH, E2, GABA and Glutamate levels in group II after treatment with isoflavone in a dose of (100 mg/kg for 90 days), when compared with that of sham group (group I).
- However, ovariectomy resulted in a significant increase (**P < 0.01**) in FSH & GABA levels and a significant decrease in E2 & Glutamate levels in group III when compared with that of sham group (group I) and sham+ isoflavone group (group II).
- On the other hand, treatment with isoflavone after ovariectomy in group IV in a dose of (100 mg/kg for 90 days), significantly decreased FSH & GABA levels (**P < 0.05**) and increased E2 & Glutamate levels when compared with group II.

Table (3): The mean of discrimination index (d1), discrimination ratio (d2) and recognition index (RI) in different experimental groups 90 days after isoflavone treatment.

	Group I	Group II	Group III	Group IV	P value
d1	39.75 ± 9.55	38.87 ± 11.75	-46.12 ± 7.73 * +	22.4±7.4 * + #	<0.05
d2	0.46 ± 0.089	0.46 ± 0.15	-0.59 ± 0.07 * +	0.29 ± 0.14 * + #	<0.05
RI	73.17 ± 4.45	73.25 ± 5.06	20.1 ± 3.97 * +	64.65 ± 7.22 * + #	<0.05

Data are represented as Mean ± SD., n=8. Group I: sham group; group II: sham + isoflavone group; group III: ovariectomy group; group IV: ovariectomy + isoflavone (100 mg/kg for 90 days). P < 0.05 is significant tested by one-way analysis of variance (ANOVA) and post hoc multiple comparison LSD method. * P < 0.05 vs. Group I; + P < 0.05 vs. Group II; # P < 0.05 vs. Group III.

From table 3:

- There was a non-significant change (**P > 0.05**) in d1, d2 & RI in group II after treatment with isoflavone in a dose of (100 mg/kg for 90 days), when compared with that of sham group (group I).
- However, ovariectomy resulted in a significant decrease (**P < 0.01**) in d1, d2 & RI in group III when compared with sham group (group I) and sham+ isoflavone group (group II).
- On the other hand, treatment with isoflavone after ovariectomy in group IV in a dose of (100 mg/kg for 90 days), significantly increased d1, d2 & RI (**P < 0.05**) when compared with group III.

Discussion:

Menopause is a normal and essential developmental and physiological process in a woman's life. Physical and psychological alterations occur during menopause (5). Estrogen deficiency is associated with the increased risk of neurological symptoms such as memory deficit. E2 has been implicated to exert neuroprotective actions in the brain, including modulation of cognition (23).

Intact glutamate and GABA signaling in hippocampus and medial prefrontal cortex (mPFC) is required for novelty recognition and intact function of the hippocampus is also necessary for intact performance in NORT (24).

In the current study, menopause was induced surgically in female albino rats via bilateral ovariectomy (OVX). After 30 days of the OVX,

serum E2 and FSH were measured to ensure the complete removal of all ovarian tissues.

30 days after OVX, the ovariectomy group showed significant increase (P value <0.05) in serum level of FSH and decrease in serum level of E2 when compared with sham group as shown in table (1). These results were in agreement with previous studies (3 & 25) who stated that decreased serum E2 and elevated serum FSH levels were strongly associated with OVX after 30 days of the operation. The ovarian artery ligation causes ovarian dysfunction and atresia of the ovarian follicles leading to decreased level of E2 (26 & 25).

Preference for novelty is an important component of learning and memory. NORT is commonly used for assessing learning and memory in rodents (20).

Isoflavones exhibits similarity to the chemical structure and/or function of E2. The hydroxyl groups on the phenolic rings of phytoestrogens correspond to the hydroxyl groups on the aromatic rings of E2, thus enabling binding of phytoestrogens to estrogen receptors (ERs) especially estrogen receptor beta (ER β) (27).

Our study revealed that isoflavone administration in a dose of 100 mg/day for 90 days by oral gavage caused significant increase (P value <0.05) in serum E2 and significant

decrease in serum FSH levels in rats in group IV (ovariectomy + isoflavone group) when compared to ovariectomized non treated rats in (group III) as seen in table (2). These results were in agreement with previous studies (18 & 28) they stated that isoflavone could increase serum level of E2 via its effects on enzymes involved in steroid metabolism, including aromatase, 17 β -hydroxysteroid dehydrogenases, steroid sulfatases and sulfotransferases, potentially resulting in alterations of the serum level of E2 and FSH.

In our study short term recognition memory was evaluated by NORT in all groups after 90 days of isoflavone treatment in group II (sham + isoflavone) and group IV (OVX + isoflavone).

The NORT indices (d1, d2 & RI) were calculated. There was a significant increase in d1, d2 and RI in group IV (ovariectomy+ isoflavone group) (P < 0.05) when compared with the group III (ovareictomy group) as shown in table (3). There was a non-significant change (P > 0.05) in d1 , d2 and RI in group II (sham + isoflavone) after treatment with isoflavone in a dose of 100 mg/kg for 90 days, when compared with that of sham ovariectomized rats in group I as shown in table (3).. However, ovariectomy resulted in a significant decrease (P < 0.01) in d1, d2 and RI in group III when compared with that of sham group (group I) and sham+

isoflavone group (group II) as shown in table (3).

The significant increase in the indices of NORT in group IV indicated significant improvement in the recognition memory in rats. These results were in agreement with (18) who stated that recognition memory improved after isoflavone treatment, and these effects could be associated with increased E2 and, consequently, activation of estrogen receptors (18). In addition, other possible mechanisms such as antioxidant properties, positive impact on the cholinergic neurotransmission and anti-inflammatory effects promoted by isoflavones could contribute to the presented data (18).

Another study (29) reported that isoflavone could dose-dependently improve spatial recognition, discrimination, and memory deficits in lipopolysaccharide induced memory dysfunction and this study explained the improving effect of isoflavone on memory by lowering hippocampal level of malondialdehyde (MDA) and increasing activity of superoxide dismutase (SOD) and catalase and glutathione (GSH) level. Furthermore, isoflavone ameliorated hippocampal acetylcholinesterase (AChE) activity in LPS-challenged rats and lowered hippocampal level of interleukin 6 (IL-6), nuclear factor-kappa Beta (NF- κ B)p65, toll-like receptor 4 (TLR4), tumor necrosis factor α

(TNF α), cyclooxygenase2 (COX2), inducible nitric oxide synthase (iNOS), glial fibrillary acidic protein (GFAP) (29).

Moreover, soy isoflavones administration for 12 weeks improved the cognitive performance of scopolamine (SCOP)-treated mice by enhancing cholinergic system function and suppressing oxidative stress levels in the hippocampus of SCOP-treated mice. Furthermore, it markedly upregulated the phosphorylation levels of extracellular signal-regulated kinase (ERK), cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) expression levels in the hippocampus, suggesting that soy isoflavones could be a good candidate for possible treatment of neurodegenerative diseases, such as Alzheimer's disease (AD) (30).

Soy isoflavones affected the synthesis of acetylcholine, and neurotrophic factors such BDNF and nerve growth factor (NGF) in the brain of the female rat. The mRNA levels of BDNF in the frontal cortex were significantly higher in rats receiving soy isoflavones than in ovariectomized rats (31).

Interestingly, the decrease in the indices of NORT in group III (the ovariectomy group) is associated with the reduction in the E2 level. In the women's health, estrogen not only plays

important roles in the estrous cycle but also has a protective role in the brain (32).

It is widely accepted that compromised memory in menopausal women emerge mainly from declines in steroid hormones levels, particularly estradiol specially in brain structures like the hippocampus which is a key player in learning and memory processes (33).

Estrogen exerts both genomic and nongenomic effects on brain tissue and there are many actions of estrogen that can underlie improved memory. Possible actions include induction of synaptogenesis, increased formation of CA1 neurons in the hippocampus, direct effects on excitatory amino acids, enhancement of cholinergic and glutamatergic neurotransmitter systems and modulation of neurotrophies (34).

Overall, research reports suggest that sexual hormones are key players in the maintenance of cognitive abilities and studies in ovariectomized animals showed significant alterations in the structure and function of hippocampal and cortical circuits accompanied by poor performance in several cognitive tasks (35). These observations were confirmed in humans, as estrogen therapy improved performances in cognitive tasks, verbal memory, and executive functions of perimenopausal women (36). The widespread presence of estrogen receptors in the hippocampus, amygdala and cerebral cortex

plays a significant protective role against the deterioration in these cognitive functions that occur with normal aging (37). It is indeed now well recognized that the functions of gonadal and adrenal steroid hormones go far beyond reproduction and that they regulate vital neuronal and glial functions by various mechanisms of action (38).

The ovariectomy group (group III) showed a significant increase ($P < 0.01$) in GABA level and a significant decrease in glutamate level when compared with that of sham group (group I) and sham+ isoflavone group (group II) as shown in table (2) and these results are in agreement with a previous study (39) who reported that OVX resulted in increased GABA level as a result of increased level of glutamate decarboxylase (GAD), which is the enzyme for GABA synthesis, in the hippocampus. On the other hand, treatment with isoflavone in group IV in a dose of (100 mg/kg for 90 days), significantly decreased GABA level and increased glutamate level ($P < 0.05$) when compared with group III (the ovariectomy group) as shown in table (2).

This can be explained by the decreased estrogen level in the ovariectomized group of rats as estrogen was found to potentiate the release of glutamate and it acts on postsynaptic membranes via the positive modulation of the ionotropic N-

methyl-D-aspartate receptor (NMDA) receptor which is related to synaptic plasticity, learning, and memory (40).

Another effect of estrogen is to act as a suppressor of GABAergic transmission through the inhibition of the L type Ca^{2+} channel required for GABA release in the presynaptic terminal (40). And in our study estrogen level was found to increase significantly in group IV (ovariectomy + isoflavone group) compared to group III as shown in table (2) so the alterations in brain levels of glutamate and GABA may be associated with the alteration in estrogen levels after the ovariectomy and after isoflavone treatment.

Estrogen also increases the expression of NMDA receptor and its sensitivity to glutamate, which then induces an increase of neuronal sensitivity to synaptic input through calcium influx (42). Hence, glutamatergic potentiation by estrogen leads to an increase in neuronal excitability and this has been presented as a mechanism through which estrogen generates morphological plasticity changes in the hippocampus, amygdala, and prefrontal cortex (PFC) (43). Estrogen exerts its effects on the glutamatergic system by modulating presynaptic glutamate release, and regulation of receptor and protein expression (44).

Moreover, isoflavone treatment in old adult menopause female rats increased hippocampal level of glutamate and stated that the improvement in recognition memory in rats could be associated with increased glutamate GABA balance in the brain (18). Glutamate enhances memory performance via increasing acetyl choline (A.Ch.) thus, glutamate can be suggested as a useful supplement for improving learning and memory performance and neurochemical status (45).

Conclusion:

From the current study, it may be concluded that ovariectomy has a deteriorate effect on recognition memory in female rats and isoflavone plays a notable role in ameliorating this deteriorate effect through modulating the hippocampal level of GABA and Glutamate. So, isoflavone can be used as a natural therapeutic and preventive drug for improving menopause associated cognitive impairment.

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