ORIGINAL ARTICLE

Therapeutic Efficacy of Fenugreek Extract or/and Choline with Docosahexaenoic Acid in Attenuating Learning and Memory Deficits in Ovariectomized Rats

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Abstract:

Background: Studies have demonstrated that estradiol influences cognitive functions. Phytoestrogens and many other estrogen-like compounds in plants have beneficial effects on cognitive performance in postmenopausal women. However, there is no evident report of fenugreek and choline-Docosahexaenoic Acid (DHA) on cognition in ovariectomized rats. *Aim and Objectives:* The present study was aimed to evaluate the therapeutic efficacy of fenugreek extract or/and choline-DHA in attenuating ovariectomy-induced memory impairment, brain antioxidant status and hippocampal neural cell deficits in the rat model. *Material and Methods:* Female Wistar 9-10 months old rats were grouped (n=12/group) as - (1) Normal Control (NC), (2) Ovariectomized (OVX), (3) OVX+FG (hydroalcoholic seed extract of fenugreek), (4) OVX+C-DHA,(5) OVX+FG+C-DHA and (6) OVX+Estradiol. Groups 2-6 were bilaterally OVX. FG, C-DHA was supplemented orally for 30 days, 14 days after ovariectomy. Assessment of learning and memory was performed by passive avoidance test. Oxidative stress and antioxidant markers were assessed by standard methods. Nissl stained hippocampal sections were analyzed to determine alterations in neural cell numbers in CA1, CA3 and dentate gyrus. *Results:* Supplementation of FG or/and choline with DHA to OVX rats, caused significant improvement in learning and memory as well as decreased neural cell deficits compared to the same in OVX rats. Further, significantly reduced levels of brain Malondialdehyde (MDA) and increased levels of Glutathione (GSH) were observed. *Conclusion:* Therapeutic supplementation of FG with choline-DHA significantly attenuates ovariectomy-induced neuro-cognitive deficits in rats.

Keywords: Menopause, Learning and Memory, Hormone Replacement Therapy, Phytoestrogen, Fenugreek, Choline, Docosahexaenoic acid

Introduction:

The rapid decline of estrogen during the period of menopause in women is known to cause neurological changes in different areas of the central nervous system [1]. Women with an onset of menopause may experience hot flushes, depression, anxiety, sleep disorder, mood swings, learning and memory disorders [2]. Estrogen acts on target tissues via interaction with its receptors, ERα and ERβ on different cell types [3]. Several animal studies have provided evidence regarding the potential effects of estrogen therapy on cognitive behavior, hippocampal synaptic circuitry, neocortex and hypothalamus, although the nature and extent of cognitive improvement vary with age [4]. Decreased levels of estrogen are also associated with elevated oxidative stress during menopause. Studies show that this can be reversed with an antioxidant mechanism of estrogen [5], where estrogen interacts with

estrogen receptors, which eventually leads to Nuclear Factor kappa B (NFκB) and Mitogen-activated Protein Kinases (MAPK) activation [6]. Studies also show that estrogen induces the Phosphatidylethanolamine-N-methyltransferase (PEMT) gene as well as facilitates increased PEMT activity in humans [7]. PEMT catalyzes the de-novo biosynthesis of Phosphatidylcholine (PC), a non-dietary source of choline [8]. Low choline intake leads to cognitive dysfunction in adults as choline is a precursor of PC, a major component of all biological membranes, including neurons and glial cells [9]. Therefore, women with lower estrogen concentration may require more choline for normal functioning of the brain. Additionally, studies also show that in PEMT -/- mice dietary supplementation of DHA helps in restoring normal fetal brain development [10]. Increasing omega-3 Polyunsaturated Fatty Acid (PUFA) intake has been proposed as a possible intervention for preventing or delaying age-associated cognitive decline [11]. The rate-limiting enzymes in PUFA biosynthesis, δ-5 and δ-6 desaturases are stimulated by endogenous estrogen. The most effective treatment for menopausal adverse symptoms currently, is Hormone Replacement Therapy (HRT). However, the side effects due to its long-term use hinder the acceptance of HRT among post-menopausal women [12]. Thus, there is a need for alternative approaches to treat menopause-induced cognitive deficits. One such alternative/complementary therapy may be the use of fenugreek extract which is known to contain phytoestrogens.

Trigonella foenum graecum, commonly known as Fenugreek (FG) belongs to the family Leguminosae [13]. FG is well known for its anti-diabetic, anti-hyperlipidemic, analgesic, antioxidant and anti-cancer activities [14, 15]. The plant contains active components such as flavonoids, alkaloids, steroids and saponins [16]. Fenugreek seeds are known to contain diosgenin, and can mimic the estrogen and act as anti-hyperlipidemic agent [17]. Moreover, choline is a key nutrient required for structural coherence and signaling function of cell membranes and neurotransmitter synthesis [18]. According to National Health and Nutrition Examination Survey (NHANES), only 4 and 2% of men and women respectively over the age of 71 years fulfill the adequate intake value [19]. Additionally, in brain DHA is the principle long-chain polyunsaturated fatty acid. DHA is involved in multiple brain functions including cell membrane fluidity, receptor affinity, and modulation of signal transduction molecules [20]. DHA has been known to have antioxidant, anti-inflammatory, and anti-apoptotic effects, which may slow brain deterioration [21].

Since post-menopausal life is prolonged as a result of enhanced female life expectancy, there is a need for new therapeutic strategies to promote successful aging in women with emphasis for improving quality of life with higher cognitive and physical abilities. Thus an attempt to delay or prevent the cognitive impairment associated with menopause is required. However, the phyto-estrogens in fenugreek seeds as a source of supplement to estrogen in post-menopausal conditions is yet to be investigated. Similarly, choline-DHA supplementation singly or in combination with fenugreek extract on cognition of estrogen-deprived postmenopausal women has also not been investigated. Therefore, this study was designed to evaluate the therapeutic efficacy of fenugreek seed extract or/and choline-DHA in

attenuating ovariectomy-induced memory impairment, hippocampal neural cell deficits and brain antioxidant status in the rat model.

Materials and Methods:

Animals

Adult healthy female albino Wistar rats of 9-10 months age were selected for this experiment. The study was carried out according to guidelines of CPCSEA and Institutional Animal Ethics Committee (IAEC/KMC/12/2015), Kasturba Medical College, Manipal Academy of Higher Education. Three rats were housed in a single polycarbonate cage. The rats were maintained under standard laboratory environmental condition (12 hours day-night cycle with a temperature of 22 ± 2°C) and allowed to access standard pellet diet and water *ad libitum*.

Fenugreek seed extract preparation

Fenugreek seeds (100% organic seeds purchased from Pro Natural, India) were coarsely powdered and refluxed three times, at 85°C with 70% ethanol (1 liter each). Hydroalcoholic extract was filtered and concentrated under vacuum. Final drying was done using a freeze dryer.

Experimental design

Rats were randomly grouped (n=12/group) as – Group 1- Normal control, fed with Normal Pellet Diet (NPD). Group 2 to 6 - bilaterally OVX under aseptic conditions, during the preoperative period, animals were anesthetized with Intra-peritoneal (IP) injection of ketamine (50 mg/kg b.w) and xylazine (5 mg/kg b.w) mixture. After checking withdrawal and blinking reflexes, a midline incision of the lower abdominal cavity was performed, followed by bilateral ovariectomy [22]. Group 2 animals were fed with NPD and served as OVX control. After two weeks of OVX, groups 3, 4, 5 and 6 were supplemented either with fenugreek seed extract or choline-DHA, or combination of fenugreek seed extract with choline-DHA, or 17β-estradiol, respectively, for 30 days.

Dosage

Fenugreek seed extract - 200mg/kg/day, **Choline -** 4.6 mmol/kg/day and **DHA -** 300 mg/kg/day were administered orally. **17β-estradiol -** 100µg/kg/day was administered subcutaneously.

Behavioural Assessment

Two compartment passive avoidance test

After 30 days of supplementation period, learning and memory of rats were evaluated by passive avoidance test [23]. In the exploration trial, rats were placed in the middle of the box facing opposite to the entrance of small (dark) compartment and permitted to explore both the compartments for 3 minutes. Total time spent in larger and smaller compartments and latency to enter into the dark compartment were noted. Each rat was subjected to 3 exploration trials with inter-trial interval of five minutes. After the last trial, rats were placed in the smaller compartment; three strong electric foot shocks (50 Hz, 1.5 mA, 2sec) with two-second interval was given. After 24 hours of exploration trail, a retention test was carried without foot shock, during which rats were left in the larger compartment and permitted to explore both the compartments for 3 minutes. Each rat was given three trials with five minutes inter-trial interval. In each trial, the time spent in the larger and smaller compartment and latency time were noted. If a rat failed to enter into the dark compartment within 180sec, then the latency value was considered as 180sec.

Biochemical Assessment

After perfusing with cold saline, brains were quickly dissected out and one cerebral hemisphere was homogenized and samples were centrifuged (at 4°C); the supernatants were used for biochemical assays.

Estimation of brain Malondialdehyde (MDA) level

Twenty µl of brain homogenate was added to 300 µl of Thiobarbituric Acid (TBA) - Trichloroacetic Acid (TCA) and placed in boiling water bath for 15 min at 100^0C. This reaction mixture was centrifuged after cooling. The pink colored supernatant was used to read the Optical Density (OD) at 535 nm [24].

Estimation of brain Glutathione (GSH) level

About 100 µl of brain homogenate was added to 100 µl of 5% TCA solution and centrifuged for 5 minutes at 5000 rpm. Further, 25 µl of supernatant was added to 150 µl of sodium phosphate buffer (PBS 0.2 M, pH 8.0) and 25 µl of 5,5–dithio-bis-(2-nitrobenzoic acid) (DTNB) (0.6mM) in 96-wells of the micro test plate. This plate was incubated at room temperature for 10 minutes and absorbance was read at 412 nm by using an ELISA reader Bio Tek Instruments ELx800- MS, (USA) [25].

Cresyl violet staining and neural cell counting

After the treatment and behavioral tests, all experimental rats were sacrificed and trans-cardially perfused with normal saline and then by 10% formalin. The brains were quickly removed, fixed in 10% formalin and processed for paraffin impregnation. Further, five micron thick sections were cut using rotary microtome and mounted serially on slides coated with gelatin.

Brain sections from n=6 rats/group were then stained with 0.1% cresyl violet stain for 20-25 min at 60°C. Surviving neural cell quantification was carried out with the help of a light microscope (Magnus MLX, Microscope). Ten sections from each rat were selected for quantification. CA1, CA3 and dentate gyrus of hippocampal formation were considered. Surviving neural cells with a clear and distinct nucleus in these sub-regions of the hippocampus were quantified. Neural cells that were darkly stained and had shrunken cell body with irregular nuclei were excluded from quantification. Quantification was carried after the calibration of a light microscope by using an ocular micrometer and stage micrometer (Erma, Japan).

Statistical analysis

Data analysis was done with one-way ANOVA followed by Bonferroni's *post-hoc* test (Graph Pad Prism). Results were expressed as mean ± SD and p-value $\leqslant 0.05$ was expressed as significant.

Results:

Passive Avoidance Test

Time spent in dark compartment

Twenty-four hours after administration of the foot shock, OVX animals showed poor memory retention and spent 66.2% of total duration in dark compartment. Control group spent around 20.5% of total duration, which is nearly three times lesser duration compared to OVX group. Whereas, OVX rats supplemented with fenugreek alone, choline-DHA alone, a combination of fenugreek with choline-DHA and estradiol spent 19.4%, 30.5%, 20.9% and 22.9% of total duration respectively. (Table 1).

Latency to enter dark compartment

In memory retention test, OVX animals have taken 17.2% of total latency time for first entry into the dark compartment. Whereas OVX rats

supplemented with fenugreek alone, choline-DHA, a combination of fenugreek with choline-DHA and estradiol have taken 58%, 49.5%, 59.8% and 48.8% of approximate total latency time respectively. Control group has taken 48.3% of total latency time, which is nearly three times longer duration compared to OVX group (Table1).

Biochemical results

Antioxidant enzyme activities

Our results revealed that the activity of GSH was 8.01% less in OVX group compared to normal control group. Whereas fenugreek alone and choline-DHA alone supplemented groups showed 5.06% and 2.33% more GSH activity compared to OVX group. However, a combination of fenugreek with choline-DHA and estradiol showed 7.21% and 6.26% more activity of GSH compared to OVX group (Table2).

Lipid peroxidation marker level

MDA concentration in brain tissue of OVX animals was 3.42% higher compared to normal control animals. Supplementation with fenugreek alone, a combination of fenugreek with choline-DHA and estradiol reduced 4.34%, 4.46% and 4.37% MDA levels compared to OVX group. Choline- DHA supplemented group also showed 3.28% reduction compared with OVX group (Table 2).

Quantitative analysis of hippocampal neurons

CA1 region

Microscopic examination of CA1 sub region of hippocampus showed 5.08% less viable neurons in OVX group compared to normal control group. Whereas OVX rats supplemented with fenugreek alone, choline-DHA, a combination of fenugreek with choline-DHA and estradiol showed 4.09%,

Table 1: Effect of Fenugreek and Choline-DHA on Learning and Memory by Passive Avoidance Test

Groups	Time spent in dark compartment (180 Secs)	Latency to enter dark compartment (180 Secs)
NC	37.00± 25.36	86.92± 40.97
OVX	112.40 ± 39.23***	31.00± 24.08*
OVX+FG	35.17 ± 23.28$^{###}$	104.50± 47.17$^{##}$
OVX+C+DHA	55.83 ± 47.45$^{\$\$}$	89.25± 48.78$^{\$}$
OVX+FG+C+DHA	37.75± 35.36$^{\&\&\&}$	107.80± 46.14$^{\&\&\&}$
OVX+E2	41.33 ± 39.06$^{@@@}$	88.17± 47.65$^{@}$

*Each value represents Mean ± SD. NC vs. OVX: *** $p<0.001$, * $p<0.05$; OVX vs. OVX+FG: $^{###}p<0.001$, $^{##}p<0.01$; OVX vs. OVX+C+D: $^{\$\$}p<0.01$, $^{\$}p<0.05$; OVX vs. OVX+ FG+C+D: $^{\&\&\&}p<0.001$, $^{\&\&\&}p<0.001$, OVX+E2: $^{@@@}p<0.001$, $^{@}p<0.05$. NC=Normal control, OVX=Ovariectomy, FG=Fenugreek, C=Choline, DHA=Docosahexaenoic acid, E2=17β-estradiol*

Table 2: Effect of Fenugreek and Choline-DHA on Brain MDA and GSH Levels

Groups	MDA mg/tissue	GSH mg/tissue
NC	1.19 ± 0.13	1.63± 0.18
OVX	1.44 ± 0.17*	0.97± 0.16**
OVX+FG	1.13 ± 0.06$^{\#\#}$	1.39± 0.25
OVX+C+DHA	1.20 ± 0.14$^{\$}$	1.16± 0.15
OVX+FG+C+DHA	1.12± 0.05$^{\&\&}$	1.57 ± 0.27$^{\&\&}$
OVX+E2	1.13 ± 0.11$^{@@}$	1.50± 0.38$^{@}$

*Each value represents Mean ± SD. NC vs. OVX: * p<0.05, ** p<0.01; OVX vs. OVX+FG: ## p<0.01; OVX vs. OVX+C+DHA: $ p<0.05; OVX vs. OVX+FG+C+DHA: && p<0.01, && p<0.01; OVX+E2: @@ p<0.01, @ p<0.05. MDA= Malondialdehyde, GSH= Glutathione, NC=Normal control, OVX=Ovariectomy, FG=Fenugreek, C=Choline, DHA=Docosahexaenoic acid, E2=17β-estradiol.*

2.93%, 5.04% and 3.49% more viable neurons with clear, distinct nucleus compared to OVX group respectively (Table 3 and Fig. 1).

CA3 region

Quantification of neurons in CA3 sub region of the hippocampus of OVX animals showed 5.72% decreased survival neurons compared to normal control group. Although, choline-DHA supplemented group showed 3.3% more viable neurons compared to OVX, it was not significant statistically. However, the animals supplemented with fenugreek alone and a combination of fenugreek with choline-DHA, as well as estradiol, showed 5%, 5.88% and 4.69% more surviving neurons with clear, distinct nucleus compared to OVX group respectively (Table 3 and Fig. 1).

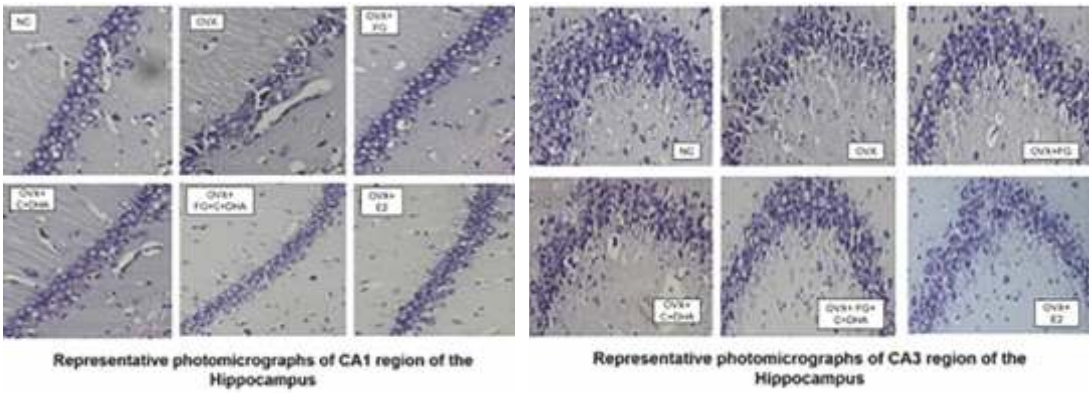

Fig. 1: Photomicrographs of CA1 and CA3 Regions of Hippocampus

Supra-pyramidal blade

Compared with the normal control group, OVX group showed 4.41% less number of viable neurons. Whereas OVX rats supplemented with fenugreek alone, a combination of fenugreek with choline-DHA and estradiol showed 3.56%, 3.96% and 3.36% more viable neurons compared to OVX group. Choline-DHA group also showed 2.13% less degeneration compared to OVX, but not to the significant level (Table 3 and Fig. 2).

Infra pyramidal blade

Quantitative analysis revealed that neuronal loss in the infra pyramidal region was 4.87% higher in OVX group than that of the control group. However, after supplementation with fenugreek alone, Choline-DHA alone and a combination of fenugreek with choline-DHA as well as estradiol showed 5.27%, 4.57%, 6.48% and 5.26% more surviving neurons with clear, distinct nucleus compared to OVX group (Table 3 and Fig. 2).

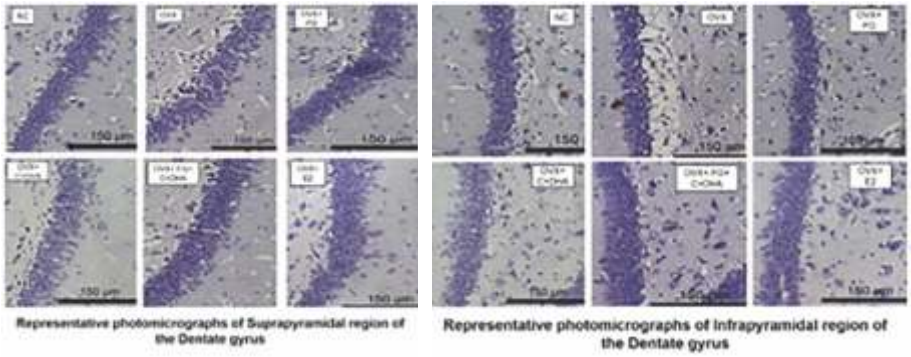

Fig. 2: Photomicrographs of Supra and Infrapyramidal Regions of Hippocampus

Table 3: Effect of Fenugreek and Choline-DHA on Hippocampal Neural Cell Quantification

Groups	CA1	CA3	Suprapyramidal Blade	Infrapyramidal Blade
NC	86.50 ± 7.09	76.83 ± 6.17	221.20 ± 23.40	211.0± 8.92
OVX	62.50± 4.59***	52.83± 15.30***	168.50± 23.29**	151.0± 36.36**
OVX+FG	81.83± 5.03$^{###}$	73.83± 4.79$^{##}$	212.00± 14.94$^{#}$	216.0± 20.59$^{###}$
OVX+C+DHA	76.33± 5.50$^{\$}$	66.67± 10.54	194.50± 21.17	207.3± 11.57$^{\$\$}$
OVX+FG+C+DHA	86.33± 7.33$^{\&\&\&}$	77.50± 4.59$^{\&\&\&}$	216.80 ± 24.44$^{\&\&}$	230.8 ± 33.83$^{\&\&\&}$
OVX+E2	79.00± 8.55$^{@@}$	72.50± 7.34$^{@@}$	209.50± 10.60$^{@}$	215.8± 18.90$^{@@@}$

*Each value represents Mean ± SD. NC vs. OVX: *** p<0.001, ** p<0.01; OVX vs. OVX+FG: $^{###}$ p<0.001, $^{##}$ p<0.01, $^{#}$ p<0.05; OVX vs. OVX+C+DHA: $^{\$}$ p<0.05, $^{\$\$}$ p<0.01; OVX vs. OVX+FG+C+DHA: $^{\&\&\&}$ p<0.001, $^{\&\&}$ p<0.01; OVX vs OVX+E2: $^{@@}$ p<0.01, $^{@}$ p<0.05, $^{@@@}$p<0.001.NC=Normal control, OVX=Ovariectomy, FG=Fenugreek, C=Choline, DHA=Docosahexaenoic acid, E2=17β-estradiol.*

Discussion:

In this study, the therapeutic efficacy of FG extract and choline-DHA on estrogen deficiency induced learning and memory impairment in OVX rats are reported. Ovariectomy model is believed to be the best tool to imitate human ovarian hormone loss. The majority of studies have shown ovariectomy can lead to early aging of the nervous system and impairment in the hippocampus-dependent learning and memory [26, 27]. The results of passive avoidance test in the current study showed a deficit in learning and memory in OVX rats. This observation is consistent with previous studies on animals and humans that report learning and memory impairment following ovariectomy [28, 29]. Moreover, in the present study significant attenuation in learning and memory retention was observed in rats treated with FG alone, choline-DHA, FG+choline-DHA and estradiol following ovariectomy compared to the same in non-treated OVX rats. This positive effect of fenugreek on learning and memory may be due to its inhibitory effect on acetylcholinesterase, which hydrolyzes the neurotransmitter acetylcholine important for cognitive events [30]. Studies show that fenugreek seed powder also reversed the memory deficits induced by scopolamine, diazepam and aluminum [31, 30]. Studies also report that Tamoxifen (TAM) and soy phytoestrogen supplementation improved memory performance in OVX rats [32, 33]. Additionally, earlier studies reported that choline supplementation might facilitate increased cholinergic nerve activity in the hippocampus, by serving as a precursor for the synthesis of the neurotransmitter acetylcholine [34]. Supplementation of fish oil or DHA can reverse learning disabilities, progressive memory loss and cognitive alterations in the aging mouse brain caused due to PUFA deficiency [35]. DHA affects brain-barrier function and has a major role in the regulation of neurotransmission systems like dopamine, serotonin, acetylcholine, and norepinephrine, which in turn are affected in various mood and neurodegenerative disorders [36]. These studies support the findings from the present study wherein choline-DHA supplementation along with FG was shown to facilitate learning and memory possibly through increased cholinergic activity mitigating the ovariectomy induced learning and memory deficits.

Estrogen depletion during menopause is found to be associated with increased brain oxidative stress, leads to memory impairment [37]. Oxidative products accumulated in the brain leads to progressive DNA damage, further decrease reduced glutathione [38]. Results of the present study showed that ovariectomy markedly increased the MDA level and decreased GSH, which is in agreement with the study by Huang & Zhang, suggested that MDA levels were changed after ovariectomy [39]. In the current study fenugreek treatment significantly reduced brain MDA levels, increased brain GSH levels but not to a significant level compared to OVX group. These findings are supported by the human study which reported a reduction in lipid peroxidation after supplementation with phyto-estrogen rich tualang honey in post-menopausal women [40]. Our results are also in consistent with findings of Dong *et al*., demonstrated phytoestrogen α-ZAL supplementation significantly enhanced the antioxidase activities induced by OVX [41]. The combined supplementation of fenugreek with choline-DHA and estradiol significantly increased GSH and decreased MDA levels in

estrogen deficient rats indicating the protective potential of fenugreek with choline-DHA against the free radicals.

The oxidative stress in neurons due to Reactive Oxygen Species (ROS) leads to oxidative damage. Further it affects neuronal cell membrane and its function by damaging proteins and lipid structure that may impair cellular stabilization [42]. Supporting this hypothesis, in the present study the probable reason for the observed degeneration and loss of neural cells in CA1, CA3 and dentate gyrus regions of hippocampus, could be due to estrogen deprivation induced oxidative stress in the brain. Accumulating evidences suggest that DHA plays a vital role in regulating the fluidity of neural cell membranes which are largely made of phospholipids. Further, DHA increases phosphatidylethanolamine and phosphatidylserine concentrations, in addition triggers neurite outgrowth by nerve growth factor and thus facilitates the neuroelectrical signal transmissions [43, 44]. The readily release of DHA, retained by neuronal membrane phospholipids, probably exerts a trophic or antiapoptotic effect plays a critical role in neuronal survival [45]. Supporting to this, the current study showed that supplementation of choline-DHA to OVX rats has significantly minimized neuro-degeneration and improved neuronal survival and cell density in CA1 and infrapyramidal dentate gyrus regions of hippocampus compared to non-supplemented OVX rats. But the neuro-degeneration was minimal and non-significant in CA3 and suprapyramidal dentate gyrus sub regions whereas, FG combined with C-DHA significantly reduced the neural cell degeneration in all the sub-regions of the hippocampus.

Conclusion:

The present study demonstrates that FG seed extract with choline-DHA effectively improved learning and memory abilities in OVX animals. Additionally, combined dietary supplementation reduces ovariectomy induced oxidative stress, hippocampal neural cell damage and enhances the brain antioxidant levels. These findings suggest that fenugreek with choline-DHA could be a potential therapeutic agent to ameliorate cognitive dysfunctions associated with menopause. However, further investigations are needed to validate the efficacy and safety of these therapeutic agents, prior to use in clinical practice.

Acknowledgement:

The authors would like to thank Manipal Academy of Higher Education for permitting and providing research facilities to conduct the research study.

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