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ORIGINAL ARTICLE

Supplementation of fenugreek with choline–docosahexaenoic acid attenuates menopause induced memory loss, BDNF and dendritic arborization in ovariectomized rats

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Abstract

Cognitive impairment due to natural or surgical menopause is always associated with estrogen deficiency leading to reduced brain-derived neurotrophic factor (BDNF). Reduced BDNF levels in menopause affect neuronal maturation, survival, axonal and dendritic arborization and the maintenance of dendritic spine density. Conventional long-term estrogen replacement therapy reported causing the risk of venous thromboembolism and breast cancer. To overcome these undesirable effects, phytoestrogens have been used in menopause-induced condition without the risk of side effects. Therefore, the aim of the present study was to investigate the effect of dietary supplementation of fenugreek seed extract (FG) either alone or in combination with choline–DHA on BDNF and dendritic arborization of pyramidal neurons in CA1 and CA3 regions of the hippocampus in ovariectomized rats. Female Wistar rats of 9–10 months old were divided into six groups as normal control (NC); ovariectomy (OVX); OVX + FG; OVX + choline–DHA; OVX + FG + choline–DHA; and OVX + estradiol. All the groups, except NC, were ovariectomized. After 2 weeks of ovariectomy, dietary supplementation was initiated for a period of 30 days. After supplementation, behavioral studies, BDNF levels and dendritic arborization were estimated. Ovariectomized (OVX) rats showed reduced BDNF levels, dendritic branching points and dendritic intersections of pyramidal neurons in CA1 and CA3 regions of the hippocampus. OVX rats supplemented with FG with choline–DHA showed significantly improved BDNF levels, dendritic branching points and dendritic intersections. These results are demonstrating that FG with choline–DHA supplementation can be an alternative for estrogen replacement therapy to modulate menopause-induced learning and memory deficits.

Keywords Menopause · Memory · Fenugreek · Choline · DHA · BDNF · Dendritic arborization

Introduction

Menopause is a middle age physiological phenomenon that marks the cessation of women's reproductive capacity due to the deficiency of ovarian hormones, mainly estrogen. It was reported that hormonal changes and aging influences the cognitive functions in female rodents (Luine 2008). The cognitive functions are associated with dendritic trees of pyramidal neurons in the hippocampus (Churchwell et al. 2010; Luine and Frankfurt 2013). In addition, studies also reported that estrogen regulates brain-derived neurotrophic factor (BDNF) levels (Scharfman and MacLusky 2006). BDNF plays a significant role in the development, maintenance and plasticity of the brain, especially in the hippocampus (Driscoll et al. 2012). BDNF increases dendrite number, outgrowth and branching in pyramidal neurons (Horch and Katz 2002). In addition, it also helps in maturation and survival of neurons, their dendritic

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arborization and maintenance of dendritic spine density (Poo 2001). The dendritic arborization pattern is critical as it determines the synaptic input field of the dendrite. Two days of estradiol treatment to OVX rats increased BDNF mRNA levels in hippocampus (Gibbs 1999). Similarly, 8 weeks of treatment of estradiol or soybean phytoestrogen to OVX rats also increased BDNF mRNA levels in the hippocampus as well as frontal cortex (Pan et al. 1999). An increased levels of TrKB mRNA in the hippocampus were observed in OVX rats treated with estradiol or phytoestrogen for 12 weeks indicating that prolonged supplementation is essential for improving BDNF levels (Pan et al. 2010). However, prolonged/chronic estrogen therapy after menopause was found to increase the risk for breast, ovarian and endometrial cancers (Chlebowski et al. 2010).

In light of these observations, there has been increasing attention to use natural/herbal alternatives that are rich in phytoestrogen as alternatives with minimum side effects. In this regard, various studies, both in rodents and post-menopausal women, have investigated the effect of non-steroidal plant compounds such as soy, flax seeds, red clover as an alternative to hormonal therapy with a resultant increase in the memory (Islam et al. 2008; Pan et al. 2010). It is interesting to note that fenugreek also contains significant amounts of phytoestrogens (Sreeja et al. 2010). The seeds of fenugreek (Trigonella foenum graecum from Leguminosae family) contains alkaloids, flavonoids, steroids, saponins and known to exhibit antihyperlipidemia, diabetes, analgesic, cancer, etc. (Ahmadiani et al. 2001; Nagamma et al. 2019). Moreover, fenugreek has also been reported for its neuroprotective effect against Parkinson's disease (Gaur et al. 2013), Aβ25–35-induced memory impairment and axonal neurite outgrowth (Tohda et al. 2005). However, there is scant literature on the efficacy of fenugreek on menopause-induced neurodegenerative disorders.

Furthermore, estrogen modulates phosphatidylethanolamine-*N*-methyltransferase (PEMT) gene, thus facilitating the de-novo biosynthesis of phosphatidylcholine (Ptd-Cho) (Zeisel 2006). It also increases the polyunsaturated fatty acid (PUFA) biosynthesis by stimulating rate-limiting enzymes like δ-5 and δ-6 desaturases. Choline and DHA are known to improve the cognitive scores by increasing the connectivity between brain regions and synaptic efficacy (Scheltens et al. 2012; de Waal et al. 2014). Hence, women with lower estrogen concentration, as is the case with a post-menopausal condition, may require supplementation of choline and DHA for normal functioning of the brain.

Materials and methods

Animals

Prior approval of the Institutional Animal Ethical Committee (IAEC) was obtained (IAEC/KMC/12/2015). The rats were handled as per the standard guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Adult healthy female Wistar albino rats of 9–10 months age, weighing about 200–250 g were maintained under standard environmental condition (12 h day–night cycle with a temperature of 22 ± 2 °C), provided with a standard pellet diet and water ad libitum at the central animal research facility, Manipal Academy of Higher Education (MAHE), Manipal. The rats were acclimatized for 2 weeks before commencement of the experiment.

Experimental design

The rats were randomly assigned into six groups of 12 rats in each group as follows: Normal control (NC), OVX, OVX + FG, OVX + C + DHA, OVX + FG + C + DHA, OVX + E2. Other than the NC group, rest of the animals were anesthetized using an intraperitoneal injection of ketamine and xylazine at 50 mg and 5 mg/kg body weight, respectively. Upon the withdrawal of blinking reflexes, bilateral ovariectomy (OVX) was carried out aseptically (Parhizkar et al. 2008). OVX group rats were served as OVX control. After 2 weeks of surgery, OVX + FG group rats supplemented with FG, OVX + C + DHA group rats, supplemented with choline–DHA, OVX + FG + C + DHA group rats supplemented with FG and choline–DHA, OVX + E2 group rats subcutaneously injected with estradiol for 30 days. After supplementation, the behavioral study was evaluated by the radial arm maze (RAM) test. Upon completion of the behavioral study, six animals from each group were sacrificed and their blood sample, brains were used for biochemical and Golgi Cox staining.

Fenugreek seed extraction

Fenugreek seeds (100% organic, PRO NATURE, India) were purchased air dried, coarsely powdered and soaked in 70% ethanol for 2 days. The extract was filtered through a Whatman #1 paper. This extraction procedure was repeated three times (1 l each) under the same conditions with a new solvent. The extract was refluxed at 85 °C. Subsequently, the collected extract was concentrated under vacuum followed by drying in a freeze dryer.

Administration of test materials

Fenugreek seed extract was dissolved in 0.5% carboxymethyl cellulose and administered orally at 200 mg/kg/day (Anjaneyulu et al. 2018). Choline (98% Choline chloride, Loba Chemie Laboratory Reagents and Fine Chemicals)

was dissolved in distilled water and administered orally at 4.6 mmol/kg/day (Thomas et al. 2007). DHA (300 mg docosahexaenoic acid/capsule, Nouveau Medicament (P) Ltd., Chennai) was administered orally at 300 mg/kg/day (Sakamoto et al. 2007). 17β-estradiol (E8515-5 G, Sigma-Aldrich) powder was dissolved in sesame oil and injected to rats subcutaneously at 100 µg/kg/day (Green et al. 2001).

Eight-arm radial maze test

This test was performed as described by Satoh et al., with slight modifications. The test was carried out in the following phases: (1) habituation phase consisting of a single exploratory trial (10 min), (2) acquisition phase consisting of one trial/day (5 min each) for 6 consecutive days, and (3) retention phase consisting of 5-min trial which was performed at 48, 72, 96 and 120 h after completion of the last acquisition trial. Two days before the habituation phase, rats were starved of food to reduce their body weight to 80–85%. In the habituation phase, reward food pellets were placed at the entrance as well as at the end of all eight arms. During acquisition and retention phase, alternate arms (arm no: 2, 4, 6 and 8) were baited. During each trial, rats were placed on the central platform and freely allowed to move for a cut-off period of 5-min. Successful entry to an arm was considered when all four paws passed over the entrance of the arm. Entry to a non-baited arm and re-entry to the previously visited baited arm was considered as reference and working memory errors, respectively. Analysis of data was performed by SMART (v 2.5.21) software.

Estimation of brain-derived neurotrophic factor (BDNF)

After the radial maze test, rats were sacrificed and brains were quickly removed and right cerebral hemisphere was homogenized with phosphate buffered saline (pH 7.4) at 1:10 w/v dilution. Subsequently, it was centrifuged at 3000 rpm for 20 min and the supernatant solution was used for BDNF analysis using ELISA kit. The BDNF levels were quantified as per the manufacturer's protocol mentioned in the ELISA kit (Catalog no: GXBR0178, Genxbio). Both samples and standards were added to BDNF antibody coated ELISA microtiter plate along with anti-BDNF antibodies labelled with biotin and ELISA solutions. The plate was covered and incubated at 37 °C for 60 min. The content of the wells was washed five times with washing buffer. After the addition of chromogen reagent A and B, the plate was incubated at 37 °C for 10 min. Stop solution was added to stop the reaction and the OD values of the solutions were read at 450 nm.

Estimation of estradiol (E2) in serum

After the supplementation (after 30 days) of test materials to the animals, their serum estradiol level was measured by Estradiol (E2) ELISA kit, as per the manufacturer's protocol (Catalog no: GXBR19541, genxbio). Briefly, 40 µl of the sample was incubated along with ten µl antibody and 50 µl of the streptavidin-HRP conjugate, at 37˚C for 60 min. The content of the wells was washed with 1 × washing buffer for five times. After the washing, the buffer was removed completely, each well was added with 50 µl of chromogen reagent A and B and incubated for 10 min at room temperature. Subsequently, equal volumes of stop solution was added to stop the development of color. The absorbance values were recorded at 450 nm.

Neuro-histological analysis: Golgi-Cox staining and dendritic study

Left cerebral hemisphere was fixed in freshly prepared Golgi-Cox fixative for 2 weeks. After fixation, tissues were carefully mounted on a tissue holder by applying a few drops of Fevikwik (adhesive). 150 µm thick coronal sections of the hippocampus were sliced using sledge microtome. The sections were further processed, as described in a previous investigation (Shankaranarayana Rao and Raju 2004). Briefly, the sections were immersed in 5% sodium carbonate for 20 min, dehydrated in ascending grades of alcohol, cleared and mounted with DPX (quick mounting media). Well-stained pyramidal neurons in CA1 and CA3 regions of the hippocampus without truncation or overlap were traced using camera lucida. From each animal, eight pyramidal neurons were traced. Both dendritic branching points (indicating dendritic arborization) and dendritic intersections (indicating dendritic length) were quantified using Sholl (1956) concentric circle method.

Statistical analysis

Data analysis was done with one-way ANOVA followed by Bonferroni's post hoc test. Results were expressed as mean ± SD and p value ≤ 0.05 was expressed as significant.

Results

Radial arm maze test

Working memory errors

Analysis of the working memory errors revealed that NC showed 14.21% of errors, whereas the OVX group showed

29.97% of errors. OVX animals repeatedly entered into the baited arms, even after consuming the food pellets, indicating the memory deficit after OVX. OVX group treated with either FG or choline–DHA or their combination or 17-β estradiol made 12.14%, 15.25%, 13.18% and 15.25% of errors, respectively, indicating that dietary supplementation significantly improved the memory retention (Fig. 1).

Reference memory errors

During the retention test, NC group made 15.36% of errors, whereas OVX animals showed 25.67% of errors. However, the groups treated with either FG or its combination with choline–DHA or 17-β estradiol showed 14.33%, 13.92% and 14.54% of errors, respectively. This indicates that treated groups remembered the baited arms and hence did not enter into the non-baited arms frequently compared to the OVX group. The choline–DHA alone treated group showed only 16.19% of error, which was less compared to the OVX group, but the same was not statistically significant (Fig. 2).

Real-time track plot of radial arm maze

During the radial arm maze retention trails, the OVX group rats were entered several times in to the baited arms even after consuming food pellets. Furthermore, they repeatedly entered in to the unbaited arms, indicating memory loss.

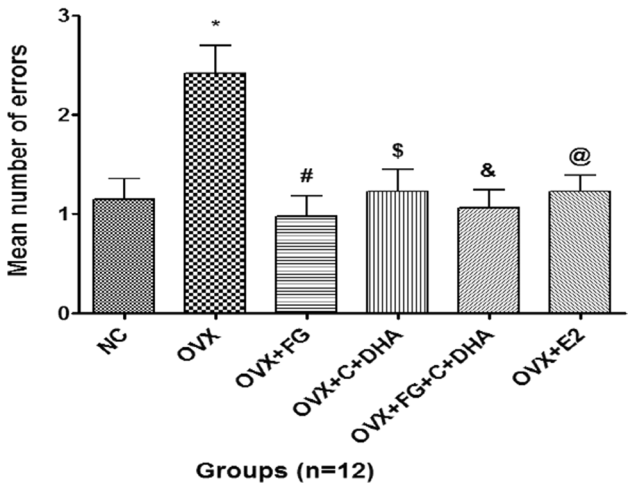

Fig. 1 Mean number of working memory errors during radial arm maze retention trails. The values are expressed in mean ± SD. NC vs. OVX: $^{*}p<0.01$; OVX vs. OVX+FG: $^{\#}p<0.001$; OVX vs. OVX+C+DHA: $^{\$}p<0.01$; OVX vs. OVX+FG+C+DHA: $^{\&}p<0.001$; OVX vs. OVX+E2: $^{@}p<0.01$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

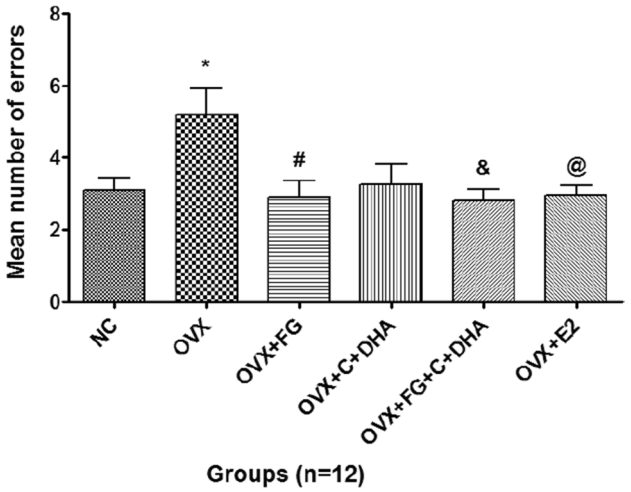

Fig. 2 Mean number of reference memory errors during radial arm maze retention trails. The values are expressed in mean ± SD. NC vs. OVX: $^{*}p<0.05$; OVX vs. OVX+FG: $^{\#}p<0.05$; OVX vs. OVX+FG+C+DHA: $^{\&}p<0.01$; OVX vs. OVX+E2: $^{@}p<0.05$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

However, compared to OVX group, the treated group rats entered less number of times into the baited and unbaited arms, indicating the memory retention after the treatment (Fig. 3).

Expression of brain-derived neurotrophic factor (BDNF)

OVX group showed 8.62% lesser BDNF expression in the brain tissue than the NC group. However, supplementation with FG, choline–DHA, FG with choline–DHA or 17β-estradiol showed 7%, 5.72%, 7.77% and 6.89% increase in BDNF levels compared to the OVX group, respectively. The elevated BDNF levels after the treatment indicate the protective effect of supplementations against the neurodegeneration (Fig. 4).

Estradiol (E2) estimation in serum

After the ovariectomy, 15.11% of estradiol concentration was reduced in OVX group compared to normal control group. On the other hand, fenugreek alone, fenugreek with choline–DHA supplemented group showed 2.27% and 2.21% increased estradiol concentration compared to OVX group. Indicating the presence of phytoestrogen components in fenugreek seed extract. However, 17β-estradiol-treated group showed 10.3% increased estradiol concentration compared to OVX group. Choline–DHA-treated group could not show any effect on estradiol concentration (Fig. 5).

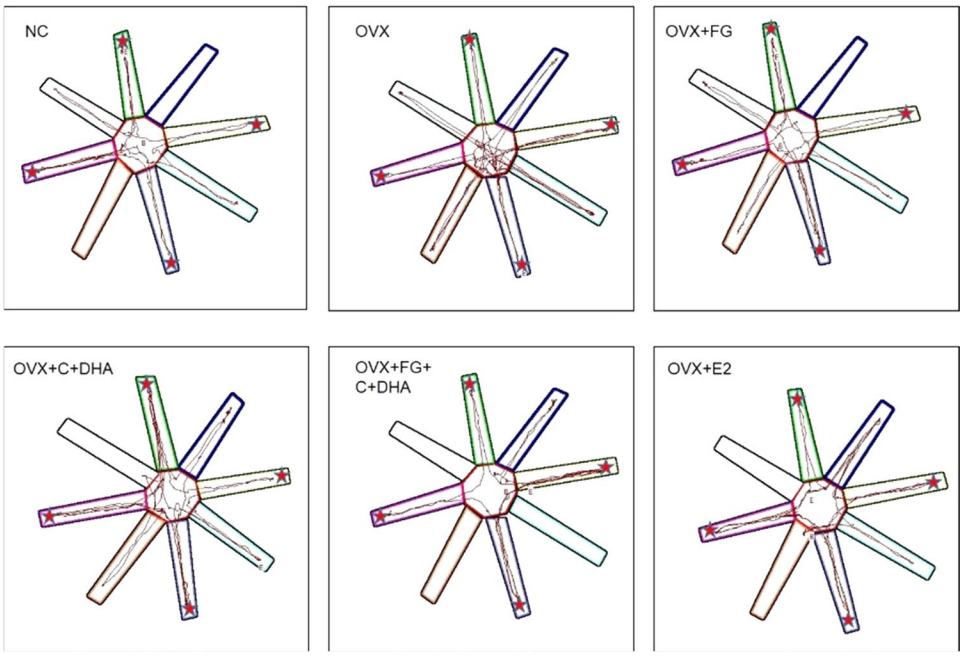

Fig. 3 Representative photograph of real-time track-plots of the animal behavior during radial arm maze test. The OVX rats were repeatedly entered in to the baited and unbaited arms, showed the higher number of errors than control and treated rats. *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

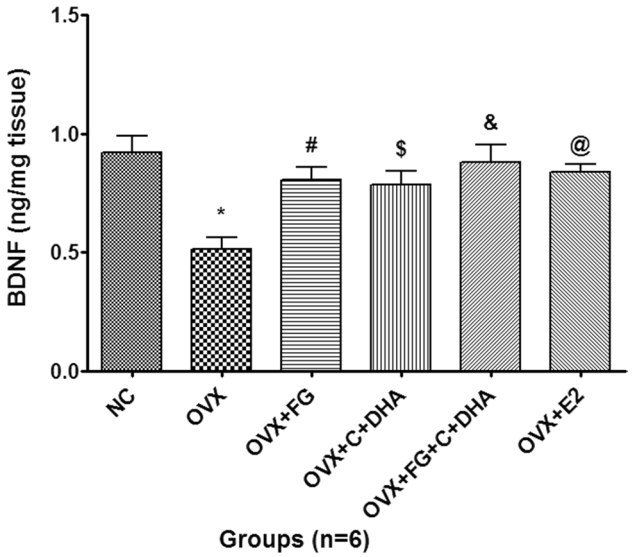

Fig. 4 Brain-derived neurotrophic factor (BDNF) concentration in brain tissue. The values are expressed in mean ± SD. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.05$; OVX vs. OVX + C + DHA: $^{\$}p < 0.05$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.01$; OVX vs. OVX + E2: $^{@}p < 0.01$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

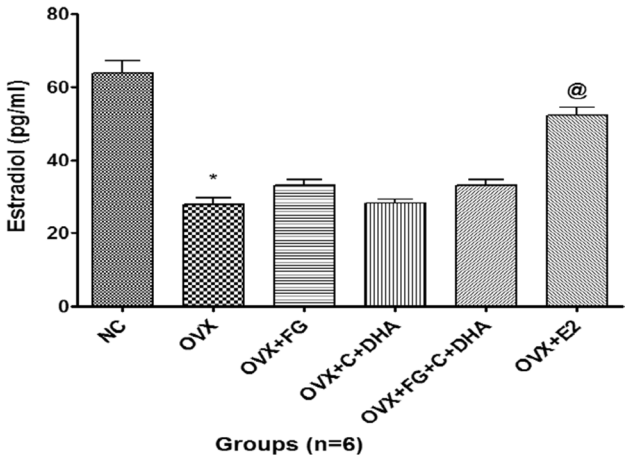

Fig. 5 Estradiol concentration in serum. The values are expressed in mean ± SD. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.01$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

Dendritic arborization of pyramidal neurons in CA1 and CA3 regions of the hippocampus with Golgi-Cox stain

CA1 pyramidal neurons in the hippocampus

Apical dendritic branching points OVX group showed significantly less number of dendritic branching points at 40–60 (2.05 ± 0.31 in NC group vs. 1.22 ± 0.41 in OVX group, $p < 0.05$), 60–80 (2.24 ± 0.44 in NC group vs. 1.24 ± 0.31 in OVX group, $p < 0.01$) and 80–100 µm (1.72 ± 0.27 in NC group vs. 0.99 ± 0.29 in OVX group, $p < 0.01$) concentric zones compared to NC group. OVX + FG group showed a significantly higher number of dendritic branching points at 60–80 (1.24 ± 0.31 vs. 2.02 ± 0.35, $p < 0.05$) and 80–100 µm (0.99 ± 0.29 vs. 1.63 ± 0.24, $p < 0.01$) concentric zones compared to OVX group. OVX + C + DHA group showed an increased number of dendritic branching points at 60–80 (1.24 ± 0.31 vs. 2.02 ± 0.41, $p < 0.05$) and 80–100 µm (0.99 ± 0.29 vs. 1.58 ± 0.31, $p < 0.05$) concentric zones compared to OVX group. Similarly, increased number of dendritic branching points were also observed in OVX + FG + C + DHA group at 40–60 (1.22 ± 0.41 vs. 1.99 ± 0.36, $p < 0.05$), 60–80 (1.24 ± 0.31 vs. 2.24 ± 0.40, $p < 0.01$) and 80–100 µm (0.99 ± 0.29 vs. 1.66 ± 0.33, $p < 0.01$) concentric zones compared to OVX group. The OVX + E2 group showed a higher number of dendritic branching points at 60–80 (1.24 ± 0.31 vs. 2.10 ± 0.32, $p < 0.01$) and 80–100 µm (0.99 ± 0.29 vs. 1.69 ± 0.24, $p < 0.01$) concentric zones compared to OVX group.

The analysis of a total number of branching points revealed that the OVX group showed 6.91% less number of branching points than the NC group. However, dietary supplementation with FG, choline–DHA or their combination or estradiol-treated groups showed 5.64%, 5.16%, 6.63% and 5.83% more dendritic branching points, respectively, compared to OVX group (Fig. 6).

Apical dendritic intersections NC group showed significantly higher number of dendritic intersections at the 40 µm (1.38 ± 0.20 in NC group vs. 0.83 ± 0.23 in OVX group, $p < 0.05$), 60 µm (2.66 ± 0.33 in NC group vs. 1.49 ± 0.29 in OVX group, $p < 0.001$), 80 µm (3.52 ± 0.35 in NC group vs. 2.80 ± 0.34 in OVX group, $p < 0.01$) and 100 µm (2.96 ± 0.57 in NC group vs. 1.80 ± 0.41 in OVX group, $p < 0.05$) concentric circles compared to OVX control group. OVX + FG group showed an increased number of dendritic intersections at the 60 µm (1.49 ± 0.29 vs. 2.27 ± 0.32, $p < 0.05$) and 80 µm (2.80 ± 0.34 vs. 3.41 ± 0.34, $p < 0.05$) concentric circles compared to OVX group. OVX + C + DHA group also showed more number of dendritic intersections at 60 µm (1.49 ± 0.29 vs. 2.19 ± 0.28, $p < 0.05$) and 80 µm (2.80 ± 0.34 vs. 3.41 ± 0.25, $p < 0.05$) concentric circles compared to OVX group. The dendritic intersections were significantly increased in OVX + FG + C + DHA-treated group at 40 µm (0.83 ± 0.23 vs. 1.38 ± 0.20, $p < 0.05$), 60 µm (1.49 ± 0.29 vs. 2.46 ± 0.51, $p < 0.01$), 80 µm (2.80 ± 0.34 vs. 3.63 ± 0.28, $p < 0.01$) and 100 µm (1.80 ± 0.41 vs. 2.85 ± 0.55, $p < 0.05$) concentric circles compared to OVX group. OVX + E2 group showed more dendritic intersections at the 40 µm (0.83 ± 0.23 vs. 1.35 ± 0.33, $p < 0.05$), 60 µm (1.49 ± 0.29 vs. 2.38 ± 0.40, $p < 0.01$) and 80 µm (2.80 ± 0.34 vs. 3.52 ± 0.35, $p < 0.01$) concentric circles compared to OVX group.

The analysis of the total number of dendritic intersections revealed that OVX group showed 6.12% less dendritic

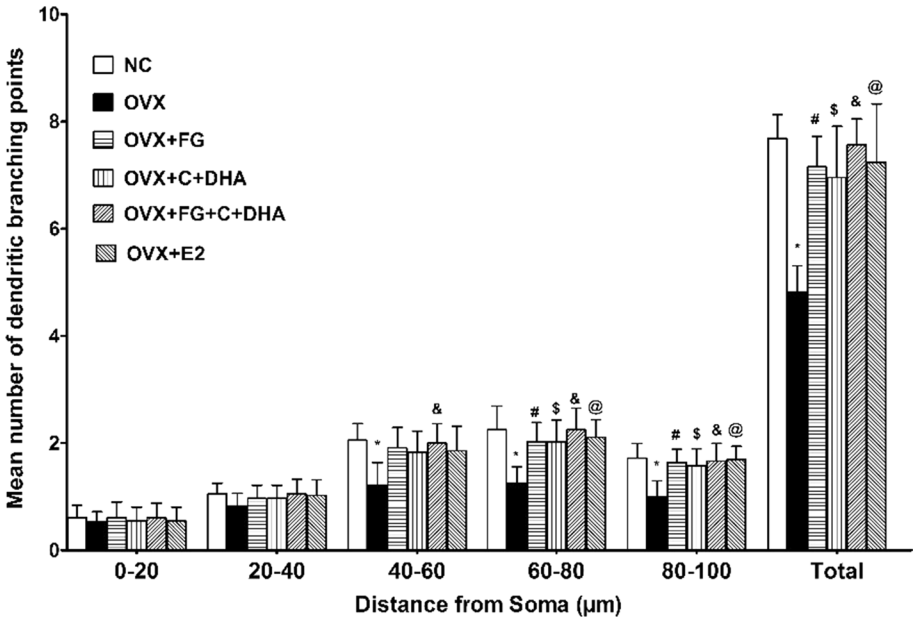

Fig. 6 Mean number of apical dendritic branching points at different concentric zones in CA1 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.001$; OVX vs. OVX + C + DHA: $^{\$}p < 0.001$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

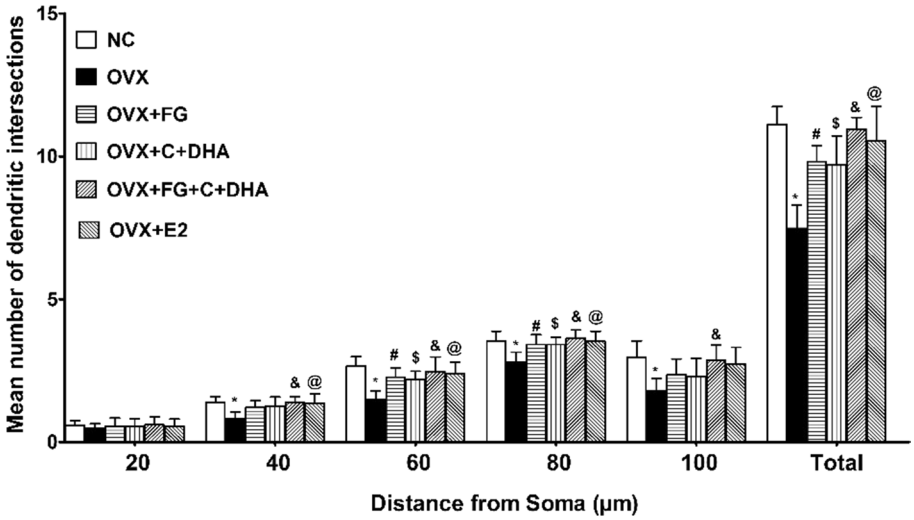

Fig. 7 Mean number of apical dendritic intersections at different radial distances from the soma in CA1 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.001$; OVX vs. OVX + C + DHA: $^{\$}p < 0.001$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

intersections than the NC group. FG, choline–DHA, or their combination or estradiol-treated groups showed 3.92%, 3.74%, 5.83% and 5.14% more dendritic intersections, respectively, than OVX group (Fig. 7).

Basal dendritic branching points The dendritic branching points were significantly less in OVX group at 20–40 (3.49 ± 0.44 in the NC group vs. 2.49 ± 0.43 in OVX group, $p < 0.01$) and 40–60 μm (4.05 ± 0.35 in the NC group vs. 3.08 ± 0.31 in OVX group, $p < 0.001$) concentric zones compared to NC group. OVX + FG group showed significantly more number of dendritic branching points at 40–60 μm (3.08 ± 0.31 vs. 3.74 ± 0.40, $p < 0.05$) concentric zone compared to the OVX group. OVX + C + DHA group showed an increased number of dendritic branching points at 40–60 μm (3.08 ± 0.31 vs. 3.74 ± 0.36, $p < 0.05$) concentric zone compared to the OVX group. However, the dendritic branching points significantly increased in OVX + FG + C + DHA group at 20–40 (2.49 ± 0.43 vs. 3.30 ± 0.41, $p < 0.05$) and 40–60 μm (3.08 ± 0.31 vs. 3.85 ± 0.35, $p < 0.01$) concentric zones compared to OVX group. The OVX + E2 group also showed more number of dendritic branching points at 20–40 (2.49 ± 0.43 vs. 3.30 ± 0.44, $p < 0.05$) and 40–60 μm (3.08 ± 0.31 vs. 3.91 ± 0.31, $p < 0.01$) concentric zones compared to OVX group.

The total number of basal dendritic branching points were 4.16% less in OVX group than NC group. FG, choline–DHA, or their combined supplementation or estradiol supplementation showed an increased dendritic branching points by 2.50%, 2.31%, 3.49% and 3.18%, respectively, than the OVX group (Fig. 8).

Basal dendritic intersections The dendritic intersections were significantly more in the NC group at 40 μm

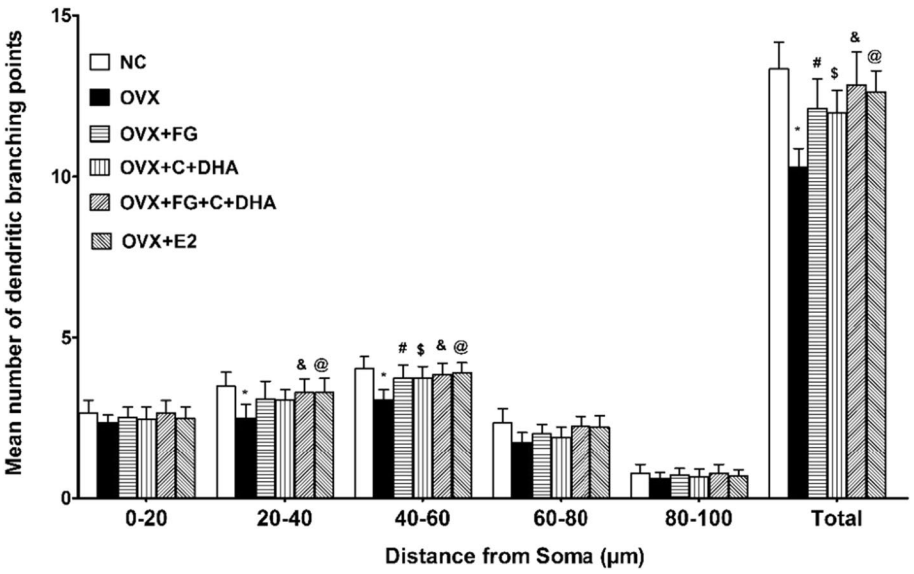

Fig. 8 Mean number of basal dendritic branching points at different concentric zones in CA1 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.01$; OVX vs. OVX + C + DHA: $^{\$}p < 0.05$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

(4.60 ± 0.54 in the NC group vs. 3.30 ± 0.38 in OVX group, $p < 0.01$) and 60 µm (5.66 ± 0.47 in the NC group vs. 3.85 ± 0.62 in OVX group, $p < 0.001$) concentric circles compared to OVX control group. OVX + FG group significantly showed more number of dendritic intersections at 40 µm (3.30 ± 0.38 vs. 4.30 ± 0.51, $p < 0.05$) and 60 µm (3.85 ± 0.62 vs. 4.96 ± 0.51, $p < 0.05$) concentric circles compared to OVX control group. OVX + C + DHA group also showed an increased number of dendritic intersections at 40 µm (3.30 ± 0.38 vs. 4.27 ± 0.41, $p < 0.05$) and 60 µm (3.85 ± 0.62 vs. 4.91 ± 0.51, $p < 0.05$) concentric circles compared to OVX control group. However, OVX group treated with FG + C + DHA showed a higher number of dendritic intersections at 40 µm (3.30 ± 0.38 vs. 4.44 ± 0.65, $p < 0.01$) and 60 µm (3.85 ± 0.62 vs. 5.21 ± 0.50, $p < 0.01$) concentric circles compared to OVX group. OVX + E2 group significantly showed more number of dendritic intersections at 40 µm (3.30 ± 0.38 vs. 4.41 ± 0.45, $p < 0.01$) and 60 µm (3.85 ± 0.62 vs. 5.08 ± 0.60, $p < 0.01$) concentric circles compared to OVX group.

The total number of basal dendritic intersections were 4.62% less in OVX group than the NC group. However, the dietary supplementation with FG, choline–DHA or their combination or estradiol increased the dendritic intersections by 2.96%, 2.53%, 3.72% and 3.30%, respectively, compared to the OVX group (Fig. 9).

Pyramidal neurons in CA1 region of the hippocampus

The Golgi-Cox stained pyramidal neuronal analysis of CA1 region of OVX group showed significant reductions in the number of basal and apical dendritic branching points and total dendritic length, indicating the neuronal damage after ovariectomy. However, rats treated with FG, Choline–DHA and their combination significantly revealed the increased number of branching points and total dendritic length (Fig. 10).

CA3 pyramidal neurons in the hippocampus

Apical dendritic branching points Dendritic branching points were significantly less in OVX group at 20–40 (1.10 ± 0.20 in NC group vs. 0.74 ± 0.17 in OVX group, $p < 0.05$), 40–60 (2.08 ± 0.27 in NC group vs. 1.30 ± 0.24 in OVX group, $p < 0.01$), 60–80 (3.47 ± 0.55 in NC group vs. 2.33 ± 0.29 in OVX group, $p < 0.001$) and 80–100 µm (3.38 ± 0.30 in NC group vs. 2.35 ± 0.30 in OVX group, $p < 0.001$) concentric zones compared to NC group. OVX + FG group showed significantly more number of dendritic branching points at 60–80 µm (2.33 ± 0.29 vs. 3.30 ± 0.24, $p < 0.01$) concentric zone compared to the OVX group. OVX + C + DHA group also showed an increased number of dendritic branching points at 60–80 µm (2.33 ± 0.29 vs. 3.13 ± 0.51, $p < 0.05$) concentric zone compared to the OVX group. However, the dendritic branching points significantly increased in OVX + FG + C + DHA group at 40–60 (1.30 ± 0.24 vs. 1.88 ± 0.36, $p < 0.05$), 60–80 (2.33 ± 0.29 vs. 3.41 ± 0.49, $p < 0.01$) and 80–100 µm (2.35 ± 0.30 vs. 3.10 ± 0.29, $p < 0.01$) concentric zones compared to OVX group. The OVX + E2 group also showed more number of dendritic branching points at 40–60 (1.30 ± 0.24 vs. 1.94 ± 0.30, $p < 0.05$), 60–80 (2.33 ± 0.29 vs. 3.38 ± 0.45, $p < 0.01$) and 80–100 µm (2.35 ± 0.30 vs. 3.10 ± 0.38, $p < 0.01$) concentric zones compared to OVX group.

Analysis of the total number of apical dendritic branching points revealed that OVX group showed 6.24% less number of branching points compared to NC group, whereas FG or choline–DHA or a combination of both or estradiol-treated

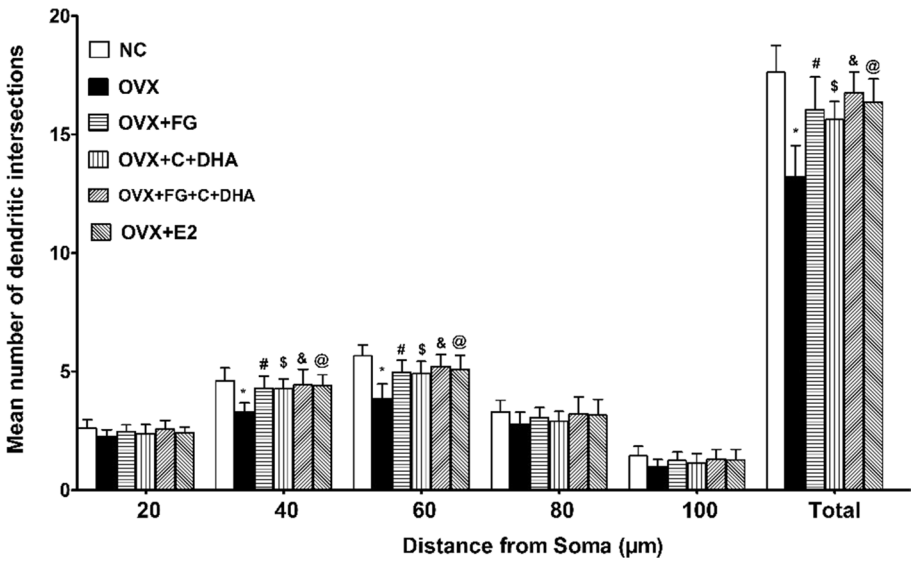

Fig. 9 Mean number of basal dendritic intersections at different radial distances from the soma in CA1 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.01$; OVX vs. OVX + C + DHA: $^{\$}p < 0.05$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

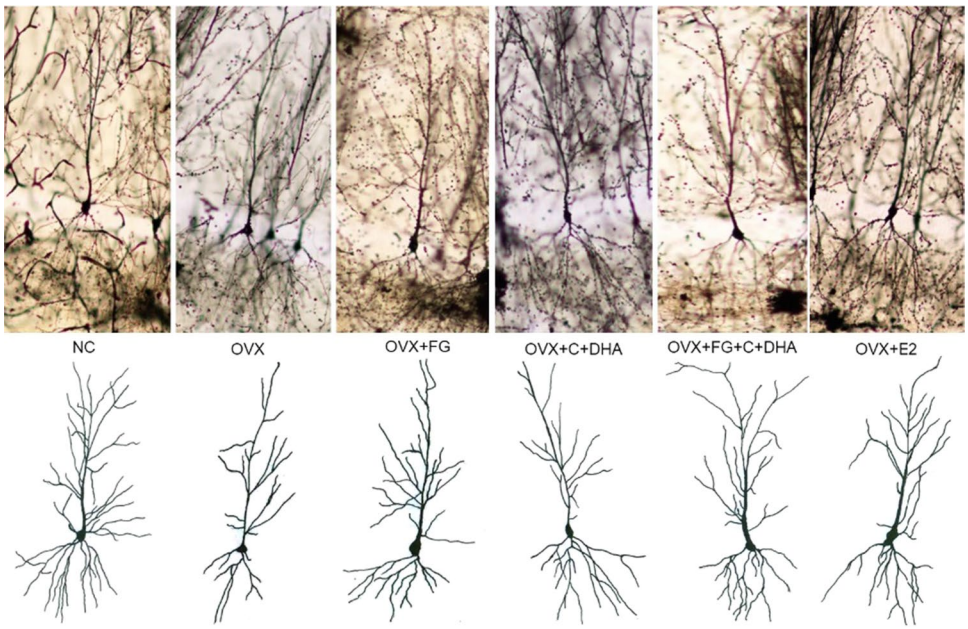

Fig. 10 Representative photomicrograph of pyramidal neurons in CA1 region of the hippocampus (Golgi-Cox stain and camera lucida tracings). A significant increase in number, length of basal and apical dendrites were observed in treated rats compared to OVX-untreated rats. *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

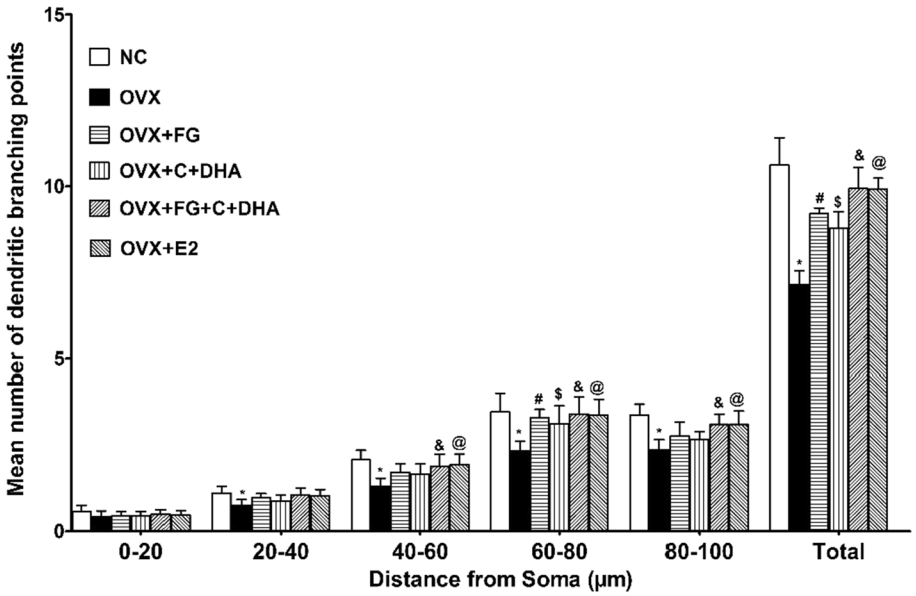

Fig. 11 Mean number of apical dendritic branching points at different concentric zones in CA3 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.001$; OVX vs. OVX + C + DHA: $^{\$}p < 0.001$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

groups had 3.69%, 2.95%, 5.02% and 4.99% more dendritic branching points, respectively, compared to OVX group (Fig. 11).

Apical dendritic intersections Control group showed significantly more number of dendritic intersections at the 40 μm (1.46 ± 0.24 in NC group vs. 0.94 ± 0.25 in OVX group, $p < 0.01$), 60 μm (2.47 ± 0.28 in NC group vs. 1.55 ± 0.29 in OVX group, $p < 0.001$), 80 μm (4.21 ± 0.72 in NC group vs. 2.74 ± 0.36 in OVX group, $p < 0.01$) and 100 μm (3.71 ± 0.51 in NC group vs. 2.60 ± 0.49 in OVX group, $p < 0.01$) concentric circles compared to OVX control group. However, OVX + FG group showed an increased number of dendritic intersections at the 60 μm (1.55 ± 0.29 vs. 2.27 ± 0.20, $p < 0.01$) and 80 μm (2.74 ± 0.36 vs. 3.85 ± 0.44, $p < 0.05$) concentric circles compared to OVX group. OVX + C + DHA group also showed more number of dendritic intersections at the 60 μm (1.55 ± 0.29 vs. 2.21 ± 0.24, $p < 0.01$) concentric circle compared to the OVX group. The dendritic intersections were significantly increased in OVX + FG + C + DHA-treated group at 40 μm (0.94 ± 0.25 vs. 1.41 ± 0.27, $p < 0.05$), 60 μm (1.55 ± 0.29 vs. 2.38 ± 0.29, $p < 0.001$), 80 μm (2.74 ± 0.36 vs. 4.07 ± 0.55, $p < 0.01$) and 100 μm (2.60 ± 0.49 vs. 3.46 ± 0.41, $p < 0.05$)

concentric circles compared to OVX group. OVX + E2 group also showed more dendritic intersections at the 40 µm (0.94 ± 0.25 vs. 1.38 ± 0.20, $p < 0.05$), 60 µm (1.55 ± 0.29 vs. 2.27 ± 0.27, $p < 0.01$), 80 µm (2.74 ± 0.36 vs. 4.16 ± 0.59, $p < 0.01$) and 100 µm (2.60 ± 0.49 vs. 3.49 ± 0.48, $p < 0.05$) concentric circles compared to OVX group.

Analysis of the total number of apical dendritic intersections revealed that OVX group showed 6.43% less dendritic intersections compared to NC group whereas FG, choline–DHA or a combination of both or estradiol-treated groups increased dendritic intersections by 4.11%, 3.56%, 5.50% and 5.46%, respectively, compared to OVX group (Fig. 12).

Basal dendritic branching points The dendritic branching points were significantly less in OVX group at 20–40 (4.41 ± 0.40 in NC group vs. 3.22 ± 0.27 in OVX group, $p < 0.01$), 40–60 (5.10 ± 0.35 in NC group vs. 3.71 ± 0.66 in OVX group, $p < 0.001$) and 60–80 µm (2.35 ± 0.58 in NC group vs. 1.46 ± 0.32 in OVX group, $p < 0.05$) concentric zones compared to NC group. OVX + FG group showed significantly more number of dendritic branching points at 40–60 µm (3.71 ± 0.66 vs. 4.74 ± 0.47, $p < 0.05$) concentric zone compared to the OVX group. OVX + C + DHA group also showed an increased number of dendritic branching points at 40–60 µm (3.71 ± 0.66 vs. 4.66 ± 0.47, $p < 0.05$) concentric zone compared to the OVX group. However, the dendritic branching points significantly increased in OVX + FG + C + DHA group at 20–40 (3.22 ± 0.27 vs. 4.22 ± 0.69, $p < 0.05$) and 40–60 µm (3.71 ± 0.66 vs. 4.97 ± 0.28, $p < 0.001$) concentric zones compared to OVX group. The OVX + E2 group also showed more number of dendritic branching points at 20–40 (3.22 ± 0.27 vs. 4.16 ± 0.45, $p < 0.05$) and 40–60 µm (3.71 ± 0.66 vs. 4.85 ± 0.44, $p < 0.01$) concentric zones compared to OVX group.

The total number of basal dendritic branching points were 5.19% less in OVX group than NC group. However, treatment with FG, choline–DHA or their combination or estradiol showed increased the number of dendritic branching points by 3.36%, 2.73%, 4.33% and 4.06%, respectively, compared to OVX group (Fig. 13).

Basal dendritic intersections The dendritic intersections were significantly more in NC group at 40 µm (4.88 ± 0.40 in NC group vs. 3.77 ± 0.43 in OVX group, $p < 0.01$), 60 µm (6.16 ± 0.78 in NC group vs. 4.16 ± 0.62 in OVX group, $p < 0.001$) and 80 µm (3.33 ± 0.66 in NC group vs. 2.19 ± 0.44 in OVX group, $p < 0.05$) concentric circles compared to OVX control group. OVX + FG group significantly showed more number of dendritic intersections at 60 µm (4.16 ± 0.62 vs. 5.58 ± 0.62, $p < 0.01$) concentric circle compared to the OVX control group. An increased number of dendritic intersections at 60 µm (4.16 ± 0.62 vs. 5.49 ± 0.43, $p < 0.05$) concentric circle compared to the OVX control group were observed in OVX + C + DHA group. However, compared to OVX group, OVX group treated with FG + C + DHA showed more number of dendritic intersections at 40 µm (3.77 ± 0.43 vs. 4.71 ± 0.54, $p < 0.05$) and 60 µm (4.16 ± 0.62 vs. 5.88 ± 0.43, $p < 0.001$) concentric circles. The OVX + E2 group also significantly showed more number of dendritic intersections at 40 µm (3.77 ± 0.43 vs. 4.77 ± 0.52, $p < 0.05$) and 60 µm (4.16 ± 0.62 vs. 5.83 ± 0.64, $p < 0.001$) concentric circles compared to OVX group.

The total number of basal dendritic intersections were 5.38% less in OVX group than NC group. The dietary supplementation of FG, choline–DHA or a combination of these or estradiol increased the dendritic intersection by

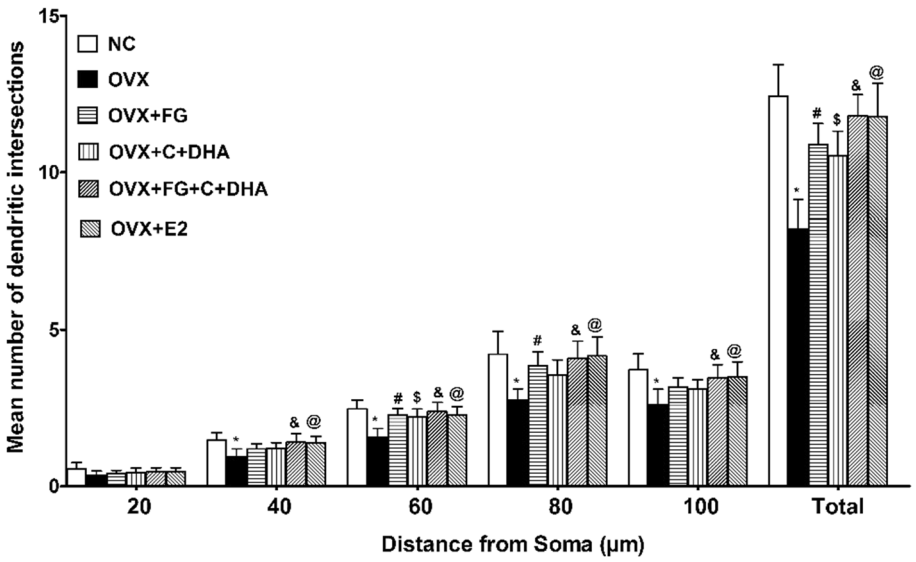

Fig. 12 Mean number of apical dendritic intersections at different radial distances from the soma in CA3 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.001$; OVX vs. OVX + C + DHA: $^{\$}p < 0.001$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

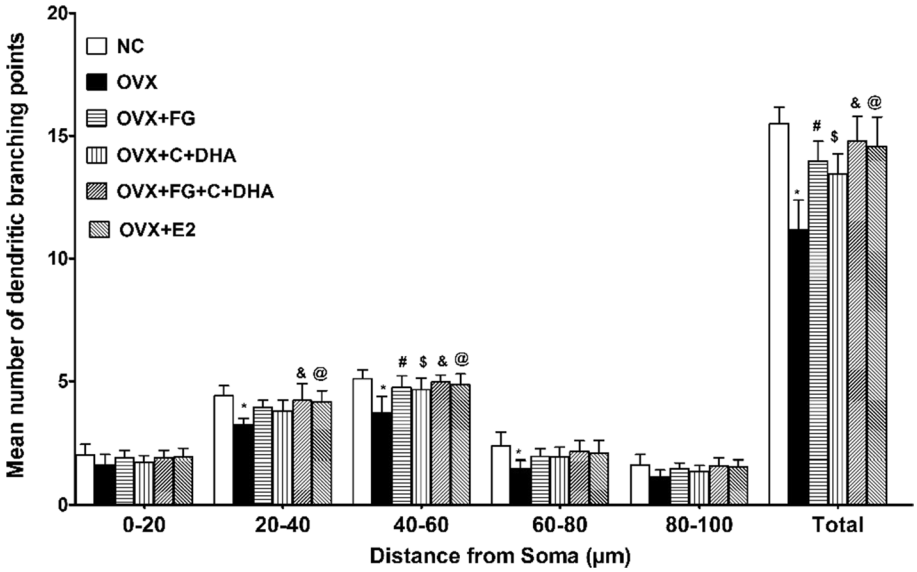

Fig. 13 Mean number of basal dendritic branching points at different concentric zones in CA3 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.001$; OVX vs. OVX + C + DHA: $^{\$}p < 0.01$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

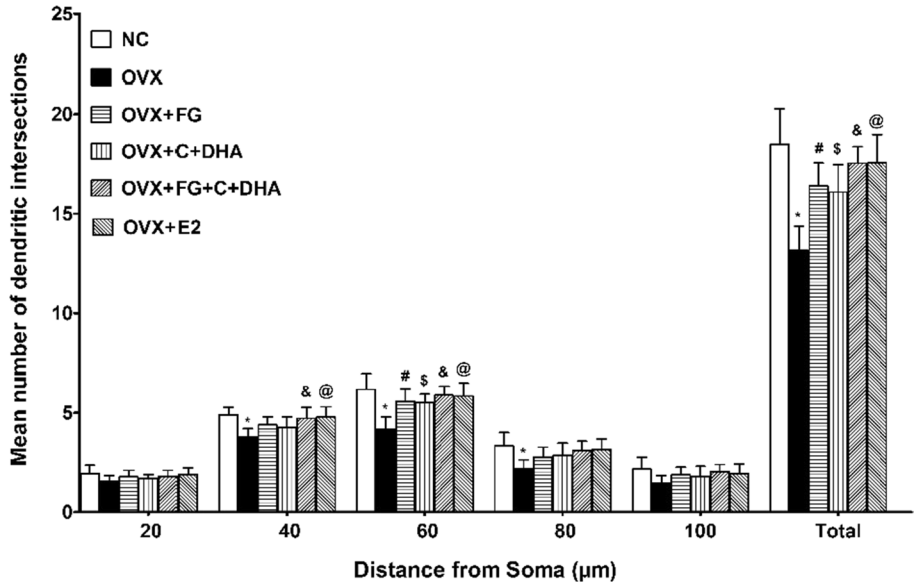

Fig. 14 Mean number of basal dendritic intersections at different radial distances from the soma in CA3 pyramidal neurons of the hippocampus. The values are expressed in mean ± SD, $n = 6$ in each group. NC vs. OVX: $^{*}p < 0.001$; OVX vs. OVX + FG: $^{\#}p < 0.01$; OVX vs. OVX + C + DHA: $^{\$}p < 0.05$; OVX vs. OVX + FG + C + DHA: $^{\&}p < 0.001$; OVX vs. OVX + E2: $^{@}p < 0.001$ (one way ANOVA, Bonferroni's test). *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

3.28%, 2.97%, 4.43% and 4.45%, respectively, compared to OVX group (Fig. 14).

Pyramidal neurons in CA3 region of the hippocampus

The Golgi-Cox stained pyramidal neuronal analysis of CA3 region of OVX group showed significantly less number of basal and apical dendritic branching points and total dendritic length, indicating the neuronal damage. However, after the treatment with FG, Choline–DHA and their combination significantly prevented the damage and improved the number of branching points and dendritic length (Fig. 15).

Discussion

The aim of this study was to investigate the role of BDNF and dendritic arborization in the memory-enhancing effect of phytoestrogens in FG and choline–DHA on menopause-induced OVX model. Our results indicate that OVX rats showed a higher number of visits to non-baited arms (reference memory errors) as well as repeated visits to baited arms (working memory errors) even after consuming food pellets during retention trails indicating memory deficit. These results are in agreement with previous investigation reporting that OVX declines working and reference memory performance (Gibbs and Johnson 2008). OVX leads

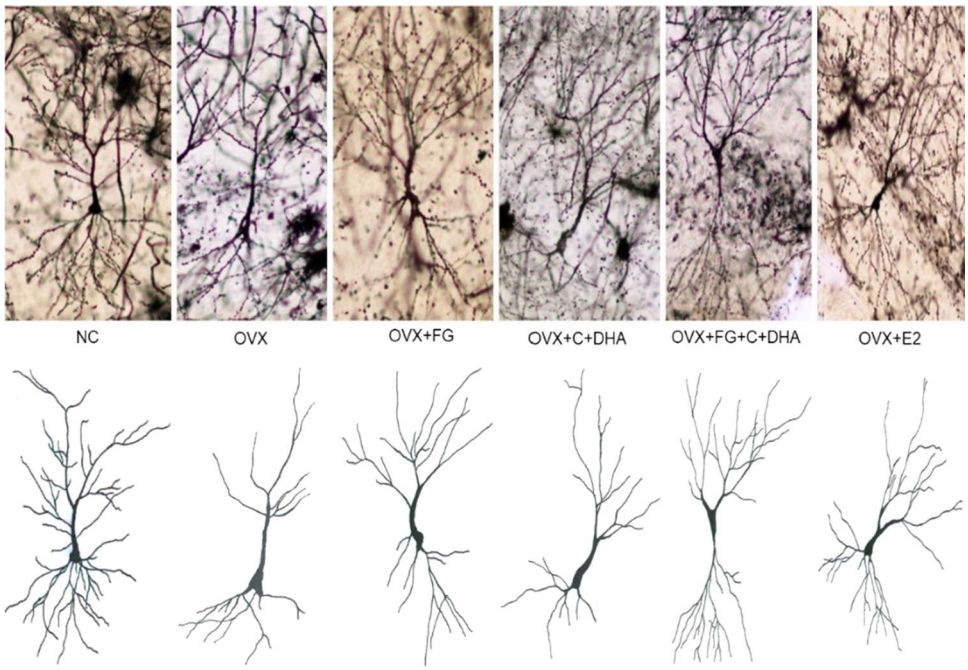

Fig. 15 Representative photomicrograph of pyramidal neurons in CA3 region of the hippocampus (Golgi-Cox stain and camera lucida tracings). A significant increase in number, length of basal and apical dendrites were observed in treated rats compared to OVX-untreated rats. *NC* normal control, *OVX* ovariectomy, *FG* fenugreek, *C* choline, *DHA* docosahexaenoic acid, *E2* 17β-estradiol

to reduced estrogen levels, as in the case of postmenopausal conditions, leading to reduced N-Methyl-D-aspartate (NMDA) receptor binding and/or calcium signaling pathways in hippocampal CA1 dendrites (Cry et al. 2000).

OVX alters the balance between kinase and phosphatase pathways due to the changes in calcium ion signals leading to changes in the CA1 response to synaptic input (Day and Good 2005). Both estrogen receptors, α (ER α) and β (ER β), mediates NMDA receptors and spatial memory. The widespread presence of estrogen receptors in the hippocampus, amygdala and cerebral cortex plays a significant role in the cognitive processes (Genazzani et al. 2007). An increase in NMDA receptors was observed after 2-day estrogen treatment, which improved novel object recognition memory and CA1 long-term potentiation (LTP) magnitude in rats (Vedder et al. 2013). In addition, estrogen modulates the LTP and the long-term depression (LTD) of neurons, which are thought to be key events of cognitive behavior (Mukai et al. 2007).

On the other hand, supplementation of FG, choline–DHA, FG + choline–DHA and E2 to OVX rats reduced working as well as reference memory errors compared to OVX rats. The reduction in the working and reference memory errors in the E2 group is due to supplementation of estrogen levels by estradiol. These results in agreement with previous investigations indicating that estradiol administration enhanced working memory performance during RAM trails (Fader et al. 1999). A significantly lower working and reference memory errors in FG, choline–DHA, FG + choline–DHA can be attributed to the presence of phytoestrogens with a chemical structure similar to estrogen in the FG seed extract. Similar improvements in learning and memory was observed with the dietary supplementation of soy isoflavones (daidzein and genistein) (Duncan et al. 2003; Huang et al. 2004).

Moreover, cholinergic neurons utilize choline for the synthesis of ACh that has a significant role in learning and memory (Klein 2000). A choline-deficient diet over 28 days showed memory impairment in rats (Nakamura et al. 2001). An in vitro study reported that choline deficiency resulted in reduced Ptd-Cho, sphingomyelin leading to increased apoptotic activity (Yen et al. 1999). On the other hand, choline supplementation prevents Ptd-Cho hydrolysis from cholinergic neurons (Klein 2000). Additionally, supplementation of DHA was found to improve learning and memory in healthy aged adults with mild memory issues (Yurko-Mauro et al. 2010). Such supplementation was also found to improve the expression of cell survival genes, inhibit the oxidative stress and inflammation in neurodegenerative animal models (Wu et al. 2011; Horrocks and Farooqui 2004). Furthermore, DHA supplementation to 9-month-old mice for 8 weeks made less number of working memory errors in 8-arm radial maze test (Sugimoto et al. 2002).

A significant reduction in BDNF levels in the brain of OVX rats was observed, which is consistent with the observations of the previous studies (Ahmed et al. 2012; Takuma et al. 2007). BDNF is a member of the neurotrophic family, plays a key role in learning and memory by regulating growth, preservation and survival of neurons (Tyler et al. 2002; Mattson et al. 2004). Studies indicate that depletion of BDNF leads to hippocampal atrophy and neuronal loss in animals (McEwen 1999). Estrogen exerts its direct modulatory effect on BDNF by regulating the estrogen response element on the BDNF gene (Jezierski and Sohrabji 2000).

In our study, FG supplementation significantly enhanced BDNF expression in the brain. Phytoestrogens were found to regulate BDNF mRNA expression by binding with ER-β receptor site (File et al. 2003). A similar increase in BDNF expression in the brains of OVX rats was observed with soybeans or diet that contained steroid for 8 weeks (Hughes and Woods 2003; Pan et al. 1999). Furthermore, learning and memory enhancing the effect of DHA could be due to its antioxidant activity and increasing concentration levels of BDNF in the hippocampus (Tian et al. 2016). As a methyl donor choline can influence cytosine residues at guanine (CpG) islands, in turn changes in gene expression through epigenetic regulation of gene promoter regions, and such as was shown to occur for BDNF (Roth et al. 2009; Newell-Price et al. 2000).

A significant reduction in serum estradiol levels was observed after OVX. However, the serum estradiol levels were found to slightly improve in OVX rats supplemented with fenugreek seed extract. Similarly, genistein, a phytoestrogen, enhanced the estrogen levels in the OVX rats (Li and Liu, 2009). However, choline–DHA supplementation alone did not show any effect on serum estradiol concentration, whereas fenugreek with choline–DHA supplementation also moderately increased serum estradiol levels. This indicates that the moderately elevated levels of serum estradiol after the supplementation of fenugreek is due to the presence of phytoestrogens in it. The phytoestrogen compounds such as steroidal saponins with a structure similar to estrogen hormone imparts estrogenic effect by binding to the estrogen receptors leading to the expression of estrogen responsive gene in vitro (Sreeja et al. 2010). Even though the significant beneficial effects are seen by phytoestrogens present in the fenugreek, the rise in the serum estradiol level by fenugreek is very minimal in our experiment. Therefore, the sensitivity and specificity of the kit used to detect various phytoestrogens present in the fenugreek, needs to be further evaluated.

Dendrites of a neuron are key areas for integrating synaptic transmission. The branch of the dendrite, dendrite length and dendritic spine can enlarge the neuron's surface area for receiving and signaling information plays a vital role in the neural transmission (Nimchinsky et al. 2002). Altering the frequency of neuronal networks is commonly thought to be one of the mechanisms by which memory is preserved and retained in the brain. Long-term synaptic plasticity dependent on activity in adult neural networks often depends on strengthening or weakening existing synapses and creating new contact sites. These effects involve structural changes due to altered shape or arborisation (Holtmaat and Svoboda, 2009; Bosch and Hayashi, 2012). Neuro-histological results of our study revealed marked neurodegeneration with reduced apical and basal dendrites in CA1 and CA3 regions of pyramidal neurons in the hippocampus. It is well reported that OVX reduces the number of both apical and basal dendrites of pyramidal neurons in hippocampus (Luine and Frankfurt 2012). Furthermore, loss or decreased number of dendritic spines, distortion of spine shape, reduction of size and immature structure has been associated with learning and memory impairment (Penzes et al. 2011). Estradiol administration showed an improvement in the number of spines on the apical dendrites of hippocampal CA1 pyramidal neurons along with better production of mRNA for NMDA receptor subunits and the density of excitatory NMDA receptors on the dendritic spines (Gazzaley et al. 1996). In the present study, dietary supplementation of FG significantly improved the apical and basal dendritic branching points and dendritic intersections of pyramidal neurons in CA1 and CA3 regions of the hippocampus. The similar neuroprotective effect was reported earlier with the use of phytoestrogen resveratrol (Hernandez-Hernandez et al. 2016).

The dietary supplementation of choline–DHA also improved basal and apical dendritic branching points and dendritic intersections in OVX rats. The reduced levels of choline may lead to the loss of membrane PtdCho and sphingomyelin leading to cell death via apoptosis (Yen et al. 1999). Furthermore, it was shown that DHA supplementation could protect the brain against centrally acting neurotoxins by upregulating BDNF and neurogenesis (Bousquet et al. 2009; Kawakita et al. 2006). In this study, FG along with choline–DHA supplementation significantly improved the number of basal and apical dendritic branching points and dendritic intersections. This can be attributed to the presence of high amounts of PC-DHA and BDNF when FG is supplemented along with choline–DHA. This PC-DHA is available for sustaining dendritic architecture despite reduced estrogen in OVX rats. These results confirm that supplementation of FG along with choline–DHA has shown a beneficial effect against OVX-induced changes in dendritic arborization.

In conclusion, our study indicated that dietary supplementation of FG, choline–DHA either alone or in combination helps in combating the adverse effects of the OVX. The synergistic effect of combined dietary supplementation of FG and choline–DHA is more evident. Further evaluation of synaptic profile and other molecules related to synaptic neurotransmission is warranted.

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Author contributions AK, KMRB and KSR conceived and designed the study, conducted the research, and performed the analysis and interpretation of the data. KG and YSRM conducted the methods and performed the analysis. All authors contributed equally and significantly to the draft of the article and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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