
ORIGINAL ARTICLE**Role of Choline-Docosahexaenoic acid and *Trigonella foenum graecum* Seed Extract on Ovariectomy Induced Dyslipidemia and Oxidative Stress in Rat Model**

Nagamma Takkella¹, Anjaneyulu Konuri², Anoop Kishore³, Kiranmai S. Rai⁴, Kumar M. R. Bhat^{5*}

¹Department of Biochemistry,⁴Department of Physiology, Melaka Manipal Medical College,

²Department of Anatomy, Kasturba Medical College, ³Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal-576104 (Karnataka)

India, ⁵Department of Anatomy, Ras Al Khaimah College of Medical Sciences, RAK Medical & Health Sciences University, UAE

Abstract:

Background: Menopause is characterized by the deficiency of ovarian hormones, mainly estrogen. The decline in estrogen hormone is contributing the cardiovascular disorders in women. Hormone replacement therapy has disadvantages especially a higher risk of breast, ovarian and endometrial cancers upon chronic use. Phytoestrogens may be used as an alternative to hormone replacement therapy. **Aim and Objectives:** This study was designed to scientifically evaluate the role of Choline- Docosahexaenoic Acid (DHA) and *Trigonella foenum graecum* (TFG) seed extract on Ovariectomy (OVX) induced dyslipidemia and oxidative stress in rat model. **Material and Methods:** Female Wistar rats were allocated into four groups (n=6): 1) Sham control, 2) ovariectomized, 3) ovariectomized+ choline-DHA and 4) ovariectomized + choline-DHA+TFG. After 30 days of treatment, fasting blood samples and liver tissues were collected. Serum was analyzed for lipid profile and liver homogenates were used for assessment of oxidative stress and antioxidant activity. **Results:** Ovariectomized rats showed significantly increased (P<0.05) Total Cholesterol (TC), Low Density Lipoprotein (LDL) levels and decreased High Density Lipoprotein (HDL) levels. Treating ovariectomized rats with choline-DHA and TFG seed extract significantly lowered (P<0.05) total cholesterol, LDL and markedly increased the HDL. Significantly increased (P≤ 0.01) Thiobarbituric Acid Reactive Substances (TBARS)

and reduced (P<0.05) glutathione levels were observed in OVX group. The synergetic effect of choline-DHA and fenugreek showed a significant reduction ((P≤ 0.01) in TBARS levels. **Conclusion:** These results showed that choline-DHA with TFG supplementation have a favorable effect on OVX induced hyperlipidemia and oxidative stress. Therefore, these components may be a therapeutic agent for treating the menopause induced hyperlipidemia or oxidative stress.

Keywords: Choline-DHA, Fenugreek, Ovariectomy, Dyslipidemia, Oxidative Stress

Introduction:

Menopause is characterized by the deficiency of ovarian hormones, mainly estrogen. Estrogen decline is one of the primary factors contributing the Cardiovascular Disorders (CVD) in post-menopausal women [1]. The hormone replacement therapy currently followed has disadvantages especially a higher risk of breast, ovarian and endometrial cancers upon chronic use [2]. Various studies have established that statins are the drug-of-choice for hyperlipidemia. Atorvastatin, rosuvastatin, and pitavastatin considered being the potent statins to lower the Low Density Lipoproteins (LDL) [3]. But the long-term consumption of hypolipidemic drugs has side effects like hyperuricemia, myositis,

diarrhea, gastric irritation, dry skin and liver failure [4]. Therefore, complementary and alternative medicines are preferred over synthetic drugs to avoid possible adverse side effects. Some of the natural plant products can show an estrogen-like effect in the body, such types of components are known as phytoestrogens. To reduce menopausal symptoms, phytoestrogens can be used as an alternative to hormone replacement therapy.

The plant *Trigonella foenum graecum* commonly known as fenugreek belongs to the family Leguminosae [5, 6]. Fenugreek has a wide range of medicinal properties such as antidiabetic, antihyperlipidemic, analgesic, anticancer and antioxidant activities [7-10]. The plant contains active constituents such as alkaloids, flavonoids, steroids and saponins [11]. Fenugreek seeds contain diosgenin, which mimic estrogen and helps in hypolipidemic activity [8].

In the present study, we have used another component known for its hypolipidemic activity, choline-DHA. Choline is obtained from phosphatidylethanolamine by de novo synthesis in the presence of the enzyme Phosphatidylethanolamine-N-methyltransferase (PEMT). The expression of PEMT gene is induced by estrogen [12]. In post-menopausal women, choline levels are declined, that is effecting the formation of phosphatidylcholine, acetylcholine, sphingomyelin and Very Low Density Lipoproteins (VLDL) [13]. Docosahexaenoic Acid (DHA) is a polyunsaturated (22:6 n₃) fatty acid present in the fish oils. DHA acts primarily on hepatic VLDL production to reduce Triglyceride (TG) and secondarily by VLDL clearance [14]. However, no studies have been reported the combined effect of choline-DHA along with or without fenugreek seed extract. We have designed the study to see the

combined effect of fenugreek seed extract along with choline-DHA on dyslipidemia in an ovariectomized rat model.

Material and Methods:

Animals:

Adult healthy female Wistar albino rats of 09-10 months age was allowed to acclimatize for two weeks before starting the experiment. Animals were handled according to the guidelines of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. Animal usage protocol was approved by Institutional Animal Ethics Committee (IAEC), KMC Manipal, Manipal Academy of Higher Education, Manipal (Protocol Approval Number: IAEC/KMC/ 12/2015). Three animals were housed in one polycarbonate cage at Central Animal Research Facility, Manipal Academy of Higher Education, Manipal. The rats were maintained under standard environmental condition (12 hours day-night cycle with the temperature of $22 \pm 2^{\circ}\text{C}$). Animals were provided with a standard pellet diet and water *ad libitum*.

Test Materials:

Fenugreek seed extract:

Trigonella foenum graecum (TFG: 200 mg/kg/day) 100% organic seeds purchased from Pro Natural were coarse powdered and refluxed for three times, at 85°C with 70% ethanol (1 litre each). The hydroalcoholic extract was filtered and concentrated under vacuum. Final drying of extract was done using a freeze dryer. Choline: 4.6 mmol/kg/day; DHA: 300 mg/kg/day.

Experimental Design:

Twenty-four rats were selected randomly into four groups of six animals each. Group 1 served as sham-operated control (subjected to midline incision without removing the ovaries) fed with

Normal Pellet Diet (NPD). Groups 2 to 4 were bilaterally Ovariectomized (OVX) under aseptic conditions. Ketamine (50 mg/kg body weight) and xylazine (5mg/kg body weight) were injected intraperitoneally to anesthetize the rats. Animals were bilaterally ovariectomized by exploring the lower abdominal cavity [15]. Animals were left two weeks to recover from surgery and for the cessation of the estrous cycle. Group 2 animals fed with NPD and served OVX control. After the recovery, group 3 and 4 animals were treated with choline-DHA and choline-DHA + *TFG* seed extract respectively for 30 days. After the treatment, blood samples (2.0 ml) were collected through the retro-orbital plexus of the medial canthus of the eyes with capillary tubes. Before collection of the samples, animals have fasted for 12-16 h. Blood samples were centrifuged at 3000 rpm for 10 minutes at 4°C to obtain serum.

Determination of Serum Lipid Profile:

Serum TC, HDL, TG were determined enzymatically using standard kits obtained from Aspen biochem, India. LDL levels were estimated by using Friedewald's equation: $LDL-C = TC - HDL - VLDL$ ($VLDL = TG/5$) [16]. Coronary Risk Index (CRI) and Atherogenic Index (AI) were calculated as TC/HDL and $\log(TG/HDL)$ respectively [17].

Collection of Liver Tissue for TBARS and GSH Estimation:

All experimental animals were sacrificed, and liver tissue was rapidly removed, washed and homogenized with ice-cold phosphate buffer (pH 7.4, 10% w/v) by using Teflon homogenizer. The homogenate was centrifuged for 10 minutes at 3000 rpm, and the resultant cloudy supernatant liquid was used for estimation of lipid peroxide and antioxidant enzyme activity. Thiobarbituric

Acid Reactive Substances (TBARS) and Glutathione (GSH) were assessed by standard methods [18, 19].

Statistical analysis:

Data was analyzed by using one-way ANOVA followed by Bonferroni's *post-hoc* test (Graph Pad Prism Version 5.0). Results were expressed as mean \pm SD. P value \leq 0.05 was expressed as significant.

Results

The mean Total Cholesterol (TC) (2%) and Low Density Lipoproteins (LDL) (1.3%) were increased significantly ($P < 0.05$) in OVX group compared to Sham (S.) OVX. Whereas fenugreek seed extract with choline-DHA treated group significantly ($P < 0.05$) decreased the TC (2.3%), TG (1%), VLDL (1%), LDL (2%) and significantly increased 1% of HDL ($P < 0.05$) compared to OVX group. Although it is not statically significant, choline-DHA treated group decreased the TC (0.2%), TGs (0.15%), VLDL and 0.5% HDL levels are increased compared to OVX group but LDL (0.5%) level was significantly ($P \leq 0.01$) decreased (Table 1).

CRI was increased significantly ($P < 0.01$) in OVX group when compared to S.OVX. However, fenugreek seed extract with choline-DHA significantly reduced AI and CRI levels when compared with OVX group (Table 1).

Statistically significant ($P < 0.01$) increase in TBARS (1.4 ± 0.2) production and a decrease of antioxidant enzyme status (GSH; 1.13 ± 0.1) were observed in the hepatic tissue of OVX rats when compared with S.OVX group. Fenugreek seed extract with choline-DHA significantly ($P \leq 0.01$) decreased the TBARS (1.15 ± 0.1) level, and GSH (1.3 ± 0.1) activity was markedly increased compared to OVX group (Table 2).

Table 1: Effect of Choline-DHA and Fenugreek Extract on Lipid Profile, Atherogenic Index and Coronary Risk Index

Groups	TC (mg/dl)	TG (mg/dl)	VLDL (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	AI	CRI
S.OVX	108±8.2	125 ±5.7	25.2±1.1	33.6 ±5.9	49±4.1	0.39 ± 0.05	2.15±0.1
OVX	126±6.4*	127 ±5.5	25.4±1.1	55.7±9.3**	45±4.5	0.43 ±0.04	2.7±0.37***
OVX+C+D	112± 12.3	125± 11	25± 2.3	36.1± 12.5 ^{##}	51±4.3	0.37 ±0.05	2.17±0.2 ^{##}
OVX+C+D+F	96.3 ± 11.3 ^{sss}	112± 7.3 ^{sss}	22 ±1.4 ^s	17.2±8.3 ^{sss}	57± 6 ^{ss}	0.28 ±0.04 ^{sss}	1.65±0.15 ^{sss}

Each value represents Mean ± SD. S.OVX Vs OVX *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; OVX Vs OVX+C+D^{##} P ≤ 0.01; OVX Vs OVX+C+D+F^s P ≤ 0.05, ^{ss}P ≤ 0.01, ^{sss}P ≤ 0.001; TC= Total Cholesterol, TG = Triglycerides, VLDL= Very Low Density Lipoprotein, LDL= Low Density Lipoprotein, HDL= High Density Lipoprotein, AI = Atherogenic Index, CRI = Coronary Risk Index, S. OVX= Sham Ovariectomy, OVX = Ovariectomy, C = Choline, D = Docosahexaenoic acid, F= Fenugreek

Table 2: Effect of Choline-DHA and Fenugreek extract on TBARS and GSH

Groups	TBARS (mg/tissue)	GSH (mg/tissue)
S.OVX	1±0.1	1.5± 0.2
OVX	1.4±0.2**	1.13±0.1*
OVX+C+D	1.2 ±0.1 [#]	1.19± 0.1
OVX+C+D+F	1.15± 0.1 ^{ss}	1.3±0.1

Each value represents Mean ± SD; S.OVX Vs OVX *P ≤ 0.05, **P ≤ 0.01; OVX Vs OVX+C+D[#] P ≤ 0.05; OVX Vs OVX+C+D+F^{ss} P ≤ 0.01; TBARS= Thiobarbituric Acid Reactive Substances, GSH = Glutathione; S. OVX= Sham Ovariectomy, OVX = Ovariectomy, C = Choline, D = Docosahexaenoic acid, F= Fenugreek

Discussion:

According to the previous study reports, estrogen deficiency in OVX animals results in elevated levels of total cholesterol and LDL [20]. Similarly, in our study, OVX animals showed high levels of TC. Although it is not significant TC, LDL levels were reduced by treating with choline-DHA, whereas fenugreek seed extract with choline-

DHA treated group significantly decreased the TC and LDL. In this study we have observed the decreased levels of HDL and elevated LDL levels. This may be due to estrogen deficiency, reduced functional activity of LDL receptor and direct secretion of LDL [21]. In another study, it was reported decreased fasting TAG levels (22-26%),

increased HDL-cholesterol concentration (8%) and lower HDL:TAG (28%) after four weeks supplementation with DHA (1.6g/d) in postmenopausal women [22, 23]. Contrary to this report observed by Wu *et al.* [24] that 42 days supplementation of DHA (2.14 g/day) in postmenopausal women decreased plasma cholesterol but not shown any significant effect on oxidative stress markers and estrogen metabolism [24]. This cholesterol lowering effect of DHA may be due to the decrease in expression of liver SREBP1 and fatty acid synthase [25]. Phosphatidylcholine is an important choline metabolite present in the liver is essential for the solubilization of bile salts and secretion and export of TGS in VLDL [26, 27]. Fenugreek, reduce fat accumulation by decreasing the activity of HMG CoA, which is involved in cholesterol synthesis and by upregulation of LDL receptors [8], which could be the reason for reduced levels of TC, TAG, LDL and increased the HDL treated with the combination.

Hypercholesterolemia is associated with ovariectomy and promotes the atherogenesis and cardiovascular disorders; this may be due to estrogen deficiency. Estrogens are known to have antiatherogenic activity by increasing the HDL levels and decreasing the LDL and VLDL levels [28]. In our study, CRI and AI were increased in OVX group. This effect was reversed with combined treatment of choline-DHA with fenugreek extract. This could be due to their hypolipidemic activity.

It was reported earlier, the protective role of estradiol against oxidative damage. Tang *et al.*

found that oxidative stress was significantly raised after eight weeks of ovariectomy in rats. Estrogen can enhance de novo synthesis of phosphatidylcholine via the PEMT pathway [12]. Low levels of choline cause hepatocyte steatosis by increased free fatty acid levels and ROS which in turn alter the composition of the mitochondrial membrane and thereby ATP production, these all lead to oxidative stress [29]. DHA inhibits the neurodegenerative process by increasing the antioxidant activity of GSH reductase, Glutathione peroxidase and catalase activity [30]. Fenugreek acts as an antioxidant due to the presence of polyphenolic components like flavonoids and phenols [31]. The synergistic effect of choline-DHA with fenugreek significantly increased GSH and decreased TBARS levels, showed the protective role against oxidative stress.

Conclusion

In this preliminary study, although choline-DHA has decreased the levels of TC, LDL, and TBARS, the combination of choline-DHA with fenugreek seed extract has been shown better results compared to individual supplementation. However, further investigation is required to understand the exact cellular and molecular mechanisms involved in these process.

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**Author for Correspondence: Dr. Kumar M R Bhat, Department of Anatomy, RAK College of Medical Sciences, RAK Medical and Health Sciences University, Ras Al Khaimah, UAE. Email: kumarbhat@rakmhsu.ac.ae
Cell: +971 05 63974278*