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Research Paper

Pharmacodynamic Evaluation of Oral Estradiol Nanoparticles in Estrogen Deficient (Ovariectomized) High-Fat Diet Induced Hyperlipidemic Rat Model

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Purpose. It is believed that estrogen deficiency contributes importantly to the pathogenesis of menopausal metabolic syndrome and symptoms can be ameliorated with estradiol therapy. The present study reports efficacy of 17-β estradiol encapsulated nanoparticles in treating the postmenopausal dyslipidemic condition.
Materials and Methods. Estradiol encapsulated poly(lactide-*co*-glycolide) (PLGA) nanoparticles were prepared by emulsion–diffusion–evaporation method and evaluated in estrogen deficient (ovariectomized) high fat diet induced hyperlipidemic rat model.
Results. The results obtained showed that estradiol nanoparticles were equally/more effective in treatment of estrogen deficient hyperlipidemic conditions at three times reduced dose and frequency in comparison to that of drug suspension administered orally.
Conclusion. Together, these results demonstrate the ability of nanoparticles in improving oral bioavailability/efficacy of estradiol.

KEY WORDS: bioavailability; estrogen therapy; lipid profile; nanoparticles; oral drug delivery.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in postmenopausal women, with approximately 50% developing CVD in their lifetime, 30% dying from the disease and 20% developing stroke (1,2). The increased incidence of CVD in postmenopausal women has been mainly attributed to menopausal metabolic syndrome manifested by obesity, dyslipidemia (increases in total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, and decreases in high-density lipoprotein (HDL) cholesterol levels), insulin resistance and hypertension (3–5). Estrogen deficiency due to loss of ovarian function at menopause is primarily responsible for the development of various metabolic abnormalities and conditions can be made reversible with estrogen replacement therapy. Numerous epidemiological studies suggested that postmenopausal estradiol treatment reduces the cardiovascular disease risk up to 50% (6–9). Estradiol cardiovascular action is mainly related with the modulation of lipids and lipoproteins. LDL cholesterol are the primary means by which cholesterol is transported from liver to peripheral tissues and hepatic LDL receptors (LDLr) are responsible for maintaining the cholesterol levels in the cells by regulating the plasma LDL levels. After menopause, the clearance rate of LDL from plasma by hepatic LDL receptors becomes slow due to decrease in their number. This leads to elevated plasma LDL levels with relatively longer circulation time, making them more susceptible to modification such as oxidation and finally, giving rise to initiation and development of atheromatous plaque. Estradiol cholesterol lowering action is mostly achieved through up-regulation of hepatic LDLr and thereby, decreasing the plasma LDL concentration (10, 11). Hyperinsulinaemia and hyperglycemia, resulting from insulin resistance are also the major postmenopausal metabolic disturbances leading to many adverse changes of the CVD. At physiological levels, estrogens are thought to be involved in maintaining normal insulin sensitivity. However, estrogen deficiency at menopause may promote insulin resistance. Improved glucose metabolism may contribute to the protective effect of estrogen in CVD as estrogen therapy after menopause has been suggested to improve the insulin sensitivity and reduce Type II diabetes risk profile (12,13).

Oxidative stress has also been implicated as a major factor in the pathogenesis of cardiovascular disease. Deprivation of endogenous estradiol after menopause leads to increased oxidative stress due to an unbalanced pro-oxidant/antioxidant equilibrium and this also thought to be a potential inducer of postmenopausal cardiovascular risk. The increased oxidative stress results in enhanced oxidative modification of LDL. Oxidized LDL are excessively taken up by the scavenger receptors on macrophages in the arterial wall, leading to cholesterol accumulation and the conversion of macrophages into foam cells, which plays a major role in the development of atherosclerotic plaque formation (14,15). The cardio-protective effect of estradiol might also be partly due to its antioxidant action, resulting in protection against lipoprotein oxidation (16,17).

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Table I. Composition of HFD (30)

Ingredients	Diet (g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
DL-Methionine	03
Yeast powder	01
Sodium chloride	01

The effect of oestrogen on lipids and lipoproteins depends on the type and dose of oestrogen used, and its route of administration. Oral route is usually favoured because apart from the patient compliance, it also provides profound beneficial effect in increasing the HDL, a change that is often considered pivotal to have cardiovascular benefit (10,18). However, oral formulations of estradiol have poor systemic bioavailability because of hepatic first pass metabolism, which requires high doses to attain the required therapeutic levels. The high dose can cause potential adverse effects, such as production of harmful active metabolites reported to cause breast cancer (19,20) in postmenopausal women receiving oral estrogen therapy and also, over expression of liver proteins leading to triglyceridemia and gall bladder disease (21). Thus, the risks associated with conventional oral formulations outweigh the benefits offered by them and therefore, demands a delivery system which alleviates the risks without compromising the patient compliance. In recent years, polymeric nanoparticles have emerged as an ideal candidate for oral delivery of many 'difficult to deliver' drug molecules because of their potential to avoid the first pass metabolism of the entrapped drugs as well as sustain their release over long periods of time. Literature reports suggest that apart from normal intestinal enterocytes, nanoparticles uptake also occurs by membranous epithelial cells (M-cells) of Peyer's patches in the gut-associated lymphoid tissue (GALT); resulting in direct entry into systemic circulation through the lymphatics without going into portal circulation and thus, preventing the drug from hepatic first pass metabolism (22–26). Therefore, the present study deals with the prospects of oral polymeric nanoparticulate approach in treatment of postmenopausal hyperlipidemia. We have evaluated estradiol encapsulated PLGA nanoparticles in estrogen deficient (ovariectomized) high fat diet induced hyperlipidemic rat model. The rationale for going for the high-fat diet, further in the estrogen deficient rat was to study the role of estrogen supplementation on its protection against dyslipidemia clinically seen in postmenopausal women. But this condition takes long time to develop in estrogen deficient rat; therefore to achieve similar condition in rat we have designed ovariectomy followed by high fat diet feeding protocol to mimic the postmenopausal dyslipidemic state.

MATERIALS AND METHODS

Materials

Poly(lactide-*co*-glycolide) (PLGA) (Resomer RG 50:50 H; molecular weight 35–40 kDa) was purchased from Boehringer Ingelheim (Ingelheim, Germany) and Didodecyldimethyl ammonium bromide (DMAB) was purchased from Aldrich (St. Louis, MO, USA). Estradiol was a gift sample from Orion Pharma (Espoo, Finland). ELISA kits were procured from DRG diagnostics (Frauenbergstr, Germany). Ultrapure water (SG Water Purification System, Barsbuttel, Germany) was used for all the experiments. All the other chemicals and reagents were of highest commercially available grade.

Preparation and Characterization of PLGA Nanoparticles

Estradiol entrapped PLGA nanoparticles were prepared by emulsion–diffusion–evaporation method using DMAB as stabilizer. The size and zeta potential of the nanoparticles were measured with Zetasizer (Nano ZS, Malvern, UK). Drug entrapment efficiency was determined by centrifuging the drug loaded nanoparticles and then calculating the drug content in the pellet using validated HPLC method (27–29).

Animals

Female Sprague–Dawley (SD) rats (200–210 g) were procured from the central animal facility of the Institute. The animals were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature (21±2°C) and humidity (55±5%) with 12 h light and 12 h dark cycle. All the rats were fed with commercially available rat normal pellet diet (NPD) (Amrut Diet, New Delhi) and water *ad libitum*, prior to the dietary manipulation. The guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Govt. of India were followed and prior permission was sought from the institutional animal ethics committee (IAEC) for conducting the study.

Experimental Protocol

The animals were divided into eight groups of $n=6$. Ovariectomy (OVX) was done in five groups by bilateral incision in the lower part of the peritoneal cavity under anesthesia and after that they were kept for 1 week to allow them to recover from the surgical stress. Thereafter,

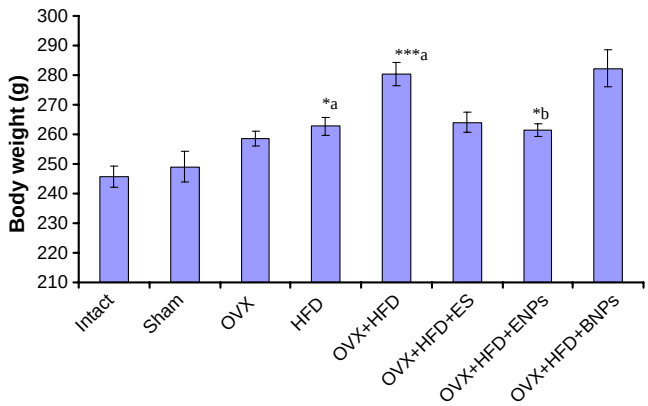

Fig. 1. Effect of estradiol treatment on body weight of estrogen deficient hyperlipidemic rats. Each data point is represented as mean± SEM ($n=6$). ***$p<0.001$, **$p<0.01$, *$p<0.05$; *a* Vs intact and *b* Vs OVX+HFD.

four out of five OVX groups and one intact ovary group were fed on high-fat diet (HFD) while other three groups (control, sham and OVX only) received normal pellet diet (NPD) through out the experimental period of 6 weeks. HFD comprises of 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal and detailed composition of various ingredients are described in Table I (30). Four OVX rats groups receiving HFD were again divided into one no treatment (OVX+HFD), and three treatment groups. After 4 weeks the animals were treated with estradiol suspension (OVX+HFD+ES), estradiol nanoparticles (OVX+HFD+ENPs), and blank nanoparticles (OVX+HFD+BNPs). Treatment was carried out for 2 weeks till the end of sixth week. Drug treatment groups (OVX+HFD+ES & OVX+HFD+ENPs) received a dose of 200 μg/kg body weight (31). However, same doses of nanoparticles (made freshly and dispersed in ultrapure water at each dosing time) were administered once in three days as compared to daily administration of the suspension. The frequency of administration for the nanoparticle group

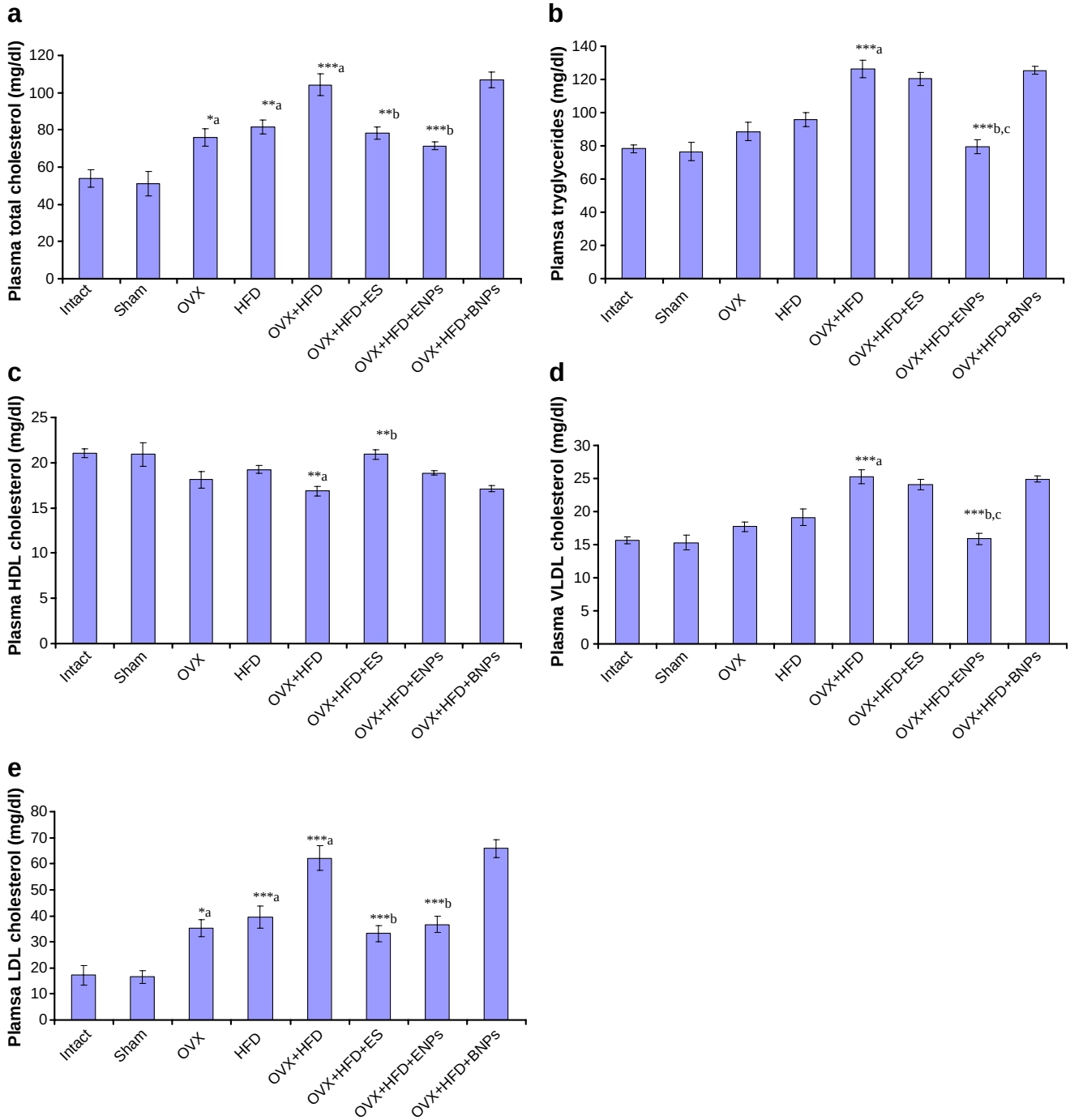

Fig. 2. Plasma levels of **A** total cholesterol (*TC*), **B** triglycerides (*TG*), **C** high density lipoproteins cholesterol (*HDL-C*), **D** very low density lipoproteins cholesterol (*VLDL-C*), and **E** low density lipoproteins cholesterol (*LDL-C*), after 6 weeks of study. Each data point is represented as mean±SEM (n=6). ***p<0.001, **p<0.01, *p<0.05; *a* Vs intact, *b* Vs OVX+HFD and *c* Vs OVX+HFD+ES.

was based on the *in vivo* pharmacokinetics of estradiol (28) and the previous pharmacodynamic effects of nanoparticles formulations in various animal/disease models (32–34). The particle size was thought to influence the circulation times, where as molecular weight/copolymer composition influences the release profile (28).

Estimation of Biochemical Parameters

Blood sampling was done after 4th and 6th weeks and plasma was analyzed for total cholesterol (TC), triglycerides (TG), high density lipoproteins cholesterol (HDL-C), low density lipoproteins cholesterol (LDL-C), very low density lipoproteins cholesterol (VLDL-C) and glucose using commercially available colorimetric diagnostic kits (Accurex Biomedical Pvt. Ltd., India).

Estimation of Oxidative Stress by TBARS Method

The malondialdehyde (MDA), an index of lipid peroxidation and oxidative stress, was measured in the form of thiobarbituric acid reacting substances (TBARS). The method works on the principle of formation of TBARS upon reaction of MDA with thiobarbituric acid, which is a pink colored complex giving peak absorbance at 532 nm (35). The amount of TBARS formed was calculated against the calibration curve prepared using MDA as standard. The MDA contents were expressed as nanomoles of malondialdehyde per milliliter of plasma.

Statistical Analysis

Experimental values were expressed as mean±SEM. Statistical significance between groups was determined by using one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons and a value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The particle size of estradiol loaded PLGA nanoparticles was found to be 115.3±2.5 nm with zeta potential values of 92.4±3.2 mV (pH range was 4.01–4.08). The entrapment efficiency at 10% (*w*/*w* of polymer) initial drug loading was 51.2±3.8%.

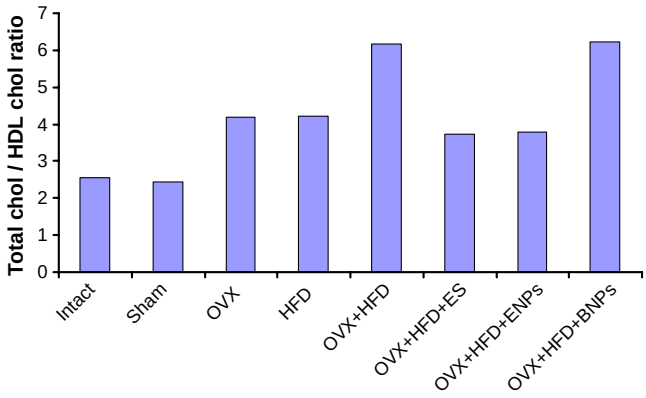

Fig. 3. Effect of estradiol treatment on TC/HDL-C ratio (indicative of cardiovascular risk factor) of estrogen deficient hyperlipidemic rats.

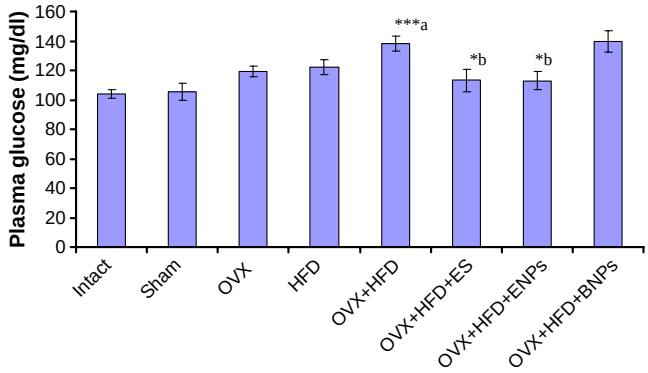

Fig. 4. Plasma glucose levels after 6 weeks of study. Each data point is represented as mean±SEM ($n=6$). ***$p<0.001$, **$p<0.01$, *$p<0.05$; *a* Vs intact and *b* Vs OVX+HFD.

Ovariectomy resulted in significant reduction in circulating plasma estradiol levels (8.4±2.7 pg/ml) compared to control (intact) and sham operated groups (32.5±5.7 pg/ml). This ensured successful ovariectomy of the animals. After 6 weeks, body weight was slightly but not significantly increased in OVX animals, whereas HFD feeding resulted in significant increase ($p<0.05$) in body weight compared to control (intact) animals. On the other hand, OVX+HFD group attained higher body weight than OVX or HFD groups alone, indicating that estrogen deficient condition further aggravated the HFD induced obesity. Treatment with estradiol nanoparticles (ENPs) significantly ($p<0.05$) decreased the body weight, but estradiol suspension (ES) did not show any significant difference (Fig. 1). The results showed that estrogen deficiency may be related to development of obesity in postmenopausal women and effects can be attenuated by estrogen treatment (36).

There was a slight/no difference in the plasma lipids levels of both OVX and HFD fed animals. Ovary intact female animals were appeared to be protected from HFD feeding by available endogenous estrogen, because male

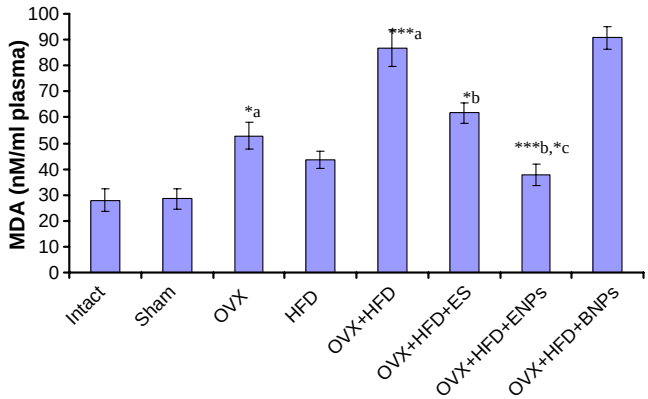

Fig. 5. Effect of estradiol treatment on plasma lipid peroxidation in estrogen deficient hyperlipidemic rats. Each data point is represented as mean±SEM ($n=6$). ***$p<0.001$, **$p<0.01$, *$p<0.05$; *a* Vs intact, *b* Vs OVX+HFD and *c* Vs OVX+HFD+ES.

Sprague–Dawley rats develop hyperlipidemia upon HFD feeding for 4 weeks (33). However, a significant difference was observed in plasma lipid profile of OVX+HFD fed groups compared to control (intact) group, closely resembling to the clinical condition of postmenopausal hyperlipidemic women having high risk of developing cardiovascular complications. 2 weeks of treatment with estradiol nanoparticles (ENPs) significantly reduced the elevated plasma levels of TC, TG, VLDL-C and LDL-C irrespective of their three times lower dose than suspension, indicating the potential of oral polymeric nanoparticles in improving the bioavailability as well as sustaining the drug release (Fig. 2A–E). Though several mechanisms are suggested for lipid lowering effect of estradiol in postmenopausal hyperlipidemic condition but up-regulation of hepatic LDL receptors is the primary means considered to be responsible for the beneficial effect (37,38). The plasma levels of HDL-C "considered to be good cholesterol" were found to be decreased in OVX+HFD fed group. ES treated group increased the HDL-C levels after 2 weeks of treatment; whereas, ENPs were found to have no effect on plasma HDL-C levels (Fig. 2C). This can be explained on the basis of "first pass effect" which is encountered when formulation is given as suspension. Large doses of drug are required to overcome this first pass effect in order to attain the clinically relevant plasma drug levels. This high dose leads to increased hepatic synthesis of many proteins such as apolipoprotein A-1 (major component of HDL-C) and therefore, increases plasma levels of HDLs. However, this overdosing also leads to severe metabolic changes such as triglyceridemia (18,21) and this could be the reason that ES treatment did not make any difference in plasma TG levels, while nanoparticulate formulation significantly ($p<0.001$) decreased them (Fig. 2B). No significant difference was observed in the lipid profile of sham operated group as compared to control (intact) group indicating that surgery did not alter lipid profile. Likewise, blank nanoparticles treated group (BNPs) did not improve the hyperlipidemic condition showing that blank nanoparticles did not possess any pharmacological activity. The cardiovascular risk factor was calculated by taking ratio of TC and HDL-C and the ratio is indicative of the degree of cardiovascular risk. This ratio was less than 3 (2.56) in ovary intact control animals which is considered safe, where as OVX and HFD alone showed risk ratio of 4.19 and 4.20 respectively. These values are above the safe limit and might cause heart ailments in long term. Further, OVX+HFD animals showed 6.17 as risk ratio, where the chances of developing CVD is considered to be double. Both ES and ENPs decreased the CVD risk by bringing down the ratio to 3.73 and 3.79 respectively (Fig. 3), indicating that estradiol supplementation is effective in the postmenopausal dyslipidemic women, who are at great risk of developing CVD in later stages.

A significant elevation was observed in the blood glucose level of OVX+HFD group. This reflects to the insulin resistance condition present in these animals, which may further lead to Type II diabetes at the advanced stages of the estrogen deficient postmenopausal condition. E2 treatment (both suspension as well as nanoparticles) decreased the blood glucose levels (Fig. 4), proving that estradiol is effective in improving the insulin sensitivity in postmenopausal condition.

MDA is an end product of lipid peroxidation and serves as oxidative stress biomarker (39). MDA levels were found to be increased in OVX groups, evidencing the induction of oxidative stress due to estrogen deficiency at menopause and this could be further related to high prevalence of cardiovascular disease in postmenopausal women. HFD feeding alone did not increase MDA levels, possibly because of the antioxidant property of estrogen present in ovary intact HFD fed animals. However, 2 weeks of treatment with estradiol suspension (ES) and nanoparticles (ENPs) significantly decreased the raised MDA levels (Fig. 5), confirming the antioxidant potential of estradiol. Nanoparticle formulation was found to be more effective ($p<0.05$) than suspension which might be because of its controlled and sustained drug release action.

CONCLUSIONS

The study has confirmed that estrogen therapy is efficient in treating the postmenopausal metabolic syndrome by preventing or reversing the weight gain, dyslipidemia and insulin resistance. Furthermore, estradiol PLGA nanoparticles were found to extend the same/better therapeutic benefits at much lower dose than suspension, indicating that they could alleviate the adverse effects of conventional oral formulations. Thus, nanoparticulate approach has a large potential to overcome the dose related problems of estradiol and can be successfully adopted for oral administration of estradiol.

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