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Prevention of Cardiac Myocyte Changes by Raloxifene, a Selective Estrogen Receptor Modulator (SERM) in Rat Model of Surgically Induced Estrogen Depleted State

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ABSTRACT

Background: *Cardiac failure, Cardiovascular diseases (CVD) including coronary artery, cerebrovascular and peripheral vascular disease are the leading causes of morbidity and mortality in both men and women, although female sex has long considered to be a "protective factor" against CVD. This study evaluated the effect of Selective estrogen receptor modulator (SERM) Raloxifene (RAL) therapy on endo-myocardium for therapeutic effectiveness in estrogen-depleted states induced by ovariectomy (OVX).* ***Methods:*** *We divided the rats into four groups: (I) Sham operated (II) ovariectomized controls without any treatment, (III) ovariectomized rats treated with vehicle sesame oil (IV) ovariectomized rats treated with raloxifene 1 mg/kg, s.c. daily for 60 days. Rats were sacrificed by transcardial perfusion. Different chambers of the heart sections were analyzed by image analysis software after hematoxylin and eosin staining.* ***Results:*** *This study reported that an increase in heart weight, cardiac wall thickness and myocytes diameter in response to estrogen depletion that can be effectively reversed by raloxifene treatment.* ***Conclusion:*** *This research revealed that the raloxifene treatment is the novel strategy to alleviate the post-menopausal problems and prevent the heart failure due to cardiac hypertrophy without the adverse effect of estrogen. This original article can give an idea to develop a cardioselective SERM`s which can be a used in both genders. The further studies are needed to find the molecular mechanisms of raloxifene over the hypo estrogenic cardiac myocytes.*

Keywords: Selective estrogen receptor modulator, Raloxifene, Ovariectomy, Estrogen-depleted states, Myocyte diameter, Cardiac hypertrophy

INTRODUCTION

Cardiovascular diseases (CVD) including coronary artery, cerebrovascular and peripheral vascular disease are the leading causes of morbidity and mortality in both men and women, although women have long been considered to be protected against them. Indeed, the incidence of CVD is lower in premenopausal women, but increases with age, especially after menopause; women develop CVD about a decade later than men [1-4]. This has been attributed to the cardioprotective effects of endogenous estrogen, whose levels decline after menopause [2,4]. Left ventricular hypertrophy caused by essential hypertension is one of the major causes of impaired cardiac function followed by heart failure [5,6]. Hormone therapy (HT) with estrogen alone or using an estrogen/progestogen combination remains the most important treatment option for menopausal symptoms. HT is useful in the prevention of cardiovascular (CV) events in post-menopausal women. Observational studies have shown that women who use HT have a 35-50% lower risk of coronary artery disease (CAD) compared with nonusers. CVD-related morbidity and mortality are low in women of reproductive age, but increase to a significant level in older women, especially after menopause; this increase in CVD risk has been attributed to the loss of estrogen at menopause [7]. Several other studies have suggested that a younger age at menopause may be associated with increased risk of CV mortality [8,9]. The Nurses' Health Study demonstrated furthermore, that bilateral ovariectomy is associated with a higher risk of CVD in women who

have never used HT, besides a younger age at natural menopause [10]. However, the randomized clinical trials have challenged the cardioprotective effects of hormone replacement [11]. Several studies have also demonstrated the carcinogenic potential of estrogen-progestin combinations in breast and un-apposed estrogens in the endometrium [12,13]. Hormone replacement therapy (HRT) in hypertensive postmenopausal women contributes by reducing left ventricular mass, improving cardiac function, and decreasing future cardiovascular events [14]. However, the application of long-term hormone replacement therapy is limited by its side effects, which include an increased risk of breast and endometrial cancer, venous thromboembolism, vaginal bleeding, mastodynia, and weight gain [15-17].

Studies have demonstrated the carcinogenic potential of estrogen-progestin combinations in breast and un-apposed estrogens on the endometrium. Selective estrogen receptor modulators (SERMs) areas, alternatives to hormone therapy. SERMs are non-steroidal estrogen receptor ligands that act as partial agonists or antagonists in a tissue pathway or isoform-specific manner. This review offers an opportunity to dissociate the favourable estrogenic effects on bone and cardiovascular system from the unfavourable stimulatory effects on breast and endometrium [18,19].

Raloxifene and Tamoxifen are the two highly characterized SERMs currently approved for use in humans [20]. Raloxifene is a second generation SERM, which was first identified for the treatment of osteoporosis. Later the Multiple Outcomes of Raloxifene Evaluation (MORE) randomized trial shown to prevent breast cancer without the Tamoxifen-associated increase in risk of endometrial cancer [21,22]. The NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 Trial compared the efficacy and safety of these two drugs on various disease outcomes [23]. It reported a significantly lower risk of thromboembolic events, endometrial cancer and cataracts in the raloxifene-treated group compared to the Tamoxifen-treated group, and equivalent reduction within the two groups in the risk of breast cancer, osteoporotic fractures, and ischemic heart disease. Raloxifene therapy is associated with improvement in the levels of serum lipoprotein cholesterol, fibrinogen, and homocysteine. The beneficial effects of raloxifene on markers of cardiovascular risk are corroborated by evidence from observational studies that raloxifene treatment and its association with a reduced incidence of coronary heart disease (CHD) in postmenopausal women [24]. The researchers demonstrated that raloxifene prevented the progression of cardiac hypertrophy and dysfunction induced by transverse aortic constriction banding in mice [25]. Thus, studies using clinical and biochemical markers in animal and human models of CVD have reported favourable changes following raloxifene treatment. It may deduce from the foregoing literature survey that the cardioprotective effects of SERMs are at best controversial and largely unsubstantiated by direct tissue evidence. While the impact of raloxifene on other disease outcomes has been established by consensus, there is some ambiguity as to whether they have a protective or neutral effect on coronary heart disease. Some researchers do attest to their beneficial effects in coronary artery disease, despite the prejudicial effects detected in clinical trials. There is a remarkable scarcity of morphological studies on the cardiovascular system of *in-vitro/in-vivo* models of estrogen insufficiency. The few references pertaining to effects of raloxifene on the cardiovascular system of ovariectomized rats use biochemical markers and electrophysiological parameters. There are no studies available in the literature within our knowledge that provides histological evidence of the cardiovascular benefits of raloxifene in surgically created hypo-estrogenic animals. It would be interesting and instructive, therefore, to observe the microscopic evidence of the cardioprotective effects of raloxifene in an estrogen depleted animal model induced by ovariectomy. A conclusive result as to the effect of Raloxifene on the cardiovascular system would be of great significance in planning management strategies for postmenopausal women.

MATERIALS AND METHODS

Experimental procedure

These investigations were carried out on adult female non-pregnant Wistar rats (*Rattus norvergicus*) (n=24), aged 4-6 months, with body weights ranging from 150-200 g. The animals were procured from the Central Animal Facility of the All India Institute of Medical Sciences, New Delhi with the approval of the Institute Ethics Committee for the use of animals in research. We monitored the estrous cycles of the experimental animals with the help of vaginal smear studies. Daily vaginal smears were obtained for over three weeks, and only those animals that exhibited three consecutive and regular 4-5-day cycles were included in the studies, presuming them to be having a physiological hypothalamo-pituitary-ovarian axis. The smears were allowed to air dry for 30 seconds, spray fixed with ethanol, and immersed in Giemsa stain for 2 minutes. The excess stain was washed with distilled water, the slides were air dried and observed under the light microscope.

Surgical procedure and grouping

We segregated the selected female rats into the following four groups with six animals per group:

- Group 1: Sham operated (NC),
- Group 2: Ovariectomized controls without any treatment (OVX),
- Group 3: Ovariectomized rats treated with vehicle sesame oil (VEH),
- Group 4: Ovariectomized rats treated with raloxifene (RAL).

An adult rat of Group 1 is the sham operated while those of Groups 2-4 shall undergo bilateral ovariectomy (OVX). The bilateral ovariectomy performed via a dorsal incision according to the procedure of Zhou, et al. [26] Animals were anesthetized by an intraperitoneal injection of a mixture of ketamine (120 mg/kg body wt.) and acepromazine maleate (1.5 mg/kg body wt.). The lower part of the back skin was shaved, and a 2-3 cm long incision was made to expose the back muscles. Ovaries were exposed by making 1-2 cm long incision in the overlying muscles. The ovaries were isolated, tied off with sterile sutures and removed in groups 2, 3, and 4 animals. In case of Group 1 (NC) rats, the ovaries are exposed and left intact before suturing the incisions. Following the surgery, the rats were allowed to recover from anaesthesia and then taken back to the animal house. Each rat received a subcutaneous injection of buprenorphine hydrochloride (30 µg/ kg) to control pain, and an intramuscular injection of benzathine penicillin G, 50,000 U/kg in aqueous suspension to control the infections. The operated animals were housed separately and allowed to recover from surgical trauma for the next two weeks. Following surgery, the animals were subjected to vaginal smear studies for the confirmation of complete bilateral removal of the ovaries.

Raloxifene treatment

Raloxifene (Procured from Sigma-Aldrich) dissolved in sesame oil was administered subcutaneously once daily from the post-ovariectomy day 15 onwards for eight weeks, 1 mg/kg dosage based on previously reported studies [26,27]. Along with the injection, rats' body weight was recorded once in two days.

Tissue processing

Twenty-four hours after the last injection, after measuring the body weight the animals (n=24, 6 from each group) were anesthetized with a heavy dose of sodium pentobarbital (60 mg/kg, intramuscularly), and sacrificed using transcardial perfusion fixation with 50 ml of 0.9% saline, followed by 200 ml ice-cold 10% formalin. The hearts were dissected out, together with the ascending aorta weighed and post-fixed in the same fixative and processed for paraffin sectioning. The preparation of paraffin block was done by already prescribed procedure [28]. From the paraffin block, the serial coronal section was cut in 10 µm sections in microtome Leica. Sections were put in hot water bath at 45ºC to spread the section by flotation. Then the sections were taken in the clean egg albumin coated slides and kept in the oven at 45ºC overnight. The sections were stained by haematoxylin and eosin Staining (H&E Staining) [29].

Analysis and measurement of stained sections

The H&E stained sections were analyzed under a light microscope and suitable fields were photographed. Myocardium was identified by the presence of branched striated muscle fibre with intercalated disc and centrally placed nucleus. The thicknesses of each chamber were measured at six different locations. In each chamber, the diameters of ten different myocytes were measured at the mid nucleus level. Sections were analyzed with the help of Image-Pro-Plus 6.2 software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism and all statistical comparisons were made using the one-way analysis of variance test followed by post hoc Bonferroni's Multiple Comparison Test. Results were expressed as mean ± standard error of the mean and $p<0.01$ to $p<0.0001$ was considered as the statistically significant difference.

RESULTS

Body weight and heart weight

From the Table 1, There was no considerable difference in body weight (BW) and total heart weight between NC

and RAL groups, however, significant increase of body weight and heart weight in OVX and OVX + VEH animals than the NC and RAL groups. Heart weight body weight ratio in RAL group is maintained as NC group ($p<0.0001$). Chamber wall thickness and myocyte diameter.

Table 1 Comparison of body and heart weight

Parameters	Group 1 (NC)	Group 2 (OVX)	Group 3 (VEH)	Group 4 (RAL)
Body weight	188.4 ± 8.67	234.0 ± 15.97 [a]	231.0 ± 16.73	198.0 ± 7.583
Heart weight/Body weight	0.0043 ± 0.000146	0.00658 ± 0.000214	0.00660 ± 0.000226	0.00450 ± 0.000148
NC: Normal Control, OVX: Ovariectomized, VEH: Vehicle Treated, RAL: Raloxifene Treated, Body weight expressed in grams (g); Mean ± S.D [a]$p<0.0001$ compared with groups A, C and D				

The wall thickness and myocyte diameters of the all the four chambers of the heart in RAL and NC groups were significantly lower than OVX and VEH groups. However, there is no significant difference between NC and RAL groups. Similarly, there is no significant difference between OVX and VEH groups. The results were summarized in Table 2, and shown in Figures 1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A, 5B, 6, 7 and 8.

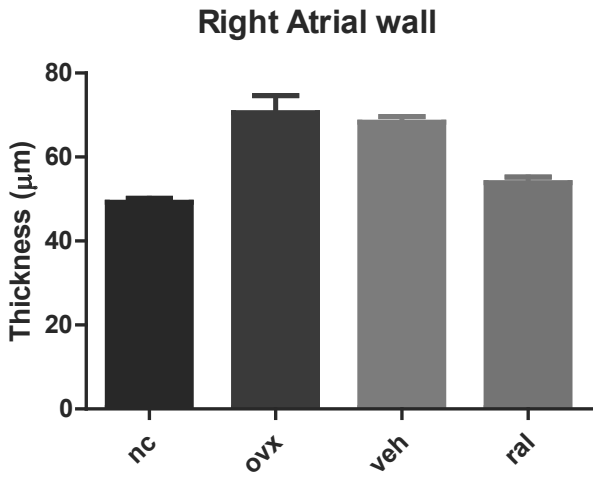

Figure 1A Right atrial wall thickness of nc, ovx, veh and RAL treated groups; $p<0.001$ compared to nc and ral treated groups

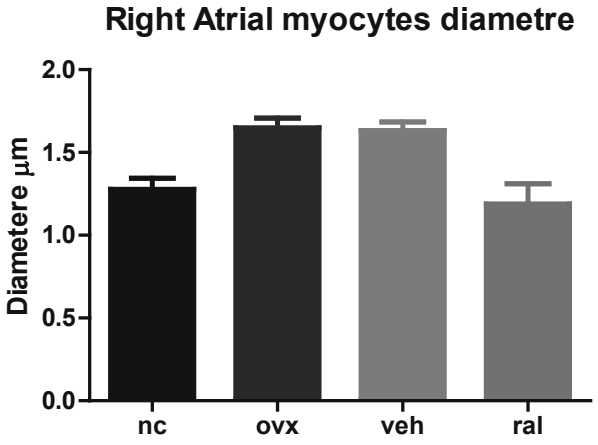

Figure 1B Right atrial myocytes diameters of nc, ovx, veh and ral treated groups. bp<0.05 compared to nc and ral treated groups. (nc- Normal control, Ovx-Ovariectomized, veh-Vehicle treated, ral-Raloxifene treated

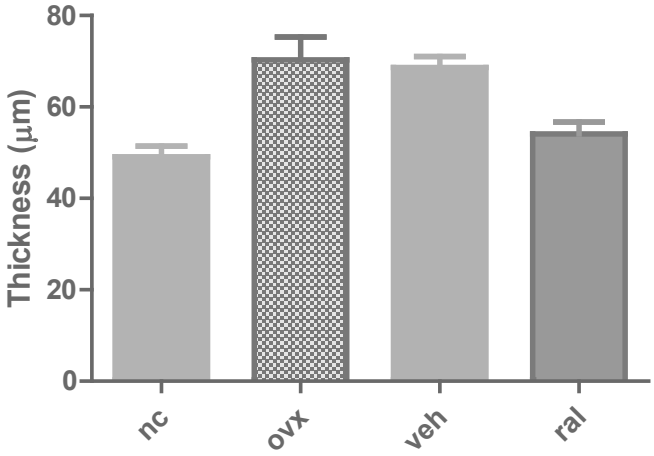

Figure 2A Left atrial wall thickness of nc, ovx, veh and ral treated groups; p<0.05 compared to nc and ral treated groups

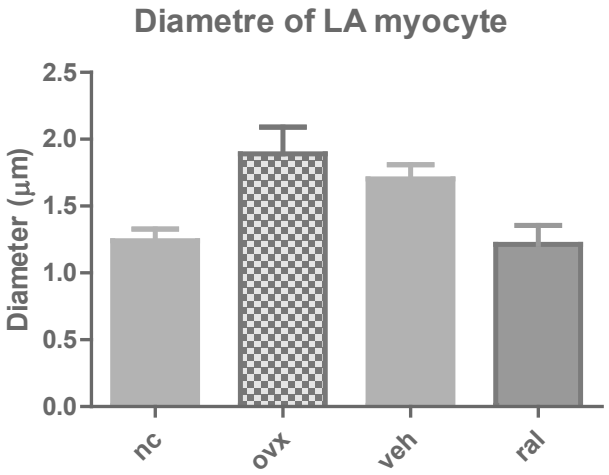

Figure 2B Left atrial myocytes diameters of nc, ovx, veh and ral treated groups. bp<0.05 compared to nc and ral treated groups (nc- Normal control, Ovx-Ovariectomized, veh-Vehicle treated, ral-Raloxifene treated)

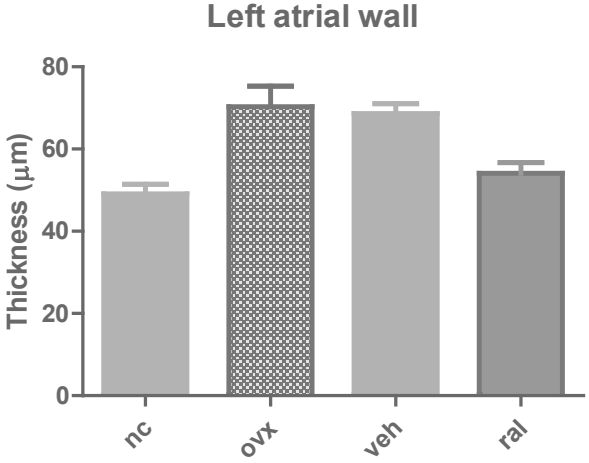

Figure 3A Right ventricular wall thickness of nc, ovx, veh and ral treated groups; p<0.001 compared to nc and ral treated groups

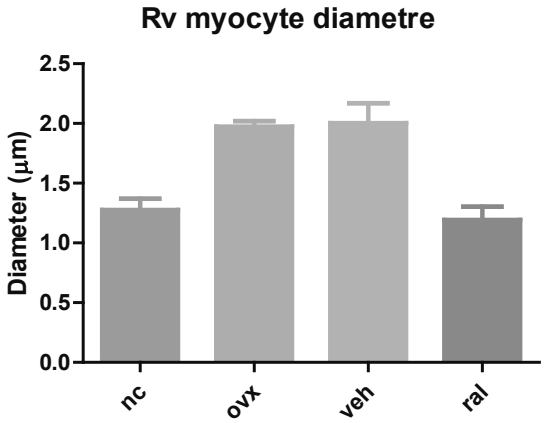

Figure 3B Right ventricular myocytes diameters of nc, ovx, veh and ral treated groups; p<0.0001compared to nc and ral treated groups (nc- Normal control, Ovx-Ovariectomized, veh-Vehicle treated, ral-Raloxifene treated)

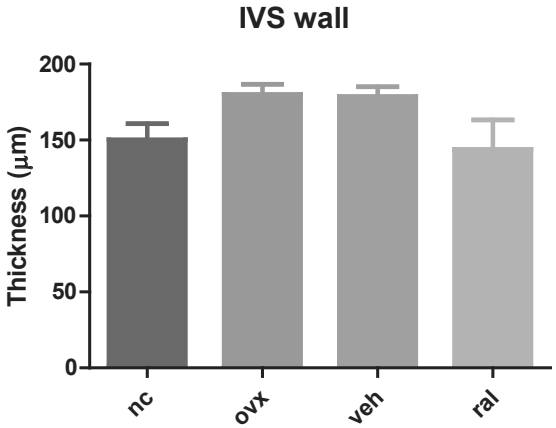

Figure 4A IVS thickness of nc, ovx, veh and ral treated groups; p<0.01 compared to nc and ral treated groups

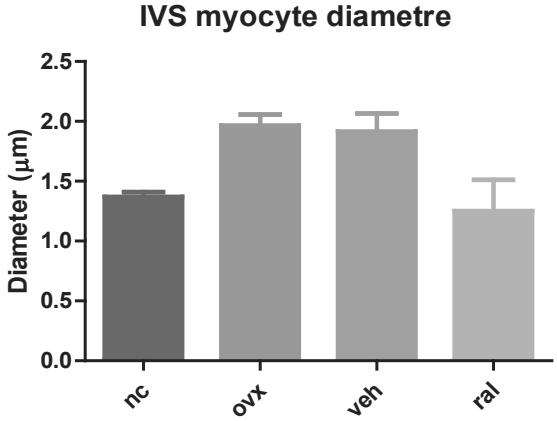

Figure 4B Diameters of IVS myocytes of nc, ovx, veh and ral treated groups; p<0.001 compared to nc and ral treated groups (nc- Normal control, Ovx-Ovariectomized, veh-Vehicle treated, ral-Raloxifene treated)

Interventricular septum

Interventricular septum (IVS) thickness also was measured at various sites and analyzed. Apart from the cardiac chambers the hypoestrogenic state induced by ovariectomy also shown the effects on IVS thickness and myocyte diameters. These results also mimic the results of heart chambers. Thickness and myocyte diameters of IVS in RAL and NC groups were appreciably lower than OVX and VEH groups. On the other hand, there is no noteworthy

difference between NC and RAL groups. In the same way, there is no significant difference between OVX and VEH groups (Table 2 and Figure 5B).

Table 2 Morphological Analysis of various chambers and myocyte diameters of different groups

Parameters	Group 1 (NC)	Group 2 (OVX)	Group 3 (VEH)	Group 4 (RAL)
RA myocytes diameter	1.27 ± 0.164	1.64 ± 0.146 [c]	1.63 ± 0 .127	1.18 ± 0 .290
L A wall thickness	49.11 ± 2.336	70.32 ± 5.012 [c]	68.62 ± 2.405	54.11 ± 2.591
LA myocytes diameter	1.240 ± 0.087	1.891 ± 0.200 [c]	1.702 ± 0.108	1.214 ± 0.140
RV thickness	58.80 ± 3.99	88.19 ± 14.55 [b]	88.58 ± 1.47	63.22 ± 14.18
RV myocytes diameter	1.27 ± 0.093	1.97 ± 0 .045 [a]	2.01 ± 0.166	1.19 ± 0.110
IVS thickness	150.25 ± 10.46	180.11 ± 6.68 [d]	178.67 ± 6.47	143.86 ± 19.34
IVS myocytes diameter	1.36 ± 0.039	1.96 ± 0.092 [b]	1.91 ± 0.148	1.25 ± 0.260
LV thickness	147.69 ± 18.53	188.81 ± 2.89 [a]	189.56 ± 4.79	150.28 ± 42.96
LV myocytes diameter	1.39 ± 0.103	2.12 ± 0.090 [a]	1.99 ± 0.098	1.29 ± 0.197

NC: Normal Control, OVX: Ovariectomized, Veh: Vehicle Treated, Ral: Raloxifene treated, RA: Right Atrium, LA: Left Atrium, IVS: Interventricular Septum, RV: Right Ventricle, LV: Left Ventricle. Various chambers of heart and myocyte diameters are expressed in micrometre (µm); Mean ± S.D; [a] P<0.0001 compared with groups A, C and D; Mean ± S.D; [b] p<0.001 compared with groups A, C and D; Mean ± S.D; [c] p<0.05 compared with group A, C and D; Mean ± S.D; [d] p<0.01 compared with groups A, C and D

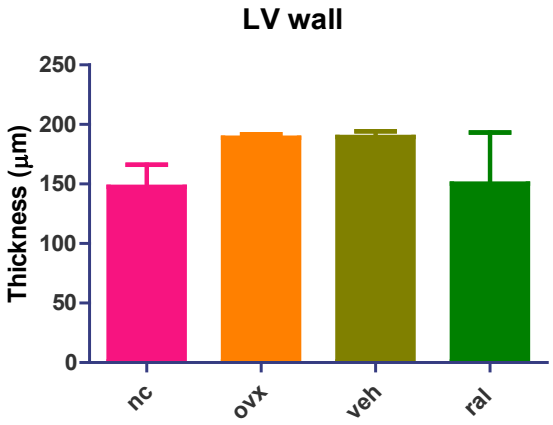

Figure 5A Left ventricular wall thickness of nc, ovx, veh and ral treated groups; p<0.0001compared to nc and ral treated groups

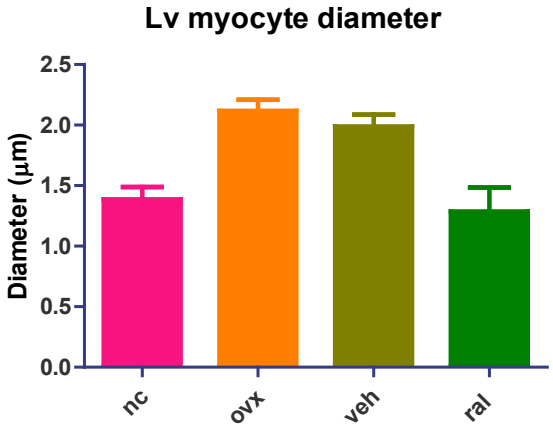

Figure 5B Left ventricular myocytes diameters of nc, ovx, veh and ral treated groups; p<0.0001compared to nc and ral treated groups (nc- Normal control, Ovx-Ovariectomized, veh-Vehicle treated, ral-Raloxifene treated)

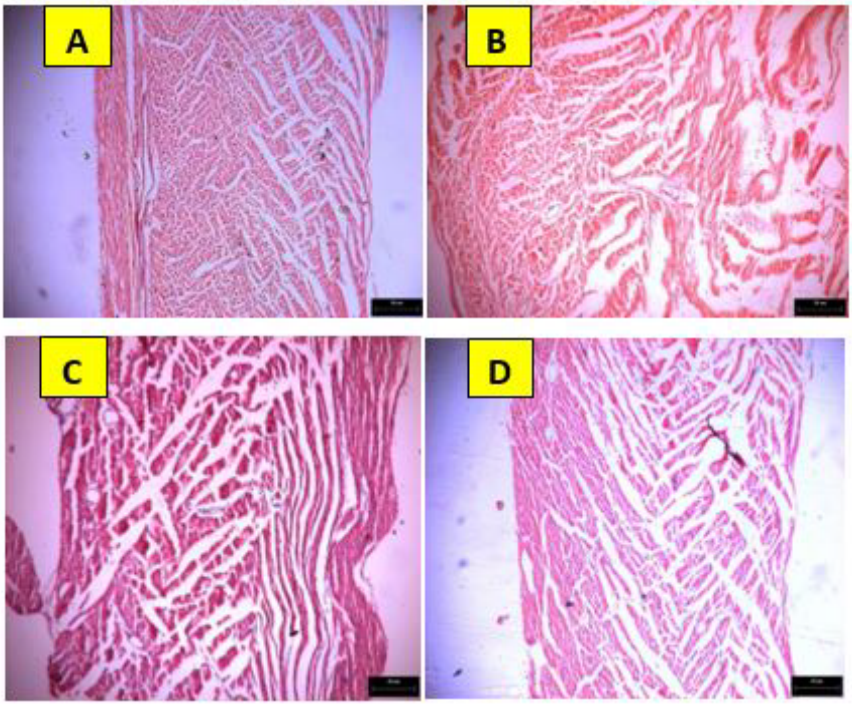

Figure 6 Photo micrographs of coronal sections of H&E stained heart showing Interventricular septum of (A) Normal control sham operated group (B) Ovariectomized control group (C) Vehicle-treated group (D) Raloxifene-treated group. Scale bar 20 µm (40x magnifications)

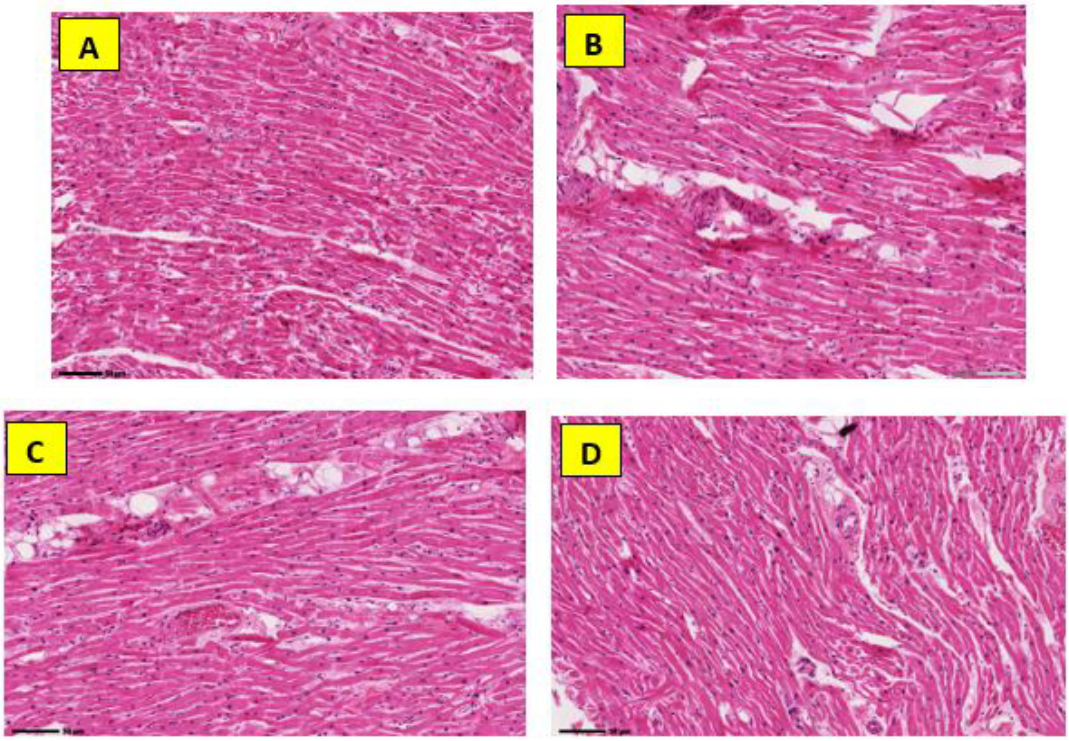

Figure 7 Photo micrographs of coronal sections of H&E stained heart showing Left ventricular wall thickness of (A) Normal control sham operated group (B) Ovariectomized control group (C) Vehicle-treated group (D) Raloxifene-treated group. Scale bar 50 µm (10x magnification)

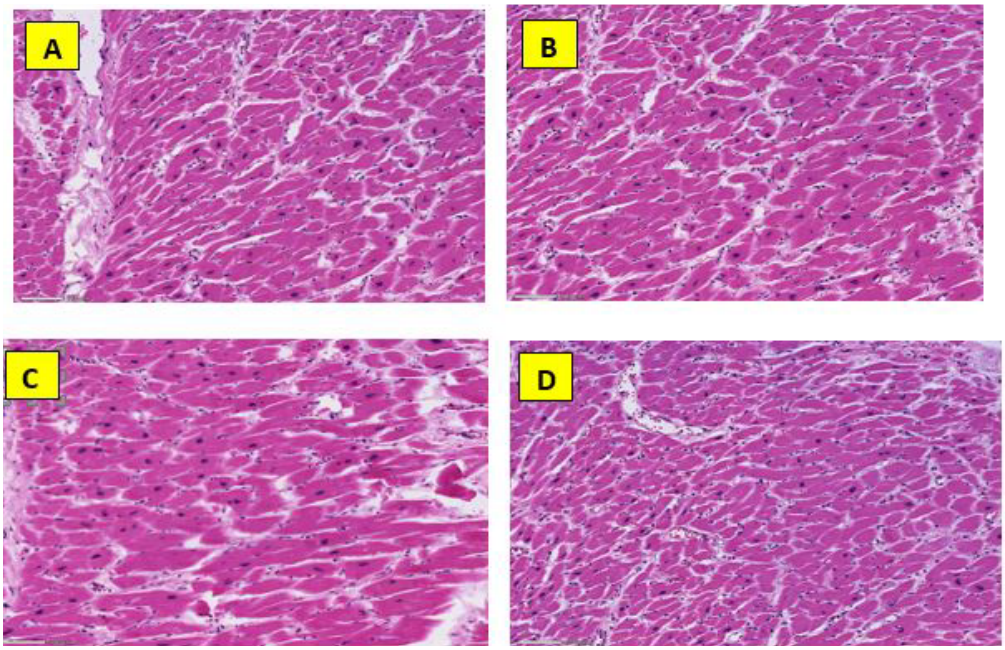

Figure 8 Photo micrographs of coronal sections of H&E stained heart showing Left ventricular myocytes of (A)Normal control sham operated group (B) Ovariectomized control group (C) Vehicle-treated group (D) Raloxifene-treated group. Scale bar 100 µm (20x magnifications)

DISCUSSION

In this study, we established that the ovariectomy causes an increase in heart weight and total body weight compared with sham-operated NC group. Raloxifene prevented the cardiac and total body weight gain. Earlier studies also have proven the increase of body weight after ovariectomy [30]. Increased left ventricular weight /BW ratio due to ovariectomy was associated with an enhancement of the expression of the fetal isoform b-MHC, a characteristic feature of hypertrophied and failing hearts. Estrogen may affect LV hypertrophy by modulating the expression of Ang II receptors on heart tissue. Myocardium contains primarily type I and type III collagen. In the heart, expression of collagen I and III were increased in ovariectomized rats. An enhancement in the concentration of collagen type I, with a 2-fold increase in the type I to type III collagen ratio that was prevented by estrogen replacement. Rat body weights were increased after ovariectomy compared with sham-operated animals. Raloxifene therapy prevented this weight gain. The previous studies have supported these results [31]. Those studies state the ovariectomy causes an increased weight gain due to estrogen depletion [32]. Rosaria Meli, et al., studied how hypo estrogenism alters adiposity [33].

In our study, we found OVX and VEH treated groups shown significantly increased the cardiac wall thickness of atrium, ventricles, and IVS compared with NC and RAL groups. There was a significant increase in the transverse diameter of cardiac myocytes in OVX, VEH treated groups compared with NC and RAL groups. It indicates that the estrogen depletion leads to cardiac hypertrophy by means increasing wall thickness and myocytes diameter. From the above results, we establish that raloxifene reverses the effect of ovariectomy on rat heart. The previous study suggests that the inhibition of p38 MAP kinase phosphorylation by raloxifene or estrogen may represent one of the cardioprotective mechanisms against cardiac hypertrophy and dysfunction caused by hypertrophic stimulation. Those studies have demonstrated that p38 MAP kinase is involved in cardiac hypertrophy and dysfunction in a spontaneously hypertensive rat model [34]. Activated p38 MAP kinase increases desmin expression in cardiomyocytes via Hsp25 and estrogen inhibits p38 MAP kinase phosphorylation induced by TAC in mice [35,36]. Ali, et al. demonstrated the prevention of cardiac hypertrophy by estrogenic compounds which act on estrogen receptor-β [37]. Previous studies have not given any morphological evidence of prevention of hypertrophy. In this study, we have proven the prevention of cardiac hypertrophy by raloxifene in estrogen depleted models.

Comparison of estradiol levels with mean arterial blood pressure (MABP) in Dahl salt-sensitive (DS) rats suggests that the OVX-induced increase in MABP is associated with decreased levels of plasma estrogen because estradiol

replacement was able to prevent the OVX-induced hypertension [38]. Both ERα and ERβ are expressed in the human myocardium and are up-regulated in MH and HF [39,40]. Imre pavo, et al. demonstrated the estrogen-depletion due to ovariectomy causes down-regulation of aortic Ca^{2+} dependent constitutive nitric oxide synthase (cNOS), which strongly suggests its involvement in the increased sensitivity of the vasculature to the vasoconstrictors like vasopressin. Both actions can be reversed by the therapy with the natural estrogen 17b-oestradiol, or the selective estrogen-receptor modulator raloxifene. Thus, raloxifene behaves as an estrogen receptor agonist with regards to both regulation of vascular cNOS and vasopressin-provoked increases in blood pressure *in vivo* [41].

When we consider the clinical implications of raloxifene for hormone replacement therapy, raloxifene is thought to be more beneficial than estrogen because, although estrogen exerts many cardioprotective effects, it increases the risk of carcinogenesis in the breasts and uterus. Previously, SERMs have been shown to improve lipid profiles and endothelial function, inhibit smooth muscle cell proliferation, and have beneficial effects on ischemic heart diseases in human subjects and experimental models [42,43]. Moreover, we have for the first time revealed that raloxifene, one of the SERMs, prevents cardiac hypertrophy and dysfunction in ovariectomized rats. Based on these findings, raloxifene may be considered as a therapeutic drug for postmenopausal women who are at high risk for cardiac hypertrophy. Raloxifene already the drug of choice for osteoporosis in females. A conclusive result as to the effect of Raloxifene on the cardiovascular system would be of great significance in planning management strategies for postmenopausal women.

Raloxifene restored the body weight gain caused by ovariectomy and prevented the cardiac hypertrophy. OVX increased wall thicknesses in all chambers, including IVS by increasing the transverse diameter of cardiac myocytes. Raloxifene treatment retained the myocytes diameter comparable to an ovary intact rat myocyte diameter. We have proven the prevention of cardiac hypertrophy by raloxifene in estrogen depleted models with morphological evidence.

CONCLUSION

This research showed that there was an increase in heart weight, cardiac wall thickness, and myocytes diameter. There was an increase of body weight after ovariectomy also noted, and that increase was prevented by the Raloxifene treatment. Finally, this study indicates that Raloxifene treatment is one of the treatment options to alleviate the post-menopausal problems and prevent the heart failure due to cardiac hypertrophy without the adverse effect of estrogen. This can give an idea to develop a cardioselective SERM`s which can be used in both females and males to give a cardio protectivity and other beneficial effects like beneficial changes in serum cholesterol levels and prevention of osteoporosis. No other studies have proven the occurrence of myocardial hypertrophy after ovariectomy by morphology. Moreover, there is no morphological evidence based study to prove the preventive effect of raloxifene on cardiac hypertrophy due to ovariectomy. The present study is the first study to be proven the beneficial effect of raloxifene on the cardiac hypertrophy in ovariectomized rat heart. Further studies are needed to find out the molecular mechanism for the aforesaid beneficial effects.

DECLARATIONS

Conflict of interest

The authors and planners have disclosed no potential conflicts of interest, financial or otherwise.

Ethical approval

The study was approved by the Institutional Ethics Committee.

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