Effect of Ovariectomy and Estrogen Replacement Therapy on the Thoracic Aorta: Light And Electron Microscopic Study

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ABSTRACT

Background: The majority of cardiovascular diseases occurs in the post-menopausal period. The loss of estrogen associated with menopause can increase the prevalence of various cardiovascular disease risk factors, including hypertension, atherosclerosis, hyper-triglyceridemia and diabetes.

Aim of the Work: This study was done to elucidate the effect of ovariectomy and estrogen replacement therapy on the wall of the thoracic aorta in the adult female albino rat. The study included light and electron microscopic methods.

Materials and Methods: 30 female adult albino rats (6 months old) were obtained from the laboratory animal house, Faculty of pharmacy, Zagazig University. These animals were equally divided into three groups; every group contains 10 animals. Control (normal) Group, Ovariectomized Group (OVx group) and treated ovariectomized Group (OVx+E2 group) received 17β-Estradiol (E2) (15 μg/ kg/day sc) after ovariectomy was done. All rats were weighed weekly during the experimental period. At 13th week after surgery, thoracic aorta specimens were taken for histological and electron microscopic examination.

Results: At the beginning of the study, the body weights of all rats did not greatly differ among the three groups. In the 13th week, the body weight of the OVx rats increased from 195±3.4 g prior to surgery to 265±8.5 g at 13th week post-operative. However, in the treated ovariectomized (OVx+E2) group, the increase in weight was regular and similar to the control group. Under light and electron microscopes, the wall of the thoracic aorta normally consisted of three tunics: Tunica intima, tunica media and tunica adventitia. In the Ovariectomized (OVx) group, ovariectomy resulted in disruption of the intimal layer, even irreversible necrosis of the endothelium, corrugation of the internal elastic lamina in zigzag form with irregularity of its inner border, swelling of endothelial cells and alteration of the endothelial cell ultra-structure (mitochondrial swelling, cytoplasmic vacuolization and collagen accumulation). However, in treated ovariectomized (OVx+E2) group, the wall of the thoracic aorta was seen similar to the normal control and showed no obvious ultra-structural changes.

Conclusions: In the ovariectomized rats, estrogen deficiency leads to increased body weight. Also, ovariectomy changes in the TI/TM ratio and these findings may be indicators of early atherosclerosis. These changes did not occur in the treated ovariectomized rats. Also, the present study suggested that the ovariectomy may induce irreversible damage to the structure of the endothelium but 17β-estradiol treatment elicits a protective effect on the endothelium.

Key Words: Ovariectomy, estrogen replacement, thoracic aorta.

Introduction

Cardiovascular diseases (CVD) are the leading cause of death in women (Mendelsohn, 2002). The vast majority of CVD occurs in the postmenopausal period. The loss of estrogen associated with menopause can increase the prevalence of various cardiovascular disease risk factors, including hypertension, atherosclerosis, hyper-triglyceridemia and diabetes (Baker et al., 2003).

Estrogen has been reported to exhibit a protective effect on the cardiovascular system during pre-menopausal stage (Deroo & Korach, 2006). For nearly 50 years, Estrogen Replacement Therapy (ERT) and Hormone Replacement Therapy (HRT) with estrogen and progestin have been extensively used to prevent cardiovascular diseases in postmenopausal women (Liu et al., 2004). However, the Women's Health Initiative (WHI)
studies of HRT and ERT failed to verify the cardiovascular protective action of such treatments. In fact, HRT recipients were found to have higher cardiovascular disease risks (Rossouw, 2002; Powledge, 2004; Speroff, 2004). Mendelsohn and Karas, (2005) thought that resolving this controversy would require a more complete understanding of the differences in the vascular biology that exist between pre-menopausal and older women.

The purpose of this study was to elucidate the effect of ovariectomy and estrogen replacement therapy on the wall of the thoracic aorta, in the adult female rats. The study included light and electron microscopic methods. Estrogen-deficient rats (ovariectomized) were used to simulate an estrogen-deficient state observed in the postmenopausal women.

MATERIALS AND METHODS

Thirty female adult albino rats (6 months old) were obtained from the laboratory animal house, Faculty of pharmacy, Zagazig University. The animals were housed in the animal cages of the farm, kept under standard laboratory conditions at 21±2 °C, fed with balanced diet and excess water and exposed to natural light-dark cycle for one week prior to the start of the experiments.

These animals were equally divided into three groups with five animals in each. Control (normal) Group (n= 10): Animals received only balanced diet and tap water. Ovariectomized Group (OVx group) (n= 10): Animals were subjected to an ovariectomy at the beginning of the experiment and received balanced diet and tap water. Treated ovariectomized Group (OVx+E2 group) (n= 10): Animals were subjected to an ovariectomy at the beginning of the experiment and then received 17β-Estradiol (E2) treatment. The dose was 15μg/kg/day by subcutaneous injection for 13 weeks (experimental period).

Ovariectomy procedure:

Under pentobarbital sodium (Sigma Chemical) anesthesia (30 mg/kg, intraperitoneal), bilateral ovariectomy was performed to the experimental groups (OVx and OVx+E) of rats in the laboratory animal house, Faculty of pharmacy, Zagazig University. Shaving of abdominal region, then the skin and musculature were incised longitudinally in midline. The peri-ovarian fatty tissue was identified and exteriorized bilaterally. Ligation of the upper part of the Fallopian tubes with 4.0 silk sutures was performed and both ovaries were excised together with surrounding fat and the oviducts. The muscles and skin were stitched separately. Postoperative care including the systemic administration of analgesics and antibiotics was done.

All rats were weighed weekly during the experimental period. The Body weight levels for all rats prior to surgery (gm) and 13 weeks post-surgery were collected for statistical analysis (by using SPSS 10) and expressed as mean±SD (Standard deviation).

At 13th week post-surgery, 3 rats/group were randomly selected and anaesthetized to obtain the thoracic aorta specimens for light and electron microscopic examination.

Preparation for Light microscopy:

The fresh aortic specimens from each experimental group were fixed in buffered neutral formalin. After routine histological laboratory procedures, tissues were blocked in paraffin and sections of 5 μm were cut and stained by two types of stains; haematoxylin and eosin and with Mallory's trichrome stains in Nasser institute. The latter stain was used to identify the connective tissue like collagen and elastic fibrils.

Preparation for Transmission electron microscopy:

Small pieces from fresh aortic specimens, (about one cubic millimeter in size) from each experimental group were immediately fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 24 hours. The specimens were then washed thoroughly in the buffer and post-fixed in 1% cold osmium tetroxide for one hour, dehydrated in graded alcohol, cleared in acetone and embedded in Epon. Semi-thin sections about 1 μm in thickness were stained with 1% toluidine blue. These sections were used to select regions for electron microscopy. Ultra-thin sections about 100 nm in thickness were cut from the selected regions, mounted on copper grids stained with uranyl acetate and lead citrate, for electron microscopic examination in the Faculty of Science, Ain Shams University.
RESULTS

Body weight of the animal models:

The success of the ovariectomy procedure was confirmed by examining body weight levels of all rats/week during the experimental period. At the beginning of the study, the body weights did not greatly differ among the three groups. In the 13th week, the body weight of the OVx rats increased from 195±3.4 g prior to surgery to 265±8.5 g at 13th week post-operative. However, in the treated ovariectomized (OVx+E₂) group, the increase in weight was regular and similar to the control group (Table).

Light microscope:

By Light micrograph using Haematoxylin and Eosin stains showed that the wall of the thoracic aorta of normal rats appeared consisting of three tunics, tunica intima, tunica media and tunica adventitia. Thin tunica intima was seen on the top. The tunica media constituted the major part of the wall of the aorta. The tunica media was composed of a large complement of smooth muscle cells and their nuclei were displayed. However, the normal tunica adventitia was composed of longitudinal elastic fibers (Fig. 1). In the Ovariectomized (OVx) group, the intimal layer appeared irregular, disrupted and showed furrows on the top. The tunica media was nearly similar in its thickness to the normal control and did not exhibit any changes. The tunica adventitia contained many large fat cells (Fig. 2). In treated ovariectomized (OVx+E₂) group, the wall of the thoracic aorta was seen similar to the normal control. The intimal layer was normal in appearance on the top. The tunica media was seen normal in thickness and the tunica adventitia contained longitudinal elastic fibers and had no fat cells (Fig. 3).

By Light micrograph using Mallory’s trichrome stain showed that the internal elastic lamina, (stained pink) and the collagen fibers, (stained blue) were well demonstrated in the tunica media by this preparation. In the normal rats, the internal elastic lamina was uniform in appearance through the tunica media of the wall of the thoracic aorta (Fig. 4). In Ovariectomized (OVx) group, the intimal layer appears irregular, disrupted and showed furrows on the top. The internal elastic lamina appears corrugated in zigzag form especially in the subendothelial layer. The collagen fibers, which were stained blue, were distributed among the internal elastic lamina (Fig. 5). In the treated ovariectomized (OVx+E₂) rats, the internal elastic laminas were normal in their uniform and showed no corrugation. The collagen fibers were seen normally distributed between the internal elastic laminae in the tunica media (Fig. 6).

Transmission electron microscope:

In the normal rats, the wall of the thoracic aorta was lined by endothelial cells which were usually elongated in shape. The endothelial cell showed oval mitochondria and large nucleus with thick spots of dense heterochromatin along the nuclear envelope. The uniform regular internal elastic lamina with smooth inner border was demonstrated in the subendothelial layer. Little collagen fibers were observed below the internal elastic lamina in the tunica media (Fig. 7).

In Ovariectomized (OVx) group, the endothelial cell was swollen and showed highly irregular nucleus with thin dense heterochromatin along the nuclear envelope. The internal elastic lamina was corrugated (curved), irregular in thickness where the curved part was thin and exhibited some irregularity in its inner border below the endothelial layer (Fig. 8). Large vacuoles and small vesicles containing a flocculent material were demonstrated in the apical cytoplasm. Swollen mitochondria were seen in the basal cytoplasm and in the next cell (Fig. 9). Abundant collagen fibers were seen accumulated above the internal elastic lamina in the subendothelial layer and below it in the tunica media and were arranged in longitudinal and transverse forms (Fig. 10). Apoptosis (necrosis) was also observed in the ovariectomized group. The necrotic cell was seen small in size, with little condensed cytoplasm. Its nucleus was irregular, shrunked and filling the whole cell with condensed peripheral chromatin. Cellular exudate was seen extravasated into the lumen and some RBCs appeared sticky to it (Fig. 11).

However, in the treated ovariectomized (OVx+E₂) group there were no any obvious ultrastructural changes. The Endothelial cell was seen similar to the normal control. The Endothelial cells contained large nucleus with thick dense spots of peripheral heterochromatin. No corrugation was
observed in the internal elastic lamina but it appeared uniformly normal with smooth inner border. Little collagen fibers were observed in the tunica media below the internal elastic lamina (Fig. 12).

**Fig. 1:** Photomicrograph of the wall of the thoracic aorta of normal rats showing normal tunica intima (TI) on the top. Normal tunica media (TM) possesses a large complement of smooth muscle cells, the nuclei of which stand out. Normal tunica adventitia (TA) is composed of longitudinal elastic fibers (arrow). Hx.&E.; X100

**Fig. 2:** Photomicrograph of the wall of the thoracic aorta of Ovariectomized (OVx) rats showing irregular intimal layer (TI) with furrows (arrows) on the top. The tunica media (TM) is nearly normal in its thickness. The tunica adventitia (TA) contains many large fat cells (F). Notice the lumen (L). Hx.&E.; X100

**Fig. 3:** Photomicrograph of the wall of the thoracic aorta of treated ovariectomized (OVx+E₂) rats showing normal intimal layer (TI) on the top. The tunica media (TM) appears normal in its thickness. Normal tunica adventitia (TA) is composed of longitudinal elastic fibers (arrow). Notice the lumen (L). Hx.&E.; X100

**Fig. 4:** Photomicrograph of the wall of the thoracic aorta of normal (control) rats showing uniform internal elastic lamina (IEL) (stained pink). The collagen fibers (C) (stained blue) are demonstrated in the tunica media (TM) by this preparation. Mallory’s trichrome; X100

**Fig. 5:** Light micrograph of the wall of the thoracic aorta of (OVx) rats showing the internal elastic lamina (IEL) (stained pink), and corrugated in zigzag form in the subendothelial layer. The collagen fibers (C) (stained blue) are seen distributed among the internal elastic lamina. Note the intimal Layer appears irregular, disrupted and shows furrows (arrows) on the top. Notice also the lumen (L). Mallory's trichrome; X100

**Fig. 6:** Photomicrograph of the wall of the thoracic aorta of the treated ovariectomized (OVx+E₂) rats showing the internal elastic lamina (IEL) uniformly normal and not corrugated. The collagen fibers (C) are seen normally distributed between the internal elastic laminas in the tunica media (TM). Mallory's trichrome; X100
Fig. 7: An electron micrograph of the wall of the thoracic aorta of normal (control) rat showing normal elongated endothelial cell (EC). The endothelial cell shows oval mitochondria (M) and large nucleus (N) with thick spots of dense heterochromatin along the nuclear envelope. Uniform regular internal elastic lamina (IEL) with smooth inner border is seen in the subendothelial layer. Little collagen (C) fibers are seen in the tunica media below internal elastic lamina. Notice the lumen (L). x 20,000

Fig. 8: An electronmicrograph of the wall of the thoracic aorta of (OVx) rats showing swollen endothelial cell (EC) with highly irregular nucleus (N) with thin dense heterochromatin along the nuclear envelope. The internal elastic lamina (IEL) is corrugated, irregular in its thickness with irregular inner border (arrows). x 10,000

Fig. 9: An electronmicrograph of the wall of the thoracic aorta of (OVx) rats showing swollen endothelial cell (EC) with highly irregular nucleus (N) with thin dense heterochromatin along the nuclear envelope. Large vacuoles (V) and small vesicles (Vs) containing a flocculent material are demonstrated in the apical cytoplasm. Note swollen mitochondria (M) in the basal cytoplasm and in the next cell. x 20,000

Fig. 10: An electron micrograph of the wall of the thoracic aorta of (OVx) rats showing abundant collagen fibers (C1) above the internal elastic lamina (IEL), in the subendothelial layer and below it (C2) in tunica media in longitudinal and transverse forms. x 20,000

Fig. 11: An electronmicrograph of the wall of the thoracic aorta of (OVx) rats showing necrotic cell (NC). The necrotic cell appears small in size with little condensed cytoplasm and shows irregular shrunk nucleus with condensed peripheral chromatin. Cellular exudate (Ex) is seen extravasated into the lumen (L) and some RBCs appear sticky to it. x 12,000

Fig. 12: An electronmicrograph of the wall of the thoracic aorta of the treated ovariectomized (OVx+E₂) rats showing elongated endothelial cells (EC) and large nucleus (N) with thick dense spots of peripheral heterochromatin. The internal elastic lamina (IEL) appears normal, uniform and shows no corrugation. Little collagen (C) fibers are observed in the tunica media below internal elastic lamina. x 20,000
DISCUSSION

This study was conducted on the female rat model to observe the ultra-structural changes in the wall of thoracic aorta after ovariectomy with or without estrogen replacement. Ovariectomy represents an estrogen-deficient state.

Previous investigations have indicated that postmenopausal women are more than twice as likely to be hypertensive as pre-menopausal women (Tremollieres et al., 1999).

In the present study, the body weight of the OVx rats increased from 195±3.4 g at the beginning of the study to 265±8.5 g at 13th post-operative week. Also, the tunica adventitia of the OVx group contained large fat cells. However, in the treated ovariectomized (OVx+E₂) group, the increase in weight was regular and similar to the control group. Also, there was no fat cells deposition in the tunica adventitia. The increase of fat cells in ovariectomized rats would explain the significant increase in body weight and which appeared to be ameliorated by estrogen therapy. These results support the hypothesis that the change in the estrogen status might be related to the development of obesity (Karjalainen et al., 2004).

An increasing level of obesity could partly contribute to the risk of hypertension and ischemic heart disease (De Lusignan et al., 2006). On the other hand, although blood pressure increases in most postmenopausal women, obese postmenopausal women have a greater predisposition to hypertension than thin postmenopausal women (Reckelhoff & Fortepiani, 2004).

Zureik et al. (2000) and Rodriguez-Macias et al. (2006) postulated that increase in the tunica intima / tunica media (TI/TM) ratio is an early indicator of atherosclerosis. Theoretically, this ratio can vary with changes of tunica intima or media width or both. In our study, the endothelial cells in the normal rat aorta were elongated in shape, while in the ovariectomized group (OVx), the endothelial cells were swollen. Also, the endothelial cells in OVx group contained abundant collagen fibers accumulated above the internal elastic lamina in the subendothelial layer. Furthermore, the internal elastic lamina of this group was corrugated with irregular inner border. Similar changes were previously reported in literatures (Flaherty et al., 1972; Xu et al., 2008). All these changes could explain the increase in the width of endothelial and subendothelial layers and in turn the increase in intimal thickness (TI layer). The tunica media (TM layer) did not exhibit any significant difference as compared with the normal group. This might contribute to increased TI/TM ratio which is considered an early stage of atherosclerosis (Zureik et al. 2000; Rodriguez-Macias et al., 2006). In contradistinction to our findings, Adam et al. (2009-b) found that the TI layer was unaffected, but the TM layer became thinner in the ovariectomized groups.

In the treated ovariectomized (OVx+E₂) group of this study, there was a uniform endothelial lining and the endothelial cell shape resembled that of the normal control. No corrugation was observed in the internal elastic lamina. These findings suggest that estrogen treatment (oestradiol 17β) protected the rat aorta from any change caused by ovariectomy.

Beside the previously mentioned features of collagen and elastic fibers of ovariectomized group, collagen fibers were found accumulated in the tunica media (indicated more by electron microscope). Also, corrugated internal elastic lamina was in zigzag form denoting vascular constriction (indicated more by Mallory trichrome stain). Little collagen fibers were observed only in the tunica media but
the corrugation was absent in the treated ovariectomized (OVx+E2) group. This may cast a shadow on the possible effect of estrogen in this issue. It has been found that estrogen has a vasodilator effect on the cardiovascular system (Sharpe, 1998) and according to our observations, we hypothesize that this effect may be via prevention of collagen accumulation. In a previous study Ito et al. (2000) mentioned that cerebral vessels could be constricted by the unopposed vasoconstriction if the vasodilator effect was damped. Further studies are required to refine the relation between collagen accumulation and estrogen deficiency.

In the ovariectomized (OVx) rats of our study, the structure of the endothelium was damaged, i.e. the intimal layer appeared irregular and disrupted with furrows. Apoptosis (necrosis) was also observed. The necrotic cell was seen small in size, having an irregular shrunken nucleus and little condensed cytoplasm with extravasation of cellular exudates into the lumen. Alterations in the structure of the swollen endothelial cells were also demonstrated in the ovariectomized group of this study (mitochondrial swelling and cytoplasmic vacuolization). These findings are compatible with the previous literatures (Xu et al., 2008; Adam et al., 2009-a&b). Vacuolization and condensation of cytoplasm were mentioned before as indicators of early stage of apoptosis (Shi et al., 2007). According to Moreno and Mitjavila (2003), an excess of oxidized low-density lipoprotein in macrophages during the progression of atherosclerosis can induce necrosis or apoptosis. Based on these findings, necrosis or apoptosis observed in this study may be attributed to the development of advanced atherosclerotic lesions.

Research reports depict that estrogen therapy is essential in postmenopausal women to protect themselves against atherosclerosis and cardiovascular diseases (Naessen & Rodriguez-Macias, 2006). On the other hand, previous studies confirmed that estrogen is of no benefit in animals that have artery damage caused either by balloon injury or an atherosclerotic diet prior to the initiation of hormone therapy (Wagner & Clarkson, 2005). Therefore, once vascular lesions occur, they can create a diffusion barrier. Thus, estrogen administration would not be able to reverse the disease process due to the loss of estrogen's interaction with its receptors (Xu et al., 2008).

The present study denoted that an estrogen deficiency (ovariectomy) may induce irreversible damage to the structure of the endothelium (disruption of the intimal layer and even irreversible necrosis in addition to alterations in the swollen endothelial cell structure). However, in the treated ovariectomized (OVx+E2) group of this study there were no obvious ultra-structural changes. We suggest that estrogen treatment can inhibit these endothelial cell changes and can offer some degree of protective effect on the endothelium from damage and in turn from the development of atherosclerosis.

The study cast shadow on the effect of estrogen deficiency over vascular wall which could be related to atherosclerosis. The nature and details of this effect need further studies.

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تأثير إستئصال المبيض والعلاج التعويضى بالإستروجين على الأورطى الصدري
دراسة بالمجهر الضوئى و الإلكترونى
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ملخص البحث
غالبية الأمراض القلبية والأوعية الدموية تحدث في فترة سن اليأس. ان نقص الإستروجين المصاحب لهذه الفترة يزيد من إنتشار بعض الأمراض مثل ارتفاع ضغط الدم وتصلب الشرايين وأمراض السكر.

هدف البحث: هو دراسة تأثير إستئصال المبيض والعلاج التعويضى بالإستروجين على جدار الأورطى الصدري في أنثى الجرذان البالغة بإستخدام المجهر الضوئى والالكترونى.

الفحصات:
أ- الفحص بالمجهر الضوئى باستخدام صبغة الهيماتوكسالين والأيوسين وصبغات أخرى مثل صبغة مالوري الثلاثية.
ب- فحص التركيب فائق الدقة باستخدام المجهر الإلكترونى.

المجموعة الأولى: استخدمت كمجموعة حساسة وقدم لها طعام متوازن.
المجموعة الثانية: خضعت لعملية إستئصال المبيض عند بداية البحث واعطيت الإستروجين (إستراديول) بجرعة 15 ميكرو جرام لكل كل بروتوم في اليوم تحت الجلد لمدة 13 إسبوع.
المجموعة الثالثة: خضعت لمجموعة المبيض عند بداية البحث واعطيت الإستروجين ثم اعطى لها علاج التعويضي (إستراديول) بجرعة 15 ميكرو جرام لكل كل بروتوم في اليوم تحت الجلد لمدة 13 إسبوع.

نتائج البحث:
1. اظهرت النتائج زياية واضحة في الوزن في المجموعة الثانية التي تم إستئصال المبيض لها عن باقي المجموعات.
2. اظهرت النتائج أن المجموعة الثانية التي تم إستئصال المبيض لها حدث لها بعض التغيرات كالآتي:
   - تدمير غشاء الطبقة المبطنة الداخلية مع التنكرز (موت موضعي بالنسيج الحي) تلتتحم مع بعضها لتكون ضفيرة عصبية تغذي الحشفة نفسها. أما الفروع الوحشية فهي تغذي الجلد والأنسجة الليفية والجسم الكهفي والجسم الإسفنجي والأحيل.
   - إعوجاج الصفيحة المتعرجة في شكل زيجزاجي مع عدم انتظام حافتها المواجهة للطبقة المبطنة.
   - إنتفاخ الحلقة الداخلية لجدار الأورطى مع وجود تغيير في تركيبتها الداخلية (إنتفاخ الميتوكوندريا) ونمو حبيبات داخل النسيج السطحي، تراك الكولاجين الليفي حول الصفيحة المعرجة الداخلية.
3. وفي المقابل فأن المجموعة الثالثة التي تم إستئصال المبيض لها ثم اعطى علاج التعويضي وجد أن جدار الأورطى فيها يشبه تماما المجموعة الأولى التي استخدمت كمجموعة حساسة ولا يوجد أي تغييرات واضحة سواء كان ذلك بالمجهر الضوئى أو الإلكتروني.