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Chronic oestrogen deficiency induced by ovariectomy may cause lung fibrosis through activation of the renin-angiotensin system in rats

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ABSTRACT

Context: Oestrogen deficiency is linked with pulmonary fibrosis. Additionally, it may lead to over-activation of the renin-angiotensin system (RAS), which worsens lung fibrosis.

Objective: The present study aims to investigate the role of RAS on lung fibrosis associated with oestrogen deficiency in ovariectomised rats.

Materials and methods: Serum 17\textsubscript{B}-oestradiol (E2), arterial blood gases, plasma angiotensin II levels, lung tissue hydroxyproline content, and transforming growth factor beta 1 (TGF-\textbeta1) concentration, the mRNA expression of angiotensin type 1 receptor (AT1R), and angiotensin-converting enzyme (ACE1) were evaluated. Moreover, lung tissues were examined by histopathology and immunohistochemistry.

Results: Hydroxyproline content, TGF-\textbeta1 concentration, plasma angiotensin II, the relative mRNA expression of ACE1, and AT1R is found to increase in ovariectomised rats. The mentioned changes can be largely rescued by administration of RAS blockers.

Conclusion: Oestrogen deficiency activates RAS, which consequently increases the expression of pro-fibrotic factors and stimulates the fibrotic cascade causing lung fibrosis.

Introduction

Menopause is a physiological event in a woman’s life. It is associated with an obvious decrease in the level of oestradiol which has vital outcomes on female biology. All receptors of sex steroid hormone are expressed in lung tissue (Triebner et al. 2017). Although menopause has been associated with respiratory disorders, lung function decline remains a serious matter to be studied in menopause (Memoalia et al. 2018).

Oestrogen, as a steroid hormone in respiratory health, is becoming largely recognised. Lung diseases, such as lung fibrosis, chronic obstructive pulmonary disease, and asthma, can be influenced by oestrogen deficiency (Macsali et al. 2013). Simultaneously, pulmonary function in menopausal women was shown to be better by oestrogen replacement therapy (Dogru et al. 2010).

The pathophysiology of lung fibrosis may result from a loss of the equilibrium between the production and absorption of the extracellular matrix (ECM). The increase in fibrotic repair in lung injury causes thickening of the alveolar wall and collapse of the alveoli. Additionally, irreversible pulmonary epithelial cell injury and accumulation of fibroblasts and myofibroblasts with collagen deposition were shown by the pathological examination (Zosky et al. 2011). However, the molecular mechanisms which contribute to the pathogenesis of lung fibrosis by oestrogen deficiency have not been elucidated.

The overactivity of the renin-angiotensin system (RAS) is an important pathogenic factor that has been implicated in the pathogenesis of lung fibrosis. The classical RAS begins with the cleavage of angiotensinogen (AGT) by renin (REN) to form Ang I, which is then hydrolysed by the action of the angiotensin converting enzyme (ACE) and the product Ang II is the major biologically active peptide of RAS (Cruz-Laguna et al. 2013). Ang II enhances the expression of transforming growth factor-beta (TGF-\textbeta) and connective tissue growth factor, two powerful pro-fibrotic factors that force lung fibroblast/myofibroblast proliferation and ECM protein expression (Uhal et al. 2012).

Previous studies investigated that oestrogen deficiency as in menopause may contribute to the overactivity of the RAS by eliciting upregulated tissue expression of ACE and AT1R in a cardiovascular system, brain, adrenal cortex, and kidney (Owonikoko et al. 2004, O’Hagan et al. 2012, Hilliard et al. 2013).

According to these findings, we hypothesise that oestrogen deficiency induced by ovariectomy of adult female rats may cause lung fibrosis and so disturb lung function by activating RAS in the lung. So, in this study, we aimed at exploring: (1) The effect of oestrogen deficiency induced by ovariectomy on lung function by assessing arterial oxygen and carbon dioxide tensions (PO2 and PCO2), arterial pH, and arterial bicarbonate (HCO3); (2) The possible pulmonary pro-fibrotic effect of oestrogen deficiency; (3) The role of RAS in this process.
Materials and methods

Animals and experimental design

Forty adult female Wistar albino rats, 12-weeks-old, weighting 180–220 g each, were purchased from Animal House, Faculty of Veterinary Medicine, Benha University, Egypt. They were housed in metallic cages and were maintained on prevailing atmospheric conditions and room temperature. They were fed with control pellet diets (Nile Company, Egypt) and drinking water ad libitum for a week, as an acclimatisation period. All the animal experiments were conducted according to the guidelines established by the Research Advisory Ethical Committee of Faculty of Medicine, Benha University, Egypt.

Experimental groups

The experimental animals were randomly allocated to five groups:

A control group (n = 8), sham-operated group (n = 8), and 3 groups consisted of ovariectomised rats, one served as untreated ovariectomised group (OVX group) (n = 8), the other received losartan – AT1 receptor blocker – (Cozar, Merck & Co., Whitehouse Station, NJ) which was dissolved in distilled water and given orally at a dose of 10 mg/kg/day for two weeks before sacrifice (OVX + losartan) (n = 8) (Liu et al. 2016). And the last group received aliskiren-renin blocker – (Raziles, Novartis, Switzerland) which was dissolved in phosphate-buffered saline (PBS) and administrated through a daily intraperitoneal injection at the dose of 20 mg/kg/day for two weeks before sacrifice (OVX + aliskiren) (n = 8) (Wang et al. 2015). The control, the sham group, and the OVX groups received only 1 ml of PBS by intraperitoneal injection for two weeks before sacrifice.

At the end of the experiment, all rats were euthanised using thiopental sodium (5 mg/kg). Then midline abdominal incision was done. Next, blood samples were drained from the abdominal aorta into a heparinised syringe and immediately used for analysis of arterial pH, arterial PO2, PCO2, and arterial HCO-3 concentrations were measured with blood gas analyser (Cargnoni et al. 2014).

For assessment of lung function for each rat, arterial pH, arterial PO2, PCO2, and arterial HCO-3 concentrations were measured with blood gas analyser (Cargnoni et al. 2014).

Functional assessment of the lung

For assessment of lung function for each rat, arterial pH, arterial PO2, PCO2, and arterial HCO-3 concentrations were measured with blood gas analyser (Cargnoni et al. 2014).

Assessment of hydroxyproline levels and TGF-β1 concentration

The lung tissues were analysed using the acid hydrolysis method with a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) for assessment of hydroxyproline levels according to the manufacturer’s instructions. For assessment of TGF-β1 concentration, the lung tissues were homogenised on ice in 2 ml of cold PBS. After centrifugation at 4 °C (10,000 rpm, 15 min), supernatants were stored at −70 °C for determination of TGF-β1. Then, the concentration of TGF-β1 was quantified using ELISA kits (DRG international company, USA) according to the manufacturer.

Plasma Ang II levels

Plasma Ang II levels were determined by an Assay Max Angiotensin II ELISA kit (AssayPro, St Charles, MO) according to the instructions.

Real-time PCR

For quantitative expression of ACE1and AT1R mRNA, the following procedure was performed. Lung tissues (50–100 mg) were disrupted in 1 ml of Trizol to collect total RNA according to the manufacturer’s instructions (Invitrogen, Grand Island, NY). Reverse transcription was performed using 1 μg total RNA and a cDNA kit (high-capacity cDNA archive kit). The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm. The amount of ACE1 and AT1R mRNA was determined with ABI Prism 7000HT quantitative real-time PCR (Applied Biosystems, Foster City, CA). The primer sequences for the targeted genes are listed in Table 1. Changes in the

intra-muscularly injected with 0.1 ml of 2.5% enrofloxacin antibiotic and ibuprofen analgesic 50 mg, 0.1 ml/kg, for 4 days and maintained under good conditions to recover (Hussien and Emam 2016). The rats were allowed 12-weeks postoperative to confirm surgically induced menopause and to assure the long-term absence of oestrogens (Fedotova 2018). Then, blood samples were collected from retro-orbital vein of all operated rats for measuring oestrogen level in the serum to confirm surgically induced menopause and oestrogen deficiency.
expression of each target gene were normalised relative to the mean critical threshold values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene by the 2^−ΔΔCt method (Wei et al. 2000).

Histopathological examination

For histological examination, lung samples were fixed in 10% formalin buffer. Sections 3–4 μm thick were cut, stained with Haematoxylin and Eosin (H&E) for microscopic observation and Masson’s Trichrome for assessing fibrotic lesions. For the quantitative histological analysis, Ashcroft score (a numerical fibrotic scale) was used to score pulmonary fibrosis, 5 sections from each group by 100 × magnification, each field was given a score ranging from 0 to 8 in which grade 0: normal lung, grade 1: minimal fibrous thickening of alveolar or bronchiolar vessels, grade 2: mild fibrous thickening of alveolar septa with knot-like formation, group 3: moderate thickening of the walls without obvious damage to lung architecture, grade 4: single fibrotic masses, group 5: confluent fibrotic masses with damage of lung architecture, group 6: large contiguous fibrous masses, group 7: severe distortion of lung architecture with honeycomb pattern, and group 8: total fibrous obliteration of lung architecture (Langhe et al. 2012).

TGF-β1 immunohistochemistry

Four-micrometre sections of paraffin-embedded samples of all lung specimens were prepared. Immunohistochemical staining was performed using primary antibodies against TGF-β1 (ready to use, TGF β1 polyclonal antibody; Catalogue Number PA1-9574; Thermo Fisher Scientific Inc., Fremont, CA). Avidin-biotin immune-peroxidase complex technique was used by applying the super sensitive detection kit (Biogenex, CA). The prepared tissue sections were fixed on poly-l-lysine-coated slides overnight at 37 °C. They were deparaffinised and rehydrated through graded alcohol series. Then, the sections were heated in a microwave oven in 10 mM citrate buffer (pH 6.0) for 10 min. After the blocking of endogenous peroxidase and incubation in Protein Block Serum-Free Solution (DakoCytomation) for 20 min, the sections were incubated with TGF-β1 antibody according to the manufacturer’s instructions. Biotinylated anti-mouse immunoglobulin and streptavidin conjugated to horseradish peroxidase were then added. Finally, 3, 3′-diaminobenzidine as the substrate or chromogen was used to form an insoluble brown product. Finally, the sections were counterstained with haematoxylin and mounted (Abuelezz et al. 2016).

Results

Results of serum oestrogen and functional assessment of the lung

All the ovariectomised rats either treated or untreated showed a significant decrease (p < 0.05) in serum oestrogen when compared with the control and sham groups. The oestrogen deficiency induced by ovariectomy is associated with a significant decrease (p < 0.05) in arterial PO2 compared to the sham and the control groups. While administration of both losartan and aliskiren caused a significant increase (p < 0.05) in arterial PO2 compared to the OVX group. On the other hand, our results showed that there was no statistically significant difference between groups regarding PCO2, HCO3 − and pH findings (Table 2).

Effect of oestrogen deficiency on hydroxyproline content and TGF-β1 concentration

For assessment of lung fibrosis, hydroxyproline content and TGF-β1 concentration were assessed. There was a significant increase (p < 0.05) in hydroxyproline content and TGF-β1 concentration in lung tissues of the OVX group when compared with the control and the sham groups. While administration of both losartan and aliskiren (OVX + losartan) group and aliskiren in (OVX + aliskiren) showed a significant decrease (p < 0.05) in their contents when compared with the OVX group (Table 3).

Effect of oestrogen deficiency on plasma Ang II levels

Plasma Ang II level was significantly increased (p < 0.05) in ovariectomised untreated rats compared with the control and the sham-operated rats after 12 weeks of surgery. RAS blockade either by losartan in the (OVX + losartan) group or by aliskiren in the (OVX + aliskiren) significantly decreased

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### Table 1. Primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′-3′)</th>
<th>Reverse (3′-5′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE1</td>
<td>AGCATCACCAAGGAGAATA</td>
<td>ACTGGAAACTGGATGATGGA</td>
</tr>
<tr>
<td>AT1R</td>
<td>GAAGAAACAGCCAGAAATG</td>
<td>AATACGCTATGCAGATGGTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTTGGAAGCCAGCATTCTG</td>
<td>AGCGATTACCTGGTACACC</td>
</tr>
</tbody>
</table>

ACE1: angiotensin-converting enzyme 1; AT1R: angiotensin type 1 receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

### Table 2. Changes in serum oestrogen and arterial PO2, PCO2, HCO3 −, and pH in the experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Sham group</th>
<th>OVX group</th>
<th>OVX + losartan group</th>
<th>OVX + aliskiren group</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 (ng/mL)</td>
<td>78.6 ± 2.8</td>
<td>79.6 ± 3.2</td>
<td>42.9 ± 3.4</td>
<td>43.6 ± 3.2</td>
<td>43.3 ± 2.9</td>
</tr>
<tr>
<td>PO2 (mmHg)</td>
<td>89.4 ± 1.4</td>
<td>90.88 ± 3.6</td>
<td>73 ± 2.9</td>
<td>80.88 ± 3.7</td>
<td>79.6 ± 6.2</td>
</tr>
<tr>
<td>PCO2 (mmHg)</td>
<td>39 ± 1.9</td>
<td>37.6 ± 3.1</td>
<td>37.8 ± 2.5</td>
<td>39.3 ± 2.9</td>
<td>41.4 ±2.1</td>
</tr>
<tr>
<td>HCO3 − (mmol/L)</td>
<td>23.1 ± 1.5</td>
<td>23.1 ± 1.3</td>
<td>23 ± 2.4</td>
<td>23.8 ± 2.3</td>
<td>22.9 ± 1.1</td>
</tr>
<tr>
<td>pH</td>
<td>7.33 ± 0.01</td>
<td>7.32 ± 0.02</td>
<td>7.33 ± 0.01</td>
<td>7.33 ± 0.01</td>
<td>7.33 ± 0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (n = 8 per group). p < .05 is significant tested by using one-way ANOVA and post hoc multiple comparisons (LSD).

*P < .05 vs. control group;

*P < .05 vs. sham group;

*P < .05 vs. OVX group.
Effect of oestrogen deficiency on pulmonary RAS expression

Real-time PCR demonstrated a significant elevation \((p < .05)\) in angiotensin-converting-enzyme 1 (ACE1) and angiotensin type 1 receptor (AT1R) mRNA levels in the lung tissues of the ovariectomised rats in the OVX group (Table 3). Furthermore, administration of both losartan in the (OVX + losartan) group and aliskiren in the (OVX + aliskiren) group showed a significant decrease in their expression when compared with the OVX group \((p < .05)\) Figure 2 (A, B).

Histopathology results

Haematoxylin and eosin (H&E) stained sections

Histological examination of lung sections from the control group and the sham-operated groups were comparable and showed normal bronchiole lined by respiratory-ciliated epithelium, normal blood vessels, the alveoli appeared patent with thin interalveolar septa and normal alveolar sacs (Figure 3(A)). With higher magnification, the alveoli wall lined by pneumocytes type I appeared flat in shape, pneumocytes type II appeared cuboidal in shape and alveolar macrophage (dust cell) (Figure 3(B)).

Histological examination of lung sections from the ovariectomised rats (OVX group) showed marked inflammatory cell infiltration around the bronchiole, patches of inflammatory cell infiltration in the alveolar wall with marked thickened alveolar septa, narrowing of alveolar sacs (Figure 3(C)). With higher magnification, there was marked inflammatory cell infiltration and extravagated red blood cells in the alveolar wall and collapsed alveoli with marked thickened alveolar septa and rupture of some interalveolar septa (Figure 3(D)).

Histological examination of lung sections from the ovariectomised rats treated with losartan (OVX + losartan group) showed moderate improvement of lung tissue, numerous patent alveoli and normal alveolar sac, mild thickened alveolar septa, moderate inflammatory cell infiltration beside the bronchiole and thick blood vessel (Figure 3(E)).

Histological examination of lung sections from the ovariectomised rats treated with aliskiren (OVX + aliskiren group) showed moderate improvement of lung tissue, numerous patent alveoli and normal alveolar sac, mild thickened alveolar septa, moderate inflammatory cell infiltration around the bronchiole and marked thickened blood vessel (Figure 3(F)).

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**Table 3.** Changes in hydroxyproline content and TGF-\(\beta\)1 concentration in the experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Sham group</th>
<th>OVX group</th>
<th>OVX + losartan group</th>
<th>OVX + aliskiren group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline Mg/g protein</td>
<td>0.50 ± 0.13</td>
<td>0.53 ± 0.15</td>
<td>2.2 ± 0.21(^{ab})</td>
<td>1.17 ± 0.16(^{abc})</td>
<td>1.04 ± 0.11(^{abc})</td>
</tr>
<tr>
<td>TGF-(\beta)1(pg/ml)</td>
<td>137.5 ± 4.9</td>
<td>134.5 ± 7.2</td>
<td>217.3 ± 11.9(^{ab})</td>
<td>154.4 ± 4.2(^{abc})</td>
<td>153.5 ± 5.2(^{abc})</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation \((n = 8\) per group). \(p < .05\) is significant tested by using one-way ANOVA and post hoc multiple comparisons (LSD).

\(^{a}\)\(p < .05\) vs. control group;
\(^{b}\)\(p < .05\) vs. sham group;
\(^{c}\)\(p < .05\) vs. OVX group.
Masson’s trichrome-stained sections
Histological examination of lung sections stained by Masson’s trichrome revealed the degree of fibrosis and the density of the collagen deposition in the lung tissue. The control group and the sham-operated groups were similar and showed minimal collagen fibres around alveoli grade (0 & 1) in the Ashcroft score (Figure 4(A,B)). In the OVX group showed marked collagen fibres deposition around the bronchioles and blood vessel with confluent fibrotic masses in the alveolar wall, numerous collapsed alveoli with a honeycomb pattern (Figure 4(C)). These findings were included in grades (5, 6, & 7) in the Ashcroft score. Masson’s trichrome-stained sections from the OVX + losartan group showed moderate collagen fibres deposition around bronchiole, some sections showed single fibrotic masse in the alveolar wall (Figure 4(D)). These findings were included in grades (2, 3, & 4) in the Ashcroft score. In OVX + aliskiren group showed moderate collagen fibres deposition around bronchi- ole and blood vessel, some sections showed single fibrotic masse in the alveolar wall (Figure 4(E)). These findings were included in grades (2, 3, & 4) in the Ashcroft score.

Results of Ashcroft grading of fibrosis
Concerning histopathological changes, Ashcroft grading of fibrosis in OVX group had a significantly higher grade than the control and sham groups (p < .05). Furthermore, treatment of the ovariectomised rats with losartan in the group (OVX + losartan) and aliskiren in the group (OVX + aliskiren) was found to improve Ashcroft scoring as compared with the OVX group without complete restoration of lung morphology to normal (Figure 4(F)).

TGF-β1 immunohistochemistry
TGF-β immunostaining of lung sections from the control group and the sham-operated groups were comparable and showed negative expression of TGF-β antibody (Figure 5(A, B)). Lung sections from the OVX group showed high positive expression of TGF-β antibody (Figure 5(C)). Which represent the accumulation of intra-alveolar fibroblasts or myofibroblasts. Whereas lung sections from the (OVX + losartan) group showed areas with high positive expression of TGF-β antibody and other areas with negative expression of TGF-β antibody (Figure 5(D)) and lung sections from the (OVX + aliskiren) group showed areas with high positive expression of TGF-β antibody and other areas with negative expression of TGF-β antibody (Figure 5(E)).

Discussion
Menopause has been associated with increased respiratory disorders and may even impair lung function (Draijer et al. 2016, Triebner et al. 2017, Memoalia et al. 2018). However, the molecular drivers of menopause-induced respiratory dysfunction are largely unknown. Meanwhile, oestrogen depletion is thought to be the underlying pathological mechanism. Here, we report that the RAS is the primary pathogenic driver of lung fibrosis in oestrogen-deficient rat. Targeting the RAS – via using the AT1 blocker losartan and the renin blocker Aliskiren – protects against oestrogen deficiency-induced lung fibrosis.

In the current study, ovariectomised rats have been used as models for studying the effects of oestrogen deficiency on lung health. To assure the long-term absence of oestrogens, all the rats after surgery remained to the housing facilities for 12 weeks (Fedotova 2018). Our results showed significant
oestrogen deficiency in all ovariectomised rats when compared with the control and the sham groups. The current study supports the interpretation that oestrogen deficiency induced by ovariectomy is associated with an increase in fibrotic markers in lung tissue. Our results showed a significant increase in collagen fibre deposition in ovariectomised rats when compared to the rats in the control and the sham groups indicated by a significant increase in hydroxyproline content. In accordance with this result, a previous study (Memoalia et al. 2018) reported that the total lung concentration and subepithelial collagen deposition were significantly increased in ovariectomised rats. The increase in collagen fibres could be clarified on the basis of fibrosis, as an outcome of chronic inflammation induced by oestrogen deficiency, because oestrogen is considered as an anti-inflammatory factor in the lung (Abd Elfattah 2012).

The hypothesis of fibrosis was reported by a previous study (Abd Elfattah 2012), which demonstrated that fibroblastic foci are composed of proliferating and migrating myofibroblasts and fibroblasts accounting for ECM deposition altering the alveolus structure. Regarding the source of myofibroblasts and fibroblasts, previous studies (Bhatia et al. 2012, Daghigh et al. 2017) have reported that injured type I alveolar epithelial cells (AECs) release TGF-β, which is a main cytokine associated with the acceleration of lung injury, and it leads to lung fibrosis through the increase in collagen

Figure 3. (A,B,C,D,E,F) Histological changes of female rat lung stained with haematoxylin and eosin (H&E). Photomicrographs of lung section from adult female rats (A) In the control group showing: normal bronchiole (Br) lined by respiratory ciliated epithelium, normal blood vessel (bv), patent alveoli (Av) with thin alveolar septa (I) and normal alveolar sac (as) (H&E × 100). (B) In sham-operated group showing: alveoli (Av) lined by type I pneumocyte appeared flat in shape (PI), type II pneumocyte appeared cuboidal in shape (PII), alveolar macrophage (arrow head), thin alveolar septa (I) and alveolar sac (as) (H&E × 400). (C) In the OVX group showing: marked inflammatory cell infiltration (arrow) around the bronchiole (Br), patch of inflammatory cell infiltration (arrow head) in alveolar wall, marked thickened alveolar septa (I) with narrowing of the alveolar sac (as) (H&E × 100). (D) In the OVX group showing: marked inflammatory cell infiltration (arrow), extravasated RBCs (arrow head), collapsed alveoli (Av), marked thickened alveolar septa (I) rupture interalveolar septa (thick arrow), and narrow alveolar sac (as) (H&E × 400). (E) In the OVX + losartan group showing: numerous patent alveoli (Av), normal alveolar sac (as), mild thickened alveolar septa (I) moderate inflammatory cell infiltration (arrow) beside the bronchiole(Br) and thick blood vessel (bv) (H&E × 100). (F) In the OVX + aliskiren group showing: numerous patent alveoli (Av), normal alveolar sac (as), mild thickened alveolar septa (I), moderate inflammatory cell infiltration (arrow) around the bronchiole(Br), and marked thickened blood vessel (bv) (H&E × 100).
gene expression or synthesis by stimulation of fibroblast proliferation. Hence, the authors’ attention was focussed on measuring the concentration of TGF-β1 as well as the immuno-histochemical examination of TGF-β1 in the lung tissue, showing a significant increase in TGF-β1 concentration and a strong positive expression of TGF-β1 in the lung tissues of ovariectomised rats when compared with the control and the sham groups. This result is in accordance with previous studies (Ito et al. 2010, Daghigh et al. 2017) which demonstrated that the reduction in ovarian function with surgical menopause has been associated with an increase in the TGF-β1 level in lung tissues. This result can be explained by Ito et al. (2010) who reported that oestrogen inhibits TGF-β signalling by preventing the recruitment of Smad – a key transducer in TGF-β signalling – to the promoter region of TGF-β responsive genes. Hence, oestrogen deficiency increases TGF-β expression in the lung tissue. After that, TGF-β1 induces fibroblast proliferation and transforming fibroblasts into myofibroblasts (Wang et al. 2015). Activated fibroblasts deposit collagen I, which causes the thickening of the alveolar walls (Yaguchi et al. 2013), as observed in our study.

Supporting the former results, the histopathological examination of lung sections from the ovariectomised rats showed marked inflammatory cell infiltration around the bronchiole, patches of inflammatory cell infiltration in the alveolar wall with marked thickening of alveolar septa and narrowing of alveolar sacs. Also, higher magnification showed marked

Figure 4. (A,B,C,D,E,F) Masson-trichrome staining for fibrosis. Photomicrographs of lung section from adult female rats (A). In control group showing minimal collagen fibres (arrow) around alveoli (masson trichrome × 400). (B) In sham-operated group showing minimal collagen fibres (arrow) around alveoli (masson trichrome × 400). (C) In O VX group showing: marked collagen fibres deposition around the bronchioles and blood vessel (arrow), fibrotic masses in the alveolar wall (arrow head), numerous collapsed alveoli with a honeycomb pattern (star) (masson trichrome × 100). (D) In the O VX + losartan group showing: moderate collagen fibres deposition around bronchiole (arrow), fibrotic masse in the alveolar wall (arrow head) (masson trichrome × 100). (E) In the O VX + aliskiren group showing: moderate collagen fibres deposition around bronchioles and blood vessel (arrow), fibrotic masse in the alveolar wall (arrow head) (masson trichrome × 100). (F) Ashcroft grading of fibrosis. Data are expressed as mean ± standard deviation (n = 8 per group). p < .05 is significant tested by using one-way ANOVA and post hoc multiple comparisons (LSD). a p < .05 vs. control group; b p < .05 vs. sham group; c p < .05 vs. O VX group.
inflammatory cell infiltration, extravagated red blood cells in the alveolar wall, collapsed alveoli with marked thickening of alveolar septa and rupture of some interalveolar septa. Then, on examination of the Masson's trichrome-stained sections by Ashcroft grading of fibrosis, ovariectomised rats had a significantly higher grade than the control and the sham groups. In accordance with these results, a previous study reported that lung tissues of ovariectomised rats are characterised by infiltration with mononuclear cells, tissue destruction, and attempts at healing by connective tissue replacement of damaged tissue, in particular, fibrosis (Alipour et al. 2018). Also, Dogru et al. (2010) studied the effect of ovariectomy and diabetes mellitus on the lungs, the later found that ovariectomy-induced bronchial hyperplasia, oedema, pulmonary capillary dilatation, mononuclear inflammatory cells infiltration, haemorrhagic area, and an increase in apoptotic cell density were observed more in the ovariectomy group compared to the diabetic group.

To assess the effect of lung fibrosis induced by oestrogen deficiency on respiratory function, we measured blood gases. Accordingly, we found that oestrogen deficiency resulted in a significant decrease in arterial PO2 with a non-significant effect on arterial PCO2, HCO3-, and pH when compared with the control and sham groups. These results can be explained by Abd Elfattah (2012) who suggested that fibrosis might decrease air spaces, which results in hypoxia and contribute significantly to the relative hypoventilation (Preston et al. 2009). Further, hypoestrogenism plays a critical role in osteoporosis, which results in the reduced height of the thoracic vertebrae which may mechanically reduce the expansion of

Figure 5. (A,B,C,D,E) Immunostaining reaction for TGF-β. Photomicrographs of lung section from adult female rats. (A) In control group showing negative expression of TGF-β antibody. (B) In sham-operated group showing negative expression of TGF-β antibody. (C) In OVX group showing high positive expression of TGF-β antibody (arrow). (D) In OVX + losartan group showing areas with high positive expression of TGF-β antibody (arrow) and other areas with negative expression of TGF-β antibody (arrow head). (E) In the OVX + ailskiren group showing areas with high positive expression of TGF-β antibody (arrow) and other areas with negative expression of TGF-β antibody (arrow head) (TGF-β immunostaining × 200).
the thoracic cage during inspiration and place the diaphragm in a suboptimal position (Abd Elfattah 2012, Memoalia et al. 2018).

To examine the mechanism by which oestrogen deficiency exerts its fibrotic effects, we investigated the role of RAS in this process. Because the activation of RAS has been reported to induce lung fibrosis both in transgenic animals and in disease models (Kumar et al. 2009, Murphy et al. 2015, Wang et al. 2015) it was reasonable to hypothesise that the lung fibrosis observed in ovariectomised rats is associated with the increased expression of RAS components. In this study, we demonstrate for the first time that oestrogen deficiency induced by ovariectomy elicits upregulated lung tissue expression of ACE and AT1R when compared with the non-ovariectomised rats. Additionally, we reported a significant increase in plasma Ang II levels in oestrogen deficient rats when compared with the non-ovariectomised rats. This finding can be explained by Brasier (2000) who showed that Ang II up-regulates AGT mRNA through nuclear factor-kappa B activation (Brasier 2000). Also, renin synthesis in collecting ducts can be directly increased by Ang II, and this increment in renin synthesis may be the cause of AT1 receptor and ACE upregulation (Peti-Peterdi et al. 2002).

To clarify this, we treated the ovariectomised rats with RAS antagonists. We used losartan which is a blocker of AT1R that prohibits angiotensin II from binding to AT1R. Also, we used aliskiren that blocks the production of renin. Either losartan or aliskiren caused a significant decrease in plasma Ang II level with a significant decrease in ACE and AT1 receptor mRNA expressions in lung tissues when compared with the ovariectomised non-treated rats. These results were in agreement with the study of Cruz-Laguna et al. (2013) who showed that inhibition of Ang II synthesis by RAS blockade is associated with renal regulation of RAS mRNA expression.

Importantly, the renin antagonist aliskiren and the AT1R blocker losartan could significantly rescue the tissue damage and alleviate lung fibrosis through inhibition of the cytokine TGF-β1. Seeing that both losartan and aliskiren resulted in a significant decrease in hydroxyproline content and TGF-β1 concentration and resulted in a significant increase in arterial PO2 compared to the ovariectomised non-treated rats. Besides, the histological examination showed mild thickened alveolar septa with areas restoring normal alveolar architecture and alveolar sac and there were numerous patent alveoli with decrease in the inflammatory cell infiltration. Furthermore, Ashcroft grading of fibrosis had a significantly lower grade than the O VX group and Masson’s trichrome-stained slides showed mild collagen deposition around the alveolar walls. Additionally, TGF-β immunostaining showed a marked decrease in the areas with positive (TGF-β) immunoreactivity within the interalveolar septa. These results were in agreement with (Murphy et al. 2015) who studied the effect of losartan treatment on lung fibrosis induced by bleomycin and they found that losartan treatment significantly reduced the interstitial collagen and fibroblast deposition and they added that the antifibrotic effect of losartan appears to be mediated by its ability to stimulate the production of prostaglandin E2. Also, Abu leuzz et al. (2016) studied the effect of aliskiren treatment on lung fibrosis induced by bleomycin and they found that Aliskiren attenuated signs of pulmonary fibrosis and decreased the overexpressed TGF-β. Also, this protective effect of RAS blockers has also been reported in radiation-induced lung fibrosis (Lang et al. 2010, Jiang et al. 2012).

The relation between RAS and lung fibrosis previously investigated by many studies which revealed that Ang II, through activation of AT1 receptor, causes stimulation of lung fibroblasts/myofibroblasts, directly or indirectly as follows; directly, through direct stimulation of ECM protein production, including fibronectin and collagen I and III. And indirectly, through an increase in TGF-β expression from fibroblasts and myofibroblasts which, in turn, causes stimulation of ECM protein production (Rockey et al. 2015, Wang et al. 2015). The absence of data concerning the protein expression levels of ACE1, AT1R, and TGF-β1 in lung tissue – represents a limitation of the study; however, these limitations do not appear to undermine the results achieved in the study. Also, we recommend this in further studies.

In summary, oestrogen deficiency induced by ovariectomy in rats may induce over-activation of RAS, which subsequently stimulates the expression and the concentration of TGF-β1 and activates the fibrotic cascade. Structural damage and collagen deposition aggravate with prolonged oestrogen deficiency. Our study draws attention to menopause, which might be a cause of lung fibrosis and for the first time reported the role of RAS in this process.

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References


