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Impact of air pollution on the estrogen and progesterone receptors in the uterus and ovary of Wistar rat

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ABSTRACT

Air pollution has become a significant global health concern. Exposure to PM2.5, particles with a diameter of less than or equal to 2.5 μm, has been linked to certain diseases, including female reproductive disorders. Several molecular mechanisms in the female reproductive system may be affected by $PM_{2.5}$ exposure. The present study investigated the effect of $PM_{2.5}$ exposure on the expression of ESR-1 and PGR steroid hormone receptors in uterine and ovarian tissues of rats as an air pollution model. Twenty-four female Wistar rats were prepared for $PM_{2.5}$ exposure for three months. Exposure was conducted for five hours per day, and four days in week. Three groups were defined, including 'Group A' with exposure to $PM_{2.5}$ plus gaseous pollutants, 'Group B' with exposure to gaseous pollutants only, and 'Group C' as a control group with clean standard air. Uterus and ovary tissues were removed after the scarification of the rats, and the mRNA level was examined for ESR-1 and PGR using quantitative Real-Time PCR. Findings showed significant overexpression of ESR-1 and PGR genes in exposure groups (A and B) in comparison with the control group (C). Significant up-regulation of ESR-1 and PGR genes in the uterus of group A was detected compared to group B. Results showed that $PM_{2.5}$ exposure may cause upregulation of the female sex hormone receptors. It seems that $PM_{2.5}$ exposure could potentially lead to several diseases related to the female reproductive system.

Keywords: $PM_{2.5}$ exposure, ESR-1, PGR, Uterus, Ovary.
Article type: Research Article.

INTRODUCTION

Particulate matter (PM) is one of the major air pollutants. Due to its complex composition and wide distribution, there is growing concern about its harmful effects on human health. Indeed, the chemical composition and size distribution of PM determine its health effects (Cai *et al.* 2014). Particles with a diameter of less than or equal to 2.5 micrometers are referred to as $PM_{2.5}$ and have a small diameter and a large surface area. The $PM_{2.5}$ mostly contains metals, carbon, organic carbon, sulphate, nitrate, ammonium and organic compounds (such as polycyclic aromatic hydrocarbons) (Srimuruganandam & Nagendra 2012). The $PM_{2.5}$ is able to carry several toxic stuffs, penetrate to the upper respiratory system and reaching the end of this system. Through the air–blood barrier, $PM_{2.5}$ passes through blood and from blood through all tissues (Schwartz *et al.* 1996). In last decade, the "Harvard six Cities Study", showed that $PM_{2.5}$ has linear relationship with non-accidental death (Schwartz *et al.* 1996). Ample evidence suggests that $PM_{2.5}$ exposure deregulates several biological systems, including the immune, male and

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female reproductive, respiratory, cardiovascular, and central nervous systems (Halonen *et al.* 2009; Pearson *et al.* 2010; Guaita *et al.* 2011; Sram *et al.* 2017, Carré *et al.* 2017). Both rat model and human studies reported that air pollutants may induce sever decline in the sperm quality and motility (Khamutian *et al.* 2015). Although $PM_{2.5}$ and other air pollutants have adverse health effects, little is known about their effects on female reproduction, and it is reported that $PM_{2.5}$-induced apoptosis of granulosa cells and oocytes in the ovary can result in severe disturbances in embryonic development and female fertility (Liao *et al.* 2020). The uterus is an important secondary reproductive organ in female mammals known as a hormone-responsive organ. The main function of the uterus is to receive the fertilized egg from the fallopian tube through the utero-tubal junction. After fertilization, the embryo attaches to a wall of the uterus leading to the formation of a placenta and the development of the fetus (Mahalingaiah *et al.* 2014). By directing blood flow to the pelvis and ovaries and to the external genitals, the uterus is also involved in sexual response (Alataş & Yağci, 2004). The rat model studies suggest that uterus functions are also involved in cognitive and spatial memory functions (Koebele *et al.* 2019). The ovary is another important organ of the female reproductive system, which is responsible for producing eggs. Ovary, is responsible for secreting increasing hormones during puberty, leading to secondary sex characteristics. Beginning with puberty, the ovary changes its structure and function (Lang-Muritano *et al.* 2018). The ovary also plays an important role in pregnancy and fertility because of its ability to regulate hormones (Durlinger *et al.* 2002). During the release of eggs from the fallopian tube, several feedback mechanisms stimulate the endocrine system, which in turn causes changes in hormone levels (Yaron & Levavi-Sivan 2011). Hypothalamus and ovary are also involved in feedback mechanisms of hormone releases during adulthood (Baskind & Balen 2016). Estrogen receptors (ERs) are recognized as proteins which activated by the steroid sex hormone, estrogen (17β-estradiol) (Wilson & Westberry 2009). Two classes of ERs have been identified including nuclear estrogen receptors (ERα and ERβ), which belong to the nuclear receptor family of intracellular receptors, in addition to membrane estrogen receptors (mERs), which are recognized as G protein-coupled receptors (Wilson *et al.* 2011). Once activated by estrogen, the ER is capable of translocating to the nucleus and binding to DNA to regulate the activity of various genes, particularly DNA-binding transcription factors, which in turn can trigger a number of changes in various molecular pathways (Helzer *et al.* 2018). Estrogen plays role in regulation of the levels of progesterone receptor (PR) in the uterus of the rat (Kraus & Katzenellenbogen 1993). The ESR1 and ESR2 genes encode two different forms of estrogen receptor, α and β, respectively. These genes are mostly co-expressed uterus and ovary and dimer forms of estrogen receptors could be homodimers (αα or ββ) or heterodimers (αβ) (Hutson *et al.* 2019). The progesterone receptor (PR) is an intracellular nuclear protein receptor which needs steroid sex hormone, progesterone for activation (Vasquez *et al.* 2018). Before progesterone binding, carboxyl terminal of PR inhibits transcription factors. Once progesterone binds to receptor, induces a structural reconfiguration which removes the inhibitory action (Grimm *et al.* 2016). Progesterone binding, leads to dimerization of the complex that enters the nucleus and binds to DNA to induce the expression of number of genes (Ogara *et al.* 2019). During sexual maturation and pregnancy, both estrogen and progesterone receptors are important (Dickinson *et al.* 2018). However, genetic and epigenetic effects and affected pathways of chronic $PM_{2.5}$ exposure especially in female reproductive system have remained largely unaddressed (Zhou *et al.* 2020). Ample evidence has indicated that epigenetic regulation of gene expression (including DNA methylation and histone modification) is a process that is highly sensitive to environmental insults, and may even be lifelong (Dang *et al.* 2018). In addition, it has been shown that environmental pollution during pregnancy has a major effect on epigenetic alternations (Li *et al.* 2003). Furthermore, it has been found that the decreased methylation level in the placenta of pregnant women was closely associated with $PM_{2.5}$ exposure (Janssen *et al.* 2013). The aim of the present study was to evaluate the effects of air pollution on the gene expression changes of the estrogen and progesterone receptors in the uterine and ovarian tissues of rats exposed to $PM_{2.5}$ for a period of three months.

MATERIALS AND METHODS

Rat model designing

The study had the approval of the central ethics committee of Islamic Azad University. All experimental procedures performed in this study were approved by the Institutional Animal Ethics Committee of Islamic Azad University, Science and Research Branch, Tehran, Iran (Ethics reference: IR.IAU.SRB.REC.1398.054). The protocols of Shahid Beheshti University of Medical Sciences "Guide for the Care and Use of Laboratory Animals" were followed in all procedures. The animal experimental room was located in the North Tehran, the

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capital of Iran (Ethics reference: IR.IAU.SRB.REC.1398.054). Twenty-four female Wistar rats in four-weeks of age (n¼18) weighing from 75 to 95 g were provided from Pasteur Institute (Pasteur Institute of Iran No. 69, Pasteur Ave, Tehran, Iran). Wistar rats were habituated in standard conditions. The condition of the rats included provision of water, food, ad libitum and balanced light time cycle (12 hours light/12 hours dark) for a week. In addition, environmental conditioning including temperature (20-25 ℃), relative humidity (40-60%), and indoor air quality were provided for one-week prior to the start of *in vivo* model. Finally, eight male rats were included in each group to minimize the number of rats. The rats were grouped as exposure group A (called "group A" that exposed by $PM_{2.5}$ plus gaseous pollutants), exposure group B (called "group B" that exposed only by gaseous pollutants) and control group (called "group C" that lived in room with clean standard air condition). Exposure was conducted for a period of three months. Exposure was performed for five hours daily (9:00 to 14:00), for four days weekly. The ambient air of the pilot animal room was continuously analyzed for $PM_{2.5}$ along with SO_2, O_3 and NO_2 during each period. $PM_{2.5}$ was measured by the beta attenuation monitoring method and gaseous pollutants were measured by UV-fluorescence (Horiba AP-370) during the exposure time. Sampling for $PM_{2.5}$ was performed continuously using the Echo PM Low Volume Sampler in the ambient air of the adjacent pilot animal room (EPA 2017a; Kattner *et al.* 2015; Triantafyllou *et al.* 2016). The $PM_{2.5}$ collection procedures, maintenance and exposure were performed based on previous studies (Sowlat *et al.* 2012). The metals and polycyclic aromatic hydrocarbons (PAHs) procedure were analyzed with at least three replications and the mean concentration was expressed. Standard reference material (SRM 1648) was used to evaluate the accuracy and precision approach of the analysis based on previous studies (Zarandi *et al.* 2019; Noshadirad *et al.* 2023).

RNA extraction from ovary and uterus tissues

All rats were sacrificed by decapitation. Afterward, the uterus and the left ovary tissues were quickly removed. Then, the extraction of RNA was carried out immediately under ice-cold conditions. Tissues were removed and became flash-frozen in liquid nitrogen, followed by tissue homogenization. Total RNA was obtained using an RNA purification kit (GeneJET™ RNA Purification Kit#K0732, Fermentas, Latvia) as described by the manufacturer and then treated with RNase-free DNase I to eliminate contamination caused by genomic DNA. Finally, prior to storage at -80 ℃, the RNA was resuspended in RNase and DNase-free water. RNA integrity and quality/quantity were determined by intact ribosomal RNA (28S and 18S bands) using 1% agarose gel electrophoresis and NanoDrop ND1000 spectrophotometer (NanoDrop Technologies).

cDNA synthesis

cDNA synthesis was done according to the instructions of the Transcription First Strand cDNA Synthesis Kit (RevertAid Premium First Strand cDNA Synthesis Kit #K1652, Fermentas, Latvia). total RNA (1 µg) was mixed with 20 µL reaction mixture containing 0.5 µg oligo (dT) as primer and 200 U of Maloney murine leukemia virus reverse transcriptase.

Quantitative Real-Time PCR

Gene expression or mRNA levels of candidate genes were quantified by quantitative PCR in all lung tissues of all groups. Specific primers for all genes were designed using "oligo7" and checked for the specificity at the NCBI website. Primers are presented in Table 1. The predicted size of PCR amplicons was verified by conventional PCR and agarose gel electrophoresis. Serial dilutions (1:4) of pooled cDNA from total RNA extracted from control samples were used to construct standard curves for each gene. The R^2 value of the standard curve was greater than 0.99 in each experiment and no detectable signal was obtained in control assays without template. SYBR Green [Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) #K0221, Fermentas, Latvia] was used for quantitative real-time PCR. For real-time PCR, the method was performed in triplicate using the 7900HT Fast Real-time PCR System with the Fast 96-well block module (Applied Biosystems, Foster City, CA, USA). PCR data were acquired using Sequencing Detector (SDS version 2.3 Rev C Patch, Applied Biosystems) and quantified using the standard curve method. Software plots a standard curve of threshold cycles versus extracted RNA volume. As a reference gene, the GAPDH gene was selected for normalization. The ratio was calculated using Pfaffl formula. The real-time PCR procedure was performed based on previous studies (Haghighatfard *et al.* 2018).

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Statistical analysis

Statistical assessments were performed using SPSS version 24. One-sample Kolmogorov-Smirnov test confirmed normal distribution for continuous variables. For statistical differences in multiple group comparisons, One-Way ANOVA analysis was used. Pearson correlation analysis was performed to detect the relationship between variables. Descriptive data were reported as the mean ± SD (range), and the level of statistical significance was defined as $p < 0.05$. For correction of multiple comparisons, the Bonferroni correction was used. Potential confounders such as RNA quality and concentration, cDNA synthesis quality, qPCR plates/runs, and primer efficiency were added as covariates, and ANOVA tested for persistence of significant main effect differences between groups.

Table 1. Primer sequences used to evaluation of gene expression by Real-time PCR.

Gene	Forward primer	Reverse primer
ESR-1	5'GCAAGTGTTACGAAGTGGGC3'	5'TCGGCCTTCCAAGTCATCTC3'
PGR	5'GTGACTTCCCAGACTGCACC3'	5'GGCTGGAATTCGCCGTAAAC3'
GAPDH	5'TCATCGTCACTGCACCTTCC3'	5'TTGCTGACAACGGTCATGGA3'

RESULTS

Real-time PCR

Gel electrophoresis of PCR products for PGR gene and ESR1 gene is presented in Fig. 1.

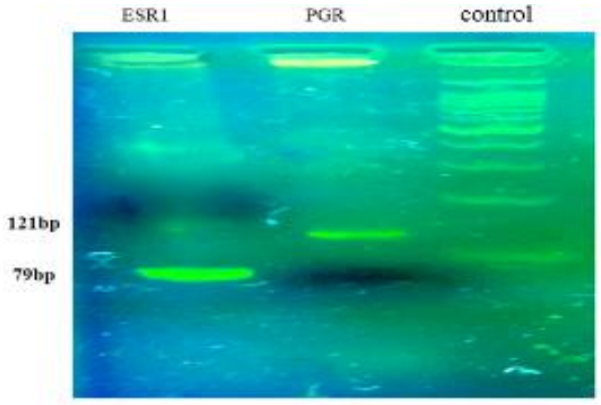

Fig. 1. Electrophoresis in agarose 1/5% gel results were obtained from the assessment of the bands created by the ESR-1 and PGR genes.

Gene expression statistical analysis in uterus tissue between groups

The gene expression analysis showed that the expression of the PGR gene in uterus tissue of the **group A** was significantly ($p<0.001$) increase compared with the control group following a three -month exposure period. However, the comparison of the PGR gene between **group B** and **group A** groups did not show any significant difference (**Fig. 2**).Also, in the study of ESR-1 gene expression in the uterus, a statistically significant increase in the expression of this gene was observed in groups A and B compared to the control ($P<0.001$ and $P<0.05$, respectively) (**Fig. 3**).

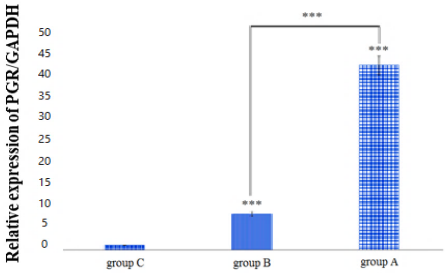

Fig. 2. The expression of PGR gene in uterus tissue in experimental groups following a three-month exposure period; the values are displayed as the means and SEM; *** $p < 0.001$.

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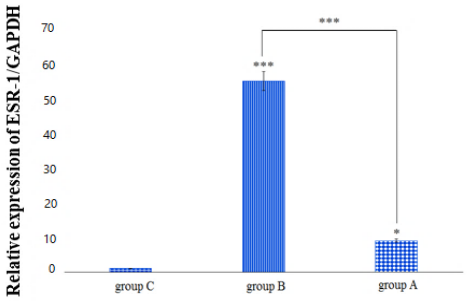

Fig. 3. The expression of ESR-1 gene in uterus tissue in experimental groups following a three-month exposure period; the values are displayed as the means and SEM; * $p < 0.05$, *** $p < 0.001$.

Gene expression statistical analysis in ovary tissue between groups

Quantitative PCR findings showed significant over expression of PGR in exposure groups (A and B) compared to control group (C) in the ovary tissues. Significant increase regulation of PGR was detected in ovary tissue of group A compared to B (Fig. 4). The gene expression analysis revealed that the expression of the ESR-1 gene in ovarian tissue of the group A was significantly ($p < 0.001$) elevated compared to the control group following a three-month exposure period. However, the comparison of the PGR gene between groups B and A did not show any significant difference (Fig. 5).

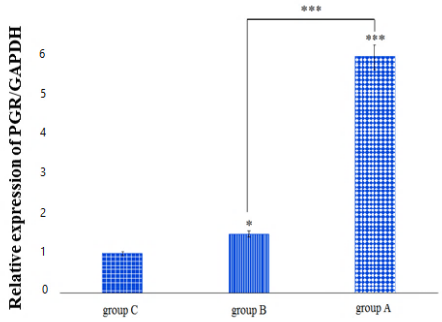

Fig. 4. The expression of PGR gene in ovary tissue in experimental groups following a three-month exposure period; the values are displayed as the means and SEM; * $p < 0.05$, *** $p < 0.001$.

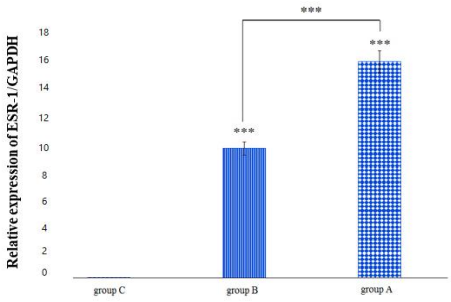

Fig. 5. The expression of ESR-1 gene in vary tissue in experimental groups following a three-month exposure period; the values are displayed as the means and SEM; *** $p < 0.001$.

DISCUSSION

In this study, the concentration of $PM_{2.5}$ was found to exceed WHO standard indicators, which can be attributed to the increased emissions from various sources, including natural factors (dust), human activities (vehicle fuel combustion), and industrial sources (Sowlat *et al.* 2012) Air pollution leads to various health problems through multiple mechanisms. In other words, it can cause issues with the production of reproductive cells (gametogenesis), resulting in reduced reproductive capacity in exposed populations (Ogara *et al.* 2019). Our results indicated the severe effects of air pollution especially $PM_{2.5}$ on expression level of steroid sex hormones receptors. $PM_{2.5}$ induced over expression of these receptors, can influence several

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molecular patterns related to female fertility, ovary and breast cancer and even cognition (Moore *et al.* 2020). It has been reported that $PM_{2.5}$ could cause global DNA methylation changes in promoter region and expression reduction of several genes related to cell signalling such as proto-oncogenes and tumour suppressing genes (Zhou *et al.* 2016). The air pollution role in alteration of Proto-oncogenes and tumour suppressing genes production may lead to a variety of cancers in different organs. Recent studies reported that exposure to $PM_{2.5}$ displays adverse impact on female reproductive system. It has been observed that exposure to $PM_{2.5}$ in mice for 28 days could cause elevation in numbers of apoptotic granulosa cells and oocytes, female infertility and severe abnormalities in embryo development (Liao *et al.* 2020). Ovarian function and female gametogenesis as well as embryo development could be disrupted by air pollution (Vander Borght & Wyns 2018). It has been repeatedly confirmed in epidemiological and experimental studies (Maluf *et al.* 2009) . The male gametogenesis reduction because of $PM_{2.5}$ exposure was detected in rat, but there was lack of studies on $PM_{2.5}$ effects on oocytes and folliculogenesis (Pires *et al.* 2011; Somers 2011). The chronic $PM_{2.5}$ exposure may cause severe physiological changes in female reproductive system such as reproductive/fetal outcomes, menstrual irregularities and step down of the primordial and primary follicular pool (Veras *et al.* 2009; Ogliari *et al.* 2013; Vizcaíno *et al.* 2016). Mechanistic studies of the hormones have shown that $PM_{2.5}$ can depress the levels of manganese, zinc, and magnesium which can affect the signalling pathway of steroid biosynthetic system, as well as the hormone distribution and maturation of eggs in the ovaries (Xia *et al.* 2018) . $PM_{2.5}$ exposure may increase reactive oxygen species (ROS) that in turn could cause ovarian inflammation, depletion of primordial follicles and subsequently apoptosis (Gai *et al.* 2017). The $PM_{2.5}$ exposure during pregnancy was reported to cause the decreased birth weight, intrauterine developmental restriction, and preterm birth in a study about the effects of $PM_{2.5}$ exposure in Northern China. In this study, the uterine estrogen receptor, mRNA and protein levels were decreased and methylation levels of CpG sites in the CpG island of ERα promoter region were elevated in the uterus (Dang *et al.* 2018). The results of an experimental study in mice conducted by Kundakovic and colleagues revealed that methylation alternations in the promoter of the ERs gene in hippocampal tissue of female offspring could be triggered by exposing to bisphenol A as an attached substance to the airborne particulates at a dose equivalent to the environment during pregnancy (Kundakovic *et al.* 2013). In another study conducted by Dos Anjos *et al.* it has been shown that exposure to $PM_{2.5}$ is responsible for endocrine disturbance including increased levels of estrogen and progesterone which ultimately can cause leiomyomas as an important type of uterine benign tumours (Dos Anjos *et al.* 2023). Our findings showed different alteration caused by $PM_{2.5}$ exposure that increased ESR1 expression level in both ovaries and uterus. It was revealed that expression of Esr1 increased by bisphenol A exposure (Bhandari *et al.* 2019). Unfortunately the number of molecular or cellular studies about air pollution effects ended by inconclusive and controversial results, which could be related to the exposure assessment misclassification, bias and lack of data monitoring (Lin *et al.* 2016). Incidentally, Esr1 overexpression as a central component of the p53-MDM2-MDM4 signal axis can lead to a large proportion of breast cancers (Holst *et al.* 2007; Swetzig *et al.* 2016), and the endometrial carcinoma development (Lebeau *et al.* 2008). The proliferative response to progesterone, independent of estrogenic signal transduction, was increased (Fleisch *et al.* 2009). Over-expression of progesterone receptor that detected in uterus and ovary of the $PM_{2.5}$ exposed rats was previously indicated as a biomarker for malignant uterine smooth muscle tumour (Mittal & Demopoulos 2001). The expression level alteration of estrogen and progesterone receptor is important in central nervous system as well as female reproduction system. Preoptic neurons, in response to estrogen and progesterone, are involved in production of luteinizing hormone (Lauber *et al.* 1991). It seems that over-expression of ESR1 and PR induced by air pollution and in particular, $PM_{2.5}$ exposure leading to several severe abnormalities in female reproduction abilities, may cause carcinogen and even cognitive and psychological problems.

CONCLUSION

The present study used gene expression assessment in rat modelling of the air pollution exposure to evaluate the effect of $PM_{2.5}$ in two most important tissues of female reproduction system, uterus and ovary. Expression alteration of the progesterone and estrogen receptors in these tissues which indicated in our results, may cause number of different abnormalities in fertilization process as well as the sex-related health of females. $PM_{2.5}$ comes from a variety of sources, such as automobiles, coal and oil combustion, the nitrogen dioxide and

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sulphur dioxide transformation products, and even biogenic organic matter and dust. It seems that policies and protocols to reduce women exposure to air pollution may lead to the reduction of global disease burden of infertility and female diseases on economy and public health.

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The data are available from the corresponding author on reasonable request.

Ethics approval

The Institutional Ethics Committee of Science and Research Branch of Islamic Azad University approved this study in accordance with the international guidelines.

Conflict of interest

The authors declare no conflicts of interest.

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