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Research Article

The effect of particulate matter (PM2.5) on the expression levels of GATA-4, GATA-6, and Aquaporin-9 genes in testes of rats

Elnaz Noshadirad [1], Kazem Parivar* [1], Saeed Motesaddi Zarandi[2], Pejman Mortazavi[3], and Batool Gorbani Yekta[4]

[1] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[2] *Department of Environmental Health Engineering, School of Public Health and safety, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[3] *Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[4] *Department of Physiology, Faculty of Medicine, Tehran Medical Sciences, Islamic Azad University of Medical Sciences, Shariati St, Tehran, Iran*

*Corresponding author: Kazem Parivar, Ph.D. Kazem.parivar41@gmail.com

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Abstract

Introduction and Aim: Particulate matter (PM) is one of the major components of air pollutants and is composed of a mixture of liquid droplets or solid particles which are suspended in the air with a diameter of 2.5µm ($PM_{2.5}$) or less. In this study, the co-exposure effect of ambient fine particulate matter ($PM_{2.5}$) and gaseous pollutants was assessed on the expression of aquaporin-9, GATA-4, and GATA-6 in testicles of male rats at the gene and protein levels.

Materials and Methods: In this study, three experimental groups were assigned; the first group was exposed to gaseous pollutants along with $PM_{2.5}$ and was called the TM group; the second group included rats exposed to only gaseous pollutants and was called the TJ group. The control group received clean air without any pollutants or PMs and was called the TC group. The exposure time was five hours per day which started from 9:00 A.M to 14:00 P.M for four days per week. The process of time exposure lasted six months. The levels of SO_2, NO_2, O_3, and $PM_{2.5}$ were routinely measured. The UV fluorescence method was utilized for the measurement of SO_2, NO_2, and O_3, while the beta- attenuation technique was employed for the analysis of $PM_{2.5}$.

Results: The results demonstrated that the concentration of $PM_{2.5}$ in the exposed groups was significantly higher than the level recommended by the WHO, after six months, whereas the levels of SO_2, NO_2, and O_3 were significantly lower than the recommended levels. The order of increment in the levels of metals in $PM_{2.5}$ was Al> Ca>Mn>Cu> Cd> Na > Fe > Cr> Ni> Mn > Pb.

Conclusion: According to our findings, it is concluded that exposure to $PM_{2.5}$ could cause male infertility problems and must be considered a health concern for males.

***Keyword*:** PM2.5 exposure; GATA-4; GATA-6; Aquaporin-9

Introduction

Pollutants are harmful solid or gaseous materials produced above the permitted concentrations, and they are able to negatively affect the environment (Weagle *et al.*, 2018). It has been shown that exposure to air pollutants is associated with impairment in the natural process of the reproductive system (Manisalidis *et al.*, 2020). Particulate matter 2.5 ($PM_{2.5}$) is composed of hazardous chemicals, particulate matter, dust particles, and pathogenic microorganisms that have become a concern of communities due to its harmful and sometimes irreversible effects on humans and the environment (Li *et al.*, 2019). PMs are divided into two groups, namely PM_{10} and $PM_{2.5}$, according to their size and importance. PM_{10} has a diameter equal to or less than 10 micrometer, while $PM_{2.5}$ possesses a diameter of 2.5 micrometer. PMs can cause inflammation in the human body because they are small and, once inhaled, can quickly enter the alveoli, deposit there, and reduce reproduction through the bloodstream (Cao *et al.*, 2017). The level of $PM_{2.5}$ in the environment is positively associated with hormonal and morphological disorders of the male reproductive system, especially spermatozoa(Hansen *et al.*, 2010), and negatively with the number of spermatozoa in the semen, their motility, and the rate of testosterone secretion (Zhou *et al.*, 2014; Jurewicz *et al.*, 2015). $PM_{2.5}$ severely impairs the functionality of the male reproductive system, as it could emerge as an increase in the frequency of abnormal and infertile spermatozoa, as well as a decrease in sperm count and the secretion of testosterone in mice (Zhang *et al.*, 2018). PMs are able to enter the human body and reach the central and sensitive tissues and organs such as the reproductive system(Watanabe and Oonuki, 1999) through the lungs (Karottki *et al.*, 2013) and circulatory system (Sicard *et al.*, 2019) (Figure 1).

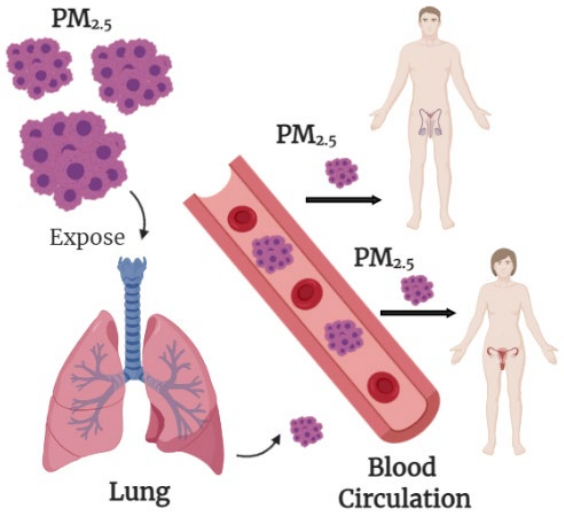

Figure 1. Routes of fine particulate matters (PM2.5) entry into the reproductive system.

Methods

Chemicals: Chloroform and Hematoxylin/Eosin staining solutions were purchased from the Merck Company. The Maxima™ SYBR® Green/ROX qPCR Master Mix (2X) was purchased from the Thermo Fisher Scientific Company. The Fast 96-Well Block Module was procured from Applied Biosystems Company. RevertAid Premium First Strand cDNA Synthesis Kit and GeneJET™ RNA Purification kits were obtained from the Thermo Fisher Scientific Company. Immune-Blot™ polyvinylidene difluoride (PVDF) membrane (Cat No: 162-017777) was purchased from the Bio-Rad Laboratories Company. Syto9 dye was procured from the Invitrogen Company. 5% BSA was obtained from the Sigma Aldrich Company (Cat No: A-7888). The primary antibodies against anti-GATA-4 (ab84593), anti-GATA-6 (ab22600), anti-aquaporin-9 (ab191056), as well as the secondary goat anti-rabbit horseradish peroxidase antibody (ab6721) and anti-beta actin-loading control antibody (ab8227) were all procured from the Abcam Company.

PM2.5 preparation

Location of sampling: The process of sampling was performed on the roof of the School of Public Health (SPH) building affiliated with Shahid Beheshti University (35.79910N, 51.39470E), Tehran, Iran. The collection of specimens was carried out at the height of twenty meters above the ground.

Preparation of $PM_{2.5}$ samples: In this experiment, microfiber sampling filters were applied to gather $PM_{2.5}$ quartz filter samples. The Echo PM low-volume sampler (47 mm diameter, Whatman International Ltd) was utilized for the sampling of $PM_{2.5}$ quartz filter samples in ambient air next to the animal room (EPA 2017a). The process of sampling was conducted at a flow rate of 20 L/min for 24 h between 9.00 AM and the following 9.00 AM). Quartz microfiber filters were rinsed with sterile distilled water and then placed in an oven at 105°C for 2 h before the beginning of the sampling process (Sowlat *et al.*, 2012). Afterward, quartz microfilters were exposed to room temperature (18-20°C) and relative humidity of 40-50% for 24 h (Zarandi *et al.*, 2019). Finally, the filters were placed in aluminum foil and kept at -10 °C in order to avoid evaporation and optical decomposition (Haghighatfard *et al.*, 2018).

Determination of metal concentrations: In this analysis, A quarter of each quartz microfilter was isolated and crushed in a 15-ml container. In parallel with that, 2.5 ml of 70% $HClO_4$ plus 2.5 ml of 69% HNO_3 were mixed and heated at 170 °C for 4 h. The resulting solution was positioned on a hot plate at 95-100 °C to dry completely. After that, 2.5 ml of distilled water was added to the solution and then agitated at 180 rpm for 30 min (Carré *et al.*, 2017). Afterward, the solution was filtered through a 125-mm Whatman filter (No. 42;), and then 10 ml of the filtered volume was transferred to a plastic vial at 4 °C and stored until use. As a control group, a clean filter was employed and exposed to all treatments (Carré *et al.*, 2017). The concentrations of metals were analyzed using ICP/MS (Model 7900, CA).

Evaluation of Polycyclic aromatic hydrocarbons (PAHs): In this assay, a quarter of each quartz microfilter was crushed in a 15-mL container. Next, 2.5 ml of CH2Cl2 and 2.5 ml of CHOH=CHOH were mixed and poured into a dish. Then, the resulting solution was sonicated at a frequency of 20 kHz in an ultrasonic bath for 30 min (Elmasonic S 80H). Finally, the mixture was filtered by a 0.22-mm Millipore PTFE filter (Hesperia, CA, USA). The GC-MS (Agilent 5890A model) method was used to analyze the concentration of 16-PAH. Similar to the previous section, a clean filter was applied as a control group and exposed to all treatments.

***In-vivo* Analysis**

Animal handling and ethics approval: All experimental procedures performed in this study were approved by the Institutional Animal Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics reference: IR.1398.055). In the present research, 48 male Wistar rats, with a mean weight of 85±10 g and an average age of 4 weeks, were purchased from the Pasteur Institute, Tehran, Iran. The animals were kept under standard laboratory conditions (45-55% humidity, 22 ±2°C temperature, 12h:12h light/dark cycle. The animals had free access to food and tap water. After that, the animals were randomly assigned to six groups (n = 8).

Study design: In the current research, the concentrations of O_3, SO_2, NO_2, and $PM_{2.5}$ were measured in part-per-billion(ppb) unit. The beta attenuation monitoring method was utilized for measuring the concentration of $PM_{2.5}$, while the ultraviolet (UV) fluorescence technique (Horiba AP-370 series) was employed for analyzing the concentration of gaseous pollutants at exposure period time of 5 hours between 9:00 A.M. and 2:00 P.M., according to methods used in a previous study(Zarandi *et al.*, 2019). Polycyclic aromatic metals and hydrocarbons (PAHs) were analyzed with 3

replications, and the average values were expressed. According to our previous study, the detailed analysis was carried out using standard reference materials (SRM 1648) (Zarandi *et al.*, 2019).

The current research was conducted over a period of six months. During the period, the animals were exposed to $PM_{2.5}$ for 5 hours per day between 9:00 A.M. and 2:00 P.M. (4 days per week). The animals were divided into three groups of control (exposed to air with clean standard conditions (TC), TJ (exposed to only gaseous pollutants), and TM (exposed to gaseous pollutants and $PM_{2.5}$) (Figure 2).

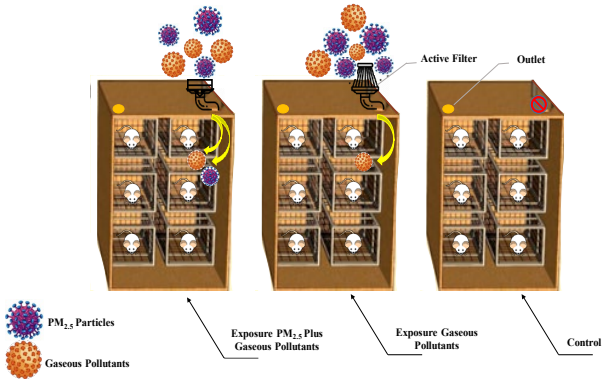

Figure 2. The assignment of experimental groups to different chambers; The assigned chambers were composed of control (exposed to air with the clean standard condition), TJ 2 (exposed only by gaseous pollutants), and TM (exposed by gaseous pollutants and $PM_{2.5}$) groups.

Gene expression analysis by real-time PCR

RNA extraction: In this experiment, the total RNA was obtained from testicular tissues of male rats using the GeneJET RNA Purification kit (Thermo Scientific). At the end of the exposure time, all animals were sacrificed, and their testicular tissues were isolated and utilized for RNA extraction (-70 °C). The extracted RNA samples were stored at -20 °C until the day of analysis.

cDNA synthesis: The extracted RNA specimens (1 µg in each vial) were reverse transcribed into complementary DNA (cDNA) (Dejakam *et al.*, 2020). This enzymatic reaction was performed at a final volume of 20 µl containing 200 U of Maloney murine leukemia virus reverse transcriptase and 0.5 µg of oligo (dT) as a primer, according to RevertAid H minus first-strand cDNA synthesis kit (Fermentas Inc, Germany).

Real-time PCR: The specific primers for *Aquaporin-9*, *GATA-4*, and *GATA-6* genes, along with the β-actin gene, as a control gene, are shown in Table 1. The primers were designed by the "Oligo 7" software, and their specificity was verified by the National Center for Biotechnology Information (NCBI).

Table 1. Specific primers used for real-time PCR

Genes	Forward primers	Reverse primers
GATA-4	3′-CTGTGCCAACTGCCACACCA-5′	3′-GGCTGACCGAAGATGCGTAG-5′
GATA-6	3′-TGTGCAATGCTTGTGGACTC-5′	3′-AGTTGGAGTCATGGGAATGG-5′
Aquaporin-9	3′-TCAGTCGAGAAAAGGCTGGT-5′	3′-GGCACGGATACAATGGTTT-5′
β-actin	3′-TCATCGTCACTGCACCTTCC3-5′	3′-TTGCTGACAACGGTCATGGA-5′

Quantitative real-time PCR: The expression of the three genes was analyzed by real-time PCR in testicular tissues of all experimental groups. The conventional PCR method, along with gel electrophoresis, was utilized to amply the corresponding genes and intactness of the PCR results, respectively. The real-time PCR technique was conducted using the SYBR Green master mix, and CT values were determined for all three genes. The β-actin gene was applied as a control gene. The procedures of the thermocycling program were in accordance with a method used in previous studies (Rahmanian *et al.*; Haghighatfard *et al.*, 2018).

Western blot: In order to conduct the western blot analysis, testicular tissue was first lysed with RIPA buffer. Next, the cell debris was discarded when centrifuged at 14,000 rpm at

4°C for 20 min. Afterward, the concentration of the protein contents was calculated by the Bradford method. Then, an equal volume of 2X Laemmli sample buffer was mixed with tissue lysates. The resulting mixture was boiled for 5 min, run on the SDS-PAGE, and transferred to a 0.2 µm immune-Blot™ polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then transferred to a container containing 0.1% Tween and 5% BSA for 1 h to block the membrane. After that, the membrane was incubated with primary antibodies against anti-GATA-4, anti-GATA-6, aquaporin-9, and anti-GAPDH proteins at room temperature for 4 h. Next, the membrane was rinsed in TBST solution three times and incubated with goat anti-rabbit IgG H&L (HRP) as a secondary antibody. In order to visualize the protein bands, enhanced chemiluminescence (ECL) was used. The expression levels of proteins were normalized against the β-actin protein, and the densitometry of protein bands was carried out by the Gel Analyzer Software version 2010.

Statistical evaluations: The Kolmogorov-Smirnov test was applied to examine whether the obtained data were normally distributed. The difference between the experimental groups was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni correction for post hoc test. The relationship between independent variables was analyzed by the Pearson correlation coefficient. The obtained values are expressed as the means and standard error of the mean (mean ± SEM), and the level of the statistical significance was set at $p<0.05$. Besides, potential confounds (e.g., the quality of cDNA synthesis, etc.) were considered covariates. The analysis of the data was performed by the SPSS software version 24.

Results

The concentrations of NO_2, O_3, SO_2, and $PM_{2.5}$: The concentrations of NO_2, O_3, SO_2, and $PM_{2.5}$ in samples after 6 months of exposure to pollutants are depicted in Table 2. The results indicated that the level of $PM_{2.5}$ was higher than the values of the WHO guideline, whereas the mean concentrations of NO_2, O_3, and SO_2, were lower than those of the WHO guideline.

Table 2. The concentrations of NO_2, O3, SO_2, and $PM_{2.5}$ in ambient air of the animal house following six-month exposure

Pollutants	Exposure time (months)	Units	Average values	Ranges	WHO guideline
O_3	6	Ppb	23.37 ± 7.72	12.00–39.00	100
NO_2	6	Ppb	53.28 ± 8.73	37.00–77.00	100
SO_2	6	Ppb	5.37 ± 1.23	3.00–8.00	20
$PM_{2.5}$	6	µg/m^3	31.61 ± 11.20	10.01–58.57	25

Ppb unite: part per billion

The levels of $PM_{2.5}$-bound heavy metals: The concentrations of $PM_{2.5}$-bound heavy metals are displayed in Table 3. According to the mean concentrations of heavy metals bound to $PM_{2.5}$ following six-month exposure period, the order of the level of heavy metals was as follows; Al> Ca> Na> Cu> Cd> Cr> Ni> Pb> Fe> Mn. Apparently, Aluminium and Manganese elements had the highest and lowest levels, respectively.

The concentration of PAHs bound with $PM_{2.5}$: The levels of $PM_{2.5}$-bound PAHs are depicted in Table 4. The average concentration of $PM_{2.5}$-bound 16 PAHs during a six-month period was reported to be 30.04 ± 25.27 ng/m^3. The level of Phenanthrene was higher than other $PM_{2.5}$-bound PAHs inhaled by the animals after six-month periods.

Real-time PCR: The gene expression analysis showed that the expression level of the *GATA4* gene in testicular tissue of the TM group was significantly (p<0.001) reduced compared with the control group following a six-month exposure period. However, the comparison of the *GATA4* gene between TJ and TM groups did not show any significant difference. Figure 3a exhibits the expression level of the *GATA-6* gene in both TM and TJ groups. The expression level of the_*GATA-6* gene was significantly (p<0.001) lower in both TM and TJ groups than that of the control group. Similar to the *GATA-4* gene, the comparison of *GATA-6* expression between TJ and TM groups did not indicate any statistically significant discrepancies (Figure 3b). The expression rate of the *aquaporin*-9 gene was significantly (p<0.001) diminished in both TJ and TM groups in comparison with the control group. Also, the tissue expression of *aquaporin*-9 was significantly (p<0.05) higher in the TJ group than that of the TM group (Figure 3c).

Table 3. The concentrations of $PM_{2.5}$-bound heavy metals after six months of exposure to pollutants (mg/m^3).

Hevy metals	Ca	Ni	Cd	Al	Cr	Cu	Pb	Mn	Fe	Na
Six-month exposure	14.70 ±12.65	0.18 ± 0.18	0.17 ± 0.10	8.09 ± 4.15	0.32 ± 0.39	0.98 ± 1.28	0.18 ± 0.13	0.13 ± 0.06	0.57 ± 0.52	0.88 ± 1.20

Table 4. The levels of $PM_{2.5}$-bound 16-PAHs (ng/m^3) at three- and six-month periods of exposure to pollutants

16-PAHs	6-month exposure
Acenaphten	1.67 ± 1.34
Florene	1.33 ± 0.34
Naphtalene	4.39 ± 11.4
Phenanthrene	9.86 ± 7.26
Anthracene	2.89 ± 2.3
Fluorantene	0.86 ± 0.38
Pyrene	0.87 ± 0.55
Benzo(a)ant	0.35± 0
Chrysene	3.05 ± 1.27
Acenaphten	1.67 ± 1.34
B(k)F	ND
B(b)F	ND
B(a)P	2.2 ± 1.45
Benzo(g,h,i) Perylene	ND
Dibenzo(a,h) Anthracene	ND
Indeno(1,2,3-cd)Pyrene	ND
Sum	30.04 ± 25.27

ND: not determined

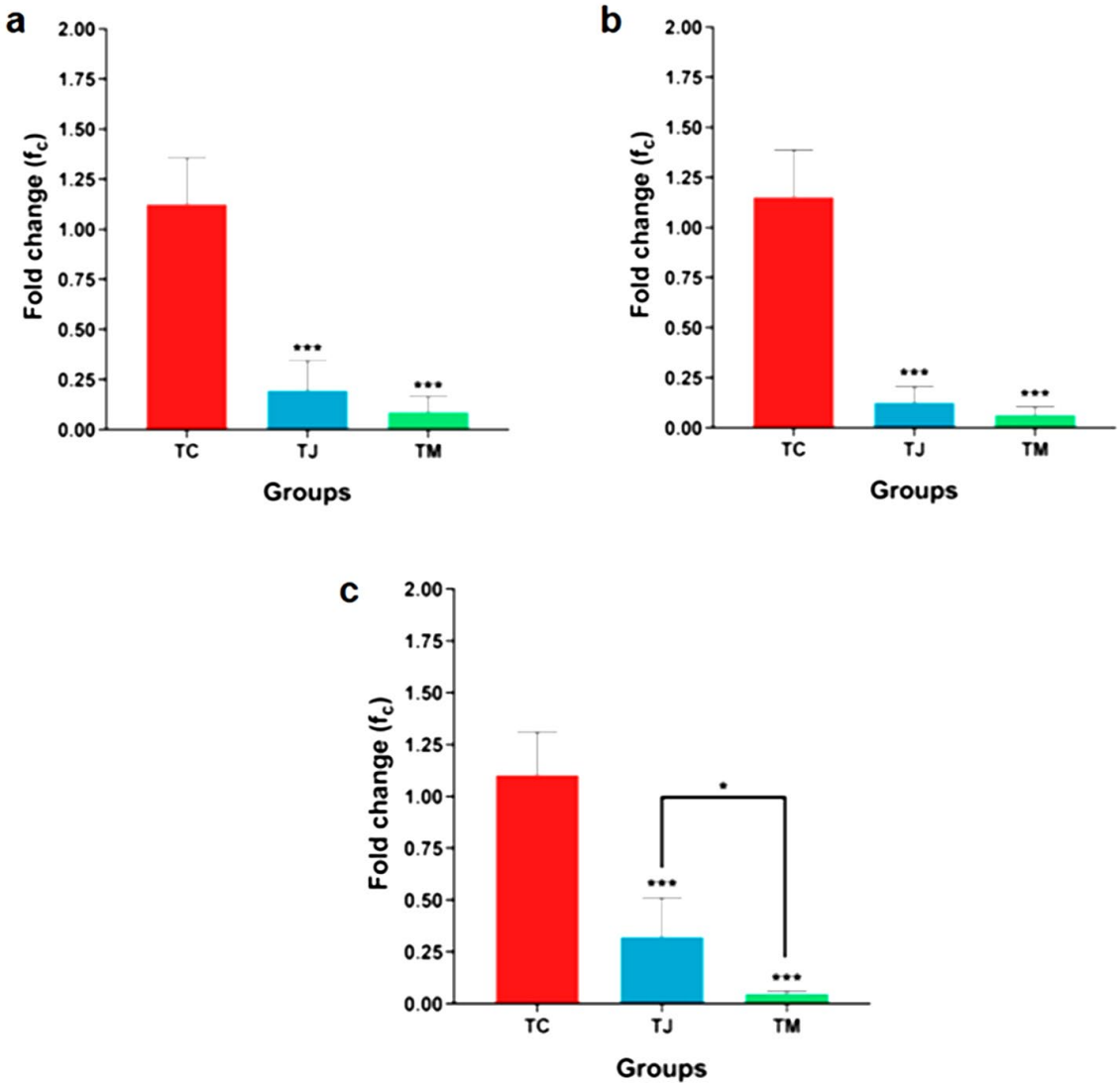

Figure 3. The expression of GATA-4 (a), GATA-6 (b), and aquaporin-9 (c) genes in experimental groups following a six-month exposure period; the obtained data are depicted as the means and SEM. *p<0.05;** p<0.01; ***p<0.001.

Western blot analysis: The expression levels of GATA-4, GATA-6, and aquaporin-9 proteins were evaluated in all experimental groups after a six-month exposure period using the western blot analysis (Figures 4a). Following a six-month exposure period, the expression level of GATA-4 was significantly (p<0.001) declined in TM and TJ groups in comparison with the control group. Also, the expression level of GATA-4 was significantly (p<0.01) lower in the TM group than that of the TJ group (Figure 4b).

The level of GATA-6 expression was significantly (p<0.001) downregulated in TM and TJ groups compared with the control group (Figure 4c). Besides, a significant (p<0.001) reduction was observed in aquaporin-9 expression in the TM group compared with the control and TJ groups (Figure 4d). Also, in the TJ group, the expression of aquaporin-9 was significantly (p<0.01) lower than that of the control group.

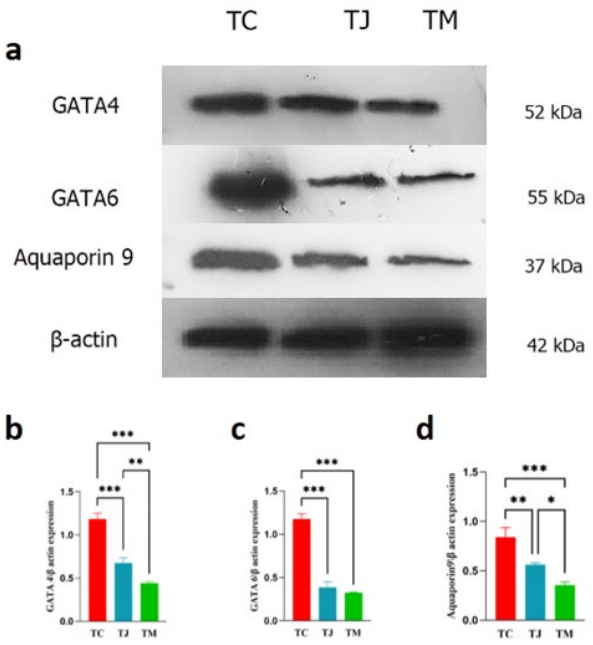

Figure 4. The western blot analysis was performed to analyze the expression levels of GATA-4 (b), GATA-6 (c), and aquaporin-9 (d) following six-month exposure. The obtained values are expressed as the means ± SEM. *p<0.05; **p<0.01; ***p<0.001.

Discussion

Air pollution is involved in many health problems through various mechanisms (Carré *et al.*, 2017). It is thought that air pollution can cause a defect in the process of gametogenesis, which leads to a decrease in reproductive capacity in endangered populations (Carré *et al.*, 2017). In the present study, it was found that the expression rates of GATA4, GATA-6, and aquaporin-9 were markedly decreased at the gene and protein levels in TJ and TM groups compared with the control group. Also, the expression levels of GATA-4, GATA-6, and aquaporin-9 were remarkably downregulated in the TM group compared with the TJ group. In the current research, the concentration of $PM_{2.5}$ was higher than that of the values allowed by the WHO guidelines. Such an increment in the concentration of $PM_{2.5}$ may stem from the over-generation of PM2.5-producing resources, such as environmental-natural (Dust), human (fuel combustion in vehicles), and industrial (factories, industrial-production centers) factors in cities (Ketola *et al.*, 2002).

It is now known that air pollution is responsible for the causation of some health problems, such as hormonal disorders, epigenetic changes, oxidative stress, and DNA injury (Carré *et al.*, 2017). Decreased sperm quality due to PM2.5 can be partly explained by the systemic inflammatory response in testicles (Zhou *et al.*, 2019). The epidemiological studies reported that the incidence rates of testicular dysplasia syndrome (low sperm production in adulthood) and testicular cancer are increasing (Liu *et al.*, 2017; Zhou *et al.*, 2019; Wang *et al.*, 2021). It has been shown that PM2.5 is able to impact gene expression patterns, including cytochromes, oxide reductase, glutathione transferase, and MAP-kinase signaling in different organs (Raymond *et al.*, 1998; Madaniyazi *et al.*, 2020). As observed in this study, $PM_{2.5}$ plus gaseous pollutants could have adverse effects on the testicular tissues of the studied groups.

GATA-4 and *GATA-6* genes are abundantly expressed in the murine ovary and testis. Studies indicated that the lack of *GATA-4* and *GATA-6* genes leads to apoptotic cell death in embryonic endoderm and cardiomyocytes (Raymond *et al.*, 1999). A number of studies indicated that GATA-1 and GATA-4 are significant regulators of gene expression in testicular tissue (Molkentin, 2000; Ketola *et al.*, 2002). Several lines of evidence showed that exposure to $PM_{2.5}$ results in male reproductive toxicity, thereby excessive ROS-induced autophagy (Wei *et al.*, 2018). A study carried out by Li and colleagues showed that uterine exposure of mice offspring to $PM_{2.5}$ led to a substantial reduction in the expression of *GATA4* in cardiomyocytes compared with mice offspring exposed to normal ambient air (Li *et al.*, 2020). In addition, a recent study has shown that during pregnancy and even before pregnancy in mice, exposure to $PM_{2.5}$ can increase myocardial cell apoptosis and impair myocardial cell growth by reducing *GATA-4* expression (Wu *et al.*, 2019). GATA-4 has been studied in the development and disease of the human testicular germ cells (Ketola *et al.*, 2000). Although the precise mechanistic role of *GATA-*

6 in human testicles has not been fully elucidated, it has been postulated that GATA-6 may participate in the regulation of apoptosis and the cell cycle process (Heikinheimo *et al.*, 1997; Morrisey *et al.*, 1998). In mice, *GATA6* is expressed in somatic cells, as well as the testicles of the human fetus, and is regulated by several gonadal genes (Robert *et al.*, 2002; Hu *et al.*, 2013).

Both *GATA-4* and *GATA-6* genes are expressed in the embryonic testis(Heikinheimo *et al.*, 1997). Limited expression of GATA-6 in the embryonic testis is confined to the seminiferous tubules, whereas GATA-4 is expressed in the seminiferous tubules and interstitial cells (Heikinheimo *et al.*, 1997; Ketola *et al.*, 2003). It has been reported that $PM_{2.5}$ exposure is capable of disrupting the integrity of the blood-testis barrier (BTB), thereby excessive ROS-induced autophagy (Wei *et al.*, 2018).

Aquaporin-9 is abundantly expressed in the testicles of rats (Tsukaguchi *et al.*, 1999; Elkjær *et al.*, 2000). The expression of aquaporin-9 is mainly expressed in Leydig cells and immature spermatocytes of rats (Pastor-Soler *et al.*, 2001; Arima *et al.*, 2003). The role of aquaporin-9 in the membrane exchange of water and neutral salts, from Sertoli cells to spermatocytes, spermatids, and sperm cells, has been identified within the BTB (Arima *et al.*, 2003). Aquaporin-9 is known to be an essential factor in the water transfer and neutral membrane exchange of neutral minerals, from Sertoli cells to spermatocytes, spermatids, and sperm cells in the male reproductive system (Arena *et al.*, 2011). Testicles mainly contain germ cells and somatic supportive cells (namely Sertoli and Leydig cells); thus, Sertoli cells reside in the seminiferous tubules with mature germ cells (Carré *et al.*, 2017; Zhou *et al.*, 2019). Our findings showed that the levels of the testicular tissue expression of *GATA-4* and *GATA-6* and *aquaporin*-9 genes were significantly decreased in Wistar rats exposed to $PM_{2.5}$ plus gaseous pollutants compared with those exposed to air with a clean standard condition.

Our results demonstrated that $PM_{2.5}$ plus gaseous pollutants had more harmful and destructive effects on the testicular tissues of rats than gaseous pollutants alone. This indicates a serious impairment in the expression of essential spermatogenesis genes, especially aquaporin-9, in men in large cities with higher levels of PM2.5-containing contaminants. The detrimental effects of gaseous pollutants and $PM_{2.5}$ might be due to a change in the expression levels of various genes in cells.

Conclusion

As a result, using animal laboratory models and molecular examinations, the present study showed that $PM_{2.5}$, as an air pollutant, is a dangerous substance that affects different signaling pathways and may lead to reproductive problems and changes in the expression of GATA-4, GATA-6, and aquaporin-9. However, the development of human activities, urbanization, and the rise of polluting industries may increase air pollution. Moreover, strict public policies and government regulations may reduce fertility and reproductive problems by reducing $PM_{2.5}$.

Declarations

Funding: The current study did not receive any research grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials: All data supporting the findings of the current study are available upon reasonable request from the corresponding author.

Ethics approval and consent to participate: There are no "human subjects" in this study. 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.1398.055).

References

Arena, S., Arena, F., Maisano, D., Di Benedetto, V., Romeo, C. and Nicòtina, P. 2011. Aquaporin-9 immunohistochemistry in varicocele testes as a consequence of hypoxia in the sperm production site. Andrologia, 43(1): 34-37.

Arima, H., Yamamoto, N., Sobue, K., Umenishi, F., Tada, T., Katsuya, H. and Asai, K. 2003. Hyperosmolar mannitol stimulates expression of aquaporins 4 and 9 through a p38 mitogen-activated protein kinase-dependent pathway in rat astrocytes. Journal of Biological Chemistry, 278(45): 44525-44534.

Cao, X.-n., Shen, L.-j., Yan, C., Zhou, Y., Xiong, G., Wang, Y.-c., Liu, Y., Liu, B., Tang, X.-l. and Guo, M. 2017. Urban fine particulate matter exposure causes male reproductive injury through destroying blood-testis barrier (btb) integrity. Toxicology letters, 266: 1-12.

Carré, J., Gatimel, N., Moreau, J., Parinaud, J. and Léandri, R. 2017. Does air pollution play a role in infertility?: A systematic review. Environmental Health, 16: 1-16.

Dejakam, A., Dejakam, A. and Hekmat, A. 2020. Evaluating of bdnf expression in blood cells of opium recovering patients with a new treatment method: A molecular marker. Research in Cell and Tissue, 1(1): 16-25.

Elkjær, M.-L., Vajda, Z., Nejsum, L., Kwon, T.-H., Jensen, U., Amiry-Moghaddam, M., Frøkiaer, J. and Nielsen, S. 2000. Immunolocalization of aqp9 in liver, epididymis, testis, spleen, and brain. Biochemical and biophysical research communications, 276(3): 1118-1128.

Haghighatfard, A., Andalib, S., Amini Faskhodi, M., Sadeghi, S., Ghaderi, A.H., Moradkhani, S., Rostampour, J., Tabrizi, Z., Mahmoodi, A. and Karimi, T. 2018. Gene expression study of mitochondrial complex i in schizophrenia and paranoid personality disorder. The World Journal of Biological Psychiatry, 19(sup3): S133-S146.

Hansen, C., Luben, T.J., Sacks, J.D., Olshan, A., Jeffay, S., Strader, L. and Perreault, S.D. 2010. The effect of ambient air pollution on sperm quality. Environmental health perspectives, 118(2): 203-209.

Heikinheimo, M., Ermolaeva, M., Bielinska, M., Rahman, N.A., Narita, N., Huhtaniemi, I.T., Tapanainen, J.S. and Wilson, D.B. 1997. Expression and hormonal regulation of transcription factors gata-4 and gata-6 in the mouse ovary. Endocrinology, 138(8): 3505-3514.

Hu, Y.-C., Okumura, L.M. and Page, D.C. 2013. Gata4 is required for formation of the genital ridge in mice. PLoS genetics, 9(7): e1003629.

Jurewicz, J., Radwan, M., Sobala, W., Polańska, K., Radwan, P., Jakubowski, L., Ulańska, A. and Hanke, W. 2015. The relationship between exposure to air pollution and sperm disomy. Environmental and molecular mutagenesis, 56(1): 50-59.

Karottki, D.G., Spilak, M., Frederiksen, M., Gunnarsen, L., Brauner, E.V., Kolarik, B., Andersen, Z.J., Sigsgaard, T., Barregard, L. and Strandberg, B. 2013. An indoor air filtration study in homes of elderly: Cardiovascular and respiratory effects of exposure to particulate matter. Environmental Health, 12: 1-10.

Ketola, I., Anttonen, M., Vaskivuo, T., Tapanainen, J.S., Toppari, J. and Heikinheimo, M. 2002. Developmental expression and spermatogenic stage specificity of transcription factors gata-1 and gata-4 and their cofactors fog-1 and fog-2 in the mouse testis. European Journal of Endocrinology, 147(3): 397-406.

Ketola, I., Pentikäinen, V., Vaskivuo, T., Ilvesmäki, V., Herva, R., Dunkel, L., Tapanainen, J.S., Toppari, J. and Heikinheimo, M. 2000. Expression of transcription factor gata-4 during human testicular development and disease. The Journal of Clinical Endocrinology & Metabolism, 85(10): 3925-3931.

Ketola, I., Toppari, J., Vaskivuo, T., Herva, R., Tapanainen, J.S. and Heikinheimo, M. 2003. Transcription factor gata-6, cell proliferation, apoptosis, and apoptosis-related proteins bcl-2 and bax in human fetal testis. The Journal of Clinical Endocrinology & Metabolism, 88(4): 1858-1865.

Li, R., Zhao, Y., Shi, J., Zhao, C., Xie, P., Huang, W., Yong, T. and Cai, Z. 2020. Effects of pm2. 5 exposure in utero on heart injury, histone acetylation and gata4 expression in offspring mice. Chemosphere, 256: 127133.

Li, X., Jin, L. and Kan, H. 2019. Air pollution: A global problem needs local fixes. Nature, 570(7762): 437-439.

Liu, X., Jin, X., Su, R. and Li, Z. 2017. The reproductive toxicology of male sd rats after pm2. 5 exposure mediated by the stimulation of endoplasmic reticulum stress. Chemosphere, 189: 547-555.

Madaniyazi, L., Li, S., Li, S. and Guo, Y. 2020. Candidate gene expression in response to low-level air pollution. Environment international, 140: 105610.

Manisalidis, I., Stavropoulou, E., Stavropoulos, A. and Bezirtzoglou, E. 2020. Environmental and health impacts of air pollution: A review. Frontiers in public health, 8: 14.

Molkentin, J.D. 2000. The zinc finger-containing transcription factors gata-4,-5, and-6: Ubiquitously expressed regulators of tissue-

specific gene expression. Journal of Biological Chemistry, 275(50): 38949-38952.

Morrisey, E.E., Tang, Z., Sigrist, K., Lu, M.M., Jiang, F., Ip, H.S. and Parmacek, M.S. 1998. Gata6 regulates hnf4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes & development, 12(22): 3579-3590.

Pastor-Soler, N., Bagnis, C., Sabolic, I., Tyszkowski, R., McKee, M., Van Hoek, A., Breton, S. and Brown, D. 2001. Aquaporin 9 expression along the male reproductive tract. Biology of reproduction, 65(2): 384-393.

Rahmanian, N., Hekmat, A. and Hajebrahimi, Z. Effect of simulated microgravity condition on cells proliferation and myostatin expression in differentiated skeletal muscle cells (c2c12).

Raymond, C.S., Parker, E.D., Kettlewell, J.R., Brown, L.G., Page, D.C., Kusz, K., Jaruzelska, J., Reinberg, Y., Flejter, W.L. and Bardwell, V.J. 1999. A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. Human molecular genetics, 8(6): 989-996.

Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J. and Zarkower, D. 1998. Evidence for evolutionary conservation of sex-determining genes. Nature, 391(6668): 691-695.

Robert, N.M., Tremblay, J.J. and Viger, R.S. 2002. Friend of gata (fog)-1 and fog-2 differentially repress the gata-dependent activity of multiple gonadal promoters. Endocrinology, 143(10): 3963-3973.

Sicard, P., Khaniabadi, Y.O., Perez, S., Gualtieri, M. and De Marco, A. 2019. Effect of o 3, pm 10 and pm 2.5 on cardiovascular and respiratory diseases in cities of france, iran and italy. Environmental science and pollution research, 26: 32645-32665.

Sowlat, M.H., Naddafi, K., Yunesian, M., Jackson, P.L. and Shahsavani, A. 2012. Source apportionment of total suspended particulates in an arid area in southwestern iran using positive matrix factorization. Bulletin of environmental contamination and toxicology, 88: 735-740.

Tsukaguchi, H., Weremowicz, S., Morton, C.C. and Hediger, M.A. 1999. Functional and molecular characterization of the human neutral solute channel aquaporin-9. American Journal of Physiology-Renal Physiology, 277(5): F685-F696.

Wang, L., Luo, D., Liu, X., Zhu, J., Wang, F., Li, B. and Li, L. 2021. Effects of pm2. 5 exposure on reproductive system and its mechanisms. Chemosphere, 264: 128436.

Watanabe, N. and Oonuki, Y. 1999. Inhalation of diesel engine exhaust affects spermatogenesis in growing male rats. Environmental Health Perspectives, 107(7): 539-544.

Weagle, C.L., Snider, G., Li, C., Van Donkelaar, A., Philip, S., Bissonnette, P., Burke, J., Jackson, J., Latimer, R. and Stone, E. 2018. Global sources of fine particulate matter: Interpretation of pm2. 5 chemical composition observed by spartan using a global chemical transport model. Environmental science & technology, 52(20): 11670-11681.

Wei, Y., Cao, X.-N., Tang, X.-L., Shen, L.-J., Lin, T., He, D.-W., Wu, S.-D. and Wei, G.-H. 2018. Urban fine particulate matter (pm2. 5) exposure destroys blood–testis barrier (btb) integrity through excessive ros-mediated autophagy. Toxicology mechanisms and methods, 28(4): 302-319.

Wu, X., Pan, B., Liu, L., Zhao, W., Zhu, J., Huang, X. and Tian, J. 2019. In utero exposure to pm2. 5 during gestation caused adult cardiac hypertrophy through histone acetylation modification. Journal of Cellular Biochemistry, 120(3): 4375-4384.

Zarandi, S.M., Shahsavani, A., Khodagholi, F. and Fakhri, Y. 2019. Co-exposure to ambient pm2. 5 plus gaseous pollutants increases amyloid β1–42 accumulation in the hippocampus of male and female rats. Toxin reviews.

Zhang, Y., West, J.J., Mathur, R., Xing, J., Hogrefe, C., Roselle, S.J., Bash, J.O., Pleim, J.E., Gan, C.-M. and Wong, D.C. 2018. Long-term trends in the ambient pm 2.5-and o 3-related mortality burdens in the united states under emission reductions from 1990 to 2010. Atmospheric chemistry and physics, 18(20): 15003-15016.

Zhou, L., Su, X., Li, B., Chu, C., Sun, H., Zhang, N., Han, B., Li, C., Zou, B. and Niu, Y. 2019. Pm2. 5 exposure impairs sperm quality through testicular damage dependent on nalp3 inflammasome and mir-183/96/182 cluster targeting foxo1 in mouse. Ecotoxicology and Environmental Safety, 169: 551-563.

Zhou, N., Cui, Z., Yang, S., Han, X., Chen, G., Zhou, Z., Zhai, C., Ma, M., Li, L. and Cai, M. 2014. Air pollution and decreased semen quality: A comparative study of chongqing urban and rural areas. Environmental Pollution, 187: 145-152.