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Gaseous pollutants and $PM_{2.5}$ Co-exposure induces BCL2/Bax apoptosis pathway activation in rat Sertoli cells: Implication of GATA4 and GATA6 interaction

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ABSTRACT

Exposure of males to particulate matter and gaseous air pollution poses a serious threat to spermatogenesis. However, the specific molecular mechanism underlying this effect remains unclear. In this *in-vivo* study on Wistar albino rats, we investigated the impact of combined exposure to ambient fine particulate matter ($PM_{2.5}$) and gaseous pollutants on the BCL2/BAX signaling pathway, as well as GATA4 and GATA6 gene and protein expression in Sertoli cells. A total of twenty-one male rats were randomly assigned to one of three groups: a control group exposed to standard air conditions, a Gas group exposed to gaseous pollutants alone, and a Gas+$PM_{2.5}$ group exposed to both $PM_{2.5}$ and gaseous pollutants. All groups were exposed for four days a week, five hours per day, for three months. Sertoli cells were isolated and analyzed for gene and protein expression. The findings revealed a significant reduction in BCL2, GATA4, and GATA6 gene and protein expression in the Sertoli cells of the Gas+ $PM_{2.5}$ group, accompanied by an elevation in the Bax gene and protein expression compared to the control group. Consequently, the combined exposure to ambient $PM_{2.5}$ and gaseous pollutants is likely to trigger the upraised Sertoli cell apoptosis via activation of the BCL2/BAX pathway and possible interaction with GATA 4 and GATA 6 proteins.

Keywords: Male reproductive, Particle matter, Sertoli cells, Testes, Apoptosis.
Article type: Research Article.

INTRODUCTION

$PM_{2.5}$ refers to suspended particulate pollutants with an aerodynamic diameter of ≤ 2.5 μm (Jeong *et al.* 2017). This complex mixture primarily consists of inorganic ions, polycyclic aromatic hydrocarbons, heavy metals, bacteria, and viruses, and its composition varies considerably depending on the location. Notably, $PM_{2.5}$ has emerged as a critical factor impacting human health (Jeong *et al.* 2017). $PM_{2.5}$, owing to its minute size, can extensively accumulate in the bronchial and alveolar regions. Furthermore, it can permeate the pulmonary blood barrier, thereby accessing the bloodstream and reaching various organs such as the brain, liver, spleen, kidney, and testicular tissues (Li *et al.* 2017). This particulate matter is linked to the development of respiratory, neurological, cardiovascular, and reproductive system ailments (Miller & Xu 2018). Recently, there has been a growing interest in the impact of $PM_{2.5}$ on reproductive organs, particularly the testes (Nassan *et al.* 2018).

Exposure to $PM_{2.5}$ has been found to heighten the risk of male reproductive system disorders by negatively affecting sperm production, quality, motility, testosterone levels, and blood-testis barrier integrity, as shown by various studies (Nassan *et al*. 2018; Liu *et al*. 2018). Sertoli cells play a crucial role in the complex process of spermatogenesis, as they are the only somatic cells that come into direct contact with spermatogenic cells (França *et al*. 2016). The tight junctions between Sertoli cells form the blood-testis barrier (BTB), which creates an immune-protective environment and shields germ cells. Additionally, studies suggest that glycolysis metabolism is a critical aspect of spermatogenesis and is essential for energy production, similar to the Warburg effect observed in tumor cells (Mateus *et al.* 2018). Sertoli cells offer both nutritional and structural assistance to developing germ cells. In addition, the growth and proliferation of germ cells are closely linked to the secretion of metabolites by these cells (Oliveira *et al.* 2015). While $PM_{2.5}$ exposure has been shown in animal experiments to damage BTB integrity, its impact on the function of Sertoli cells remains uncertain (Liu *et al*. 2019). At typical physiological conditions, a balance exists between reactive oxygen species (ROS) and antioxidants. Research has consistently demonstrated that exposure to $PM_{2.5}$ can disrupt this equilibrium by increasing ROS, leading to oxidative stress, cellular harm, and activation of redox-sensitive apoptotic signaling pathways in the testes (Ren *et al.* 2022). Previous studies have shown that exposure to $PM_{2.5}$ can damage male reproductive health by impairing BTB integrity along with inducing oxidative stress and apoptosis (Liu *et al.* 2020). However, the specific mechanisms underlying oxidative stress in response to $PM_{2.5}$ exposure are not yet fully understood, and further research is needed to investigate the potential pathways of the Sertoli cell damage induced by oxidative stress. The regulation of apoptosis in testicular cells involves BCL-2 proteins, including BCL-2 and Bax (Zakariah *et al.* 2022). These proteins are present in the adult testis, and also contribute to cell differentiation and maturation (Beumer *et al.* 2000). In addition, GATA zinc finger transcription factors play a role in regulating cell fate, including differentiation, adrenogonadal development, proliferation, and apoptosis in the testis (Deng *et al.* 2017). The expression of endodermal factors such as GATA4 and GATA6 is high in mammalian gonads and may contribute to cell development (Ketola *et al.* 2003). While the exact involvement of GATA proteins in testicular cell death remains unclear, evidence suggests that they may have a role in apoptosis. Specifically, studies have shown that regulatory cis-elements, including GATA proteins, are present in the promoters of genes involved in apoptosis such as BCL2 (Suzuki 2011). There are some reports about air pollution around the world (Masoudi *et al.* 2019; Atiya & Abdulhay 2022). This study aimed to investigate the role of transcription factors, GATA4 and GATA6, and apoptosis regulators, BCL2 and BAX in the development of testicular cells following exposure to $PM_{2.5}$. Sertoli cells were evaluated in a rat model to determine the effect of $PM_{2.5}$ exposure on these factors and their contribution to induced apoptosis in the cells.

MATERIALS AND METHODS

Experimental design

The earlier study provided a detailed description of the collection, processing, and analysis of $PM_{2.5}$ chemical composition (Motesaddi Zarandi *et al.* 2019). Wistar albino rats (n = 21; 6 weeks old; 175-200 g) were obtained from the Pasteur Institute in Tehran, Iran and acclimatized for one week under specific pathogen-free conditions before being randomly divided into three groups: the control group ($T_{Control}$), the gaseous pollutants group (T_{Gas}), and the $PM_{2.5}$ plus gaseous pollutants group ($T_{Gas + PM2.5}$; n = 7 in each group). The experiment was carried out during the winter heating period over a 3-month exposure time (Zarandi *et al.* 2019). The exposure chambers, designed according to Zarandi *et al*. (2019) method, maintained a 12:12-hour light-dark cycle with a humidity level of $55 \pm 5\%$ and a temperature of 24 ± 1 °C. The control group ($T_{Control}$) was exposed to HEPA-filtered ambient air with standard conditions, while the gas group (T_{Gas}) was exposed to gaseous pollutants alone. In contrast, the Gas+ $PM_{2.5}$ group ($T_{Gas + PM2.5}$) was exposed to urban ambient air containing both $PM_{2.5}$ and gaseous pollutants at a flow rate of 12 L min^{-1}. In order to carry out sampling $PM_{2.5}$ in ambient air, an LVS was used, allowing to collect fine dust in the exhaust air when the sampling filter was removed (TCR 2014). The inlet valve of the vacuum pump used in T_{Gas} had a model H13 HEPA filter to remove PM, ensuring that only gaseous pollutants were present in the exhaust air. Noteworthy, the discharged ambient air contained ambient $PM_{2.5}$ and gaseous pollutants due to using $PM_{2.5}$ warhead in this study. This study adhered to the Animal Welfare Act regarding experimental animal principles (AWR 2013). The Institutional Animal Ethics Committee of Islamic Azad University, Science and Research Branch, Tehran, Iran approved all experimental procedures (Ethics reference: IR.IAU.SRB.REC.1398.055), with efforts made to minimize the number of animals used.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

The study involved exposing Sertoli cells to 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) for 10 min, followed by transfering the extracted RNA to a 1.5 mL microtube. The quality of the RNA was assessed using NanoDrop1000, and the extracted RNA was stored at -80 °C. A high-capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM) was used for the reverse transcription of the extracted RNA. This study analyzed the expression of several genes, namely BCL2, Bax, GATA4, and GATA6, using RT-qPCR. The β-actin gene served as an internal control to ensure successful reverse transcription and cDNA template calibration. The reaction mixture contained 10 μL SYBR® Premix Ex TaqTM II (2×) (TaKaRa), 2 μL of 1:5 diluted cDNA, 0.8 μL of each 10 μmol L^{-1} forward and reverse primers (Table 1), 0.4 μL ROX Reference Dye II (50×)*3, and 6 μL DEPC-treated water in a volume of 20 μL. The PCR program comprised an initial incubation step at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 34 sec. Subsequently, a melt curve analysis was performed by heating the samples to 95°C for 15 sec, cooling them to 60°C for 1 min, and then heating them again to 95°C for 15 sec. Negative controls were included using DEPC-treated water instead of a template. Data from three replicates were used to quantify transcript copy numbers with the ABI 7300 system SDS Software (Applied Biosystems). The relative gene expression level was calculated using the 2-ΔΔCT comparative CT method, and the results were presented as fold-changes relative to the internal control gene.

Table 1. Primer sequences in this study.

Primer name	Sequence (5'-3')	length (bp)	Source
β-actin	F- TTG CTG ACA GGA TGC AGA AG R- TAG AGC CAC CAA TCC ACA CA	108	(Takayasu *et al.* 2008)
Bcl2	F-TATATGGCCCCAGCATGCGA R -GGGCAGGTTTGTCGACCTCA	136	(Jafari *et al.* 2015)
Bax	F-ATCCAAGACCAGGGTGGCTG R-CACAGTCCAAGGCAGTGGGA	150	(Jafari *et al.* 2015)
GATA4	F-TCA AAC CAG AAA ACG GAA GC R- GCA TCT CTT CAC TGC TGC TG	143	(Takayasu *et al.* 2008)
GATA6	F- GCC AAC TGT CAC ACC ACA AC R- TTC ATA TAA AGC CCG CAA GC	106	(Takayasu *et al.* 2008)

Western blot

To extract the total protein of Sertoli cells, we used cell lysis buffer (DNAbioTech, Iran) and measured the concentration using the BCA protein assay kit (DNAbioTech, Iran). We loaded 40-60 μg protein onto an SDS-PAGE gel and transferred it to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, USA) for western blot analysis. The membranes were blocked with 3% bovine serum albumin (BSA) before being probed with primary antibodies overnight at 4 °C. The primary antibodies utilized were: Anti-beta actin as control antibodies (1:1000, Cat No: ab8227), Anti-BCL2 (1:1000, Cat. No: 15071), Anti-BCL2-associated Xprotei (Bax; 1:1000, Cat.No:5023), Anti-GATA4(1:1000, Cat. NO: ab8459) and Anti-GATA6 (1:1000, Cat. No: ab22600). Horseradish peroxidase-conjugated secondary antibodies (Abcam) were used for incubation following three washes with Tris-buffered saline containing 0.15% Tween 20. The enhanced chemiluminescence system was employed to visualize protein bands, and the grey value of the blots was analyzed using ImageJ software.

Statistical analysis

The data's normality was assessed using the Shapiro-Wilk test, and variance homogeneity was verified using the Levine test. Mean ± standard deviation was used to represent quantitative data. To compare groups with normally distributed data, a one-way analysis of variance (ANOVA) was employed, followed by Tukey's test for multiple comparisons. For non-parametric data, OriginPro 2016 software programs were utilized for all analyses. The probability of observed differences between groups was considered significant at $p \leq 0.05$.

RESULTS

Gene expression

In this study, $T_{Gas + PM2.5}$ displayed a notable and significant decrease in the expression of the BCL2, GATA4, and GATA6 genes ($p < 0.05$) compared to the control group (Fig. 1). Conversely, the expression of the Bax gene was significantly higher than that of $T_{control}$ and T_{GAS} ($p < 0.05$; Fig. 1).

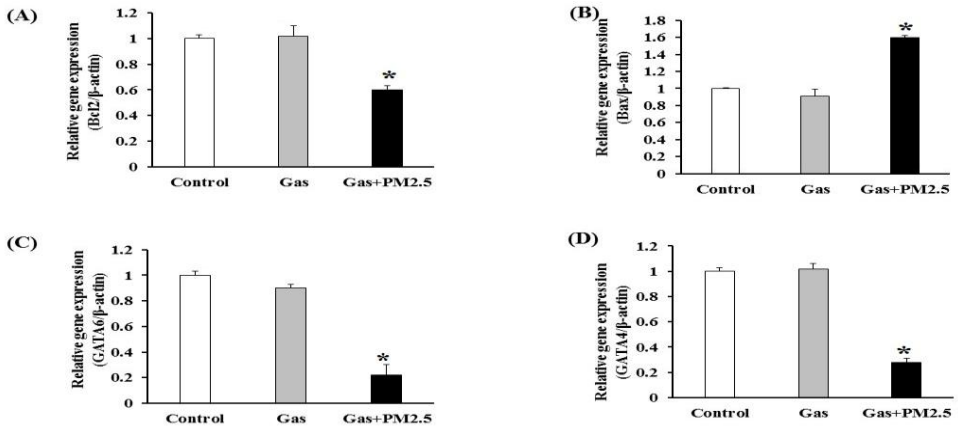

Fig. 1. Quantitative analyses of gene expressions for BCL2, BAX, GATA4, and GATA6 in Sertoli cells of rat testes following a 3-month exposure to gas pollution (T_{Gas}) and gas pollution plus $PM_{2.5}$ ($T_{Gas + PM2.5}$), with normalization to β-actin. Significant differences with $T_{Control}$ ($p < 0.05$) are indicated by "*". All experiments were performed at least thrice (n = 7). The mean ± SD values are presented.

Protein expression

The T_{Gas} and $T_{Gas + PM2.5}$, exhibited a significant reduction in the expression of BCL2, GATA4, and GATA6 proteins ($p < 0.05$) when compared to the control group (Fig. 2). Conversely, the expression of Bax protein was significantly higher than that of the control group ($p < 0.05$; Fig. 2).

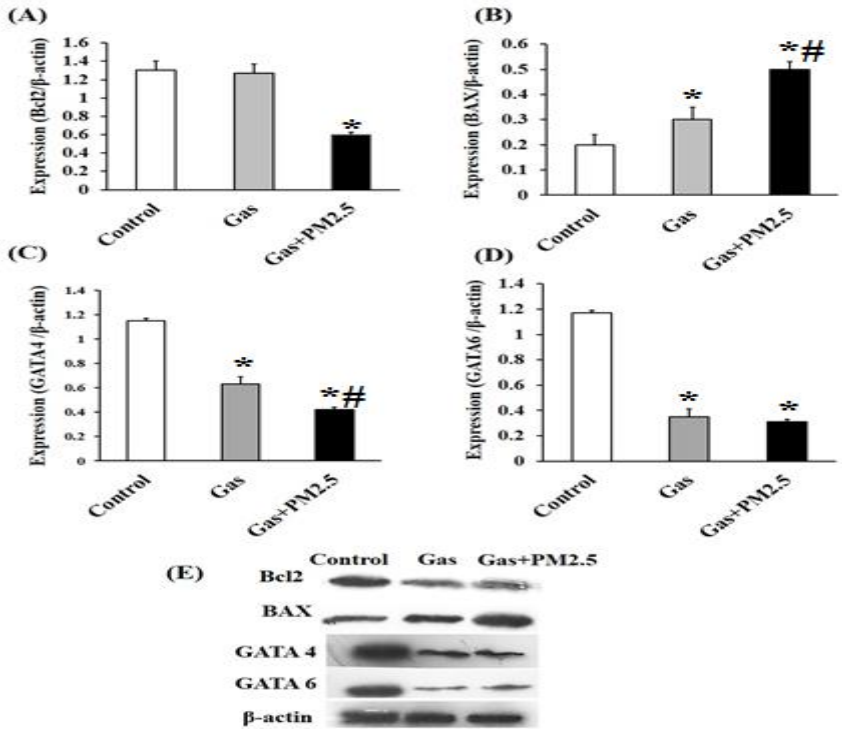

Fig. 2. Quantitative analyses of protein expression levels of BCL2 (A), BAX (B), GATA4 (C), and GAT6 (D) in Sertoli cells of rat testes following exposure to gas pollution (T_{Gas}) and gas pollution plus $PM_{2.5}$ ($T_{Gas + PM2.5}$), for three months. The protein expressions were normalized to β-actin; Statistical significance differences with $T_{Control}$ ($p < 0.05$) are indicated by "#" while with T_{Gas} ($p<0.05$) by "*" ($p < 0.05$). The experiments were conducted in triplicate (n = 7), and the data is presented as mean ± SD. Panel E shows the representative protein bands of BCL2, BAX, GATA4, and GATA6 with the corresponding β-actin as determined by Western blotting.

DISCUSSION

$PM_{2.5}$ has been identified as an environmental factor that negatively affects reproductive competence (Qiu *et al.* 2020). Numerous epidemiological studies have established that $PM_{2.5}$ from automobile exhaust adversely affects sperm quality and quantity, resulting in male infertility (Wang *et al.* 2021). However, the specific mechanism remains unclear. Previous study has shown that exposure to $PM_{2.5}$ damages the blood-testis barrier (BTB), leading to testicular germ cell apoptosis and infertility, and has identified signalling pathways that may associate with its toxic effects (Liu *et al.* 2020). Nevertheless, earlier studies have focused on *in vivo* experimentation and overlooked the role of Sertoli cells (SCs), which act as "nurse cells" for germ cells and perform key functions in BTB. These cells provide both nourishment and protection throughout spermatogenesis. Thus, investigating the SCs function alteration is vital for comprehending the underlying mechanism of BTB impairment due to $PM_{2.5}$ exposure. This study examines the impact of gaseous pollutants and $PM_{2.5}$ exposure on gene and protein expression in the Sertoli cells of rat testes. Results show a significant decrease in the expression of the BCL2, GATA4, and GATA6 genes and proteins, while the expression of the Bax gene and protein significantly increased in this group. In the $T_{Gas + PM2.5}$, significant alterations in the expression levels were observed. These alterations included a marked change in the levels of BCL-2 family proteins, which are key regulators of apoptosis and serve as an indicator for air pollution-mediated apoptosis in the rat testes. The observed elevation in the reactive oxygen species (ROS) probably resulted from the upraised oxidative stress levels within the testes. This, in turn, led to a decline in the BCL-2 protein expression and an elevation in Bax protein expression (Kalia & Bansal 2008). Previous studies have reported on the potential inhibitory function of GATA-6 in testicular cell apoptosis and its association with the apoptosis-related proteins, i.e., BCL-2 and Bax (Ketola *et al.* 2003). In addition, the GATA-4 protein has been shown to function as a cell survival factor in gonadal cells and act as an anti-apoptotic agent (Heikinheimo *et al.* 1997), although the underlying mechanisms are not well understood. Co-expression of GATA-4 and GATA-6 proteins has been observed in various tissues, including the testes and ovaries. Studies have documented the co-expression of GATA-4 and GATA-6 proteins in various tissues, including the testes and ovaries (Bouchard *et al.* 2022). The overlap in their transcript distributions suggests a potential interplay between these transcription factors. Reduction in protein expression of GATA-4 and GATA-6, coupled with an elevation in gene and protein expression of the box, may indicate their involvement in ROS-mediated apoptosis of Sertoli cells following exposure to $PM_{2.5}$ in rats. GATA-4 and GATA-6 have been suggested to play a functional role in hormonal signalling and cell proliferation in testes. This is thought to be achieved through the formation of homodimeric and heterodimeric complexes with other transcription factors, including steroid hormones (Heikinheimo *et al.* 1997). Possible ROS produced due to $PM_{2.5}$ exposure might indirectly decline the expression of GATA 4 and GATA 5 transcription factors by reducing sexual hormones, although direct evidence supporting this hypothesis is lacking.

CONCLUSION

This study demonstrated that exposure to $PM_{2.5}$ in rat models activates the apoptosis signalling pathway in the Sertoli cells of the testes. Additionally, it decreases the expression of BCL2, GATA4, and GATA6 genes and proteins, while elevating the expression of the Bax gene and protein. The resultant upraise in apoptosis may lead to the decreased fertility.

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