



Investigating Lead Nitrates Effects on Mouse Reproduction via Ovarian FSHR Gene Expression Analysis and Immune Response



Suaad S. Mukhlif¹, Huda S. Naser², Mohammed Taha³

¹Assistant Lecturer, Scientific Research Commission, Research and Technology Center of Environmental, Water and Renewable Energy, Baghdad, Iraq; ²Lecturer, Scientific Research Commission, Research and Technology Center of Environmental, Water and Renewable Energy, Baghdad, Iraq; ³Lecturer, Department of Medical Laboratory Technologies, Al-Rafidain University, Baghdad, Iraq

Abstract

Background: There are many diseases or disorders that can arise as a result of exposure to harmful chemicals like PbNO₃. It has been observed in many studies that there are adverse effects on reproduction caused by exposure to environmental toxins. **Objective:** The purpose of the present study was to investigate the effect of PbNO₃ exposure on the FSHR gene correlated with immune response. **Methodology:** Twelve female Balb/c mice were housed from January 2025 to March 2025 in the Research and Technology Center of Environmental, Water and Renewable Energy, Baghdad, Iraq. Under standard environmental conditions, including a 12-hour light/dark cycle at 220 C and 55.0% humidity. Divided into a control group (4 mice) and an experimental group (8 mice) that received 10 mg/kg/day of lead nitrate intraperitoneally for six weeks. At the end of the exposure period, mice were euthanized to carefully dissect, clean, and collect ovarian tissues from both groups. The ovaries were immediately flash-frozen in liquid nitrogen or preserved in TRIzol solution for subsequent gene expression analysis using Rt-PCT. **Results:** The data presented the gene expression levels of the FSHR gene standardized against the Gapdh-mis gene using the 2- $\Delta\Delta C_t$ (folding) method. The average fold change of the treated samples is 0.60, showing downregulation of FSHR expression compared to the control samples. The control samples had a standard baseline fold change of 1.00. Generally, the majority of the treated samples, such as Sample 1, Sample 3, Sample 4, Sample 8, and Sample 9, showed downregulation below 1.00. **Conclusion:** Analysis of mRNA can provide an accurate quantitative means for assessing the effects of lead nitrate on the expression of FSHR genes, which indirectly shows the change in the translated receptor protein, and analysis would provide insights on other cellular processes involved in the toxicity of lead through disruptions in the pathways associated with the production of FSHR. [Bangladesh Journal of Infectious Diseases, June 2026;13(1):221-227]

Keywords: Fertility; FSHR gene; lead nitrate; gene expression; Immunity

Correspondence: Suaad S. Mukhlif, Assistant Lecturer, Scientific Research Commission, Research and Technology Center of Environmental, Water and Renewable Energy, Baghdad 10011, Iraq; Email: suaad_b1978@yahoo.com;
ORCID: <https://orcid.org/0009-0007-3678-1282>

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Introduction

Chemicals are required for the life of people and economic growth, yet toxicants from the environment (ETs) have been shown to have detrimental effects on ovaries, leading to a variety of reproductive issues. According to the latest

assessments, the size of the global chemical industry exceeded US\$ 5 trillion in 2017. Between 2000 and 2017, the global chemical industry's production almost doubled, from about 1.2 billion tons to 2.3 billion tons. It is projected to double by 2030 and to triple by 2050¹. World health

organization Directs The process of implementing procedures and policy development to reduce and prevent environmental health risks resulting from chemicals, especially among Vulnerable populations. Priority hazardous chemicals that are particularly concerned for public health include toxicants, carcinogens, and mutagens that affect reproduction and brain growth. To help achieve the Objectives for Sustainable Development (SDGs), Many actions related to responsible chemical management are recommended by the 2030 Agenda for Sustainable Development and well-health for all by contributing to poverty elimination, food security, and health security².

SDG 6.3 aims to enhance water quality by decreasing pollution, ending dumping, and minimizing the release of hazardous chemicals and materials. SDG 3.9 calls for significant reductions in the number of deaths and diseases caused by hazardous chemicals by 2030. Within the framework of internationally recognized agreements, SDG 12.4 aims to manage chemicals and their waste sustainably throughout their entire cycles³. Exposure to hazardous chemicals can lead to health disorders and diseases. The 2021 WHO data addendum estimated that 2 million lives and 53 million disability-adjusted life-years were lost in 2019 through exposure to selected chemicals. This is higher than the estimate of the previous data addendum for 2016 of 1.6 million lives and 45 million disability-adjusted life-years lost. Even this latest calculation has been done for limited number of chemicals for which there are enough scientific data and is underestimated⁴. Follicle-Stimulating Hormone (FSHR) is a key hormone that binds to specific receptors (FSHR) on ovarian cells. This interaction is vital for proper follicle development and maturation, ultimately leading to the release of a mature egg during ovulation.

Disruptions in FSHR signaling can have severe consequences, including infertility. Environmental pollutants, particularly heavy metals like Lead Nitrate (PbNO₃), are emerging threats to reproductive health. Studies suggest that PbNO₃ exposure can disrupt hormonal functions in various organisms. However, the specific effects of PbNO₃ on FSHR expression and function in mouse ovaries remain unclear. By studying genes at the molecular level, researchers can zoom in on the very instructions that dictate how much and what kind of FSHR protein is being produced in the ovary⁵. This study focuses on how PbNO₃ exposure affects the FSHR gene in lab mice's ovaries. We use gene expression analysis with a focus on the FSHR gene.

Methodology

Study Settings: Long-term exposure to heavy metals on the reproductive system of laboratory animals was evaluated using FSHR gene expression analysis. To achieve this, twelve female Balb/c mice were housed from January 2025 to March 2025 in the Research and Technology Center of Environmental, Water and Renewable Energy, Baghdad, Iraq.

Animals and Treatment: Varies were harvested from Twelve balb/c female mice (25 to 37 g, 8 to 12 weeks) were obtained from the Industrial Research and Development Authority/Al-Razi Center/Ministry of Industry and Minerals/Baghdad/Iraq. All standard conditions for animal keeping were provided, including water, food, transparent and separated cages, an environmental temperature (220 C), humidity of 55.0% # 3.0%, and a 12-hour light/12-hour dark photo-cycle⁶. The mice were randomly divided into groups (control and exposed groups); the first group included 4 mice as a control group, and the second group included 8 mice as an experimental group. Experimental mice received heavy metal (PbNO₃) at a concentration of 10 mg/kg per day lead nitrate, intraperitoneal i.p), for a defined long-term period (six weeks)⁷. PbNO₃ was dissolved in suitable solvents (d H₂O₂), The solutions were sterilized by filtration using a 0.22 µm syringe filter. The metal solutions were either used immediately or stored at 40 C for further use.

Tissue Collection and Preparation: At the end of the exposure period, euthanize all animals according to ethical guidelines, so carefully dissect and collect ovarian tissues from both control and experimental groups, and then immediately flash-freeze the ovarian tissues in liquid nitrogen to preserve their structure and minimize potential degradation. For gene expression of FSHR, Midline laparotomy was then performed, and the ovaries were dissected from both sides of the body; the surrounding adipose tissue was separated, and the ovaries were quickly washed in normal saline and then preserved in TRIzol solution for gene expression analyses.

RNA Extraction: RNA was isolated from the sample according to the protocol of TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions -Cat No.11667165001).

Ethics Approval: The study was conducted in accordance with the ethical standards of the institutional research committee. Ethical approval was obtained from the relevant institutional ethics committee (approval code: CSEC/1025/0148

October 27, 2025). Written informed consent was obtained from all participants before inclusion in the study.

Statistical Analysis: The Statistical Package for the Social Sciences (SPSS) application V 21 (2019) was utilized to assess the impact of various groups on study parameters. The T-test and Least Significant Difference (LSD) were employed to compare means significantly. The Chi-Square test was employed to compare percentages significantly. Estimation of the correlation coefficient among various factors in patient groups within this study.

Results

Reaction Setup and Thermal Cycling Protocol: Mouse GAPDH gene is used as a housekeeping



Figure 1: Experimental mice were given heavy metal (PbNO₃) at a concentration of 10 mg/kg per day *(intraperitoneal i. p), for a defined long-term period (six weeks)

control gene for normalization of gene expression levels. Lyophilized primers provided by Macrogen were dissolved in nuclease-free water to make a stock concentration of 100 pmol/μl, A working solution of 10 pmol/μl was obtained by diluting 10 μl stock with 90 μl water and storing it at -20°C. Experimental mice received heavy metal (PbNO₃) at a concentration of 10 mg/kg per day lead nitrate, intraperitoneal i.p), for a defined long-term period (six weeks) figure 1.

Table 1: Distribution of Patients according to Age Group (n=974)

FSHR_exp-F	300	100
FSHR_exp-R	300	100
Gapdh_mice-F	300	100
Gapdh_mice-R	300	100

RT-PCR Component Calculation: The RT-PCR experiment was designed for 12 reactions, using a 10 μL reaction volume, two primers, with a 5% safety margin, and running at an annealing temperature of 58°C for 40 cycles (Table 3, Table 4).

Treated Samples (1 to 12) detail the extracted RNA concentration (measured in ng/μl) for twelve treated samples and six control samples. Sample 7's concentration was as low as 85 ng/μl, while Sample 8's concentration was as high as 786 ng/μl. In the

Table 2: Primer Sequences of Control and Sample Genes

Primer Name	Seq.	Annealing Temp. (°C)
FSHR_exp-F	CAGGCTGAGTCGTAGCATC	58
FSHR_exp-R	GGCGGCAAACCTCTGAACT	
Gapdh_mice-F	CATGGCCTTCCGTGTTCC TA	
Gapdh_mice-R	CATGGCCTTCCGTGTTCC TA	

Table 3: qPCR Master Mix Calculation

Master mix components	Stock	Unit	Final	Unit	Volume
					1 sample
qPCR Master Mix	2	x	1	x	5
RT mix	50	x	1	x	0,25
MgCl ₂					0,25
Forward primer	10	uM	0.5	uM	0,5
Reverse primer	10	uM	0.5	uM	0,5
Nuclease-free water					2.5
RNA		ng/μl		ng/μl	1
Total volume					10
Aliquot per single rxn	9μl of Master mix per tube and add 1μl of Template				

Table 4: Real-Time PCR Program

Steps	Temperature	m: s	Cycle
RT. Enzyme Activation	37°C	15:00	1
Initial Denaturation	95°C	05:00	
Denaturation	95°C	00:20	40
Annealing	58°C	00:20 Acquiring on Green	
Extension	72°C	00:20	

corresponding Control Samples (C1 to C7), sample C3 had a concentration of 178 ng/μl, whereas sample C5 had a concentration of 700 ng/μl (Table 5).

Table 5: RNA Concentration (ng/μl)

Samples	Concentration	samples	Concentration
1	659	C1	281
2	636	C3	178
3	702	C5	700
4	631	C6	676
5	307	C7	540
6	745	C4	490
7	85		
8	786		
9	600		
10	725		
11	90		
12	380		

The Relative Quantification ($\Delta\Delta C_t$ method) is a common approach for quantifying relative gene expression using RT-PCR data (Table 6).

Reverse transcription was performed to convert the extracted RNA to cDNA for gene expression analysis, normalized against the reference gene (Gapdh-mis), the threshold cycle (C_t) values, ΔC_t , $\Delta\Delta C_t$, and fold change (Folding) calculated using $2^{-\Delta\Delta C_t}$ method so, Treated Group Mean Folding the average fold change in gene expression for the treated samples (Samples 1–12) was calculated to be 0.60. The average fold change in gene expression for the untreated control samples (C1 to C7) was calculated to be 1.00. Therefore, comparing the treated group (0.60) to the control group (1.00) reveals a clear down-regulation of FSHR gene expression; this down-regulation is likely caused by Pb(NO₃)₂ (lead nitrate) interference or accelerated protein breakdown, which may ultimately impair follicle development

Table 6: Relative Quantification ($\Delta\Delta C_t$ method) and Fold Change

Sample	Gapdh-mis	FSHR	dct	ddct	Folding		
1	19.41	32.38	12.97	2.48	0.18	0.67 the total of the samples' folding values divided by the total number of samples	0.67/1.12= 0.60
2	23.02	32.72	9.70	-0.79	1.73		
3	20.15	33.27	13.12	2.63	0.16		
4	22.23	34.36	12.13	1.64	0.32		
5	20.49	31.54	11.05	0.57	0.67		
6	21.73	32.11	10.38	-0.10	1.07		
7	23.02	33.55	10.53	0.04	0.97		
8	21.12	32.35	11.22	0.74	0.60		
9	21.12	33.31	12.19	1.70	0.31		
10	22.12	33.27	11.14	0.66	0.63		
11	20.35	31.23	10.88	0.40	0.76		
12	21.30	32.36	11.06	0.58	0.67		
C1	22.15	32.23	10.08	-0.40	1.32	1.12 the total of the controls samples folding values divided by the total number of controls samples	1.00 Gene expression levels are consistently compared using a value of one.
C3	21.94	32.96	11.02	0.53	0.69		
C5	22.86	32.12	9.26	-1.23	2.34		
C6	21.43	32.04	10.61	0.12	0.92		
C7	19.41	32.38	12.97	2.48	0.18		
C4	23.02	32.72	9.70	-0.79	1.73		
			10.485				

Relative quantification: (dct =CT gene - CT House Keeping gene) : (ddCT =dCT Treated or Control - dCT Control): (Folding = $2^{(-\Delta\Delta C_t)}$)

(folliculogenesis) and reduce female fertility. Fold change was calculated using $2^{(-\Delta\Delta Ct)}$ (Table 6).

Discussion

A typical technique for deproteinizing RNA that was established very recently is TRIzol solubilization and extraction (TRIzol (or TRI Reagent) is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures protein)⁸. This technique is especially useful when it is not possible to separate nuclear RNA from cytoplasmic RNA or when cells or tissues have high endogenous RNase concentrations⁹. In all areas of biological and medical research, the identification of appropriate reference genes is essential for precise gene expression analysis¹⁰, mostly due to the numerous differences across various tissues and specimens¹¹. In addition, reverse transcription was used to convert RNA to cDNA for gene expression analysis¹². A negative control ensures no DNA contamination¹³; melt curve analysis verifies specific amplification and excludes non-specific products. Comparing gene expression between samples, normalized to a reference gene, calculates fold-change using Ct value differences¹⁴. Down-regulated FSHR expression¹⁵, likely caused by PbNO₃ interference or accelerated protein breakdown, may affect follicle development and fertility¹⁶.

There are two main reasons why gene expression analysis is a valuable tool in this research on the impact of PbNO₃ on FSHR in mice. Quantifying FSHR Levels: Gene expression analysis allows researchers to measure the level of activity of the FSHR gene in the ovary. This provides a quantitative measure of how much FSHR protein is being produced by the cells. If PbNO₃ exposure disrupts the FSHR gene's function, the gene expression analysis will reveal changes in the amount of mRNA (messenger RNA) produced, which in turn gets translated into protein. By measuring mRNA levels, researchers can indirectly assess how PbNO₃ might be affecting FSHR protein production¹⁷. Identifying Potential Mechanisms: Gene expression analysis can also identify other genes whose expression is altered by PbNO₃ exposure. This broader analysis might reveal potential pathways or mechanisms by which PbNO₃ disrupts FSHR function. For example, PbNO₃ exposure might be affecting genes involved in the signaling cascade leading to FSHR production or genes involved in the stability of the FSHR mRNA. By looking at a wider range of gene expression changes, researchers can get a more comprehensive picture of how PbNO₃ might be

impacting ovarian function¹⁸.

Gene expression analysis specifically targets the FSHR gene, allowing researchers to quantify its activity level directly. This provides a focused measure of how PbNO₃ might be affecting FSHR production. Mechanism insights: Gene expression analysis can identify other genes whose expression is altered by PbNO₃. This broader analysis can shed light on potential pathways or mechanisms by which PbNO₃ disrupts FSHR function¹⁹. Risks from heavy metal pollution are primarily attributed to their ability to persist in the environment. Continuous exposure to these contaminants may result in bioaccumulation in the biota, causing them to move up the food chain. After intake by an organism, heavy metals are distributed through body systems via molecule-binding and deposit in different tissues and organs. While excretion of heavy metals can extend over months, metal residues may remain within tissues throughout the animal's life. These residues may cause distinct physiological disruptions and adverse health effects. Following absorption of heavy metals, mammalian cells phagocytose, partition, and metabolize the heavy metal particles. This allows metal ions to form complexes with specific biological molecules intracellularly²⁰.

The accumulation of heavy metals in some tissues can also provide a persistent source of low-dose toxicity through constant intracellular bioavailability of the toxic metal²¹, who observed a correlation between lead accumulation in the ovary and damage to olliculogenesis. So, this requires the development of effective biological treatment methods²² to remove these pollutants from the environment²³. Understanding the effects of PbNO₃ on FSHR in mice provides valuable insights that can be extrapolated to inform research on human health. The mechanisms regulating ovarian function and the role of FSHR are largely conserved across mammals, including humans. While the specific effects of PbNO₃ might differ in dosage and response between mice and humans, this study can serve as a steppingstone for future investigations into the potential impact of lead exposure on female fertility in humans²⁴.

Conclusion

FSHR expression was Down-regulated indicates that mice's cells are not producing as much FSHR protein as they should, this could be caused by PbNO₃ interfering with the cellular processes that produce FSHR or by PbNO₃ exposure accelerating the breakdown of FSHR protein, and the variations in FSHR expression may have an effect on follicle

development, which is essential for fertility and egg production. This study can serve as a stepping stone for future investigations into the potential impact of lead exposure on female fertility in humans.

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Conflict of interest:

No competing interests exist by the authors.

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Authors' contributions

Conceptualization, methods and literature review was done by Suaad S. Mukhlif; preparation of the draft manuscript was done by Huda Sajir Naser and Mohammed Yousuf Taha.

Data Availability

Any questions regarding the availability of the study's supporting data should be addressed to the corresponding author, who can provide it upon justifiable request.

Ethics Approval and Consent to Participate

All the participants involved in this study were informed, and consent was obtained verbally from each participant before sample collection. This study was approved by a local committee of publication ethics in the medical city, Baghdad, Iraq, under reference number 0214 on July 11, 2024.

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ORCID:

Suaad S. Mukhlif: <https://orcid.org/0009-0007-3678-1282>

Huda S. Naser: <https://orcid.org/0009-0005-2188-8058>

Mohammed Taha: <https://orcid.org/0000-0002-9151-6381>

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