

Placental Lead (Pb) Accumulation and Maternal Metabolic Disturbance in Pregnant Rats: An *In Vivo* and *In Silico* Study

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Abstract

Prenatal Pb exposure is an important environmental health concern because it may disrupt maternal metabolic homeostasis during pregnancy. However, experimental evidence linking placental Pb accumulation with metabolic and inflammatory alterations relevant to gestational diabetes mellitus (GDM)-associated pathophysiology remains limited. This study evaluated the effects of prenatal Pb exposure on placental Pb accumulation and maternal metabolic-inflammatory biomarkers in pregnant rats and explored a supportive placental metal-handling mechanism using *in silico* analysis. A post-test control group design was conducted using pregnant Sprague-Dawley rats allocated into nine groups, including a control group and treatment groups exposed continuously to Pb acetate in drinking water at concentrations of 0.05% and 0.2% from gestational day 1 (GD1) to GD7, GD14, or GD21/parturition. Placental Pb, maternal blood glucose, methylglyoxal (MG), and TNF- α were measured. Placental Pb differed significantly among treatment groups and was highest in the 0.2% Pb group (1.044 ± 0.052 mg/g, $p = 0.007$). After adjustment for exposure duration, maternal blood glucose in the 0.2% Pb group was 62.333 mg/dL higher than in controls ($p < 0.001$), while MG and TNF- α were also consistently highest in the 0.2% group. No significant interaction between exposure duration and treatment was observed, indicating that Pb concentration exerted a stronger effect than exposure duration on biomarker changes. *In silico* analysis showed higher predicted Pb interaction with DMT1 than with MT2A, supporting a model of transporter-related Pb entry followed by intracellular sequestration. Overall, prenatal Pb exposure promoted placental Pb accumulation and maternal metabolic-inflammatory alterations in pregnant rats. While these biomarkers are associated with GDM in humans, this study does not directly diagnose GDM.

Keywords: Gestational diabetes mellitus, Glucose metabolism, Methylglyoxal, Lead exposure, Placenta, Pregnancy, TNF- α

Introduction

Pb is still considered a major environmental toxicant of concern for maternal and fetal health because of its lasting presence in the environment, tendency to bioaccumulate, and ability to interfere with multiple biological systems during pregnancy [1]. This problem has been reported in many parts of the world, including Indonesia, indicating that exposure through the environment and food chain is a continuing public health concern [2-4]. The issue is especially important during pregnancy, as Pb can cross the placental barrier, accumulate in gestational tissues, and possibly disrupt both maternal metabolic homeostasis and fetal development [1].

In addition to its well-established toxic effects, Pb has increasingly been linked to metabolic disturbance [5-8]. Findings from both experimental and observational studies suggest that Pb exposure may alter glucose homeostasis through various interrelated processes, including oxidative stress, mitochondrial dysfunction, inflammatory activation, and disruption of pathways involved in carbohydrate metabolism [5,9-12]. Pb-induced redox imbalance may further intensify inflammatory signaling and carbonyl stress, providing a biologically plausible explanation for how metal exposure could contribute to metabolic disturbance during pregnancy. These mechanisms are particularly relevant to the pathophysiology of GDM, in which impaired glucose regulation, inflammation, and oxidative stress are central features.

Interest in the relationship between heavy metal exposure and GDM has increased in recent years, although the available evidence remains largely epidemiological. Cohort and case-control studies have reported associations between prenatal exposure to heavy metals and GDM, as well as altered maternal glycemic markers, while studies focusing specifically on Pb have also suggested associations with dysglycemia-related indicators such as glycosylated hemoglobin [13-15]. However, these studies provide limited insight into causality and essential mechanisms. Experimental studies directly examining whether prenatal Pb exposure leads to placental Pb accumulation, along with maternal metabolic and

inflammatory changes relevant to GDM, remain scarce. As a result, the mechanisms by which Pb may contribute to gestational metabolic dysfunction remain poorly understood.

The placenta is a biologically plausible target in this context because it can act as both a transport interface and a temporary reservoir for toxic metals during pregnancy. Among placental metal-handling proteins, DMT1 is relevant because it participates in the transport of divalent metals across placental cells, whereas MT2A is a cysteine-rich intracellular metal-binding protein that may contribute to metal sequestration and detoxification. Therefore, evaluating Pb interaction with these proteins may help explain how placental Pb accumulation occurs and how it may be linked to downstream metabolic and inflammatory disturbance. In the present study, two graded Pb acetate concentrations (0.05% and 0.2%) were used as experimental toxicology exposures to assess dose-dependent mechanistic effects rather than to reproduce a single quantitative human environmental exposure level.

The placenta is a plausible target in this setting because it acts not only as a transport interface between mother and fetus, but also as a temporary reservoir for toxic metals during pregnancy. Among placental metal-handling proteins, DMT1 is of particular interest because it participates in the transport of divalent metals across placental cells [16-18]. MT2A, by contrast, is a cysteine-rich intracellular metal-binding protein that may contribute to metal sequestration and detoxification [16,19,20]. Examining the interaction between Pb and these proteins may therefore help explain how placental Pb accumulation occurs and how it may be connected to downstream metabolic and inflammatory disturbance. In the present study, two graded concentrations of Pb acetate (0.05% and 0.2%) were used as experimental toxicology exposures to examine dose-dependent mechanistic effects rather than to mimic a single quantitative level of human environmental exposure.

Accordingly, this study was designed to evaluate whether prenatal Pb exposure promotes placental Pb accumulation and maternal metabolic-inflammatory alterations in pregnant rats, as reflected by blood glucose, MG, and TNF- α , and whether *in silico* analysis of DMT1 and MT2A supports a plausible placental

metal-handling mechanism underlying these changes. By combining *in vivo* biomarker assessment with *in silico* metal-binding analysis, this study aimed to provide mechanistic insight into placental Pb handling and its relevance to gestational metabolic dysfunction. Although the biomarkers assessed are relevant to GDM-associated pathophysiology, this study does not directly diagnose GDM.

Materials and methods

Study design

This experimental study employed a Post-Test with Control Group Design. It was structured as a Completely Randomized Design (CRD) according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [21]. Nine experimental groups were included: Three control groups without Pb exposure and six treatment groups exposed to $\text{Pb}(\text{CH}_3\text{COO})_2$ (Pb acetate). Pb exposure was applied at two concentrations, 0.05% and 0.2% Pb acetate (w/v), over three gestational exposure durations: Up to day 7 of gestation; up to days 10 - 14 of gestation; and up to day 21 of gestation or at parturition.

Experimental animals and sample size

The experimental animals were white rats (*Rattus norvegicus*) of the Sprague-Dawley strain, both male and female, aged 2 - 3 months, with body weights ranging from 120 to 180 g. All animals were healthy and exhibited regular activity. Rats were obtained from the Veterinary Research and Investigation Center (BPPV), Banjarbaru, Indonesia. A total of 36 female rats and 18 male rats were used. The number of female rats was calculated using the Federer formula. Because this study included 6 treatments based on exposure type and duration, the minimum sample size calculated using the Federer formula was 27 female rats. Furthermore, due to the possibility of death during treatment, the number of subjects was increased by 20%, resulting in a total of 36 female white rats, divided into 4 female rats per treatment group. Each experimental group consisted of 4 pregnant rats ($n = 4$ per group), serving as the experimental units for the *in vivo* analyses. No formal a priori power calculation was performed. The sample size was determined based on the exploratory nature of the study, ethical considerations regarding animal use, feasibility of the nine-group design, and allowance for

potential attrition during the experimental period. This approach was used to balance methodological feasibility and animal welfare.

Housing, husbandry, and acclimatization

All rats underwent a 7-day acclimatization period prior to the experiment. During this period, animals were housed under standard laboratory conditions and received the same diet and drinking water, consisting of C-05 standard pellets and distilled water *ad libitum*. Environmental conditions were kept consistent throughout the study to minimize external variability [8].

Induction of pregnancy

Following acclimatization, female rats underwent superovulation induction to synchronize estrous cycles prior to mating. Superovulation was induced via intraperitoneal (IP) injection of Pregnant Mare Serum Gonadotropin (PMSG) followed by Human Chorionic Gonadotropin (HCG) at a dose of 10 IU (0.2 mL). PMSG was administered first, followed by HCG 48 h later. After hormonal induction, female rats were maintained for 3 days and subsequently co-housed with male rats at a 1:2 ratio, except in one cage with a 1:1 ratio. Co-housing was conducted for one overnight period. Pregnancy was confirmed the following morning through vaginal smear examination under a light microscope. The presence of a vaginal copulatory plug, observed as a whitish-gray mass within or around the vaginal opening, was used as confirmation of successful mating. The day on which the copulatory plug was detected was defined as gestational day 1 (GD1) [22].

Treatment allocation and exposure period

Pb acetate was administered continuously via drinking water *ad libitum* from GD1 until the endpoint of each experimental group. Pb acetate exposure may be expressed as estimated daily intake using the following formula: estimated daily intake (mg/kg bw/day) = drinking-water concentration (mg/L) $\times$ daily water intake (L/day) / body weight (kg). Where relevant, conversion to elemental Pb should be based on the molecular-weight fraction of Pb in the specific lead acetate form used. Pregnant female rats were randomly assigned to 9 experimental groups based on Pb exposure

concentration and exposure duration. The control groups received distilled water without Pb acetate. The treatment groups received Pb acetate at concentrations of 0.05% (w/v) or 0.2% (w/v). In the D1 groups, exposure was given continuously from GD1 to GD7, and animals were euthanized at the end of GD7. In the D2 groups, exposure was given continuously from GD1 to GD14, and animals were euthanized at the end of GD14. In the D3 groups, exposure was continuous from GD1 to GD21 or until parturition, after which the animals were euthanized, and biological samples were collected. Each experimental group consisted of 4 pregnant rats ($n = 4$ per group). The selected Pb acetate concentrations of 0.05% (w/v) and 0.2% (w/v) in drinking water were used as graded experimental toxicological exposures to generate a measurable exposure gradient. The lower concentration was intended to represent a biologically active exposure sufficient to detect early metabolic disturbance, whereas the higher concentration was included to evaluate whether a greater toxicological burden would produce more pronounced placental accumulation and metabolic-inflammatory changes. These concentrations were selected for mechanistic dose-response assessment and were not intended to represent a direct quantitative equivalent of a single human environmental exposure level.

Blood and placental sample collection

Blood and placental samples were collected through surgical procedures under ketamine-xylazine anesthesia administered intraperitoneally (IP). Blood samples were obtained via intracardiac puncture using a 3 mL syringe and collected into Ethylenediaminetetraacetic Acid (EDTA)-containing tubes. Placental tissue was also collected following surgery. Both blood and placental samples were washed with 20% trichloroacetic acid (TCA) and fixed in phosphate buffer solution (pH 7). The tissues were then cut into small pieces and homogenized using a mortar until a uniform liquid homogenate was obtained. Placental Pb concentrations were measured only in the D2 groups. Placental tissue at the D1 endpoint was insufficient in size for reliable analysis, whereas placental tissue at the D3 endpoint was generally unavailable after delivery because the placenta is typically expelled and may be consumed by the dam.

Therefore, placental Pb analysis was restricted to the D2 time point, and temporal comparisons of placental accumulation across all exposure durations were not possible within this design.

Measurement of placental Pb levels

Placental Pb concentrations were determined using a spectrophotometric method as previously described by Suhartono *et al.* [6]. Sample preparation involved reaction with citric acid, potassium thiocyanate (KSCN), ammonia solution, and sodium sulfide (Na_2S). Placental Pb concentration was determined spectrophotometrically after reaction with citric acid, potassium thiocyanate, ammonia solution, and sodium sulfide, with absorbance measured at 430 nm.

Measurement of blood glucose levels

Blood glucose levels were measured using an Easy Touch Blood Glucose Test Meter with manufacturer-calibrated test strips. The glucometer was calibrated according to the code of the test strips used. A drop of blood was applied to the test strip, and glucose levels were recorded after 6 seconds. Results were expressed in mg/dL [23].

Measurement of blood MG levels

Blood MG levels were measured according to the method described by Suhartono *et al.* [8]. The principal procedural steps, reagent concentrations, incubation conditions, and absorbance wavelengths used in the present study are described below to facilitate reproducibility. Briefly, 25 μL of homogenate was reacted with 350 μL 2,4-Dinitrophenylhydrazine (DNPH) solution (0.1% DNPH in 2 N HCl) and 2.125 mL distilled water, followed by incubation at 37 °C for 15 min. Subsequently, 1.5 mL of 10% NaOH was added, and absorbance was measured at 576 nm (A_1). Further processing involved incubation with DNPH, precipitation using 20% TCA, centrifugation at 1,400 rpm, repeated washing with ethanol-ethyl acetate, and final incubation with 9 M urea. After centrifugation, absorbance was measured at 390 nm (A_2).

Measurement of Blood TNF- α Levels

Blood TNF- α concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. A TNF- α

ELISA kit (NB-E30635, Novatein Biosciences, Massachusetts, USA) was used, with an assay range of 15.6 - 1,000 pg/mL and an analytical sensitivity of 1.0 pg/mL. Absorbance was measured at 450 nm using a BMG LABTECH SPECTROstar Nano microplate reader [24].

***In silico* molecular docking analysis**

In silico metal-binding site prediction and modeling were performed to explore the potential interaction of Pb^{2+} with two placental metal-handling proteins, namely DMT1/ Solute Carrier Family 11 member 2 (SLC11A2) and MT2A. The three-dimensional structures of DMT1 and MT2A were obtained from the Protein Data Bank (PDB IDs 9F6N and 2MHU, respectively) [25]. Prior to analysis, receptor structures were prepared in UCSF Chimera 1.16 by removing non-essential molecules, including water and irrelevant heteroatoms, and adding polar hydrogens and assigning partial charges. Metal-binding prediction/modeling was then performed using the MIB2 server (<https://combio.life.nctu.edu.tw/MIB2/>). MIB2 is a template-based metal ion-binding site prediction and modeling platform that uses fragment transformation, structural comparison with known metal-binding templates, and a metal ion type-specific scoring function to predict probable metal-binding residues and modeled ion positions. MIB2 was selected because the objective of the present analysis was the prediction/modeling of metal-ion-binding sites rather than conventional small-molecule docking. MIB2 is a template-based metal ion-binding site prediction and modeling server that applies an expanded metal-binding residue template library and a metal ion type-specific scoring function to identify probable binding residues and modeled ion positions. Therefore, the output score was interpreted as a predicted metal-binding support score/probability rather than a classical thermodynamic docking free energy. Pb^{2+} was analyzed against both proteins, whereas Mn^{2+} for DMT1 and Cd^{2+} for MT2A were used as comparator/native-reference metals. The highest-scoring, biologically plausible interactions involving residues within or near known metal-handling regions were selected for descriptive comparison. Visualization of predicted binding sites and bond distances was performed using UCSF Chimera 1.16 [26].

Statistical analysis

Data analysis was conducted in a stepwise manner. Descriptive analyses of placental Pb, blood glucose, $\text{TNF-}\alpha$, and MG levels were performed and presented as tables, graphs, or figures in Microsoft Excel 2019 for Windows 11. Statistical analysis was performed using SPSS version 27. Data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variance was evaluated using Levene's test. Outcomes that met parametric assumptions were analyzed using two-way ANOVA to assess the main effects of Pb treatment and exposure duration and their interaction. When overall effects were significant, Tukey's post hoc test was used for pairwise comparisons. For outcomes that did not satisfy parametric assumptions, a non-parametric factorial approach appropriate for independent-group data was applied, followed by adjusted pairwise post hoc comparisons. Placental Pb concentration, measured at a single gestational time point, was analyzed separately across treatment groups using one-way analysis, with parametric or nonparametric testing selected based on data distribution and variance characteristics.

Ethical approval

All experimental procedures involving animals were reviewed and approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, Universitas Lambung Mangkurat (FKIK ULM). This study was declared ethically feasible as documented in the Ethical Clearance Certificate No. 132/KEPK-FKIK ULM/ECNI/2025. All animal handling, treatment, and experimental procedures were conducted in accordance with relevant institutional guidelines and regulations, and were reported in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines to ensure ethical integrity and transparency in animal research.

Results and discussion

Placental Pb concentration was assessed at the D2 time point. The mean placental Pb level was 0.150 ± 0.010 mg/g in the control group, 0.037 ± 0.017 mg/g in T1, and 1.044 ± 0.052 mg/g in T2 (**Table 1**). Shapiro-Wilk testing indicated that the data were not normally distributed ($P = 0.001$); therefore, group differences were analyzed using the Kruskal-Wallis test. Placental

Pb levels differed significantly among treatment groups ($P = 0.007$). Pairwise comparisons showed significant differences between C and T1, C and T2, and T1 and T2 (all $p < 0.05$), with the highest placental Pb level observed in T2.

These findings indicate that placental Pb levels differed significantly among treatments. Exposure to Pb at a concentration of 0.2% significantly increased placental Pb levels compared with 0.05% Pb exposure. This finding indicates that higher Pb concentrations are associated with greater placental accumulation of Pb. The observed increase in placental Pb levels can be attributed to the placenta's strong tendency to accumulate heavy metals such as Pb as part of its protective function for the fetus. Recent studies have demonstrated that the placenta contains numerous metal-binding proteins, including metallothioneins and other proteins with reactive sulfhydryl groups, which actively bind divalent cations such as Pb [27-29]. Through this mechanism, a substantial proportion of Pb entering the maternal circulation is retained within trophoblastic tissue before reaching the fetus.

A biologically plausible explanation for this pattern is that Pb may enter placental tissue via pathways normally involved in divalent metal transport, then be retained by intracellular metal-binding mechanisms [30,31]. In addition, the placenta contains multiple sulphhydryl-rich proteins and detoxification systems that may initially buffer incoming metal ions. However, as exposure burden increases, these buffering systems may become less efficient, leading to greater net accumulation in placental tissue [32-34]. Although the present study did not directly measure placental transporter expression, metallothionein induction, or oxidative damage in placental cells, the observed placental Pb pattern is consistent with previous reports describing placental metal retention during toxic exposure. Accordingly, the present findings should be interpreted as evidence of altered placental Pb handling under higher maternal Pb exposure rather than as direct proof of a specific transporter or sequestration mechanism [35-37].

Table 1 Placental Pb level in different group of treatments.

Group of treatments	Placenta Pb Level (mg/g)	Kruskal wallis test result	
		<i>p</i> Value	Interpretation
C	0.150 ± 0.010	0.007	Statistically Significant
T1	0.037 ± 0.017 ^a		
T2	1.044 ± 0.052 ^{b,c}		

Data are presented as mean ± SD; n = 4 dams per treatment group, with one placental homogenate analyzed per dam. Statistically significant between ^a C vs T1; ^b C vs T2; and ^c T1 vs T2.

Blood glucose concentrations were lowest in the control group and highest in T2 across exposure durations (**Figure 1**). The data were normally distributed ($p = 0.204$) and homogeneous ($p = 0.132$); therefore, they were analyzed using two-way ANOVA. No significant interaction between treatment and exposure duration was observed ($p = 0.848$), indicating that the treatment-related pattern of blood glucose change was broadly similar across the tested gestational

endpoints. A significant main effect of treatment was identified ($p < 0.001$). In the duration-adjusted model, the estimated mean blood glucose level in T2 was 62.333 mg/dL higher than that in the control group. Post hoc analysis showed significant differences between C and T2 ($p < 0.001$) and between T1 and T2 ($p = 0.019$), whereas the difference between C and T1 was not statistically significant ($p = 0.071$).

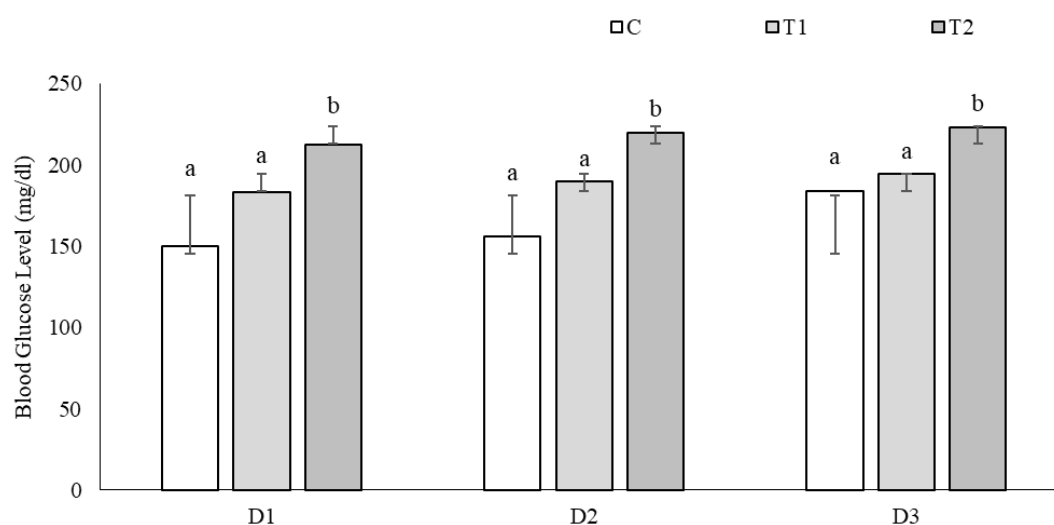


Figure 1 Blood glucose levels in the control and Pb-exposed groups across different exposure durations. Data are presented as mean $\pm$ SD. Bars with different lowercase letters indicate significant differences among treatment groups ($p < 0.05$). C: control group; T1: 0.05% Pb exposure group; T2: 0.2% Pb exposure group; D1: GD1 - GD7; D2: GD1 - GD14; D3: GD1 - GD21 or parturition; $n = 4$ pregnant rats per group.

Maternal blood glucose levels were consistently higher in Pb-exposed animals, particularly in the high-exposure group. This finding indicates that prenatal Pb exposure was associated with disruption of maternal glucose homeostasis in the present rat model. Mechanistically, Pb disrupts glucose metabolism by damaging β cells, leading to reduced insulin secretion and impaired glucose sensing [38]. High-dose Pb exposure has been shown to suppress the expression of Glucose Transporter-2 (GLUT-2) [39] and pancreatic and duodenal homeobox-1 (PDX-1) [40], both of which are critical regulators of glucose homeostasis. Disruption of these molecules leads to hyperglycemia [41,42]. Because the study did not include direct assessment of insulin, oral glucose tolerance, pancreatic histology, or insulin resistance indices, the observed glucose alteration should be interpreted as evidence of gestational metabolic disturbance rather than direct diagnosis of GDM.

Pb exposure can also disturb glucose regulation through oxidative stress, mitochondrial dysfunction, inflammatory activation, and impairment of pathways involved in insulin action [43-46]. Inflammatory mediators such as TNF- α , which were also elevated in the present study, have been linked in other models to reduced insulin signaling efficiency and decreased peripheral glucose utilization. In addition, Pb-related

cellular stress has been reported to affect pancreatic and hepatic metabolic regulation [15,47-51]. However, these mechanisms were not directly measured here. Therefore, the current data are more appropriately interpreted as showing that Pb exposure was associated with hyperglycemia-related biomarker changes during pregnancy, with possible relevance to GDM-associated pathophysiology, rather than confirming a diabetogenic diagnosis.

With respect to mechanism, the present data are more directly compatible with impaired maternal metabolic regulation and possible insulin resistance than with primary β -cell failure. This interpretation is based on the combined elevation of blood glucose and TNF- α , together with the absence of direct pancreatic functional measurements. Nevertheless, β -cell dysfunction remains a plausible contributory pathway reported in previous literature. Further studies, including insulin measurement, HOMA-IR, pancreatic histology, and glucose tolerance testing, are required to distinguish more clearly between these potential mechanisms.

MG levels in the control and T1 groups remained around 101% across exposure durations, whereas higher values were observed in T2, particularly at D1 (103.2%) and D3 (102.1%) (**Figure 2**). Because the MG data were neither normally distributed nor homogeneous (both $p < 0.001$), a nonparametric analysis was performed.

Friedman testing showed significant differences across exposure durations within the control group ($p = 0.024$) and T2 ($p = 0.018$), but not within T1 ($p = 0.223$). Pairwise comparisons indicated a significant difference between D1 and D2 in the control and T2 groups, whereas D1 vs D3 and D2 vs D3 were not statistically significant.

The increase in MG, particularly in the high-exposure group, suggests that Pb exposure was associated with enhanced carbonyl stress during pregnancy. MG is a reactive dicarbonyl compound generated mainly from glycolytic intermediates, and increased MG is commonly interpreted as an indicator of dysregulated glucose metabolism and carbonyl imbalance [52-54]. In the present study, the MG pattern was directionally consistent with the blood glucose findings, supporting the possibility that Pb exposure contributed to a broader disturbance in maternal metabolic homeostasis [41,43,44].

A plausible explanation is that hyperglycemia-related metabolic overload and oxidative stress together may favor increased MG formation from triose phosphate intermediates [56-59]. In addition, impaired glutathione-dependent detoxification of MG has been

described in other experimental settings and could further contribute to MG accumulation. Previous studies have implicated reduced activity of the glyoxalase system, including glyoxalase-1 (GLO-1), in conditions of oxidative stress and toxic exposure [60,61]. However, neither glyoxalase activity nor glutathione status was directly measured in the present study. Therefore, pathways involving GLO-1 suppression or impaired MG detoxification should be regarded as literature-supported mechanistic possibilities rather than direct findings of this experiment.

MG may also have pathophysiological relevance beyond being a passive biomarker. In other biological systems, MG has been associated with advanced glycation end-product formation, amplification of oxidative stress, and inflammatory signaling. Within that framework, the elevated MG observed here may represent one component of a broader metabolic-inflammatory disturbance induced by Pb exposure during pregnancy. Nonetheless, because downstream AGE-related signaling pathways were not directly evaluated, this interpretation should remain cautious and mechanistically inferential.

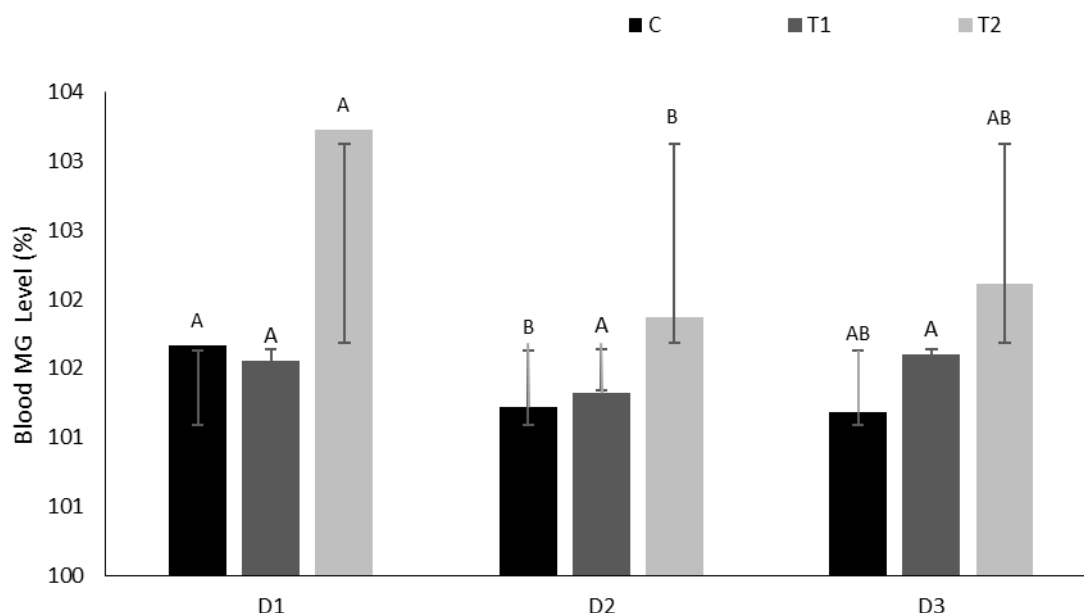


Figure 2 Blood methylglyoxal (MG) levels in the control and Pb-exposed groups across different exposure durations. Data are presented as mean $\pm$ SD. Bars with different uppercase letters indicate significant differences among exposure durations within the same treatment group ($p < 0.05$). C: control group; T1: 0.05% Pb exposure group; T2: 0.2% Pb exposure group; D1: GD1 - GD7; D2: GD1 - GD14; D3: GD1 - GD21 or parturition; $n = 4$ pregnant rats per group.

TNF- α concentrations were highest in T2 and lowest in the control group across exposure durations (**Figure 3**). The data were normally distributed ($p = 0.200$) but not homogeneous ($p = 0.029$); therefore, non-parametric analysis was used. Friedman testing showed significant differences across exposure durations within C ($p = 0.018$), T1 ($p = 0.039$), and T2 ($p = 0.039$). However, post hoc comparisons showed significant pairwise differences only in the control group. In the control group, all pairwise comparisons among exposure durations (D1 vs D2, D1 vs D3, and D2 vs D3) were significant, whereas no significant pairwise differences were detected in T1 or T2.

The increase in TNF- α in Pb-exposed animals indicates that maternal Pb exposure was accompanied by inflammatory activation [62,63]. TNF- α is a key pro-inflammatory cytokine with established relevance to gestational metabolic disturbance and insulin resistance [64]. In the present study, the TNF- α pattern paralleled

the elevation in blood glucose and, to a lesser extent, MG, supporting the interpretation that inflammatory processes may be involved in the metabolic effects associated with Pb exposure during pregnancy [65,66].

One plausible explanation is that Pb-induced oxidative stress may activate inflammatory signaling pathways, thereby increasing TNF- α production. Previous studies have implicated pathways such as NF- κ B and MAPK in this context [67,68]. These pathways provide a biologically coherent framework for linking Pb exposure with inflammatory mediator release and altered metabolic regulation [68-70]. However, neither NF- κ B activation, MAPK signaling, nor other intracellular inflammatory pathways were measured directly in the present study. Accordingly, these mechanisms should be interpreted as literature-based explanations consistent with the TNF- α findings, rather than as pathways experimentally demonstrated here.

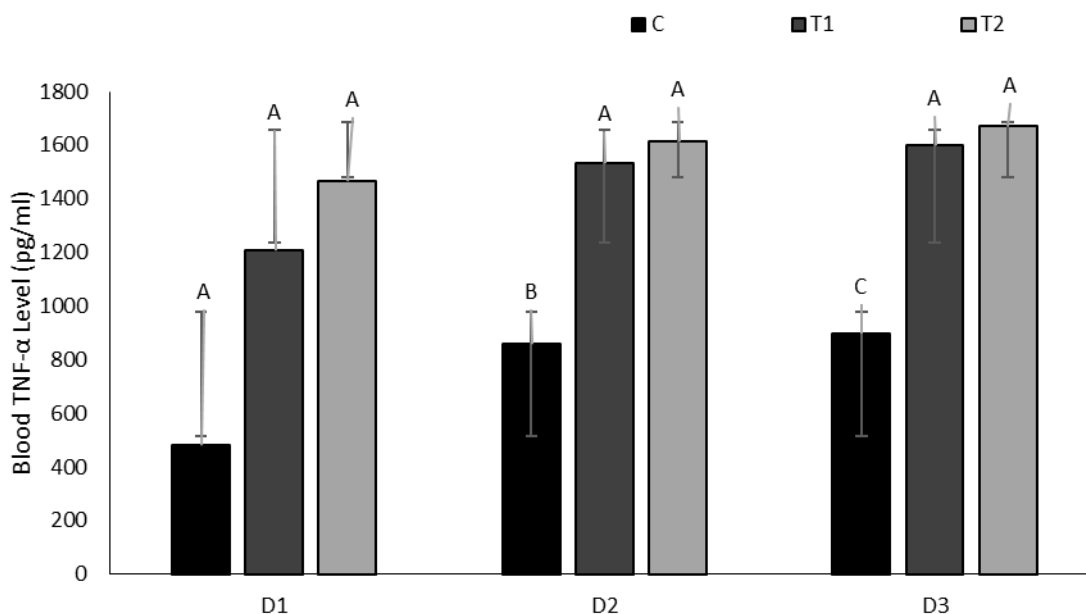


Figure 3 Blood TNF- α levels in the control and Pb-exposed groups across different exposure durations. Data are presented as mean $\pm$ SD. Bars with different uppercase letters indicate significant differences among exposure durations within the same treatment group ($p < 0.05$). C: Control group; T1: 0.05% Pb exposure group; T2: 0.2% Pb exposure group; D1: GD1 - GD7; D2: GD1 - GD14; D3: GD1 - GD21 or parturition; $n = 4$ pregnant rats per group.

A broader mechanistic interpretation may explain the concurrent increase in blood glucose, MG, and TNF- α after Pb exposure. Pb is known to disrupt cellular redox homeostasis through calcium mimicry, mitochondrial dysfunction, and enhanced reactive

oxygen species generation, thereby promoting oxidative stress. Increased oxidative stress can activate NF- κ B-dependent inflammatory signaling, which contributes to elevated TNF- α expression. With respect to the mechanism of Pb-induced diabetogenic change, the

present findings are more directly compatible with insulin resistance than with primary β -cell failure. This interpretation is supported by the concurrent elevation of TNF- α , which is mechanistically linked to impaired insulin signaling and reduced peripheral glucose utilization. Nevertheless, β -cell damage remains a plausible contributory pathway reported in previous studies. Because this study did not assess pancreatic histology, circulating insulin, or insulin resistance indices such as HOMA-IR, the relative.

Hyperglycemia and oxidative stress together may further increase MG formation from glycolytic intermediates, while impaired glutathione-dependent glyoxalase activity may reduce MG detoxification. Accumulated MG can then enhance carbonyl stress and AGE-related inflammatory signaling, creating a self-amplifying cycle linking oxidative stress, inflammation, and metabolic dysregulation. Although these pathways were not directly measured in the present study, they provide a biologically plausible framework for interpreting the observed biomarker profile and its relevance to GDM-associated pathophysiology.

Although this integrative interpretation is biologically plausible and supported by prior literature, it is important to emphasize that the present study did not directly measure oxidative stress markers, mitochondrial function, glyoxalase activity, NF- κ B signaling, MAPK activation, or insulin signaling intermediates. Therefore, these pathways should be understood as mechanistic hypotheses consistent with the observed biomarker profile, rather than as direct experimental findings. Within the present design, the data support the presence of Pb-associated gestational metabolic-inflammatory alteration, but not a complete molecular pathway model. Importantly, while the measured biomarkers are relevant to GDM-associated pathophysiology, this study does not directly diagnose GDM. No oral glucose tolerance testing, insulin secretion assessment, insulin resistance index, or functional fetal metabolic outcome was included. Accordingly, the findings are more appropriately framed as evidence of metabolic-inflammatory biomarker changes during pregnancy that may be relevant to gestational metabolic dysfunction.

Also, across the measured outcomes, the findings consistently indicate that Pb exposure concentration had a more pronounced effect than exposure duration in the

present experimental design. The higher Pb concentration was associated with greater placental accumulation and more pronounced alterations in metabolic-inflammatory biomarkers, whereas variation across exposure duration produced a less consistent pattern. This observation should not be interpreted as evidence that exposure duration is biologically unimportant, but rather as indicating that, within the tested gestational windows and outcome panel, concentration was the stronger determinant of the observed response profile.

An in-silico metal-binding analysis was performed using MIB2 to compare the predicted interaction of Pb with MT2A and DMT1 (**Table 2, Figures 4 and 5**). Because MIB2 does not provide a universal threshold for absolute binding strength, the scores were interpreted comparatively within each target protein and against the selected comparator/native-reference metals. In MT2A, the predicted Pb-MT2A score was 1.825, compared with 1.695 for Cd-MT2A. In DMT1, the predicted Pb-DMT1 score was 6.524, compared with 5.474 for Mn-DMT1. Within this comparative modeling framework, Pb showed a higher predicted interaction score with DMT1 than with MT2A [71].

At the residue level, the Cd-MT2A complex was associated with Cys15, Cys26, Cys21, and Cys7, with interaction distances of 3.538, 4.147, 3.370, and 3.384 Å, respectively. The Pb-MT2A complex involved Cys7 and Cys5, with interaction distances of 2.750 and 2.946 Å. In DMT1, the Mn-DMT1 complex involved Asp122 and Asp340, with interaction distances of 1.894 and 1.269 Å, whereas the Pb-DMT1 complex involved Asp122 and Asn340, with interaction distances of 2.739 and 1.746 Å (**Table 2 and Figures 4(a) and 4(b)**). These shorter distances suggest a closer predicted interaction between Pb and the sulfur-containing residues of MT2A, consistent with the known metal-binding role of the cysteine-rich metallothionein domain [72,73].

In the DMT1 model, the comparator Mn-DMT1 complex was predicted to interact with Asp122 and Asp340, whereas the Pb-DMT1 complex was predicted to interact with Asp122 and Asn340 (**Table 2, Figures 5(a) and 5(b)**). This indicates that the Pb-predicted binding pattern retained Asp122 but differed at the second interacting residue from the Mn comparator model. Given that DMT1 is a human SLC11-family divalent metal transporter involved in metal ion uptake

and distribution, the markedly higher MIB2 score and residue-level interaction pattern indicate that Pb is predicted to interact more strongly with DMT1 than with MT2A, while MT2A may still serve as an intracellular Pb-binding protein after Pb enters placental cells [74].

Table 2 Predicted metal-binding scores and interacting residues of MT2A and DMT1 based on MIB2 analysis.

Target protein	Metal complex	MIB2 Score	Interacting residues	Binding distance (Å)
MT2A	Cd-MT2A	1.695	Cys15	3.538
			Cys26	4.147
			Cys21	3.370
			Cys7	3.384
MT2A	Pb-MT2A	1.825	Cys7	2.750
			Cys5	2.946
DMT1	Mn-DMT1	5.474	Asp122	1.894
			Asp340	1.269
DMT1	Pb-DMT1	6.524	Asp122	2.739
			Asn340	1.746

Cys: Cysteine, Asp: Aspartate; Asn: Asparagine; MT2A: Metallothionein-2A; DMT1: Divalent Metal Transporter 1.

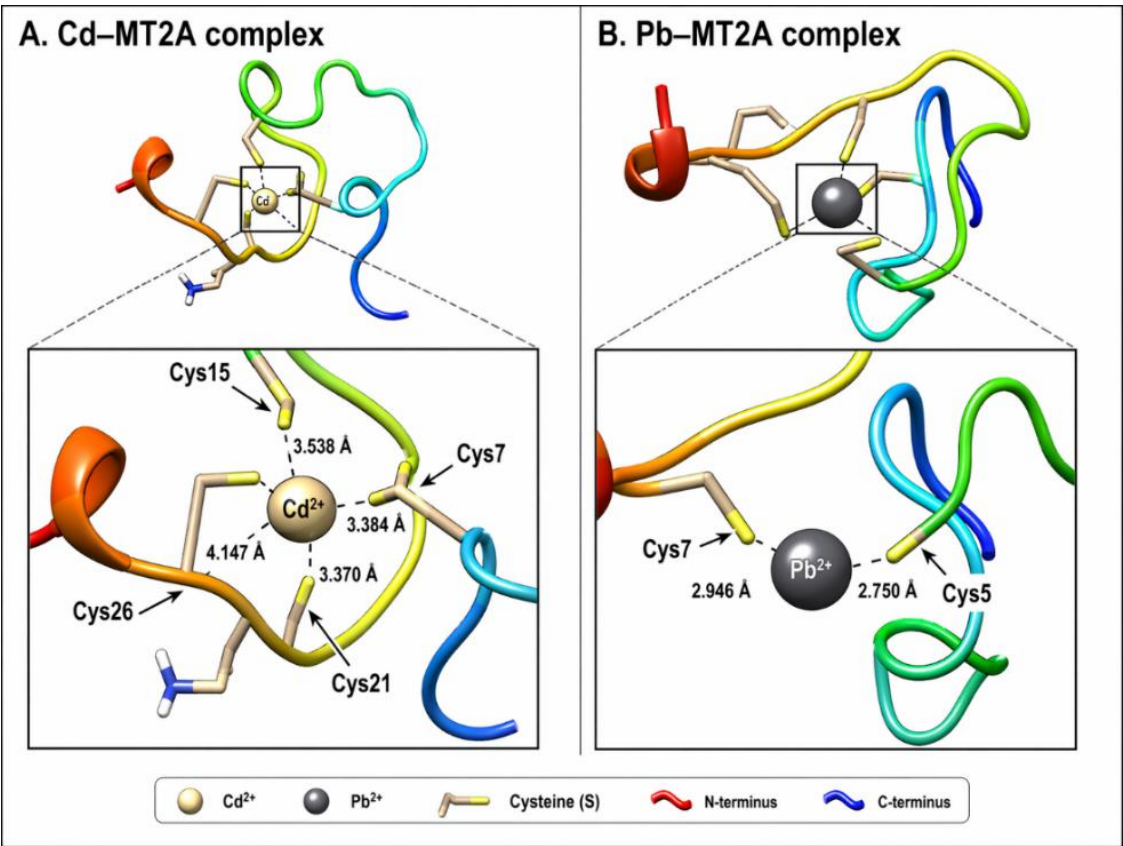


Figure 4 Predicted binding sites of (a) Cd and (b) Pb in MT2A based on MIB2 analysis. The figure illustrates the localization of metal ions relative to key interacting amino acid residues. In MT2A, Pb was predicted to interact mainly with cysteine-rich residues, particularly Cys5 and Cys7 within or near the divalent metal transport-associated region.

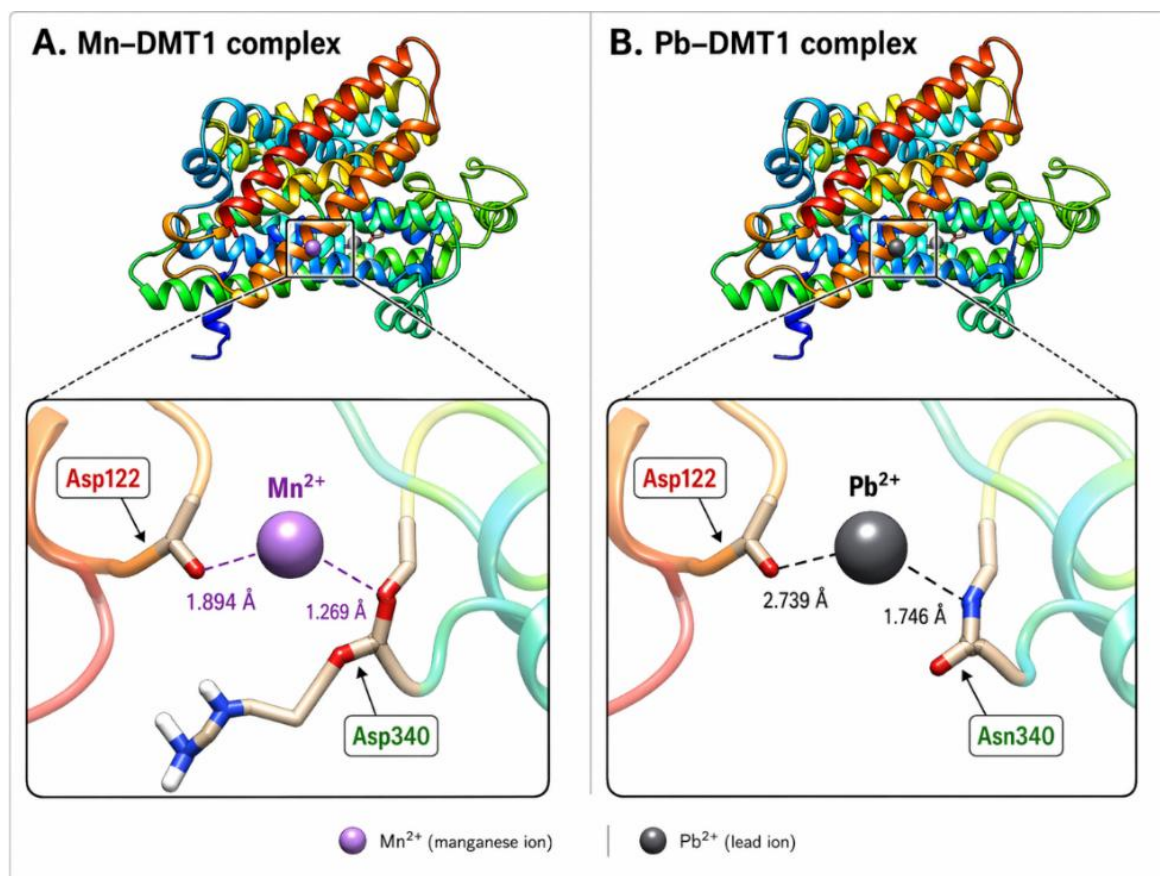


Figure 5 Predicted binding sites of (a) Mn and (b) Pb in DMT1 based on MIB2 analysis. The figure illustrates the localization of metal ions relative to key interacting amino acid residues. In DMT1, Pb was predicted to interact with Asp122 and Asn340 within or near the divalent metal transport-associated region.

These *in silico* findings strengthen the biological interpretation of the *in vivo* results by providing a plausible framework for placental Pb handling. The markedly higher Pb-DMT1 MIB2 score (6.524) compared with Mn-DMT1 (5.474), together with the interaction with functionally relevant residues such as Asp122 and Asn340, supports the possibility that Pb may enter placental cells, at least in part, through transporter-related pathways involved in divalent metal handling [74]. This supports the possibility that DMT1 contributes to placental Pb uptake, thereby facilitating Pb accumulation in placental tissue. In contrast, the Pb-MT2A score (1.825) was lower in absolute terms but still higher than Cd-MT2A (1.695), and the interaction of Pb with Cys5 and Cys7 at relatively short distances (2.946 and 2.750 Å) suggests that MT2A may participate in intracellular Pb sequestration after metal entry. Taken together, this two-step interpretation—transporter-facilitated entry through DMT1 followed by intracellular retention or buffering by MT2A—offers a

plausible explanation for the marked increase in placental Pb concentration observed in the higher-exposure group. In turn, persistent placental Pb accumulation may contribute to trophoblast stress, inflammatory signaling, and dysregulated maternal metabolic homeostasis, thereby supporting the observed increases in blood glucose, methylglyoxal, and TNF- α . Because elevated glucose reflects disturbed maternal metabolic regulation, MG indicates carbonyl stress associated with impaired glucose handling, and TNF- α represents inflammatory activation linked to insulin resistance, the combined *in vivo* and *in silico* findings support the interpretation that placental Pb accumulation may contribute to biological pathways relevant to GDM risk. Nevertheless, because the computational analysis was predictive and no placental expression or functional assays of DMT1 or MT2A were performed, these findings should be interpreted as supportive mechanistic evidence rather than direct proof of causality or binding affinity [71,75].

In contrast, although the absolute Pb-MT2A score was lower (1.825), it remained higher than that of Cd-MT2A (1.695), and the predicted interaction of Pb with Cys5 and Cys7 at relatively short distances is biologically consistent with intracellular sequestration by cysteine-rich metal-binding proteins. Taken together, these results support a biologically plausible two-step mechanism of placental Pb handling, involving transporter-mediated uptake through DMT1 followed by intracellular retention or buffering by MT2A. Such a mechanism is consistent with the present experimental findings, which show that higher Pb concentration led to greater placental Pb accumulation and was accompanied by increased maternal glucose, MG, and TNF- α levels. Because elevated glucose reflects disturbed maternal metabolic homeostasis, MG indicates carbonyl stress associated with dysregulated glucose metabolism, and TNF- α represents inflammatory activation linked to insulin resistance, the combined *in vivo* and *in silico* findings support the interpretation that placental Pb accumulation may contribute to metabolic and inflammatory pathways relevant to GDM risk. Since MIB2 provides a predictive metal-binding score rather than a true free-energy binding estimate, these docking findings should be interpreted as supportive mechanistic evidence rather than definitive proof of binding affinity [71,74].

This study has several limitations that should be acknowledged. First, the findings were derived from an experimental pregnant rat model; therefore, direct extrapolation to human pregnancy should be made with caution. Second, the biomarker panel was limited to blood glucose, MG, and TNF- α . Although these markers were selected to reflect glycaemic disturbance, carbonyl stress, and inflammatory activation, they do not fully capture the broader metabolic phenotype relevant to GDM, particularly insulin resistance, oxidative stress status, and fetal functional outcomes. Third, placental Pb concentration was assessed only at the D2 endpoint because placental tissue at D1 was insufficient for reliable analysis, and tissue at D3 was generally unavailable after delivery. Consequently, temporal variation in placental Pb accumulation across all exposure durations could not be evaluated.

An additional limitation is that no direct measurements of circulating insulin, HOMA-IR, oxidative stress markers, placental transporter

expression, or metallothionein response were performed. Likewise, the *in silico* analysis was predictive and lacked functional validation. Therefore, the proposed mechanistic pathways should be interpreted as biologically plausible models rather than definitive causal pathways. While the biomarker profile observed here is relevant to GDM-associated pathophysiology, this study did not apply clinical diagnostic criteria for GDM and does not directly diagnose GDM.

Future studies should incorporate direct molecular analyses of insulin signaling, oxidative stress, placental transporter expression, and metallothionein responses, together with longitudinal assessment of placental Pb accumulation throughout gestation. Also, another limitation is that this study did not include direct measurements of circulating insulin, insulin resistance indices such as HOMA-IR, oxidative stress markers, or placental expression of relevant transporters and metal-binding proteins. Inclusion of these parameters in future studies would allow more precise discrimination between insulin resistance and β -cell-related mechanisms and would strengthen the mechanistic evaluation of oxidative stress-mediated placental Pb handling. Further translational studies integrating experimental findings with human epidemiological and clinical data are needed to clarify the relevance of Pb-induced placental and metabolic disturbances to GDM in humans. Such efforts may contribute to improved risk assessment, preventive strategies, and public health policies to reduce Pb exposure during pregnancy and minimize its adverse effects on maternal metabolic health.

Conclusions

In conclusion, prenatal Pb exposure promoted placental Pb accumulation and was accompanied by increased maternal blood glucose, MG, and TNF- α levels in pregnant rats. Within the present experimental design, the higher Pb concentration was associated with greater placental accumulation and more pronounced alterations in metabolic-inflammatory biomarkers than variation in exposure duration. These findings support the relevance of placental Pb handling to gestational metabolic disturbance in an experimental rat model. While the observed biomarker profile is relevant to

GDM-associated pathophysiology, this study does not directly diagnose GDM.

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Declaration of Generative AI in Scientific Writing

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CRedit Author Statement

Iskandar Iskandar: Conceptualization; Methodology; Writing - Original draft preparation. **Eko Suhartono:** Data curation; Visualization; Investigation; Supervision. **Rizmi Yunita:** Writing - Reviewing and Editing. **Didik Dwi Sanyoto:** Supervision; Software; Validation. **Isna Syauniah:** Writing - Reviewing and Editing; Supervision. **Hapsari Lintang Sekartaji:** Writing - Reviewing and Editing.

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