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Moringa Oleifera Supplementation for Reducing Heavy Metals Toxicity and Oxidative Stress in Pregnant Women: A Non Randomized Trial Study Protocol

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Submitted to: JMIR Research Protocols
on: February 27, 2025

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Abstract

Background: Heavy metals present in the environment, including lead, cadmium, and mercury, pose significant health risks to pregnant women and fetal development through food, water, and air contamination. Exposure to these metals has been linked to miscarriage, low birth weight (LBW), preterm birth, and developmental issues in children. The mechanism of oxidative stress, characterized by increased 8-OHdG and MDA levels, contributes to DNA damage, genomic instability, and adverse pregnancy outcomes. Additionally, DNA methylation changes induced by metal exposure may further exacerbate these risks. Certain micronutrients play a crucial role in heavy metal detoxification, and Moringa oleifera, a locally available plant rich in antioxidants and chelating compounds, has demonstrated protective effects against Hg, Pb, and Cd toxicity in experimental studies. However, intervention studies on pregnant women remain scarce.

Objective: This study protocol aims to evaluate the effect of Moringa Oleifera supplementation in reducing heavy metal toxicity and oxidative stress biomarkers, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and malondialdehyde (MDA), in pregnant women exposed to high levels of heavy metals.

Methods: A quasi-experimental, non-randomized pre-post test design is used. Pregnant women with elevated heavy metal levels, identified through initial screening, will be included in the intervention group, receiving Moringa Oleifera supplementation for two months. The control group will consist of women from similar geographical regions who will not receive the intervention. Primary outcomes will include changes in heavy metal concentrations, measured using inductively coupled plasma mass spectrometry (ICP-MS). Secondary outcomes will focus on reductions in oxidative stress biomarkers, measured via enzyme-linked immunosorbent assay (ELISA). Statistical analyses, including ANCOVA, will be used to adjust for baseline differences between the groups.

Results: The protocol anticipates that the intervention group will show a significant reduction in both heavy metal levels and oxidative stress biomarkers compared to the control group, suggesting the potential efficacy of Moringa oleifera in detoxifying heavy metals and reducing oxidative stress.This study will contribute to the understanding of Moringa Oleifera as a preventive intervention for pregnant women in regions with high environmental exposure to heavy metals, offering a natural, scalable public health solution to improve maternal and fetal health outcomes

Conclusions: This study will contribute to the understanding of Moringa Oleifera as a preventive intervention for pregnant

women in regions with high environmental exposure to heavy metals, offering a natural, scalable public health solution to improve maternal and fetal health outcomes. Clinical Trial: ANZCTR.org.au ACTRN12625000162415

(JMIR Preprints 27/02/2025:73201)
DOI: https://doi.org/10.2196/preprints.73201

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Moringa Oleifera Supplementation for Reducing Heavy Metals Toxicity and Oxidative Stress in Pregnant Women: A Non Randomized Trial Study Protocol

Abstract

Background: Heavy metals present in the environment, including lead, cadmium, and mercury, pose significant health risks to pregnant women and fetal development through food, water, and air contamination. Exposure to these metals has been linked to miscarriage, low birth weight (LBW), preterm birth, and developmental issues in children. The mechanism of oxidative stress, characterized by increased 8-OHdG and MDA levels, contributes to DNA damage, genomic instability, and adverse pregnancy outcomes. Additionally, DNA methylation changes induced by metal exposure may further exacerbate these risks. Certain micronutrients play a crucial role in heavy metal detoxification, and Moringa oleifera, a locally available plant rich in antioxidants and chelating compounds, has demonstrated protective effects against Hg, Pb, and Cd toxicity in experimental studies. However, intervention studies on pregnant women remain scarce.

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Trial Registration: ANZCTR.org.au ACTRN12625000162415

Keywords: Moringa oleifera; Heavy metal poisoning; Oxidative stress; Maternal health; detoxification; DNA damage

Introduction

Heavy metals exist in different environmental media such as water, air, and food, and can therefore lead to excessive human exposure through the contamination of food, drinking water, and air.[1,2] Exposure to several hazardous metals, including cadmium (Cd), mercury (Hg), and lead (Pb), is known to increase the risk of negative consequences on pregnancy, including miscarriage, premature births, and developmental issues in children.[3] Heavy metal levels in pregnant mothers correlate directly with adverse pregnancy outcomes, such as stillbirths, Low Birth Weight (LBW) and preterm labor.[4] Conversely, the exceed of essential metals including iron (Fe), zinc (Zn), copper (Cu), and selenium (Se) during pregnancy is linked to pregnancy outcome.[5,6]

According to data provided by the Makassar City Health Office, the number of cases of low birth weight (LBW) in Makassar was 717, 347, and 816 in 2021, 2022, and 2023, respectively. An initial investigation of pregnant women in Makassar revealed significantly elevated levels of heavy metals in their blood. The levels ranged from 1.35 to 385.00 μg/L for lead (Pb), and 0.55 to 135.00 μg/L for cadmium. These concentrations exceeded the reference values in various countries. A previous study [7] found that metals such as lead, cadmium, and mercury accumulate in the maternal blood, cord blood, and placenta, suggesting the need for preventive measures and public health to reduce exposure.

Oxidative stress, which refers to an imbalance between oxidation and anti-oxidation processes that can harm DNA, lipids, and proteins and cause cell death [8] , is one possible mechanism by which metal exposure can adversely affect fetal birth outcomes [9]. Experimental studies have shown that essential and non-essential metals play a role in oxidative stress [10,11]. Essential metals can undergo redox cycle reactions, which makes them reactive radical generators [12]. Non-essential metals such as Hg, As, Cd, have the ability to directly attach to protein sulfhydryl groups and reduce the levels of glutathione [13,14]. Multiple studies have demonstrated that exposure to metals can increase oxidative stress levels.[15–17]

DNA methylation, a type of epigenetic modification, is sensitive to environmental factors [18] and may be another mechanism that links metal exposure to adverse effects on birth. DNA hypermethylation or hypomethylation associated with genomic instability and altered gene expression [19,20] negatively impacts pregnancy outcomes [21], and in vivo and in vitro studies have shown that metal exposure induces global DNA methylation changes [22–26]. Human studies have also shown that prenatal exposure to toxic metals is associated with changes in global and specific gene DNA methylation.[15,21,27,28] Previous studies on pregnant women in Takalar, Indonesia showed that the average levels of Malondealdehyde (MDA) as a biomarker of DNA damage were 26.30 nmol/ml and 8-OHdG as a biomarker of oxidative stress were 47.62 ng/ml.[29]

Certain micronutrients have been identified for detoxification of heavy metals, such as zinc and cysteine.[30] Micronutrients may also act as enzyme cofactors or components of protein complexes (metalloenzymes) involved in DNA synthesis and repair, protection against oxidative damage, and DNA methylation [31–33]. Micronutrient supplements have been widely used to prevent complications during pregnancy, including those caused by environmental pollution leading to oxidative stress.[34–39]

One of the locally available natural resources in Indonesia that has been proven to be rich in micronutrients but has not been fully utilized by the community, including pregnant women, is Moringa oleifera leaves. Moringa leaves are rich in antioxidants such as flavonoids, phenolic acids, phytates, glucosinolates, and carotenoids, which work together to neutralize free radicals produced by heavy metals. The chelating property of this substance prevents the absorption of heavy metals into the body, while its phytate and glucosinolate content binds

heavy metals in the intestines, so reducing their accumulation and toxic effects in the body. Several animal studies have found that moringa leaves have protective effects against heavy metal toxicity, such as Hg [40,41], Pb [42,43], dan Cd [44,45]. Mishra also highlighted the ability of Moringa oleifera to detoxify heavy metals through the renal process and reduce oxidative stress.[46]

Research on interventions to mitigate the effects of heavy metal exposure during the prenatal period on DNA damage and oxidative stress in pregnant women in Indonesia remain scarce, making this study crucial. The majority of the studies examined were observational and primarily documented the consequences of heavy metal exposure during pregnancy. There is a considerable dearth of intervention trials targeting the reduction of heavy metal levels in pregnant women.

Administering Moringa oleifera leaf extracts for detoxification could address this issue by providing a preventative or therapeutic remedy. Previous research [47] has investigated the impact of combined exposure to several heavy metals. However, further investigation is required to understand the cumulative effect of several metals (such as lead, mercury, and arsenic) on the health of fetuses.

The objective of this study is to investigate the impact of Moringa oleifera on the combined detoxification of several metals, rather than concentrating on individual metal species. The expected outcome is intended to serve as a foundation for designing a programs to protect the health of mothers and their children. Hence, this study aims to analyze the influence of heavy metal exposure on oxidative DNA damage and the effect of Moringa leaf extract on heavy metal intoxication, 8-OHdG, and MDA in pregnant women. Furthermore, this study contributes to SDG 3: Good Health and Well-being, by exploring interventions to mitigate the adverse health effects of heavy metals exposure in pregnant women, which directly addresses maternal and child health outcomes. This research also emphasizes the importance of SDG 12: Responsible Consumption and Production by investigating the role of Moringa Oleifera as a sustainable, natural supplement that can mitigate the toxic effects of heavy metal exposure.

Methods

Study Design

The present study is on-going quasi-experimental investigation that employ a non-randomized pre-post test methodology. Expectant mothers have been segregated into two cohorts, one receiving an intervention and the other serving as a control. This study applies a non-randomized pre-post test design due to practical considerations related to the geographical setting, management feasibility, and time constraints. Several factors necessitate a non-randomized approach, such as:

1. Practical Management
   a. Simplified Logistics: Managing the study is easier when we designate specific locations for the intervention group and other locations for the control group. This approach ensures that the intervention (Moringa oleifera supplementation) can be efficiently delivered and monitored in each location without the complexity of randomizing participants across multiple sites.
   b. Grouping participants by location helps streamline logistical operations such as monitoring compliance and ensuring consistent follow-up over the course of the intervention period.
2. Geographical Differences
   a. Although the geographical settings of the intervention and control groups may differ, they are comparable in terms of demographic characteristics and environmental

conditions. This allows us to evaluate the effects of Moringa supplementation in different geographical settings while maintaining comparability between groups.

b. By focusing on distinct locations for intervention and control, we ensure better resource allocation and minimize logistical challenges that would arise from attempting to randomize participants across a broader region.

3. Time Constraints

a. Heavy metal screening results. Due to limitation in the laboratory, we cannot receive heavy metal analysis results from the initial screening at the same time for all participants. Therefore, women with high levels of heavy metals in the first screening were immediately included in the intervention group to avoid delays. This ensures that these participants can begin the 2-month intervention with Moringa oleifera as soon as possible after obtaining the result, considering the limited research timeframe.

b. Limited Research Period. Since we have limited overall time for the study, randomizing participants could cause delays in implementing the intervention and complicate the process of ensuring that those with high levels of heavy metals receive timely treatment. A non-randomized approach allows for a more timely intervention, while still enabling an effective comparison between the intervention and control groups.

Hence, while randomization is typically preferred to reduce selection bias, a non-randomized design is justified in this study because of the need for manageable logistics, time constraints, and the necessity of starting the intervention as soon as the heavy metal analysis results are available. By grouping participants based on location and heavy metal levels, we were able to ensure the efficient management and timely delivery of the intervention. Statistical methods will be used to control for baseline disparities and account for potential differences between the groups.

Participant recruitment

This research has been conducted in the Makassar city, specifically at six community health centers where expectant women receive prenatal services. The six health facilities are representative of a variety of areas in the district of Makassar, including industrial zones, waste disposal sites, and coastal areas, each of which has its own unique characteristics.

The study will include 40 pregnant women in each group of intervention and control who underwent an examination at health centers in Makassar and fulfilled the following inclusion criteria:

1. Pregnancy during the second or third trimester
2. Intention to deliver in Makassar
3. Not enduring twin pregnancy
4. Absence of chronic diseases
5. Living in Makassar for a period exceeding one year
6. Blood levels of mercury, lead, or cadmium exceed the reference value

The sample size of 40 per group is based on a moderate effect estimate (Cohen's d = 0.5), with a significance level of 5% and a test power of 80%. The outcomes will be assessed using Cohen's d = 0.5 as an effect size because this is a moderate effect size that is frequently employed in public health interventions. The study anticipates a significant decrease in heavy metal levels and the decrease in oxidative stress biomarkers which are indicators of lipid peroxidation and DNA damage. A moderate effect size is sufficient to detect significant changes, clinical relevance, and feasibility, despite the constraints of time and population heterogeneity.

Ethical Clearance

This study was approved by the Ethics Committee of th Public Health Faculty (Number: 1549/UN4.14.1/TP.01.02/2024). All participants provided informed consent prior to their involvement.

Research Procedure

The research step is presented in Figure 1.

Data Collection

An interview has been conducted with pregnant women using a questionnaire that contains questions about their characteristics and potential exposure sources. Data on food/supplement intake will also be obtained using a 24-hour recall form and a Food Frequency Questionnaire (FFQ) to assess exposure to heavy metals through food. Each participant provided 10 mL of venous blood samples acquired using tubes lined with ethylenediaminetetraacetic acid (EDTA) and kept at temperature of 2-8 °C until analysis. For MDA and 8-OHdG analyse, blood samples were centrifuged to obtain plasma and stored at temperatures of -80°C until analysis.

Heavy Metals Analysis

The measurement to be conducted is the determination of Hg, Pb, and Cd levels in the blood of pregnant women during the second or third trimester. A total of 200 pregnant women will be included in the screening.

Samples were extracted from the EDTA tube using a 1 ml micropipette and subsequently transferred to a test tube. Each sample is treated with 10 ml of 98% nitric acid (HNO3). At a temperature of 200-300°C, the samples were deposited on a hotplate for destruction until the vapor at the tube's edge dissipated. Each sample was treated with 49 ml of distilled water (Amidis). The sample is homogenized after the mixture then transferred to a 50 ml measuring glass and covered with parafilm. It was filtered using filter paper before analysis. Heavy metals were analyzed using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Fisher Scientific, Bremen, Germany).

MDA Analysis

The plasma samples will be added to an ELISA (Thermo Fisher Scientific, Bremen, Germany) plate pre-coated with MDA-specific antibodies. The MDA-TBA adduct in the sample binds to the specific antibodies on the plate. A secondary antibody, conjugated to an enzyme will be added, which binds to MDA-TBA adducts. A substrate solution will be added, and the enzyme reaction produces a color change proportional to the amount of MDA in the sample. The color intensity will be measured using a plate reader at a specific wavelength, and the MDA concentration in the sample will be quantified by comparison with a standard curve.

8-OHdG (8-hydroxy-2'-deoxyguanosine) Analysis

The 8-OHdG-specific ELISA kit (Bioassay Technology Lab, Shanghai Korain Biotech Co Ltd) includes plates pre-coated with antibodies that bind to 8-OHdG in plasma samples. Plasma samples will be added to the wells to allow 8-OHdG to bind to the antibodies. After washing the unbound substances, a secondary antibody conjugated to an enzyme was added to detect the bound 8-OHdG. A substrate will be introduced, that reacts with the enzyme to produce a colorimetric change. The color intensity will be measured at a specific wavelength using a plate

reader. The concentration of 8-OHdG is calculated by comparing the absorbance values to a standard curve.

Moringa Leaves Extract Supplementation

Collecting Moringa Leaves

Moringa leaves used in this study were collected from moringa trees growing in the Gowa and Takalar regencies. Fresh and healthy leaves were selected for supplementation. The leaves chosen were mature, dark green leaves from the tenth stalk down from the tip of the branches. Previous research[48] has shown that mature moringa leaves exhibit higher antioxidant activity than younger leaves.

Washing and Drying

The harvested moringa leaves were washed by dipping them in water and rinsing them with running water several times to remove any dirt or impurities. After washing, the leaves were air-dried for 2h to remove excess water. Leaves were separated from the stalks. The drying process was carried out in an oven at a temperature of 30-40°C for 3-4 hours or until the leaves reached a moisture content of less than 10%.

Extraction

The dried moringa leaves were ground into a fine powder using mesh 60. The powder was extracted with distilled water (Aquadest). The extraction process involved soaking the moringa powder in distilled water, after which the mixture was squeezed and filtered through a muslin cloth to separate the extract from the residue. The extract obtained was further processed using a rotary evaporator and dried using a freeze dryer until a dry extract was produced.

Capsule Supplement Production

The dry extract was ground again using mesh 100 to obtain a fine extract powder. This powder was then mixed with corn starch as a filler in a 4:1 ratio (400 mg Moringa extract and 100 mg corn starch per capsule). Each capsule contained 400 mg of moringa leaf extract, which will be administered twice dayly, resulting in a total daily dosage of 2000 mg.

Quality Control

After production, supplementation with moringa leaf extract capsules was tested to ensure weight uniformity and quality, including checks for moisture content, microbial presence, and organoleptic tests to ensure the safety and quality of the product. The dosage of moringa leaf supplementation in this study was determined based on several previous safety studies [49–52], which indicated that the consumption of moringa leaves is safe at doses of up to 2000 mg/day for humans and has the potential to be a safe source of antioxidants for pregnant women.

Intervention group

The intervention group will receive moringa extract supplement (2000 mg/day) consisting of two capsules twice per day. We collaborate with the health cadres at the health center. We ask them to supervise, remind, and ensure the mother take the capsules. We created a WhatsApp group for cadres to monitor and remind them to supervise the participants.

Control group

Mothers in the control group will receive iron supplement. The supplement is a standard supplement provided by Health Centers as part of their routine program.

Data Analysis

Analysis of group differences (pre-and post-intervention). For each group, analysis will be conducted using the paired t-test or Wilcoxon signed-rank test, depending on data distribution, to assess changes in heavy metals, MDA, and 8-OHdG levels before and after intervention.

Comparison between the groups (intervention vs. control). Analysis will be conducted using the difference-in-differences (DiD) method to measure the difference in changes between the intervention and control groups. The results will provide an overview of the impact of moringa leaf supplementation on the reduction of heavy metals level and prevention of DNA damage in pregnant women. To minimize bias, confounding factor such as nutritional status, environmental exposure, and diet will be analyzed using Analysis of Covariance (ANCOVA)

Expected Outcome

Heavy Metals Detoxification

The primary outcome of this study will be heavy metals detoxification. The hypothesis is that supplementation with Moringa oleifera will result in a reduction in the blood levels of heavy metals in pregnant women compared to the control group. Hence, the expected result are as follows:

a. A measurable decrease in blood heavy metal concentration will be observed in the group receiving Moringa oleifera supplementation.
b. The intervention group is expected to show a greater reduction in blood heavy metal levels than the control group, based on pre- and post-test analyses.

Scientific Implications expected is that the result would provide as evidence that Moringa Oleifera has the potential to detoxify, thereby demonstrating that it is a safe and effective approach to reducing heavy metal accumulation in expectant mother, who are at a higher risk of exposure to environmental pollutants.

DNA Damage prevention

Hypothesis**:** Moringa oleifera will reduce oxidative stress and DNA damage in pregnant women, as indicated by decreased levels of biomarkers such as 8-OHdG and MDA, compared to the control group.

The expected result are as follows:

a. A significant reduction in 8-OHdG and MDA levels in the intervention group, indicating a protective effect against oxidative stress and DNA damage.
b. The intervention group will show lower levels of oxidative stress and DNA damage after the intervention than the control group.

These finding will indicate that the antioxidant compounds of Moringa oleifera are effective in inhibiting DNA damage and protecting cells against oxidative stress. This is especially important for pregnant women to ensure fetal health and reduce complications.

Safety and Tolerability of Moringa oleifera

Hypothesis: Moringa oleifera supplementation will be safe for pregnant women, with no significant adverse effects reported during the intervention period. Hence, the **e**xpected results are as follows:

a. Minimal to no adverse effects in the group receiving Moringa Oleifera supplementation.
b. This safety profile is consistent with previous studies indicating that 2000 mg/day is a safe dosage for pregnant women.

This will provide further evidence that Moringa oleifera is a safe supplement for pregnant women, supporting its use in future clinical applications and maternal health programs.

Discussion

The potential implications for the health of the mother and child are the increase in breast milk production and the presence of oxidative stress biomarkers such as 8-OHdG and MDA in the mother. High levels of heavy metals, such as mercury, lead, and palladium, result in unfavorable outcomes, including premature birth, severe birth defects, and neurological complications in newborns. Through the implementation of this intervention, the risk of this complexity can be reduced, thereby resulting in a more favorable outcome and long-term improvement in the health of the mother and child.

In addition, the detection of oxidative stress biomarkers reduces the protective effect against DNA damage and lipid oxidation, which are associated with serious health risks such as preeclampsia, gestational hypertension, and chronic kidney disease in children. An increase in oxidative stress can result in a healthier population, increase the risk of obesity, and improve the neurodevelopmental outcomes of children.

Public Health Implication

From a public health perspective, this intervention has the potential to serve as a preventive strategy that can be implemented in regions with high levels of environmental pollution. This intervention can be implemented in areas with limited resources where conventional deforestation methods may not be available or are prohibitively expensive by using Moringa oleifera, a cheap and easily accessible substitute. The use of moringa also aligns with the Sustainable Development Goals (SDGs), particularly in the promotion of maternal health (SDG 3) and environmental health (SDG 6).

Strength and Limitations

The non-randomized design of this study has several limitations, particularly in terms of internal validity and potential for selection bias. Without randomization, it is possible that there are differences in the core characteristics of the intervention and control groups that could influence the results. However, a non-randomized design was chosen owing to time and logistics constrain.

To minimiz the potential for bias, statistical fitting techniques such as Analysis of Covariance (ANCOVA) will be implemented to mitigate the disparity in distribution between the groups. In addition, the aim is to select intervention and control teams from regions with unique demographic and environmental characteristics.

Conflict of Interest

The authors have no conflicts of interest associated with the material presented in this paper.

Abbreviations

8-OHdG: 8-hydroxy-2'-deoxyguanosine
Cd: Cadmium
Cu: Copper
DNA: Deoxyribonucleic Acid
Fe: Iron
Hg: Mercury
ICP-MS: Inductively Coupled Plasma Mass Spectrometry
LBW: Low Birth Weight
MDA: Malondialdehyde
Pb: Lead
SDG: Sustainable Development Goals
Se: Selenium
Zn: Zinc

Funding

This research is funded by Directorate of Research, Technology, and Community Service of the Ministry of Education, Culture, Research, and Technology, Indonesia (Number 02035/UN4.22.2/PT.01.03/2024)

Acknowledgements

This research is supported by Directorate of Research, Technology, and Community Service of the Ministry of Education, Culture, Research, and Technology, Indonesia, Environmental Toxicology and Health (EtoxH) Research Group and Laboratory of Public Health Makassar I, Ministry of Health Indonesia.

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