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PREVENTIVE EFFECT OF CILNIDIPINE ON CYCLOPHOSPHAMIDE-INDUCED LUNG TOXICITY IN *WISTAR* ALBINO RATS

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ABSTRACT

Cancer remains one of the leading global causes of death, accounting for approximately one in every six deaths, with its prevalence steadily rising due to factors like aging populations, lifestyle habits such as smoking, unhealthy diets, obesity, physical inactivity, and air pollution. The present study is driven by the need to find effective treatments for cyclophosphamide (CP)-induced organ toxicity, especially lung damage caused by its toxic metabolite acrolein. CP's use is limited due to oxidative stress, antioxidant depletion, and increased pro-inflammatory cytokines leading to cell death and tissue injury. Existing therapies are inadequate, highlighting the need for safer, more effective protective strategies.

The comprehensive evaluation of biochemical and histological markers reveals that cilnidipine, particularly at a higher dose, mitigates oxidative damage, reduces pro-inflammatory cytokine levels, and maintains tissue integrity. Its dual calcium channel blocking ability, coupled with its capacity to activate antioxidant defenses and suppress inflammatory pathways, underscores its therapeutic potential beyond hypertension. The findings suggest that cilnidipine can be repurposed as an adjunctive treatment in cancer therapy to reduce organ toxicity associated with cyclophosphamide and possibly other chemotherapeutic agents.

Keywords: Cilnidipine; prevention; albino rats; cyclophosphamide.

INTRODUCTION

Cancer remains one of the leading global causes of death, accounting for approximately one in every six deaths, with its prevalence steadily rising due to factors like aging populations, lifestyle habits such as smoking, unhealthy diets, obesity, physical inactivity, and air pollution. By 2050, cancer cases are expected to increase by around 77%, putting significant pressure on healthcare systems worldwide. Cancer is fundamentally characterized by abnormal and uncontrolled cell growth that can spread to other parts of the body. Historically observed as tumors, cancer was later understood as a genetic disease caused by mutations and evolutionary processes within cells, leading to drug resistance and metastasis. Based on tissue type, cancer is classified into carcinomas, sarcomas, lymphomas, leukemias, and adenomas; and based on the affected organ, types include lung, liver, colorectal, and others. Symptoms vary but commonly include fatigue, weight loss, and unexplained bleeding. Causes are multifactorial, involving genetic predisposition, chemicals, infections, and lifestyle factors. Cyclophosphamide (CP), a widely used chemotherapy drug, is effective against various cancers and autoimmune disorders. However, its therapeutic use is limited by serious side effects including lung, heart, brain, liver, bone marrow, and gonadal toxicity.[1,2] CP is a prodrug activated in the liver to phosphoramide mustard, which fights cancer, and acrolein, which causes organ damage. CP induces oxidative stress, reduces antioxidant defense, increases inflammatory cytokines like TNF-α and IL-6, and promotes apoptosis in multiple organs. Lung toxicity from CP is a major concern as it can lead to pulmonary fibrosis. Cardiotoxicity affects patient survival, with oxidative stress and inflammation damaging cardiac tissue. Neurotoxicity leads to cognitive impairment, commonly termed "chemo brain," as acrolein crosses the blood-brain barrier. Hepatotoxicity occurs via oxidative stress, inflammation, and apoptosis, damaging liver cells and altering enzyme levels. Bone marrow toxicity manifests as myelotoxicity, reducing blood cell counts due to disrupted hematopoietic growth factors. Gonadal toxicity affects fertility and reproductive health through oxidative stress-induced cellular damage in reproductive organs. In addressing CP's multi-organ toxicity, cilnidipine emerges as a potential protective agent. Cilnidipine is a fourth-generation calcium channel blocker acting on both L- and N-type channels, offering benefits beyond blood pressure control. It reduces oxidative stress, inflammation, and apoptosis through its antioxidant and anti-inflammatory properties, making it suitable for patients with hypertension, diabetes, kidney disease, and even neurodegenerative concerns. Cilnidipine helps regulate pro-inflammatory cytokines like TNF-α and IL-6, improves antioxidant enzyme levels such as SOD and GPx, and prevents calcium overload-related cell death. Its dual action may protect against CP-induced toxicities, suggesting a promising role in integrative cancer therapy approaches aimed at minimizing chemotherapy side effects while maintaining therapeutic efficacy.[3]

The present study is driven by the need to find effective treatments for cyclophosphamide (CP)-induced organ toxicity, especially lung damage caused by its toxic metabolite acrolein. CP's use is limited due to oxidative stress, antioxidant depletion, and increased pro-inflammatory cytokines leading to cell death and tissue injury. Existing therapies are inadequate, highlighting the need for safer, more effective protective strategies.

MATERIALS AND METHODS

Materials

The drugs were used in the research are mentioned in Table 1.

Table 1: Drugs used in this research**.**

Drugs	**Source**
CIL	Sigma Aldrich, USA
CP	Sigma Aldrich, USA

The chemicals and reagents were used as analytical grade in the research.

Animals

The details of the animal were used in the research is mentioned in Table 2.

Table 2: Details of animal used in the research.

Wistar Albino Rats	CAHF, Jamia Hamdard, New Delhi
Weight	150 – 200 gm
Sex	Female
Strain	Wistar Rats
Animal Diet	Amrut Rat Feed, Nav Maharastra Chakan Oil Mills Ltd.

Methodology

The proposed experimental protocol was approved by Institutional Animal Ethics Committee of Jamia Hamdard, New Delhi, India (Registration no. JH/1484/CPCSEA and JH/1610/CPCSEA) and as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Preparation of drug solutions

- **CIL Solution:** Weighed amount of CIL is dissolve in 0.5% CMC solution.
- **CP Solution:** Weighed amount of CP was dissolved in normal saline solution.

Phase I – Experimental protocol

Table 3: Experimentation groups of aminals

S. No.	Groups	Dose	Route & duration
1	Control	Normal saline	Oral, daily 14 days
2	Toxic	CP 200 mg/kg	i.p., once on 7th day
3	CIL + CP	CIL 1mg/kg + CP 200 mg/kg	p.o., daily 14 days + i.p once on 7th day
4	CIL + CP	CIL 2mg/kg + CP 200 mg/kg	p.o., daily 14 days + i.p once on 7th day
5	CIL *per se*	CIL 2mg/kg	p.o., daily 14 days
CP: Cyclophosphamide, **I.P:** Intraperitoneal, **p.o:** per os			

Methodology for in vivo study

Blood collection

Blood sample from the experimental animals were collected in sterile centrifuge tubes, after the last treatment dose and the serum was separated for the estimation of the following biochemical parameters.[4]

Biochemical estimations in tissue

(a) Protein Estimation

(b) Thiobarbituric acid reactive substances (TBARS)

(c) Reduced glutathione (GSH)

(d) Catalase (CAT)

(e) Superoxide dismutase (SOD)

Inflammatory parameters from Kits:

(a) Tumor Necrosis Factor α (TNF-α)

(b) IL-1B

(c) NF-κB

(d) Nfr-2

Histopathology Study

Biochemical Estimation in Tissue (Lungs)

(a) Protein Estimation[5]

Principle: - Protein reacts with Folin's ciocalteau phenol reagent to give a colored complex. The color so formed is due to the reaction of alkaline copper with protein as in the burette test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Reagents

(i) Alkaline sodium carbonate (2% Na2CO3 in 0.1 N NaOH)

(ii) Copper sulphate- sodium potassium tartrate (0.5% CuSO4.5 H2O in 1 % sodium potassium tartrate)

(iii) Alkaline copper solution

(iv) Folin's ciocalteau phenol reagent

(v) Bovine serum albumin (BSA) solution (0.5 mg/ml)

Preparation of reagents[6,7]

(i) **Alkaline sodium carbonate (2% Na2CO3 in 0.1 N NaOH):** 0.1 N NaOH was prepared by dissolving 400 mg of NaOH in double distilled water and the volume was made upto 100 ml with double distilled water. Then 2 g of Na2CO3 was added in 100 ml of 0.1 N NaOH solution.

(ii) **Copper sulphate- sodium potassium tartrate:** 0.5 g of CuSO4.5 H2O was dissolved in 100 ml in double distilled water to prepare 0.5% copper sulphate solution and 1 g of sodium potassium tartrate solution. Then the CuSO4.5 H2O solution was added to the sodium potassium tartrate solution with through mixing.

(iii) **Alkaline copper solution:** It was prepared on the day of use by mixing 50 ml of reagent

(i) and 1 ml of reagent (ii).

(iv) **Folin's ciocalteau phenol reagent:** The commercial reagent was diluted with 2 folds of double distilled water on the day of use.

(v) **Bovine serum albumin (BSA) solution (1mg/ml):** 3 mg of BSA was dissolved in 3 ml of double distilled water to get a solution of 1 mg/ml of protein.

Procedure: 500 mg Lung tissue was homogenized in 5 ml 0.15 M KCl and centrifuged at 10,000 rpm for 10 minutes. 1 ml of supernatant was mixed with 5 ml of alkaline copper solution and allowed to stand at room temperature for 10 minutes. 0.5 ml diluted Folin's reagent (1:2) was then added the tubes were shaken to mix the solution. After 30 minutes the absorbance was read at 750 nm against appropriate blank. The protein content was expressed in mg.[8]

Preparation of standard curve: 5 ml of BSA solution (1 mg/ml) was prepared and different volumes were taken in 5 tubes. To all tubes distilled water was added to make up the volume in each tube to 1 ml. The protein concentration in the above 5 tubes was

estimated in same way as for the sample. A graph was plotted between concentrations of protein and optical density (OD). The calibration standard plot was used to calculate the concentration of protein in each ml suspension of the sample.[9]

(b) Thiobarbituric Acid Reactive Substances (TBARS) (Deleon et al., 2020; Ghani et al., 2017; Nowak D et al., 2001)

Principle: - Lipid peroxidation is a process where fats in the body get damaged by free radicals, which are highly reactive molecules. This damage creates a mix of chemicals, including something called malondialdehyde (MDA). MDA is one of the main byproducts of lipid peroxidation.To measure how much lipid damage has happened, scientists use a test that involves a chemical called thiobarbituric acid (TBA). When TBA reacts with MDA, it produces a color change, which can be measured. This color change gives an estimate of how much MDA (and therefore lipid damage) is present in a sample. This test is known as the TBARS (Thiobarbituric Acid Reactive Substances) test. It's a simple and sensitive way to measure lipid damage in the body.[10]

Reagents required: -

1. **0.8% TBA Solution**:

- Take **0.8 grams** of **Thiobarbituric Acid (TBA)**.
- Dissolve it in **distilled water**.
- Add water until the total volume reaches **100 ml**.

2. **30% TCA Solution**:

- Take **30 grams** of **Trichloroacetic Acid (TCA)**.
- Dissolve it in **distilled water**.
- Add water until the total volume reaches **100 ml**.

3. **Standard TEP Solution**:

- Take **0.02 grams** (with a possible error of ±0.004 grams) of **TEP (1,1,3,3-tetraethoxypropane)**.
- Dissolve it in **40% ethanol** (a mixture of ethanol and water).
- Once dissolved, add **distilled water** to make the total volume **1000 ml**.
- Then, take **100 ml** of this solution and dilute it further with **distilled water** until the total volume reaches **500 ml**.
- This final solution will have **4 micrograms (µg)** of TEP per **milliliter (ml)**.

Procedure: -

Take 1 ml of the supernatant from the 10% tissue homogenate and place it in a tube. Centrifuge this tube at 10,000 rpm to separate the components. Add 0.5 ml of 30% TCA (Trichloroacetic acid) and then 0.5 ml of 0.8% TBA (Thiobarbituric acid) to the sample. Cover the tubes with aluminum foil

and place them in a shaking water bath at 80°C for 30 minutes. After 30 minutes, remove the tubes and place them in ice-cold water for 10 minutes to cool. Centrifuge the tubes again at 3000 rpm for 15 minutes to separate the contents. Measure the absorbance (how much light the sample absorbs) at 540 nm (a specific wavelength of light) using a spectrophotometer. This will be done at room temperature.[11]

Preparation of standard MDA Curve

0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ml of standard TEP solution was taken in 6 test tubes. 0.5 ml of 30 % TCA and 0.5 ml of 0.8% of TBA reagents will be added to each of the tubes and the same procedure as for the sample will be followed. A graph will be plotted between concentration of TEP and optical density.

Calculation:

The amount of MDA present in the sample will calculated according to the equation:

$$\text{n moles of MDA} = \frac{\text{O.D. at 540 nm x Volume of test solution}}{0.156}$$

(c) Tissue Glutathione (Reduced GSH Level)

Principle: -

This spectrophotometric procedure is based on Ellman's method, which involves the use of 5,5'-dithiobis-2-nitrobenzoic acid (DNTB). In this method, –SH groups (sulfhydryl groups) in a sample react with DNTB and reduce it, forming one molecule of 2-nitro-5-mercaptobenzoic acid for each mole of –SH group present. The resulting product, 2-nitro-5-mercaptobenzoic acid, produces a bright yellow color. This yellow color can be measured spectrophotometrically at a wavelength of 412 nm, and the intensity of the color is directly proportional to the amount of –SH groups in the sample. This method is commonly used to quantify thiol groups in proteins and other biological molecules.[12,13]

Reagents Required: -

(a) EDTA (0.2 M)

(b) EDTA (0.02 M)

(c) DTNB (0.01 M)

(d) Tris buffer (0.4 M, pH 8.9)

(e) TCA (50%)

Preparation of Reagents: -

a) EDTA (0.2 M): 22.3g of EDTA will be dissolved in 300ml of warm double distilled water.
b) EDTA (0.02 M): 20 ml of above solution will be diluted to 200ml of double distilled water
c) DTNB (0.01 M): 99mg of DNTB will be dissolved in 25 ml of absolute methanol.
d) Tris buffer (0.4 M, pH 8.9): 24.2 g of Tris buffer will be dissolved in 100 ml of double distilled water, 100 ml of 0.2 M EDTA will be added to it and the volume of solution will be made up to 1000ml with double distilled water. The pH of the solution was adjusted to 8.9 with 1 N HCl.
e) TCA (50%): 50 g of TCA will be dissolved in 100 ml of double distilled water.

Procedure: -

Test

To prepare the heart tissue sample, a known amount of tissue (between 300-600 mg) will be blended in 5-8 ml of 0.02 M EDTA solution to break it up. Then, 4.0 ml of cold distilled water will be added to dilute the mixture. After mixing, **1 ml of 50% trichloroacetic acid (TCA)** will be added to help separate proteins from the sample. The mixture will be shaken intermittently for **10 minutes** using a vortex mixer.

Once the shaking is done, the mixture will be transferred to centrifuge tubes (which have been rinsed with EDTA) and spun at **6000 rpm** for **15 minutes** to separate the liquid from the solid parts. After centrifugation, **2 ml of the liquid (supernatant)** will be taken and mixed with **4.0 ml of 0.4 M Tris buffer (pH 8.9)**. This solution will be thoroughly mixed, and then **0.1 ml of 0.01 M DTNB** (a chemical that reacts with sulfhydryl groups) will be added. Finally, the absorbance (how much light the solution absorbs) will be measured at **412 nm** using a spectrophotometer within **5 minutes** of adding DTNB. The reading will be compared against a reagent blank to calculate the results.[14]

Blank

The method will be same as for the test expect that 0.02 M EDTA will be added in place of tissue homogenate**.**

Standard

The method will be same as for the test expect that standard glutathione solution (50µg/ml) will be used in place of tissue homogenate.

(d) Catalase

Principle: -

Hydrogen peroxide (H_2O_2) absorbs light in the ultraviolet (UV) range, with its absorption increasing as the wavelength decreases. When hydrogen peroxide breaks down, it causes a decrease in absorption at **240 nm**. By measuring how much the absorption changes (ΔA) over time, we can determine the activity of **catalase**, the enzyme that breaks down hydrogen peroxide. The faster the decrease in absorbance, the higher the catalase activity.[15]

Reagents: -

(i) Potassium phosphate buffer (50Mm; pH 7.4)

(ii) Hydrogen peroxide30% (19 mM/L)

Procedure: -

To measure catalase activity in heart tissue, the tissue will first be blended in a **50 mM potassium phosphate buffer (pH 7.4)**, with a ratio of **1 part tissue to 10 parts buffer**. The mixture will then be spun at **10,000 rpm** at **4°C** for **20 minutes** in a cooling centrifuge. After centrifugation, the liquid part (called the **supernatant**) will be used to measure catalase activity. In the next step, **50 µl of the supernatant** will be added to a cuvette containing **2.95 ml of a 19 mM hydrogen peroxide (H_2O_2)** solution made in potassium phosphate buffer. The absorbance (how much light the solution absorbs) will be measured at a wavelength of **240 nm** every minute for **3 minutes**. Since catalase breaks down hydrogen peroxide, the absorbance will decrease as the enzyme decomposes the hydrogen peroxide. This decrease in absorbance indicates the level of catalase activity.[16]

Calculation: Catalase activity will be calculated as

$$\text{Catalase} = \frac{(\Delta A)/\text{minute} \times \text{volume of assay}}{0.081 \times \text{volume of homogenate} \times \text{mg of protein}}$$

(e) Estimation of Superoxide Dismutase (Sun YI et. al., 1988; Weydert CJ et. al., 2010; Mockett et. al., 2002)

Principle: -

Pyrogallol, a chemical compound, naturally undergoes **auto-oxidation** (it reacts with oxygen) when dissolved in water. The process happens faster at **higher pH levels** and produces several intermediate products. At first, the solution turns **yellow-brown** and absorbs light in the range of **400-425 nm**. After a few minutes, the color changes to **green**, and eventually, after a few hours, it becomes **yellow**. To study the auto-oxidation process, we focus on the first stage, where the absorbance at **420 nm** increases in a straight line over several minutes, starting after a brief delay (about 10 seconds). The **superoxide anion radical** helps speed up the oxidation of pyrogallol. A simple and quick method to measure **superoxide dismutase (SOD)** activity is based on the enzyme's ability to **inhibit** the auto-oxidation of pyrogallol. In other words, the more active the SOD enzyme, the slower the pyrogallol oxidation, which can be measured by the change in colour.

Reagents Required: -

- Tris HCl buffer (pH 8.5)
- EDTA (1mM)
- Pyrogallol (24 mM)

Procedure: -

To measure **superoxide dismutase (SOD)** activity, we will test the **supernatant** (the liquid part after centrifuging the tissue) by looking at how it affects the **auto-oxidation of pyrogallol**. First, **100 µl** of the supernatant will be added to a **Tris HCl buffer** (with a pH of 8.5). The total volume of the mixture will be made up to **3 ml** using the same buffer. Next, **at least 25 µl of pyrogallol** will be added to the

mixture. The absorbance (how much light the sample absorbs) at **420 nm** will be measured every minute for **3 minutes**. Normally, after adding pyrogallol, the absorbance increases as it oxidizes. However, if **SOD** is present, it will **slow down the oxidation**, reducing the increase in absorbance. The more SOD there is, the less the absorbance will increase, indicating higher enzyme activity.[17,18]

Calculation: -

1 unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3ml of assay mixture and given by the formula:

$$\text{Unit of SOD per ml of sample} = \frac{(A-B) \times 100}{A \times 50}$$

Where, A is the difference of absorbance in 1 minute in control.

A is the difference of absorbance in 1 minute in test sample.

Data will be expressed as SOD units per mg of protein.

5.3.3.3 Inflammatory parameters from Kits:

(a) Tumor Necrosis Factor α (TNF-α) (El-Kashef et al., 2018)

Principle:

Tumor Necrosis Factor-alpha (TNF-α) is a key pro-inflammatory cytokine that plays a central role in mediating inflammatory responses, including those associated with cyclophosphamide-induced lung injury. Its quantification is crucial to assess the extent of inflammation and the protective effects of therapeutic agents such as cilnidipine.

The measurement of TNF-α is typically performed using the Sandwich Enzyme-Linked Immunosorbent Assay (ELISA). In this method, a monoclonal antibody specific to TNF-α is pre-coated onto a microtiter plate. Samples containing TNF-α bind to this capture antibody. A biotinylated secondary antibody specific to TNF-α is then added, forming an antibody-antigen-antibody sandwich. This is followed by the addition of an enzyme (usually horseradish peroxidase, HRP) conjugated with streptavidin, which binds to the biotin on the secondary antibody. A substrate solution, typically tetramethylbenzidine (TMB), is added, leading to a color change. The intensity of the color is directly proportional to the amount of TNF-α in the sample and is measured spectrophotometrically at 450 nm.[19]

Reagents and Materials:

- Pre-coated TNF-α ELISA 96-well microplate
- TNF-α standards
- Sample diluent
- Biotinylated detection antibody
- Streptavidin-HRP conjugate

- TMB substrate solution
- Stop solution (usually 2N sulfuric acid)
- Wash buffer (e.g., PBS with 0.05% Tween 20)
- Lung tissue homogenates or serum samples
- Microplate reader set to 450 nm
- Micropipettes and tips
- Plate washer or manual washing setup

Procedure:

1. **Sample Preparation:**
    - Collect blood samples via retro-orbital or cardiac puncture and separate serum via centrifugation.
    - Alternatively, prepare lung tissue homogenates in PBS or lysis buffer, followed by centrifugation to obtain clear supernatant.
2. **Plate Setup:**
    - Bring all reagents and samples to room temperature.
    - Prepare the required number of microplate wells for standards, samples, and blanks.
3. **Standard Curve Preparation:**
    - Reconstitute TNF-α standard as per kit instructions.
    - Perform serial dilutions to generate a standard curve covering the assay's dynamic range (e.g., 15.6–1000 pg/mL).
4. **Addition of Standards and Samples:**
    - Add 100 µL of standards and samples into the appropriate wells.
    - Incubate at 37°C for 90–120 minutes or as per kit instructions.
5. **Washing:**
    - Aspirate each well and wash with 300 µL wash buffer 3–5 times.
    - Tap the plate gently on absorbent paper to remove excess liquid after each wash.
6. **Addition of Detection Antibody:**
    - Add 100 µL of biotinylated anti-TNF-α antibody to each well.
    - Incubate at 37°C for 60 minutes.
7. **Washing:**
    - Repeat washing steps as above.

Data Analysis:

- Construct a standard curve by plotting the absorbance values of the standards against their known concentrations.
- Determine the concentration of TNF-α in each sample by interpolating absorbance values on the standard curve.
- Express the results as pg/mL of TNF-α in serum or lung homogenate.

(b) Interleukin-1 beta (IL-1β) (El-Kashef et al., 2018)

Reagents and Materials:

- Pre-coated anti-IL-1β antibody ELISA plate (96-well)
- IL-1β standard solution (lyophilized or liquid)
- Sample diluent
- Biotinylated anti-IL-1β detection antibody
- Streptavidin-HRP conjugate
- TMB substrate solution
- Stop solution (e.g., 2N H_2SO_4)
- Wash buffer (PBS with 0.05% Tween-20)
- Lung tissue homogenate or serum samples
- Micropipettes and tips
- ELISA plate reader (450 nm)

Procedure:

1. **Sample Preparation:**
    - For serum: Collect blood samples, allow clotting, centrifuge at 3000 rpm for 10 minutes, and collect clear serum.
    - For lung tissue: Homogenize lung tissue in cold PBS or lysis buffer. Centrifuge at 10,000 rpm for 10 minutes at 4°C. Collect the supernatant for assay.[20]
2. **Plate Setup:**
    - Bring all ELISA kit components and samples to room temperature before use.
    - Allocate wells for standards, samples, and blank.
3. **Standard Curve Preparation:**
    - Reconstitute IL-1β standard as per the kit's instructions.
    - Perform serial dilutions (e.g., 1000 pg/mL down to 15.6 pg/mL) to create a standard curve.
4. **Addition of Standards and Samples:**

- Add 100 µL of standards and samples to respective wells in duplicate or triplicate.
- Incubate the plate at 37°C for 1.5–2 hours or as specified in the kit protocol.

5. **Washing:**
    - Aspirate contents and wash each well 4–5 times with wash buffer.
    - Tap the plate on absorbent paper to remove excess liquid.
6. **Addition of Detection Antibody:**
    - Add 100 µL of biotinylated detection antibody solution to each well.
    - Incubate for 1 hour at 37°C.

Data Analysis:

- Generate a standard curve by plotting absorbance vs. standard IL-1β concentration.
- Calculate IL-1β concentrations in samples by interpolating from the standard curve.
- Express results as pg/mL in serum or lung homogenate.

(c) Nuclear Factor kappa B (NF-κB) (Gupta et. al., 2019)

Principle:

Nuclear Factor kappa B (NF-κB) is a key transcription factor involved in regulating immune and inflammatory responses. In cyclophosphamide-induced lung toxicity, NF-κB is activated due to oxidative stress and pro-inflammatory signaling, leading to the upregulation of inflammatory cytokines such as TNF-α and IL-1β. Its estimation helps in understanding the extent of inflammatory pathway activation and the anti-inflammatory potential of therapeutic agents like cilnidipine.

NF-κB can be quantified using a specific Transcription Factor ELISA, which is based on the ability of activated NF-κB (mainly the p65 subunit) to bind its consensus DNA sequence. The wells of the ELISA plate are coated with oligonucleotides containing the NF-κB binding site. Nuclear extracts from lung tissues are added, and active NF-κB binds to the immobilized DNA sequence. This complex is then detected using a specific antibody against the NF-κB subunit followed by an HRP-conjugated secondary antibody. The enzymatic reaction with a chromogenic substrate (TMB) results in a color change measured at 450 nm. The intensity is directly proportional to the amount of active NF-κB present in the sample.[21]

Reagents and Materials:

- NF-κB p65 Transcription Factor ELISA kit (pre-coated 96-well plate with consensus sequence)
- Lung tissue nuclear extracts
- Lysis buffer (for nuclear protein extraction)
- Capture oligonucleotide plates (pre-coated)
- Anti-NF-κB p65 detection antibody
- HRP-conjugated secondary antibody

- TMB substrate
- Stop solution (2N H_2SO_4)
- Wash buffer (PBS + Tween-20)
- ELISA reader set at 450 nm

Procedure:

1. **Preparation of Nuclear Extracts:**
    - Homogenize lung tissue samples in a hypotonic buffer and centrifuge to pellet nuclei.
    - Resuspend nuclei in extraction buffer containing protease inhibitors.
    - Incubate on ice for 30 minutes with intermittent vortexing.
    - Centrifuge at high speed (14,000 rpm) at 4°C for 10 minutes.
    - Collect the supernatant containing nuclear proteins.
2. **Plate Setup:**
    - Bring all kit components to room temperature.
    - Set up wells for standards, samples, and blanks.
3. **Sample and Standard Addition:**
    - Add 20–30 µL of nuclear extract to each well.
    - Add binding buffer as per the kit's instructions.
    - Incubate for 1 hour at room temperature to allow NF-κB binding to the oligonucleotide.
4. **Washing:**
    - Wash each well 4–5 times with wash buffer to remove unbound material.
5. **Addition of Detection Antibody:**
    - Add 100 µL of anti-NF-κB p65 detection antibody to each well.
    - Incubate for 1 hour at room temperature.
6. **Washing:**
    - Wash the wells as before.

Data Analysis:

- Prepare a standard curve using NF-κB p65 standards provided in the kit or relative absorbance units if no standard is available.
- Calculate the relative NF-κB activity by comparing sample absorbance with the standard or control group values.
- Express results as relative optical density (OD) or normalized values against control.

(d) Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2) (Murakami S. et. al., 2023)

Principle:

Nrf2 is a critical transcription factor that regulates cellular antioxidant defense mechanisms. Under oxidative stress conditions, such as those induced by cyclophosphamide (CP) in lung tissues, Nrf2 dissociates from its inhibitor Keap1, translocates into the nucleus, and activates antioxidant response element (ARE)-driven genes. These genes encode enzymes such as heme oxygenase-1 (HO-1), superoxide dismutase (SOD), and catalase. Estimating Nrf2 levels helps assess the activation of antioxidant pathways and evaluate the protective role of agents like cilnidipine.

In an Nrf2 ELISA, wells are pre-coated with an antibody specific to Nrf2. Lung tissue nuclear or total cell lysate samples are added, allowing Nrf2 present in the sample to bind to the immobilized antibody. A secondary antibody conjugated with horseradish peroxidase (HRP) binds to captured Nrf2, and color development occurs through a substrate like TMB. The color intensity measured at 450 nm correlates directly with Nrf2 concentration in the sample.

Reagents and Materials:

- Nrf2 ELISA kit (pre-coated 96-well plate)
- Lung tissue nuclear extracts or total lysate
- Lysis buffer (with protease and phosphatase inhibitors)
- Sample diluent
- Biotinylated anti-Nrf2 detection antibody
- Streptavidin-HRP conjugate
- TMB substrate
- Stop solution (e.g., 2N sulfuric acid)
- Wash buffer (PBS + 0.05% Tween-20)
- ELISA reader set at 450 nm
- Micropipettes and tips

Procedure:

1. **Preparation of Tissue Lysate or Nuclear Extract:**
    - Homogenize lung tissues in ice-cold lysis buffer with protease inhibitors.
    - For nuclear extracts, use a nuclear extraction kit or hypotonic lysis method.
    - Centrifuge at 10,000–14,000 rpm for 10–20 minutes at 4°C.
    - Collect the supernatant (cytoplasmic or nuclear fraction as needed).
2. **Plate Setup:**
    - Bring all ELISA kit reagents and samples to room temperature.
    - Assign wells for standards, samples, and blank.
3. **Standard Curve Preparation:**

- Reconstitute and serially dilute the Nrf2 standard according to kit instructions to generate a standard curve (e.g., 5000–78 pg/mL).

4. **Addition of Standards and Samples:**
   - Add 100 µL of standards and samples to each well.
   - Incubate for 1–2 hours at room temperature or as per kit protocol.
5. **Washing:**
   - Aspirate wells and wash 4–5 times with wash buffer to remove unbound substances.
6. **Addition of Detection Antibody:**
   - Add 100 µL of biotinylated anti-Nrf2 detection antibody.
   - Incubate for 1 hour at room temperature.
7. **Washing:**
   - Wash the wells again as described.

Data Analysis:

- Plot a standard curve using the known concentrations of Nrf2 standards versus their corresponding absorbance values.
- Calculate the concentration of Nrf2 in tissue samples using the standard curve.
- Express the results as pg/mg protein or pg/mL.

5.3.3.4 Histopathology Study (Suddek GM et.al., 2013)

Method: Eosin - Hematoxylin

The right lungs were rapidly removed from the animal, sliced transversely, paraffin embedded, and prepared as 3-lm thick sections that were then stained with hematoxylin and eosin (H&E) for light microscopic evaluation. Histopathological examination of the lung sections was performed without knowledge of the treatment protocol, as described previously

5.3.3.5 Statistical Analysis

Data are expressed as mean ± SEM. (Significance was calculated at p 0.05.) Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. Paired Student's t test was also used as a test of significance for comparison between two arithmetic means of the same subject before and after treatment (Daniel et. al.,1991). Statistical calculations were carried out using Instat-2 computer program (V2.04, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Body Weight

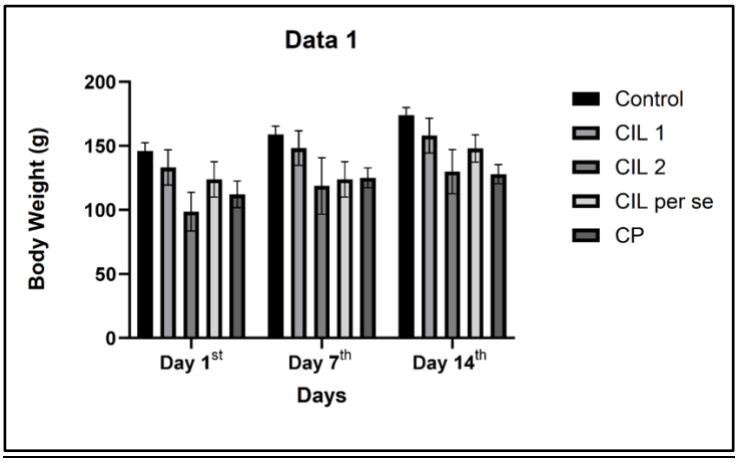


Fig. 1: Graph of Body weight

The graph illustrates the changes in body weight (g) over 14 days across five experimental groups: Control, CIL 1, CIL 2, CIL per se, and CP. The Control group exhibited a steady increase in body weight from Day 1 to Day 14, indicating normal growth. In contrast, the CP group (likely treated with cyclophosphamide) showed a significant reduction in body weight on Day 1, with only slight recovery by Day 14, suggesting CP-induced toxicity and growth suppression.

Glutathione (GSH)

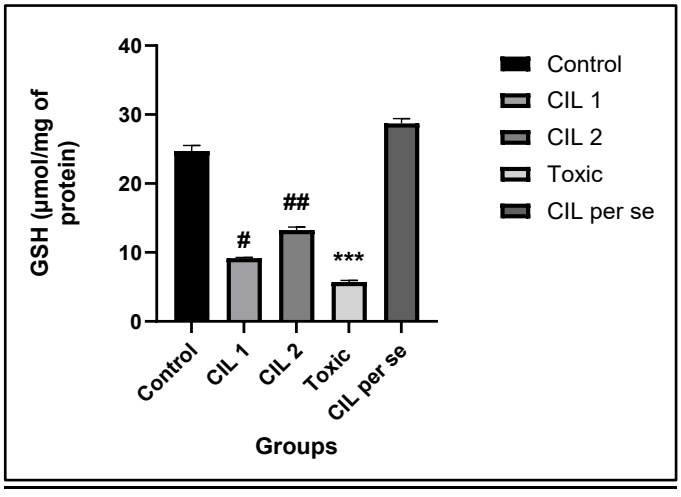


Fig 2: Effect of different treatments on tissue GSH of Different Groups. * Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.

The bar graph illustrates the levels of GSH (glutathione), a key antioxidant marker, across five experimental groups: Control, CIL 1, CIL 2, CIL per se, and CP. The Control group shows a high GSH

level, indicating a normal oxidative balance. In contrast, the CP group exhibits a marked reduction in GSH, suggesting significant oxidative stress or damage. Treatment with Cilnidipine shows a dose-dependent improvement in GSH levels. The CIL 1 group shows moderate restoration, while the CIL 2 group demonstrates near-normal GSH levels, indicating a stronger protective effect. Interestingly, the CIL per se group maintains GSH levels similar to the Control. Overall, the data suggest that Cilnidipine, particularly at higher doses, may help counteract oxidative damage by restoring GSH levels.

Superoxide Dismutase (SOD)

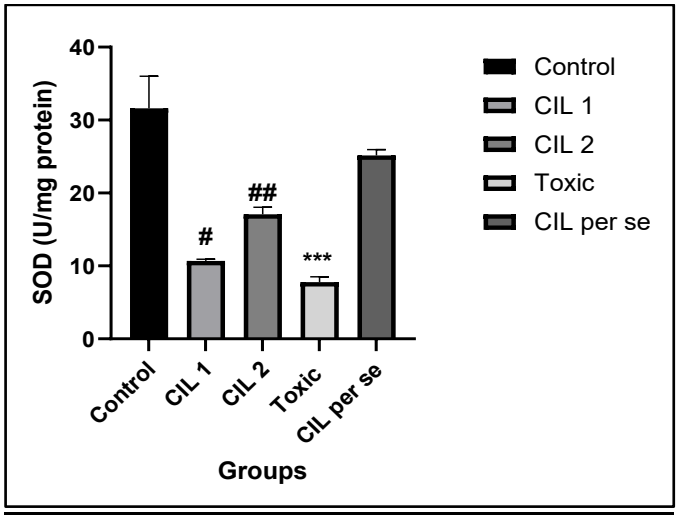


Fig 3: Effect of different treatments on tissue SOD of Different Groups. * Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.

The bar graph illustrates the activity of superoxide dismutase (SOD), a key antioxidant enzyme, across five distinct groups: Control, CIL 1, CIL 2, CIL per se, and CP. The Control group shows elevated SOD levels, indicating normal antioxidant functioning. A notable decline in SOD levels is observed in the CP group, suggesting compromised oxidative defense. Administration of Cilnidipine appears to counteract this effect, with CIL 2 producing a stronger enhancement of SOD activity compared to CIL 1, implying a concentration-dependent benefit. Additionally, the CIL per se group maintains SOD values similar to those of the Control group. These results point to the potential of Cilnidipine in restoring antioxidant balance under oxidative stress conditions

Catalase (CAT)

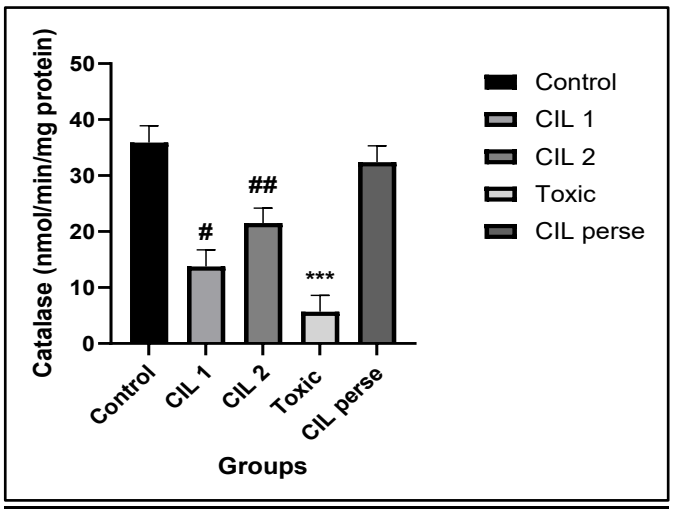
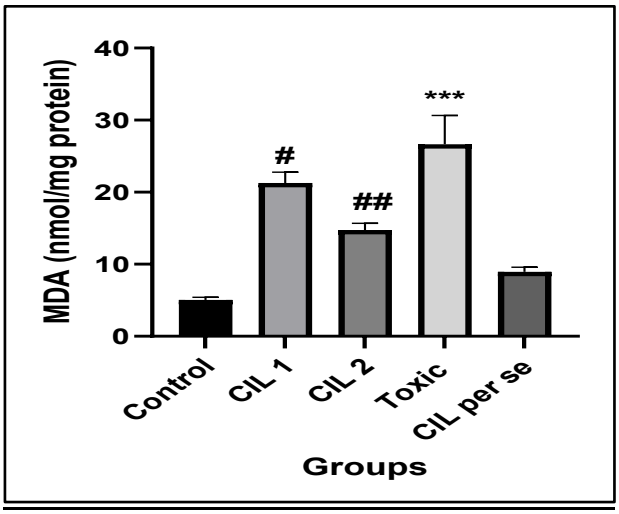


Fig 4:_Effect of different treatments on tissue Catalase of Different Groups. * Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.

This bar graph displays catalase enzyme levels in liver tissue across five groups: Control, CIL 1, CIL 2, CIL per se, and CP. Catalase is a key antioxidant enzyme responsible for detoxifying hydrogen peroxide in cells. In the Control group, liver catalase levels are high, indicating a normal antioxidant state.

Malondialdehyde (MDA)



<u>Fig 5:</u> Effect of different treatments on tissue MDA of Different Groups. * Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.

The graph represents the levels of malondialdehyde (MDA), a well-established marker of lipid peroxidation and oxidative stress, in liver tissue across five experimental groups: Control, CIL 1, CIL 2, CIL per se, and CP. The Control group shows relatively low MDA levels, indicating minimal oxidative damage under normal conditions. In contrast, the CP (cyclophosphamide-treated) group exhibits a significant increase in MDA levels, reflecting pronounced lipid peroxidation and severe oxidative stress caused by the toxic metabolites of cyclophosphamide. Treatment with Cilnidipine at

two different doses (CIL 1 and CIL 2) leads to a noticeable reduction in MDA levels compared to the CP group, suggesting that Cilnidipine confers a protective effect against CP-induced oxidative liver damage.

Tumor Necrosis Factor-alpha (TNF-α)

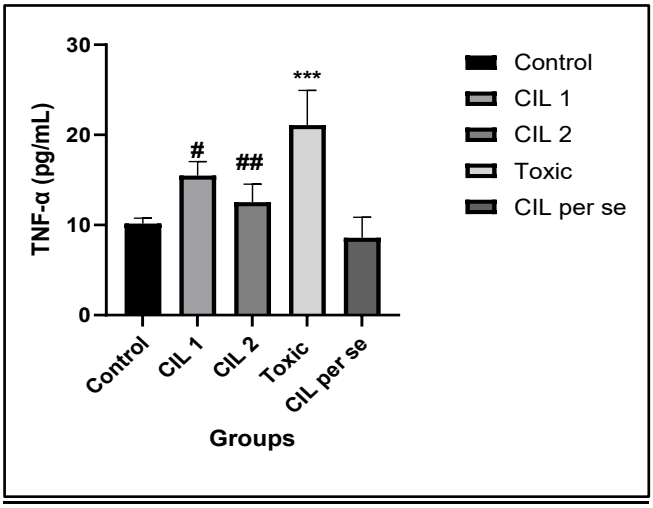


Fig 6: Effect of different treatments on tissue TNF-α of Different Groups. * Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.

The bar graph illustrates the effect of various treatments on TNF-α levels (pg/mL) across different experimental groups. The toxic group exhibited a significant increase in TNF-α levels compared to the control group, indicating a pronounced inflammatory response (denoted by $p < 0.001$). In contrast, both CIL 1 and CIL 2 treatment groups showed reduced TNF-α levels compared to the toxic group, suggesting an anti-inflammatory effect. Among them, CIL 2 demonstrated a greater reduction (##$p < 0.01$*) than CIL 1 (#$p < 0.05$), indicating a dose-dependent response.

Interleukin-1β (IL-1β

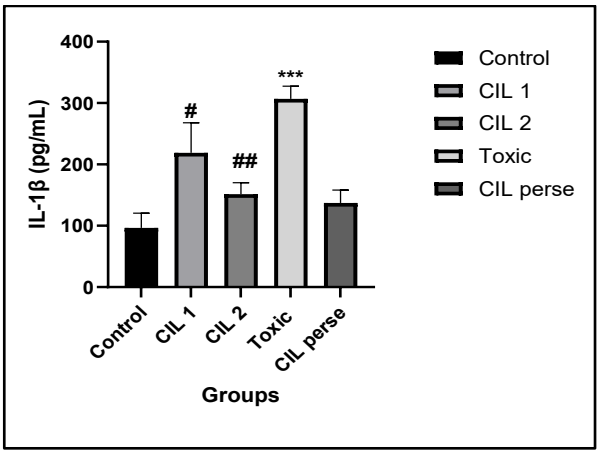


Fig 7: **Effect of different treatments on tissue IL-1β of Different Groups. * Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.**

The graph represents the levels of IL-1β (pg/mL) across different treatment groups. The toxic group exhibited a significant elevation in IL-1β levels compared to the control group (***$p < 0.001$), indicating a strong pro-inflammatory response following toxic exposure. Treatment with CIL 1 resulted in a moderate reduction in IL-1β levels compared to the toxic group (#$p < 0.05$), while CIL 2 showed a more pronounced reduction (##$p < 0.01$), suggesting a dose-dependent anti-inflammatory effect of the treatment.

Nuclear Factor Erythroid 2–Related Factor 2 (Nrf2)

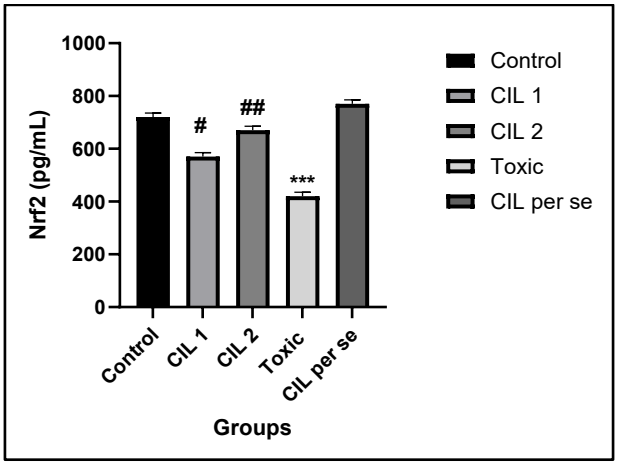


Fig 8: Effect of different treatments on tissue Nrf-2 of Different Groups. * Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.

The graph shows the expression levels of Nrf2 (pg/mL) across different experimental groups. The toxic group displayed a significant decrease in Nrf2 levels compared to the control group (***$p < 0.001$), indicating oxidative stress and impaired antioxidant defense. Treatment with CIL 1 and CIL 2 led to a noticeable restoration of Nrf2 levels, with CIL 2 producing a greater effect (##$p < 0.01$) than CIL 1 (#$p < 0.05$), suggesting a dose-dependent activation of the antioxidant pathway. Interestingly, the CIL per se group showed even higher Nrf2 levels than the control,

Nuclear Factor kappa B (NF-κB)

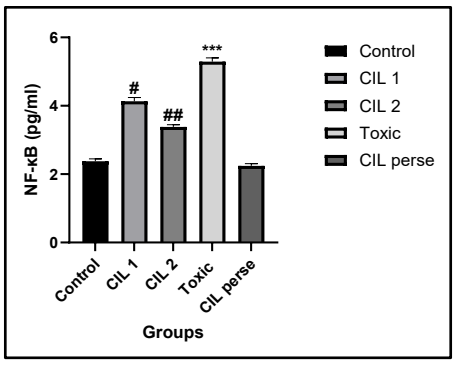


Fig 9: Effect of different treatments on tissue NF-κB of Different Groups. *Significantly different from the control group (p<0.001), # Significantly Different from Toxic groups.

The bar graph displays the levels of NF-κB (pg/mL) in different experimental groups. The toxic group showed a significant elevation in NF-κB levels compared to the control group (***$p < 0.001$), reflecting an intense inflammatory response. Treatment with CIL 1 resulted in a partial reduction in NF-κB levels (#$p < 0.05$), while CIL 2 produced a more pronounced decrease (##$p < 0.01$), indicating a dose-dependent anti-inflammatory effect of CIL. The CIL per se group displayed NF-κB levels similar to the control group, suggesting that CIL alone does not activate inflammatory signaling. Overall, these results suggest that CIL treatment effectively attenuates NF-κB activation, especially at higher doses, thereby potentially mitigating inflammation caused by toxic exposure.

Histopathology

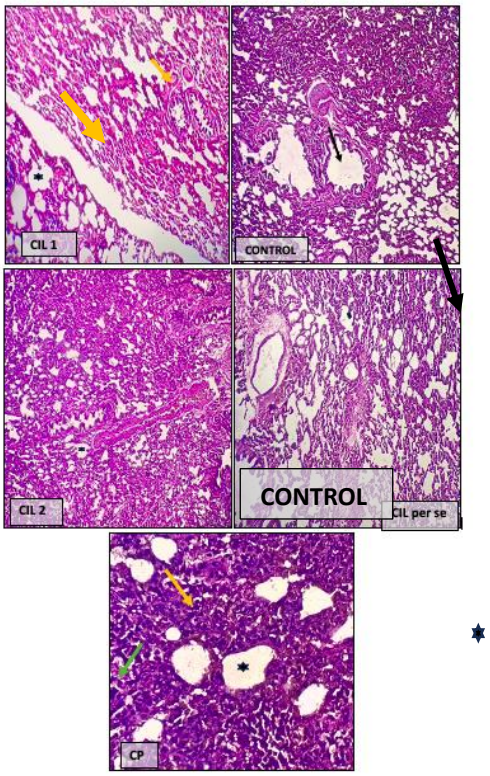


Fig 10- Histopathology images of lungs tissues

Histological analysis of lung tissues revealed significant differences among the experimental groups. The **control group** exhibited normal pulmonary architecture with intact and thin interalveolar septa, absence of inflammatory infiltration, and well-maintained alveolar spaces, indicative of healthy lung morphology. In contrast, the **cyclophosphamide (CP)-treated group** demonstrated severe histopathological alterations characterized by marked emphysema [★], prominent infiltration of inflammatory cells [yellow arrows], arterial hypertrophy [green arrows], and thickened intra-alveolar septa [black arrows], indicating substantial lung injury induced by CP administration.

The **CIL per se group**, which received cilnidipine alone, showed lung histoarchitecture comparable to that of the control group, with no evident pathological changes, suggesting that cilnidipine does not exert any adverse effect on lung tissue integrity when administered independently.
In the **CIL 1-treated group**, moderate attenuation of CP-induced damage was observed. There was a partial reduction in inflammatory cell infiltration and vascular changes, suggesting a protective but limited therapeutic effect at this dose. In contrast, the **CIL 2-treated group** demonstrated a near-complete preservation of normal lung architecture. Minimal signs of emphysema, negligible inflammatory infiltration, and maintained alveolar and vascular structure were noted, indicating a robust protective effect of cilnidipine at a higher dose.

DISCUSSION

The research offers a profound and meticulously conducted preclinical investigation into the protective potential of cilnidipine against the adverse toxic effects induced by cyclophosphamide (CP), a commonly used chemotherapeutic and immunosuppressive drug. Cyclophosphamide, though effective in treating various malignancies and autoimmune disorders, suffers a major limitation due to its serious side effects, particularly its damaging impact on vital organs like the lungs and liver.

The research was methodically structured, with Wistar albino rats divided into five groups: control, toxic (CP only), cilnidipine low dose + CP, cilnidipine high dose + CP, and cilnidipine per se. The experimental protocol included the administration of CP to induce lung and liver toxicity, followed by treatment with cilnidipine at two different doses to assess its protective effects. Biochemical parameters such as TBARS (indicating malondialdehyde levels), GSH, SOD, and CAT were analyzed, alongside pro-inflammatory markers measured using ELISA kits. Histopathological examinations of lung tissues provided further insight into structural integrity and cellular damage.

The results were striking and consistently demonstrated that CP treatment led to significant reductions in antioxidant enzyme levels, increased lipid peroxidation, elevated inflammatory cytokines, and severe histopathological alterations such as alveolar thickening, congestion, and infiltration of inflammatory cells. Conversely, cilnidipine treatment, particularly at the higher dose of 2 mg/kg, significantly reversed these effects. It restored antioxidant defenses, reduced MDA and inflammatory markers, and preserved lung architecture, thus highlighting its potent protective role.

Overall, this thesis successfully demonstrates the organoprotective efficacy of cilnidipine and advocates for further translational research to evaluate its utility in clinical oncology settings, where it may significantly enhance patient quality of life and expand the therapeutic window of essential but toxic chemotherapeutic agents like cyclophosphamide.

Conclusion

In conclusion, this thesis successfully establishes cilnidipine as a promising protective agent against cyclophosphamide-induced lung toxicity in experimental animal model. The comprehensive evaluation of biochemical and histological markers reveals that cilnidipine, particularly at a higher dose, mitigates oxidative damage, reduces pro-inflammatory cytokine levels, and maintains tissue integrity. Its dual calcium channel blocking ability, coupled with its capacity to activate antioxidant defenses and suppress inflammatory pathways, underscores its therapeutic potential beyond hypertension. The findings

suggest that cilnidipine can be repurposed as an adjunctive treatment in cancer therapy to reduce organ toxicity associated with cyclophosphamide and possibly other chemotherapeutic agents. Given its proven safety profile and effectiveness in this study, cilnidipine holds clinical promise for improving patient outcomes by reducing treatment-related complications, and future clinical trials are warranted to validate these findings in human subjects.

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