

# NEPHROPROTECTOR EFFECT OF MURAMYLDIPEPTIDE IN CADMIUM INDUCED WISTAR RATS

G. REVATHI\*, P. SUARNALATHA DEVI, P. JAYALAKSHMI, N. D. PRASANNA, AND S. K. SHAHEEN

Department of Applied Microbiology, S. P. M. V. V., Tirupati - 517 502

E-mail: drrevathiphd@gmail.com

## KEY WORDS

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\*Corresponding author

## ABSTRACT

Cadmium is a toxic element which enters body via a number of routes including food, water, air and through cigarette smoke. The cadmium may inhibit lipogenesis by binding with the thiol group (SH) of coenzyme A. N-acetyl muramyl L-alanyl D-isoglutamine (MDP), is the minimal structural sub unit obtained from the gram positive bacteria like *Mycobacterium tuberculosis*. After 10<sup>th</sup> day of injection CDIR (Cadmium chloride induced rats) group showed cystic dilatation of tubules, focal inter tubular infiltration of mononuclear cells, dilatation of bowmen's capsules and focal areas of hemorrhages. These changes were slightly aborted to normal condition after the treating with of MDP to CDIR group which were designated as MDPT. In the present study MDP showed both immunomodulating and nephroprotector effects.

## INTRODUCTION

Heavy metals which released by industries are immutable and non- biodegradable in nature. Among them, Cadmium is one which is used extensively in batteries, coating, electroplating and alloys. Although some cadmium- containing products can be recycled, a large share of the general cadmium pollution is caused by dumping and incineration of cadmium containing wastes. Cadmium is not only industrial pollutant but also found in ores with zinc, copper, and lead. Cadmium targets human prostate glands indicating a link between cadmium exposure and cancer. It causes many toxic effects like kidney damage, lungs edema, Itai-Itai disease. Maternal exposure to cadmium is associated with low birth weight and an increase of spontaneous abortions. The World Health Organization (WHO) has recommended that the provisional permissible intake of cadmium should not exceed 0.4 to 0.5 mg/week or 0.057 to 0.071 mg/day (Copenhagen 1972), with maximum acceptable concentration of cadmium in drinking water is therefore 0.005 mg/L (WHO 1984). Cadmium and cadmium compounds are carcinogenic in experimental animals. They cause neoplasms in rats after subcutaneous, intramuscular, and intra prostatic injection. In rats cadmium will induce a variety of tumors including malignant tumors at the site of injection and causes respiratory tumors by inhalation. Cadmium treatment in rats will induce benign tumors of the testis and ventral prostate (IARC, 1993; Waalkes *et al.*, 1997a; Waalkes, 1995). Epidemiological studies are available on nickel-cadmium battery workers and metal smelting workers. Cadmium is a metallic toxin of great environmental and occupational concern and it is one of the major organic

carcinogens.

N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP) is a class of muramyldipeptide and it is a minimum structural unit of peptidoglycans in acid fast Gram positive bacteria responsible for immunopotentiating ability (Ellouz *et al.*, 1974 and Kotani *et al.*, 1975). MDP and its derivatives have important biological activities like adjuvant activity, Stimulation of non- specific resistance against bacterial, viral and parasite infections and against tumors, Somnogenic activity, *i.e.*, increase of the duration of slow wave sleep (Lefrancier and Lederer, 1987). In the study the nephroprotector effect of MDP on cadmium induced Wistar rats has been reported.

## MATERIALS AND METHODS

### Separation of Peptido glycan (PG) from *M. tuberculosis*

*M. tuberculosis* cells at mid-log phase were harvested by centrifugation and washed with phosphate buffered saline (PBS) to remove growth medium of isolation of PG. The bacilli were resuspended in 10 mM NH<sub>4</sub>HCO<sub>3</sub> containing 1 mM phenylmethylsulfonyl fluoride and disrupted by intermittent probe sonication with an MSE Soniprep 150 (MSE-Sanyo; Integrated Services) for 30 cycles (60-s bursts separated by 60 s of cooling). The sonicate was digested with 10g each of DNase and RNase/mL for 1hr at 4°C. A cell wall-enriched fraction was obtained by centrifugation at 27,000g for 30 min. The pellet containing cell walls was resuspended in PBS containing 2% sodium dodecyl sulfate (SDS) and the suspension was incubated for 1hr at 50°C with constant stirring. The suspension was recentrifuged at 27,000g for 30 min, and the

supernatant was discarded. This process was repeated twice. The resulting pellet was resuspended in PBS containing 1% SDS and 0.1mg of self-digested proteinase K/mL, and the suspension was incubated at 45°C for 1hr with constant stirring. The mixture was then heated at 90°C for 1hr before centrifugation at 27,000g for 30 min. The supernatant was discarded, and the 1% SDS extraction procedure was repeated twice to remove proteinase K. The pelleted material was washed twice with PBS and four times with deionized water to remove SDS. The resulting Mycolyl-arabinogalactan-peptidoglycan complex (MAPc) was extracted with ethanol-diethyl ether (1:1) and dried under a vacuum. In order to hydrolyze the mycolic acids, the MAPc was resuspended in 0.5% KOH in methanol and stirred at 37°C for 4 days. The mixture was centrifuged, and the pellet was washed twice with methanol and twice with diethyl ether and dried under a vacuum. The resulting arabinogalactan-PG was digested with 0.05 N H<sub>2</sub>SO<sub>4</sub> at 37°C for 5 days to remove the arabinogalactan. The resulting insoluble PG was washed four times by centrifugation with deionized water and dried under a vacuum (Mahapatra *et al.*, 2008).

#### Solubilization of Peptidoglycan (PG)

The purified PG (2 mg) was suspended in 0.5 mL of 10 mM sodium acetate (pH 5.0) containing 25g of purified muramidase and the suspension was incubated at 37°C for 16 hr with stirring. Digests were centrifuged at 27,000g for 30 min, and the supernatant was filtered through a 10-kDa-cutoff ultrafiltration membrane (Millipore) to remove muramidase and dried under a vacuum. The muropeptides were resuspended in 0.5 M sodium-borate buffer (pH 9.0), and sodium borohydride was added to achieve a final concentration of 8 mg/mL. The mixture was incubated for 30 min at room temperature to reduce the sugar moieties. The reaction was stopped by the addition of orthophosphoric acid, and the pH was adjusted to 4.0 prior to fractionation by size exclusion chromatography on a Superdex peptide 10/300 GL column with a model 600 controller connected to a model 600 pump and a model 2487 UV detector. The column was equilibrated and eluted with 30% acetonitrile containing 0.1% trifluoroacetic acid with a flow rate of 0.5 ml/min. The absorbance of the effluent at 214 nm was monitored. (Mahapatra *et al.*, 2008).

#### Purification of muramyldipeptide by High Pressure Liquid Chromatography (HPLC)

The fractions containing muropeptides were dried under a vacuum and resuspended in high-performance liquid chromatography (HPLC)-grade water at an approximate concentration of 10 M. An aliquot (20µL) was applied to a 2-by-150-mm Hypersil octyldecyl silane (C18) column connected to an Agilent 1100 HPLC system. The muropeptides were eluted with a 2 to 30% linear gradient of aceto nitrile containing 0.5% formic acid at 320 l/min (Mahapatra *et al.*, 2008).

#### Selection, procurement and maintenance of experimental animals

The experiment involved 120 healthy, weighing about 120-150g male Wistar rats which were purchased from BROS Enterprises, Tirupati. After procurement they were thoroughly

examined and acclimatized to lab conditions prior to start the experiment. These Wistar rats were maintained at laboratory conditions (26 ± 2°C; 12hr light and 12hr dark cycle) throughout the course of study. They were kept in well cleaned and sterilized cages. The animals had free access to standard laboratory chow food which was supplied by BROS Enterprises, Tirupati and sterilized water in hygienic conditions. All care and management procedures for maintaining rats were in accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals.

#### Experimental design

After well acclimatization to lab conditions, 120 male Wistar rats were divided in to two groups 10 animals as control Wistar rats (CWR) and the other 110 animals as cadmium chloride induced rats (CDIR). CWR group rats were received normal saline and the CDIR group received cadmium chloride (16µM). After administration of 10 doses in 10 weeks *i.e.*, the total dose of the cadmium chloride reaches to 160µM. After well determination of tumor/cancer, the tumorous rats were divided into two groups, 10 were kept as cancer control rats (CCR) group and the remaining 100 animals as MDP treated (MDPT) group. Tumor induced CCR group rats received normal saline and the MDPT group received 10µM of MDP dissolved in normal saline. At the end of the doses total amount of MDP injected is 100µM. As the carcinogenicity of the heavy metal depends upon several biotic and abiotic factors like age, weight, developmental stages of the animal, period of exposure, temperature and sex, the specimens were maintained uniformly throughout the experimental study.

#### Selection and administration of carcinogen

Cadmium Chloride (LR) 98.0% (CdCl<sub>2</sub>.H<sub>2</sub>O; M.W.201.32) was selected as the carcinogen, which was obtained from S. d. Fine-Chemicals Ltd, Mumbai, India.

Rats were injected subcutaneously in the dorsothoracic mid line with CdCl<sub>2</sub> once in a week for the period of 10 weeks with dosage of 16µM/Kg body weight. This result in total dosage of 160µM/Kg. 0.85% saline solution was used for control rats. After 70 days the histopathological, Ultrastructural studies was done (Waalkes *et al.*, 1997).

#### Histopathological studies

##### Tissue collection, sample preparation and Staining of tissue sample

Tissues were collected by sacrificing the rats by cervical dislocation procedure. The fixed tissue were dehydrated by immersion and gentle agitation in baths of 50%, 70% and three times of 95% ethanol extract, allowing 90 minutes in each. paraffin wax blocks were prepared and 4 to 6µ thick sections were cut with the help of microtome. Permanent silds were prepared and stained with Ehrlich's haematoxylin and Eosin following the standred protocol.

##### Ultra structural studies by Transmission Electron Microscope (TEM)

The Transmission Electron Microscope (TEM) studies of the tissue were performed by using Electron Microscopy (Bozzola and Russell 2<sup>nd</sup> Edition 1999).

##### Tissue collection, Sample preparation and staining procedure

For microscopic studies, samples were transferred to vials and fixed in 3% Glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 24hrs at 4°C and post fixed in 2% aqueous Osmaium tetraoxide in the same buffer for 2hrs.

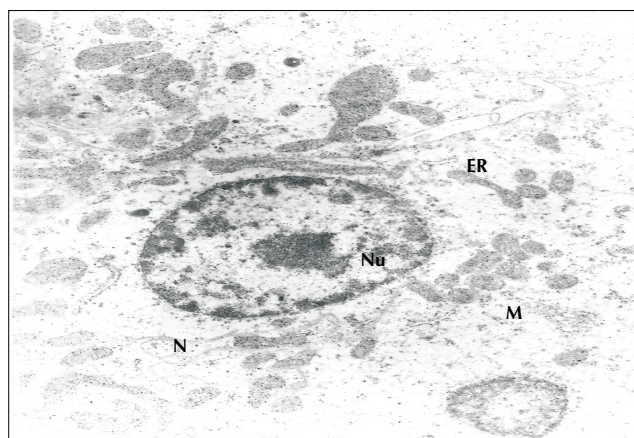
Samples were dehydrated in a series of graded acetone, infiltrated and embedded in Araldite 6005 resin. Semi thin section (300-500nm) and ultra thin sections (50-70 nm) were cut with a glass knife on a Leica Ultra cut (UCT-GA-D/E-1/00) microtome. Ultra thin sections were mounted on grids and stained with saturated aqueous Uranyl acetate, counter stained with 4% lead citrate, observed under transmission electron microscope (Model: Hitachi,H-7500) (Glauert, and Glauert, 1958; Mollenhauer, 1959).

## RESULTS AND DISCUSSION

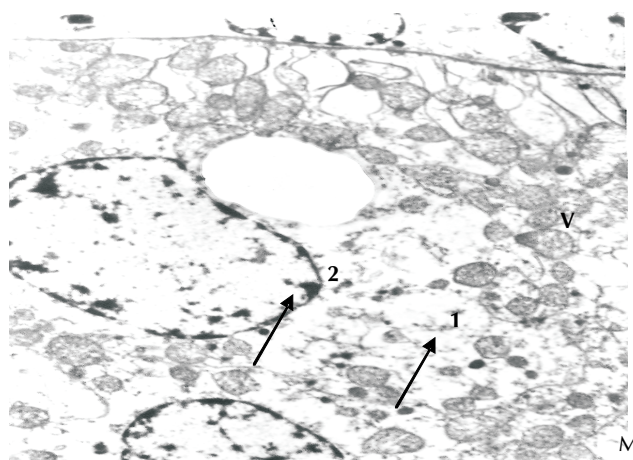
Electron microscopic pathological changes observed in Cadmium induced rats from the control rats (Fig. 1) kidney were: injured brush border microvilli, swollen mitochondria in the proximal convoluted tubular cells, thinning of nuclear membrane, chromatolysis of nucleus and development of vacuolation (Fig. 2). MDP treated Kidney showing pignotic, chromatolysis of nucleus and narrowing of intracellular junction with blood cells and development of monocytes (Fig. 3).

According to Massanyi *et al.*, (2007) most frequent ultrastructural alterations are undulation of external nuclear membrane, dilation of perinuclear cistern and endoplasmic reticulum. In all studied types of cells mitochondria with altered structure were observed. An electron microscopic analysis showed dilation of perinuclear cistern. The intercellular spaces were enlarged and junctions between cells were affected. Mainly after long term cadmium administration nuclear chromatin disintegration was present. Endoplasmic reticulum was dilated. Ord *et al.*, (1988) found the damaged organelle were cell membrane, mitochondria, golgi cisternae and tubular network, chromatin, nucleoli, microfilaments and ribosomes. Mitochondria distortion and some damage to golgi were also observed.

According to Fidler (1985), activated monocytes that



**Figure 1: Control Kidney**-showing cell organelle like Mitochondria (M), Nucleolus (Nu), Nucleus(N), Endoplasmic reticulum(ER) (7280X)



**Figure 2: Induced Kidney**- showing 1. Thinning of nuclear membrane, 2. Chromatolysis of nucleus and it also shows the changes in cell like Swollen Mitochondria (M) and Vacuolation (V) (6370X)



**Figure 3: Treated Kidney**-showing the development of monocytes (12740X)

phagocytosed liposomes containing MTP-PE clustered around melanoma cells at a higher density than did control monocytes. This initial clustering of the tumoricidal monocytes around melanoma cells was followed by the establishment of numerous focal points of binding, and some areas actually exhibited discontinuous membrane which was confirmed by stereophotography. The same results like, activation of monocytes was observed in the ultra structural changes of Muramyldipeptide treated (MDPT) group rat's kidney. Monocytes play a central role in immune regulation and inflammation. When activated, monocytes produce and release a number of inflammatory mediators, such as IL-1, IL-6, IL-8, TNF- $\alpha$ , and arachidonic acid metabolites. This leads to the recruitment and activation of other immune cells into sites of injury and infection.

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