

Effect of Cadmium on Macrophage U937 Cell Proliferation and Activation

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Abstract

The effect of cadmium concentration on the proliferation and activation of U937 cells was studied. Cadmium at different concentrations was incubated with the cell line U937 in culture. Three days later, it was observed that cadmium inhibited the proliferation of the cells in a concentration-dependent manner which may be attributed to the formation of adducts with some growth factors. Later, U937 cells were also pre-treated with phorbol 12-myristate, 13-acetate to transform the cells from the monocyte-like morphology to the macrophage form. The macrophage U937 cells were then incubated with different concentrations of cadmium and the supernatants of the cell cultures were analyzed for the production of nitric oxide via the Griess reaction. Tumour necrosis factor alpha (TNF- α), and the interleukins 6 and 1 (IL 6 and IL 1) were also analyzed by enzyme linked immunosorbent assay. The results reveal that cadmium significantly enhanced the macrophages to produce nitric oxide and the cytokines when compared to lipopolysaccharide. This shows that cadmium significantly activated macrophages therefore the heavy metal has the capacity of initiating the inflammatory response. It is thus considered as an immunotoxicant.

Keywords: cadmium, hepatotoxic, nephrotoxic, inflammatory, nitric oxide, lipopolysaccharide.

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Introduction

Cadmium is one of the environmentally known toxic heavy metals hence its occurrence in appreciable quantities is of utmost concern to man. Till date, cadmium has no known function in the human body. The accumulation of cadmium in the body causes serious health problems such as nephrotoxicity, hepatotoxicity and cardiovascular aberrations (Bremner, 1978; Afshar *et al.*, 2000). In natural ecosystems, there are only trace quantities of cadmium however, excess load has been detected as a consequence of urbanization and industrialization (Cook, 1977). A high level of cadmium has been found in sedimentary rocks since marine phosphates and phosphorites could contain high concentrations of cadmium (WHO, 1992). The occurrence of cadmium in the environment constitutes a potential danger because it is easily biomagnified (Mance, 1987).

Most organisms could tolerate trace levels of heavy metals including cadmium however this depends on the availability of metallothionein – a cysteine-rich protein (molecular weight 6000 – 8000 Da) which participates in the detoxification process in the liver and kidney but serious pathological consequences may arise when the endogenous concentration of the protein has been overwhelmed (Klassen *et al.*, 1999). Cadmium has a very long half-life in humans during which it may impair the intrinsic antioxidant system by causing oxidative stress (Goering *et al.*, 1987; Thijssen *et al.*, 2007). The antioxidant system is part of the innate immunity hence cadmium may have the propensity of impairing gross immunity. Macrophages and neutrophils are major players of the human immune system which destroy micro-organisms and damaged cells via

oxidative burst. This current study investigates the potential of cadmium in affecting the

proliferation and differentiation of the macrophage cell line U937.

Materials and methods

Chemicals: These include cadmium chloride (CdCl_2) obtained from Merck (Germany), cell line U937 was obtained from the European collection of cell cultures (Salisbury, UK). Fetal calf serum, phorbol 12-myristate 13-acetate, L-glutamate, streptomycin, extravidin-horse raddish peroxidase conjugate (extravidin-HRP) and trypan blue were purchased from Sigma Chemicals (USA). Penicillin was a product from Euroclone (Italy). Others are purified rat anti-human $\text{TNF-}\alpha$, purified rat anti-human IL6, mouse anti-human IL6, and the biotinylated forms obtained from Pharmingen (Beckton Dickinson). The culture media RPMI-1640 was obtained from the Invitro life technologies (UK). All other chemicals were analytical reagents hence were used without further purification. All buffers were prepared in double glass-distilled water while all dilutions were made in RPMI-1640.

Propagation of U937 cells: Cell propagation was carried out in a biosafety cabinet unless otherwise stated. U937 cells were grown in complete medium which is RPMI-1640 medium supplemented with 50 mL fetal calf serum (heat inactivated), 5 mL of penicillin (100 U/mL), 5 mL of 100 $\mu\text{g/mL}$ streptomycin, and 5 mL of 0.02 M glutamine. In brief, the vial of U937 cells was allowed to thaw (as they were preserved in liquid nitrogen) and delivered into 10 mL of the complete medium and washed thrice with RPMI-1640. The cells were later resuspended in 10 mL of complete medium at a density between 2×10^5 and 1×10^6 cells mL^{-1} . The cells were later kept in a humidified incubator maintained at 37°C and gassing up to 5 % CO_2 .

Cell incubation with cadmium: Cells were seeded at 5×10^5 cells mL^{-1} and 5 mL of each cell culture was delivered into sterile tubes. One microlitre of 1 μmol , 2 μmol , 4 μmol , 8 μmol , and 16 μmol CdCl_2 was delivered into each of the cell culture and kept in a humidified incubator for three days. Thereafter, each cell culture was agitated and viable cells were identified by trypan blue dye staining and

counted using a haemocytometer. Viable cell count was expressed as percent viability over the cells that were not incubated with cadmium.

Cell transformation and incubation with cadmium: Two hundred microlitres of cells in culture resuspended at 5×10^5 cells mL^{-1} was delivered into each well of a 96-well culture plate. Twenty microlitres of phorbol 12-myristate, 13 acetate (100 ng/mL) was added into each well and kept in the incubator for 48 hours. Thereafter, the supernatants of the cell culture were removed and 200 μL of each of the cadmium solution were delivered into the wells that were split into groups. Two hundred microlitres of lipopolysaccharide (5 ng/mL) was also added to some wells as positive controls. Thus the various treatments were:

- PMA and 1 μmol Cadmium Chloride
- PMA and 2 μmol Cadmium Chloride
- PMA and 4 μmol Cadmium Chloride
- PMA and 8 μmol Cadmium Chloride
- PMA and 16 μmol Cadmium Chloride
- PMA and 5 ng/mL lipopolysaccharide

They were then incubated for 24 hrs and the supernatants were analysed for the production of tumour necrosis factor 1 ($\text{TNF-}\alpha$), interleukin 1 (IL 1), interleukin 6 (IL 6) and nitric oxide.

Nitric oxide and cytokine production assays: Nitric oxide production was determined in the supernatants by the Griess reaction according to Hwang *et al.* (2002). The levels of the cytokines $\text{TNF-}\alpha$, IL 1, and IL 6 were analyzed via enzyme linked immunosorbent assay at 25°C as described (Okoko and Oruambo, 2008).

Statistical analysis: Data were expressed as mean \pm standard error of the mean. Where appropriate, a two-way analysis of variance was used to test for significant differences between the treatments. Minitab Statistical Software (version 14) was the package used and values of $P < 0.05$ was considered statistically significant.

Results and Discussion

The viability of the U937 cells when incubated with the different cadmium concentrations are shown in figure 1. Viability was 54.57 ± 4.19 % when incubated with 16

μmol cadmium and 88.21 ± 3.22 % when incubated with 1 μmol cadmium. Also the results for the production of the cytokines and nitric oxide by the transformed cells are shown in

table 1. It was observed that cadmium enhanced the release of nitric oxide and the cytokines however, this tendency was not as effective as lipopolysaccharide. Nitric oxide production was $0.412 \pm 0.12 \mu\text{M}$ when the transformed cells were incubated with $1 \mu\text{mol}$ cadmium and $3.392 \pm 0.24 \mu\text{M}$ when incubated with $16 \mu\text{mol}$ cadmium. The production tumour necrosis factor alpha in the transformed cells was $9.25 \pm 2.55 \text{ pg/mL}$ when incubated with $1 \mu\text{mol}$ cadmium and $63.17 \pm 4.17 \text{ pg/mL}$ when treated with $16 \mu\text{mol}$ cadmium. Similar trend was also observed for the release of interleukins 6 and 1.

It has been proposed that the toxicity of cadmium may involve oxidative stress principally by impairing the antioxidant defence system in the organism with the resultant production of reactive oxygen species (Wang *et al.*, 2004; Thijssens *et al.*, 2007). Thus the release of nitric oxide (a major ROS) which is a signal compound in the inflammatory response may be attributed partly to this property of cadmium. Nitric oxide is mutagenic because it attacks DNA and also interferes with DNA repair mechanisms (Keefer and Wink, 1996). This implies that cadmium acts like a catalyst in carcinogenesis in addition to forming adducts with various biomolecules (Rikans and Yamano, 2000).

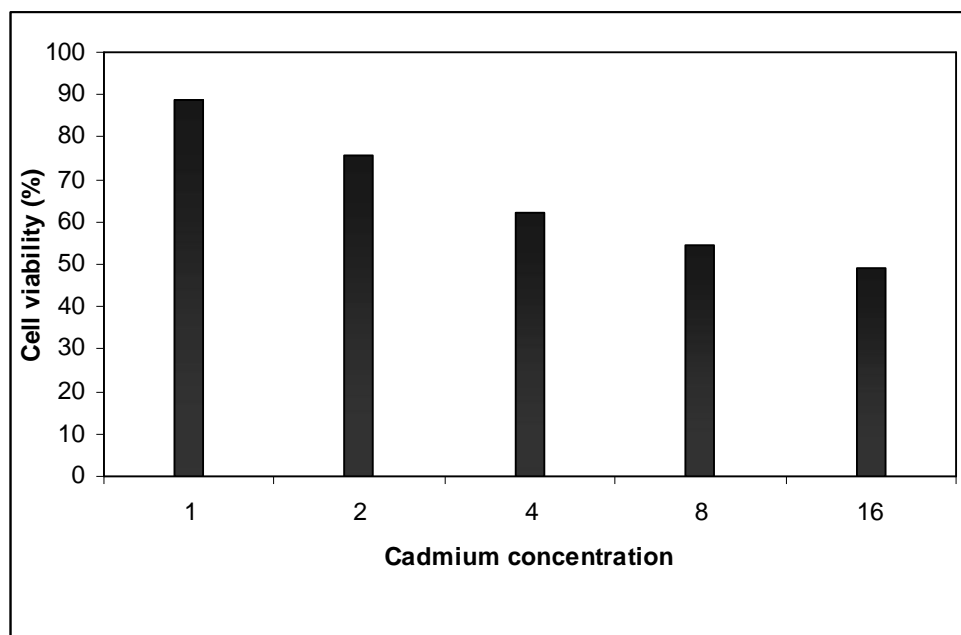


Fig 1. The effects of cadmium concentration on the viability of U937 cells

Table 1. The effect of cadmium concentration on nitric oxide, TNF- α , IL6 and IL1 production by U937 cells

Cadmium	NO ($\times 10^{-1} \mu\text{M}$)	TNF- α (pg/mL)	IL6 (pg/mL)	IL1 (pg/mL)
0 (μmol)	0.98 ± 0.03	0.36 ± 0.04	0.17 ± 0.02	0.15 ± 0.02
1 (μmol)	4.12 ± 1.22	9.25 ± 1.66	10.12 ± 2.76	11.52 ± 1.98
2 (μmol)	12.37 ± 2.06	14.55 ± 3.06	13.26 ± 1.01	19.47 ± 4.21
4 (μmol)	18.25 ± 2.76	31.67 ± 2.32	21.33 ± 4.66	43.19 ± 4.29
8 (μmol)	27.61 ± 3.11	56.21 ± 4.74	36.17 ± 3.18	51.63 ± 5.56
16 (μmol)	33.92 ± 2.35	63.17 ± 4.17	41.22 ± 5.76	65.26 ± 3.83
^a LPS (5 ng/mL)	57.12 ± 1.97	85.25 ± 5.12	59.92 ± 4.44	83.87 ± 4.32

Results are presented as mean \pm SEM of readings from 12 wells. (^a LPS is Lipopolysaccharide was used as a reference compound; NO is nitric oxide; TNF- α is tumour necrosis factor alpha; IL6 is interleukin 6; and IL1 is interleukin 1)

Phorbol esters especially phorbol 12-myristate 13-acetate enhance the differentiation of U937 cells from the monocyte form to the macrophage form which peaks at 48 hrs (Liu

and Wu, 1992; Okoko and Oruambo, 2008). The activation of macrophages especially by lipopolysaccharide leads to the release of nitric oxide and some proinflammatory cytokines such

as interleukin 6, interleukin 1, and tumour necrosis factor alpha (Meng and Lowell, 1997; Hsieh *et al.*, 2007). The ability of cadmium to induce the production of nitric oxide and other proinflammatory cytokines in the transformed U937 cells shows that it is an important activator of macrophages thus may trigger the inflammatory process (Roitt *et al.*, 2002; Goldsby *et al.*, 2003). This implies that the activation of macrophages is associated with the toxic process ascribed to cadmium.

It was observed that cadmium has the propensity of reducing the viability of the cells in a concentration dependent manner. This may be attributed to the effect on specific growth factors. Cadmium has the ability to disrupt the function of certain biological molecules such as proteins. For instance, it has been postulated that cadmium causes primary injury to the cell by binding to sulphhydryl groups on critical molecules in mitochondria. This inactivation causes oxidative stress and to an appreciable extent mitochondrial dysfunction (Rikans and Yamano, 2000).

This current work reveals that cadmium is an immunotoxicant in addition to the reported nephrotoxic and hepatotoxic effects. Further studies to elucidate the mechanism of cadmium-induced toxic effects are solicited.

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