Cadmium reduces the efficiency of Sindbis virus replication in human cells and promotes their survival by inhibiting apoptosis

Etienne Frumence, Marjolaine Roche *, Pascale Guiraud *

Université de la Réunion, UM 134 Processus Infectieux en Milieu Insulaire Tropical (PIMIT), INSERM U1187, CNRS UMR9192, IRD UMR249, Plateforme Technologique CYROI, 97490 Sainte Clotilde, France

**Abstract**

Arthritogenic alphaviruses are emerging arthropod-borne viruses that occasionally cause sporadic to global outbreaks all over the world. Many environmental factors including xenobiotics have been identified as capable of influencing the spread, the susceptibility and the outcome of viral infection. Among them cadmium is a toxic non-essential heavy metal and a prevalent environmental contaminant. In the present study we evaluated the effect of cadmium exposure on alphavirus infection in vitro. We infected Human Embryonic Kidney (HEK) 293 cells in the presence of cadmium chloride (CdCl 2 ) with Sindbis virus. Cell viability, apoptosis and viral growth were then examined. Our data show that effective doses of cadmium decreased the virus mediated-cell death by inhibition of apoptosis. Moreover, virus growth in HEK 293 cells was also reduced by CdCl 2 treatment. Altogether our results demonstrate that cadmium triggers a protective response which renders HEK 293 cells resistant against Sindbis virus infection.

**Keywords:**
Cadmium
Alphavirus
Sindbis virus
Viral growth
Apoptosis

**1. Introduction**

Arboviruses or ARthropod-BOrne viruses transmitted by arthropod vectors, represent a major group of infectious viruses that infects over 100 million people each year with viruses such as Dengue virus, Chikungunya virus, West Nile virus and Zika virus. Among them, arthritogenic alphaviruses from the Togaviridae family occasionally cause sporadic to global outbreaks all over the world [1]. Since 2004, the Chikungunya virus has been responsible for large epidemic affecting millions in Kenya, Islands of the Indian Ocean, India, parts of the Southeast Asia, the Caribbean and South America [2,3]. Understanding the complex interactions between host, arthropod vectors, infectious agents and environment is becoming necessary to fight against these diseases. Many environmental factors including xenobiotics have been identified as capable of influencing the spread, the susceptibility and the outcome of viral infection [4]. Among them, heavy metals have been shown to have a potential effect on several virus infections.

Cadmium is a toxic non-essential heavy metal and a prevalent environmental contaminant. Cadmium is used in electroplating, in some industrial paints and in the manufacturing of batteries [5]. In humans, cigarette smoke is the major source of cadmium exposure via inhalation [6]. Cadmium exposure occurs also from ingestion of contaminated food through bioaccumulation or water [7]. This metal accumulates in the kidney and the liver throughout the entire lifetime. In humans and animals, cadmium causes toxic effect in a variety of tissues and induces a number of adverse health effects and diseases, including cancer, renal dysfunction, osteoporosis and hepatotoxicity [7,8]. At the cellular level, cadmium affects cell proliferation, survival and cell death, differentiation, and many biological mechanisms [9].

Several reports showed that cadmium may have a beneficial or deleterious effect on the outcome on viral diseases. Cadmium treatment in vitro results in an increase influenza virus replication whereas Sendai virus and Human Immunodeficiency Virus type 1 (HIV-1) are inhibited [10–12]. In mouse models, it has been shown that cadmium exposition reduces the efficiency of antiviral innate immune responses directed against cytomegalovirus [13].

In a mouse model of alphaviral encephalitis, it has been reported that a single oral dose of cadmium can enhance the neuropathogenicity of Semliki Forest virus and Venezuelan equine encephalitis virus, two members of the alphavirus genus [14]. Sindbis virus (SINV), which is a widely distributed zoonotic alphavirus distributed in Eurasia, Africa and Australia, has been extensively studied as a model for alphavirus replication [15]. In the present study we investigated the effects of cadmium exposure on the efficiency of SINV replication in HEK 293 cells. We showed that cadmium chloride (CdCl 2 ) treatment inhibits viral growth and promotes the viability of infected human cells.

* Correspondence address: GIP CYROI, Université de la Réunion, 2 Rue Maxime Rivière, 97490 Sainte Clotilde, Réunion, France.

E-mail addresses: marjolaine.roche@univ-reunion.fr (M. Roche), pascale.guiraud@univ-reunion.fr (P. Guiraud).

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2. Materials and methods

2.1. Reagents and antibodies

Cadmium chloride (CdCl₂, Sigma Aldrich) was prepared in sterile water as 100 mM solution and stored at −20 °C. The pan-caspase inhibitor, Z-VAD-fmk was purchased from Promega. Etoposide (Sigma Aldrich) was used as an inducer of apoptosis. Annexin V-FITC was purchased from BD Biosciences and propidium iodide (PI) was from Biolegend.

2.2. Virus and cell lines

Sindbis Virus (SINV, Strain AR-339) was obtained from the National Collection of Pathogenic Viruses (NCPV). In this study, SINV was amplified in Vero cells (ATCC, CCL-81) and stored at −80 °C. Human Embryonic Kidney cells, (HEK 293, ATCC, CRL-1573), NIE115 and Vero cells were cultured at 37 °C in a 5% CO₂ atmosphere in MEM Eagle medium, supplemented with 10% fetal bovine serum, 1 mg mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin and 0.5 µg mL⁻¹ fungizone (PAN Biotech). For infection, cells were infected for 2 h at 37 °C with SINV at a multiplicity of infection (MOI) of 1 PFU/cell. The inoculum was then removed and replaced with fresh medium. The cells were incubated at 37 °C and 5% CO₂ for the rest of the experiment.

2.3. Cytotoxicity assays

Cell viability was assessed with the colorimetric MTT metabolic activity assay. Briefly, 20 µL of a 5 mg mL⁻¹ MTT (3-[4,5-di-methylthiazol-2-yl]-2,5- diphenyltetrazolium bromide, Sigma Aldrich) solution was added to HEK 293 cells cultured in a 96-well plate at a density of 10,000 cells per well. Following 1 h incubation, MTT medium was removed and the insoluble formazan salt was solubilized with 100 µL of DMSO. Absorbance of converted dye was measured at 570 nm with a background subtraction at 690 nm. For Trypan Blue staining, cells grown in 6 well plates at a density of 6 × 10⁶ cells per well were trypsinized and mixed with 0.4% trypan blue solution (1:10). Viable cells (unstained) and nonviable cells (stained) were then counted using a hemacytometer. The percentage of viable cells was calculated as the number of unstained cells divided by the total number of cells.

2.4. Flow cytometry assay

HEK 293 cells were grown on 100-mm Petri dishes. About 10⁶ cells were harvested and resuspended in Annexin V binding buffer. Then cells were incubated with Annexin V-FITC (BD Biosciences) and propidium iodide (PI) for 15 min in the dark at room temperature. Cells were washed and fixed with 3.7% formaldehyde solution for 10 min and subjected to a flow cytometric analysis using FACScan flow cytometer (Becton Dickinson). Results were analyzed with FCS Express 5 software (De Novo Software).

2.5. Caspase activity

HEK 293 cells were cultured in 96-well plates at a density of 10⁴ cells per well. Caspase 3/7 activity in crude cell lysates was measured using Caspase Glo® 3/7 Assay Kit (Promega) according to the manufacturer’s protocols. Caspase activity was quantified by luminescence using a FLUOstar Omega Microplate Reader (BMG LABTECH).

2.6. Virus titration

Virus titration assay was run using standard plaque assay. Vero cells seeded in 24-well culture plates were incubated with tenfold dilutions of virus samples. The plates were incubated for 2 h at 37 °C and unabsorbed virus was removed. Then culture medium supplemented with 0.8% carboxymethylcellulose (CMC) was added to each well, followed by an incubation at 37 °C for 2 days. The CMC overlay was removed and the cells were stained with 0.5% crystal violet. Plaques were counted and expressed as plaque-forming units per mL (PFU mL⁻¹).

2.7. Statistical analysis

Results are expressed as mean ± SEM of at least 3 independent experiments. Comparisons between different treatments have been analyzed by a one-way ANOVA test. Values with p < 0.05 were considered statistically significant for a post-hoc Tukey’s test. All statistical tests were done using the software Graph-Pad Prism version 6.0. Degrees of significance are indicated in the figure captions as follow: *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, n.s = not significant.

3. Results

3.1. Cytotoxicity of CdCl₂ on HEK 293 cells

Cytotoxicity of CdCl₂ on HEK 293 cells was assessed by MTT assay (Fig. 1). The estimated CC50 value (cytotoxic concentration that reduced cell viability by 50%) was 30 µM for 24 h of treatment. The relative cell viability evaluated by Trypan Blue staining was similar to that obtained by MTT assay (data not shown). The administration of high doses of CdCl₂ (≥ 40 µM) caused cytotoxicity and reduced dramatically cells metabolic activity and viability. At concentrations lower than 10 µM, there was no significant difference in terms of cell viability and cell morphology between the non-treated control cells and the CdCl₂ treated cells. Thus, concentrations of CdCl₂ up to 10 µM were considered non-cytotoxic and were used for further experiments.

3.2. Effect of CdCl₂ on SINV-mediated cell death

To evaluate the effect of CdCl₂ treatment on the infected cells viability, HEK 293 cells were infected with SINV at a multiplicity of infection (MOI) of 1 PFU/cell and cell viability was analyzed by MTT assay (Fig. 2A). We observed that 24 h post SINV infection, the
infected cell viability decreased by more than 50% compared to non-infected cells. A treatment with 2.5–10 μM of CdCl₂ applied 2 h after SINV exposure led to a significant increase of cell viability. More than 70% of SINV-infected cells treated with 10 μM of CdCl₂ were still alive 24 h post-infection. CdCl₂ concentrations below 2.5 μM did not change the infected cells viability. Interestingly, these results showed that CdCl₂ treatment could partially rescue HEK 293 cells from SINV-mediated cell death. Thereafter, the activation of executioner caspases-3 and -7, hallmarks of apoptosis, was evaluated using a caspase-3/7 assay kit. Results revealed that SINV infection in HEK 293 cells leads to a significant increase in caspase 3/7 activity (Fig. 2B). Treatment with CdCl₂ markedly reduced caspase 3/7 activity in infected HEK 293 cells. Flow cytometric analysis performed 18 h post-infection also confirmed that SINV infection leads to apoptosis in HEK 293 cells as cells were both stained with both Annexin V and PI, as a hallmark of apoptosis (Fig. 2C). CdCl₂ exposure decreased the proportion of infected cells in the apoptotic state. These results suggest a causal relationship between the reduction of apoptosis and the increases viability of HEK 293 cells infected with SINV and treated with CdCl₂.

3.3. Effect of CdCl₂ on SINV growth

Given that CdCl₂ can rescue the viability of SINV-infected cells, it is important to determine whether the protective effect of the CdCl₂ might be the direct consequence of a lower efficiency of virus replication in HEK 293 cells. To investigate this issue, SINV-infected HEK 293 cells were incubated with CdCl₂ at different time points of
infection. Treatment with 10 μM of CdCl₂ at 2 h post-infection has a moderate effect on viral growth (~0.5 log) (Fig. 3A). In contrast, addition of CdCl₂ 24 h prior infection resulted in a marked reduction by at least 2 log of the progeny virus production (Fig. 3B). This result suggest that cadmium can impair SINV growth in HEK 293 cells. Similar results were obtained using NIE115 cells, a mouse neuroblastoma cell line (Supplemental Fig. 1).

To examine whether CdCl₂ could inactivate the virions, SINV inoculum was incubated with CdCl₂ at 37 °C 2 h before cell titration in Vero cells. No change in plaque formation was observed after incubation with CdCl₂, indicating no direct virucidal activity (Fig. 3C). Moreover, to determine if CdCl₂ could interfere with viral adsorption and entrance, Vero cells were infected with SINV in the presence of CdCl₂ for 2 h. Results showed that CdCl₂ did not significantly prevent virus adsorption (Fig. 3D).

3.4. Effect of CdCl₂ on etoposide-mediated apoptosis

To investigate if cadmium has a general inhibitory effect on apoptosis in HEK 293 cells, we exposed cells for 24 h to etoposide, an anti-tumor agent that is commonly used in vitro as an apoptosis inducer. Results showed that etoposide markedly reduced the viability of HEK 293 cells (Fig. 4A). CdCl₂ co-treatment rescued cells from etoposide-mediated cell death. Moreover, using a caspase 3/7 assay kit, we also confirmed a significant increase in caspase 3/7 activity in HEK 293 cells treated with etoposide (Fig. 4B). CdCl₂ co-treatment inhibited caspase 3/7 activity in HEK 293 cells treated with etoposide. Taken together, these results suggested that cadmium reduced etoposide-mediated cell death through reduction of apoptosis.

3.5. Effect of apoptosis on SINV growth

To determine if apoptosis inhibition could contribute to the reduction of SINV growth, cells were exposed to ZVAD-fmk, a pan-caspase inhibitor. Treatment with ZVAD-fmk (30 μM) protected cells from SINV-mediated cells death (Fig. 2A) through reduction of caspase 3/7 activity (Fig. 2B). SINV progeny production in supernatant of HEK 293 cells were examined after treatment with ZVAD-fmk 2 h post infection. No significant reduction of viral
progeny was observed in the presence of ZVAD-fmk (30 μM) (data not shown). These results suggested that inhibition of apoptosis does not impact SINV growth in HEK 293 cells.

4. Discussion

The heavy metal cadmium is a widespread environmental contaminant that may adversely affect human health [7,16]. To date very little information is available on the effect of cadmium exposure on viral infections. It has been reported that an orally administrated dose of cadmium enhanced the growth and the neuropathogenicity of alphaviruses in a mouse model of viral encephalitis [14]. In the present study, we investigated whether cadmium can modify the replication of SINV, the prototype of alphavirus genus, in cultured human and mouse cell lines. Surprisingly, at non-cytotoxic doses, addition of 10 μM CdCl₂ on cells after SINV infection resulted in a moderate reduction in virus progeny production. In this condition cadmium had no virucidal activity and did not prevent virus adsorption and entry. Very interestingly addition of cadmium prior SINV infection suppressed viral growth in infected cells by 2 log. It has been reported that cadmium can induce oxidative stress, autophagy or heat-shock response [17–20]. Such cellular stress responses could contribute to prevent SINV replication in the host cells. Autophagy induction has been shown to protect cells against SINV in vitro [21]. Also, heat shock protein synthesis may be linked to the inhibition of SINV replication in Vero cells [22].

An unexpected finding was that CdCl₂ does not exert a marked antiviral effect when SINV replication has been initiated but it could rescue the viability of SINV-infected cells. In our in vitro infection model, we confirmed that SINV can trigger apoptosis [23]. We have investigated whether cadmium promotes cell survival through an inhibition of apoptosis. The analysis of caspase 3/7 activity, showed that apoptosis was significantly delayed in SINV-infected cells. It has been previously published that cadmium can inhibit apoptosis induced by several stressor such as chromium, hygromycin B and actinomycin D [24]. Furthermore, cadmium, like zinc, can reduce caspase 3 activity in a cell-free system [24,25]. Cadmium is a known inductor of metallothionein, a family of metal-binding proteins that has been shown to act as a negative regulator of apoptosis in HepG2 cells [26]. In this regard, it remains to be determined their role in the reduction apoptosis induced by cadmium in the infected HEK 293 cells. Our results suggested that cadmium may have a general inhibitory effect on apoptosis in HEK 293 cells as it reduced also etoposide-mediated cell death. Although alphaviruses such as Chikungunya virus could mobilize the apoptotic machinery to spread and invades host cells through hiding in apoptotic blebs [27]. Our results showed that apoptosis inhibition using a pan-caspase inhibitor did not contribute to the reduction of SINV growth in HEK 293 cells.

To conclude, our results showed that cadmium exposure may have a collateral beneficial effect by mediating cell protection against SINV infection in HEK 293 cells through reduction of viral growth and apoptosis, by mechanisms that are still misunderstood necessitating the need for further investigation. It is worth cautioning that these in vitro results may not hold under in vivo conditions [14]. Many studies have described positive beneficial dose-response relationships (also known as hormesis or pre/post-conditioning) induced by toxins and stressors such as cadmium [28–30]. Low cadmium oral exposure has been shown to reduce the susceptibility of infectious colitis and to reduce arthritis disease progression in mice [31,32]. However, despite these potentially positive side effect of cadmium one should not forget the harmful long-term toxicogenic effects of this heavy metal in vivo (immunotoxicity, nephrotoxicity, cancer).

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.023.
Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.023.

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