Antiviral Research 140 (2017) 13–17

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Heparin prevents Zika virus induced-cytopathic effects in human neural progenitor cells

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ARTICLE INFO

Article history:
Received 3 November 2016
Received in revised form
22 December 2016
Accepted 29 December 2016
Available online 5 January 2017

Keywords:
Zika virus (ZIKV)
Neural progenitor cells
Heparin
Cell death

ABSTRACT

The recent Zika virus (ZIKV) outbreak, which mainly affected Brazil and neighbouring states, demonstrated the paucity of information concerning the epidemiology of several flaviruses, but also highlighted the lack of available agents with which to treat such emerging diseases. Here, we show that heparin, a widely used anticoagulant, while exerting a modest inhibitory effect on Zika Virus replication, fully prevents virus-induced cell death of human neural progenitor cells (NPCs).

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1. Introduction

Zika virus (ZIKV) is a member of the *Flaviviridea* family comprising *ca.* 70 viruses sharing common structural features, and takes its name from the Zika Forest in Uganda where it was first isolated (Dick, 1953; Dick et al., 1952). It is the causative agent of an emerging infection arising from bites of the *Aedes aegypti* species for which, as yet, the full implications for human health remain unclear. Widespread infection and serious birth defects, including neurological diseases and microcephaly in Brazil have been reported (Rasmussen et al., 2016). In adults, ZIKV infection has been linked epidemiologically to increased prevalence of Guillain-Barré Syndrome (Cao-Lormeau et al., 2016; Parra et al., 2016). Thus, both *in utero* and adult, ZIKV-associated pathological conditions suggest that the virus could infect nerves (Bell et al., 1971) and neural cells (Garcez et al., 2016). Additional modes of transmission to mosquito bites have also emerged, including sexual transmission (D'Ortenzio et al., 2016; Foy et al., 2011; Musso et al., 2015) and blood transfusion (Motta et al., 2016). These could further expand the epidemic impact of ZIKV infection, increasing the need for an effective vaccine and antiviral agents with which to treat infected individuals.

Conventional antiviral drug-discovery pipelines involve complex, expensive, and time-consuming processes, militating against their rapid and widespread deployment, particularly in developing countries. One attractive alternative for combating emerging and rapidly spreading infectious diseases is drug repurposing (Cheng et al., 2016), the application of an existing licensed pharmaceutical agent for another medical need. This has already been proposed for the treatment of both hepatitis C (He et al., 2015) and Ebola virus (Sakurai et al., 2015) infections. Heparin, the widely used anticoagulant, has potentially attractive features including

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http://dx.doi.org/10.1016/j.antiviral.2016.12.023
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activity against attachment and entry of the enveloped viruses, human simplex (HSV) (Herold et al., 1996; Nahmias and Kibrick, 1964), human immunodeficiency (HIV) (Baba et al., 1988), SARS coronavirus (Vicenzi et al., 2004), and influenza (H5N1) (Skidmore et al., 2015). Here, we explore the effects of heparin on ZIKV infection in human neural progenitor cells (hNPCs) with particular regard to virus replication and induction of cytopathic effects (CPE) resulting in cell death.

2. Materials and methods

2.1. Cells

Human neural progenitor cells (hNPCs) were obtained by the reprogramming of human adult skin fibroblasts (Movement Disorders Bio-Bank, Carlo Besta Institute, Milan, Italy) and were reprogrammed (CytoTune-iPS 2.0 Sendai Kit, Life Technologies) to obtain human induced pluripotent stem cells (hiPSCs), then maintained in feeder-free conditions (mTeSR1 culture medium, Stem Cell Technologies), as reported (Orellana et al., 2016). For embryoid bodies (EBs), dissociated hiPSCs were seeded into low-adhesion plates in mTeSR1 medium supplemented with N2 (1:200), (ThermoFisher Scientific), human Noggin (0.5 μg/ml, R&D System), SB431542 (5 μM, Sigma), Y27632 (10 μM, Miltenyi Biotec) and penicillin-streptomycin (100 μg/ml, Sigma). For rosettes, EBs were plated after 10 days onto matrigel-coated plates (1:100, matrigel growth factor reduced, Corning) in DMEM/F12 (Sigma) with N2 (0.5%), non-essential amino acids (1%, ThermoFisher Scientific) and penicillin-streptomycin. After 10 days, cells were passaged with Accutase (Sigma) and seeded into matrigel coated-flasks in media containing DMEM/F12, N2 (0.5%), B27 (0.5%), (ThermoFisher Scientific), penicillin-streptomycin (1%) and basic fibroblast growth factor (FGF2) (20 ng/ml, ThermoFisher Scientific).

2.2. Viruses

The viruses employed were the historical ZIKV strain (MR766), (EVAg - European Virus Archive), or recent Brazilian strain (INMI-1; GenBank Accession # KU991811), isolated from an Italian citizen who had travelled in Brazil in January 2016. Both viral strains were

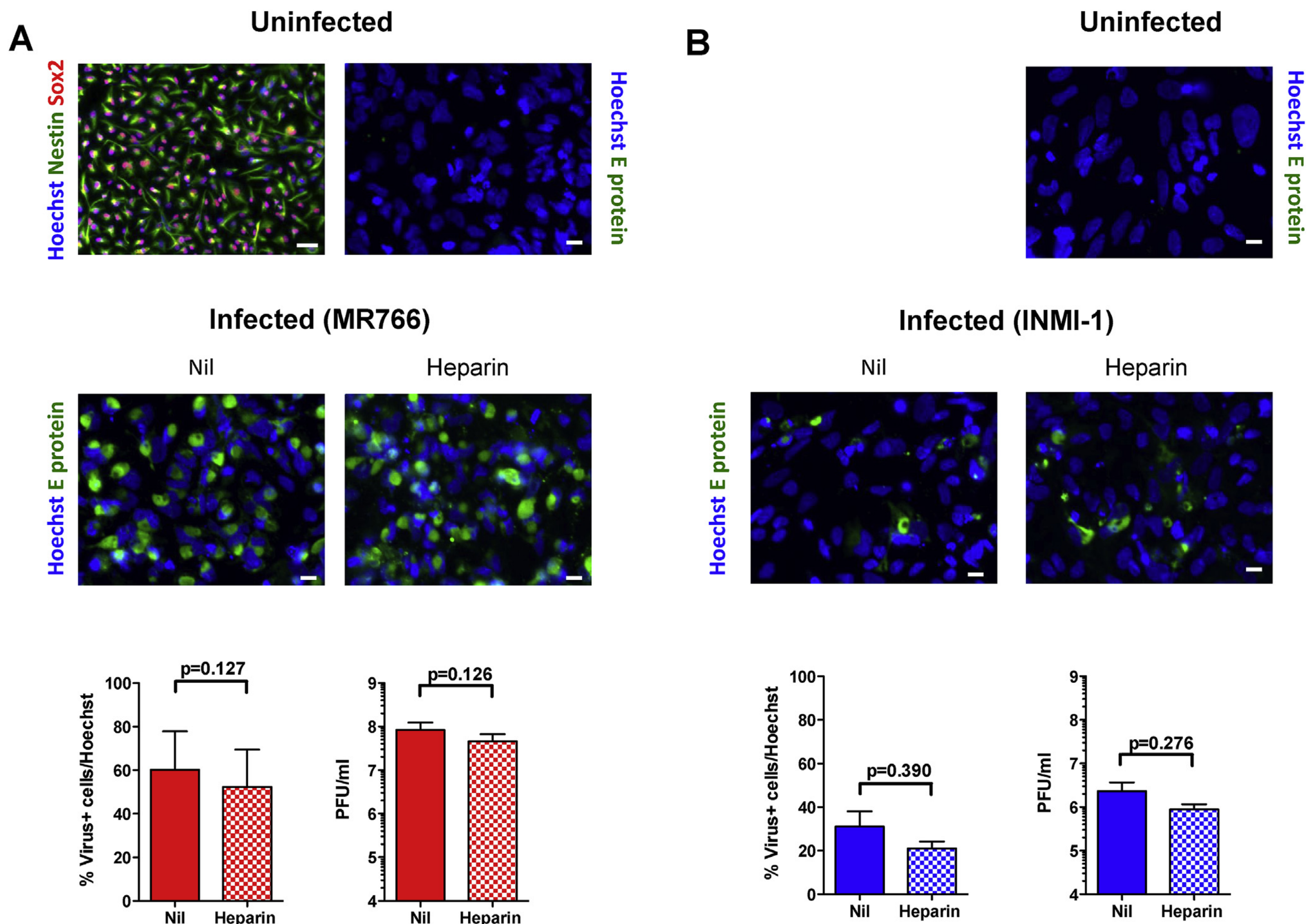

Fig. 1. Heparin does not inhibit ZIKV infection of hNPCs. **A.** Infection of hNPCs with the MR766 strain with (middle two panels) and without (upper two panels) heparin treatment (100 μg/ml). To determine that the cells were *bona fide* hNPCs, cells were stained with Sox2 (red), Nestin (green) and Hoechst (blue). Uninfected and infected cells were fixed after 3 days with paraformaldehyde solution and stained with a mAb specific for Flavivirus E protein (green). After PBS washes, cells were washed again, mounted and examined by microscopy. Quantification of infection efficiency and viral titers released into the culture supernatant are reported in the two lower panels (left and right, respectively). Scale bar of upper left panel: 20 μm, scale bar of remaining panels: 5 μm. Bar graphs indicate the mean ± SEM of 4 independent experiments. P values were calculated by Student's paired *t*-test. **B.** Infection of hNPCs by the INMI-1 strain, with (middle two panels) or without (upper panel) heparin treatment (100 μg/ml). Uninfected and infected cells were fixed after 7 days with paraformaldehyde solution and stained with a mAb specific for Flavivirus E protein (green). Quantification of infection efficiency and viral titers released into the culture supernatant are reported in the lower two panels (left and right, respectively). Scale bar: 5 μm. Bar graphs indicate the mean ± SEM of 2 fields including more than 1000 cells in 3 independent experiments. P values were calculated by Student's paired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expanded in Vero cells and titred in a Plaque Forming Assay (PFA) (Hamel et al., 2015).

2.3. Infection

Cells were seeded at 3×10^4/ml in 48 well plates. After 3 days of culture, heparin (Celsus, Cincinnati, OH) was added 1 h prior to infection at 100 µg/ml and then virus-containing supernatant was added, providing multiplicity of infection (moi) of 1. The efficiency of infection was evaluated by immunofluorescence staining with either an anti-flavivirus envelope monoclonal antibody (mAb) (Millipore, mAb 10216) or double-stranded RNA (1:300, English and Scientific Consulting Kft, Hungary) 3 and 7 days post-infection. To evaluate apoptosis, an Ab against cleaved-caspase-3 (1:200, Cell Signaling, 9661) was used. Cells were then washed with PBS and incubated for 1 h with Hoechst and either anti-mouse Alexa Fluor-488 or anti-rabbit Alexa Fluor-594 secondary Abs (1:1000 in blocking solution, ThermoFisher Scientific). Viral supernatants were collected and viral titers were determined in the PFA. Cell death was detected in 10 µl samples of culture supernatant transferred into half-black 96 microwell plates (Costar). To each well, 50 µl of adenylate kinase detection reagent (ToxiLight® BioAssay, Lonza) was added and the plate incubated (10 min, room temperature). Luminescence was measured in a Mithras LB940 Microplate Reader (Berthold Technologies).

3. Results

3.1. Effect of heparin on infection

Immunofluorescence staining with Nestin (a neuroectodermal stem cell marker) and SOX2 (a transcription factor maintaining pluripotency or self-renewal) confirmed the nature of the hNPCs employed (Fig. 1A, upper panels). hNPCs were incubated with heparin (100 µg/ml, 1 h prior to infection (see Supplementary Table 1 for details of heparin characterization and Supplementary Figs. 2 and 3 for ^{1}H and ^{13}C NMR spectra.) with historical ZIKV strain MR766 and recent Brazilian strain, INMI-1. The proportion of MR766 infected hNPCs was *ca*. 60% (Fig. 1A, middle panels). Modest decreases (not statistically significant) in infected cell numbers were observed in cultures pretreated with heparin. Progeny infectious virion production was measured in culture supernatants using standard PFA on Vero cells, indicating that heparin reduced the production of progeny virus by 2–3 fold (Fig. 1A, lower panels).

We next evaluated the effect of heparin on hNPCs infection by the INMI-1 strain, which is characterized by lower virus replication efficiency (35%) than MR766. Nonetheless, the antiviral effect of heparin was modest and, as for MR766, not statistically different from untreated cultures, as determined by quantification of infection efficiency and viral titers released into the culture supernatant in the PFA on Vero cells (Fig. 1B, lower panels).

3.2. Heparin modulates cytopathic effects

Then, we analyzed the virus-induced cytopathic effects by measuring the levels of adenylate kinase released into the culture supernatant after damage of the cell membrane (Crouch et al., 1993; Olsson et al., 1983). Heparin did not exert toxic effects on uninfected cells (Fig. 2). Unexpectedly and strikingly, however, heparin prevented fully the virus-induced cytopathic effects of cultures infected with either the MR766 or INMI-1 ZIKV strains (Fig. 2A and B, respectively). Thus, heparin partially uncoupled the virus replication (that was only modestly inhibited) from the virus-induced death of hNPCs.

ZIKV infection of cranial neural crest cells has been linked to the release of vascular endothelial growth factor (VEGF) and leukaemia inhibitory factor (LIF) exerting proapoptotic effects on adjacent cells (Bayless et al., 2016). Thus, we determined the proportion of cells that were positive for caspase-3 staining, as an indicator of apoptotic cell death (Lavrik, 2005). The proportion of caspase-3$^+$ cells in uninfected and untreated cell cultures increased from 9.5± 3.4% at day 3 after infection (day 6 after the initial seeding) to 29± 1.1% at 7 days after infection. At the same time points, heparin decreased the proportion of caspase-3$^+$ cells to 5.9± 2.7% and 12.1± 3.7%, respectively. In infected cultures, the proportion of caspase-3$^+$ cells increased to 31.1± 5.5% at 7 days post-infection with the INMI-1 strain, and decreased to 16.2± 0.3% when treated with heparin (data not shown). These results suggest that the activation of the master executor of apoptosis, i.e. caspase-3, is activated in hNPC cultures independently of virus infection and

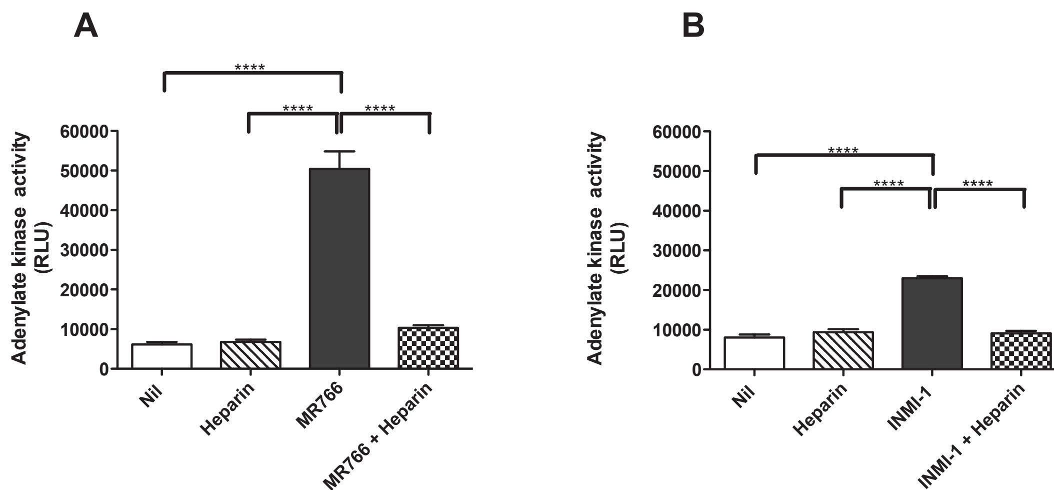

Fig. 2. Heparin prevents virus-induced CPE. **A.** Supernatant of infected hNPCs with the MR766 strain was collected 3 days post infection. The results are expressed as relative luminescent unit (RLU). Data are expressed as mean ± SEM of 3 independent experiments. Repeated measures Anova was used with the Bonferroni correction. *Represents statistical comparison among groups (**, $p < 0.01$; *, $p < 0.05$). **B.** Supernatant of infected hNPCs with the INMI-1 strain was collected 7 days post infection. The cell death was analyzed as reported in **A.** Results are expressed as relative luminescent units (RLU). Data represent the mean ± SEM of 3 independent experiments. Repeated Measures ANOVA was used with the Bonferroni correction. *Represents statistical comparison among groups (**, $p < 0.01$; *, $p < 0.05$).

that heparin probably prevents its activation by stabilizing FGF2 for the duration of the culture, as reported (Caldwell et al., 2004). In contrast, heparin fully prevented the virus-induced necrotic death of cells, as measured by the release of adenylate kinase in the culture supernatant (Fig. 2), although the mechanism of action remains unknown. Since the culture condition induced the apoptosis of uninfected hNPCs, we tested whether heparin affected ZIKV cytopathic effects, and in particular, prevented caspase-3 activation in Vero cells that are highly permissive to ZIKV replication. The background level of apoptosis in the absence of viral infection was negligible (Supplementary Fig. 1). Indeed, heparin treatment did not alter the levels of viral replication compared to control Vero cells, however, it significantly decreased the percentage of caspase-3^{+} cells, suggesting that heparin can also prevent ZIKV-induced apoptosis.

4. Discussion

In terms of drug repurposing, in addition to caspase-3 inhibitors (Cheng et al., 2016; He et al., 2015), heparin represents both a clinically-approved pharmacological agent capable of preventing ZIKV-induced necrosis of neural cells, as observed with hNPCs, and a lead compound to help discover novel agents preventing virus replication and cytopathic effects.

Independently of the obvious heparin anticoagulant effect, there is now considerable evidence that heparin possesses anti-inflammatory, anti-apoptotic and proliferative activities that rely on a variety of mechanisms (Katsi et al., 2016). However, the polyanionic nature of heparin favours the neutralization of cationic mediators of inflammation and apoptosis but, also viral envelopes (Rusnati et al., 2009). The mechanism through which heparin derivatives inhibit infectivity in other viral infections, such as herpes simplex virus (Shukla and Spear, 2001), is most likely by competing with host cell surface glycoproteins or proteoglycans, or in other cases, through binding of low specificity to several proteins by virtue of their polyanionic nature (Skidmore et al., 2015). In the present case, however, the effect of heparin on infectivity is less striking than its ability to protect infected cells from cell death. The most likely mechanism to explain this phenomenon is that heparin is binding several proteins that promote inflammation and cell death. Heparin has been documented to bind and, in many cases neutralise, a range of relevant growth factors and enzymes, including tissue necrosis factor-α (TNF-α) (Salas et al., 2000; Spratte et al., 2013; Veraldi et al., 2015).

In conclusion, since heparin is used safely during pregnancy it could serve as a potential adjunct therapy in the presence or likelihood of ZIKV infection. While ultra-low molecular weight heparin has been reported to cross the blood brain barrier (FDA guidelines, accessed 05/10/2016), heparin does not cross the placenta http://www.fda.gov/Safety/MedWatch/SafetyInformation/ucm219000.htm. Improved delivery systems may facilitate the administration of heparin and heparin derivatives.

Acknowledgments

MAL and EAY gratefully acknowledge gs1:Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, gs2:Fundação de Amparo à Pesquisa do Estado de São Paulo (15/08782-3) and gs3:Conselho Nacional de Desenvolvimento Científico e Tecnológico for financial support. MAS and EAY acknowledge the BBSRC/EPSRC for a New Investigator Award (BB/L023717/1) and Keele University for Seed Funding Award (MAS, EAY and MAL). GI acknowledges the European Union, Seventh Framework Programme and European Union's Horizon 2020 Research and Innovation Programme.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.12.023.

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