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Research Report

Melatonin, minocycline and ascorbic acid reduce oxidative stress and viral titers and increase survival rate in experimental Venezuelan equine encephalitis

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ABSTRACT

Venezuelan equine encephalitis (VEE) virus causes an acute central nervous system infection in human and animals. Melatonin (MLT), minocycline (MIN) and ascorbic acid (AA) have been shown to have antiviral activities in experimental infections; however, the mechanisms involved are poorly studied. Therefore, the aim of this study was to determine the effects of those compounds on the viral titers, NO production and lipid peroxidation in the brain of mice and neuroblastoma cultures infected by VEE virus. Infected mouse (10 LD_{50}) were treated with MLT (500 μg/kg bw), MIN (50 mg/kg bw) or AA (50 mg/kg bw). Infected neuroblastoma cultures (MOI: 1); MLT: 0.5, 1, 5 mM, MIN: 0.1, 0.2, 2 μM or AA: 25, 50, 75 μM. Brains were obtained at days 1, 3 and 5. In addition, survival rate of infected treated mice was also analyzed. Viral replication was determined by the plaque formation technique. NO and lipid peroxidation were measured by Griess' reaction and thiobarbituric acid assay respectively. Increased viral replication, NO production and lipid peroxidation were observed in both, infected brain and neuroblastoma cell cultures compared with uninfected controls. Those effects were diminished by the studied treatments. In addition, increased survival rate (50%) in treated infected animals compared with untreated infected mice (0%) was found. MLT, MIN and AA have an antiviral effect involving their anti-oxidant properties, and suggesting a potential use of these compounds for human VEE virus infection.

Abbreviations: VEE, Venezuelan equine encephalitis; MLT, Melatonin; MIN, Minocycline; AA, Ascorbic acid; NO, Nitric oxide; LD_{50}, Median lethal dose; bw, Body weight; MDA, Malondialdehyde

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1. Introduction

Venezuelan equine encephalitis (VEE) virus produces an acute infection in human and murine central nervous system (CNS) with neuronal damage (Weaver, 1998; Schoneboom et al., 2000a, 2000b). During an anti-viral immune response a balance between two opposing pathways is observed: the production of pro-inflammatory cytokines and cytotoxic cells to limit the viral infection and regulators to control the excessive immune response and avoid tissue damage. Nitric oxide (NO) can mediate immunopathology and/or inhibit the antiviral immune response to promote chronic infection (Burrack and Morrison, 2014). In this regard, NO has been implicated in the experimental spread of Venezuelan equine encephalitis (VEE) virus and other viruses (Bonilla et al., 2004; Perrone et al., 2013; Mannick, 1995). The increased presence of NO in VEE infection is probably due to the activation of induced nitric oxide synthase (iNOS) (Valero et al., 2006; Schoneboom et al., 2000a, 2000b). Oxidative stress and/or altered immune response could be involved in VEE viral infection. In this regard, pro-oxidant and immune modulator properties of NO have been reported (Burrack and Morrison, 2014; Mannick, 1995); therefore, approaches to inhibit the effect of NO on viral replication and oxidative stress need to be used appropriated compounds. Melatonin (MTL), a molecule with antioxidant properties, has shown to have protective effects on experimental murine VEE virus infection (Bonilla et al., 2003, 2004; Valero et al., 2004, 2007, 2009). In addition to anti-oxidant properties, MLT has the capability to regulate leukocyte function and contributes to the control of inflammation in tissues acting as both an activator or inhibitor of the inflammatory and immune responses (Silvestre and Rossi, 2013). MLT also decreases the toll like receptor mediated downstream gene expression and the subsequent NFkB-dependent gene expression, such as those encoding for TNF-α and iNOS produced during viral replication, preventing injury through the inhibition of the oxidative stress and of the production of pro-inflammatory cytokines (Huang et al., 2008). Minocycline (MIN), a tetracycline derivative, and ascorbic acid (AA) have shown antiviral activities in cell cultures and animal model of infections by Japanese encephalitis, human immunodeficiency and measles viruses (Dutta et al., 2010a, 2010b; Bhaskar et al., 2012; Mishra et al., 2009; Zink et al., 2005). Beside the capacity to inhibit iNOS (Garrido-Mesa et al., 2013), minocycline combines both immunomodulatory and anti-microbial properties that can be neuroprotector. It has also been shown that MIN reduces virus infection and immune responses in several experimental models. This anti-viral effect seems to be linked to its ability to reduce the activation of monocytes and their permissiveness to viral infection. Anti viral mechanisms of MIN include, decreased p38 activation and viral replication in lymphocytes (Zink et al., 2005), reduced production of MCP-1 altering chemotaxis (Zink et al., 2005), blunting changes in activation/proliferation markers and cytokine secretion necessary for the activation pathway that regulate viral replication, decreasing viral production by inhibition of DNA integration or transcription (Si et al., 2004; Zink et al., 2005; Follstaedt et al., 2008; Ratai et al., 2010; Szeto et al., 2010). Several mechanisms of action have been proposed for the potential beneficial effect on viral infections of AA. The anti-oxidative ability of ascorbate, reducing oxidative stress could be important in detoxification and neutralization of reactive oxygen species associated with infection (Wintergerst et al., 2006). AA is also necessary for neutrophil function (Wintergerst et al., 2006) and can stimulate the production of interferon and other anti-viral cytokines, down regulate inflammation, and reduce the ability of several virus to infect cultured cells (Bissell et al., 1980; White et al., 1986; Gerber, 1975; Mandl et al., 2009; Mikirova et al., 2012; Furuya et al., 2008). These data suggest a potential beneficial effect of these compounds in VEE virus infection. Therefore, the aim of the present study was to analyze the effect of MLT, MIN and AA on the viral replication, NO production and lipid peroxidation in the brain of VEE virus infected mice and in infected neuroblastoma cell cultures.

2. Results

2.1. *Effect of MLT, MIN or AA on the survival rate of VEE virus infected animals*

MLT, MIN or AA was capable of decreasing the mortality rate of mice infected by VEE virus. Analysis of survival curves showed that 100% of mortality rate was reached earlier in untreated infected mice (day 7); time when, 50% of MLT, MIN or AA treated infected mice were alive and remained in this way until the end of experiment (day 10) (Fig. 1). Log rank test analysis showed significant differences when untreated animal curve were compared with treated animal curves ($p=0.001$). Uninfected animals treated with those compounds remained with 100% of survival during all experiment. Very high titers of IgM anti-VEE virus antibodies were found in survived animals (dilution range: 1:2560 to 1:5120 at week 3 postinfection and 1:640 to 1:1280 at week 7 postinfection).

2.2. *Effect of MLT, MIN and AA on brain VEE virus infection and oxidative stress*

Brain homogenates from infected mice showed increased viral replication over time. Significant reduction of viral titers was observed with MLT, MIN or AA treatments. This effect was more effective with MLT and MIN treatments (Fig. 2). Increased nitrite production (NO) at day 3 and 5 post-infection in untreated infected animals was found. MLT (days 3 and 5) and AA (day 5) were capable of reducing NO production (Fig. 3). MDA content in infected mice was found increased. MLT (days 3 and 5), MIN (days 1, 3 and 5) or AA (days 1, 3 and 5) reduced lipid peroxidation (Fig. 4).

2.3. *Effect of MLT, MIN and AA on VEE virus infected neuroblastoma cultures and oxidative stress*

VEE virus was capable of replicating in neuroblastoma cell cultures as shown by the increased PFU observed in Vero cell cultures. Culture of VEE virus-infected cells in the presence of MLT, MIN or AA significantly reduced viral titers. MLT had the highest inhibitory viral effect at low concentrations when compared with low concentration of MIN or AA (Fig. 5A). VEE virus induced increased production of NO by neuroblastoma