Virus Research 236 (2017) 1–8

Contents lists available at ScienceDirect

Virus Research

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

Oxidative stress in *Mayaro virus* infection

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ARTICLE INFO

Keywords:
Mayaro virus
ROS
Oxidative stress
Antioxidant defences

ABSTRACT

Mayaro virus (MAYV) is a neglected tropical arbovirus that causes a febrile syndrome that is sometimes accompanied by incapacitating arthritis/arthralgia. The pathogenesis of MAYV has not been completely defined and oxidative stress mediated by an increase in reactive oxygen species (ROS) and/or depletion of antioxidant defences has been found to contribute to several aspects of viral disease. To investigate whether MAYV induced oxidative stress in host cells, we monitored ROS production, oxidative stress markers and antioxidant defences at different time points after infection. Our results show that MAYV induced significant oxidative stress in infected HepG2 cells, as indicated by the increase of malondialdehyde (MDA) and protein carbonyl levels, and by a significant decrease of the reduced versus oxidized glutathione (GSH/GSSG) ratio. Generally, MAYV-infected HepG2 cells also showed an increase in antioxidant defences. We observed an increase in the superoxide dismutase (SOD) and catalase (CAT) activities and the total glutathione content. To determine whether similar effects occurred in other cell types, we evaluated the ROS, MDA and SOD activity levels in J774 cells after MAYV infection. Similar to our observations in HepG2 cells, the J774 cells showed an increase in ROS, MDA and total SOD activity following MAYV infection. Thus, since the cellular redox environment is influenced by the production and removal of ROS, we hypothesize that the overproduction of ROS was responsible for the oxidative stress in response to the MAYV infection despite the increase in the antioxidant status. This study is the first report on the involvement of oxidative stress during MAYV infection. Collectively, our data shed light on some mechanisms that are operational in host cells following exposure to MAYV.

1. Introduction

The MAYV is an arbovirus belonging to the *Togaviridae* family and the *Alphavirus* genus. It was originally isolated in 1954 from the blood of five febrile rural workers near the town of Mayaro, Trinidad and Tobago (Anderson et al., 1957). Since its first isolation, the number of MAYV infections has increased in several countries, including Peru, Brazil, Suriname, French Guiana, Guyana, Venezuela, Colombia, Ecuador, Panama, Bolivia, Costa Rica, Guatemala and Mexico (Schaeffer et al., 1959; Forshey et al., 2010; Muñoz and Navarro, 2012; Halsey et al., 2013). Reports have also described MAYV infections in German, Dutch, French and Swiss tourists who visited the Amazon Basin; thus, this virus poses a concern regarding its spread to other continents (Hassing et al., 2010; Receveur et al., 2010; Neumayr et al., 2012; Theilacker et al., 2013; Llagonne-Barets et al., 2016).

In Brazil, MAYV was initially isolated in 1955 (Aitken et al., 1960) and has since been responsible for several outbreaks in the northern region, including the Pará and Amazonas states where the virus can be considered endemic (Figueiredo, 2015). Additionally, MAYV presence was reported in the central-west region, including Goiás, Mato Grosso and Mato Grosso do Sul states (Batista et al., 2013; Zuchi et al., 2014; Pauvolid-Correa et al., 2015; Vieira et al., 2015).

After the bite of an arthropod vector (mainly mosquitos of the genus *Culex* sp. and *Aedes* sp.), the virus causes Mayaro fever, which presents with acute symptoms including fever, myalgia, headache, rash, arthralgia, vomiting, and diarrhoea that last an average of 3–5 days (Tesh et al., 1999). During the convalescent phase of the disease, the arthralgia and arthritis may persist for several weeks, months or years.

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http://dx.doi.org/10.1016/j.virusres.2017.04.017
Received 2 November 2016; Received in revised form 18 April 2017; Accepted 24 April 2017
Available online 25 April 2017
0168-1702/

No cases of death due to Mayaro fever have been reported, but infection can cause significant morbidity, leading to a loss of productivity and overloading of public medical services (Assunção-Miranda et al., 2013; Halsey et al., 2013; Mota et al., 2015). The generic nature of the clinical manifestations of MAYV fever results in misdiagnosis with other viral fevers, mainly dengue fever (Forshey et al., 2010; Mota et al., 2015). Forshey et al. (2010) confirmed this underreporting by showing that 1% of all febrile illnesses with the same symptoms as dengue in northern South America were caused by MAYV.

Because Mayaro fever reaches the poorest regions and receives little government attention, the disease is still very much neglected. The urbanization of forest areas and the possibility of *Aedes aegypti* becoming a vector for MAYV have increased the risk of MAYV emerging in urban areas (Long et al., 2011; Mota et al., 2015). Moreover, the recent entry of the *Chikungunya virus* (CHIKV) in Brazil (CDC, 2014), which is another component of the Semliki Forest Complex of the *Alphavirus* genus, has increased the need to implement health surveillance related to this infection and strengthened the potential risk of MAYV mimicking the epidemiological progression of CHIKV. Consequently, more studies investigating MAYV are urgently needed to obtain a better understanding of its epidemiology and pathology.

Although the mechanisms underlying MAYV-induced acute disease and the associated long-term arthralgia remain incompletely defined, the inflammatory response is thought to play a fundamental role (Santiago et al., 2015). Furthermore, recent studies have demonstrated that oxidative stress via RNA virus infection can contribute to several aspects of viral disease pathogenesis, including the inflammatory response and viral replication (Reshi et al., 2014).

Oxidative stress is established when there is a disruption/dysregulation of signaling and redox control caused by the increase of ROS and/or a reduction in the antioxidant defence system (Jones, 2006). ROS are well known oxidants and mediators of both cell injury and intracellular signaling. ROS are formed by several physiological processes and are currently thought to be involved in the pathogenesis of several disorders, sometimes as causes and sometimes as effects (Halliwell and Gutteridge, 1999). In the context of viral infections, ROS are produced by activated neutrophils to destroy viruses and neighbouring cells via oxidative bursts; however, recent studies have shown that ROS are produced in all types of cell and serve as messengers in cell signaling and various signal transduction pathways (Reshi et al., 2014). ROS play important roles in fighting infections and are viewed as a protection mechanism of the host cell that contributes to its apoptosis. However, more ROS are formed with the advancement of viral multiplication, causing an imbalance in cellular homeostasis (Jacobson, 1996).

To protect the cells against exposure to different ROS, several defence mechanisms have been developed, such as the enzymatic and non-enzymatic antioxidant systems including SOD, CAT, and the glutathione-dependent enzymes, thioredoxin and peroxiredoxins (Halliwell and Gutteridge, 1999). The first ROS produced in the oxygen reduction pathway is the superoxide anion ($O_2\cdot^-$), which is metabolized to hydrogen peroxide (H_2O_2) by the SOD family enzymes. The glutathione redox cycle is complementary to catalase in converting H_2O_2 to water and oxygen. Changes to the body's antioxidant defence system in relation to SOD, ascorbic acid, selenium, carotenoids, and glutathione have been reported in various tissues of RNA virus-infected patients (Reshi et al., 2014). Thus, because oxidative stress is related to pathogenesis of a variety of viral diseases and little is known about the various pathological aspects of MAYV infection, this study aimed to investigate whether MAYV caused oxidative stress in infected cells and modulated host antioxidant defences associated with this cellular stress condition.

Our results showed that MAYV infection of a human hepatocyte cell line (HepG2) induced ROS production and caused significant oxidative stress as indicated by the increase in the MDA and protein carbonyl levels, which are biomarkers of lipid peroxidation and oxidative modification in proteins, respectively, and by a significant decrease of the GSH/GSSG ratio. In relation to the antioxidant status, the enzymatic assay results showed that the total SOD and CAT activities and the total glutathione content were increased in MAYV-infected cells. However, this increase in antioxidant defences after MAYV infection was not sufficient to restore the physiologic redox status because the oxidative stress biomarker levels were elevated. Thus, since the cellular redox environment is influenced by the production and removal of ROS, we suggest that ROS overproduction is responsible for the oxidative stress observed in MAYV-infected cells. This study provides novel insights into MAYV infection. Therefore, the identification of the importance of oxidative stress on MAYV pathogenesis requires further studies.

2. Material and methods

2.1. Virus and cells

MAYV was kindly provided by Professor Maurício Lacerda Nogueira (Faculty of Medicine of São José do Rio Preto/FAMERP/SP). This MAYV strain (BeAr20290) was originally isolated from a pool of 93 *Haemagogus* spp. in Belém (Pará, Brazil) in 1960. The virus was propagated in Vero cells, and virus pools were aliquoted and stored at −80 °C. The virus titer was 10^8 plaque-forming units (PFU)/ml using a methylcellulose plaque assay. The HepG2 (human liver carcinoma cell line), Vero (African green monkey kidney cell line) and J774 (mouse reticulum sarcoma cell line) cells were maintained in Dulbecco's modified Eagle's medium (Cultilab) supplemented with 10% foetal bovine serum (FBS; Cultilab) in a humidified incubator at 37 °C with 5% CO_2.

2.2. Detection of ROS production

The production of intracellular of ROS was measured using the Image-iT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Invitrogen). The technique utilizes a fluorogenic marker [(5-and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA)], which when broken by nonspecific intracellular esterases generates the carboxy-molecule DCFH; in turn, DCFH reacts with ROS to generate fluorescence.

The assay was performed in a white 96-well plate seeded with approximately 2,5 × 10^4 HepG2 or J774 cells per well. The dosing was performed as recommended by the manufacturer. The cells were infected with MAYV at a multiplicity of infection (m.o.i.) of 5, and at different times points, 1, 2, 4, 6, 15 and 24 h (each time point included 8 infected samples and 8 control samples), cells were washed with Hank's balanced salt solution and loaded with 25 µM carboxy-H_2DCFDA for 30 min at 37 °C, protected from light. The cells were then washed three times and fluorescence intensity was determined at 485 nm excitation and 535 nm emission, using the VICTOR™ X3 Multilabel microplate reader (Perkin Elmer) with the Perkin Elmer 2030 workstation and workout 2.5 software.

2.3. Measurement of lipid peroxidation products

To measure the lipid peroxidation marker MDA, HepG2 or J774 cells were seeded at a density of 1 × 10^6 cells/well in 6-well plates. After infecting the cells or not with MAYV (m.o.i. of 5) for different times (6, 15 and 24 h, with 8 infected samples and 8 control samples per time point), the cells were washed with PBS, scraped and lysed. The cell lysates were combined with 8.1% SDS, 2.5 M acetic acid and 0.8% thiobarbituric acid. The mixture was heated at 95 °C for 1 h and 30 min, and the absorbance was taken at the 532 nm wavelength. The results were expressed as the concentration of MDA participating in the reaction (nmol/ml).

2.4. Measurement of the protein carbonyl content

The protein carbonyl levels were determined according to the method described by Levine et al. (1994). HepG2 cells were seeded in 6 well-plates at a density of 1×10^6 cells/well, including 8 control samples and 8 infected samples (m.o.i. of 5). At 6, 15 and 24 h, the protein carbonyl content was measured by derivatising the protein carbonyl with 2,4-dinitrophenylhydrazine (DNPH), which resulted in the generation of the dinitrophenyl (DNP) hydrazone product. The absorbance of the samples was determined at 370 nm. The concentration of the DNPH-derivatised proteins was calculated using a molar absorption coefficient of 22.000 M^{-1} cm^{-1}. The results were expressed in nmol of DNPH incorporated/mg of protein. The total protein content was determined according to the method described by Bradford using bovine serum albumin (BSA) as the standard.

2.5. Growth kinetics of MAYV and cell viability assays

HepG2 and J774 cells were incubated with MAYV at an m.o.i. of 5 for 1 h at 37 °C in 5% CO_2. Then, the medium containing the nonadsorbed virus was removed, the cells were washed twice with 1X phosphate buffered saline (PBS) and cultured in DMEM supplemented with 10% FBS, at 37 °C in 5% CO_2. Culture supernatants were collected at desired periods of infection, and viral growth was assessed by plaque assay on Vero cell monolayers. The viability of the HepG2 and J774 cells after MAYV infection was analysed by assessing the cellular morphology and by staining with 0.4% trypan blue.

2.6. Biochemical assay for the SOD and CAT activities

To determine the total SOD activity, we used the Superoxide Dismutase Assay kit (Cayman Chemical Company), which generates superoxide anions, xanthine and xanthine oxidase. This kit measures the ability of the test solution to inhibit the reaction of superoxide anion with WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-5-tetrazolium). The reaction forms a compound called formazan, which absorbs light at 450 nm. To measure the CAT activity, we used the ECAT-100 kit (BioAssay Systems), which directly measures the breakdown of H_2O_2 using a redox dye. The absorbance was taken at the 570 nm wavelength.

For both dosages, HepG2 and/or J774 cells were seeded into 6-well plates at a density of 1×10^6 cells/well and infected with MAYV at an m.o.i. of 5 (8 control samples and 8 infected samples per time point). At 6, 15 and 24 h p.i., the cells were washed, scraped, lysed and stored at −80 °C prior to dosing according to the manufacturer's recommendations. The SOD and CAT activities were expressed as U/ml.

2.7. Determination of the total glutathione content and reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio

The total glutathione content was determined using a kinetic method based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB) as proposed by Griffith, 1980. For the assay, HepG2 cells were seeded into 6-well plates at a density of 1×10^6 cells/well and infected with MAYV at an m.o.i. of 5. After 6, 15 and 24 h (with 8 control samples and 8 infected samples per time point), the cells were washed with PBS, scraped and lysed. After these procedures, the dosage and absorbance of the samples were read in an ELISA plate reader at 412 nm. The glutathione content was expressed as nmol/ml. For GSSG measurement, 2-vinylpyridine was added to the sample with TEA reagent, which was incubated at room temperature for 1 h and assayed for GSSH concentration. The concentration of reduced glutathione was obtained by subtracting the total concentration of the oxidized glutathione.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. The results are expressed as mean ± SD. Student's *t*-test at 95% confidence was used to determine the level of differences between the MAYV-infected cells and the uninfected cells, where $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. The letters a, b, and c represent differences between the groups of MAYV-infected cells using one-way ANOVA and Tukey's post-test. Sample size was provided in the respective figure legends.

3. Results

3.1. MAYV induces reactive oxygen species formation in human hepatocyte cells

To determine whether MAYV infection induced ROS production, HepG2 cells were grown to 90% confluence and then infected with MAYV at a m.o.i. of 5. At different time points after infection (1, 2, 4, 6, 15 and 24 h), the cells were loaded with the fluorogenic marker (carboxy-H_2DCF-DA), which is trapped intracellularly following cleavage by cellular esterase's. DCF oxidation was measured by changes in the mean fluorescence intensity in the control versus infected cells. MAYV infection of HepG2 cells induced an increase in ROS generation at all tested time points starting between 1 and 2 h post-infection (p.i.), reaching a plateau approximately 4 h p.i. and extending to 24 h p.i. (Fig. 1).

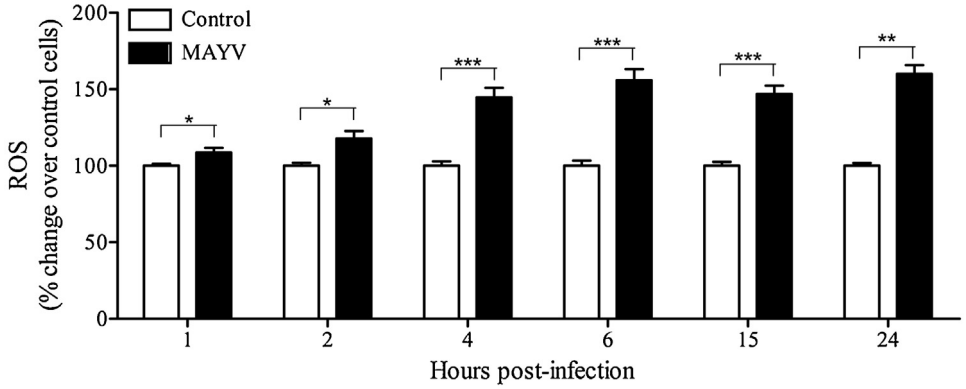


Fig. 1. MAYV induces ROS formation in HepG2 cells. HepG2 cells were infected with MAYV (m.o.i. of 5) and at various time points after infection, cells were washed with Hank's balanced salt solution and loaded with 25 µM carboxy-H_2DCFDA for 30 min at 37 °C, protected from light. The cells were then washed three times and fluorescence intensity was measured in control and infected cells. Mean fluorescence intensity is reported as% increase over control cells. The results include data from three experiments (mean ± SD, $n = 24$). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with control cells, Student's *t*-test.

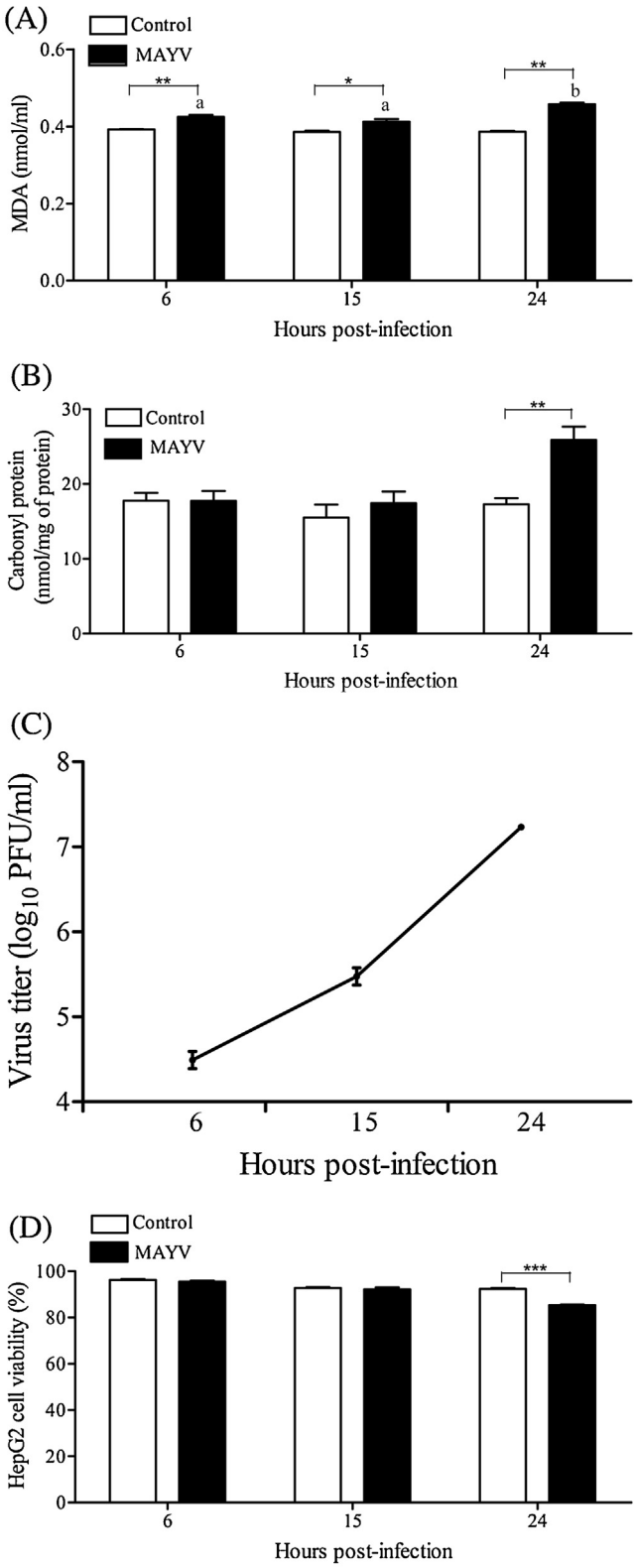


Fig. 2. MAYV induces oxidative stress in HepG2 cells. HepG2 cells were infected with MAYV (m.o.i. of 5) and 6, 15 and 24 h p.i., cells were harvested to measure oxidative stress biomarkers MDA (A) and carbonyl protein (B). (C) Viral growth kinetics in HepG2 cells assessed by plaque assay. (D) Viability of HepG2 cells during infection with MAYV. The results include data from two experiments (mean ± SD, $n = 16$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control cells, Student's *t*-test. Different letters indicate differences between MDA levels on MAYV-infected cells, using one-way ANOVA and Tukey's post-test.

3.2. MAYV induces oxidative stress in human hepatocyte cells

To investigate whether oxidative stress is induced during MAYV infection, two oxidative stress biomarkers (MDA and protein carbonyl) were monitored in the control and MAYV-infected HepG2 cells at 6, 15 and 24 h p.i. MAYV infection of HepG2 cells resulted in a significant increase in the MDA level at all time points tested compared with the control cell (Fig. 2A). Although no change in the protein carbonyl level was detected in the MAYV-infected cells at the early times points of infection (6 and 15 h), the levels of this marker was increased at 24 h p.i. compared to the uninfected cells (Fig. 2B). The MDA and protein carbonyl levels increased in parallel with the viral titer at 24 h p.i. (Fig. 2C). We investigated HepG2 viability during time course of infection and no differences were observed in early stages (6 and 15 h) of MAYV infection when compared to control cells (Fig. 2D). However, a loss of cellular viability was observed as infection progresses, with 7% decrease of viable cells after 24 h p.i. Altogether, the increases in the MDA and protein carbonyl levels during infection confirmed that MAYV infection induced oxidative stress in HepG2 cells.

3.3. MAYV increases the antioxidant status in human hepatocyte cells

Since MAYV induces an increase in ROS production and causes oxidative stress, we investigated whether MAYV infection modified the enzymatic antioxidant defences in HepG2 cells. Total lysates were prepared from uninfected or infected HepG2 cells at 6, 15 or 24 h to measure the SOD and CAT enzymatic activities. A significant increase in the total SOD activity was observed at all-time points in the MAYV-infected cells (Fig. 3A) compared with the uninfected cells. No change in CAT activity was detected at 6 and 24 h p.i., but we observed an increase in CAT activity at 15 h p.i. in the MAYV-infected cells compared with the uninfected cells (Fig. 3B).

Next, because the glutathione redox cycle is complementary to CAT in scavenging H_2O_2, we also investigated the total glutathione content in the MAYV-infected cells. As shown in Fig. 3(C), the total glutathione content in the MAYV-infected cells was increased by approximately 4-fold and 2.5-fold at 6 and 15 h p.i., respectively, compared with the uninfected cells. However, the total glutathione levels returned to the control cell levels at 24 h p.i. (Fig. 3C). Glutathione is present in cells mainly in its reduced form (GSH), which represents approximately 90% of the total glutathione in the cell. The remaining amount is in the form of oxidized glutathione (GSSG). Following oxidation by ROS, modification of the reduced GSH results in the formation of the oxidized GSSG. Then, we investigated changes in GSH/GSSG ratio in HepG2 cells either uninfected or infected with MAYV at various time points after infection (Fig. 3D). At 6 h p.i., the intracellular glutathione content was increased without substantial increase in GSSG content, since the GSH/GSSG ratio was significantly higher in MAYV infected HepG2 cells. On the other hand, we observed a progressive decrease of the GSH/GSSG ratio in HepG2 cells after MAYV infection, with a 35 and 37% reduction of the ratio at 15 and 24 h after infection, respectively, compared with uninfected cells (Fig. 3D), showing an increase in GSSG content.

3.4. MAYV also causes oxidative stress in murine macrophage cells

To determine whether similar effects of MAYV infection occurred in other cell types, murine macrophage J774 cells were utilized. We chose this cell type because studies have shown involvement of these cells in the pathogenesis of the arthritis induced by *Alphavirus* (Assunção-Miranda et al., 2010; Assunção-Miranda et al., 2013). Following infection of the J774 cells with MAYV, we assessed the ROS and MDA dosages. Additionally, since the first and most abundant ROS produced in the oxygen reduction pathway is $O_2\cdot^-$, which is metabolized by the SOD enzymes, we evaluated the total SOD activity in the infected J774 cells. Similar to our observations in the HepG2 cells, ROS production was increased in the MAYV-infected J774 cells. However,

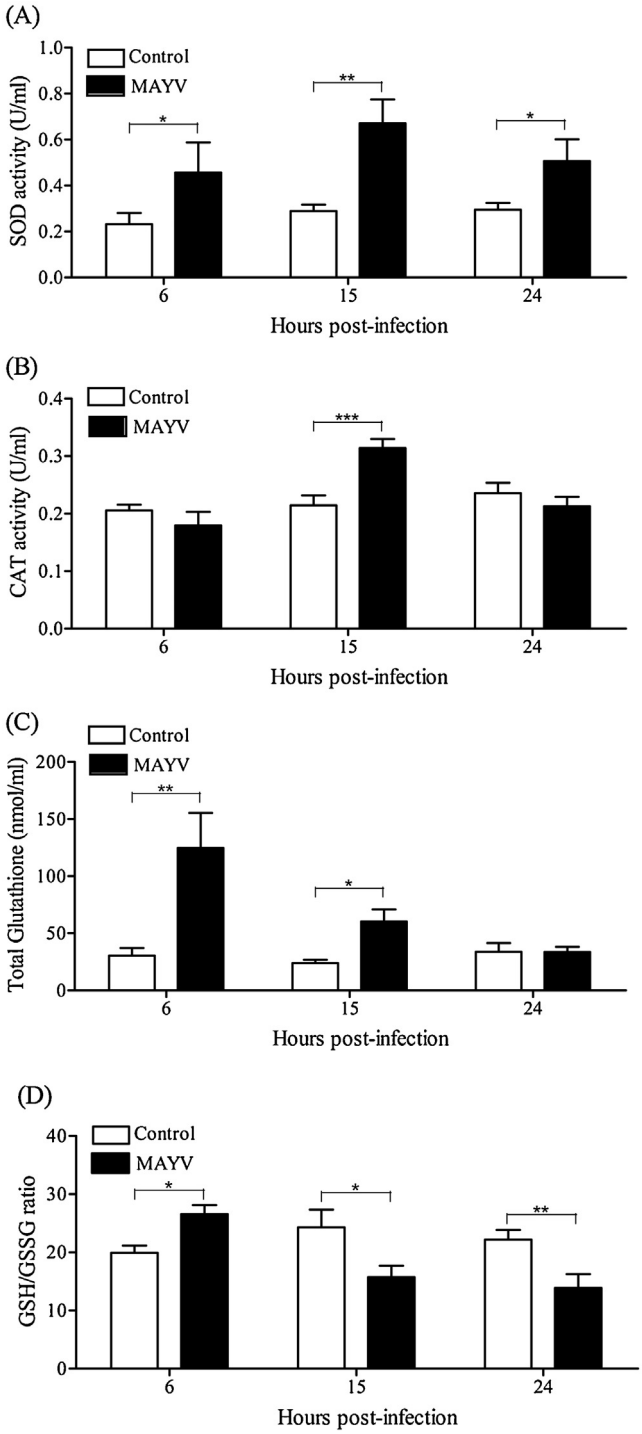
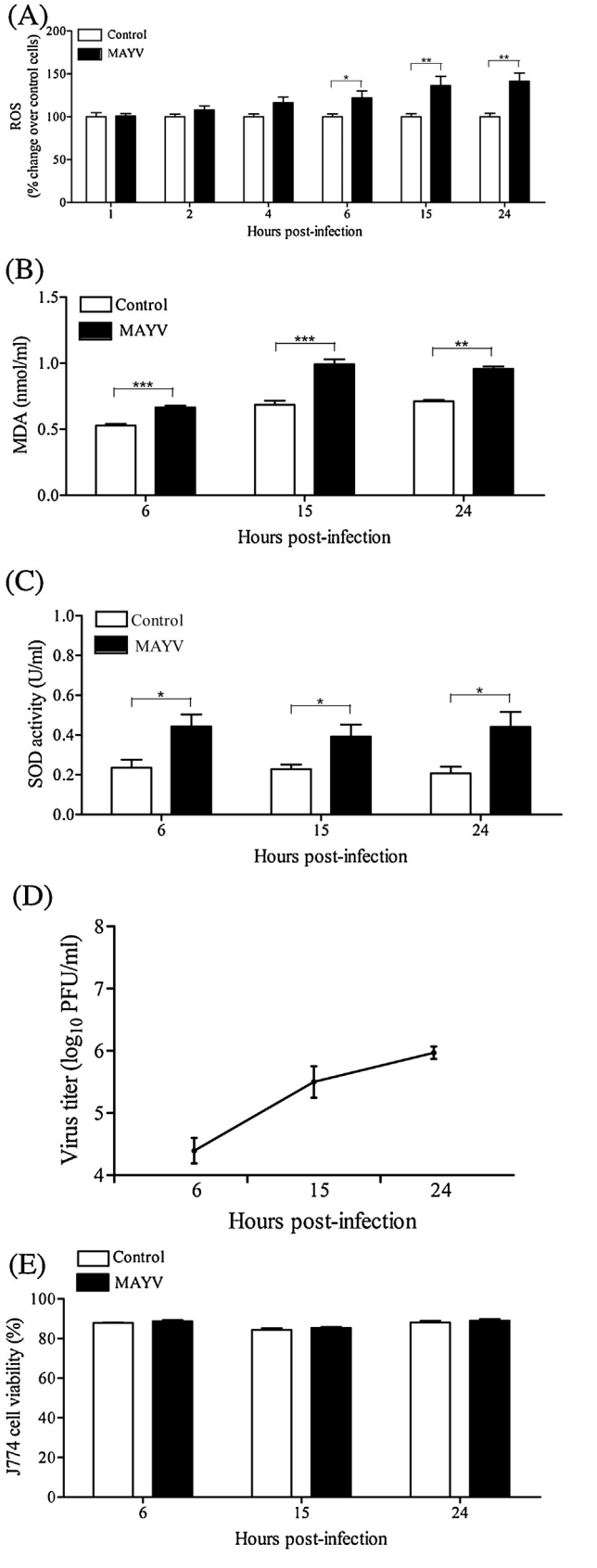


Fig. 3. MAYV increases antioxidant status in HepG2 cells. Total cell lysates were prepared from uninfected and MAYV infected HepG2 cells for 6, 15, and 24 h to measure total SOD (A) and CAT enzyme activities (B), total glutathione content (C), and GSH/GSSG ratio (D). The results include data from two experiments (mean ± SD, $n = 16$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control cells, Student's *t*-test.

MAYV infection of HepG2 cells induced an increase in ROS generation at all-time points tested (1, 2, 4, 6, 15 and 24 h p.i.), whereas an increase at the later time points following infection was observed in the J774 cells (6, 15 and 24 h p.i.) (Fig. 4A). Next, we found that similar to our observations in HepG2 cells, the J774 cells showed an increase in MDA and total SOD activity at all-time points following infection (6, 15 and 24 h p.i.) (Fig. 4B,C). MAYV presents a similar replication profile in HepG2 and J774 cells, although in HepG2 cells, at 24 h p.i., MAYV



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titers were one order of magnitude higher than those obtained in J774 cells (Fig. 4D). No differences were observed in J774 cell viability after MAYV infection when compared to control cells (Fig. 4E). Overall, the

Fig. 4. Effects of MAYV infection on ROS, MDA and total SOD activity in J774 cells. J774 cells were infected with MAYV (m.o.i. of 5) and, at various time points after infection, were measured in control and infected cells ROS production (A), MDA levels (B), and total SOD activity (C), as previously described. (D) Viral growth kinetics in J774 cells assessed by plaque assay. (E) Viability of J774 cells during infection with MAYV. The results include data from three (a) experiments (mean ± SD, $n = 24$) or two (b,c) experiments (mean ± SD, $n = 16$). *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control cells, Student's *t*-test.

data from the HepG2 and J774 cells suggest that oxidative stress may be a common phenomenon following MAYV infection.

4. Discussion

The first evidence that a virus could induce oxidative stress by increasing ROS levels was published in 1979 (Peterhans, 1979). Since then, many studies have shown that different viruses can induce oxidative stress via different pathways, which can directly influence viral pathogenesis. Some examples of important viruses that can induce oxidative stress are *Human papilloma virus* (HPV), *Hepatitis B virus* (HBV), *Hepatitis C virus* (HCV), *Dengue virus* (DENV) and *Human immunodeficiency virus* (HIV) (Greenspan and Aruoma, 1994; Sumida et al., 2000; Mahmood et al., 2004; Bolukbas et al., 2005; Yen et al., 2008; Higgs et al., 2014; Williams et al., 2014).

We demonstrated that MAYV infection induced a significant increase in the ROS levels in the HepG2 and J774 cells. In the HepG2 cells, the increase in ROS production was observed at all-time points, starting 1 h p.i. and extending to 24 h p.i., whereas, this increase was observed from 6 to 24 h p.i. in the J774 cells. Similar to our results, Cavalheiro et al. (2016) demonstrated that MAYV induced an increase in ROS generation in murine macrophage RAW 264.7 cells at 6 h p.i. The authors demonstrated that the increase in ROS production 6 h after MAYV infection coincided with the peak of virus replication and preceded TNF secretion; moreover, treatment of the cells after infection with the antioxidants *N*-acetyl-L-cysteine (NAC) and apocynin abolished TNF secretion, thereby supporting the involvement of ROS in inflammation during MAYV infection. Other studies have demonstrated that cultured cells infected with *Herpes simplex virus type I* (HSV-1), *Sendai virus* (SEV), HIV and DENV increased the generation of ROS (Palamara et al., 1995; Ciriolo et al., 1997; Wang et al., 2013). Similar to our findings, infection of HepG2 cells with DENV-2 increased ROS production at 24 and 48 h p.i. (Wang et al., 2013).

The effect of ROS on cellular functions depends on the amount of ROS and the length of time the cell is exposed to the ROS (Reshi et al., 2014). At first, these ROS fight infection and are seen as a protection mechanism of the host cell that may contribute to apoptosis (Jacobson, 1996). However, more ROS are formed with the advancement of viral replication, causing an imbalance in cellular redox homeostasis and oxidative stress. In this condition, the cells suffer DNA, lipid and protein damage, leading to the loss of cellular integrity and functionality (Halliwell and Gutteridge, 1999). Moreover, oxidative stress has been found to enhance viral replication in different viral infections (Reshi et al., 2014).

Thus, to assess whether the increase in ROS after MAYV infection caused oxidative stress, two oxidative stress markers were monitored in the control and MAYV-infected cells. MDA is a by-product of lipid peroxidation, and protein carbonyl is a product of the irreversible nonenzymatic oxidation or carbonylation of proteins, which often leads to a loss of protein function (Dalle-Donne et al., 2006). Using these markers, we demonstrated that oxidative stress occurred during MAYV infection. In the HepG2 and J774 cells, the MDA levels were elevated in the MAYV-infected cells at all analysed time points (6, 15 and 24 h p.i.). Additionally, an increase in the protein carbonyl level was observed in the MAYV-infected HepG2 cells at 24 h p.i. We also verified that a significant increase in viral titers coincided with the largest MDA and carbonyl protein values in the HepG2 cells. Similar to our results, Dhanwani et al. (2012) found increased MDA levels at 36 and 48 h after infection with CHIKV in the neuroblastoma SH-SY5Y cell line. Oxidative stress is generally explained by two hypotheses: the presence of high amounts of ROS and an impaired enzymatic and/or non-enzymatic antioxidant system (Dröge, 2002). First, we evaluated the antioxidant SOD enzyme after MAYV infection. A significant increase in SOD activity was observed in the two infected cell lines at all-time points analysed. Yoshinaka et al. (1999) demonstrated that the alphavirus *Sindbis virus* caused persistent infection in the human lung foetal cell line MCR-5 and that this persistence was due to the accumulation of large amounts of mitochondrial Mn-SOD in the infected cells. The authors suggested that a cellular factor that regulated the oxidative pathway modulated the outcome of *Sindbis virus* infection, highlighting the significance of oxidative stress in *Alphavirus* infections.

Since SOD enzymes convert $O_2\cdot^-$ to H_2O_2 and the CAT and glutathione redox cycle convert H_2O_2 to water and oxygen, we also evaluated CAT activity, total glutathione content, and GSH/GSSG ratio in the MAYV-infected cells. The observed increase in total SOD activity after MAYV infection suggested that the cells displayed high H_2O_2 levels. Therefore, CAT and glutathione should be responsible for inactivating this ROS. In the HepG2-infected cells, no change in CAT activity was observed at 6 and 24 h p.i.; however, an increase in CAT activity occurred at 15 h p.i. The total glutathione content in the MAYV-infected cells increased at 6 and 15 h p.i. and returned to the control cell levels at 24 h p.i., while GSH/GSSG ratio increased at 6 h p.i. and decreased at 15 and 24 h p.i. Therefore, we suggest that the cells produced large amount of $O_2\cdot^-$ after MAYV infection to try to combat the viral infection and that SOD enzyme activity increased to reverse this excess of $O_2\cdot^-$ to H_2O_2. At the early time points of infection (6 and 15 h p.i.), the observed increase in CAT activity and/or the glutathione content may have been an attempt to maintain the oxidant/antioxidant balance by inactivating the excess H_2O_2. However, this increase in antioxidant defences at the early time points of MAYV infection was not sufficient to prevent oxidative stress. With the progression of MAYV infection (24 h p.i.), the decrease in CAT activity and the depletion of the glutathione content suggest that the infected cells accumulate H_2O_2, which may have contributed to a higher level of oxidative stress as confirmed by the largest MDA and protein carbonyl values. In addition, a progressive reduction of GSH/GSSG ratio at 15 and 24 h p.i. reinforces an evidence of increased oxidative stress in MAYV-infected HepG2 cells. Thus, the oxidative stress observed during MAYV infection could be explained by the presence of high amounts of ROS and the failure of the antioxidant system to fight them.

Regarding to the enzymatic antioxidant system, various studies have shown different types of changes in the SOD and CAT enzyme levels after viral infections. Dhanwani et al. (2012) showed that the neuroblastoma SH-SY5Y cell line infected with CHIKV displayed a marked gradual decrease in the transcriptional SOD and CAT levels with the increasing infection time. Kumar et al. (2009) observed that *Japanese encephalitis virus* (JEV) infection increased the SOD levels in the brains of rats in an attempt to suppress the high O_2^- levels. HPV infection confers cells the ability to survive in an oxidizing environment through different mechanisms, such as increases in the SOD and CAT enzymes (Foppoli et al., 2015). Infection with *Rift valley fever virus* (RVFV) causes an early decrease in SOD1 protein expression and significant oxidative stress in the infected cells (Narayanan et al., 2011). Duygu et al. (2012) found high CAT levels in patients with chronic hepatitis B and increased oxidative stress.

In relation to the glutathione redox cycle, Dhanwani et al. (2012) observed a significant decline in the glutathione levels (36 and 48 h p.i.) in SH-SY5Y cells infected with CHIKV. Tian et al. (2010) demonstrated that DENV-2 infection significantly decreased the glutathione levels in HepG2 cells and that the production of new viral particles decreased considerably after treatment with exogenous glutathione. Wang et al. (2013) also showed that the exogenous administration of glutathione could prevent oxidative stress and liver injury in an

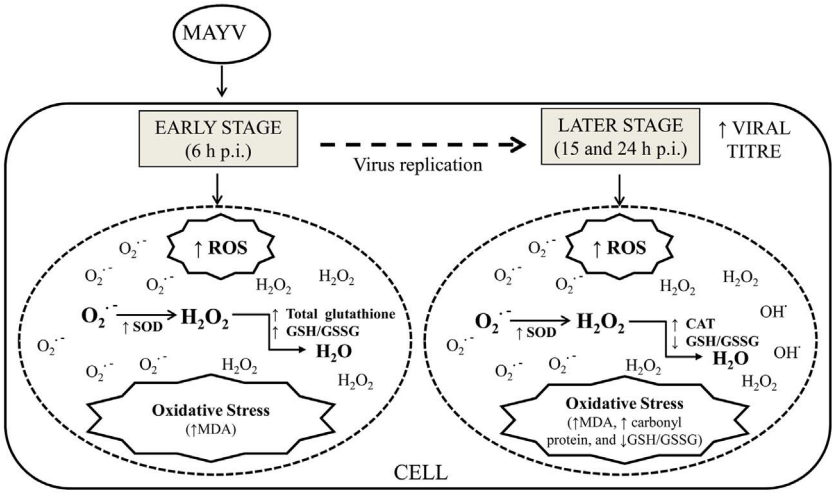

Fig. 5. Schematic representation of possible factors that contributed to the development of oxidative stress after MAYV infection.

experimental animal model of DENV-2. In other studies, treatment with glutathione had an inhibitory effect on the replication of HSV-1 and *Influenza A virus* (Palamara et al., 1995; Cai et al., 2003). Recently, we demonstrated that endogenous glutathione might be involved in neutralizing ROS and constitute an important cellular system to maintain a reductive intracellular environment in the livers of mice infected with *Caraparu virus* (*Bunyaviridae* family) (Camini et al., 2014).

5. Conclusions

According to the results presented in this study, we propose a model based on the possible factors that contributed to the development of oxidative stress at different time points of MAYV infection (Fig. 5). When the cell is infected, ROS production increases in an attempt to combat the infection. An increase in ROS production was observed at 6, 15 and 24 h after MAYV infection. The first ROS produced in the oxygen reduction pathway is $O_2\cdot^-$, which must be metabolized to H_2O_2 by the SOD enzymes. We observed an increase in SOD activity at the early time point after infection (6 h), which was responsible for the conversion of $O_2\cdot^-$ to H_2O_2. However, overproduction of $O_2\cdot^-$ may have occurred, and consequently part of this species was not sufficiently inactivated by the SOD enzymes, resulting in its accumulation inside the cell. SOD enzymes convert $O_2\cdot^-$ to H_2O_2 and the CAT and glutathione redox cycle convert H_2O_2 to water and oxygen. At 6 h p.i., no change in CAT activity was detected and the intracellular glutathione content was increased without substantial increase in GSSG content. Then, at 6 h p.i., an accumulation of H_2O_2 inside the cell may also have occurred, contributing to oxidative stress (demonstrated by increased of MDA). With the progression of infection and increase of viral titer, we observed an increase in oxidative stress biomarkers (MDA and protein carbonyl) and a reduction of GSH/GSSG, provide a strong evidence of increase oxidative stress in MAYV-infected cells. At 15 h p.i., simultaneous with the increase in SOD, we verified an increase in CAT activity and a decrease of GSH/GSSG ratio, as a result of the conversion of reduced GSH to oxidized GSSG. However, at 24 h p.i., the increase in the total SOD activity was not accompanied by increase in CAT activity, and the total glutathione levels returned to the control cells, with decrease in GSH/GSSG ratio. Therefore, the MAYV infection likely resulted in enhanced intracellular H_2O_2 production. H_2O_2 has limited reactivity to organic molecules but plays an important role in oxidative stress via its ability to easily cross cell membranes. Moreover, together with $O_2\cdot^-$ in the presence of transition metals, H_2O_2 can produce the highly reactive OH· through the Fenton and Haber and Weiss reactions (Halliwell et al., 1992). In summary, on MAYV infection, an imbalance in the production of ROS and the cell's inability to detoxify these reactive species may have been responsible by oxidative stress.

Several methods exist to change cellular redox homeostasis. These changes depend on the pathophysiological mechanism of each virus and the host cell response. Thus, different viruses may alter cell homeostasis in various ways, which can generate oxidative stress and its deleterious effects on the host cell. From the results presented in this work, we can infer that MAYV infection induces oxidative stress and that this event may be important for its pathogenesis. Therefore, further studies are needed to better characterize oxidative homeostasis during MAYV infection and the role of oxidative stress in viral pathogenesis.

Conflict of interests

The authors declare that there is no conflict of interests.

Acknowledgements

This work received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 443293/2014-7] and Universidade Federal de Ouro Preto [grant numbers 23.109.006271/2014-70 and 23109.003753/2105-59]. We thank Universidade Federal de Ouro Preto (UFOP) and the Research Center in Biological Sciences (NUPEB/UFOP), Brazil. The authors are grateful to colleagues from the Laboratory of Biology and Technology of Microorganisms and Laboratory of Metabolic Biochemistry (UFOP) for their technical and scientific support.

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