



## OPEN Vaccinia virus modulates the redox environment by inhibiting reactive oxygen and nitrogen species with increased activity of endogenous antioxidant enzymes

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The vaccinia virus (VACV) is the most studied and well-characterised member of the *Poxviridae* family. However, the mechanisms through which it modulates redox homeostasis in host cells remain unclear. Although oxidative stress, which is marked by elevated levels of reactive species, contributes to the pathogenesis of many viral infections, poxviruses may adopt distinct strategies. VACV has redox effector proteins that are released into the cytosol when the virus penetrates the host cell. This study demonstrates for the first time that VACV infection leads to the activation of the nuclear factor erythroid 2 (Nrf2)/antioxidant response element pathway, a key regulator of cellular antioxidant responses, a mechanism not previously described for any poxvirus. Using BSC-40 cells, we observed that VACV significantly reduced reactive oxygen and nitrogen species levels, downregulated inducible nitric oxide synthase, and enhanced the activity of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. This antioxidant shift is correlated with increased *Nrf2* activity and the upregulation of its downstream targets. This virus-induced antioxidant state may be an immunomodulatory mechanism that facilitates viral replication by dampening host defence. Thus, our findings expand the current understanding of virus–host interactions in poxvirus infections.

**Keywords** Vaccinia virus, Poxviruses, Antioxidant, Redox homeostasis

Poxviruses are large double-stranded DNA viruses belonging to the family *Poxviridae*, which includes pathogens that infect a wide range of vertebrate and invertebrate hosts<sup>1,2</sup>. Among these, the vaccinia virus (VACV) is the

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most extensively studied member of the genus *Orthopoxvirus* because of its historical use in smallpox vaccination and continued relevance in zoonotic infections<sup>1,3</sup>.

Recent outbreaks of VACV in Brazil, particularly in the southeastern region, have drawn attention to bovine vaccinia, a zoonosis that affects dairy cattle and milkers, leading to vesicular pustular lesions and systemic symptoms in humans<sup>4,5</sup>. VACV also circulates in Asia, the Middle East<sup>6</sup>, and several South American countries including Argentina, Uruguay, and Colombia<sup>4</sup>. VACV was initially introduced during the vaccination campaigns. After the smallpox vaccine was discontinued, cross-protective immunity declined. Consequently, the global population has become more susceptible to *Orthopoxvirus* infections<sup>6</sup>. Several strains and/or sublineages of VACV have been identified globally, including *buffalopox virus*, which circulates in South Asia and affects buffaloes, cattle, and humans, and the rabbitpox virus, which is associated with infections in domestic rabbits in the Netherlands and the United States<sup>4</sup>.

Although VACV is the most studied poxvirus, information on how it modulates the cellular redox state after infection is limited. Poxviruses have in their structure two viral protein substructures called lateral bodies, composed of approximately 100 structural proteins that deliver effector proteins into the host cell cytosol after viral entry<sup>7,8</sup>. Of these, five are redox, including thiol oxidoreductase, a superoxide-like dismutase, and a human homologue of glutaredoxin-1 and 2<sup>7</sup>. Owing to the presence of these antioxidant enzymes in the lateral bodies, investigation of redox homeostasis during infection has become even more attractive.

Redox homeostasis is critical for maintaining cellular functions. Although reactive oxygen and nitrogen species (RONS) play physiological roles in signalling, their accumulation can lead to oxidative stress, cellular damage, and impaired immune responses<sup>9–12</sup>. The host counters these effects through a sophisticated antioxidant defence system, prominently regulated by the nuclear factor erythroid 2 (Nrf2)/antioxidant response element (ARE) signalling pathway<sup>13</sup>, which modulates the expression of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)<sup>12</sup>.

VACV infection reduces RONS levels<sup>7,14</sup>. However, modulation of the antioxidant environment during poxvirus infection, particularly the potential activation of the Nrf2/ARE pathway, remains unclear. Therefore, this study aimed to investigate whether VACV infection leads to a redox imbalance in host cells. To date, no studies have reported activation of the Nrf2/ARE antioxidant pathway by any member of the *Poxviridae* family. Elucidating the mechanisms and host responses triggered by infection may provide valuable insights into disease progression, especially considering that the re-emergence of poxviruses poses an ongoing public health concern.

## Results

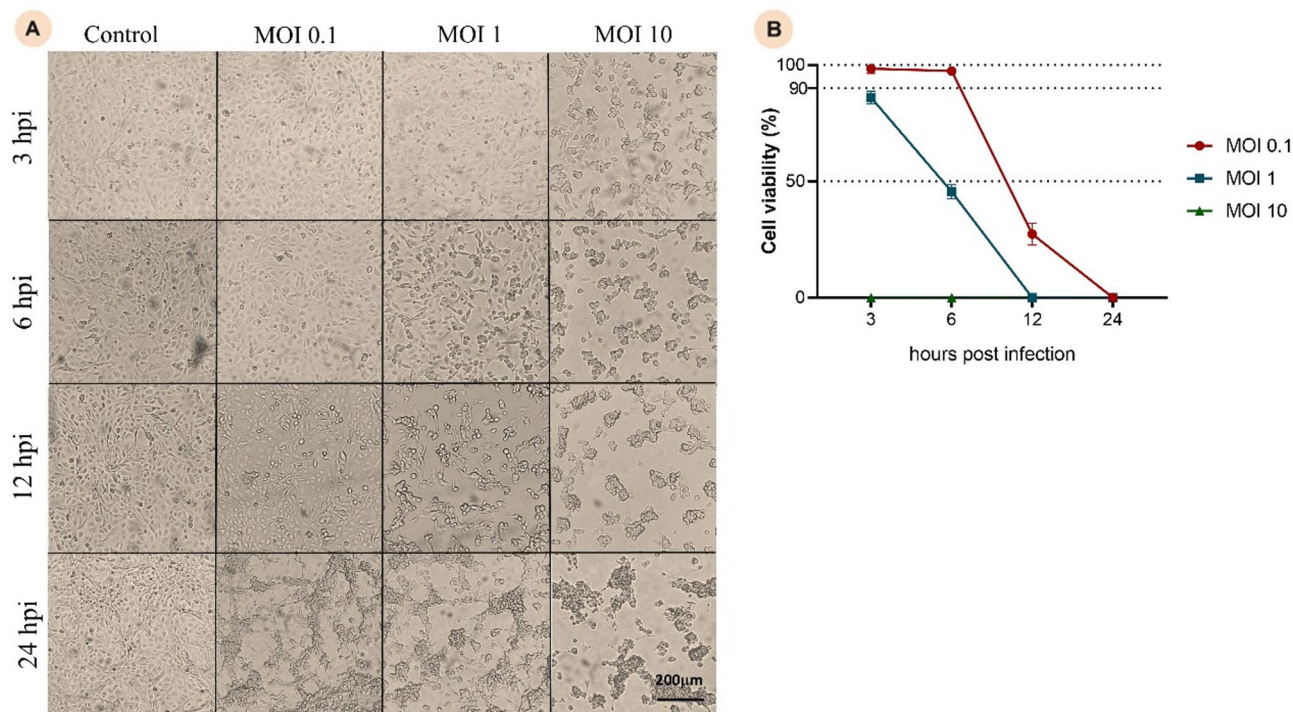
### Characterisation of VACV infection in BSC-40

The first step in this study was to monitor VACV infection in BSC-40 cells to define the best conditions for the evaluation of redox homeostasis. When evaluating three multiplicities of infection (MOI) (0.1, 1, and 10) and four different times post-infection (3 h, 6 h, 12 h, and 24 h), BSC-40 cells were permissible and susceptible to VACV infection. Cytopathic effect (CPE) was apparent at 3 h post infection (hpi) when using an MOI 10 (Fig. 1A). This was confirmed by the percentage of viable cells (Fig. 1B). At lower MOI, such as 0.1, cell viability remained high up to 6 hpi, and CPE was evident only at 12 hpi and 24 hpi. The viability was reduced to 27.3% and 0%, respectively, when compared to cell control (CC, 100%) and cell death (CD, 0%). At MOI 1, the viability was approximately 90% at 3 hpi, decreasing to 44.2% at 6 hpi, where observing CPE was already possible. Complete cell death was observed 12 hpi onwards. By contrast, cells infected with MOI 10 already showed 0% cell viability at 3 hpi.

### VACV infection reduces intracellular RONS levels and inducible nitric oxide synthase (iNOS) gene expression

To evaluate the redox environment of VACV infection, reactive oxygen species (ROS) production was first measured in BSC-40 cells at different time points: 3, 6, 12, and 24 hpi for MOI 0.1, 3, and 6 hpi for MOI 1. These parameters were selected based on cell viability. VACV infection induced a significant decrease in ROS production, which was observed from the first 3 hpi, with an MOI of 1 (Fig. 2A). From 6 hpi onwards, ROS production continued to be significantly reduced in both MOI analysed compared to basal (control) fluorescence levels (Fig. 2B–D). In addition, a positive control for ROS induction, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), was included. As expected, the H<sub>2</sub>O<sub>2</sub> group showed a significant increase in ROS levels at all evaluated time points (Fig. 2A–D). In parallel, an antioxidant control, N-acetylcysteine (NAC), which is a well-known and potent antioxidant<sup>15</sup>, was included. At all-time points analysed, ROS levels in the NAC-treated group were significantly lower than basal levels. When comparing the antioxidant positive control with the VACV-infected group for both MOI, although VACV infection reduced ROS levels, NAC treatment led to an even greater reduction. *BSC-40 cells* infected with UV-inactivated VACV were included in this study. The inactivated virus maintained basal ROS levels equal to those of the control group at all evaluated times (Fig. 2A–D). Furthermore, this UV-control showed that the decrease in the oxidative environment observed in cells infected with VACV (MOI 1 and 0.1) was generated owing to the process of infection and viral multiplication and not only because of the presence of VACV viral particles.

Cell viability and reduction in ROS production were established for the following experiments: VACV infection using MOI 1, and the parameters were evaluated at 3 hpi. To evaluate the reactive nitrogen species (RNS), nitrite (NO<sub>2</sub><sup>-</sup>) content was measured. In addition to ROS production, a significant reduction in NO<sub>2</sub><sup>-</sup> concentration was observed in VACV-infected cells compared to that in the control (Fig. 3A). In addition, the expression of iNOS significantly decreased during the same period (Fig. 3B). Thus, VACV infection induced an antioxidant environment as early as 3 hpi, decreasing RONS and iNOS expression, which is responsible for catalysing NO production.



**Fig. 1.** Cell viability of BSC-40 cells infected with VACV. Cells were infected at different multiplicities of infection (MOI) (0.1, 1 and 10) and followed for 3, 6, 12, and 24 h post infection (hpi). (A) Cell morphology. The cytopathic effect observed (cell death) is proportional to the MOI used. (B) Cell viability. Cells were fixed with formalin, stained with 1% violet cristal, and desitometry was measured in ImageJ software.

### VACV infection prevents oxidative damage

VACV induced a decrease in RONS levels. The next step was to evaluate whether the oxidative damage generated by the reactive species would also be altered during viral infection. Therefore, the lipid peroxidation and protein oxidation levels were measured. Malondialdehyde (MDA) is a product of membrane lipid oxidation, and the levels of carbonyl derivatives refer to protein oxidation. As expected, VACV infection did not alter MDA concentration (Fig. 4A) or carbonyl protein (Fig. 4B) at 3 hpi, which remained at basal levels.

### VACV infection increased activity of the endogenous antioxidant enzymes

To evaluate the antioxidant system and elucidate how VACV alters redox homeostasis, the activities of the main endogenous antioxidant enzymes, SOD, CAT, and GPx, were measured. All three enzymes showed a significant increase in activity compared with the control group (Fig. 5A–C). Therefore, the decrease in ROS levels induced by VACV infection may also be related to the increased activity of antioxidant enzymes that neutralise free radicals and unstable molecules, preventing damage to cellular constituents.

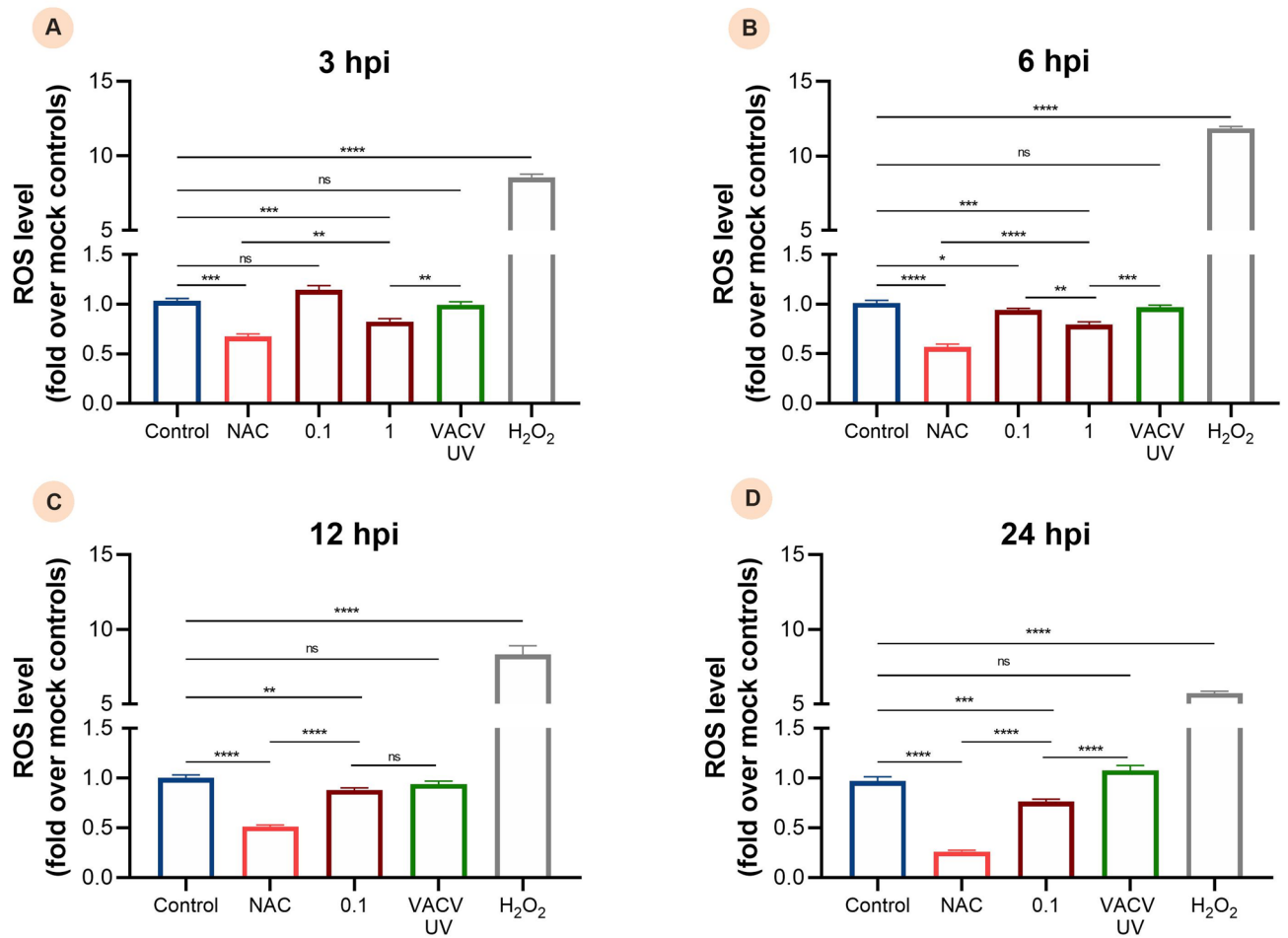
### VACV infection activates the signalling pathway of the endogenous antioxidant system

Once the increase in antioxidant enzymatic activity was confirmed, the next step was to evaluate whether VACV infection altered the signalling pathway and expression of these enzymes. The Nrf2 pathway is an essential regulator of the antioxidant defence gene, responsible for the transcription of these antioxidant enzymes<sup>13</sup>. The regulation of the Nrf2/ARE pathway after VACV infection was investigated using a luciferase assay, which demonstrated a significant increase in the ratio between ARE-dependent Firefly luciferase activity and ARE-independent Renilla luciferase activity (Fig. 6A).

Next, we investigated whether the expression of SOD 1 (the most abundant SOD isoform), CAT, and GPx increased during infection. VACV infection did not alter SOD1 expression at 3 hpi (Fig. 6B). However, the expressions of CAT (Fig. 6C) and GPx (Fig. 6D) significantly increased. Thus, the increased activity of antioxidant enzymes may be related to the upregulation of Nrf2 associated with the increased gene expressions of CAT and GPx after VACV infection.

## Discussion

Poxviruses are viruses of medical importance, and their emergence is a concern for public health agencies. Recently, the World Health Organization has declared monkeypox (Mpox) a global emergency<sup>16,17</sup>. Considering that VACV is genetically similar to Mpox<sup>18</sup>, smallpox, and cowpox viruses<sup>19</sup>, the mechanisms involved in its pathogenicity can be extrapolated to other members of the viral family. Although extensively studied, the



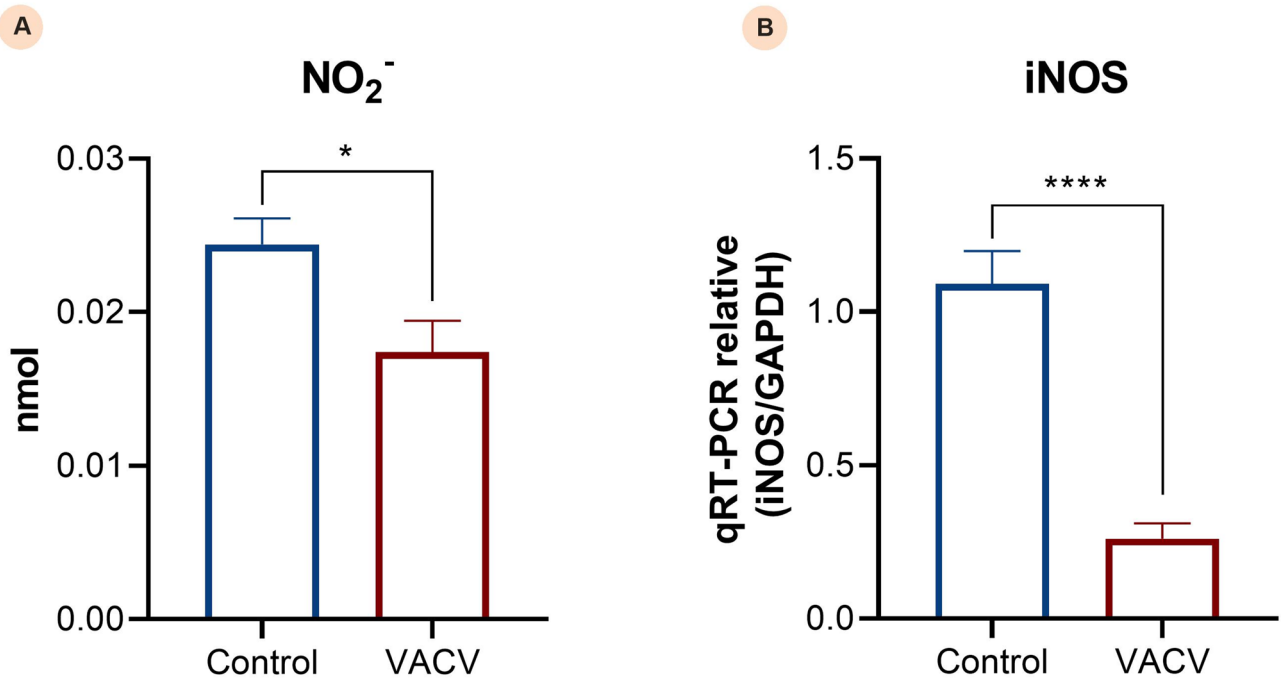
**Fig. 2.** VACV infection exerts antioxidant effect and decreases intracellular reactive oxygen species (ROS). ROS were measured using the fluorogenic marker carboxy-H2DCFDA at 4 post-infection times (A: 3 hpi), (B: 6 hpi), (C: 12 hpi), (D: 24 hpi) and 2 different MOI (0.1 and 1) of VACV. Cell control (CC), antioxidant control (N-acetylcysteine NAC; 10mM) ROS-inducing positive control (hydrogen peroxide H<sub>2</sub>O<sub>2</sub>; 1000μM), and UV-inactivated VACV were included. The results are expressed as mean ± SEM, where \*  $p \leq 0.05$  \*\*  $p \leq 0.01$  \*\*\*  $p \leq 0.001$  \*\*\*\*  $p \leq 0.0001$  compared with the control group ( $p < 0.05$ , one-way ANOVA with Tukey's post-test).

mechanisms that contribute to the pathogenicity of VACV, including those that guide redox homeostasis, remain unclear.

For several viral infections, whether RNA (e.g. Zika virus<sup>20</sup>, Mayaro virus<sup>21</sup>, Oropouche virus<sup>22</sup>, Yellow Fever virus<sup>23</sup>, HIV<sup>24</sup>, or DNA viruses (e.g. Hepatitis B virus and human papillomavirus<sup>25</sup>, oxidative stress is involved and contributes in the pathogenesis. It is a consequence of an increase in RONS levels, which is detrimental to antioxidant activity. Generally, viral infections enhance RONS production, leading to both favourable and unfavourable effects on the host or pathogen<sup>26</sup>. However, the opposite is true for poxviruses. In this study, we evaluated the intracellular RONS production and the endogenous antioxidant defence system in BSC-40 cells infected with VACV.

In this study, VACV infection induced an antioxidant effect by decreasing the intracellular ROS and NO levels during the first 3 hpi. In addition to reducing nitrite levels, VACV infection significantly downregulated iNOS expression at 3 hpi. iNOS generates NO via two-step oxidation of the terminal guanidine nitrogen of L-arginine. The nitric oxide radical (NO•) gives rise to reactive nitric oxide species (RNOS) and reacts with superoxide anion (O<sub>2</sub><sup>-</sup>) to form the peroxynitrite radical (ONOO<sup>-</sup>), which is reduced to nitrite and nitrate (NO<sub>3</sub><sup>-</sup>)<sup>26,27</sup>. Our data are consistent with those of other investigators who have previously demonstrated that VACV infection in human lung cells (A549) decreased ROS levels at 1 and 8 hpi<sup>7</sup> and maintained basal nitrite levels in infected RAW 264.7 macrophages when evaluated at 24 hpi<sup>14</sup>.

VACV carries viral effector proteins that are delivered into the host cytosol during viral entry into its structure, more specifically into the lateral bodies. Among these proteins, five were putative viral redox proteins (A2.5 L, a thiol oxidoreductase; A19L, a protein with two putative thioreductase motifs; A45R, an SOD; O2L and G4L, a human homologue of glutaredoxin-1 and 2, respectively)<sup>7</sup>. Reduced RONS levels may also be attributable to the redox enzymes present in the VACV lateral bodies, which are released when the virus enters the cell. Glutaredoxin 1 (Grx1) is upregulated during influenza A virus infection to modulate the redox environment.

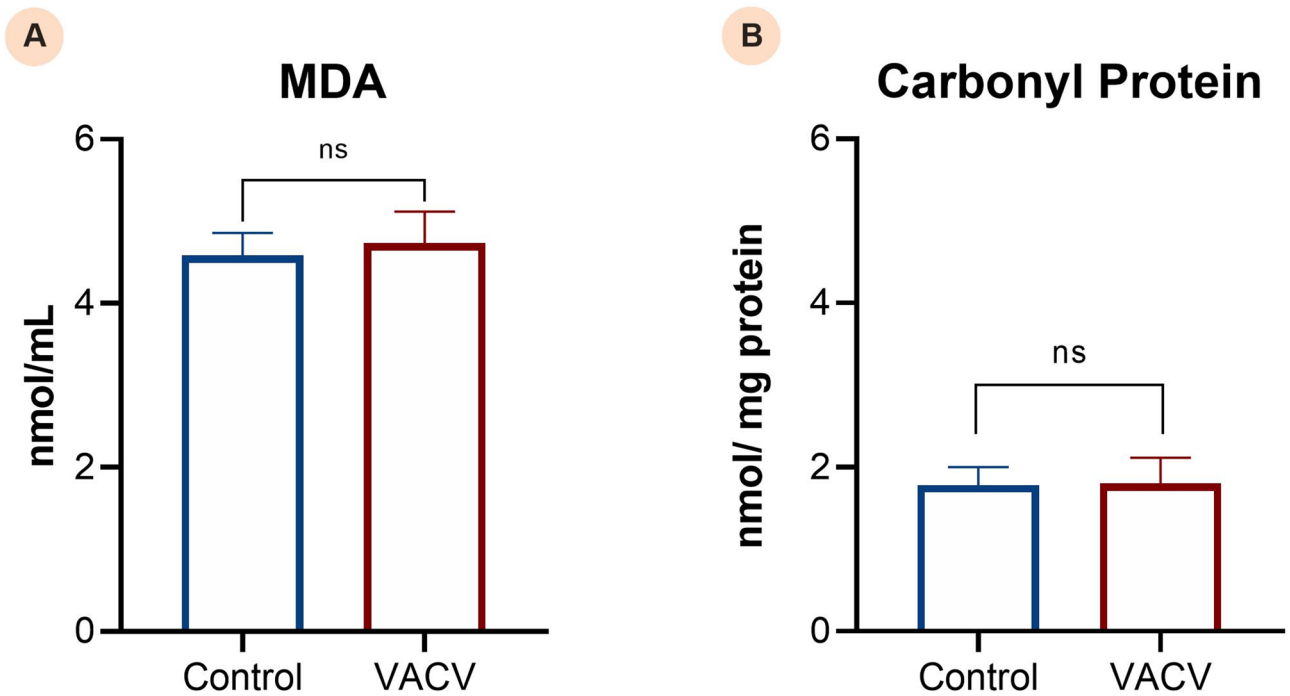


**Fig. 3.** VACV infection downregulates nitric oxide (NO) production. BSC-40 cells were infected with VACV (MOI 1) and the parameters were evaluated at 3 hpi. Nitrite (NO<sub>2</sub><sup>-</sup>), generated from the oxidation of nitric oxide (NO) was measured according to the Griess test (A). Expression of inducible nitric oxide synthase (iNOS) (B) was determined by RT-qPCR using specific primers. Relative expression was determined as the ratio of expression of each of the enzymes to the constitutive GAPDH gene. Results are expressed as mean ± SEM, analyzed using Student's t-test, where \* $p \leq 0.05$  \*\* $p \leq 0.01$  \*\*\* $p \leq 0.001$  \*\*\*\* $p \leq 0.0001$  compared with the control group.

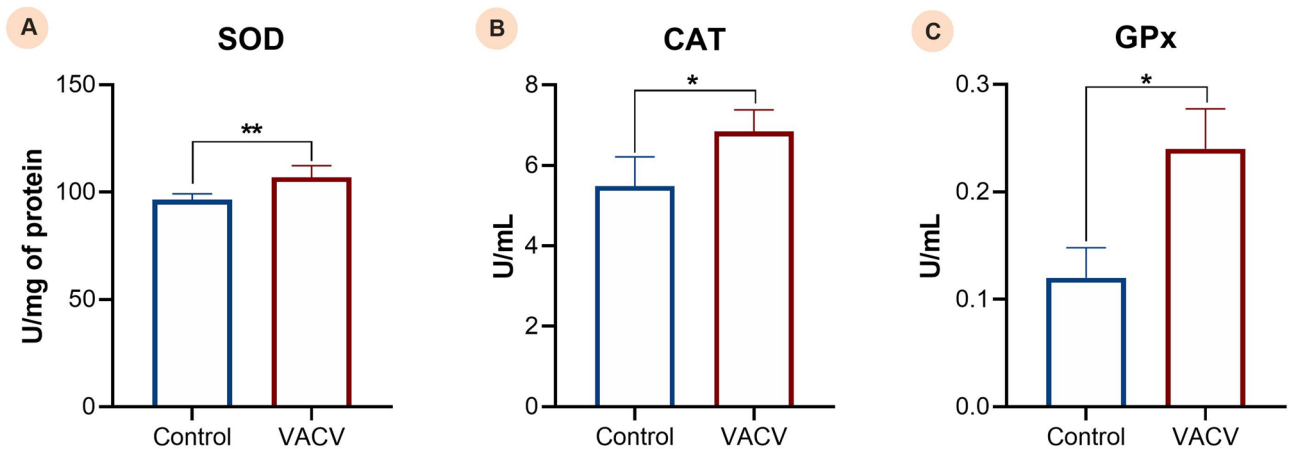
Furthermore, Grx1-mediated deglutathionylation is crucial for efficient viral replication because Grx1 inhibition significantly reduces viral titres<sup>28</sup>. Poxviruses are highly adept at disrupting host cellular processes, notably by remodelling microtubule (MT) networks. Seo et al.<sup>29</sup> demonstrated that the poxvirus protein A51R, which is expressed after viral entry, is directly associated with and stabilises MTs. This interaction suppressed the ROS-dependent antiviral pathway and facilitated viral replication. One possible mechanism by which A51R–MT binding reduces ROS levels is the potential enzymatic activity of A51R in scavenging ROS, which may depend on its interaction with microtubules. Alternatively, the A51R–MT association could interfere with the positioning of ROS-related factors, thereby impairing their antiviral function.

To better characterise the antioxidant profile resulting from VACV infection, the activities of the endogenous antioxidant enzymes SOD, CAT, and GPx were measured. VACV infection increased the activity of these three endogenous enzymes. VACV positively regulated the Nrf2 pathway, which consequently led to the increased expression of CAT and GPx genes, resulting in the highest activity. ARE play an important role in detoxification and are responsible for low-level basal gene expression to mitigate ROS produced by cellular respiration. Thus, regulating redox reactions under stress and non-stress conditions is crucial<sup>13,30</sup>. The ARE is activated when the Nrf2 protein is released and binds to its receptor, activating the transcriptional pathway of antioxidant enzymes<sup>13</sup>. Therefore, the dysregulation of redox signalling in favour of antioxidants may be related to the upregulation of the ARE/Nrf2 pathway.

As mentioned above, viral infections cause a redox imbalance in host cells and intracellular RONS production. This, in turn, leads to the activation of antiviral immune responses such as promotion of cell death, inhibition of the mammalian target of rapamycin, and RONS-coupled pattern recognition receptor signalling<sup>7</sup>. Macrophages play a significant role in host defence against invading viruses by activating autophagy. ROS and NO are critical for antiviral activity as they signal phagocytosis<sup>14,31</sup>. In this study, we confirmed that VACV infection inhibited RONS production and concomitantly increased antioxidant enzyme levels. These findings support the notion that the regulation of intracellular RONS levels and maintenance of a reducing environment may play a critical role in VACV replication. To clarify how an oxidant milieu can affect VACV replication, Bidgood et al. have demonstrated that high ROS levels induced by tert-butyl hydroperoxide affect VACV transcription or transcript stability, resulting in impaired or delayed protein expression and a moderate impact on virion production<sup>7</sup>. Regarding RNS, IFN $\gamma$ -induced NO production suppresses the replication of VACV in mouse macrophages and viral replication was restored in IFN $\gamma$ -treated macrophages exposed to iNOS inhibitors<sup>32</sup>. By contrast, epithelial cells that do not naturally produce detectable levels of NO can inhibit viral replication when transfected with cDNA encoding iNOS or treated with organic compounds that generate NO<sup>32</sup>. Thus, the potential target



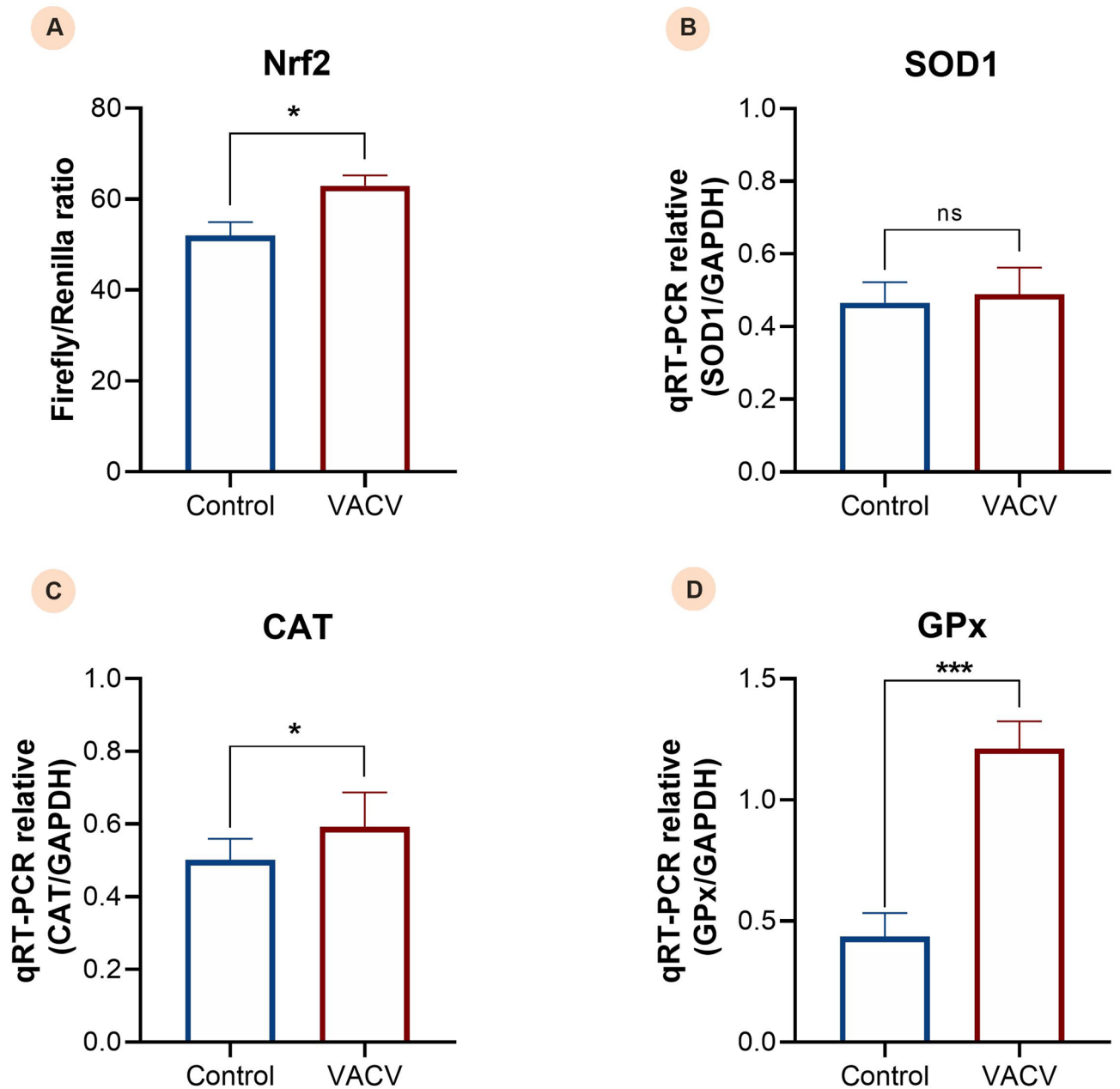
**Fig. 4.** VACV infection maintains basal levels of oxidative damage biomarkers. BSC-40 cells were infected with VACV (MOI 1) and the parameters were evaluated at 3 hpi. Malondialdehyde (MDA) (A) is a byproduct of lipid peroxidation by ROS, while Protein carbonyl (B) is due to protein oxidation by ROS. Results are expressed as mean  $\pm$  SEM, analyzed using Student's t-test. In this case, no significant (ns) differences were found between groups.



**Fig. 5.** VACV infection increases the activity of endogenous antioxidant enzymes. BSC-40 cells were infected with VACV (MOI 1) and the parameters evaluated at 3 hpi. The activities of the enzymes (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) glutathione peroxidase (GPx) were measured. The results are expressed as mean  $\pm$  SEM, analyzed using Student's t-test, where \*  $p \leq 0.05$  \*\*  $p \leq 0.01$  compared with control group.

RONS-producing pathways and Nrf2 signalling as antiviral targets may be promising in pharmacological approaches to poxvirus infections.

Influenza A, Dengue virus, and HIV increase intracellular ROS levels as part of their pathogenesis<sup>33–35</sup>. For example, influenza A virus promotes oxidative stress by downregulating antioxidant defences, such as glucose-6-phosphate dehydrogenase and Nrf2, leading to ROS accumulation that facilitates viral replication and suppresses antiviral signalling pathways<sup>36</sup>. Similarly, Dengue infection also leads to ROS accumulation, resulting from the degradation of Nrf2 by a viral protein (NS2B3), leading to increased viral replication and inflammatory or apoptotic gene expression<sup>35</sup>. HIV infection is associated with chronic oxidative stress due to the activity of viral



**Fig. 6.** VACV infection modulates the antioxidant signaling pathway. (A) Increase of the ARE/Nrf2 pathway. BSC-40 cells were transfected with plasmids and infected with VACV (MOI 1) and analyzed at 3 hpi. Luciferase reporter gene (Nrf2/ARE) activity was measured by chemiluminescence intensity. Data were analyzed as the ratio of ARE-dependent Firefly luciferase activity to ARE-independent Renilla luciferase activity. (B) Superoxide Dismutase isoform 1 (SOD1) or Cu/Zn-SOD gene expression. (C) Catalase (CAT) and Glutathione Peroxidase (Gpx) (D) gene expression. For gene expression analysis, BSC-40 cells were infected with VACV (MOI 1), harvested at 3 hpi and total RNA extracted. Expression was determined by RT-qPCR using specific primers. Relative expression was determined as the ratio of expression of each of the enzymes to the constitutive gene GAPDH. Results are expressed as mean  $\pm$  SEM, analyzed using Student's t-test, where \* $p \leq 0.05$  \*\* $p \leq 0.01$  \*\*\* $p \leq 0.001$ ; ns: non significant compared with the control group.

proteins (e.g. Tat, Nef, and gp120), which stimulate ROS production and contribute to immune dysfunction and inflammation<sup>34</sup>. By contrast, despite promoting increased ROS production, human cytomegalovirus (HCMV) infection positively modulates Nrf2 expression. Increased ROS production promotes viral establishment during acute infections, whereas Nrf2 activation may be beneficial to the virus by increasing the host cell's ability to cope with the oxidative stress resulting from viral infection and/or inflammation. Furthermore, HCMV evades the latent immune response and suppresses ROS production. The ability to regulate host ROS production may allow HCMV to remain latent and undetectable by the host immune system<sup>37,38</sup>.

Our findings suggest a distinct strategy for VACV. Rather than exploiting oxidative stress, VACV may suppress it to avoid triggering immune responses activated by ROS, such as proinflammatory cytokine production or apoptosis. Therefore, although other viruses may benefit from a pro-oxidant environment, VACV may adopt a more discreet approach, suppressing oxidative cues to evade detection, and supporting the early stages of infection. These differences emphasise the diversity of redox modulation strategies among viruses and highlight the potential for therapies to be adapted to specific viral contexts. This reinforces the biological relevance of our findings and highlights VACV as a model for studying viral redox manipulation.

Several strategies have been developed to evade the host immune response against VACV. VACV secretes approximately 100 immunomodulatory proteins that neutralise cytosolic nucleic acid sensors such as cGAMP synthase, along with several other antiviral response pathways<sup>39</sup>. Some cytokines that are inhibited by the secretion of these viral proteins are essential for NO production<sup>14</sup>. In the mechanism “host shutoff”, viruses are instigated to induce a rapid and profound decrease in host protein synthesis. Simultaneously, viral proteins are continuously synthesised. This is the mechanism by which viruses prevent, modulate, and evade host antiviral responses and escape the immune system<sup>40</sup>. Additionally, shutoff leads to the reapportioning of cellular machinery and critical host processes to confer a replication advantage to the virus. Viruses encode proteins that shutoff their hosts. VACV is a clear example, and Dhungel<sup>40</sup> analysed several mechanisms by which VACV induces shutoff. In this context, the observed reduction in RONS levels, suppression of iNOS expression, and activation of the Nrf2/ARE pathway suggests that VACV actively promotes an antioxidant state early during infection. This may function as a form of shutoff by preventing the activation of pro-inflammatory pathways such as NF- $\kappa$ B and interferon responses, which are typically triggered by oxidative stress. By limiting oxidative burst, VACV may avoid premature detection by the host immune system, minimise tissue damage that could hinder viral dissemination, and create an intracellular environment favourable for viral replication. Furthermore, cell proliferation can be induced by low ROS levels, and many viruses exploit this cell division process for efficient multiplication<sup>21,25</sup>. In view of what was observed in this study, we hypothesised that VACV promotes a reduction in the redox environment to initiate host shutdown to escape the host’s antiviral immune response.

Thus, our work elucidates some aspects of the antioxidant and immunomodulatory effects of VACV on host cells and contributes to the understanding of the mechanisms involved in the pathogenesis of poxviruses. However, the absence of *in vivo* data limits the extrapolation of our conclusions to organism-specific responses. Understanding the mechanism by which VACV modulates the host oxidative response as an immune evasion strategy is fundamental. Therefore, future studies should validate and extend our findings to *in vivo* models and further explore the immunomodulatory effects at the level of immune signalling pathways. These efforts may open new avenues for the development of medical interventions targeting diseases caused by poxviruses.

## Methods

### Cells lines and virus

BSC-40 cells (a continuous epithelial lineage from the kidney of the African green monkey, ATCC CRL-2761) were used for viral propagation and all other experiments. Vero cells (African green monkey kidney; ATCC CCL-81) were used for the titration. Both cells were cultured in Dulbecco’s Modified Eagle’s Minimal Medium (Sigma-Aldrich, USA) in a humidified incubator at 37 °C with 5% CO<sub>2</sub>. The media was supplemented with 5% foetal bovine serum (FBS; Sigma-Aldrich, USA), penicillin/streptomycin (200 U/mL), and amphotericin B (2.5 µg/mL) (Sigma-Aldrich, USA). Sub-culturing was performed when the cell monolayer reached 90–100% confluence. The VACV strain Western Reserve was kindly provided by Professor Flávio Guimarães da Fonseca, Universidade Federal de Minas Gerais. For the viral stock, the virus was propagated in BSC-40 cells cultured in T175 cm<sup>2</sup> flasks at an MOI 0.01. After 48 h of incubation at 37 °C with 5% CO<sub>2</sub>, the cells were harvested, and viral particles were purified by ultracentrifugation on a 2% sucrose cushion, as previously described by Kroon et al.<sup>41</sup>. The purified viral stock was aliquoted and stored at –80 °C until use. Viral titration was performed using Dulbecco’s plaque assay<sup>42</sup>. Vero cells were seeded in 6-well plates (1 × 10<sup>6</sup> cells/well) and infected with 10-fold serial dilutions of the viral sample. After viral adsorption, the inoculum was removed, and the culture medium was added to a final volume of 2 mL containing 1% FBS<sup>41</sup>. The plates were incubated for 24 h. Finally, the monolayers were fixed in 10% formalin and stained with 1% crystalline violet. Viral titres were expressed as plaque-forming units per millilitre<sup>41</sup>. All experiments were conducted in a Biosafety Level 2 laboratory, in accordance with the biosafety guidelines established by the Brazilian Ministry of Health<sup>43</sup>.

### Cell viability assay

BSC-40 cells were seeded in 96-well plates (2.5 × 10<sup>4</sup> cells/well) and infected with VACV at MOI of 0.1, 1, and 10. CC and CD groups were included, to which were added fresh culture medium and Triton X-100 (10% v/v), respectively (six samples for each group). At 3, 6, 12, and 24 hpi, the cells were observed and photographed using an optical microscopy (100×) to evaluate the CPE. Then, the cells were fixed in 10% formalin and stained with 1% crystal violet. For cell densitometry analysis, the plates were scanned using a ChemiDoc MP Imaging System (Bio-Rad, Brazil) and analysed using the ImageJ software by measuring the optical density of each well. To determine cell viability, values were interpolated such that the control group (no VACV infection) was equivalent to 100% cell viability, whereas the cell death control (Triton 10%) was equivalent to 0%.

### ROS assay

Intracellular ROS production was measured using the Image-iT LIVE Green Reactive Oxygen Species Kit (Invitrogen). The assay was performed in a black 96-well plate, where the BSC-40 cells were seeded (2.5 × 10<sup>4</sup> cells/well) and infected with 100 µL of VACV at MOI of 0.1 and 1. Some control groups were included: (i) a cell control (without infection), which received only culture medium (5% SFB); (ii) a positive ROS inducer control, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 1000 µM); (iii) a positive antioxidant control, NAC (10 mM); and (iv) an infected

group with VACV inactivated by UV light. After 3, 6, 12, and 24 hpi for MOI 0.1 and after 3 and 6 hpi for MOI 1, the ROS levels were determined. The assay was performed according to the manufacturer's instructions. Briefly, the cells were washed with HBSS buffer and incubated with 25  $\mu\text{M}$  carboxy-H<sub>2</sub>DCEFDA for 45 min at 37 °C, protected from light. The fluorescence intensity at 485/535 nm (excitation/emission) was measured using a Victor X3 Multilabel microplate reader (PerkinElmer). The assay was independently performed twice (five samples/group). The results are expressed as fold-change and normalised to the average fluorescence intensity of the control cells.

### Measurement of NO production

The NO has a short half-life. Therefore, the production of this radical was measured based on the formation of nitrite, a stable and nonvolatile degradation product. For this purpose, BSC-40 cells were previously cultured in a 6-well plate ( $1 \times 10^6$  cel/well), four samples/group (control and infected), infected with 300  $\mu\text{L}$  of VACV (MOI 1). The control group received 300  $\mu\text{L}$  of culture medium (0% FBS) as inoculum. After adsorption, the culture medium of each well was completed, obtaining a final volume of 2 mL/well, 5% FBS, followed by incubation at 37 °C, 5% CO<sub>2</sub>. After 3 h of infection, the cells were washed with 1 $\times$  PBS and removed from the monolayer using a scraper. The cells were collected in 400  $\mu\text{L}$  of 1 $\times$  PBS. The samples were lysed by freezing and thawing (three times), followed by centrifugation at 13,000  $\times g$  for 10 min, and the supernatant was collected for subsequent analysis. Nitrite was measured using a modified Griess method, in which the reaction principle is based on the formation of an azo compound<sup>44,45</sup>. Briefly, 100  $\mu\text{L}$  of sample was mixed with 50  $\mu\text{L}$  of a solution containing 1% sulphanilamide in 5% phosphoric acid and incubated for 10 min. Then, 50  $\mu\text{L}$  of a solution of 0.1% naphthylethylenediamine in 2.5% phosphoric acid was added. Absorbance was recorded at 540 nm using a spectrophotometre. The standard curve for quantification of nitrite production was constructed using 0.1–100  $\mu\text{M}$  sodium nitrate (Sigma, USA). This assay was performed twice independently.

### Oxidative damage biomarker assays

For lipid peroxidation and protein carbonylation assays, BSC-40 cells were cultured in 6-well plates ( $1 \times 10^6$  cells/well). Two groups were analysed: control and infected (eight samples/group). The control group received 300  $\mu\text{L}$  of culture medium (0% FBS) as inoculum. The infected group was infected with 300  $\mu\text{L}$  VACV at MOI of 1. After adsorption, the culture medium of each well was completed, obtaining a final volume of 2 mL/well, 5% FBS, followed by incubation at 37 °C, 5% CO<sub>2</sub>. After 3 h of infection, the cells were washed with 1 $\times$  PBS and removed from the monolayer using a scraper in 300  $\mu\text{L}$  of the lysis buffer (for lipid peroxidation assay) or 500  $\mu\text{L}$  of cold 1 $\times$  PBS (for protein carbonylation assay). The samples were lysed by freezing and thawing (three times), followed by centrifugation at 13,000  $\times g$  for 10 min, and the supernatant was collected for subsequent assays.

#### Lipid peroxidation

ROS production leads to lipid degradation. To evaluate whether VACV infection induces oxidative damage, a lipid peroxidation assay was performed. An MDA assay kit (Invitrogen, USA #MAK085) was used to detect lipid peroxidation in living cells through the reaction of malondialdehyde with thiobarbituric acid. Supernatants from infected and non-infected cells were collected (as described above), and the assay was performed according to the manufacturer's instructions.

#### Protein carbonylation

The method described by Levine<sup>46</sup> was used to determine carbonyl protein levels. Briefly, 2,4-dinitrophenylhydrazine can react with carbonyl groups to generate 2,4-dinitrophenylhydrazone, which is diluted in sodium dodecyl sulphate and can be detected at 370 nm using a UV-visible spectrophotometre. For the assay, supernatants from infected and non-infected cells were collected (as described above), and the procedure was conducted following the Levine protocol<sup>46</sup>. The total protein in each sample was measured using the Bradford method, and the carbonyl protein content was expressed as nmol/mg protein.

### Endogenous antioxidant activity

To evaluate the profiles of the endogenous antioxidant enzymes, the activities of SOD, CAT, and GPx were quantified. The infection procedure was the same as described in the present study. BSC-40 cells were seeded in 6-well plates ( $1 \times 10^6$  cells/well), eight samples for group. Wells designated for the control group (non-infected) received 300  $\mu\text{L}$  of culture medium with 0% FBS, whereas the infected group received 300  $\mu\text{L}$  of VACV inoculum at an MOI of 1. After viral adsorption at 37 °C and 5% CO<sub>2</sub>, wells were completed with culture medium containing 5% FBS and incubated for an additional 3 h. Finally, the cells were washed with 1 $\times$  PBS and detached from the monolayer using a cell scraper in 500  $\mu\text{L}$  (for SOD and CAT assays) or 300  $\mu\text{L}$  (for the GPx assay) of cold 1 $\times$  PBS. The cells were then lysed by three freeze-thaw cycles, and the supernatant was collected and used for enzymatic activity assays.

#### SOD assay

To determine total SOD activity, a spectrophotometric biochemical assay proposed by Marklund and Marklund<sup>47</sup> was performed. This method is based on the inhibition of pyrogallol auto-oxidation, the colour intensity of which can be determined at 570 nm. One unit of the enzyme SOD (U SOD) was defined as the amount of enzyme that reduces the auto-oxidation rate of pyrogallol by 50%. The total protein content of each sample was measured using the Bradford method, and SOD activity was expressed as U SOD/mg protein.

#### CAT assay

CAT activity was determined using supernatants from infected and non-infected cells. The assay was performed using a Catalase Colorimetric Activity Kit (#E1ACATC; Invitrogen) according to the manufacturer's instructions.

#### GPx assay

GPx activity was measured using GPx (GPx Assay Kit #ab102530; Abcam, USA) according to the manufacturer's recommendations.

#### Luciferase assay

ARE is a protein sequence in the promoter regions of several antioxidant genes. Induction of antioxidant enzymes by ARE is mediated by Nrf2. The Nrf2/ARE signalling pathway plays an important role in ameliorating cellular oxidative stress. Thus, a luciferase assay using a reporter gene of the Nrf2/ARE pathway was performed. BSC-40 cells were cultured in 24-well plates ( $1 \times 10^5$  cells/well). Plasmids pGL4.37 and pRL-TK were transfected using the Lipofectamine™ 3000 Transfection Reagent (Invitrogen, USA), according to the recommendations by the manufacturer. After 24 h of transfection, the cells were infected with VACV (MOI 1) or with culture medium as a control, and at 3 hpi, the cells were collected for further analysis. ARE activity was detected by measuring the luminescence intensity using the *Renilla*-Glo Luciferase Assay System kit (Promega, USA) according to the manufacturer's recommendations. The data obtained were analysed for relative intensity, which is the ratio of ARE-dependent Firefly luciferase activity to ARE-independent *Renilla* luciferase activity. The experiments were independently performed twice (four samples per group).

#### Expression of antioxidant enzymes by reverse transcription quantitative polymerase chain reaction (qRT-PCR)

To quantify the expression of iNOS, SOD1, CAT, and GPx, BSC-40 cells were seeded in 6-well plates and infected with VACV (MOI 1) or with culture medium as a control (seven samples/group). In 3 hpi, the cells were washed twice with  $1 \times$  PBS and lysed with 500  $\mu$ L of Trizol (Sigma-Aldrich, USA) for extraction of total RNA, according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesised using reverse transcriptase and random primers using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The expression levels of the enzymes and normalised glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were evaluated using real-time PCR (qRT-PCR). The iNOS primer sequences (forward 5'-ATCTCCTTTGTTACCGCTTCC-3' and reverse 5'-GTTTGACCAGAGGACCCAG-3') were derived from the *Cercopithecidae* genome available in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) and constructed using the Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primers for SOD1, CAT, GPx, and GAPDH were constructed as previously described<sup>23</sup>. Reactions were performed using the SYBR Green PCR Master Mix kit (Applied Biosystems, USA), according to the manufacturer's recommendations.

#### Statistical analyses

The data were analysed using GraphPad Prism version 8 and are presented as means  $\pm$  standard errors of the mean. According to the Kolmogorov–Smirnov normality test, differences between groups were considered statistically significant when  $p \leq 0.05$ . Statistical significance is indicated as follows:  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*), and  $p < 0.0001$  (\*\*\*\*). Statistical comparisons were performed using an unpaired *t*-test (for comparisons between two groups) or one-way analysis of variance followed by Tukey's post-hoc test (for comparisons among three or more groups).

#### Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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#### References

- Silva, N. I. O. et al. There, and everywhere: the wide host range and geographic distribution of zoonotic orthopoxviruses. *Viruses* **43**, 1–20 (2021).
- McInnes, C. J. et al. ICTV virus taxonomy profile: poxviridae 2023. *J. Gen. Virol.* **104**, 1–2 (2023).
- Fonseca, F. G., Kroon, E. G., Nogueira, M. L. & Trindade, G. S. Zoonotic vaccinia virus outbreaks in Brazil. *Future Virol.* **6**, 697–707 (2011).
- Oliveira, J. S. et al. Vaccinia virus natural infections in Brazil: the good, the bad, and the ugly. *Viruses* **9**, 340 (2017).
- Trindade, G. S., Emerson, G. L., Carroll, D. S., Kroon, E. G. & Damon, I. K. Brazilian vaccinia viruses and their origins. *Emerg. Infect. Dis.* **13**, 965 (2007).
- Silva, P. H. B. et al. Geographic distribution of vaccinia virus, diagnosis and demographic aspects of affected populations, Minas Gerais, Brazil, 2000–2023. *Viruses* **17**, 22 (2025).
- Bidgood, S. R. et al. Poxviruses package viral redox proteins in lateral bodies and modulate the host oxidative response. *PLoS Pathog.* **18**, e1010614 (2022).
- Schmidt, F. I. et al. Vaccinia virus entry is followed by core activation and proteasome-mediated release of the immunomodulatory effector VH1 from lateral bodies. *Cell. Rep.* **4**, 464–476 (2013).
- Ursini, F., Maiorino, M. & Forman, H. J. Redox homeostasis: the golden mean of healthy living. *Redox Biol.* **8**, 205–215 (2016).
- Meo, S. D., Reed, T. T., Venditti, P. & Victor, V. M. Role of ROS and RNS Sources in Physiological and Pathological Conditions. *Oxid. Med. Cell Longev.* (2016). (2016).
- Weidinger, A. & Kozlov, A. V. Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction. *Biomolecules.* **5**, 472–484 (2015).

12. Jomova, K., Alomar, S. Y. & Alwasel, S. H. Several lines of antioxidant defense against oxidative stress: antioxidant enzymes, nanomaterials with multiple enzyme-mimicking activities, and low-molecular-weight antioxidants. *Arch. Toxicol.* **98**, 1323–1367 (2024).
13. Wei, Z. et al. Cell-Based assays to identify modulators of Nrf2/ARE pathway. *Methods Mol. Biol.* **2474**, 59–69 (2022).
14. Bellows, C. F., Garry, R. F. & Jaffe, B. M. Vaccinia Virus-Induced Inhibition of nitric oxide production. *J. Surg. Res.* **111**, 127–135 (2003).
15. Lima, R. L. S. & Et Sylmarin exerts antioxidant and antiviral effects on Zika virus infection. *J. Virol. Methods.* **335**, 115133 (2025).
16. Tiwari, A. et al. Monkeypox outbreak: wastewater and environmental surveillance perspective. *Sci. Total Environ.* **856**, 159166 (2023).
17. Sah, R. et al. Mpox strikes once more in 2024: declared again as a public health emergency of international concern. *Travel Med. Infect. Dis.* **61**, 102753 (2024).
18. Wang, Y., Yang, K. & Zhou, H. Immunogenic proteins and potential delivery platforms for Mpox virus vaccine development: A rapid review. *Int. J. Biol. Macromol.* **245**, 125515 (2023).
19. Molteni, C., Forni, D., Cagliani, R., Clerici, M. & Sironi, M. Genetic ancestry and population structure of vaccinia virus. *Npj Vaccines* **7**(1), 92 (2022).
20. Almeida, L. T. et al. Zika virus induces oxidative stress and decreases antioxidant enzyme T activities in vitro and in vivo. *Virus Res.* **286**, 19804 (2020).
21. Camini, F. C. et al. Oxidative stress in Mayaro virus infection. *Virus Res.* **236**, 1–8 (2017).
22. Menegatto, M. B. S. et al. Oropouche virus infection induces ROS production and oxidative stress in liver and spleen of mice. *J. Gen. Virol.* **104**, 001857 (2023).
23. Ferraz, A. C. et al. Yellow fever virus infection in human hepatocyte cells triggers an imbalance in redox homeostasis with increased reactive oxygen species production, oxidative stress, and decreased antioxidant enzymes. *Free Radic Biol. Med.* **212**, 266–273 (2024).
24. Ivanov, A. V. et al. Oxidative Stress during HIV Infection: Mechanisms and Consequences. *Oxid Med Cell Longev.* (2016). (2016).
25. Camini, F. C. Implications of oxidative stress on viral pathogenesis. *Arch. Virol.* **162**, 907–917 (2017).
26. Molteni, C. G., Principi, N. & Esposito, S. Reactive oxygen and nitrogen species during viral infections. *Free Radic Res.* **48**, 1163–1169 (2014).
27. Szabó, C., Ischiropoulos, H. & Radi, R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nat. Rev. Drug Discov.* **6**, 662–680 (2007).
28. Checconi, P. et al. Influenza virus replication is affected by glutaredoxin1-mediated protein deglutathionylation. *FASEB J.* **37**, e22729 (2023).
29. Seo, D. et al. Poxvirus A51R proteins regulate microtubule stability and antagonize a cell-intrinsic antiviral response. *Cell Rep.* **43**, 13882 (2024).
30. Ngo, V. Y. & Duennwald, M. L. Nrf2 and oxidative stress: A general overview of mechanisms and implications in human disease. *Antioxidants* **11**, 2345 (2022).
31. Deramaut, T. B., Dill, C. & Bonay, M. Regulation of oxidative stress by Nrf2 in the pathophysiology of infectious diseases. *Med. Mal Infect.* **43**, 100–107 (2013).
32. Karupiah, G. et al. Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. *Science* **10**, 1445–1448 (1995).
33. Liu, M. et al. The role of oxidative stress in influenza virus infection. *Microbes Infect.* **19**, 580–586 (2017).
34. Ivanov, A. V. & Et al. Oxidative Stress during HIV Infection: Mechanisms and Consequences. *Oxid Med Cell Longev.* 8910396 (2016). (2016).
35. Ferrari, M. et al. Dengue virus targets Nrf2 for NS2B3-Mediated degradation leading to enhanced oxidative stress and viral replication. *J. Virol.* **94**, 1–21 (2020).
36. De Angelis, M. et al. Influenza virus Down-Modulates G6PD expression and activity to induce oxidative stress and promote its replication. *Front. Cell. Infect. Microbiol.* **11**, 804976 (2022).
37. Lee, J., Koh, K., Kim, Y. E., Ahn, J. H. & Kim, S. Upregulation of Nrf2 expression by human cytomegalovirus infection protects host cells from oxidative stress. *J. Gen. Virol.* **94**, 1658–1668 (2013).
38. Planchon, M. S., Fishman, J. A. & El Khoury, J. Modulation of monocyte effector functions and gene expression by human cytomegalovirus infection. *Viruses* **16**, 1809 (2024).
39. Meade, N. et al. The poxvirus F17 protein counteracts mitochondrially orchestrated antiviral responses. *Nat. Commun.* **14**, 7889 (2023).
40. Dhungel, P., Cantu, F. M., Molina, J. Á. & Yang, Z. Vaccinia Virus as a Master of Host Shutoff Induction: Targeting Processes of the Central Dogma and Beyond. *Pathogens*, 9 (2020).
41. Kroon, E. G. et al. Natural vaccinia virus infection: diagnosis, isolation, and characterization. *Curr Protoc. Microbiol.* **42**, 14A.5.1–14A.5.43 (2016).
42. Dulbecco, R. Production of Plaques in Monolayer Tissue Cultures by Single Particles of an Animal Virus. *Proc. Natl. Acad. Sci. USA.* **38**, 747–752 (1952).
43. Brasil. Ministério Da saúde. Secretaria de ciência, tecnologia e insumos estratégicos; departamento do complexo industrial e Inovação Em Saúde. *Classificação De Risco Dos Agentes Biológicos* 1–78 (2022).
44. Green, L. C. et al. Analysis of nitrate, nitrite, and [<sup>15</sup>N]nitrate in biological fluids. *Anal. Biochem.* **126**, 131–138 (1982).
45. Sun, G., Zhang, X., Broderick, M. & Fein, H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors* **3**, 276–284 (2003).
46. Levine, R. L., Williams, J. A., Stadtman, E. R. & Shacter, E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **233**, 346–357 (1994).
47. Marklund, S. & Marklund, G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* **47**, 469–474 (1974).

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## Author contributions

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ogy; J.C.M.: Resources; J.G.A.C.R.: Investigation; F.G.F.: Investigation; G.S.T.: Investigation, Resources, Funding acquisition; E.G.K.: Investigation, Resources, Funding acquisition; C.L.B.M.: Conceptualization; Methodology, Formal analysis, Writing - Original Draft, Supervision, Project administration, Funding acquisition.

## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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