Genotoxic effects of foot-and-mouth disease virus serotypes A and O in IBRS-2 cells

Aidin Rahim Tayefeh[1*], Pegah Goodarzi[2], Farzam Ghahramani[3], Rouhollah Keshavarz[1], Mohsen Lotfi[4]

*Corresponding author: Aidin Rahim Tayefeh

[1] Department of Animal Biotechnology, Faculty of Agriculture Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), P.O. Box. 14965/161, Tehran, Iran.

aidin rahim tayefeh: https://orcid.org/0000-0001-8583-2321

Rouhollah Keshavarz: https://orcid.org/0000-0003-4976-1508

[2] Faculty of Veterinary Medicine, Ka.C., Islamic Azad University, Karaj, Iran.

Pegah Goodarzi: https://orcid.org/0009-0007-6828-423X

[3] Department of Clinical Sciences, Faculty of Veterinary Medicine, Ka.C., Islamic Azad University, Karaj, Iran

Farzam Ghahramani: https://orcid.org/0000-0002-8358-3306

[4] Department of Quality Control, Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization (AREEO), Karaj, Iran

mohsen lotfi: https://orcid.org/0000-0001-9902-7999

Tel: +98 (021) 44787421

E. mail: tayefe@nigeb.ac.ir

Abstract

Introduction: Foot-and-mouth disease virus (FMDV) is a major viral pathogen of herds. The evidence shows that FMDV disrupts host-cell homeostasis by inducing oxidative stress and apoptosis that may contribute to genomic instability. However, FMDV-associated DNA damage at the cellular level remains insufficiently characterized.

Objective: This study aimed to evaluate the cytotoxic, oxidative, apoptotic, and genotoxic effects of FMDV serotypes A and O in IBRS-2 cells *in vitro*.

Materials and Methods: The IBRS-2 were contaminated with FMDV serotype A or O at a concentration of 10^3 $TCID_{50}$ mL^{-1} and compared with the control. Apoptosis was assessed using flow cytometry. Intracellular reactive oxygen species (ROS) generation was measured using the DCFH-DA fluorescence assay. Genomic instability was evaluated by alkaline single-cell gel electrophoresis (SCGE). Statistical analysis was achieved using one-way ANOVA followed by Tukey's post hoc test, with significance set at $p \leq 0.05$.

Results: Infection with both FMDV serotypes significantly augmented apoptosis, ROS production, and DNA damage compared with the control ($p \leq 0.05$). Serotype O showed a more cytopathic effect than serotype A, ROS signals, and TM, OTM, and DI values. TM and OTM increased from 16.00 ± 2.13 and 9.00 ± 1.01 in the control to 55.00 ± 6.19 and 27.00 ± 2.46 in the serotype A and to 66.00 ± 6.84 and 34.00 ± 4.03 in the serotype O groups.

Conclusion: These findings highlight genomic instability as a component of FMDV cellular pathogenesis and provide a reason for future studies of virus–host interactions.

Keywords: Apoptosis, FMD, Genotoxicity, IBRS-2, SCGE.

1- Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease, and recognized as one of the most economically damaging diseases for livestock worldwide. Foot-and-mouth disease virus (FMDV), belongs to the Picornaviridae family and shows a range of antigenic variation including A, O, C, SAT1, SAT2, SAT3, and Asia1 serotypes. While these serotypes cause similar clinical symptoms, there are differences in severity depending on factors like the virus strain, the host species, and their immune reactions [1]. FMDV infection is known for causing vesicular lesions in epithelial tissues, but some research suggests that the virus's impact extends much further [2]. For instance, earlier investigations have proved that FMDV triggers strong cytopathic effects and disrupts cellular balance [3]. It also seems to change host defense mechanisms in ways that help the virus replicate and persist [4].

Maintaining the integrity of the genome is essential for normal cell function and survival. When this integrity is compromised, it can lead to mutations, decreased cell viability, and changes in how viruses interact with their host cells. DNA damage caused by viruses may happen through various routes. Virus components might directly or indirectly interact with host DNA. It can increase reactive oxygen species (ROS) production, which harms DNA, and it can disrupt DNA repair machinery. These factors play a role in how the disease progresses. Then, it is important to examine DNA damage in cells infected by viruses.

One method for detecting these events is the alkaline single-cell gel electrophoresis (SCGE) assay, called the comet assay. It can detect breaks in DNA strands at the level of individual cells. This technique stands out because it is inexpensive, and sensitive. For these reasons, it uses not only for studying genotoxicity caused by chemicals or environmental factors but also in analyses of viral

infection effects [5]. However, careful thought of apoptosis is necessary for correct understanding of SCGE results since DNA fragmentation during programmed cell death can produce many strand breaks that resemble primary genotoxic lesions. If apoptosis is not assessed simultaneously, endonuclease-mediated cleavage of genomic DNA during apoptosis may yield comet patterns that could be mistaken as direct virus-induced genotoxicity. Thus, in investigations of virus–host interactions, a thorough examination of apoptotic reactions and DNA integrity has been advised to resolve the source of DNA damage.

The current study performed to evaluate genomic instability and DNA integrity in IBRS-2 cells infected with FMDV serotypes A and O. This study aims to clarify the genotoxic potential of FMDV and clarifies understanding of virus–host interactions by combining apoptosis analysis with semi-quantitative SCGE assay. It can provide a more image of FMD pathogenesis.

2- Material and Method

All chemicals and reagents used the study were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Foot-and-mouth disease virus (FMDV) serotypes A and O, as well as the IBRS-2 cell line, were kindly supplied by the University of Tehran (Tehran, Iran).

2-1- Cell Culture Conditions

Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS) was used to culture the IBRS-2 cells under proper incubation conditions (37 °C, 5% CO_2, humidified atmosphere). Regular cell subculture was done to keep exponential growth, and these cells were used for tests at ideal confluency (70% confluency). The control group consisted of the uninfected IBRS-2 cells.

2-2- Virus Preparation and Titration

Foot-and-mouth disease virus (FMDV) serotypes A and O were cultured in the IBRS-2 cells. Four successive passages increased the viral concentration to achieve enough titers (Fig. 1). The cell supernatants were collected, centrifuged to clarify, aliquoted, and stored at −80 °C till use.

The Reed and Muench method [6] was used for virus titration. Briefly, frozen viral stocks were thawed and serially diluted ten-fold (10^{-4} to 10^{-8}) in serum-free DMEM. Quadruplicate replicates of each dilution were induced onto confluent IBRS-2 cell monolayers in 96-well plates (SPL Life Sciences, Korea). After 72 hrs. of incubation, the plates were microscopically checked for virus-induced cytopathic effects (CPEs). Finally, a final concentration of 10^3 $TCID_{50}$ mL^{-1} was chosen for all experiments.

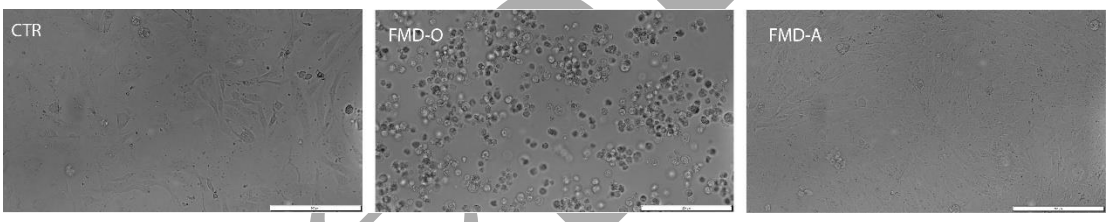

Fig. 1. Cytopathic effects of foot-and-mouth disease virus (FMDV) serotypes A and O on IBRS-2 cells.

2-3- Apoptosis Assessment by Flow Cytometry

Using an Annexin V–FITC/propidium iodide (PI) dual-staining assay, apoptosis in IBRS-2 cells infected with FMDV serotypes A and O was measured [7]. This approach helps to differentiate between viable, early apoptotic, late apoptotic, and necrotic cells based on phosphatidylserine externalization and membrane integrity. Briefly, the IBRS-2 cells were seeded into culture vessels and left to grow exponentially. While control cells leaved in virus-free medium, treatments were

infected with FMDV serotype A or O. Following the incubation period, the cells were gathered by mild trypsinization, twice rinsed with cold phosphate-buffered saline (PBS, pH 7.4), and centrifuged at 1500 rpm for 5 min. The Cell pellets were dissolved in 1× binding buffer, and samples were transferred into flow-cytometry tubes. Annexin V–FITC was added, then kept at 4 °C in the dark for 15 min. Just before analysis, PI was used to detect cells with impaired membranes. Using a calibrated flow cytometer, flow-cytometric analysis was carried out; FITC was read in the FL1 channel and PI in the FL3 channel. Data were examined to ascertain the percentages of viable (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells. The sum of early and late apoptotic populations was utilized to measure the total apoptotic response.

2-4- Measurement of Intracellular Reactive Oxygen Species (ROS)

Using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), intracellular ROS levels in IBRS-2 cells infected with FMDV serotypes A and O were assessed [8]. The oxidation-dependent conversion of non-fluorescent DCFH-DA into fluorescent dichlorofluorescein (DCF) inside cells forms the basis of this test. Twelve-well plates (SPL Life Sciences, Korea) were seeded with IBRS-2 cells at an appropriate density and kept overnight under regular. FMDV serotypes A or O were then applied to cells while untreated cells acted as controls. The cells were incubated with DCFH-DA (final concentration of 10 μM) for 45 min at 37 °C in the dark. The medium was then removed and the cells were washed with PBS to get rid of any extra dye. After adding trypsin, the cells resuspend in PBS, and read by a fluorescence spectrophotometer with excitation at 490 nm and emission observed between 495 and 550 nm.

125 **2-5- Evaluation of DNA Damage by Alkaline Single-Cell Gel Electrophoresis (SCGE)**

126 Using the alkaline single-cell gel electrophoresis (SCGE) test, DNA damage in IBRS-2 cells
127 infected with FMDV serotypes A and O was evaluated [9]. Following incubation and infection,
128 the cells were gathered, rinsed with PBS. Microscope slides precoated with a layer of 1% normal-
129 melting-point agarose made in PBS, and were let to solidify. At 37 °C, cell suspensions were
130 combined with low-melting-point agarose and promptly distributed onto the precoated slides. The
131 slides were submerged in freshly made cold lysis solution to eliminate cellular membranes and
132 proteins, leaving nucleoids of supercoiled DNA. The slides were deposited in alkaline buffer (pH
133 > 13). Under basic conditions at a steady voltage, electrophoresis allowed broken DNA to travel
134 toward the anode and create comet-like formations. Following electrophoresis, the slides were
135 stained with Siber Green dye (Fig. 2). A fluorescence microscope (Eclipse Ti; Nikon Tokyo, Japan)
136 was used to view comets. Tail Moment (TM) and Olive Tail Moment (OTM) were used to measure
137 DNA damage. a semi-quantitative Damage Index (DI) was determined using visual comet

morphology grading.

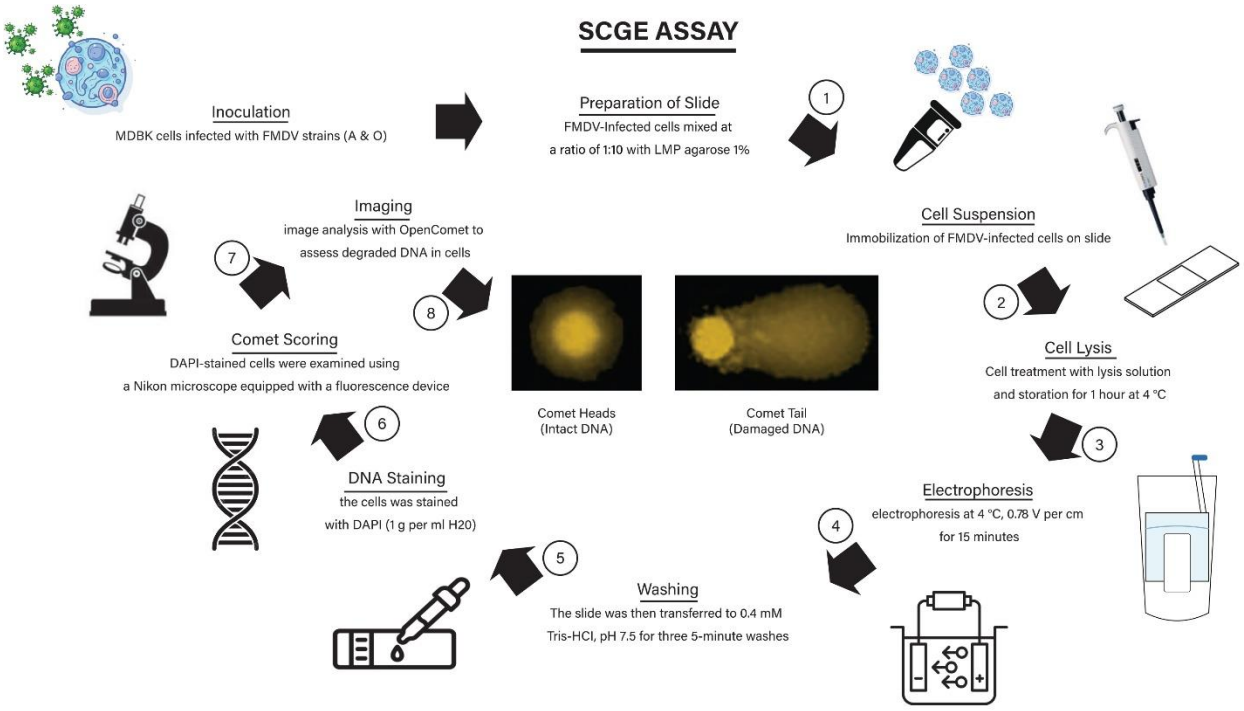

Fig. 2. Schematic workflow of the SCGE assay used to evaluate DNA damage in IBRS-2 cells following FMDV infection.

2-6- Statistical Analysis

Statistical calculations were done with SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). Data are provided as mean ± SD. One-way analysis of variance (ANOVA) was used to examine variations among experimental groups, then Tukey's post hoc test when appropriate. Statistical significance was indicated by a p-value of ≤ 0.05. The combined proportion of early and late apoptotic cells was used for apoptosis analysis.

3- Result

3-1- Flow-Cytometric Evaluation of Apoptosis

In uninfected IBRS-2 cells, the majority of the population remained viable, with 80.3% of cells residing in the viable quadrant (Q4). Basal levels of cell death were minimal, as evidenced by small proportions of early apoptotic (12.7%, Q3), late apoptotic (5.09%, Q2), and necrotic cells (1.98%, Q1), indicating preserved membrane integrity under control conditions.

In contrast, infection with foot-and-mouth disease virus (FMDV) serotype O induced a pronounced shift towards apoptosis. The viable cell population declined markedly to 21.6% (Q4). This was accompanied by a substantial increase in early apoptotic cells (60.2%, Q3) and a rise in late apoptotic cells (14.5%, Q2). The proportion of necrotic cells remained low at 3.68% (Q1). Overall, the combined apoptotic fraction (early + late) reached 74.7%, reflecting a strong cytopathic and pro-apoptotic effect induced by this serotype.

Cells infected with FMDV serotype A exhibited an intermediate apoptotic profile. The viable cell population decreased to 39.3% (Q4), while early apoptosis increased to 51.5% (Q3). Late apoptotic cells constituted 6.16% (Q2), and necrotic cells remained at a low level (3.03%, Q1) comparable to that observed in the serotype O infection. The total apoptotic fraction reached 57.7%, demonstrating that while serotype A also induces significant apoptosis in IBRS-2 cells, its effect is less pronounced than that of serotype O.

Collectively, flow cytometric analysis revealed a clear serotype-dependent apoptotic response. FMDV serotype O exerted the most potent pro-apoptotic effect, followed by serotype A, whereas minimal apoptosis was observed in control cells. Importantly, necrosis remained a minor contributor to cell death across all experimental groups, consistently accounting for less than 4% of the total cell population, which confirms that the observed reduction in viability is primarily

attributable to apoptosis (Fig. 3).

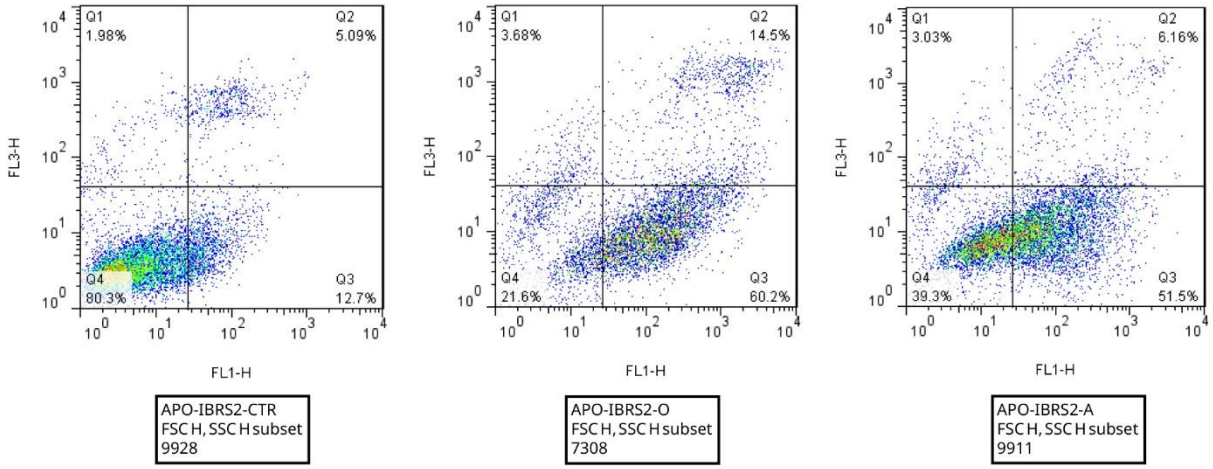

Fig. 3. Flow-cytometric assessment of apoptosis in IBRS-2 cells infected with foot-and-mouth disease virus (FMDV) serotypes A and O.

3-2- Intracellular ROS Production

Infection with FMDV significantly elevated intracellular ROS generation compared to uninfected control cells. Control IBRS-2 cells had the lowest DCF fluorescence intensity for all wavelengths measured, ranging from 500 to 550 nm, indicating a low basal level of ROS production.

The induction of ROS production was significantly higher by both FMDV viral serotypes, but its magnitude varied between different serotypes. The highest production of ROS was observed in the cells infected with the O serotype of FMDV. The peak levels of ROS in this group were substantially higher compared to both the control and serotype A groups, which indicated robust induction of oxidative stress.

By comparison, FMDV serotype A infection resulted in a significant elevation in ROS output compared to the controls, but the average fluorescence intensity was lower than that recorded in

the serotype O group. Thus, these results confirmed that FMDV serotypes induced intracellular oxidative stress in a serotype-dependent manner in IBRS-2 cells (Fig. 4).

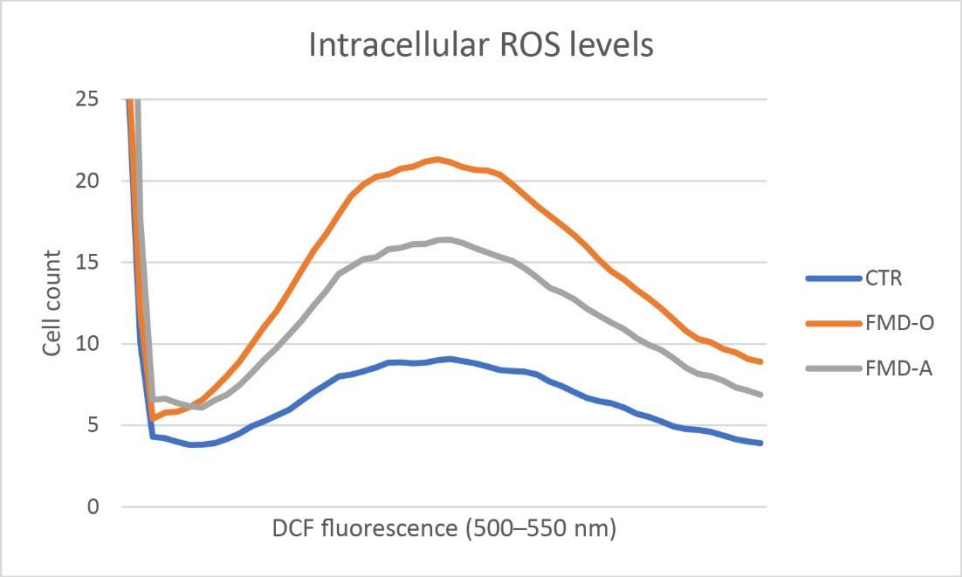

Fig. 4. Intracellular ROS levels in IBRS-2 cells infected with FMDV serotypes A and O.

3-3- DNA Damage Quantification and Damage Index

As indicated in Table 1, an infection with both serotypes of FMDV significantly increased DNA strand breaks compared to uninfected control cells. The control cells had minimal DNA migration as indicated by TM and OTM values of 16.00 ± 2.13 and 9.00 ± 1.01, respectively, which is consistent with intact genomic integrity. In contrast, a very sharp increase in the DNA damage of IBRS-2 cells infected with FMDV serotype A was observed. TM values increased to 55.00 ± 6.19 and OTM values to 27.00 ± 2.46. The infection with FMDV serotype O produced the highest degree of DNA fragmentation, which is reflected by TM and OTM values of 66.00 ± 6.84 and 34.00 ± 4.03, respectively. Statistical analysis showed that both infected groups were significantly different from the control group ($p \leq 0.05$). There is no statistically significant difference between the two serotypes as determined by superscript lettering, yet there is a definite trend of greater

genotoxicity in the serotype O compared to serotype A. These results indicate that FMDV infection compromises the integrity of DNA in IBRS-2 cells in a serotype-dependent manner (Fig. 5).

The DNA damage was also analyzed quantitatively using the DI based on the visual analysis of the comets (Tab. 2). In the control cells, the majority of nucleoids belonged to low damage classes (Classes 0-2), and the DI value of 72 represented low basal DNA damage. In FMDV-infected cells, there was a significant increase in the damage classes. In cells infected with FMDV serotype A, the number of comets belonging to Classes 2 and 3 increased, giving a high DI value of 194, indicating moderate to high genomic DNA damage. In cells infected with the FMDV serotype O virus, the maximum DNA damage occurred, where the majority of comets belonged to Classes 3 and 4, giving the maximum DI value of 235. Totally, the DI analysis supported the quantitative SCGE findings that FMDV infection causes high levels of genomic instability in IBRS-2 cells, with the strongest genotoxic effect in the serotype O.

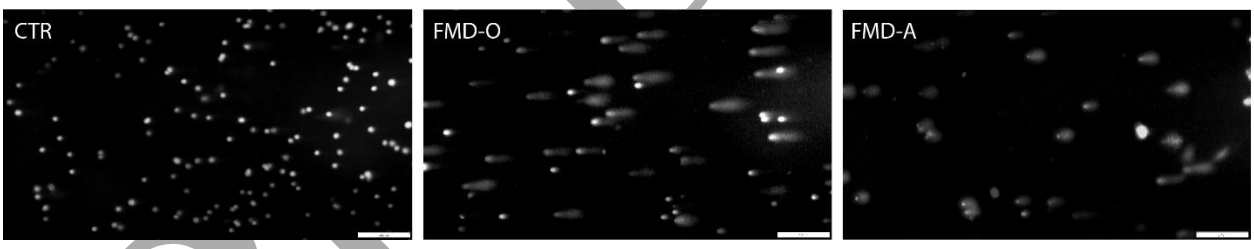

Fig. 5. SCGE images of IBRS-2 cells infected with FMDV serotypes A and O.

Table 1. Quantification of DNA damage in FMDV-infected IBRS-2 cells

Group	Time (h)	TM (Mean ± SD)	OTM (Mean ± SD)
Control	24	16.00 ± 2.13[a]	9.00 ± 1.01[a]
FMDV-A	24	55.00 ± 6.19[b]	27.00 ± 2.46[b]
FMDV-O	24	66.00 ± 6.84[b]	34.00 ± 4.03[b]

Tail Moment (TM) and Olive Tail Moment (OTM) values in IBRS-2 cells following 24 h exposure to foot-and-mouth disease virus (FMDV) serotypes A and O. Data are presented as mean ± SD calculated from SCGE assay measurements. Statistical differences among groups were determined by one-way ANOVA followed by Tukey's post hoc test. Different superscript letters indicate significant differences at $p \leq 0.05$.

Table 2. Semi-quantitative evaluation in FMDV-infected IBRS-2 cells

Group	Class 0	Class 1	Class 2	Class 3	Class 4	DI (0–400)
Control	48	16	22	4	0	72
FMDV-O	0	10	33	29	18	235
FMDV-A	0	20	42	22	6	194

Visual scoring distribution of 90 comets per group (n = 3) in five DNA damage classes (0–4) in IBRS-2 cells following infection with foot-and-mouth disease virus (FMDV) serotypes A and O. Damage Index (DI) was calculated as the sum of number of nuclei in each class × class value. DI ranges from 0 (no DNA damage) to 400 (maximum damage). Both FMDV serotypes induced markedly higher DI values compared with the control group, with serotype O producing the most pronounced genomic damage.

4- Discussion

FMDV is known for its ability to disrupt host immune responses. This disruption helps the virus replicate, spread, and persist. Previous studies have shown that FMDV weakens both innate and adaptive immune systems. It occurs through cytokine imbalance, slowing down lymphocyte growth, and impairing the functions of macrophages and dendritic cells. These effects together weaken antiviral defenses [10]. This study builds on the hypothesis that FMDV infection damages

host genomic integrity. Increased intracellular ROS generation exhibited especially by serotype O–infected cells is well corroborated by more pronounced apoptosis and DNA damage documented in parallel assays, indicating that at least part of this increased genomic instability takes a path linked with virus mediated cytopathogenicity via oxidative stress. This damage represents another area of virus-related cellular damage that hasn't been reported much. The flow cytometry technique showed that serotype O was responsible for up to three times higher levels of both early and late apoptosis than serotype A. This corroborated previous observations that FMDV was able to induce programmed cell death, via direct action of viral proteins and through indirect pathways that include oxidative stress and immune modulation [11]. Although apoptosis is thought to function as a host defense mechanism to limit the spread of the virus, unchecked or improperly regulated apoptotic responses can worsen the damage, and this might lead to increased severity of the disease.

Oxidative stress is becoming more and more recognized as a main factor mediating the virus-caused damage to the cells [12]. The current study showed that ROS levels intracellularly were very high in FMDV-infected IBRS-2 cells especially in those infected with serotype O. Similar ROS-related processes have been reported for many RNA viruses such as poliovirus [13], influenza virus [14], enteroviruses [15], and SARS-CoV-2 [16]. Excessive ROS production leaded to DNA damage, disruption of cellular signaling, and the activation of apoptosis [17]. This study proved a very strong correlation among the increased ROS production, which suggested very clearly that oxidative stress is the main factor in FMDV-induced cytopathogenicity.

The evaluation of genotoxicity through the alkaline SCGE assay revealed a considerable rise in DNA strand breaks and genomic instability due to FMDV. The quantitative comet parameters (TM

and OTM) and the semi-quantitative DI demonstrated the infected cells as significantly more damaged compared to normal cells. Among the serotypes, the serotype O showed the highest genotoxicity, as evidenced by the greatest values of TM, OTM, and DI. Thus, these indicates a serotype-dependent genotoxicity pattern. Such findings are consistent with earlier studies reporting similar outcomes of viral DNA damage in infections with poliovirus [18], influenza virus [19], enterovirus A71 [20], and parvoviruses [21].

As far as we know, this study gives the first direct application of the SCGE assay to measure DNA integrity in cells with FMDV. In Previous report the genotoxicity of BVDV performed by the classical cytogenetic methods such as sister chromatid exchange, chromosomal aberration analysis, fragile site detection, and micronucleus assays [22]. While these methods provide useful information, they are usually quite labor-intensive and not sensitive. On the contrary, the SCGE assay is a quick, sensitive, and quantitative method of evaluating a wide range of DNA damage at the single-cell level. The significant differences seen between the infected and control groups in this study confirm the appropriateness of this assay for assessment of FMDV-related genomic instability.

The mechanisms responsible for the FMDV-induced DNA damage are varied. One of these is the excessive ROS production which can directly result in oxidative DNA lesions, strand breaks, and the formation of alkali-labile sites [23]. Furthermore, the viral modulation of the host DNA damage response and repair pathways might make the resolution of the DNA lesions inefficient, resulting in the accumulation of the lesions over time [24]. Although limited DNA damage might allow the virus to replicate faster by the alteration of the cellular checkpoints, extensive genomic injury could affect the cellular functions that are crucial and eventually lead

to apoptosis [25]. The interaction between these contradictory processes might determine the degree of viral replication and the extent of virus-induced cellular damage.

5- Conclusion and Future Perspectives

According to the present findings, FMDV is shown to induce oxidative stress, apoptosis, and DNA damage in IBRS-2 cells specially with serotype O. The findings offer new perspectives on the cellular pathogenesis of FMD. Research in the future can determine the individual role of viral proteins, host DNA repairs and their impact on viral replication pathways. Gaining more insight on these mechanisms can lead to novel antiviral strategies targeting virus-induced cellular damage that may improve disease control.

Author Contributions

All aspects of the research were conducted by the author. The author has read and approved the final version of the manuscript.

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Competing Interests

The author declares that there are no competing interests.

Authors' Contributions

A.R.T. Conceptualization, Methodology, Software, Visualization, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **P.G.** Funding acquisition, Methodology, Investigation, Writing - original draft. **F.G.** Resources, Funding acquisition, Methodology, Investigation, Writing - original draft. **R.K.** Methodology, Conceptualization. **M.L.** Methodology, Conceptualization.

Ethics Approval

This study did not involve human participants or live animals. All procedures were performed using established in vitro cell culture techniques. Therefore, ethics approval was not required.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Declaration

OpenAI's ChatGPT (GPT-5.2) was used to refine grammar, improve sentence clarity, and ensure consistency in scientific writing. No AI tools were used for data generation, statistical analysis, or image production.

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