EFFICACY OF GRAPHENE OXIDE AS AN ANTIVIRAL ALTERNATIVE AGAINST EQUINE HERPESVIRUS-1 (EHV-1)

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CERTIFICATION

This is to certify that we have read this research paper entitled 'Efficacy of Graphene Oxide Compound as an Antiviral Alternative against Equine Herpesvirus-1 (EHV-1)' by Muhammad Shahin bin Kamarul-Anuar and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course DVT 55204 - Research Project.

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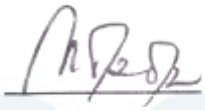

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DEDICATIONS

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ABBREVIATION

GO	**-**	**Graphene Oxide**
EHV-1	**-**	**Equine Herpesvirus-1**
µg/mL	**-**	**Microgram per milliliter**
nm	**-**	**Nanometer**
rpm	**-**	**Revolution per minute**
NT	**-**	**Non-Treated**
µM	**-**	**Micromolar**
URT	**-**	**Upper Respiratory Tract**

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 55204 - Research Project.

Equine herpesvirus-1 (EHV-1) is one of the most important and prevalent viral pathogens of horses and a major threat to the equine industry throughout most of the world. This infectious virus, especially strains of EHV-1 and 4 pose the most serious health risk for domestic horses. EHV-1 primarily causes respiratory disease but viral spread to distant organs enables the development of more severe sequelae; abortion and neurologic disease. The treatment has been partially successful due to the nature of virus that replicates within the host cells, restricting drug permeation. Therefore, it is important to find alternatives to the existing treatment. Graphene oxide (GO) is a carbon based nanoparticle that has been investigated for application in biomedical application. For this research, we aimed to demonstrate the virucidal and prophylactic activity of GO and toxicity impact of the compound towards mammalian cell growth. Procedures performed include Resazurin assay for cytotoxic assay, GO treatment and polymerase chain reaction (PCR). Based on the results, GO demonstrated virucidal activity at 400 ug/mL but not prophylactic capability. Also, at 400 ug/mL GO also demonstrated less toxicity effect towards the mammalian cells, evidenced by cell growth at the concentration tested. Due to the high concentration required for virucidal activity, ultimately it is not effective to be used as a treatment against EHV-1. Therefore, GO is not recommended to be used against EHV-1 infections.

Keywords: *Graphene Oxide, Vero cells, virucidal activity, antiviral, Equine herpesvirus-1, toxicity cell growth*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada kursus DVT 55204 - Projek Penyelidikan.

Herpesvirus kuda-1 (EHV-1) adalah salah satu patogen virus kuda yang paling penting dan lazim dan merupakan ancaman besar bagi industri kuda di seluruh dunia. Virus berjangkit ini, terutamanya strain EHV-1 dan 4 menimbulkan risiko kesihatan yang paling serius bagi kuda domestik. EHV-1 terutamanya menyebabkan penyakit pernafasan tetapi penyebaran virus ke organ yang jauh memungkinkan perkembangan sekuel yang lebih teruk; pengguguran dan penyakit neurologi. Rawatan tersebut telah berjaya sebahagiannya disebabkan oleh sifat virus yang mereplikasi di dalam sel inang, yang menyekat penyerapan ubat. Oleh itu, penting untuk mencari alternatif untuk rawatan yang ada. Graphene oxide (GO) adalah nanopartikel berasaskan karbon yang telah disiasat untuk digunakan dalam aplikasi bioperubatan. Untuk penyelidikan ini, kami bertujuan untuk menunjukkan aktiviti virucidal dan profilaksis GO dan kesan ketoksikan sebatian terhadap pertumbuhan sel mamalia. Prosedur yang dilakukan termasuk ujian Resazurin untuk ujian sitotoksik, rawatan GO dan tindak balas rantai polimerase (PCR) Berdasarkan hasilnya, GO menunjukkan aktiviti virucidal pada 400 ug / mL tetapi bukan kemampuan profilaksis. Juga, pada 400 ug / mL GO juga menunjukkan kesan ketoksikan yang lebih sedikit terhadap sel mamalia, yang dibuktikan oleh pertumbuhan sel pada kepekatan yang diuji. Oleh kerana kepekatan tinggi yang diperlukan untuk aktiviti virucidal, akhirnya tidak berkesan digunakan sebagai rawatan terhadap EHV-1. Oleh itu, GO tidak digalakkan digunakan untuk menentang infeksi EHV-1.

Kata Kunci: *Graphene Oxide, Sel Vero, aktiviti antiviral, Equine Herpesvirus-1, ketoksikan pertumbuhan*

1.0 INTRODUCTION

Horses in particular are frequently infected with the equine herpesvirus-1 (EHV-1) subtype of the disease worldwide (Khusro et al., 2020). Clinical signs of the virus include respiratory distress, abortion in pregnant mares, neurological problems, and neonatal foal fatalities. It also causes humoral and cellular immunological responses (Khusro et al., 2020). This virus' illness is frequently linked to rare epidemics among horse populations. Due to its recurrent outbreaks and significant financial harm to the global equine industry, EHV-1 has gained attention during the past ten years. (Laval et al., 2012).

EHV-1 is an alphaherpesvirus belonging to the Alphaviridae family. Currently, the Herpesviridae family has 120 viruses, of which 9 are known to infect humans, while the remaining 120 are known to infect animals, such as the EHV-1 virus (Oladunni et al., 2019). They belong to the same family as enveloped DNA viruses, it has an approximately 150kbp linear, double-stranded DNA genome with 80 open reading frames (ORFs), four of which are duplicated (Telford et al., 1992). The most prevalent subtypes are EHV-1 and EHV-4 (Khusro et al., 2020). Similar to the other herpesvirus family strains, EHV-1 tends to be immune-protected from elimination and can reactivate under stressful circumstances. It also has quite the longevity with chronic infection in its latent form following primary infection (Oladunni et al., 2019).

Within the current medicine world, especially in veterinary medicine, there are no direct treatment or antiviral drugs targeting entry for infections of EHV-1 (Laval et al., 2021). Supportive care is usually provided such as antivirals, non-steroidal and antibiotic treatment for secondary infections (prophylaxis) can be given instead to help the horse as much as possible (Laval et al., 2021). Since the virus often stays latent within the cells, the compound must be able to kill the virus and transverse the mammalian cells. One of the alternative is to opt for nanoparticle-based compounds such as graphene based compounds that is known to be able to penetrate mammalian cells (Zhang et al., 2016).

An oxidized version of graphene called graphene oxide contains carbon, oxygen, and hydrogen in varying amounts (Lerf et al., 1998). In carbon-based constructions, graphene is a corrugated material that is frequently employed. Its variant, graphene oxide (GO), has just lately

been tested and used in biomedicine and biotechnology for its usage as pathogen inhibitors and biosensors (Ziem et al., 2013). GO's current medical applications include cancer treatment, medication delivery, cellular imaging, and antibiotic substitutes (Rhazouani et al., 2021).

The inability to adjust to the size of virus particles is one of the major problems with developing an effective virus inhibitor system. As a result, GO are made in the form of different-sized nanoarchitectures that can be coupled with polyglycerol to make a programmable polyvalent nanocompound (Ziem et al., 2013). Ultimately. Viral infection will be prevented by the nanomaterials by preventing cellular absorption (Ziem et al., 2013). However, because GO's cytotoxic effects are size-dependent and its biological properties may be modified by serum (Song et al., 2020), the use of nanoGO in therapy has been restricted thus far. Thus, based on the great potential of antiviral activity of the compound mentioned above, this study aims to investigate the application of carbon-based nanoparticles against Equine herpesvirus type-1 in Vero cells in vitro.

1.1 Research Problem

EHV-1 infections outbreak commonly occur within the equine industry and cause serious damage to the economy. Currently there are no specific antiviral drugs that could target EHV-1 within hosts. GO is a compound that is known to enter mammalian cells and therefore may have a chance to interact with the intracellular virus. Thus, this study aims to investigate the efficacy of graphene oxide against Equine herpesvirus type-1 in Vero cells in vitro.

1.2 Research Questions

I. Can Graphene Oxide (GO) prevent EHV-1 infections in Vero cells in vitro?
II. Can Graphene Oxide (GO) kill EHV-1 are latent within Vero cells in vitro?
III. What concentration of Graphene Oxide (GO) can cause toxicity to the Vero Cells in Vitro?

1.3 Research Hypothesis

H_0: Graphene Oxide (GO) compound is effective against EHV-1 infections in Vero cells in vitro.

H_1: Graphene Oxide (GO) compound is not effective against EHV-1 infections in Vero cells in vitro.

1.4 Research Objectives

I. To determine the effective concentration of graphene oxide for prevention of EHV-1 infections in Vero cells in vitro.
II. To determine the virucidal activity graphene oxide against EHV-1 infections in Vero cells in vitro.
III. To determine the concentration of graphene oxide that causes toxicity in Vero cells in vitro.

2.0 LITERATURE REVIEW

2.1 Equine Herpesvirus-1 (EHV-1)

One of the most significant and pervasive viral diseases of horses, equine herpesvirus-1 (EHV-1), poses a serious danger to the global equine industry (Oladunni et al., 2019). The majority of horses around the world have EHV, which are DNA viruses. Virtually all horses have the viruses, and most of the time there are no major adverse effects (Oladunni et al., 2019). This virus family is identified by numerals like EHV 1, 2, 3, 4, and 5. There are additional members of this family of viruses, however domestic horses are primarily at danger from EHV 1 and 4. (Oladunni et al., 2019). EHV-1 mainly causes respiratory illnesses, but when it spreads to other organs, it might result in more serious side effects including abortion and neurological disorders (Oladunni et al., 2019). The virus has the ability to go into latency, a state in which viral genes are barely expressed, and then reactivate at any time to cause lytic infection. EHV-1 infection does not currently have any specific therapies, however immunisations have been established, and owners are encouraged to maintain good health and hygiene (Oladunni et al., 2019).

2.2 Biological properties of EHV-1

EHV-1 is a member of the Herpesviridae subfamily Alphaherpesvirinae. It has an approximately 150kbp linear, double-stranded DNA genome with 80 open reading frames (ORFs), four of which are duplicated (Telford et al., 1992). Although the mechanism of EHM development is unknown, EHV-1 strains with a single nucleotide point (SNP) mutation in the ORF30, which encodes a catalytic subunit of viral DNA polymerase, have a significantly higher potential for neuropathogenicity (Nugent et al., 2006). The A to G mutation in nucleotide (nt) position 2254 of the virus genome results in the substitution of asparagine (N) by aspartic acid (D) at amino acid position 752 of the viral DNA polymerase's catalytic subunit.When compared to animals infected with EHV-1 without this specific mutation, this single amino acid mutation in the viral polymerase causes higher tropism to lymphocytes and longer viremia in experimentally infected horses (Goodman et al., 2007).

2.3 Pathogenesis of EHV-1

2.3.1 EHV-1 Primary Replication in the Upper Respiratory Tract (URT)

The EHV-1 enters the host via the respiratory route, where it first replicates in a restricted plaque-wise manner within the epithelial cells lining the upper respiratory tract (URT). This includes the nasal septum, nasopharynx, and trachea (van Maanen, 2002; Gryspeerdt et al., 2010). Primary EHV-1 infection of several tissues within the URT would result in epithelial destruction and erosion (Gryspeerdt et al., 2010). To summarize EHV-1's mechanism within the URT, the virus spreads through the respiratory epithelium and viral shedding (Gryspeerdt et al., 2010). EHV-1 then passes through the basement membrane and into the lamina propria via infected leukocytes (Gryspeerdt et al., 2010). Finally, the EHV-1 virus enters the blood circulation and draining lymph nodes, where it enters peripheral nerve endings and spreads retrogradely to the trigeminal ganglia (Gryspeerdt et al., 2010). Finally, the destruction of respiratory epithelial cells and local inflammation in the horse results in mild clinical symptoms such as serous nasal discharge and fever (Allen and Bryans., 1986).

2.3.2 EHV-1 Replication in Draining Lymph Nodes and Cell-Associated Viremia

As previously stated, EHV-1 infects the URT and then crosses the basement membrane via infected leukocytes (Vandekerckhive et al., 2010; figure 1.1c). EHV-1-infected leukocytes penetrate connective tissues and reach the bloodstream and draining lymph nodes after crossing the basement membrane (Vandekerckhive et al., 2010; figure 1.1c). EHV-1 antigens and infectious virus can be found in the submandibular, retropharyngeal, and bronchial lymph nodes within 24-48 hours of immunisation. The infection is exacerbated in the draining lymph nodes, which contain the discharge of infected leukocytes into the bloodstream via the efferent lymph (Kydd et al., 1994). This causes cell-associated viremia in peripheral blood mononuclear cells to spread throughout the host (van der Muelen et al., 2000; Figure1.2). EHV-1 requires cell-associated viremia to infect other vital organs such as the central nervous system (CNS) and/or the pregnant uterus (Patel et al., 1982; Allen and Bryans, 1986).

2.3.3 Latent EHV-1 Infection in the Peripheral Nervous System (PNS) and in Respiratory Associated Lymphoid Tissues.

Following primary infection and replication within the horse respiratory epithelium, EHV-1 is able to enter the PNS nerve endings, including the trigeminal ganglia (TG), sympathetic and parasympathetic neurons that innervate the epithelium (Van Cleemput et al., 2017). The virus has also been shown to establish latency in lymphoid tissues associated with the respiratory system, such as mandibular, retropharyngeal, and bronchial lymph nodes (Welch et al., Kydd et al., 1994; Slater et al., 1994). Finally, circulating T lymphocytes are known to be a primary site for EHV-1.

2.3.4 Secondary EHV-1 Replication in the Pregnant Uterus, CNS and/or Eye

Infected leukocytes can adhere to and directly transfer EHV-1 to endothelial cells lining the blood vessels of targeted organs such as the pregnant uterus or the CNS within the bloodstream (Goehring et al., 2011). Cell-to-cell contacts between infected peripheral blood mononuclear cells and endothelial cells in the late-gravid uterus or CNS mediate infection of endothelial cells (Goehring et al., 2011). Secondary replication within the endothelial cells of the pregnant uterus causes vasculitis and multifocal thrombosis, especially in small arterial branches within the glandular layer of the endometrium at the base of the microcotyledons, resulting in avascular necrosis and endometrial oedema (Edington et al., 1991; Smith et al., 1992, 1993). A single widespread endothelial cell infection causes the foetal membrane to detach, resulting in the abortion of a virus-negative foetus. Because of less extensive uterine vascular pathology, EHV-1 can infiltrate the fetus through the uteroplacental barrier, resulting in the abortion of a virus-infected foetus. Infected EC are found in blood vessels of the allantochorion and umbilical cord of EHV-1-positive foetuses (Smith et al., 1997).

2.3.5 Current Treatment and Management of EHV-1 infections in horses

There is currently no specific treatment for EHV-1 infections in horses, but supportive care is provided. However, effective management and sanitation procedures, as well as symptomatic treatment for affected horses, may aid in preventing the viral illness from spreading (Wilson et al., 1991). Recumbent horses should be treated with supportive care, nutritional assistance, rehydration, routine bladder and rectal evacuation to prevent colic, and CNS inflammation

reduction (Lunn et al., 2009). Corticosteroids and immunomodulatory drugs can also be used to treat early EHM symptoms. However, no studies have been conducted to demonstrate the efficacy of any treatment class; therefore, precautions must be taken to prevent latently infected horses from reactivating their virus shedding (Lunn et al., 2009). Corticosteroids, which are thought to be protective against the cellular response to CNS infection, may prevent the development of common early symptoms of EHM such as haemorrhage, edoema, vasculitis, and thrombosis. Their application should be restricted to severe cases of EHM (Lunn et al., 2009).

2.4 Nanoparticles and graphene-based compound

Nanoparticles are defined as materials with at least one external dimension measuring 1-100nm. Nanoparticles can occur naturally, as byproducts of combustion reactions, or be purposefully produced through engineering to perform a specific function. Nanoparticles have been widely used in medicine and pharmaceuticals due to their unique mechanical, optical, and electrical properties, as well as their antiviral properties (Damodharan., 2021). Graphene is a carbon compound nanostructure that exists as graphite building blocks and is defined as an atomically two-dimensional layer of hexagonally bonded carbon atoms (Huang et al., 2012). These carbon structures have remarkable properties such as a large specific surface area, high mechanical strength, and high electron conductivity (Huang et al., 2012). Graphene-based materials are those that contain a few layers of graphene or graphene oxide (Alemi et al., 2020). Graphene and graphene oxide's unique properties have made these nanostructured materials suitable for a wide range of biological and medical applications, including anti-pathogenic applications, biosensors, bioimaging, tissue engineering, and drug delivery (Liu et al., 2017). Graphene oxide (GO), in contrast to graphene, is the oxidised form of graphene with hydroxyls, epoxides, diols, ketones, or carboxyls functional groups on its surface. In comparison to graphene, the presence of oxygen on the edges and basal planes of GO increases its hydrophilicity, water dispersibility, and attachability (Aliyev et al., 2019).

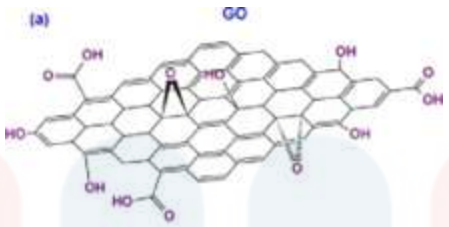


Figure 1: Chemical structure of Graphene Oxide (GO)

2.5 Antiviral Properties of Graphene Oxide (GO)

Graphene compounds, such as graphene oxide, have demonstrated excellent antiviral inhibitory capabilities against enveloped and non-enveloped viruses, including RNA and DNA viruses (Seifi and Kamali., 2021). Its antiviral properties could be attributed to the physiochemical properties of these materials' surfaces (Seifi and Kamali., 2021). Graphene and its derivative compounds, such as GO, have the ability to inactivate various virus strains via various mechanisms, including photothermal activity, and to inhibit infections on a cellular level by binding nanomaterials to virus surface proteins or host cell receptors (Seifi and Kamali., 2021). One of the most important properties of graphene compounds like GO is their ability to be functionalized (Seifi and Kamali., 2021). They can also be used as a substrate to load other antiviral agents uniformly (Seifi and Kamali., 2021). Given this, the surface area, charge density, and concentration of graphene materials, as well as the type and size of loaded particles, and the type and degree of functional groups, are all factors influencing their antiviral performance (Seifi and Kamali., 2021). It should be noted that virus characteristics such as enveloped or non-enveloped viruses, as well as the time of use of nanomaterials in cases of virus pre-treatment, virus co-treatment, cell pre-treatment, and cell post-treatment, play important roles in determining the antiviral activity of graphene-based materials (Seifi and Kamali., 2021). It is important to note that carbon materials have multifunctional properties that can be used for organic pollutant decomposition (Lin et al., 2021), heavy metal adsorption, and infectious pathogen killing (Khurshid., 2012).

2.6 Mechanism of action of Graphene Oxide against Virus

Graphene oxide has been shown in numerous studies and research to be effective against viruses and bacteria. It was discovered that before viruses can bind to and enter host cells, they must first interact with host cell surface receptors (Spear and Longnecker., 2003). Heparin sulphate proteoglycans and chondroitin sulphate proteoglycans are cell surface receptors found within viral envelope glycoproteins (Banfield et al., 1995). Based on the interaction of negatively charged functional groups that mimic cell surface heparan sulphate, functionalized graphene materials can prevent cellular viral infections (Ziem et al., 2016). To summarise, GO and viral pathogens will physically/chemically react with each other to inhibit viral protein binding to host cell surface receptors (Chen et al., 2016).

3.0 MATERIALS & METHODS

3.1 Graphene Oxide (GO)

Graphene oxide (GO) was obtained from GO Advanced Solutions Sdn. Bhd. in Malaysia. For stock preparation, GO was dried, weighed, and suspended in water. GO was produced at different concentrations by dispersing GO nanoparticles in water, which acts as a dispersing medium, and then sonicating the suspension for one hour with probe sonication at 50% strength to break down the GO to a monolayer sheet.

3.2 Cells and Virus stocks

The virology laboratory at Fakulti Perubatan Veterinar provided the Vero cell lines (FPV). These cells were grown in Dulbecco's Minimum Essential Medium (DMEM) containing Earle's salts, L-glutamine, non-essential amino acids, Sodium bicarbonate, Sodium pyruvate (Sigma Chem. Co., St. Louis, USA), 10% heat-inactivated foetal calf serum (Gibco BRL Co., Germany) and antibiotics, penicillin (100 IU/mL), streptomycin (100 µL/mL). EHV-1 viral stock has previously been isolated and detected using Polymerase Chain Reaction (PCR). After that, the viral samples are inoculated into the vero cell line until the cytopathic effect on the cells is clearly visible. The virus then exhibits cytopathic effect and is harvested for PCR. The virus with the thickest PCR band is used for anti-infectivity and prophylaxis against EHV-1 testing.

3.3 TCID50 Assay

To estimate the amount of infectious virus particles, the Median Tissue Culture Infectious Dose (TCID50) assay is widely used. To perform the test, the virus sample is serially diluted and added to cells in a 96-well plate configuration. The cell type is specifically chosen to exhibit a cytopathic effect (CPE), or morphological changes caused by viral infection or cell death. After an incubation period, each well is classified as infected or not infected after the cells are tested for CPE or cell death. It is also possible to use colorimetric or fluorometric readouts to improve the assay's sensitivity. The viral sample's TCID50 is calculated as the dilution at which 50% of the wells exhibit a CPE. Virus titer is expressed as TCID50/ml.

3.4 Resazurin Assay

Vero cells (1.8 x 10^5) were grown in a 96-well plate for 24 hours at 37°C with a titrated concentration of GO. As controls, only untreated cells and mediums were used. Resazurin sodium salt was prepared in 50 mL PBS (Sigma-Aldrich, UK) as a 544 M stock solution, and 50 L of the stock solution was injected into each well. The plate was then incubated for another 24 hours. The optical density was then measured at 550 and 630 nanometers with a microplate reader (BMG Labtech 96). The number of live cells is directly proportional to the change in OD value (or percentage dye reduction).

3.5 Cell treatment

After EHV-1 inoculation, Vero cell lines were treated with GO. This is referred to as an anti-infectivity assay. GO was also used to pre-treat another set of Vero cell lines before viral inoculation in two wells of a 12-well cell culture plate. This is done to determine the efficacy of GO as a prophylaxis against EHV-1 and is classified as a prophylaxis assay. Vero cells that have not been treated serve as the negative control.

3.5.1 Cell line preparation

Vero cell with concentration of 1.8 x 10 5 cell/ml was cultured in two 12-well culture plates with growth media and was incubated for 24 hours.

3.5.2 Anti-infectivity assay

The growth medium was discarded after 24 hours of incubation and replaced with a 900 L medium containing 100L EHV-1. The cell culture was then incubated at 37°C for 2 hours. After 2 hours of incubation, the growth medium was discarded and the medium was replaced with GO. The cell associated virus has gone through three freeze-thaw cycles after 48 hours of incubation at 37°C. The cell suspension was then transferred into the tube, and DNA extraction for the PCR test was performed.

3.5.3 Prophylaxis assay

The growth media was discarded after cell line preparation, and GO was inserted and reincubated for two hours. The medium was eventually discarded and replaced with 900 L medium containing 100L virus. The cell culture was then incubated at 37°C for 2 hours. The growth medium was discarded after 1-2 hours of incubation and replaced with 2% medium. After 48 hours of incubation at 37°C, the associated virus will have gone through three cycles of freeze-thaw. The cell suspension was then transferred to a tube and used for DNA extraction for the PCR test.

3.6 DNA extraction and PCR assay

The presence of EHV-1 in the Vero cell was detected using PCR. The virus was extracted by collecting the contents of all wells. For the DNA extraction procedure, presumptive EHV-1 was pelleted by centrifugation at 6000xg for 2 minutes. Gsync TM DNA Extraction Kit Quick Protocol is used to extract DNA (General Biotech Ltd., Taiwan). A 200 L GSB buffer was added to the pellet to lyse it. A 200 L sample of the elution buffer was added to a 1.5ml centrifuge tube and inoculated in a 60°C bath at the same time. Following that, 200 L of absolute ethanol was added and immediately mixed by vigorously shaking for ten seconds. Two mI collection tubes were filled with GS columns. The solution was then transferred to the GS column. The mixture was then centrifuged for one minute at 15000xg. The collection tubes, along with the flow-through, were discarded.

The GS columns were then placed in new collection tubes. The GS column was filled with 400 L of W1 buffer. The GS columns were then centrifuged for 30 seconds at 15000xg, and the flow-through was discarded. The GS columns were then returned to the collection tubes and centrifuged at 15000xg for eight minutes. After discarding the collection tubes, the GS columns were placed in centrifuge tubes. The columns were then filled with 30 L of pre-heated elution. After three minutes, the GS columns and centrifuge tube were spun at 15000xg for 30 seconds to elute the purified DNA. The DNA samples in the collection tubes were kept at -20 °C until they were used.

3.7 Amplification of EHV-1 specific genes

A standardised FPV virology laboratory procedure was used for PCR analysis. To begin, a PCR mastermix of 25.0uL was prepared and used for PC, containing 5.5uL nuclease-free water, 12.5uL of mastermix solution, 1.0uL each of 20pmol forward and reverse primer, and 5.0uL of extracted DNA. For viral DNA extraction, the PCR product was run on 1.5% (w/v) agarose gel electrophoresis at 100 volts for 40 minutes.

Table 1: List of Primers used in the study

Primer	Target Gene	Primer Sequence	Reference
MGB F1	1247-1264	CATGTCAACGCA CTCCCA	(Lechmann et al., 2019)
MGB R1	1293-1309	GGGTCGGGCGTT TCTGT	(Lechmann et al., 2019)
MGB probe	1277-1290	CCCTACGCTGCT CC	(Lechmann et al., 2019)

Table 2: PCR amplification protocol

Steps	Settings
Initial Denaturation	92°C at 300 seconds
Final Denaturation	92° at 60 seconds
Annealing	50°C at 30 seconds
Initial Extension	72°C at 30 seconds
Final Extension	72° at 300 seconds
Hold	12°C

4.0 RESULTS

4.1 Viral titration and calculation

EHV-1 virus stock was titrated along in a 96-well plate that contains 1 x 10^5 Vero cells in each well. The sample was inoculated in Vero cells in order to detect presence of EHV-1 virus through the highest cytopathic effect (CPE) after 48 hours of incubation.

Figure 2: Normal Vero cell in cell culture plate (40x magnification)

Under 40x magnification, Figure 2 depicts the normal appearance of Vero cells. The cells grow in monolayers, and their shape is elongated and similar to that of fibroblast cells (Adam et al., 2015). CPE was observed in infected Vero cells 48 hours after incubation as the cell distribution was reduced by 70% of the cell culture plate. Cell morphology changes, and the cells appear to be multinucleated (syncytium).

The 2nd well dilution was seen with the most significant CPE. TCID50 assay calculation was done and 10^3 TCID50/ml EHV-1 titre was obtained based on Figure 3.

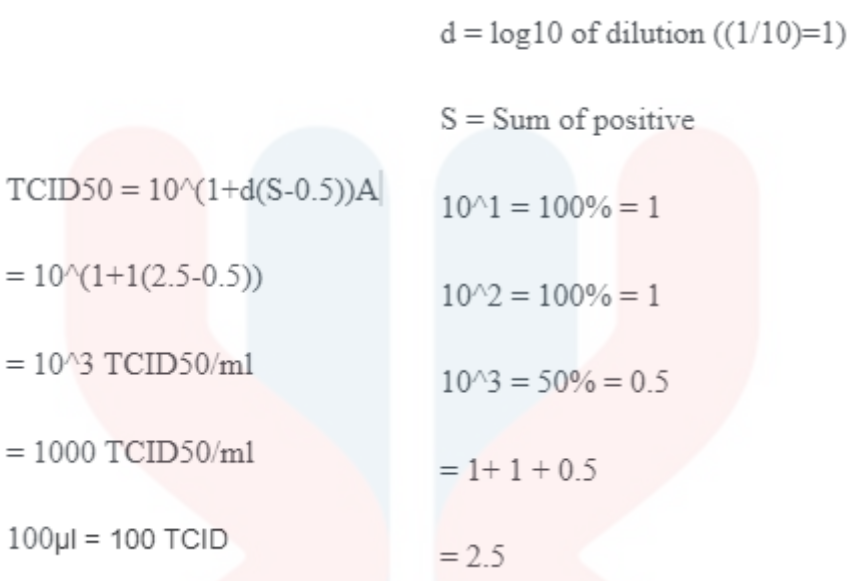


Figure 3: Calculation of TCID50 of EHV-1

4.2 Resazurin Assay

Table 3: Absorbance Value of Vero Cells treated with Graphene Oxide after 48 hours

Graphene Oxide (µg/ml)	Net Absorbance	Net Viability (%)
NT	0.478	100.0
400	0.57	119.2
200	0.454	95.0
100	0.487	101.9
50	0.485	101.5
25	0.469	98.1
12.5	0.475	99.4
6.25	0.483	101.0

Table 3 shows the absorbance value of mammalian cells after being treated with Graphene Oxide (GO) after 48 hours of incubation. The net absorbance shows the viability of the mammalian cells. Absorbance value of those that are less than the non-treated cells suggest a slower rate of cell proliferation. Vice versa, greater absorbance value suggests increase in cell proliferation. The highest marked an outstanding 119.2% increase.

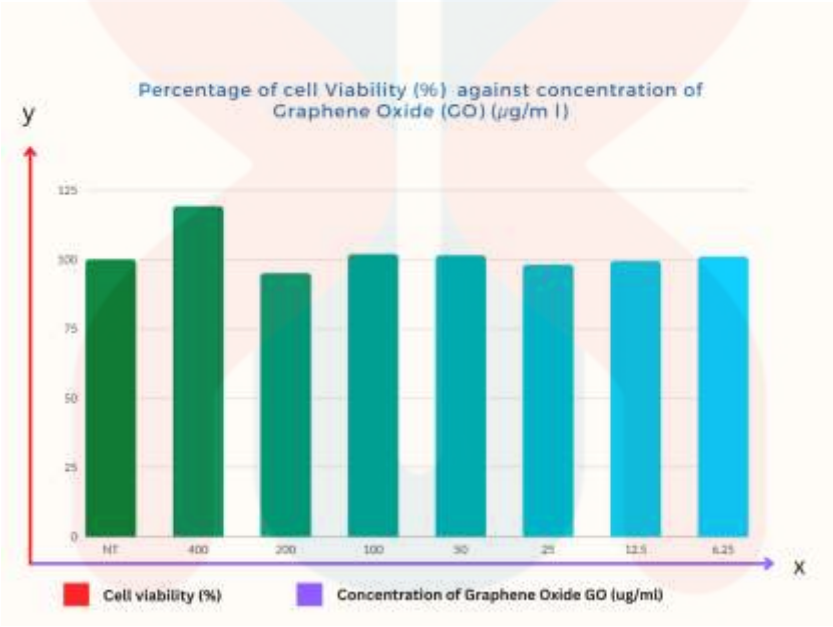


Figure 4: The percentage of cell viability against concentration of Graphene Oxide (GO)

The horizontal X-axis represents the different concentrations of GO in µg/ml, while the vertical y axis represents the cell viability in percentage. The only notable statistical difference is in concentrations 400µg/ml and 200µg/ml which shows the highest viability at 119.2% and 95% respectively. For statistical comparison, treatment using 400 ug/ml of GO shows a 19.2% increase in cell viability from 100% of non-treated control. This shows that usage of 400 ug/ml of Go promotes cell proliferation as well within Vero cells. While for treatment using 200 ug/ml of GO, there is a drop of 5% in cell viability from 100% of non-treated control. This shows that the concentration shows mild toxicity and reduces cell proliferation in Vero cells.

4.3 Treatment and prophylaxis assay

Figure 5 below shows the appearance of cells for both treatment and prophylaxis assay in which we are able to observe an increased number of confluency of vero cells along with a build of dead cells. There is also brown pigmentation which represents GO. Thus, the CPE method is not useful for this experiment. To determine the prophylactic effect of GO, PCR was used.

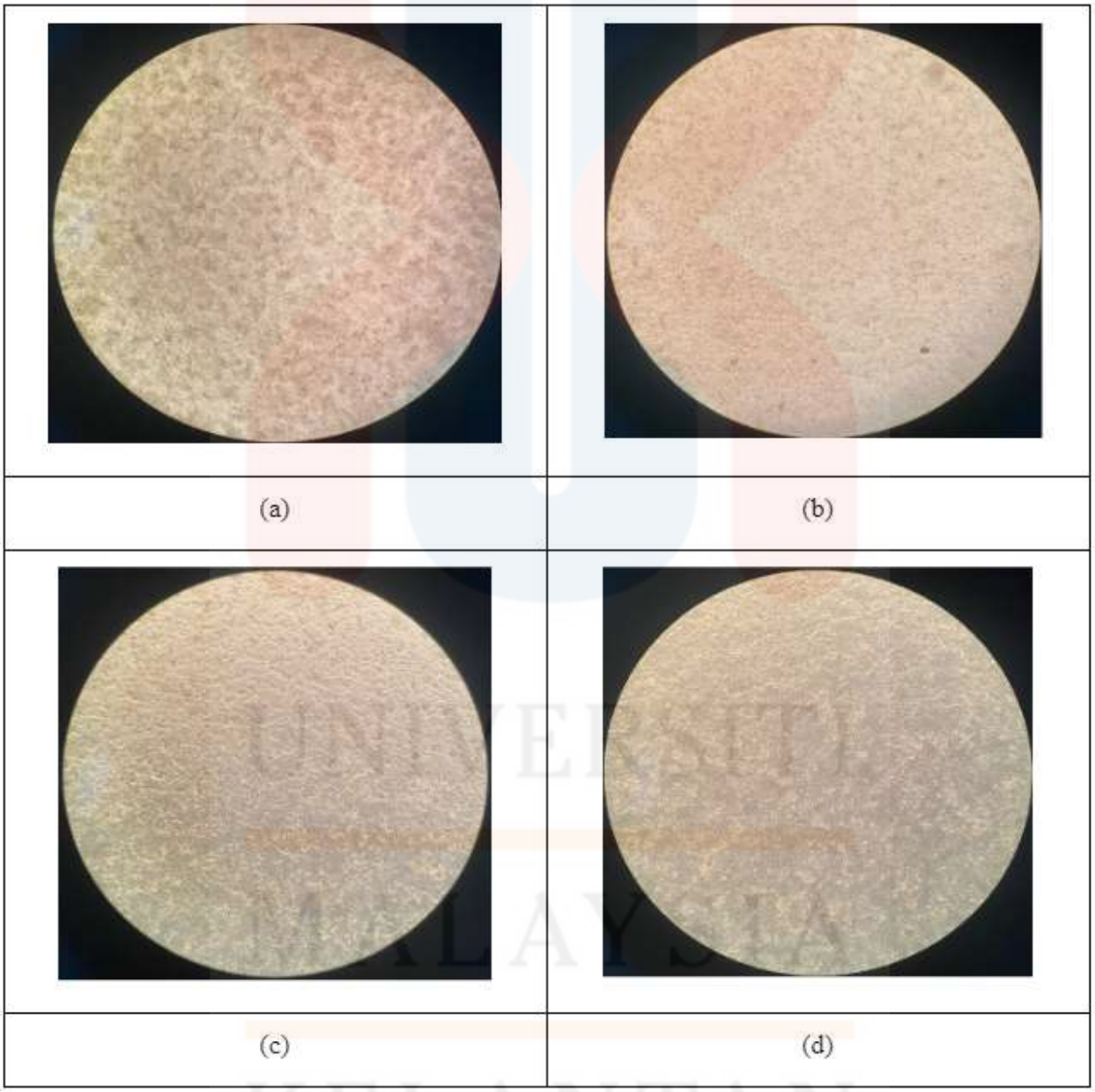


Figure 5: (a): Treatment assay 400µg/ml, (b): Prophylaxis assay 400µg/ml, (c): Treatment assay 200µg/ml, (d): Prophylaxis assay 200µg/ml

4.4 PCR detection of treatment and prophylaxis assay

From the results of PCR of gel electrophoresis, only treatment assay using 400µg/ml of GO shows slight reduction in intensity compared to the band of sample (positive control) that have been infected solely with EHV-1. This indicates that there is a reduction of virus concentration within cells infected with EHV-1 that have been treated with 400µg/ml GO. As for the other assays, there barely shows any reduction in band size compared to the positive control, thus giving insignificant results.

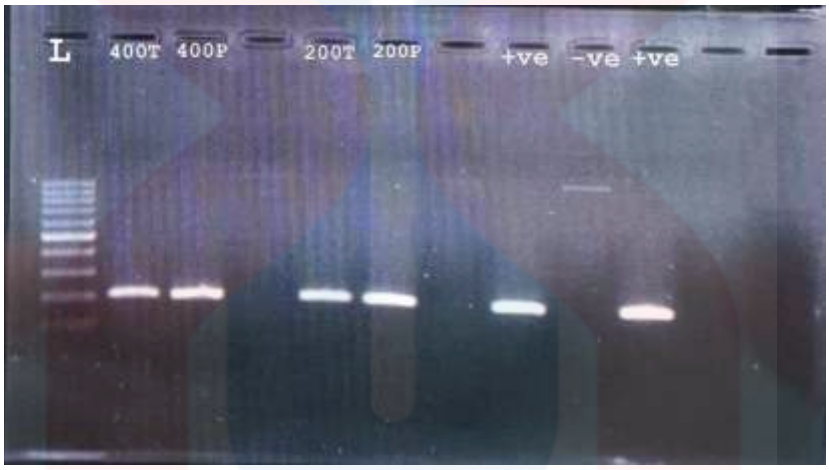


Figure 6: Results of PCR of Gel Electrophoresis

L:100bp DNA ladder, 400T: Treatment assay 400µg/ml, 400P: Prophylaxis assay 400µg/ml, 200T: Treatment assay 200µg/ml, 200P: Prophylaxis assay 200µg/ml, +ve: positive control with EHV-1, -ve: negative control

5.0 DISCUSSION

This research was done in order to investigate the antiviral properties and its toxicity effect on mammalian cells in which we used Vero cells specifically. From the cytotoxic assay done using resazurin assay, we concluded to use 400µg/ml and 200µg/ml concentration to treat mammalian cells that are infected with EHV-1. Both concentrations were used in treatment and prophylaxis assay. Based on the PCR results, there was only a slight reduction in the band thickness of treatment assay using 400µg/ml GO while other assay and concentrations showed no significant reduction in size. This probably shows that GO is poor in adhering to viruses, or in this case EHV-1 and unable to destroy the pathogens.

As the mechanism of action of Graphene Oxide is to react with the viral pathogens physically/chemically to inhibit the binding of viral proteins to the host surface proteins, this proves that functionalized graphene materials can prevent cellular EHV-1 infections based on the interaction between negatively charged functional groups which mimic cell surface heparan sulfate (Chen et al., 2016). Thus, it is safe to say that while GO possesses mild antiviral properties at 400 ug/ml of GO, it ultimately is not significantly viable to use to treat EHV-1 infections.

This is also supported by the data we obtained from the cell viability data we've obtained from ELISA reader on the 96 plate well we used for resazurin assay. Using 400µg/ml of GO concentration on Vero cells, there is a 119% increase in cell viability while using 200µg/ml of Go shows 95% decrease in cell viability. As for other concentrations used, most of the results are very near to the non-treated value. From the data, although some GO concentrations show a difference in cell viability percentage, ultimately there is no significant difference among the cells when treated with different concentrations of GO. This then supports the theory that GO is poor in promoting cell proliferation.

Since graphene oxide is hydrophilic in nature, it can prevent cell aggregation on culture media. This inhibition can cause oxidative stress, limit nutrient absorption, and cause the cell to self-destruct. On the other hand, because it can alter protein adsorption in the extracellular matrix, which enhances cell adhesion and proliferation, it can also cause cell proliferation

(Ghulam et al., 2022). We may be able to determine the precise effect of graphene oxide on human cells if concentrations of the material are employed that are higher than 400 g/mL.

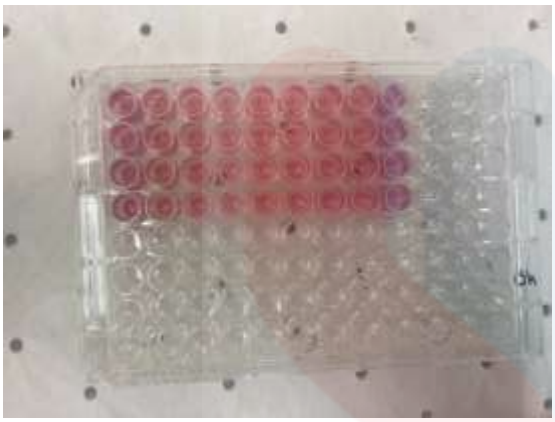
6.0 CONCLUSION

In conclusion, Graphene Oxide (GO) shows mild significance in cell proliferation along with mild capability of antiviral properties at 400 ug/ml but is ultimately not effective to be used as a treatment against EHV-1 as higher concentrations of GO is required to promote virucidal activity. Therefore, GO is not recommended to be used against EHV-1 infections.

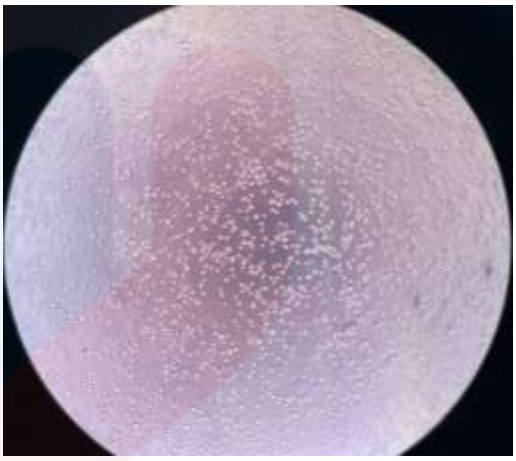
7.0 RECOMMENDATIONS AND FUTURE WORK

For recommendations, we could observe and test the viability of Graphene Oxide against other viruses. This could help us further conclude whether GO is only ineffective in EHV-1 infections or viruses in general. We could also test for cytotoxicity on other mammalian cells to determine the safest concentration to be used on them. The concentration of GO used in this research is also limited. Higher concentrations of GO could be used to observe its effectiveness in treating EHV-1 or different viruses as the highest concentration of GO used in this research shows the best targeted results.

APPENDICES



Appendix A: Resazurin Assay

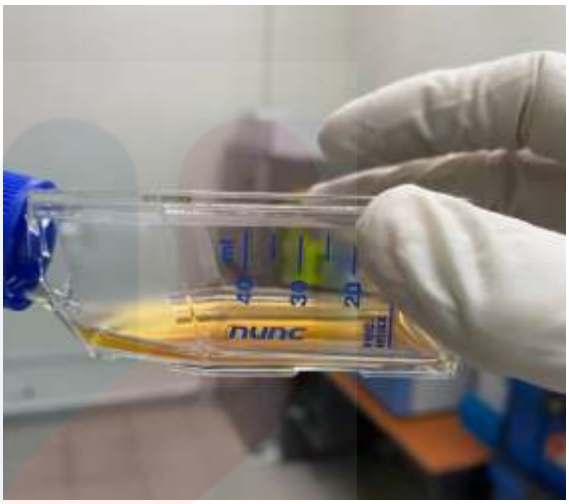


Appendix B: Individual Vero cells

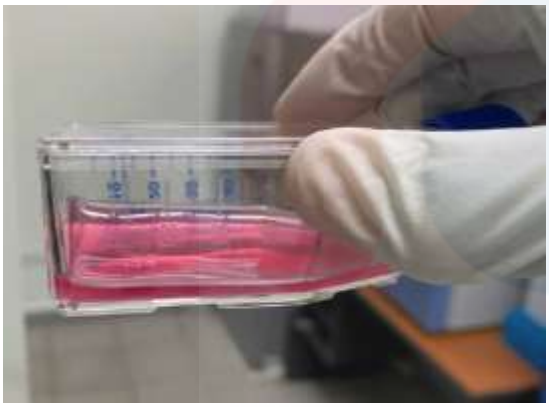
Appendix C: Cell growth Media

Appendix D: Phosphate Buffer Saline

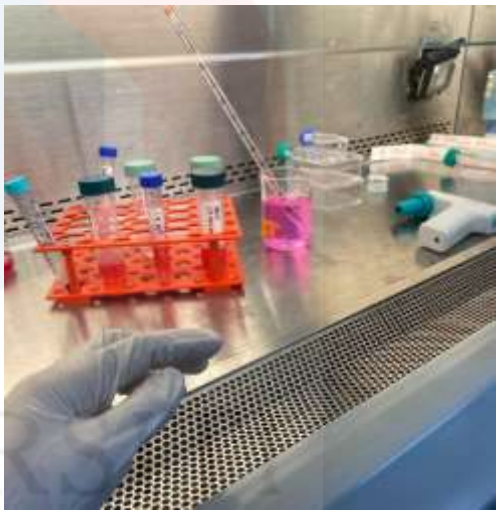
Appendix E: Trypsin



Appendix F: Yellow colored media due to used up nutrient within media



Appendix G: Pink coloured media which indicates enriched nutrient within media



Appendix H: Biosafety chamber

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