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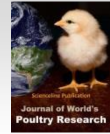

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The Importance and Challenges of Primary Chicken Embryo Liver Cells in Studies of Poultry Viral Diseases: A Review

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ABSTRACT

Primary chicken embryo liver (CEL) cells are derived from the liver tissue of chicken embryonated eggs (CEE) using an aseptic isolation technique and growth under a controlled atmosphere in an artificial environment for cell attachment and proliferation. Although this primary cultured cell has been established for more than six decades, utilization of primary cells is still the preferable medium nowadays as the "gold standard" due to several advantages over other diagnostic techniques. Cells provide better adaptability of the viruses and easily mimic the natural host environment with high virus titration. The volume of virus suspension could be increased by applying an immortal chicken embryo liver-derived cell line. The current review aimed to highlight the importance and challenges of using primary chicken embryo liver cells in poultry virus studies. Primary CEL cells are widely used as an alternative host for diagnosis of infectious poultry viruses, cultivation and passaging of virus isolates, and vaccine production. Yet, there are some challenges and limitations in handling this primary cell, which requires appropriate facilities and environment to sustain the rapid growth of confluent monolayer cells, as highlighted in this paper. The availability of specific pathogen-free CEE is a major concern due to limited resources globally, thus creating a challenge for vaccine manufacturers to upscale the cultured cells. Future improvement of primary cell culture preparation necessitates new technology by applying cellular microcarrier in the bioreactor machine for efficient cell growth and subsequent routine virus cultivation. This study can help the researchers understand the advantages of primary CEL cells and their applications due to their significant impact on poultry viruses.

Keywords: Chicken embryonated eggs, Embryo liver cells, Poultry, Viruses, Vaccine

INTRODUCTION

Cell culture consists of a complex process that is initiated by the isolation of cells from animal tissues (*in vivo*) and growth under a controlled atmosphere in an artificial (*in vitro*) environment (Verma et al., 2020; Dubovi and Rankin 2021; Zhao, 2023). Primary chicken embryo liver (CEL) cells are derived from liver tissue of chicken embryonated eggs (CEE), which involves explant and tissue dissociation under aseptic conditions prior to incubation into a controlled atmosphere for attachment, growth, and proliferation (Swain et al., 2014). Cell culture systems had been established for more than six decades by the early 1960s, mainly for isolating and detecting viruses (Leland and Ginocchio, 2007). Since then, the usage has largely expanded dramatically because commercial cell lines and highly purified reagents are readily available (Pandurangan and Hwang, 2014). Since cell culture systems essentially retain the same properties as the natural tissue, this virus isolation method is considered "gold standard".

As the availability of a live host, such as permissive cell cultures, is a requirement for the isolation of infectious viruses from chickens, monolayer culture of CEL cells is frequently used as a diagnostic and research tool, especially in the diagnosis of viral diseases affecting poultry (Li et al., 2018; Liebhart et al., 2023). In recent years, this cell culture has exhibited excellent substrate for the propagation of viruses needed for vaccine production

and manufacturing (Kim et al., 2014; Sohaimi et al., 2019; Ugwu et al., 2020). The liver in chicken embryos is comprised of hepatocytes and high glycogens, with less connective tissue, and it lacks a true lobular structure (Zaefarian et al., 2019; Bao et al., 2023). Upon dissociation, the liver cells yield monolayer CEL cells comprised of irregular epithelial islands, which consist of hepatocytes surrounded by a network of fibroblasts (Sohaimi et al., 2019). These cells are supported for effective proliferation by the provision of suitable growth media supplemented with a fetal bovine serum to provide nutrients and equipment to provide *in vivo*-like conditions *in vitro,* such as 37°C temperature, 5% CO_2, and 85 to 90% humidity (Barua and Rai, 2003; Soumyalekshmi et al., 2014).

Cell culture systems are more convenient and economical than eggs and animals, which can easily be examined microscopically for cytopathic effect (CPE) as an indicator of viral replication (Leland and Ginocchio, 2007). Additionally, there are benefits to cell culture, such as lower contamination, product purity, efficient use of wild-type viruses, decreased immunogenic changes, large-scale production, and rapid response to pandemics like the recent COVID-19 pandemic or influenza outbreaks (Whitford, 2010; Haredy et al., 2013; Yazawa et al., 2023). Primary CEL cells are the most susceptible cell culture for various poultry viruses, mainly fowl adenovirus (Kumar et al., 2003; Sohaimi et al., 2019). In primary cell culture, cells are taken from the organs of animals, insects, or plants, grown *in vitro* (Swain et al., 2014), and then kept in a medium to express therapeutics, enzymes, and antibodies. Additionally, viruses are grown to develop vaccines (Moreira, 2007; Marquis, 2019). Moreover, anchorage-dependent cells—such as liver cells—need surfaces to adhere to to stabilize and promote growth. Several viruses are routinely isolated using this monolayer cell, including reoviruses, infectious laryngotracheitis virus (ILTV), and Newcastle Disease Virus (NDV) strains from collected samples in the field outbreaks. Due to high sensitivity to these viruses, early CPE formation is recorded within 24 to 48 hours post-infection (pi) and indicated as a superior medium for virus isolation than other cell cultures (Barua and Rai, 2003; Mao et al., 2022).

Virus passaging and cultivation have been conducted globally to increase the virus suspension volume for further analysis (Leland and Ginocchio, 2007). To achieve this with less laborious processes than traditional methods, the use of microcarriers, especially for anchorage-dependent cells like liver cells, has been the major focus of interest for rapid cell proliferation with the purpose of large-volume production and upscaling of viruses for vaccine production (Ugwu et al., 2020) which can only be achieved by using cell cultures. An overview of primary CEL cells used in poultry virus research highlights the significant function that these cells perform for both virus propagation and research, which will help develop and produce vaccines. This could be useful since studying other viruses has been limited worldwide. This paper aims to review the literature on the significance of primary CEL cells, emphasizing their advantages and applications while highlighting the challenges of handling this cell culture.

THE IMPORTANCE OF PRIMARY CHICKEN EMBRYO LIVER CELLS

Advantages of chicken embryo liver cells over chicken embryonated eggs and other continuous cell lines

Primary CEL cells are routinely used as an alternative medium for cultivating poultry viruses and exhibit a significant impact in various studies due to several advantages over continuous cell lines. Cell culture methods are more economical and convenient than egg inoculation. Moreover, cell culture is superior to the egg-based method for the large number of viruses needed for adequate vaccines to meet the continually expanding animal population (Whitford and Fairbank, 2011). Further advantages of cell culture include less contamination, increased efficiency with the use of wild-type viruses, reduced immunogenic changes, fast pandemic response, product purity, higher doses produced in a shorter period of time, and a more reliable, flexible, and expandable process (Whitford, 2010). On the other hand, primary CEL cells could offer a better medium than continuous cell lines being a direct derivative of live chicken embryos which could provide better adaptability of the viruses and easily mimic the natural host environment (Verma et al., 2020).

Basically, primary cells originate from specific pathogen-free (SPF) CEE and are free from extraneous agents compared to commercial chicken eggs (Jungbäck and Motitschke, 2010). Both sterility and safety of virus inoculum are critically important for working on vaccine production (Ibrahim et al., 2019; Cahyani et al., 2020). Preparation of these cells under the aseptic environment with appropriate facilities is important to minimize contamination (Verma et al., 2020). The simple procedure involved harvesting and dissociation of liver tissue with larger size compared to other complex tissue, such as chicken embryo kidney (CEK) cells from the embryo`s

kidney (Soumyalekshmi et al., 2014; Styś-Fijoł et al., 2017). For virus passaging, viruses grown in every monolayer cell culture flask are uniformly maintained in an adequate volume of growth media, and the virus suspension is stored directly for further analysis (Leland and Ginocchio, 2007). In the egg inoculation procedure, the volume of the virus inoculum is variable depending on the size of the embryo's tissues and subsequently involves tissue processing, which is more laborious and time-consuming (Alemnesh et al., 2012).

Primary CEL cells exhibit rapid cell proliferation within 24 hours due to a high metabolic rate that causes a more rapid confluent period than other primary cells and cell lines (Ugwu et al., 2020). The selection of a growth medium is critical for cells to adapt and grow from the original host into an artificial condition (Yao and Asayama, 2017). L-glutamine-supplemented cells can function as a source of energy for cells that reproduce rapidly as well as those that utilize glucose inadequately (Yusof and Jainul, 2019).

Virus identification and detection were rapidly observed less than 7 days post-inoculation (pi) by cytopathic effect (CPE) formation compared to the egg incubation period. There is a possible superior sensitivity of CEL cells over chorioallantoic membrane (CAM) inoculation, as indicated by Sohaimi et al. (2019). Furthermore, in comparison with CK or CEK monolayers, the epithelial cells found in CEL monolayers are smaller and have a greater cell density per unit area, which increases the potential that the virus will invade susceptible cells and cause detectable CPE earlier (Nwajei et al., 1988).

High virus titration is normally achieved in this monolayer cell following virus passaging than in other cell culture systems due to high sensitivity for most poultry viruses (Sohaimi et al., 2019; Ugwu et al., 2020). Indeed, it is the major reason for selecting this medium specifically for vaccine production and is more appropriate than CEE (Wambura et al., 2006). Interestingly, the cell has a high potential to be used as a substrate for vaccine production (Ugwu et al., 2020). An upscaling technique like applying a complex bioreactor with microcarriers enhances cell proliferation within 24 hours for a confluent monolayer and improves virus titer. More than sixty different cell lines were cultivated in cell culture systems using the Cytodex™ 1 microcarrier, which was developed with all the features that contribute to a good microcarrier (Yang et al., 2019). But this technique, which is capable of increasing cell and virus yield for large volume production of vaccines, can only operate with cell cultures and is not adaptable to egg-based inoculation. This scale-up technology in vaccine production is more economical as it can be reused, is less laborious, and accommodates a high capacity of cell volume and virus suspension (Lawal et al., 2018). It was found that primary CEL cells are well adapted to Cytodex™ 1 microcarriers with rapid cell proliferation at 24 hours for confluent monolayer cells (Ugwu et al., 2020). Subsequently, FAdV strain UPM08136 from the 20th passage in primary CEL cells propagated in the microcarrier by stirring in a bioreactor, producing a virus titer of $10^{6.5}$TCID$_{50}$ /mL.

Routine applications of primary chicken embryo liver cells

Diagnosis of infectious avian viruses

Isolation and identification of viruses were routinely performed by inoculation into cell culture for confirmation of the aetiological agent from field outbreak (Leland and Ginocchio, 2007). This approach has been regarded as the "gold standard" to diagnose viral diseases for more than six decades, although extensive technical expertise is required. Indeed, primary CEL cell culture has proved to be an appropriate and sensitive substrate for various poultry virus isolation (Kumar et al., 2003)

Several poultry viruses can be isolated in CEL cells with clear CPE, while others failed to replicate due to differences in tissue tropism (Hofle et al., 2012). In earlier research, the isolation of adenoviruses from disease outbreaks in chicken farms occurred in primary CEL cells with CPE formation, followed by an electron microscopy examination (Gough et al., 1988). Recently, FAdV isolates from inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), and gizzard erosion outbreaks have been routinely diagnosed by isolation in CEL due to it being highly sensitive, which took only 24 to 48 hours post-infection (pi) at the second passage to produce CPE (Soumyalekshmi et al., 2014; Radwan et al., 2019; Sohaimi et al., 2019). This could be attributed to the abundance of Coxsackievirus-adenovirus receptor (CAR) in the liver tissue, which aids virus attachment as the major reason for the cell's high susceptibility to FAdV replication (Wang et al., 2014).

In India, FAdV was confirmed in CEL cells from liver samples of dead chickens. Cells began to round, clump, and detach at the second passage, and within 48 hours pi, the CPE demonstrated cell swelling and rounding. The cells started to detach from the monolayer at 72 hours pi, as well as the cell monolayers completely detached at 96 hours pi (Soumyalekshmi et al., 2014). The type of CPE produced is compatible with human

adenoviruses (HAdV) in chicken embryo fibroblast (CEF) cells (Alameedy, 2016). The protein involved in the adenoviruses seems to utilize a similar virus epitope located at the penton base protein and induces cell rounding activity in the infected cells prior to lysis and monolayer detachment (Zhang and Bergelson, 2005; Russell, 2009).

On the other hand, avian reoviruses were successfully isolated into CEL cells as the medium for virus isolation. It seems that this primary cell exhibits excellent media quality which could be better than Vero cells based on virus titration produced after several passages (Zhang et al., 2019). Sample from hock joints obtained from arthritis and tenosynovitis in chickens were isolated into monolayer cells and produced the highest virus titer compared to other tested primary cells with early CPE formation within 24 to 72 hours pi (Zhang et al., 2019). A similar finding was also stated in earlier work (Barta et al., 1984), which indicates that the primary CEL cell is the most sensitive medium for reoviruses (Kort et al., 2013; Zhang et al., 2019).

Similarly, in cases of the ILT virus, the CEL cells are the most sensitive and rapid method for the isolation of the virus from the infected tracheal samples than other cell cultures and chicken embryos (Parra et al., 2016). CPE was detected as early as the first passage in the form of large syncytial or formation of the multinucleated giant cell due to fusion cell nuclei (Hughes and Jones, 1988). In addition, this monolayer culture cell is highly permissive to the Newcastle Disease Virus (NDV) as well (McGinnes et al., 2006; Bello et al., 2018). Isolation of astroviruses from chickens was conducted in CEL cells and produced a marked CPE after four to five passages (Baxendale and Mebatsion, 2004).

Avian influenza (AI), infectious bronchitis (IB), infectious bursal disease virus (IBDV), and Marek's disease are not very susceptible to CEL cells (Rekha et al., 2014; Han et al., 2017; Wu et al., 2020) regardless of the fact that these cells are highly susceptible for the isolation of FAdV, Reoviruses, ILT, NDV, and chicken astroviruses (CAstV). Some viruses target specific tissues, whereas others have a wide range of cell targets. Therefore, in CEL cells, only particular viruses are able to replicate and exhibit CPE. Nevertheless, the monolayer culture of CEL cells has high potential to be used for the diagnosis of other avian viruses since the CEL is rapidly growth due to a high metabolic rate compared to chicken embryo kidney (CEK) and chicken embryo lung (CELu) cells (Prasad et al., 2018).

To overcome this issue, the immortal CEL cell line developed by Lee et al. (2013) is capable of isolating some other viruses, such as avian metapneumovirus (AMPV) and Mareks`s disease virus serotype 1 (MDV-1). The cell culture has the potential to be used as an alternative host for primary CEL cells in the future.

Cultivation of poultry viruses

The aim of virus cultivation is to increase the volume of virus suspension, mainly for viruses originating from poultry farms for ultrastructural studies, development of vaccines, and as a reference strain for molecular work (Sohaimi et al., 2019; Ugwu et al., 2020). Therefore, virus propagation procedure necessitates a suitable alternative host for continuous growth and multiplication.

FAdV isolates obtained from hepatitis-hydropericardium syndrome (HHS) outbreaks in chickens were propagated in the CEL cells and produced CPE at first passage (Kumar et al., 2003). A similar finding was observed by Barua and Rai (2003), starting in the third passage onwards. It was demonstrated that CEL cells are highly susceptible to FAdV replication regardless of serotypes and strains from field outbreaks (Al Naguib et al., 2021). Interestingly, the CPE produced is identical to human adenoviruses (HAdV) in the form of refractile, rounding, clumping of cells, and detachment of monolayer cells from the flasks as terminal stage of viral infection (Adair et al., 1979; Barua and Rai, 2003). A similar finding was observed in FAdV serotype 8b from cases of IBH and gizzard erosion in commercial layer chickens, in which the virus isolate was propagated for 35th passages in primary CEL cells with early CPE formation within 24 to 48 hours pi from second passage onwards (Sohaimi et al., 2019).

The selection of CEL cells as an alternative host for the passaging of viruses was attempted in different works due to the high sensitivity of the cells for FAdV replication. In addition, the rapid formation of CPE within 24 to 48 hours pi is a major concern for virus adaptability for a high number of passages. The CPE produced is consistent, which is beneficial for virus propagation and attenuation for high virus titer production (Sohaimi et al., 2019). A large volume of viral suspension was produced compared to liver embryo tissues from SPF CEE, which is suitable for preparing virus seeds for vaccine production.

However, the susceptibility of CEL cells towards a wide range of poultry viruses is limited due to different tissue tropism for virus infectivity into host cells. Since hepatocytes are the major target site for FAdV replication, almost all pathogenic FAdV strains are propagated in this

cell (Ahmad et al., 2011; Shah et al., 2017). A study on Egg drop syndrome (EDS) disease from duck samples revealed varieties of replication activities of the *Atadenovirus* isolates in CEL cells (Kang et al., 2017). Although the virus is from a different group under the *Adenoviridae* family, the tropism toward CEL cells is similar to FAdV strains.

On the other hand, propagation of NDV in CEL cells produced CPE although the strain, namely, V4, is non-cytopathogenic to other conventional avian cell cultures (Nwajei et al., 1988; Uruakpa, 1997). There are similar findings for serial passages of infectious laryngotracheitis (ILT) from the UDCEOD1 strain caused by herpesvirus (Taylor, 2013). It shows that primary CEL cells are adaptable and highly permissive to various avian viruses from field outbreaks for extended passage level.

As an alternative option, the immortal CEL cells (CEL-im) served as a continuous cell line and were used for the passaging of avian viruses such as avian metapneumovirus (AMPV), Marek`s disease virus serotype 1 (MDV-1), and ILT virus. The virus titer was high for AMPV, which was more than 105pfu/mL; however, the lower titers were for the MDV-1 and ILT viruses. It suggests that the CEL-im could be tested for permissiveness to other avian viruses (Lee et al., 2013).

Application of chicken embryo liver cells in vaccine development

Production of vaccine viruses on a large scale is necessary in the poultry industry as a strategy for disease control and prevention (Gomez and Robinson, 2018; Sohaimi et al., 2019; Ugwu et al., 2020). Vaccines are important to stimulate antibody response to confer protection against the disease in commercial poultry farms (De Luca et al., 2020). Throughout the review on purposes of primary CEL cells, it is shown that they are useful for virus passaging for the production of a large volume of virus suspension and for further analysis in chickens for vaccine development. Furthermore, the virus attenuated in CEL cells produced high virus titer up to $10^{6.8}$ $TCID_{50}$/ml and was useful for the development of vaccine candidates in chickens (Sohaimi et al., 2019).

Nowadays, research on vaccine development in primary CEL cells has become a major focus of attempts due to high sensitivity for virus replication and virus titration determination. Previous reports mostly focused on FAdV instead of other viruses due to tissue tropism in the hepatocytes (Sohaimi et al., 2019; Ugwu et al., 2020).

Attenuation of FAdV isolate, UPM1137 was performed at 35th passages in primary CEL cells with virus titer of $10^{6.8}$ $TCID_{50}$/ml and induced antibody response in both SPF and commercial broiler chickens (Sohaimi et al., 2019; Sohaimi et al., 2021). The process exhibited several molecular changes in hexon and fiber gene proteins, which were crucial for the virus to continuously survive and replicate for serial passages in artificial conditions (Sohaimi et al., 2019). A recent study conducted by De Luca et al. (2020) successfully developed a fiber-based vaccine against IBH in chickens. Both FAdV-8a and 8b strains have been propagated into primary CEL cells according to the previous protocol by Schat and Sellers (2008), and the vaccine stimulates humoral immunity by type-specific virus neutralization associated with T and B cell responses.

High antibody response was induced by inactivated oil-emulsion vaccine from FAdV serotype 4 and effectively provided cross-protection against FAdV serotype 5, 8a, 8b, and 11 (Kim et al., 2014). The vaccine was developed by infecting the CEL cells with FAdV isolate and harvested prior to inactivation by formaldehyde (Kim et al., 2014). In previous work, the IBH vaccine oil-adjuvanted cell culture conferred high protection against IBH disease in chickens when compared to the autogenous vaccine (Shah et al., 2017). It seems that primary CEL cells have been used extensively worldwide for virus propagation and the production of inactivated vaccines (Kim et al., 2014; Junnu et al., 2015; Norfitriah et al., 2019).

The FAdV inactivated oil-emulsion vaccine consists of serotype two, which induces a high antibody response and confers full protection against the challenged FAdV strain. The vaccine was prepared from liver isolate passaged into primary CEL cells, and the infected CEL cell supernatant was inactivated by binary ethyleneimine (BEI) (Junnu et al., 2015). Those findings on the efficacy trial with a high protection rate were compatible with a recent study using FAdV serotype 8b strain from the 5th passage at titer $10^{7.5}$$TCID_{50}$/ml (Norfitriah et al., 2019).

In other poultry viruses, propagation and attenuation of the ILTV strain, UDCEOD1 was attempted in CEL cells for 29 passages. It was shown that it is necessary to continuously pass the strain approximately 100 times to achieve attenuation (Taylor, 2013). Based on previous research, there is still a lack of studies on other poultry viruses in primary CEL cells for poultry vaccine development. This monolayer cell could be very useful for the attenuation of other permissive viruses, such as avian reoviruses and NDV, for significant outcomes in the future.

CHALLENGES AND LIMITATIONS OF THE PRIMARY CHICKEN EMBRYO LIVER CELLS

Ongoing research revealed that the utilization of this primary cell has decreased due to some challenges and issues in handling primary cell culture. Literally, primary CEL cells have a finite lifespan with time-consuming and tedious preparation (Lee et al., 2013; Swain et al., 2014; Shittu et al., 2016). A specific age of embryos is needed prior to harvesting the liver tissues rather than the continuous cell lines, which are easier to propagate at an unlimited passage number (Swain et al., 2014). However, the sensitivity of cell lines towards various poultry viruses is limited compared to primary CEL cells (Lawal et al., 2018; Verma et al., 2020). To overcome this issue, the application of current technology could be considered using a bioreactor with less handling, cost-effectiveness, and improved volume yield. Growing cells in suspension facilitated by microcarrier in a bioreactor will increase FAdV titration (Ugwu et al., 2020). The study could be expanded for other viruses for vaccine manufacturing purposes.

Availability of the SPF eggs could be a major concern for CEL cell preparation since the primary cells rely on fresh liver tissues. Some countries have the capability to sustain an ongoing supply of eggs, yet since SPF eggs are pricey, the availability of eggs is limited in developing countries (Shittu et al., 2016). Thus, the intervention of these cells has been performed as an immortal cell line, as reported in previous literature, for the propagation of certain avian infectious viruses (Lee et al., 2013).

On the other hand, cell preparation needs appropriate facilities and environment to sustain the rapid growth of confluent monolayer cells (Coté, 2001; Swain et al., 2014). Some primary cells are reluctant to adapt and proliferate into cell culture flasks due to mishandling, shortage of electricity supply, or possibly inadequate CO_2 concentration in the cell incubator. Lack of experience for technical persons may contribute to this issue, and perhaps, it could be prevented by appropriate training under supervision with adequate arrangements. Furthermore, optimization of the right media should be attempted to obtain rapid confluent monolayer cells following 24 hours post-cultured.

Technically, the preparation of primary cells for vaccine production necessitates a sterile working area throughout the procedure until the product is reached. There are still high chances for microbial contamination in cell culture flasks or bioreactors due to expired media, improper techniques, unsterilized materials, or even through contaminated virus inoculum (Coté, 2001; Prasad et al., 2020). Although this technical concern could be prevented by adequate sterilization or treatment with antibiotic and antimycotic solutions, the quality control and adequate monitoring system of the working area and equipment should routinely be tested based on the standard operating procedure prior to the handling of cells and virus samples (Roth et al., 2020; Weiskirchen et al., 2023). Care and maintenance of the cell culture laboratory equipment in line with biosafety and biosecurity protocols are critical aspects of maintaining excellent primary cell culture procedures throughout the research process (Ochiai et al., 2021).

CONCLUSION

Primary CEL cells exhibit excellent performance for adaptation, passaging, and attenuation of poultry viruses. Perhaps the CEL cells have a high potential to be used for various poultry viruses in the future due to several advantages and significant impacts, as highlighted in this paper. For future recommendations, the immortal CEL cells should be tested for the adaptability of other avian viruses for diagnosis and vaccine development. It could be suggested that the price of SPF eggs could be slightly reduced globally by the supplier, mainly for vaccine producers and research institutes in developing countries such as Africa and Southeast Asia.

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Availability of materials and data

All data of the current study are available according to reasonable request.

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Authors' contribution

Norfitriah Mohamed Sohaimi was involved in the conception and design, carried out data collection, and drafted the manuscript. Ugwu Chidozie Clifford provided grammatical revisions and helped draft the manuscript. All authors read and approved the final manuscript.

Ethical considerations

The authors checked the manuscript for evidence of plagiarism, consent to publish, misconduct, data

manipulation or deception, double publication or submission, or redundancy.

Competing interests

There are no conflicts of interest in accordance with the authors.

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