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Farah

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4 Conclusions and Recommendations

4.1 Conclusions:

- Tissue culture infection dose 50% was 3.5×10^5.
- Live attenuated measles virus Schwarz (MV) vaccine induces oncolytic cytopathic effect in Iraq tumor cell line GBM and in RD cell line.
- MV vaccine strain induces the formation of multinucleated giant cell (syncytia) in tumor cells which may one of causes of cell death.
- MV vaccine strain has ability to insert its H protein to tumor cells which may lead to modified antigenic surface of tumor cell.
- MV vaccine strain induce cell killing by direct cytolysis and apoptosis induction.

4.2 Recommendation:

- Studying the cytotoxic effect of measles virus *in vivo* by using xenograft animal model will be highly sensitive to infection with wild type MV.
- Studying the apoptosis pathways, and investigated on the virus which mechanism and caspase activated in MV-infected cells.
- Studying the cytotoxic effect of measles virus in combination with chemotherapies.
- Using genetically modified measles virus to improve the efficacy, safety and applicability of virotherapy.

Dedication

To my parents who have supported me all the way since the beginning of my studies.

To the angel of kindness my husband.

To my sisters and brother who have been a great source of motivation and inspiration.

To all those who believe in the richness of learning.

Farah

1 Introduction and Literature review

1.1 Introduction

Cancer is one of the major health problems of our time (Jemal *et al* 2004). During the past two decades, the paradigm for cancer treatment has evolved from relatively nonspecific cytotoxic agents to selective, mechanism-based therapeutics. Cancer chemotherapies were initially identified through screens for compounds that killed rapidly dividing cells. These drugs remain the backbone of current treatment, but they are limited by a narrow therapeutic index, significant toxicities and frequently acquired resistance. Now, an improved understanding of cancer pathogenesis has given rise to new treatment options, including targeted agents and cancer immunotherapy (Ahmedin *et al.,* 2005).

Targeted approaches aim to inhibit molecular pathways that are crucial for tumour growth and maintenance; whereas, immunotherapy endeavours to stimulate a host immune response that effectuates long-lived tumour destruction. Targeted therapies and cytotoxic agents also modulate immune responses, which raises the possibility that these treatment strategies might be effectively combined with immunotherapy to improve clinical outcomes. Hence, a variety of new approaches is currently being explored, one of which is based on the use of viruses (Vanneman and Dranoff, 2012).

Oncolytic viruses are defined by their ability to specifically kill tumor cells, but to leave the normal tissues unharmed. Their most characteristic features are their target specificity and their cytolytic capacity. Ideally, they exhibit additional features including, but not limited to, a high reproductive capacity *in vivo*, the ability to recruit uninfected neighboring

cells (syncytia formation), the ability to infect both dividing and non dividing cells, a high stability *in vivo*, the inability of chromosomal integration, the lack of disease induction, and the general absence of preexisting antibodies to the virus in the host population (Verheije and Rottier, 2012).

The use of replicating viruses for cancer therapy is attracting increasing interest. Numerous viruses are now being considered as potential cancer therapeutics, including the vaccine strain of measles virus (Moss and Griffin, 2006).

The attenuated strain of measles readily lyses transformed cells, whilst replication and lysis are limited in normal human cells. It has a number of features which make it highly suitable for further development as an oncolytic agent, among them stability and a long history of safety in human use. These features are being combined with its ready potential for genetic manipulations to generate recombinant MV with desirable therapeutic attributes (Fielding, 2005).

Peng *et al.* (2006) reported that a growing interest in oncovirotherapy using a number of replication-competent (oncolytic) viruses like MV for cancer therapy has emerged. Previous studies proved that different MV strains demonstrated potent oncolytic efficacy with minor or none side effects against multiple primary and established cancer cell lines and several preclinical animal tumor models including both solid tumors and hematologic malignancies such as ovarian cancer.

Indeed, tumors develop many different strategies to grow, especially by conditioning their microenvironment to escape the immune system and to promote neoangiogenesis. They produce immunosuppressive cytokines, notably TGF-β, and recruit immunosuppressive cells, such as

T regulatory cells (Tregs), to maintain immune tolerance within the tumor (Eitan, 2013).

Thus, MV may also act as an immunoadjuvant to overcome the immunosuppressive tumor environment. It has already been demonstrated for reovirus, other oncolytic viruses, that innate and adaptive immunity-mediated antitumor activity is necessary for successful virotherapy, independent of direct oncolysis (Errington *et al.*, 2008; Prestwich, *et al*., 2009).

To investigate the oncolytic activity of live attenuated measles virus strain, this study was aimed to:

- Propagation of live attenuated measles virus strain on Vero cell line.
- Studying the oncolytic cytopathic effect of live attenuated measles virus on Vero cell line.
- Characterazation of live attenuated measles virus by using some viral markers.
- Studying the cytotoxic effect of live attenuated measles virus on tumor cell lines *in vitro.*
- Studying the apoptotic effect of live attenuated measles virus on tumor cell lines *in vitro*.

1.2 Literature review

1.2.1 Cancer

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death (Patyar *et al.,* 2010). Cancer is caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism) and these causal factors may act together or in sequence to initiate or promote the development of cancer (Paul and Penny**,** 2013). Cancer is the second most common cause of death in the world, exceeded only by heart disease, accounting for nearly 1 of every 4 deaths (ACS, 2013).

Clearly, new therapies are needed that are capable of treating such advanced cancers in addition to preventing their formation. Although conventional anticancer therapies, consisting of surgical resection, radiotherapy and chemotherapy, are effective in the management of many patients but for about half of cancer sufferers these are ineffective, so alternative techniques are being developed to target their tumors (Douek and Taylor, 2003). Experimental cancer treatments are medical therapies intended or claimed to treat cancer by improving, supplementing or replacing conventional methods. These include photodynamic therapy, immunotherapy, gene therapy, telomerase therapy, hyperthermia therapy, dichloroacetate (DCA), complementary and alternative therapy, diet therapy, insulin potentiating therapy and bacterial treatment (Jain, 2001). But many of these therapies are controversial due to lack of evidence, efficacy, feasibility, availability, specificity and selectivity (Ravindra *et al.,* 2009).

The goal of cancer therapy is to promote the death of cancer cells without causing much damage to normal cells (Gerl and Vaux, 2005). A number of viruses with an ability to kill cancer cells while sparing normal cells have been discovered. Such viruses are referred to as oncolytic viruses (Nelson, 1999). Many viruses, like vaccinia virus, Newcastle disease virus, measles virus, reovirus and adenovirus, which are intended to achieve selective replication and killing of tumor cells have been investigated. **(**Douek and Taylor, 2003).

Viruses have shown the most potential to carry altered genes to cancer cells, to find target cells in body and ability to latch onto these cells. Oncolytic viruses cause lysis (rupture) of cancer cells, which can then be processed by the adaptive immune system, and target similar cells in other parts of the body. But the effective use of such viruses is sometimes hindered by the production of potentially neutralizing antibodies generated against them (Parato *et al.*, 2005).

1.2.2 Oncolytic virotherapy

The idea of using viruses in the treatment of cancer is not new. Observations made in the early 1920s indicated that viruses replicated in and lysed murine and other experimental tumors (Christopher and Ring, 2002). Amongst the earliest reports by Bluming and Ziegler (1971) on regression of human tumors in the case of cervical carcinoma that regressed after inoculation of the patient with attenuated rabies vaccine. In addition, there are reports of remissions of Burkitt's and Hodgkin's lymphomas following natural infections with measles virus (Taqi *et al.*, 1981). Intentional inoculations of live viruses into tumor patients were initiated in the late 1940s; however, these very seldom resulted incomplete remissions (Sinkovics and Horvath, 1993).

The use of viruses for therapy was introduced by the field of gene therapy, mainly as vehicles for nucleic acid transfer. Several modalities of gene therapy are aimed to treat cancer, most of them with replication deficient viral vectors to avoid the risk of virus systemic dissemination (Patel *et al.*, 2004). These non-replicating vectors may intend to reestablish wild-type copies of mutated tumor-suppressor genes, affect the metabolism of tumor cells, attract the immune response, or sensitize the neoplastic tissues to standard therapies (Wadhwa *et al*., 2002).

Virotherapy resurged in the late 90's as an innovative alternative for oncolysis with a simple idea to create new vectors with capacity to propagate in deregulated tumor cells and minimum adverse effects in healthy tissues (García *et al.,* 2008).

Al-Shammari (2010) concluded that, Iraqi strain of Newcastle Disease Virus (NDV) had the ability to replicate in different cell type of tumor and insert their HN protein in tumor cell surface that lead to modify the antigenic determinants and increase the immunogenicity of tumor cell surface. Thus, the modification of tumor induces the attachment of CD4 and CD8 T cells on infected tumor cell surface. Another study of Al-Shammari *et al.* (2011) revealed that Iraqi strain of NDV when used as oncolytic agent by intratumoral injection showed a significant stimulation in immune response by increasing the level of IL-2 and IFN-gamma and the percentage of CD8 and CD56.

This therapeutic modality is called oncolytic virotherapy and is divided into two approaches: oncolytic wild viruses, or natural occurring viruses with preferential replication in human cancer cells; and gene-modified viruses engineered to achieve selective oncolysis (Everts and Poel, 2005).

Oncolytic viruses engineered to selectively replicate within, and thus kill tumor cells. Studies of the interactions of viruses with their host cells have found that cells transformed with some oncogenes or in active cell-cycling state were more permissive to the replication of some viruses (Imperiale *et al.,* 1984; Farassati *et al.,* 2001). Since viruses normally propagate at the expense of their host by causing lysis, the ability to harness and regulate this process could be very beneficial. Many wild-type viruses induce cells into active states, resulting in upregulation of the cellular machinery necessary for replication (Southam, 1960). In such viruses, mutations or genetic modifications of the genome can alter the regulation of early, replication-essential genes, rendering the viruses dependent on their host for an environment conducive to replication. Such mutated or altered viruses are unable to replicate in quiescent or normal, non dividing cells; they replicate almost exclusively in cells that are actively dividing, such as tumor cells. These viruses, termed oncolytic viruses, are essentially tumor-specific, self-replicating, and lysis-inducing cancer killers. They are self-perpetuating in cancerous, rapidly dividing tissue and will continuously infect and replicate as long as the host's cell population is permissive (Nelson, 1999).

Many oncolytic viruses belonging to several viral families have been identified, they include wild type virus such as adenoviruses, orthomyxoviruses, poxviruses, picornaviruses, myxomaviruses, reovirus, New Castle Disease virus (NDV), Coxsackievirus, Vesicular stomatitis virus (VSV), parvoviruses and engineered virus such as Herpes simplex viruses (García *et al.,* 2008).

These can be categorized into different groups on the basis of their oncolytic restriction, typically caused by natural mechanisms, large or small deletions, or complete replacement of critical viral proteins and

promoters (Davis and Fang, 2005). These viruses are made, or naturally evolved to replicate tumor-specifically in a number of ways by altering the normal expression patterns or protein interactions between a virus and its host. For several decades, clinicians have observed that natural viral infections can lead to tumor suppression and clearance (Wheelock and Dingle, 1964). These natural or attenuated viruses have demonstrated a significant amount of tumor specificity both in research and clinical studies, especially viruses such as Vesicular stomatitis virus (VSV) (Balachandran *et al.*, 2001; Lichty, 2004), Mumps (Asada, 1974; Okuno, 1978), Newcastle disease virus (Csatary, 1993; Pecora, 2002; Al-shammari, 2003; Al-shammari, 2010), Reovirus (Coffey *et al.*, 1998; Norman *et al.*, 2004) and Measles virus (Bluming and Ziegler, 1971; Grote, 2001; Msaouel *et al.*, 2009; Zhou *et al*., 2012).

Zhang *et al.*,*(*2012) showed that a large variety of oncolytic viruses have been engineered, among the many oncolytic virus systems, the attenuated Edmonston vaccine strain of the Measles virus (MV-Edm) has proven safe and effective. In addition to its capacity to lyses infected tumor cells, MV has also the ability to activate the immune system. This ability may participate in the efficiency of antitumor virotherapy based on MV.

1.3 Measles virus (MV)

Measles virus (MV) is the type species for the genus *Morbillivirus*, belong to subfamily *paramyxovirinae*, family *paramyxoviridae* , the order *mononegavirales* and only infects humans(Navaratnarajah *et al*., 2009).

1.3.1 Measles virus structure

Measles virus is a spherical, enveloped, non-segmented, single stranded, negative-sense, liner RNA virus and found as pleomorphic particles with a size of 120-270 nm (Communie *et al*., 2013). The MV

RNA genome consists of about 16 000 nucleotides includes six genes and encodes eight proteins are a nucleocapsid protein (NP), a phosphoprotein (P), virulence factors (V and C), matrix protein (M), membrane fusion protein (F), the hemagglutinin/receptor-binding protein (H), and large protein (viral RNA polymerase) (L) (Figure 1-1).

The fusion (F) and the hemagglutinin (H) represent the surface glycoproteins that project from the lipid envelope and mediate viral entry and exit from the host cell (Norrby and Oxman, 1990). These surface projections are 9-15 nm long and spaced 7-10 nm apart. Inside the envelope lies a helical nucleocapsid core containing the RNA genome and the nucleocapsid (N), phospho- (P) and large (L) proteins that initiate the intracellular virus replication .The matrix (M) protein, which lies between the envelope and the core, is important in viral structure and is released from the core during viral entry (Cathomen *et al*., 1998).

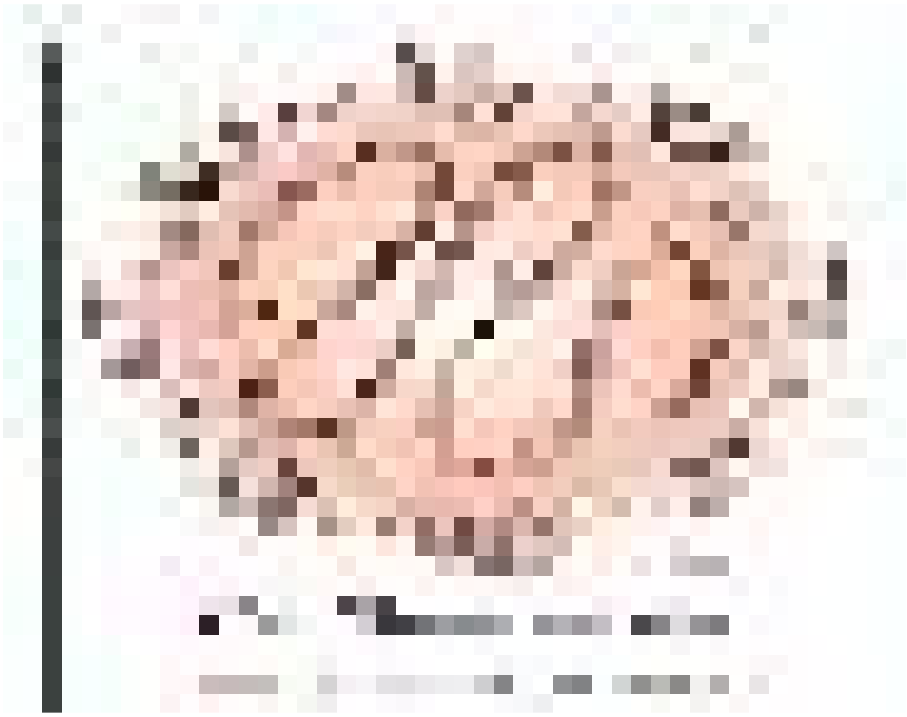

Figure (1-1): Measles virus structure (Moss and Griffin, 2006).

1.3.2 Nucleocapsid Protein

The N protein of the paramyxoviruses ranges from 489 to 553 amino acids, is elongated by helical symmetry and has a filamentous morphology with a length of approximately 600-800 nm (Compans *et al.*, 1972; Mountcastle *et al*, 1974). The N protein associates with Phosphoprotein and large protein, the result of this association, the ribonucleoproteic complex, constitutes the template for the transcription and replication of the viral genome and N protein associates with the M protein during virus assembly. However, N has also the ability to self-assemble onto cellular RNA in the absence of viral RNA and viral proteins (Jacobson *et al.*, 1989; Bhella *et al.*, 2002).

1.3.3 Phosphoprotein

For morbilliviruses, the P gene mRNAs are translated into two non-structural proteins V and C. But P protein is the only P gene that is structural and essential for viral RNA synthesis in all aspects. The P protein is an essential component in the viral RNA phosphoprotein complex, nascent chain assembly complex, RNA synthesis and the L protein needs to bind to the nucleocapsid RNA template via the P proteins that maps to the C-terminal end that is relatively conserved and is never expressed by itself, but the module is translated from the unedited mRNA (Horikami *et al.*, 1992; Curran *et al.*, 1995 and Communie *et al.*, 2013).

1.3.4 Cystein-rich V and C Open Reading Frames (ORFs)

The V and C accessory proteins play a role in regulating viral RNA synthesis and countering host defenses to viral infection (Gotoh *et al*, 2001).

1.3.5 Matrix Protein

The M protein is considered to be critical in viral morphogenesis by interacting with the lipid bilayer (envelope) and the cytoplasmic tails of the glycoproteins F and H (Cathomen *et al.*, 1998). The abundance of basic residues in the M protein and their interaction with the N proteins may be the driving force in forming a budding virus particle and, therefore, viral assembly (Iwasaki *et al.,* 2009; Hiroshi *et al.,* 2013).

1.3.6 Fusion Protein

The fusion or F protein is a surface glycoprotein that mediates viral entry into the host cell by fusion of the virion envelope and the host cell plasma membrane at a neutral pH (Wild and Buckland, 1997). F proteins are synthesized as inactive precursors that must be cleave by host cell proteases. The fusion of the virion with the plasma membrane allows the nucleocapsid to be delivered into the cytoplasm. As the virus is replicating, the F protein is expressed on the surface of the infected host cell and mediates fusion with the neighboring cells to form syncytia or "multinucleated giant cells." Syncytia are characteristic cytopathic effects of morbilliviruses that can ultimately lead to tissue necrosis and spreading of the virus. The F protein on the surface of infected cells also serves as new F proteins for new viruses that will obtain their envelope as they bud from the plasma membrane (Norrby and Oxman, 1990).

1.3.7 Hemagglutinin Protein

The hemagglutinin or H protein is a surface glycoprotein that projects from the lipid membrane of the virus. Therefore, the H protein serves as an attachment protein and lacks detectable neuraminidase activity. Knipe and Howley (2001) reported that for MV vaccine strains, the cellular receptor molecule is CD46, a natural complement cofactor

(Naniche *et al.*, 1993; ori! *et al.*, 1993). CD46 is likely to cause virus aggregation during virus budding. Therefore, morbilliviruses in general do not need neuraminidase activity to free the virus from the cell surface (Lamb and "ar#s, 2007; Ader *et al.*, 2012a and 2012b).

The H glycoprotein is an antigenic protein because it projects from the surface of the virus and therefore the host's immune system directly recognized. This protein needs to evolve to avoid the host immune response. Therefore, neutralizing antibody responses are directed mostly against the H glycoprotein and sometimes the F surface protein even though non-neutralizing antibodies are still made against the other viral proteins, especially the N protein (Santibanez *et al*., 2005). Neutralizing antibodies can't be made against the N protein because in a fully assembled virion particle the N protein is not exposed on the surface of the virion like the two glycoproteins, H and F. During an investigation of a natural infection of MV, antibodies to the H protein were the second most abundant to the N protein (Graves *et al.*, 1984).

1.3.8 Large Protein

The L gene is thought to function as the viral polymerase because of its large size, low abundance and its localization to the transcriptionally active viral core. The P and the L protein form a complex, which is required for polymerase activity with the nucleocapsid: RNA templates, but L protein is thought to contain the majority of the catalytic activity and is named the ribonucleoprotein complex (Hamaguchi *et al.*, 1983; Horikami *et al.*, 1992 and Curran *et al.*, 1995).

1.4 Viral Transmission

Morbilliviruses is transmitted via the respiratory route and causes systemic disease. MV enters the host by infection of alveolar macrophages and/or dendritic cells in the airways, and is amplified in local lymphoid tissues (Rory *et al.,* 2012). Yanagi *et al* (2006) reported that viremia mediated by infected CD150+ lymphocytes results in systemic dissemination and infection of lymphocytes and dendritic cells in the respiratory submucosa facilitates basolateral infection of epithelial cells via the receptor Nectin-4. Concomitant and extensive epithelial damage may contribute to efficient transmission to the next host. The primary infected blood cells are the monocytes and lymphocytes, which further transport the virus to various organs of the host. It has been described that the lungs, skin, conjunctivae, gastrointestinal tract, liver, kidney and genital mucosa are all affected by the viral infection (Hall *et al.,* 1971; Sakaguchi *et al.,* 1986; Kobune *et al.,* 1996 and McChesney *et al.*, 1997).

In humans, MV has an incubation time of 10- 14 days before clinical symptoms become detectable and can cause immune suppression, lymphopenia and various neurological disorders (Griffin, 2007). Subacute sclerosing panencephalitis (SSPE) can occur several years after acute infection from a persistent MV infection in the central nervous system (Permar *et al*$, 2001; %ima and u&re' , 200( )$

1.5 Viral Absorption and Penetration

The Edmonston vaccine strain of MV is able to use human CD46 as a cellular receptor for attachment and entry. CD46 is expressed on almost all nucleated human cells and is a member of the regulators of complement activation family (Liszewski *et al.,* 1991). As studies progressed, evidence began to accumulate that not all MV strains used

CD46 as a receptor (Buckland and Wild, 1997). Signaling lymphocyte activation molecule (SLAM or CD150) is a glycoprotein of the immunoglobulin super family and a regulator of antigen-driven T-cell responses and macrophage function (Tahara *et al.,* 2007). SLAM is a co-stimulatory molecule and therefore, its expression is restricted to B and T lymphocytes, mature dendritic cells and macrophages (Sidorenko and Clark, 1993; Cocks *et al.,* 1995 and Aversa *et al.,* 1997). Wild type MV strains have been shown to use SLAM, not CD46, as cellular receptors (Tatsuo *et al.,* 2000a; Ono *et al.,* 2001 and Yanagi *et al.,* 2006).

Edmonston, the vaccine and laboratory strain of MV uses both CD46 and SLAM. Use of two receptors is the result of the adaptation of the vaccines strains, as they are passed and grown efficiently in mammalian cell lines like Vero cells (African green monkey kidney cells) and MDCK cells (Madin-Darby canine kidney cells), by acquiring a number of mutations in their genomes (Parks *et al.,* 2001a; "ar#s *et al.,* 2001b). Amino acid residue 481 on the H gene has been specifically found to play an important role in determining the receptor usage of MV strains (Tatsuo *et al*$, 2000*; +ielsen *et al.,* 2001 and Vongpunsawad *et al*., 2004).

The virus uses its own H surface glycoprotein to attach and absorb to the surface receptor CD46, SLAM and perhaps another receptor. Morbilliviruses do not have neuraminidase on their surface as a glycoprotein; therefore they can't use sialic acid as a receptor. After attachment, morbilliviruses use the other surface glycoprotein, the F protein, to fuse with the cellular plasma membrane found on the cell surface at a neutral pH and release the helical nucleocapsids into the cytoplasm of the host cell (Anthony *et al.,* 2012; Lu *et al.,* 2013).

1.5.1 Ribonucleoprotein (RNP) Complex

Inside the envelope lies the RNA genome encapsidated by the N protein within a helical nucleocapsid to form the N: RNA template (Kingston *et al.,* 2004). The phospho- (P) and large (L) proteins make up the RNA-dependent RNA polymerase (RdRp) that carries out transcription and replication on the N: RNA template. The P protein binds to the N protein in the C-terminal moiety of the N protein to direct template synthesis, and the N-terminal moiety encodes for all regions necessary for self-assembly and RNA binding (Figure 1-2) (Bankamp *et al.*, 199(; , arlin *et al.,* 2003). The RdRp is a part of the RNP complex and works in transcription and translation (Robinson, 1971).

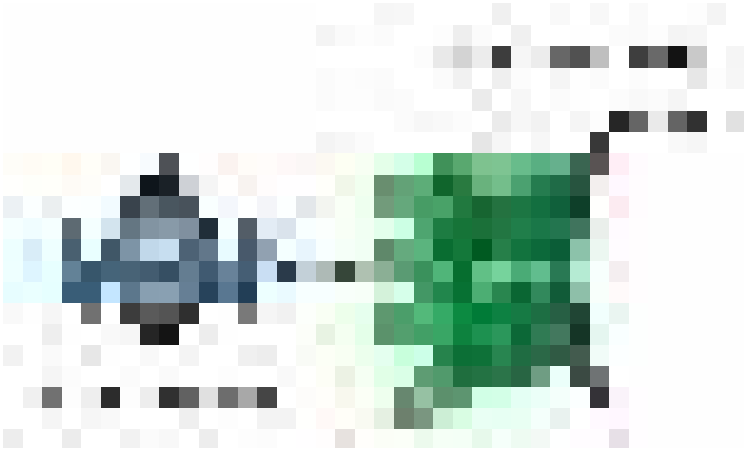

Figure (1-2): Schematic diagram of the ribonucleoprotein complex which is involved in all aspects of viral transcription and replication. The nucleocapsid binding domain, located in the C-terminal region of the N protein, binds the phosphoprotein which associates with the large protein. All together, this complex comprises all the catalytic activity of the virus (Kingston *et al.,* 2004).

1.5.2 Viral Transcription (mRNA Synthesis)

Transcription of the paramyxovirus genome RNA occurs from the 3' end to the 5' end of the genome such that the mRNAs are synthesized sequentially in the order N, P/C, M, F, H, and L (Vulliemoz and Roux, 2001). Before the synthesis of the N mRNA the negative-strand

RNAvirus, have been shown to synthesize both *in vivo* and *in vitro* a short leader RNA (45 to 55nucleotides) which was initiated precisely at the 3' end of the genome (Leppert *et al.,* 1979). In contrast, no leader RNA has been detected in measles virus-infected cells. Instead, Castaneda and Wong (1990) have shown that a polyadenylated leader-N read through RNA was present in measles virus-infected cells. This RNA was, however, encapsidated in N protein and was apparently not translated, since it was not associated with polysomes. These observations led to the hypothesis that, unlike other negative-strand viruses, measles virus may have two RNA initiation sites, one directly at the 3' end for the synthesis of the positive-strand replicative RNA and a second at the beginning of the N gene for productive mRNA synthesis (Grant, 2008). The editing activity results in the insertion of a non template G nucleotide at a specific site during transcription of the P gene which results in a frame shift and access to a different coding region. For measles, the P gene mRNA codes for the P protein, while the edited mRNA encodes the cysteine-rich V protein (Brown *et al.,* 2005).

In the other paramyxoviruses, Simian virus 5, Mumps virus, and the Parainfluenza viruses, the P gene mRNA codes for the V protein and the edited version of the mRNA is translated into the P protein. Cattaneo *et al*. (1989) have deduced a consensus editing sequence: 3' UURRR CCC GUXRCR 5', in which R is any purine and X is any nucleotide, based on the various viral P gene sequences, some of which were subsequently shown to be edited *in vivo.* It is postulated that the G insertion(s) occurs via a stuttering mechanism by the viral polymerase at the site of at least three consecutive C nucleotides in the genomic sequence (Vidal *et al.,* 1990).

1.5.3 Genome Replication

The genome (negative polarity) replicates by a full-length antigenome, which is a complimentary copy and the antigenome is only found in an assembled from inside the nucleocapsid, similar to the genome (Garcin *et al.,* 1999). Antigenomes can represent 5% to 20% of the genome-sized RNAs in virus particles, but antigenomes contain no open reading frames (ORFs) and no mRNAs are known to be transcribed from them. Antigenomes are thought to serve the specific purpose by acting as an intermediate and act as the template for genome replication. Also, short trailer RNAs that are expressed from the antigenome 3' end are thought to prevent the host cell from undergoing programmed cell death. The nucleocapsid plays an important role in mediating the interaction between the RdRp and the genome (Galinski, 1991). It has been demonstrated in an experiment with Sendai viruses that "N associates with P protein to prevent non-specific binding of cellular RNA and co-expression of N and P prevent the encapsidation of non-specific RNA by N" (Curran *et al.*,1995).

1.5.4 Virion Assembly and Release

The site of intracellular nucleocapsid assembly is in the cytoplasm, just like all other events in the life cycle of the viruses in the family *Paramyxoviridae*, it is theorized that there are two steps in the assembly of nucleocapsids (Kingston *et al.,* 2004). The first step is the association of free N subunits with the genome or template RNA to form the helical RNP structure. The second step is the formation of the association between the P-L protein complexes that binds with the N: RNA complex to from the RNP structure or polymerase Figure (1-2).

Since paramyxovirus mRNAs are encapsidated, unlike the antigenomes, it has been concluded that the GP and AGP contain regions that encode for initiating encapsidation. Paramyxoviruses obtain their envelope by budding from the apical surface of the cell. The glycoproteins, H and F, are synthesized in the endoplasmic reticulum (ER) and undergo conformational maturation before they are transported by the secretory pathway to the surface to be incorporated on the outside of the envelope with newly budding virions. The mechanism of viral glycoprotein folding and conformational maturation is complicated and assisted by numerous folding enzymes and molecular chaperones. Generally, only correctly folded and assembled proteins are transported out of the ER to the Golgi apparatus where the carbohydrate chains may be modified and cleavage occurs before transport to the plasma membrane.

The mechanism for virus assembly at the plasma membrane is unknown, but the M protein is thought to play a major role in bringing the assembled ribonucleoprotein core to the appropriate areas on the plasma membrane to form a budding virion. Cellular proteins are highly excluded in virion assembly and with measles virus it has been demonstrated that the N protein distinguishes viral RNAs destined for encapsidation by the presence of a leader sequence (Castaneda and Wong, 1990). Therefore, it is assumed that the protein-protein interactions and the interaction of M with the cytoplasmic tails of the glycoproteins of F and H are extremely specific and critical for proper virion assembly and budding (Richard *et al.,* 2001).

1.6 Pathogenesis of measles

Measles is a classical disease, an important cause of childhood morbidity and mortality in developing countries and causes a contagious,

acute, and infectious disease characterized by high fever, cough, maculopapular rash and conjunctivitis (Rima and Duprex, 2006). Paradoxically, measles is also associated with the induction of strong MV-specific humoral and cellular immune responses, resulting in lifelong immunity (Duke and Mgone, 2003).

1.7 Measles virus vaccine strain

The MV vaccine strain was isolated in 1954 from the throat washings of an 11-years-old measles &atient named avid - dmonston .- "/, 1980; Hilleman, 2001). Although the wild type MV can result in potentially serious infectious disease, a live-attenuated measles virus (MV) strain derived from the Edmonston vaccine lineage (Schwarz strain) were derived from a clinical isolate by extensive passages through culture of chick embryo fibroblasts (Schwarz, 1962). This vaccine has been administered to hundreds of millions of children since the 1970s and is considered as one of the most effective and safe human vaccines. Furthermore, the MV genome is very stable and the reversion of vaccine strains to pathogenic forms has never been recorded (Cutts and Mar#owit0, 1994; 1 illeman, 2001; Griffin and Pan 2009).

1.8 Measles virus as oncolytic virotherapy

Historically, evidence of measles oncolytic activity was first provided by several case reports in the mid-twentieth century that documented the spontaneous regression of hematological cancers (ie, leukemias, Hodgkin's disease and Burkitt's lymphoma) after wild-type MV infection Figure (1-3) (Hernandez, 1949; Bluming and Ziegler, 1971; Gross, 1971; Zygiert, 1971; Pasquinucci, 1971 and Mota, 1973).

Liu *et al.*, (2007) noted that a number of viral strains including certain derivatives of the attenuated live measles virus Edmonston (MV-Edm) vaccine strain, demonstrate a propensity to preferentially infect, propagate in, and destroy cancerous tissues.

Infection by wild-type MV can result in potentially serious disease while on the other hand the measles vaccine strains have an excellent safety record with millions of vaccine doses having been safely administered in over 40 years of use (Cutts and Markowitz, 1994). In contrast to the wild-type MV which enters cells exclusively via the SLAM receptor, the attenuated Edmonston MV vaccine strain (MV-Edm) enters via CD46 and preferentially infects cells that overexpress the CD46 receptor, without significant cytopathic effect against cells expressing low receptor levels (Anderson *et al.*, 2004).

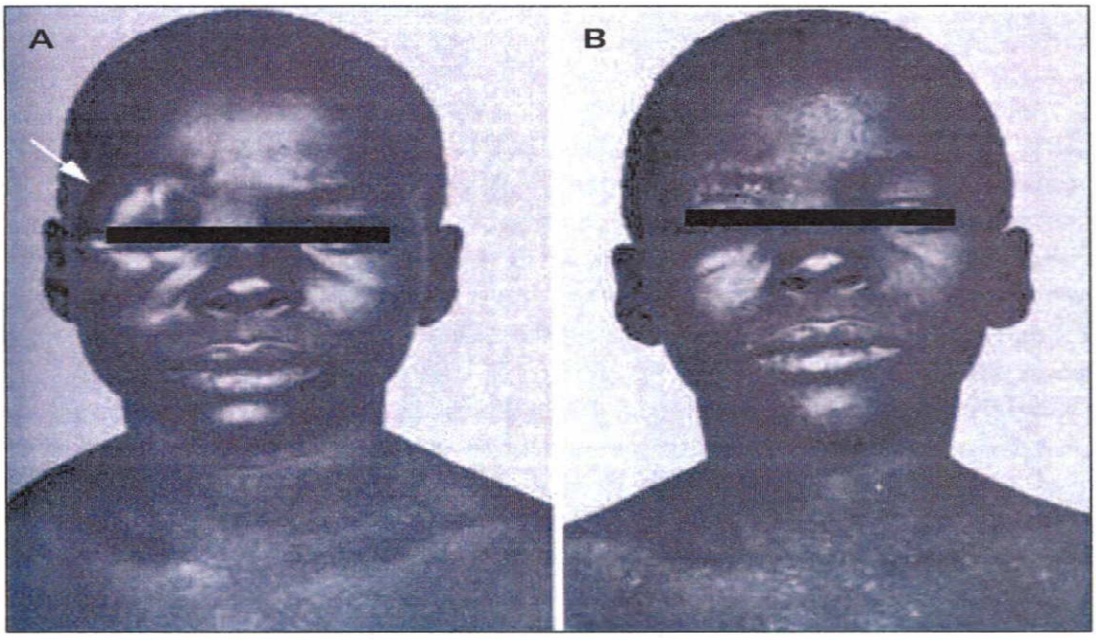

Figure (1-3): Burkitt's lymphoma regression following measles virus infection (A) an eight-year-old African boy with painless right orbital swelling due to Burkitt's lymphoma. **(B)** The appearance of generalized measles exanthema coincided with complete tumor regression. The patient remained in complete remission for 4 months after the measles infection (Bluming and Ziegler, 1971).

Measles virus has shown promising oncolytic properties (Fielding, 2005). The use of vectors derived from the Edmonston vaccine strain of measles virus (MV-Edm) have potent and selective oncolytic activity against a wide variety of human tumors including non-Hodgkin lymphoma (Grote *et al.*, 2001), multiple myeloma (Peng *et al.*, 2001), ovarian carcinoma (Peng *et al.*, 2002), cerebral glioma (Phuong *et al.*, 2003) and breast carcinoma (McDonald *et al.*, 2006) without any harmful effects on normal tissues. This tumor selectivity is partly dependent on high-level expression of CD46 by tumor cells (Anderson *et al.*, 2004; Ong *et al.*, 2006), the receptor preferentially used by these vectors for entry into cells (Schneider *et al.*, 2002).

It is known that CD46 is a trans-membrane complement regulatory protein that protects human cells against autologous complement lysis by acting as a cofactor in the proteolytic inactivation of C3b and C4b complement products. The overexpression of CD46 in tumors (Jurianz *et al.*, 1999; 2ishelson *et al.*, 2003), serves as a mechanism of tumor cell protection against complement mediated lysis. Thus, it has been shown that MV-Edm derivatives are tumor-selective, but cause minimal cytopathic effect in non-transformed cells, with low expression level of CD46 including normal ovarian surface epithelial cells, hepatocytes, dermal fibroblasts, astrocytes, mesothelial cells, peripheral blood lymphocytes and coronary artery smooth muscle cells. So the difference in CD46 expression levels between tumor and normal cells represents a key mechanism explaining oncolytic specificity of measles derivatives (Peng *et al.*, 2002; Phuong *et al.*, 2003; Anderson *et al.*, 2004 and Blechacz *et al.*, 2006).

1.9 Apoptosis and Cancer

Apoptosis is a physiological process that eliminates harmful and severely damaged cells and maintains tissue homeostasis in multicellular organisms (Young *et al.,* 1991). The process is defined based on changes in cellular morphology and biochemical features, including chromatin condensation, DNA fragmentation, cytoplasm vacuolation, plasma membrane blebbing, and cell shrinkage. Eventually, the cells breaks into small membrane surrounded fragments (apoptotic bodies), which are cleared by phagocytosis without inciting an inflammatory response (Kerr *et al* ., 1912). The release of apoptotic bodies is what inspired the term "apoptosis" from the Greek, meaning "to fall away from" and conjuring notions of the falling of leaves in the autumn from deciduous trees (Kerr *et al.,* 1972).

In mammalian cells, apoptosis occurs through two distinct molecular pathways, the intrinsic or mitochondrial pathway which activated by intracellular events and depends on the release of proapoptotic factors from the mitochondria. Standard chemotherapy and radiotherapy for cancer predominately initiate apoptosis via the intrinsic pathway and thus may positively select for cancer cells that can evade intrinsic apoptosis signaling (Estaquier *et al.,* 2012). By contrast, the extrinsic apoptosis pathway receives signals through the binding of extracellular protein death ligands to proapoptotic death receptors (DRs). In some cancer cells following extrinsic apoptosis signaling, cell death can still occur in the absence of intrinsic apoptosis. Both pathways lead to the hierarchical activation of specialized proteases called caspases (Sayers, 2011). Thomberry and Lazebnik (1995) stated the apoptotic process is executed mainly by a family of cysteine proteases called caspases. The activation of a family of intracellular cysteine proteases which cleave their

substrates at aspartic acid residues, known as caspases for Cysteine Aspartyl specific Proteases (Alnemri *et al.,* 1996). These proteases are present as inactive zymogens in essentially all animal cells, but can be triggered to assume active states, generally involving their proteolytic processing at conserved aspartic acid (Asp) residues.

Apoptosis, being a gene controlled process is susceptible to disruption by mutations (Lowe and Lin, 2000). Hence any deregulation in apoptosis might lead to uncontrolled cellular growth (cancer), such as loss of proapoptotic factors p53 and Bax, or the over expression of antiapoptotic factors like Bcl-2, will reduce intrinsic apoptosis signaling, thus preventing the efficient elimination of transformed cells. This critical relationship between apoptosis and cancer signifies that, any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells will have potential therapeutic effect (Reed, 1999). So apoptosis is essential to maintain tissue homeostasis for all organ systems in the human body (Thongrakard & Tencomnao, 2010). Suppression of apoptosis in carcinogenesis plays a central role in the development and progression of cancer. Tumor cells use a variety of molecular mechanisms to suppress apoptosis. Hence, induction of apoptosis in tumor cells is a specific therapeutic approach towards cancer therapy (Elmore, 2007).

1.9.1 Measles virus and Apoptosis

A number of viruses have recently been shown to cause cell death by induction of apoptosis (Meyaard *et al.,* 1992; Jeurissen *et al.,* 1992; Levine *et al.,* 1993; Gougeon *et al.,* 1993 and Lisa *et al.,* 1995). Often, viruses that cause persistent infection have developed mechanisms to prevent or delay the induction of apoptosis or infect cells resistant to virus-induced cell death (Rao *et al.,* 1992; Henderson *et al.,* 1993). Measles virus causes both lytic and persistent infections *in vivo* and *in*

vitro. The characteristic cytopathic effect of measles virus infections *in vitro* and *in vivo* is the production of syncytia, or multinucleated giant cells .- nders and "ee*les, 1934; 1enderson *et al.*, 1993). Measles virus induces apoptosis of infected cells and that nuclei of cells recruited into syncytia are initially normal and then develop evidence of endonucleolytic cleavage of chromosomal DNA. When Enders and Peebles first isolated measles virus in 1954, they described the cytotoxicity as including a ‘‘‘redistribution of the chromatin,’’’ which then ‘‘assumed a marginal position where it formed a dense ring or crescent’’ (Enders and Peebles, 1954). In 1958, examination of autopsy tissue obtained from patients dying with measles revealed condensed peripheral chromatin and shrinking and clumping of the chromatin at the base of the syncytial cytoplasm (Sherman and Ruckle, 1958).

Additionally, Nichols *et al.*, (1965) studied chromosome damage with measles virus *in vitro* in 1965 and reported pulverization of chromosomes particularly within the syncytia. Darzynkiewics *et al.*, (1992) showed that measles virus-infected cells cause distinctive condensation and margination of chromatin, with mitochondrial preservation characteristic of apoptosis. The helical nucleocapsid structure of measles virus was readily seen in the cytoplasm of some cells which also showed the membrane associated condensed chromatin. Cells fragmented into apoptotic bodies (clusters of membrane-bound segments), some of which contained dense balls of chromatin (Bhaskar *et al.*, 2011).

Danhua *et al.*, (2012) showed that MV treatment resulted in statistically significant reduction in tumor growth, increased apoptosis of lung cancer cells *in vitro* and *in vivo*, and increased infiltration of lymphocytes, while significantly prolonging the survival of tumor-bearing animals. In addition, there was no obvious undesired toxicity

following treatment. The intratumoral delivery of MV may be a promising strategy for the treatment of lung cancer. Further studies involving this treatment strategy, used alone or in combination with chemotherapy and biotherapy, warrant consideration. Live attenuated measles vaccine may be used as a novel type of anticancer drug.

2 Materials and Methods:

2.1 Materials:

2.1.1 Apparatus and equipments:

The following apparatus and equipments were used in this study:

No.	Equipment or Apparatus	Company	Origin
1	96-well flat-bottomed micro titration plates	Iwaki	Japan
2	Plastic tissue culture tube (15) ml		
3	Pap pen	Daido sangyo-tokyo	
4	Inverted microscope	Olympus	
5	Light microscope		
6	Beaker	Santa Cruze	USA
7	Glass tissue culture Petri dish		
8	Glass culture bottle		
9	Graduated cylinder		
10	Fluorescent microscope	Lumin	
11	Fluorescent microscope digital camera		
12	Nalgene filter units, pore size 0.22 µm	Nalge	
13	Nalgene Millipore filters, pore size 0.22 µm		
14	Light microscope digital camera	LW-Scientific	
15	Liquid nitrogen container	Union Carbide	
16	Whatman filter papers No.1	What man	
17	Magnetic stir bar	Science Lab	
18	Distillator	Running waters	
19	Parafilm	Bemis	
20	Plastic tissue culture flasks (25 cm^2)	Nunc	Denmark
21	Plastic tissue culture Petri dish		
22	Disposable sterile syringes (5 ml)	Medeco	UAE

No.	Equipment or Apparatus	Company	Origin
23	Disposable sterile syringes (1 ml)	Medeco	UAE
24	Cooled Centrifuge	Hettich	Germany
25	Water bath	Memmert	
26	Incubator		
27	Laminar air flow cabinet	K&K	S. Korea
28	Electrical oven		
29	Magnetic stirrer	Gallenkamp	UK
30	pH-meter		
31	ELISA micro well system micro plate reader	Asyshitech	
32	Sensitive balance	Ohous	Swisland
33	Cover slips (22*22mm)	Apel	China
34	Microscope glass slides	Afco	
35	Shaker	Cyan	Belgium
36	Micro- pipette (2-20 µl)	Cypress	
37	Micro- pipette (10-100 µl)		
38	Micro- pipette (100-1000 µl)		
39	Micro- pipette (1000-5000µl)		
40	Disposable Tips		
41	Deep freeze (-80ºC)	Nuve	Turkey
42	Autoclave	GSL	
43	Disposable sterile cell scraper	Geriner bio one	Germany
44	Improved Double Neubauer Ruling Counting Chamber	Assistent	
45	Cryo tube	Simport	Canada
46	Refrigerator	-	-
47	Vacuum pump	Franklin elective	-

2.1.2 Chemicals and biological materials:

No.	Chemical or biological material	Company	Origin
1	Streptomycin	TROGE	Germany
2	Ampicillin		
3	PDX mountant	Fluka	
4	Ethanol 99%	BDH	UK
5	DMSO (Di Methyl Sulf Oxide)		
6	Di sodium hydrogen phosphate (Na_2HPO_4)		
7	Potassium chloride (KCl)		
8	Sodium bicarbonate (NaHCO3)		
9	Trypan blue stain	Pharmacia Fine Chemical Uppsala	Sweden
10	Potassium dihydrogen phosphate (KH_2PO_4)	GCC	
11	Methyl thiazolyl tetrazolium (MTT)	Sigma-Aldrich	USA
12	Fetal calf serum	Cellgrew	
13	Trypsin EDTA	USBiological	
14	Formaldehyde 37%	Santa Cruze	
15	Hydrogen peroxide (H_2O_2)	-	Iraq
16	Hematoxylin	Syrbio	SAR
17	Eosin		
18	Xylene	Scharlau	Spain
19	Sodium chloride (NaCl)	Thomas baker	India

2.1.3 Tissue culture media:

No.	Culture medium	Company	Origin
1	Rosswell Park Memorial Institute, RPMI-1640	US Biological	USA
2	Minimum Essential Medium, MEM		

2.1.4 Kits:

No.	Kit	Company	Origin
1	Measles H monoclonal antibody	Santa Cruze Biotechnology	USA
2	ImmunoCruz mouse ABC Staining System		
3	Apoptosis detection, Mitochondria	US Biological	

2.1.5 Vaccine strain:

No.	Vaccine strain	Company	Origin
1	Live hyper attenuated virus vaccine against measles (Schwarz strain)	Aventis Pasteur S.A.	Lyon France

2.1.6 Cell Lines:

2.1.6.1 Rhabdomyosarcoma (RD) Cell Line:

This human cell line was derived from a biopsy specimen obtained from a pelvic rhabdomyosarcoma of a 7-year-old Caucasian girl (McAllister *et al.*, 1969; Johnston and Siegel, 1990). Passages 75-77 of RD cell line were used throughout this study and MEM media with 10% FCS was used in maintaining the cells. The culture of these cells was supplied by Iraqi Center for Cancer and Medical Genetic Researches (ICCMGR), experimental therapy department.

2.1.6.2 Glioblastoma-Multiform Cell Line:

This human tumor culture has come from a human cerebral glioblastoma multiform (GBM) obtained from a 72-year-old Iraqi male who underwent surgery for intracranial tumor. Passage 38 of Glioblastoma Multiform cell culture was used in this study and the cells were grown in RPMI-1640 with 20% FCS. The culture of these cells was supplied by Iraqi center for cancer and medical genetic researches (ICCMGR), experimental therapy department.

2.1.6.3 Vero Cell Line:

The Vero cell line was initiated from the kidney of a normal adult African green monkey in 1962. In this study, passages 88-89 were used and the cells were maintained in MEM with 10% FCS. The culture of these cells was supplied by Iraqi center for cancer and medical genetic researches (ICCMGR), experimental therapy department.

2.2 Methods:

2.2.1 Preparation of solutions for cell cultures:

2.2.1.1 Antibiotics:

- Streptomycin: 1 g of streptomycin was dissolved in 5ml triple distilled water, and 0.5ml of it was added to 1 litter of culture media.
- Ampicillin: 1000000 IU of ampicillin was dissolved in 5ml triple distill water, and 1ml of it was added to 1 litter of culture media (Freshney, 2000).

2.2.1.2 Sodium Bicarbonate:

The solution was prepared by dissolving 2.2 g of Sodium bicarbonate in one liter of culture medium (Freshney, 2000).

2.2.1.3 Phosphate buffer Saline PBS (pH 7.2):

It was prepared by dissolving 8 g NaCl, 0.2g KCl, 0.92 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1L triple distilled water and stirred constantly on a magnetic stirrer at room temperature; the pH was adjusted to 7.2 and autoclaved at 121^oC for 15 min and stored at 4^oC until used (Freshney, 1994).

2.2.1.4 Fetal calf serum:

The serum was used directly for tissue culture media as required.

2.2.1.5 Trypsin-EDTA Solution:

Trypsin-EDTA Solution was prepared as recommended by manufacturing company, by dissolving 10.1g of trypsin-EDTA powder

and 1g sodium bicarbonate in one liter of triple distal water. One ml of ampicillin and 0.5 ml of streptomycin were added. The solution was stirred constantly on a magnetic stirrer at room temperature and sterilized by Nalgen filter 0.22µm unit. Finally the solution was stored in the refrigerator and used within a short period of time (1-3 months).

2.2.2 Preparation of stain solutions:

2.2.2.1 Trypan Blue Solution:

A concentration of 1% was prepared as described by Yaseen (1990). One gram of trypan blue was dissolved in 100 ml PBS. Excess solid residue was filtered off using Whatman No.1 filter paper and stored at 4°C till use.

2.2.2.2 Methyl thiazolyl tetrazolium (MTT) Solution:

A quantity of 200 mg of Methyl thiazolyl tetrazolium was dissolved in 100 ml of PBS in order to prepare a concentration 2 mg/ml of the dye (Galvis *et al*., 2002). The solution was filtered through 0.22 µm Nalgene Millipore filter to remove any blue formazan product as recommended by Denizot and Lang (1986), and then stored in sterile, dark, screw-capped bottles at 4°C. The solution was used within no longer than week of preparation.

2.2.2.3 Hematoxylin:

Hematoxylin stock solution was ready to use.

2.2.2.4 Eosin:

Eosin stock solution was ready to use.

2.2.3 Preparation of Tissue Culture Media:

Tissue culture media were prepared according to ICCMGR guidelines.

2.2.3.1 Rosswell Park Memorial Institute (RPMI)-1640 Medium:

RPMI-1640 culture media was prepared by dissolving 16.35 g powder of RPMI-1640 with HEPES buffer and L-glutamine in approximately 600

ml of triple distilled water (TDW) and then 2 g of sodium bicarbonate powder, 1 ml of ampicillin, 0.5 ml of streptomycin and 100 ml of fetal calf serum (FCS) were added. The volume was completed to one liter with TDW and the medium was sterilized using Nalgen filter unit (0.22 µm) and aliquot into sterile containers.

2.2.3.2 Minimum Essential Medium (MEM):

MEM culture medium was prepared by dissolving 16.65 g MEM powder with HEPES buffer and L-glutamine in approximately 600 ml of TDW. 2.2 g of sodium bicarbonate powder, 1 ml of Ampicillin, 0.5 ml of Streptomycin and 100 ml of fetal calf serum (FCS) were added. The volume was completed to one liter with TDW and the medium was sterilized using Nalgen filter (0.22 µm) unit. Finally, the medium was aliquot into sterile containers.

2.2.3.3 Preparation Serum Free Medium (SFM):

It was either RPMI-1640 or MEM (prepared as described in 2.2.3.1 and 2.2.3.2) without FCS.

2.2.4 Viable Cell Count:

Method of Freshney (2000) was used to count the cell viability by using trypan blue dye which allows distinguishing between live cells with uncompromised membrane integrity (unstained) and dead ones (stained blue) as below:

- Cell suspension was prepared by trypsinization and resuspension in culture medium.
- Cover slip was fixed on a clean heamocytometer Improved Double Naubauer Ruling Counting Chamber.
- Two hundred µl of cell suspensions were mixed with 200µl of trypan blue solution and 1600 µl of PBS, twenty µl of the diluted cell suspension was transferred to the edge of the cover slip, along running into the counting chamber.

- A light microscope was used to count the cells under magnification powers 40X.
- The following equation was then used to calculate the number of cells per unit volume (cells/ml):

$$\mathbf{C = N \times D \times 10^4}$$

Where C is the cell concentration per milliliter, N is the number of viable cells counted, and D is the dilution factor (= 10) (Freshney, 1994).

2.2.5 Maintenance of cell lines *in vitro*:

Cell lines were sub cultured when monolayers were confluent. The growth medium was decanted off and the cell washed once with 2 ml of trypsin-EDTA solution. Two to three ml of trypsin - EDTA were added to the cell and the flask rocked gently, part of it was decanted again to obtain about one millilitre of trypsin-EDTA solution covering cell surface then the cells were incubated at 37°C for 1-2 minute until they had detached from the flask. Cells were dispensed in growth medium and then redistributed at the required concentration into culture flasks and incubated at 37°C (Al-Shammari, 2003).

2.2.6 Virus propagation:

2.2.6.1 Virus preparation:

Each vaccine dose contains lyophilized live hyper attenuated measles virus Schwarz strain at least 1000 $CCID_{50}$ (cell culture infection dose 50 percent) and human albumin.

Virus prepared as recommended by manufacturing company by adding 5 ml of sterile D.W to dissolve the lyophilized powder and shake well before use. The five ml of vaccine solution was distributed in ten cryo tubes each tube containing 0.5 ml to avoid freezing and thawing then stored at -80 ^{O}C and protects from light. Each 0.1ml of vaccine solution

was diluted with 9.9 ml of serum free media immediately before virus first inoculation.

2.2.6.2 Cell infection:

In this study lives attenuated measles virus Schwarz strain was propagated in Vero, RD and GBM cells line after 70% confluence. After 1×10^5 Vero cells, 2×10^5 RD cells and 138×10^4 GBM cells in T25 flask at 37°C were formed, the medium was removed and 3 ml of diluted hyper attenuated live measles virus Schwarz strain solution was added for 2 h at room temperature with shaking to allow virus attachment and penetration. At the end of incubation period, the virus was discarded and the cells were maintained in standard medium. The cells were re-incubated at 37°C for 3-7 days (Msaouel *et al.*, 2009).

2.2.6.3 Virus harvesting:

Cells were scraped and freeze thawed three times when syncytia reached 80% to 90% confluence after 3-7 days of virus infection. Vero, RD and GBM cells in SFM centrifuged 3000 rpm for 30 minute at 4C˚. The pellet cellular debris was discarded and supernatant (concentrated inoculums) was distributed in several cryo tubes and stored at -80^{o}C (Al-Shammari, 2003).

2.2.6.4 Virus passaging:

Measles virus supernatant (concentrated inoculums) of first cycle was inoculated onto 10^6 Vero cells, 12×10^5 RD cells and 3×10^5 GBM cells in T25 flask at 37°C. The medium was removed and 3 ml of concentrated inoculums was added for 2 h at room temperature with shaking to allow virus attachment and penetration. At the end of incubation period, the virus was discarded and the cells were maintained in standard medium. The cells were re-incubated at 37°C for 3-7 days. The second cycle of viral replication was accomplished within 24 h after infection when cell fusion was started. The whole monolayer appeared to be covered with

syncytia after three to seven days (Al-Shammari, 2003). Five cycles of viral replication was made to increase the quantity of measles virus supernatant. The titers of viral stocks were determined by 50% end-point dilution assays (TCID50) on Vero cells in 96-well plates (Anne *et al.*, 2008).

2.2.7 Calculation of 50% end-point dilution assays (TCID50): (Spearman, 190% Ka¨rber, 1931)

2.2.7.1 Cell seeding:

- Vero cells were detached from their flask when they reached to confluent monolayer by trypsinization as described previously (2.2.5.). Then 20 ml of culture medium with 10% FCS were added to the flasks and mixed gently to prepare cell suspension.
- Two hundred µl of cell suspension was transferred by using micropipette to each well in micro titration plate (each well contain approximately $1.5x10^5$ cell/well) then the plate was covered with a sterile adhesive film, was shaked gently for few minutes and incubated at 37^oC for 24 h.

2.2.7.2 Virus exposure:

The micro titer plate was examined under the inverted microscope to be sure that the confluent monolayer was formed. Two hundreds of serially diluted virus in MEM media at ratio 1:2 to 1:256 was added to each well. All tubes were fixed on ice pack during the treatment. Virus was added to the titer plate as follows: six wells were used for each dilution and control, since the ends of the plate was left. Plates were incubated in humidified incubator at 37^oC for 6 days of infection, each well with syncytia was marked as positive.

- Cell infection with MV was done by removing the medium from the micro titration plate first by pouring it off aseptically. Then, 0.2 ml of each dilution of MV was added to each well, six replicates were used

for each dilution as well as the control cells which were treated with cold SFM only.

- MV was added for 2 h at room temperature with shaking to allow virus attachment and penetration. After that, the solution was removed from each well using micropipette as fast as possible. Cells were washed with PBS and 0.2 ml of warmed SFM was added. The plate was covered again with a new sterile adhesive film, and sealed with parafilm.
- The plates were re-incubated at 37°C for six days or until the syncytia were observed.

2.2.7.3 Calculation of TCID50% dose:

Virus was titrated on Vero cells, and the 50% tissue-culture infectious dose (TCID50) was calculated according to the method of Spearman (1908) and Ka¨rber (1931). It was achieved by using 96- well flat bottomed micro titration plates. When the syncytia appeared after six days of virus infection, each well with syncytia is marked positive and Virus titer calculated by use this equation:

$\log10\ (TCID_{50}/ml) = L + d\ (s-0.5) + \log\ (1/v)$

L = negative log10 of the most concentrated virus dilution tested in which all wells are positive

d =log10 dilution factor

s = sum of individual proportions pi

pi = calculation proportion of an individual dilution

(Amount of positive wells/total amount of wells per dilution)

v = volume of inoculum (ml/well).

TCID 50 % for MV was 3.4×10^5.

2.2.8 Studying the cytopathic effect of Measles virus and hemadsorption test: (Haas *et al.*, 1998).

2.2.8.1 Cells seeding:

Cancer cells (RD and GBM) and Vero cells were dispersed with trypsin-EDTA, and suspended in growth media with 10% FCS, and 1-3 ml of cell suspension ($1x10^5$cells/dish) was seeded in plastic tissue culture Petri dishes that containing sterile cover slips. Cell were incubated at 37^{o}C for 24 h or until confluent monolayer is formed (Freshney, 2010).

2.2.8.2 Cells infection:

The procedure of infection with MV was carried out by removing the old media that containing serum and adding 1 ml of the virus solution concentrated inoculums ($3.4*10^5$) for 2 h at room temperature with shaking to allow virus attachment and penetration. After that, cells were washed with PBS and serum free medium was added and incubated for three days or until cytopathic effect was appeared (Al-Shammari, 2010).

2.2.8.3 Preparation of 1% human red blood cells solution:

Blood was collected from healthy person in heparinized tube and washed three times with PBS by centrifugation at 1000 rpm at 4C˚, the supernatant was discarded and 0.1 ml of cell pellet was transferred to sterile graduated tube and the volume was completed to 10 ml with PBS (Bell *et al.*, 1985).

2.2.8.4 Hem-adsorption of cell culture infected with Measles virus:

This procedure was carried out according to Haas *et al.* (1998) and as follows

- RD, GBM and Vero cell line infected with measles virus were washed three times with PBS after removing the old media.
- Aliquot of 1ml of 1% human red blood cells solution was added to cell sections.

- Cells were incubated with the RBCs solution for 30 min at 4^{O}C, and then washed two times with PBS to remove non adsorbed RBCs.
- Finally adsorbed RBCs were fixed by adding 1ml of 4% formaldehyde and cells were incubated for 30 min at 4^{O}C.
- This experiment was done in the present of control groups of cancer cells that not infected with MV.

2.2.8.5 Hematoxylin and Eosin staining: as ICCMGR guidelines' cancer researches department.

- Cell sections were immersed in hematoxylin solution for 5 minutes and washed in distilled water for three changes.
- Cell sections were immersed in eosin solution for 2-3 minutes.
- Cell sections were dehydrated by immersed in 95% ethanol for 1 minute. Then two changes were made in absolute ethanol for 2 minutes each.
- Clearing was made by adding xylene for 1minutes. Then absolute ethanol was added for two minutes to remove xylene. Finally cell sections were mounted with xylene based mounting medium (DPX).

2.2.9 Detection of measles virus H protein:

2.2.9.1 Preparation of ABC staining system working solution:

- **Blocking serum:** It was prepared by mixing 75 µl of normal blocking serum stock with 5 ml of PBS.
- **Biotinylated secondary antibody:** Seventy five µl of normal blocking serum stock was mixed with 5 ml of PBS and 25 µl biotinylated secondary antibody stock.
- **AB enzyme reagent:** It was prepared by mixing of 50 µl of reagent A (avidin), 50 µl of reagent B (biotinylated HRP) and 2.5 ml of PBS and left to stand for approximately 30 minutes.

- **Peroxidase substrate:** It was prepared by mixing of 1.6 ml of distilled water, 5 drops of 10x substrate buffer, 1 drop of 50x DAB chromogen and 1 drop of 50x peroxidase substrate.

2.2.9.2 Preparation of monoclonal antibody: Measles H (6016) is a mouse monoclonal antibody raised against the Edmonston strain of Measles virus. It was prepared by mixing 10 µl of monoclonal antibody stock solution with 490 µl of blocking serum working solution.

2.2.9.3 Preparation 1 % of Hydrogen peroxide H_2O_2: To prepared 1% H_2O_2 1ml of H_2O_2 stock solution was mixed with 99 ml of PBS.

2.2.9.4 Counter stain: Hematoxylin which was ready to use.

2.2.9.5 Mounting medium: PDX mounting ready to use.

2.2.9.6 Preparation of fixing reagent: Formalin solution (4%) was prepared by mixing 4 ml of stock Formaldehyde with 96ml PBS.

2.2.9.7 Preparation of washing solution: PBS was used for washing, and prepared as described in item 2.2.1.3.

2.2.10 Immunoperoxidase staining procedure for the detection of cell lines infected with measles virus:

This was done by using immunoperoxidase kit for staining cell lines infected with measles virus. This kit was designed for demonstrating the presence of MV.

The principle of the test is that the primary antibody of measles H (6016) monoclonal antibody binds to the H- protein of measles virus, and the secondary antibody bind to determinant on the primary antibody. Then the avidin containing the horseradish peroxidase enzyme was allowed to bind to the biotin molecule attached to the secondary antibody.

2.2.10.1 Cell infection:

After cancer cells (RD and GBM) and Vero cells were dispersed with trypsin-EDTA, and suspended in 10 ml growth media with 10% FCS the cells were seeded at 1 x 10^5 cells/dish in plastic tissue culture Petri dishes

that containing sterile cover slips, after 24 h or confluent monolayer is achieved. Cancer cells (RD and GBM) and Vero cells were infected with measles virus(concentrated inoculum), by adding 1 ml of MV to each Petri dish for 2 h at room temperature to allow virus attachment and penetration, then cells were washed with PBS and re-incubated with warmed serum free medium. After three days or until cytopathic effect appeared, cells were fixed with 4% formaldehyde for 10 min.

2.2.10.2 Staining procedure:

In all steps the sections were placed in the humid chamber and at room temperature (20-25 ºC)

- To inhibit endogenous peroxidase, cells were incubated with 1% hydrogen peroxide for 10-15 minutes and wash with PBS for 5 min three times.
- Aliquot of 1.5% blocking serum was added to cell section for one hour and then washed with three changes of PBS for 5 minutes. To decrease background staining.
- Cell sections were incubated with 125 µl of diluted primary antibody at a ratio(1:50) for one hour at room temperature or over night at 4ºC then washed with three changes of PBS for 5 minutes .
- Cell sections were incubated for 30 min with 1.2 ml of biotinylated secondary antibody (which prepared in 2.2.8.1 item) and washed with three changes of PBS for 5 minutes.
- A liquot of 650 µl of AB enzyme reagent (which prepared in 2.2.8.1 item) was added to cell section and washed two times with PBS for 2 min.
- Three drops of peroxidase substrate (which prepared in 2.2.8.1 item) was added to cells for 10 min, or until desired stain intensity develops and washed with distilled water for 5 minutes.

- Hematoxylin stain was added to cell section for 5-10 seconds and immediately washed with distilled water. Finally 1-2 drops of permanent mounting medium was added and examined by light microscopy at magnification powers 40x and 100x.

2.2.11 Studying the oncolytic effect of MV on tumor cells:

It was achieved by using 96- well flat-bottomed micro titration plates. This procedure includes three stages as previously described by Al-Shammari (2010).

2.2.11.1 Cell seeding stage:

- Cells of different cell lines (RD and GBM) were detached from their flask when they reached to confluent monolayer by trypsinization as described previously (2.2.5 item). Then 20 ml of culture medium with 10% FCS were added to the flasks and mixed gently to prepare cell suspension.
- Two hundred µl of cell suspension was transferred by use micropipette to each well in micro titration plate (each well contain approximately $1.5x10^5$ cell/well) then the plate was covered with a sterile adhesive film, shaked gently for few minutes and incubated at 37^oC for 24 h.

2.2.11.2 Exposure stage:

- The micro titer plate was examined under the inverted microscope to be sure that the confluent monolayer was formed. Concentrated inoculums and two fold dilutions of virus were prepared starting from 1:2 to 1:64. The dilutions of MV were done in sterile tubes using cold serum free medium (RPMI-1640 for the virus propagated in GBM cell line and MEM for the virus propagated in RD cell line). All tubes were fixed on ice pack during the treatment.

- Cell infection with MV was done by removing the medium from the micro titration plate by pouring it off aseptically. Then, 0.2 ml of each dilution of MV was added to each well, three replicates were used for each dilution as well as the control cells which were treated with cold SFM only.
- MV was added for 2 h at room temperature with shaking to allow virus attachment and penetration. After that, the solution was removed from each well using micropipette as fast as possible. Cells were washed with PBS and 0.2 ml of warmed SFM was added. The plate was covered again with a new sterile adhesive film, and sealed with parafilm.
- The plates were re-incubated at 37°C for 72 and 120 h.

2.2.11.3 Staining stage:

Cell viability was calculated after 72 and 120 h of infection by removing the medium, adding 28 µl of (2 mg/ml) MTT solution and incubating for 1.5 h at 37°C. After removing the MTT solution, the crystals remaining in the wells were dissolved by adding of 130 µl of DMSO (Dimethyl Sulphoxide). Plate was incubated at 37°C for 15 min with shaking and read at 550 nm by ELISA reader. The assay was performed in triplicate (Galvis *et al.,* 2002).

Endpoint parameters that were calculated for each individual cell line included:

1- Percentage of cell growth or percentage of cell proliferation (PR) = mean optical density of treatment / mean of optical density control X 100(Kamuhabwa *et al*., 2000).

2- The inhibiting rate of cell growth (the percentage of cytotoxicity) was calculated as G.I% = (A-B)/A X100, Where A is the mean optical density of untreated wells and B is the optical density of treated wells (Gao *et al*., &00' ; Galvis *et al*., 1999).

3- The lowest concentration that kills 50 % of cells was (LC50) (Takimoto, 2003).

2.2.12 Mitochondrial permeability transition apoptosis test:

Principle

Disruption of the mitochondrial transmembrane potential is one of the earliest intracellular events that occur following induction of apoptosis.The Bioassay™ Apoptosis Detection; Mitochondrial Kit provides a simple, fluorescent-based method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial transmembrane potential. The kit utilizes a cationic dye that fluoresces differently in healthy cells and in apoptotic cells. In healthy cells, the dye accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, the dye cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form giving green fluorescing. The fluorescent signals can be easily detected by fluorescence microscopy using a band-pass filter (detects FITC and rhodamine).

2.2.13 Apoptosis induction *in vitro*:

- RD and Glioblastoma cells were cultured in plastic tissue culture Petri dishes. After 70% confluence (18-24 h) RD, Glioblastoma cell lines were infected with measles virus for 48h, 72 h and 120 h as described in item (2.2.9.1). Each virus incubation period was done with three replicas.
- Prior to use: MitoCapture was diluted at a ratio (1:1000) by mixing 1µl of MitoCapture to 1 ml pre-warmed incubation buffer and mixing well by vortex. MitoCapture is poorly soluble in aqueous solutions. To remove non soluble particles the dye solution was centrifuged for 1

minute at 13,000 rpm and carefully transferred the supernatant without disturbing pellet debris, then 0.4 ml of the diluted MitoCapture solution was added to adherent cells and incubated at 37C° for 15-20 minutes in the dark.

- Cells were washed with pre-warmed incubation buffer three times, then 0.4 ml of the pre-warmed incubation buffer was added.
- Cells were examined immediately under a fluorescent microscope using a band-pass filter (detects fluorescein and rhodamine).

  MitoCapture aggregated in the mitochondria of healthy cells and giving red light. In apoptotic cells, MitoCapture cannot accumulate in the mitochondria, it remains as monomers in the cytoplasm, and giving green light.
- The results were calculated by counting apoptotic cells and using Image J program version 1.47. This program is image processing and analysis program. It can create density histograms and converted the images of MitoCapture to histograms by showing the number of pixels of each value, regardless of location, and the mean and max values. The log display allows for the visualization of minor components.
- It displays a histogram of the distribution of green values or red values in the active image. The X-axis represents the possible green values or red values and the Y-axis shows the number of pixels found for each green values or red values. The horizontal LUT bar below the X-axis is scaled to reflect the display range of the image that includes the total pixel Count, as well as the Mean, standard deviation (Std- Dev), minimum (Min), maximum (Max) and modal (Mode) green values or red values (Hand *et al.,* 2009).

2.2.14 Statistical Analysis:

One way ANOVA followed by Duncan was used within the SPSS version 20 program to analysis of data and studying the effect of MV on tumour cell lines at different incubation period and dilutions. The difference was considered significant when the probability value (P (0.05) (IBM, 2011).

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Summary

This study was carried out to evaluate the antitumor effect of live attenuated measles virus Schwarz (MV) vaccine strain on tumor cell lines *in vitro.* Live attenuated measles virus Schwarz vaccine strain was obtained from Aventis Pasteur, France. It was propagated on Vero, human Rhabdomyosarcoma **(**RD**)** and human Glioblastoma-Multiform (GBM) cell lines which were supplied by Iraqi Center for Cancer and Medical Genetic Researches (ICCMGR). Results revealed that cell fusion was occurred after 24 h of infection. The infected confluent monolayer appeared to be covered with syncytia with granulation and vaculation of cells after 72 to 120 h of infection. Moreover, the formation of large round empty plaque spaces was observed in infected cells. Results showed that after 72 h of exposure, alterations in morphology of Vero, RD and GBM cells were observed. Cells were rounded, shrinkage, clustered cells and large empty space with cell debris as a result of cell lysis and death. Haemadsorption effect of MV was studied and result recorded that all cell lines infected with virus have the ability for haemadsorption to human red blood cells after 72 h of infection. Detection of MV H protein by monoclonal antibodies in infected cells of all cell lines by immunocytochemistry assay gave positive results in cytoplasm of infected cells. Assessment of the MV antitumor effect on RD and GBM cell lines was carried out by using different dilutions of virus starting from 1:2 to 1:64 for 2 h at 37 ^{0}C. Cell viability was measured after 72 and 120 h of infection by MTT assay. Results showed a significant cytotoxic effect for measles virus ($P \leq 0.05$) on growth of RD and GBM cell lines at the dilution 1:2 and the 1:4 dilutions after 72 and 120 h of infection. When the dilution increases, there was a significant decline in the inhibitory effect with a significant cytotoxic effect as

compared with the control. Also concentrated inoculums of measles virus showed a significant cytotoxic effect when compared with the control. Induction of apoptosis by MV virus was assessed by measuring mitochondrial membrane potentials in RD and GMB after 48, 72 and 120h of infection by Mitocapture kit. Healthy cells gave a bright red fluorescent light. Apoptotic cells gave green fluorescent light. Apoptotic cells were counted and the mean percentage of dead cells was significantly higher (87.42, 93.69 and 97.36 *Vs.* 20.83) % after 48, 72 and 120 h of infection for RD when compared with control. A significant difference was recorded in the percentage of dead cells in GBM after 48, 72 and 120 h in comparison with control (89.62, 95.43 and 97.97 *Vs.* 20.83). Also results were analyzed by imageJ program and results exhibited that MV induce apoptosis in RD and GBM cell lines.

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List of abbreviations

MV	Measles virus
P protein	Phosphoprotein
L protein	Large protein
N protein	Nucleoprotein
M protein	Matrix protein
F protein	Fusion protein
H protein	Hemagglutinin protein
Tregs	T regulatory cells
DCA	Dichloroacetate
NDV	Newcastle disease virus
HN	Hemagglutinin-neuraminidase
VSV	Vesicular stomatitis virus
MV-Edm	Measles virus – Edmonston vaccine strain
NP	Nucleocapsid
V	Virulence factor
SSPE	Subacute sclerosing panencephalitis
SLAM	Signaling lymphocyte activation molecule
MDCK cells	Madine – Darby canine kidney cells

RdRp	RNA – dependent RNA polymerase
ORFs	Open reading frames
RNP	Ribonucleoprotein
ER	Endoplasmic reticulum
DRs	Death receptors
Asp	Aspartic acid
RD	Rhabdomysarcoma
GBM	Glioblastoma - multiform
MEM	Minimum essential medium
RPMI	Rosswell park memorial institute
ICCMGR	Iraq center for cancer and medical genetic researches
FCS	Fetal calf serum
DMSO	Di methyl sulfo oxide
MTT	Methyl thiazolyl tetrazolium
PBS	Phosphate buffer saline
TCID	Tissue culture infection dose
RBCs	Red blood cells
ELISA	Enzyme linked immuno-sorbent assay
FITC	Fluorescein isothiocyanate
H&E	Hematoxylin and eosin
ROS	Reactive oxygen species
IL-2	Interlukin-2
IFN-δ	Interferon - gamma
TGF-β	Transforming growth factor -beta

Supervisors Certification

We, certify that this thesis entitled **" *Role of Live Attenuated Measles Virus Schwarz Vaccine Strain as Anti Tumor Agent in Vitro* "** was prepared by **"Farah Essa Ismaeel"** under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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We, the examining committee certify that we have read this thesis entitled **" *Role of Live Attenuated Measles Virus Schwarz Vaccine Strain as Anti Tumor Agent in Vitro* "** and examined the student **"Farah Essa Ismaeel"** in its contents and that in our opinion, it is accepted for the Degree of Master of Science in Biotechnology,

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Title: Dean of College of Science
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3 Results and discussion

3.1 Propagation of measles virus on Vero, RD and GBM cell lines:

Results revealed that the first cycle of viral replication was not completed within the first 24 h of infection and at this time point only cell fusion was observed. Figures (3-1B, C, 3-2B, C and 3-3B, C) showed that, after 72 to 120 h the monolayer appeared to be covered with syncytia, granulation and vaculation of cells. Moreover, the formation of large round empty plaque spaces were observed in infected cells as compared with control cells (Figure 3-1A, Figure 3-2A and Figure 3-3A).

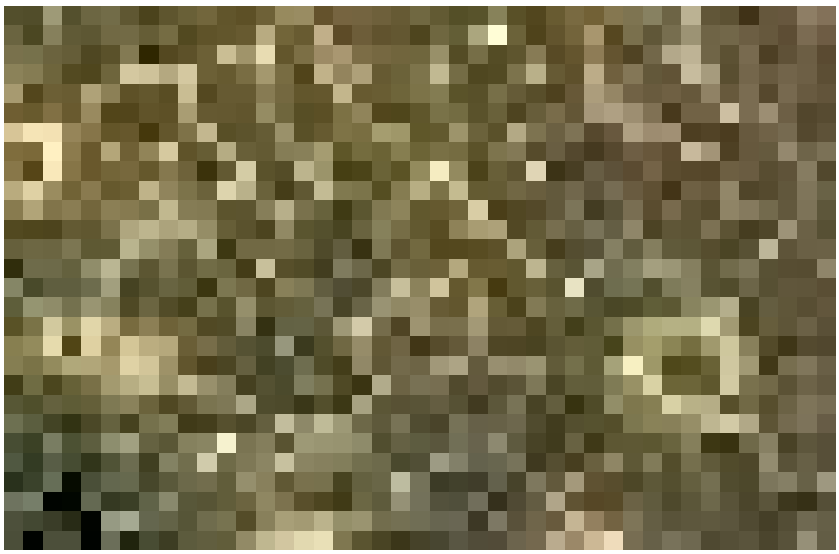


Figure (3-1A): Vero cell line untreated with measles virus 100X (Control).

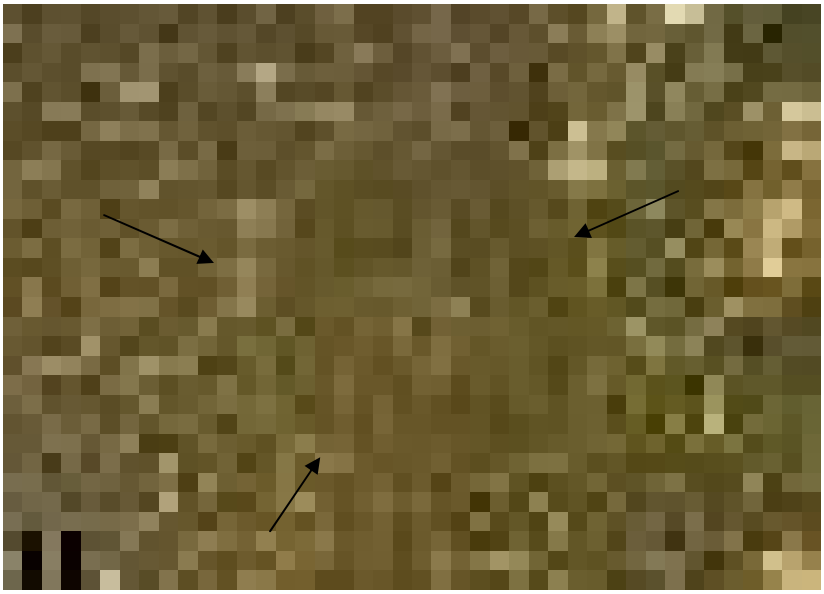


Figure (3-1B): Vero cells infected with measles virus showing syncytial formation and cell granulation after 72h of infection (Arrows) 40X.

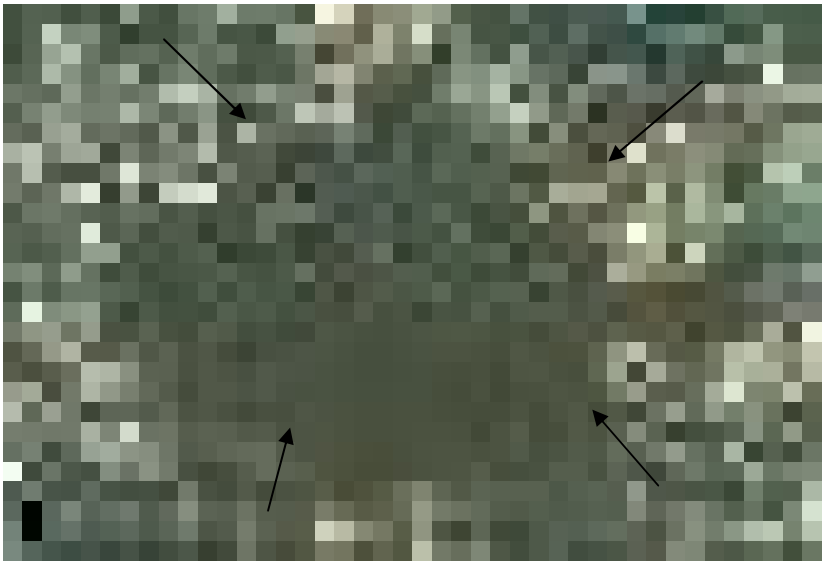


Figure (3-1C): Vero cells infected with measles virus showing syncytial formation and cell granulation after 120 h of infection (Arrows) 40X.

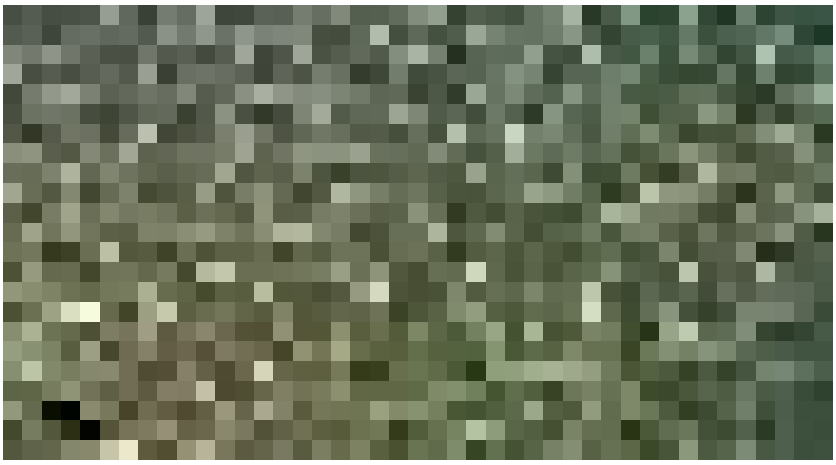


Figure (3-2A): RD cell line untreated with measles virus 100X (Control).

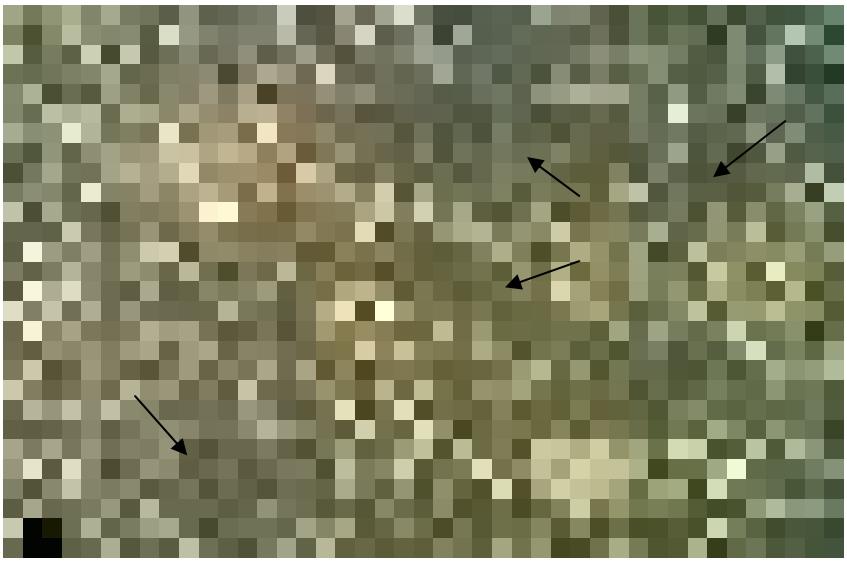


Figure (3-2B): RD cell line infected with measles virus showing syncytial formation and cell granulation after 72h of infection (Arrows) 40X.

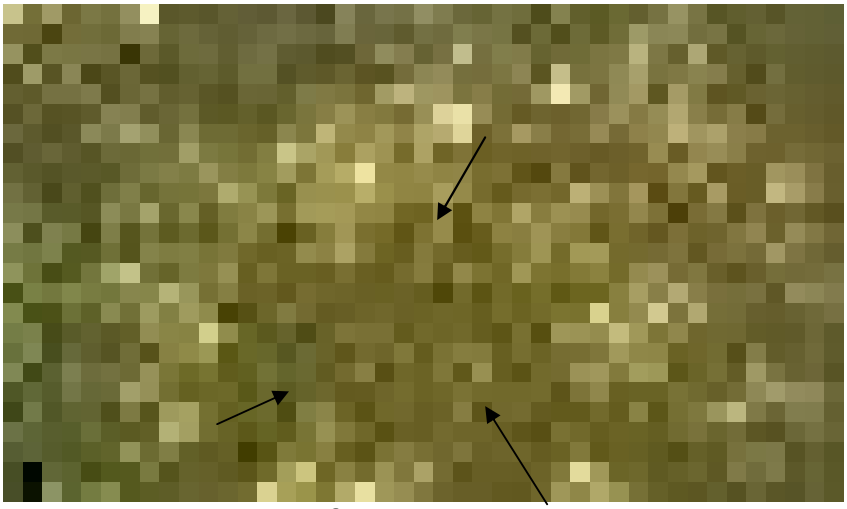


Figure (3-2C): RD cell line infected with measles virus showing syncytial formation and cell granulation after 120h of infection (Arrows) 40X.

Figure (3-3A): GBM cell untreated with measles virus 20X (Control).

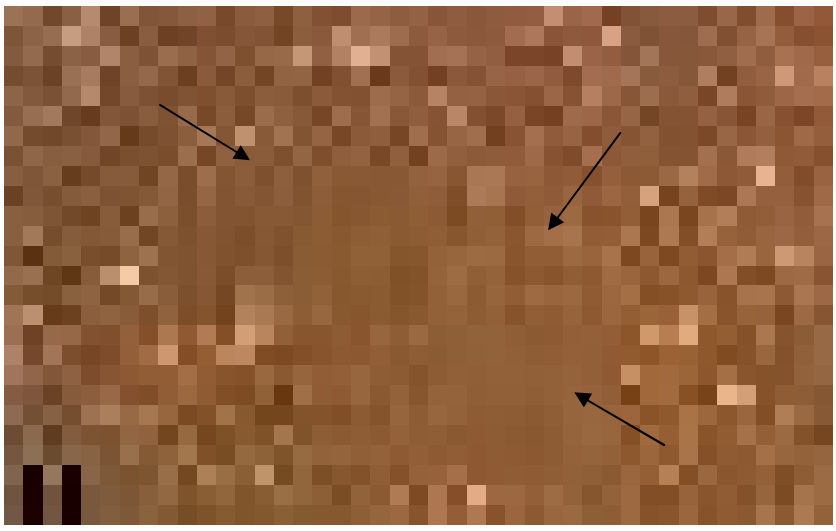


Figure (3-3B): GBM cell line infected with measles virus showing syncytial formation and cell granulation after 72 h of infection (Arrows) 40X.

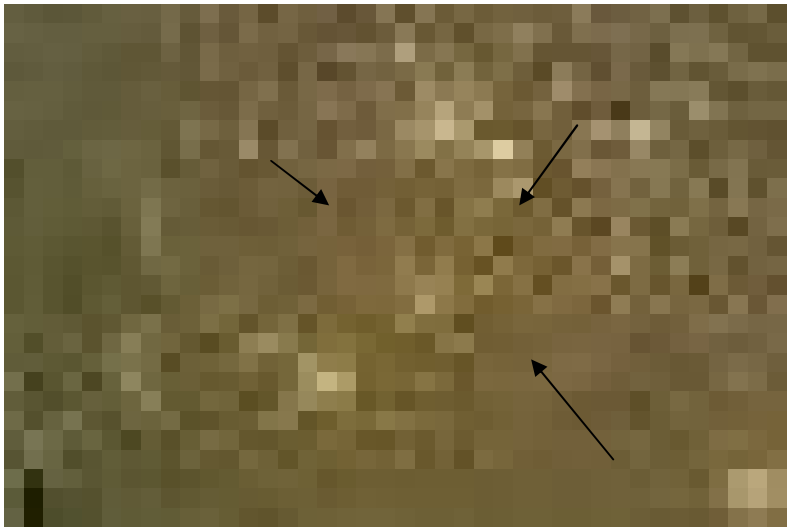


Figure (3-3C): GBM cell line infected with measles virus showing syncytial formation and cell granulation after 120 h of infection (Arrows) 40 X.

Results of virus propagation showed that the live attenuated measles virus vaccine was successfully cultivated and has been passed on Vero cells, RD cells and GBM cells. Vero cells are susceptible to infection by measles virus (Norrby, 1990). These viruses enter through the natural measles receptors CD46 and spread by cell–cell fusion causing giant syncytia formation and killing the tumor cells (Yanagi *et al*., 2006).

3.2 Cytopathic effect (CPE) of measles virus on tumor cell line *in vitro*:

The typical cytopathic effect of MV was the formation of mononuclear cell aggregates (syncytia) caused by cell–cell fusion (Wild *et al.,* 1991). The measles virus showed a high ability for propagation and lysis of the following Vero cells. After 72 h of exposure the alterations in Vero cells were included morphological characteristics: cell rounded shrinkage, cell clustering and large empty space appeared with cell debris as a result of cell lysis and death. While there was no morphological alteration was observed in uninfected cells (Figure 3-4A, B, C and D). MV infection was conducted to the same effect on RD cells (Figure 3-5A, B, C and D) and GBM cells (Figure 3-6A, B, C and D) as on Vero cells.

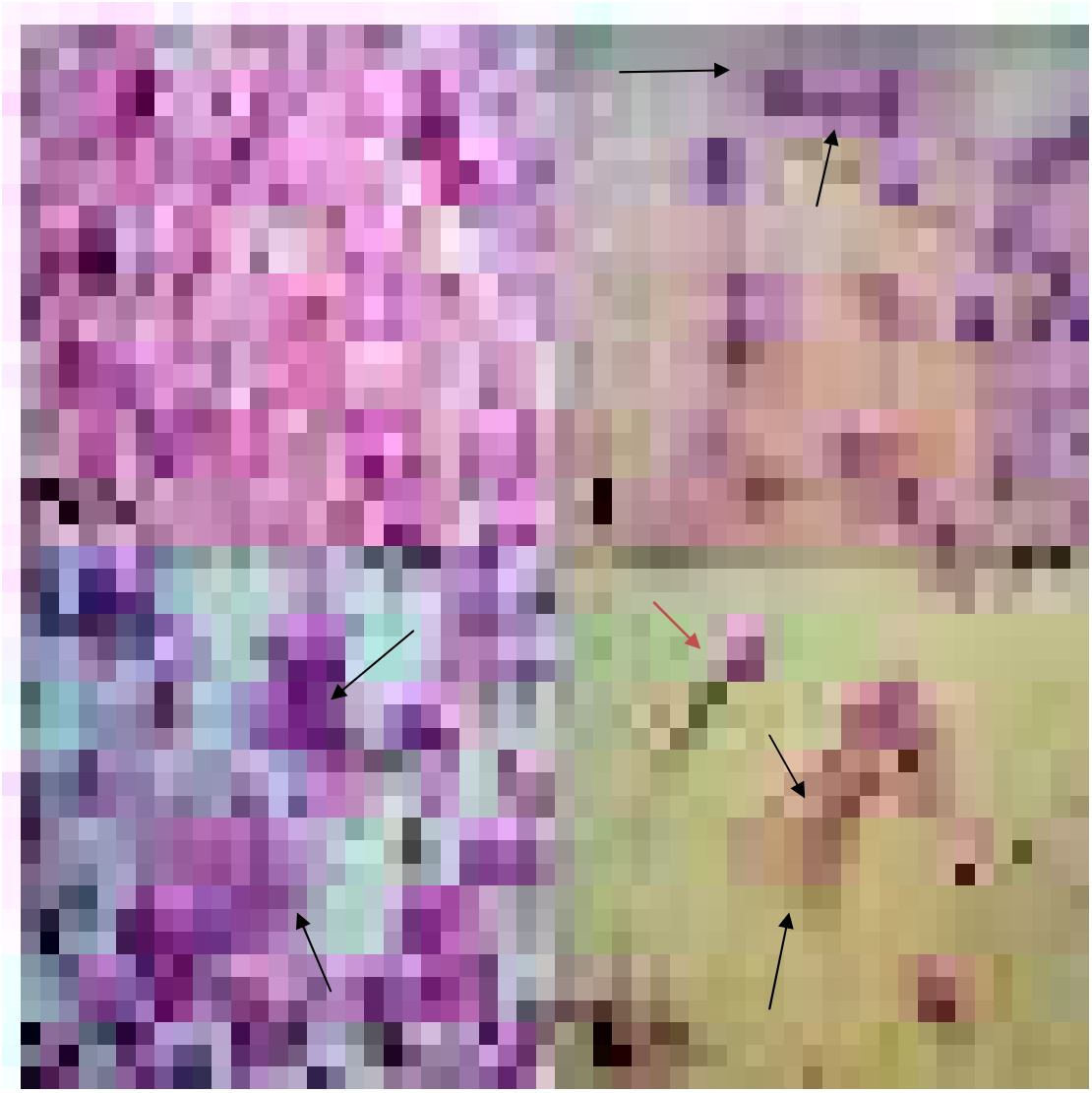


Figure (3-4): CPE of measles virus on Vero cell line. A. Vero cells uninfected with virus. B. showing cells fusion after 48 h of infection C. showing syncytia formation after 72 h D. Black Arrows showing syncytia formation and large empty space with cell debris after 120 h, while Red Arrows showing picnotic cells (H&E) 40X.

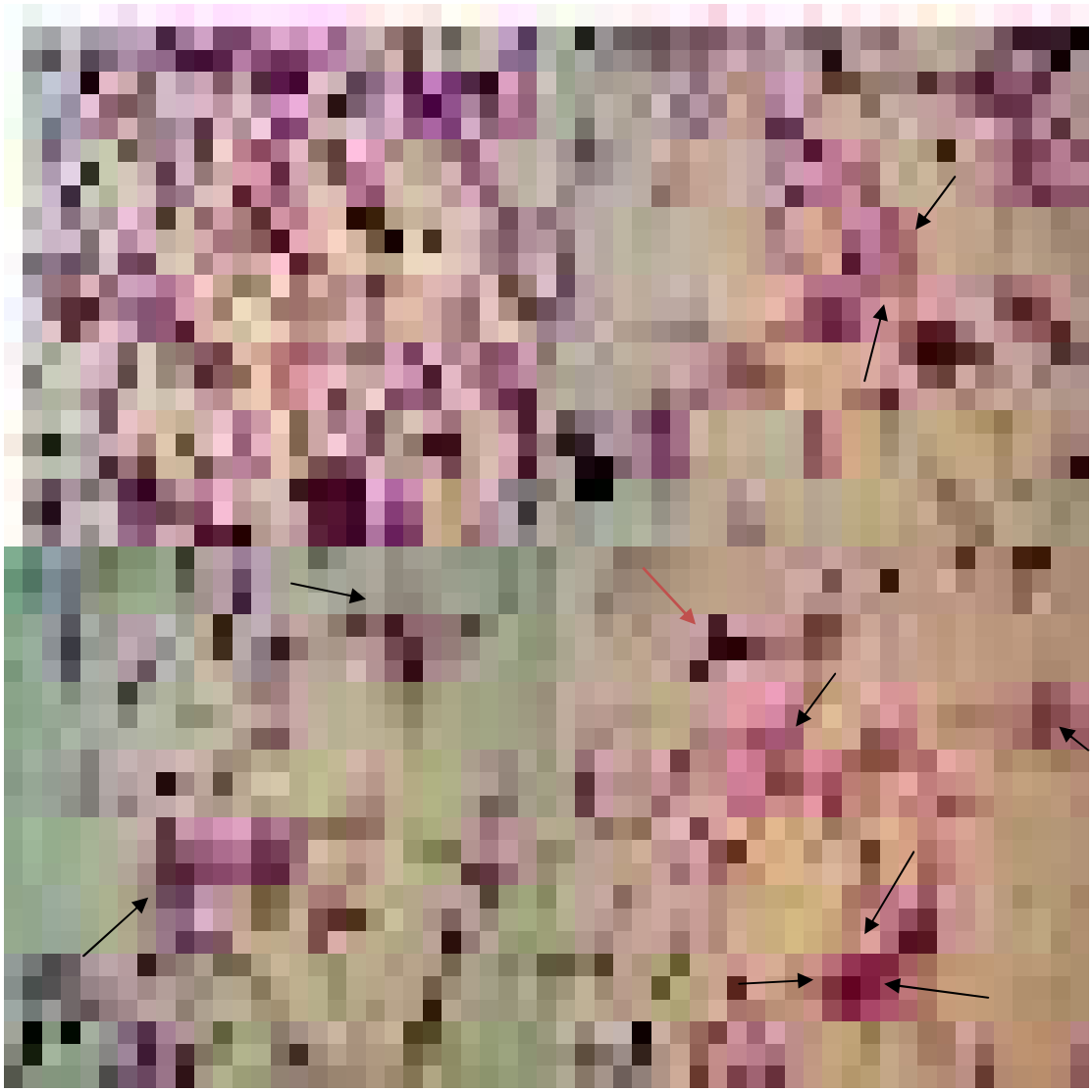


Figure (3-5): CPE of measles virus on RD cell line. A. RD cells uninfected with virus. B. showing cells fusion after 48 h. of infection C. Black arrows showing syncytia formation after 72 h D. Black arrows showing syncytia and large empty space with cell debris after 120 h, while Red Arrows showing picnotic cells (H&E) 40X.

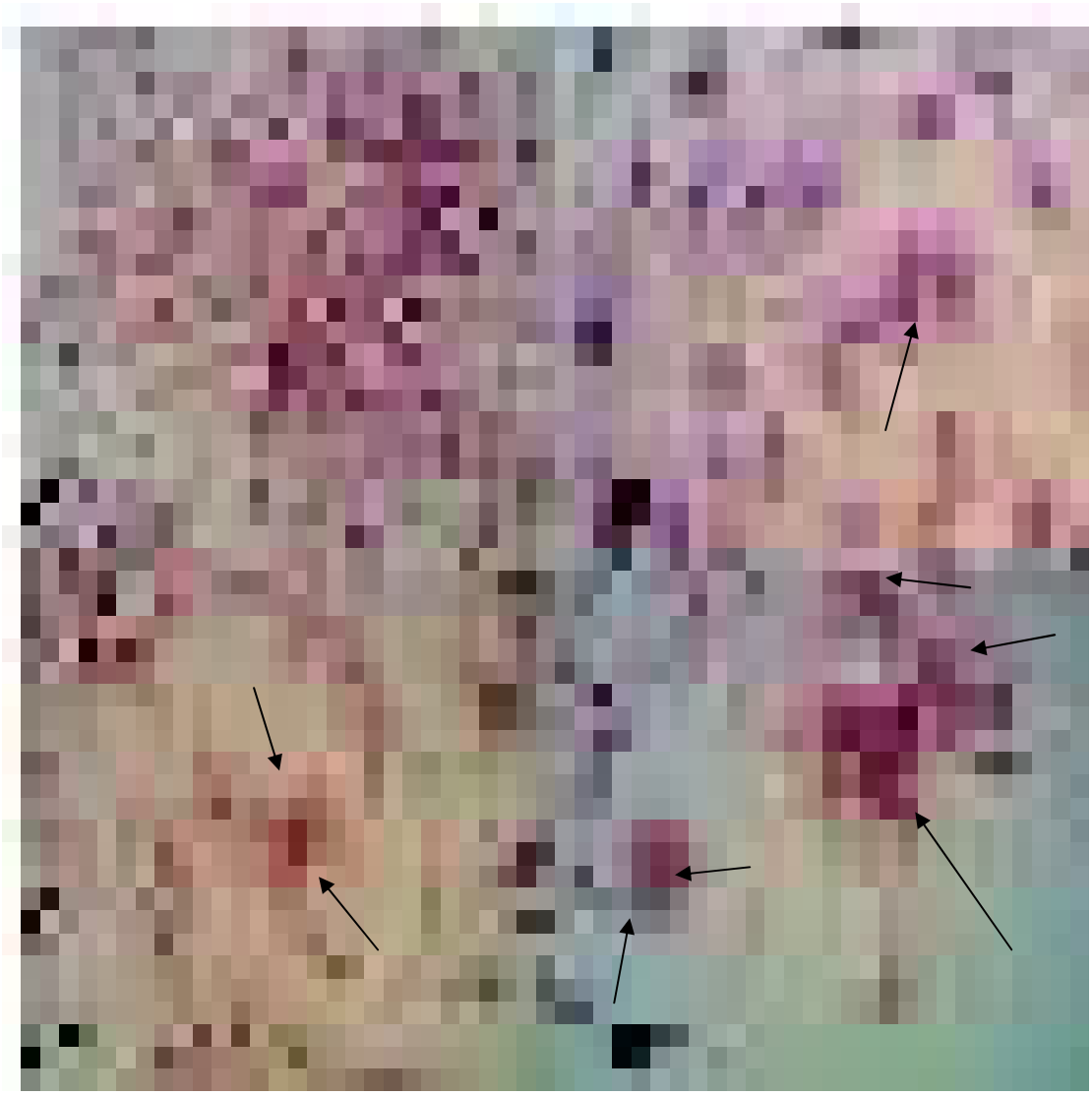


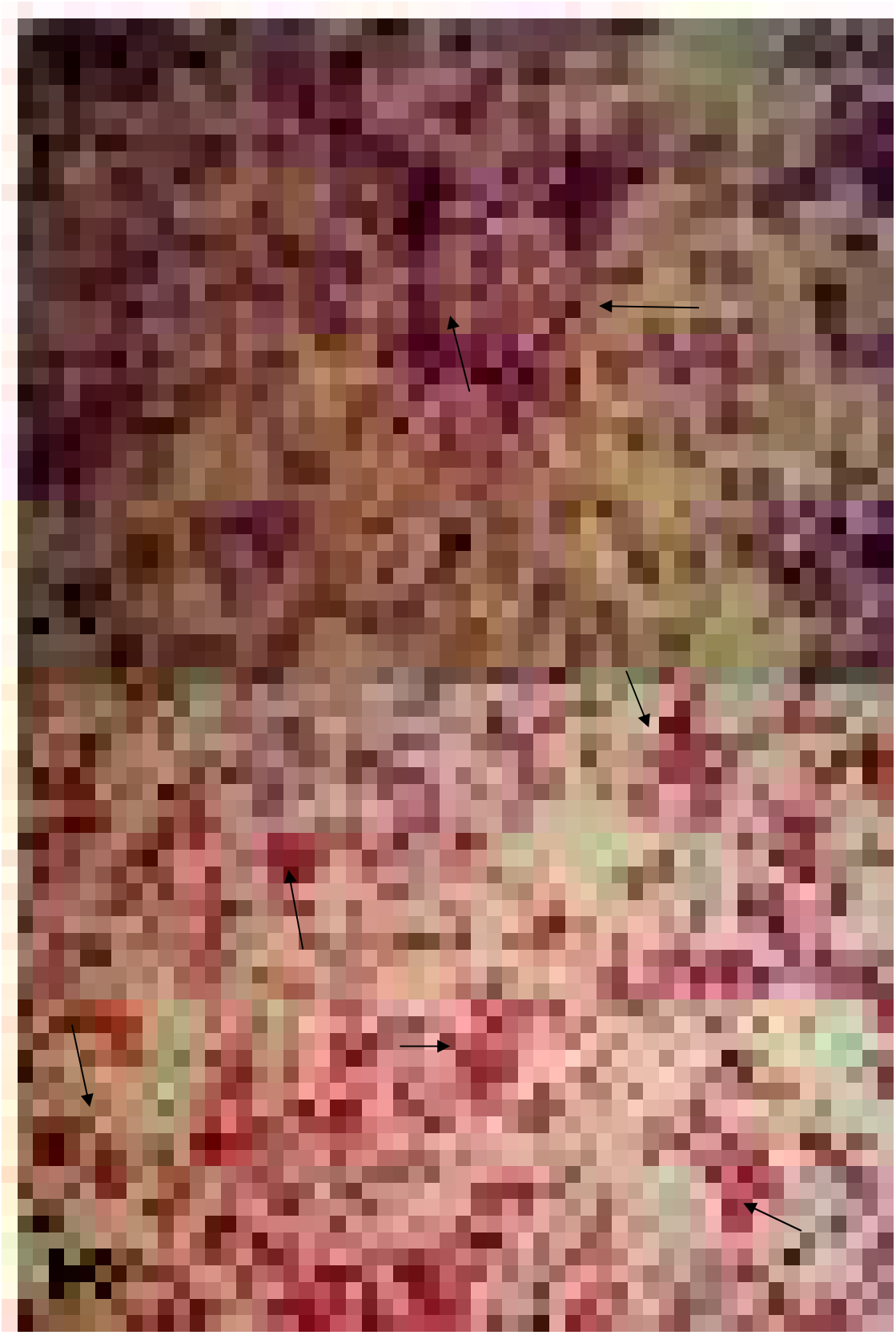
Figure (3-6): CPE of measles virus on GBM cell line. A. GBM cells uninfected with virus. B. showing cells fusion after 48 h of infection C. showing syncytia formation after 72 h D. showing syncytia and large empty space with cell debris after 120 h (Arrows) (H&E) 40X.

Results showed that MV Schwarz strain efficiently killed tumor cells, and causing obvious cytopathic effect on infected cell lines after 72 to 120 h of infection. The cytopathic effect of MV was beginning after the viral hemagglutinin (H) protein interacts with its receptor (CD46) on target cell. This interaction triggers conformational changes in the viral fusion (F) protein that leads to fusion of the viral and cell plasma membranes, allowing the viral nucleoprotein complex to enter into the cell. The H protein interacts with CD46 on neighboring cells and can

trigger membrane fusion between cells. As the expression of the viral H and F proteins increases, the probability that an infected cell fuses with a neighboring tumor cell increases. Once two cells fuse, there is an effective decrease in the concentration of viral proteins expressed on the cell surfaces and therefore there is a delay in spread of fusion until additional proteins are expressed on the surface. In time, this spread of cell-to-cell fusion leads to the formation of giant cell syncytia that normally die after a few days (Peng *et al.,* 2001; 2002). Infected cells that have been incorporated into syncytia stop replicating and do not contribute to further growth of the tumor population. In addition, once infected cells die, they might release free virus particles that can infect surrounding cells (Russell *et al.,* 2008).

3.3 Haemadsorption effect of MV:

Results showed that all cell lines (Vero, RD and GBM) infected with virus have the ability for haemadsorption human red blood cells after 72 h of infection. While uninfected cells gave negative results for haemadsorption (Figure 3-7A and B, Figure 3-8A and B, Figure 3-9A and B).



Figure(3-7): Haemadsorption test of Vero cell line. A. Vero cells uninfected with measles virus and treated with human RBCs. B. Haemadsorption of human RBCs after 72 h of MV infection. (Arrows) (H & E) 40X.

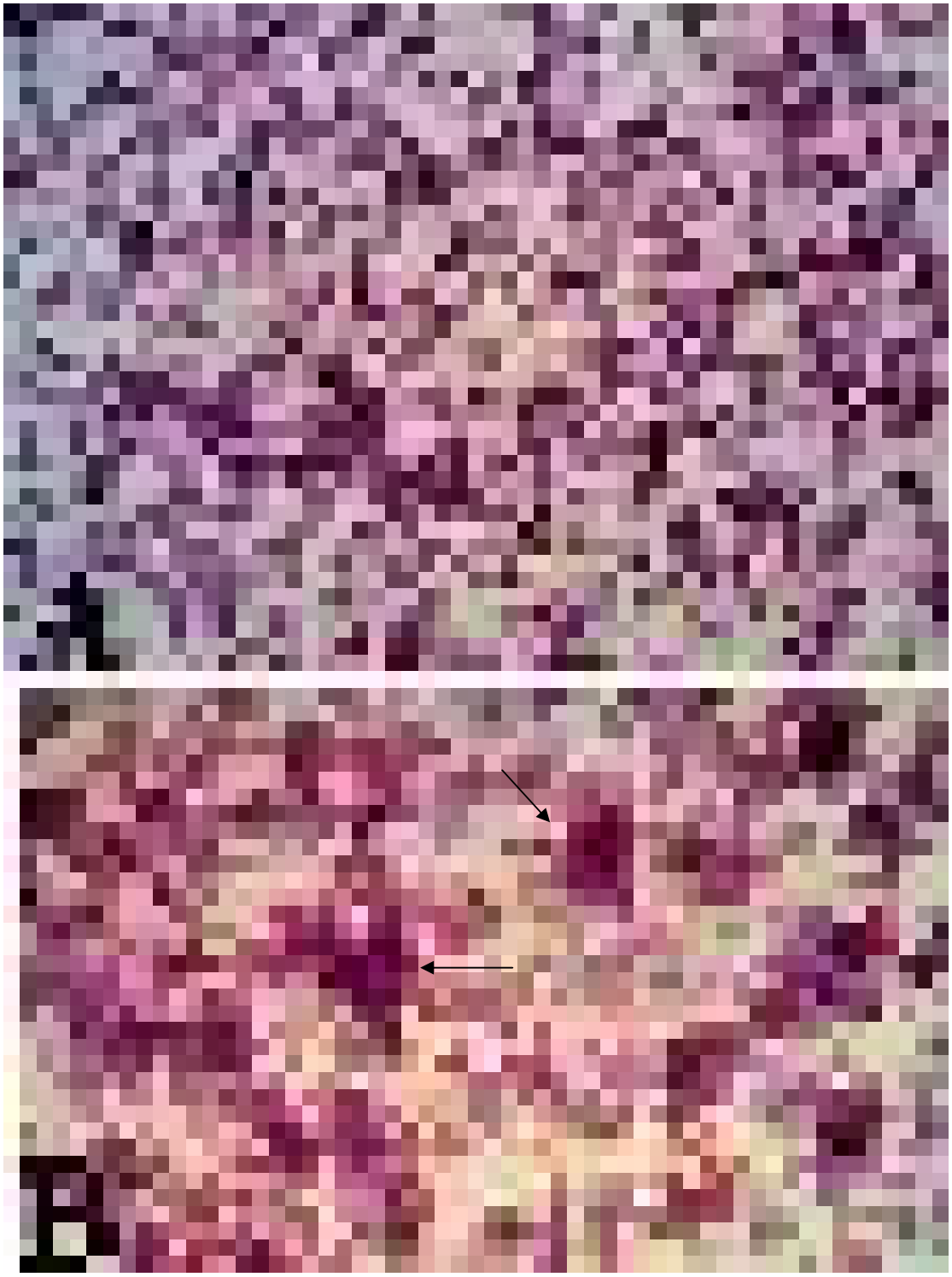


Figure (3-8): Haemadsorption test of RD cell line. A. RD cells uninfected with measles virus and treated with human RBCs. B. Haemadsorption of human RBCs after 72 h of infection. (Arrows) (H&E) 40X.

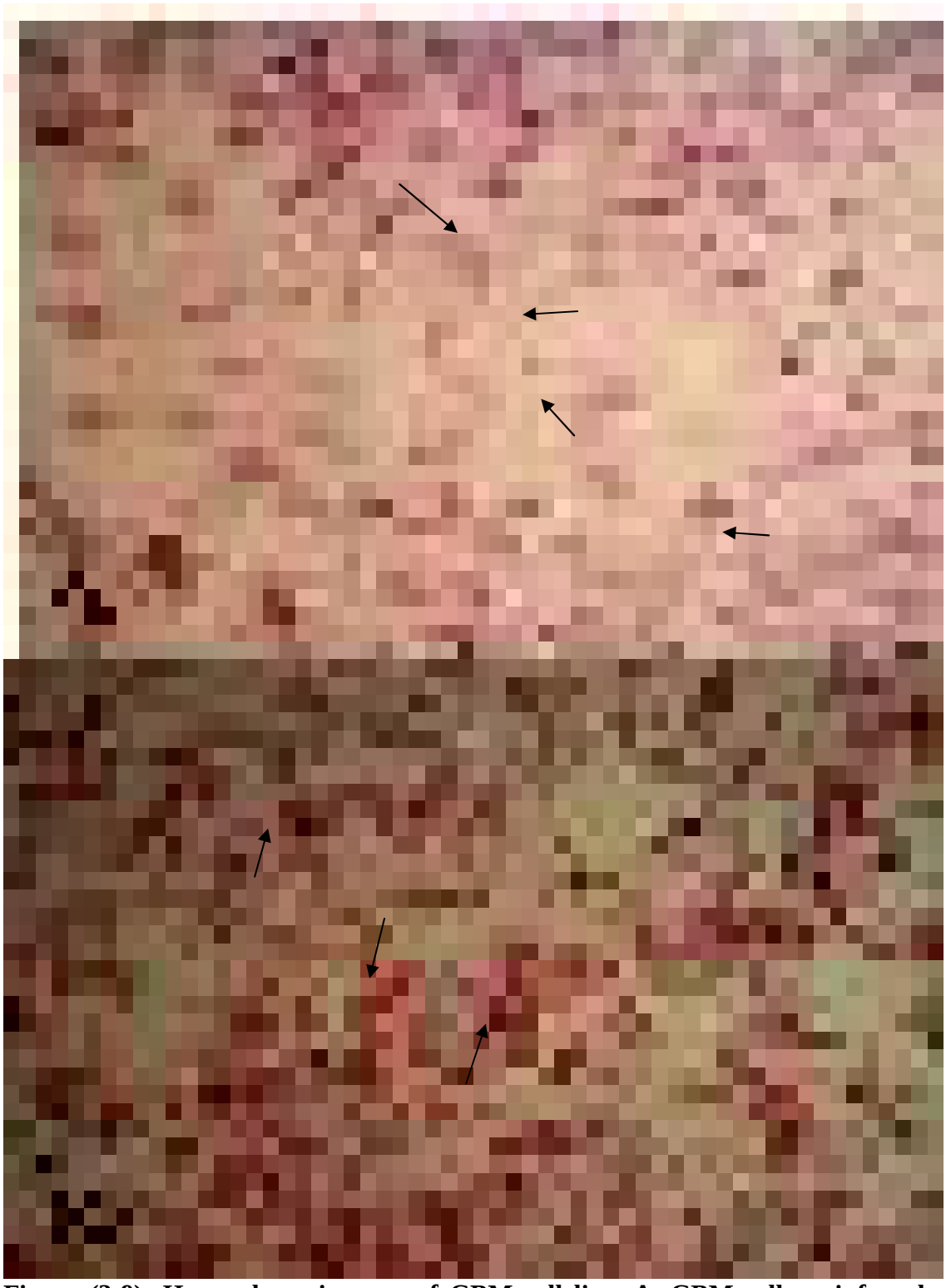


Figure (3-9): Haemadsorption test of GBM cell line. A. GBM cells uninfected with virus and treated with human RBCs. B. Haemadsorption of human RBCs after 72 h of infection. (Arrows) (H&E) 40X.

Results of hemadsorption test showed that, the cells infected with virus for 72h have a positive result because measles virus contains surface glycoprotein known as haemagglutinin (H). These are capable of binding red blood cells. When virus replicate on cell culture, H molecules appear on the cell surface. If human red blood cells are added to the cell culture in which the virus is replicating, they will adhere to the cell sheet a phenomenon known as haemadsorption. The presence of haemadsorping viruses can therefore be detected several days before a cytopathic effect becomes apparent (Koneman *et al*., 1997). While non infected cells have negative result to hemadsorption test.

3.4 Detection of measles virus H- protein in infected cell lines:

The application monoclonal antibodies specific for H protein of measles virus in Vero, RD and GBM cell lines after 72h and 120 h of virus infection showed that H protein was found in cytoplasm of infected cells as it stained with brown color. While uninfected cells cytoplasm gave negative stained. Nuclei of infected and uninfected gave blue color (figure 3-10A, B, C and D), (figure 3-11A, B, C and D) and (figure 3-12A, B, C and D).

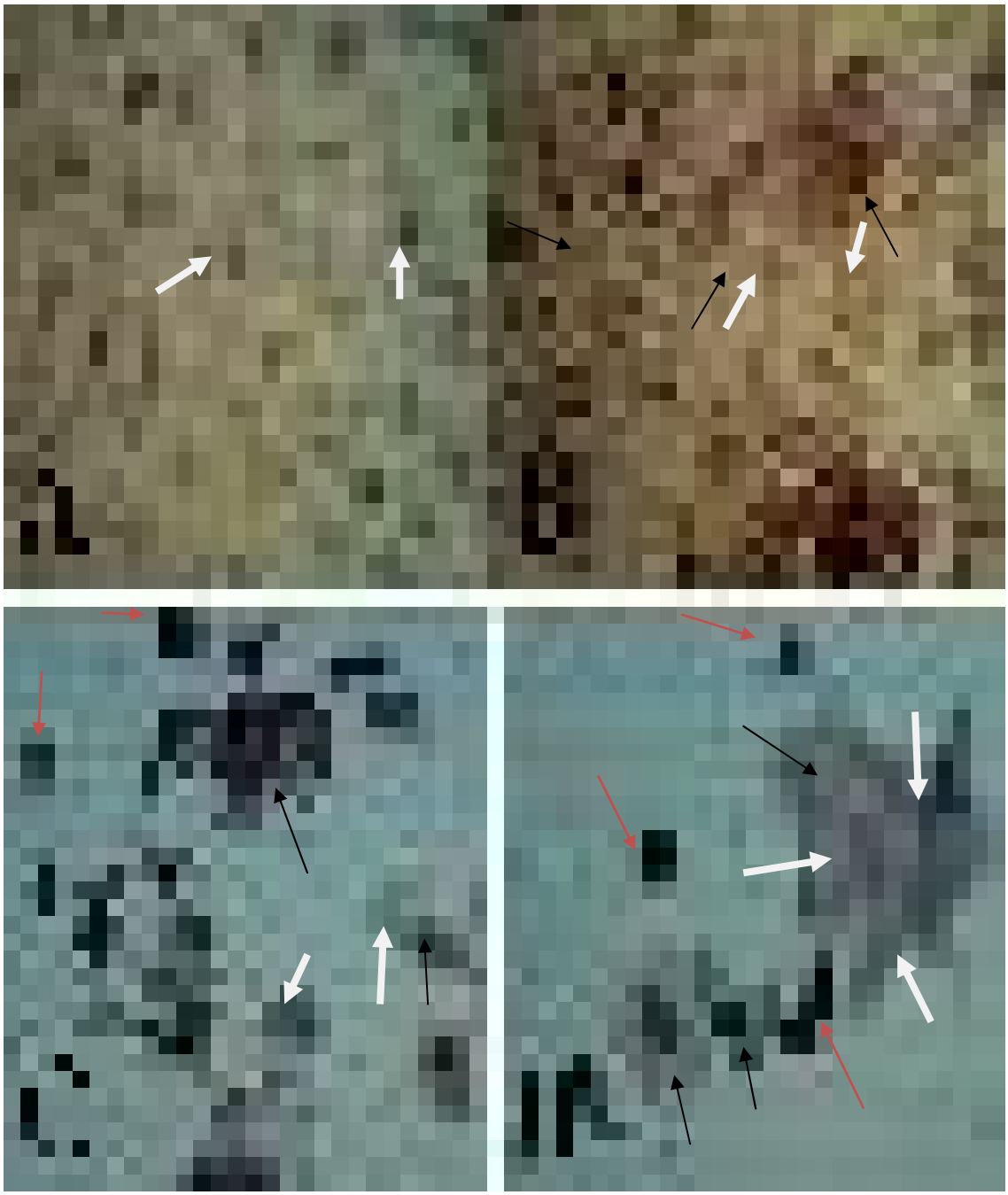


Figure (3-10): Detection of MV H protein in Vero cell line. A. Cytoplasm of uninfected Vero cells showing no color with blue nuclei 40X. B. Cytoplasm of Vero infected with MV showing brown color and blue nuclei after 72 h of infection 40X. C. Cytoplasm of Vero cells showing brown color (black arrow), blue nuclei (white arrow), and dead cells with black color (red arrows) after 120 h of infection 40X. D. Vero infected with MV for 120h cells cytoplasm stains brown, nucleus stained blue 100X.

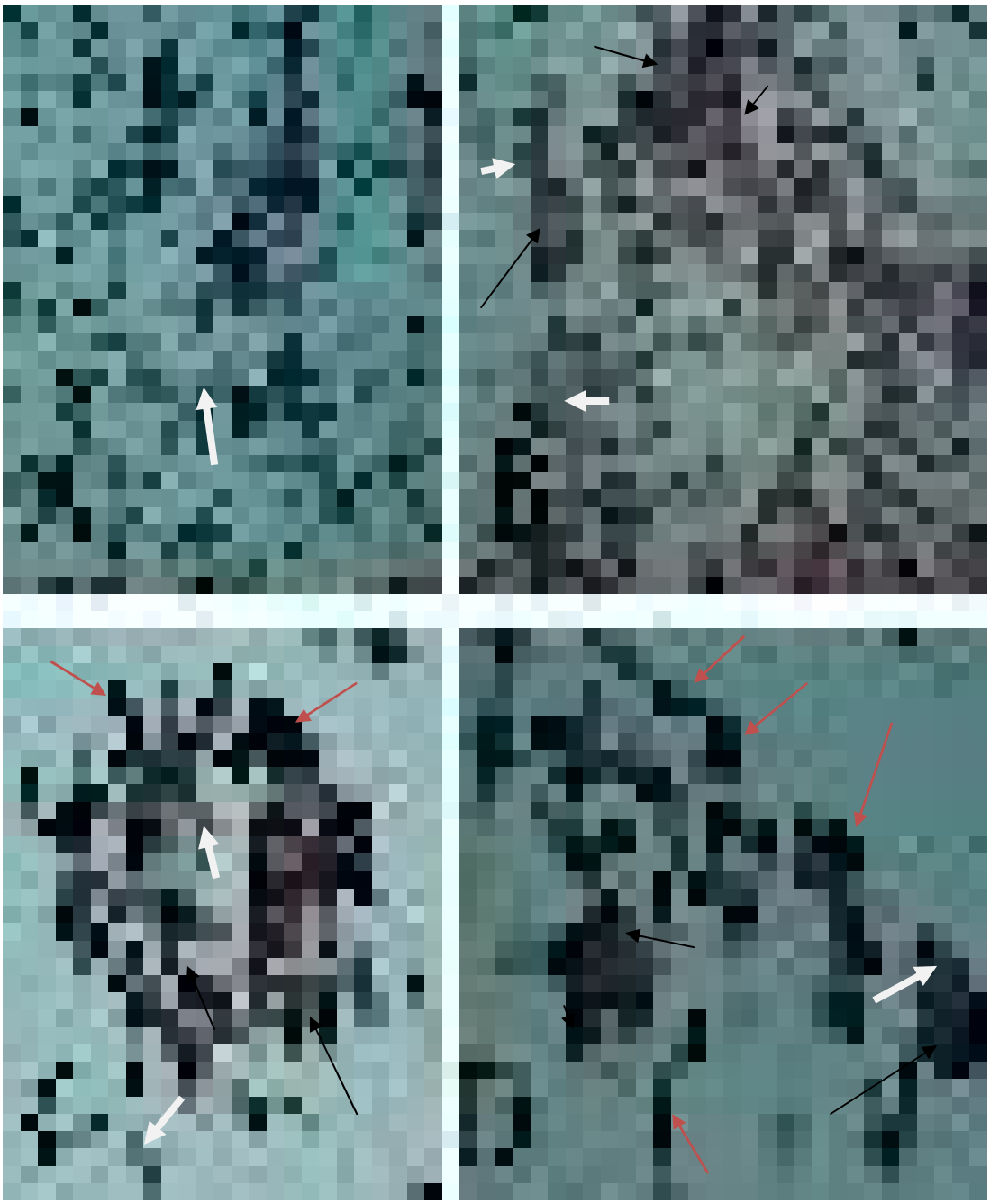


Figure (3-11): Detection of MV H protein in RD cell line. A. Cytoplasm of uninfected RD cells showing no color with blue nuclei 40X. B. Cytoplasm of RD infected with MV showing brown color and blue nuclei after 72 h of infection 40X. C. Cytoplasm of RD cells showing brown color (black arrow), blue nuclei (white arrow), and dead cells with black color (red arrows) after 120 h of infection 40X. D. Cytoplasm of RD cells showing brown color (black arrow), blue nuclei (white arrow), and dead cells with black color (red arrows) after 120 h of infection100X.

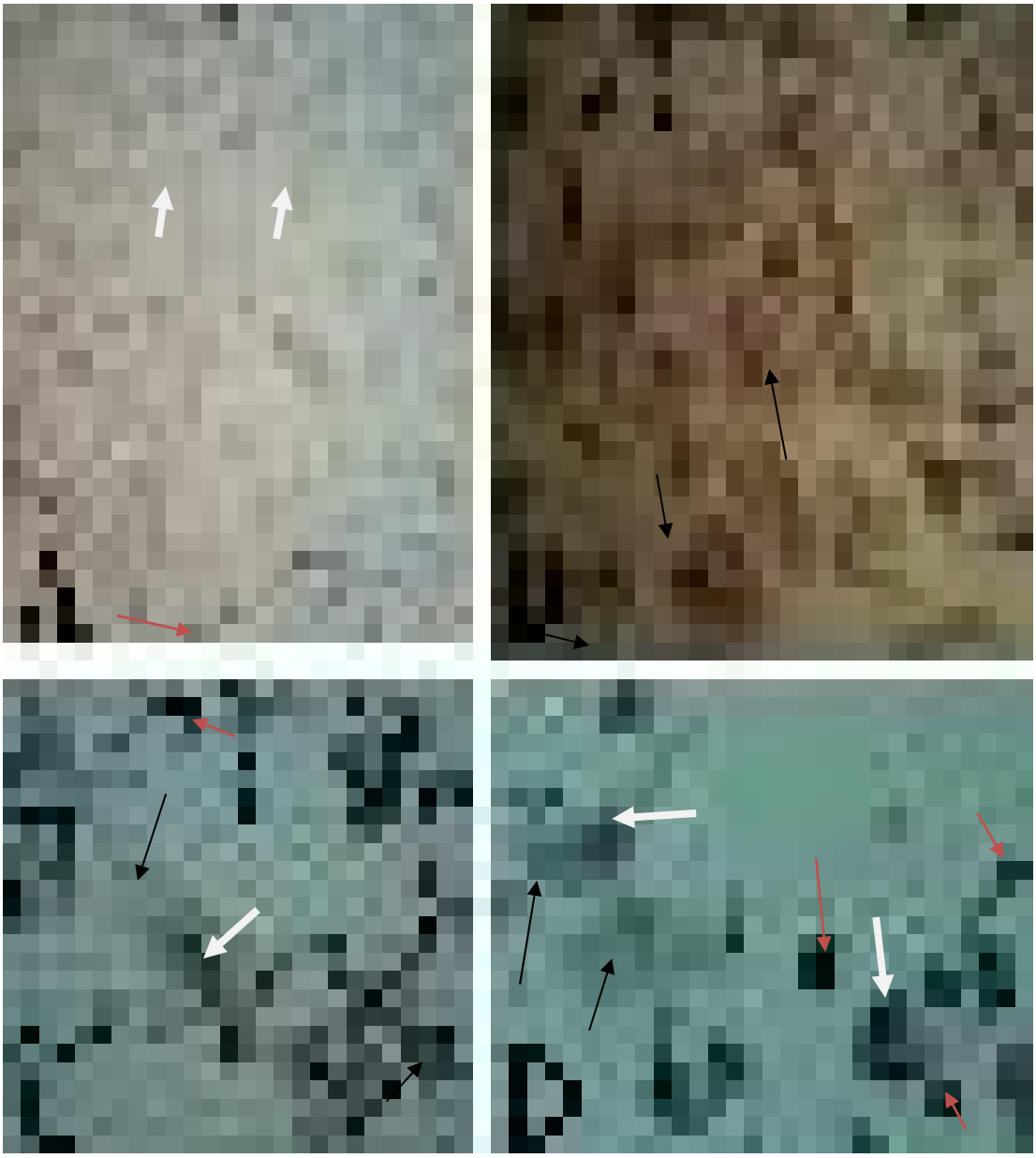


Figure (3-12): Detection of MV H protein in GBM cell line. A. Cytoplasm of uninfected GBM cells showing no color with blue nuclei 40X. B. Cytoplasm of GBM infected with MV showing brown color and blue nuclei after 72 h of infection 40X. C. Cytoplasm of GBM cells showing brown color (black arrow), blue nuclei (white arrow), and dead cells with black color (red arrows) after 120 h of infection 40X. D. Cytoplasm of GBM cells showing brown color (black arrow), blue nuclei (white arrow), and dead cells with black color (red arrows) after 120 h of infection 100X.

The identification of live attenuated measles virus in cancer cell lines was achieved by the detection of H protein (hemagglutinin protein). H protein is readily identified in fixed cells sections by The ABC staining

technique using most effective measles H (6016) mouse monoclonal antibody for localization of measles H antigen (Ulrike *et al.*, 2013).

3.5 Cytotoxic effect of measles virus on tumor cell lines (RD and GBM):

Results in table (3-2) showed a significant cytotoxic effect for measles virus ($P \leq 0.05$) on growth of RD cell line at the first dilution and the second one with growth inhibition percentage was (56.778 and 60.439%) respectively after 72 h of infection. When the dilution increase, there was a significant decline in the inhibitory effect with a significant cytotoxic effect when compared with the control with GI % were (38.616, 31.039 and 19.801) % respectively. Also, concentrated inoculum of measles virus showed a significant cytotoxic effect when compared with the control with growth inhibition percentage was 41.298% as shown in table (3-1).

Table (3-1): Cytotoxic effect of concentrated and diluted inoculum of measles virus on RD cell line after 72 h of infection.

dilution	O.D. mean± S.E.*	GI%
Control	1.103±0.007 a	----
Concentrated inoculums (TCID50/ml =$3.4*10^5$)	0.761±0.004 b	41.298
Dilution1 (TCID50/ml =$1.7*10^5$)	0.591±0.005 c	56.778
Dilution 2(TCID50/ml =$8.5*10^4$)	0.550±0.005 d	60.439
Dilution3 (TCID50/ml =$4.25*10^3$)	0.676±0.006 e	49.074
Dilution4(TCID50/ml =$2.125*10^2$)	0.791±0.003 f	38.616
Dilution5 (TCID50/ml =1.06*10)	0.875±0.004 g	31.039
Dilution 6 (TCID50/ml =5.312)	0.999±0.007 h	19.801

***different letters= significant differences (P≤0.05) between means of treatments and control**.

Results in table (3-2) showed the cytotoxic effect of measles virus on RD cells after 120 h of infection. A significant cytotoxic effect ($P \leq 0.05$) on growth of RD cell line at the dilutions 1, 2 and 3 when compared with the control with GI % were (53.110, 69.954 and 53.717) % respectively. A concentrated inoculum of measles virus showed a significant cytotoxic effect when compared with the control with growth inhibition percentage was 13.050%.The cytotoxic inhibition rate was significantly decrease when the dilution increased. There were significant effects at the dilutions 4, 5 and 6 with GI % were (31.714, 15.174 and 5.918) % respectively.

Table (3-2): Cytotoxic effect of concentrated and diluted inoculum of measles virus on RD cell line after 120h of infection.

dilution	O.D. mean ± S.E.*	GI%
Control	0.659±0.002 a	----
Concentrated inoculums (TCID50/ml =$3.4*10^5$)	0.573±0.001 b	13.050
Dilution1 (TCID50/ml =$1.7*10^5$)	0.309±0.002 c	53.110
Dilution 2(TCID50/ml =$8.5*10^4$)	0.198±0.003 d	69.954
Dilution3 (TCID50/ml =$4.25*10^3$)	0.305±0.002 e	53.717
Dilution4(TCID50/ml =$2.125*10^2$)	0.450±0.001 f	31.714
Dilution5 (TCID50/ml =1.06*10)	0.559±0.001 g	15.174
Dilution 6 (TCID50/ml =5.312)	0.620±0.001 h	5.918

***different letters= significant differences (P≤0.05) between means of treatments and control**.

The measles virus had a significant effect on GBM cells after 72 h of exposure when compared with the control at the dilutions 1, 2 and 3 with GI % were (46.047, 73.183 and 58.012) % respectively. A significant

decrease in cytotoxic inhibition rate was recorded when the dilution increased with a significant cytotoxic effect at the dilutions (4, 5 and 6) with PGI were (27.350, 19.658 and 14.529) % respectively. A concentrated inoculum of measles virus showed a significant cytotoxic effect when compared with the control with growth inhibition percentage was 46.047% as shown in table (3-3).

Table (3-3): Cytotoxic effect of concentrated and diluted inoculum of measles virus on GBM cell line after 72 h of infection.

dilution	O.D. mean ± S.E.*	GI%
Control	0.936±0.003 a	----
Concentrated inoculums (TCID50/ml =$3.4*10^5$)	0.505±0.003 b	46.047
Dilution1 (TCID50/ml =$1.7*10^5$)	0.302±0.001 c	67.735
Dilution 2(TCID50/ml =$8.5*10^4$)	0.251±0.001 d	73.183
Dilution3 (TCID50/ml =$4.25*10^3$)	0.393±0.003 e	58.012
Dilution4(TCID50/ml =$2.125*10^2$)	0.680±0.000 f	27.350
Dilution5 (TCID50/ml =1.06*10)	0.752±0.001 g	19.658
Dilution 6 (TCID50/ml =5.312)	0.800±0.001 h	14.529

***different letters= significant differences (P≤0.05) between means of treatments and control**.

The table (3-4) showed the cytotoxic effect of measles virus on GBM cells after 120 h of exposure was significantly higher ($P \leq 0.05$) on growth of GBM cell line at the dilutions 1, 2 and 3 when compared with the control with GI % were (87.817, 90.101 and 74.746)% respectively. A concentrated inoculum of measles virus showed a significant cytotoxic

effect when compared with the control with growth inhibition percentage was 35.279%. The cytotoxic inhibition rate was significantly decrease when the dilution increased with a significant effect at the dilutions 4, 5 and 6 with GI % were (48.477, 36.421 and 23.477) % respectively.

Table (3-4): Cytotoxic effect of concentrated and diluted inoculum of measles virus on GBM cell line after 120 h of infection.

dilution	O.D. mean ± S.E.*	GI%
Control	0.788±0.004 a	----
Concentrated inoculums (TCID50/ml =$3.4*10^5$)	0.510±0.005 b	35.279
Dilution1 (TCID50/ml =$1.7*10^5$)	0.096±0.003 c	87.817
Dilution 2(TCID50/ml =$8.5*10^4$)	0.078±0.000 d	90.101
Dilution3 (TCID50/ml =$4.25*10^3$)	0.199±0.000 e	74.746
Dilution4(TCID50/ml =$2.125*10^2$)	0.406±0.003 f	48.477
Dilution5 (TCID50/ml =1.06*10)	0.501±0.001 g	36.421
Dilution 6 (TCID50/ml =5.312)	0.603±0.003 h	23.477

***different letters= significant differences (P≤0.05) between means of treatments and control**.

Results of cytotoxicity test showed that the live attenuated measles virus on human cancer cells (RD and GBM) after 72h and 120h at concentrated inoculums and higher dilutions of measles virus have low cytotoxic inhibition rate. While the low dilutions of measles virus caused higher cytotoxic inhibition rate. This result highlights the competition between viral replication, tumor cell growth and the death rate of infected tumor cells (Wodarz, 2001; Wu *et al.,* 2001; Wein *et al.,* 2003; Wu *et al.,* 2004; Tao and Guo, 2005; and Friedman *et al.,* 2006). Wodarz (2003)

reported that very low concentrations of virus which have infected cells are not available to infect additional cells, leading to a potential imbalance in the virotherapy. Moreover, Dingli *et al* (2006) reported that the virus in high concentration cannot infect tumor cells because the surface receptors of tumor cells will be saturated with virus ligands.

3.6 Mitochondrial permeability transition apoptosis test:

Results as shown in figure (3.13) indicated that MV induces apoptosis in RD cell lines stained with MitoPT and the means percentage of apoptotic cells were significantly higher (87.42, 93.69 and 97.36) % respectively after 48, 72 and 120 h of infection in comparison with uninfected RD cells (20.83) %. Also the amounts of green and red color of cells were statically analysis to histogram in the images by using program ImageJ (figures 3.14a, b, c and d).

Also the means percentages of apoptotic cells were significantly lower in uninfected GBM cells (22.99) %. A significant difference was recorded (89.62, 95.43 and 97.97) respectively of GBM cells infected with MV after 48, 72 and 120 h of infection (figure 3.15) and (figures 3.16 a, b, c and d).

Figure (3-13): RD cell line infected with MV at different incubation period.

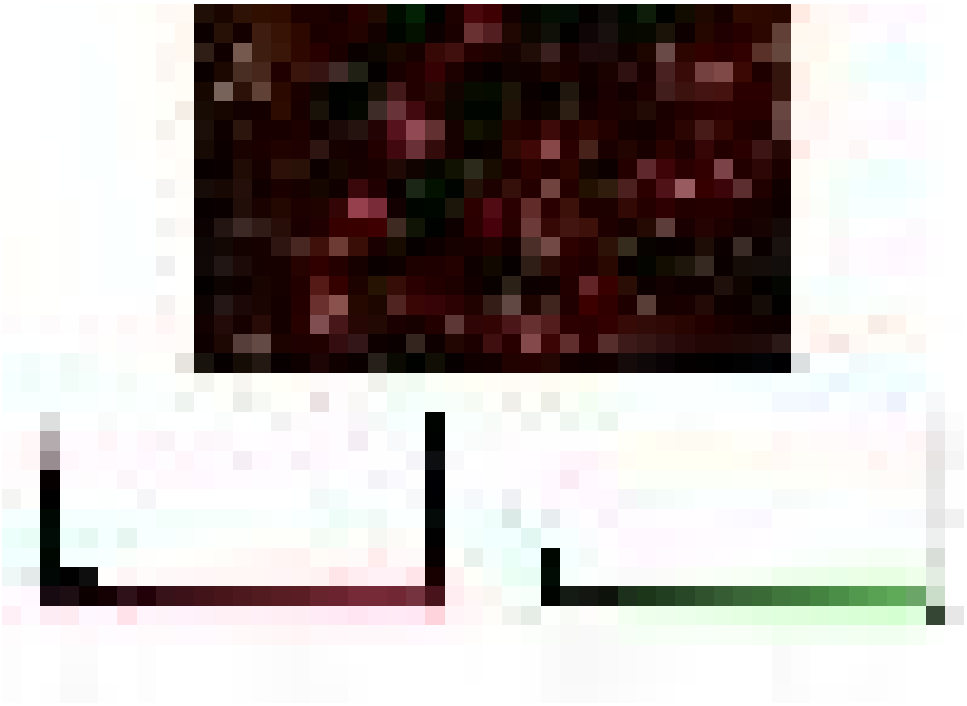


Figure (3-14a): Mitochondrial permeability transition apoptosis test for control of RD cells 40X. RD cells uninfected with measles were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram by was peformed by using program ImageJ. The mean of red color of non-apoptotic cells was (35.66). The mean of green color of apoptotic cells was (8.40), so the percentage of green color (apoptotic cells) was 23.5%.

Figure (3-14b): Mitochondrial permeability transition apoptosis test for RD cells infected with measles virus after 48 h 40X .RD cells were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram was performed by using program ImageJ. The mean of red color for non-apoptotic cells was (4.93). The mean of green color for apoptotic cells was (40.56), so the percentage of red color (viable cells) was 12.15%.

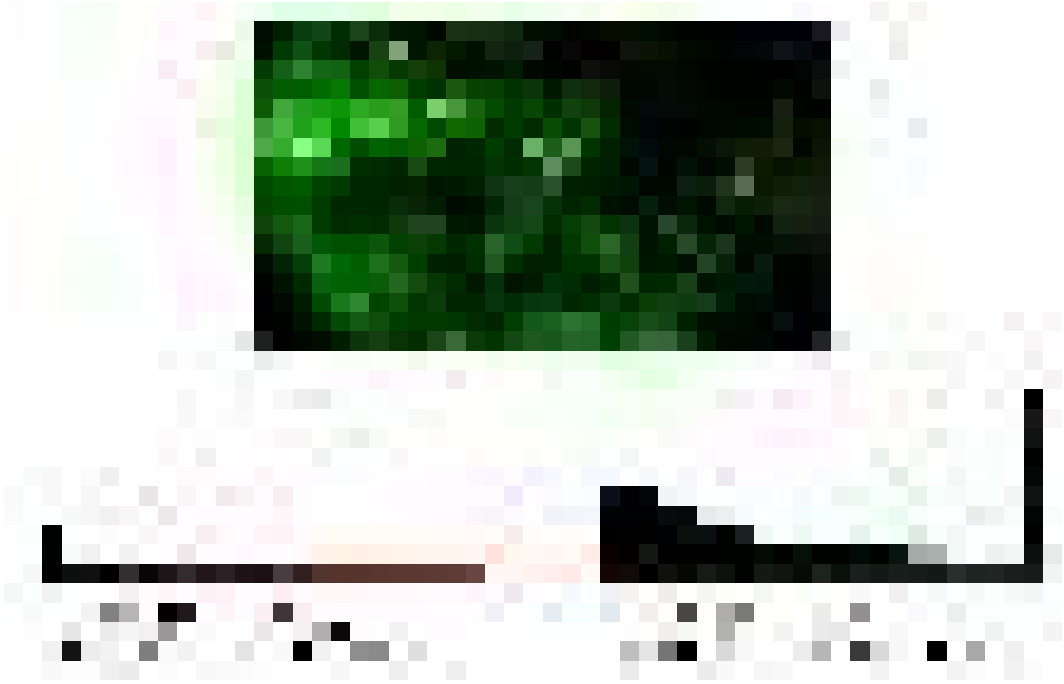


Figure (3-14c): Mitochondrial permeability transition apoptosis test for RD cells infected with measles virus after 72 h 40X. RD cells were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram was performed by using program ImageJ. The mean of red color for non-apoptotic cells was (2.54). The mean of green color for apoptotic cells was (48.29), so the percentage of red color (viable cells) was 5.25%.

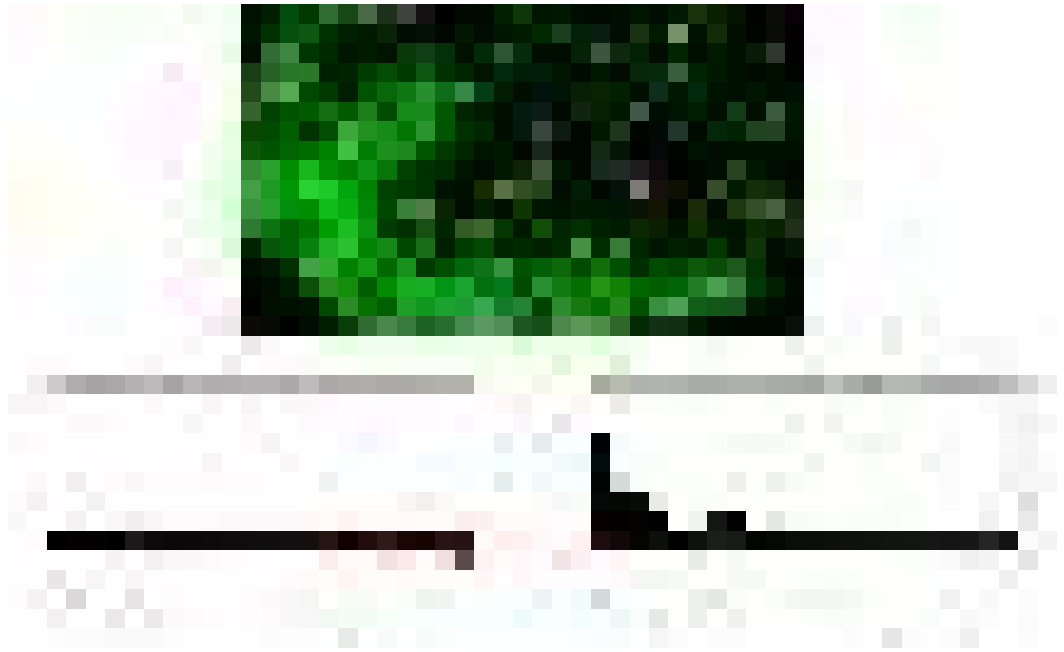


Figure (3-14d): Mitochondrial permeability transition apoptosis test for RD cells infected with measles virus after 120 h 40X. RD cells were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram was performed by using program ImageJ. The mean of red color for non-apoptotic cells was (1.125). The mean of green color for apoptotic cells was (64.883), so the percentage of red color (viable cells) was 1.73%.

Figure (3-15): GBM cells infected with MV at different incubation period.

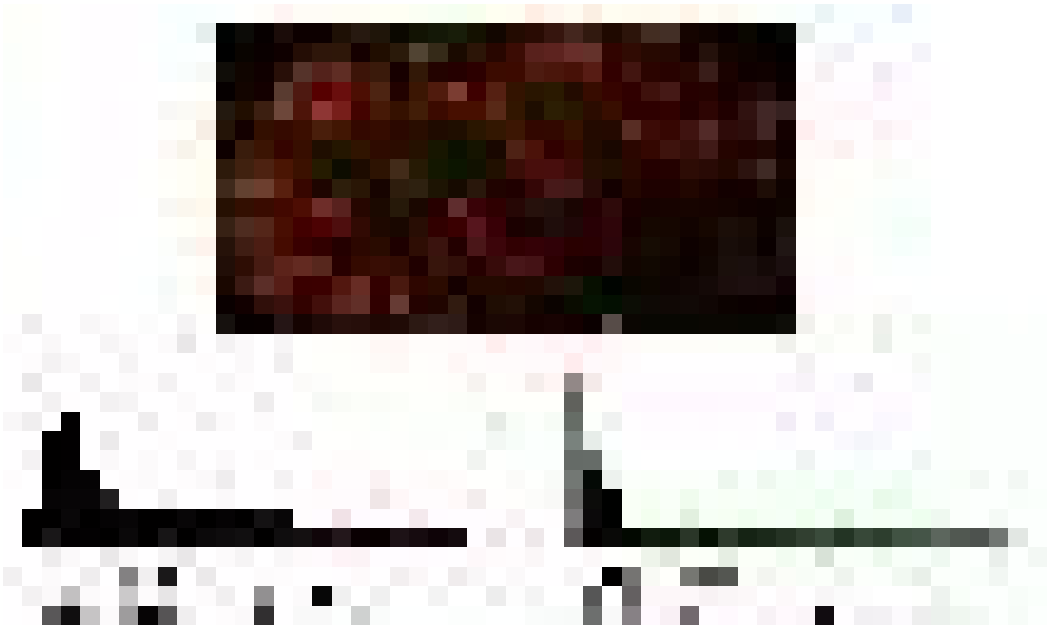


Figure (3-16a): Mitochondrial permeability transition apoptosis test for GBM cells 40X. GBM cells uninfected with measles virus were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram was performed by using program ImageJ. The mean of red color for non-apoptotic cells was (44.979). The mean of green color for apoptotic cells was (12.90), so the percentage of green color (apoptotic cells) was 28.68%.

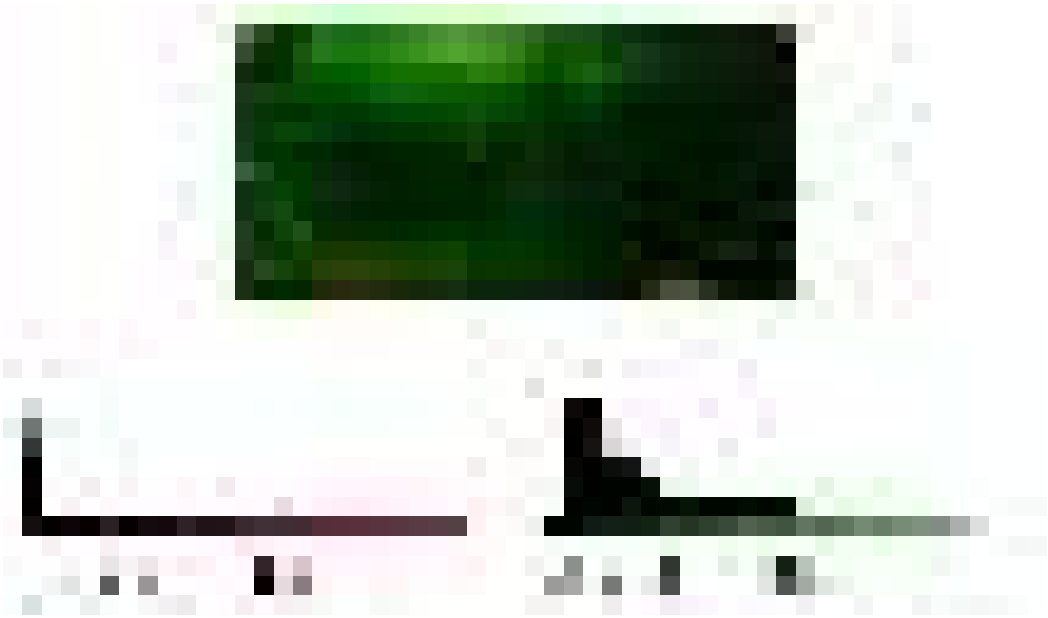


Figure (3-16b): Mitochondrial permeability transition apoptosis test for GBM cells infected with measles virus after 48 h 40X. GBM cells were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram was performed by using program ImageJ. The mean of red color for non-apoptotic cells was (5.46). The mean of green color for apoptotic cells was (52.66), so the percentage of red color (viable cells) was 10.36%.

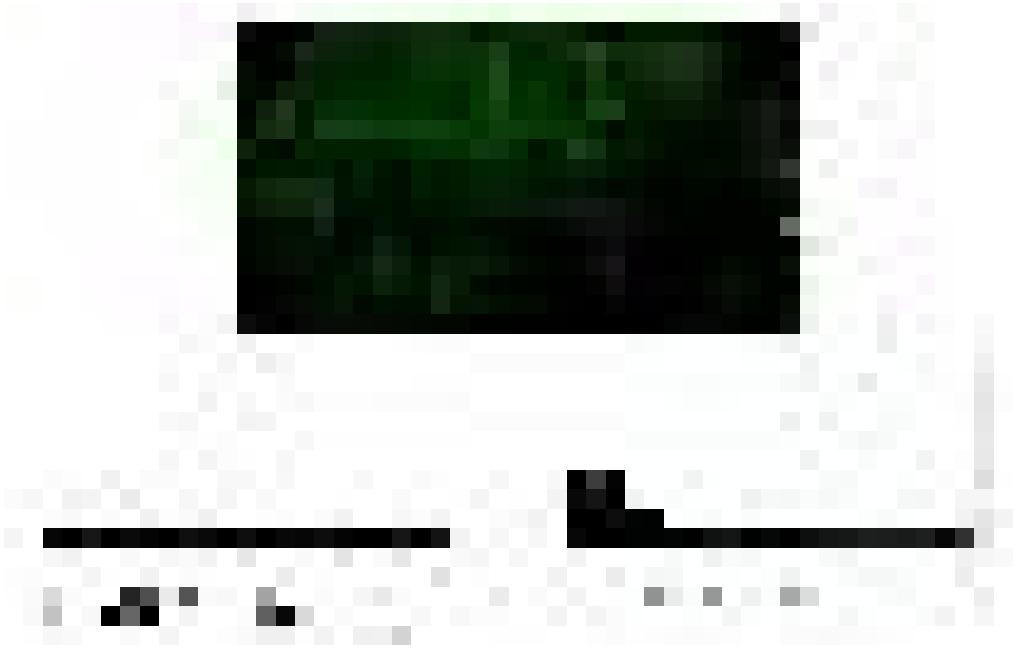


Figure (3-16c): Mitochondrial permeability transition apoptosis test for GBM cells infected with measles virus after 72 h 40X. GBM cells were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram was performed by using program ImageJ. The mean of red color for non-apoptotic cells was (1.04). The mean of green color for apoptotic cells was (23.09), so the percentage of red color (viable cells) was 4.50%.

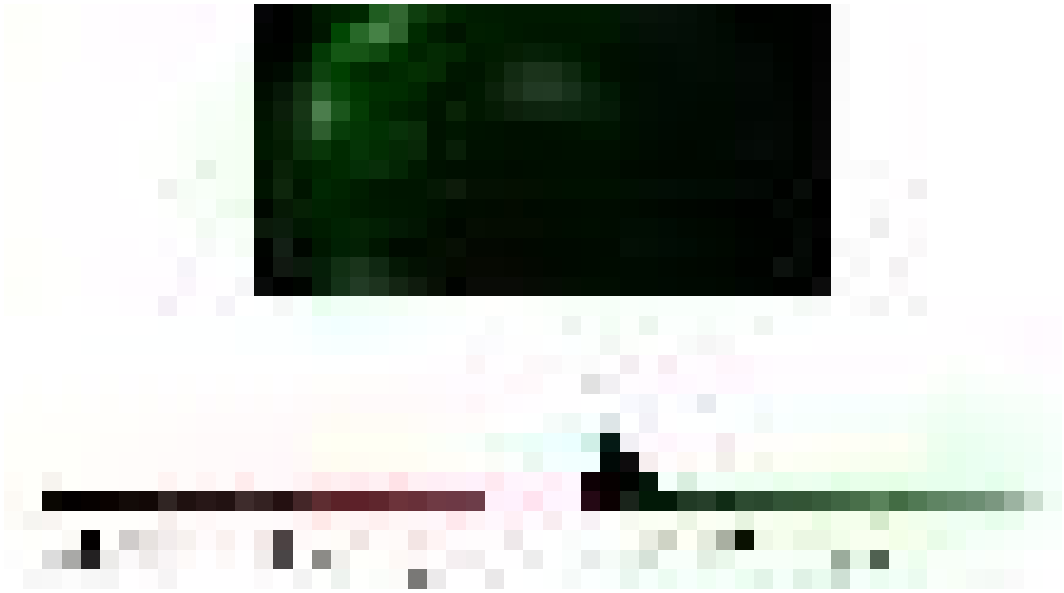


Figure (3-16d): Mitochondrial permeability transition apoptosis test for GBM cells infected with measles virus after 120 h 40X. GBM cells were stained with MitoPT and viewed through a fluorescence microscope. Analysis the image to histogram was performed by using program ImageJ. The mean of red color for non-apoptotic cells was (0.62). The mean of green color for apoptotic cells was (21.42), so the percentage of red color (viable cells) was 2.89%.

Result of the cytotoxic effect and apoptosis of MV showed that the live attenuated measles virus vaccine have natural ability to specifically kill variety of human tumor cells. It exerts its cytopathic effect (CPE) by fusing infected cells with the surrounding cells, forming multinucleated syncytia, which is followed by cell death by apoptotic or nonapoptotic mechanisms to a variety of human tumor cells (Gauvrit *et al.*, 2008). This selectivity may attribute to the entry mechanism of MV. Attenuated measles virus enters the cell via the receptor CD46, which is over expressed on tumor cells (Davis and Fang, 2005). MV is a lytic virus, in which replication in tumor cells lead to cell lysis of cancer cells. Also MV induces the program cell death, apoptosis. Zhou *et al* (2012) reported that the live-attenuated MV vaccine could potently induce apoptosis in ovarian cancer cells because the live-attenuated MV vaccine possesses antitumor activity against ovarian cancer *in vitro* through aberrant ROS activation-mediated epigenetic silencing of E-cadherin and lays the

theoretical foundation for the clinical application of the live-attenuated MV vaccine in the treatment of ovarian cancer.

Grote *et al*. (2001) proved that live attenuated measles virus is a potential antitumor agent for lymphoid malignancies, and reported that MV may be particularly promising as oncolytic virus for number of reasons, First**,** a nonpathogenic strain of MV is available, well characterized, and safe live attenuated MV vaccines. Second, although many human cell types are permissive for MV infection *in vitro*, in the presence of an intact immune system, virus replication after natural infection is limited to a few cell types *in vivo*. Lymphoid organs are prominent sites of MV replication multinucleated giant cells develop during infection in lymph nodes as a result of gross cell-cell fusion (Warthin, 1931). Third, recently shown that expression of virally derived fusogenic membrane glycoproteins in tumor cells after viral infection, including MV fusion (F) and hemagglutinin (H) glycoproteins (Fielding *et al*.,2000; Bateman *et al*.,2000), results in a potent cytopathic effect mediated by massive cell-cell fusion.

There are several reported cases of regression of Hodgkin disease and of non-Hodgkin lymphoma (NHL) after natural MV infection (Taqi *et al.*, 1981). Msaouel *et al*. (2009) demonstrated that the considerable antitumor potency of recombinant MV-Edm as a virotherapy agent against prostate cancer.

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Ministry of Higher Education and Scientific Research
Al- Nahrain University
College of Science

Role of Live Attenuated Measles Virus Schwarz Vaccine Strain as Antitumor Agent *In vitro*

A Thesis

Submitted to the College of Science /Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

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February 2014 Rabi'II 1435

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