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RESEARCH ARTICLE

Intratumoral Co-Administration of Oncolytic Newcastle Disease Virus and Bacterial Hyaluronidase Enhances Virus Potency in Tumor Models

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Abstract

Purpose: Bacterial hyaluronidase in co delivery evaluation effect with oncolytic Newcastle Disease Virus to determine spreading of the virus throughout Adenocarcinoma tumors Models in mice, and evaluate the reduction in tumor mass by efficacy of co delivery injection in tumor models. This study aimed to define the success of Combination effect between bacterial origin hyaluronidase and Newcastle Disease Virus and compare the invasion of this combination versus treatment with hyaluronidase enzyme or with oncolytic Newcastle disease virus only.Experimental Design:Newcastle disease virus (NDV) titration $6.3x10^5$ injected in combined dose with 200 international unit (IU) bacterial origin hyaluronidase to provide a mass invasion efficiency and spread of Newcastle disease virus within tumors that detected through the measurement of growth inhibition of tumor mass and immunofluorescence detection assay. The experiment performed *in vivo* in mice bearing AMN3 tumor cell line model. Conclusion: The data presented in this study show that combination treatment of solid tumors with an NDV and bacterial origin hyaluronidase significantly improves spread of virus through the tumor mass, resulting in prolonged survival of the tumor-bearing animals and in vivo tumor mass reduction without increasing the metastatic potential. Thus, this combination of NDV and hyaluronidase may offer a novel and promising therapy for the treatment of patients with cancer.

Keyword: *NDV (Newcastle Disease Virus), HA (Hyaluronic Acid), AMN3 (Ahmed Murtatha Nahi 2003 Cell Line), ECM (Extra Cellular Matrix).*

Introduction

Conventional cancer therapy is achieved by many methods including radiotherapy, chemotherapy, surgery or a combination of them. Surgery and radiotherapy are most preferred in localized tumors but the other therapy like chemotherapy is applied when cancer cells are spread through the body [1].Virotherapy is a method used for cancer therapy that utilizes the replication competent oncolytic viruses to kill tumor cells [2].

The transformed phenotype of tumor cell provides an attractive condition and environment for some viruses or functions to complement viral mutations. Oncolytic viruses have the tendency to selectively replicate in tumor cells and kill them that given a major advantage of such replication in situ amplification and subsequent spread within the tumor [3].The main idea to achieve the success therapy by making oncolytic virus infects and replicate only in tumor cells without harming the healthy neighbouring cells [4].

Hyaluronan or Hyaluronic acid (HA) are an insoluble Glycosaminoglycan unbranched polysaccharides which is long chain of repeating disaccharide units, this polysaccharide is protein-free, and contain a ratio of uronic acid which first isolated from hyaloid [5]. HA containing solutions that makes molecule functioning to maintain the physical volume and rigidity of connective tissue [5].

In epithelial and connective tissue cancers, have shown greater hyaluronic acid enrichment of extracellular matrices surrounding tumors than found in parenchymal regions with higher concentration in tumors than in normal tissues [6]. It is known that hyaluronan levels are elevated during embryological development wound healing and tumorigenesis [7].

Also high HA levels have been detected at the invasive front of growing tumors, signifying that HA may be involved not only in cell proliferation, but possibly in invasion as well [5]. Many types of bacteria especially the pathogenic bacteria produce hyaluronidase that play an important role as a virulence factor involved in pathogenesis and disease progression caused by this Bacteria. The productions of hyaluronidase for defense mechanism for conceal the bacterial surface from host-defense mechanisms or for directly interact with host tissues.

The action of hyaluronidase on extracellular matrix by enzymatic degradation of their components inside host tissues facilitates in enhancing the invasion of pathogens in tissues that appears to play a major role in wound infections [8]. Also the fragment that formed from HA oligomers generated by microbial Hyaluronidase are potent inflammatory agents and promote microbial-invasion, Hyaluronidase are produced by many species of gram positive bacteria like various species of *Streptococcus, Staphylococcus, Peptostreptococcus, Propionibacterium, Streptomyces* and *Clostridium*.

Also many species of gram-negative bacteria have the ability to produce the hyaluronidase but is less likely to play a role in pathogenesis. Hyaluronidase and chondroitin lyase activities have been reported from *Aeromonas, Vibrio, Beneckea and Proteus. Bacteroides fragilis, Bacteroides vulgatus, Bacteroides ovatus, prevotella melaninogenicus and Fusobacterium mortiferum* are also reported to produce hyaluronidase [9].

Newcastle disease virus (NDV) is a negative-sense single-stranded RNA virus causes severe disease in several avian species and come under the *Paramyxoviridae* family [10].

Newcastle disease is one of fatal and contagious disease that affects all species of birds, Newcastle disease has major industry threat among the birds [11].The important features that make this virus to used widely in therapeutic process is its oncolytic properties, so it is used as a cancer vaccine and also as an oncolytic agent in several clinical trials in different human cancers [12].

The replication of NDV in cancer cells is due to defects in the type I interferon (IFN) response of cancer cells which enhance replication and increase their destruction of cancer cells that give the specificity of NDV as oncolytic viruses to cancer [13].Combination therapies have shown to be successful in the treatment of cancer. This therapy has been found more effective than single-drug procedures [14].

Oncolytic viral therapy is one of many viral-based therapies which come under investigation for cancer therapy, including, tumor suppressor-gene replacement therapy, "suicide" pro-drug gene therapy, immunomodulatory therapy.

The aim of Virotherapy To increase the efficacy of combination therapy, and permit lower doses of each agent to reduce the cost and toxicity of the drugs used in combination [15].

Material and Methods

Bacterial Origin Hyaluronidase

The ready prepared hyaluronidase enzyme was purchased from Sigma-Aldrich Company which isolated from *Streptococcus pyogenes* and has international unit or equal to > 8 unit/mg protein (5.0-15.0 mg/ml) with lot result 13.5 U/mg and has the reference code 56177-10MG and was preserved in ammonium sulphate suspension form. Other type of Hyaluronidase used in this study was imported from Santa Cruz biotechnology Company with code (CAS 37259-53-3). This enzyme is lyophilized and isolated from *Streptomyces hyalurolyticus* with 300IU per ampule.

Newcastle Disease Virus Propagation

Virus was previously isolated in Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) from suspected birds and purified formerly preserved in deep freezing at -85 C°.

For proliferation of virus, embryotic chicken eggs have age 10 days brought from Al-Hanaa Hippocampus located in Abo Ghareeb district-Baghdad province Iraq, the egg incubated at 37 C° with humidity nearly 40% before the injection with virus. Before the injection, the site of embryo was marked along with air sac, so the site of injection marked in opposite site of embryo to prevent the death of embryo during injection, then the egg cover was cleaned with iodine to prevent the contamination before the injection.

The virus thawed slowly from the deep freeze storage before injection, after thawing the viral supernatant injected (0.1ml) into allantoic fluid according to. Injected egg with virus incubated at 37 C° and approximately 40% humidity, through the incubation period the egg was surveillance for mortality of egg's embryo by the effect of injected virus, the first 24 hours after the injection of virus, the death embryo was discarded due to accidental death through injection procedure, Second period that represent the second 24 hours after injection, the incubated egg is monitoring in interval time (4-6 hours) for dead embryo which collected and, transferred to the refrigerator to be preserved at (4C°) before collection.

After 6-8hours of preservation at (4C°) the allantoic fluid of dead embryo was collected by sterile syringe and collected in 50ml tubes for centrifugation (3000 rpm, 30 minute, 4C°) to be free of debris. Collected allantoic fluid purified with Nalgene Filter of 0.2 mm Millipore filter and then distributed in 1ml Eppendorf tubes to be stored at -85C°.

Tumor Cell Line

Tumor cell line used in this study were cultured in RPMI 1640 with 10% fetal bovine serum.AMN3 tumor cell line were provided from Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) Baghdad-Iraq.Single cell suspension was made through mechanical disaggregation of the cells by vigorous pipetting.Tumor suspension aspirated by syringe with needle gage 18 and inoculated with S/C injection of 1 x 10^4 viable cells in 0.1ml cell suspension into shoulder region through puncture in thigh region.

In-Vivo Experimental Study

Female mice (8-10) weeks old, (15-29 g) weight housed that has been brought from Iraqi centre for drug monitoring – Baghdad and all purchased mice were female, then maintained in Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) animal house, with controlled conditions of temperature (23 ± 5 C°) and tested for any accidental infection before inoculation with cancer.

Mice were injected subcutaneously in the right flank with 1x10^4 AM3 tumor mass (injected volume of 100µl). When the mass tumor reach the desired mean volume nearly 1.2mm^3 based on formula, Volume = W x $(L)^2/2$ which represent the approximately the standard procedure for tumor mass measurement, these animals distributed randomly into 2 treatment groups added to them one control group nude mice tumor model of female mice, each group consist of at least 5 mice.

First group of mice injected with oncolytic NDV at dose 6.5x10^6 viral particles, Second group were injected with combination dose of virus 6.5x10^6 virus particles and Bacterial Hyaluronidase Enzyme at dose 200U in 100µl.tumor volume of treated mice checked once every other day and compared to control group to yield the tumor growth value was compared by linear regression analysis for 30 days experiment. At day 30 of single dose treatment, tumor progression was monitored with survival mice, animals from each group euthanized and tumors collected.

Mass of tumor was separated into two halves, one half used for immunohistochemistry assay and other was used for detection of viral replication. For histologic examination the preserved section of tumor in 10% neutralized buffered formalin followed according to (Luna, 1968) for tissue preparation, after paraffin sections they carried out in Shandon automated histokinate system ,samples were fixed and prior to process they cut and marked and put in plastic box.

Dehydration, embedding, sectioning and staining were done as described by Luna (1968), then xylene added 3 times to fixed tissue on charged slide to remove paraffin followed washing with ethanol 100% and 90% to remove excess xylene, next step involving treat fixed tissue with blocking media (10%BSA in PBS) before adding of primary monoclonal antibody specific binding to

Neuraminidase and haemagglutinase receptors of NDV(Santa Cruz, USA)which incubated overnight at 4C then subsequently adding of secondary fluorescent antibody after washing with PBS to remove excess not binding primary antibody, after incubation time , the slide magnified by fluorescent microscope under 20x to show fluorescent sites which represented the virus particles.

Results

Combination Dose Effect of Bacterial Hyaluronidase with Oncolytic Newcastle Disease Virus to Enhances the Spreading of Virus within Adenocarcinoma Tumor Mass

Effectiveness of bacterial hyaluronidase to degrade Extra Cellular Matrix (ECM) component hyaluronan that enhance spreading of oncolytic NDV in solid tumor can be determined through the combination dose of NDV and bacterial hyaluronidase in AM3 tumor model.

Tumor Volume Measurement

after 23 day of experiment, during this period, tumor volume measured day after two days using formula L*(W)2/2 then the tumor values compared between groups based on relative tumor volume among the groups to show the growth inhibition achieved by the combination treatment between the oncolytic virus and bacterial hyaluronidase against values result from group treated with NDV alone or group of bacterial hyaluronidase dose, the (Figure 1) showed values of tumor mass inhibition, based on comparison between values the treatment effect of combination dose show reducing in tumor growth when compared with NDV dose alone that improve the degradation influence of bacterial hyaluronidase with dose 200IU on ECM component of Hyaluronic acid in tumor mass that made a good chance to utilized by oncolytic virus to invade the tumor mass and replicate in more number of cancer cells.

The growth inhibitory effect as cleared in Figure (1 and2) show48.8% in the third day after the injection then the inhibition was only 81.4% at the day 9, to be continuously increasing slowly to become 72% in day 14 which have the same stationary phase of stability in growth inhibition until day 29 when the growth inhibition little stabilized at 73% to growth in the mice till the end of the experiment at day 30 to 80% growth inhibition.

Combination effect of Hyaluronidase enzyme and virus significantly improved virus spread that also was reflected in a significant survival advantage of the mice infected with cancer and undergo treatment by combination effect than control or cancerous mice treated with oncolytic NDV alone.

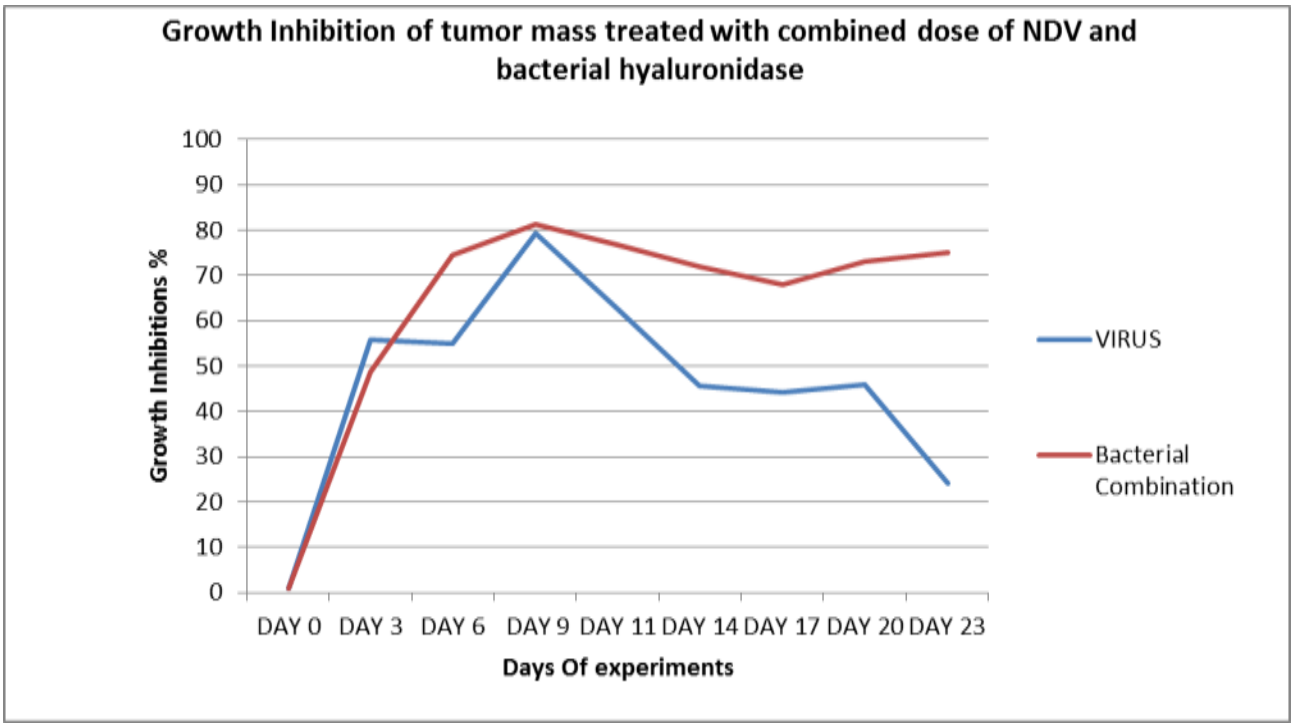

Figure 1: Growth inhibition treated with combination treatment of NDV in combined with bacterial hyaluronidase NDV treatment dose starting from day zero to day 23

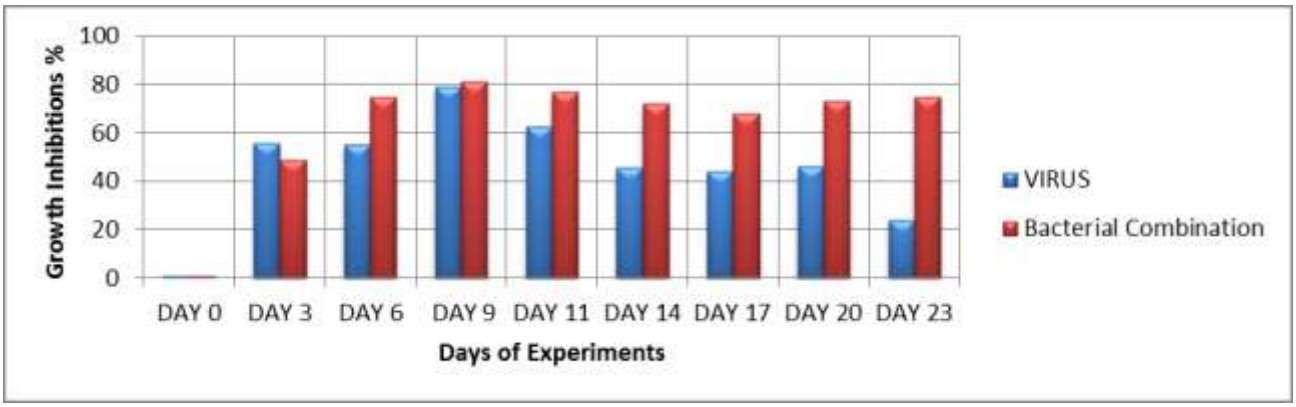

Figure 2: Growth inhibition values of two tumorous mice group, first group treated NDV dose at titration 6.3x105 which indicate in graph as blue columns against values of group treated with combination effect of bacterial hyaluronidase 200IU and NDV titration 6.3x105.Experiment lasts for 23 days

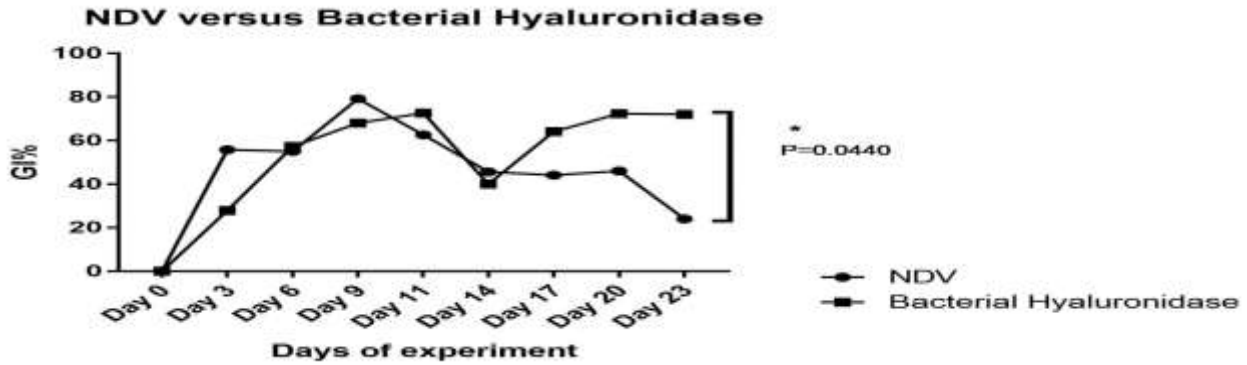

Figure 3: Growth inhibition of two tumorous mice group, one group treated with bacterial hyaluronidase 200IU and other group treated with NDV titration 6.5x10^5

These values show the peak reached group treated with bacterial hyaluronidase 70% at day 11 compared to nearly 60% for group treated with NDV dose with significant comparison p=0.0440.

Combination effect of bacterial hyaluronidase and NDV significantly improved as explained in (Figure 4) that virus spread that also was reflected in a significant survival advantage of the mice infected with cancer and undergo treatment by combination effect than control or cancerous mice treated with oncolytic NDV alone.

Virus Spread Enhancement

We evaluated the possible use of 2 different origin of hyaluronidase, one of ovine origin clinically versus bacterial origin in combination therapy. The dose of hyaluronidase was 200IU for both enzyme versions co-injected at the same time with the NDV into transplanted mammary adenocarcinoma tumor in mice that show the variation in result or effect of inhibition between two types of hyaluronidase which explained (in Figure 5) determine that ovine hyaluronidase showed more percentage in growth inhibition than bacterial origin hyaluronidase which depend on structure and mechanism of enzyme and mode of action of hyaluronic acid of tumor mass..

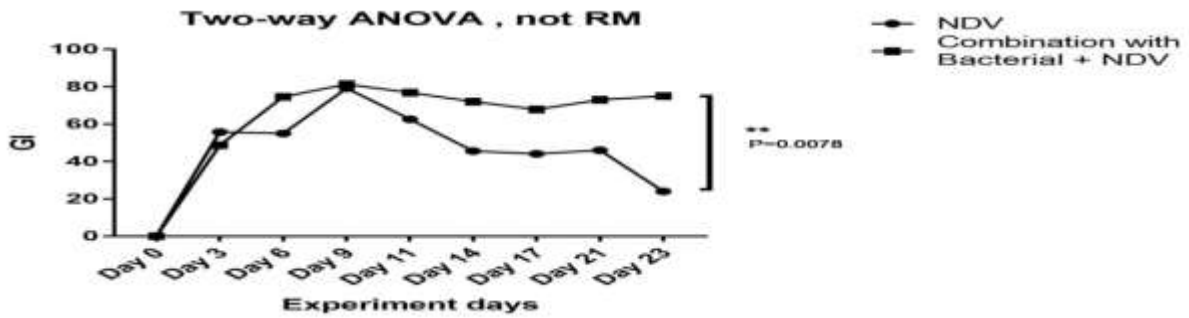

Figure 4: values analysis between two groups of tumorous mice by graph pad analysis software that show significant difference between the values of two groups which represent the growth inhibition of tumor mass after treatment with combination dose versus NDV dose

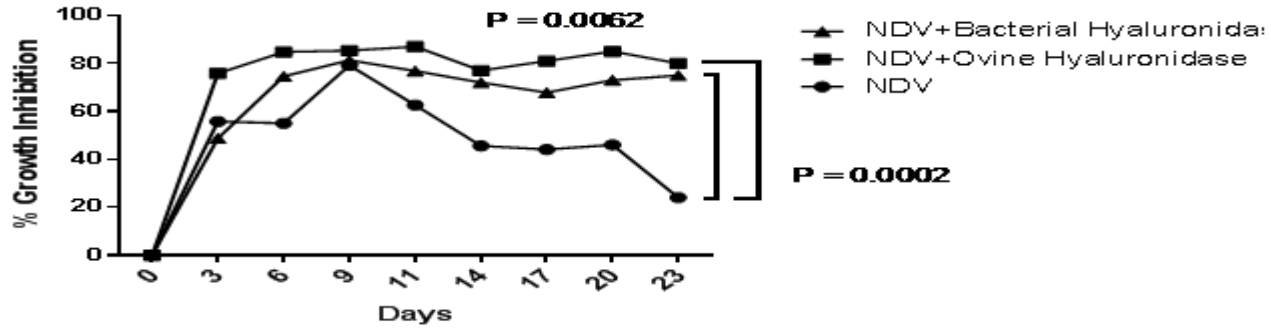

Figure5: growth inhibition analysis between three values of groups treated with combination effect (Bacterial hyaluronidase + NDV), (ovine hyaluronidase + NDV) and NDV doses with significant variation p=0.0002 among the three groups

Immunohistochemistry Detection

tumor were harvested at day 23 after initiation of treatment,Immunofluorescence detection of the tumor tissue isolated from the mice treated with combination effect of virus and bacterial hyaluronidase show the invasion of virus in the tissue when compared to the control that show non-binding antibody in the tissue due to absence of the virus in control tumor tissue, in (Figure 6) immune fluorescence results of the combination treatment of virus and bacterial hyaluronidase show the fluorescent yellow color tissue indicate the invasion of virus inside the tumor cells against the dark tissue which represent the control unreached area of tissue. In summary, the mice treated with combination effect show the increase in virus potency correlates with an improved spread of virus within the tumors, as shown by the presence of virus invasion when detection of viral particles using immune fluorescence techniques, which in turn correlates with an increase in the amount of infectious virus present within these tumors.

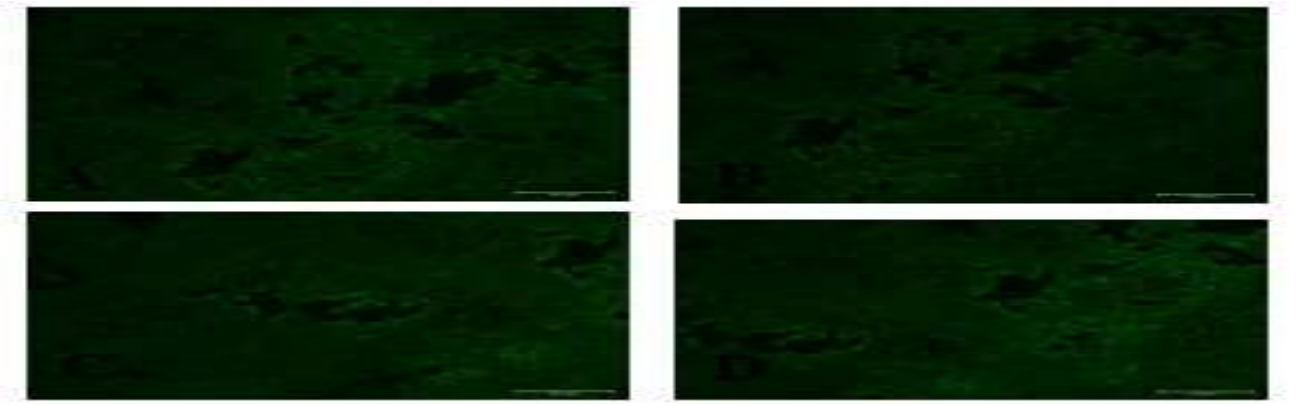

Figure 6: Immunofluorescence assay results of AMN3 tissue section stained with primary antibody has affinity to Hemagglutinin and Neuraminidase proteins of NDV and second fluorescent antibody that show affinition to primary antibody

This section from group of tumorous mice treated with combination dose of bacterial origin hyaluronidase 200IU and NDV titration $6.5x10^5$ under 20x magnification power of fluorescent microscope.

Discussion

As mentioned in (Guedan *et al.,* 2010) the main obstacle to the successful application of oncolytic viruses in cancer verotherapy is their inability to effectively spread in the solid tumors so utilizing the treatment of combination between the Hyaluronidase and oncolytic NDV can improves viral spread through the tumor mass and enhance the overall antitumor efficacy without increasing its toxicity [16].Combination of hyaluronidase and NDV may be a more optimal therapy because these results are improves the reduction in volume of primary tumors treated with the combination compared with tumors treated with either virus or enzyme alone, because metastatic spread is believed to be related to the size of the primary tumor in this model. Hyaluronidase by itself, even at a 5-fold higher dose, does not increase the metastatic spread of tumor. This observation is in agreement with a report from Bookbinder et al. [17], The data collected from this experiment show that Combination

of hyaluronidase at a concentration of 200 U with NDV significantly improves viral replication in the AM3 tumor mass growth in the swiss albino mice, so by Intratumoral injection for virotherapy was used in the present experiment as it was proved to be the most effective way and the possibility to be applicable on human tumors when used by Hecht *et al*. (2003) to deliver oncolytic virus to human pancreatic carcinoma through intratumoral endoscopic ultrasound injection [17].

These results are most likely due to the reduced growth of primary tumors treated with the combination compared with tumors treated with either virus or enzyme alone, because metastatic spread is believed to be related to the size of the primary tumor in this model.

Conclusion

The data presented in this study show that combination treatment of solid tumors with an NDV and bacterial origin hyaluronidase significantly improves spread of virus through the tumor mass, resulting in prolonged survival of the tumor-bearing animals and in vivo tumor mass reduction without increasing the metastatic potential. Thus, this combination of NDV and hyaluronidase may offer a novel and promising therapy for the treatment of patients with cancer.

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