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2-Deoxyglucose Glycolysis Inhibitor Augment Oncolytic Virotherapy to Induce Oxidative Stress and Apoptosis in Breast Cancer (Part III)

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A B S T R A C T

One of the "hallmarks of cancer" is altered energy metabolism, which is increased glycolysis in cancer cells, the primary source of energy that uses this metabolic pathway to generate ATP. Oncolytic virotherapy with aerobic glycolysis inhibitor smart therapeutic approach to induce apoptosis in cancer cells. The current study aimed to use the 2-Deoxyglucose (2DG), a specific glycolysis inhibitor, to enhance the Newcastle disease virus (NDV). In this study, a mouse model of breast cancer allograft with mammary adenocarcinoma tumor cells (AN3) was used and treated with 2DG, NDV, and a combination of both. Anti-tumor efficacy and glycolysis analysis (hexokinase -1 (HK-1), pyruvate, and ATP) were determined. The induction of oxidative stress was investigated by reactive oxygen species (ROS) and total glutathione assay examination. Apoptosis induction was investigated using immunohistochemistry (cleaved Caspase-3) and histopathology. The result showed that combination therapy enhances anti-tumor efficacy (decrease in relative tumor volume and increase in tumor growth inhibition) of NDV against breast cancer. This effect was accompanied by a reduction in HK-1 concentration, pyruvate, and ATP (glycolysis products). Moreover, NDV+2DG therapy induces oxidative stress (decreases total glutathione and increases ROS). Immunohistochemistry and histopathological examination showed the apoptotic area in tumor tissues in treated groups. In conclusion, the present study found that the combination therapy could be considered as an effective cancer therapy through induction of glycolysis inhibition, oxidative stress, and apoptosis selectively in cancer cells.

Keywords: glycolysis inhibitor, Newcastle disease virus, oxidative stress, apoptosis

INTRODUCTION

Cancer cells use a variety of strategies to proliferate and invade other tissues, including apoptosis avoidance, resistance to growth inhibitors, independence from growth signals, unlimited replication potential, chronic inflammation induction, genetic instability, immune escape, angiogenesis, and changes in cell metabolisms (1, 2). Otto Warburg, a Nobel laureate, discovered what is now known as the Warburg effect, or

aerobic glycolysis, when cancer cells use glycolysis, even when oxygen is present (3). Thus, the alteration in glucose metabolism in the cancer cell can be used as a goal for cancer therapy through chemotherapeutic agents such as 2-deoxyglucose (2DG) (3).

2DG is a relatively specific glycolysis inhibitor, as it prevents glucose from being further metabolized by hexokinase. Hexokinase converts 2DG to phosphorylated 2DG, which accumulates in the cell, resulting in non-competitive inhibition of hexokinase, decreased ATP and lactate synthesis, and ultimately cell growth suppression and death (4). 2DG induces glucose deprivation without altering other metabolic or nutrients pathways (5). It is induced cell cycle arrest, inhibited proliferation and apoptosis in cancer cells (6). 2DG exhibits oxidative stress by glucose deprivation that selectively kills cancer cells (7). Therefore, using 2DG as anti-metabolites therapy to target cancer cell metabolism could improve cancer therapeutics (8, 9).

Oncolytic Newcastle disease virus (NDV) is an Avulavirus belong to the Paramyxoviridae family (10). NDV exhibits three mechanisms to kill tumor cells: apoptosis induction, virus replication in cancer cells leading to cytolysis, and specific immune stimulation against cancer cells through tumor cells surface antigenic modification by virus infection (11-13). In addition, it was reported that NDV suppresses glyceraldehyde-3-phosphate and inhibition the glycolysis pathway in cancer cells (14). In animal models and clinical trials, oncolytic virotherapy as monotherapy has not been totally effective in eradicating cancer. Combination therapy is the greatest technique for attacking tumor cells through different mechanisms to prevent cancer cells from acquiring resistance to therapy (15). Thus, this study investigated using 2DG, a hexokinase inhibitor to synergize oncolytic NDV for inducing metabolic oxidative stress, and study mechanisms action of this combination through glycolysis products analysis and apoptosis in breast cancer tissue.

MATERIALS AND METHODS

NDV Propagation

Cell Bank Unit, The Experimental Therapy Department, Iraqi Center of Cancer and Medical Genetics Research (ICCMGR), Mustansiriyah University, Baghdad, Iraq, kindly provided the attenuated AMHA1 NDV Iraqi strain (16) used in this study. The NDV Iraqi strain was propagated in embryonated chicken eggs (Al-Kindi Company, Baghdad, Iraq), harvested from allantoic fluid, and purified by centrifugation at 3000 rpm for 30 min at 4°C. The concentration of NDV was determined using a hemagglutination test, aliquoted, and kept at -80°C. Viral titers were established using a conventional approach involving a 50% tissue culture infective dose titration on Vero cells (17).

Experimental Animals

Twenty Swiss Albino female mice (6-8 weeks old) weighing 20-24 g were purchased and housed at ICCMGR. The scientific committee at the College of Veterinary Medicine, University of Baghdad, Institutional Animal Care and Use Committee of Mustansiriyah University, College of Science, and ICCMGR reviewed and approved all procedures in this study.

Experimental Design

The mice were injected with 1×10^6 murine mammary adenocarcinoma tumor cells (AN3) in100 µL per site in the right flank in each mouse (18, 19). Tumor size is measured using a vernier caliper every three days for eighteen days. After reaching the tumor size to about 5-10 mm in diameter, the animals were randomly divided into four groups (five animals per group). Control group: mice in this group were received 0.9% normal saline i.p.; 2DG group: mice in this group were received 2DG i.p. 500 mg/kg for five days each week (20). NDV group: mice in this group were injected NDV at 70×10^6/animal intratumorally in a single dose (14). NDV+2DG group: mice in this group were received 2DG i.p. at 500 mg/kg for five days each week and NDV at 70×10^6/animal intratumorally in a single dose. The mice were anesthetized and sacrificed with inhalation of chloroform after eighteen days.

Anti-tumor Efficacy Assessment

On every third day, tumor diameters were measured, and from those measurements, the tumor volume (length × width × width) was calculated using the equation (product of 0.5 × length × width × width) (21). This process was utilized to calculate mean±SD values for each group. To determine the tumor growth inhibition (TGI), the tumor volume was standardized to the volume of each tumor at time zero, the time point at which therapy began. The following formula was used to determine tumor growth inhibition twice weekly over the period of time (22):

$$TGI\% = \frac{A\text{-}B}{A} \times 100$$

Where, TGI=tumor growth inhibition, A=tumor volume of untreated group, and B=tumor volume of treated group. A tumor growth inhibition >50% was considered meaningful.

Glycolysis Pathway and Glycolysis Products Analysis

The tissue sample was weighed and homogenized in phosphate buffer solution on ice, then centrifuged at 5000 × g for 5 min to collect the supernatant. The concentration of the hexokinase enzyme was quantified by ELISA kit according to the manufacturer's protocol (ElabScience, USA) for a mouse hexokinase-1 (HK-1).

Glycolysis products (pyruvate and ATP) were measured. Pyruvate content was determined through a colorimetric assay using a pyruvate assay kit (ElabScience, USA). ATP levels were determined using a colorimetric method by utilizing an ATP assay kit (ElabScience, USA).

Oxidative Stress Assessment

Oxidative stress was assessed through the measurement of the total glutathione and reactive oxygen species (ROS). Total glutathione was measured in tissue using a colorimetric method through a total glutathione assay kit (ElabScience, USA). According to the manufacturer's recommendations, ROS was measured through a fluorometric method using ROS assay kit (ElabScience, USA).

Cleaved Caspase-3 Immunohistochemistry

To analyze apoptosis in breast cancer tissue, the Immunohistochemistry technique was used in this study. Breast cancer tissues were fixed in10% neutral buffered formalin and embedded in paraffin for 18–24 h. The tissue sections were exposed to 3% H_2O_2 for 10 min, then soaked into citrate buffer (pH 6) at 98℃ for 40 min. These sections were incubated with anti-cleaved caspase-3 rabbit polyclonal antibody (1:50 dilution; ElabScience, USA) for 30 min at room temperature, then probed with a labeled streptavidin-biotin reagent according to the manufacturer's protocol. Immunoreactive products were visualized with the DAB reaction. Sections were counterstained with hematoxylin for 2 min. Using NIH Image J (Fiji, Version 1.53), the optical density (OD) of cleaved caspase-3 was measured (23). The OD values were determined using the following formula:

$$OD = \log \frac{M}{S}$$

Where, OD=optical density, M=maximum intensity (225), and S=mean intensity.

Statistical Analysis

Data were analyzed using GraphPad Prism version 8.01 (GraphPad software, Inc., CA, USA). Data were subjected to one-way ANOVA and significant means were detected using the post hoc Fisher's lease significant differences (LSD). The value of significant was set at $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$.

RESULTS

Anti-tumor Efficacy of NDV and 2DG

The result of the effect of 2DG and NDV on relative tumor volume and tumor growth inhibition in female mice bearing breast cancer was summarized in Figure 1. Significant elevation (P<0.05) in tumor volume was observed starting from the third day of the experiment in the control group as compared to NDV and NDV+2DG treated groups (Figure 1A). At the end of the experiment, a significant reduction (P<0.05) in relative tumor volume was observed in NDV, 2DG, and combination therapy groups (NDV+2DG) when compared to the control group. Figure 1B clarified the effect of 2DG and NDV synergize on tumor growth inhibition (%) in female mice bearing breast cancer at different treated groups. On the eighteenth day of the experiment, a significant (P<0.05) elevation in tumor growth inhibition was observed in combination therapy group NDV+2DG comparing to monotherapy groups NDV and 2DG.

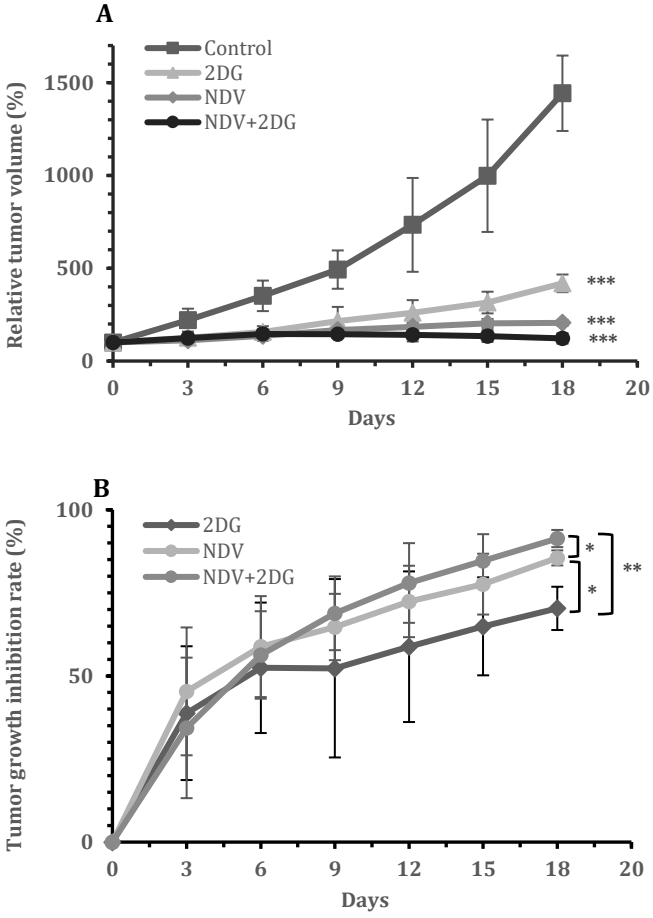

Figure 1. Anti-tumor efficacy of NDV, 2DG, and combination of both against breast cancer model of female mice. **A)**. Relative tumor volumes. **B)**.Tumor growth inhibition. $^{***}P$<0.001, $^{**}P$<0.01 and $^{*}P$<0.05 versus control

NDV and 2DG Effect on Glycolysis Pathway in Breast Cancer Model

At the end of the experiment, there was a significant (P<0.05) decrease of hexokinase-1 enzyme concentration in 2DG, NDV, and NDV+2DG treated groups compared to the control group (Figure 2A). The value of pyruvate and ATP concentration was illustrated in Figures 2B, and 2C. The result indicated that NDV and 2DG significantly (P<0.05) decreased in glycolysis products (pyruvate and ATP) compared to the control group.

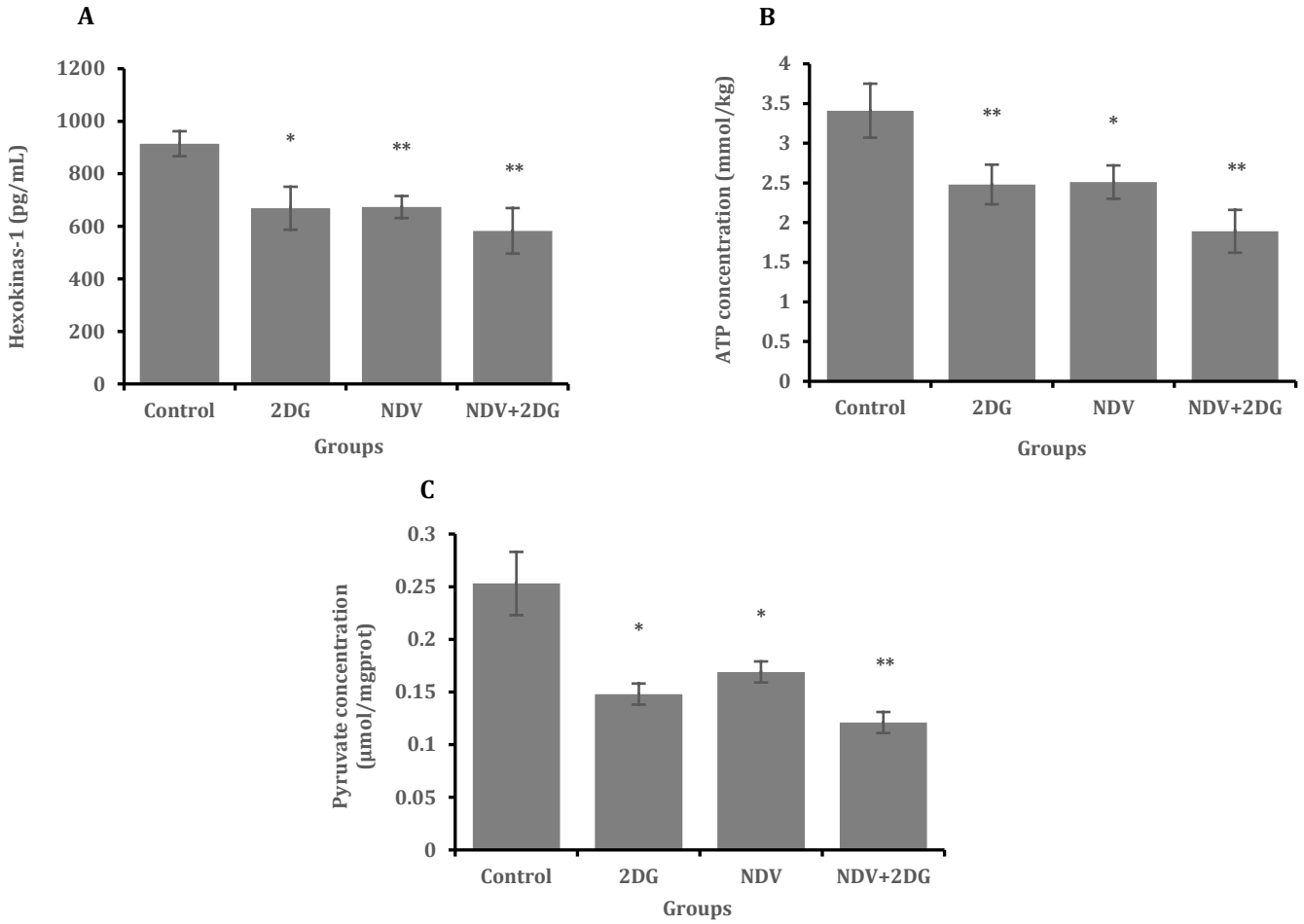

Figure 2. NDV and 2DG effect on Glycolysis pathway and products on breast cancerin female mice. **A)** hexokinase-1 (HK-1) quantification. **B)** ATP concentration. **C)** pyruvate contents.$^{**}P< 0.01$ and $^{*}P< 0.05$ versus control

NDV and 2DG effect on Metabolic Oxidative Stress in Breast Cancer

Figures 3A, B summarizes the results of the effect of 2DG and NDV on ROS and total glutathione. The results revealed that there was induction of oxidative stress through a significant (P<0.05) elevation in ROS levels in all treated groups when compared with the control group (Figure 3A). Moreover, the results indicated a significant (P<0.05) decrease in total glutathione level in all treated groups compared to the control group. Comparing to monotherapy groups (2DG and NDV), a significant (P<0.05) reduction was observed in total glutathione level in the NDV+2DG group (Figure 3B).

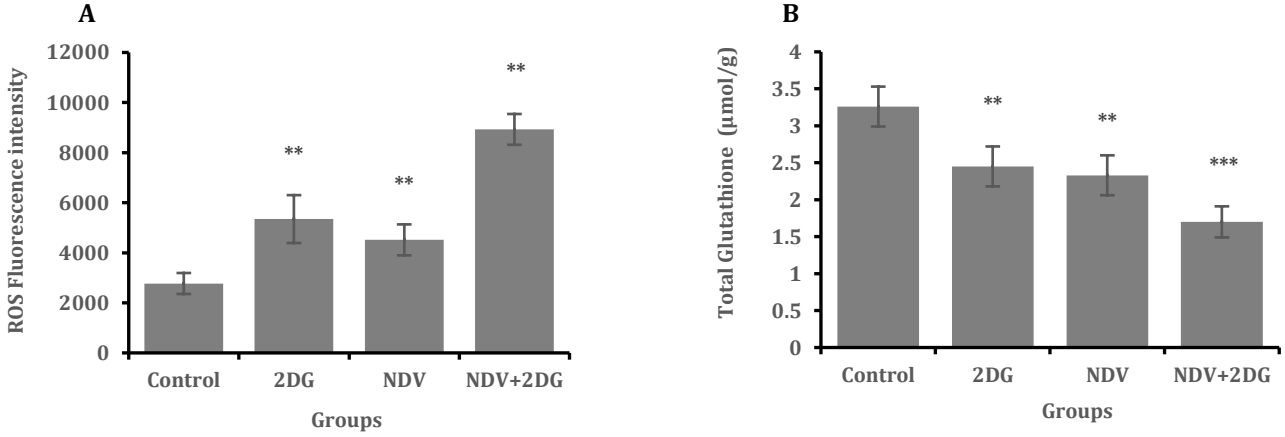

Figure 3. Effect of 2DG and NDV on metabolic oxidative stress on breast cancer in female mice. **A)**, ROS Levels. **B)**, Total glutathione concentration

NDV+2DG Induces Apoptosis in Breast Cancer Model

There was a significant elevation (P<0.05) in optical density of cleaved caspase-3, which was observed in the NDV treated group as compared to the untreated group. NDV+2DG combination therapy has a higher level of cleaved caspase-3optical density than monotherapy (Figure 4).

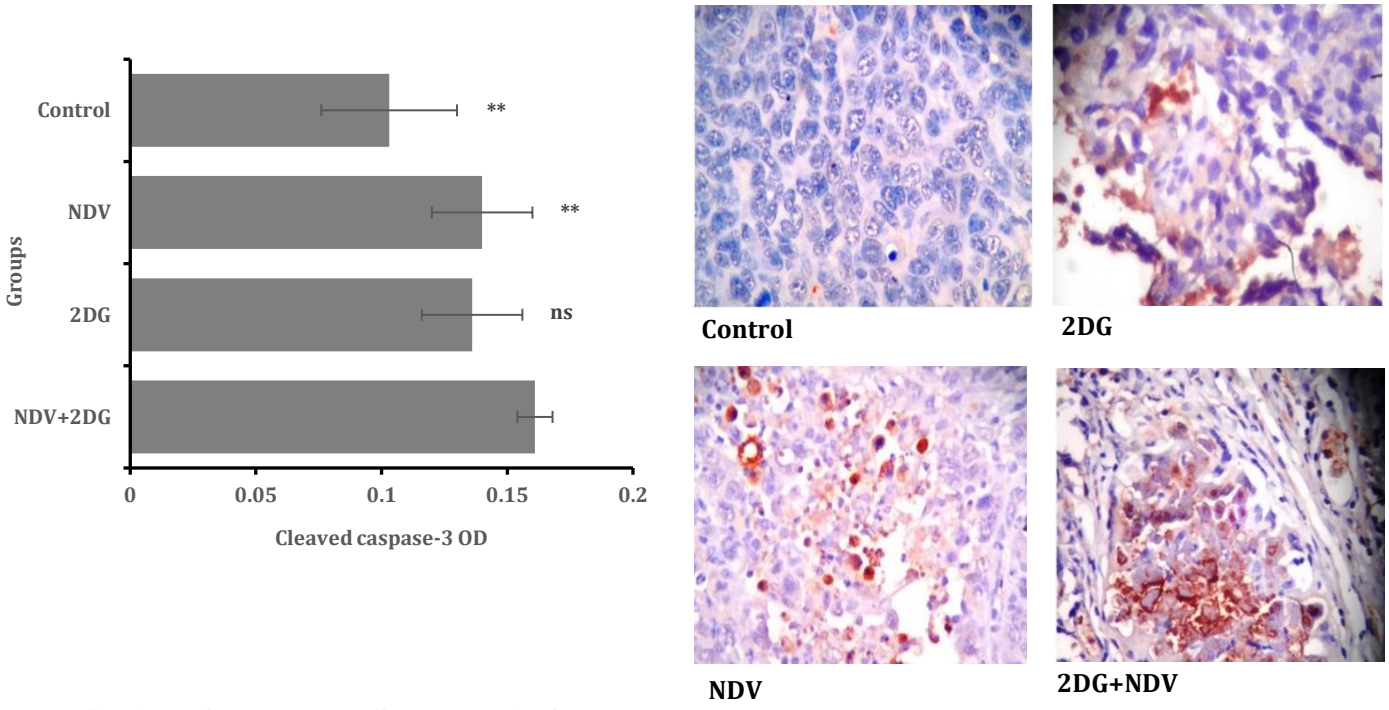

Figure 4. Effect of 2DG and NDV on apoptosis of breast cancer in female mice

DISCUSSION

Cancer cell is characterized by defects in the respiratory chain and highly ROS generation. Therefore, it relies on aerobic glycolysis to avoid oxidative phosphorylation and utilize high quantities of glucose foranabolic processes (24). Otto Warburg described the Warburg effect (aerobic glycolysis) before 70 years ago (25). In addition, cancer cells increase glucose metabolism in the pentose cycle pathway for NADPH production, which acts as detoxification ROS production from mitochondria (26). Therefore, this study used 2DG as a glycolysis inhibitor to enhance NDV and overcome cancer cells' resistance to therapy.

The result of the current study showed that the decrease in relative tumor volume in treated groups compared with untreated group and NDV+2DG combination had more efficient tumor growth inhibition than monotherapies. NDV attacks and kills cancer cells with no effect on normal cells, and NDV inhibits the subcutaneous tumor growth in SCID mice after intratumorally virotherapy (27). Previous researchers demonstrated the mechanism of NDV as anti-tumor efficacy. One mechanism is immune system activation and cytokine secretion (IL-2 and IFN-γ) to attract cytotoxic lymphocytes and natural killers into infected tumor tissue (21). Other mechanisms, it's able to replicate within tumor cells and activation of apoptosis in cancer cells (28). Finally, NDV showed to act as anti-tumor effect through glycolysis pathway inhibition and GAPDH downregulation (14).

Moreover, 2DG enhances anti-tumor efficacy of NDV through tumor bioenergy deprivation and promotes apoptosis in cancer cells (29). 2DG reduced the angiogenesis of tumors by decrease the number of blood vessels and inhibiting oncogene activity (HIF-1α, PDK1, and c-Myc) (20). It induces autophagy and oxidative stress and has a limited therapeutic effect on cancer, but it exhibits a synergistic anticancer effect when combined with other therapeutic agents (30).

The present study demonstrated that NDV-2DG suppresses the glycolytic pathway in breast cancer tissue by inhibiting the HK concentration. Previous works documented that glycolysis-related gene, such as HK-I and HK-II, overexpression in many cancer (31, 32). The current study is shown that reduction in Hexokinase-1 protein concentration in 2DG treated groups compared with the control group. HK is phosphorylated 2-DG to form 2-DG-P, which cannot metabolize by phosphohexose isomerase,

leads to accumulation of 2-DG-P, and inhibits hexokinase (4). Current results agreed with previous research that showed Iraqi NDV strain acts to inhibit HK activity (33).

The result confirms the effect of the combination agent on glycolysis inhibition through decreased glycolysis product (pyruvate and ATP levels) in the treated group compared with the untreated group.2DG is inhibited pyruvate level in breast cancer tissue treated with 2DG due to accumulation of 2-DG-P and without further metabolism in cell (14), leading to deficient ATP levels in treated groups compared to the untreated group (6). NDV reduces HK activity, resulting in diminishing pyruvate levels, thus a decline in pyruvate formation accompanying ATP depletion (33, 34).

In correlation with the mechanism of the combination therapy, the results indicated that induction of metabolic oxidative stress by increasing intracellular ROS formation and decreasing total glutathione in treated groups compared with the control group. 2-DG is acted to activate the AMPK pathway and stimulate ROS production in cancer cells (35). In addition, breast cancer cell which exposed to 2DG showed a reduction in total glutathione and elevation in glutathione disulfide and lipid peroxidation (36). Recently, work documented that NDV impedes glutathione synthesis, glutathione peroxidase down-expression, and ROS accumulation in the cancer cells (37). Thus, current results indicate that combination therapy decreases the metabolism of glucose lead to T-GSH depletion and elevation of ROS.

The apoptotic pathway inhibition in cancer cells was suggested through impairment of mitochondrial pathway, antiapoptotic proteins overexpression, and proapoptotic proteins under-expression (38). Therefore, the present study uses combination therapy to activate the apoptotic pathway by a different mechanism in breast cancer tissue. The current result showed that combination therapy induces a higher apoptosis rate than NDV alone or 2DG alone. NDV had several mechanisms for inducing apoptosis, including endoplasmic reticulum pathway, ferroptosis, caspase-dependent and caspase-independent pathways (28, 37, 39). In addition, 2DG induces ER stress-induced apoptosis by protein N-glycosylation disrupting (40). Other mechanisms activate apoptosis which enhances by 2DG through down-regulating Bcl-2 expression, activating p53 and TRAIL-induced apoptosis (41, 42).

In conclusion, 2DG glycolytic inhibits or enhances virotherapy through promoting metabolic oxidative stress and induction of apoptosis. This study is distinguished by its intelligent targeting therapy that can be used in clinical cancer therapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ديوكسي كلوكوز مثبط تحلل السكريقوي العلاج الفيروسي المضاد للأورام السرطانية لاحداث الاجهاد التاكسدي والموت المبرمج للخلايا في سرطان الثدي

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[١]قسم الإنتاج الحيواني، كلية الزراعة، جامعة سومر، ذي قار، العراق، العراق، [٢]فرع الفسلجة والكيمياء الحياتية والأدوية، كلية الطب البيطري، جامعة بغداد، العراق، قسم العلاج التجريبي، المركز العراقي للسرطان والبحوث الوراثية الطبية، جامعة المستنصيرية، بغداد، العراق،

الخلاصة

واحدة من "السمات المميزة للسرطان" هي التغيير في ايض الطاقة ، وهو زيادة تحلل السكر في الخلايا السرطانية التي تعد المصدر الأساسي للطاقة التي تستخدم هذا المسار الأيضي لإنتاج أدينوسين ثلاثي الفوسفات. العلاج الفيروسي للأورام السرطانية مع مثبط التحلل السكر يعتبر طريقة علاج ذكية للحث على موت الخلايا المبرمج في الخلايا السرطانية. تهدف الدراسة الحالية إلى استخدام 2-ديوكسي كلوكوز وهو مثبط محدد لتحلل السكر لتعزيز التأثير المضاد للسرطان لفيروس مرض نيوكاسل . في هذه الدراسة ، تم استخدام فئران حقنت بخلايا سرطان غدد الثديمأخوذة من نفس النوع وعولجت باستخدام 2-ديوكسي كلوكوز وفيروس مرض نيوكاسل و كليهما. تم قياس الفعالية المضادة للورم وتركيز انزيم هيكسوكيناز -1، البيروفات و أدينوسين ثلاثي الفوسفات. احداث الإجهاد التأكسدي تم فحصه بواسطة قياس تركيز مركبات الاوكسجين الفعالة وفحص الجلوتاثيون الكلي. تم التحقيق من حدوث الموت المبرمج للخلايا باستخدام الكيمياء النسيجية المناعية (المشقوق كاسبيس 3) . أظهرت النتائج أن 2-ديوكسي كلوكوز يعزز الفعالية المضادة للأورام (انخفاض في حجم الورم النسبي وزيادة في تثبيط نمو الورم) لفيروس مرض نيوكاسلضد سرطان الثدي. ترافق هذا التأثير انخفاض في تركيزانزيم الهكسوكينيز، وانخفاض في نواتج التحلل السكر (البيروفات و) أدينوسين ثلاثي الفوسفات. علاوة على ذلك، فإن العلاج ل2-ديوكسي كلوكوز وفيروس مرض نيوكاسل سويتا يؤدي إلى احداث الإجهاد التأكسدي (يقلل من الجلوتاثيون الكلي ويزيد من مركبات الاوكسجين الفعالة). أظهر الفحص الكيميائي النسيجي المناعي ان نسيج الورم في المجاميع المعالجة يوجد فيه مناطق متنخرة نتيجة الموت المبرمج. يمكن الاستنتاج مما ورد في نتائج الدراسة الحالية أن العلاج المركب يمكن اعتباره علاجًا ذكيًا من خلال تثبيط تحلل السكر واحداث الإجهاد التأكسدي والموت المبرمج للخلايا.

الكلمات المفتاحية: مثبط تحلل السكر، فيروس مرض نيوكاسل، الإجهاد التأكسدي، الموت المبرمج للخلايا