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Caspase-3 apoptotic induction by Iraqi Newcastle disease virus on mammary adenocarcinoma transplanted in mice

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Abstract:

This study aimed to investigate the ability of Iraqi strain of Newcastle Disease Virus (NDV) to induce apoptosisin vivothrough Caspase 3 activation when administered intratumoral and compared to intra-peritoneal injection.Immunohistochemistry test was used for detecting apoptosis by using mAb against Caspase-3. Histopathological sections for the treated tumor mass showed proliferation of granulation tissue with extensive area of necrosis (mixture of apoptotic and necrotic cells).This revealed that NDV infection induce apoptosis significantly in mammary adenocarcinoma (AM3) when compared to control group proved by high expression of caspase-3.In vivo immunohistochemical detection of Caspase 3in mammaryadenocarcinoma which give brown stain revealed a significantly($p<0.05$)increase in the mean percentage of cells expressing caspase 3 in NDV treated group compared with low increase in the mean percentage of cells expressing caspase 3 in untreated control group at day 1,2,3,7, and at 14 day. These results revealed that NDV had powerful effect on inducing apoptosis in mammary adenocarcinoma (AM3) during its replication inside the tumor mass for long time after one single injection. This study indicate the role of NDV Iraqi strain in inducing apoptosis as confirmed by caspase-3 activation in cancer cells which is interesting confirmed feature that make NDV Iraqi strain as anti-tumor agnet.

Key words: *Newcastle disease virus Iraqi strain, Apoptosis, Caspase 3, 8, 9*

Introduction:

Induction of apoptosis is the most important mechanism of NDV killing for tumor cells. Newcastle disease virus Iraqi Strain is interesting oncolytic agent with promising anti-tumor properties. One of the major anti-tumor properties is apoptotic induction.Apoptosis can be defined as a carefully regulated process characterized by specific morphologic and biochemical features. It is initiated by both physiologic and pathologic stimuli, and its full expression requires a signaling cascade in which caspase activation plays a central role (1). Apoptosis may be essential for the prevention of tumor formation, and its deregulation is widely believed to be involved in pathogenesis of many diseases, including cancer (2, 3). There are three major caspase-dependent apoptotic pathways: First one isextrinsic pathway whichthe receptor triggered (4, 5).The receptor induced pathway use caspase-8 or - 10 (initiator caspases) (6). In accordance with a pivotal role of caspase-8 in CD95- or TRAIL induced cell death, mice or cell lines deficient in these molecules are completely protected from the apoptotic action of TRAIL or CD95L (7,8), Activated caspase-8 then directly cleaves pro-caspase-3 or other executioner caspases, eventually leading to the apoptosis (9).The second pathway was the intrinsic or mitochondrial pathway which activated by a variety of extra- and intracellular stresses, including oxidative stress, irradiation, and treatment with cytotoxic drugs (10, 11). Unlike the death receptor dependent pathway, the mitochondria dependent pathway is mediated by Bax/Bak insertion into mitochondrial membrane, and subsequent release of cytochrome c from the mitochondrial inter-membrane space into the cytosol (12, 13, 14, 15, and 16). Activated caspase-9 in turn activates caspase-3 and initiates the proteolytic cascade (17, 18, 19,20 and21), while endonuclease G cause DNA damage and condensation (22). The third pathway was en-

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doplasmic reticulum (ER) pathway in which is triggered by ER stress and involves activation of upstream caspases, caspase -12. Caspase -12 subsequently activates downstream executioner caspase, including caspase -3 and caspase -7, which induce apoptosis (23). Rao and co-workers (2001) (24) proposed that any cellular insult that causes prolonged ER stress may induce apoptosis through caspase-7 mediated caspase -12 activation (25, 26, 27).Newcastle disease virus is interesting oncolytic agent with promising anti-tumor properties. One of the major anti-tumor properties is apoptotic induction.Al-Shammary et al. (28) found Iraqi strain to cause internucleosomal DNA fragmentation on Rhabdomyosarcoma and Glioblastoma cells which is the most characteristic feature of programmed cell death. The apoptosis induction was dose –dependant manner (29). Apoptosis was accompanied by virus replication in tumor cell lines tested and signs of endoplasmic reticulum stress were also detected in tumor cells (30).Al-Shammari et al. (31),demonstrate that NDV triggers apoptosis and necrosis as proved by morphological features. So this experiment aimed to confirm theability of Iraqi strain of NDV to induce apoptosis in vivo and to give preliminary look about the caspase-3activation of apoptosis that NDV induce through virus replication in the tumor mass over 14 days of one single injection in 2 different rout of administration.

Material and methods:

1.Ahmed Majeed-2003 (AM3) Transplantable mammary adenocarcinoma line:

This transplantable tumor line was established from Spontaneous murine mammary adenocarcinoma of aged female mouse that transplanted into immunosuppressed mice and successfully adapted for grown in immunocompetent mice for more than 50 passages in vivo. And used as animal tumor model in the development and testing of new anticancer agents in ICCMGR (32).

2. Experimental Animals:

Inbred Albino Swiss mice female mice (8-10) weeks old, (20-25g) weight housed and maintained in ICCMGR animal house, with controlled conditions of temperature (23 ± 5ºC). The animals were fed on special formula food pellets and given water ad libitum. Throughout the experiments, each five animals were housed in a plastic cage containing hardwood chip as bedding. The bedding was changed weekly to ensure a clean environment.

3. virus Isolation and propagation:

1. Sample preparation and Virus propagation:

Newcastle disease virus Iraqi strain (Iraq/Baghdad/Najaf/ICCMGR/2012) was provided by experimental therapy department / ICCMGR, it was directly thawed then antibiotics where added to the virus sample, Ampicillin (200µg/ml) and Streptomycin (200µg/ml), the sample was centrifuged at 3000 rpm for 30 min 4°C this will initially remove any debris and large particulate matter,The supernatant was injected (0.1ml) into 10days embryonted chicken eggs by allantoic sac inoculum. Theeggs were observed daily for mortality, immediately after the death of embryo, it was transferred to the refrigerator (4˚C). After 12-24hrs the allantoic fluid was collected bysterile syringe purified from debris by centrifugation (3000 rpm, 30 minute,4C˚). Then it dispensed into small tubes and stored at -20C˚.

2.Hemagglutination test:

Newcastle disease virus was quantified in which one hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination as described in (30).

4.Transplantation of AM3 mammary adenocarcinoma Tumor Cells:

A method established by (32):Mice were anesthetized by intraperitoneal (I.P) injections of zylazine (40mg/kg) (laboratories Calier, Barcelona, Spain).The tumor mass region was well disinfected with 70% ethanol.Implantations of tumor tissue were carried out by aseptically aspirating the subcutaneous tumors using needle gage 18.The tissue fragments were placed immediately in sterile PBS and the tumor cells were allowed to settle down and the supernatant was discarded, and then the tumor fragments were resuspended in PBS at appropriate volume (100uI).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 10 x 106 viable cells in 0.1ml cell suspension into shoulder region through puncture in thigh region.

5.Treatmeant of animal with Newcastle disease virus :

Once tumor reached the suitable volume at least 5 mm in dimension, mice were randomized into three treatment groups (each contains 15 adult female albino swiss mice).

1-Group one: Intratumoral injection (IT) with NDV (2 x 109HAU) (0.1ml)(one injection).

2-Group two:intraperitoneal injection (IP) with NDV (2 x 109HAU)(0.1ml)(one injection).

3- Group three:used as control (+) group receive no treatment.

The experiments were ended 14 days after initiation of treatment, and the mice were sacrificed. Tumor of the treated and control groups were carefully dissected and fixed in 10% neutralized buffered formalin, paraffin embedded, and sectioned at 5µm thickness for histology and immunohistochemistry.

6.Histopathological samples processing:

The steps were followed according to (33) for tissue preparation, paraffin sections and carried out in Shandon automated histokinate system (Thermo, USA), samples were fixed in 10% neutralized buffered formalin and prior to process they cut and marked then they put in plastic box. Dehydration, embedding, sectioning and staining were done as described by(33).The slides used for routine H&E staining were usual slides while those for Immunohistochemistry were coated with gelatin, all slides were kept in clean dray place until stained.

7. Apoptosis determination in tumor sections

For detection of apoptosis in tumor tissues, Immunohis-

tochemistry assay that the following mAbs were used: Primary mAb: mouse anti- caspase-3, concentration 200 mg/ml diluted at (1:50) (USBiological, USA). Secondary antibody: mouse anti-human IgG, (Biotin), (concentration 2mg/ml) (USBiological, USA). Immunohistochemistry was performed according to USbiological recommended procedure. the quantitative scoring for assessment of caspase-3 staining was doneaccording to (34) by counting the number of positive and negative cells in several randomly selected fields in each section .More than 1200 cells evaluated under 40X high power and the percentage of positive cells was graded.

Statistical analysis:

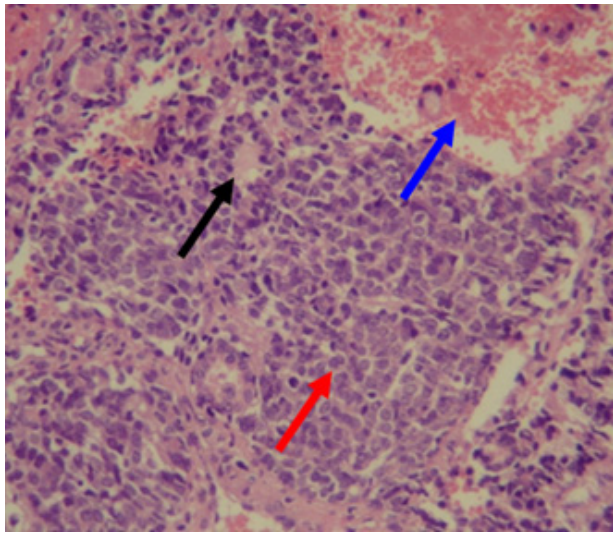
Statistical analysis of data was performed by using (SPSS) Version 13, and for determination of significant differences using ANOVA two way to analysis of date.The difference was considered significant when the probability value ($P \leq 0.05$).

Results:

1.Histopathological study :

The histopathological study in control group showed that the tumor mass characterized by the formation of acinar like structure. Cancer cells are pleomorphic, with large hyperchromatic nuclei, giant cells and numerous mitotic figure were seen (fig4) the sections showed that the tumor cells are separated into variable sizes lobules by interlacting strands of connective tissue or solid masses which undergo necrosis, there is an extensive hemorrhage can be seen. In the treated group (intraperitoneal treatment) at 24hr the histopathological section of tumor mass showedvaculation of cancer cells and area of necrosis.At 48hr there is vaculation of tumor cells with slight fibrosis. After 72hr of NDV treatment the histopathological examination revealed area of granulation tissue infiltrated by mononuclear cells. After 1week of NDV treatment the microscopical section showed interlobular fibrosis infiltrated with mononuclear cells and the tumor mass encapsulated with granulation tissue which infiltrated down word between the tumor cells.After 2weeks of NDV treatment the histopathological section showed marked fibrosis with complete dissolution of cancer cells with congestion of blood vessels (Figure-5).

The histopathological section in the treated group (intratumoral treatment)at 24hr showed extensive area of necrosis with remaining of small nest of cancer cells with vaculation. At 48hr the sections showed wide necrotic area contain debris of cancer cells.After 72hr of treatment with NDV the histopathological section showed granulation tissue infiltrated with mononuclear cells. After 1 week of treatment the histopathological section revealed extensive necrotic area with congestion of blood vessels. After 2 week of treatment the microscopic section revealed proliferation of granulation tissue with extensive area of necrosis (Figure-6).



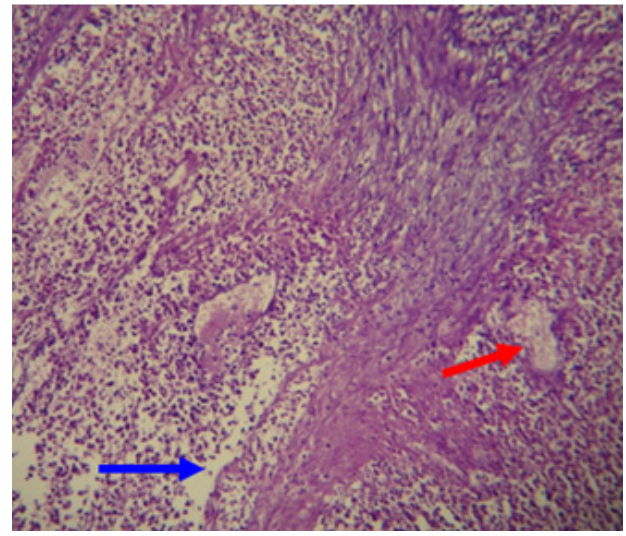


Figure (4) Histopathological section of tumor massFigure (5) histopathological sectiontumor masstransplanted transplanted in mice for control group showing acinirin mice of treated group (IP) after 2week showingmarked like structure (black arrow) with proliferation of pleo-fibrosis (blackarrow)withcomplete dissolution of cancer morphic cells with hyperchromatic nuclei (red arrow) cells (blue arrow) with congestion of blood vessels (red with extensive areas of hemorrhages (blue arrow) arrow)(H and E stain 400x). (H and E stain400x).

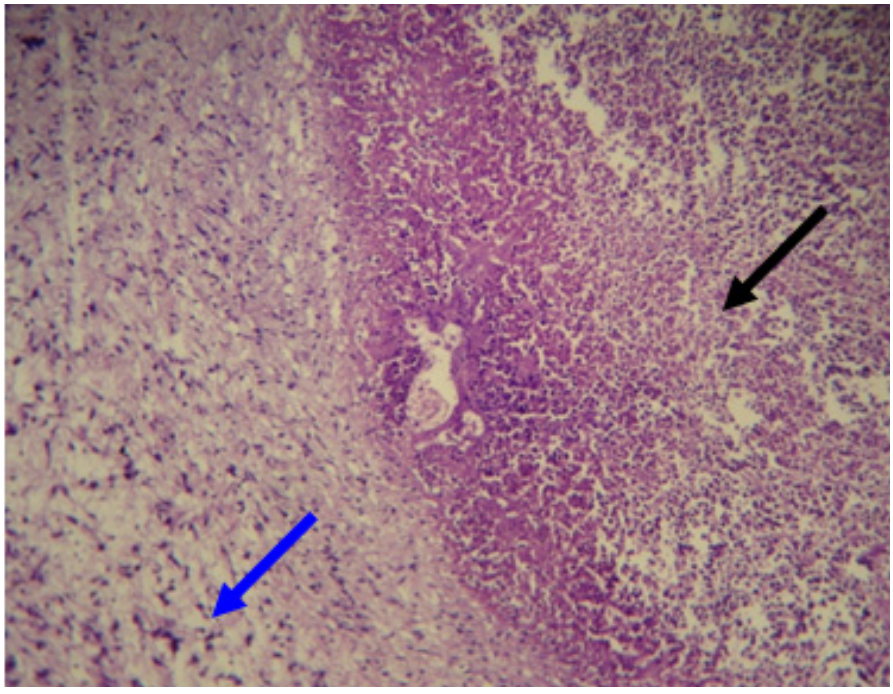


Figure (6) histopathological section of tumor mass transplanted in mice of treated group (IT) after 2 week showing proliferation of granulation tissue (blue arrow) with extensive area of necrosis (mixture of apoptotic and necrotic cells) (black arrow) (H and E stain 400x).

2. Immunohistochemistry study:

To assess apoptosis induced by treatment caspase 3 expression was evaluated in tumor specimens by immunohistochemistry.

The immunohistochemistry section in the control group showed pleomorphic cancer cells take different arrangement , high cellularity proliferation of cancer cells which appear soiled masses also there is mitotic figure present and it negative to Dap stain (Fig7).

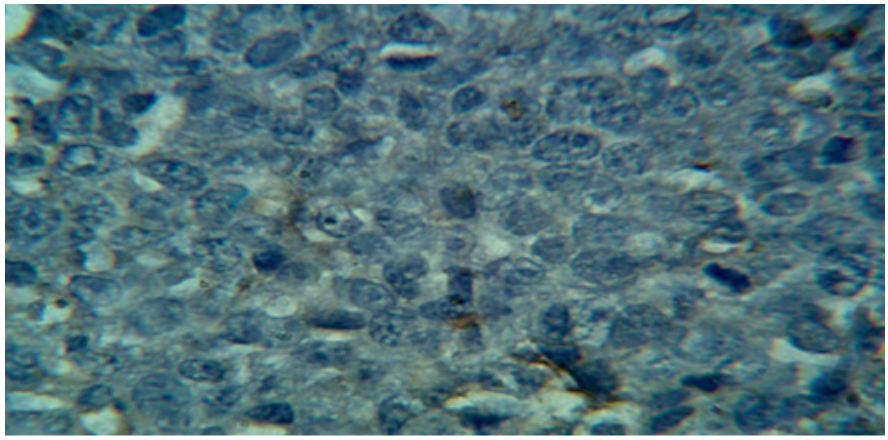


Figure (7) Immunohistochemistry section of tumor mass in transplanted mice of control untreatedgroup showing pleomorphic cancer cell taken different arrangement negative to DAB stain(DAB stain 1000x).

Immunohistochemistry section in the treated group (intraperitoneal injection of NDV) at day 1,2,3,7, and day 14 showed a number of apoptotic cells which take brown in color for caspase 3 protein as illustrated in table (1) and (Figure-8)
the result which shown in table (1) revealed a significantly ($P>0.05$) marked increase in the mean percentage of cells expressing caspase 3 in the Newcastle disease virus treated group at day 1, 2, 3, 7dayand day 14 compared with untreated control group, the expression of caspase 3in treated group was increasing significantly($P<0.05$)when compared to all treated groups

Table (1) The mean percentage of caspase 3 expressions and the frequency of distribution of expression scores in mammary adenocarcinoma tissues in treated mice injected (IP) with NDV and control group :

Type of Antibody	1day	day 2	3day	day 7	14day
Caspase 3	12.6+0.005 B,e	15.0+0.003 B,d	20.0+0.005 A,c	21.4+0.004 B,b	21.7+0.003 B,a
Control Caspase 3	5.1+0.003 D,e	5.5+0.003 D,d	5.7+0.005 D,c	6.0+0.003 D,b	6.3+0.005 D,a

Different capital letter represents significant differences (P≤0.05) between meansof the same column. Different small lettersrepresent significant differences(P≤0.05) between means of the

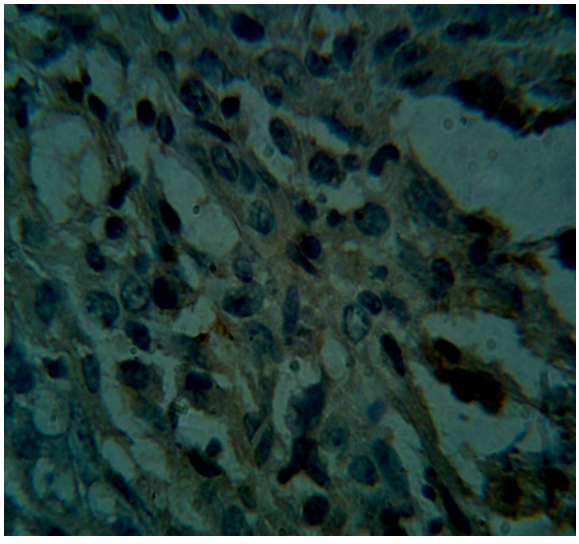


Figure (8) Immunohistochemistry section of tumor mass of transplanted mice of treated group(IP) after 14 day showing apoptotic cells (DAB stain1000x)

The stained sections in the treated group (intratumoral injection of NDV) atday 1,2,3,7, and day 14 showed a number of apoptotic cells which take brown in color in caspase 3 protein as illustrated in table (2)and (Figure-9).The result which shown in table (2) revealed a significantly (P>0.05) marked increase in the mean percentage of cells expressing caspase 3 in the Newcastle disease virus treated group at day 1, 2, 3, 7dayand day 14 compared with untreated control group,At day 14 the expression of caspase 3in treated group was more significant (P<0.05) when compared control groups.

Table (2) The mean percentage of caspase 3,8,9 expressions and the frequency of distribution of expression scores in mammary adenocarcinoma tissues in treated mice injected (IT) with NDV and control group :

Type of Antibody	1day	2day	3day	7day	day 14
Caspase 3	11.2±0.003 B,e	15.0±0.003 A,d	16.5±0.003 B,c	18.3±0.003 A,b	19.1±0.003 B,a
Control Caspase 3	5.1±0.003 D,e	5.5±0.003 D,d	5.7±0.005 D,c	6.0±0.003 D,b	6.3±0.005 D,a

Different capital letter represents significant differences (P≤0.05) between means of the same column. Different small letters represent significant differences (P≤0.05) between means of the same rows.

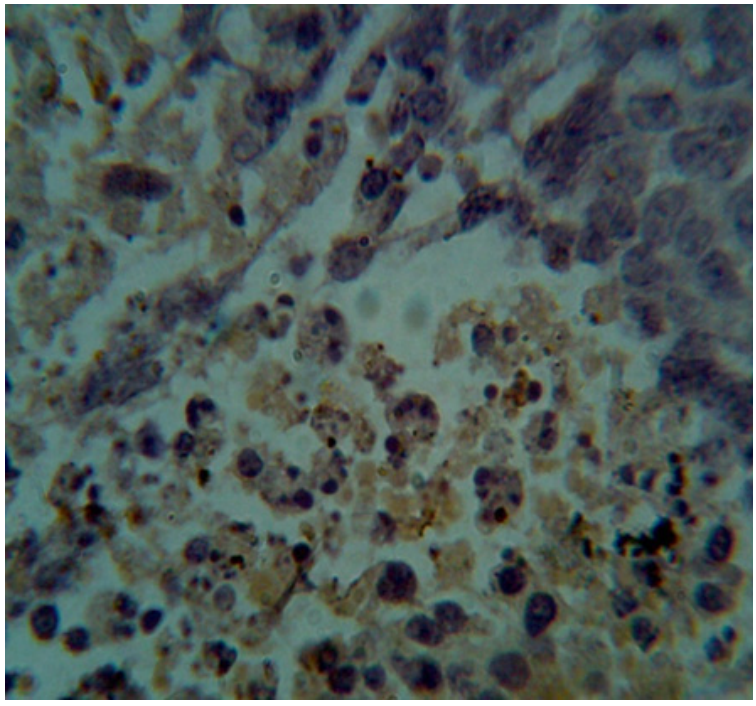


Figure (9) Immunohistochemistry section of tumor mass of transplanted mice of treated group(IT) after 14days showing apoptotic cells (DAB stain 1000x).

Table (2) The mean percentage of caspase 3,8,9 expressions and the frequency of distribution of expression scores in mammary adenocarcinoma tissues in treated mice injected (IT) with NDV and control group :

Discussion:

Histopathological examination of the sections prepared from tumor masses of the treated and control groups was performed to analyze the histological process of anti-tumor effect after treatment. The histopathological features of this tumor was previously described by (32) as aggressive metastatic adenocarcinoma with poorly differentiated cells. Al-Shamery, et al (35) used this model of tumor cells to test anti-tumor activity of NDV Iraqi strain and it showed strong growth inhibition as well as prolong surviving for the treated animals.

Notably, the tumor regression induced by treatment with Newcastle disease virus was accompanied by increasing numbers of infiltrating lymphocytes (cytotoxic), natural killer cells, macrophage and increase TNF-α expression and by increasing numbers of apoptotic cells in tumor tissues.. These finding further confirmed by histopathological examination. Infiltrating lymphocytes were markedly observed in and around the tumor mass in the NDV treated groups. Where we can find extensive necrosis in the tumor mass infiltrated with T-lymphocyte, Natural killer cells as well as macrophages and plasma cells. The histopathological finding confirmed by (31) and (36) results in histopathological examination of treated mammary adenocarcinoma by Iraqi strain of NDV. (37) found Tumor inflammation (presence of mononuclear inflammatory cells) in response to PV701-NDV therapy shortly after dosing and before some of the tumor responses; lesion became inflamed or swollen.Further more direct cytolytic action second to virus replication in the tumor cells as well as apoptosis induction which proved earlier explain more of antitumor action in treatment groups. The infiltration of inflammatory cells in NDV-infected tumor has the complimentary function of killing infected and surrounding tumor cells. Control untreated group showed progressive tumor mass growth with less necrotic areas,(38) explained that that the blood vessels within tumors are highly irregular and tortuous, often leading to blind ends, leading to poor circulation, This also give rise to hypoxic and nutrient poor regions, which are not as susceptible to the many types of chemotherapies in use that target rapidly proliferating cells.

From the result which shown in table (1),(2) we showed that Newcastle disease virus treatment was the most effective modality in inducing apoptosis by increase expression of caspase 3 by both rout of administration, IT and IP.The downstream caspases (like caspase-3) induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, and finally, destruction of “housekeeping” cellular functions. Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing (36). (28, 39and 40) found NDV caused internucleosomal DNA fragmentation which is most characteristic feature of late

event of programmed cell death. (41) reported induction of apoptosis by inactivated NDV, this report together with (42) result about M protein of VSV virus which induces apoptosis via the mitochondrial-associated pathway due to inhibition of host gene expression, we can propose that one of NDV proteins can play rule in apoptosis induction which needs more investigation.As final conclusion, we can see from the results of current work that caspase-3 expression increased gradually over the 14 days of first and only injection by both rout of administration (IT and IP) which indicate continues virus replication as there was low expression in the control group.

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آلية استحداث الموت المبرمج لفايروس مرض النيوكاسل العترة العراقية على سرطان الغدة اللبنية المغروس في الفئران

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2 كليةالطب البيطري /جامعة بغداد

الخلاصة:

يعد استحداث الموت المبرمج اهم الية لاصابة الخلايا السرطانية بفايروس النيوكاسل. يعد فايروس النيوكاسل العترة العراقية عاملمضاد لنمو الاورام ويمتلك خواص مثيرة للاهتمام . اهم هذه الخواص هي الموت المبرمج . هدفت دراستنا الى التحقق من قابلية العترة العراقية لفايروس النيوكاسل لاستحداث الموت المبرمج في لاورام المزروعة في المختبر والتعرف على الالية والمسار التي يستحدث بها الموت المبرمج. تم استخدام اختبار الكيمياء المناعية النسيجية لتحديد الموت المبرمج وذلك باستخدام الجسم المضاد الاحادي النسيلة المضاد للكاسبيس 3والجسم المضاد الاحادي النسيلة المضاد للكاسبيس 8والجسم المضاد الاحادي النسيلة المضاد للكاسبيس 9 . النتائج اضهرت ان فايروس النيوكاسل يستحدث الموت المبرمج بشكل مهم احصائيا عند القارنة بالخلايا الغير مصابة . الدراسة الكميائية المناعية النسيجية للاورام المزروعة في الحيوانات اضهرت تعبيرا عاليا للعامل كاسبيس9واذا اعطى اللون الجوزي وبشكل مهم احصائيا للمقاطع الورمية للفئران المعالجة بفايروس النيوكاسل بالقارنة مع التعبير المنخفض للعامل كاسبيس 3و8 والفئران الحاملة للاورام وغير المعالجة وذلك بعد 1و2و3و7ويوم و14 يوم بعد الحقن.هذه النتائج اظهرت ان فايروس النيوكاسل يمتلك خواص استحداث الموت المبرمج في الاورام بشكل قوي وان المسار المسلوك لاستحداثه مسار المايتوكوندريا (الداخلي) .هذه الدراسة سلطت الضوء على ان فايروس النيوكاسل العترة العراقية هي اضافة مهمة للعوامل المضادةللاورام.