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Confirming intrinsic pathway apoptosis event in cervical carcinoma cells (HeLa) treated with hybrid nanoliposomes

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Abstract:

Cancer targeted nanotherapy represent an exciting field in the search for new cancer specific therapies to avoid conventional chemotherapy side effects. Because cancer cells usually have malfunctioning apoptotic machinery which favors survival pathways and drug resistance. Cancer cell apoptosis is the favorable event to be induced in any new anticancer agent development. Nanotherapy goals are to elevate therapeutic efficiency, selectivity, and overcome drug resistance as major obstacle in cancer treatment. Hybrid nanoliposomes (nHLs) may fulfill all these features in cancer therapeutics. We have previously demonstrated the ability of in house synthesized nHLs to inhibit HeLa cell line proliferation and study preliminary the induction of apoptosis as a consequences of that inhibition. In order to confirm the event of apoptosis in HeLa cell line incubated with the synthesized nHLs we exposed HeLa cells to inhibition concentration 50 (IC50) of previously synthesized hybrid nanoliposomes. Mechanism of apoptosis induction was determined using mitochondrial membrane potential disruption, caspase-3 activity and single cell gel electrophoresis as well as DNA fragmentation assay. All apoptosis detection procedures used gave a clear defined significant indication that nHLs was capable of induce apoptosis in HeLa cells through intrinsic pathway. This result needs further investigation to confirm nHLs as potential nanotherapy.

Key words: *apoptosis, nanoliposome, liposome, nanobiotechnology, HeLa cells.*

Introduction:

Conventional cancer therapies such as chemotherapy and radiation always combined with undesirable side effects as a result of their nonspecific cancer cell targeting. More than two decades know researchers around the world put time, effort, and huge budget investments on the line in order to shape old cancer therapies and invent new ones toward high specificity targets against cancer cells(1). Studies on the molecular levels were able to recognized mutated proteins, aberrant pathways and cell surface markers expressed or/and over expressed specifically in cancer cells, differentiate them from normal cells and makes them favorable candidate to targeted therapy of cancer (2 and 3). In this regard nanotechnology contributes to this effort considerably through constructing targeted nanoparticles getting use of those differentiated markers (4). This came in different strategies, such as the conjugation of cancer cell specific antibody, protein or peptide to nanoparticles loaded with anticancer agents, and constructing nanocarriers (5 and 6) or using the unique optical properties of nanomaterials (7). Hybrid nanoliposomes (nHLs) came in as a cancer cell membrane targeted nanoparticles (8), not like other conventional nanoliposome which used as chemotherapy encapsulation vessels, nHLs can serve as chemotherapy by themselves (9 and 10). These nanoliposomes were able to induce apoptosis in different cancer cells in vitro (11-15). In previous research our team at the Iraqi Center for Cancer and Medical Genetics Research (ICCMGR) was able successfully to synthesis, characterize nHLs, and test them in vitro against cervical carcinoma cells (HeLa cells) (16). The growth inhibition concentration 50 (IC50) was determined and apoptosis event in the challenged cells was observed with one method of detection. In this research the same team goes further and used the same cells with the same conditions of synthesizing and characterizing nHLs using the same IC50 to provide more evident about the induction of apoptosis in the HeLa cell.

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Materials and Methods:

Hybrid nanoliposome was synthesized and characterized exactly as described previously, with materials supplied from the same sources (16). Cervical cell carcinoma cell line (HeLa) supplied from Iraqi Center for Cancer and Medical Genetics Research (ICCMGR). Cells were grown on RPMI-1640 tissue culture media with HEPES, L-glutamate (USBiological, USA), 10% fetal calf serum (Capricorn, Germany) , Streptomycin and Gramycine (Ajantaa pharm, India). Cells were grown in tissue culture vessels and 3.5mm Petri dishes (Santa Cruz, USA). The IC50 of nHLs determined previously was used (0.2mM) to assess the apoptosis event in HeLa cell. Apoptosis was detected in both cells incubated with nHLs and control untreated cells. Mitochondrial potential disruption was assayed using the procedure of Castedo et.al, (2002) (17) on adherent cells with minor modifications using Apoptosis Detection Mitochondria Bioassay Kit (USBiological, USA). Cells imaged with CCD camera (Micros, Switzerland) under fluorescence microscope (Micros, Switzerland) and 25 images for treated and control untreated cells analyzed suing ImageJ® analyzing software (NIH, USA). Caspase-3 activity was determined using Caspase-3 Apoptosis Detection Colorimetric Bioassay Kit (USBiological, USA) according to the kit manufacturer method. Single cell gel electrophoresis was carried out according to Rojas et.al, (1999) (18), three parameters were used. They were tail moment length, %DNA on tail, and olive head moment, cells were imaged with CCD camera (Micros, Switzerland) under fluorescence microscope (Micros, Switzerland) and results analyzed with Comet Assay IV software (Perceptive, England). DNA fragmentation assay carried out using mixed fluorescence dyes acridine orange and propidiume iodide according to Petit et.al, (1999) (19). Statistical analyses carried out using ANOVA and post hoc t-test on exel Microsoft office ($p<0.05$).

Results:

Three concentrations of nHLs contain 0.1, 0.2, 0.3 mM of DMPC have growth inhibition activity against HeLa cells ($p<0.05$) and the IC50 laid in the 0.2mM of nHLs (16), a representative images of these cells are presented in figure (1).

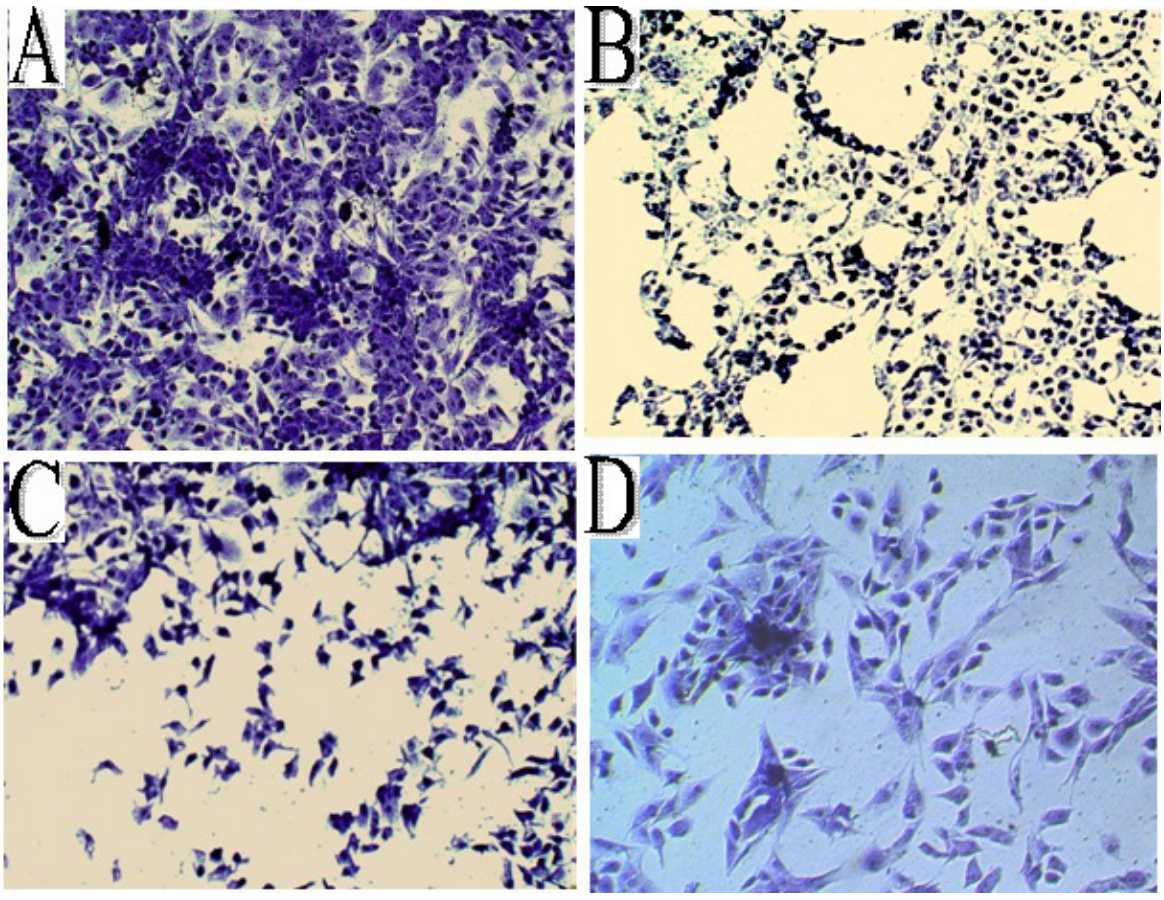

Figure 1: representative images for human cervical carcinoma cells (HeLa) after exposure to different concentrations of hybrid liposomes (HLs) for 24hr at 37ºC. A, control untreated cells 200X, B; cells treated with 10mM of HLs 200X, C; cells treated with 20mM HLs 200X, D; cells treated with 30mM of HLs 400X.

To investigate if the apoptosis induced in treated cells and in order to explore the mechanism of this induction, mitochondrial membrane potential disruption was determined in different timing. Results indicated that apoptosis take place in the nHLs treated cells just after 4hrs of incubation time through distraction of mitochondrial membrane (Figure 2). The intensity of green color fluoresces was much higher in treated cells after this time of incubation with nHLs compared to control untreated cells. As indicated using ImageJ ® software the mean green color fluorescent was 60 (mean of 25 images analyzed) in nHLs treated cells. Whereas the mean red color fluorescent was 12 in the same cells. The situation was in reverse with the control untreated cell, where the mean red color fluorescent was 55.5, while mean green color fluorescent was 7.8 (Figure 3). The differences between these fluorescence intensities were significant ($p<0.05$). These results indicate that apoptosis was initiated just after 4hrs of incubation period. In order to confirm the induction of apoptosis with more certainty the activity of caspase-3 enzyme in the cell line was detected in different time intervals of incubation with nHLs. Results can be presented as follows, at 2.5 hr of nHLs exposure the caspase-3 activity was significantly higher ($p<0.05$) in cells treated with nHLs than control untreated, this difference was 0.26 time fold greater in treated HeLa cells. After extending the exposure time to 5 hr the activity of caspase-3 in both HL treated cells and control untreated cells was declined and there was no significant difference between them ($p<0.05$). At 10 hr of exposure time the activity of caspase-3 was notable elevated in the nHLs treated cells up to 4.5 times fold compared to the activity of this enzyme in control untreated cells this increment was significant ($p<0.05$). Extending the incubation time to 24hrs the caspase-3 activity declined significantly in treated cells and it was similar to the activity of this enzyme in the control untreated cells (Figure 4).

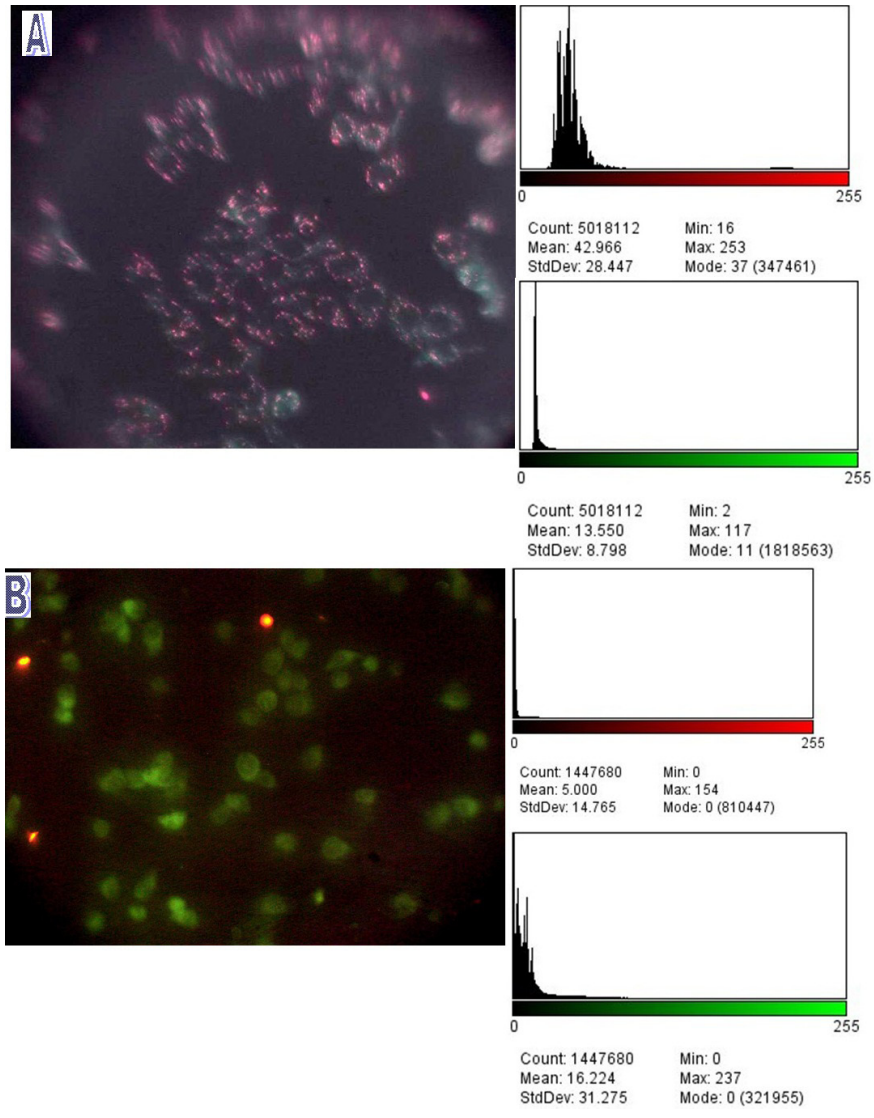

Figure 2: fluorescence intensity of red and green color as detected under fluorescent microscope, A;control untreated HeLa cells, B; HeLa cells incubated with HLs for 4 hrs. Images analyzed with ImageJ® software.

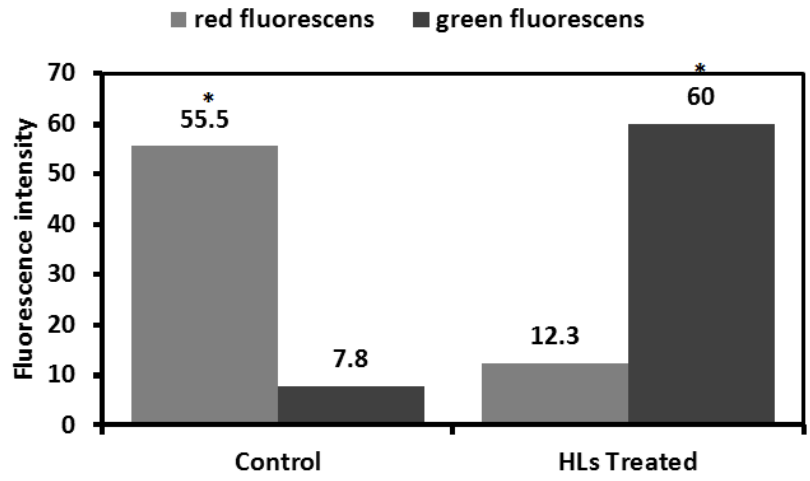

Figure 3: Fluorescence intensity of red and green color as detected under fluorescent microscope for HeLa cells (control untreated and nHLs treated) and analyzed with ImageJ® software, columns represent means of 25 image analysed, * represent significant differences ($p<0.05$).

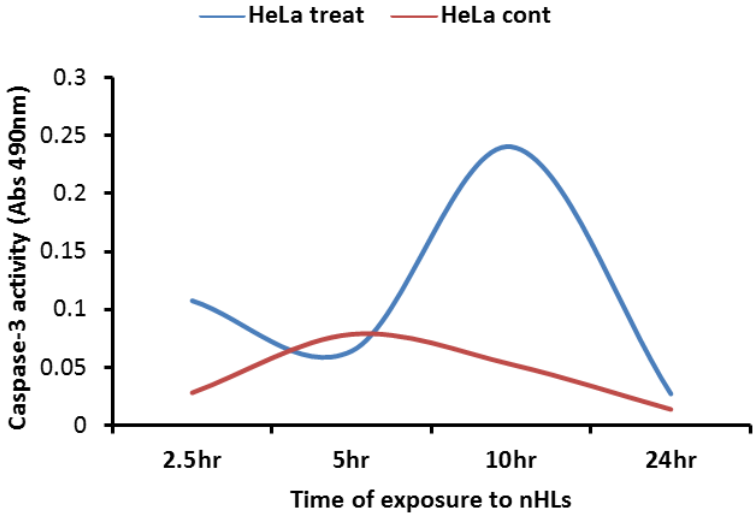

Figure 4-: Caspase-3 activity in HeLa cells of control untreated and cells treated with nHLs for three time interval 2.5 hr, 5 hr and 10 hr. enzyme activity determined in cell lysate of both control and nHLs treated cells.

Single cell gel electrophoresis conducted on both treated and untreated control HeLa cell line cells revealed that cells incubated with nHLs were having 1.21 times fold comet length than the control untreated cells (significant $p<0.05$).Whereas the percentage of DNA in the tail and olive moment increased in the treated cells by 2.85 and 5 times respectively compared to control untreated cells (significant $p<0.005$). (Figures 5 and 6). A DNA fragmentation assay result clearly shows the nuclear DNA condensation and fragmentation after 24 hr of HeLa cells exposed to nHLs (Figure 7).

HeLa cell line	Comet Length	DNA in Tail %	Olive moment
Control untreated	114.94±28.85	6.17±6.49	1.74±1.83
Incubated with HLs	139.22±68.26*	17.61±16.86*	8.85±10.68*

mean±SD, n=50

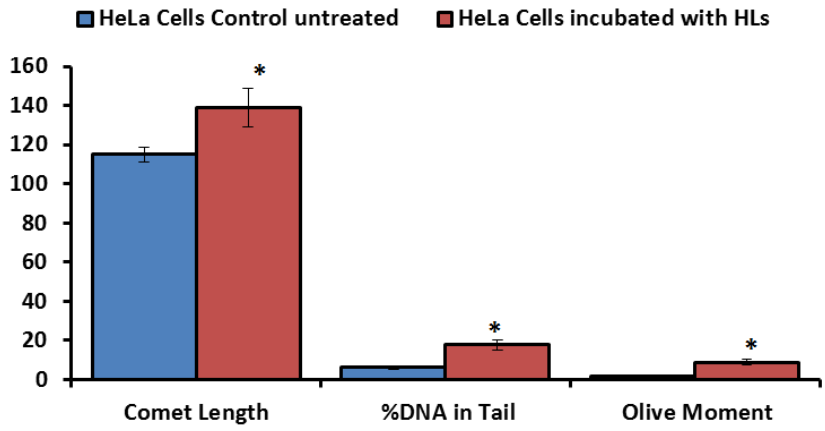

Figure 5: Comet assay for HeLa cells, DNA damage assessment, three parameters of DNA damage comparison between control untreated cells and cells incubated with HLs for 24hrs at 37ºC, * represent significant difference between the means ±S.E.

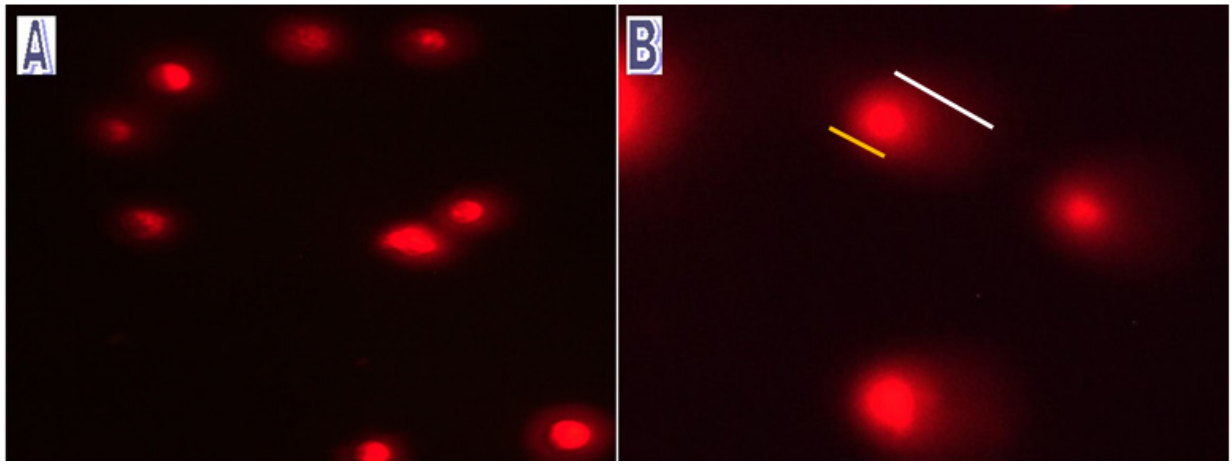

Figure 6: A representative image of comet assay for HeLa cells for DNA damage assessment, A; control untreated cells, B; cells incubated with nHLs for 24hr at 37ºC. White line indicates tail length, yellow line indicates olive moment, and the red color intensity indicate % DNA in tail.

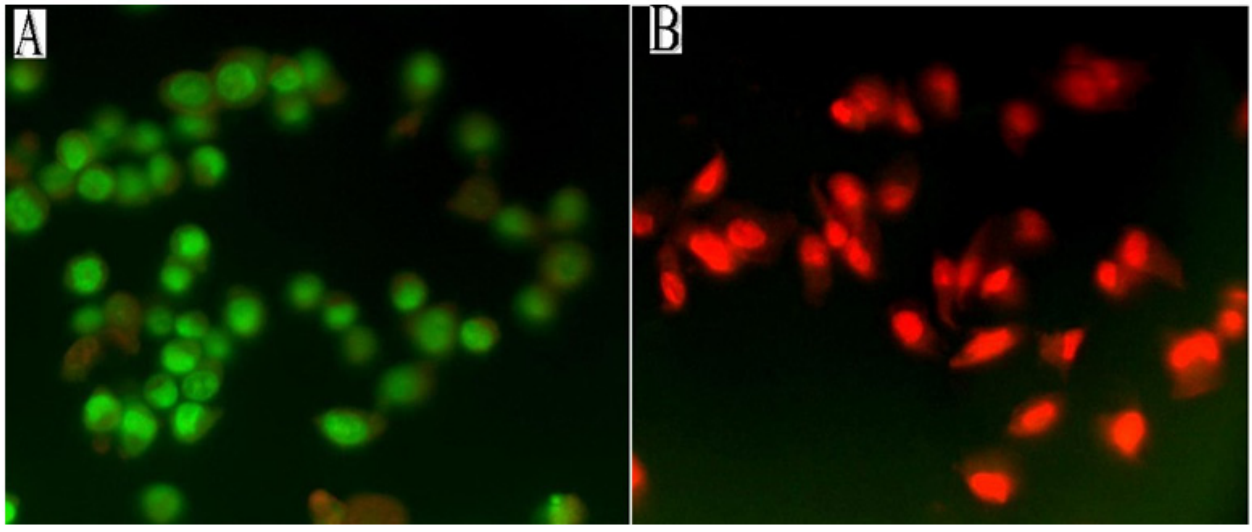

Figure 7: A representative images of DNA fragmentation assay for HeLa cells, A; control untreated cells, B; cells incubated with nHLs for 24hr at 37ºC. Cells were stained with fluorescence dyes mix acridine orange and propidium iodide.

Discussion:

Conventional liposomes are closed vesicles consist of two bilayer chambers, inner core chamber with hydrophilic properties and outer or external chamber with lipophilic properties. These liposomes used in many experiment as drug delivery system, they synthesized to encapsulate different chemotherapeutic agents that should be deliver directly to the site of cancer in order to spare the body all unwanted side effects of the chemotherapeutic. Most of these liposomes prepared with mix of phospholipids and cholesterol in different ratios to form particles with different membrane rigidity (20). Hybrid liposomes contain the same inner core chamber and external lipophilic surface as the conventional liposomes, but it different in there lipid bilayer construction. Hybrid liposomes contain phospholipid (DMPC) and polyoxyethylene(n) dodecyl ethers (C12(EO)n), this structure makes these type of liposome capable of integrating in to the cancer cell membrane. This makes the cancer cell membrane less fluidic and more rigid which prevent them from being capable to grow, as a result apoptosis evoked (14 and 21). This type of liposomes was first developed by Ueoka et. al, in Japan (8). They differ from conventional liposomes in that they do not encapsulate any therapeutic chemical compound, so they do not categorized in the field of drug delivery systems. Hybrid liposome intended to be a therapeutically by themselves (9). Nano-hybrid liposomes are constructed by same procedure but their size should be under 100nm to gain the character of nanoliposome (9). This nano-size provides a privilege of longer existing in the circulation (14). In previous research our team synthesized successfully and characterized hybrid nanoliposome and tested its antiproliferative activity against HeLa cell line. This liposome did not have a detectable cytotoxicity against normal human lymphocytes (16).Since evading growth suppressing and sustained proliferating signaling are major hallmarks of cancers, the induction of apoptosis considered to be the main goal of all drug development maneuvers (22-25). After exposing HeLa cells to the synthesized hybrid liposome, it's vital to confirm the induction of apoptosis event. In previous work Komizu et.al (2011) used TUNEL assay, revealed that apoptosis was occurred in HeLa cells treated with HL consist of 90 mol % DMPC and 10 mol % C12(EO)n (n = 21, 23, 25) mixture in 5% glucose solution. In this research we try to confirm the induction of apoptosis using four different techniques. The selection of assays used was designed to determine the apoptosis event timing and the pathway its take. Mitochondrial membrane potential disruption represent the first event in the sequence of apoptosis (26). Our study indicated that apoptosis process started just after 4hr of exposing HeLa cells to nHLs. Mitochondrial membrane potential distraction represents the earliest event for apoptosis (27). The result of this assay indicated that apoptosis was through the intrinsic pathway. In order to confirm the intrinsic pathway occurrence for the apoptosis a second assay was the evaluation of caspase-3 activity (28). As can be seen from the results the caspase-3 activity during the 24hr of cells exposure to nHLs the maximum activity for this machinery was after 10hr of exposure. Now we tried to determine the end stage of apoptosis event in the nHLs exposed cells. Since DNA fragmentation was the well defined character of apoptosis end stage, we thought comet assay is the perfect tool to detect this event in the exposed cells. Our results indicated the collective characters of DNA damage in the nHLs treated cells after 24 hrs. In order to confirm the end stage DNA damage via apoptosis, another method was conducted to have a firm consideration about the induction of apoptosis. Staining cells with mixed fluorescence dyes helps to differentiate between apoptotic, necrotic, and live cells. Acridine orange and propidium iodide dye mix is well documented method for such detection (29). Our results clearly indicate the DNA fragmentation of HeLa cells exposed for 24 hrs to nHLs.

In conclusion the results of this research confirm the event of apoptosis in the HeLa cells treated with nHLs. This event takes place throughout the intrinsic pathway and starts in mitochondria membrane destruction just after four hours of the time of exposure. The primary hallmark of this pathway, caspase-3, was at its peak activity in the treated cells just after 10 hrs of cells exposure to nHLs. The end of apoptosis was confirmed by cellular DNA fragmentation after 24 hr of exposing treated cells to the nHLs.

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تأكيد حدوث ظاهرة الموت المبرمج في خط خلايا سرطان عنق الرحم HeLa المعالجة بالجسيمات الدهنية النانوية الهجينة

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الخلاصه:

تعد عملية إستحداث العلاجات النانوية المستهدفه للخلايا السرطانية مثار لإهتمام الباحثين في هذا المجال لغرض التخلص من الأثار الجانبية الناتجة عن إستخدام العلاجات الكيميائية التقليدية . تميل الخلايا السرطانية نتيجة للخلل الحاصل في مكنونها الوراثي الى الحيود عن المرور في سبل الموت المبرمج ونتيجة لنفس الخلل تكتسب تلك الخلايا قدرة على مقاومة العلاجات الكيميائية التقليدية لذا فان من أفضل الطرائق التي يتم فيها تحديد كفاءة العلاجات الجديدة المقترحة هي مقدرة تلك العلاجات على حث الموت المبرج في الخلايا السرطانية . تطمح عملية تطوير العلاجات النانوية إلى رفع كفائة وإنتخابية العلاجات المتوفرة فضلا عن تجاوز عقبة ظاهرة مقاومة الخلايا السرطانية للأدوية ، هنا تأتي أهمية إستخدام الجسيمات الدهنية النانوية الهجينة كعلاج مقترح يحقق كل تلك الطموحات . قام الفريق الذي أعد هذا البحث في بحث سابق بتصنيع الجسيمات الدهنية النانوية الهجينة ودرس بشكل أولي قدرة الجسيمات الدهنية النانوية المصنعة على تثبيط نمو خلايا سرطان عنق الرحم HeLa في الزجاج كما درس بشكل أولي إمكانية حث الموت المبرمج في تلك الخلايا نتيجة لذلك التثبيط ، ولغرض التثبت تماما من إمكانية حث الموت المبرمج في خلايا HeLa بواسطة الجسيمات الدهنية النانوية الهجينة المصنعة جرى إستخدام أربعة تقنيات مختلفة لدراسة هذه العملية وشملت تحديد مدى تأثر مكنون غشاء المايتوكوندريا وقياس فعالية أنزيم caspase-3 وتقطع الدنا في الخلايا المعرضة من خلايا إستخدام تقنية هجرة الخلايا المنفردة في المجال الكهربائي comet assay وتقنية التلوين بالصبغ المتفلورة . أشارت نتائج كل تلك التقنيات المستخدمة إلى قدرة الجسيمات الدهنية النانوية المصنعة على إحداث ظاهرة الموت المبرمج في خلايا HeLa بشكل واضح وذلك من خلال المسار الداخلي intrinsic pathway ، تحتاج النتائج المعروضة إلى دراسات إضافية بهدف تدعيم هذه الفرضية .