# EXOGENOUS CERAMIDE INHIBITS SOME CANCER CELL LINES GROWTH, INDUCES APOPTOSIS, AND REDUCES MAMMARY ADENOCARCINOMA TUMORS IN MICE

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## Abstract

Fresh bovine brain and spinal cord samples were collected for separation ceramide. Tissues were homogenized with organic solvents to extract ceramide which purified by using silicic acid column and obtained one fraction with yellow color. Subsequently, the purified extract analyzed and identified by using TLC, spectrophotometer, infrared assay. The results confirmed that the purified extract was identified as ceramide in comparison with commercially standard ceramides. In addition, this work included several genetic and histopathological assays to demonstrate the antitumor activity of ceramide.It was found that 30 μg/ml of exogenous ceramide had antiproliferative activity against cancer cell lines (RD, Hep–2, AMN–3, and AMGM5) particularly against cancer cells model AMGM5; the percentage of growth inhibition was 80%. Moreover, ceramide was significantly induced apoptosis in cancer cell lines (RD, Hep–2, AMN–3, and AMGM5), the percentage of apoptotic cells was 18, 28, 30, and 50.3% respectively. Furthermore, histopathological study was carried out; it was found that administration of exogenous ceramide particularly at 250 mg/kg was revealed a putative regression of tumor growth and volume in adenocarcinoma mice.

**Keywords: exogenous ceramide, cytotoxic activity, apoptosis, tumor growth inhibition, transplanted mice.**

## Introduction

During the last two decades an explosive studies were carried out to understanding the sphingolipid biology. Initially considered as inert structural constituents of the cell membranes or precursors thereof, sphingolipids have emerged as key messenger and bioactive molecules in a wide range of biological processes [1]. The sphingolipid ceramide can be formed by the breakdown of sphingomyelin or through *de novo* synthesis. It is intimately involved in growth, differentiation, senescence, and death of cancerous cells. Several inductors of cell death, for example, TNFα [2], anthracyclines [3], or irradiation [4] involve ceramide signaling. Administration of exogenous ceramide also causes cell death [5]. It is noteworthy that, many cancer cells have a specific "sphingolipid–phenotype", including lower endogenous ceramide levels [6] and a higher sensitivity to the effects of exogenous ceramide [7]. This offer the opportunity to selectively target cancer cells with ceramide compounds.

Despite many pleas for ceramide–based treatment regimens against cancer, progression from cell-culture to *in vivo* application has been slow, and no critical trails have been reported to date. Thus, this study was aimed to investigate the role of locally purified exogenous ceramide on proliferation of cancer cells and detect its efficacy on apoptosis, then translate its influence on mammary adenocarcinoma mice.

## Materials and Methods

### Separation, extraction, and purification of ceramide

Fresh bovine brain and spinal cord were collected from a local abattoir. White and gray matters were carefully dissected from brain. Tissues (30–50mg) were homogenized with chloroform: methanol: water (2:4:1 v/v/v), stirred for 1 h. and then centrifuged. The pellets were re-extracted with the above solvent and this step repeated twice.

The extract was pooled, dried, and suspended in a minimum volume of chloroform. Sphingolipids [e.g. ceramide, monoglycosylceramide (MGC), and glycosphingolipid (GSL)] were purified as individual fraction as previously described [8]. Briefly, the chloroform suspension was applied to a silicic acid column (0.5 × 5 cm) and washed with 15 volume of chloroform to remove non-polar lipids. The column was then eluted successively with chloroform: acetone (9:1 v/v). The obtained fraction was then dried and stored at 4ºC until use. Commercially available ceramides (NC 16: O-, NC 18: O-, and NC 24:1-D 18:1, CinaGene, Iran) were used for comparison.

**Detection of ceramide**

*Thin layer chromatography (TLC) analysis*.

Ten microgram of dried extract was dissolved in chloroform: methanol (1:1 v/v) and applied to silica gel TLC plate. The plate was run twice with chloroform : methanol : acetic acid (95: 4.5 : 0.5 v/v/v), air dried , and stained by dipping in 1% Benzedrine solution [0.5ml of Benzedrine was added to one small crystal of potassium iodide( KI) dissolved in 50ml of 50% ethanol], then the plate was heated to 170ºC.Therefore, the fraction was scraped off from silica gel with chloroform : methanol (2:1 v/v) by shaking overnight, and the organic phase sucked off and dried under nitrogen and then used for further assay [9,10]. The results were compared with standards ceramides mentioned above.

*Spectrophotometer and infra red assay*s After dissolving the dried extract in chloroform: acetic acid (9: 1 v/v), the optical density was read under wide range of wavelengths (200–1100 nm) as well as the test extract was assessed using infra red spectrometer assay and compared with commercially ceramide mentioned above [11].

**Cell lines**

Cancer cell lines included human larynx epidermoid (Hep-2), rhabdomyosarcoma (RD), human cerebral glioblastoma muliforme (AMGM5), and two types of murine mammary adenocarcinoma (AMN-3 and AM–3) were used in this study. Cell lines were kindly provided by Iraqi Center for Cancer and Medical Genetics Research (ICCMGR).

**Cell culture**

Cells were cultured in RPMI -1640 medium supplemented with 10% fetal calf serum (FCS), 2% L-glutamine (200mM) and 1% penicillin–streptomycin (100U to 100mg/ml) in humidified incubator at 37ºC and 5% carbon dioxide–95% air mixture [12,13].

**Detection of cytotoxicity of ceramide**

The cytotoxicity was measured as previously described [14]. Briefly, $10^6$ cells were seeded in 96-well microplate and routinely cultured in humidified incubator at 37ºC for 24 h. Cell culture medium was removed with micropipette and different concentrations of exogenous ceramide (7, 15, 30, and 60µg/ml), dissolved in 1µl/ml of dimethylsulphoxide (DMSO), was added and reincubated for 72 h. The control (RPMl–1640 medium without ceramide) was also tested. Therefore, medium with or without ceramide was discarded, 50µl of crystal violet solution (0.01%) was added to every well for 20 min. and the plates then were read on a microplate reader (Organon Teknika, Austria) at 492 nm. In this test four replicates were used for each concentration and the percent of cytotoxicity was expressed as percent of growth inhibition as compared with control (0% cytotoxicity).

**Measurement of apoptotic cells**

To assess whether ceramide induces apoptosis, mitochondrial BioGene$^{TM}$ kit with serial no. A2299.92 (USBilogical, lran) and mitoCapture reagent were used as a marker for apoptosis. Cancer cells ($1.5\times10^6$) were seeded in 6–well dishes and treated with 30 µg/ml of ceramide for 24 h. The control (cells without treatment) also tested. After incubation, adherent and floating cells were washed twice with PBS and then treated with monoclonal antibody (mAb) anti apo A2299.92–phycoerythrin (clone A2299.92 A6 A3) according to the supplier's recommendations [15].

**Animal study**

The use of animals for determination the maximum tolerated dose (MTD) and therapeutic efficacy of exogenous ceramide

were performed according to protocol that was approved by the Institutional American Care. The MTD of ceramide was determined by dose escalation studies. In brief, 7–week old BALB/c mice (obtained from ICCMGR, IQ) were treated with increasing concentrations of ceramide for various time intervals. Possible toxicity of exogenous compound to the vital organ of mice was analyzed by both gross examination and histopathology. The role of exogenous ceramide in the inhibition of tumor growth *in vivo* was examined as follows: AM–3 cell engrafts were obtained by subcutaneously injection of $4\times10^6$ cells in the posterior flank of the female mice. After tumor were grown to at least $100mm^3$ (approximately 2 weeks after implantation), the mice were treated with ceramide by intrapertoinal injection every 4 days for 30 days. Tumor volumes were calculated using the formula: $lengh\times width^2\times0.52$ [16]. Each experiment included five mice per treatment. The concentrations of the ceramide use in this study were 250 and 500 mg/kg.

**Statistical analysis**

Data were statistically analyzed using a two-tailed Student's ***t*** test for statistical analysis. Differences among three or more groups were analyzed using a one-way ANOVA test. A *P*- value below 0.05 was considered to indicate statistical significance.

**Results and Discussion**

**Identification of ceramide**

Ceramide was separated from bovine brain and spinal cord. Purification of crude extract on silicic acid column was showed one fraction with yellow color as compared with standards. Subsequently the purified fraction was analyzed using TLC assay. A blue spot with $R_f$ value 1.2 was observed. Furthermore, the purified extract was qualitatively identified using spectrophotometer, the result revealed one peak at 326nm Fig.(1).

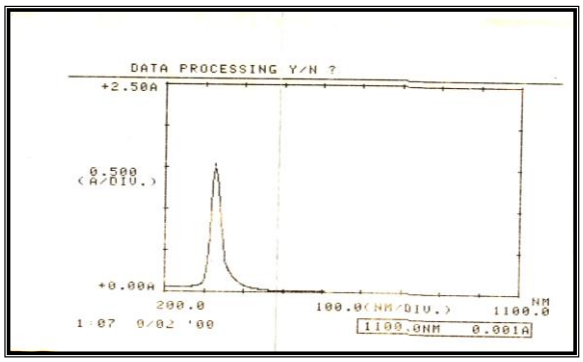


***Fig. (1): Spectrophotogram of extracted ceramide.***

Whereas different peaks of absorbance were recorded by infra red assay Fig.(2), value of each absorbance was represented a functional group in purified extract as follows: 748.34(O–disubstituted), 964.34 $RCH=CH_2$), 11126.35 (–C–C–), 1280.65 (–O–C–ether), 380.94–1458.8 ($CH_2$), 1650 (C=C), 1728.10(C=O), 2854 (C–H strong), 2923.88 (C–H alkane), 2970–3140 (N–H) and 3363.62 (O–H alcohol) as compared with commercially standards of ceramide.

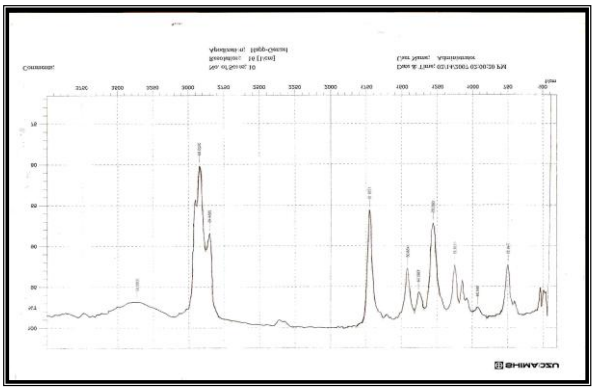

***Fig. (2): Different of absorbent peaks of Purified ceramide by infra red assay.***

Based upon these results, it can be concluded that the purified extract is belonged to ceramide [11].

**Cytotoxic activity of exogenous ceramide on cancer cell lines**

Fig.(3) indicates that low concentrations of exogenous ceramide (7 and 15 µg/ml) as well as high concentration (60 µg/ml) had low toxic effect on cancer cell lines used in this experiment. Obviously, 30 µg/ml of exogenous ceramide was significantly ($P<0.05$) inhibited proliferation of cancer cells.

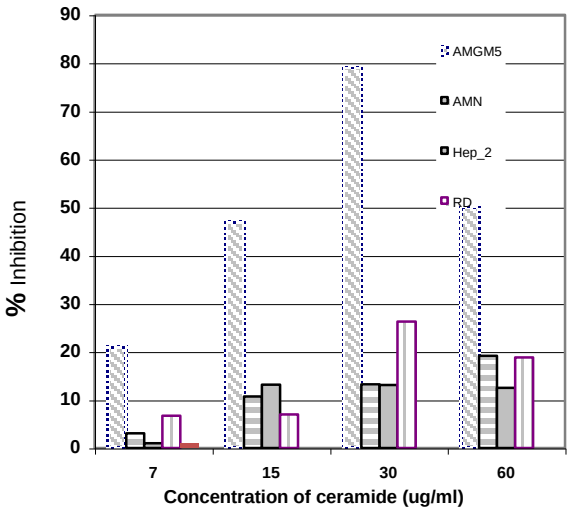


***Fig. (3): Cytotoxic activity of exogenous ceramide on cancer cell lines.***

For certain, this concentration showed a putative inhibition especially against cancer cells model AMGM5, the percentage of inhibition rate was 80%. This result may due to the cellular nature of AMGM5 model, as mentioned above they were derived from human cerebral glioblastoma, so their specify may support the engagement of ceramide to death receptors on cell membrane surface.

On the other hand, the accumulation of ceramide at limited concentrations may alter the activity of members of the mitogen–activiated protein kinase (MAPK) cascades such as the classic intracellular signal-regulated kinase (ERK) 1 and 2 or p38/ re–activating kinase subfamily and these parallel signaling pathways regulate such fundamental aspects of cell function as metabolism, secretion, and gene expression [17].

**Exogenous ceramide induces apoptosis**

The effect of 30 µg/ml of exogenous ceramide on chromatin of cancer cells was examined. Apoptotic cells were appeared with fluorescent green color while non–apoptotic cells were fluorescent red Fig.(4).

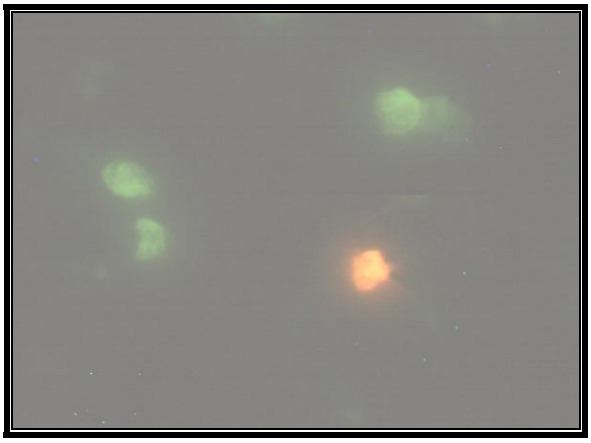

***Fig. (4): Effect of exogenous ceramide on AMN-3 cells. Apoptotic cells were appeared with greenfluorescence while non-apoptotic cells were red fluorescence.***

Moreover the ability of exogenous ceramide to fragment DNA and induce apoptosis in cancer cells was expressed by calculation the percentage of apoptotic cells Fig.(5). The percentage of apoptotic cells was 18, 28, 30, and 50.3% for RD, AMN–3, Hep–2, and AMGM5, respectively.

Exogenous ceramide demonstrated a significant ($P$<0.05) efficacy of cell death especially against cancer cells model AMGM5 in comparison with other models.

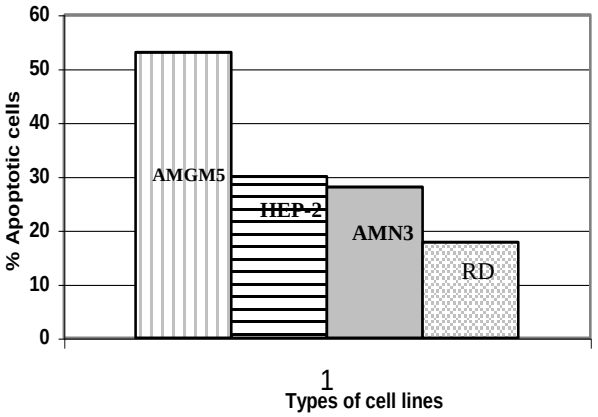


***Fig. (5): percentage of apoptotic cells in Cancer lines treated with 30µg/ml of exogenous ceramide.***

Obviously, the results indicated that ceramide was able to induce apoptosis in all models of cancer cells used in this study. Indeed, the key events implicated in ceramide–triggered apoptosis remain unknown. Recently, it was demonstrated that the reactive oxygen species (ROS) play an important role in ceramide-induced apoptosis through

production of intracellular $H_2O_2$ followed by DNA fragmentation in the cells [18,19].

**Exogenous ceramide induced tumor regression in implanted mice**

Mammary adenocarcinoma cells (AM–3) were successfully established in BAB/c mice. The mass of tumor was enlarged indurate and softened in consistency and paralysis was observed in some advanced cases furthermore continuous increasing in tumor volume was seen. The implanted mice were divided into three groups, the first group was considered as control, the second group was treated with 250 mg/kg of ceramide while the third group was treated with 500 mg/kg of ceramide. After 30 day, the inhibition of tumor growth as well as reduction of tumor volume was observed. Significantly the exogenous ceramide was showed inhibition in tumor growth (70.31% and 56.1%) in transplanted mice treated with either 250 or 500mg/kg of ceramide Fig.(6).

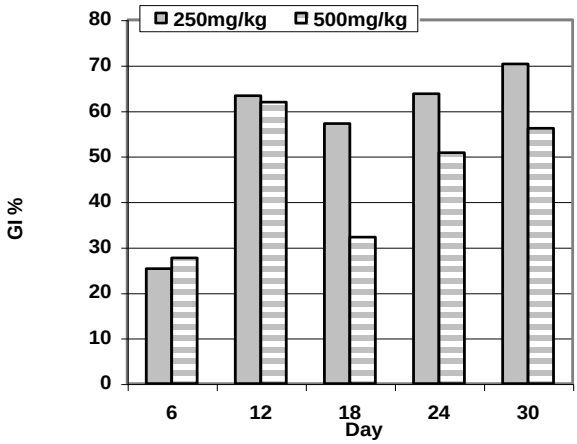


***Fig. (6): Tumor growth inhibition (GI %) in treated mice.***

On the basis of observation the efficacy of 250 mg/kg ceramide to inhibit tumor growth and reduce tumor volume, a histopathological study concerns with regression of mass tumor in the flank region besides other organs such as kidney and liver in ceramide–transplanted mice was done Fig.( 7 and 8). In general, the exogenous ceramide showed thin mass of tumor in the flank Fig.(7A) and cells were underwent apoptosis Fig.(7B).

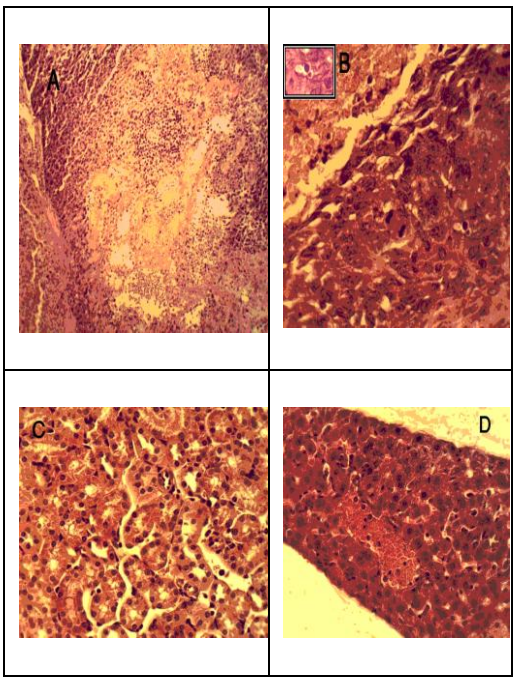


***Fig. (7): Histopathological changes in ceramide-treated mice.(A), thin mass of tumor with wide massive area of necrosis (100×).(B), cells undergoing apoptosis (600×).(C), normal tubules with myeloid cloudy swelling in kidney (400×). (D). Congestion of the blood vessels in liver (400×). Stain (H&E)***

In addition a cross sections of kidney were revealed normal tubules with myeloid cloudy swelling Fig.(7C) while tumor metastasis in veins was absent and congestion of blood vessels in liver was seen Fig.(7D).

Whereas untreated implanted mice showed tumor mass with malignancy features Fig.(8A and B), tubular degeneration and lining cells of tubules undergoing degeneration in kidney Fig.(8C), and metastasis of Adenocarcinoma in liver Fig.(8D).

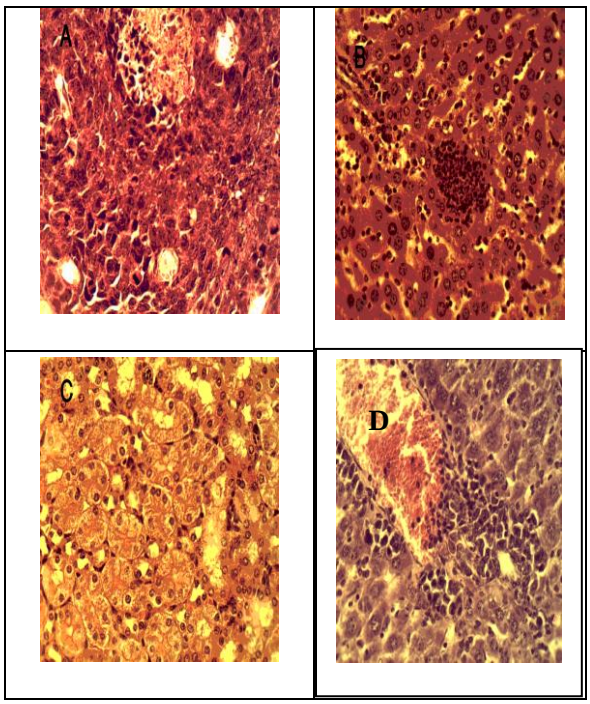


***Fig.(8): Histopathological examination in untreated mice. (A), solid mass of tumor with many mitotic figure (400 ×). (B), blocked vein begins to invasion the surrounding tissue (400 ×). (C), tubular degeneration and the lining cell of tubules undergoing degeneration and myeloid cloudy swelling kidney (600 ×). (D), Metastasis invasion and filtration in the adjacent tissues of blood vessels in liver (400×).Stain (H&E).***

Remarkably, ceramide-transplanted mice inhibited adenocarcinoma tumor growth and regression its volume. The explanation for this result was that the administration of exogenous ceramide mediates apoptosis through release of cytochrome *c* by two pathways: one involves mRNA increase of pro-apoptotic BcI-2 members, while the other directly affecting the integrity of the mitochondria [7, 19].

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**الخلاصة**

استخدمت عينات من دماغ البقر و الحبل الشوكي لفصل السيرامايد، وقد أستخلص السيرامايد بعد مجانسة الأنسجة المعزول منها باستخدام المذيبات العضوية، ونقي بوساطة عمود السيلليست، وتم الحصول على جزء واحد ذو لون أصفر. تلا ذلك أجراء تحليل و توصيف للسيرامايد المنقى باستعمال TLC، ومقياس الطيف الضوئي و الأشعة تحت الحمراء؛ و أثبتت النتائج أن المستخلص المنقى شخص كسيرامايد مقارنة مع السيرامايدات القياسية التجارية. ومن جانب أخر تضمنت الدراسة أجراء العديد من الفحوصات الوراثية والنسيجية لإثبات الفعالية ضد ورمية للسرامايد. فقد وجد أن السيرامايد بتركيز 30 مايكرو غرام/مل يمتلك فعالية ضد تضاعف خطوط الخلايا السرطانية ( RD، Hep–2، AMN–3، AMGM5) خصوصا ضد الخط الخلوي AMGM5 حيث بلغت النسبة المئوية لتثبيط نمو خلاياه 80% . علاوة على ذلك فقد تمكن السيرمايد من حث عملية الموت المبرمج للخلايا السرطانية ( RD،Hep–2، AMN–3 و AMGM5) وبشكل معنوي؛ اذ بلغت نسبة الخلايا المنتحرة 18 ،28 ،30، 50% على التوالي. وفضلا عن ذلك أجريت دراسة نسيجية مرضية وجد أن تجريع الفئران المغروس فيها سرطان الغدة اللبنية بالسيرامايد الخارجي وبتركيز 250 ملغم/كغم ساعد على تراجع نمو الورم وحجمه.