Journal of Taibah University Medical Sciences (2023) 18(3), 579–586

Taibah University

Journal of Taibah University Medical Sciences

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Original Article

Anticancer activity of retinoic acid against breast cancer cells derived from an Iraqi patient

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Received 8 August 2022; revised 27 September 2022; accepted 3 December 2022; Available online 17 December 2022

الملخص

أهداف البحث: يعتبر سرطان الثدي من أكثر الأمراض المميتة للإناث في جميع أنحاء العالم، ويظهر في العراق نفس النمط. يعزى معدل الوفيات المرتفع في الغالب إلى المقاومة الكيميائية للعلاجات التقليدية. يعد البحث عن علاج فعال وآمن ضرورة، وأحد العوامل الواعدة التي أظهرت نشاطا ضد أنواع السرطان المختلفة هو حمض الريتينويك.

طريقة البحث: تم اختبار حمض الريتينويك ضد مجموعة من خطوط خلايا سرطان الثدي الدولية ومقارنته بخلايا سرطان الثدي المشتقة من المريض العراقي والتي هي خلايا مستقلة عن الهرمونات باستخدام اختبار قياس لوني لتقييم نشاط الخلية الاستقلابي. تم تقييم الأمراض الخلوية تحت المجهر المقلوب، كما تم تقييم تحريض موت الخلايا المبرمج باستخدام اختبار يوديد بروبيديوم أكريدين البرتقالي.

النتائج: أظهرت النتائج أن خلايا سرطان الثدي "ايه ام جيه 13" كانت حساسة لتأثير القتل الذي يسببه حمض الريتينويك أكثر من خلايا "ام سي اف -7" و "سي ايه إل -51". في المقابل، أظهرت خلايا "اتش بي إل -100" الطبيعية تأثيرا ضئيلا. لوحظت تغيرات خلوية في جميع الخلايا السرطانية المعالجة بحمض الريتينويك، مع عدم وجود تغيرات في خلايا "اتش بي إل -100" الطبيعية. أظهرت خلايا سرطان الثدي المشتقة من المرضى العراقيين نسبة أعلى من الخلايا التي خضعت لموت الخلايا المبرمج عند معالجتها بحمض الريتينويك مقارنة بخلايا سرطان الثدي الأخرى.

الاستنتاجات: نقترح استخدام حمض الريتينويك كعلاج محتمل لسرطان الثدي مع تطبيق سريري محتمل وأمان عالي.

الكلمات المفتاحية: سرطان الثدي؛ خلايا سرطان الثدي؛ حمض الريتينويك

Abstract

Objective: Breast cancer is one of the most lethal diseases in women, both worldwide and in Iraq. The high mortality rate is attributed primarily to the chemoresistance to conventional therapeutics. The search for effective and safe treatments is critical. One promising agent that has shown activity against various cancer types is retinoic acid (RA).

Methods: RA was tested against a panel of international breast cancer cell lines and compared with Iraqi patient-derived hormone-independent breast cancer cells through MTT viability assays. Cytopathology was assessed under an inverted microscope, and apoptotic induction was evaluated with acridine orange propidium iodide assays.

Results: AMJ13 breast cancer cells were more sensitive to killing induced by RA than MCF-7 and CAL-51 cells. By contrast, normal HBL-100 cells showed a negligible effect. Cytological changes were observed in all cancer cells treated with RA, whereas no changes were observed in normal HBL-100 cells. Iraqi patient-derived breast cancer cells showed a higher percentage of cells undergoing apoptosis after RA treatment than the other breast cancer cells.

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Peer review under responsibility of Taibah University.

ELSEVIER Production and hosting by Elsevier

 https://doi.org/10.1016/j.jtumed.2022.12.002

Conclusion: We suggest RA as a possible breast cancer treatment with potential for clinical application with high safety.

Keywords: AMJ13; Breast cancer; CAL-51; HBL-100; MCF-7; Retinoic acid

Introduction

Traditional cancer treatments, such as chemo/radiotherapy and surgery, have several limitations and fail to treat many advanced cancer forms in humans and animals.[1] Vitamin A is highly important but is not produced by animals and must be obtained through a plant-based diet.[2] Some carotenes, isotretinoin, retinal, retinol and retinoic acid (RA) are all vitamin A derivatives.[3] The ability of RA to treat cancer is well known, such as its influence on cancer cell growth through differentiation and death.[4] RA has been found to stimulate the mitochondrial pathway, thus resulting in cell differentiation arrest and apoptosis.[5] All-*trans* retinoic acid (ATRA) appears to be a potential drug for human leukemia cell growth inhibition, whereas RA shows excellent promise as a chemotherapeutic agent in the treatment of acute promyelocytic cancer.[6,7] A lack of RA signaling has been proposed to obstruct normal differentiation in response to carcinogenic stimuli, thus resulting in pancreatic cancer, whereas restoring retinoid signaling may be a unique therapeutic option. One study has indicated that 30 days of treatment with RA eliminates tumorigenicity in human hepatoma cells.[8]

The AMJ13 cell line (Ahmed, Mahfoodha, Mortadha, Jabria-2013) was established in 2014 as the first Iraqi breast cancer cell line. AMJ13 cells were isolated from the main tumor of an Iraqi woman with breast cancer.[9] This cell line expresses BRCA1 and BRCA2, but not vimentin, or estrogen or progesterone receptors. It is also weakly positive for HER2/neu gene expression.[10] Many prior studies have investigated the potential effects of RA against international breast cancer cell lines, such as MDA and MCF-7, and have found that it inhibits their growth.[11,12] This study was aimed at comparing the effects of RA on two international breast cancer cell lines (MCF-7 and CAL-51) and a local breast cancer cell line (AMJ13), to determine whether morphological changes, growth inhibition, cell proliferation and apoptotic effects might be achieved in vitro.

Materials and Methods

Cell lines

The human cell lines HBL-100, MCF-7, AMJ13 and CAL-51 were provided by the Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, cell bank unit. HBL-100, MCF-7 and CAL-51 cells were maintained as monolayer cultures in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), whereas AMJ13 cells were grown in RPMI-1640 enriched with 10% FCS. The cells were frequently tested for typical growth features and were validated. Passage 28 AMJ13 cells were used in this investigation.

Retinoic acid

RA (Haihang Industrial (Jinan) Co., Ltd, China) was prepared by dissolving 7 mg of RA powder in 5 ml (1% DMSO Plus serum-free medium) and filtering through a 0.2 μm Nalgene syringe filter to prepare a 5 mM stock solution.[13]

Exposure of the cell lines to RA

The confluent cell monolayers were treated with serial dilutions (400, 300, 200, 100, 50 or 25 μg/ml) of RA stock solution (5 mM) prepared with RPMI-1640 medium. In addition, negative controls treated with serum-free medium were included to ensure that the test was valid for each experiment. HBL-100 cells (non-cancerous cells) underwent the same treatment. For each concentration examined, three replicates were used. The cells were incubated at 37 °C for 48 h in a humidified environment with 5% CO_2.[13]

Cytotoxicity of RA

We used 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays (Bio-world, USA) to assess the viability of breast cancer (MCF-7, AMJ13 and CAL-51) and normal (HBL-100) cells. MTT is a water-soluble yellowish dye that is quickly absorbed by living cells and reduced by mitochondrial dehydrogenase. A blue formazan is produced from the water-insoluble reduction product, which is subsequently dissolved in dimethyl sulfoxide (DMSO) solution for colorimetric detection. Cells were cultivated on 96-well plates (10,000 cells per well) and treated with different concentrations of RA (400, 300, 200, 100, 50 or 25 μg/ml). After 48 h incubation, the cells were washed with phosphate-buffered saline (PBS), and then 0.5 μg/ml MTT solution was added for up to 2 h. After the supernatant was discarded and cells were dried, 100 μl DMSO was applied to each well. Photometric spectrophotometric analysis was used to determine the extinction values at 570 nm.[14] The following formula was used to calculate the rate of growth inhibition (cytotoxicity percentage) as follows: growth inhibition percentage = $(A - B)/A \times 100$, where A represents the mean optical density of control wells, and B represents the mean optical density of treated wells.[15] The inhibitory concentration for eliminating 50% of infected cells (IC_{50}) was determined in GraphPad Prism 7.0 (version 2016) program.

Morphological study

Hematoxylin stain was poured onto formalin-fixed cells for 3–5 min, then washed with distilled water. Subsequently, adherent cell slides were soaked with eosin stain for 1–2 min. After dehydration of stained cells with 90% ethanol, slides

were soaked with absolute alcohol. Finally, cell sections were maintained with a xylene-based mounting medium.

Apoptosis assays

The apoptosis triggered by RA in breast cancer cell lines was detected with acridine orange-propidium iodide (AO-PI) stain. The AO-PI stain was prepared by mixture of 1 ml acridine orange and 1 ml propidium iodide in 1 ml PBS in a 1:1 ratio. Cell lines were grown in 96-well plates. After 80% proliferation, cancer cells were treated with RA for 48 h (on the basis of the IC_{50} for each cell line). Control wells were left untreated. The medium was then removed, and the cells were stained with 50 µl/well AO-PI stain. The stain was removed from the wells after 20 s. An inverted fluorescence microscope was used to investigate and photograph morphological differences in live and apoptotic cells.[16] Live and dead cells were stained with AO dye. Cells with damaged membranes were stained with PI. The color of living cells was uniformly green. Apoptotic cells incorporate PI, thus resulting in orange staining.[17] The results were calculated in ImageJ version 1.47 software, thus distinguishing between living and apoptotic cells.

Statistical analysis

For all triplicate readings, data are reported as mean ± standard deviation (SD). Multiple comparisons of one-way variance assessment were used to elucidate differences among groups on the basis of statistical significance estimation (GraphPad Prism version 7.0 for Windows, GraphPad Software, San Diego, CA, USA) to evaluate the cytotoxicity and identity of the effects of RA on apoptosis in MCF-7, AMJ13 and CAL-51 cell lines in vitro. Statistical significance was defined as $p < 0.05$.

Results

Cytotoxicity of RA in breast cancer cell lines

To calculate the IC_{50}, we treated proliferating MCF-7, AMJ13, CAL-51 breast cancer cell lines and normal HBL-100 cells with a serial dilution of RA (400, 300, 200, 100, 50 or 25 µg/ml). MTT assays was conducted after a 48-h incubation period. The growth inhibition and IC_{50} were calculated as a percentage of viable cells after RA

Table 1: Cytotoxic effects of retinoic acid on different cell lines.

No.	Cell line	IC_{50} (µg/ml)	R^2 value
1	HBL-100	454.8 ± 5.7	–
2	CAL-51	169.1 ± 8.2	0.9825
3	MCF-7	139.9 ± 4.6	0.9798
4	AMJ-13	104.7 ± 3.8	0.9861

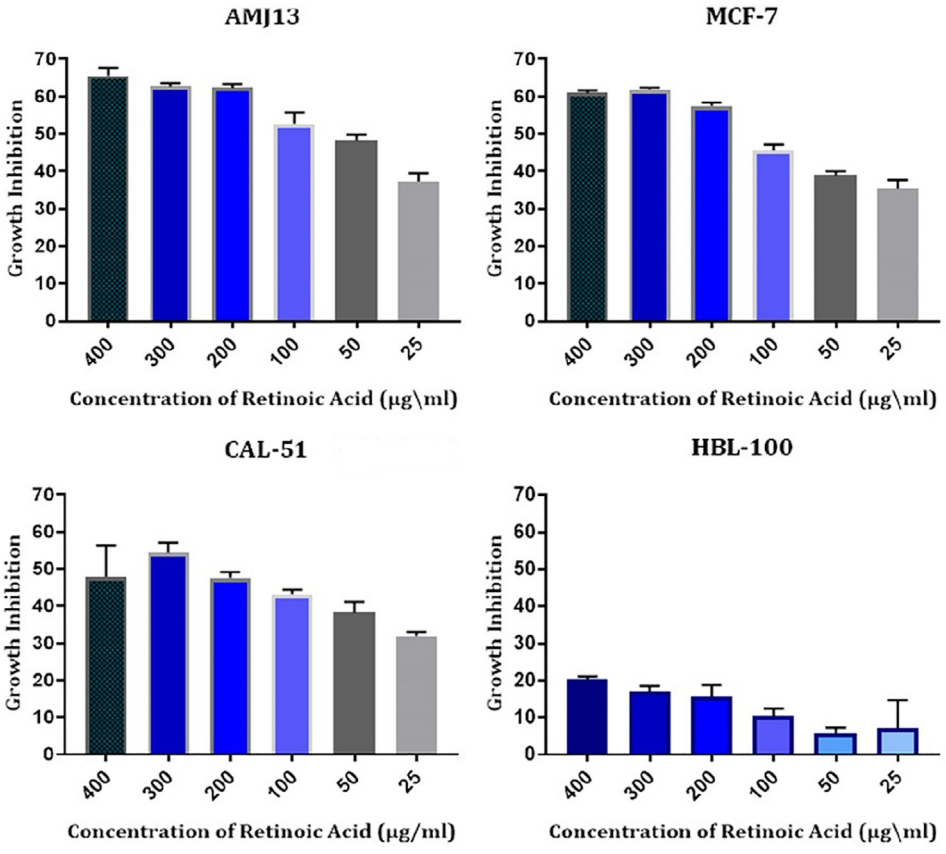

Figure 1: In vitro growth inhibition of different concentrations of retinoic acid on MCF7, AMJ13 and CAL51 breast cancer cells, and HBL-100 cells.

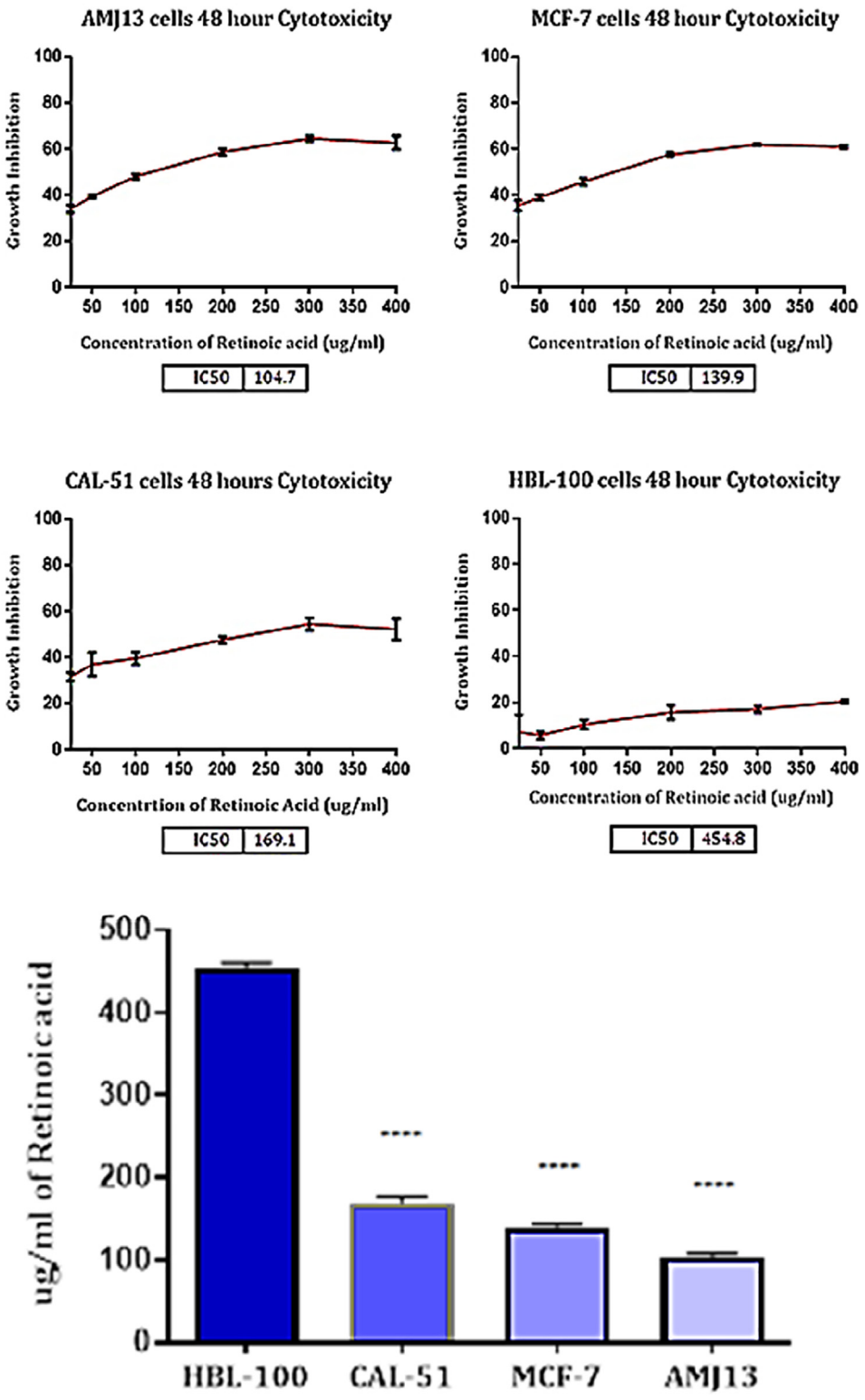

Figure 2: Cytotoxic effect of retinoic acid on MCF-7, AMJ-13, CAL-51 cancer cell lines and the HBL-100 normal cell line, determined by MTT assays. Values are mean and SD for five replicates from three experiments (IC_{50}). (****$p < 0.05$ compared with HBL100).

treatment with respect to control cells that had not been exposed to RA. The growth inhibition was dose dependent, and the inhibitory effect of RA differed among cell lines (Figure 1).

The cytotoxic effects of RA were calculated (Table 1). RA had a cytotoxic effect on breast cancer cell lines (MCF-7, CAL-51 and the local breast cancer cell line AMJ13), with IC_{50} values of 139.9 ± 4.6, 169.1 ± 8.2 and 104.7 ± 3.8 μg/ml,

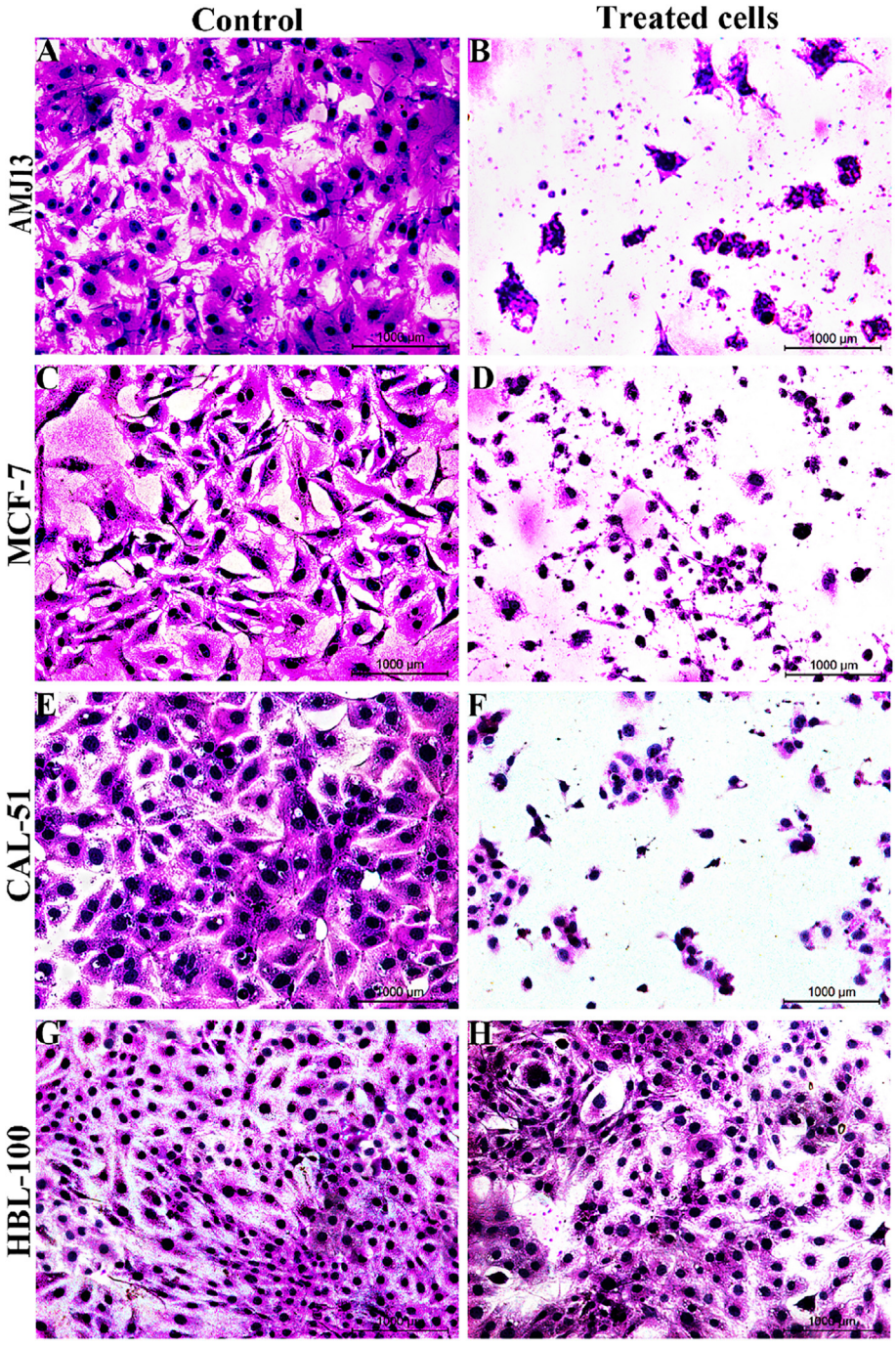

Figure 3: Retinoic acid's cytopathic effect on breast cancer cell lines and the HBL-100 cell line. Untreated control cells maintained their characteristic morphology (A, C, E, G). After 48 h of treatment with retinoic acid, cytotoxic effects were observed (B, D, F, H). Cancer cells appeared as shrunken clumps of dead cells with wide flatulent spaces and cell debris. HBL-100 cells appeared to be unaffected (20 ×, H&E).

respectively. However, at these concentrations, RA had a less significant effect on HBL-100, with an IC_{50} of 454.8 ± 5.7 µg/ml (Figure 2). The growth inhibition of cancer cells by RA appeared to be dose dependent, with $R^2 > 0.97$. Normal cell lines showed minimal growth inhibition, whereas cancer cell lines showed significant growth suppression ($p < 0.05$).

Morphological characterization

On the basis of hematoxylin and eosin staining, the morphology of treated and untreated cells was observed in microphotographic images (Figure 3). The cellular features of untreated cells were similar to those of the origin cell line. The morphological changes in treated cells were

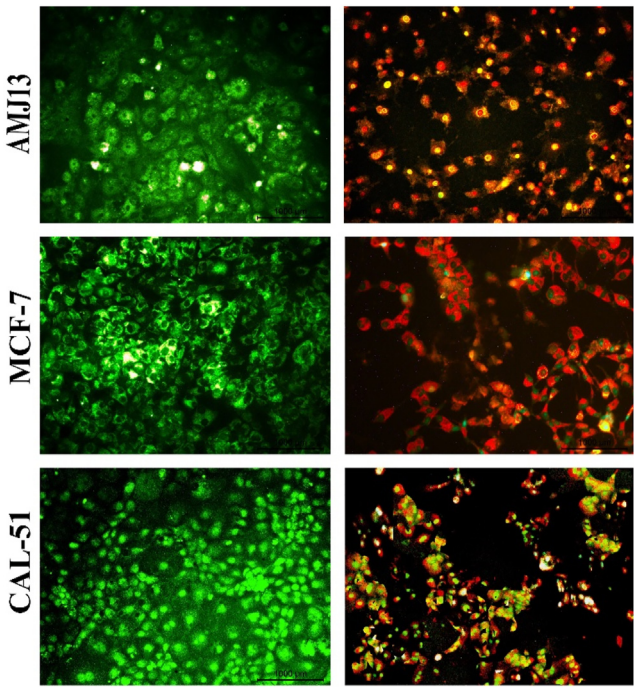

Figure 4: Fluorescence microscopy images of mitochondrial permeability transition apoptosis tests in breast cancer cells. After 48 h, the non-treated MCF7 (A), AMJ13 (C) and CAL51 (E) cells stained green, whereas the treated MCF7 (B), AMJ13 (D) and CAL51 (F) cells stained red (20 ×).

characterized by damaged cell membranes and cell shrinkage. Cell damage leads to the loss of shape and the formation of spherical bodies, both of which are characteristics of apoptosis.

Apoptosis induction by RA

Fluorometric cell viability assays with AO-PI were performed to detect morphological alterations, as well as the proportions of apoptotic, necrotic and normal viable cells among the treated and untreated breast cancer cells. In live cells, the nucleus and cytoplasm were tagged with green fluorescence. Apoptotic cells accumulated propidium iodide, thus resulting in red or orange fluorescence, along with condensed nuclei that were frequently fragmented. Necrotic cells were orange, lacked condensed chromatin and exhibited a similar nuclear structure to that of viable cells.

The in vitro results indicated that RA treatment resulted in significantly greater destruction of breast cancer cells and induction of apoptosis than observed in the control group (Figure 4). The proportion of apoptotic AMJ13 cells was 73.6 ± 0.73%, $n = 3$, whereas that of MCF-7 and CAL-51 cells was 66.5 ± 1.88%, $n = 3$ and 64.02 ± 4.02%, $n = 3$ respectively, after treatment with RA (200 µg/ml) (Figure 5). Furthermore, Image-J software was used to construct histograms of the apoptosis test results for the different cell lines (see Figure 6).

After treatment of each cell line with RA (200 µg/ml), significant differences were observed in the means percentages of apoptotic cells ($p < 0.05$) between the tested breast cancer cell lines; AMJ13 and CAL-51 (**p; 0.0021),

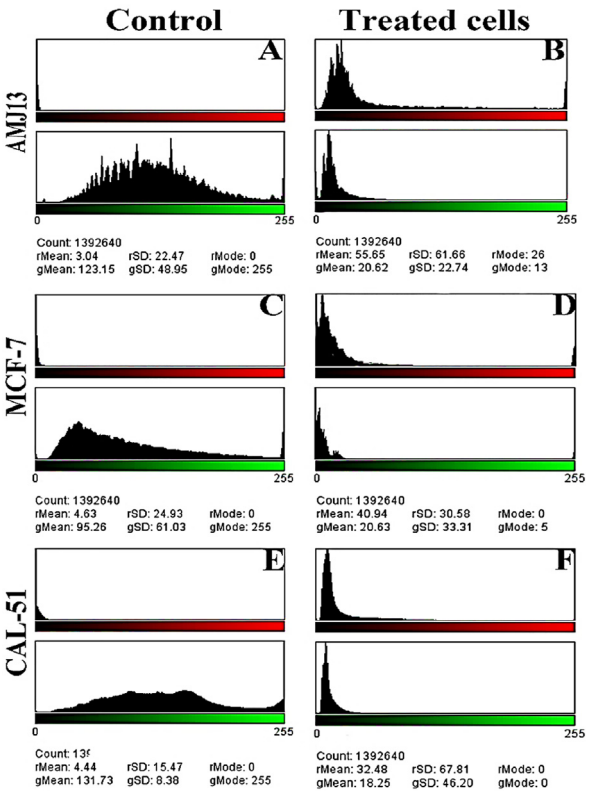

Figure 5: Apoptosis test histogram of (AMJ13, MCF-7 and CAL-51) cell lines (20 ×). (A) The proportion of apoptotic cells was 2.4% in untreated AMJ13 cells. (B) The proportion of apoptotic cells in the AMJ13 cell line was 73.6% 48 h after treatment with retinoic acid (RA). (C) The proportion of apoptotic cells was 4.6% in untreated MCF-7 cells. (D) The proportion of apoptotic cells in the MCF-7 cell line was 66.5% 48 h after treatment with RA. (E) The proportion of apoptotic cells was 3.3% in untreated CAL-51 cells. (F) The proportion of apoptotic cells in the CAL-51 cell line was 64.02% 48 h after treatment with RA.

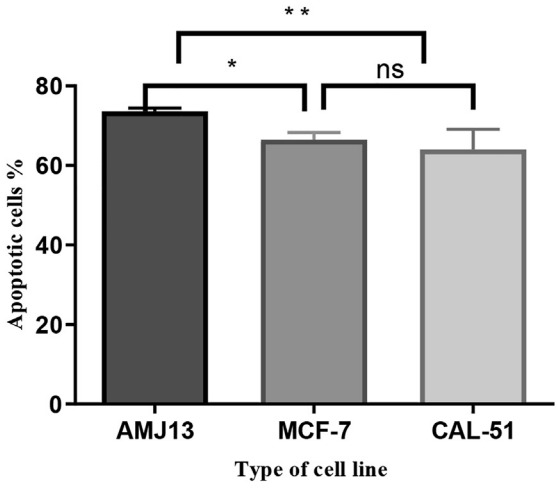

Figure 6: Proportions of apoptotic cells after 48 h treatment with retinoic acid. Graph represent the variation in mean of apoptotic cell between AMJ13, MCF-7 AND CAL-51. Statistical analysis carried with paired t test, mean ± SD, 0.033(*), (**)0.002; (*ns*) not significant.

AMJ13 and MCF-7 (*p; 0.0332), but not between MCF-7 and CAL-51 (Figure 6).

Our findings suggested that the AMJ13 breast cancer cell line was most sensitive to RA treatment. Consequently, RA is a more successful therapy in the treatment of localized breast cancer.

Discussion

The current study supports the use of RA as breast cancer therapy, according to the in vitro results indicating effective killing against most breast cancer types tested, particularly in cells derived from an Iraqi patient.

The notion of using RA to treat cancer is not new. RA has been demonstrated to be active in cancer therapy, and synthetic retinoids have been investigated to treat numerous malignancies, owing to their efficacy in cancer treatment.[4,11] The cytotoxicity effects of RA on three breast cancer cell lines (MCF-7, CAL-51 and AMJ13) as well as the HBL100 normal cell line were investigated. The results indicated a dose-dependent increase in cell killing percentage, but the effects on CAL-51 and MCF-7 cells were lower, and no noticeable increase in the effect of 400 μg/ml RA on AMJ13 cells was observed (Figure 1). Experimental and/or clinical studies on human breast cancer have demonstrated that RA inhibits the functions of antioxidant enzymes, including peroxidase, catalase and glutathione.[18,19] High concentrations of RA cause molecules to clump together, thus increasing their size and hindering their passage across cellular membranes exposed to RA, as observed in experiments. By contrast, increasing the RA concentrations within rather than outside cells prevents the entry of additional molecules, according to laws of diffusion.[20] Because the intracellular RA transporters CRABP2 and FABP5 have been found to play opposing roles in modulating the cellular response to retinoids, their quantity inside cells may suggest breast cancer cell responsiveness to ATRA.[21]

The local cell line (AMJ13) was most affected by RA, and showed a lower IC_{50} value (104.7 μg/ml) than the international cell lines (MCF-7 and CAL-51); the CAL-51 cancer cell line was more resistant to RA. Furthermore, at the tested concentrations, RA had a diminished effect on HBL-100, with an IC_{50} of 454.8 μg/ml. Breast cancer is a heterogeneous tumor type that can be classified into subtypes on the basis of the presence of biological markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).[22] Moreover, RA has a variety of receptors, such as retinoic acid receptors (RAR), retinoid X receptors (RXR) and retinoic acid receptor gamma (RARγ), which interact with diverse pathways such as the Wnt/β-catenin and β-catenin pathways. The RAR and RXR receptors suppress Wnt/β-catenin, whereas the RARγ receptor functions as a tumor oncogene, thus leading to the activation of the Wnt/β-catenin pathway.[23] Consequently, RA works in two opposing ways, depending on its receptors.

The morphological changes in the cancer cell lines after RA treatment indicated the cytopathic effects observed in infected cells 48 h later. Many confined cells showed granulation and shrinking, thus resulting in opaque foci due to increased cell granulation.

In vitro, the cytotoxic effects and apoptosis of RA appeared to result in significantly lower cancer cell viability and more apoptosis than those observed in control cells. ImageJ quantification indicated that the proportion of apoptotic AMJ13 cells was $73.6 \pm 0.73\%$, $n = 3$, whereas that of MCF-7 and CAL-51 cells was $66.5 \pm 1.88\%$, $n = 3$ and $64.02 \pm 4.02\%$, $n = 3$, respectively, after treatment with RA (200 μg/ml) (Figure 5). These findings suggest that the AMJ13 breast cancer cell line was most sensitive to RA treatment. Consequently, RA appears to be a more successful therapy in the treatment of localized breast cancer.

Apoptosis is a well-organized process in which a cell's genome is broken into smaller fragments and consumed by neighboring (phagocytic) cells).[24] If apoptosis does not occur, these damaged cells may survive and grow into cancer cells. When cells are not connected to other cells or the extracellular matrix, they "self-destruct".[25] Pro-death proteins (BH3) accumulate in malignant cells but are not sufficient to counteract the increase in anti-apoptotic proteins (Bcl-2). Medications that mimic BH3 proteins can also boost pro-death signals, thus triggering apoptosis pathway forward and triggering apoptosis.[26] Several agents directly targeting the anti-apoptotic proteins Bcl-2 and IAP are being tested, whereas other agents preserve pro-apoptotic components that have been removed, such as caspase activity or p53 gene expression.[27]

RA receptor beta mediates retinoid activity in breast cancer cells by inducing apoptosis.[28] According to Chen et al., HOXA5 functions immediately downstream of RARβ and may play a role in retinoid-induced apoptosis and growth inhibition.[29]

Conclusions

Our observations suggest that RA controls cancer cell proliferation through cell death. In breast cancer cells, RA induced apoptosis, particularly in the local breast cancer cell line (AMJ13), which was most susceptible to RA treatment. Consequently, RA may be a promising treatment for breast cancer with high clinical potential and safety.

Source of funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical approval

Ethical approval was not applicable because we used ready-made cancer cell lines (in vitro study) (Approval number: 130; date: 25/11/2021).

Authors contributions

SAA and AMA proposed the presented idea. SAA formulated the hypothesis and performed the calculations.

SAH confirmed the analytic approaches. Both SAA and SAH conducted the experiments and contributed to the final version of the manuscript. AMA supervised the project and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Acknowledgements

The authors thank the Department of Experimental Therapeutics, Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq, for the support and use of their laboratory facilities during this study.

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How to cite this article: Abdullah SA, Hassan SA, Al-Shammari AM. Anticancer activity of retinoic acid against breast cancer cells derived from an Iraqi patient. J Taibah Univ Med Sc 2023;18(3):579–586.