Retinoic Acid Treatment of Human Hematological Malignancies Induces Caspase Dependent and Independent Apoptotic Cell Death

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Abstract

The unprejudiced of this education is to gauge the ability of the retinoic acid to induce apoptotic cell death in hematological tumors through caspase dependent or independent apoptotic pathway, The cytotoxicity effects of retinoic acid of different concentrations (400,350,300,250,200,150,100,50,25,12.5 µg\ml) and exposure for all hematological malignancy cell lines (Human non-Hodgkin lymphoma SR and human multiple myeloma (COLO 677) and Human Monocytic Leukemia THP1 and Acute promyelocytic leukemia NB4) have been determined using a microtetrazolium (MTT) assay. Propodeum iodide and alcidine orange (AO/PI) paired discoloration was used to study the ability of retinoic acid to induce apoptosis in the infected cells and examined under fluorescence microscope and quantified for the percentage of apoptosis induction. Quantitative immunocytochemistry assay was used to study the caspase dependent and independent proteins expression in infected and control cells. Cells treated with Retinoic Acid showed increased cell death percentage compared to the untreated cells as quantified by MTT assay. AO/PI results revealed that Retinoic Acid had powerful effect on inducing apoptosis significantly ($p<0.001$) in human cancer cell lines tested, compared to control cell. Immunocytochemistry in Retinoic Acid infected human hematological cell lines revealed remarkable increase in expression of caspase 8,9 (dependent pathway) and AIF, ENDOG (independent pathway) induces a significant ($p<0.002$) as compared untreated cell.

This study, which shows the role of the Retinoic Acid in inducing apoptosis through a dependent and independent pathway in cancer cells, we anticipation these annotations will shanty light on the impending exploration of retinoic acid in cancer hindrance and rehabilitation.

Keywords: *Retinoic Acid, SR, NB4, THP1, COLO677, Apoptosis*

Introduction

A trademark variation from the norm of leukemia cells is that they are hindered at a beginning period of their advancement and neglect to separate into useful develop cells. During the 1970s and 1980s, a few logical accomplishments promoted the procedure of actuating harmful cells to defeat their square of separation and enter the apoptotic pathways as an exquisite option in contrast to murdering disease cells by cytotoxic therapies[1]. This mediation could hypothetically confine introduction to undesirable reactions of cytotoxic chemotherapy, and all the more significantly, improve total reduction and fix rates, endeavors to clarify substances to control the separation of myeloid leukemia,2 and the principal proof of the separating properties of retinoic acid.[2, 3]. The potential for separating treatment to improve fix rates in leukemia is exemplified by the advancement of all-trans retinoic corrosive for the focused on treatment

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of intense promyelocytic leukemia . One of the most amazing aftereffects of starting in vitro analyses was accomplished in separating Hl-60 cells per ATRA, which bent terminal separation in 90% of cells per 10−6 M retinoic acid[2].

Retinoids are a class of normally happening aggravates that are fundamentally identified with nutrient An (or retinol). Retinoids control a wide scope of organic procedures, including improvement, separation, multiplication, and apoptosis [4]. ATRA is the dynamic metabolite of nutrient An and intercedes its organic impacts by enacting at least one of the firmly correlated retinoic corrosive receptorse (RARα, RARβ, and RARγ) that aptitude as ligand-subordinate transcriptional controllers. These receptors structure heterodimers with rexinoid receptors (RXRα, β, and γ) and tie to retinoid responsive reaction components situated in the advertiser district of retinoid target qualities to animate quality translation [4].

A functioning common retinoid, all-trans retinoic corrosive, is viable in separation treatment for intense promyelocytic leukemia [5]. is a subtype of intense myeloid leukemia, which is portrayed by a particular chromosomal variation from the norm t(15,17) related with a hereditary revision between retinoic corrosive receptor α (RARα) (quality image, RARA) and the promyelocytic leukemia quality PML[6]. RARα assumes a job in granulocytic separation of hematopoietic cells and the irregular illusory receptor PML-RARα has been involved in APL pathogenesis by obstructing the myeloid separation program and upgrading self-reestablishment of leukemic cells[7, 8]. Pharmacological portions of ATRA instigate separation of APL cells into granulocytes through corruption of PML-RARα and recuperation of physiological RARα flagging [7]

Nutrient An is particularly significant in light of the fact that it can't be combined by creatures and must be provided from an eating regimen that incorporates plants [9], suppress malignant transformation in vitro [10]. Sure of these mixes restrain the development of changed cells [11] and actuate separation of mouse embryonal carcinoma cells in vitro [12]. The antineoplastic impacts of retinoids recommend that these mixes could be utilized restoratively for the chemoprevention of malignancy [2]. ATRA actuates terminal separation of leukaemic cell lines, so ATRA-based treatment can prompt total reduction in APL [13]. Tumor improvement, angiogenesis and metastasis are the organic capacities hindered by retinoic acid [14]. It has likewise been found that retinoic corrosive directs mitochondrial porousness, passing receptors, ubiquitination, and types of responsive oxygen, etc.[15]. The inhibitory effects of retinoic corrosive are believed to be cultivated by initiating the receptor of retinoic corrosive (RAR) or the receptor of retinoic X. rar and rxr structure ligand restricting heterodimers and aptitude, o change downstream quality articulation, rar and rxr travel into cell cores and tie to the retinoic corrosive response components (Uncommon) set in the 5 ' downstream qualities of retinoic corrosive[16, 17] . Actuating the old style pathway above will bring about cell separation, capture and eventually apoptosis [16], The pathways on that RA demonstrations need adjusted convergences of this retinoid, and distortion on the degree of retinoic corrosive from ordinary outcomes in irregular development and advancement [18]. The activity of retinoic corrosive relies upon different procedures, just as combination and degeneration of RA, phosphorylation and debasement of RA receptors, and enrolling of chromatin remodelers and procedures worried inside the vehicle, spread, and cell ingestion of retinoids [19], notwithstanding the previously mentioned great pathway, retinoic corrosive can likewise control downstream quality articulation by adjusting other interpretation factors, for example, NF-b, IFN-J, TGF-b, MAPK, and even chromatin rebuilding [20]. RARs/RXRs heterodimerize and control the motioning of these accomplice receptors, including non-exemplary or non-genomic forms, These accomplice receptors some of the time have opposite capacities to RARs/RXRs, including retinoid flagging and digestion, cell bond, cell-grid collaboration, and cytoskeleton renovating in their appearance microarray assessment[21].

Materials and Methods

Preparation of cells and cell culture

This investigation remained affirmed via Baghdad College, School of Science, biotechnology office. The human cell line non-Hodgkin lymphoma SR (CD20 –) was generously given by Dr S.J. Russell, Mayo Center, Atomic Drug Office (Rochester, M N, USA) and refined in altered Falcon's media (US Organic, Salem, Mama,

USA) with 5% fetal ox-like serum (Capricorn Logical.

The human various myeloma (COLO 677) was initially portrayed as being gotten from a tumor in the left axillary lymph hub of a 39 - years old male with little lung cell carcinoma in 1989. Be that as it may, DNA fingerprinting recommends cross - sullying with cell line RPMI - 8226; RPMI-8226 was built up from the fringe blood of a 61-year-old male with various myeloma in 1966. The THP1 (Human monocytic leukemia) Got from the fringe blood of a 1-year-old male with intense monocytic leukemia[22].

The NB4 cell line Intense promyelocytic leukemia (APL) is a well-characterized substance among intense leukemia, cytogenetically described by a t(15;17) (q22;q11-12) translocation [23], was acquired from the Division of Test Treatment, Iraqi Community for Disease and Restorative Hereditary Exploration (ICCMGR; Baghdad, Iraq). Every one of the cells were refined in Roswell Park Commemoration Establishment 1640 medium (US Natural) with fetal calf serum 10%, penicillin 100 u/ml, and streptomycin 100 µg/ml, and brooded at 37°C.

Retinoic Acid

In this study Retinoic acid (RA) was used as a chemotherapeutic agent by dissolving 7 mg of RA powder in 5 ml (1%DMSO and complete free serumsmedium RPMI and filtered by syringe filter 0.2 µm to prepare stock solution 5mM. Then diluted with 3 ml of serum free medium until before using for in vitro studies[24]

Cytotoxicity Assay

The cells remained seeded in 96well saucers and were eroded with phosphate cradled saline before vaccinating with and without the Retinoic Corrosive at various focus (400,350,300,250,200,150,100,50,25,12.5). Following 72 hours brooding, the intermediate was suctioned and an absolute of100 µl of MTT arrangement (5 mg/mL in PBS, pH 7.2) remained supplementary to separately well and the plates were hatched for 2 hours at 37 °C. After hatching, 50 µl of dimethyl sulfoxide was supplementary to each well, trailed by delicate shaking for 45 min to dissolve the color of formosan. The receptiveness was resolved on a microplate peruser at 584 nm wavelength. The examine was achieved in triplicate for each of the ailment[25], The repressing pace of cell development (the cytotoxicity level was determined as (IG%) = (a-b)/ax100, where a is the mean optical thickness of untreated wells and b is the optical thickness of treated wells [26].

Apoptosis quantification using acridine orange and propidium iodide double staining:

Acridine orange is an interposing color that can infiltrate equally animate and dead cells. AO will recolor every single nucleated cell to create green fluorescence.

Propidium iodide can just arrive departed cells with poor layer honesty, so it will recolor all dead nucleated cells to produce red fluorescence. Cells recolored with both AO and PI fluoresce red because of extinguishing, so all live nucleated cells fluoresce green and all dead nucleated cells fluoresce red. 1µl Acridine orange stock (5mg\ml) and 1 µl propidium iodid stock (3mg\ml) blending with 1 ml PBS (phosphate cushion arrangement). We included AO/PI to the tried wells after media expulsion from each well and including 50 µl of blend arrangement. Following 20 second we expelled the stain from the well, and quickly saw under fluorescent microscope[27].

Immunocytochemistry

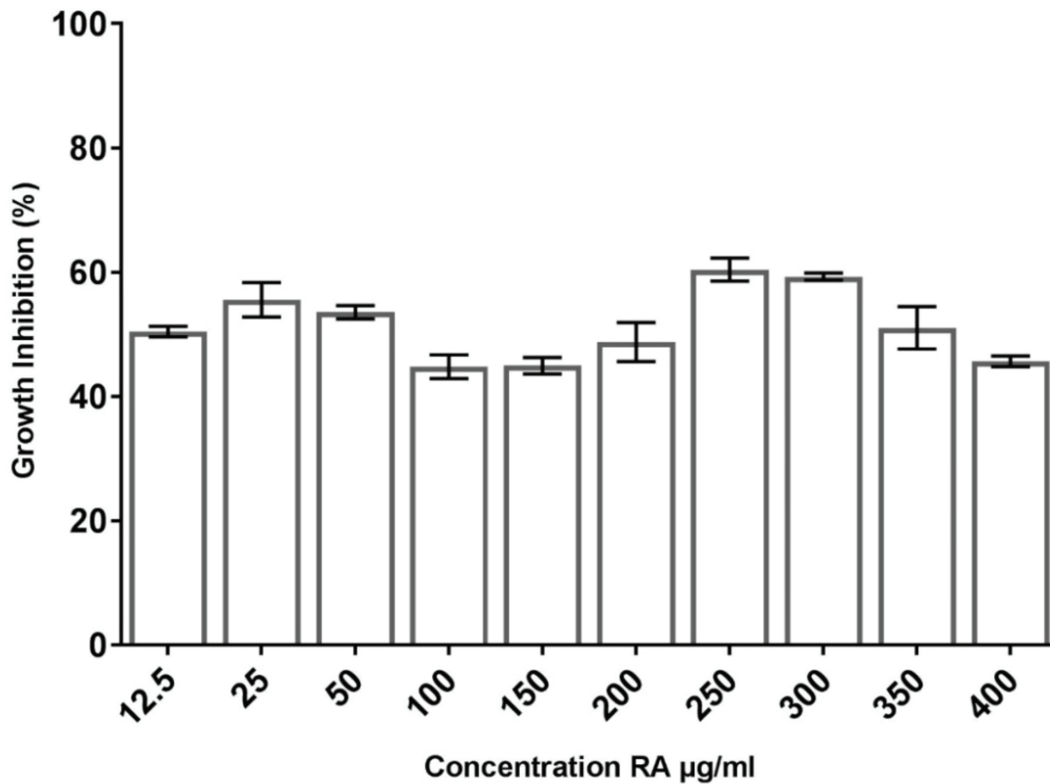
For the disciple cell lines (NB4 and SR) were refined on coverslipes. The cells were permitted to build up a one layer. After this introduction of RA (IC50), after 72hrs. At that point, the cells were fixed with cold CH3)2CO at 2 – 5 minutes, at that point expulsion cold CH3)2CO and eroded with phosphate-cushioned saline for multiple times and late to dry. Then slides were hatched in a humidified chamber for 10 minutes with H_2O_2 1% after obsession, eroded a few times with PBS, and brooded with 1.5% blocking reagent for thirty to forty minutes at room temperature. At that point, the essential immune response of the CAS-8, CAS-9, AIF and ENDO G counter acting agent for 1 - 1:30 hrs., eroded a few times with PBS, A while later, include auxiliary immune response and remain for two hrs. It was recolored by the ImmunoCruz™ mouse ABC recoloring framework. Later counterstained with Hematoxcylin for thirty to sixty seconds after eroded widely with PBS, and. The slides were mounted with Distyrene, a plasticizer, and xylene, investigated utilizing light microscopy, and

captured utilizing a computerized camera[28].

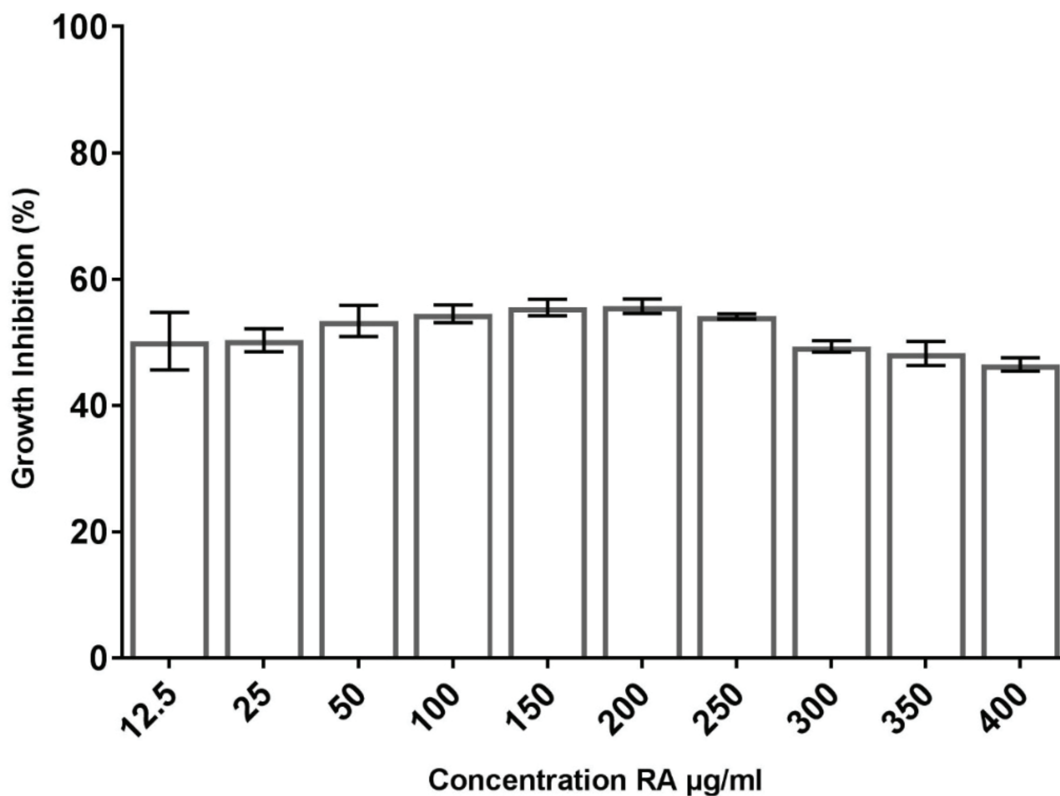
Results

Cytotoxicity of Retinoic Acid:

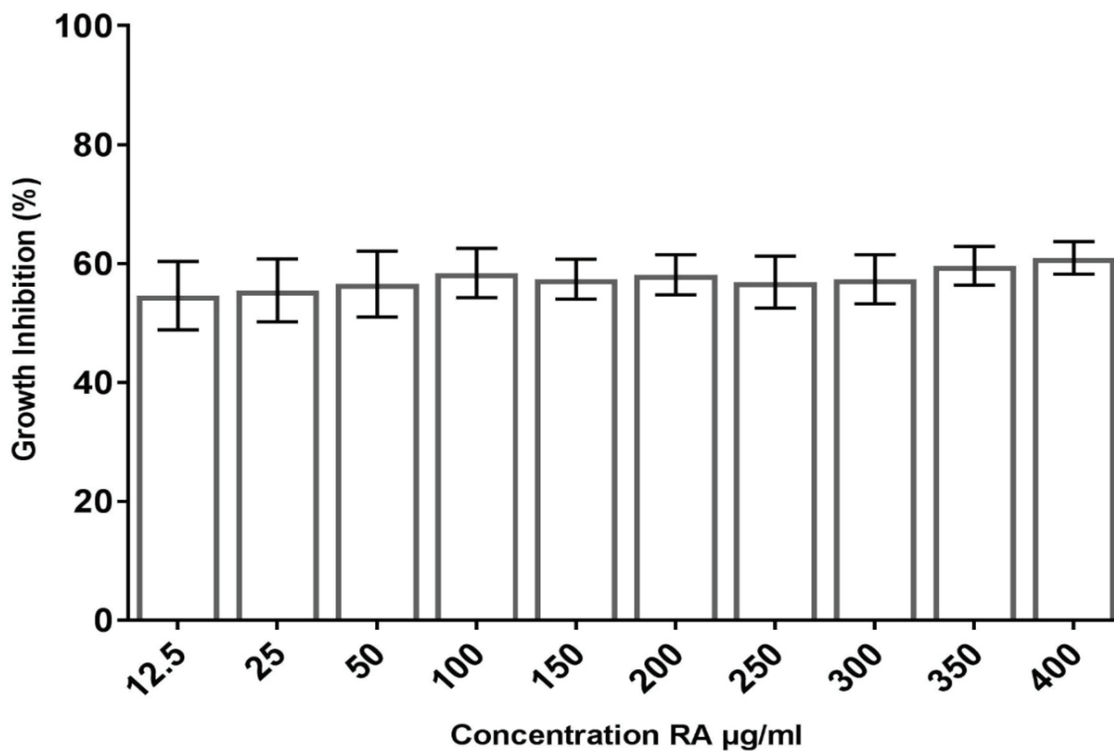
In this examination, the cytolytic impacts of Retinoic Corrosive on hematological danger cell lines, ordinary HBL – 100 cell line, were dictated by estimating the cytotoxic portion that execute half of the cell populace when contrasted with the untreated control for 72 hr. utilizing colorimetric cytotoxicity test (MTT). The examine was rehashed multiple periods. The level of feasible cells was conspired against RA and the various fixations (400, 350,300,250,200,150,100,50,25,12.5 µg\ml). qualities were resolved after 72 hr. RA indicated cytotoxicity impact on lymphoma SR cell line and human different myeloma (COLO 677) and Human monocytic leukemia (THP1) and Intense promyelocytic leukemia (NB4) cell lines and HBL-100 ordinary cell line in portion subordinate way. The outcome was a noteworthy ($p<0.0001$) increment in RA cell passing and cytotoxicity sway on hematological threat and low cytotoxic on HBL-100 typical cell line. (Figure 1, A, B, C, D and E)



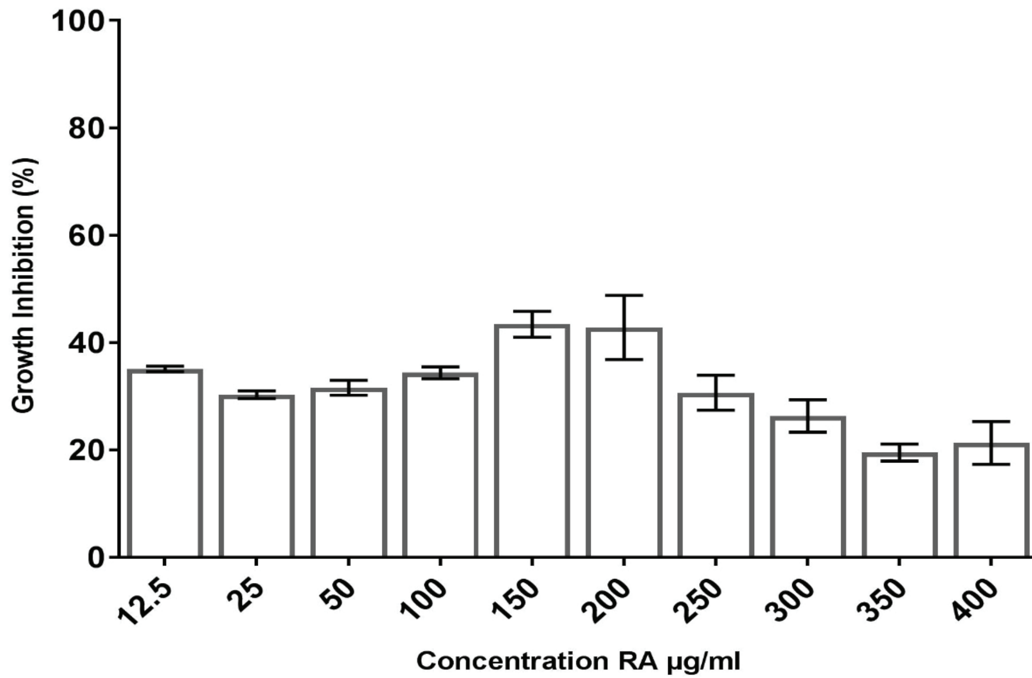
A:Showing the RA's cytotoxicity impact on the cell line COLO677



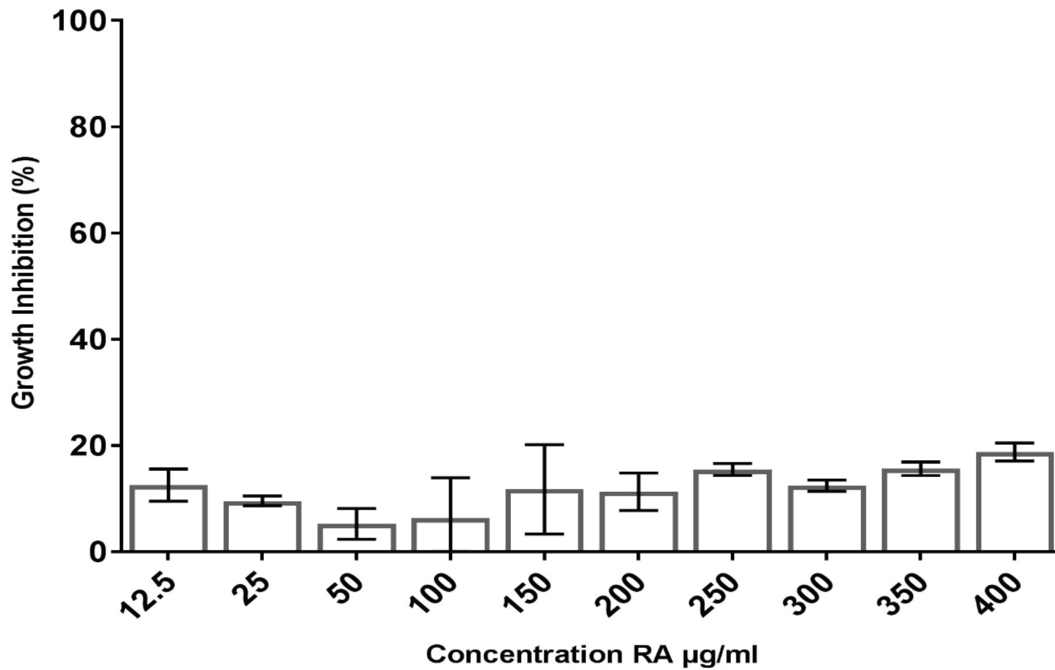
B: Showing the RA cytotoxicity impact on NB4 cell line



C: Showing the RA cytotoxicity impact on the SR cell line



D: cytotoxicity effect of the RA on THP1 cell line



E: Cytotoxicity effect of the RA on HBL-100 normal cell line.

Figure 1: Cytotoxicity effect of the Retinoic Acid on Five different cell lines, represented by (A, B, C, D, and E).

Quantification of apoptosis using propidium iodide and acridine orange double staining:

This examination was done to recognize the changes in morphology and the extents of apoptotic, necrotic and typical feasible cells in the number of inhabitants in SR and NB4 disciple cell lines presented to RA at IC50 for 72 h contrasted with untreated cells. AO will recolor every single nucleated cell to create green fluorescence. Propidium iodide can just arrive departed cells with pitiable film intergrity, so it will recolor all dead nucleated cells to produce red fluorescence. Cells recolored per equally AO and PI fluoresce red because of extinguishing, so all live nucleated cells fluoresce green and all dead nucleated cells fluoresce red. The percentage of viable SR cells unprocessed with RA gave 76.615% at 72 h but the percentage of apoptotic cells had significantly ($p < 0.001$) and gave 117.519%, at 72 h, On the other hand, The percentage of viable NB4 cells untreated with RA gave 75.105% at 72 h but the apoptotic cells percentage had significantly ($p < 0.0001$) and gave 105.62% at 72 h. as shown in (Figure 2).

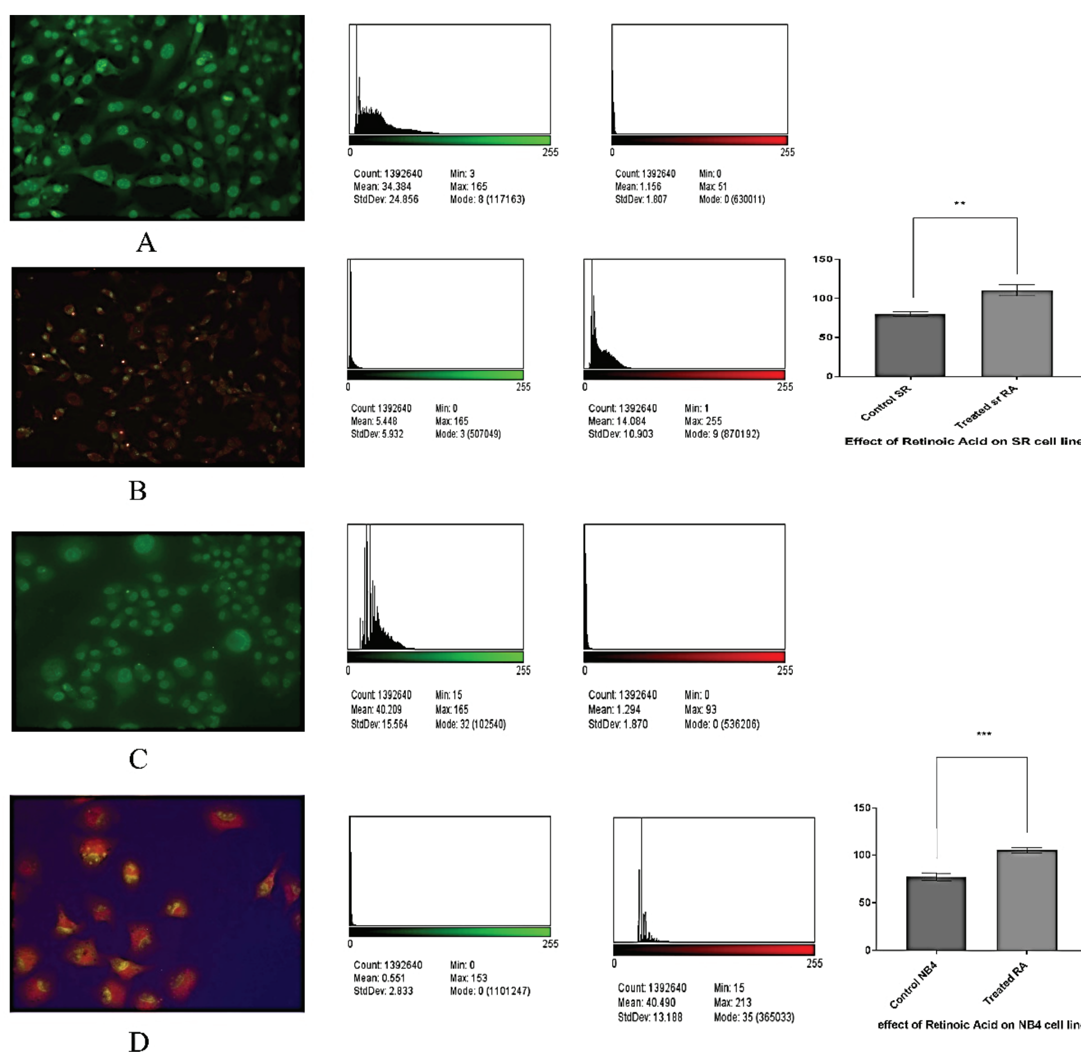


Figure 2: The apoptotic by use acridine orang stain: (A) control viable green cells SR cell line, (B) cell infected with RA red cell SR cell line, (C) control viable green cells NB4 cell line, (B) cell infected with RA red cell NB4 cell line

Immunocytochemistry

Immunocytochemistry results showed that NB4 and SR cell lines were sure for ward pathway CAS-8 and CAS-9 and positive for free pathway AIF and Endonuclease G this test was done to affirm that focused treatment utilizing Retinoic Corrosive is compelling and the focused on antigen is available. The outcomes exhibited that the action of caspase-8 was altogether ($P>0.0001$) and caspase - 9 was essentially ($P>0.001$) (expanded in both cell lines treated with RA (Figure 3). This affirmed by utilizing explicit monoclonal antibodies for caspase 8, 9 (ward) and AIF and Endonuclease G proteins for apoptosis. . lymphoma SR cell line, Intense promyelocytic leukemia NB4 cell line were recolored positive for articulation of caspase 8,9, AIF and Endonuclease G proteins in the tumor cells, This test was done to distinguish RA prompting apoptosis component in various cells in vitro after 72 hr. from contamination, (Antigens in tainted cells stains dark colored). Uninfected cells do not stain. (Nuclei stain blue by counter stain).

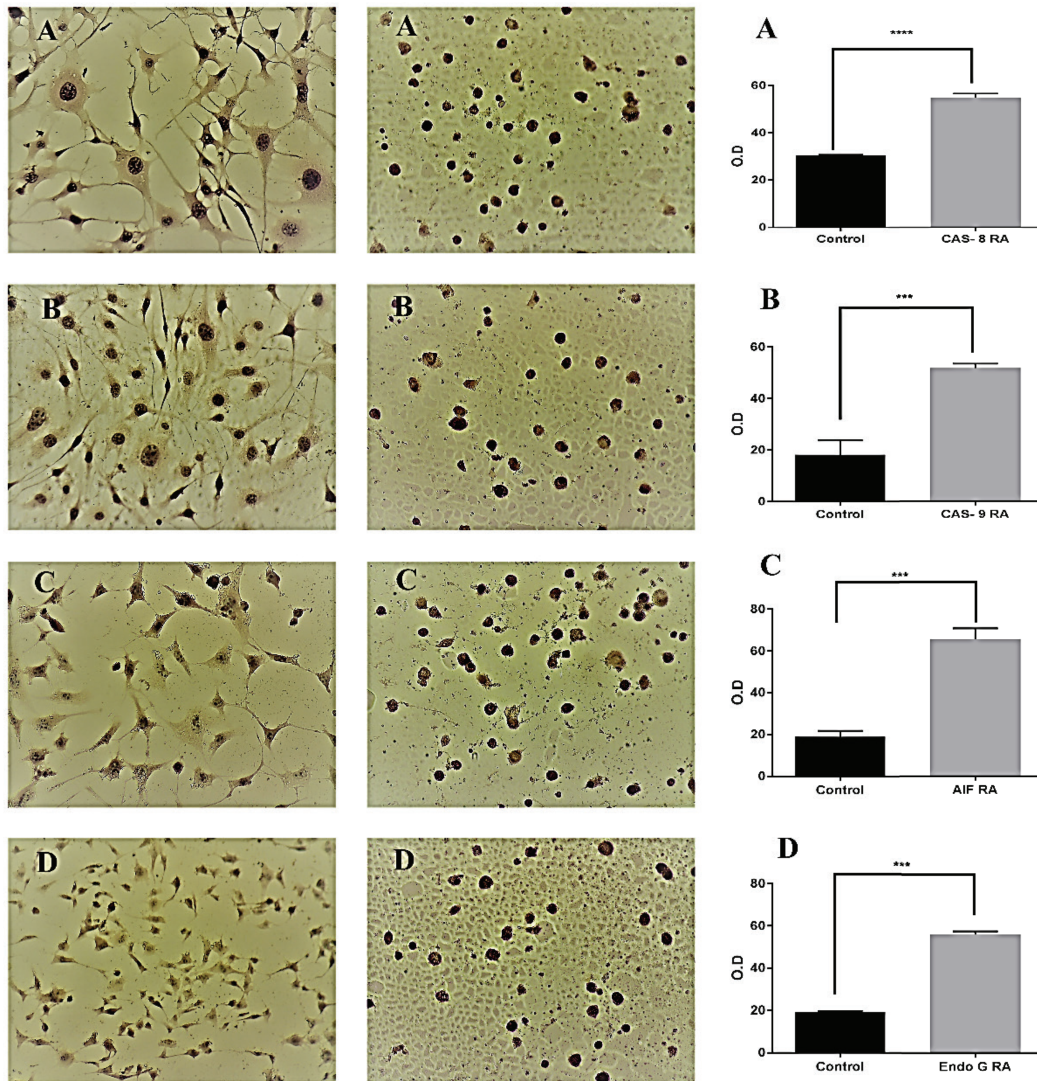


Figure 3: Immunocytochemical study of lymphoma SR cell line (A1) control, (A2) CAS-8 antibody, (A3) Analysis, (B1) control, (B2) CAS- 9 antibody, (B3) analysis, (C1) Control, (C2) AIF antibody, (C3) Analysis, (D1) Control, (D2) EndoG antibody, (D3) Analysis, (DAB stain) Magnification: 20×.

Discussion

The aim of this study was to investigate the involvement of caspase dependent or independent way of apoptosis through infection by Retinoic Acid.

In this investigation, we have assessed the potential antitumor impacts of the RA in leukemic cell lines. Our information demonstrate that RA applies antiproliferative and proapoptotic or separation initiating impacts in hematological cell lines. High convergences of RA gave low cytotoxicity practically identical with low Focuses, which was trailed by cell passing following 72 hours. Conversely, low convergences of RA instigated apoptosis. These information demonstrated that RA applies portion subordinate proapoptotic and antiproliferative impacts in leukemic cells. Moreover, a factually huge lower cell executing of typical HBL-100, It is discovered that, RA have been explored widely for their utilization in strong tumor disease counteractive action and treatment[29].

Retinoic destructive may be gained from either through the consistently use of plants in a reasonable eating regimen or through supplement upgrades. Under common conditions in the body, retinoic destructive does preventive kill threatening development course of action. After dangerous development course of action, retinoic destructive transforms into an attacker to illness cells, one that discourages their advancement and division and moreover triggers their partition and going through express pathways[30].

In general, RA causes a square in the cell cycle stage G1, with an expansion in the extent of cells in the stage G0/G1 and an abatement in the extent of cells in the stage S [31]. RARβ2 is inducible to RA and is the prevalent receptor that intercedes the inhibitory effects of RA on cell proliferation [32, 33].

In this study, we showed that RA repress the development and actuate the apoptosis of RA-safe hematology tumor, gave that they are additionally fit for initiating the caspase ward or caspase free pathway. The apoptotic cells concoction and morphological changes can be separated from any other cell demise through numerous strategies. In this investigation, affirmation of apoptosis started by RA was finished utilizing the minute assessment. AO/PI recoloring affirms that RA in cell societies instigates cell passing by means of the apoptotic pathway, the quantity of apoptotic cells uncovered that in treated hematology cell lines the level of apoptotic cells relatively expanded with post-immunization time. Identification of the acridin orang and propidium iodid strategy (AO\PI) gives an early sign of the commencement of cell apoptosis[27] .

RA is viable at the phone multiplication and separation just as the counter malignant growth capacities during the procedure of carcinogenesis (Zhu and Luo, 2016). RA have various receptors RAR, RXR and RARγ act with various pathways as Wnt/β-catenin and β-catenin/TCF, the RAR and RXR receptors restrain Wnt/β-catenin, while RARγ receptor goes about as a tumor oncogene lead to the actuation of Wnt/β-catenin pathway [34], these methods the RA demonstrations in two inverse ways, relied upon its receptors. While, RA repress malignancy cell expansion [34].

Retinoic acid likewise initiates the NF-κB endurance pathway [35, 36], ATRA prompts the outflow of cIAP2 and TRAF1 within the sight of elevated levels of cIAP1 and TRAF2 mRNAs, The attending articulation of these variables is thought to build up a complex at the TNF-R1 that prompts actuation of NF-κB by means of the NIK-IKK-IκB flagging course with the outcome that TNF-α, rather than inciting apoptosis, sets up a NF-κB–interceded autocatalytic endurance loop[37].

Quantitative picture examination for the immunocytochemistry test uncovered expanding in articulation of caspase-8 and caspase-9 (caspase subordinate pathway) in SR and NB4 cell lines. Caspase-8 was distinguished as an initiator caspase activated by death receptors. Along these lines, the initiation of caspase-8 recommended that RA may instigate apoptosis through the outward demise receptor-pathway. cells was seen in the immunocytochemistry measures, as contrasted and untreated cell Examinations of the component of RA-prompted cell passing demonstrated mitochondrial depolarization, translocation of phosphatidylserine to the cell surface, enactment of caspases, and DNA discontinuity, proposing apoptotic cell demise. To explicitly inspect the job of caspase-8,9 in RA-prompted apoptosis[38],. Late investigations have demonstrated that RA instigated Ca2 discharge from the endoplasmic reticulum in skin, bosom, and lung

malignancy cells, and that Ca2 chelation hindered the apoptogenic impacts of CDDO [38]. Strangely, Ca2 can trigger AIF discharge from the mitochondria [39], which could conceivably provide a link between endoplasmic reticulum stress and caspase-independent cell death.

Notwithstanding the counter apoptosis exercises of BCL2A1 and NFκB, retinoic corrosive invigorates the articulation of the apoptosis inhibitors cIAP2 as well as of NAIP. Overexpression of IAPs restrains apoptosis instigated by master apoptotic BCL-2 relatives through their immediate official to the effectors casp-3 and casp-7, the initiator casp-9, and its expert enzyme19. Additionally, the retinoic-corrosive induced39 articulation of casp-8, which transfers TRAIL activity in the Plate, may encourage TRAIL–interceded destruction of leukemia blasts[40].

In conclusion, our study suggests that the Retinoic Acid is a very promising anti-hematological malignancy agent that found to act by inducing apoptosis through caspase dependent and caspase independent pathways of apoptosis.

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Conflicts of Interest: Nil.

Ethical Clearance: Nil.

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