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The Impact of Combinational Treatments (Botulin Toxin A and Anthrax toxin) on Proliferation Apoptosis in Normal (MRC5) and Lung Cancer (A545) Cells

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ABSTRACT

In the both males and females, the occurrence of lung cancer as the second highest in the world. The aim of this study was performed to assess the activity of the botulin toxin A (BtxA) and Anthrax lethal factor (ATx) alone and together on normal (MRC5) and cancer lung (A545) cells. To examine BtxA and LF effect on cell viability used MTT assay. The determination of cellular sensitivity to BtxA, LF, and the synergistic combination of both BtxA and LF was accomplished through the utilization of clonogenic assays. The type of cells death was measured by acridine orange/ethidium bromide. Induction of apoptosis in normal (MRC5) and lung cancer (A549) cells were also determined with flow cytometric analysis. MTT assay illustrated that BtxA and LF inhibited MRC5 and LF cell growth in a time as dose dependent manner. After 24 h, the IC_{50} values calculated. In MRC5 cells this Inhibition concentration were 0.1 ng ml-1 and 310 ng ml-1 for BtxA and LF, respectively. Treatment of A545 cells with 0.08 ng ml-1 BtxA and 310 ng ml-1 LF for 24 h was found to inhibit cell growth with IC_{50} values. Surviving fraction reduced when the cells exposed IC_{50}. Thereby resulting, in apoptosis studies, Combinational treatments was also induced apoptosis more than separately. Our results indicate that combinational treatment had significant anti-tumor activity in A545 cells.

Keywords: Botulinium toxin, Anthrax toxin, Lung cancer, Apoptosis

INTRODUCTION

Cancer factors leading deaths have been an enormous challenge in recent decay. In men and women malignant tumors, lung cancer morbidity ranks second, whereas the fatality ranks first [1]. Approximately 80% cause of lung cancer is caused by cigarette smoking, uptake and cessation that is distinct among men and women. Occurrence and mortality rates are higher im men rather than woman [2].

Recent advances in understanding the tumor microenvironment have led to the development of targeted therapies and immunotherapies for lung cancer [3]. Recent progresses in non-small cell lung cancer (NSCLC) management include histology-directed, molecular-directed, and maintenance therapies [4]. However, small cell

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lung cancer (SCLC) handling has seen fewer advances, and treatment outcomes have plateaued [5]. Clostridium botulin is a bacterium that causes botulism. The bacterium produces botulin toxin, which is classified as a Category A of bioterrorism due to its ability to interrupt cholinergic nerve transmission [6]. Botulinum toxin type A, also known as Botox A, is widely used therapeutic applications [7]. Botulinum toxin is a dichain polypeptide that inhibits acetylcholine release by cleaving the SNARE complex. As well as, the toxin can block synaptic vesicle exocytosis by cleaves SNAP-25 [8]. Botulinum toxin A inhibits the growth of LNCaP human prostate and T47D breast cancer cell lines [9,10]. Anthrax toxin is a tripartite protein toxin constructed by Bacillus anthracis. This bacterium consists of three proteins such as protective antigen (PA), lethal factor (LF), and edema factor (EF). PA is responsible for binding receptors on the surface and internalization of the toxin complexes [11]. The toxin complex Trans located from the endosome to the cytosol, where it exerts its toxic effects Understanding the mechanisms of anthrax toxin is crucial for the development of medical countermeasures against anthrax bioterrorism [12]. Anthrax toxin (AT) specifically increased ovarian carcinoma [13]. Previous studies assessed the inhibited breast cancer cell migration, adhesion, and invasion [14]. Anthrax toxin receptor 1 (ANTXR1) plays a role in tumor angiogenesis, this toxin barricade angiogenesis and reducing tumor growth rate [15]. The aim of our investigation is to survey BtxA and LF against normal and cancer lung cells. This bioterrorism factor can be a novel drug to treat lung cancer cells.

MATERIAL AND METHODS

Cell Culture

In this in vitro experimental study, normal lung cells MCR-5 (ATCC, CCl-171) and lung cancer cell lines A545 (ATCC, CCl-185) were obtained from the national cell bank (Pasteur Institute, Iran). The cells were cultured in DMEM medium (Gibco, United Kingdom) with 4.5% of glucose, supplemented with 10% heat-inactivated FBS (Gibco, United Kingdom) and 0.1 mg penicillin/streptomycin solution (Sigma-Aldrich, USA). The growth stages completed to stored flask into an incubator with 10% FBS at 37 °C in a humidified 5% CO2 atmosphere. The cell sub-cultured upon is was reaching 80-90% confluence. Botulinum toxin A (Ontario, Canada), Lethal factor (LF) (quadratech-United kingdom) used according to the manufacturer's protocols.

Cytotoxicity Assay

MTT assay can investigated the level of vitro cytotoxic effects of toxin on cell lines. MTT assay is subservient in mitochondrial dehydrogenase enzyme to assess the cellular energy capacity of a cells. The effect of BtxA and LF on MRC5 and A545cells viability were evaluated by 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, USA) assay. MRC5 and A545 were seeded in 96-well. Afterwards, both cells were treated with specific concentration of BtxA and LF for 24, 48 and 72 h. After these 3 particular times, the cells were washed with phosphate buffered saline (PBS) then added MTT (Merck, Germany) solution (0.5 mg ml-1 PBS). Afterwards, added 100 µl DMSO (Sigma-Aldrich, USA) to each well separately and incubated for 3 h to dissolve the formazan crystals. The absorbance optical density (OD) of each well was read at 590 nm with ELISA plate (BioTek, USA). The inhibition concentration was calculated following formula: (Atest/Acontrol) × 100.

Colony Forming Efficiency Assay

The efficiency of colony formation is prominent assay for assessing the quality of cell preparations after treatment with cytotoxic reagents. The normal and cancer cells as MRC5 and A545 respectively, were seeded in 6-well diver's densities of 10, 50, 100, and 200 cells/well. When, the cells were in exponentially curve treated with IC_{50} concentrations. After that, the well chambers were filled with DMEM medium and 10 % of FBS in a humidified atmosphere of 5 % of CO2 at 37 C. Colonies were fixed formaldehyde (Merck, Germany), stained with crystal violet solution (Merck, Germany) Plating efficiency (PE) was calculated, using the following equations:

$$\frac{\text{The number of colines}}{\text{The number of seeded cells}} \text{ X100}$$

Double Fluorescent Staining

To distinguish the normal, apoptotic and necrotic cells from early to late stage used Double-staining fluorescence. This method is rapid and statistically substantial test. Acridine orange (AO)-(Chadwell Heath, England)/ethidium bromide (EB) (Merck, Germany)-double staining demonstrated morphological changes in nuclei structure. Brifely, the cells were treated with IC_{50} concentrations of the tested compounds. Thereupon, added to the cell suspension two fluoresce dye (100 µM and AO and 250 µM EB). The cellular morphology was considered with Axoscope 2 plus fluorescence Spmicroscopy from Zeiss (Germany). At least 200 cells were counted to determine the viable, apoptotic, and necrotic cells percentage.

Flow Cytometry Studies

Flow cytometry demonstrated apoptosis in various celltypes and highlight the importance of validating. In this significant test two probs play rolls such as, Annexin-V is a protein that binds to phosphatidylserine, which is exposed on the outer membrane of apoptotic cells and propidium iodide is a fluorescent molecule that binds to DNA and can be used to discriminate between live cells with intact membranes and dead cells with permeable membranes. For this objective, firstly the cells (MRC5&A549) were treated with BTXA and LF concentrations of IC_{50} for 24 h then trypsinized and washed with PBS. In the next step, centrifuged and adding the binding buffer. The treated cells were stained with PI (Merck, Germany) and Annexin-V (Merck, Germany), each solution incubated in the dark room for 15 min. Eventually, the cells were incubated at 25 °C for 10 min in dark. The cells were investigated with flowcytometry (Partec, Germany). Image j software accomplished data analysis.

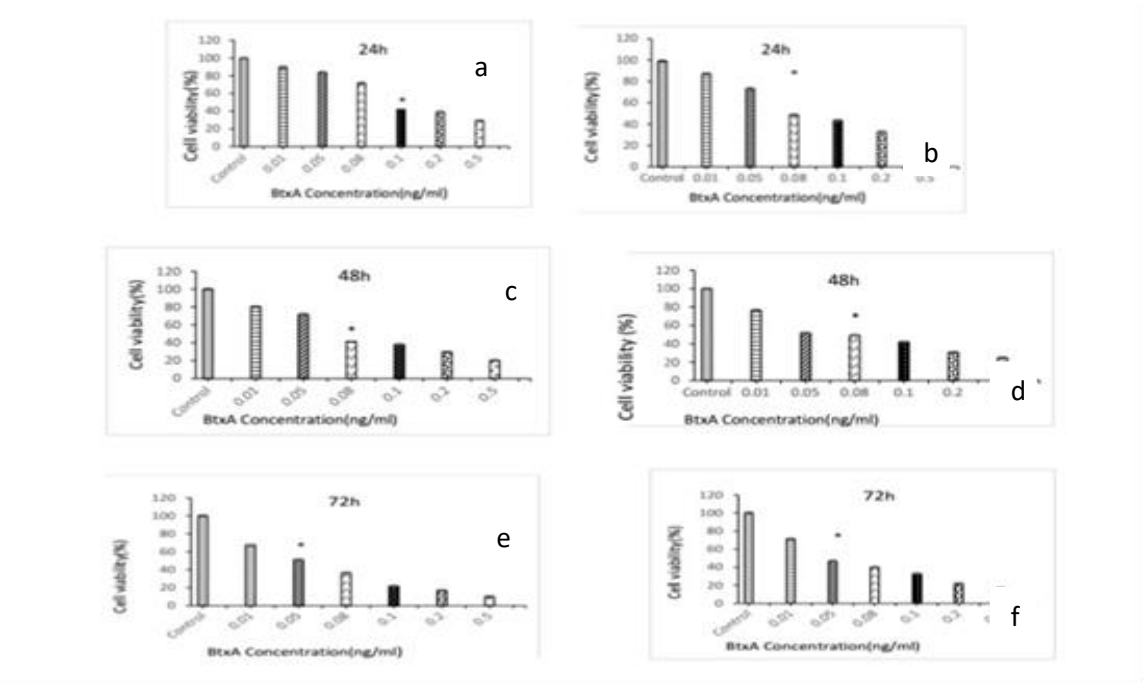
Statistical Analysis

Data in this research represented three independent experiments as mean ± SEM and analyzed by one-way ANOVA by t-test. P-value < 0.05 was considered to be significant. To calculated theses data was used MATLAB software and Image J.

RESULTS

Cytotoxicity Effects on Normal (MRC5) and Malignant (A545) Cell Lines

MRC-5 and A549 cell lines were treated



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Figure1. Effect of BtxA on the viability of MRC5 and A549. The cells were treated with different concentration BTXO for 24, 48, and 72 h and cell viability was evaluated using the MTT assay. Treated MRC5 and A549 cells with BTXO after a,b (24 h), c,d (48 h), e,f (72 h).).BtxA reduced the cell viability in time- and dose-dependent manners. The data represent the mean ± SD of three independent experiments. BtxA = Botulinum toxin A.

for 24, 48 and72 h with various concentrations of BTXO (0.01, 0.05, 0.08, 0.1, 0.2 and 0. 5 ng ml-1) and LF (50,100, 210, 310, 350, 410 ng ml-1). The cytotoxic effect on cells was determined by the tetrazolium colorimetric test (MTT test). As it is seen in the Fig. 1a-1b at 24 h the cell viability of MRC5 and A545 cell line were 99.6%, 89.6%, 83.7%, 71.6%, 55.1%, 38.9%, 29.1%, and 99.1%, 87%, 73.7%, 48.6%, 43.4%, 32.5% and 25.8%, respectively.
Figure 1c, d showed, treatment MRC5 and A545 with BTXO at 48 h, decreased cell viability from 100% to 25.8% and with BTXO decreased significantly in 72 h (Fig. 1e-f). Based on result in Fig. 2a, b treated cells with LF decreased viability. Cell viability of MRC5 and A545 cell line were 98.9%, 97.3%, 79.7%, 69.7%, 50.8%, 38.9%, 35.8%, and 99.3%, 93.3%,73.7%, 55.7%, 48.8%, 33.6% and 28.4%, respectively. At 48 h (Fig. 2c-d), the viability of MRC5 and A549 reduced when treated with LF from 100% and 99.6% to 24.4% and 23.8% for MRC-5 and A549 respectively. Fig 2e-f, demonstrated the cell viability that treated with LF decreased significantly in

72 h. All treatments presented significant effects on MRC5 and A549.

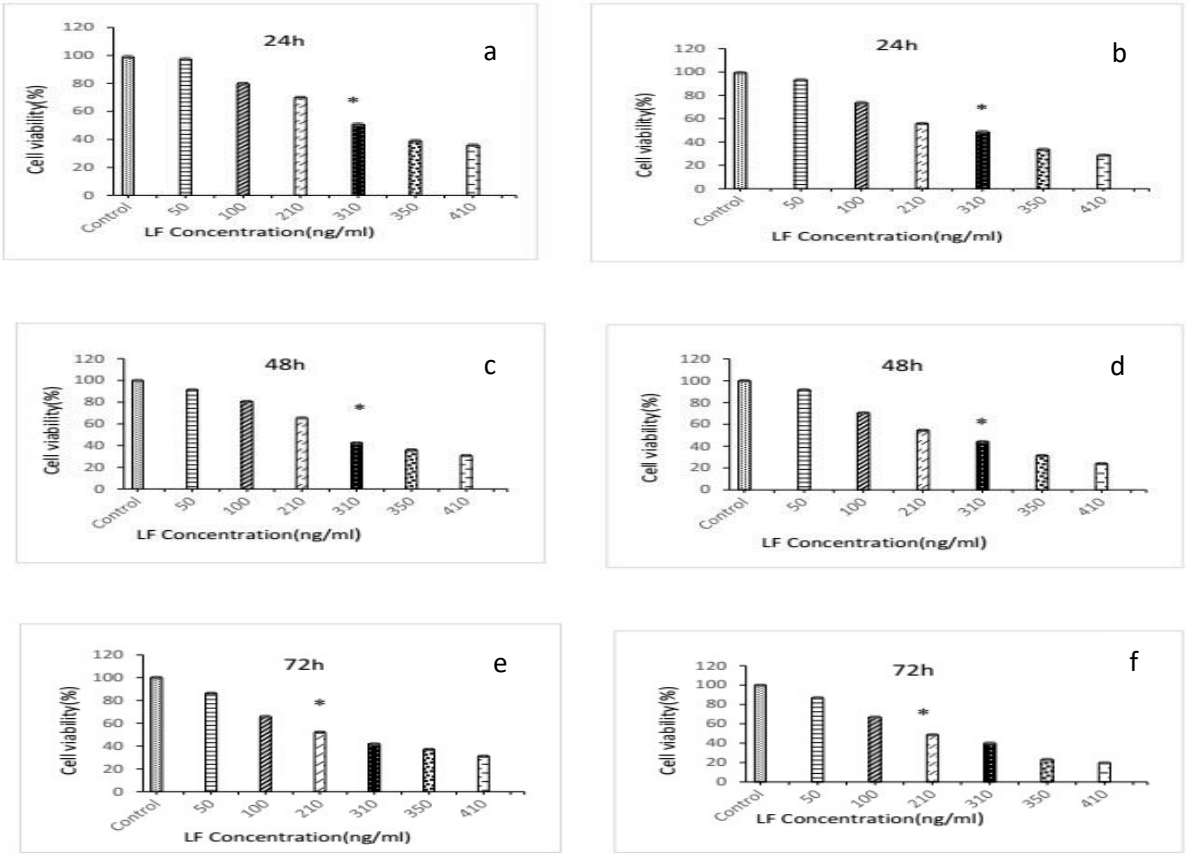


Figure2. The cell viability of MRC5 and A549 treated with various concentrations of LF O after a,b (24 h), c,d (48 h), e,f (72 h) LF reduced the cell viability in time- and dose-dependent manners. The data represent the mean ± SD of three independent experiments. LF = lethal factor.

In this study BTXO and LF 50% inhibitory concentration (IC_{50}) values occurred in MRC5 at (0.1-0.08-0.05 ng ml-1 and 0.08- 0.05-0.05 ng ml-1) for 24, 48 and 72 h respectively. LF actuated to 50% both normal and cancer cell death at 310-310 and 210 mg ml-1 for 24, 48 and 72 h respectively (Table 1).

Based on the results presented in Fig. 3a-b and 3c, d the IC_{50} values it can declare that the A549 cell line is the most sensitive to the treatment while MRC-5 cells are the least sensitive.

Table 1. IC_{50} Values of BtxA and LF in MRC5 and A549 for Different Incubation Time

Cells	Treat with BtxA-after 24 h	Treat with BtxA-after 48 h	Treat with BtxA after 72 h	Treat with LF after 24 h	Treat with LF after 48 h	Treat with LF after 72 h
MRC5- IC0 (ng ml^{-1})	0.1	0.08	0.05	310	310	210 1
A549- IC0 (ng ml^{-1})	0.08	0.05	0.05	310	310	310

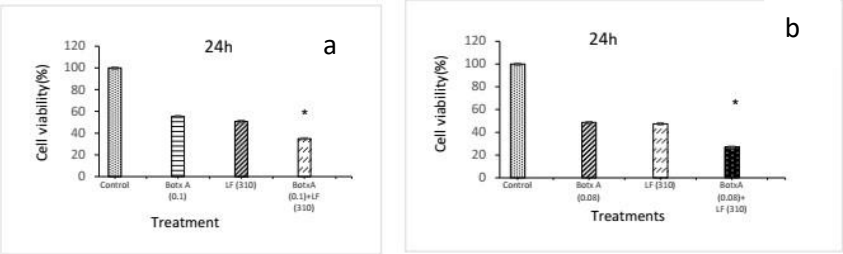


Figure 3. Response of Combinational treatment with BtxA and LF. Figure 3a, combinational treatment induced inhibitory effect on Proliferation of MRC5 and b, A549 Cells. Both cells were incubated during 24 h in presence of IC_{50} concentration of BtxA and LF after 24 h. The data represent the mean ± SD of three independent experiments. BtxA = Botulinum toxin A, LF = lethal.

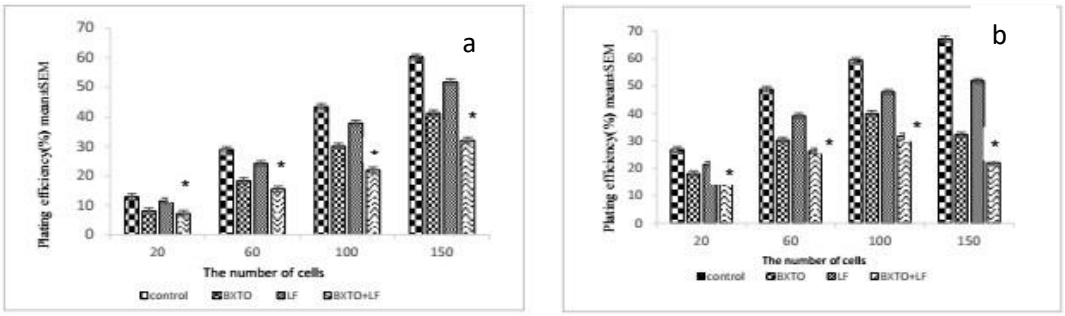


Figure 4. Relative colonogenic formation BtxA, LF and combinational treatment on MRC5 (4a) and A545 (b) cells were evaluated by a clonogenic assay for 24 h. The data represent the mean ± SD of three independent experiments. BtxA = Botulinum toxin A, LF = lethal.

Evaluation of Normal and Cancer Cell Line Colony Forming Efficiency by BtxA and LF

Colony formation ability distinguishes the resumption of cancer cells. In this investigation MRC5 and A549 treated with BtxA and Lf separately and mixtures of these two compounds (IC_{50}, 24 h). Based on the result in Fig. 4a, b the number of colonies increased in untreated cells whereas, in treated cells decreased. In the first column, the number of colonies were the same as incipient cell density. Similar results were acquired on MRC5 and A549 cell lines. Column two and three illustrated, BtxA and LF separately, this toxin can reduce the number of colonies whereas this ability effected more prone to be clonogenic in A545 compared to MRC5.The number of colonies of normal and cancer cells significantly decreased after treatment with combinational treatment (BtxA and LF) at IC_{50} concentration.

BtxA, LF and Combinational Treatments Determine the Type of Cell Death Induced in MRC5 and A549

Cytotoxic effects of the BtxA, LF and synergistic cytotoxic effects of cell death investigated with double Fluorescent Staining. The Cells were treated under the specific time and IC_{50} in each cell. Eventually, stained with EB-AO and analyzed (Fig. 5). Apoptosis induction by BtxA&LF in either of the cell lines examined. In A545 compare to MRC5, compression of apoptotic cells (early and late stage) increased. As declared in Tables 2 and Fig. 6a-b and, combinational treatments cause of cancer cells death more significant than normal cells. The necrotic cells seemed to stay moderate.

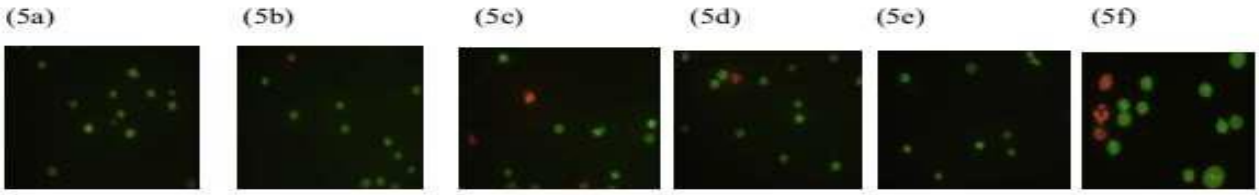


Figure 5. Representative the treated cells by fluorescence microscopy. MRC5 and A545 were treated with BtxA and LF IC_{50} for 24 h then imaged by fluorescence microscope. Treated MRC5 and A545 with IC5 BtxA (5a,5d), LF (5b,5e) and combinational (5c,5f) respectively. In normal, early, late and necrotic showed green, yellow-green, orange nuclei and red continuously.

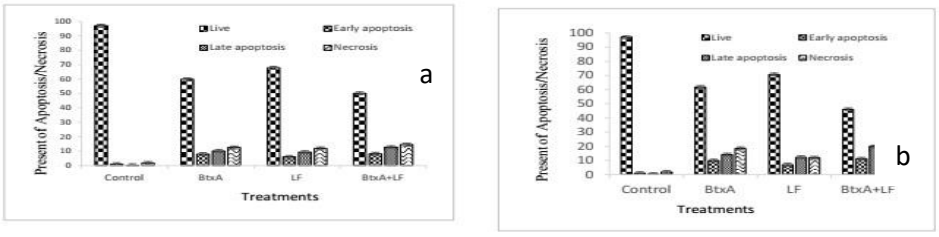


Figure 6. Quantity of apoptotic and necrotic cells in 6a (MRC5) and 6b (A545) cells with BtxA, LF, BtxA & LF treatment. The data represent the mean ± SD of three independent experiments. BtxA = Botulinum toxin A, LF = lethal.

Table 2. Percentage of Apoptotic Cells. Increased Number of Counted MRC5 and A545 Cells Undergoing Apoptosis Following BtxA, LF Alone and Combinational

Treatments	Live cells	Early apoptosis	Late apoptosis	Necrosis
MRC5 (control)	97.04%	0.91%	0.27%	1.78%
MRC5+BtxA	59.87%	7.9%	10.1%	12.5%
MRC5+LF	67.87%	6.01%	9.2%	11.9%
MRC5+BtxA&LF	50.13%	8.2%	12.8%	14.5%
A549(control)	97.3%	0.90%	0.27%	1.78%
A549+BtxA	61.87%	9.8%	14.1%	18.5%
A549+LF	70.78%	7.02%	12.2%	11.9%
A549+BtxA&LF	46.13%	11.1%	19.8%	20%

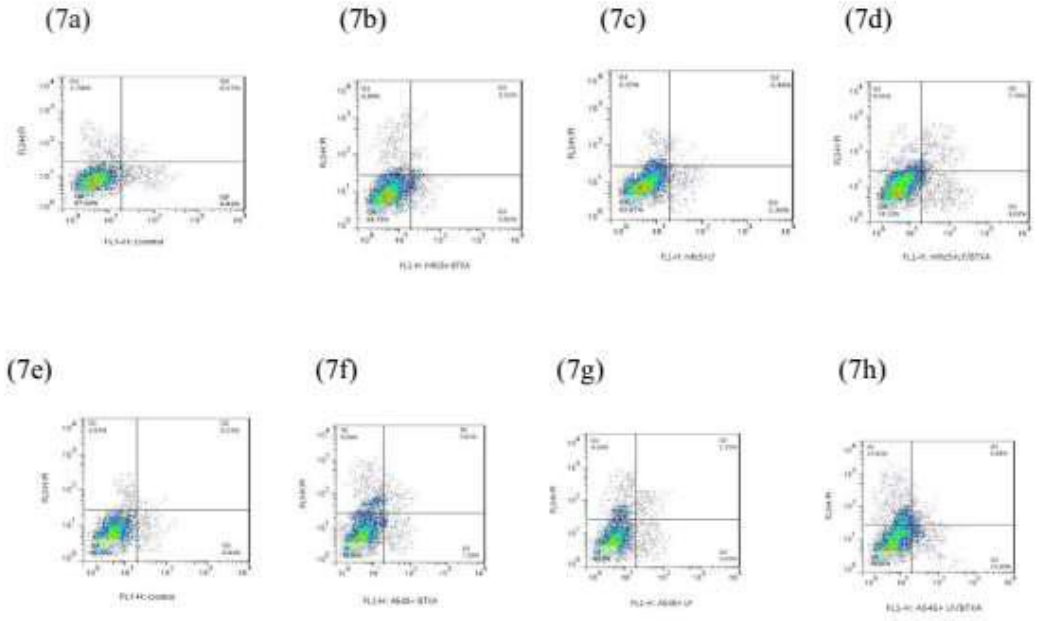


Figure 7. Apoptotic effect of BtxA, LF and combinational treatments on two cell lines. 7a-h represented MRC5 control, MRC5+BtxA, MRC5+LF, MRC5+BtxA/LF, A545 control, A545+BtxA, A545+LF, A545+BtxA/LF treatments. Q1 to Q4 illustrated necrotic, late apoptotic, and live and early apoptotic cells respectively.

Determine the Inhibition of MRC5 and A545 cell Proliferation Mediated by Flowcytometry To explore the apoptotic cells used flowcytometry. Following 24 h of treatment with particular time and IC_{50} concentration, the cells were treated with annexin V-FITC then apoptotic cells were counted. Flowcytometry absolutely corroborate the microscopy result. Treated MRC5 and A545 cells with BtxA and LF (together) marked increase in apoptosis. Whiles treated cells with separate toxin had little effect on cell lines. Although both cell lines, early and late-stage apoptosis were detected. On the other hand, it was apparent that late-stage apoptosis exhibited greater.

DISCUSSION

Lung carcinoma stands as the primary culprit behind cancer-related fatalities in all around the world. The number of people caused by lung cancer annually nearly rivals the combined death toll of prostate, breast, and colon cancer [16]. The scarcity of studies conducted on lung cancer cells is also noteworthy, underscoring the significance of creating preclinical [17]. Toxins that induce bioterrorism, such as Botulinum toxin, Anthrax toxin can enhance the destruction of cancer cells by enabling the opening of tumor vessels [18]. The priori survey demonstrated, botulinum toxin decreases the apoptotic volume due to massive efflux of K+ and Cl- through their specific channels [19]. Previous investigation has provided Anthrax toxin is comprised of three distinct proteins. Among these proteins, one prominent component, referred to as protective antigen, demonstrated an affinity for two specific receptors found on neovascularization within tumors [20]. MRC5 and A549 the ideal cells for normal and cancer lung cells respectively, to study these treatments used BtxA, LF and combinational. The exert inhibition of cell growth by BtxA and LF were time and dose dependent for both cell lines. Combinational treated illustrated A549 more severe growth reduction than MRC5. According to MTT data, for MRC5 cells BtxA (0.1 ng ml-1) caused 50% ofcell death in these cells at 24 h, While 310 ng ml-1 LF caused the same cell death. As reports shed light in A549, BtxA IC_{50}% was 0.08 ng ml-1 but this inhibition concentration increased in LF (310 ng ml-1). Shebl RI. Showed the fatal dose for HCT116 and DU145 cells were 7.2 and 6.4 U/ml at 24 h [21]. Moreover, there have been various in vitro and in vivo studies which have reported a comparable pattern of growth suppression cancer cell lines, some researchers in 2020, displayed the viability of Glioblastoma U87-MG cell line treated by BtxA at doses of 0.03 ng ml-1 after 24 to 48 h, significantly decreased [22]. Based on previous study, N.Y. Kimetal, investigated anthrax lethal toxin on the viability of cell lines in the human lungs. Added 0.5 µg ml-1 LF enforce the viability of HSAEC [23]. However, the treatment time required to induce cell death in A545 cells by BtxA is higher than that of MRC5 cells. The distinct variations in responsiveness can be attributed to the inherent biological and genetic disparities among different cell types. In this particular investigation, we have presented substantial evidence that combinational treatment (BtxA&LF) effect on cell proliferation of two lung cell lines such as MRC5 and A549. This test was the first to describe the MRC5 and A545 colony ability of BtxA and LF alone and

together. In this invitro studies, combination treatment means BtxA +LF at IC_{50} concentration significantly extinguish the colony forming ability additionally, combinational compound effect in A545 cells more than MRC5. Our result, revealed that late apoptosis and necrosis increased in A549 cells treated with BTXA 0.08 ng ml-1 and LF310 ng/ml compared the MRC5 cells. The final phase of apoptosis is distinguished by the division of DNA, which commences subsequent to the conclusion of nuclear condensation. This process is facilitated by both caspase dependent and independent pathways. Notably, during the latter stage, substantial alterations in morphology and phenotype [24]. Necrotic cell death, a type of cell demise, can be identified by the swift deterioration of the integrity of the plasma membrane. This particular mode of cell death can arise from the activation of various signaling pathways, with the most extensively studied pathway being reliant on the functionality of the protein kinase RIP3 [25]. In 2009, scientists showed cell proliferation of PC-3 and LNCaP cell lines compared to the presence and absence of BtxA, this compound specially reduced LNCaP colony formation ability and increased apoptosis [26]. A recent study by Chen Cai,etal described that in gastric cancer, Anthrax toxin induced proliferation and induced suppressed apoptosis. It seems extrinsic and intrinsic pathway activated by PI3K/AKT/mTOR [27]. The current investigation was undertaken to examine the consequences of combinational treatments on lung and normal cell lines. findings undeniably demonstrate that BtxA and LF induced apoptotic in MRC5 less than A545. This discovery exhibited

a resemblance to a separate investigation that demonstrated the restraint of LNCaP human prostate cancer cells' proliferation in vitro and in vivo by BtxA [26]. In another study reported that BTX-A has the effect of diminishing the growth and proliferation of cellular activity in both LNCaP and PC-3 prostate cell lines, as well as in cells that have been altered by the overexpression of phospholipase C-Gamma 1. [28]. Zhu H,etal showed the assessment of the effectiveness of captopril and BTX-A in treating cancer is highly significant compared to the pursuit of novel medications. DU145 is a type of prostate cancer cell line called androgen independent, which is distinguished by the existence of two mutations in the p53 gene [29]. Restoring the apoptotic potential of p53 through the action of BTX-A may contribute to drug resistance in cancer treatment by inducing self-destruction of cancer cells while minimizing impact on adjacent healthy tissues [21]. Apoptosis divided into intrinsic and extrinsic pathway. BtxA induced Caspase 9 (intrinsic pathway) and cause apoptosis [30]. Caspase-9 plays an essential role in facilitating alterations in mitochondrial morphology and the generation of reactive oxygen species (ROS) through its cleavage activity. Following activation by caspase-9, caspase-3 impedes ROS production and is indispensable for the effective progression of apoptosis. Furthermore, the involvement of effector caspase-7 is crucial for the detachment of apoptotic cells [31]. LF cause cell death and suppressing angiogenesis in human cells by inactivating the MAPK

signaling pathway. The phenomenon of gradual cytotoxicity, observed the complete fragmentation of MAP kinase, kinases within a span of 5 h following the introduction of the toxin, and a seemingly abundant supply of the toxin. The gradual progression of cell death is likely a consequence of the inherent diversity present within primary endothelial cultures, including the existence of various subtypes of cells [32]. LF induced apoptosis cells by inhibition of MAPK and AKT signaling pathways [33]. The induction of apoptosis in human melanoma cells can be achieved by inhibiting the MAPK signaling pathway used eanthrax lethal toxin. Normal melanocytes do not undergo apoptosis when exposed to MAPK inhibition; instead, they arrest in the G1 phase of the cell cycle. Notably, when human melanoma xenograft tumors are treated with LF in vivo, significant or complete regression of the tumors is observed. This suggests that targeting the MAPK signaling pathway could serve as a beneficial approach for the treatment [34, 35]. The ability of BtxA and LF to cause apoptosis was also investigated. Taken together, the impacts being examined in this study reported that A545 cells are more sensitive to growth inhibition by BtxA and LF. The diverse between the responses of the two cell lines could be related to normal and cancer cell lines. Additional research is required to identify the underlying molecular mechanism of the reparative process that is affected by BtxA and LF.

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