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ER chaperone *GRP78* regulates autophagy by modulation of p53 localization

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TABLE OF CONTENTS

1. ABSTRACT

GRP78 (glucose regulated protein 78) is a major Endoplasmic Reticulum (ER) chaperone that plays a pivotal role in normal ER functioning. Its increased expression also works as an indicator of ER stress. Its anti-apoptotic and pro-autophagic activity makes it an intriguing target to study the relationship between GRP78 and p53, which is also a major regulator of apoptosis and autophagy. Here, we studied the effect of Rotenone and Parathion on human lung cancer cells (A549 cell line) specifically with respect to ER stress and its association with different cell death pathways. In our study, we observed that both compounds increase reactive oxygen species (ROS) generation, down regulate mitochondrial membrane potential (MMP) and affect DNA integrity. Our results indicate that Parathion causes ER stress, up regulates the expression of *GRP78*, leads to nuclear localization of p53 and induces autophagy while Rotenone down regulates *GRP78*, causes cytoplasmic localization of p53 and inhibits autophagy. Therefore, it may be concluded that *GRP78* affects p53 localization which in turn regulates autophagy.

2. INTRODUCTION

Endoplasmic Reticulum (ER) is an organelle where folding and modification of newly synthesized proteins takes place by a machinery of foldases and molecular chaperones in the ER lumen. A well-organized system termed “quality control” manages the rate of protein synthesis, folding, and trafficking and confirms that only correctly folded proteins leave the ER. Misfolded proteins are either maintained within the ER or subjected to degradation by the proteasome-dependent ER-associated protein degradation (ERAD) pathway or by autophagy (1). It is a highly dynamic organelle which responds to environmental stress and developmental signals through a sequence of signalling cascades known as the unfolded protein response (UPR) (2).

As a stress response pathway, the UPR regulates the shape, size (3), and components of the ER to respond to fluctuating demands on protein folding in coordination with diverse physiological and pathological conditions. Recent work on the relationship between ER stress signaling pathways and metabolic stress, oxidative stress, and inflammatory response signalling pathways provide new ideas into the diverse cellular processes that are regulated by the UPR (4). Unfolded protein response pathway activates in response to the accumulation of unfolded or misfolded proteins within the ER with the help of three signal pathways: protein kinase RNA-like ER kinase (PERK)/eukaryotic translation initiation factor alpha (eIF2a), serine/threonine kinases inositol-requiring enzyme-1 (IRE1), and activating transcription factor 6 (ATF6) (5). Upon ER stress UPR may provide either survival or death signals depending on the extent of ER stress. Each of above UPR sensor binds to major ER chaperone, glucose regulated protein 78 (*GRP78*), also known as BiP (6) to prevent further accumulation of unfolded protein, thus promoting cell survival (7). UPR activation in response to chronic or severe ER stress leads to apoptotic cell death. A large amount of data support that PERK–eIF2α–ATF4 signalling is a primary determinant for apoptosis (8). Autophagy may be a mechanism induced by UPR as a cell survival pathway in stress conditions by enabling the use of intracellular resources (9). Autophagy or self-degradation, which is structurally and functionally conserved in all eukaryotes is a mechanism used by all living organisms to efficiently respond and adapt their growth and metabolism to different types of stresses. Through autophagy (macroautophagy), cytoplasmic components like proteins, membranes, and organelles are nonselectively encircled inside a double-membrane vesicle known as autophagosome which fuses with lysosome for degradation of toxic or damaged components and recycling of desired nutrients (10). Generally autophagy plays a survival role under stress conditions however with respect to cancer, it may play a dual role (tumor suppressive role by stabilizing cellular integrity during tumor growth and by possible contribution to cell death and may also employ oncogenic effects by helping tumor cell survival and preventing cell death, for example, upon anticancer treatment). Modulation in autophagy might be promising in anticancer therapies; though, it is a context-dependent matter that whether inhibition or activation of autophagy leads to tumor cell death (11). Research from different origins showed that autophagy function depends on several factors like step of tumor formation, tissue origin, and gene mutations existing in specific cancer type. Macintosh and Ryan. (2013) showed that some cancer types like human pancreatic cancers through constitutive Ras activation have elevated levels of autophagy which serve in their growth and survival (12). Conversely, tumors like human breast, prostate and ovarian cancers have allelic deletions of the crucial autophagy regulator BECLIN1, indicating that decreased autophagy may promote tumor development (13).

Several proteins that play important role in autophagy regulation are actually described as oncosuppressors. For example, a protein UVRAG which is mono-allelically deleted at high frequency in human colon cancers interacts with BECLIN1 to generate a class III PI3-K signaling complex, which is an initial step in autophagosome formation (14). An additional function of autophagy in inhibition of tumor progression is its ability to regulate cell death processes. It is well described that autophagy and apoptosis share some regulatory pathways which includes proteins such as BCL-2, BCL-XL, cFLIP, CASPASE-3, tBID, BAD, and PUMA (15). In many cases implementation of apoptosis even depends on autophagy, e.g., during DNA damage, macroautophagy regulator DRAM-1 is required for p53 mediated apoptosis (16).

It has been commonly accepted that apoptosis and autophagy are systematically linked (17). Several reports prove that one of the principal pro-apoptotic transcription factor p53 can stimulate autophagy (18). Nuclear p53 is known for its autophagy inducing (19) activities whereas cytoplasmic p53 exerts autophagy inhibition (20). The research of Tsademir *et al*. (2008) suggests that switching autophagy ‘on’ or ‘off’ is not only a matter of basal level versus stress level but it also depends on the nature of stimulus (21). It is also shown that not only does inhibition of basal p53 induce autophagy, but also the proteasome mediated p53 depletion appears to be a criterion for autophagy induction in response to physiologically significant stress stimuli, like starvation or ER stress. Thus, it is possible that the inhibition or induction of autophagy by p53 is in part determined by the nature of the stress stimulus, with divergent actions of oncogenic and genotoxic stress versus starvation and ER stress (22). Increase in level of p53 gene expression during ER stress is well described, but it is unclear whether and how p53 gene expression is regulated under ER stress conditions (23).

The present study aims to investigate the role of endoplasmic reticulum stress on p53 localization and its possible effect on autophagy induction after exposure to different types of stresses. For this, we used two different types of environmental stressors namely Rotenone and Parathion and exposed the lung cancer cell lines A549 to study the possible mechanism followed by these stressors in regulation of autophagy. This study may help in understanding the mechanism responsible for anti-apoptotic and autophagy inducing activities of GRP78 which may give new targets for anticancer therapy to induce apoptosis and inhibit/induce autophagy to increase the efficacy of chemotherapeutic drugs.

3. MATERIALS AND METHODS

3.1. Cell culture and maintenance

A549 cells were grown in Dulbecco's modified eagle's medium (DMEM):F12 supplemented with 10 % fetal bovine serum (FBS), 0.2. % sodium bicarbonate, and antibiotic/antimycotic solution (1009, 1 ml/100 ml of medium, Invitrogen, Life technologies, USA). The cells were maintained in 5 % CO2, 95 % atmosphere under high humidity at 37°C.

3.2. Compounds/Drugs used

Rotenone was purchased from MP Biosciences, and dissolved in di-methylsulfoxide (DMSO) at 1mM in stock and Parathion was purchased from Sigma-Aldrich and stored at -20°C. The final concentration of DMSO was 0.1.%. The compounds were diluted in the appropriate medium immediately before use.

3.3. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay to assess the effect of compounds on cell viability

Cell viability was assessed by the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method (24). It is a colorimetric assay of the action of cellular enzymes that reduce the tetrazolium dye, MTT, into insoluble formazan, giving a purple colour. Briefly, cells were seeded in 96-well plates at a density of $1x10^4$ cells/well in 100 µl medium, and incubated in a 37°C humidified incubator for attachment. After overnight incubation, the cells were treated with the different concentrations of Rotenone (2.0. µM to 12 µM) and Parathion (1.0. µM to 5 µM). After the 24 h of treatment, the medium was removed and MTT (20 µl of a 5 mg/ml solution) was added to each well, after that plates were incubated at 37°C for 4 hours to allow the formation of purple formazan crystals. Then, 100 µl of the dimethyl sulfoxide (DMSO) was added into each well and were left for 30 min at room temperature for colour development. Lysates were spectro-photometrically measured for absorption at λ570 nm. Cell viability was expressed as a percentage of the value in untreated control cells.

3.4. 2', 7'-diclorodihydrofluorescein di-acetate (DCFH-DA) to check the effect of compounds on reactive oxygen species (ROS) generation

Intracellular ROS generation in the presence of both stressors was estimated using 2', 7'-diclorodihydrofluorescein di-acetate (DCFH-DA) dye by calculating the conversion of non-fluorescent DCFHDA to fluorescent dichlorofluorescein (DCF). Briefly, following the exposure of Rotenone and Parathion for 24 h, cells were washed with PBS (phosphate buffered saline) and incubated for 30 min in DCFH-DA (10 µM) containing complete culture medium in dark at 37°C. Then, the cells were analyzed for intracellular fluorescence using fluorescence microscope. Pictures were captured using a Zeiss Axioplan 2 fluorescence microscope under 20X objective at 485 nm excitation and 535 nm emission wavelength. Fluorescence strength of stained cells was quantified by Image J software.

3.5. Rhodamine staining to check the effect of compounds on mitochondrial membrane potential (MMP)

MMP was measured using the procedure described by Zhang *et al*. (25). In brief, after 24h exposure of compounds, the untreated control and treated cells were washed twice with PBS. Then, the cells were stained with 10 µg/ml of Rhodamine-123 fluorescent dye for 1 h at 37°C in dark. After washing with PBS fluorescence intensity of Rhodamine-123 was calculated by fluorescence microscope (Zeiss Axioplan 2) at 485 nm excitation and 535 nm emission wavelength under 20 X objectives. Fluorescence intensity of stained cells was quantified by Image J software.

3.6. 4, 6-diamidine-2-phenylindole (DAPI) staining to check the effect of compounds on DNA integrity

DAPI staining was performed according to the protocol as described early with minor modifications (26). Briefly cells were cultured in a 24-well tissue culture grade plate for 24h. After 24h incubation, Rotenone and Parathion treated cells were washed in PBS, fixed with 4% PFA for 15 min and were treated with 0.2.% triton X-100 in PBS for 15 min at room temperature. After washing with PBS cells were stained with DAPI (2 µg/ml) and incubated in dark for 30 min. The cells were then examined and photographed using a Zeiss Axioplan 2 fluorescence microscope at 20X objective. Imaging of stained cells was done by fluorescence microscope with an excitation wavelength of 352 nm and an emission wavelength of 460 nm and fluorescence intensity of stained cells was quantified by Image J software.

3.7. Reverse transcriptase-polymerase chain reaction (RT-PCR) to check the effect of compounds on expression of marker genes

The relative expression levels of *BECLIN1*, *ATG5*, *LC3*, *P53*, *BCL2* and *GRP78* in the presence of

Table 1. Details of primer sequence used in the study

Gene	Primer (5'-3')	PCR Product Size (base pair)
BECLIN1	F-CAAGATCCTGGACCGTGTCA R-TGGCACTTTCTGTGGACATCA	180
ATG5	F- AGTATCAGACACGATCATGG R- TGCAAAGGCCTGACACTGGT	530
LC3	F- AAAGCTGTGGATGATCCACG R- AGCAGGTGACAGGAACTCCT	480
GRP78	F- GCTCGACTCGAATTCCAAAG R- TTTGTCAGGGGTCTTTCACC	380
p53	F-GGGTGGTTGGGAGTAGAT R-CGGCATTTTGAGTGTTAGA	460
BCL2	F-AGGAAGTGAACATTTCGGTGAC R-GCTCAGTTCCAGGACCAGGC	266
β-ACTIN	F-AGGAAGTGAACATTTCGGTGAC R-GCTCAGTTCCAGGACCAGGC	500

compounds were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Briefly, cells were seeded in six -well plates at a density of 30-40% in 2 ml medium, and incubated overnight in a 37°C humidified incubator for attachment. After 24h of treatment, cells were harvested with 0.2.5% trypsin-EDTA for RNA extraction. Total RNA was extracted from untreated and treated cells using total RNA extraction kit (Himedia, Cat-MB602) according to manufacturer's instructions. Total RNA was reverse transcribed using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, cat- K1622).

Primer sequences used are shown in Table 1. cDNA was amplified with an initial denaturation of 3 minutes at 94°C, followed by 35 cycles at 94°C for 30 seconds, annealing (55°C for *BECLIN1* and *GRP78*, 60°C for *ATG5* and *BCL2*, 52°C for *LC3*, 58°C for *P53*, and 56°C for *β-ACTIN*) for 30 seconds, and 72°C for 45 seconds, followed by one last step of extension at 72°C for 10 minutes. The expressions of genes were normalized using *β-ACTIN* as an internal control. PCR products were separated in a 1% agarose gel and visualized by ethidium bromide staining.

3.8. Immunofluorescence microscopy to check the effect of compounds on *p53* localization

A549 cells were grown on coverslips and incubated in 10% FBS supplemented DMEM-F12 for 24h. Cells were treated with Rotenone and Parathion in the same medium. For immunofluorescence microscopy of p53, cells were fixed with 4% PFA, permeabilized in 0.5.% Triton X100 in PBS and blocked with 2% BSA (bovine serum albumin). Cells were probed overnight for p53 using anti-p53 primary antibody (1:150 mouse monoclonal antibody, Santa Cruz Biotechnology, sc-126) at 4°C, washed thrice with PBS and incubated with corresponding Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody (1:750 goat anti-mouse, Thermo Fisher Scientific, A11001), for 1h at RT. Images were acquired using a Zeiss Axioplan 2 fluorescence microscope under 20X objective at 485 nm excitation and 535 nm emission wavelength.

3.9. Statistical analysis

Statistical analysis was done by *t* test and one-way ANOVA through Graph Pad prism 5. Data were presented as mean ± S.D of at least triplicate determinations. *p < 0.05, ** p < 0.01, ***p < 0.001.

4. RESULTS

4.1. Rotenone induces cell death whereas Parathion mediates cell proliferation in A549 cells

To examine the effect of compounds on cell viability, A549 cells were treated with different concentration of Rotenone (2μM to10μM) and Parathion (1μM to5μM) for 24h and the viabilities of these cells were determined using the MTT assay. In our study we found that Rotenone depicted a dose-dependent cytotoxic response in A549 cells (Fig1 A). Dose-dependent cytotoxic response of Rotenone is corroborated by various studies. Siddiqui *et al* (2013) reported dose dependent cytotoxic activity of Rotenone in HepG2 cells (27). Whereas in Parathion treated cells we found an increase in cell proliferation (Fig1 B). Increase in cell proliferation after Parathion treatment is also reported by GLORIA *et al (2007)* where they found increase in MCF-10F cell proliferation after exposure of Parathion (28). To examine the mechanism of cytotoxicity or cell proliferation followed by these compounds we used sublethal dose of Rotenone i.e. 2 μM and 1μM of Parathion where cell proliferation was initiated, in rest of experiments.

4.2. Rotenone and Parathion treatment leads to increase in ROS levels in A549 cells

To explore the role of Rotenone and Parathion on generation of oxidative stress, we examined the alteration in levels of reactive oxygen species (ROS) in A549 cells after 24h of treatment. As shown in fig 2 Rotenone and Parathion treated cells showed significant increase in level of ROS but among these two compounds, Rotenone treated cells showed comparatively more increase in levels of ROS as compared to untreated cells. We quantified the images for fluorescence intensity of ROS generation using Image J software and the increase being 4.8 ($p < 0.001$) and 2.6 fold ($p < 0.05$) in Rotenone and Parathion treated cells respectively as compared to untreated control.

It is well described that ROS contributes as one of the major signalling molecule in induction of apoptosis (29) and autophagy (30) in cells. In our study we found that Rotenone induced ROS may signal

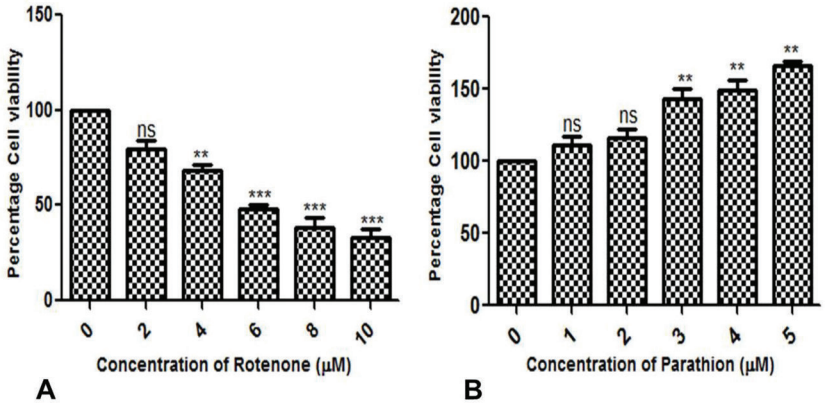

Figure 1. Rotenone and Parathion affects the viability of A549 cell line. Cells viabilities were determined by MTT assay. Cells (1×10^4 cells/well/ 96-well plates) were plated in DMEM F12 medium + 10% fetal bovine serum (FBS) with different concentrations of both compounds. (A) Rotenone inhibited the proliferation of A549 cells in a dose-dependent manner, whereas (B) Parathion treated cells showed cell proliferation activity. Nonsignificant (ns), *p < 0.05, ** p < 0.01, *** p< 0.001 versus untreated control.

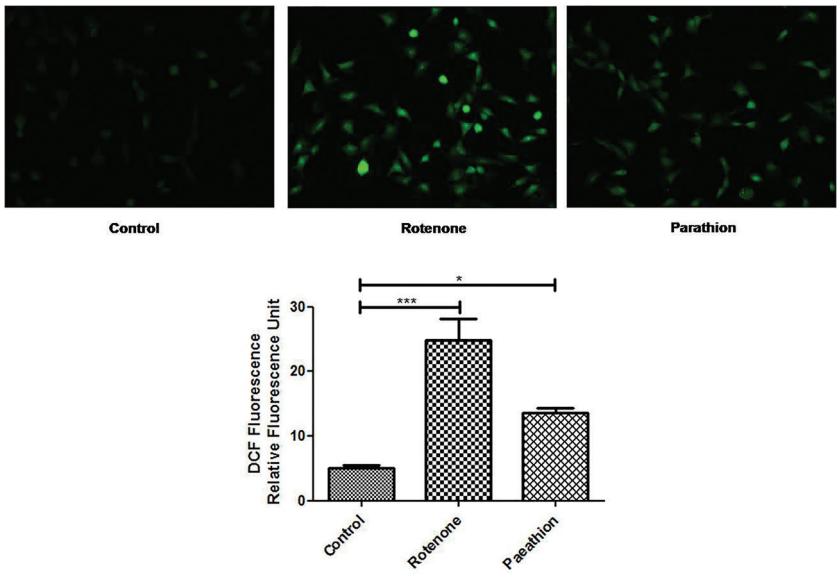

Figure 2. Change in level of reactive oxygen species was determined by H2-DCFDA staining. After 24h of treatment with Rotenone and Parathion the cells were stained with H2-DCFDA and the relative intensity of DCF was employed to evaluate the ROS level. Both compounds shows increase level of ROS in comparison to untreated control but Rotenone treated cells shows much more increase in ROS than Parathion treated cells.*p < 0.05, ***p < 0.001.

for induction of apoptosis which works as a cell killing mechanism whereas ROS in response to Parathion may induce autophagy as a cell survival mechanism. In a specific manner, under nutrient starvation conditions, induction of autophagy requires the production of H2O2 that oxidizes ATG4 leading to an increased formation of LC3 associated autophagosomes (30). Whereas in an indirect way, ROS can regulate autophagy through AMP-activated protein kinase (AMPK) which leads to the inhibition of mTORC1 and induces autophagy (31). ROS can also disturb autophagy by affecting the ubiquitin process catalysed by ATG3, ATG7 and ATG10 like proteins. These proteins use cysteine residues in their catalytic sites and ROS may cause oxidative modifications in the thiol group of these cysteine residues (32) and affect the progression of autophagosomes development catalysed by these proteins (33).

4.3. Rotenone and Parathion treatment causes loss of mitochondrial membrane potential (MMP) in A549 cells

As principal sites of ROS production, mitochondria are the organelles that are able to turn on and tune both apoptosis (34) and autophagy (35).

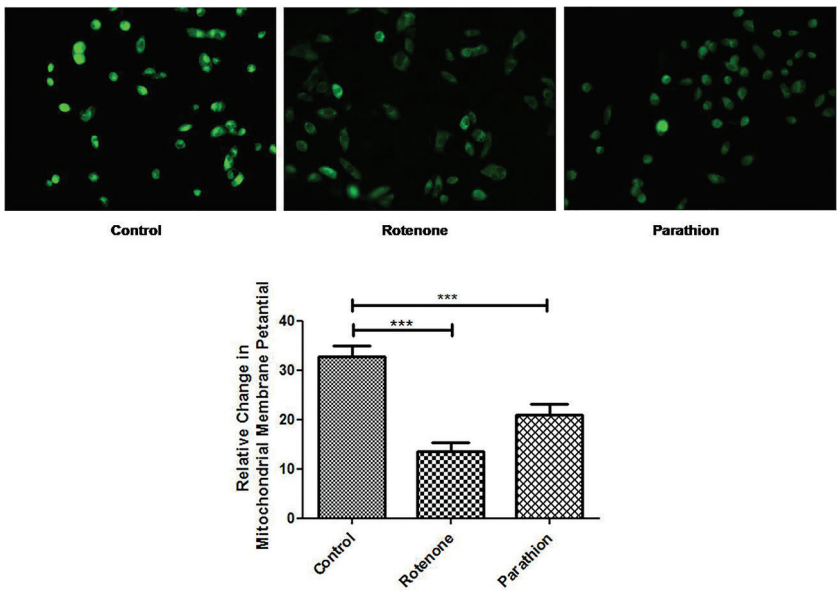

Figure 3. Change in level of mitochondrial membrane potential in A549 cells was measured using Rhodamine-123 staining. The cells were stained with Rhodamine-123 after 24h of treatment with Rotenone and Parathion and the relative fluorescence intensity was used to evaluate the change in MMP. We found that both compounds decrease the MMP in comparison to untreated control. ***p < 0.001.

In our study we found that both compounds altered mitochondrial membrane potential which is found to be down regulated after 24h of treatment in comparison to untreated control (fig 3). We quantified the images for fluorescence intensity of MMP using Image J software as a result Rotenone and Parathion treated cells showed 2.4 ($p < 0.001$) fold and 1.5 fold ($p < 0.001$) reductions respectively in fluorescence intensity of MMP as compared to untreated control. It has been shown that decrease in mitochondrial membrane potential results in an rise in ROS production (35). It has been shown that mitochondrial membrane depolarization induces autophagy under nutrient deprivation conditions in hepatocytes, and also shows that the depolarized mitochondria co-localize with autophagosomes (36).

Autophagy dependent mitochondrial turnover declines with age and is found to be dysfunctional in diseases like neurodegenerative diseases (37). The relationship between mitochondrial dysfunction, redox signalling and autophagy is not well understood. It has been found that autophagic disruption leads to further increase in oxidative stress in response to mitochondrial dysfunction and accumulation of toxic proteins (38). Mitochondria-dependent regulation of $O2^{\bullet-}$ may also play an important role in the induction of autophagy (39).

4.4. Chromatin condensation was observed after treatment with Rotenone and Parathion in A549 cells

We assessed the chromatin alteration by DAPI staining in A549 cells in the presence of both compounds as it is known that DNA damage promotes apoptosis (40) and autophagy in a p53 dependent fashion (41).

Results of DAPI staining showed more condensed chromatin after 24h of both compounds treatment in A549 cells as compared to untreated cells (Fig 4). The quantification of change in fluorescent intensity in the absence and presence of both compounds was analyzed by using Image J software indicating that Rotenone treated cells showed 2.1 fold ($p< 0.001$) increases whereas 1.4 fold ($p< 0.01$) increase in fluorescent intensity was observed in Parathion treated cells. Hence Rotenone induced more chromatin condensation as compared to Parathion. In response to DNA damage, macroautophagy regulator DRAM-1 is required for p53 mediated apoptosis (16). DNA damage response (DDR) pathway is the mechanism used by the cells in order to maintain genomic integrity in response to DNA damage by ROS and RNS (42).

Although p53 is the principle regulator of DDR (43) a number of works in recent years indicate that DNA damage event is transduced in order to activate the DDR, and concomitantly signalled to the autophagic pathway. Poly ADP-ribose polymerase 1 (PARP1) a nuclear enzyme that catalyses polyribosylation of nuclear proteins by converting NAD+ into polymers of poly ADP-ribose (PAR), and deeply participates in SSB repair, is among the proteins directly linking the DDR and autophagy (44). ROS-induced DNA damage activates PARP1 leads to NAD+ consumption and ATP depletion and results in the activation of autophagy via AMPK pathway (44).

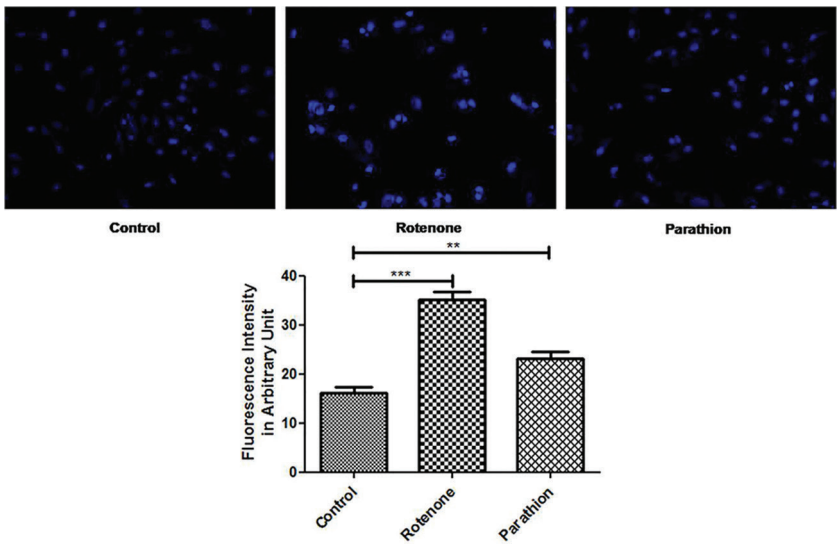

Figure 4. Effect of Rotenone and Parathion on chromatin condensation in A549 cells was explored by DAPI staining. The effect of 24h of exposure of Rotenone and Parathion on chromatin condensation in A549 cells was measured using change in the relative fluorescence intensity after DAPI staining. We found that both compound damage DNA in comparison to untreated cells whereas damage caused by Rotenone is much more than Parathion. ***p < 0.001, **p < 0.01.

Another DNA repair protein linking the DDR to autophagy is ATM, a DNA damage sensor coordinating the cell cycle with damage response checkpoints and DNA repair to safeguard the integrity of the DNA (45). It has been demonstrated that under ROS-induced cellular damage, cytosolic ATM, using LKB1/AMPK pathway, can activate TSC2 tumour suppressor to inhibit mTORC1 and induce autophagy (46).

4.5. *GRP78* affects the expression of autophagic marker genes after treatment with Rotenone and Parathion in A549 cells

Endoplasmic Reticulum (ER) is an organelle where about 30% of the total newly translated cellular proteins are modified and folded into their mature forms for membrane incorporation and secretion (47). ER chaperones play vital roles in the normal ER functioning (48). One of the best studied ER chaperones is the78-kDa glucose-regulated protein (GRP78), which is also referred to as BiP whose increased expression may functions as a sign of generation of ER stress in response to different stress conditions (49).

In our study we found up-regulation in mRNA expression of *GRP78* after treatment with Parathion while Rotenone treated cells shown down-regulation in *GRP78* indicating ER stress generation in A549 cells after exposure of Parathion (fig 5 A). Quantification of mRNA level expression of gene in the presence of both compounds was done by Image J software indicating that the mRNA levels of *GRP78* was found to be 0.6 (*p < 0.05) fold decrease and 1.5 (*p < 0.05) fold increased after treatment with Rotenone and Parathion respectively as compared to untreated control.

GRP78 is known to induce autophagy (50) and inhibit apoptosis (51). Our results are in direct correlation with the autophagic inducing activity of *GRP78* as we observed an up-regulation in expression of autophagic marker genes *LC3 (*1.5 fold) (p < 0.05), *ATG5 (*1.2 fold) (p < 0.05) and *BECLIN1 (*1.8 fold) (p < 0.05) (fig 5 B) indicating induction of autophagy in A549 cells after Parathion treatment which also causes increase in expression of *GRP78*. We also found cell proliferating activity of Parathion in A549 cells indicating prosurvival and antiapoptotic role of autophagy in response to ER stress. Whereas Rotenone treated cells shows decreased *GRP78*, (Fig 5 A) down regulation in expression of autophagic marker genes, (Fig 5 B) up-regulation in expression of proapoptotic *P53 (*2.5fold) (p < 0.01), down-regulation in anti-apoptotic *BCL2 (*0.3 fold) (p < 0.01) (Fig 5 C) and dose dependent cell death (fig 1) indicating induction of apoptosis.

We also found increase in expression of *P53 (*2.0 fold) (p < 0.01) and decrease in *BCL2 (*0.7 fold) (p < 0.05) in response to Parathion as compared to untreated control but did not observe cell death indicating generation of resistance against apoptosis by increased expression of *GRP78* and subsequent effects on autophagy regulation. Ogata *et al. (2006)* have shown that ER stress promotes cell survival by inducing autophagy and enabling the usage of intracellular resources during starvation conditions (9). In a study by Li *et al*. (2008) revealed that GRP78 is vital for UPR activation and inducing autophagy in HeLa cancer cells. Their study has shown the blockage of autophagosome formation upon ER stress or nutrition

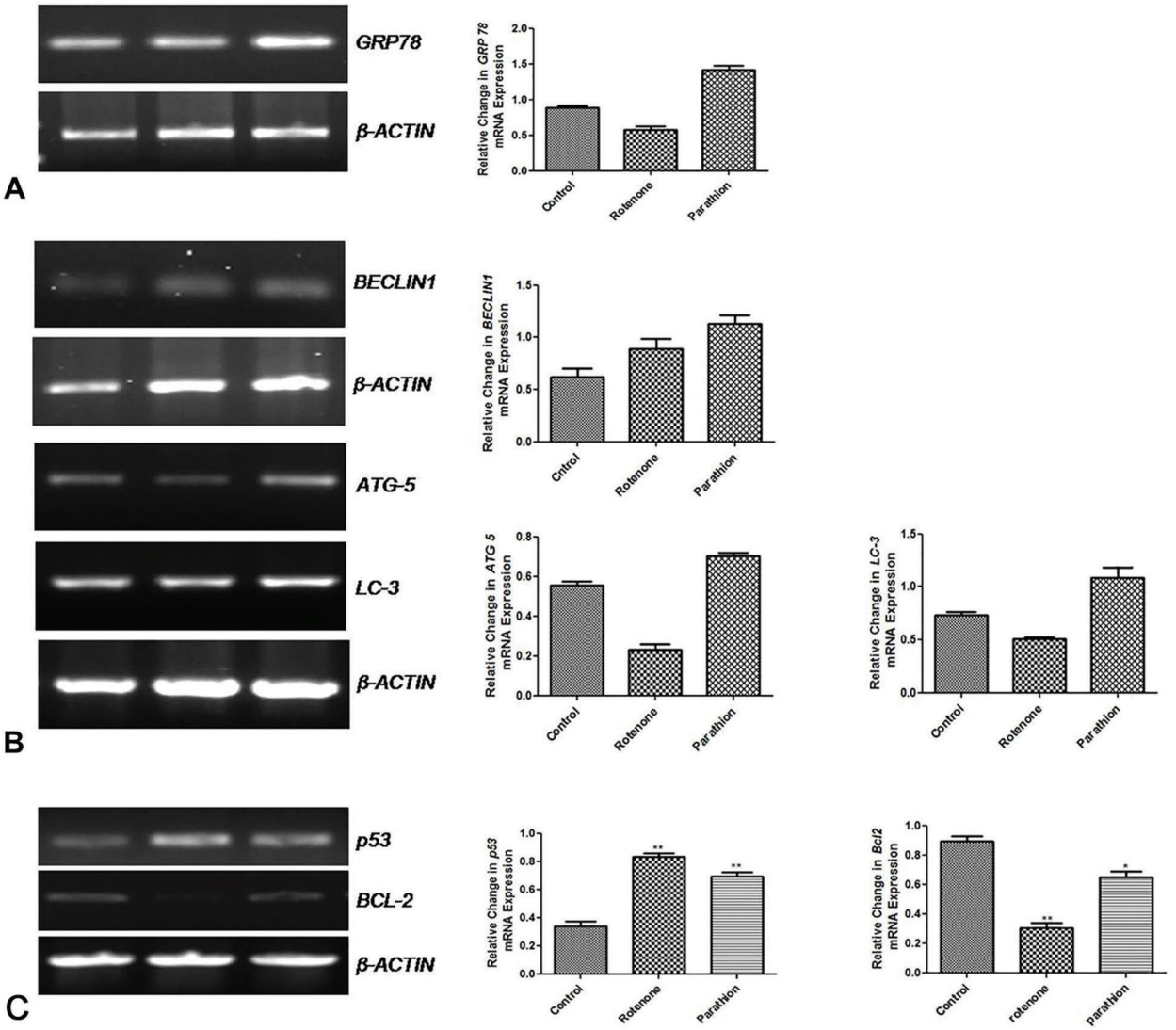

Figure 5. Change in expression of marker genes after treatment with Rotenone and Parathion was observed by RT-PCR analysis. After 24h of treatment with Rotenone and Parathion the total RNA was extracted and reverse transcribed and RT-PCR was performed with specific primers to check the effect of compounds on mRNA expression of marker genes. (A) Relative change in expression of *GRP78* was studied to check the effect of compounds on generation of Endoplasmic Reticulum stress. We found that Rotenone down regulate *GRP78* expression whereas Parathion induce the expression of *GRP78* and thought to cause endoplasmic reticulum stress. (B) Relative change in expression of autophagic marker genes. We found that Rotenone inhibit autophagy as it shown down regulation in autophagic marker genes whereas Parathion exhibit up-regulation in autophagic marker genes and shown induction of autophagy. (C) Relative change in expression of p53 and *BCL2*. We found that both compounds upregulate the expression of *p53* and BCL2. Nonsignificant (ns), * $p < 0.05$, ** $p < 0.01$ versus untreated control.

starved GRP78 silenced cells. Impaired autophagy was found to have recovered after simultaneous knockdown of GRP78 and XBP-1, that are known to control ER functions (50). However, Bennett *et al*. (2010) reported androgen receptor mediated temporary up-regulation of GRP78 in prostate cancer LNCaP cell line upon chronic serum starvation, which contributed to ER stability and the delay in onset of autophagy and cell death execution (52).

4.6. Nucleo-Cytoplasmic localization of p53 was affected in response to Rotenone and Parathion in lung cancer cell line

p53 (guardian of genome) tumor suppressor is a nuclear protein which functions as a regulator of transcription and in response to various forms of stress it mediates several biological effects like growth arrest, cell senescence, and apoptosis (53). In addition to apoptosis, p53 play a dual role in autophagy regulation

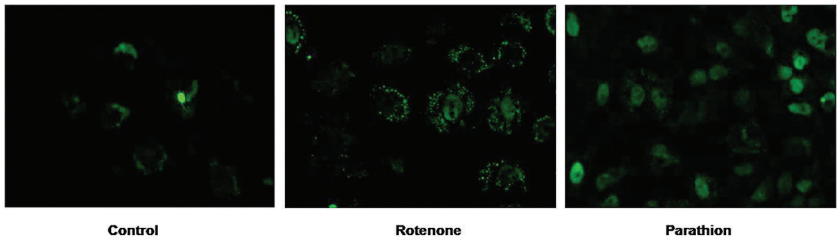

Figure 6. Nucleo-Cytoplasmic localization of p53 was determined by immunofluorescence microscopy. After 24h of treatment with Rotenone and Parathion, p53 in untreated and treated cells were localized using anti-p53 antibody. In untreated A549 cells, p53 emits a diffuse fluorescence from the entire cell, whereas it manifests with bright cytoplasmic dots after Rotenone treatment and bright nuclear localization after treatment with Parathion.

and has been found to play significant role in zebularine induced ER stress and autophagy (54). Elevation in level of p53 expression during ER stress in MEFs, MCF-7 and HCT116 cell lines has previously been reported (55).

In our study we found an up-regulation in the expression of mRNA level of *P53* after treatment with Rotenone and Parathion (fig 5 C). We also found that in addition to the expression both compounds also affect the nucleo-cytoplasmic localization of p53. We observed that Rotenone treated cells exhibit cytoplasmic localization of p53 while in case of Parathion p53 was found to be localised in the nucleus (fig 6). Dual role of p53 in autophagy regulation maybe due to differential localisation of the protein, cytoplasmic p53 inhibits autophagy (21) while nuclear p53 induces autophagy (19). Our results are in direct correlation with this as we found that Rotenone causes cytoplasmic localization of p53 and we also found down-regulation in expression of autophagic marker genes after Rotenone treatment showing inhibitory effect of cytoplasmic p53 on autophagy induction. Whereas in case of Parathion treatment nuclear localization of p53 was observed which lead to up-regulation in autophagic marker genes exhibiting pro-autophagic role of nuclear p53 in response to Parathion.

p53 also play an important role in dysregulation of ER (56). Up-regulation in expression of GRP78 functions as ER stress indicator (49), and its nuclear localization (57) and interaction with p53 has been verified (58). In our study, we found that Parathion causes up-regulation in expression of *GRP78* and exhibits nuclear localization of p53 where as in Rotenone treated cells we found down-regulation in *GRP78* and cytoplasmic localization of p53. Activation of AMPK kinase may be one of the mechanism used by nuclear p53 for induction of autophagy which subsequently activates TSC1 and TSC2 kinases, which leads to inhibition of mTOR kinase (mammalian target of rapamycin) an autophagy inhibitor. In addition to the inhibition of mTOR, p53 activation also leads to up-regulation of PTEN and TSC2 at the transcriptional level, which may cause long-term inhibition of mTOR subsequently leading to induction of autophagy.

Autophagy-suppressing function of cytoplasmic p53 was found to be separated from its cytoplasmic apoptosis inducing function, since several p53 mutants unable to interact with BCL2/Bcl-xL and to induce mitochondrial apoptosis can still inhibit autophagy. Thus, autophagic inhibitory activity of cytoplasmic p53 is drastically distinct from both its usual transcriptional function in the nucleus and its proapoptotic function in mitochondria. Moreover, the autophagy-inhibiting action of cytoplasmic p53 does not appear to associate with tumor suppressing activity, as both tumor suppression competent and deficient, or even oncogenic p53 mutants can inhibit autophagy, till they are localized into the cytoplasm (59). This maybe the reason for pro-apoptotic and anti-autophagy effects observed after Rotenone treatment in our study. Therefore, nucleo-cytoplasmic localisation of p53 regulated by GRP78 may be the molecular machinery applied by cells to survive under ER stress conditions and the effects maybe stimulus dependent.

5. CONCLUSION

The high level of connectivity between ER stress, p53, apoptosis and autophagy in tumor cells before and after chemotherapy makes this area of research attractive to those attempting to modulate these relationships in order to increase the sensitivity of cancer cells to treatment. GRP78 is well known for its antiapoptotic (51) and autophagy inducing (50) activities and increase in its expression also serves as an indicator of ER stress (60). In our study we found that Parathion up-regulates the expression of *GRP78*, increases the expression of *p53*, promotes autophagy and shows cell proliferation activity. Whereas Rotenone treated cells show down-regulation in *GRP78*, up-regulation in *p53*, inhibition of autophagy and exhibit dose dependent cell death. We also found that both compounds affect the localization of p53 as Parathion shows nuclear localization of p53 with up-regulated *GRP78* and Rotenone cause its cytoplasmic localization with down-regulated *GRP78*. So, from the results it may be concluded that ER stress affect the p53 localization and up-regulation of *GRP78* may be the reason for its nuclear localization and autophagy inducing activity by inhibiting mTOR (Autophagy inhibitor).

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