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LIMITED EXPOSURE OF CANCER CELL LINE HEPG2 TO MITOCHONDRIAL RESPIRATORY COMPLEX I INHIBITOR ROTENONE ENHANCES GLUTAMINE DEPRIVED CELL DEATH

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ABSTRACT

Cancer cells display enhanced utilisation of nutrients such as D-glucose (Glu) and L-glutamine (Q) for their growth. This high dependence of cancerous cells on nutrients has been widely utilised for developing anticancer strategies in different *in vitro* and *in vivo* models. In this study, we explored use of combination of low-dose mitochondrial complex I inhibitor, rotenone and extracellular glutamine deprivation as an antitumor approach in HepG2, an *in vitro* cellular model for studying human hepatocellular cancer. We found that exposure of HepG2 cells to low-dose of rotenone or to glutamine deprivation alone for 24 h resulted in non significant and less significant reduction in cell viability respectively. However, highly significant reduction in cell proliferation was observed on their combined treatment for 24 h as detected by MTT assay and morphological examination. Further investigation revealed involvement of generation of oxidative stress condition due to excess production of reactive oxygen species (ROS) and mitochondrial dysfunction as a result of mitochondrial membrane potential ($\Delta\psi_m$) loss, in inhibitory proliferation due to this combined treatment as confirmed by fluorescent probes based flow cytometric assays. This study thus gives an insight into a new combinatorial strategy to better control growth of human hepatocellular carcinoma *in vitro by* utilising their dependency on extracellular glutamine.

Keywords: Cancer, Glutamine, Mitochondrial ETC complex inhibitor, Reactive oxygen species, Mitochondrial membrane potential, HepG2

1. INTRODUCTION

There has been constant progress in cancer management and cure methods.Yet worldwide cancer incidence and mortality rates are on the rise. Cancer still remains the second leading contributor of global casualties accounting for 9.6million deaths in 2018 (WHO fact sheets). Among 36 most frequently diagnosed cancer, hepatic cancer ranks seventh with incidence rate of 4.7% and are the third leading cause of cancer death with mortality rate of 8.2% [1].

To sustain uncontrolled proliferation and survive under unfavourable conditions, cancer cells undergo metabolic reprogramming [2]. During metabolic reprogramming, cancer cells rewire their metabolism and networks related to energy production [3]. Metabolic requirements change right through cancer progression and accordingly vulnerabilities of cancer cells also vary. Enhanced nutrient uptake and macrmolecular biosynthesis are required by tumors which are in their initial stages of growth [4]. L-glutamine (Q) is one of the two main nutrient substrates together with D-glucose that fulfil the altered metabolic demands of many tumors [5]. In the human bloodstream, glutamine is the most abundant amino acid [6]. Glutamine functions as a source of reduced nitrogen and carbon. As reduced nitrogen source, catabolism of Q results into biosynthesis of nucleotides, nonessential amino acids, proteins and glucosamine 6-phosphate [7-10]. As carbon source, glutamine serves the bioenergetic and biosynthetic demands of cancer cells through production of ATP, replenishment of the intermediates of TCA cycle, production of glutathione and synthesis of lipids via reductive carboxylation [7,11-13].

Though most of the cancer cells show high rate of aerobic glycolysis which is famously known as “the Warburg effect”, some cancer cell lines are dependent on glutamine for their survival, which is referred to as “glutamine addiction” [14-16]. Human liver cancer cell line-HepG2 has been reported to be addicted to glutamine [17]. Mitochondrial oxidative phosphory-

lation (OXHPOS) has also been found to be upregulated in HepG2 cells [18-20]. Rotenone, a naturally occurring & lipophilic compound which is mainly found in the roots and stems of *Derris* and *Lonchocarpus* species, is a mitochondrial electron tansport chain complex I inhibitor [21]. Rotenone inhibits mitochondrial function through inhibition of OXPHOS [21-23].

A combination therapy targeting both, inhibition of glutaminolysis and OXPHOS might be deadly for cancer cells. Therefore, in this study we explored the *in vitro* effect and degree of dependence of liver cancer cell line HepG2 on one of the major nutrient substrates- Q for its proliferation and survival and utilised that finding to design a combinatorial strategy to target Q addiction by using Q deprivation and ETC complex I inhibitor for further enhancing the antitumor effects in HepG2 cells. We also determined variation in cellular phenomena like generation of cytosolic reactive oxygen species and mitochondrial membrane potential which play key roles in determining a cell's proliferation capacity. This study may extend the existing understanding related to design of effective therapeutic strategies to treat different cancer type, especially those which show Q addiction.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

HG (high-glucose), LG (low-glucose) and glutamine free DMEM (Dulbecco's modified Eagle's media), DMSO (dimethyl sulfoxide), H_2DCFDA (2′,7′-Dichlorofluorescin diacetate) and CCCP (Carbonyl cyanide 3-chlorophenylhyrazone were purchased from Sigma-Aldrich, USA. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] was purchased from SRL, India. FBS (Fetal bovine serum), trypsin and R123 (rhodamine 123) were procured from Thermo Fisher Scientific.

2.2. Cell culture and treatments

HepG2 cell line was procured from, National Centre for Cell Science, Pune, India. Cells were maintained in HG DMEM (25.5 mM Glucose + 4mM Glutamine + 1mM Pyruvate) supplemented with 10% FBS, penicillin (100U/mL)-streptomycin (100 µg/mL) and amphotericin B (100 µg/mL) at 37°C in 5% CO_2 atmosphere in a humidified incubator. Cells were subcultured using PBS- trypsin/EDTA. After the first cycle of growth in HG DMEM containing 10% FBS, desired number of cells were grown in experimental plates in fresh HG DMEM containing 2.5% FBS and maintained overnight to adapt them to the changed serum concentrations. Reduction in FBS from 10% to 2.5% was for better evaluation of effect of glutamine deprivation and to minimise the effect of FBS, since FBS is a rich source of growth factors and nutrition. There was a non-significant variation in cell viability when HepG2 cells were cultured in HG DMEM with 2.5% FBS as compared to 10 % FBS for 24 h (data not shown).

Cells grown in 2.5% HG DMEM, after PBS washing, were treated with 20 nM rotenone in LG DMEM (containing physiological concentration of Glucose- 5.5 mM + 4mM Glutamine + 1mM Pyruvate) or glutamine depleted LG DMEM (Glucose-5.5mM+1mM Pyruvate) either alone or in combination for 24 h. Cells cultured in LG DMEM was used as control.

2.3. Cell viability assay (MTT assay)

Effect of rotenone and glutamine deprivation, alone or in combination on viability of HepG2 cells was determined by MTT assay. MTT assay was performed as previously described with slight modifications [24,25]. Briefly, 4 x 10^3 cells were seeded in 96 well plate in 2.5% FBS-HG DMEM and grown overnight. Next day, cells were treated as mentioned in "Cell culture and treatments" for 24 h. On completion of treatment hours, media were removed and 4 µL MTT (5mg/mL) was added in each well in 200 µL serum-free media (SFM) and incubated for 3 h. Then 100µl DMSO was added per well and absorbance reading was taken at 570 nm in 96-well plate reader (spectraMax M5-Molecular Devices, USA). Percentage change in cell viability was calculated using the following formula.

$$\% \text{ Cell Viability} = \frac{\text{Mean Absorbance of Treatment @570 nm}}{\text{Mean Absorbance of Control @570 nm}} \times 100$$

2.4. Morphological analysis

For study of changes in morphology of cells, approximately 5 × 10^4 HepG2 cells were grown overnight in a 6 well plate and then exposed to the treatment for 24 h, as mentioned in "Cell culture and treatments". On completion of time point, images for cell morphology were captured at 10x magnification using inverted phase contrast microscope (EA-Prime, Lmi microscopes,UK).

2.5. Evaluation for ROS generation

Effects of low-dose rotenone, a mitochondrial ETC complex I inhibitor and glutamine starvation on cytosolic reactive oxygen species generation in HepG2 cells were analysed by flow cytometry. A cationic free

radical probe H_2DCFDA which readily diffuses inside the cell, is used for measuring the extent of cytosolic ROS. H_2DCFDA is a non fluorescent dye but it becomes highly green fluorescent in its 2',7'-dichloro-fluorescein (DCF) form on its de-esterification when it interacts with intracellular ROS. This assay was performed following a protocol with some modification as previously described [26]. Briefly, $2x10^5$ cells were seeded in 6 well plate, grown overnight and treated as described in "Cell culture and treatments" for 24 h. Post treatment, cells were washed with 1x PBS kept at room temperature and then loaded with 10μM H_2DCFDA in SFM for 30 minutes in dark. After that, cells were collected using trypsin, washed with cold 1x PBS and fluorescence of 10,000 cells was acquired immediately by flow cytometer (BD FACS Verse) in triplicate for each sample in FITC (Fluorescein-5-isothiocyanate) Channel .The data were analysed using FCS Express 7 software. Cell populations gated by marker M1 and M2 represent the percentage of DCF positive cells (cells showing high DCF fluorescence thus containing high level of ROS) and DCF negative cells (cells showing low DCF fluorescence thus containing low level of ROS) respectively.

2.6. Mitochondrial membrane potential ($\Delta\psi_m$) assay

Quantification of relative changes in mitochondrial membrane potential in HepG2 cells due to treatment with limited concentration of rotenone and Q deprivation was performed by flow cytometry following a standard protocol which ultilises rhodamine 123 [26, 27]. Rhodamine 123, a cationic, lipophilic and green fluorescent fluorochrome gets selectively permeabilised and retained inside mitochondria with intact membrane potential. This dye leaks out of the cell when there is a loss of $\Delta\psi_m$. Thus measurement of rhodamine 123 fluorescence emitted by cells is correlated with the $\Delta\psi_m$. Briefly, $2x10^5$ cells were seeded in 6 well plate, grown overnight and treated as described in " Cell culture and treatments" for 24 h. Post treatment, cells were, washed with 1x PBS kept at room temperature and then loaded with 10μg/mL of Rhodamine 123 (R123) in SFM for 30 minutes in dark. After that, cells were collected using trypsin, washed with cold 1x PBS and fluorescence of 10,000 cells was acquired immediately by flow cytometer (BD FACS Verse) in triplicate for each sample in FITC Channel. The data were analyzed using FCS Express 7 software. Cell populations gated by marker M1 and M2 represent the percentage of R123 positive cells (cells showing high R123 fluorescence thus containing hyperpolarised mitochondria) and R123 negative cells (cells showing low R123 fluorescence thus containing depolarised mitochondria) respectively.

2.7. Statistical analysis

Each experiments were carried out in triplicates and independently. Changes were represented as mean ± SD. Significance of changes in means between groups was analysed by one way ANOVA combined with Tukey's post hoc test using GraphPad Prism 5 software (San Diego CA, USA). $p<0.05$ versus control (LG DMEM) was considered significant.

3. RESULTS AND DISCUSSION

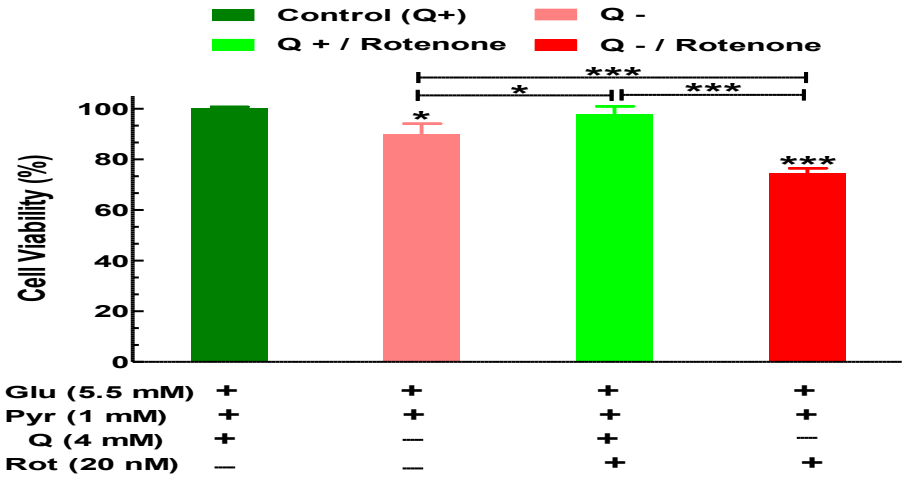
3.1. Evaluation of effect of glutamine depletion and mitochondrial ETC complex I inhibitor on cell viability in HepG2

To determine the extent of reliance of HepG2 cells on Q for growth and proliferation, firstly we conducted MTT assay for cells cultured in Q starved media, keeping the concentration of other two major nutrient substrates- glucose and pyruvate constant. MTT assay revealed that complete depletion of Q for 24 h from the growth media resulted in less significant decrease in cell viability by 9.98 ± 3.31 % compared to control ($p< 0.05$) whereas low-dose (20 nM << IC_{50}) exposure of mitochondrial electron transport chain complex I inhibitor, rotenone did not bring any significant change in HepG2 cell viability (decrease by 2.02 ± 2.12 %). In contrast, when cells were treated with the same low dose rotenone in Q deprived growth media, a highly significant reduction ($p< 0.001$) in cell viability by 25.66 ± 1.25 % was observed (Fig. 1). These results were similar to our previous study with the glycolytic human cervical cancer cell line-HeLa, where a combination treatment of diminished glucose (5.5 mM) and 1 nM rotenone for 72 h led to decreased cell viability, increased production of ROS, S-phase cycle arrest and apoptosis. [25]

3.2. Examination of morphological variations

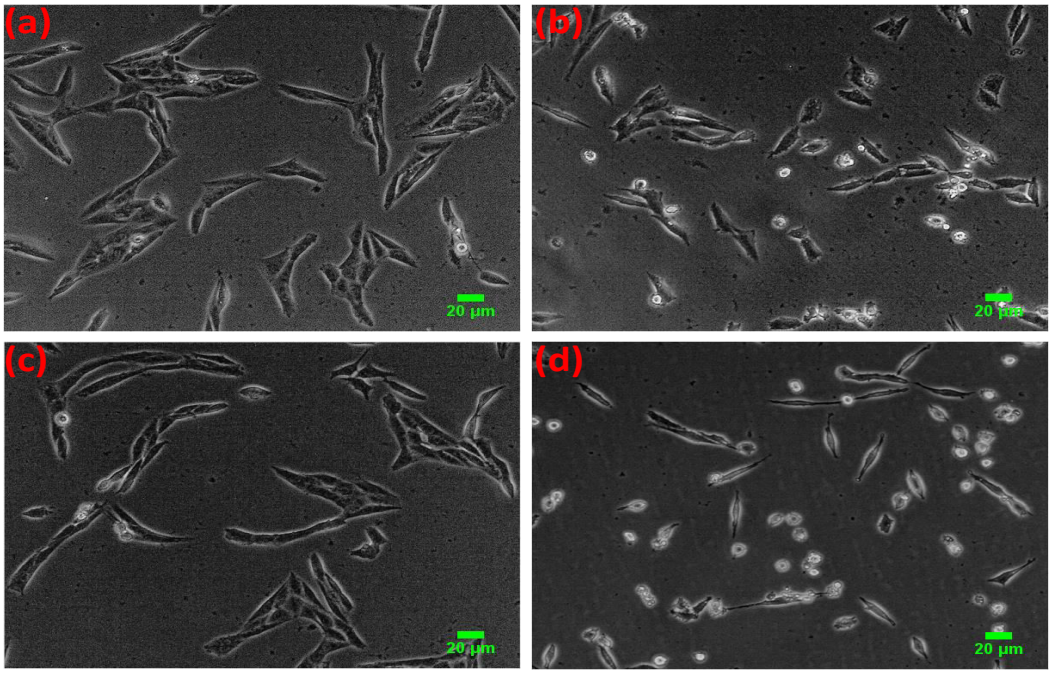
On morphological analysis, it was found that, compared to control cells which exhibited characteristic elongated morphology with tapering ends (Fig. 2a), Q deprivation for 24 h led to alteration in morphology of HepG2 cells to some extent in the form of decrease in cell size due

to cell shrinkage and presence of some round & dead cells (Fig. 2b). However, 20 nM of rotenone did not bring any change in the cell morphology and cells were similar to control (Fig. 2c). The highest reduction in growth and alterations in morphology was observed on treatment of HepG2 cells with 20 nM rotenone in Q deprived growth media, where many rounded, floating & dead cells and some highly shrunken cells were seen and there were very few cells which showed normal morphology (Fig. 2d).



Glu = D-glucose; Pyr = Sodium pyruvate; Q = L-glutamine; Rot = Rotenone. * $p<0.05$, *** $p<0.001$ vs Control after significance testing of data represented as mean ± S.D by one way ANOVA-Tukey's multiple comparison test, n=3

Fig. 1: Effect of glutamine deprivation and rotenone either alone or in combination on cell viability in HepG2 cancer cells at 24 h.

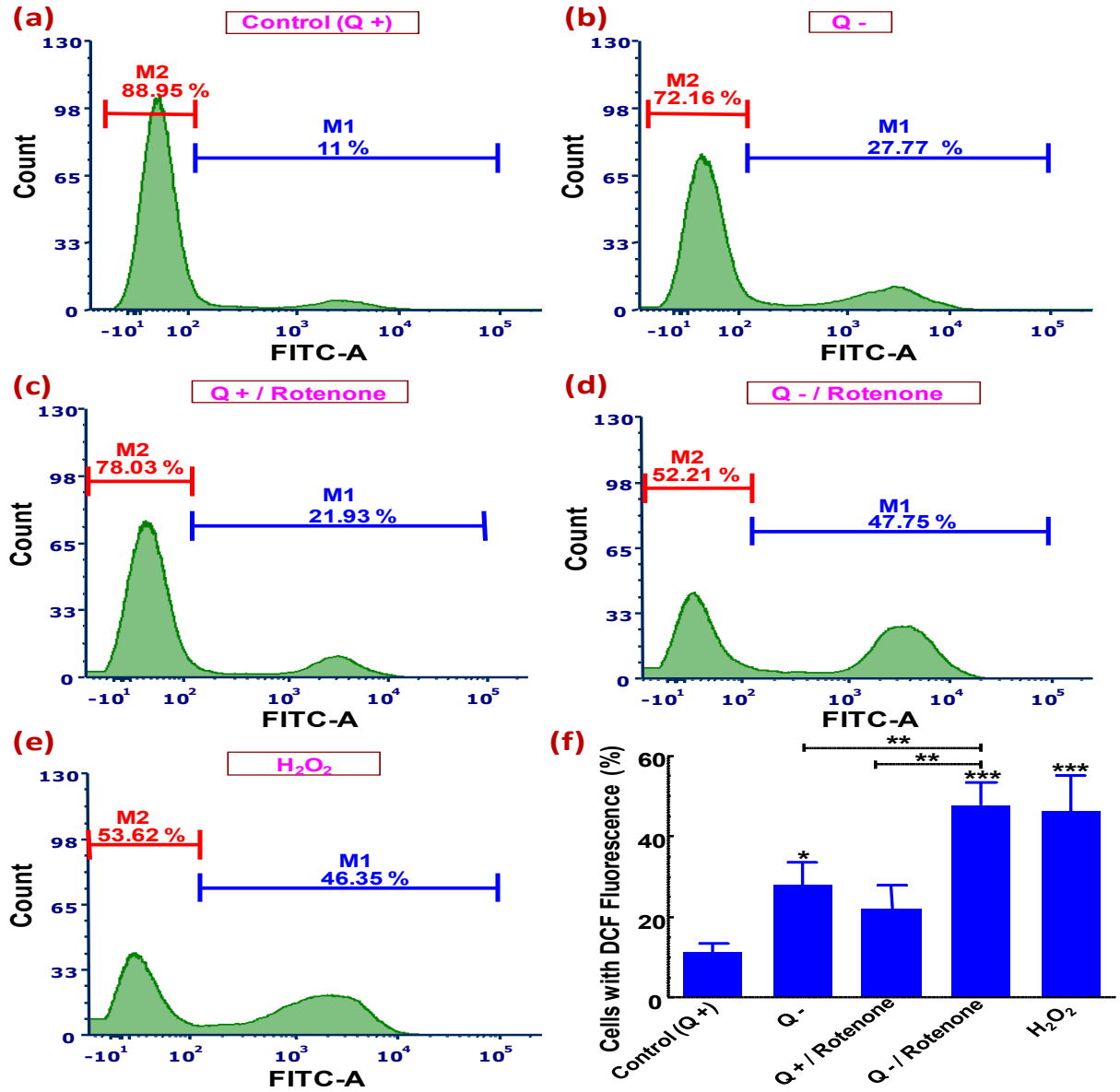


(a) Control (Q +) ; (b) Q - ; (c) Q + & Rot combination (d) Q - & Rot combination; + = Present; - = Absent; Q = L-glutamine (4 mM); Rot = Rotenone (20 nM), Scale bar = 20 µm at 10x magnification

Fig. 2: Effect of glutamine deprivation and rotenone either alone or in combination on cellular morphology in HepG2 cancer cells at 24 h.

3.3. Evaluation of cytosolic ROS generation

Nutrient deprivation and mitochondrial respiratory complex I inhibitors have been shown to be associated with excess ROS mediated cell killing in a variety of cancer cells [28-32]. So, we were also willing to know the status of cytosolic ROS after exposing HepG2 cells to rotenone and Q starvation. To determine the status of cytosolic ROS, we conducted H_2DCFDA probe based flow cytometric assay. On analysis of DCF fluorescence of each sample, it was found that control sample contained only 11% cells with high ROS (Fig. 3a), whereas Q deprivation for 24 h led to increase in percentage of cells with high ROS by 16.8 % compared to control (less significant, $p<0.05$) (Fig. 3b). Limited exposure of cells to rotenone (20nM for 24 h) did not cause any significant change in cytosolic ROS production (increase in high ROS cells by only 10.9 % compared to control) (Fig. 3c).



*(a) Control (Q+) ; (b) Q- ; (c) Q+ &Rot combination; (d) Q- &Rot combination ; (e) 0.5 mM H_2O_2 for 2 h (positive control) ; (f) Bar diagram for comparative study of changes in data shown in fig.3(a)-3(e) and significance of those changes; + = Present; - = Absent; Q = L-glutamine (4 mM); M1 = % of DCF fluorescence positive (high ROS) cells; M2 = % of DCF fluorescence negative (low ROS) cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs Control after permorming significance testing of data represented as mean ± S.D (n=3) by one way ANOVA-Tukey's multiple comparison test.*

Fig. 3: Effect of glutamine deprivation and rotenone either alone or in combination on cytosolic reactive oxygen species generation in HepG2 cells at 24 h.

Combined treatment of cells with 20 nM rotenone and Q depletion for 24 h resulted in enhanced & highly significant generation of cytosolic ROS (increase in cells containing high ROS by 36.7 % relative to control, $p<0.001$) (Fig. 3d, 3f). Cells exposed to 500 μM H_2O_2 for 2 h was used as positive control for this assay which showed increment in ROS generation similar to the treatment with 20 nM rotenone in Q deprived growth media (Fig. 3e). Percentage of cells showing high and low ROS in control and each treatment conditions together with significance level of those changes has been shown in figure 3f and Table 1.

Table 1: Percentage variation in DCF fluorescence positive and negative cell population and associated p value as an indicator of level of cytosolic ROS and oxidative stress

Sample name	% of DCF fluorescence positive cells (M1)	% of DCF fluorescence negative cells (M2)
Control (Q +)	11 ± 2.31	88.95 ± 2.31
Q -	27.77 ± 5.84*	72.16 ± 5.84*
Q + / Rotenone	21.93 ± 5.89	78.03 ± 5.89
Q - / Rotenone	47.75 ± 5.72***	52.21 ± 5.72***
H_2O_2	46.35 ± 8.72***	53.62 ± 8.70***

*$p<0.05$, *** $p<0.001$ vs Control*

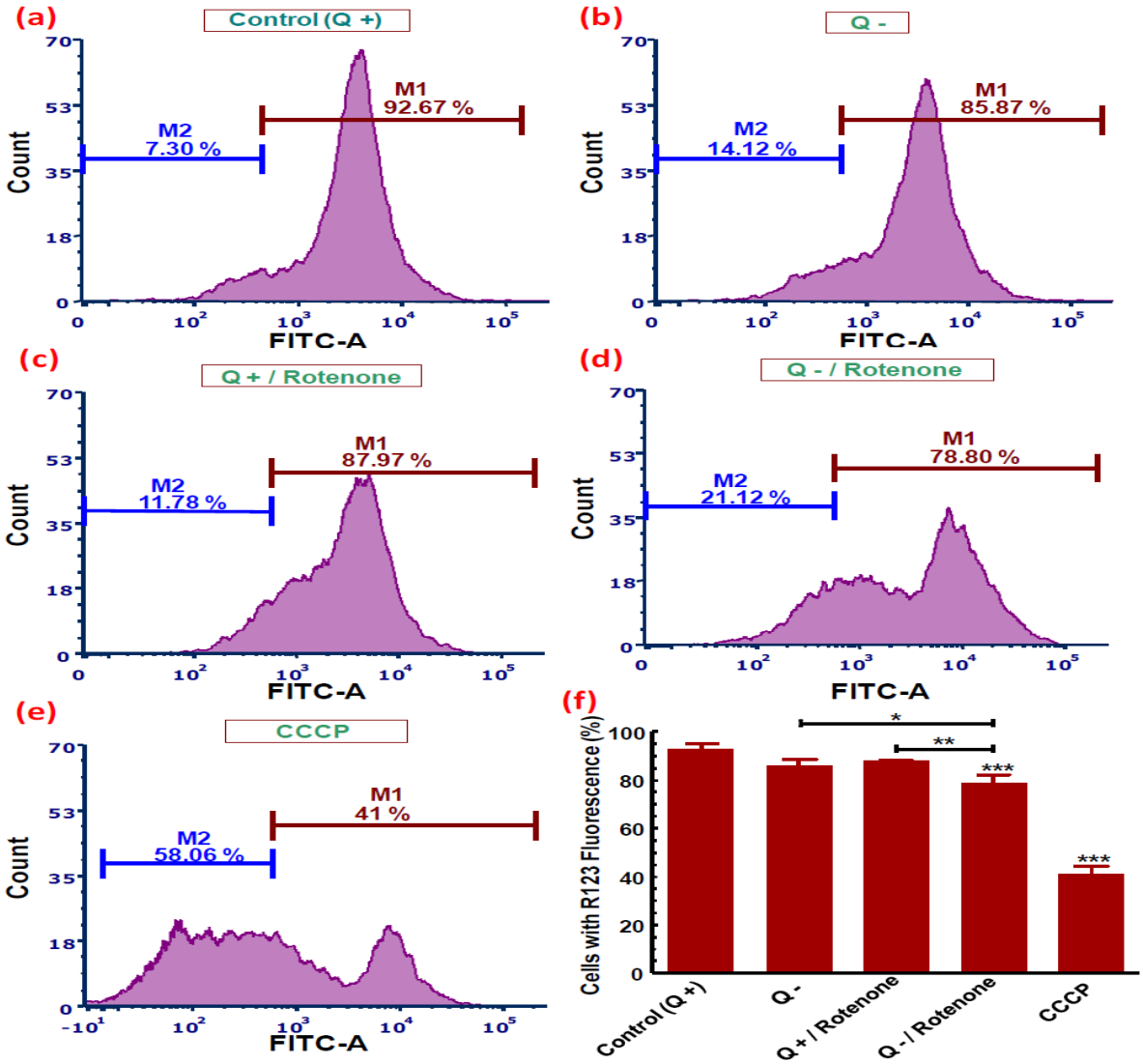
3.4. Determination of status of mitochondrial membrane potential ($\Delta\psi_m$)

Under physiological conditions, healthy mitochondria maintain a high trans-membrane potential.[33]. Excess ROS leads to mitochondrial permeability transition by targeting the permeability transition pore of mitochondrial membrane which may result into dissipation of $\Delta\psi_m$ [34-36]. So, to determine whether the high ROS obtained due to exposure of HepG2 cells to limited dose of rotenone and Q starvation, led to collapse in membrane potential across mitochondria, we conducted rhodamine123 (R123) probe based flow cytometric assay. On analysis of R123 fluorescence of each sample, it was found that control sample contained 92.7 % cells with high $\Delta\psi_m$ (Fig. 4a), whereas Q deprivation for 24 h led to non significant decrease in percentage of cells with high $\Delta\psi_m$ by 6.8 % compared to control ($p>0.05$) (Fig. 4b). This observation was partly in concurrence with Gwangwa et al. where they reported that in triple negative breast cancer cells MDA-MB-231, $\Delta\psi_m$ remain unaffected at 24 h even after a less signifiacnt ($p<0.05$) increase in ROS production [28]. Limited exposure of cells to rotenone (20 nM for 24 h) also did not cause any significant change in mitochondrial membrane potential (decrease in high $\Delta\psi$m cells by only 4.7 % compared to control) (Fig. 4c). Combined treatment of cells with 20 nM rotenone and Q depletion for 24 h resulted in enhanced & highly significant loss in $\Delta\psi_m$ (decrease in cells containing high $\Delta\psi_m$ by 13.9 % relative to control, $p<0.001$) (Fig. 4d, 4f). Cells exposed to 50 μM CCCP for 1 h was used as positive control for this assay which showed the highest and the most significant fall in $\Delta\psi$m (decrease in cells containing high $\Delta\psi_m$ by 51.7 % relative to control, $p<0.001$) (Fig. 4e). Percentage of cells showing high and low $\Delta\psi_m$ in control and each treatment groups together with significance level of those changes has shown in figure 4f and Table 2.

Table 2: Percentage variation in R123 fluorescence positive and negative cell population and associated p value as an indicator of status of $\Delta\psi$m.

Sample name	% of R123 fluorescence positive cells (M1)	% of R123 fluorescence negative cells (M2)
Control (Q +)	92.67 ± 2.15	7.30 ± 2.19
Q -	85.87 ± 2.49	14.12 ± 2.48
Q + / Rotenone	87.97 ± 0.14	11.78 ± 0.14
Q - / Rotenone	78.80 ± 3.19***	21.12 ± 3.18***
CCCP	40.99 ± 3.44***	58.06 ± 3.10***

****p<0.001 vs Control*



*(a) Control (Q+) ; (b) Q- ; (c) Q+ &Rot combination ; (d) Q- &Rot combination ; (e) 50 μM CCCP for 1 h (positive control) ; (f) Bar diagram for comparative study of changes in data shown in fig.4(a)-4(e) and significance of those changes; + = Present; - = Absent; Q = L-glutamine (4 mM); M1 = gate representing % of R123 fluorescence positive (high Δψm) cells; M2 = gate representing % of R123 fluorescence negative (low Δψm) cells; R123 = Rhodamine123; CCCP = Carbonyl cyanide 3-chlorophenylhyrazone. *p<0.05, **p<0.01, *** p<0.001 vs Control after testing the significance of variation in data represented as mean ± S.D (n=3) by one way ANOVA followed by Tukey's post-hoc test.*

Fig. 4: Determination of changes in mitochondrial membrane potential due to glutamine deprivation and rotenone either alone or in combination in HepG2 cells at 24 h.

4. CONCLUSION

Taken together, we presented a combinatorial approach for enhanced antitumor effect in hepatocellular cell line by targeting oxidative phosphorylation through utilisation of non toxic dose of mitochondrial complex I inhibitor, rotenone and inhibiting glutaminolysis through glutamine deprivation. The improved antitumor effect achieved in this study due to the above mentioned combination treatment were attributed to the excess ROS induced oxidative stress and mitochondrial depolarisation. Thus, this study may be a valuable addition to the existing knowledge of nutritional alternation based anticancer signalling in hepatocellular or other cancers.

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Conflict of interest

The authors declare that they have no conflict of interests.

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