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RESEARCH ARTICLE

PROTECTIVE EFFECT OF QUERCETIN IN LPS INDUCED MITOCHONDRIAL CELL INJURY

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ABSTRACT

We all know proper cell functioning needs energy which will be provided by Mitochondria. Sometimes some bacterial infections like Lip Opolysaccharide (LPS) affects these functions of cell by degrading mitochondrial biogenesis. LPS infection increases ROS (Reactive oxygen species) production which affects H9C2 cells contractility and functions and leads to degraded heart health. But as protective agent quercetin pre-treatment to cells provides safety from these types of infections in H9C2 cells. In our study we have focused on increased ROS production due to LPS induced inflammation and found protective effects of quercetin on pre-treated H9C2 cells.

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INTRODUCTION

Mitochondria play important role in cell survival and its damage. Circular mitochondrial genome is lacking with protective histones and is located near electron transport complexes that produces reactive oxygen species (ROS). That is why mitochondrial constituents are more susceptible to oxidation (Anderson *et al.*, 2012). This unopposed mtDNA oxidation interferes with mitochondrial transcription and OXPHOS protein synthesis not only impairs respiratory capacity but also include oxidant leakage. Mitochondrial biogenesis regulate the mitochondrial density in the cell which depends on physiological and pathogenic factors. Mitochondrial biogenesis is regulated by a complex network of factors which can enhance cellular function and survival in vivo and in vitro and promote cellular recovery from damage. Lipopolysaccharide (LPS) is known endotoxin which induces bacterial infection and generate host mediators. It is well known that Tumour necrosis factor-α (TNF-α) is pro-inflammatory cytokines which gets activated when the reactive oxygen species (ROS) increases. LPS triggers septic shock which stimulates production of TNF-α and increases ROS, RNS leading to oxidative stress, mitochondrial dysfunction and organ failure (Mittal *et al.*, 2014). Quercetin (Q) is a naturally occurring flavonoid which has a broad spectrum of bioactive effects. Quercetin is present in variety of foods (apples, peppers, red wine, dark cherries, blueberries, tomatoes, broccoli, cabbage, sprouts and leafy green veggies, including spinach and kale) which has a broad spectrum of bioactive effects. It is a potent phenolic antioxidant which can modulate mitochondrial biogenesis by modulating enzymes and transcription factors in the inflammatory signalling cascade in cells by scavenging free radicals. It can impact mitochondrial biogenesis by modulating enzymes and transcription factors in the inflammatory signalling cascade. Quercetin, a potent phenolic antioxidant can modulate mitochondrial biogenesis and reduce ROS production in the cell by scavenging free radicals and protects cell apoptosis (Ratliff *et al*., 2016).

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MATERIALS AND METHODS

Cell line

For in vitro assays H9C2 cell line was kindly provided by NCCS cell repository.

Chemicals

Ethylenediaminetetra-acetic acids(EDTA), fetal bovine serum (FBS), penicillin, Streptozotocin (STZ), 2', 7'-dichlorodihydroflurocein diacetate (DCFHDA), Phosphate buffer saline (PBS), Lipopolysaccharide (LPS), Quercetin, Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma biochemical and reagents (USA).

Methodology

Cell line culture and treatments

H9C2 cells were cultured in culture flask after poly D –lysine treatment in Dulbecco's modified eagle's medium (DMEM) containing 4mM glutamine, 1. 5 g/L sodium bicarbonate, 100 U/ml of penicillin, 100 µg/ml of streptomycin with 10% FBS in 5% CO_2 at 37^0C in a humidified incubator, H9C2 cells were subcultured into ⅓ in 3-4 days. The cultures were exposed to LPS and Quercetin after cell population reached 60-70% confluency with different concentrations at the interval of 6 and 12 h.

Emulsion preparation of Lipopolysaccharide

Lipopolysaccharide is difficult to emulsify because of its fatty nature. Therefore, little bit of vortexing and heating was required to prepare homogenous mixture in the media for further use.

Emulsion preparation of Quercetin

Quercetin is water soluble antioxidant. Thus, simple vortexing gives homogenous mixture with media and final volume is adjusted to use.

Experimental design for inflammation

Cell lines	Treatment	Concentration	Duration
H9C2 cell	H9C2+LPS	5µl of LPS	6 h
H9C2 cell	H9C2+LPS	10µl of LPS	6 h
H9C2 cell	H9C2+LPS	5µl of LPS	12 h
H9C2 cell	H9C2+LPS	10µl of LPS	12 h

Experimental design for recovery

Cell Lines	Treatment 1 (24 hr before)	Treatment 2	Concentration	Duration
H9C2 cell	H9C2+Q	QH9C2 +LPS	5µl of LPS	6 h
H9C2 cell	H9C2+Q	QH9C2 +LPS	10µl of LPS	6 h
H9C2 cell	H9C2+Q	QH9C2 +LPS	5µl of LPS	12 h
H9C2 cell	H9C2+Q	QH9C2 +LPS	10µl of LPS	12 h

Treatments

Analysis of Reactive oxygen species (ROS)

ROS production was determined by oxidation of 2', 7' - dichlorodihydroflurocein diacetate (DCFH-DA). The probe DCFH-DA enters the cell and the acetate group of DCFH-DA is cleaved by cellular esterase's trapping the non-fluorescent 2', 7'- dichlorofluorescein (DCFH) inside. Subsequent oxidation of ROS, particularly hydrogen peroxide (H_2O_2) and hydroxyl radical, yields the fluorescent product DCF. Thus, increase in DCFH oxidation to DCF is suggestive of H_2O_2 or hydroxyl generation. Briefly a stock solution of DCFH-DA (5µM) was prepared in DMSO and stored at -20^0C in the dark. H9C2 cells ($2x10^5$) were seeded into 6 well plates. The intracellular H_2O_2 was detected with inverted microscope. H9C2 cells ($2x10^5$) were plated on glass cover slips in 12 well plates. Out of 12/6 plates were pre-treated with quercetin. After 24 h cells were exposed to different concentrations ofLPS with or without quercetin. After 6 and 12 h of treatment, cells were stained for 30 min in the dark with 5µM of DCFH-DA and afterwards cells were washed with PBS and fixed with 3. 7% paraformaldehyde in PBS. The glass coverslip was mounted on a glass slide and observed using inverted microscope.

Analysis of Cell apoptosis

H_9C_2 cells were seeded in 96 well micro titre plates (5×10^3 cells per well) and left to adhere to the plates for 24 hr before being exposed to different concentrations of test material. Cells were exposed to different concentrations of LPS and quercetin in CO_2 incubator for required time intervals (6 & 12 h). Thereafter, the medium was removed and DAPI (300nM) was added to the cells by dissolving it in solubilizing reagent DMSO for 1-5 mins and afterwards the stain was removed by washing the cells with PBS for 3 times and cells was calculated with the help of haemocytometer and observed with inverted microscope.

RESULT AND DISCUSSION

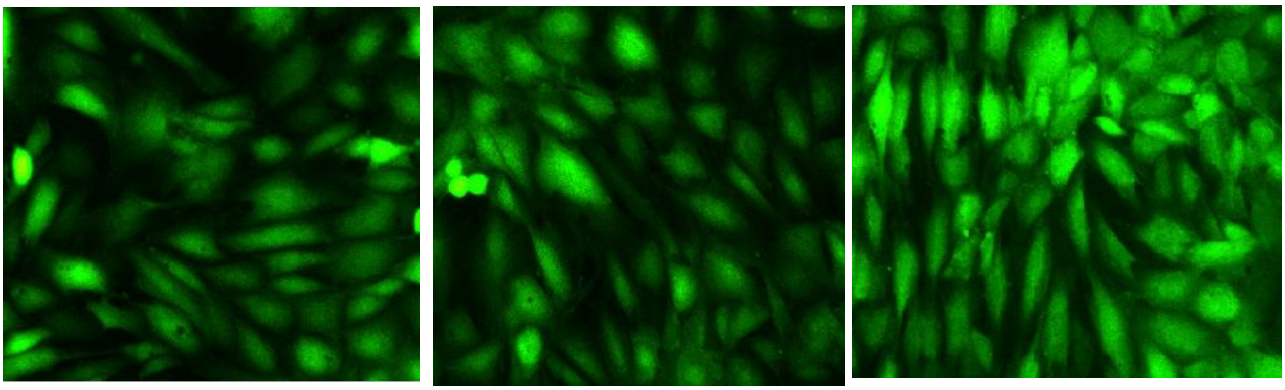
Different studies have proved that phagocytosis of LPS by macrophages give rise to increase in ROS production, finally contributing to disturbed respiratory chain of mitochondria. We found that 5µL (50ng)& 10µL (100ng) of LPS concentration for 6 h and 12 h incubation remarkably increase ROS production which leads to cell apoptosis. We also found that 24 h pre-treatment of Quercetin (30µL) (300ng) abrogate the LPS induced overproduction of ROS and reduces the LPS induced apoptosis.

Effect of LPS on H9C2 cells by analysing ROS

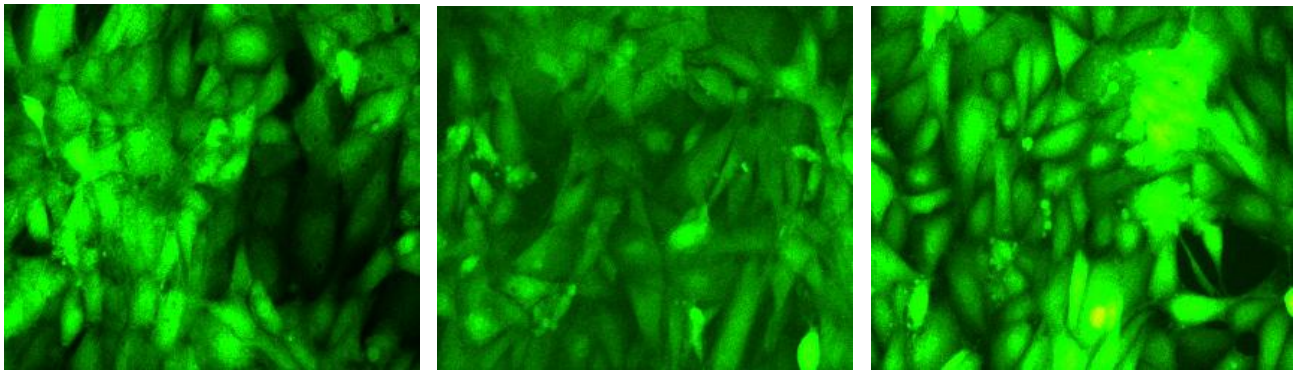
Mitochondria generate superoxide via univalent electron transfer to $O2^{\bullet-}$. This superoxide may be converted to H_2O_2 by superoxide dismutase (SOD) in the mitochondria or in the cytosol. To test whether LPS disturbs this respiratory chain and increase superoxide in mitochondria or in cytosol, we assessed ROS generation by using fluorescent probe DCFH-DA (5µM) by inverted microscopyas shown in Figure 1. Exposure of DCFH-DA increases fluorescence as compared to control cells significantly with concentration of 5µl for 6h (46. 91314±0. 816 Vs 21. 68061±0. 842, n=5 p > 0. 001)(Figure 2). The 10µlof LPS treatment caused further increase in the green fluorescence as compared to control group and recorded as in H9C2 cells for 6h (24. 40494±0. 757 Vs 15. 60861±0. 508, n=5, p > 0. 001)(Figure 3). The ROS estimated by LPS with 5µl concentration for 12h treatment increased significantly in H9C2 cells compared to control cells (19. 6157±0. 685 Vs 7. 9777332±0. 492, n=5 p>0. 0001) (Figure 4) while at concentration of 10µl of LPS for 12h the ROS level significantly increased in H9C2 cells as compared to control cells (41. 11532±2. 535 Vs 14. 74781±1. 020, n=5 p > 0. 001) (Figure 5).

Effect of Quercetin on LPS induced H9C2 cells by analysing ROS production

Fluorescence microscopic examination showed increase in DCFH-DA fluorescence upon exposure to LPS which was eliminated by quercetin 30µM as in Figure 4. This study showed that ROS production which was dramatically increased in LPS treated cells but quercetin was able to repudiate this fluorescence. DCFDA fluorescence decreases significantly in 6h, 5µlconcentration of 24 h quercetin pre-treated H9C2 cells as compared to inflamed cells (12. 403±0. 434 Vs 22. 299±0. 710, n=5, p>0. 001) (Figure 5) whereas DCFDA fluorescence



(a) Control 6h (5µl) (b) LPS 6h (5µl) (c) LPS 6h (10µl)



(d) Control 12h (5µl) (e) LPS 12h (5µl) (f) LPS 12h (10µl)

Figure 1. Images a, b, c demonstrate the effect of LPS induced ROS production on H_9C_2 cells for 6h treatment. Images d, e, f shows the impaired effect of LPS on H_9C_2 cells for 12 h treatment

Figure 2. DCFDA fluorescence increases significantly (6h, 5µl) as compared to control in LPS induced cells (21.680±0.842 Vs 46.913±0.816, n=5, p>0.001)

Figure 3. DCFDA fluorescence increases significantly (6h, 10µl) as compared to control in LPS induced cells (15.608±0.508 Vs 24.404±0.757, n=5, p>0.001)

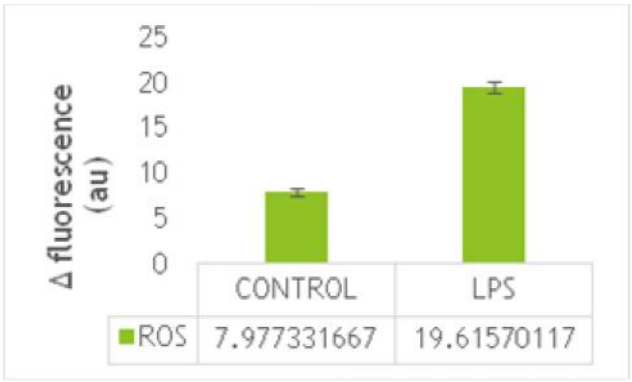


Figure 4. DCFDA fluorescence increases significantly (12h, 5µl) compared to control in LPS induced cells (7.977±0.492 Vs 19.615±0.685, n=5, p>0.001)

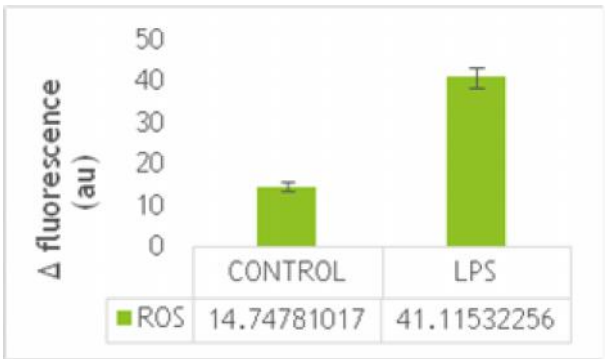
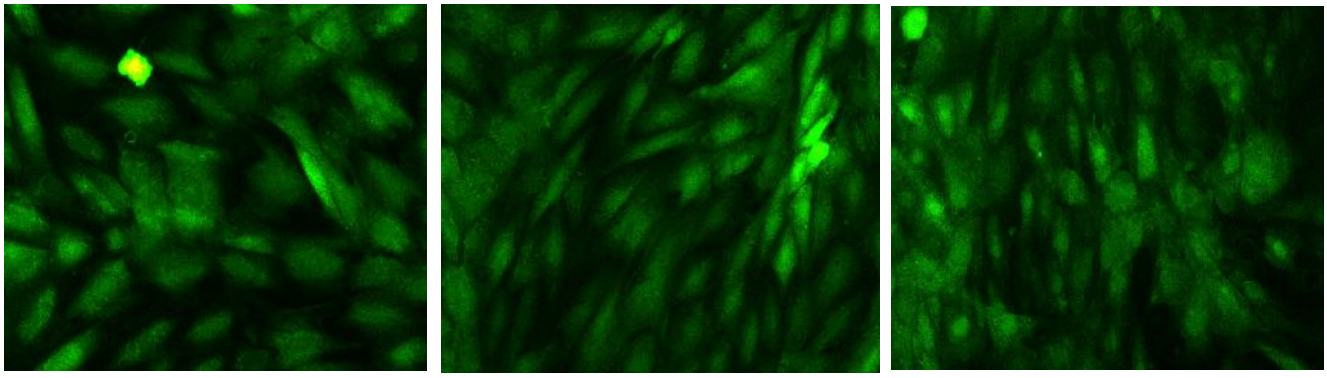
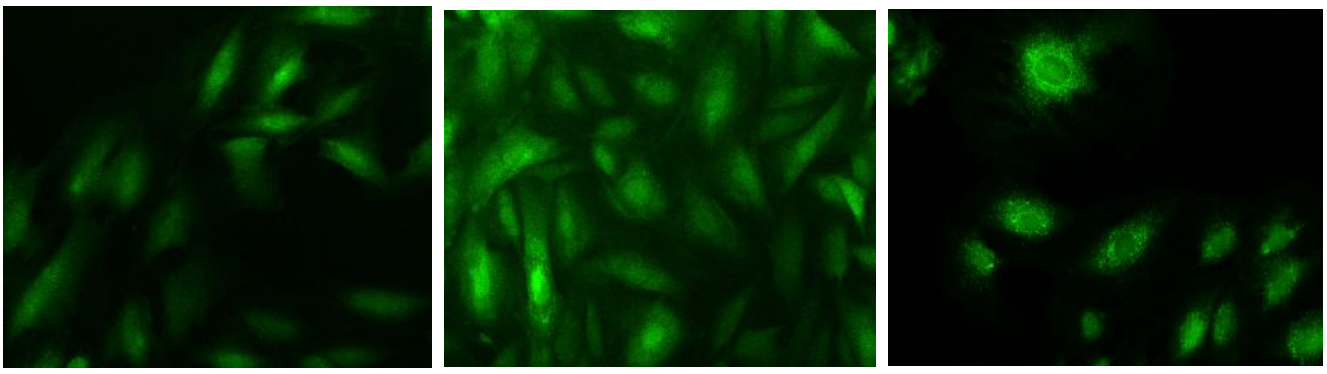


Figure 5. DCFDA fluorescence increases significantly (12h, 10µl) as compared to control in LPS induced cells (14.747±1.020 Vs 41.115±2.530, n=5, p>0.001)



(a) Q + Control 6hr (5µl) (b) Q + LPS 6hr (5µl) (c) Q + LPS 6hr (10µl)



(d) Q + Control 12hr (5µl) (e) Q + LPS 12hr (5µl) (f) Q + LPS 12hr (10µl)

Figure 4. Images a, b, c, demonstrate the protective effects of Quercetin on impaired effect of LPS on H9C2 for 6 hours. Images d, e, f shows the repaired effect of Quercetin on H9C2 cells for 12 hours

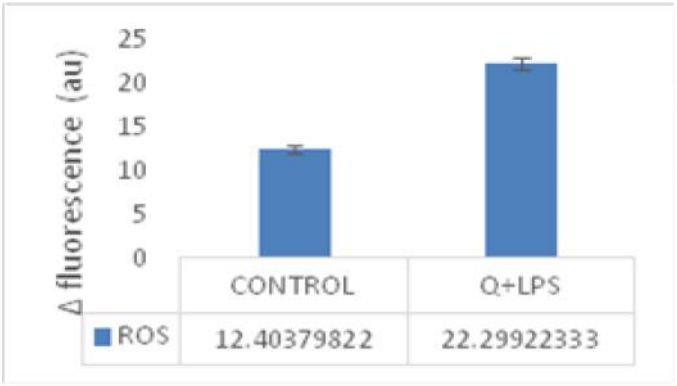


Figure 6. DCFDA fluorescence decreases significantly (6h, 5µl) as compared to control and LPS induced inflamed cells (12.403±0.434 Vs 22.299±0.710, n=5, p>0.001)

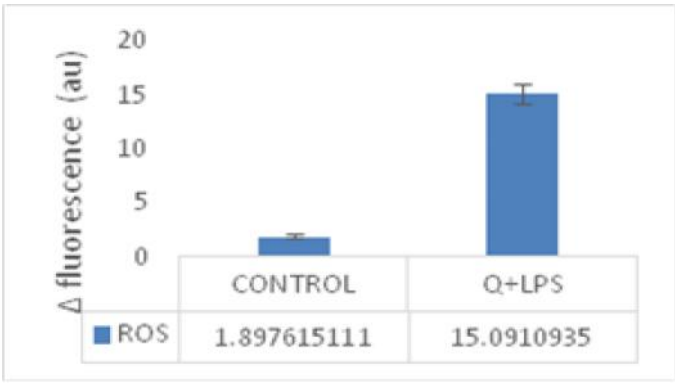


Figure 7. DCFDA fluorescence decreases significantly (6h, 10µl) as compared to control and LPS induced inflamed cells (1.897±0.155 Vs 15.091±0.889, n=5, p>0.001)

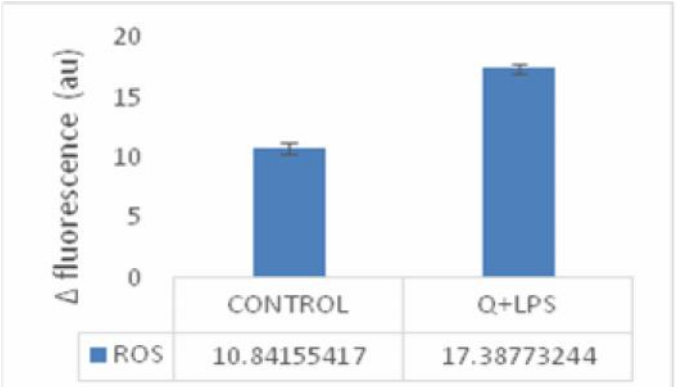


Figure 8. DCFDA fluorescence decreases significantly as compared to control and LPS induced inflamed cells (12h, 5µl) (10.841±0.522 Vs 17.387±0.377, n=5, p>0.001)

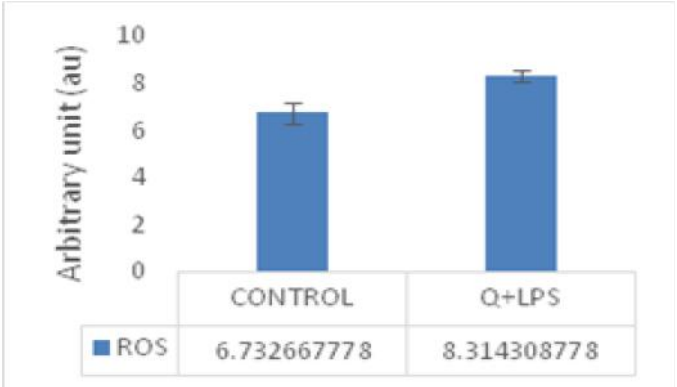


Figure 9. DCFDA fluorescence decreases significantly (12h, 10µl) as compared to control and LPS induced inflamed cells (6.732±0.485Vs8.314±0.222, n=5, p>0.001)

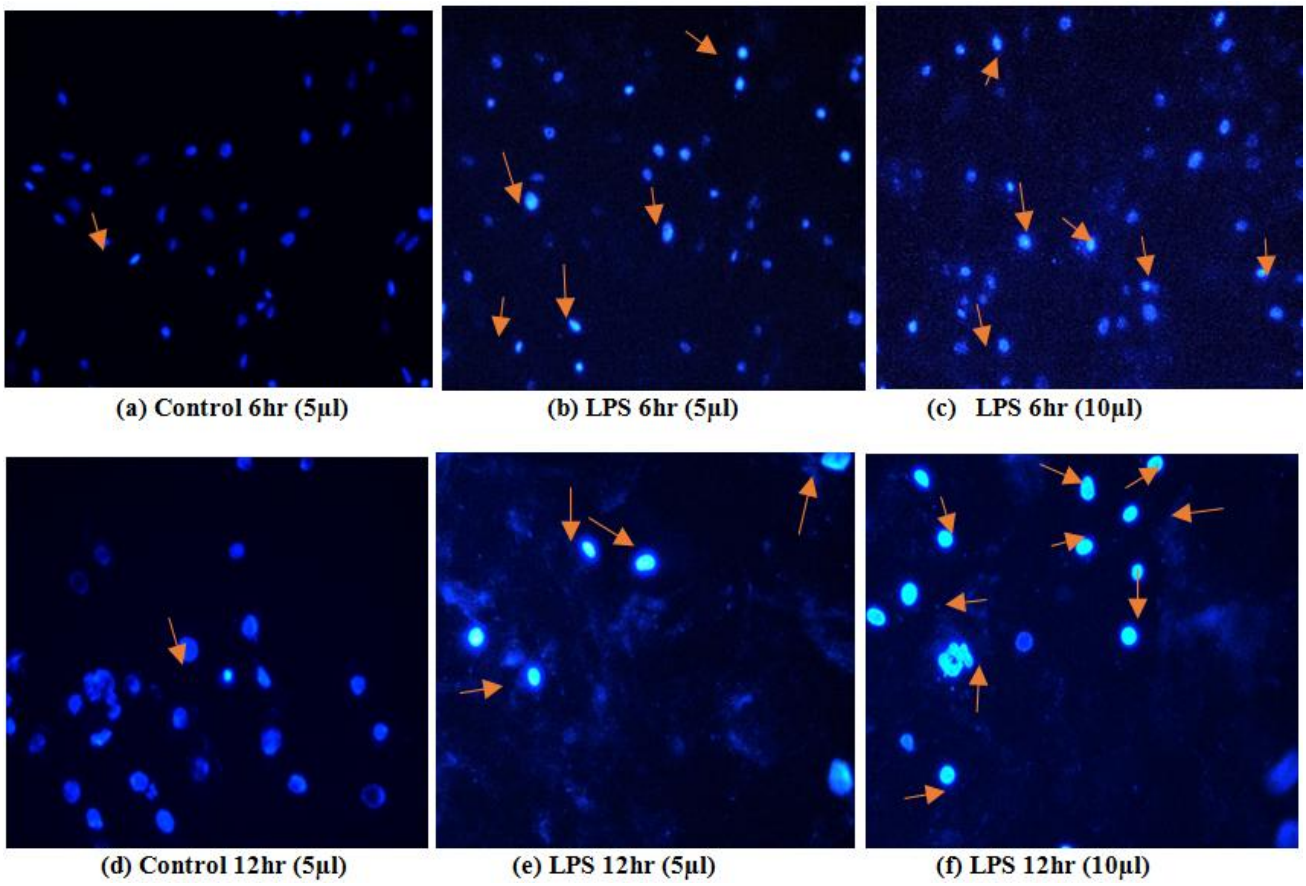


Figure 10. Images a, b, c demonstrate the apoptotic effect of LPS on H9C2 cells for 6 hours whereas images d, e, f shows the apoptotic effect of LPS on H9C2 cells for 12 hours

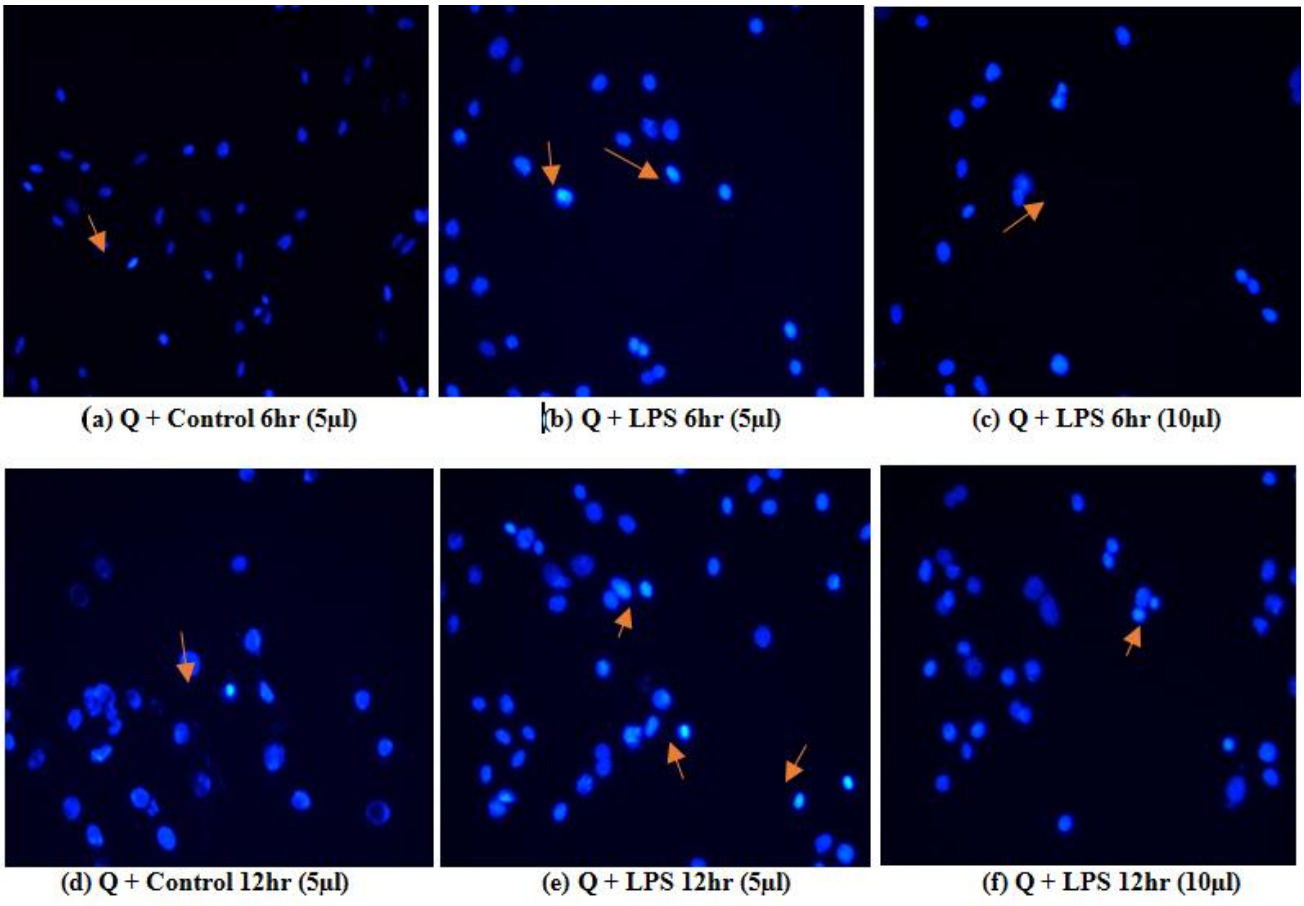


Figure 11. Images a, b, c, demonstrate the protective effects of Quercetin on apoptotic effect of LPS on H9C2 for 6 hours and images d, e, f shows the repaired effect of Quercetin on H9C2 cells apoptosis for 12 hours

decreases significantly also in 6h, 10µl concentrationas compared to control and LPS induced cells of inflammation (1. 897±0. 155 Vs 15. 091±0. 889, n=5, p>0. 001) (Figure 6). Whereas DCFDA fluorescence again shows a significant decrease in 12h, 5µl concentration of 24 h quercetin pre-treated H_9C_2 cells as compared to inflamed cells (10. 841±0. 522 Vs 17. 387±0. 377, n=5, p>0. 001) (Figure 7) whereas DCFDA fluorescence decreases significantly also in 12h, 10µl concentration as compared to control and LPS induced cells of inflammation (6. 732±0. 485Vs8. 314±0. 222, n=5, p>0. 001) (Figure 8).

Effects of LPS on cell apoptosis

LPS induces ROS production in H9C2 cells which may lead to nuclear damage and ultimately to cell death. Cell apoptosis was measured by DAPI (Figure 9) shows cells of control group which was treated with vehicle only. Treatment with 5µl of LPS exhibits cell death which increased significantly as compared to control cells (48±1. 154 Vs 2±0. 577, n=3 p >0. 0001) whereas 10µl LPS treatment also shows significant increase in cell death than control cells (63. 3333±2. 027 Vs 3±0. 577, n=3 p >0. 0001).

DISCUSSION AND CONCLUSION

Abruptly increased oxidative stress (ROS) is an early event in inflammation and its response by the cell. LPS is independent risk factor directly initiates inflammatory cascade which leads to cell death. In this study, we have evaluated the anti – inflammatory role of quercetin in H9C2 cells which shows that this polyphenol attenuated LPS-induced inflammatory events by inhibiting ROS production and counteracting apoptosis. Production of ROS leads to activation of pro-inflammatory cytokines which are involved in depression of cardiac function. Quercetin inhibited ROS production in LPS-stimulated H9C2 cells in a time dependent manner and counteracted LPS-induced apoptosis. Therefore, these results suggest that quercetin might serve as a valuable protective agent in cardiovascular diseases.

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