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Toxic effect of high glucose on cardiomyocytes, H9c2 cells: Induction of oxidative stress and ameliorative effect of trolox

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Abstract

Oxidative stress (OS) has been implicated in a variety of pathological conditions, including diabetes mellitus, characterized by hyperglycemia. In the present study, OS induced by hyperglycemia and the effect of trolox, a vitamin E analog, were studied in cardiomyocytes and H9c2 cells exposed to 15 to 33 mM glucose (HG) for 24 to 72 hours in Dulbecco modified Eagle medium. Cells treated with 24 or 33 mM glucose for 24 hours or above showed decreased viability and adenosine triphosphate (ATP) content with a concomitant increase in radicals of oxygen species, calcium (Ca^{2+}), mitochondrial permeability transition, and oxidative markers, confirming that the cells were under stress. However, upon exposure to 15 mM glucose for 24 hours, H9c2 cells maintained homeostasis and ATP generation. Pretreatment of cells with trolox reduced HG-induced OS to control levels. Here, we report that the toxic effect of HG is highly regulated and that OS induction can be prevented with Trolox, a potential inhibitor of membrane damage.

KEYWORDS

cardiomyocytes, cellular adenosine triphosphate (ATP), hyperglycemia, intracellular Ca^{2+} (Ca^{2+}), mitochondrial permeability transition (MPT)

1 | INTRODUCTION

Hyperglycemia has been studied to inflict cell injury in several cells, and chronic hyperglycemia causes diabetes and diabetes-related complications.^[1] Although high glucose exposure causes persistent alterations in macromolecules,^[2] brief exposure to hyperglycemia induces acute changes in cellular metabolism. High glucose mediates cardiovascular complications, and oxidative stress (OS) is implicated in the pathogenesis of diabetic patients.^[3,4] OS has been defined as an imbalance between pro-oxidants and antioxidants, resulting in excess production of radicals of oxygen species (ROS). Experimental evidence indicated ROS as the causative agent for tissue damage associated with diabetes.^[5,6] Different metabolic pathways have been proposed, including enhanced mitochondrial electron-transport chain, for hyperglycemia-induced ROS accumulation.^[7-9] High levels of intracellular glucose-derived pyruvate facilitate an increase in the flux of NADH and FADH_2 into the electron-transport chain, thereby causing depolarization of mitochondrial membrane potential ($\Delta\psi_m$). Cardiac tissue is known for the abundant presence of mitochondria and is known to provide continuous energy for cardiac

function.^[10] As a result, diabetic individuals often show an impairment of mitochondrial membrane function with a reduction in adenosine triphosphate (ATP) synthesis before the onset of glucose intolerance.^[11] This metabolic disorder also lessens the cardiac mitochondrial bioenergetics, resulting in decreased contractile function in diabetic hearts.^[12,13]

Hyperglycemia-induced ROS causes opening of the mitochondrial permeability transition (MPT) pore, thereby causing changes in $\Delta\psi_m$ and eventually releasing a number of apoptotic factors from the mitochondria.^[14] Furthermore, earlier studies have indicated that the impairment of mitochondrial function is partially due to decreased mitochondrial transcription factor A activity or expression.^[15,16] Apart from excessive ROS accumulation influenced by hyperglycemia, high glucose also causes an overload of Calcium (Ca^{2+}),^[17,18] and this abnormality in Ca^{2+} handling significantly reduced contractile function in diabetic hearts. Also, the intracellular retention of Ca^{2+} is markedly associated with this metabolic disorder.^[19] As a consequence, Ca^{2+} channel blockers in conjunction with antioxidants have been proved to be effective in the cardiac performance in the diabetic state.^[20]

Maladaptive signaling pathways induced by increased ROS may contribute to the pathogenesis of diabetic cardiomyopathy^[21]; however, the pathophysiological relevance of increased myocardial ROS production in diabetes-related complications needs to be characterized. Since OS results in the reduction of myocardial antioxidants, the loss of $\Delta\psi_m$ and increased superoxide,^[22] it is of interest to precisely assess the role of antioxidants that can minimize potential oxidative damage. Vitamin E is considered to be a universal participant of the antioxidant defense mechanism. Among the vitamin E analogs that are used, Trolox seems to have an advantage over others because of its moderate water solubility and the fact that it can be added directly to an intact system.^[23]

Cardiovascular cell injury is one of the complications of diabetes. The present study aims to gain insights into hyperglycemia-induced cell mortality. We examined the crosstalk among ROS, Ca^{2+} , and MPT, which are reflected by high glucose concentrations and the duration of exposure. A strategy that focuses on decreasing OS as well as enhancing mitochondrial function may provide an important option to overcome diabetic complications. To this end, we tested Trolox, a water-soluble vitamin E analog, for its antioxidant property and protection against mitochondrial damage. Here, we investigated hyperglycaemic (HG) episodes at different concentrations and exposure periods in an in vitro model to define the probable critical situation where glucose can be toxic to cardiac cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals

ATP determination kit, Fluo-4-AM cell permeant, H2DCFDA, and Mitotracker orange were obtained from Molecular probes, (Invitrogen, Carlsbad, CA). D-glucose, dimethyl sulfoxide (DMSO), dinitro phenyl hydrazine (DNPH), Dulbecco modified Eagle medium (DMEM) cell culture media, fetal bovine serum (FBS), mannitol, penicillin, sodium dodecyl sulfate (SDS), streptomycin, thiazolyl blue tetrazolium bromide (MTT), thiobarbituric acid (TBA), trichloro acetic acid (TCA), 1,1,3, 3-tetra methoxy propane (TMP), and 6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid (trolox) were obtained from Sigma-Aldrich, (St. Louis, MI). Common chemicals were of reagent grade.

2.2 | Cell culture and treatment

Rat cardiomyocyte (H9c2) cell lines were obtained from the National Centre for Cell Science, Pune, India. Cells were cultured in DMEM with 10% FBS, and 1% antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin), 5% CO₂, at a temperature of 37°C with 95% humidity.

When 60% to 70% cell confluence was reached, hyperglycemia was induced by adding 15, 24, and 33 mM of D-glucose to the media. To evaluate the protective role of trolox, cells cultured in similar concentrations were treated with 100 μ mol of trolox for 24 hours before hyperglycaemic (HG) treatment (pretreatment). Trolox-treated HG cells and HG cells in the aforementioned concentrations along with control cells (HG-C), and the trolox-treated control were maintained for 24, 48, and 72 hours.

2.3 | Cell viability assay

The viability of the cells was assessed by MTT (3-(4, 5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2-4-tetrazolium bromide) following the method described by Hansen et al.^[24] Briefly, 3×10^3 cells were seeded in 96-wells plate and incubated overnight at 37°C with 5% CO₂. After reaching confluence and the subsequent treatments, the cells were washed with phosphate buffer (PBS). MTT (2 mg/mL) was added to each well, and cells were incubated for 4 hours at 37°C. Formazan crystals were dissolved with DMSO, and optical density was measured at 570 nm, using a multimode plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland).

To confirm that the change in cell viability due to high glucose was not a consequence of high osmolarity, H9c2 cells were incubated with 15, 24, and 33 mM mannitol for 24, 48, and 72 hours. The cells were assessed for viability as mentioned above.

2.4 | Measurement of cellular ROS generation

Total ROS in H9c2 cells were measured by exposing them to the aforementioned HG conditions for 24, 48, and 72 hours, with and without trolox pretreatment. Briefly, after treatment, the cells were washed in PBS and incubated for 15 minutes in 20 μ M H2DCFDA. The cells were then washed, and fluorescence intensity was measured using a fluorescent microscope (Olympus IX, Olympus, Tokyo, Japan) under Ex/Em-495/527 nm and quantified using Image Pro Express version 6.3 software (Olympus, Tokyo, Japan).

2.5 | ATP measurement

Cells were plated in 96-well plates, and ATP content was determined using luciferin and luciferase with the ATP determination kit, as described by the manufacturers. Luminescence was read by using a multimode plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland).

2.6 | Assessment of MPT

To measure the changes in mitochondrial (mt.) permeability transition due to HG conditions, H9c2 cells were stained with a fluorescent mitochondrion-selective probe mitotracker orange. Under a fluorescent microscope (Olympus IX, Japan) using a green excitation filter (Ex/Em 554/576 nm), the fluorescent intensity was measured and quantified by Image Pro Express version 6.3 software (Olympus IX, Olympus, Tokyo, Japan). Briefly, the cells were exposed to HG concentrations for 24, 48, and 72 hours. Trolox-treated and untreated cells were washed in PBS and incubated for 20 min in mitotracker orange. After incubation, the cells were washed in PBS and subjected to fluorescence recordings.

2.7 | Measurement of cellular Calcium

The intracellular Ca^{2+} concentrations in cultured H9c2 cells were measured using the Fluo-4/AM dye under Ex/Em 491/516 nm in a

fluorescent microscope (Olympus IX, Japan). Cells were given HG treatments with and without trolox pretreatment for 24, 48, and 72 hours. After treatment, the cells were washed in PBS and were incubated in 5 μ M Fluo-4/AM containing 5 mM CaCl_2 in PBS for 20 minutes at 37°C and were resuspended in PBS. Cell fluorescence was quantified using the Image Pro Express version 6.3 software (Olympus).

2.8 | Lipid peroxidation in H9c2 cells

Malondialdehyde (MDA) generation was spectrophotometrically assayed to understand the extent of lipid peroxidation by the method of Okhawa et al.^[25] Briefly, cell homogenates containing 8% SDS were vortexed and incubated for 10 minutes at room temperature. Then, 20% acetic acid and 0.6% TBA were added, and the cells were incubated for 50 minutes in a boiling water bath. After cooling, a butanol and pyridine (15:1) mixture was added to the cocktail, which was then vortexed. The thoroughly mixed cocktail was centrifuged for 5 minutes. The organic layer was separated, and absorbance was read at 532 nm against a blank. TMP was used as a standard, and the extent of lipid peroxidation was expressed as nmol of MDA/mg protein.

2.9 | Protein carbonylation in H9c2 cells

Protein carbonylation was assayed as a marker of protein oxidation spectrophotometrically by the method of Levine et al.^[26] Briefly, cell homogenate was centrifuged for 10 minutes at 4°C. The collected supernatant was equally divided into DNPH-treated and untreated fractions, which were incubated for 1 hour at room temperature. TCA (equal volume) was added to both fractions and recentrifuged at 4°C for 10 minutes. The supernatant was discarded, and the precipitate was washed in an ethyl acetate and ethanol mixture (1:1), dissolved in 6 M guanidine hydrochloride, and centrifuged for 10 minutes at 4°C. The supernatant was collected, and absorbance was read at 365 nm against respective DNPH untreated fractions for each replicate. The molar extinction coefficient $2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was used to obtain the extent of protein carbonyl incorporation and expressed as nmol DNPH incorporated/mg protein.

2.10 | Statistical analyses

Data are presented as the mean $\pm$ standard error of the mean. Statistical analysis was performed using Prism 6.01 software (GraphPad Inc., La Jolla, CA) and SPSS statistics 20 (IBM, Armonk, NY). Data were initially tested for homogeneity (Leven's test) to determine the suitable analysis. Two-way analysis of variance followed by Tukey's post hoc tests with a single pooled variance was used to assess the differences in means between different HG concentrations and trolox treatment. Two-tailed Pearson's correlation was used to determine the degree of relativity between ROS, OS markers, mitochondrial structural, and functional integrity with cell viability in H9c2 cells. Probability values $P < 0.05$ were selected as the point of minimum significance.

3 | RESULTS

3.1 | Viability of H9c2 cells subjected to high glucose

The viability of cardiomyocytes under high glucose stress was assessed by MTT test. Incubation in high glucose for 24 hours induced a significant decrease in the viability of H9c2 cells, by 20% at the highest concentration 33 mM ($P < 0.001$), but not at lower concentrations ($P > 0.05$) (Figure 1A). A concentration-dependent decrease in viability to higher glucose was significantly evident in H9c2 cells incubated for a longer period of 48 hours. Cell viability was reduced by 13% ($P < 0.05$), 16% ($P < 0.01$), and 26% ($P < 0.001$) after incubation with 15, 22, and 33 mM glucose, respectively (Figure 1B), while at 72 hours, similar concentrations induced a decrease in viability of 18% ($P < 0.01$), 27% ($P < 0.0001$), and 48% ($P < 0.0001$), respectively. However, the cells that were exposed to mannitol, an osmotic control, did not cause significant changes in viability (Supporting Information Figure S1), suggesting that the high glucose-induced decrease in viability was not a consequence of high osmolarity. Further, prior exposure to the vitamin E analog trolox obliterated the decrease in viability caused by high glucose treatment. The role of trolox in H9c2 cell survival in HG conditions is very evident at 72 hours, with the increased viability of 28% ($P < 0.0001$), 24% ($P < 0.01$), and 64% ($P < 0.0001$) after treatment with 15, 24, and 33 mM glucose, respectively (Figure 1C).

3.2 | High glucose-induced ROS

The intracellular ROS levels were measured in terms of $\text{O}_2^{\bullet-}$. After the treatment of cells with various concentrations of glucose, an increase in the production of free radicals was seen in both dose- and time-dependent manners (Figure 2A, 2C, and 2E). Figure 2B shows that HG significantly increased the level of $\text{O}_2^{\bullet-}$ by 9% ($P < 0.0001$) and 15% ($P < 0.0001$) at 24 mM and 33 mM glucose, respectively, after incubation for 24 hours. A similar trend in ROS was also observed in cells exposed to HG for 48 hours with an increase of 23% ($P < 0.0001$) and 60% ($P < 0.0001$) at 24 and 33 mM, respectively, and the maximal effect was recorded at 72 hours with an ROS increase of 63% and 138% ($P < 0.0001$) for the two higher glucose concentrations (Figure 2D). An increase in $\text{O}_2^{\bullet-}$ upon exposure to HG was attenuated by Trolox at 5 mM under all experimental conditions. The rise in ROS levels at 24 and 33 mM HG was reversed by 15% and 27% ($P < 0.0001$) at 48 hours, respectively, and 30% and 33% ($P < 0.0001$) at 72 hours (Figure 2F), respectively. HG conditions induced by 15 mM HG failed to evoke the generation of $\text{O}_2^{\bullet-}$, irrespective of the duration of treatment.

3.3 | High glucose-induced mitochondrial dysfunction by decreasing ATP

Exposure to various concentrations of HG for 24 hours had no impact on the ATP content in cardiac cells (Figure 3A). However, exposure

FIGURE 1 Variation in cell viability at 24 hours (A), 48 hours (B), 72 hours (C) in hyperglycemic and trolox-treated H9c2 cells. Values are mean $\pm$ SEM (octaplicate). Two-way ANOVA with Tukey's multiple comparison test was used and significance in mean difference was represented as **** $P < 0.0001$; ** $P < 0.01$ with respective hyperglycemic subgroup, #### $P < 0.0001$; ### $P < 0.001$; ## $P < 0.01$; # $P < 0.05$ with control. ANOVA, analysis of variance; SEM, standard error of the mean

for 48 hours in 15, 24, and 33 mM HG decreased the ATP content by 20% ($P < 0.01$), 40% and 55%, respectively ($P < 0.0001$). Trolox pretreatment resulted in elevated ATP levels by 30% ($P < 0.0001$) in 15 mM and 45% ($P < 0.0001$) in 24 and 33 mM glucose (Figure 3B). Also, 72 hours of exposure to HG resulted in a significant depletion of ATP levels. The ATP level decreased by 17% ($P < 0.05$) in 15 mM HG and 53% in 24 and 33 mM ($P < 0.0001$) HG. Interestingly, an ameliorative effect of trolox was revealed by post hoc tests, as there was a significant increase in ATP levels by 21% in 15 mM ($P < 0.01$) and 50% in 24 and 33 mM ($P < 0.0001$) (Figure 3C). The decreased ATP concentration compared with the decreased viability of the cell population suggests the possibility of extended incubation to HG inducing mitochondrial dysfunction.

3.4 | Changes in MPT in HG treatments

Mitochondrial ATP synthesis is dependent on the proton-motive force, in which $\Delta\psi_m$ has a major role. Since prolonged exposure to HG caused decreased ATP concentrations in H9c2, we examined the MPT using mitotracker orange dye. After 24 hours of exposure to HG at 15, 24, and 33 mM concentrations, an increase in MPT by 11% ($P < 0.01$), 23% and 28% ($P < 0.0001$), respectively, was evoked. This observed increase in MPT after 24 hours of HG stress was reversed by pretreatment with trolox. A significant reduction of 13% and 16% ($P < 0.0001$) was evident in 24 and 33 mM glucose, respectively (Figure 3D and 3E). HG exposure for 48 hours caused an increase in permeability by 40%, 56%, and 75% ($P < 0.0001$) with 15, 24, and 33 mM HG, respectively. Trolox pretreatment restored the membrane permeability as a reduction in cell fluorescence by 27%, 35%, and 45% ($P < 0.0001$) in HG concentrations (Figure 3F and 3G). MPT significantly increased in the three concentrations of high glucose by almost 80% to 90% ($P < 0.0001$) and trolox pretreatment successfully restored the MPT by 48% ($P < 0.0001$) (Figure 3H and 3I).

3.5 | High glucose-induced intracellular calcium elevation

Intracellular calcium (Ca^{2+}_i) is closely related to ROS accumulation. Thus, ROS increase is likely to influence Ca^{2+} regulation. The exposure of H9c2 cells to high glucose caused a concentration- and time-dependent increase in (Ca^{2+}_i). The exposure of cells to HG for 24 hours resulted in a significant increase in the fluorescent intensity of the Fluo-4 in cells at 33 mM by 8% ($P < 0.01$). However, pretreatment with trolox before HG exposure blunted the hyperglycemia-induced intracellular calcium increase in cardiomyocytes by 6% ($P < 0.001$) (Figure 4A and 4B). At 48 hours of HG exposure, a concentration-dependent increase in Ca^{2+} by 14% and 37% ($P < 0.0001$) was evident in 24 and 33 mM glucose, respectively. An ameliorative effect of trolox became evident in cells pretreated with trolox and a decrease in Ca^{2+} levels of 11% ($P < 0.001$) and 22% ($P < 0.0001$) was found in 24 and 33 mM,

FIGURE 2 Total ROS in H9c2 cells microphotographs of 24, 48, and 72 hours post treatment are presented in panel A, C, and E. Values are mean $\pm$ SEM (triplicate) represented in panel B, D, and F, respectively, as fluorescence intensity in A.U. Two-way ANOVA with Tukey's multiple comparison test was used and significance in mean difference was represented as **** $P < 0.0001$ with respective hyperglycemic subgroup, ##### $P < 0.0001$ with control. Scale 10 μm $\times$ 40. ANOVA, analysis of variance; ROS, radicals of oxygen species; SEM, standard error of the mean

respectively (Figure 4C and 4D). In addition, 72 hours exposure to 15, 24, and 33 mM HG caused a significant increase in Ca^{2+} by 14% ($P < 0.05$), 26% and 47%, respectively ($P < 0.0001$). Trolox effectively reduced intracellular Ca^{2+} by 10% ($P < 0.05$), 15% ($P < 0.001$) and 23% ($P < 0.0001$) in high glucose concentrations (Figure 4E and 4F).

3.6 | MDA and protein carbonyl content levels as biomarkers of OS induced by high glucose

The determination of the MDA level is a marker of lipid oxidation, while carbonyl levels are used as an index of the extent of oxidative damage to proteins during stress induced by HG exposure. High

glucose caused concentration- and duration-dependent increases in the mean MDA level (nmol/mL) of H9c2 cells. Tukey's post hoc test revealed a 2- to 2.3-fold ($P < 0.0001$) increase in lipid peroxidation (LPO) in 24 and 33 mM HG, whereas trolox pretreatment caused a reduction in LPO levels by 18% ($P < 0.01$) during 24-hour HG exposure (Figure 5A). A concentration-dependent increase in LPO levels of 90% ($P < 0.001$), 185%, and 220% ($P < 0.0001$) was seen after 48 hours of HG exposure, whereas trolox exposure reduced LPO by 28% ($P < 0.001$) (Figure 5B). Similarly, 72 hours of HG stress caused an increase in LPO by 52% ($P < 0.01$), 170% ($P < 0.0001$), and 221% ($P < 0.0001$) at 15, 24, and 33 mM HG, respectively, and trolox reduced LPO levels by 24% ($P < 0.05$) in 33 mM HG (Figure 5C).

FIGURE 3 Mitochondrial integrity in H9c2 cells ATP levels are represented as mean $\pm$ SEM (octaplicate) at 24 hours (A), 48 hours (B), 72 hours (C) after HG stress and trolox pretreatment. Microphotographs of mitochondrial permeability transition after treatment at 24 hrs, 48 hrs, and 72 hrs are presented in panels D, F, and H and fluorescence intensity in A.U. is represented in panel E, G, and I as mean $\pm$ SEM (pentaplicate) respectively. Two-way ANOVA with Tukey's multiple comparison test was used and significance in mean difference was represented as **** P < 0.0001; ** P < 0.01; * P < 0.05 with respective hyperglycemic subgroup, ##### P < 0.0001; ### P < 0.01; # P < 0.05 with control. Scale 10 μ m $\times$ 40. ANOVA, analysis of variance; ATP, adenosine triphosphate; HG, hyperglycaemic; SEM, standard error of the mean

FIGURE 4 Intracellular Ca^{2+} in H9c2 cell microphotographs of 24, 48, and 72 hours after treatment are presented in panels A, C, E. Values are mean $\pm$ SEM (triplicate) represented in panels B, D, F, respectively. The fluorescence intensity is presented in A.U. Two-way ANOVA with Tukey's multiple comparison test was used, and significance in mean difference was represented as **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ with respective hyperglycemic subgroup, #### $P < 0.0001$; ## $P < 0.01$; # $P < 0.05$ with control. Scale $10 \mu\text{m} \times 40$. ANOVA, analysis of variance; SEM, standard error of the mean

The protein carbonyl content (PCC) of H9c2 was significantly higher with high glucose treatment in a dose- and duration-dependent manner (Figure 5D-F). The profound effect of HG seen in cells as PCC was more evident for 48- and 72-hour-stressed cells ($P < 0.0001$). Trolox, a water soluble derivative of vitamin E that partitions well into the lipid layer significantly reduced the high glucose-induced oxidation of membrane lipids and proteins.

A two-tailed Pearson correlation analysis revealed that a high degree of negative correlation existed between cell survivability with ROS generation ($r = -0.84$), MPT ($r = -0.81$), $(\text{Ca}^{2+})_i$ levels ($r = -0.88$), MDA ($r = -0.83$), and PCC ($r = -0.84$). A similar negative correlation

existed between ATP levels with ROS ($r = -0.82$), MPT ($r = -0.87$), $(\text{Ca}^{2+})_i$ ($r = -0.92$), MDA ($r = -0.83$), and PCC ($r = -0.69$). A further positive correlation was observed between ATP level and cell survivability ($r = 0.78$) and between ROS with MPT ($r = 0.73$), $(\text{Ca}^{2+})_i$ ($r = 0.91$), MDA ($r = 0.88$), PCC ($r = 0.79$). Such a positive correlation was found between MPT with $(\text{Ca}^{2+})_i$ ($r = 0.87$), MDA ($r = 0.71$), PCC ($r = 0.58$) and also between $(\text{Ca}^{2+})_i$ with MDA ($r = 0.85$), PCC ($r = 0.78$), between MDA and PCC ($r = 0.91$). The above analysis strengthen our hypothesis that HG stress-associated cell death in H9c2 cells is due to increase in OS and the associated mitochondrial dysfunction (Table 1).

FIGURE 5 Extent of lipid peroxidation and protein carbonylation at 24 hours (A,D), 48 hours (B,E), 72 hours (C,F), respectively, in hyperglycemic- and trolox-treated H9c2 cells. Values are mean $\pm$ SEM (quadruplicate). Two-way ANOVA with Tukey's multiple comparison test was used, and significance in mean difference was represented as **** P < 0.0001; *** P < 0.001; ** P < 0.01; * P < 0.05 with respective hyperglycemic subgroup, #### P < 0.0001; ### P < 0.001; ## P < 0.01 with control. ANOVA, analysis of variance; SEM, standard error of the mean

TABLE 1 Correlation between stress and mitochondrial parameters with cell survivability in hyperglycemic and trolox pretreated hyperglycemic H9c2 cells

	Cell viability	ROS	ATP	MPT	(Ca ²⁺) _i	MDA	PCC
Cell viability	–						
ROS	–0.84****	–					
ATP	+0.78***	–0.82****	–				
MPT	–0.81****	+0.73***	–0.87****	–			
(Ca ²⁺) _i	–0.88****	+0.91****	–0.92****	+0.87****	–		
MDA	–0.83****	+0.88****	–0.83****	+0.71***	+0.85****	–	
PCC	–0.84****	+0.79***	–0.69***	+0.58**	+0.78***	+0.91****	–

Abbreviations: ATP, adenosine triphosphate; (Ca²⁺)_i, intracellular Ca²⁺; MDA, malondialdehyde; MPT, mitochondrial permeability transition; PCC, protein carbonyl content; ROS, reactive oxygen species. Data represented as Pearson's correlation with two-tailed test between ROS, ATP, (Ca²⁺)_i, MDA, and PCC with cell survivability as "r" values (n = 3–8).

****P < 0.0001.

***P < 0.001.

**P < 0.01.

4 | DISCUSSION

The current study evaluates the relationship between hyperglycemia and OS in H9c2 cells exposed to different HG concentrations and time durations. Our results showed a significant decrease in cell viability induced by 33 mM glucose for 72 hours and are consistent with earlier findings.^[9,27] Exposure to various concentrations of mannitol failed to increase mortality, indicating that cell death is due to HG per se.^[28,29] A majority of cells in HG incubations, except for 15 mM, exhibited increased ROS levels, which resulted in approximately 15% mortality, indicating that increased ROS was not directly translated into cell death. Apparently, the ROS levels at a certain threshold induce changes in the redox state, leading to OS. Apart from mitochondrial, additional sources of ROS production are gaining importance in hyperglycemia-induced cardiomyopathy.^[30–32]

Mitochondria are the redox-active compartments generating ATP mostly through oxidative phosphorylation. In this study, the intracellular ATP levels remained the same after 24-hour HG exposure; however, the ATP levels were significantly lowered in all HG concentrations at 48 and 72 hours. We presume that ATP levels are maintained for 24 hours at low glucose concentrations due to altered electron flux, but that these are not sufficient to normalize the proton gradient across the mitochondrial membrane.

Mitochondrial dysfunction is an important manifestation of ROS generation induced by hyperglycemia. High ROS and low ATP induce MPT, which in turn dissipates the $\Delta\psi_m$ by providing protons with an alternate route to the matrix.^[33–35] Although glucose at 15 mM failed to evoke a response in $\Delta\psi_m$ at 24 hours, a steady increase in dye intensity was evident at later durations and at higher concentrations. Apparently, the decreased mitochondrial hyperpolarisation, along with the increased ROS and decreased ATP levels and a reversal of these changes by trolox clearly, indicated that hyperglycemia induced OS in cardiomyocytes. Our results are in agreement with earlier studies on the hearts of STZ-induced diabetic mice and H9c2 cells treated with 33 mM glucose, which

showed that the mitochondrial pathway mediated cell loss and restoration of $\Delta\psi_m$ through taxifolin.^[36]

Interactions between ROS and calcium signaling can be bidirectional. In our study, alterations in (Ca²⁺)_i followed the same pattern as seen in ROS generation under the same experimental conditions, indicating a probable crosstalk between the two messengers (Table 1). Trolox prevented hyperglycemia-induced Ca²⁺ levels. The end products of free radicals serve as indicators of OS, which cause macromolecular damage and contribute to the pathogenesis of diabetes.^[37,38] Concentration- and duration-dependent HG-induced changes in PCC and MDA levels and reversal by trolox in H9c2 cells further confirmed that hyperglycemia induced toxicity. Although the transfer of Trolox into the cell is possibly hampered by its partial hydrophilic nature, the residual trolox that might be retained in the membrane would have lessened the PCC and MDA in high glucose-treated cells. Although numerous cell lines and animals were used in the hyperglycemia-induced OS as an important feature in diabetic cardiomyopathy, its relevance to adult human cardiomyocytes was less contemplated upon. In the present study, we used H9c2 cells, which are undifferentiated myoblasts derived from rat left ventricle and, more importantly, are alternatives to animal-free studies.

Our study demonstrates that H9c2 cells are more relevant for study on energy metabolism and mitochondrial function as of intact heart.^[39–41] Both H9c2 cells and human cardiomyocytes AC16 exhibit significant decreased mitochondrial function, cell viability, and increased ROS production, whereas H9c2 cells are more resistant to toxicants due to less mitochondrial content than AC16 cells.^[42] Furthermore, human AC16 cardiac cells exposed to different concentrations of high glucose at 24 hours have shown decreased viability^[43] similar to H9c2 cells in the present study. Although H9c2 cells and primary neonatal cardiomyocytes show similar responses in viro,^[44] the cell line aging process plays an important role in cardiotoxicity studies in H9c2 cells.^[45] Nevertheless, uncertainty exists in determining the maximum number of passages required for in vitro studies.

In summary, HG induces OS in cardiomyocytes. However, the detrimental effect of HG is highly regulated. Lower concentrations of glucose and shorter episodes maintained cell homeostasis and ATP generation, whereas longer exposure could be harmful. Trolox exerted cardioprotection against HG-induced OS through prevention of ROS accumulation, thereby ensuring optimal MPT and ATP levels. Hence, Trolox can be used as a daily supplement with other drugs in terms of the treatment and management of hyperglycemia-induced cardiomyopathy.

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