European Review for Medical and Pharmacological Sciences 2023; 27: 2953-2963

Ranolazine exhibits anti-inflammatory and antioxidant activities in H9c2 cardiomyocytes

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Abstract. – OBJECTIVE: The aim of this study was to evaluate the effectiveness of ranolazine on hypoxia-inducible factor-1α (HIF-1α) and oxidative stress in H9c2 cardiomyocyte cells.

MATERIALS AND METHODS: We have assessed the effects of increasing concentrations of methotrexate (MTX) and ranolazine on proliferation of H9c2 rat cardiomyocyte cells by MTT assay. Malondialdehyde (MDA) protein oxidation [advanced oxidation protein products (AOPPs)], lipid hydroperoxide (LOOH) and xanthine oxidase (XO) activity as oxidative stress markers and HIF-1α levels increased and total thiol (T-SH), catalase (CAT) activity and total antioxidant capacity (TAC) antioxidant capacity markers decreased in MTX-treated cells compared to control cells.

RESULTS: Oxidative stress markers decreased, and antioxidant capacity markers increased in cells treated with ranolazine alone compared to control cells. For all parameters, we showed that the levels of oxidant, antioxidant markers and HIF-1α in cells treated with MTX and ranolazine together reached the level of the control group, and ranolazine reversed the oxidative damage caused by MTX.

CONCLUSIONS: The cell viability increased the levels of oxidant and prooxidant markers and decreased the levels of antioxidant markers decreased in H9c2 cardiomyocytes induced by oxidative stress. These results suggest that ranolazine may protect the cardiomyocytes from MTX-induced oxidative damage. The effects of ranolazine could result from its antioxidant properties.

Key Words:
H9c2 cardiomyocyte cells, Methotrexate, Ranolazine, Hypoxia-inducible factor-1α, Oxidative stress, Antioxidant.

Introduction

Ranolazine is an orally active piperazine derivative. Opinions and information about the mechanism of action of ranolazine have changed over time. Originally considered a "metabolic modulator", the drug is now considered a "selective late-sodium current-blocker (late-INa)[1]". Ranolazine was approved for use as an antianginal drug in 2006 by the US Food and Drug Administration (FDA)[2].

Initially, the first known action mechanism of ranolazine is a 'metabolic modulator' that enables the cell to perform glucose oxidation by preventing anaerobic glycolysis of cells that cannot perform oxidative phosphorylation in the event of ischemia. The need for adenosine triphosphate (ATP) in the myocardium is met in three ways: anaerobic glycolysis, fatty acid oxidation and glucose oxidation. Since more ATP is synthesized by glucose oxidation than anaerobic glycolysis in case of insufficient oxygen, aerobic glycolysis is more efficient[3]. There are also studies[4-11] demonstrating its antioxidant, anti-inflammatory and reducing effects on ischemia-reperfusion injury.

Methotrexate (MTX) is from the group of antimetabolites, which is an antineoplastic drug group. It acts as a folic acid antimetabolite. However, the cytotoxic effects of MTX not only affect tumor cells, but also affect vital organs such as the liver, kidney, and heart. Although the toxic effect mechanism of MTX has not been fully elucidated, various mechanisms are thought to play a role. Recent toxicity studies in literature with anticancer drugs have focused on oxidative stress. Despite these wide usage indications, MTX presents with side effects such as cardiotoxicity, nephrotoxicity and hepatotoxicity and oxidative damage caused by reactive oxygen species (ROS) has been blamed for these side effects. Therefore, regulating oxidative stress is a potent target for preventing organ damage caused by MTX[12-14]. For this purpose, various antioxidant agents have been tried to protect tissues from MTX-induced liver, kidney and heart damage and have been significantly successful[12-20].

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Rat H9c2 (embryonic rat ventricular cardiomyoblast) is a widely used cell line model for the evaluation of cardiac injury mechanisms. In this study, the effects of ranolazine on hypoxia-inducible factor-1α (HIF-1α) levels, lipid peroxidation [malondialdehyde (MDA), lipid hydroperoxide (LOOH)], protein oxidation (advanced oxidation protein products, AOPP), pro-oxidant enzyme [xanthine oxidase (XO)] and antioxidant effects [total thiol, T-SH catalase (CAT); total antioxidant capacity (TAC)] in inflammatory pathways during cell death, in cells under hypoxic conditions due to oxidative stress induced by MTX, were investigated in H9c2 cardiomyocytes.

Materials and Methods

Reagents and Antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-MTT, Ripa Lysis Buffer and Protease Inhibitor Coctail set were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). High-glucose Dulbecco's modified Eagle's (DMEM), fetal bovine serum (FBS), D-PBS, 0.25% Tripsin-EDTA and antibiotics (100 U/mL penicillin and 100 U/mL strepto¬mycin) were purchased from Euroclone (Milan, Italy). Methotrexate (MTX, Submex; Abdi Ibrahim) and ranolazine (Latixa, Menarini, Florence, Italy) were also used in the study.

Cell Culture and Treatment

H9c2 (2-1) cardiomyocyte cells were purchased from the American Type Culture Collection (ATCC, Manassas, MA, USA). Cells were cultured in DMEM supplemented with 10% heat inactivated FBS and antibiotics (100 U/mL penicillin and 100 U/mL strepto¬mycin) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were subcultured with 0.25% Tripsin-EDTA. The trypsin-EDTA-cell suspension was centrifuged at 120 xg for 5 min. After centrifugation, the supernatant was discarded, fresh medium was added to the cell pellet, and cells were seeded into 96 well plates at $1x10^4$/well. The effects on cell viability were analyzed by applying MTX and ranolazine at different concentrations and times.

Cell Viability Assay

Cell viability was determined using the MTT reduction assay. Briefly, H9c2 cells were incubated with DMEM containing 10% FBS overnight in 96 well plates at a density of $1x10^4$ cells/well. After reaching 80% confluence, the cells were washed twice with D-PBS and incubated with medium containing various concentrations of MTX (10-5-2.5-1.25-0.625, 0.312 and 0.156 μM) and Ranolazine (100-50-25-12.5-6.25, 3.125 and 1.562 μM) for 24, 48 and 72 hours at 37°C in a humidified atmosphere containing 5% CO_2. The medium was removed, and 100 μL DMEM and 20 MTT (5 mg/mL) was added to each well for 3 hours. The formazan crystals that formed in intact cells were dissolved in 100 μL dimethyl sulfoxide (DMSO). Absorbance was recorded at a wavelength of 490 nm, and at a reference wavelength of 570 nm, using a microplate reader (Multiskan GO-Thermo, Waltham, MA USA). Using optical density (OD) values, IC_{50} and EC_{50} values for MTX and Ranolazine were calculated as 2.2 μM and 10 μM for 48 hours respectively with the GraphPad Prism 9 program (La Jolla, CA, USA).

Experimental Groups

Groups were formed to form control, 2.2 μM MTX, 10 μM Ranolazine and 2.2 μM MTX + 10 μM Ranolazine. Cells were seeded at $3\text{-}4x10^5$ cells/well on 6 well plates for each group. 24 hours after cell seeding, the control group was replaced with the normal medium and the others with the medium containing the MTX and Ranolazine alone or in combination at the given concentrations. While the other groups were incubated for 48 hours, the MTX + Ranolazine group was treated with Ranolazine for 48 hours after 48 hours of MTX. At the end of the experiment, cell lysates were prepared for the measurement of HIF-1α, oxidant and antioxidant parameters from all groups.

Cell Lysate Preparation

At the end of the experiment, cell lysate was prepared from all groups using 1xRipa Lysis Buffer and Protease Inhibitor Coctail set. After washing the cells 2 times with cold 1xPBS, 300 μL of Ripa lysis buffer (0.5 M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) and protease inhibitor cocktail (1:200) was inserted. Cells were blasted by pipetting on ice, and the cell suspension was incubated for 30 min in a shaking water bath at +4°C. Afterwards, it was centrifuged at 14,000 xg at +4°C for 30 minutes. At the end of centrifugation, the supernatants were transferred to new Eppendorf and the obtained cell lysates were stored in a deep freezer at -80°C until measurement.

Measurement of Hypoxia-Inducible Factor 1α (HIF1α) Levels

Cell lysate HIF-1α levels were measured by a commercially available sandwich enzyme linked immunoassay kit (Enzyme Linked Immunosorbent Assay, Cat No: E0422Hu, Bioassay Technology Laboratory, Shanghai, China). The coefficients of intra and inter assay variation were 5.8% (n=6) and 6.7% (n=6), respectively.

Measurement of Malondialdehyde (MDA) Levels

Lipoperoxidation was estimated by the formation of MDA, which was analyzed using the modified thiobarbituric acid method[21]. The coefficients of intra and inter assay variation was 3.3% (n=6) and 3.9% (n=6), respectively.

Measurement of Lipid Hydroperoxide (LOOH) Levels

The spectrophotometric determination of LOOH levels were also performed using the method of ferrous oxidation with xylenol orange version 2 (FOX2)22. The coefficients of intra and inter assay variation was 4.2% (n=6) and 5.1% (n=6), respectively.

Measurement of Advanced Oxidation Protein Products (AOPPs) Levels

A modification of the Gelişgen et al[23] method was used for spectrophotometric determination of concentration of AOPPs. The coefficients of intra and inter assay variation was 4.7% (n=6) and 5.8% (n=6), respectively.

Measurement of Xanthine Oxidase (XO) Activity

The measurement of XO activity is based on the fact that XO in the sample forms uric acid from xanthine. To determine XO activity, xanthine and 100% trichloroacetic acid (TCA) solution was used, as introduced by Prajda and Weber[24]. The coefficients of intra and inter assay variation was 5.5% (n=6) and 6.7% (n=6), respectively. The coefficients of intra and inter assay variation was 5.5% (n=6) and 6.7% (n=6), respectively.

Measurement of Total Thiol (T-SH) Levels

To determine plasma T-SH concentration, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) was used, as introduced by Hu[25]. The intra- and inter-assay variation coefficients were 2.9% (n=5) and 3.5% (n=6), respectively.

Measurement of Catalase (CAT) Activity

CAT activity was measured in terms of the extent of hydrogen peroxide breakdown, as catalyzed by the enzyme[26]. The coefficients of intra and inter assay variation was 4.9% (n= 6) and 6.1% (n=6), respectively.

Measurement of Total Antioxidant Capacity (TAC) Levels

Ferric reducing antioxidant power (FRAP) assay was used for the nonenzymatic antioxidant level of cells and the method of Benzie and Strain[27] was used. The coefficients of intra and inter assay variation was 5.1% (n=6) and 6.9% (n=6), respectively.

Statistical Analysis

The distribution of all analyzed parameters was confirmed using the Shapiro-Wilk test. All parameters were normally distributed and expressed as mean ± standard deviation. One-way ANOVA and LSD test as post-hoc were used in the comparison of groups. Correlation analysis was performed using Pearson's correlation analysis. A *p*-value below 0.05 was expressed as significant. All statistical analyses were carried out using Statistical Product and Service Solutions (SPSS) v. 21.0 (IBM Corp., Armonk, NY, USA) package program.

Results

MTX Inhibits Cell Viability in H9c2 Cells

H9c2 cells were treated with various concentrations of MTX for 24, 48 and 72 hours. The results of an MTT assay demonstrated that the number of viable cells decreased in response to the increased concentration and times of MTX treatment (Figure 1).

Protective Effects of Ranolazine on H9c2 Cells

H9c2 cells were treated with various concentrations of ranolazine for 24, 48 and 72 hours. The results of an MTT assay demonstrated that the number of viable cells increased in response to the increased concentration and times of ranolazine treatment (Figure 2-3).

Ranolazine Reverses the Oxidative Stress Caused by MTX in H9c2 Cells

Oxidative stress markers (AOPP, MDA, LOOH and XO activity) and HIF-1α increased and antioxidant capacity markers (T-SH, CAT activity

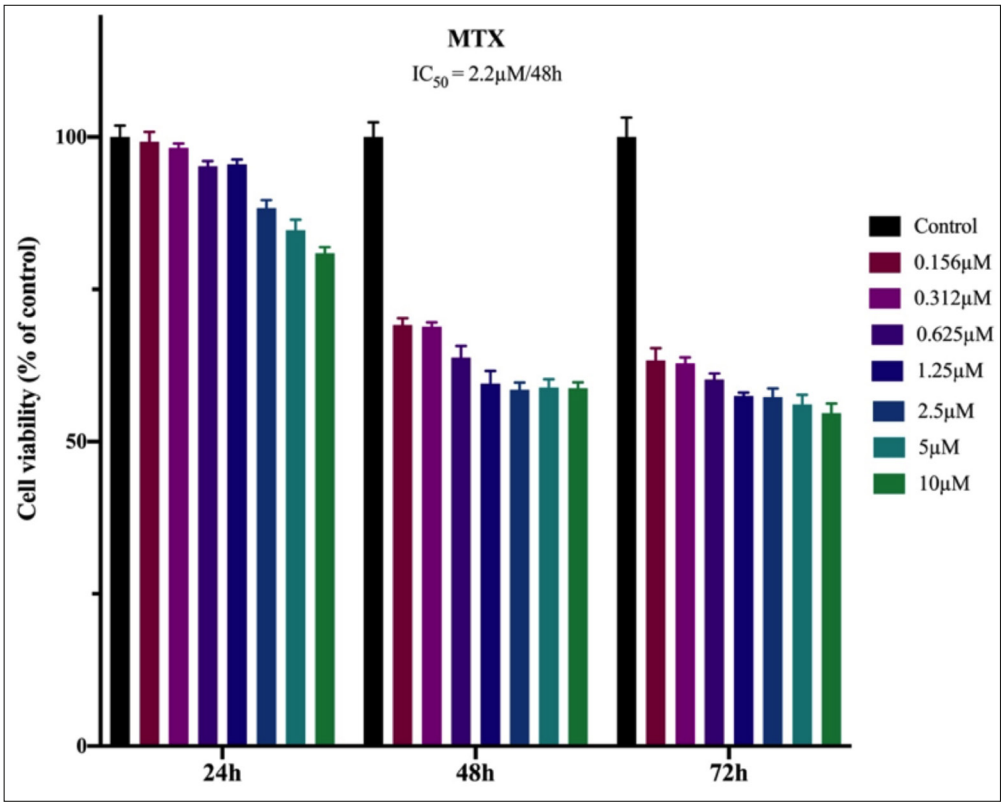

Figure 1. Time- and dose-dependent cytotoxic effect of MTX on the H9c2 cells. The effects of MTX on the viability of H9c2 cells were measured using an MTT assay. Cells were treated with various concentrations of MTX (10-5-2.5-1.25-0.625, 0.312 and 0.156 µM) for 24, 48 and 72 hours. IC_{50} value was calculated as 2.2 µM for 48 hours. Data are presented as the mean ± standard error of the mean (n=6).

and TAC) decreased in MTX-treated cells compared to control cells. On the contrary, oxidative stress markers decreased and antioxidant capacity markers increased in cells treated with ranolazine alone, compared to control cells (Figure 4-5).

Correlation of Levels of HIF1α with Oxidative Stress Markers in H9c2 cells

To calculate the correlation of level of HIF-1α and oxidative stress markers, Pearson's correlation coefficient was used. Correlation results showed that HIF-1α increase with oxidative stress and decrease with antioxidant increase. The change of HIF-1α in each group was examined separately and it was found that it showed positive correlation with AOPP, MDA and LOOH in both the control, MTX and MTX+ Ranolazine group. While XO activity showed a significant positive correlation with HIF1α in the other three groups, it was found close to the limit of significance in the Ranolazine group (Figure 6). Similarly, there was a negative correlation between antioxidant parameters and HIF-1α levels. While the correlation between CAT activity and HIF-1α could not reach the limit of significance in the Ranolazine group, there was a negative correlation in all other groups separately, while T-SH and TAC showed a significant negative correlation with HIF in all groups (Figure 7). Finding the correlation data especially strongly negative ($r < -0.8$) or strongly positive ($r > 0.8$) provided us with strong evidence for the change of HIF-1α according to oxidant and antioxidant status.

Discussion

Ranolazine is a piperazine derivative that has been approved as an antianginal agent to treat patients with chronic angina in clinical practice. The most important feature of ranolazine is that it only acts on ischemic cells rather than normal myocytes at therapeutic doses[1]. In the current study, MTX-induced oxidative stress in H9c2 cardiomyocytes decreased cell viability and the

levels of antioxidants, while increasing the levels of oxidants and hypoxia. Treatment with ranolazine increased cell viability and reduced oxidative stress in both H9c2 cardiomyocytes and oxidative stress-inducing cells. This study suggests that ranolazine, a P-glycoprotein inhibitor, can be used as an antianginal drug as well as a drug that reduces oxidative stress and hypoxia, which have an important role in the etiology of heart diseases. Since there is no study in the current literature on oxidative stress and hypoxia of ranolazine in cell culture, our study suggests that it is a pioneering study on this subject. However, the mechanism by which it acts needs to be revealed by comprehensive studies at the cellular level in experimental and human study.

It has been shown[28] that MTX, widely used in the treatment of several cancers, causes a significant decrease in cell viability and cell migration in glioblastoma cells, leading to cell cycle arrest in S phase. In previous studies[29-31], it has been reported that ranolazine protects heart function against cardiotoxicity caused by both doxorubicin and trastuzumab by reducing the effects of oxidative stress. Aldasoro et al[32] showed that ranolazine significantly increased cell viability and proliferation over 24 hours in cultured astrocytes. Also, ranolazine has been shown[33] to promote cell viability in C2C12 skeletal muscle cells. To determine whether ranolazine could protect from MTX-induced cardiotoxic effects in H9c2 cardiomyocytes, cells were pretreated with MTX in the presence or absence of ranolazine. Following the administration of MTX for 48 hours, cell viability was examined. It was found that MTX decreased cell viability and when treated with ranolazine, cell viability was increased compared to the MTX group alone.

In hypoxic conditions, two main signaling systems are activated in the body: AMP activated protein kinase (AMPK) and "hypoxia-inducible factor" (HIF) pathways[34]. When the intracellular ATP level decreases, the AMPK pathway is activated, inhibiting anabolic processes while accelerating catabolic processes. HIF plays a key role in the cellular response to hypoxia in all mammalian cells[35]. Majumder et al[36] reported ranolazine treatment reduced HIF-1α in ischemic cells. In another study[37], ranolazine reversed the oxidative metabolic shift in ApoE-/-/LDLR-/- mice and reduced cardiac damage induced by hypoxia. While HIF-1α degrades rapidly in normoxia (21%

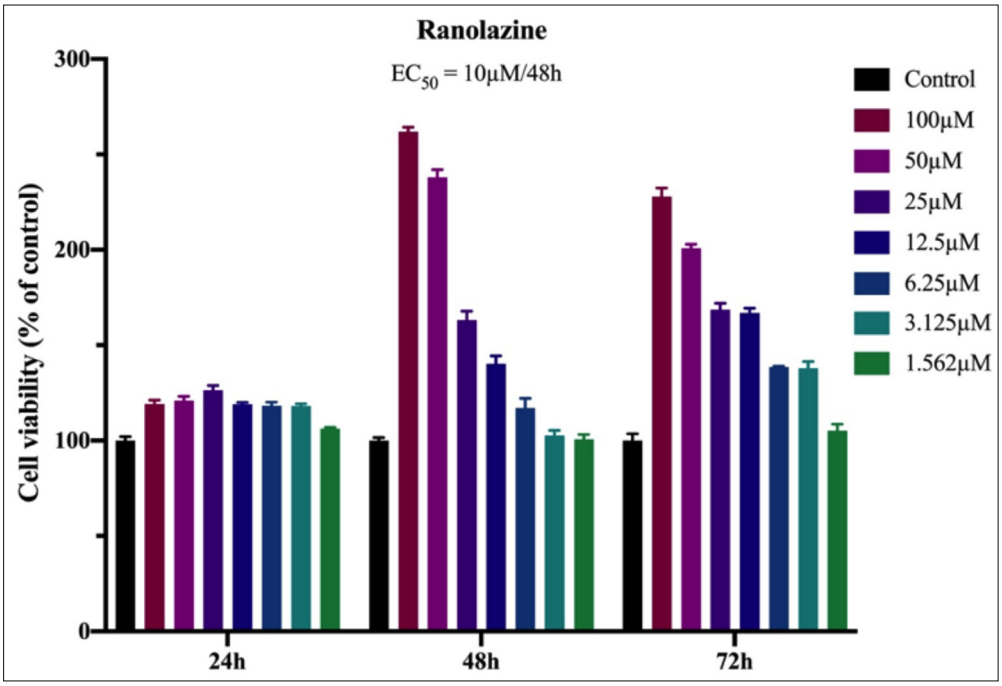

Figure 2. Time- and dose-dependent effect of ranolazine on the H9c2 cells. Time- and dose-dependent protective effect of Ranolazine on the H9c2 cells. The effects of ranolazin on the viability of H9c2 cells were measured using an MTT assay. Cells were treated with various concentrations of ranolazine (100-50-25-12.5-6.25, 3.125 and 1.562 μM) for 24, 48 and 72 hours. The EC_{50} value was calculated as 10 μM for 48 hours. Data are presented as the mean ± standard error of the mean (n=6).

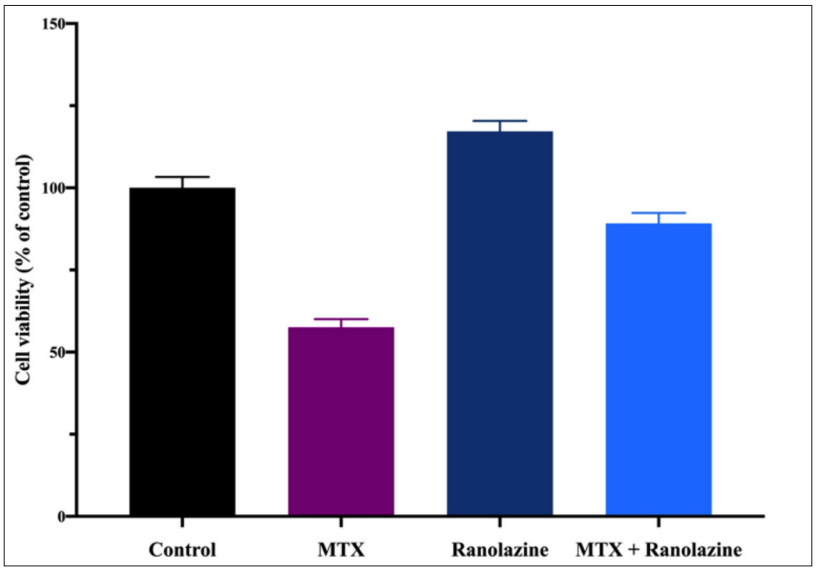

Figure 3. Protective role of ranolazine against MTX-induced damage on the H9c2 cells. The effects of MTX and ranolazine on the viability of H9c2 cells were measured using an MTT assay. After 48 hours of 2.2 µM MTX treatment, cells were treated with 10 µM ranolazine for 48 hours. Data are presented as the mean ± standard error of the mean (n=6). MTX: Methotrexate.

oxygen), its degradation slows down when the oxygen concentration in the environment drops to 5%. For this reason, HIF-1α measurement is accepted as an indicator of hypoxic conditions in *in vitro* studies[38,39]. In the current study, HIF-1α levels were investigated in order to show that the

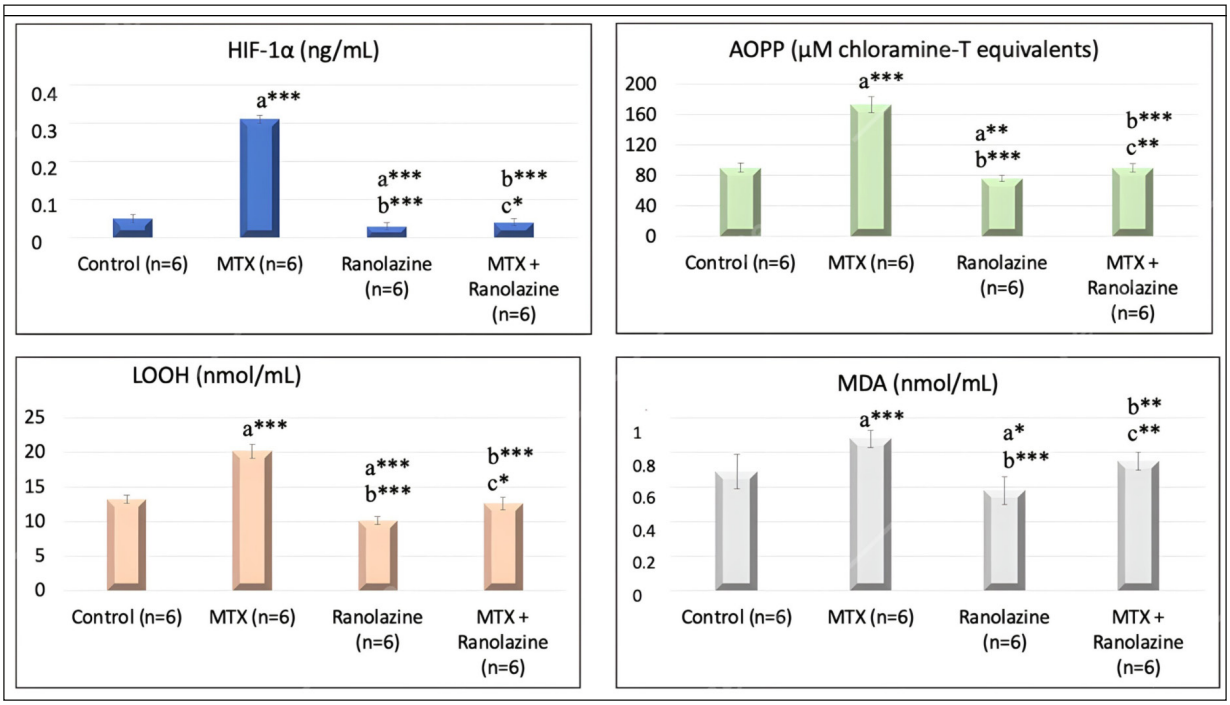

Figure 4. **A**, HIF-1α levels, (**B**) AOPP levels, (**C**) LOOH levels, (**D**) MDA levels in H9c2 cardiomyocytes under hypoxic conditions due to oxidative stress-induced hypoxia. *p<0.05; **p<0.01; ***p<0.001; a: *vs.* control; b: *vs.* MTX; c: *vs.* Ranolazine. MTX: Methotrexate; HIF-1α: Hypoxia-inducible factor 1-alpha; MDA: malondialdehyde; LOOH: lipid hydroperoxide; AOPP: advanced oxidation protein products; XO: xanthine oxidase; T-SH: total thiol; CAT: catalase; TAC: total antioxidant capacity.

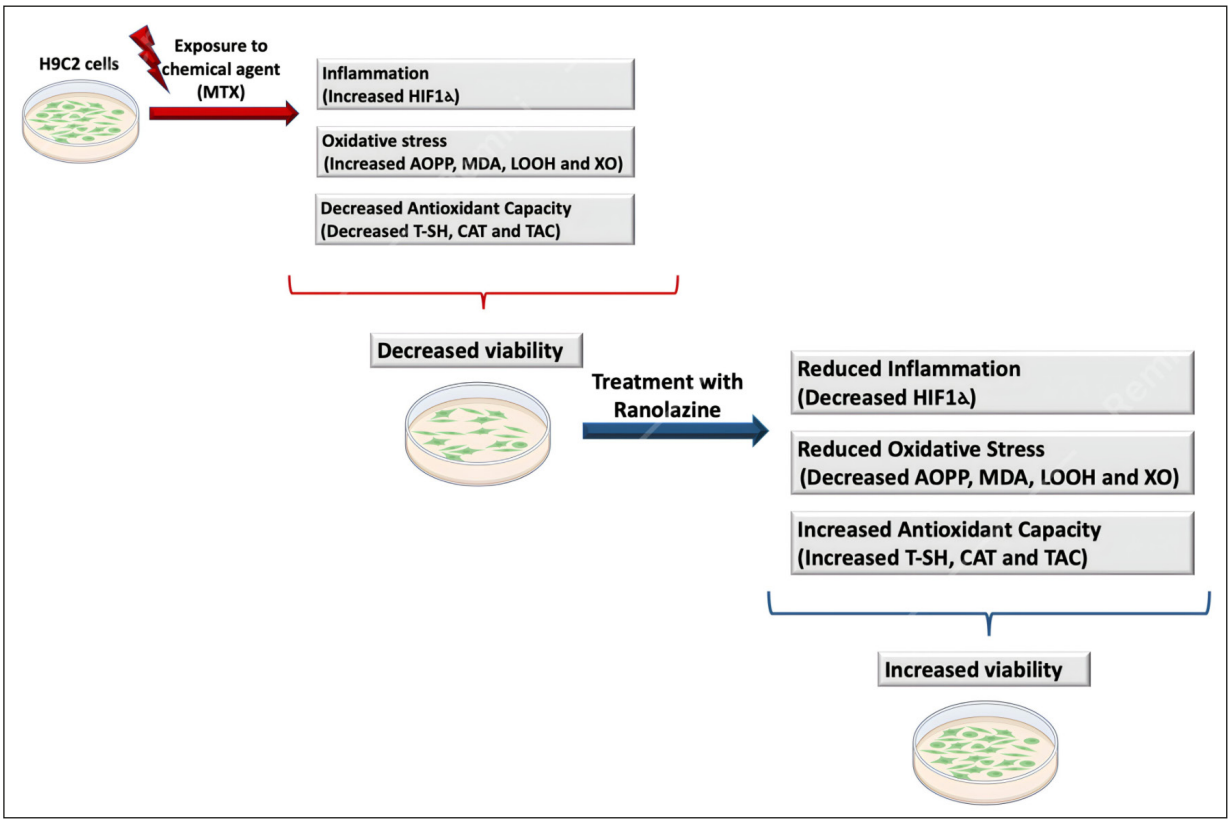

Figure 5. Ranolazine increases cell viability by reversing the oxidative and inflammatory damage caused by MTX.

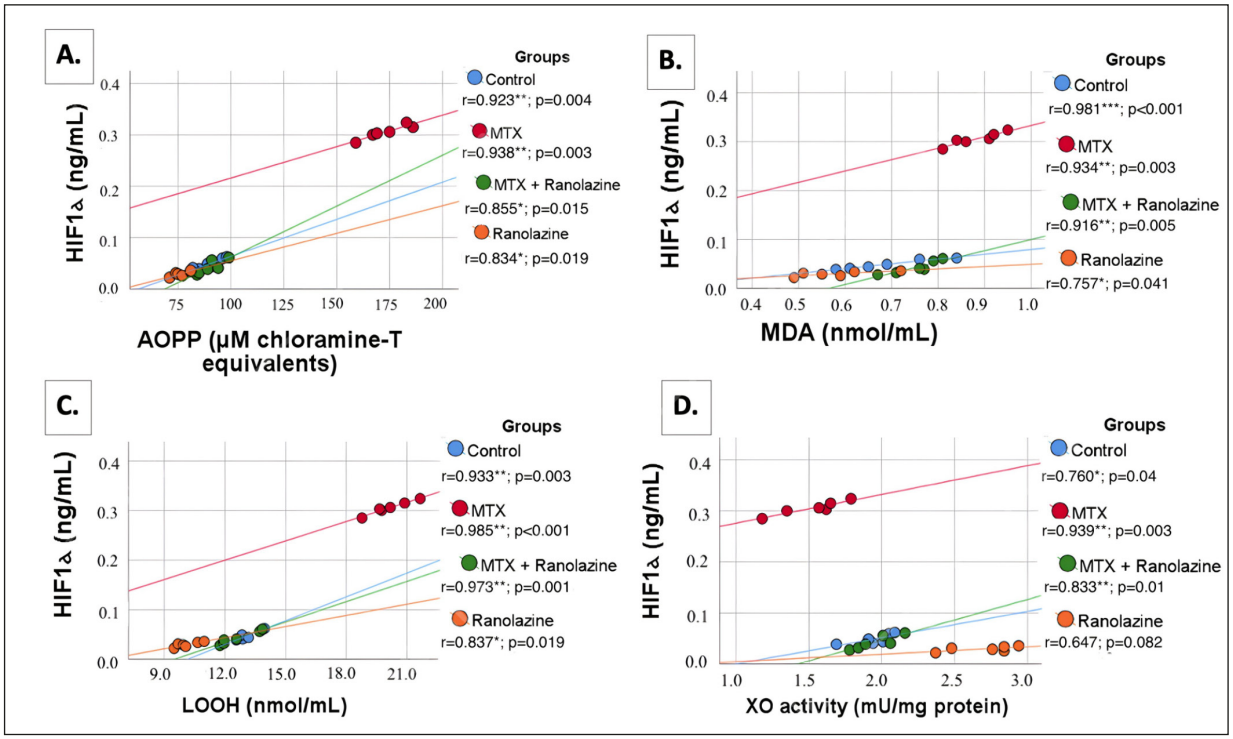

Figure 6. Pearson's correlation scatter plot of the H9c2 cells lysate level of HIF-1α and oxidative stress markers in all groups. Correlation between the level of HIF-1α and AOPP (**A**), MDA (**B**), LOOH (**C**) levels and XO activity (**D**) in H9c2 cells lysate (n=6). (Correlation is significant at 0.05 levels). MTX: Methotrexate; HIF-1α: Hypoxia-inducible factor 1-alpha; MDA: malondialdehyde; LOOH: lipid hydroperoxide; AOPP: advanced oxidation protein products; XO: xanthine oxidase. *p<0.05; **p<0.01; ***p<0.001.

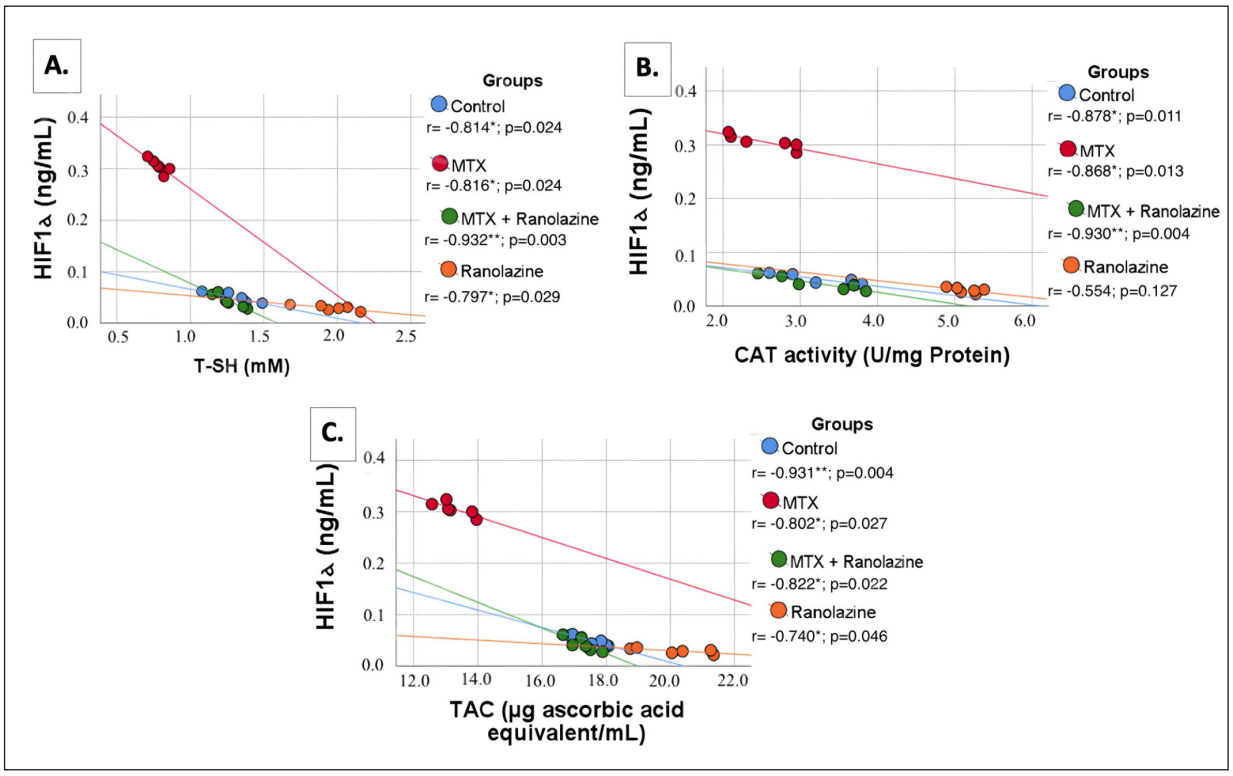

Figure 7. Pearson's correlation scatter plot of the H9c2 cells lysate level of HIF-1α and antioxidant capacity markers in all groups. Correlation between the level of HIF-1α and T-SH (**A**) levels, CAT activity (**B**) and TAC (**C**) levels in H9c2 cells lysate (n=6). (Correlation is significant at 0.05 levels). MTX: Methotrexate; HIF-1α: Hypoxia-inducible factor 1-alpha; T-SH: total thiol; CAT: catalase; TAC: total antioxidant capacity. *p<0.05; **p<0.01; ***p<0.001.

environment remains hypoxic due to oxidative stress at the cell level. It was found that HIF-1α levels were increased in MTX-treated cells compared to control cells, and HIF-1α levels were decreased after the addition of ranolazine. While the HIF-1α levels is also positively affected by oxidant molecules (AOPP, MDA and LOOH), HIF-1α is negatively regulated by antioxidants (especially T-SH and TAC). Oxidative stress is an important pathophysiological process in the cardiomyocytes. HIF-1α plays an important role in hypoxia after oxidative stress. Oxidative stress may increase HIF-1α production to create a positive feedback loop. These findings show that hypoxic conditions occurred in our study, and that ranolazine reduced oxidative stress-induced hypoxia in H9c2 cardiomyocytes. New studies are needed to reveal its effectiveness, the mechanisms of action and dose-dependent effects.

Oxidative stress occurs as a result of decreased activity of antioxidant enzyme systems and excessive production of ROS. Cardiac toxicity after MTX administration has been previously reported in studies[40,41] of arrhythmias, hypotension, and cardiac arrest. The drug has various toxic effects on the liver, kidneys, heart, gastrointestinal and nervous system. MTX induces the formation of free radical species and ROS, which is formed in excess, and causes lipid and protein damage in the cell[12-14]. Therefore, it is important to evaluate agents with cardiac protective activities against MTX-induced oxidative damage. Abdel-Daim et al[12] investigated the effects of diosmin, a biologically active flavonoid, against MTX-induced liver, kidney, and heart damage in mice. They reported[12] that MTX caused a decrease in antioxidant as SOD, CAT, glutathione peroxidase (GSH-Px) and glutathione (GSH) levels and an increase in MDA levels in tissues. Abo-Haded et al[14] stated that MTX-induced liver toxicity caused a decrease in SOD and GSH levels in liver tissue and a significant increase in MDA levels in the MTX-administered group compared to the control group. The current results indicated that oxidative stress markers (AOPP, MDA, LOOH and XO activity) and antioxidant capacity markers (T-SH, CAT activity and TAC) decreased in MTX-treated cells compared to control cells. The results of previous studies[12,14] are in agreement with our

study. In 1998, Matsumura et al[42] reported that ranolazine reduced the mechanical and metabolic disorders induced by H_2O_2, but the mechanism is unknown. This action of ranolazine may contribute to its protective action on the myocardium from ischemia-reperfusion damage. El Amrani et al[43] reported that ranolazine decreases inflammatory mediators IL-1β and TNF-α and increases anti-inflammatory PPAR-γ as well as the antioxidant SOD in primary cultured astrocytes. These results suggest that ranolazine could be useful as a neuroprotective drug in pathologies inducing inflammatory damage and oxidant processes. Aldasoro et al[32] showed that ranolazine significantly increased cell viability, the activity of Cu/Zn-SOD and Mn-SOD as antioxidant proteins significantly increased in cultured astrocytes. In the current study, oxidative stress markers decreased, and antioxidant capacity markers increased in cells treated with ranolazine alone, compared to control cells. This study demonstrated that the levels of oxidant and antioxidant markers in cells treated with MTX and ranolazine together reached the level of the control group, and ranolazine reversed the damage caused by MTX. Ranolazine attenuates MTX-induced oxidative toxicity in H9c2 cardiomyocytes partly through ameliorating oxidative stress by decreasing the MDA, LOOH, AOPPs, XO activity and preserving the level of T-SH, TAC, and activity of CAT. As in other studies[44-46] and our study, ranolazine is a novel pleiotropic drug with a new mechanism of action that seems to abolish most of the adverse hemodynamic effects.

Limitations of the Study

Although our study has strengths, it has some limitations. The lack of transmission electron microscopic data is the shortcoming of the study. It needs to be performed with *in vivo* data to make the study more rigid.

Conclusions

To generate oxidative stress, toxic agents like MTX are generally used in experimental studies. Oxidative stress and hypoxia have been proved to induce oxidative stress by MTX. Our results suggest that ranolazine is potentially protective against MTX-induced cardiotoxicity. Ranolazine attenuates MTX-induced oxidative toxicity in H9c2 cardiomyocytes partly through ameliorating oxidative stress by decreasing the MDA, LOOH, AOPPs, XO activity and preserving the level of T-SH, activity of CAT and TAC levels as well as inhibiting the expression of HIF-1α inflammatory pathway. Results of the study demonstrated the pharmacological effectiveness of ranolazine on H9c2 cardiomyocytes toxicity and explored its potential mechanism, which provided a new insight into the prevention of oxidative stress and hypoxia.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Availability of Data and Materials

The data underlying this article are available in the article. If needed, please contact the corresponding author.

Ethics Approval

Ethics Committee approval is not required for cell culture studies.

Informed Consent

Not applicable.

Funding

None.

Authors' Contributions

ZD and HU wrote the manuscript and DDE, SD, and RG prepared tables and figures. All authors reviewed the manuscript.

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