

Comparative Study of the Effect of Macrolide Antibiotics Erythromycin, Clarithromycin, and Azithromycin on the ERG1 Gene Expression in H9c2 Cardiomyoblast Cells

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Abstract

Background: Macrolides are a clinically well-established class of antibiotics. Macrolides induce cardiotoxicity by blocking ERG potassium channels in cardiac myocytes. The aim of this study was to compare the effects of erythromycin, clarithromycin and azithromycin on cell viability and expression of ERG1 gene in H9c2 cells.

Methods: Cell viability and ERG1 gene expression of H9c2 cells in 3 different concentrations, 1, 10 and 25µg/ml, after 48 and 72 hours were determined by MTT test and Real time-PCR method respectively.

Results: According to the results, these antibiotics decreased cell viability in a concentration and time-dependent manner. After 48 hours, the growth of H9c2 cells treated with erythromycin and clarithromycin (at 1 and 25 µg/ml) were inhibited significantly compared to control group ($p < 0.05$). All three groups of antibiotics showed toxic effects on cells after 72 hours in all concentrations. Azithromycin-inhibiting effects were significantly higher than two other groups after 72 hours of treatment. The expression of ERG1 gene increased in all three groups of antibiotics by increasing the concentration and duration of treatment. Azithromycin had the most pronounced effect on ERG1 expression in 48 and 72 hours.

Conclusions: This study indicated that these macrolides affect ERG1 expression due to their potential cardiac adverse effects. Further investigations are required to understand the exact mechanism of cardiotoxicity associated with macrolides.

Background

Macrolides represent one of the most frequently prescribed groups of antibiotics characterized by a distinctive large macrocyclic lactone ring varying in size from 14 to 16 atoms [1]. The antimicrobial spectrum of these antibiotics such as azithromycin, clarithromycin, and erythromycin is slightly broader than that of penicillin [2]. They can be appropriate alternatives to penicillin for patients with a penicillin allergy [3–4]. A wide variety of infections including respiratory, skin, and soft tissue bacterial infections are commonly treated by macrolides with a potential impact against pathogens, such as Legionella, Chlamydia, Streptococci, Campylobacter spp., and Branhamella spp. [5–6]. They are administered in both long term and short-term therapeutic approaches. Although this class of antibiotics is more often known as bacteriostatic drugs, they can exhibit the bactericidal effects at high concentrations [7]. In fact, they terminate protein synthesis in bacteria and result in bacterial growth prevention by binding to the 50 s ribosomal subunit [8]. The safety profile of macrolides requires accurate scrutiny due to the popularity and widespread use of them. Despite the obvious benefits in the treatment of several infectious diseases, some studies suggest that these antibiotics may induce cardiac toxicity recognized most commonly in patients already have experienced cardiovascular diseases [9, 10]. This rare adverse cardiovascular toxic effect is mainly manifested by prolongation of the QT interval on the electrocardiogram (ECG). The QT prolongation and cardiac arrhythmias that are attributed to macrolides may be presenting as fatal Torsade de Pointes (TdP), and sudden cardiac death especially in patients with coronary heart disease.

Even though the reports indicate that macrolide treatment may cause low incidence of cardiotoxicity, these side effects have raised more attention recently [9, 10 &11].

The Food and Drug Administration (FDA) has warned the public and health care professionals regarding the potentially life-threatening arrhythmias, QT prolongation, TdP, and sudden cardiac deaths with azithromycin, clarithromycin, and erythromycin [12–17].

Previous studies have presented that the delayed rectifier potassium current (I_K), playing a pivotal role in ventricular repolarization, is inhibited by erythromycin and this results in QT prolongation [18, 19]. Additionally, it has been reported that clarithromycin and erythromycin induced long QT syndrome and TdP by suppressing hERG (Human ether-a-go-go related gene) potassium ion channel function [20, 21]. In patients diagnosed with QT prolongation and in healthy patients with normal QT interval, azithromycin has been shown to cause TdP [22, 23].

Ionic channels have a key role in heart functions. A network of ionic channels, including potassium channels, regulates action potential of cardiac cells. The K₁ voltage-gated potassium channels are critical for their contribution in cardiac action potential repolarization [24]. The hERG1 gene encodes the K₁ voltage-gated potassium channel, which regulates the duration of cardiac action potential. Its mutation is associated with abnormal heart rhythms, and sudden death.

The cardiac safety profiles of erythromycin, azithromycin, and clarithromycin have not been extensively investigated. The aim of this study was to quantify and compare the risk of cardiotoxicity among these three macrolides using MTT assay for evaluating cell viability, and PCR (Polymerase chain reaction) method for measuring expression of ERG1 gene in the H9c2 cell line derived from embryonic rat heart tissue. According to our results, erythromycin, clarithromycin and azithromycin decrease cell growth and increase ERG1 expression in a time/concentration-dependent manner. Our findings may help health care professionals to prescribe the most appropriate types of macrolides in order to minimize the potential risk of cardiotoxicity related to these drugs particularly in patients with a previous diagnosis of cardiovascular diseases.

Methodology

Chemicals

High glucose Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA (0.25%), and Dulbecco's Phosphate-buffered saline (PBS) were purchased from BIO-IDEA (Tehran, Iran). Dimethyl Sulfoxide (DMSO) and 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium (MTT) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Penicillin-streptomycin was purchased from Gibco (USA). Erythromycin, clarithromycin and azithromycin were obtained from Shifa Pharmed Industrial Group (Tehran, Iran).

Cell Culture and Cell Treatment

H9c2 rat cardiomyoblast cell line was obtained from Pasteur Institute Cell Bank (Tehran, Iran). The cells were cultured in high glucose DMEM, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin antibiotics. Cells were incubated at 37 °C in a humidified cell culture incubator (5% CO₂). Cells were proliferated in 75 cm² cell culture flasks and sub-cultured once they reached 70 % confluency. They were washed with PBS solution, trypsinized with 0.25% trypsin and EDTA solution, and centrifuged at 1800 rpm for 5 min. In order to prepare cells for treatment, the suspension of 2 × 10⁴ cells in DMEM added into each well of a 96-well plate and incubated for 24 hours. After 24 hours, various concentrations (1, 10 and 25 µg/ml) of azithromycin, erythromycin or clarithromycin were added to wells. To prepare the considered concentration, all three antibiotics were dissolved in DMSO solvent. Then, three considered dilutions were made with culture medium. After diluting and adding the drugs, the plates were placed in an incubator and examined at 48 hours and 72 hours after incubation using MTT test.

Cell Viability Test

Following treatment, cell viability was evaluated using a MTT assay. After removal of the culture medium, cells were washed with PBS and incubated with 20 µl/well MTT solution (5 mg/ml MTT dissolved in PBS) for 4 hours at 37°C. After the incubation time, the top solution was removed and 150 µl/well of DMSO solution was added to all wells. Cells were shaken at room temperature for 10 minutes until formazan crystals were dissolved and yielded a blue-purple color. The plate was placed in Elisa reader and the absorbance of wells was measured in 570 nm. The results were reported as percentage of the control. All experiments were conducted in triplicate wells and repeated twice.

Real-time polymerase chain reaction

Total RNA was extracted from H9c2 cells using the YT9063 kit and purified according to the manufacturer's instructions. First-strand cDNA synthesis was performed also using the YT4502 kit purchased from Yekta Tajhiz Azma. (Tehran, Iran). Real-time reverse transcriptase PCR was performed using a Corbett RG-6000 realtime PCR machine. Master Mix (primer 0.8 µl, H₂O 2.2 µl, cDNA 0.2 µl & Cyber 5 µl) was prepared and added to strip cap micro tube. Temperature-time cycle was as follow, step1: temperature 95°c for 15 second, step2: temperature 95°c for 5 second and step3: temperature 60°c for 30 second. The reactions were performed in 45 cycles and then samples were run for the dissociation protocol [i.e. melting curve analysis]. The expression levels of the tested genes were determined by the $\Delta\Delta C_t$ method. All results were presented as mean \pm S.D. or SE. The internal control was β -actin.

The sequences of primers used for qPCR were as follows:

β -actin-F: 5'-TTCTACAATGAGCTGCGTGTG-3'

β -actin -R: 5'-GGGGTGTGGAAGGTCTCAAA-3'

ERG1-F: 5'-GACACCATCATCCGCAAGTT-3'

ERG1-R: 5'-CGAGAGTAGCCGCACAGT-3'

Statistical analyses

The data, expressed as means \pm SD and means \pm SE, were compared using one-way analysis of variance (ANOVA). Where a significant difference was detected by ANOVA, the treated groups were compared with the control one or with each other using Dunnett and Tukey post-tests respectively. $P < 0.05$ was considered statistically significant.

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [...].

Results

Evaluation of the inhibitory effect of erythromycin, clarithromycin and azithromycin on growth of H9c2 cardiac myoblast cells

In this research, three concentrations of 1, 10 and 25 $\mu\text{g/ml}$ were used for each drug. After adding the drugs, the plates were placed in an incubator and cell viability was examined at 48 and 72 hours after incubation using MTT test. The data obtained from the MTT test after 48 and 72 hours revealed that the erythromycin and clarithromycin antibiotics had cytotoxic effects on the H9c2 myoblasts.

As shown in figure 1, erythromycin and clarithromycin antibiotics at concentrations of 1 and 25 $\mu\text{g/ml}$ showed a significant inhibitory effect on growth of H9c2 cells compared to control group (non-treated) after 48 hours (* $p < 0.05$). No cytotoxic effects were observed at any of the concentrations, 48 hours after treatment in the group treated with azithromycin (Figure 1).

The cytotoxic impact of antibiotics was examined with extending time duration, after 72 hours. The results demonstrated that increasing the concentration and duration of treatment in all three groups augmented toxic effects on cell viability and inhibited the growth of the H9c2 cells compared to non-treated group (Figure 2).

Based on the data presented in Figure 2 and 3, it seems that azithromycin has higher toxicity effects with increasing concentration and treatment duration (72 hours), compared to groups treated with clarithromycin and erythromycin, so that significant difference (**** $p < 0.0001$) was appeared after 72 hours in group treated with azithromycin at all three concentrations.

Examining the expression of ERG1 gene in cardiac myoblast cells H9c2 treated with erythromycin, clarithromycin and azithromycin antibiotics.

Ion channels, including potassium channels, perform the regulation of action potential in the heart cells. ERG or KV11 is a subfamily of the voltage-gated potassium channel superfamily that consists of three members: KV11.1 (ERG1), KV11.2 (ERG2) and KV11.3 (ERG3). Mutation in this channel is related with heart arrhythmias and sudden death [7]. Thus, the effects of three mentioned antibiotics at different concentrations on ERG1 expression in H9c2 cells were investigated in this study using Real Time-PCR.

Based on obtained data (Figure 4), elevated ERG1 gene expression level was determined in all three groups by increasing the drugs concentrations, and statistical analyses revealed that the expression of ERG1 gene were higher level after 48 hours in the azithromycin group, at concentrations of 10 and 25 µg/ml, compared to 1 µg/ml, and from 25 to 10 concentrations with a significant difference of (*p <0.05) and (** p <0.01), respectively. Moreover, by increasing duration of treatment to 72 hours, the expression of ERG1 gene enhanced at concentrations of 10 and 25 µg/ml compared to that at the concentration of 1 µg/ml. Accordingly, increased ERG1 gene expression was recognized in two groups of erythromycin and clarithromycin after 48 hours at two concentrations of 10 and 25 µg/ml (Figure 4). In addition, the expression of ERG1 gene enhanced with extending treatment duration to 72 hours. In other words, ERG-1 gene showed higher level of expression in all three groups of antibiotics by increasing the concentration and duration of treatment in comparison with non-treated group.

The expression of the ERG1 gene was enhanced in azithromycin treated H9c2 cardiac myoblast cells

Based on the data obtained from evaluating gene expression using Real Time-PCR test, increased expression was noticed in all groups over time. In addition, by comparing the data derived from all three groups, it seems that in the group treated with azithromycin, a remarkable increase (***p<0.001) and (****p<0.0001) in ERG1 gene expression was observed after 48 hours at all three concentrations of 1, 10 and 25 µg/ml compared to erythromycin and clarithromycin (Figure 5). Significant increase (**** p <0.0001) in the group treated with azithromycin was seen after 72 hours too (Figure 5). Furthermore, it is noteworthy to mention that the increase in the expression of ERG1 gene after 72 hours was more significant in azithromycin group, compared to erythromycin and clarithromycin groups.

Discussion

Macrolides including azithromycin, clarithromycin, and erythromycin are considered as a broad-spectrum class of antibiotics with an expanding role in treating various bacterial infections. Therefore, their efficacy and safety should be carefully investigated [25–27]. Macrolides are generally considered safe antibiotics. However, like all other drugs, this group of antibiotics also has unwanted adverse effects, the most important of which is cardiotoxicity [28]. Several studies have shown a potential association between macrolides and cardiac toxicity including probability of QT interval prolongation, ventricular tachycardia, TdP arrhythmia, and sudden cardiac death [29–31]. According to considerable evidence, there is a relationship between drug-induced QT interval prolongation and blockade of the hERG cardiac potassium channel [32, 33].

Macrolides such as erythromycin and clarithromycin may contribute in QT interval prolongation and TdP arrhythmia by inhibiting the cardiac hERG potassium channels, which mediate the rapid delayed rectifier K⁺ current (IKr) in cardiac myocytes [34]. QT interval represents the duration between ventricular depolarization and repolarization on the ECG [35, 36]. The prolonged QT interval can be due to congenital heart defects or QT-prolonging drugs, involved in extending the time of the cardiac muscle action potential [37–39]. A network of ion channels, including potassium channels, regulates the action

potential in the heart cells. The ERG or KV11 is a subfamily of the voltage-gated potassium channels superfamily with three members: KV11.1 (ERG1), KV11.2 (ERG2), and KV11.3 (ERG3) channels. Many studies have been conducted on ERG1 channel, which regulates the cardiac action potential duration. Mutation in the ERG1 channel is associated with increased risks of arrhythmia and sudden cardiac death [40–44].

Previous studies have indicated that dysfunction of hERG channel as an adverse effect of macrolides can lead to life-threatening arrhythmias caused by long QT interval syndrome [31, 42–44].

hERG leads to rapid potassium current, which is an important regulator of repolarization of cardiac potential of action. The most known mutations in hERG lead to reduction in potassium current and finally loss or reduction in the function of potassium fast channels. Previous studies have provided evidence suggest that improper function of the hERG channel leads to both inherited and acquired types of long QT interval syndrome [45].

Some studies also suggest that increased reactive oxygen species (ROS) production will change the kinetics of hERG potassium conductivity. In a study conducted by Salimi and his colleagues, the isolated heart mitochondria from cardiomyocytes were exposed to erythromycin, azithromycin and clarithromycin. Their results showed that macrolides induced the production of reactive oxygen species, mitochondrial membranes permeability, mitochondrial swelling and, finally, the release of cytochrome C from the mitochondria of cardiac myocytes. According to these results, damage to heart mitochondria is the starting point for the cardiotoxic effect of the macrolides [1]. Although the mechanisms underlying the cardiotoxicity of macrolides has been investigated to some extent, their effects on the expression of potassium channels genes involved in the QT interval prolongation process have not been completely elucidated yet.

In this research, the cardiotoxic effects of three macrolides antibiotics, azithromycin, erythromycin and clarithromycin, have been evaluated at three concentrations of 1, 10 and 25 µg/ml on H9c2 cell line at 48 and 72 hours. The results demonstrate that toxic effects of these antibiotics on H9c2 cells are depend on drug concentration and exposure time. While they showed cytotoxicity effect after 48 hours, the toxicity of the drugs increased 72 hours after the treatment. It is noteworthy that their significant differences in toxicity compared to the control group were occurred at two concentrations of 10 and 25 µg/ml after 72 hours. Among the three antibiotics, azithromycin showed more severe toxic effects than two other antibiotics, and the highest toxicity was observed at 72 hours. It should be noted that all three drugs had inhibitory effects of less than 30% at three concentrations, and not all three concentrations of drugs did show inhibitory effects led to 50% loss in cellular population (IC50).

The results of this study are partly consistent with those of the study conducted by Ray et al. In a cohort study, they examined the increased risk of death associated with short-term cardiac effects of azithromycin, amoxicillin, ciprofloxacin and levofloxacin drugs in a 5-day course of treatment. Their results revealed that patients received the azithromycin had a greater risk of cardiovascular death and death for any reason in comparison to those who did not received the antibiotic, but an increase in the

risk of death was not reported for patients received the amoxicillin during this period. Compared to amoxicillin, azithromycin was more associated with the risk of cardiovascular death. The cardiovascular death risk was significantly higher with azithromycin than that of ciprofloxacin, but it showed no significant difference with levofloxacin [46].

Antzelevitch and colleagues also reported that erythromycin prolongs QT intervals. Using the whole-cell patch clamp techniques on cardiomyocytes isolated from the pig heart, they suggested that erythromycin had a strong effect on inhibiting the rapidly activating component (IKr) but not the slowly activating component (IKs) of the delayed rectifier potassium current (IK). The inward rectifier current (IK1) was also unaffected [18].

In another study, Ohtani, et al. examined the effect of macrolide antibiotics on cardiac arrhythmias quantitatively. They analyzed the effects of clarithromycin, roxithromycin, and azithromycin on QT interval in terms of pharmacokinetics and pharmacodynamics in comparison with erythromycin in male rats. Their results showed that the rank order of these four antibiotics potencies for QT interval prolongation in rats was as follows: erythromycin > clarithromycin > roxithromycin > azithromycin [47]. The rank order of these antibiotics-induced cardiac arrhythmias is not consistent with the one that we obtained regarding to their effect on cell death and ERG channel gene expression in the H9c2 cell line. This inconsistency may be due to the change of experimental design from animal model in their work to cell line model in our study. On the other hand, azithromycin may enhance the efficiency of the potassium channel by increasing its gene expression and thereby reducing inhibitory effects on the channel, therefore, the toxicity caused by potassium channel inhibition was reduced.

Milberg et al. realized that erythromycin, clarithromycin and azithromycin led to QT interval prolongation, erythromycin and clarithromycin caused TdP and Early after depolarization (EAD) after reducing the potassium concentration. This arrhythmia was not seen in azithromycin case [48]. In addition to their direct effect on the QT interval, erythromycin and clarithromycin have inhibitory effects on the metabolism of some other drugs by inhibiting CYP3A. The incidence of sudden cardiac death was three times more in patients received CYP3A inhibitors in addition to erythromycin compared to those who received erythromycin alone [16, 49].

Han et al. found that mutations in drug-binding sites of the hERG channel could attenuate hERG current obstruction by roxithromycin, but did not significantly modify the disruption of trafficking [50]. According to Hancox et al. study, D85N KCNE1 mutations demonstrated an increase in the sensitivity of IKr/hERG to inhibition with clarithromycin in vitro models [51].

In the present research, H9c2 cells were treated with azithromycin, erythromycin and clarithromycin. In all three cases, after 48 and 72 hours, the expression of ERG1 gene was enhanced at two concentrations of 10 and 25 µg/ml compared to 1 µg/ml. The effect of azithromycin on ERG expression in all three concentrations and after 48 and 72 hours was higher than that of other two drugs, and this result has been confirmed by other studies [52]. According to the results of MTT assay and considering the study

conducted by Salimi et al, it seems that azithromycin has an inhibitory effect on the growth of heart cells through a distinct pathway.

Moreover, our research revealed that the inhibitory effect of clarithromycin is higher than that of other two macrolides. Potassium channels may be more sensitive to clarithromycin and this may play a key role in clarithromycin-induced cardiac arrhythmia development.

Conclusions

Study of cytotoxic effects of erythromycin, azithromycin and clarithromycin elucidates that their effects on cell death can be augmented by increasing the duration of treatment in the following order azithromycin > erythromycin > clarithromycin. Their effects on ERG gene expression and their impacts on potassium channel function are ranked as azithromycin > erythromycin > clarithromycin. As a result, it may be concluded that, primary toxic effect caused by azithromycin can be reduced due to its enhancement of ERG gene expression by increasing the treatment time. Obviously, future investigations are necessary to validate these observations.

Abbreviations

ECG: electrocardiogram; TdP: Torsade de Pointes; FDA: Food and Drug Administration; hERG: Human ether-a-go-go related gene; RT-PCR: Real-time polymerase chain reaction; IKr: rapid delayed rectifier K⁺ current; IKs : slowly activating component; EAD: Early after depolarization; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; PBS: Phosphate-buffered saline; DMSO: Dimethyl Sulfoxide ;MTT: 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium; IC50: Half-maximal inhibitory concentration

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

There is no data other than the data given in the article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, F.T.; methodology, F.T and F.S.; investigation, N.H.; B.B.; writing—original draft preparation, N.H.; N.P.; A.P.; writing—review and editing, N.P.; F.T.; supervision, F.T.; project administration, F.T.; funding acquisition, N.H.

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Figures

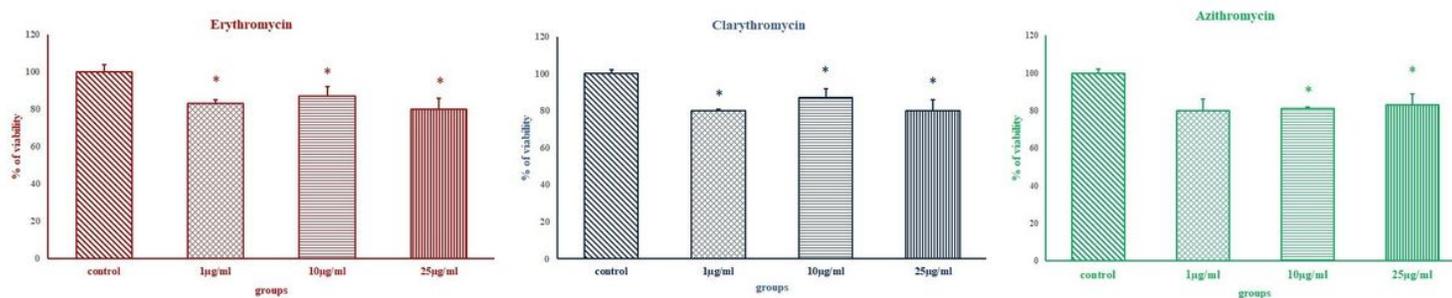


Figure 1

Cytotoxic effects of (a) erythromycin; (b) clarithromycin and (c) azithromycin on H9c2 cardiac myoblast cells after 48 hours of treatment. Cells were treated with 1, 10 and 25 µg/ml of three drugs, and cell

viability was evaluated by using MTT test. The data are presented as three independent tests mean \pm SE. * $p < 0.05$.

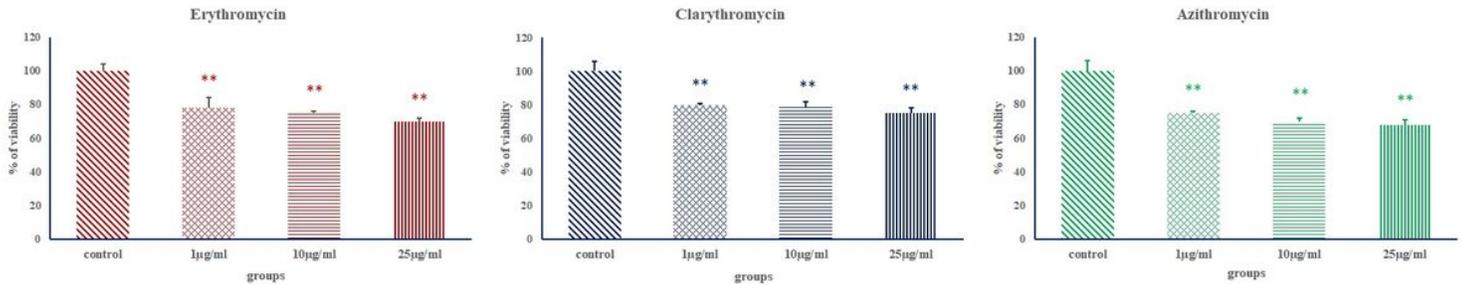


Figure 2

Cytotoxic effects of (a) erythromycin; (b) clarithromycin and (c) azithromycin on H9c2 cardiac myoblast cells after 72 hours of treatment. Cells were treated with 1, 10 and 25 $\mu\text{g/ml}$ of three drugs, and cell viability was evaluated by using MTT test. The results are presented as three independent tests mean \pm SE. * $p < 0.05$ and ** $p < 0.01$.

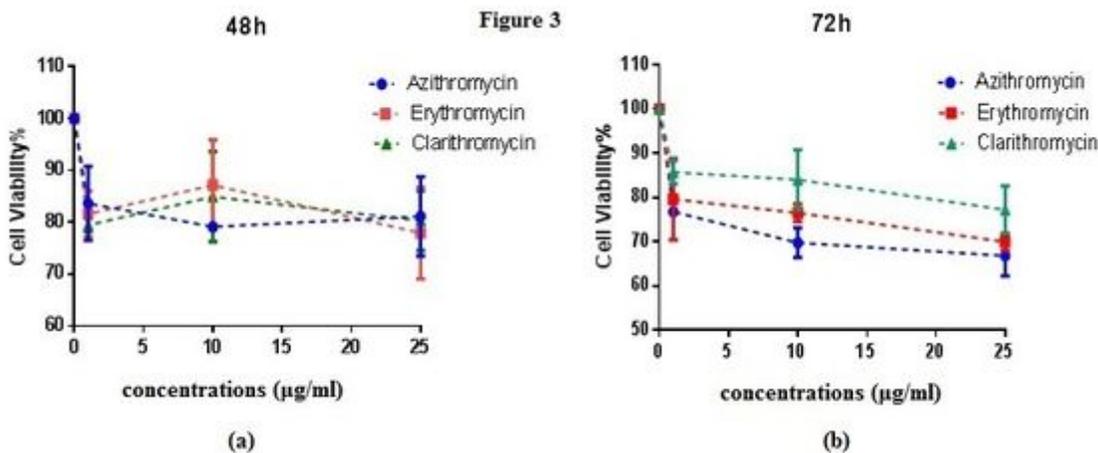


Figure 3

Comparisons of cytotoxicity effects of three antibiotics including azithromycin, erythromycin and clarithromycin at concentrations of 1, 10 and 25 $\mu\text{g/ml}$ on cardiac myoblast cells (H9c2) after (a) 48 and (b) 72 hours of treatment. Cells were treated with concentrations of 1, 10 and 25 $\mu\text{g/ml}$ of three drugs and cell viability was quantified by MTT test. The results are reported as three independent tests mean \pm SE.

Figure 4

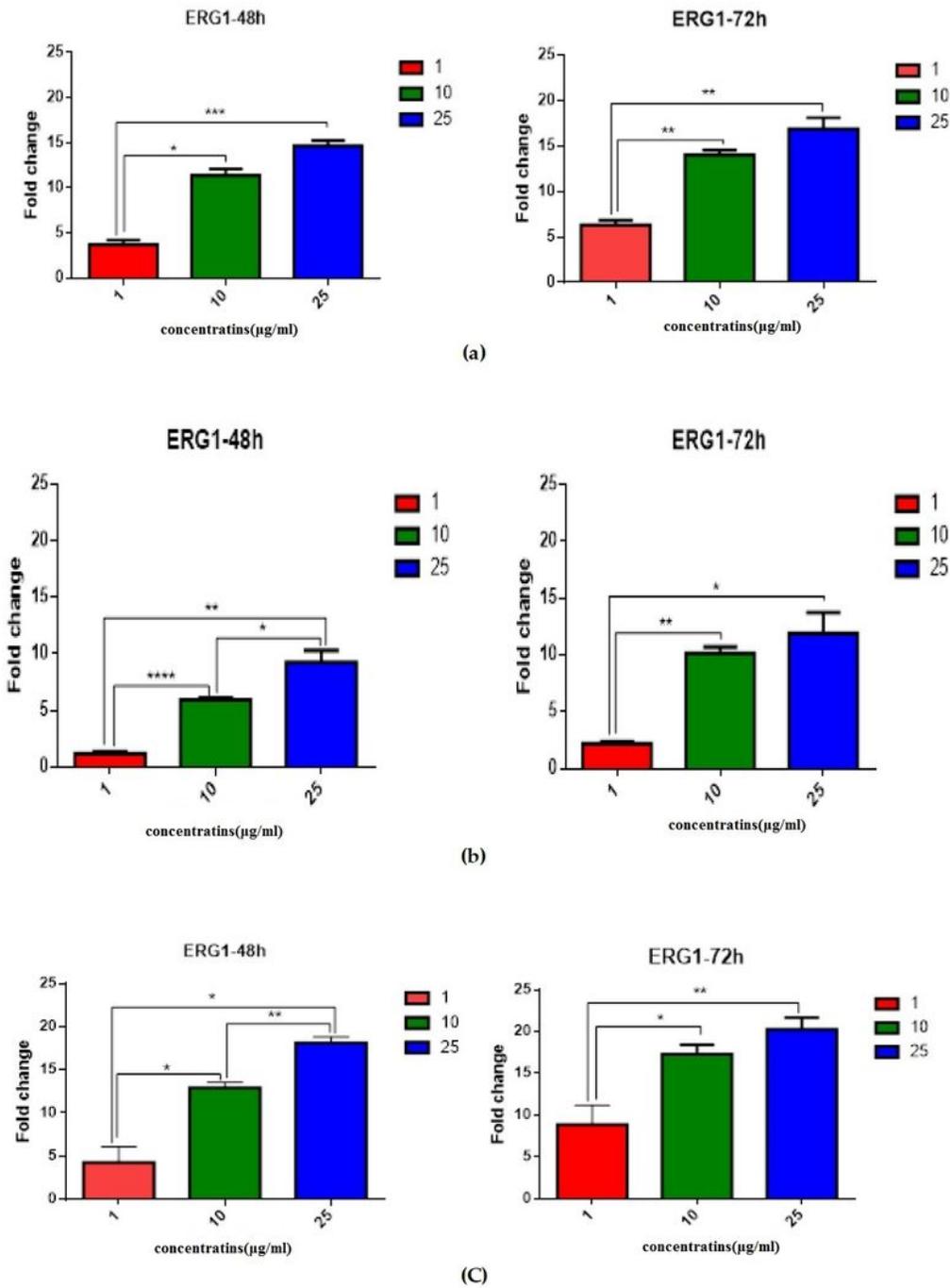


Figure 4

Comparison of ERG1 gene expression in H9c2 cardiac myoblast cells treated with three antibiotics (a) erythromycin; (b) clarithromycin, and (c) azithromycin at three different concentrations of 1, 10 and 25 µg/ml after 48 and 72 hours. Data were analyzed using $\Delta\Delta C_t$ analysis method. The results are presented as three

independent tests mean \pm SE. Significant differences were reported as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

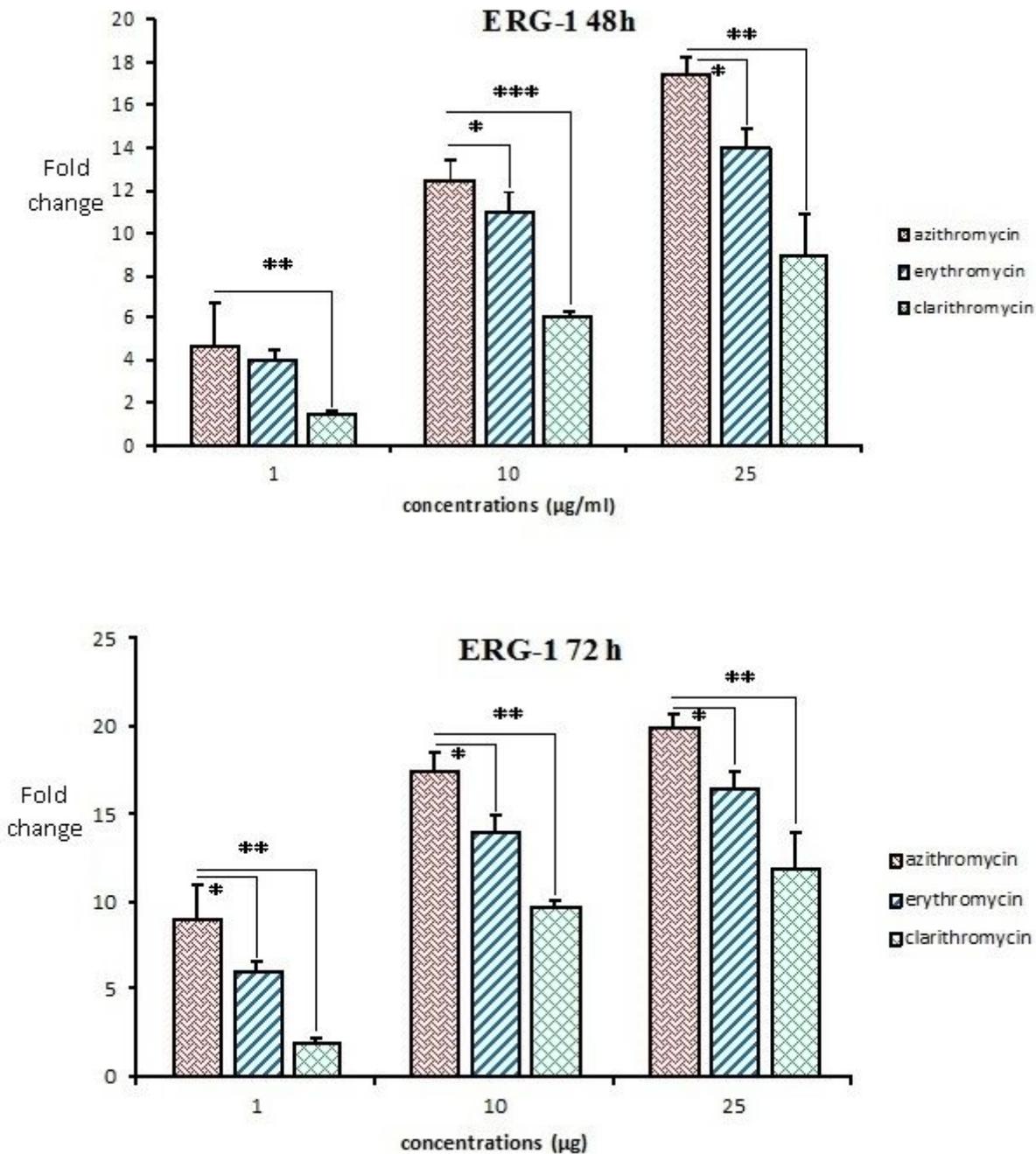


Figure 5

Concurrent comparison of ERG1 gene expression in H9c2 cardiac myoblast cells treated with three antibiotics including azithromycin, erythromycin and clarithromycin at concentrations of 1, 10 and 25 µg/ml after 48 and 72 hours: Data were analyzed using $\Delta\Delta C_t$ method. The results are presented as three independent tests mean \pm SE. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.