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# 토끼 심장에서 단상활동전위의 TRIaD 분석을 이용한 약물에 대한 심장 안전성 평가

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# Cardiac safety evaluation on drugs using the TRIaD assay in the rabbit cardiac monophasic action potential

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ABSTRACT. Drug-induced torsades de pointes (TdP) have been linked to a sudden cardiac death. Prolongation of the action potential duration (APD) and the QT interval (PQT), in nonclinical assays, have been investigated before drug development. However, TdP can often occur without any appreciable QT prolongation, and drug development companies and researchers may ask better accurate models or methods. We wish to verify whether it is effective to determine the cardiac toxicity as a result of the measurement using the TRIaD assay in the rabbit cardiac monophasic action potential. '**TRIaD**' means **T**rianguation, **R**everse use dependence, **I**nstability and **D**ispersion. In this study, to confirm this assay, control, negative control and positive control were classified based on the degree of cardiotoxicity. Control group was perfused by only normal Tyrode's solution. Diltiazem and Amoxicillin were used as negative controls. Terfenadine, Quinidine, Amiodarone, and Nifedipine were used as positive controls. Based on these, proarrhythmic scores were calculated as follow: instability 20 points, triangulation 10 points, and RUD 5 points. Both in control and negative groups, proarrhythmic score did not exceed the 25 points, but exceeded in all positive groups. These data propose that this is robust and reliable assay that can add value to drug-induced prolongation/TdP risk assessment. Additionally, this assay provides detailed information on the overall profile of drug-induced electrophysiological effects. Therefore, TRIaD assay can be a valuable to establish for the cardiovascular risk assessment of drugs.

**KEY WORDS: Proarrhythmic, MAP, TRIaD assay, rabbit cardiac monophasic action potential**

## Introduction

Proarrhythmia often occurs by a pre-existing arrhythmia or paradoxically by antiarrhythmic drugs (Horowitz, Zipes et al. 1987) Horowitz, Zipes et al. 1987). Anti-arrhythmia drugs-induced prolongation of the cardiac QT interval is often connected with torsades de pointes (TdP), which means it is a side effect (Li and Ramos 2017) Li and Ramos 2017), and occasionally threatens a patient's life. To reduce the risk of TdP, the pre-clinical assessment of QT prolongation and human ether-à-go-go-related gene (hERG) potassium channel assay is conducted through the process of drug development according to the ICH S7A/B

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guidelines (2000); ICH S7A/B guidelines (2005) (ICH 2000, ICH 2005) ICH 2005). Prenylamine, calcium channel blocking analog of amphetamine used for the treatment of angina, was first drug to be withdrawn from the market due to QT prolongation and sudden cardiac damage (Lester and Olbertz 2016) Lester and Olbertz 2016). In response to these events, proarrhythmia experiment thorough the evaluation of arrhythmia risk was needed (Lester and Olbertz 2016) Lester and Olbertz 2016), and commonly used gold standard to predict TdP before widely used clinical development. However, an increasing mass of evidence between clinical outcome and QT prolongation suggests that QT interval (PQT) does not necessarily lead to TdP (Antzelevitch and Shimizu 2002, Milberg, Eckardt et al. 2002, Lawrence, Bridgland-Taylor et al. 2006) Lawrence, Bridgland-Taylor et al. 2006). The QT prolongation positive drugs are sometimes abandoned for drug development even if they may offer significant benefits because of the potential risk of TdP. Therefore, more precise tools and analyses are needed to develop non-clinical and clinical makers of TdP.

*Lawrence et al.* have suggested that **TRIaD assay** is used as part of non-clinical integrated cardiovascular risk assessment for drug discovery process (Lawrence, Bridgland-Taylor et al. 2006) Lawrence, Bridgland-Taylor et al. 2006). It suggested that clinicians would be well advised to consider not only monophasic action potential (MAP) 60 prolongation but also triangulation changes, including reverse use dependency (RUD) (Hondeghem 2005, Dumotier, Deurinck et al. 2008) Dumotier, Deurinck et al. 2008). Indeed, clinical correlates of non-clinical observations of MAP 60 prolongation, triangulation and RUD would be seen as changes APD (Lawrence, Bridgland-Taylor et al. 2006) Lawrence, Bridgland-Taylor et al. 2006). We have reproduced on a single proarrhythmia model on the rabbit Langendorff heart model, and then investigated this model accuracy to estimate the proarrhythmic risk by the positive drugs on the marketed drugs.

In the present study, we demonstrate that the cardiac toxicity of drugs in the rabbit heart was related to the changes in the MAP by the drugs as Terfenadine, Quinidine, Amiodarone, and Nifedipine using the Langendorff heart system. The toxicity was absent on Diltiazem and Amoxicillin, suggesting a role for the assessment of the TRIaD assay. Our results highlight the importance of this model for providing the assessment and robust conclusions of drug effects.

## Materials and Methods

### 1. Materials

Rabbit hearts were exposed to increasing concentrations of Diltiazem (Sigma-Aldrich, St. Louis, MO, 1205003), Amoxicillin (Sigma-Aldrich, St. Louis, MO, A8523), Terfenadine (Sigma-Aldrich, St. Louis, MO, T9652), Quinidine (Sigma-Aldrich, St. Louis, MO, Q3625) and Amiodarone (Sigma-Aldrich, St. Louis, MO, A8423). All chemicals used were analytical grade reagents from general laboratory suppliers.

### 2. Langendorff system preparation

New Zealand white rabbit (female; age 12~14 week; weight, about 2.6~3.0 kg) were obtained from a commercial breeder (Orient Bio Inc., Seongnam, Korea). All study procedures were approved by the Institutional Animal Care and Use Committee of Korea Institute of Toxicology (1711-0435).

The method has been described previously in detail (Lawrence, Bridgland-Taylor et al. 2006) Lawrence, Bridgland-Taylor et al. 2006). Briefly, New Zealand white rabbits were fixed in a rabbit restrainer. Rabbits were sacrificed by intravenous injection of sodium pentobarbital (50 mg / kg) with 2000 U heparin. After thoracic cavity is quickly opened and the hearts were removed, immediately placed in Tyrode solution. The ascending aorta was quickly cannulated (16-gauge

cannula) and perfused with warm Tyrode solution (36.5±0.5° C, pH 7.4±0.5). The heart was equilibrated with 95% O2 and 5% CO2 to maintain the pH at 7.35. The MAP recording electrode (Hugo Sachs Elektronik, March, Germany) was pushed into the left ventricle and reached below the subendocardium area, where the Purkinje fibers were abundant. The stimulator electrode was placed in the right atrium through the left ventricle.

### 3. Experimental protocol

The amplitude of APD at 30, 60 and 100% repolarization were determined from the recordings. Purkinje fibers were stimulated at 1 Hz, 2 Hz and 3 Hz via electrodes connected to the stimulator. Data analysis means triangulation, RUD, instability, MAP 60% duration and Short-term volatility (STV) analysis. Triangulation was measured as repolarization time from 30% APD to 100% APD. The RUD was measured as the difference between of APD 60% for the first 2 min of the

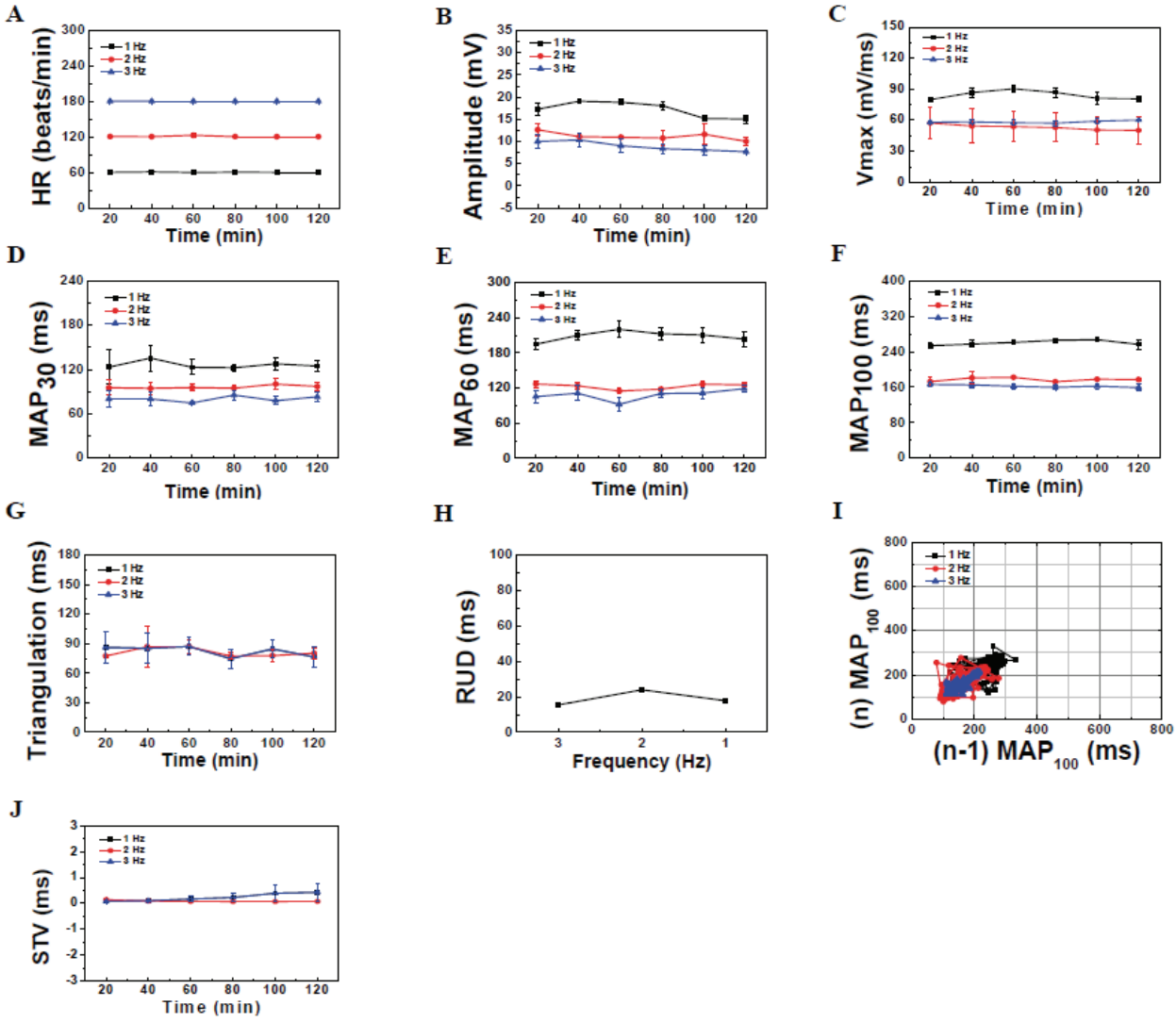


**Fig. 1. Summary of the effect of control, normal tyrode solution on rabbit cardiac MAP.** These results displayed (A), heart rate (1 Hz, 2 Hz, 3 Hz) by electric stimulate injection, (B), MAP of amplitude, (C), Vmax, (D ~ F), action potential duration (30%, 60%, 100%) in MAP and (G ~ J), analysis of TRIaD assay. * P < 0.05, ** P < 0.001 vs. Control.

last 2 minutes on a beat train. Instability is to compare the difference between APD100% and n−1 values to the average. If the duration value of MAP 60% is more than 10% of that at the end of the experiment, the MAP60% value is added as a toxicity evaluation index. STV is obtained by substituting the n−th value and the (n + 1) th value of the APD 100% value into [n− (n−1)] / √2 * 30.

### *4. Statistical analysis*

Triangulation (T) = 10 points, RUD (R) = 5 points, Instability (I) = 20 points, MAP60 (M) = 10 points (〉10% change), Incidence of overt proarrhythmia = 100 points (P); EADs, VT, VF, TdP and S = proarrhythmic score

Statistical analysis was performed by ANOVA. All data are expressed as means ± SEM. Comparison between two means was done using Student's *t*−test; Data are *p*−values 〈0.05 were considered statistically significant. All experiments were conducted in triplicate.

## Results

### 1. The effect of the normal tyrode solution on rabbit cardiac MAP

The main challenge of data analysis in this study was the merging of several proarrhythmia variables and the proarrhythmic score. Hearts were stimulated at a pulse rates of 1, 2 and 3 Hz throughout the experiment with an electrode attached to the right ventricle. Control group was perfused by only normal Tyrode solution (Fig. 1A∼J). In these experiments, the parameters used to define the arrhythmia risk (MAP 60 extension), triangulation, and RUD were treated as discrete variables, but in fact each is contiguous.

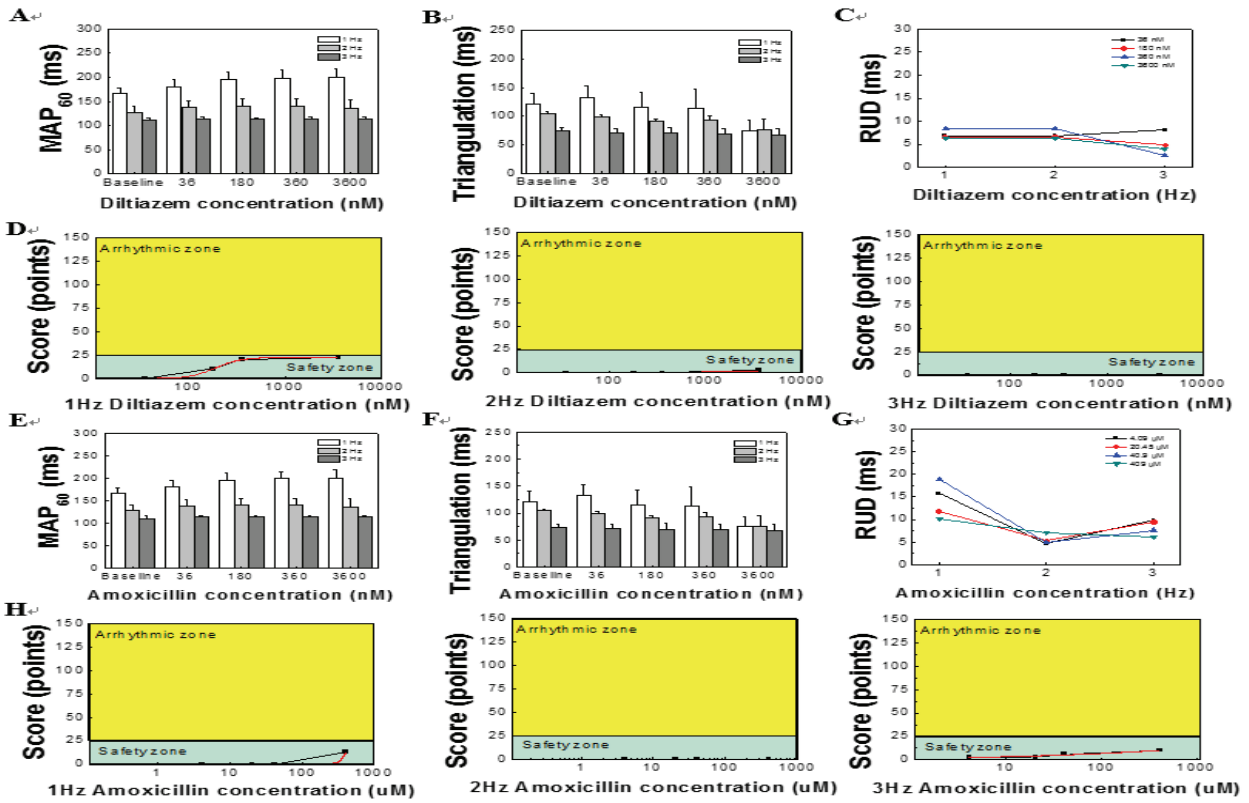


**Fig. 2.** The effects of negative drugs, diltiazem and amoxicillin in rabbit cardiac MAP. These results displayed (A, E), APD60 of negative drugs (B, C), TRIaD of diltiazem, (F, G), TRIaD of amoxicillin, (D, H), proarrhythmic score >25 (1Hz, 2Hz, 3Hz) of negative drugs. * P < 0.05, ** P<0.001 vs. Control.

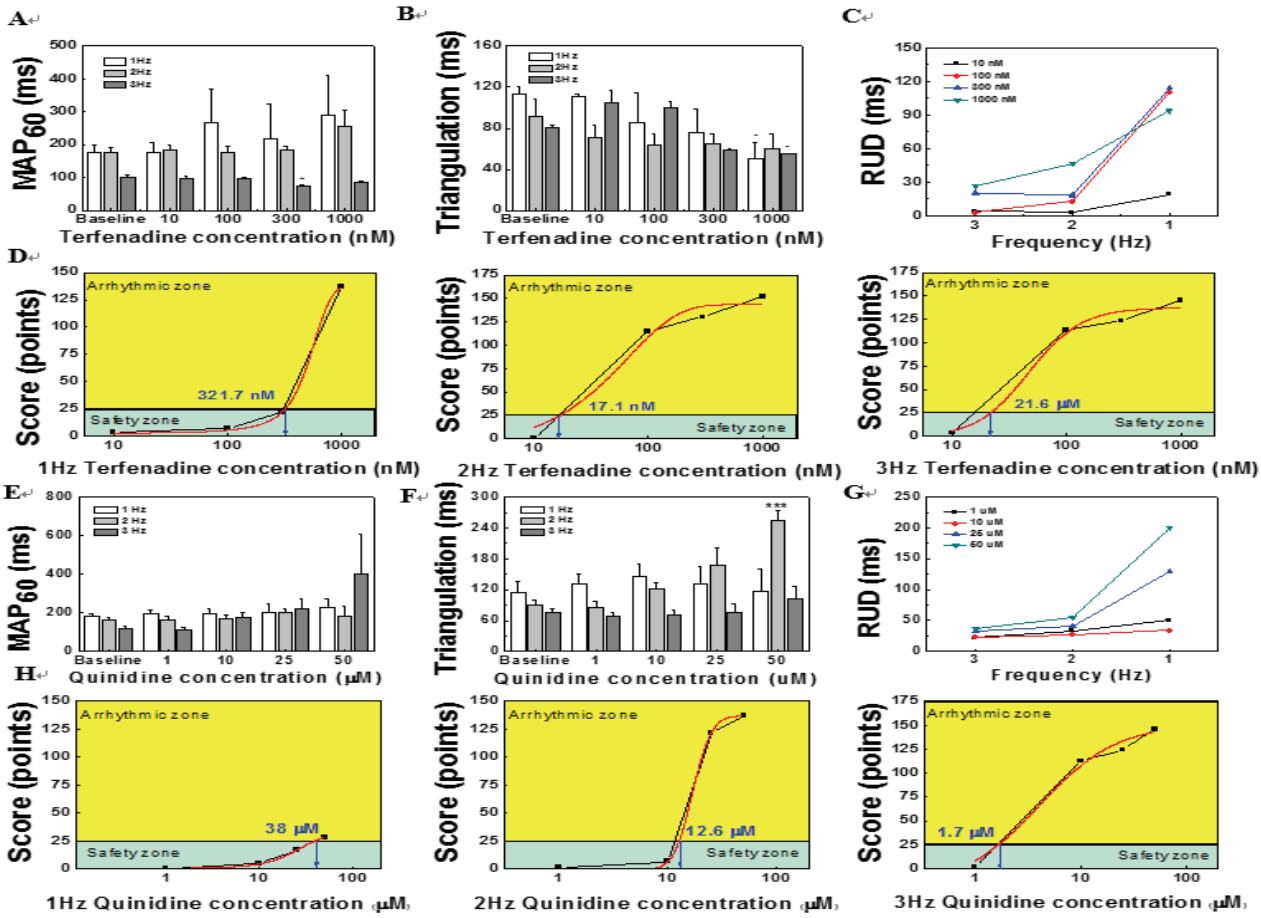


**Fig. 3.** The effects of positive drugs, terfenadine and quinidine in rabbit cardiac MAP. These results displayed (A, E), APD60 of negative drugs (B, C), TRIaD of terfenadine, (F, G), TRIaD of quinidine, (D, H), proarrhythmic score >25 (1Hz, 2Hz, 3Hz) of negative drugs. * P < 0.05, ** P<0.001 vs. Control.

## 2. Diltiazem and Amoxicillin were used as heart negative controls

Calcium channel blocker diltiazem is very effective at controlling ventricular rate and can also reduce the risk of recurrence of atrial fibrillation after successful cardioversion (Personett, Smoot et al. 2014) Personett, Smoot et al. 2014). Amoxicillin is a penicillin antibiotic that fights bacteria (Fang, Tolkoff-Rubin et al. 1978) Fang, Tolkoff-Rubin et al. 1978).

In negative drug experiments in which MAP 60, triangulation and RUD were present, proarrhythmia decreased with prolongation of APD, but this effect was not large enough to be an arrhythmia (Fig. 2). In these studies, diltiazem and verapamil were without proarrhythmic potential in studies.

## 3. Terfenadine and Quinidine were used as positive controls

Terfenadine is an antihistamine used in the treatment of allergic diseases (Olasińska-Wiśniewska, Olasiński et al. 2014) Olasińska-Wiśniewska, Olasiński et al. 2014). Terfenadine is associated with TdP because of its potent IKr blocking effect even at low doses (Nachimuthu, Assar et al. 2012) Nachimuthu, Assar et al. 2012). Quinidine is an agent that acts as a class I antiarrhythmic agent in the heart (Grace and Camm 1998) Grace and Camm 1998). However, quinidine causes QT interval prolongation as well as increased action potential duration (Wroblewski, Kovacs et al. 2012) Wroblewski, Kovacs et al. 2012).

Other concentrations of terfenadine (100, 200 and 1000 nM) increased MAP 60, including only two stimulated at 1 and 2 Hz pulses (Fig. 3A). At 321.7 nM terfenadine increased the MAP 60 (ms) proarrhythmic score (Fig. 3D). The triangulation value decreased with the concentration of terpenadine (Fig. B). Terfenadine (17.1 nM) induced triangulation in hearts proarrhythmic score (Fig. 3D). RUD for terfenadine was detected, with a more pronounced drug effect at 1 Hz compared to 2 Hz or 3Hz (Fig. 3C). RUD reached changes at 21.6 μM terfenadine in a proarrhythmic score (Fig. 3D). MAP60 values did not change with quinidine concentration (Fig. 3E). At 38 μM quinidine increased the MAP 60 (ms) proarrhythmic score (Fig. 3H). The triangulation value for quinidine showed a more significant drug effect at 2 Hz (Fig. 3 F). Quinidine (12.6 μM) induced significant triangulation in hearts proarrhythmic score (Fig. H). The RUD value varies greatly at 3 Hz depending on the concentration of quinidine. (Fig. 3G). RUD reached changes at 1.7 μM quinidine in a proarrhythmic score (Fig. 3H). It has been established that terfenadine and quinidine contributes to the proarrhythmic (Lawrence, Bridgland-Taylor et al. 2006, Nachimuthu, Assar et al. 2012, Wroblewski, Kovacs et al. 2012) Wroblewski, Kovacs et al. 2012). These results indicated that proarrhythmic potential of terfenadine and quinidine was well evaluated in both the drug.

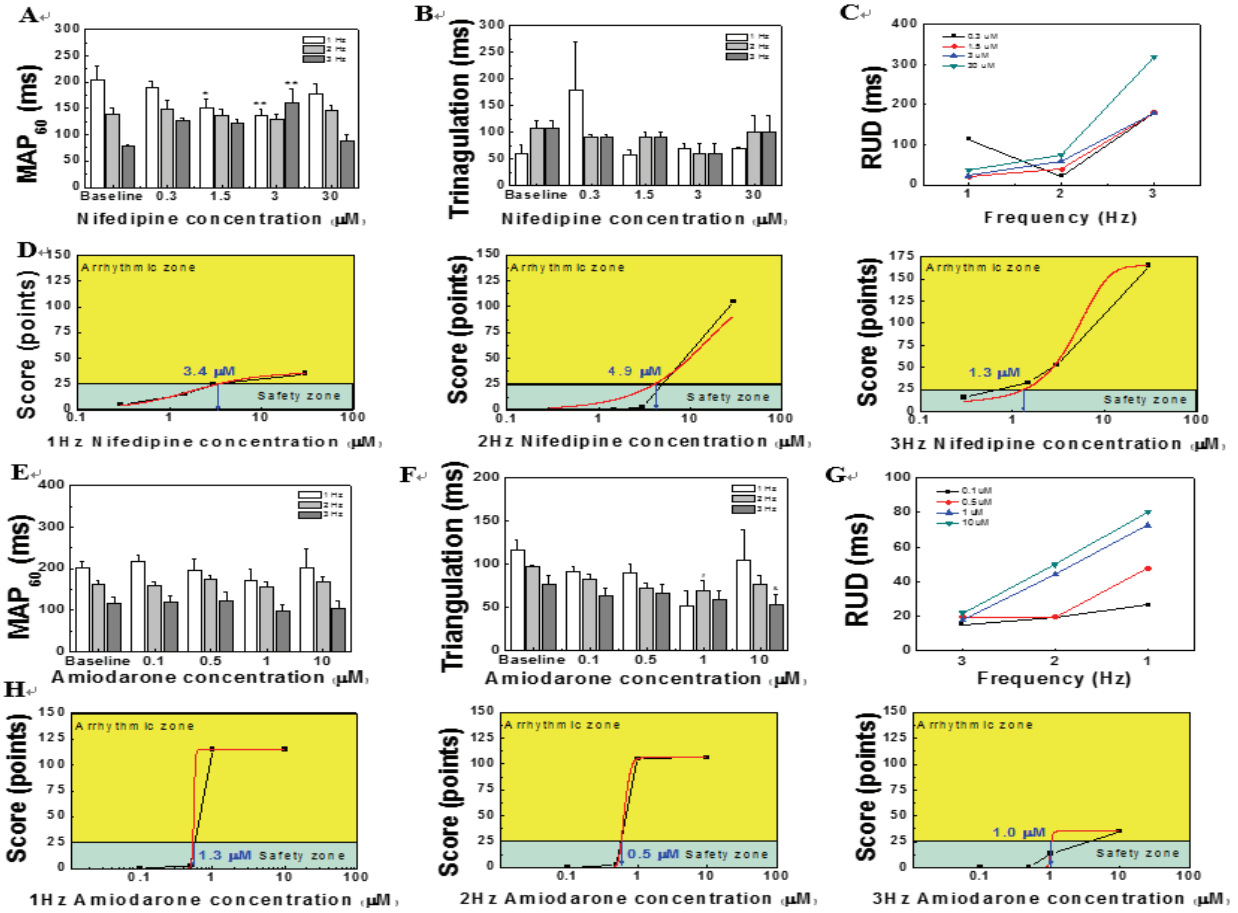


**Fig. 4.** The effects of positive drugs, nifedipine and amiodarone in rabbit cardiacMAP. These results displayed (A, E), APD60 of negative drugs (B, C), TRIaD of nifedipine, (F, G), TRIaD of amiodarone, (D, H), proarrhythmic score >25 (1Hz, 2Hz, 3Hz) of negativedrugs. * P < 0.05, ** P<0.001 vs. Control.

### 4. Nifedipine and amiodarone were used as positive controls

Nifedipine is a drug that treats angina pectoris, hypertension, Raynaud's phenomenon and premature labor (Frishman, Charlap et al. 1985) Frishman, Charlap et al. 1985). However, excessive administration of nifedipine results in severe hypotension and reflexes tachycardia. (Herrington, Insley et al. 1986) Herrington, Insley et al. 1986). Amiodarone is an antiarrhythmic agent used prevent irregular heartbeats (Roy, Talajic et al. 2000) Roy, Talajic et al. 2000).But, amiodarone is considered to be a class III drug, which indicates that it prolongs the QT interval (Hii, Wyse et al. 1992) Hii, Wyse et al. 1992).

At 3.4 μM Nifedipine increased the MAP 60 (ms) proarrhythmic score (Fig. 4A, D). Nifedipine (4.9 μM) induced significant triangulation in hearts proarrhythmic score (Fig. 4B, D). RUD reached changes at 1.3 μM Nifedipine in a proarrhythmic score (Fig. 4C, D). At 1.3 μM Amiodarone increased the MAP 60 (ms) proarrhythmic score (Fig. 4E, H). Amiodarone (0.5 μM) induced significant triangulation in hearts proarrhythmic score (Fig. 4E, H). RUD reached changes at 1.0 μM Amiodarone in a proarrhythmic score (Fig. 4E, H). It has been established that nifedipine and amiodarone contributes to the proarrhythmic (Herrington, Insley et al. 1986, Hii, Wyse et al. 1992, Lawrence, Bridgland-Taylor et al. 2006) Lawrence, Bridgland-Taylor et al. 2006). These results indicated that potential of nifedipine and amiodarone was well evaluated in both the drug.

## Discussion

TRIaD assay is predominantly useful for non-clinical assessment of TdP with a rabbit Langendorff heart. We also proved that the positive control drugs observed in the present experiments was a marked prolongation of APD. On the contrary, in negative control drugs, there were not identified any APD prolongation. Our data not only show the robust and reliable assay of this model, but also provide decision-making on the risk of proarrhythmia.

Diltiazem is in a class of calcium-channel blockers (Shantsila, Watson et al. 2007) Shantsila, Watson et al. 2007). It works by relaxing the blood vessels so that you do not have to pump your heart effortlessly and increases the supply of blood and oxygen to the heart (Boden, Vray et al. 2001) Boden, Vray et al. 2001). Amoxicillin can cause serious side effects such as shortness of breath, decreased urine volume, pressure in the chest, wheezing, swelling, diarrhea, nausea and vomiting. However, heart palpitations cannot usually be found using. It is only a myth among people that amoxicillin causes cardiac palpitations (Anderson and Muhlestein 2004) Anderson and Muhlestein 2004). Diltiazem and amoxicillin not induced significant TRIaD and has an upper range of safety zone concentration of 10000 nM. Thus, its safety ratio computes same below the proposed safety range.

Use of the Terfenadine and quinidine has been associated with QT prolongation and torsade de pointes (Nawrath, Sack et al. 1984, Woosley, Chen et al. 1993, Wiśniowska, Tylutki et al. 2016) Wiśniowska, Tylutki et al. 2016). Amiodarone is used as an antiarrhythmic agent to prevent ventricular arrhythmias. But, it is contraindicated in patients with polymorphism ventricular tachycardia, and the longer the QT interval, the worse it may be caused by the arrhythmia (Grace and Camm 1998) Grace and Camm 1998). Nifedipine was shown to delay the need for aortic valve replacement in patients with aortic regurgitation (Kloner 1995) Kloner 1995). But, observed side effect of nifedipine was tachycardia (Baykal and Avc i oğlu 2015) Baykal and Avc i oğlu 2015). It has been established that Positive drugs (terfenadine, quinidine, amiodarone and nifedipine)-induced significant TRIaD and has an upper range of arrhythmic zone concentration (Lawrence, Bridgland-Taylor et al. 2006) Lawrence, Bridgland-Taylor et al. 2006).

In this study, we investigated the clinical relevance and degree of reproducibility to the

TRIaD risk assessment. These data score of the arrhythmia calculated for each category of drugs provides a basic framework to support decision-making on the risk of proarrhythmic scores related to new chemicals. This consistency is an indication that the cardiac safety assessment is very stable and is appropriate as an appropriate analytical system for risk assessment of new drug drugs. However, a complete validation will have to await a variety set of drug which is test for TRIaD assay. In addition, verification through Patch-clamp electrophysiological analysis should be accompanied.

In conclusion, we showed that the assessment of potential proarrhythmic effects of drug entities on the safety scores requires the measurement of TRIaD assay in rabbit heart. This model can be a robust and reliable assay to validate the side effects of new drug discovery.

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