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Effects of bosentan, sildenafil and their combination on T-wave amplitude and QT ınterval in a rat model of pulmonary hypertension

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Abstract

Aim: Although, bosentan and sildenafil have been used to treat PAH, the effect of these drugs on electrocardiographic (ECG) changes are still unclear. The aim of this study is to evaluate the effects of bosentan, sildenafil, and their combination on T-wave amplitude and QT interval in rats with monocrotaline (MCT)-induced PAH.

Materials and Methods: Saline (control) or MCT were applied to sixty-six male Wistar rats. By the development of PAH (4th week), MCT-given rats were randomized into 4 groups and were treated orally with bosentan, sildenafil and combination of sildenafil and bosentan or placebo for 3 weeks. Echocardiographic (ECHO) and ECG examinations were performed three times on baseline, 4th and 7th week.

Results: ECHO results showed that the application of MCT developed PAH in all injected rats. Usually, T-wave changes and QTc prolongation in ECG began after PAH and T-wave amplitudes, T-repolarization time as well as QTc intervals significantly increased in all groups after treatment ($p<0.001$).

Conclusion: Our experimental study suggest that T-wave changes and QTc prolongation to be more clear in combined treatment than the single use of sildenafil or bosentan in MCT-induced PAH. The most interesting finding in this research was that it could be predicted serious arrythmia with ECG changes (probably QTc interval) - clearly a future direction.

Keywords: Pulmonary Arterial Hypertension; Rat Model; Sildenafil; Bosentan; Electrocardiography; Echocardiography.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is characterized by progressive obliteration of the small pulmonary vascular bed as a result of vascular proliferation and remodeling of the vessel wall leading to permanently increased pulmonary vascular resistance and elevated pulmonary artery pressures. Progressive increase in pulmonary artery (PA) pressure results in right ventricular dilatation and wall thickening, right heart failure and premature death. Developing right heart failure is the major factor for determining the prognosis and causing death in these patients (1). Animal models are important tools for the study of the pathogenic mechanisms of pulmonary hypertension, and for the development of novel therapeutic strategies. Monocrotaline (MCT), the most preferred agent in experimental models of PAH, is a plant-derived alkaloid prolizidin which results with progressive PAH, right ventricular hypertrophy and failure (2-4).

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Structural, mechanical and electrical remodeling on the right ventricle led to increase the risk of arrhythmia even lethal forms (5-8). Despite of the fact that ECG changes in patients with PAH are well known, the effects on the ECG change of PAH therapy is still unclear. The aim of this study is to evaluate the effects of bosentan, sildenafil, and combined treatments on ECG waves in rats with monocrotaline-induced pulmonary hypertension.

MATERIALS and METHODS

Experimental design

Sixty-six three-month old male Wistar rats (200-300 g body weight) were given a subcutaneous injection of saline (healthy control; HC; n=10) or monocrotaline (MCT; 60 mg/kg; n= 56). After four weeks, by the development of PAH, MCT-injected rats were separated into four groups randomly and were treated orally by gavage with bosentan (MCT-BOS; 300 mg/kg/day; n=14), sildenafil (MCT-SIL; 100 mg/kg/day; n=14), sildenafil and bosentan combination (MCT-SIL-BOS; 100 mg/kg/day, 300 mg/kg/day; n=14) or placebo (saline) (MCT; n=14) for 3 weeks. Experiments were approved by the local ethical committee.

Echocardiographic measurements

Echocardiographic (ECHO) examination was performed for all experimental groups for three times (baseline, 4th

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week and 7th week). The rat's chest was shaved to reduce ultrasound attenuation. Rats were laid supine on a platform and body temperature was maintained at 36.5°C to 37.5°C using a lamp. A warm ultrasound gel was spread over the chest wall. Transthoracic 2-dimensional, M-mode and Doppler imaging were performed with a Vivid I (S/N: 001651, General Electric, Tirat Convel, Israel) echocardiographic color-system equipped with a 10-MHz transducer was performed to spontaneously breathing rats under anesthesia with 50 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi Pharmaceutical Co.-Warner Lambert, Istanbul, Turkey). Right ventricular free-wall thickness (RVWT) was measured in the modified parasternal long-axis view using 2D mode from parasternal short-axis window. Right ventricular end-diastolic diameter (RVDD) was measured from apical four-chamber window between mid-point the right ventricular free-wall and interventricular septum while the tricuspid valve closed. Tricuspid regurgitation (TR) measurements were used for estimating PAH levels. TR flow was assessed from apical four-chamber window where can be seen the best in color and continuous-wave Doppler tricuspid regurgitation. *All ECHO* analyses *were performed* by the same person.

Electrocardiographic measurements

Electrocardiographic (ECG) measurements were performed for all experimental groups three times (baseline, 4th week and 7th week). The electrical activity of heart was recorded using the BIOPAC MP 100 Acquisition System (Biopac System Inc., Santa Barbara, USA). Prior to ECG recordings the rats were anesthetized with 50 mg/kg ketamine hydrochloride (Ketalar, Eczacibasi Pharmaceutical Co.-Warner Lambert, Istanbul, Turkey), administered intramuscularly. All the rats were situated in the supine position on the ECG recording platform. Then the extremities were shaved and the lead I electrodes were connected to an amplifier (BIOPAC ECG 100B; Biopac System Inc., Santa Barbara, USA) by a shielded three electrode lead set. The signals were digitized with a 16-bit analog-to-digital converter at a sampling rate of 1000 samples/second. BIOPAC Acknowledge Analysis Software (V3.5.7, Biopac System Inc., Santa Barbara, USA) was used to measure the amplitude and duration of the QRS complex, T-wave, T depolarization (T-dep time) and repolarization time (T-rep time), heart rate (RR interval) and QT_c. QT interval was measured manually from each ECG tracing and the QT_c (the QT interval corrected for the heart rate) were calculated using Fredericia's formula: $QT_c = QT / (RR)^{1/3}$.[9]

Statistical analysis

The data were processed and analyzed using STATA and MedCalc statistical software. Descriptive data were presented as means ± standard deviations (SD). The distributions of T-wave amplitude, T-rep time and QT_c interval were investigated by using Shapiro Wilk's normality test and the comparisons were done using one way ANOVA and post-hoc Tukey tests. The differences (two-tailed p) less than 0.05 were regarded as significant.

RESULTS

In the HC, MCT and MCT-BOS groups, no rats died in the course of the study. However, five rats in the MCT-SIL and one rat in the MCT-SIL-BOS group died approximately 2,5-3 weeks after from the injection of MCT. Therefore, at the 4th week and 7th week measurements of the study, groups of HC, MCT, MCT-BOS, MCT-SIL and MCT-SIL-BOS contained number of 10, 14, 14, 9, and 13 rats, respectively.

Echocardiographic results

The mean values and standard deviations of TR, RVDD, and RVWT of all groups are listed in Table 1.

Table 1. Echocardiographic measurement of the groups (Data were presented as means ± standard deviations (SD))

Groups	HC (n=10)	MCT (n=14)	MCT-BOS(n=14)	MCT-SIL (n=9)	MCT-SIL-BOS(n=13)
TR (ms^{-1})					
Baseline	1.391±0.146	1.261±0.109	1.343±0.098	1.300±0.065	1.368±0.145
4th week	1.740±0.075	3.006±0.104[a†]	3.154±0.476[a†]	3.099±0.237[a†]	3.310±0.628[a†]
7th week	1.586±0.151	3.688±0.380[a†]	3.553±0.424[a†]	2.326±0.399	3.593±1.256[a†]
RVDD (mm)					
Baseline	3.651±0.515	3.693±0.502	3.922±0.532	3.705±0.190	3.523±0.243
4th week	3.860±0.420	5.659±0.582[a†]	5.032±0.551[a†]	5.227±0.526[a†]	5.598±0.702[a†]
7th week	3.958±0.347	6.352±0.829[a†]	5.145±0.703[a†,b‡]	4.774±0.459[a†,b‡]	5.584±0.819[a†]
RVWT (mm)					
Baseline	0.978±0.186	0.926±0.157	0.910±0.123	1.008±0.083	0.992±0.126
4th week	0.969±0.087	1.117±0.119[a†]	1.011±0.130	1.197±0.803[a†]	1.146±0.193[a†]
7th week	0.946±0.093	1.190±0.101[a†]	0.987±0.067	1.066±0.058	1.114±0.196[a†]

HC: Rats that were treated with subcutaneous injection of saline; MCT: Rats that were treated with subcutaneous injection of 60 mg/kg monocrotaline; MCT-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan; MCT-SIL: Rats that were treated orally by gavages with 100 mg/kg/day sildenafil; MCT-SIL-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan and 100 mg/kg/day sildenafil combination. TR: Tricuspid regurgitation; RVDD: Right ventricular end-diastolic diameter; RVWT: Right ventricular free-wall thickness. [a]Compared to HC; [b]Compared to MCT. [†]$P<0.001$, [‡]$P<0.01$. The cases, where statistically significant differences were found, are given in the Table.

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Tricuspid regurgitation (TR)
Initially, the TR values which reflecting right ventricular pressure were similar between the groups. Following the 4th week measurement, after PAH developed, TR values were significantly increased in all groups compared with HC group (p<0.001). In the 7th week, after treatment, while TR values were increased significantly in MCT, MCT-BOS and MCT-SIL-BOS groups, only MCT-SIL group was similar to the HC group (p<0.001).

Right ventricule diastolic diameter (RVDD)
While there was no significant difference between groups in terms of RVDD measurements, after PAH developed, these values were significantly increased in all groups compared with the HC group (p<0.001). RVDD values still significantly increased in all groups after treatment (p<0.001). Inter-groups comparing demonstrated that RVDD measurements in MCT-SIL and MCT-BOS groups were lower than the MCT group (p<0.01; respectively); however, this difference was not observed in comparison to the MCT-SIL-BOS group.

Right Ventricle Wall Thickness (RVWT)
RVWT measurements were similar at the beginning. Following PAH development, RVWT values were significantly increased in MCT, MCT-SIL and MCT-BOS groups compared with the HC group (p<0.001). Although, slight increase in the MCT-BOS group was detected, the difference was not significant (p>0.05). RVWT values were significantly thicker in MCT and MCT-SIL-BOS groups than the HC group after treatment (p<0.001). There were no significant differences in MCT-BOS and MCT-SIL groups (p>0.05).

Electrocardiographic (ECG) Results
The mean values and standard deviations of T-wave amplitudes, T-dep durations, T-rep durations, and QT_c of all groups are summarized in Table 2.

Table 2. ECG parameters of the groups (Data were presented as means ± standard deviations (SD))

Groups	HC (n=10)	MCT (n=14)	MCT-BOS (n=14)	MCT-SIL (n=9)	MCT-SIL-BOS (n=13)
T-wave amplitude (mV)					
Baseline	0.114±0.040	0.082±0.036	0.128±0.059	0.096±0.044	0.095±0.037
4th week	0.121± 0.040	0.103±0.032	0.132±0.065	0.107±0.054	0.136±0.075
7th week	0.093±0.037	0.227±0.048[a†]	0.206±0.082[a†]	0.235±0.099[a†]	0.228±0.077[a†]
T-dep time (ms)					
Baseline	0.013±0.004	0.012±0.002	0.012±0.002	0.010±0.002	0.010±0.002
4th week	0.012±0.002	0.012±0.003	0.013±0.002	0.013±0.003	0.015±0.002
7th week	0.011±0.002	0.020±0.004	0.021±0.012[a†]	0.017±0.004	0.023±0.008[a†]
T-rep time (ms)					
Baseline	0.033±0.017	0.039±0.045	0.042±0.017	0.034±0.013	0.030±0.008
4th week	0.038±0.019	0.039±0.018	0.054±0.016	0.041±0.016	0.060±0.012[a*]
7th week	0.033±0.013	0.080±0.017[a†]	0.087±0.022[a†]	0.081±0.017[a†]	0.100±0.028[a†]
QTc interval (ms)					
Baseline	0.148±0.030	0.156±0.083	0.153±0.043	0.147±0.020	0.133±0.021
4th week	0.154±0.034	0.173±0.037	0.180±0.029	0.171±0.034	0.196±0.018
7th week	0.138±0.024	0.238±0.021[a†]	0.246±0.058[a†]	0.222±0.021[a†]	0.251±0.037[a†]

HC: Rats that were treated with subcutaneous injection of saline; MCT: Rats that were treated with subcutaneous injection of 60 mg/kg monocrotaline; MCT-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan; MCT-SIL: Rats that were treated orally by gavages with 100 mg/kg/day sildenafil; MCT-SIL-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan and 100 mg/kg/day sildenafil combination. T-dep time: T-wave depolarization time; T-rep time: T-wave repolarization time; QT_c: QT interval corrected for the heart rate. [a]Compared to HC. [†]P<0.001, [*]P<0.05. The cases, where statistically significant differences were found, are given in the Table.

There were no significant differences at QRS duration, QRS amplitude and RR interval values between the groups in each of the three measurements.

T-wave amplitudes were similar between groups in initial and 4th week measurements. On the other hand, T-wave amplitudes were significantly increased in all groups comparing to the HC group after treatment (p<0.001). There were no differences between treatment groups (Figure 1).

T-dep durations of the groups were similar in the beginning and 4th week measurements. On the other hand, T-dep time was longer in all groups than HC group, but only MCT-BOS and MCT-SIL-BOS groups were statistically significant following the treatment (p<0.001). While T-rep durations were similar initially and following the PAH development, only T-rep time of MCT-SIL-BOS group significantly increased slightly (p<0.05). T-repolarization durations were increased in all groups after treatment (p<0.001) (Figure 2).

QT_c measurements were similar between groups in initial and 4th week of the study. Following the treatment, QT_c measurements were increased in all groups (p<0.001) (Figure 3).

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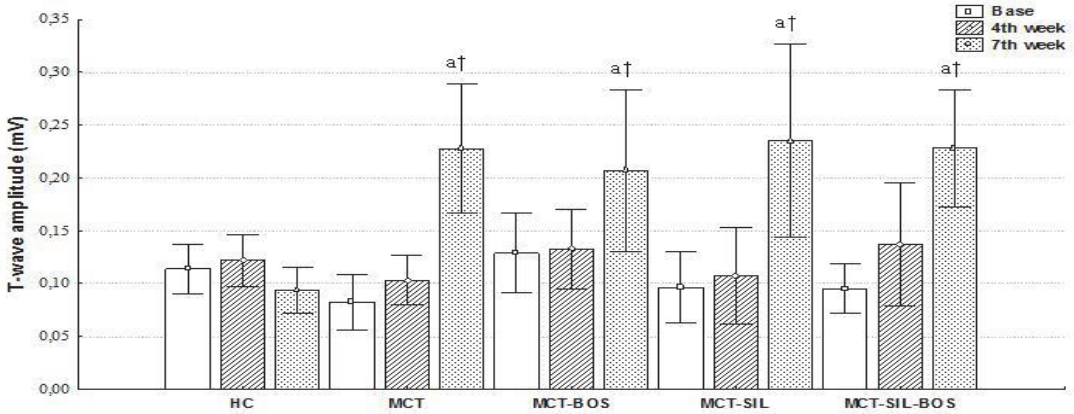

Figure 1. T-wave amplitude values of the groups. HC: Rats that were treated with subcutaneous injection of saline; MCT: Rats that were treated with subcutaneous injection of 60 mg/kg monocrotaline; MCT-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan; MCT-SIL: Rats that were treated orally by gavages with 100 mg/kg/day sildenafil; MCT-SIL-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan and 100 mg/kg/day sildenafil combination. [a]Compared to HC. [†]$P<0.001$. The cases, where statistically significant differences were found, are given in the figure.

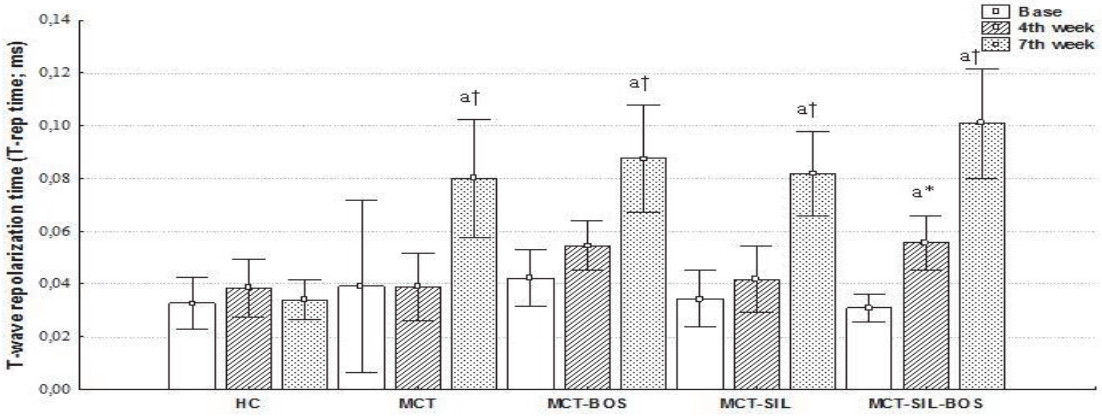

Figure 2. T-wave repolarization time values of the groups. HC: Rats that were treated with subcutaneous injection of saline; MCT: Rats that were treated with subcutaneous injection of 60 mg/kg monocrotaline; MCT-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan; MCT-SIL: Rats that were treated orally by gavages with 100 mg/kg/day sildenafil; MCT-SIL-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan and 100 mg/kg/day sildenafil combination. [a]Compared to HC. [†]$P<0.001$, [*]$P<0.05$. The cases, where statistically significant differences were found, are given in the figure.

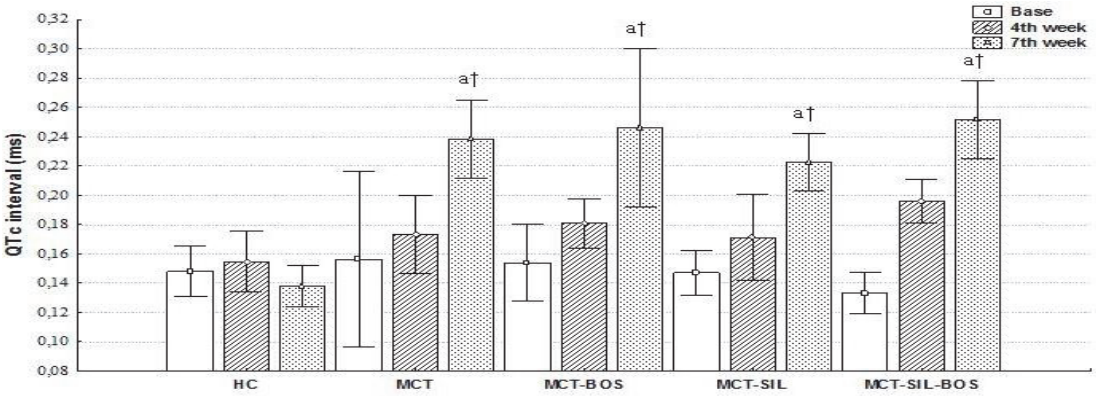

Figure 3. QTc interval values of the groups. HC: Rats that were treated with subcutaneous injection of saline; MCT: Rats that were treated with subcutaneous injection of 60 mg/kg monocrotaline; MCT-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan; MCT-SIL: Rats that were treated orally by gavages with 100 mg/kg/day sildenafil; MCT-SIL-BOS: Rats that were treated orally by gavages with 300 mg/kg/day bosentan and 100 mg/kg/day sildenafil combination. QT_c: QT interval corrected for the heart rate. [a]Compared to HC. [†]$P<0.001$. The cases, where statistically significant differences were found, are given in the figure.

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DISCUSSION

This is the first study to assess the effect of the bosentan, sildenafil, and combined treatments on ECG waves in monocrotaline-induced PAH in rats. Highly effective dosages of bosentan (300 mg/kg/day) and sildenafil (100 mg/kg/day) on MCT induced (60mg/kg) PAH were used (10). ECHO that was used in this study is the most commonly used non-invasive diagnostic tool to demonstrate the development of PAH (11,12). In the 4th week measurement, the values of TR which estimated the right ventricular pressure, RVDD and RVWT values significantly increased. It was assumed that increasing in TR values seemed to be the evidence for the development of PAH in all groups which were given MCT.

The effects of the treatments on the ECHO parameters were investigated on the experimental PAH models. Hypertrophy of the right ventricle and RV dysfunction secondary to increasing RV pressure and volume expansion occur in patients with PAH. It is well known that, bosentan is an effective inhibitor of cardiomyocyte hypertrophy and sildenafil is known to reduce the myocardial hypertrophy by increasing Cgmp (13,14). Mouchaers et al. reported that RVDD values, which reflect volume overload, decreased in combined therapy and sildenafil group but it was statistically significant in only combined therapy group (15). However, they could not find any connection between RVDD values and bosentan therapy. In our study, on the other hand, RVDD values of both sildenafil and bosentan group were less than MCT group and RVWT values were found to be similar to HC group. Nevertheless, these values were found to be close to the MCT group in combined therapy group. Also our findings demonstrated that TR values were lower in sildenafil and bosentan groups where they were only significant in sildenafil group. Clozel et al. reported that despite pulmonary artery pressure was decreased by bosentan and sildenafil, a combination therapy induced a *more distinct decrease (10).* However, our results suggest that sildenafil and bosentan are more effective than combination therapy on RVDD, RVWT and TR values.

In studies on experimental PAH models, early ECG differences have been reported in relation with PAH and anatomical and functional RV changes. But the effects on ECG waves of these drugs or the combined use of them have not been studied before. In this study, ECG parameters were analyzed in terms of response to treatment; the QT_c interval, T-dep time and T-rep time parameters were lower in sildenafil group comparing with the others. The value of T-wave amplitude was found to be the lowest in the bosentan group, as well. The increase in T-wave amplitude, T-rep time and QT_c prolongation began after the development of PAH and continued as progressive and significant differences were observed following treatment period. TR values which reflect the RV pressure increased after PAH development and continued even in post-treatment period. This result has been assumed that the drugs were not able to prevent the development of PAH and ECG changes became more clear on the basis of higher RV pressure in the 7th week. On one hand; clinically, treatment strategies improve in *life expectancy* of patients with PAH, on the other, this event is known to be progressive in most patients.

Comprising complex changes in active and passive electrical properties of myocardium is called electrical remodeling. As a result of degradation of expression or function of ion channels in ventricular myocytes during hypertrophy, voltage-gated K channels which are responsible for repolarization is down-regulated. As a result of this, remodeling of the right ventricle causes some electrophysiological impacts such as prolonged QT_c interval and T-wave changes (16). Also, in patients with PAH, right ventricular hypertrophy and ischemia lead to increase the intracellular calcium and/or acidity which inhibits repolarized Kv channels resulting as a potential risk for prolong the QT_c which is demonstrated, as well (17,18). Similarly, prolonged T-wave duration, T-wave changes, action potential mismatch and increase in calcium levels in myocytes in MCT induced PAH have been shown (19). As in severe left ventricular failure, in patients with serious RV failure, QT_c duration increase as a result of neuroendocrine activation (20,21). Prolonged QT_c interval was found to be the determining mortality and even to be associated with NT-pro BNP level which is an important bio-marker in assessing the function of the right ventricle and clinical deterioration.[9] In this study, especially T-wave and QT_c interval changes occurred after PAH development and it was observed that neither bosentan nor sildenafil were able to cure exactly. Besides, both ECHO and ECG changes in combined therapy were found out to be more clear instead of single use of sildenafil or bosentan.

It is known that, microwave T-wave alternans abnormalities and minor T-wave changes cause life-threatening ventricular arrhythmia and seem to be important for mortality (22.23). Although, arrhythmogenic effect of endothelin-1 in the pathogenesis of PAH was defined a long time ago, the underlying mechanism was not fully elucidated (24,25). It was found that endothelin-1 increased calcium transition through inositol 1,4,5 triphosphate and caused triggered fatal tachyarrhythmias by extending the duration of the action potential (24,25). Also the anti-arrhythmic effect of selective ET-A receptor blockade has been shown (26). On the other hand, Crockett et al. demonstrated the anti-arrhythmic efficiency of ET-B receptor stimulation after myocardial ischemia (27). Oikonomidis et al. reported that the levels of catecholamines and sympathetic activation increased due to deficiency of ET-B (28). Therefore, it can be said that increase in arrhythmogenesis by ET-B receptor blockade is secondary to enhanced sympathetic activation. So, the effect of bosentan, which is dual receptor antagonist, is not clear. Sildenafil is an agent that reduces arrhythmias by inhibiting of excessive sympathetic activity with the activation of the ATP-sensitive potassium channels (29). Although anti-arrhythmic effects of both drugs have been reported, our results suggest that T-wave changes have continued increasingly after treatment. In this study, although QRS and T-wave changes were

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investigated, the relation of these changes with arrhythmia was not studied.

CONCLUSION

In conclusion, our findings suggest that with combined treatment, the T-wave parameters changed more than the single use of sildenafil or bosentan in MCT induced PAH. Nevertheless, based on the highest TR values detected in combined therapy group, it is difficult to say whether these changes are due to the high pulmonary pressure or drug interactions. Especially, T-wave changes are assumed to be important for determining mortality by triggering arrhythmias which are very crucial to emphasize ECG changes during PAH and treatment. Also, in this model, many of the findings may simply be due to insufficient time from MCT to first observation and the only 3 weeks of treatments. Further studies are needed to find out the effects on ECG changes of drugs or combinations used in the PAH treatment.

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