Cardiomyocyte lethality by multidirectional stimuli

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Abstract

Multidirectional defibrillation protocols have shown better efficiency than monodirectional; still, no testing was performed to assess cell lethality. We investigated lethality of multidirectional defibrillator-like shocks on isolated cardiomyocytes. Cells were isolated from adult male Wistar rats and plated into a perfusion chamber. Electrical field stimulation threshold (E_T) was obtained, and cells were paced with suprathreshold bipolar electrical field (E) pulses. Either one monodirectional high-intensity electrical field (HEF) pulse aligned at 0° (group Mono0) or 60° (group Mono60) to cell major axis or a multidirectional sequence of three HEF pulses aligned at 0°, 60°, and 120° each was applied. If cell recovered from shock, pacing was resumed, and a higher amplitude HEF, proportional to E_T, was applied. The sequence was repeated until cell death. Lethality curves were built by means of survival analysis from sub-lethal and lethal E. Non-linear fit was performed, and E values corresponding to 50% probability of lethality (E50) were compared. Multidirectional groups presented lethality curves similar to Mono0. Mono60 displayed the highest E50. The novel data endorse the idea of multidirectional stimuli being safer because their effects on lethality of individual cells were equal to a single monodirectional stimulus, while their defibrillatory threshold is lower.

Keywords Isolated ventricular myocytes • Defibrillation • Electrical field stimulation

1 Introduction

Anomalies that affect the electrical activity of the heart, congenital or not, can lead to cardiac arrhythmias, being ventricular fibrillation (VF) the most serious of them, which can kill an individual within minutes. The only known therapy to terminate VF is defibrillation [1], which consists in the application of electrical fields (E) onto the chest to stimulate or to temporarily turn refractory a significant portion of the cardiac tissue. High-intensity electrical fields (HEF) are required in order to extinguish the uncoordinated wavefronts pertaining to the VF, consequently allowing the heart to restore its regular rhythmicity.

Still, from previous cohort studies, 40% [2] and 56% [3] of patients suffered VF rearrests. Besides the underlying cardiac disorder, one possible cause for this high recurrence can be attributed to the defibrillation itself, which may expose the heart to HEF higher than 100 V/cm [4] causing several cell injuries, associated with electroporation, which may result in cell death [5–9].

Cell stimulation and electroporation are closely related to the variation of the transmembrane potential (∆V_m) induced by E. The amplitude profile of ∆V_m depends on several factors: cell geometry and size, membrane electrical properties, the electrode–electrolyte interfaces, electrode size, the presence of neighboring cells (which may distort E), and the amplitude and direction of E, reaching higher levels when E is parallel to cell major axis [10, 11]. The optimal situation would be to apply a minimal E able to induce a ∆V_m high enough to stimulate all cells but not too high as to damage them.

In the heart, myocytes are arranged in multiple directions, which means that a monodirectional stimulus will stimulate preferentially cell aligned with E, and a higher E amplitude would be required to stimulate all cells, which would inevitably damage some. A multidirectional stimulus can optimize this situation by stimulating cells that happen to be aligned
with each E pulse direction \[12\]. Moreover, single cell stimulation may also be improved if we consider \(\Delta V_m\) temporal summation, which under certain conditions, can yield a lower electrical field stimulation threshold \(E_T\) for multidirectional stimuli against monodirectional, even with the same number of pulses \[12\].

This may help to explain the greater efficiency of multidirectional defibrillation protocols. Higher rates of successful defibrillation in dogs were observed for multidirectional stimuli in relation to monodirectional stimuli \[13\]. In humans, the defibrillatory threshold for stimuli formed by two orthogonal monopolar pulses was found 10% smaller than the threshold for monodirectional bipolar stimuli \[14\]. Recently, a three-way defibrillator was designed and achieved a 30% decrease in the energy required to terminate VF in 50% of a pig population \[15\].

Although displaying better performance regarding defibrillation threshold, data on cardiac injury and cell lethality for multidirectional stimuli is essential to ensure safety because more shocks are applied. Since myocytes are oriented in the most varied directions in the heart, determining the damage caused by multidirectional stimulation to myocytes and cardiac tissue is critical. In this work, we determined the lethal E of rat isolated cells in vitro for mono and multidirectional stimuli.

### 2 Materials and methods

An experimental setup composed of a microscope and electrical stimulators was employed. Figure 1 shows the schematic representation of the setup. Cells were plated at a stimulation chamber, and their image was captured by a camera and shown at the computer screen. The multidirectional low-intensity stimulator (MLIS—three mono or biphasic pulses in three different channels, up to 50 V, 1 ms interval between pulses, 5 ms duration for each pulse, developed at the Center of Biomedical Engineering of UNICAMP) was used for cell pacing, and the multidirectional high-intensity stimulator (MHIS—three monophasic pulses in three different channels, up to 1 kV, 1 ms interval between pulses, 5 ms duration for each pulse, developed at the Center of Biomedical Engineering of UNICAMP) was used for lethal E determination. The current through the stimulation chamber was measured by recording the voltage drop across a power resistor \((4\ \Omega/10\ W)\) in series with the chamber by means of an oscilloscope (model TDS 2014C, 100 MHz, Agilent Technologies Inc., CA, USA). E amplitude is directly proportional to the current and was calculated as previously reported for a smaller version \((20\text{-mm diameter})\) of the stimulation chamber \[16\].

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Fig. 1 a Schematic representation of the experimental setup. A computer is connected to a video camera to allow visualization of the myocytes plated on the stimulation chamber. Cells could be stimulated by the multidirectional low-intensity stimulator (MLIS), for cell pacing, or by multidirectional high-intensity stimulator (MHIS) for lethal electrical field determination. Oscilloscope was used for current measurement during the experiment. b Top view of the stimulation chamber with electrodes separated from each other by 60°. The work area is bounded by a piece of opaque acrylic, represented by the shaded area.
2.1 Isolated ventricular myocytes

The cells were extracted from adult male Wistar rats. The animals lived in shared cages and were offered water and food ad libitum. They lived under a 12:12 light-dark cycle and were not manipulated previously.

The rats were anesthetized with isoflurane, and their hearts were removed. The cardiac myocytes were isolated by coronary perfusion with collagenase I at 37 °C [17]. The experimental protocols for animal care were approved by the institutional Committee of Ethics in Animal Use (CEUA/IB/UNICAMP, protocol numbers 4093-1(I) and 4093-1(K)).

2.2 Experimental protocol

The experimental protocol is shown in Fig. 2. We inserted 100 μL of the solution containing the cells in the stimulation chamber and waited around 20 min for cell adhesion to the coverslip. The chamber was filled with 1.47 mL of Tyrode’s solution (composition (mM): 140 NaCl, 6 KCl, 1.5 MgCl2, 5 HEPES, 1 CaCl2; 11 glucose, pH 7.4).

We selected a cell that was able to contract when stimulated and was aligned to any pair of electrodes. Its image was captured by the video camera (Webcam 3810 Leadership; 5 MP) and used to measure cell length and width. The cell was stimulated with 0.5 Hz symmetric biphasic pulses with 10-ms total duration. We determined E_T by reducing the stimulus amplitude applied longitudinally to cell major axis (except for group Mono60, whose stimuli were applied 60° to cell major axis) until the myocyte stopped contracting, and we stored the lowest amplitude of the positive phase still able to evoke contractions. After E_T determination, the stimulation was resumed with an amplitude of 20% larger than E_T. A high-intensity stimulus was applied to the chamber (truncated exponential monophasic; depending on the experimental group, stimulus was either one pulse of 5 ms in one direction or three pulses of 5 ms each, in succession, in different directions; see Section 2.3), 2 s after the last low-intensity pulse. If the cell suffered hypercontracture, the experiment was finished. If it survived, it was left to rest for up to 10 min; the low-intensity stimulation was resumed, and a high-intensity stimulus, with an amplitude higher than the previous one, was applied to the chamber. The high-intensity pulse amplitudes started at 8 × E_T (a starting level of null response regarding cell damage) and were risen up to 35 × E_T (a level capable of causing cell death in an entire cardiomyocyte population [18]) with steps of 4.5 × E_T.

2.3 Experimental groups

The experimental groups were defined and named according to the high-intensity stimulus direction and waveform. We studied the lethality for five different groups (N is the number of cells in each group), as shown in Fig. 3:

- Mono0: single pulse applied at 0°, parallel to the cell major axis (N = 15).
- Mono60: single pulse applied at 60°, relative to the cell major axis (N = 8).
- Multi0: three pulses applied at 0°, 60°, and 120°, respectively, relative to the cell major axis (N = 12), 1 ms interval between pulses.
- Multi60: three pulses applied at 60°, 120°, and 180°, respectively, relative to the cell major axis (N = 10), 1 ms interval between pulses.

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![Fig. 2 Experimental protocol flowchart](image-url)
Multi120: three pulses applied at 120°, 180°, and 240°, respectively, relative to the cell major axis ($N=14$) with 1 ms interval between pulses.

We used the model proposed by Klee and Plonsey [10] to estimate the maximal transmembrane potential induced by $E_T$ ($\Delta V_T$). Briefly, it assumes the myocyte has the geometry of a prolate spheroid and that the cellular membrane has a dielectric behavior. Hence, the $\Delta V_T$ induced by an electrical field with amplitude $E_T$ depends on cell dimensions, $E$ amplitude and the angle $\theta$ relative to the cell major axis.

2.4 Statistical analysis

All calculations were performed with Prism 5.03 (GraphPad Software, San Diego, USA). The obtained data relative to length, width, $E_T$, and $\Delta V_T$ are shown as mean accompanied by the standard error of the mean. The values for each group were analyzed by three normality tests (Kolmogorov-Smirnov, D’Agostino and Pearson, and Shapiro-Wilk) and a group distribution was considered normal if at least two tests resulted in $p > 0.05$. Means were compared using one-way analysis of variance, and values of $p < 0.05$ were considered evidence of statistical significance. Bonferroni’s multiple comparison test was applied when statistical significance was found.

The values obtained for the lethal and sub-lethal $E$ were used as input to a survival analysis (Kaplan-Meier), followed by Mantel-Cox test and a non-linear regression in order to determine the lethality curves. The data was modeled by the following equation:

$$P(E) = \frac{1}{1 + \left(\frac{E_{50}}{E}\right)^h}$$

where $P(E)$ is the probability of cell death when subjected to an electrical field with amplitude $E$, $E_{50}$ is the amplitude of $E$ correspondent to a probability of lethality of 50%, and $h$ is the Hill coefficient. The parameters calculated by the fitting are shown accompanied by their 99% confidence interval (CI 99). A $p < 0.05$ from Mantel-Cox test followed by non-overlapping CI 99 was considered evidence of statistical significance.

3 Results

3.1 Comparison among cells from experimental groups

$E_T$, $\Delta V_T$, length, and width are presented on Table 1. All group distributions were normal. Values of $\Delta V_T$, length, and width were not statistically different between the experimental groups. Values of $E_T$, however, were significantly different between every experimental group when compared to Mono60 group ($p < 0.0001$ from one-way analysis of variance; ****means $p < 0.0001$ from Bonferroni’s multiple comparisons test with any other group).
3.2 Lethality curves

The lethality curves relating probability of lethality and E are presented in Fig. 4 and were significantly different (Mantel-Cox, \( p < 0.05 \)). Symbols represent means and vertical bars represent standard errors of the means. The continuous lines are the fitting from sigmoid function (Eq. 1). The mean values and the CI 99 of the fitting parameters are presented in Table 2. Cells in each group received an average of five shocks. E50 was significantly different for Mono60 compared to any others. Hill coefficient was not statistically different in any group.

4 Discussion

The present study showed, for the first time, lethality curves for multidirectional stimuli on isolated cardiomyocytes. Cell population used to build these curves was homogeneous (Table 1), and cell parameters were similar to those previously found [16, 18, 19]. The difference observed for ET in Mono60 group was also already shown [16] and can be derived from the Klee and Plonsey model [10] if we consider that \( \Delta V_m \) for cell stimulation is approximately the same, invariant to cell orientation.

We found no significant statistical difference for E50 among Mono0, Multi0, Multi60, and Multi120 groups. At first, a comparison for Mono0 and Multi0 groups is straightforward, since both had their first E pulse applied parallel to cell major axis. Considering that lethal injury is caused by electroperoration [6] and that this phenomenon reduces cell resistivity by two orders of magnitude [20], if the membrane was porated by the first shock, we would expect shocks applied after the parallel shock to induce insignificant \( \Delta V_m \). This would make groups Mono0 and Multi0 quite similar. On the other hand, if the membrane was not porated by the first shock, the following shocks could help to further increase \( \Delta V_m \). From our data, we believe that this second scenario was less likely to occur; otherwise, we would have gotten a higher lethality for Multi0 group against Mono0.

We calculated, through Klee and Plonsey model [10] for an average cell (length, 120 \( \mu \)m; width, 30 \( \mu \)m), that the maximal depolarization induced by a fixed amplitude E applied at 60° or 120° with respect to cell major axis is about 62% of that caused by the same E applied parallelly. If we consider that this same degree of depolarization happened in all our cell membranes for non-longitudinal shocks, the most probable explanation for our results would be that the first shocks applied in the Multi60 and Multi120 groups must have a reduced effect when compared to the shock applied parallelly after. At first, it was reasonable to expect that, in some membrane regions, the first pulses in these groups could facilitate \( \Delta V_m \) buildup for the following parallel pulse due to temporal and spatial summation [21], thus decreasing E50. However, since we are unable to measure electroperoration in our experimental design, and based on the fact that no multidirectional group differed from Mono0, we hypothesize that the shock at 0° would have been majorly responsible for

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**Table 1** Cell parameters

|                | Mono0   | Mono60  |
|----------------|---------|---------|
| E_T (V/cm)     | 2.82 ± 0.10 | 5.43 ± 0.35**** |
| \( \Delta V_T \) (mV) | 19.3 ± 0.7 | 22.9 ± 2.4 |
| Length (µm)    | 126.6 ± 4.1 | 137.6 ± 14.0 |
| Width (µm)     | 32.6 ± 2.0 | 27.4 ± 2.6 |

**Fig. 4** Probability of lethality (P(E)) in function of applied electrical field (E) for each group. Symbols represent means, and vertical bars represent standard errors of the means from survival analysis. Continuous lines are the fitting from sigmoid function, \( R^2 > 0.90 \) in all cases.
cell death, and that the first shock in the Multi120 group and the first two shocks in Multi60 group would not have caused membrane electroporation. Upon these considerations, we could frame the Mono0, Multi0, Multi60, and Multi120 groups as a single group that had the main shock applied longitudinally to cell major axis.

As expected, Mono60 group exhibited a much larger E50 than all the other groups. Since the ΔVm estimated is approximately 62% than that caused if cell major axis was parallel to the E direction, a higher E is needed to generate a similar ΔVm and, consequently, to cause cell death when compared to the other studied protocols.

Our data corroborate with other studies that assert factors like E amplitude, duration, and number of pulses being more important in determining cell viability after shock than the total energy delivered [22, 23]. The groups Multi0, Multi60, and Multi120 received three times the energy amount of Mono0 and Mono60. Still, cell death was similar between multidirectional and Mono0, but was higher when compared to Mono60. Thus, our results reckon stimulus direction as another important factor for isolated cardiomyocyte lethality and reject the total energy applied as a good predictor.

Some considerations must be taken into account in this study, such as the progressive increase of the HEF intensity applied during the lethality protocol, as it was a function of Ei. It results in a several number of shocks in each cell before death, which activates cell repair mechanisms, reducing cell vulnerability to subsequent shocks [24–26]. This means that all cells were in a state of facilitated membrane resealing. Also, damage was observed from non-lethal HEF, promoting reductions in cell length through reversible hypercontracture. A reduction in cell length increases E50 [19]; therefore, the effect of repeated shocks cannot be neglected, and our E50 values might be overestimated if compared to a situation in which a single HEF was applied.

Extrapolations to clinical trials are very complicated, and several extra considerations must be made. Firstly, the results obtained in this work were taken from isolated rat cardiomyocytes oriented at 0° or 60° with respect to E direction. However, an entire heart contains several cells oriented in different directions and connected through gap junctions, creating an anisotropic medium, so that E distributes heterogeneously in the cardiac tissue, which means that each cell will perceive the applied E and respond to it differently. Because of this heterogeneous distribution of E, it is expected that only a reduced fraction of the myocardium is exposed to a same amplitude of E for the three pulses of the multidirectional stimulus. Thus, the lethality curves obtained in this work could be considered an upper boundary for the probability of lethality of cells in the heart, and in order to accurately determine the injuries caused to the myocardium, further studies on the distribution of E by defibrillator-like shocks [4, 27, 28] are needed for multidirectional stimuli. Moreover, experiments were taken at 25 °C (room temperature). It would be important to understand how lethality would behave at physiological temperatures since electroporation seems to have a decreasing threshold as temperature rises [29, 30]. If verified that electroporation threshold is lower as temperature rises to physiological levels, then we would expect lower values for probability of lethality than those reported in this work. However, our protocol does not allow confirming this hypothesis.

5 Conclusion

In conclusion, considering the lethal E values obtained in this work for cells submitted to multidirectional stimuli compared to monodirectional and their lower defibrillation threshold obtained from the literature, one would expect that multidirectional defibrillatory protocols could increase the chances of cell recruitment by exciting more cells through longitudinal stimulation without increasing the potential to cause myocardium injury when compared to monodirectional, even though multidirectional apply more pulses. These results might help to develop further works in multidirectional defibrillation protocols and can also extend the knowledge of defibrillation effectiveness.

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Compliance with ethical standards

All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional Committee of Ethics in Animal Use (CEUA/IB/UNICAMP; protocol numbers 4093-1(I) and 4093-1(K)).

Conflict of interest The authors declare that they have no conflict of interest.

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