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Abstract

The neurological damage after cardiac arrest (CA) constitutes a big challenge of hospital discharge. The therapeutic hypothermia ($34\,^{\circ}\mathrm{C} - 32\,^{\circ}\mathrm{C}$) has shown its benefit to reduce cerebral oxygen demand and improve neurological outcomes after the cardiac arrest. However, it can have many adverse effects, among them the cardiac arrhythmia generation represents an important part (up to 34%, according different clinical studies). Monolayer cardiac culture is prepared with cardiomyocytes from new-born rat directly on the multi-electrodes array, which allows acquiring the extracellular potential of the culture. The temperature range is $37\,^{\circ}\mathrm{C} - 30\,^{\circ}\mathrm{C} - 37\,^{\circ}\mathrm{C}$, representing the cooling and rewarming process in the therapeutic hypothermia. Experiments showed that at $35\,^{\circ}\mathrm{C}$, the acquired signals are characterized by period-doubling phenomenon, compared to signals at other temperatures. Spiral waves, commonly considered as a sign of cardiac arrhythmia, are observed in the reconstructed activation map. With an approach from nonlinear dynamics, phase space reconstruction, it is shown that at $35\,^{\circ}\mathrm{C}$, the trajectories of these signals formed a spatial bifurcation, even trifurcation. Another transit point is found between $30\,^{\circ}\mathrm{C} - 33\,^{\circ}\mathrm{C}$, which agreed with other clinical studies that induced hypothermia after cardiac arrest should not be below $32\,^{\circ}\mathrm{C}$.

The process of therapeutic hypothermia after cardiac arrest can be represented by a Pitchfork bifurcation type process, which could explain the different ratio of arrhythmia among the adverse effects after this therapy. This nonlinear dynamics suggests that a variable speed of cooling / rewarming, especially when passing $35\,^{\circ}\mathrm{C}$, would help to decrease the ratio of post-hypothermia arrhythmia and then improve the hospital output.

1 Introduction

The cardiovascular diseases are the leading cause of death in the world. The research in this field has drawn so much attention of physicians, engineers and researchers since years. However, the cardiovascular diseases progress overtakes the development of the related treatments : in 2030, the annual number of deaths due to cardiovascular diseases will be 25 million, according to the World Health Statistics (2012) by World Health Organization. The majority of patients survived Out-of-Hospital Cardiac Arrest usually develop some degree of neurological problems, caused by ischemia-reperfusion cerebral injury [1]. Even though many clinical trials with specific drugs have been conducted against neurological damages after cardiac arrest, there are no remarkable successes reported. The therapeutic hypothermia seemed as the only therapy available which can improve the neurological recovery of the patients.

The general temperature for human being is maintained within the range of 36.1 °C and 37.8 °C [2]. The lower temperature (hypothermia) can be abnormal. However if administered in a controlled way, it could be therapeutic. In fact, the moderate hypothermia (28 °C to 31 °C) has been used successfully during some open-heart surgeries since years. It is just until recently that it began to be used in post-resuscitation care. The main benefit here is that the therapeutic hypothermia helps to reduce cerebral oxygen demand and to improve neurological outcomes after the cardiac arrest [3, 4, 5, 6]. When cardiac arrest happens, the heart ceases abruptly and unexpectedly to function, so the normal blood flux is considerably disturbed. The heart is no longer able to pump enough blood to the rest of the body. Without immediate treatment, the brain death can occur in six minutes. After a successful resuscitation, the brain could still suffer from some reperfusion injury. The induced hypothermia is almost the only therapy that could reduce the risk of brain damage. The unconscious patient with spontaneous circulation is cooled to 32 °C to 34 °C for 12 to 24 hours after the cardiac arrest.

In fact, the use of therapeutic hypothermia following cardiac arrest was reported in the late 1950s [7, 8]. The results showed some benefits but uncertain. It was also clinically difficult to control the interval between arrest and cooling which is very important. Therefore, hypothermia for cardiac arrest has waned for years. Over the past few years, the therapeutic hypothermia began to redraw attentions of researchers, clinicians etc.. In 2003, The American Heart Association (AHA) endorsed the use of therapeutic hypothermia following cardiac arrest. The International Liaison Committee on Resuscitation (ILCOR) recommended also its use [5]. Currently, the hypothermia therapy (HT) was most recommended

for out-of-hospital cardiac arrest due to ventricular fibrillation [9]. But according to ILCOR, there was possibility for cardiac arrest from other causes, but the benefit is not always confirmed. For example, the studies showed that hypothermia did not improve the outcome in non-ventricular-fibrillation cardiac arrest [10, 11].

The exact mechanism of therapeutic hypothermia is unclear and still under studies. It can be globally summarized as following : in case of resuscitation after cardiac arrest, reperfusion can continue for up to 24 hours, which could cause significant inflammation in the brain. The induced hypothermia can decrease intra-cranial pressure, reduce cerebral metabolic rate (even to $40\% - 50\%$ [12]) and the demand for oxygen consumption of the brain as well [13, 14, 9]. It helps possibly to suppress many of the chemical reactions associated with reperfusion injury, including free radical production, excitatory amino acid release, and calcium shifts, which can in turn lead to mitochondrial damage and apoptosis [15]. In addition, it can decrease cardiac output by 7% for each $1\,^{\circ}\mathrm{C}$ decrease in core body temperature with a stable stroke volume and the mean arterial pressure [16].

Despite the fact that induced hypothermia after cardiac arrest has been shown to increase the rate of neurologically intact survival, it can have many adverse effects. The whole treatment must be adequately monitored, particularly the controlled cooling. The adverse events of hypothermia therapy include pneumonia, metabolic and electrolyte disorders, sepsis and bleeding, which are often reported. There is one another adverse event : the cardiac arrhythmia, which is under discussion. Some investigators state that there is no significant cardiac arrhythmia after HT [4, 17]. However, other studies did show that cardiac arrhythmia occurs after HT. According to studies, the cardiac arrhythmia represents 6% ([18]) to $7\% - 14\%$ [19], even reaches or $33\% - 36\%$ [20, 21] of the total adverse events. So it is important to understand how the post-hypothermia-arrhythmia raises.

Here comes the motivation of this paper, with an experimental model in vitro : to study the arrhythmia generation by hypothermia in order to explore its triggering mechanism.

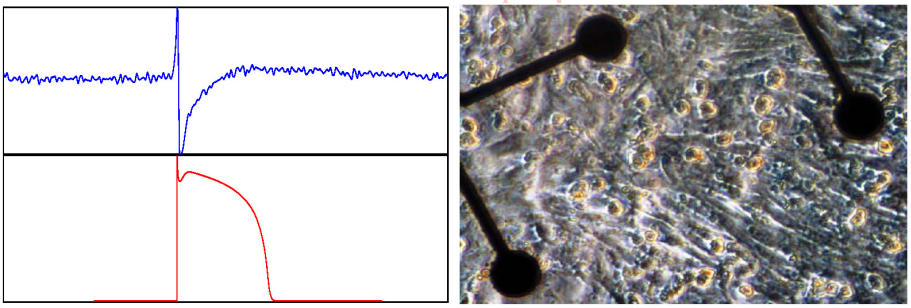
2 Materials and Methods

2.1 Cardiac cells culture

The experimental model used here is primary cardiomyocytes (CM) culture prepared from cardiomyocytes of new-born rats. One of the major advantages of the CM culture is, that myocardial contractions

of isolated muscle cells do not affect the stability of intracellular and extracellular electrodes, so the extracellular potential can be easily recorded by electro / optical sensors. Since there is no surrounding tissue to the culture, hemodynamic and neurohumoral influences are also absent, the functional and biochemical responses of cells depend only on cells themselves. Furthermore, isolated CM can be used under carefully controlled experimental conditions and be reproducible as well. Compared to other rat heart models, the engineered model in this study featured a mean period of action potential $0.50 - 0.72$ second in normal conditions. The culture beating ($83 - 120$ bpm) is similar to normal human heart, which is different from other similar models (normal rat heart beats at 600 bpm).

The acquired extracellular potential (EP) shares very similar electrophysiological properties of cardiac muscular cell in situ [22]. This model has been a good tool to study related cardiovascular problems, for instance, experiments on arrhythmogenic drugs, ischemia, hypoxia etc. [23, 24, 25, 26, 27].



(a) (b)

Figure 1. (a) Schematic illustration of the correlation between the extracellular potential and the action potential. Upper signal : extracellular potential; lower signal : action potential. (b) Cardiomyocytes culture under microscopy, zoom 40X. The black spots are electrodes of MEA, the light-yellow transparent objects are the nucleus of the CM.

EP signals have a close correlation of the depolarization and repolarizing phase of the action potentials (Figure 1a). This relationship allows to interpreting indirectly the results at "action potential propagation" level [28, 29]. Therefore, the CM cultures have the potential to reproduce in vitro a wide range of pathological conditions such as ischemia reperfusion, the radical stress or thermal shock, and any combination of these conditions.

Ethics Statement

This facility has ongoing approval to perform extraction of rat cardiomyocytes; protocols are approved by the French government, No. 00775, and are in accordance with the US National Institutes of Health guide for the Care and Use of Laboratory Animals. All animal handling and procedures are performed by individuals who are appropriate trained, and licensed by the government of France.

2.2 Multi-electrodes Array recording

The extracellular potential of the CM culture is acquired with the multi-electrode array (MEA) system. The MEA technology allows synchronously recording the extracellular potential. In addition, when applied to the cardiac cultures, it can provide a better spatial resolution than the mapping procedure by fluorescence and is less invasive than the conventional electrophysiology methods (intracellular recording or by patch-clamp) [30, 31].

The MEA, has typically 60 electrodes, aligned in a matrix form 8×8 with an inter-electrodes distance of $100\,\mu$m (the four corners are for fixation, so in total 60 electrodes). The electrodes have a diameter of $30\,\mu$m. The working region of a typical MEA is about 2.5mm^2. Every acquisition consists of 60 EP signals with a sampling frequency of maximum 50 kHz per channel and a 12 bits resolution. As for the whole MEA platform, it comprises four parts (more details in [32]: (A) pre-amplification module (PAM) of extracellular potential signal. (B) Faraday cage where the PAM is placed, in order to reduce environmental (mainly, electromagnetic) noise. (C) temperature controller, allowing to control the temperature of the CM culture. Temperature range is $20\,^\circ\text{C} - 50\,^\circ\text{C}$, temperature response is less than 30 s to 5 min according to different system. To reduce medium evaporation and dehydration induced by heating of the MEA plate, the MEA is covered by a Petri dish. (D) signal acquisition / processing system, developed under LabView and MATLAB.

The Figure 1b shows a CM culture on the MEA under optical microscopy, with 40X zoom. The black spots are electrodes of MEA, the light-yellow transparent objects are the nucleus of the CM. The cells form an inter-connected mono-layer cardiac tissue. Since the contraction of cells is synchronized, the tissue itself contracts automatically.

3 Results

The therapeutic hypothermia after cardiac arrest consists in two general processes : cooling and rewarming of patient. We have simulated these two procedures in vitro. For an in vitro model with mono-layer cardiac cell culture, its cooling and rewarming are much faster than the equivalent procedures in vivo. The obtained results are similar for both cases, but the "cooling" procedure showed more significant changes. The details will be discussed lately. After every change of temperature, to ensure that the culture is well stabilized, it is kept for 8 minutes before the recording. Since the tissue is monolayer culture, the reach of steady-state is quick. Generally, the signals become stable after 2 or 3 minutes.

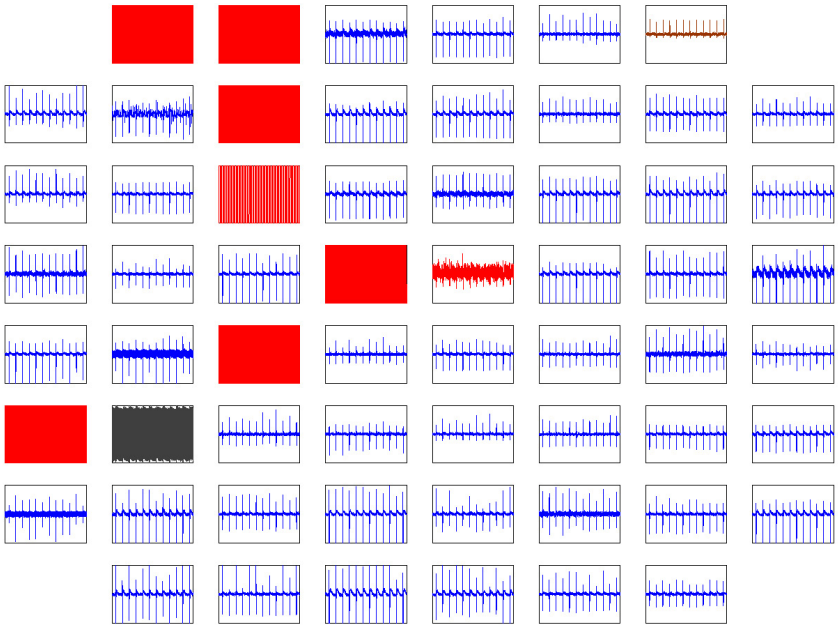


Figure 2. Examples acquired signals (matrix 8×8. No electrode at the 4 corners which are for fixation). Each panel shows an acquisition from an electrode, the position corresponds to its real position on the MEA. Signals in blue are good ones, others are excluded.

As shown in Figure 2, the acquired signals (each sub-panel) are presented in a matrix form 8×8, according to the positions of corresponding electrode on the MEA. Not all signals meet the quality requirements, only selected signals are used (in blue). The others signals are then eliminated. In normal condition, the culture activates spontaneously, beating regularly and showing stable behavior of regular period.

3.1 Single signal : period-doubling

Decreasing the temperature of the culture, the periods of EP signals increase in general (Figure 3). The periods variation at each temperature could be the consequence of junction-gap remodeling [33]. From

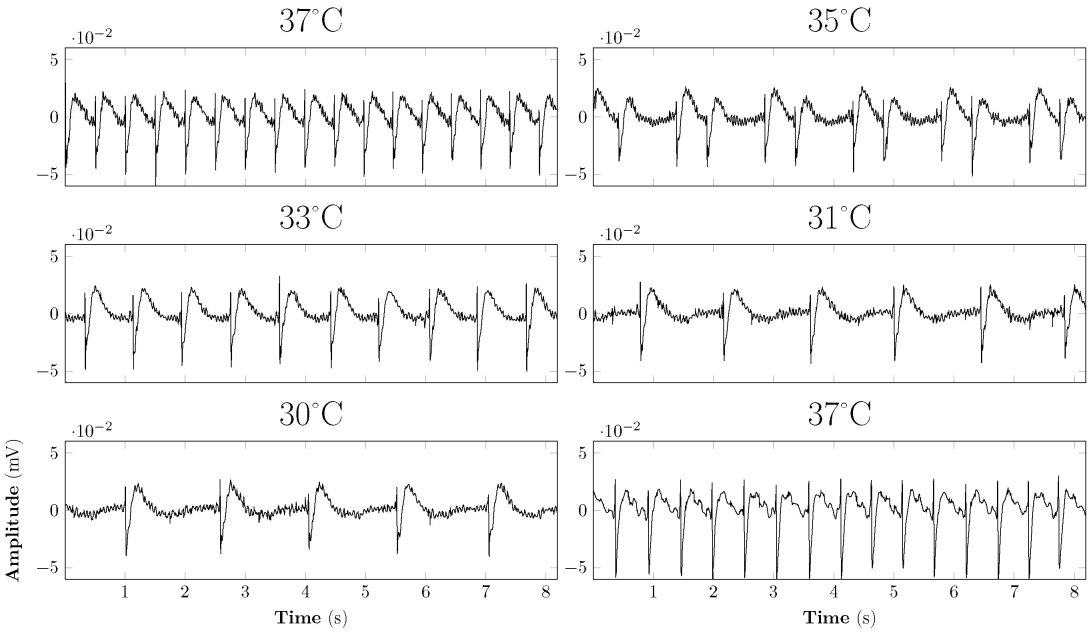
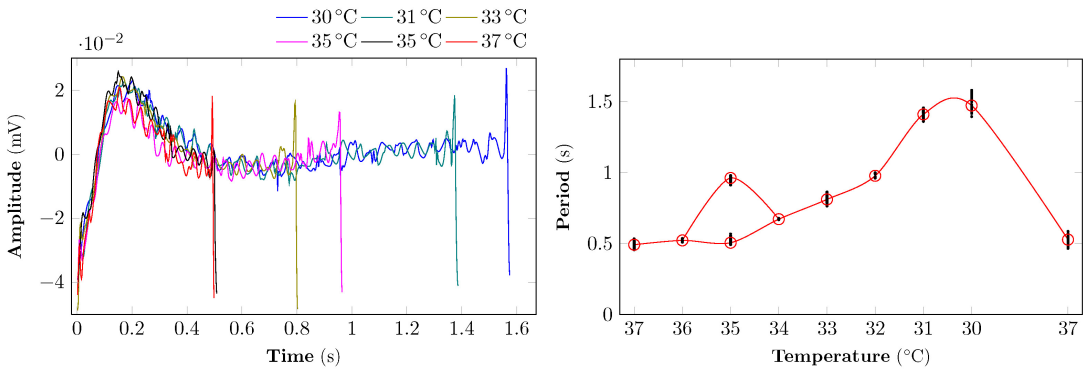


Figure 3. EP signals at different temperatures (cooling from 37 °C to 30 °C and rewarming back to 37 °C).

37 °C to 30 °C, the period is increased from about 0.5 s to 1.58 s. All periods are regular, except for 35 °C, which showed presence of different period values. In fact, the periods alternate with time in this case. Taking typical single potential at each temperature (Figure 4a), two main period values are shown at 35 °C : the lower value is almost exactly the same as the one at 37 °C, higher value is roughly the double. To better show the evolution of periods, a period diagram for different temperature is presented in Figure 4b. At 35 °C, a period-doubling phenomenon is observed. From a point view of nonlinear dynamics, this period-doubling is a marker when a system is conducted to stat "chaos" [34]. Change temperature down to 32 °C, the periods' value changed more quickly after this temperature. Entering in 31 °C $\sim$ 30 °C, the periods reach to their maximum values. Since this study focused on mild hypothermia, the experiments are stopped at 30 °C.

According to the recommendation from ILCOR, the hypothermia therapy should at first pass 35 °C.



(a) (b)

Figure 4. (a) Comparison of typical single period EP signals. (b) Periods digram, periods-doubling at T = 35 °C. Black points are periods values, red ones are median vales of periods at corresponding temperature.

The patient's temperature should above the threshold 32 °C, otherwise below which there is a risk of rebound hyperthermia, dysrhythmias and infections [35]. The result here showed that the 35 °C is a troubling temperature point. A transit point could exist around 32 °C. These results agreed with the clinical recommendation.

3.2 2D activation map : plane waves vs. spiral waves

The basic characteristics of EP signal, period, has been presented. It analyzes the signal from every electrode. However in the tissue, the action potentials are propagated from on cell (fiber) to anther (cell / fiber). As a result, it would be interesting to know how these signals act globally, in a two-dimensional space. One of the most used tools in this case, is the activation map : a map reconstructed spatially in function of arriving time of signal. Since in this study, a 8×8 MEA is used, the reconstructed map here is then 8×8 pixels. Though the image resolution is low (8×8 pixels), the main activities in the tissue can be captured. It is better shown in the real-time video, so please refer to the supplemented videos.

When temperature is different from 35 °C, plane waves propagation is observed (supplementary data : movie_plane_wave_sup01.mp4, movie_plane_wave_sup02.mp4). In Figure 5, nine snapshots have been presented to show the propagation of plane waves. Their trajectories fluctuate slightly (see the white arrows in Figure 5), the global propagation is stable and regular, which confirmed that the CM cultures have highly synchronized electrical activities like those reported for conventional endocellular recordings

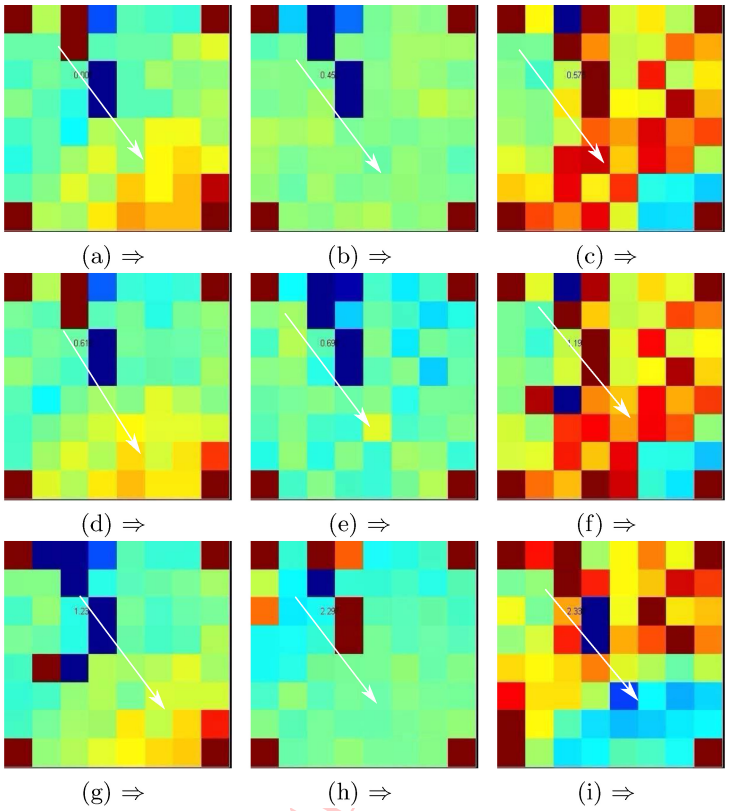


Figure 5. Activation map in normal condition T = 37 °C. Plane wave propagation, from time (a) to (i). White arrows indicate the propagation direction of plane wave.

[36]. However, at 35 °C, the activation map is marked by the presence of spiral waves (supplementary data : movie_spiral_wave_sup01.mp4, movie_spiral_wave_sup02.mp4, movie_spiral_wave_sup03.mp4).

Relevance of spiral waves to the arrhythmogenicity

The spiral waves are commonly considered as a sign of cardiac arrhythmia [37, 38]. In the pioneer work by [39], they validated experimentally (in vivo) Moe's hypothesis [40] of multiple wavelet (spiral waves / micro-reentry) of atrial fibrillation. They showed that there is a critical number of micro-reentries below which the cardiac arrhythmia can self-terminate. A large number of studies based on this "defibrillation concept" and its extension "sub-threshold stimulation" have been conducted [41, 42, 43]. These defibrillation methods could provide better termination of fibrillation with less harm to patient, which is a hot research point in recent years.

For the model in this study, the observation of spiral waves has been reported in previous studies

with different provocation methods. Arrhythmias could be induced by anomalous conduction, erratic pacemaker driving [44, 45] or by electrical stimulation [46]. Otherwise, in regular regime, a dominant pacemaker spontaneously activates the tissue (propagation of plane waves of action potential). The generation of spiral waves in this model induced by hypothermia, corresponds to clinical studies shown that in case of cardiac arrest, the therapeutic hypothermia could provoke arrhythmia cardiac [18, 19, 20, 21].

Therefore, the arrhythmogenicity of here-used model has been confirmed.

3.3 Bifurcation from phase space reconstruction

As previously shown, signals at hypothermia 35 °C have featured the period-doubling phenomena as well as spiral waves generation. A closer look at these phenomena reveals further insight on their behavior. Biological systems depend on many parameters. Under most dynamical regimes, conventional linear methods predict their behaviors only from one-dimensional time series. In this case, methods from chaos theory and nonlinear dynamics are therefore suitable to study the unpredictable behaviors of physiological signals. Among them, the method of phase space reconstruction [47] is a valuable tool for the studies of this kind of dynamical systems. The principle of this method is to transform the properties of a time series into topological properties of a geometrical object which is embedded in a space, wherein all possible states of the system are represented, each state corresponds to a unique point. So that the reconstructed space sharing the same topological properties as the original one. These states points form then a set of typical trajectories of the system, which allows showing information such as the existence of an attractor or limit cycles etc.

Mathematically, under the approach phase space reconstruction, the system can be represented as :

$$\vec{X}(\tau, m) = [x(t), x(t+\tau), \ldots, x(t+\tau(m-1))], t = 0, 1, 2, \ldots \quad (1)$$

where $\vec{X}$ denotes the system states and is a function of τ and m, $x(t)$ is the time series, m is the embedding dimension and τ is the time lag. m is estimated by the method False Nearest Neighbor (FNN) [48], which intends to find the minimal embedding dimension. As for time lag τ, a method based on autocorrelation function is used to find optimal values (largest one so that the resulting coordinates for m are relatively independent) [49].

In this study, since the signals are regular whatever the temperature is, the difference of their τ is quite small. The determining parameter is the embedding dimension m. As shown in Figure 6, during the cooling process, the mean embedding dimension initially decreases when temperature drops from 37 °C to 35 °C. It increases then slowly up to 3.52 at 31 °C to another transition point. The m increases abruptly after 31 °C. When rewarming, the temperature returns to 37 °C and m recovers its original value. This

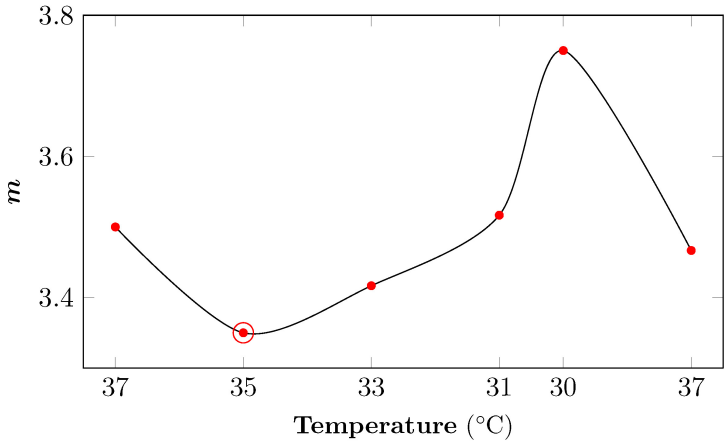


Figure 6. Mean value of embedding dimension m vs. temperature.

reinforces the evidence that a temperature around 35 °C contains a transition point for the system. In case of arrhythmia (spiral waves generation), the embedding dimension decreases, just like other studies (heart rate variability [50], electroencephalogram [51] etc.) reported that in case of pathology the related nonlinear parameters are often decreased. The stated transition point 31 °C for m re-confirmed the conclusion found in Figure 6 that in the range of 30 °C and 33 °C, there exists also a critical temperature.

Typical reconstructed phase spaces at different temperature are presented in Figure 7 and Figure 8. The trajectories in phase space have all been well-formed. Apart from 35 °C, though the original signals are different, the global forms of their trajectories are similar with just a little variation. This can be explained by the embedding dimension $m = 3.5 \pm 0.3$: despite the fact that there is a variation ± 0.3 of m, it is still relatively small compared to it median value $m = 3.5$ in the range of 37 °C − 30 °C. The widths of the trajectories are relatively tight, which proves from another point of view that the system is stable. Interesting trajectories are found at T = 35 °C, for most signals, the trajectories formed a bifurcation as in Figure 7, which reflect the period-doubling phenomena (Figure 4b) at T = 35 °C.

Rare trifurcation of trajectories (Figure 8) is also captured at this temperature. This bifurcation /

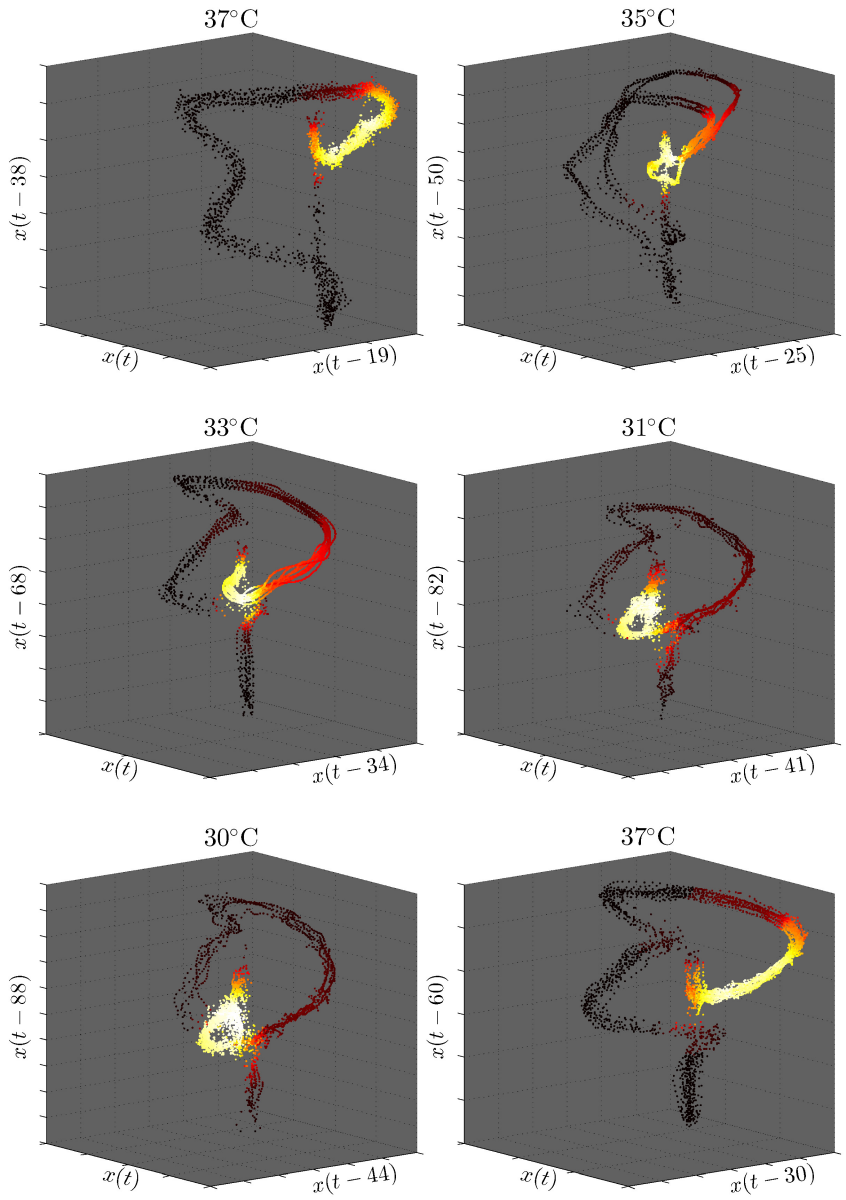


Figure 7. Phase space reconstruction for EP signals, bifurcation at T = 35 °C (time lagging unit: samples; darker color means looser points density, brighter color denotes higher points density).

trifurcation can be interpreted as supercritical pitchfork bifurcation (Figure 9), with the central path as a metastable state [52]. The bold black line denotes the stable states of the system. In the dashed line, the system is in an unstable dynamic state, but it could sustainedly remain there if quasi-static perturbations are not strong enough to make it fall to the (dynamical) lower energy states. This makes

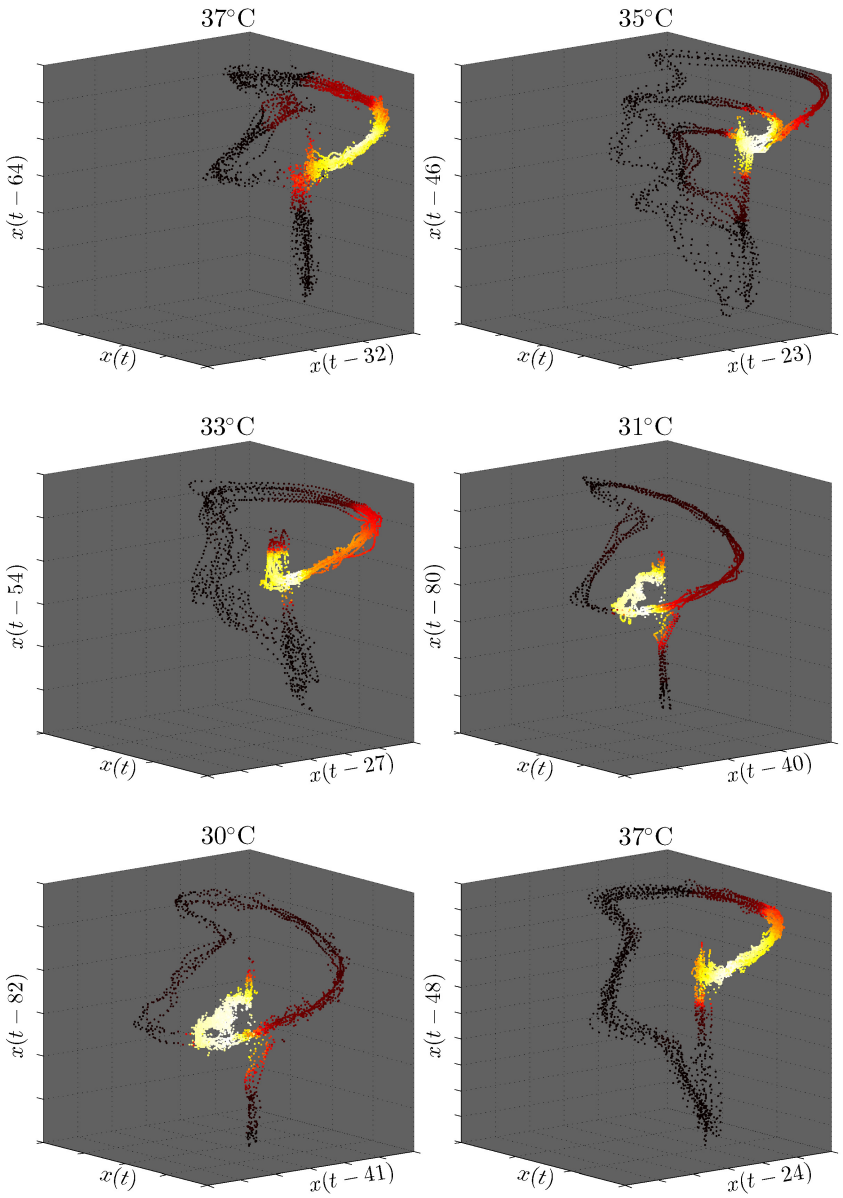


Figure 8. Phase space reconstruction for EP signals, trifurcation at T = 35 °C (time lagging unit: samples; darker color means looser points density, brighter color denotes higher points density).

that state a metastable state. From T = 35 °C, the system enters the unstable region. For example, if the system follows the black trajectory (double-line), it will cross twice stability line, which makes the period-doubling and so bifurcation in phase space will occur. If the system follows the red trajectory (triple-line), it will cross the stability line three times and exhibit trifurcation. Since in this region the

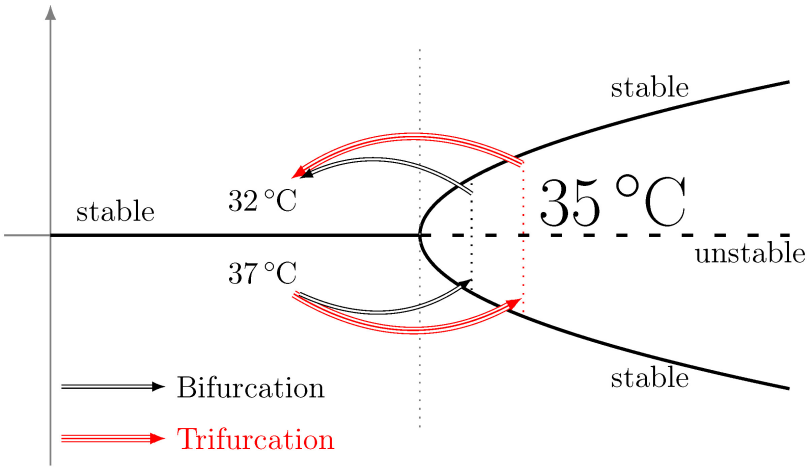


Figure 9. Illustration of Bifurcation or Trifurcation (type Pitchfork) of hypothermia effect on EP. The bold black line denotes stable state of the system. For bold dashed line, the system could show "stable" (line parts) or "unstable" (empty space) dynamics. Following the black trajectory (double-line), the system passes twice "stable" state, which makes the bifurcation in their phase space happen. if the system follows the red trajectory (triple-line), the system would pass three times "stable" state. There will be a trifurcation for the trajectories in phase space.

transition between stable and unstable states is fast and in most cases it is the unstable states which dominate the system dynamics, the metastable line is not always observed in practice.

4 Conclusion

The therapeutic hypothermia is considered as a safe and effective therapy after cardiac arrest. It is recommended for unconscious adult patients with spontaneous circulation after out-of-hospital ventricular fibrillation cardiac arrest. The outcome is positive.

In this article, the hypothermia effect of extracellular potential with a cardiac model in vitro has been studied. The object is to study the arrhythmia generation after therapeutic hypothermia to improve neurological outcome of cardiac arrest. The results showed that at T = 35 °C, spiral waves (arrhythmia) are observed. These spiral waves would disappear when the tissue's temperature returned to 37 °C. However, in realistic situation, once the arrhythmia happens, it will self-maintain and develop. This will provoke the global arrhythmia. From a point of view of nonlinear dynamics, it seemed that the process of hypothermia on cardiac tissue followed a pitchfork bifurcation. According to this diagram (Figure 9), the arrhythmia generation depends on the "stable" state that the system crossed in this "unstable" region.

Consequently, it is not certain that staying at 35 °C would create surely arrhythmia. This corresponds to the ratio diversity of arrhythmia among the adverse effects after hypothermia (from 6% up to 33% − 36%). Another finding is that there exists a transit temperature in the range of 33 °C − 30 °C, which is in accord with the clinical trials that in therapeutic hypothermia the temperature should not be bellow 32 °C.

Cooling the tissue with mild hypothermia ($\geq 30\,^{\circ}\mathrm{C}$) can induce spiral waves in the tissue. If the tissue is cooled $< 35\,^{\circ}\mathrm{C}$, the spiral waves are auto terminated and transformed into regular plane waves. This agreed with another study of mild hypothermia effect on termination of spiral waves [53]. And at lower temperature (deeper hypothermia $5.9\,^{\circ}\mathrm{C} \pm 1.3\,^{\circ}\mathrm{C}$), local cooling facilitates termination of spiral waves [54]. The latter two studies [54, 53] showed the positive effect of hypothermia in macro / organ scale. Our study provided instead another evidence at micro / cellular level. In another recent study of the ECG changes of out-of-hospital cardiac arrest during and after mild therapeutic hypothermia [55], they reported similar results as ours : the RR-interval is almost doubled during hypothermia (1019 ± 332 ms) compared to normal ECG (660 ± 161 ms). This study proved the relevance of our study, at a macro-scale. So our results would also be applicable to human data.

5 Discussion

In all our experiments, the cooling procedure induced arrhythmia more easily than the rewarming procedure. The potential mechanism would be that when cooling down the tissue, the action potential became longer and propagated slower. This would disturb the precedent plane waves and made them transform to spiral waves. However, in the reverse case (rewarming), the new generated waves propagates faster so that the precedent waves are swept away. In consequence, there is few chance that the current plane waves could be transformed into spiral waves.

The observed change of dynamics at 35 °C looked like the alternans change from 1:1 to 3:2 in case of Atrio-Ventricular Block (type Wenckebach [56]). Following the Atrio-Ventricular Block explanations, this could be interpreted as a propagation block which provoked the spiral waves in the tissue at 35 °C. Here, there is one important difference between the studies with paced models and this one. We used here primary cardiomyocytes tissues which are self-excitable and have spontaneous and regular electrical / mechanical activities. If under external electrical stimulation (like the pacing of perfused ventricular preparations in optical mapping study), the culture's electrical activities will be disturbed and the normal

plane wave propagation would be transformed into spiral waves. The propagation block and the period-doubling could be related one to the other. It is difficult to experimentally justify this point, since the intrinsic factors are unknown. This would be a limitation of this study. What's more, the experimental model differences could be also an obstacle to draw common conclusion.

A possibility of the 1:1→3:2 transition is in case where the cells are not well spatially-coupled, i.e. important heterogeneity preventing the waves from propagating when the cells are not coupled. For example, if the third wave is blocked, the 1:1→3:2 transition would thus happen. In this study, the prepared cells are characterized by their ability to proliferate after dissociation and inoculation. This helps to obtain a homogeneous monolayer in vitro of cardiac muscle cells population. One should notice, however, that the homogeneity of the tissue implies that secondary pacemakers can appear anywhere. If the dominant pacemaker region does not fire at some point (e.g., a 3:2 block) then a secondary pacemaker that is only slightly longer than the dominant one would drive during the blocked third wave. The resulting behavior would be complex, by interaction of these two regions, and far from a clear period doubling like the one observed. Our observations suggest hence a collective behavior, not a local one. The influence of heterogeneity can be nevertheless a potential way of 1:1→3:2 transition which needs further studies.

Other study showed indeed that 35 °C could be a troubling temperature. Chudin et al. [57] reported that rapid pacing of rabbit ventricular myocytes at 35 °C can lead to increased intracellular Ca^{2+} levels and then provoke complex action potential pattern, including the alternans change from 1:1 to 3:2. This provides another supporting evidence of the current result.

A recent work [58] on the role of temperature on nonlinear cardiac dynamics (alternans in paced ventricular tissues) showed very interesting results. They found that in case of hypothermia (26 °C), "... the sigmoidal shape of the restitution curves ... gives rise to a rejoining of the bifurcation diagram during progressive reduction of the circuit length ...". In our study, we observed similar bifurcation (temperature-period) at cellular level compared to their study at ventricular level. Even though there is no straightforward way to compare two different dynamics (auto-excitable vs. pacing; restitution curve vs. temperature-periods curve), the similarity in both studies implies indeed that there would exist a hypothermia threshold triggering this potential bifurcation mechanism. More work is needed to determine hypothermia threshold.

6 Limitations

One of the main concerns about in vitro studies with rat model is the "difference among species". While a rat heart largely differs from a human heart, it is one of the most used animal models to study human cardiovascular diseases. E.g., it has been successfully used to validate drug targets and to determine efficacious and safe dosage schemes for combination treatments in humans. The experimental models do not aim to fully model a disease or disease mechanisms at system level, but rather set out to obtain specific functional information.

Should be mentioned once again, our experimental model is different from other rat models. It has similar characteristics as human data, as previously discussed. The objective of the presented manuscript is to use this model to explore the potential mechanisms of PHA. Nothing in these mechanisms looks *a priori* specific to the rat, neither the hypothermal nor the arrhythmic ones. Size and anatomy are not relevant as we are considering a cultivated tissue. We could of course expect significant quantitative differences in parameters between rat and human myocytes : The reported bifurcation in the dynamics could possibly take place at a slightly different temperature. This requires a further extended study, which is one of the objectives in an on-going project.

The diagram (Figure 9) proposes that the longer that the culture is staying at 35 °C, the higher the probability of arrhythmia would be. It implies also that a variable speed of cooling / rewarming would help to reduce the possibilities of arrhythmia. However, due to the fact that cooling and rewarming of the mono-layer cardiac culture is almost instantly, it is not possible to study the effect of speed of cooling / rewarming with the current culture. To better understand the mechanism, other studies with a full model (isolated heart or heart in situ) are required. Further experiments and developments are in progress.

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