METHOD FOR STUDY MYOCARDIAL TISSUE IN VITRO: APPLICATION OF NANOSECOND PULSED ELECTRIC FIELDS (nsPEFs)

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Abstract Nanosecond pulsed electric fields (nsPEFs) were used to investigate the different responses arising in myocardial tissues when exposed to a 100 ns electrical pulse and to understand the influence of the electrical parameters on induced phenomena. Nanosecond pulse electric fields can affect the intracellular structures of cells in vitro. Nanosecond pulse electric fields applied to the cardiac tissue are considered to be a possible tool for treatment of arrhythmias or to improve blood circulation inside the myocardial tissue. For this study were used myocardial tissue surgically removed from Wistar rats. The tissue was suspended in Krebs-Henseleit solution, placed under an electrode and subjected to 100 ns voltage pulses (2.5 – 10 kV/cm). 300 and 500 pulses at 0.5 Hz were applied. For an early indication in particular, histological investigations of the myocardial tissues exposed to nanosecond pulsed electric fields (nsPEFs) were performed to determine the extent of the morphological modifications produced at the cellular level.

After exposure, tissues were prepared for optical microscopy, in order to observe any changes in cell morphology. Histopathological assessment has shown important modifications, such as significant capillary congestion, an aspect which may be explored as a potential application for treating the myocardial ischemia.

Key words: Nanosecond pulsed electric fields (nsPEFs), myocardial tissue

INTRODUCTION

Exposure of the cellular membrane to electrical currents of various intensities it is well known to induce changes in the electrical characteristics and damages to the lipid bilayer, both transient and permanent. These changes, that alter in various ways the permeability of the cell membrane, not all of them lethal, were later summed under the name of electroporation (1). Nanosecond pulsed electric fields (nsPEFs) are ultra short pulses with high electric field intensity (kV/cm) and high power (megawatts), but low energy density (mJ/cc) (2).Pulsed electric fields induce different kinds of biological effects that are different from the normal electric fields. These effects appear due to the interactions between nsPEFs and cells. When biological cells are exposed to high power electrical pulses, the observed effects may either be caused by the direct interaction of the pulsed electric field with the cell, or caused by the increased temperature of the suspension due to Joule heating, or a combination of both (3)

How the nsPEFs acts on the cells at the molecular level as well as the mechanisms inducing these effects are still poorly understood. Exposures of cells to pulsed electric fields of ultrashort duration (nanoseconds) and high field strengths (tens of kilovolts per centimeter) can alter cell morphologies and functions. On this basis, applications can be devised for bacterial decontamination, wound healing, and cancer
The use of nanosecond pulsed electric fields has found applicability in different domains, such as medicine, pharmacy and pharmacology. Therefore nsPEFs were used for: induction of apoptosis (5), reversed penetration of the cellular membrane (6), gene transfer (7, 8), chemotherapeutic drugs delivery (9) or in different types of cancer therapy (2,10-11).

Nanosecond pulse electric fields affect plasma membrane and can also affect the intracellular structures of cells in vitro. Nanosecond pulse electric fields applied to the cardiac tissue are considered to be a possible tool for treatment of arrhythmias or to improve blood circulation inside the myocardial tissue. In this study, the effect of 100 nanoseconds pulsed electric fields were applied on myocardial tissue collected from Wistar rats to investigate the influence of the electrical parameters on the intracellular structures of cells in vitro. Histopathological assessments were carried out to investigate the morphological modifications of the cells after exposure to nsPEFs. Current and voltage measurements were performed to monitor the variations in liquid.

MATERIAL AND METHODS

Materials
Pulsed electric fields

The myocardial tissues were collected from the apex of the left ventricle of a Wistar rat of 250 g weight. The tissue suspended in Krebs-Henseleit solution was exposed 300 consecutive pulse electric fields at a repetition frequency of 0.5 Hz. The nsPEFs were produced using DC power supply (Spellman, High Voltage Electronics Corporation) and a Blumlein line generator matched with a 200 MΩ resistor to deliver a singular rectangular 100-ns pulse generator (Fig.1 left). The details of the in-house pulse generator were presented in a previous work (12). The average value of the applied electric field to the Krebs-Henseleit solution was around 2500 V/cm (Fig.1 right). Voltage waveforms were acquired using a Tektronix 6015A probe connected to a Tektronix 2024 B oscilloscope. After the myocardial tissue was immersed in the solution, the external electrical circuit impedance (taking into account that the myocardial tissue is strongly vascularised, therefore its impedance differs from that of the Krebs solution) is modified and the electric field maximum value increased to 3300 V/cm.

Figure 1. 100 ns pulse generator (left); voltage wave forms acquired during the myocardial tissue exposure to nsPEFs (right)
**Myocardial tissue preparation**

**Stage I: Sampling of processed fragments**
- Wistar rat is anesthetized with ether
- The heart was collected through a median incision of the rib cage
- Identification of three myocardial fragments from the tip of the heart (each fragment is 1 mm thick, 4 mm wide and 3 mm wide) to be processed (one fragment for the control and the other two fragments to be stimulated with 300 and 500 pulses)
- The total injected energy applied during the treatment was 6J.

**Stage II: Processing and fixation in 10% formalin.**
- Immediately after sampling, the control fragment is introduced in formalin solution (10% concentration), while the other two fragments received 300 nsPEFs pulses respectively 500 nsPEFs pulses.
- Immediately after the application of pulses, the fragments on which were applied pulses are introduced in 10% formalin solution. Fixation in 10% formalin aims to stop the vital phenomena in tissues and cells, in order to capture at microscopic level some histophysiological or histopathological aspects existing in the collected product at harvest moment. Formalin fixation prevents post-mortem alterations of tissue or cell structures and strengthens the piece, so that it can be subjected to further processing.

**Stage III: Inclusion in paraffin** – this operation prepare the piece in order to create conditions that enable it to be cut in thin sections (5 μ) that can be stained and examined under a microscope.
After 24 hours, the fragments fixed in formalin solution are subjected to the following steps:
1. Dehydration (removal of water from fixed parts). Acetone was used as dehydrating agent. Three dehydration bathrooms in smoked glass were employed. The pieces were maintained 1 h in every acetone bath.
2. Clarification. During this step the piece is impregnated with paraffin solvent. This is performed at laboratory temperature, in high glass containers, sealed with an airtight stopper. Three baths are used for clarification (50% benzene + toluene mixture), allowing rapid penetration of the piece.
3. Parafining (penetration of melted paraffin in pieces and their solidification). This operation is run hot in thermostat at 58-60°C. Three paraffin bathrooms were used, each lasting 60 minutes.
4. The inclusion (embedding parts well soaked with paraffin in a paraffin block). Special metal boxes were used for inclusion of the blocks in paraffin. The piece that will be included was placed in a metal box with face designed for sectioning down, then was covered with a plastic box. The liquid paraffin was added until the plastic box was covered completely. It will be left until total solidification of the paraffin. The pieces included in paraffin blocks were prepared for microtome sectioning.

**Stage IV: Microtome sectioning of the pieces included in paraffin.**
The piece fixed in paraffin was included in the fixation device of the microtome, the thickness of the sections being set to 5 μm. Once sectioned, the samples were collected with warm water using a fine brush and a needle for dissociation.
After elimination of the folds from ribbon of paraffin, the sections were displayed from water on the slide. Sections displayed on the slide were subjected to staining after the paraffin was removed. This was performed by keeping the slide in thermostat at 58-60°C for 1 h. Then the blades were exposed for 60 minutes in a mixture compose of 50% benzene/ toluene. Subsequently, the blades were immersed in
three successive baths of 96° alcohol. From alcohol baths, slides were rinsed with tap water, and then placed in dye baths.

**Stage V: Staining**

Two types of staining were used: Haematoxylin Eosin (H-E) staining and van Gieson (VG) staining.

For H-E staining, all the steps were respected (deparaffinize in a mixture compose of 50% benzene/ toluene, 96% alcohol bath for three times, hydration, haematoxylin staining, rinse, dehydration in 98% alcohol bath for three times, drying, clarification in benzene/ toluene bath, mounting with Canada balsam). The nuclei and elastic fibers were colored in gray-black, cytoplasm in yellow, collagen fibers in red.

For van Gieson staining, all the steps were followed as well (deparaffinize in a mixture compose of 50% benzene/ toluene, 96% alcohol bath for three times, hydration, staining, rinse, staining with fuchsine, rinse, dehydration in 98% alcohol bath, drying, clarification in benzene/ toluene bath, mounting with Canada balsam). The nuclei and elastic fibers were colored in gray-black, cytoplasm in yellow, collagen fibers in red.

**Stage VI: Study under the optical microscope**

The slides with the samples were studied with ocular of 10X and objective of 10X (magnification of 100 times) and with objective of 40X (magnification of 400 times).

**RESULTS**

**For the control fragment:** Capillaries, muscle striations and nuclei of normal appearance were observed (Fig. 2, 3)

![Figure 2: capillary vessels, nuclei and integral striations (Objective 40X, H-E staining)](image1)

**Figure 2: capillary vessels, nuclei and integral striations (Objective 40X, H-E staining)**

![Figure 3: capillary vessels and integral nuclei (Objective 40X, van Gieson staining)](image2)

**Figure 3: capillary vessels and integral nuclei (Objective 40X, van Gieson staining)**

**For the fragment treated with 300 pulses** — two types of acute cell lesions were seen:

A. **Reversible lesions:** cell edema characterized by cellular hyperhydration. This is the first lesion that appears in all kinds of cell aggression at small intensity. It occurs when cells can no longer maintain hydro-ionic balance by altering the Na-K pump in the cell membrane. Microscope evidenced vacuolar degeneration (Fig. 4, 5):
B. Irreversible lesions (cell necrosis): occurs when the etiologic agent of high intensity will continue its action and causes irreversible acute changes (necrosis): lysis of muscle fibers by destroying the membrane (cellular lysis appear due to degradation of lysosomal membranes and discharging of lysosomal enzyme), disappearance of striations, agglutination of nuclear chromatin with the appearance of nuclear dysplasia (Fig. 6):

Figure 5: vacuolar degenerescence with perinuclear vacuoles (Objective 40X, H-E staining)

Figure 6: Nuclear dysplasia, nuclear pyknosis (nuclei are much darker and shrink / become pyknotic) and there are no visible chromatin details (Objective 40X, H-E staining)

Figure 7: nuclear dysplasia, homogenization of muscle fibers (Objective 40X, H-E staining)
For the fragment treated with 500 pulses – the same modifications determined by cellular stress were observed, such as:

A. **Reversible lesions**: cell edema and capillary vasocongestion with preservation of integrity of vascular walls (capillary congestion can be an interesting subject to study in acute / chronic myocardial ischemia) (Fig. 8).

![Figure 8: cellular edema, capillary vessels, nuclei and integral striations (Objective 40X, H-E staining)](image)

B. **Irreversible lesions**: partial lysis of muscle fibers (partial disappearance of striations and nuclear pyknosis) (Fig. 9).

![Figure 9: partial lysis of muscle fibres (Objective 40X, van Gieson staining)](image)

**DISCUSSIONS**

Myocardial tissue is a potent generator of electrical currents and it is also highly sensitive to electrical currents. It’s impressive array of channels and pumps maintain a highly regulated electrical “atmosphere” around and within the myocardial cell. The application of nanosecond pulse discharges in the close vicinity of those cells has been studied to the purpose of tumor ablation (13), gene transfer to the living heart (14-16), prevention or treatment of arrhythmias (17), but what major changes are induced in the myocardial tissues, and the physiological effects of pulsed electrotherapy on the heart remain to be studied.

Studies made in cancer research shows that cytotoxic efficiency cannot be correlated with cell size, nuclear size, cell morphology, metabolism level, or the extent of membrane disruption by nsPEF. Matters only the parameters of PEF (18). At cellular level, nsPEFs determine changes in voltage of plasma membrane (19) and influence elasticity of cell membrane due to disruption produced in actin cytoskeleton and due to irreversible membrane damage (20). The mode of cell death induced by nsPEFs is cell-type dependent and extracellular Ca$^{2+}$ is a critical factor for nsPEF-induced necrosis (21). We found that for a different number of pulses (300 or 500) applied on the myocardial tissue, the results are not very different.
We obtained reversible and irreversible lesions in both cases. In future we consider that using a smaller number of pulses, results can be different. Our results may be correlated with other studies made on cell culture which have shown that the principal cause of cell death induced by nsPEFs is the loss of the plasma membrane integrity, leading to water uptake, cell swelling, and membrane rupture (22).

Coronary ischemia is one of the major causes of morbidity and mortality worldwide. Although the progress made by coronary revascularization techniques is enormous, there are types of patients who do not benefit from it, particularly those suffering from diseases of the microcirculation.

In a rabbit model, scientists have demonstrated that nsPEFs improves blood flow in large surgical skin wounds and in ischemic wounds (23). In our study, we found that nsPEFs might induce vasodilatation at microcapillary level. This finding could be used in an attempt to enhance blood flow in ischemic myocardial tissue.

CONCLUSIONS

Although many sophisticated methods were used in order to study heart or myocardial tissue, there are few studies where the influence of nsPEFs on myocardial tissue is reported. Because we found significant alterations in cells treated with nsPEFs, we consider this like a proof that nsPEFs can also be a method that can be used in order to study myocardial tissue.

The total injected energy applied during the treatment was 6J, compared to that administered during defibrillation shocks (60 -360 J). After histological examinations were identified reversible and irreversible morphological modifications for the samples exposed to nsPEFs. Significant capillary congestion that was observed and this aspect may be explored as a potential application for treating the myocardial ischemia.

Further investigations will be carried out to elucidate the mechanism of the morphological modifications observed for the myocardial tissues exposed to nsPEFs.

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REFERENCES

2. Hall EH, Schoenbach KH, Beebe SJ. Nanosecond pulsed electric fields (nsPEF) induce direct electric field effects and biological effects on human colon carcinoma cells. DNA and Cell Biology. 2005;24(5):283-291.