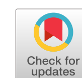
Bioelectrochemistry 125 (2019) 79–89

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Molecular and histological study on the effects of electrolytic electroporation on the liver



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ARTICLE INFO

Article history:
Received 4 July 2018
Received in revised form 24 September 2018
Accepted 28 September 2018
Available online 30 September 2018

Keywords:
Eelectrolysis
Electroporation
Tissue ablation
Necroptosis
Pyroptosis

ABSTRACT

This study examined the temporal physiological and molecular events following the treatment of the liver with a tissue ablation modality that combined electroporation with electrolysis (E2). Rat liver was treated with an E2 waveform and the tissue examined, 1 h, 3 h, 6 h and 24 h with: H&E, Masson Trichrome, TUNEL stains and Western blot. H&E and TUNEL stains have shown that cell death began to be evident 3 h and hepatocyte regeneration was seen 24 h after treatment. H&E and Masson trichrome have shown that the extracellular matrix and the large lumens, appeared intact after E2. Western blot has shown the following molecular events after E2: cleaved caspase 3–downgraded at 1 h, upgraded at 24 h (apoptosis); cleaved Caspase 1 and cleaved GSDMD–upgraded at 6 h (pyroptosis), RIP3–upgraded at 1 h, MLKL–upgraded at 3 h (necroptosis). The mechanism of cell death was possible initiated by necroptosis pathway. Pyroptosis pathway was also activated. The observation that cell death from E2 was by programed necrosis and the details on the temporal molecular pathways, may have value for the recent attempt to combine electroporation mediated ablation with immunological treatment, by demonstrating that the cell death from E2 involves an inflammatory response and by providing data that could be used to design the optimal timing for the injection of immunological adjuvants.

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1. Introduction

Tissue ablation by minimally invasive surgery has become an important field in modern medicine. Many of the minimally invasive tissue ablation techniques use the biophysical and biochemical effects of electricity. The effects used for tissue ablation by electricity are of two types, thermal and non-thermal. The thermal modalities employ the temperature elevation caused by dissipation of electrical energy, the so-called Joule heating effect. Various electromagnetic frequencies are used for thermal tissue ablation, including direct current, radio frequency and microwave frequency [1]. Electrolysis [2] and various modes of electroporation [3–5] produce nonthermal mechanisms of tissue ablation. The nonthermal modalities affect only the cells in the tissue and spare the extracellular matrix [6,7]. There are several applications in which nonthermal ablation is considered advantageous, particularly in the treatment of tumors near sensitive sites, such as in the liver near large blood vessels [8] and the pancreas [9].

In electrolytic ablation, electric currents are delivered through two electrodes brought to the vicinity of the targeted tissue. The current is delivered in such a way as to produce an electrochemical reaction, electrolysis, at the surface of the electrodes submerged in an ionic conducting medium. New chemical species are generated at the interface of the electrodes and diffuse away from the electrodes into the tissue by diffusion along a concentration gradient and by electrophoresis. These species are able to create a cytotoxic environment, which can induce cell death. A leading mechanism of cell death is due to local changes in pH [10–12]. During the last two decades, substantial research was done on tissue ablation by electrolysis [5,8–17]. Electrolytic ablation requires low voltage and current, providing advantages relative to other ablation techniques, including reduced instrumentation complexity. The goal of electrolytic ablation is to produce a large volume of ablation around the electrodes, and continuous ablation between the electrodes is desired. Because the products of electrolysis are produced at the electrodes, because they reach the tissue that is distant from the electrodes by diffusion and electrophoresis, and because certain minimal concentrations of electrolytes and times of exposure are needed to cause cell ablation, the complete ablation of large volumes of tissue between the electrodes is usually a lengthy procedure that can take tens of minutes to hours.

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https://doi.org/10.1016/j.bioelechem.2018.09.007
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Electroporation is the permeabilization of the cell membrane by a pulsed electric field delivered across the cell [18–20]. The effect on the cell membrane is a function of the electric field strength, pulse time duration and pulse frequency. Lower electric fields produce reversible electroporation, in which case the cell returns to its original state several seconds or minutes after the electric field has ceased [20]. This phenomenon is used for gene delivery [21,22], uptake of drugs or genetic materials into cells, inserting proteins into the cell membrane, and fusing between individual cells [23]. Electrochemotherapy, the combination of reversible electroporation and anticancer drugs, such as bleomycin, has been used successfully for tumor ablation in the clinic [24–26]. Electrochemotherapy usually employs eight 100 microsecond long pulses with electric fields of between 1 kV/cm and 1.5 kV/cm. While the technology is highly effective at treating cancer, it requires the injection of drugs, making it a regulatory drug therapy [27,28].

A combination of higher electric field and longer exposure time electroporation pulses results in cell death through a mechanism broadly referred to as irreversible electroporation (IRE), i.e., the cells succumb to the membrane permeabilization by electroporation [29]. IRE has also gained success in clinical tumor ablation [30]. IRE can ablate tissues without the need for drug injections and without resorting to thermal damage, which is why the procedure is also known as nonthermal irreversible electroporation (NTIRE) [31]. The NTIRE procedure is considerably faster than conventional electrolytic ablation. NTIRE ablates only the cells while preserving the extracellular matrix. This approach spares important parts of the treated tissue, such as large vessels and other lumen structures [29]. However, the high electric fields and the large number of pulses used in NTIRE have several drawbacks. NTIRE pulses induce muscle contractions that require the use of a muscle relaxant and deep anesthesia during surgery [32]. The muscle contractions may also move the electrodes during treatment resulting in possible complications [33]. The large number of pulses is usually delivered at a frequency of 1 Hz to fit into the refractory period of the heart cycle. While the actual electroporation part of the procedure is brief, the logistic complications associated with the placement of the electrodes and the large number of pulses substantially lengthen the procedure.

Recently, we have shown that by judiciously designing tissue ablation protocols that combine electroporation with electrolysis (E2), we can produce a synergistic effect that enhances tissue ablation [34–36]. First, we have shown that complete ablation between the electrodes by a combination electrical protocol of electroporation and electrolysis requires doses of electroporation and doses of electrolysis which, when delivered separately, cannot produce complete ablation between the electrodes [34,35,37]. Therefore, the combination has certain advantages over tissue ablation by either electroporation or electrolysis alone [34,35]. One advantage of this combination is that it requires substantially fewer electric pulses and a considerably lower electric field than conventional NTIRE. Because lower amounts of electrolytes are needed for complete ablation between electrodes, the procedure is faster than tissue ablation by IRE alone and considerably faster than electrolysis alone. E2 is also nonthermal and does not require the injection of drugs. Our earlier studies on E2 employed waveforms that delivered electrolysis and electroporation sequentially and separately [34–36]. The mechanistic explanation of the results is thought to be related to the permeabilization of the cell membrane by all modes of electroporation. The products of electrolysis can thus gain access to the interior of the cell by the electroporation-permeabilized cell membrane and cause cell death at a lower dose than conventional electrolysis. Subsequently, we have shown that the combination of electroporation and electrolysis can be achieved through the design of a special waveform that delivers simultaneously electroporation and electrolysis [38–40]. For small volumes of tissue, this waveform can take the form of an exponential decay, while for larger volumes of tissue, the waveform requires several modifications, particularly in the electrolysis delivering tail of the waveform [38,39]. The primary effect of the initial voltage is to cause electroporation, and the primary effect of the lower voltage is to produce increasing amounts of electrolysis to cause complete ablation of the entire tissue between electrodes. Furthermore, the trailing lower voltage also provides an electrophoretic force to propel these products into the cells. Previous preliminary studies in a rat liver model have shown that single, short, millisecond-long exponential decay voltage waveforms can ablate the treated volumes of tissue without attendant muscle contraction [38]. Moreover, the larger animal experiments (in pigs) have shown that lesions of a clinically significant size can be achieved by single-millisecond-long exponential-like decay voltage waveforms [41].

While the previous studies have focused on the potential clinical value of the combination of electroporation with electrolysis (E2), to date, there is no study on the fundamental physiological and molecular processes, which occur in tissue treated with E2. The goal of this study is to advance the clinical use of E2 by generating fundamental information on the physiological and molecular events, which occur within the first 24 h after the liver was treated by an E2 waveform. To the best of our knowledge, this study is the first of its kind for the combination of electrolysis and electroporation, E2. In the study reported in this paper, the rat liver was treated with an E2 (exponential decay-like) waveform, and the liver was examined at 1 h, 3 h, 6 h and 24 h after the E2 treatment. The H&E, Masson trichrome and TUNEL staining were used for histology. Several of the highlights of our findings are that the hepatocytes begin to disintegrate 3 h after the E2 treatment, while the extracellular matrix appeared intact. Western blotting was used to examine the pro- and cleaved caspase-3 (marker for apoptosis), pro- and cleaved caspase-1 and gasdermin D (markers for pyroptosis), and RIP3 and MLKL (marker for necroptosis). Interestingly, there is no evidence of apoptotic cell death from E2 treatment. However, the molecular pathways of pyroptosis and necroptosis, programed necrosis associated with inflammation, are activated as early as 1 h after the E2 treatment.

2. Materials and methods

2.1. In vivo experimental procedure

Sprague–Dawley rats weighing 250–350 g were used in this study. All animals received humane care from properly trained professionals in compliance with both the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (NIH publication no. 85–23, revised 1985).

The animals were anaesthetized with 2 mg/kg meloxicam and 0.05 mg/kg buprenorphine followed by chamber induction with isoflurane. Bupivacaine (up to 8 mg/kg) was administered subcutaneously along the midline of the abdomen as local anesthesia. Anesthesia was administered throughout the procedure with vaporized isoflurane. The depth of anesthesia was assessed prior to surgery and during the surgery by monitoring the rats' reflexes to pressure on their tail and feet. The breathing rate and the color of the nose and tongue were also monitored and recorded. The depth of anesthesia was assessed before surgery and throughout the surgical procedure. After the level of anesthesia was verified, the abdominal skin was shaved. Sterile surgical techniques were used throughout the entire surgery. After the level of anesthesia is verified, the peritoneal cavity is entered via a midline incision of the abdomen. Three lobes of the liver were treated in each animal and each lobe was treated once. After partial mobilization of the liver from adjacent tissue, the treated liver lobe was gently clamped between two 10 mm diameter electrodes (Harvard Apparatus, Holliston, MA, USA), as shown in Fig. 1-a. The treated liver lobe was gently clamped between two 10-mm diameter stainless Tweezertrode™ electrodes (BTX/Harvard Apparatus, Holliston, MA, USA). The measured distance between the two electrodes is 3.0 ± 0.2 mm. The distance between the electrodes was measured with a caliper at a resolution of 0.1 mm. The E2 waveforms

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