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Single exponential decay waveform; a synergistic combination of electroporation and electrolysis (E2) for tissue ablation

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Background: Electrolytic ablation and electroporation based ablation are minimally invasive, non-thermal surgical technologies that employ electrical currents and electric fields to ablate undesirable cells in a volume of tissue. In this study we explore the attributes of a new tissue ablation technology that simultaneously delivers a synergistic combination of electroporation and electrolysis (E2).

Method: A new device that delivers a controlled dose of electroporation field and electrolysis currents in the form of a single exponential decay waveform (EDW), was applied to the pig liver and the effect of various parameters on the extent of tissue ablation was examined with histology.

Results: Histological analysis shows that E2 delivered as EDW can produce tissue ablation in volumes of clinical significance, using electrical and temporal parameters which, if used in electroporation or electrolysis separately, cannot ablate the tissue

Discussion: The E2 combination has advantages over the three basic technologies of non-thermal ablation: electrolytic ablation, electrochemical ablation (reversible electroporation with injection of drugs) and irreversible electroporation. E2 ablates clinically relevant volumes of tissue in a shorter period of time than electrolysis and electroporation, without the need to inject drugs as in reversible electroporation or use paralyzing anesthesia as in irreversible electroporation.

1 **Single exponential decay waveform; a synergistic combination of** 2 **electroporation and electrolysis (E2) for tissue ablation**

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16 **Abstract**

17 Background: Electrolytic ablation and electroporation based ablation are minimally invasive, non-
18 thermal surgical technologies that employ electrical currents and electric fields to ablate
19 undesirable cells in a volume of tissue. In this study we explore the attributes of a new tissue
20 ablation technology that simultaneously delivers a synergistic combination of electroporation
21 and electrolysis (E2).

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25 histology.

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29 Discussion: The E2 combination has advantages over the three basic technologies of non-thermal
30 ablation: electrolytic ablation, electrochemical ablation (reversible electroporation with injection
31 of drugs) and irreversible electroporation. E2 ablates clinically relevant volumes of tissue in a
32 shorter period of time than electrolysis and electroporation, without the need to inject drugs as
33 in reversible electroporation or use paralyzing anesthesia as in irreversible electroporation.
34

35 **Keywords**

36 tissue ablation, liver, electrolytic ablation, reversible electroporation, irreversible
37 electroporation, synergy electroporation and electrolysis
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41 Introduction

42

43 A number of biophysical and biochemical phenomena occur simultaneously when electrical
44 potentials are applied across biological matter. These include Joule heating due to electrical
45 current energy dissipation, electrolytic reactions at the interface between the electrodes and the
46 biological milieu, and cell membrane permeabilization known as electroporation. All these
47 electrical phenomena are used for tissue ablation. Usually the electrical potential delivery
48 protocol is designed in such a way as to maximize one phenomenon, while minimizing the others.
49 For example, in non-thermal irreversible electroporation (NTIRE) the electrical potential profile
50 is designed to maximize irreversible electroporation while minimizing Joule heating (Davalos et
51 al. 2005). The non-thermal aspect of NTIRE was found to be beneficial in tissue ablation
52 treatments, in which it is desired to spare vital sites in the treated lesion, such as blood vessels
53 and nerves.

54

55 In electrolytic tissue ablation, cell death is caused by the chemical interaction between the
56 products of electrolysis and cells (Nilsson et al. 2000),(Czymek et al. 2011). Because the ablation
57 is caused by a chemical reaction, it is a function of compounds concentration and time of
58 exposure. One drawback of tissue ablation by electrolysis is the need for high concentrations of
59 electrolytes and lengthy times of exposure. An advantage is the very low currents and voltages
60 used.

61

62 In ablation by electroporation, brief, pulsed, high electric fields are used to permeabilize the cell
63 membrane. Lower electric fields and small numbers of pulses yield reversible electroporation, in
64 which the cell membrane permeabilization is temporary. Higher electric fields with larger number
65 of pulses yield irreversible electroporation in which the cell membrane permeabilization is
66 permanent, which results in cell death. Both reversible and irreversible electroporation are used
67 for tissue ablation, each with their advantages and disadvantages. Reversible tissue
68 electroporation is used for tissue ablation in combination with cytotoxic additives, in a procedure
69 known as electrochemotherapy (Mir et al. 1991), (Marty et al. 2006). One advantage of ablation
70 by means of irreversible electroporation over electrochemotherapy is that no chemotoxic drugs
71 are injected into the tissue (Rubinsky et al. 2007), while the advantage of electrochemotherapy
72 over irreversible electroporation is the use of fewer pulses and lower electric fields. The need to
73 inject cytotoxic additives adds a complicating step to the electrochemotherapy procedure. Cell
74 death through electrochemotherapy is dependent on mitosis cycle rendering and is possibly
75 more tissue selective, while irreversible electroporation induces apoptosis and necrosis
76 instantaneously over the whole volume exposed to sufficiently high fields. However, the high
77 electric fields and the large number of pulses used in conventional irreversible electroporation
78 protocols cause some undesirable effects. They induce muscle contractions that require the use
79 of a muscle relaxant and deep anesthesia during surgery. It should be noticed that in clinical
80 practice, reversible electroporation is mostly used without muscle relaxants and with topical
81 anesthesia (Marty et al. 2006). Every clinical electroporation protocols, reversible or irreversible,
82 generates some products of electrolysis, and some heat. We have recently shown that if
83 substantial amounts of products of electrolysis are inadvertently generated during an

84 electroporation protocol, a highly detrimental electrical discharge across the layer of gas formed
85 on the electrodes can occur (Guenther et al. 2015).

86

87 In several recent papers we have shown that combining electroporation and electrolysis (E2)
88 sagaciously yields a new technology of tissue ablation with certain advantages over tissue
89 ablation by electroporation (reversible or irreversible) or electrolysis alone (Phillips, Raju et al.
90 2015) (Phillips et al. 2016) (Stehling et al. 2016). We have developed several possible synergistic
91 electroporation and electrolysis (E2) protocols. One effective combination entails delivering first
92 several (eight) reversible electroporation type pulses followed by the injection of a low voltage
93 direct current to generate products of electrolysis. While effective, this combination requires two
94 different power supplies, one for electroporation and the second for electrolysis (Phillips et al.
95 2015) (Phillips et al. 2016) (Stehling et al. 2016). The combined voltage profile of electroporation
96 pulses followed by low voltage electrolysis reminded us of an exponential decay waveform
97 (EDW), generated by the discharge of a capacitor; a type of pulse which was rather common in
98 the early stages of electroporation research (Sale and Hamilton 1967). The shape of the capacitor
99 discharge exponential decay waveform is a high initial voltage followed by a rapid decay towards
100 a trailing low voltage. This type of waveform is still used in cell electroporation. We thought that
101 with a properly chosen set of capacitor discharge parameters, the initial high voltage over a
102 suitable timeframe could serve for electroporation, while the trailing lower voltage could
103 generate sufficient charge for the generation of electrolytic products. The feasibility of tissue
104 ablation with a EDW, was shown in the liver of a small rodent (Phillips et al. 2016). Here, we
105 extend the study to a large animal model, pig liver, and show that EDW has the ability to ablate
106 tissue volumes of clinical significance. The experimental study was supported by a mathematical
107 analysis to evaluate the electric fields and extent of thermal damage generated by the
108 exponential decay waveform.

109

110

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112

113 **Materials and methods:**

114

115 Animal protocol:

116

117 The study was approved by Sir José Antonio Rodríguez Correa, Director of Animal Health
118 Programs and General Director of Department of Agriculture and Livestock, Ministry of
119 Environment and Rural, Agricultural Policies and Territory, Government of Autonomous
120 Community of Extremadura (Spain), with application form number: 2015209030009567 and
121 study register number: 100370001499. The experiment was conducted on *in vivo* pig liver, which
122 was in accordance with Royal Decree Law 53/2013 (Feb.1st). According to the study protocol,
123 three female pigs between 90-110 kg were treated. After being fasted for 24 hours, animals were
124 pre-medicated with a combination of diazepam (0.4mg/kg) and ketamine (15mg/kg) injected
125 intramuscularly (IM). Anesthesia was induced with intravenous (IV) Propofol (3mg/kg).
126 Endotracheal intubation was performed and anesthesia was maintained with sevoflurane in
127 oxygen (adjusted to 1.8-2% End tidal sevoflurane). Possible postoperative pain was treated with

128 Buprenorphine 0.01 mg/kg IM Pre-med at recovery and Carprofen 4 mg/kg at
129 extubation/recovery. Cefazolin 25 mg/kg IV was administrated every 2 hours. If found to be
130 needed during the procedure, the study had the ability to deliver pancuronium (0.1 mg/kg, at a
131 dose of 1 mg/ml) through an IV to reduce muscle contractions during the application of the
132 electrical pulses. The liver was exposed via a midline incision. The treatment was delivered using
133 two 18-gauge Titanium needles (Inter Science GmbH, Ch) with a variable length (1-4 cm exposed
134 treatment length) insulating sheath inserted in the liver. Titanium was chosen, because, unlike
135 steel or aluminum it is chemically inert, and does not introduce toxic metals in tissue, during the
136 electrolysis stage. In addition, Ti is MRI compatible. The 18-gauge variable length electrodes were
137 custom designed for the delivery of both electroporation and electrolytic pulse sequences.

138
139 Two electrodes were inserted in the liver under ultrasound monitoring, in a roughly axial parallel
140 configuration, normal to the liver surface. Ultrasound images were also taken throughout the
141 procedure. Since no apparatus is currently available to produce the exponential decay voltage
142 waveform needed for the SEE procedure conceived by us, we have designed and built a new
143 power supply described in the following device section. The parameters varied in this study were:
144 the initial voltage and the time constants of the exponential voltage waveform. In addition, we
145 varied the number of exponential voltage waveforms delivered. A total of 23 lesions were
146 produced, in three pigs, in separate experiments. Animals were sacrificed at 24 hours. The pigs
147 were euthanized using Euthasol 1 ml/lb IV.

148
149 To fix the liver for microscopic viewing, a Foley catheter was placed into the descending aorta
150 and the hepatic vein was snipped off for drainage of the affluent. The liver was flushed with
151 physiological saline for ten minutes at a hydrostatic pressure of 80 mmHg from a pressurized IV
152 drip. Immediately following saline perfusion, a 10% formalin fixative was perfused in the same
153 way for ten minutes. The liver lobe in which the SEE lesion was made was removed and stored in
154 the same formalin solution. For microscopic analysis, the tissue was bread loafed perpendicular
155 to the capsule surface and parallel to the needle tracts. All cassettes were processed routinely
156 from 10% phosphate buffered formalin to wax blocks. Five micrometer sections were made from
157 each block and stained with Masson's trichromatic stain for histologic examination. The stained
158 samples were examined and analyzed by an independent histology service company and reports
159 were prepared (Narayan Raju, Inc, South San Francisco, CA). The focus of the histology was to
160 verify the extent and nature of tissue ablation with E2. To produce information of practical clinical
161 value, the focus of the analysis was on verifying the ability to produce a continuous lesion
162 between the electrodes.

163 164 **Device**

165
166 We were unable to find a power supply that can produce the waveform parameters, required for
167 an EDW protocol in tissues with the dimensions of the pig liver. Therefore, we designed a power
168 supply that operates in the modality of capacitor discharge electroporation systems (e.g. Gene
169 Pulser Xcell™ Electroporation System, BioRad, Hercules, CA) with an enhanced performance. The
170 conventional type capacitors used were replaced with a 100 microfarad capacitor to provide the
171 charge required for electrolysis. Similar to the Gene Pulser Xcell™, the generator has an output

172 of up to 3kV. Because of the larger capacitors it can generate exponential decay waveforms up
173 to time ranges of hundred milliseconds, depending on tissue conductivity and thereby
174 simultaneously deliver electrolysis and electroporation. The apparatus selects and matches the
175 internal components needed to produce the time constants selected for the specific tissue
176 conductivity of the treatment area. The apparatus is able to produce and deliver the exponential
177 decay voltage profile in the time and voltage range for the specific treatment area.

178

179 **Mathematical Analysis**

180

181 The thermal and electrical field simulations were performed using a finite element solver (Comsol
182 Multiphysics 5.2) for the Laplace equation (electrical field) and Pennes Bioheat equation, in a way
183 identical to that described in (Davalos et al. 2005). The setup was approximated as two parallel
184 titanium cylinders in a large volume of liver tissue with the parameters shown in Table 1. In case
185 of discharging capacitors, the amount of Joules heating in tissue is prescribed by the dissipation
186 of the charge energy, Q , ($Q=C*U_0$). Therefore, specifying only, the initial voltage (U_0) and capacity
187 (C) is sufficient to simulate the experiment. Permanent tissue damage can occur instantaneously
188 due to temperatures above 90°C, but also chronically with temperatures above 45°C over a
189 period of time depending on cell type and temperature. The latter mechanism is the only effect
190 for pulse-based treatments in the energy magnitude discussed here where distances of more
191 than a millimeter from the electrode could take thermal damage. Therefore, all temperature
192 graphs in the figures show the temperature after 30 seconds of heat dissipation. The waveform
193 delivered to the electrodes was assumed to be a perfect exponential decay in time, t , ($U= U_0*\exp$
194 (t/τ)), where U_0 is the initial voltage and the time constant is, τ . The time constant was taken
195 from the experimental data, through the analysis of the voltage trace during the delivery of the
196 waveform.

197

198

199 **Results**

200

201 A series of 23 lesions were generated in experiments in which we studied the effects of the E2
202 waveform parameters on tissue ablation. The study examined the effects of the initial voltage,
203 the time constant and the number of exponential decay voltage waveforms delivered. To
204 facilitate a systematic and well defined analysis of the E2 phenomenon, we will focus on the
205 results at midline between the two electrodes.

206

207 Figure 1 shows results from a series of studies in which the initial voltage between electrodes
208 was 750 V, the distance between electrodes was 15 mm, the exposed length was 10 mm and the
209 depth of penetration was 20 mm. This configuration produces an initial voltage over distance of
210 500 V/cm. The calculated electrical field norm is displayed in panel A and the calculated
211 temperature in panel B. Geometrically, both graphs represent the 1d-cutline through the
212 perpendicularly induced electrodes with the electrical field and the temperatures respectively on
213 the y-axis. Panels C and D are the macroscopic histology from lesions treated with a voltage
214 difference of 750 V between the electrodes and time constants of 50 ms and 100 ms, respectively.
215 If tissue resistance and conduction between electrode and tissue were constant, the discharge

216 could be fully described using the time constant of the EDW. However, secondary effects like thin
217 layers of burned tissue, can cause insulation and hence disrupt the ideal exponential decay. This
218 does not necessarily have any negative effect on the ablation, but will limit τ to adequately
219 describe the delivered waveform. The panels show the formalin embedded samples, sectioned
220 in a plane that is transverse to the centers of the two electrodes. In all the different experiments
221 with 750 V (500 V/cm voltage to distance between electrodes) there was no configuration in
222 which the lesion between electrodes became continuous. Panels A and B show that at the line
223 midway between the electrodes the electric field is less than 200 V/cm and the temperature is
224 below 40°C.

225

226 Figure 2 shows results from an experiment in which the initial voltage between electrodes was
227 1000 V, the distance between electrodes was 15 mm, the exposed length was 10 mm, the depth
228 of penetration was 20 mm and the time constant was 70 ms. The slides were prepared with
229 Masson's trichrome staining. Fig A gives an overview of the evaluated slide. The image is taken
230 in a plane that transverses the centers of the two electrodes. The area of the probe is clearly
231 visible, with a deep blue color at the site of the probes, representing the cellular damage caused
232 by thermal necrosis, surrounded by areas of coagulated blood (deep red color). 10x magnification
233 at the anode (Fig 2B) illustrates an area of thermal necrosis, where the hepatocytes have
234 sustained more intense cellular ablation injury resulting in denaturation of the cytoplasmic
235 organelles. At the cathode (Fig 2D) we can witness the gradual effect of the treatment: Around
236 the macroscopically visible lesion there is a pale area which represents less affected cells
237 immediately adjacent to the severely affected hepatocytes (marked with an arrow). The
238 sinusoidal spaces are dilated due to edema and/or hepatocellular swelling, while the nuclei are
239 condensed. The space between the electrodes is not fully ablated, as the microscopic images
240 show areas of unaffected cells (Figure 2C). Figure 2E shows the calculated electric field for a
241 voltage of 1000 V and figure 2F shows the calculated temperature distribution. Panels 2E and 2F
242 show that, for these experimental conditions, the minimal electric field midway between the
243 electrodes is calculated to be about 240 V/cm and the temperature midway between the
244 electrodes is well below 40°C.

245

246 Figure 3 illustrates the pathology of liver, from a treatment in which two voltage exponential
247 decay waveforms with similar parameters as those that produced Figure 2, were delivered at an
248 interval of 30 seconds. The macroscopic image taken from a plane between the center of the two
249 electrodes (Fig. 3A) shows that the partial electrode pathway (tunnel) is filled with coagulated
250 blood. This is confirmed by the deep red linear region in the histological slides stained with
251 Masson's trichrome staining in Figure 3B to 3D. The dark blue zone around that region (Figs 3B-
252 3D) represents the more severely ablated hepatocytes, by virtue of being closest to the point of
253 energy release. Figure 3E shows the calculated electric field for an exponential decay waveform
254 with an initial voltage of 1000 V and figure 3F shows the calculated temperature distribution at
255 the onset of the second pulse. There are two aspects to notice in panels 3E and 3F. Figure 3E is a
256 copy of Figure 2E. It is obvious because we have used the electrical parameters of normal liver.
257 However, it is known that the electrical conductivity of electroporated tissue changes after
258 electroporation (Ivorra and Rubinsky 2007), and therefore this panel may not be correct. The
259 second aspect relates to the temperature distribution. Figure 3E shows that the calculated

260 temperature distribution, when the second pulse is delivered is substantially elevated over the
261 initial temperature when the first pulse is delivered, and thermal damage may be induced near
262 the electrodes.

263

264 Figure 4 shows 10x magnified images of the histological slide from Figure 3. Fig. 4A shows the
265 space between the electrodes. Fig. 3B gives a 10x magnification of that area, showing a full
266 ablation zone, with affected cells throughout the area. Hepatocytes both at the cathode (Fig 4C)
267 and anode (Fig 4D) show condensed nuclei, with hemorrhage in the spaces between, however
268 with intact vessels (Fig 4C).

269

270 Figure 5 shows the histological results of exponential voltage profile in which the initial voltage
271 between electrodes was 1500 V, the distance between electrodes was 15 mm, the exposed
272 length was 20 mm and the depth of penetration was 30 mm. It is important to notice that the
273 top 10 mm of the electrode was insulated. The slides were prepared with Masson's trichrome
274 staining. Fig 5A shows the cells on the center line between the electrodes at the level of the top
275 10 mm insulated part of the electrodes. Here we see that the cells are not affected by the
276 treatment. The next panel (Fig. 5B), however, shows the lesion which was caused by the
277 treatment in the uninsulated part of the tissue between the electrodes. The lesion is continuous
278 between electrodes at this level. Figure 5C displays the calculated electric field for a voltage of
279 1500 V, and figure 5D shows the calculated temperature distribution. The electric field midway
280 between the electrodes is about 550 V/cm. The midway between electrodes temperature is
281 about 40°C.

282

283 Figure 6 displays a 10x magnification of the pathological slide shown in Figure 5. Panel 6 A is a
284 magnification of the cathode, showing swollen and necrotic hepatocytes and a disrupted
285 sinusoidal pattern. Between the electrodes (Fig. 6B) a bridged ablation with affected cells was
286 observed, with a complete loss of cellular structure. At the anode (Fig. 6C) there is an affected
287 cellular architecture with hemorrhage. Panels 6A-C show open and undamaged large blood
288 vessels within the treatment field.

289

290 A very important observation is that there was no need for muscle paralysis in any of the 23
291 lesions produced with various E2 protocols. This evaluation is based on the assessment of a
292 physician (MS) with an experience of close to 450 NTIRE procedures in which muscle contraction
293 can occur even with deep muscle relaxations.

294

295 Discussion

296

297 Our experiment was designed to be performed without a muscle relaxant, however, in such a
298 way as to allow for an immediate use of a muscle relaxant as soon as an undesirable level of
299 muscle contraction is noted. From among the 23 experiments with the exponential decay voltage
300 waveform done in this pig liver study, a muscle contraction requiring the use of a muscle relaxant
301 (pancuronium) was detected in none. In fact, the muscle contraction was negligible, and at times
302 unnoticeable. The E2 treatment was delivered by a physician with an experience of close to 400
303 irreversible electroporation treatments (MS). It was noted that the observed response is clinically

304 acceptable and the exponential decay voltage waveform procedure can be carried out without
305 the use of muscle paralyzing drugs. Obviously this observation is relevant only to the parameters
306 used in this study, in which the maximal voltage was 1500 V (1000 V/cm voltage over distance)
307 and the maximal time constant 148 ms.

308
309 The E2 protocol requires a special waveform comprised of an exponential decay shape with a
310 steep decrease in voltage to values that will not induce an electrical discharge across the
311 electrolytically product near the electrodes and a longer low voltage tail, that can generate
312 sufficient products of electrolysis for the E2 ablation. To the best of our knowledge currently
313 available electroporation systems cannot deliver exponential decay waveforms with the desired,
314 electrolytic products generating time constants. To this end we have modified existing
315 commercial designs (e.g. Gene Pulser Xcell™ Electroporation System, BioRad, Hercules, CA), as
316 described in the methods and materials section. The key difference is the use of larger
317 capacitance, in essentially the same circuit.

318
319 Our main criteria for evaluating the exponential decay voltage waveform ability to ablate tissue
320 in a clinically significant manner was the ability to induce the ablation throughout the gap
321 between the electrodes. Therefore, the histological and mathematical analysis is focused on the
322 tissue found midway between the electrodes. This is the part of the treated tissue in which the
323 lowest electric fields and lowest temperatures occur. Figures 1 and 2 show that there are
324 parameters of initial voltage and time constant for which the tissue midway between the
325 electrodes is not ablated. Figure 1A shows that for an initial voltage of 750 V and a distance of
326 1.5 cm between the electrodes (500 V/cm distance between electrodes), the electric field
327 strength midway between the electrodes is lower than 200 V/cm. This value is substantially
328 below the reversible electroporation threshold for the rabbit liver, which was measured to be
329 362 +/-21 V/cm (Miklavcic et al. 2000). Since the calculated temperature midway between
330 electrodes is below 40°C, there is no mechanism to induce damage between the electrodes. The
331 conditions in the region between the electrodes are below the levels required for irreversible
332 electroporation ablation, reversible electroporation or thermal ablation.

333
334 Figure 2 shows that increasing the initial voltage of the exponential decay waveform to 1000 V
335 will also increase the extent of the damage near the electrodes. The distance between the
336 electrodes is 1.5 cm and therefore the initial voltage to distance ratio is 750 V/cm. Figure 2E
337 shows that the electric field midway between electrodes is calculated to be below 300 V/cm. This
338 value is below the 362 +/- 21 V/cm reversible electroporation threshold (Miklavcic et al. 2000).
339 Tissue damage by heat can be also excluded, since the temperature between electrodes does not
340 exceed 40°C (Figure 1B and 2F). In this case also, the conditions in the middle between the
341 electrodes are below the levels required for irreversible electroporation ablation, reversible
342 electroporation or thermal ablation.

343
344 Figures 3 and 4 show that it is possible to ablate the entire zone between electrodes by using two
345 consecutively delivered exponential decay waveforms with the same parameters as those used
346 to produce the results in Figure 2. The initial voltage of the exponential decay waveform was
347 1000 V. The distance between the electrodes is 1.5 cm and therefore, the initial voltage to

348 distance ratio is 750 V/cm. The mechanism of ablation may be related to the possibility that the
349 second exponential waveform has brought the tissue midway between electrodes to the
350 threshold of E2 ablation. Figure 3F shows that the temperature prior to the delivery of the second
351 exponential waveform is elevated relative to that prior to the delivery of the first waveform.
352 Elevated temperatures favor electroporation and may reduce its threshold. Furthermore, it is
353 known that electroporation changes the electrical conductivity of tissue. While Figure 3E was
354 obtained for the electrical conductivity of the normal liver, the second waveform may generate
355 a somewhat modified electric fields. Last, the second waveform has delivered twice the level of
356 electrolytic compounds than in the experiment whose results are depicted in Figure 2. This
357 tentatively suggests that the mechanism of tissue ablation in the middle part of the tissue
358 between electrodes is the synergy between reversible electroporation and electrolysis.

359
360 The results displayed in Figures 5 and 6 produce stronger evidence of the E2 mechanism of tissue
361 ablation. Here, an increase of the exponential decay waveform initial voltage to 1500 V has
362 produce ablated tissue between the electrodes. Calculations show that the electric field midway
363 between the electrodes is about 550 V/cm (Figure 5E). This value is below the irreversible
364 electroporation threshold for the rat liver (637 V/cm +/- 43 V/cm)(Miklavcic et al. 2000). The
365 temperature midway between electrodes is about 40°C, (Figure 5F), which is below the threshold
366 of thermal damage. The mechanism of tissue ablation at the midpoint between electrodes is
367 neither irreversible electroporation nor thermal. The most likely possible mechanism is the
368 synergistic effect of electrolysis and reversible electroporation.

369
370 This is a first large animal study on the use of the synergy between electrolysis and reversible
371 electroporation to enhance tissue ablation by electroporation. However, the E2 combination
372 seems promising. It has the ability to create comparable clinically relevant areas of tissue
373 ablation, in a much shorter period of time than irreversible electroporation, with lower voltages
374 and single waveforms, without the need to inject drugs and without the need for paralyzing
375 anesthesia.

376

377

378

379 **Acknowledgements**

380

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382 assistance on the pathological examination and analysis.

383

384

385 **Author contributions statement**

386

387 B.R. conceived the experiment, M.S., P.M., E.G., N.K. and B.R. conducted the experiment, E.G.
388 B.R. and N.K. analyzed the results. All authors reviewed the manuscript.

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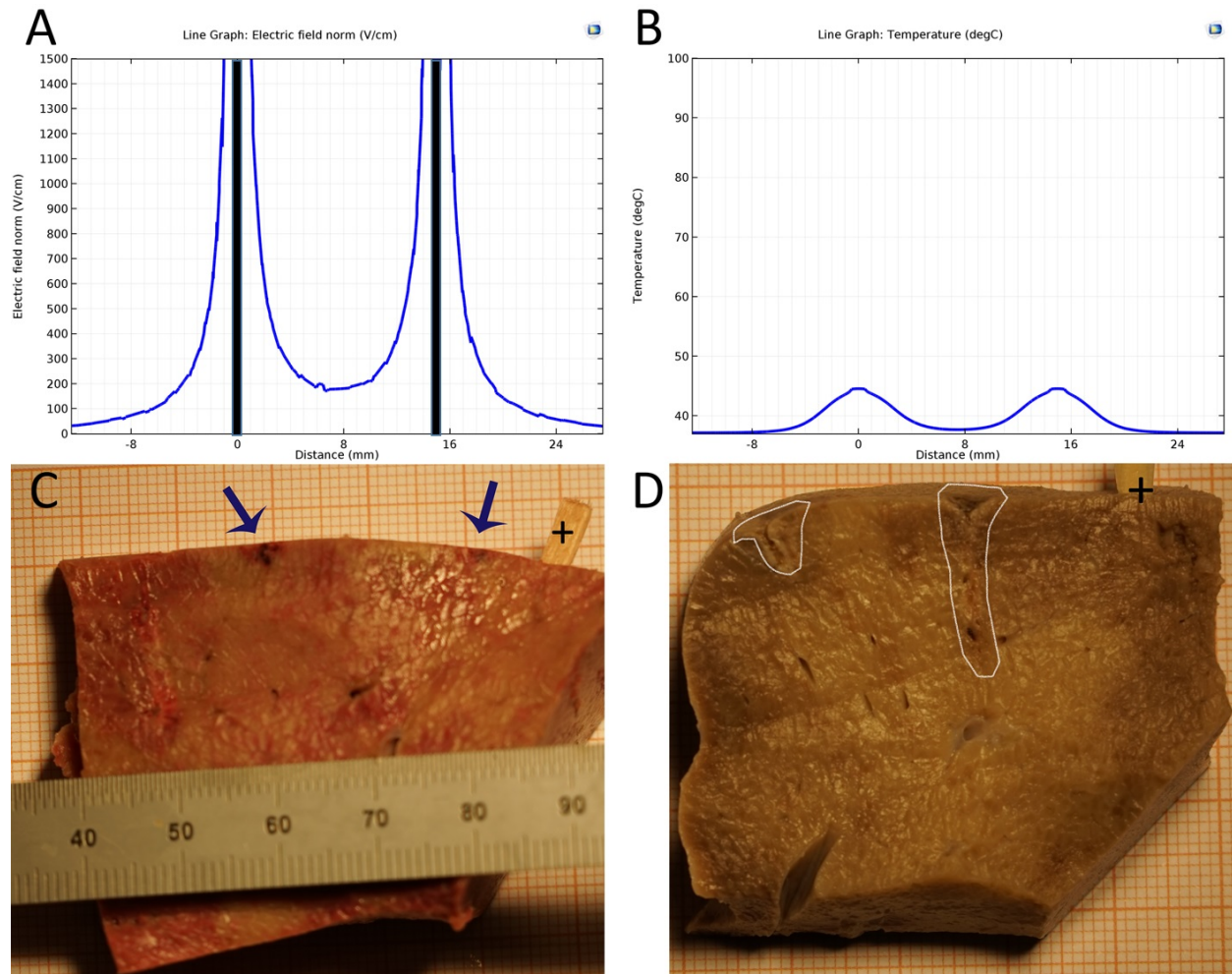
Initial Voltage U_0	750V (Fig.2), 1000V (Fig.3,4), 1500V (Fig.5)
Exposure length	1cm (Fig.2,3,4) 2cm (Fig.5)
Decay	Exponential capacitor discharge
Power supply capacitance	Listed in Figures
Distance between electrodes	1.5cm
Electrode diameter	1mm
Liver: electrical conductivity	0.286 S/m
Liver: heat capacity	3750 J/(kg*K)
Liver: density	1000 kg/m ³
Liver: thermal conductivity	0.52 W/(m*K)
Titanium: electrical conductivity	7.4e5 S/m
Titanium: heat capacity	710 J/(kg*K)
Titanium: density	4940 kg/m ³
Titanium: thermal conductivity k	7.5 W/(m*K)

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431 Table 1: Parameters used for the thermal and electrical field calculations.

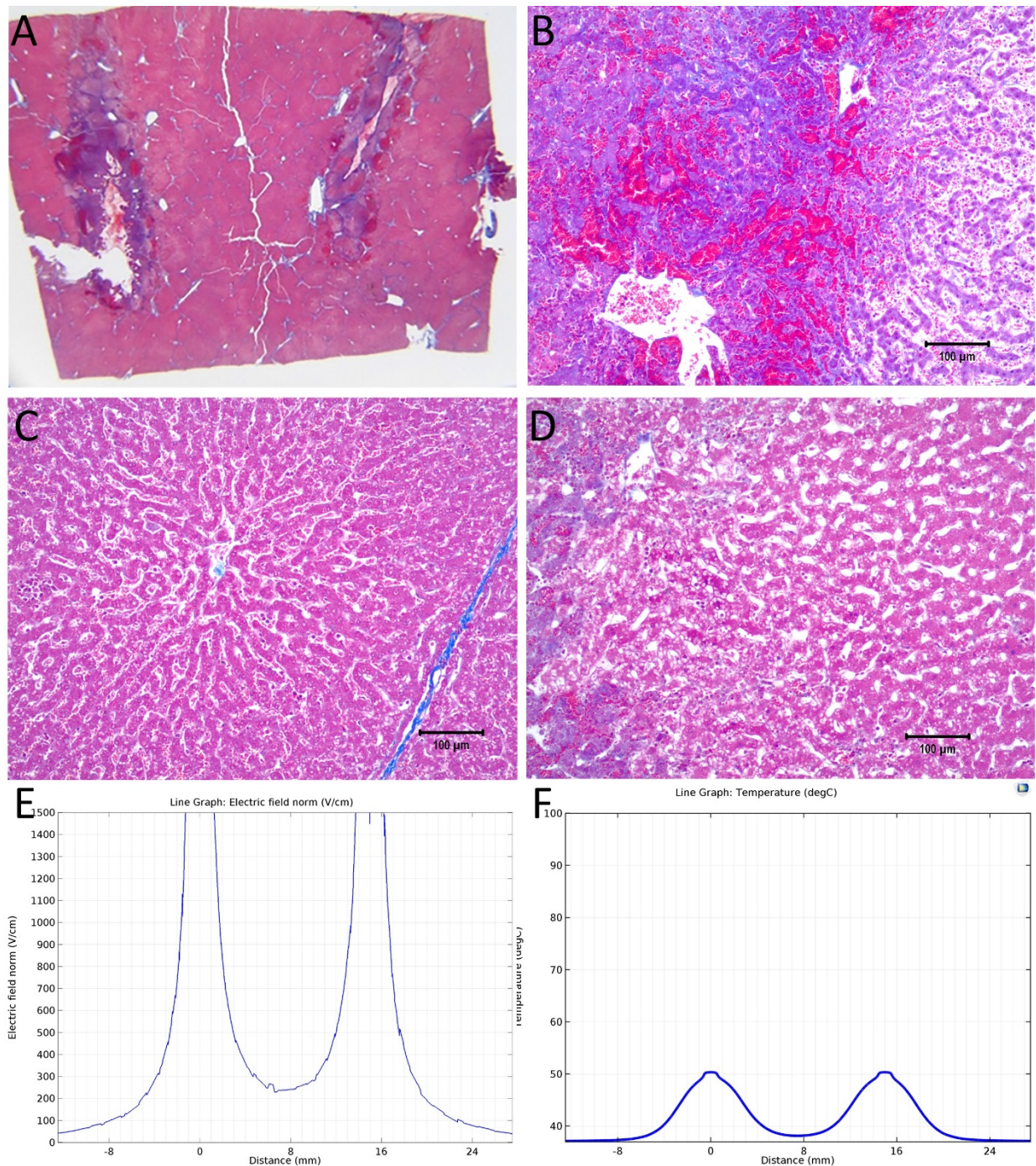
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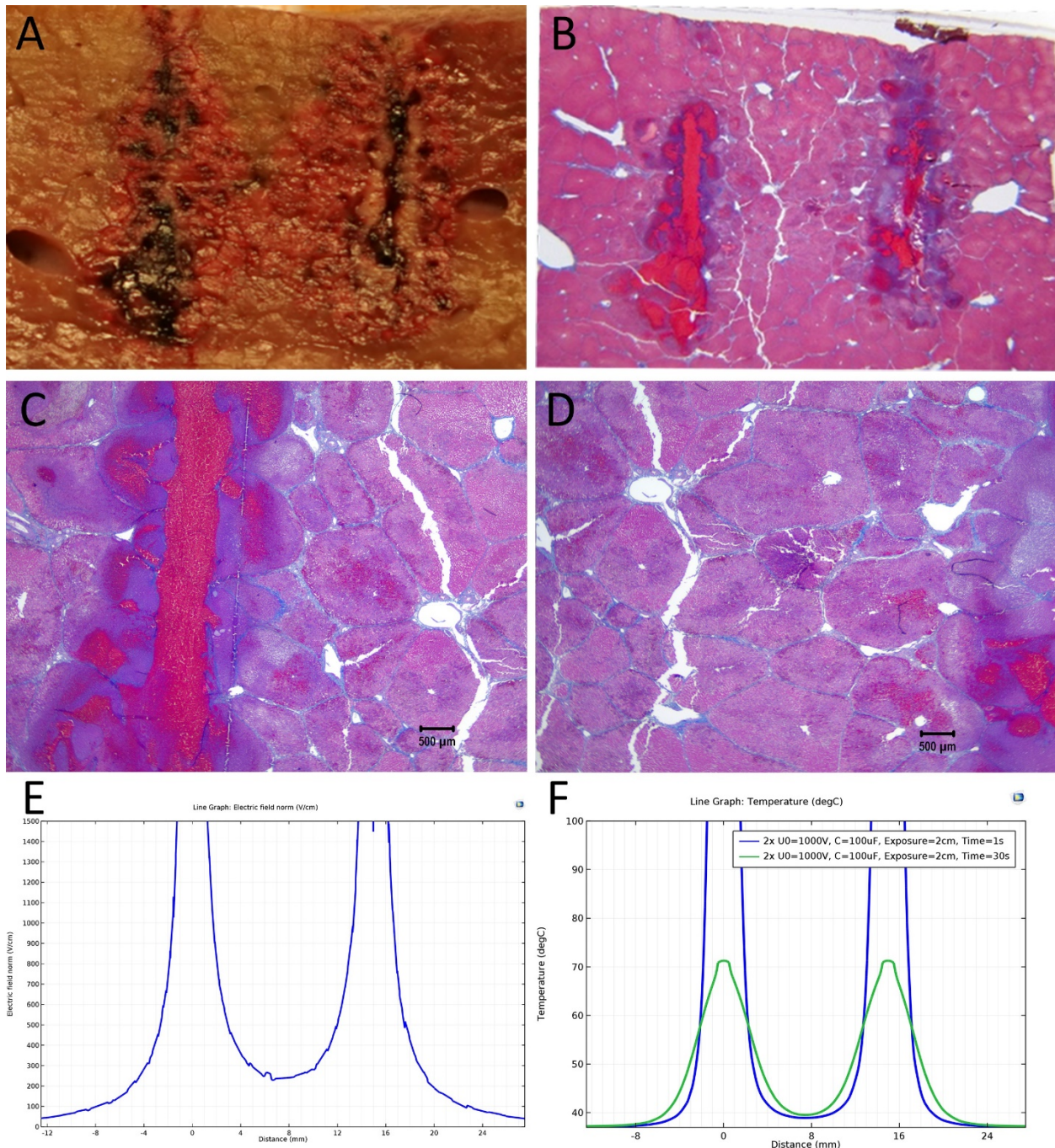
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Figure 1: Study with an EDW with initial voltage difference between electrodes of 750 V and various time constants. **A.** calculated electric field. **B.** Calculated thermal field after 30 seconds. **C.** Macroscopic image - 50 ms time constant – no ablation was noticed. **D.** Macroscopic image - 100 ms time constant – some ablation near electrodes.



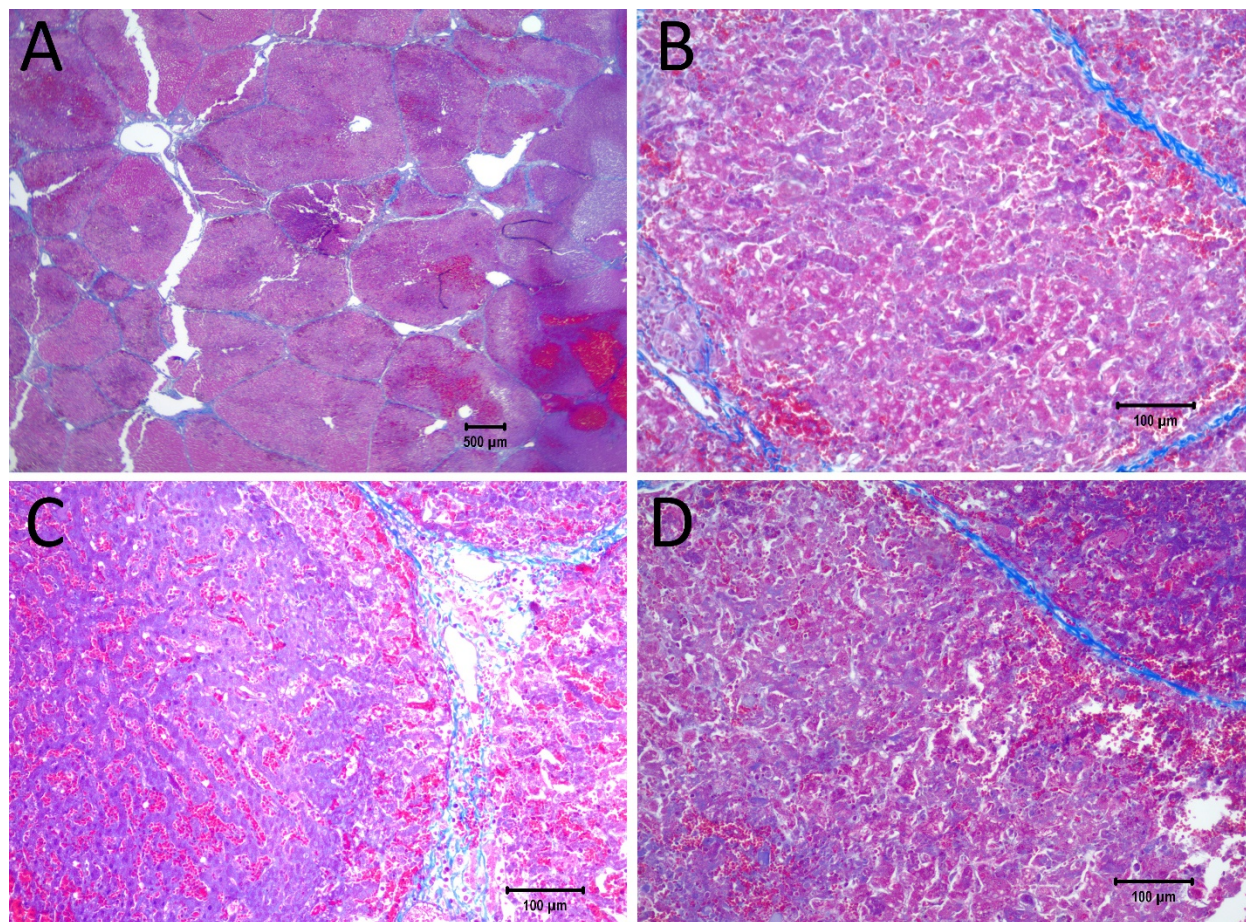
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443 **Figure 2:** Study with one EDW with initial voltage difference between electrodes of 1000 V and time
444 constant of 70 ms. **A.** Histological slide with Masson's trichrome staining. **B.** 10x magnification of the right
445 lesion, which is the anode. We see severe acute hepatocellular necrosis with coagulated blood
446 (hemorrhage) in the sinusoids. **C.** 10x magnification between the electrodes. The cells do not appear to
447 be affected. **D.** 10x magnification at the margin of the left lesion, which is the cathode. Here we see the
448 borderline between the necrotic tissue on the left and partially affected cells on the right. **E.** Electric field
449 strength distribution. **F.** Temperature distribution after 30 seconds. (scale bar 100 μm)



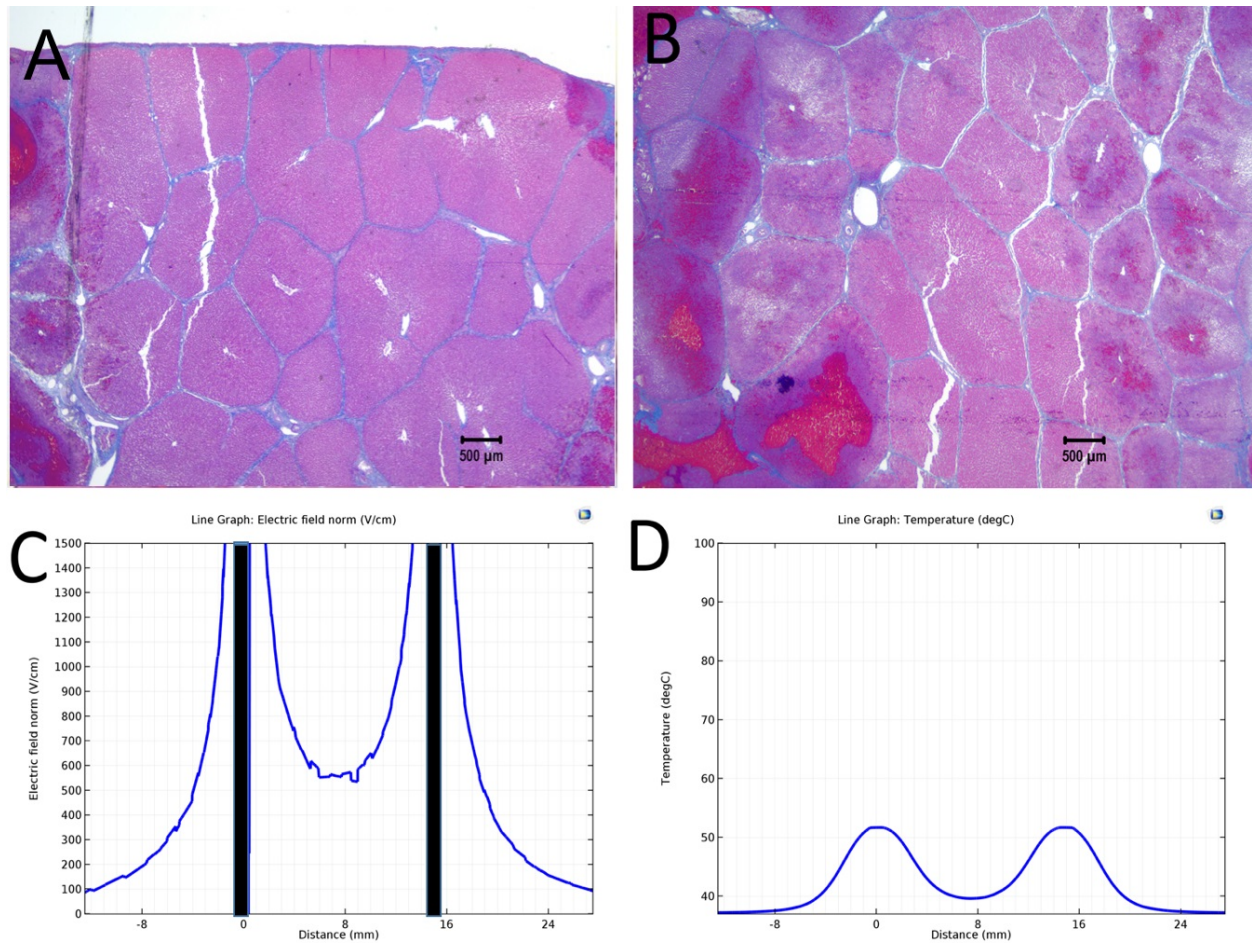
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Figure 3: Study with 2 EDW separated by 30 s with time constants of 79 and 92 ms, the first and second pulse respectively, 1000 V difference between electrodes placed at a distance of 15 mm between them, 10 mm exposed length, 100 μF capacitor. Liver was extracted 18.5 h after treatment. **A.** Macroscopic histological slide (cathode left electrode anode right electrode) **B.** Masson's trichrome staining reveals blood coagulation (red) and ablation both around and in between electrodes. **C.** Close-up of the cathode, which is the left electrode. **D.** Close-up of the right electrode, which is the anode. (scale bar 500 μm) **E.** Electric field strength distribution. **F.** Temperature distribution prior to the delivery of the second waveform at 1 and 30 seconds.



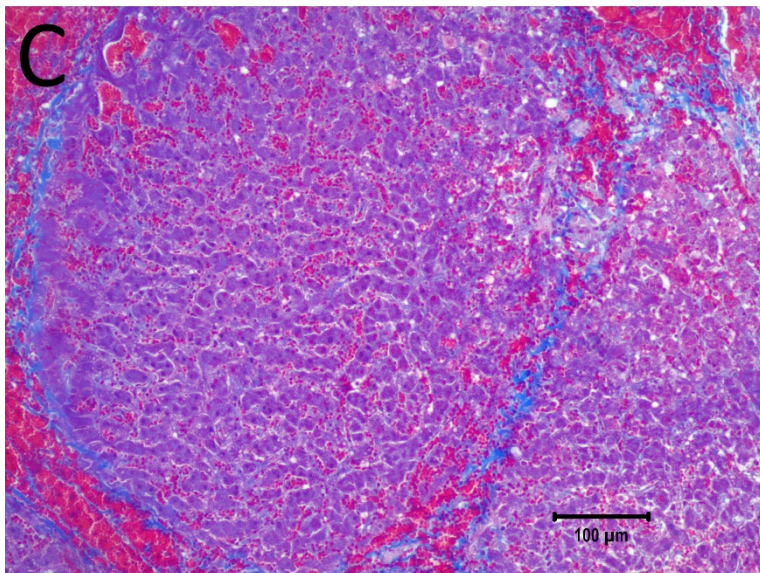
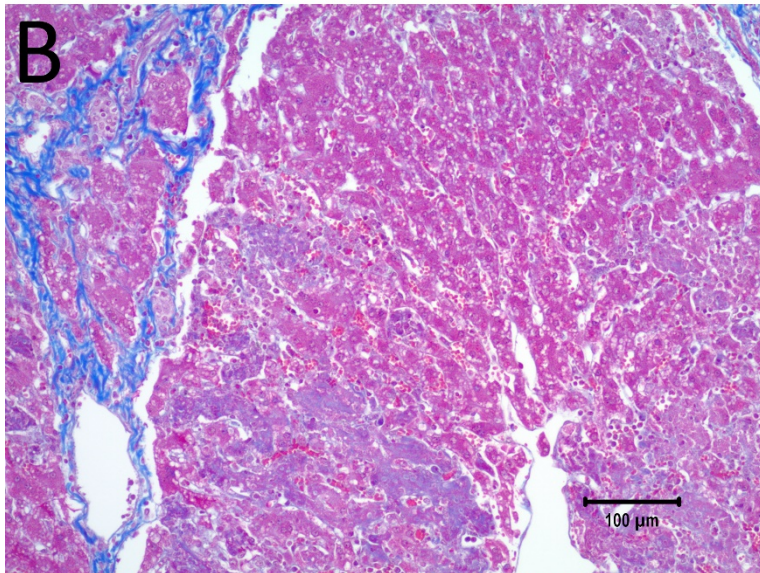
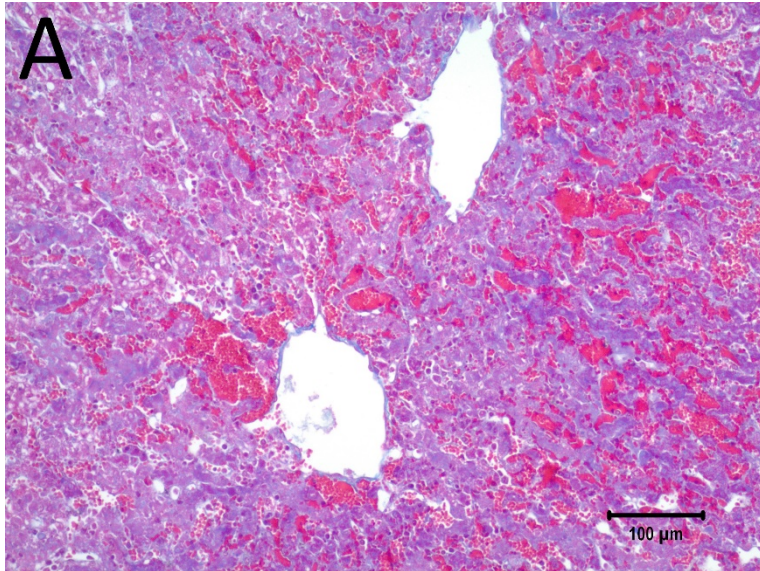
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Figure 4: Details from Figure 3. **A.** Space between the electrodes in Figure 3. Bar indicates 500 μm . **B.** 10x magnification of cells between the electrodes, showing the details of the ablated area. **C.** 10x magnification of the cathode, showing edema and cellular ablation injury. **D.** 10x magnification of the area by the anode, showing the margin of affected and non-affected cells. All images show Masson's trichrome staining. Bars in B-D indicate 100 μm .



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Figure 5: Study with a EDW with a time constant of 69 ms, 1500 V difference between electrodes placed at a distance of 15 mm between them, 200 mm exposed length, 100 μF capacitor. **A.** Macroscopic cross section in a plane through the axis of the electrodes. Image taken between electrodes at the part where the electrodes were insulated, showing that the cells are not affected. **B.** Image taken between electrodes where the electrodes were not insulated showing that the lesion was bridged. (500 μm bar). **C.** Electric field strength distribution. **D.** Temperature distribution after 30 seconds.



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480 **Figure 6:** 10x magnification of the pathological slides shown in Fig 5. **A.** Image taken at the right
481 electrode, which was the cathode, showing necrotic, swollen hepatocytes and a disrupted sinusoidal
482 pattern. **B.** Image taken between the electrodes, illustrating a complete loss of cellular structure with
483 swollen hepatocytes. **C.** Left electrode, which was the anode, showing an affected cellular architecture
484 and hemorrhage. Note that the large blood vessels are open and unaffected. Scale bar 100 μm
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