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Research paper:

Exploration of Electro-Enhanced-Chemotherapy II

Uptake of radioactive tracer in rat Muscle tissue at 6 and 24 hours after applied electric pulses of 1000 V/cm field-strength, 100μs pulse-length, and various number of pulses. (ver. 1.0)

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Executive Summary

The aim of the study is to explore the enhanced uptake of the radioactive tracer Technetium-99m-DTPA (^{99m}Tc-DTPA) in rat Muscle tissue in Fischer 344 rats after applied electric pulses of 1000 V/cm field-strength, 100μs pulse-length, and various number of pulses.

Methods: ^{99m}Tc-DTPA (total, 150 MBq) was administered intramuscularly (i.m.) at the shoulder as a bolus in several fractions of 50 μl each in 1 minute intervals. Images of the radioactivity distribution in the rats was recorded with a gamma-camera at 6 and 24 hours after electroporation. EP treatment was performed with 2 needle electrodes separated 8 mm inserted in the right back thigh muscle, through which electric pulses of 600;800;1000;1200 V/cm field-strength, and 100;250;500 μs pulse-length were applied.
Bioimpedance measurements were performed at 2 and 20 kHz through the needle electrodes in the right back thigh muscle. Before applying the EP treatment pulse, two measurements established the reference level R_{before}. Then N_p consecutive pulses (Np = 2,4,6,10,12) of field strength amplitude,1000V/cm and pulse-length 100μs were applied in 1 s interval and the impedance was recorded between each pulse. In order to study the relaxation of the poration the conductance measurements were continued 15 times after the last pulse in 1 s interval.
Statistical analysis and modelling of the data is performed using multivariate data processing methods such as Principal Component Analysis PCA, and modelled with the method of Projection to Latent Structures, PLS, also called PLSR Partial Least Square Regression.
Results: The f the uptake ratios was predicted by tissue impedance measurements at various frequencies 2- 50 kHz. This is shown by the outcome of the PLS-modelling equations of Uptake ratio at 6 and 24 h versus tissue Conductance change Index at 2 and 50 kHz after the last pulse:**UR6h = 4.22 + 0.027*CCI(2); UR6h = 4.45 + 0.040*CCI(50)**
UR24h = 3.77 + 0.07*CCI(2); UR24h = 0.44 + 0.24*CCI(50)
Conclusion: The most optimal scenario to predict the outcome of the electrochemotherapy session i.e. to achieve highest uptake ratio of bleomycin would be to use the relaxation time (T1/s) of the tissue conductivity after treatment and the delivered absorbed energy (W J/kg).

$$UR6h = -8{,}097+2.04*(T1/s)+1.88\cdot10^{-3}*(W\ J.kg^{-1})$$

$$UR24h = -25.78+7.63*(T1/s)-1.36\cdot10\text{-}3*(W\ J.kg^{-1})$$

1. Introduction

The principles for application of high voltage impulses in vivo for tumor therapy and gene therapy has previously been described in detail (Persson 2000). The aim of the present study is to explore the enhanced uptake of the radioactive tracer Technetium-99m-DTPA (^{99m}Tc-DTPA) in rat Muscle tissue in Fischer 344 rats after applied electric pulses of 1000 V/cm field-strength, 100μs pulse-length, and various number of pulses.

2. Exploration of Radioactivity Uptake

Animals

Healthy Fischer-344 rats (B&K; Stockholm, Sweden) and Wistar rats (Taconic M&B; Ry, Denmark) were used in the experiments. The animals were housed in polycarbonate cages with access to food and fresh water *ad libitum*. Both male and female rats were used, weighing 300–400 and 150–200 g, respectively. Before electric pulse treatment, the animals were anesthetized with either chloral hydrate or isoflurane (Forene; Abbott Scandinavia AB, Solna, Sweden) by applying "Univentor 400 anesthesia unit."

The Animal Ethical Committee in Malmö/Lund (Permit M171-04; Lund, Sweden) approved all experimental animal procedures.

Radiopharmaceutical

Technetium-99m (^{99m}Tc) is a radioisotope with physical characteristics suitable for *in vivo* tracer experiments. It has a half-life of 6.0 hours and emits gamma photons of 140 keV (87% per decay), which results in a low absorbed dose per activity unit (Bq) and high detection efficiency in thin NaI(Tl) crystals used in gamma cameras.

The radiopharmaceutical ^{99m}Tc-DTPA is a stable, water-soluble compound (MW 416) used clinically in radionuclide angiography, static brain imaging, and kidney and urinary tract studies. ^{99m}Tc-DTPA was chosen as the tracer in this study because its pharmacokinetic behavior is very similar to bleomycin (MW 1400).

^{99m}Tc-DTPA is prepared from a kit of TechneScan® (Mallinckrodt Medical B.V.; Petten, Holland). This kit is a freeze-dried sterile mixture of 25 mg Ca-Na-3-diethylene-triamine-pentaacetate (DTPA), 0.21 mg stannous-chloride-dihydrate ($SnCl_2 \cdot H_2O$), 0.25 g Gentisic acid that is a di-hydroxy-benzoic acid, used as an antioxidant excipient, and 12 mg sodium chloride. By adding 300 MBq ^{99m}Tc-sodiumpertechnetate in 0.75 mL of sterile, pyrogen-free physiological saline, mixed until the powder is dissolved, ^{99m}Tc-DTPA is formed. After 15 minutes at room temperature, the ^{99m}Tc-DTPA solution is ready for injection and is stable for 8 hours. The labeled compound is a slightly opalescent and colorless aqueous solution with a pH of 4.0–5.0, with a labeling efficiency 95%. In the present study the ^{99m}Tc-DTPA (total, 150 MBq) was administered intramuscularly (i.m.) at the shoulder as a bolus in several fractions of 50 μl each in 1 minute intervals.

Radioactivity measurement

Images of the radioactivity distribution in a typical rat under the gamma-camera (GK) is shown in **Figure 1** at 6 hours after electroporation in **Figure 2** after 24 hours. The administration site at the shoulder appear as a dark spot and the uptake in electric pulse treated region at the thigh is seen as a dark spot. The corresponding area at the opposite untreated side is used as reference for extracellular activity. Kidney(K), bladder(B) with urine activity are seen as dark areas in the picture.

The present study investigate the effect of applied electric pulses of 600; 800; 1000: 1200: V/cm 100 and 500 μs pulse length, and 2, 4, 6 or 12 pulses, on the accumulation of the radiolabeled pharmaceutical ^{99m}Tc-DTPA that mimics the Bleomycin, in rat muscular tissue after *in vivo* electropermeabilization. Gamma camera measurements is applied to noninvasively quantify the accumulation of ^{99m}Tc-DTPA in the region treated with electrical pulses as previously described.

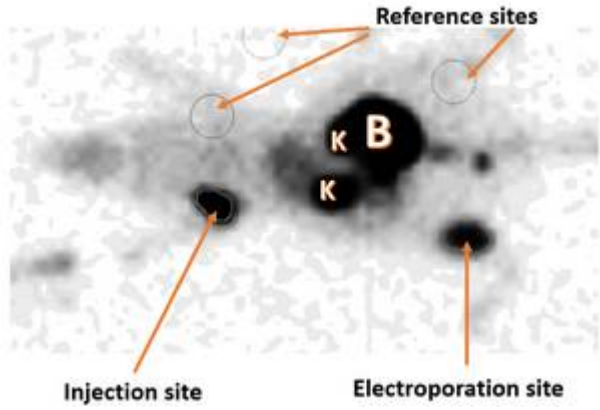

Figure 1
GK-image of a rat at 6 h after electroporation treatment with 800 V/m; 0.25 ms; 12 pulses; K= kidney, B= bladder

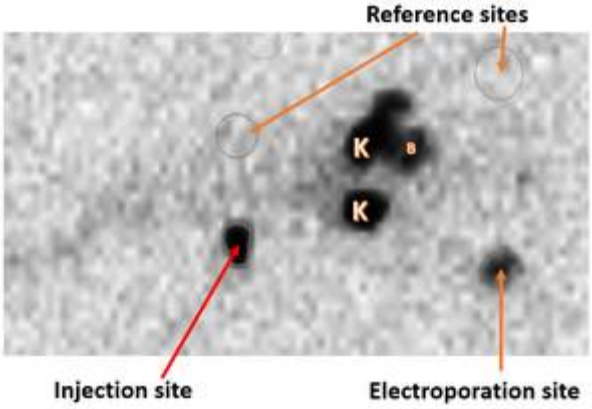

Figure 2
GK-image of the same rat as in 1a at 24 h after electroporation treatment with 800 V/m; 0.25 ms 12 pulses. K= kidney, B= bladder

In **Table 1** is displayed for each rat: applied field strength (V/cm), pulse-length (μs), number of applied pulses N, and uptake-ratio of ^{99}Tc^{m}-DTPA in the target region after 6 h (UR6h) and after 24 hours (UR24h).

Table 1 Electroporation variables and results of Activity Uptake measurements

Rat ID	E V/cm	PL μs	N pulses	TcUR6h	±Sd	TcUR24 h	±Sd
1001	1000	100	6	1,45	0,07	1,61	0,36
1002	1000	100	6	3,58	0,16	4,44	0,39
1003	1000	100	6	3,26	0,16	11,59	4,27
1004	1000	100	12	9,72	0,56	5,77	0,83
1005	1000	100	12	10,63	0,72	12,84	1,23
1006	1000	100	12	6,66	0,40	11,99	2,79

Statistical analysis and modelling of the data is performed using multivariate data processing methods such as Principal Component Analysis PCA, and modelled with the method of Projection to Latent Structures, PLS, also called PLSR Partial Least Square Regression. Herman Wold introduced the method of Partial least squares (Wold, 1982). His son Svante Wold, who

was a chemist has then developed the method to be used in chemometrics, and according to him, the *projection to latent structures* should be the correct name of the method (Wold et al., 2001). These methods are nowadays commonly used in chemometrics, bio-pharmacology and related areas. *Principal component analyses* PCA and *clustering* are used to study the quality and structure of the original database. PCA can also be used to find outliers and to find out if the data can be divided into various classes. In order to find an equation to predict the dependent variables from the descriptors, the model of *Projection to Latent Structure regression* (PLSR) was used (XLSTAT, 2015).

3. Exploration of conductance measurements

Electrical Impedance and Admittance

Tissue can be considered as a dielectric with losses, modeled as a parallel RC-circuit with admittance Y= 1/abs(Z).

$$Y = G + j \cdot \omega \cdot C \quad \text{where} \quad G = \frac{A}{d} \cdot \sigma = k \cdot \sigma \text{ and } C = \frac{A}{d} \cdot \varepsilon = k \cdot \varepsilon$$

where

Z is the impedance;

Y is the admittance =1/abs(Z);

G is the conductance (Ω^{-1});

σ is the conductivity (S);

C is the capacitance (F);

ε is the permittivity;

A is the cross section of the tissue (m^2);

d is the thickness of tissue (m);

k is the geometric constant of the electrode arrangement in question.

Impedance Powering Parameters of electroporation

Various parameters derived from the impedance or admittance data are used for prediction the outcome of electroporation in terms of uptake of ^{99m}Tc-DTPA and DNA expression in the treated tissue volume.

Conductance measurements were performed in 3 Fischer 344 rats with 2 needle electrodes separated 8 mm inserted in the right back thigh muscle. Before applying the EP treatment pulse, two measurements were performed to establish the reference level. Then 6 or 12 consecutive voltage pulses of field strength amplitude 1000 V/cm and pulse-length 100 μs were applied in 1 s interval and the impedance was recorded between each pulse. In order to study the relaxation of the poration the conductance measurements were continued 15 times after the last pulse in 1 s interval.

6 pulses 1000 V/cm and pulse-length 100 µs

The results of a selected rat (R001) are displayed in **Figure 2.**

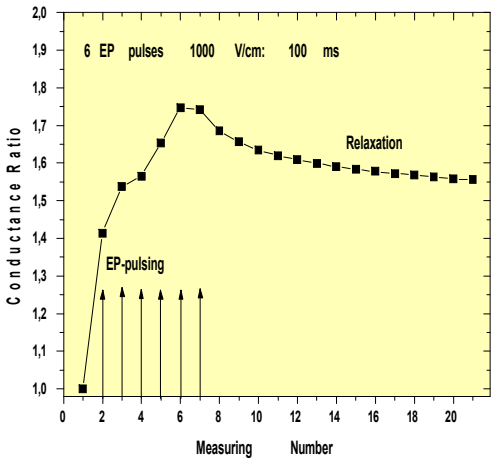

Figure 3
Conductance ratio before, during and after 6 consecutive pulses of 1000 V/cm amplitude and 100 µs pulse length Rat No. R001

The Conductance change index GCI

The Conductance change index GCI is equal to the ratio between difference in admittance between each pulse and the admittance before the first pulse and the admittance before the first pulse .

$$GCI_p = \left(\frac{\mathbf{G_p}}{\mathbf{G_0}} - 1\right)$$

$\mathbf{G_0}$ is the conductance (Ω^{-1}) before the first pulse.
$\mathbf{G_p}$ is the conductance after pulse **no. p**

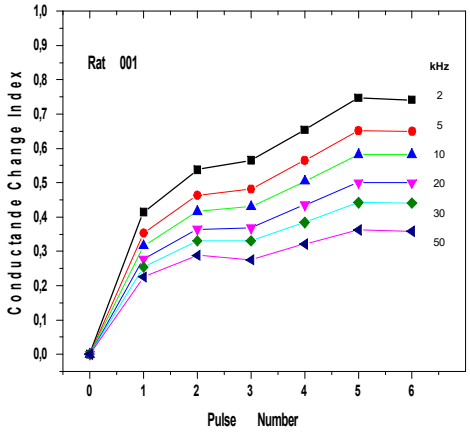

Figure 4
Conductance change index after each EP pulse measured at 2,5,10,20,30,50 kHz

Table 2
Conductance change Index in % after 6 pulses

Rat	2	5	10	20	30	50
R001	74,1	65,0	58,2	50,0	44,1	35,8
R002	29,7	26,2	23,2	20,1	18,0	14,7
R003	46,7	44,3	43,7	43,5	43,9	42,8

Conductance difference between consecutive pulses

Conductance difference between consecutive pulses

$$\Delta G = G_{i+1} - G_i$$

Where i is the pulse number $i = 0, 1, 2, 3, 4, 5, 6$

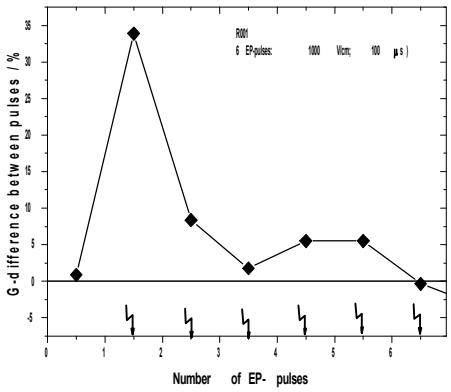

Figure 5
Conductance difference at 2 kHz between consecutive 6 pulses: 1000 V/cm, 100 µs.

Table 3
Conductivity change between the first 3 pulses and the declination

Rat	Δσ1 %	Δσ2 %	Δσ3 %	k
R001	33,89	8,38	1,76	-3,83
R002	20,09	7,23	1,21	-2,61
R003	19,34	8,15	4,36	-1,84

12 pulses 1000 V/cm and pulse-length 100 µs

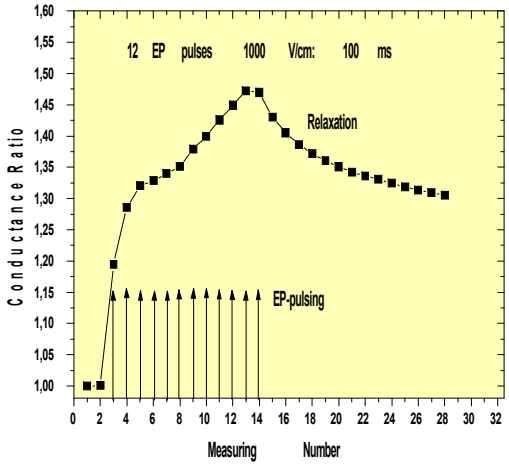

Figure 6
Conductance ratio before, during and after 12 consecutive EP pulses of 1000 V/cm amplitude and 100 µs pulse length. Rat No. R004

Conductance measurements was performed with 2 needle electrodes separated 10 mm inserted in the right back thigh muscle in Fisher 344 rats. Consecutive voltage pulses of amplitude 1000 V and pulse length 100 µs were applied and the conductance was recorded between each pulse and 15 times after the last pulse.

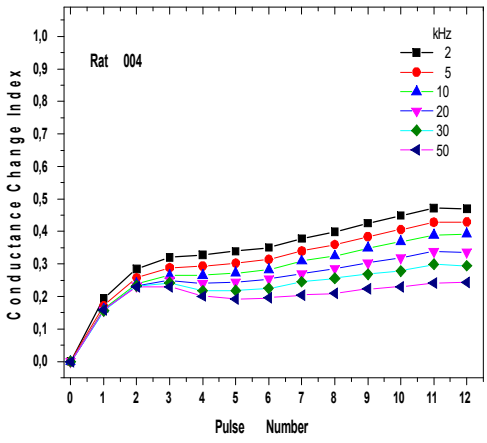

Figure 7
Conductance change index after each EP pulse Rat No. R004

Table 4
Conductance change Index in % after 12 pulses

Rat	2 kHz	5 kHz	10 kHz	20 kHz	30 kHz	50 kHz
R004	46,9	42,9	39,3	33,6	29,5	24,3
R005	73,3	69,2	64,5	57,5	52,7	44,3
R006	99,8	92,2	85,1	73,8	64,8	53,1

Conductance difference between consecutive pulses

Conductance difference between consecutive pulses

$$\Delta G = G_{i+1} - G_i$$

Where i is the pulse number: $i = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12$

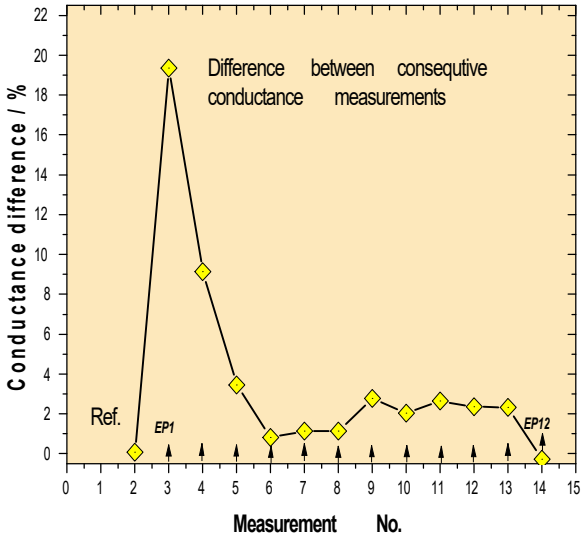

Figure 8
Conductance difference between consecutive measurements. Rat No. R004

Table 5
Conductivity change between the first 3 pulses and the declination k.

Rat	Δσ1 %	Δσ2 %	Δσ3 %	k
R004	17,64	7,36	2,66	-2,03
R005	21,20	10,12	2,18	-1,88
R006	38,90	10,70	4,78	-3,19

Summary of uptake prediction from conductance measurements

The main effect on the conductance in achieved already after the first EP-pulse as shown in the diagrams of **Figures 5** and **8**. The effect of the following pulses varies a lot from animal to animal. In some cases there is a steadily increase and in some case there is even a decrease. The correlation of the radioactivity uptake ratio and the conductance change index after the last pulse indicate a slight positive correlation coefficient for the 24 h uptake ratio.

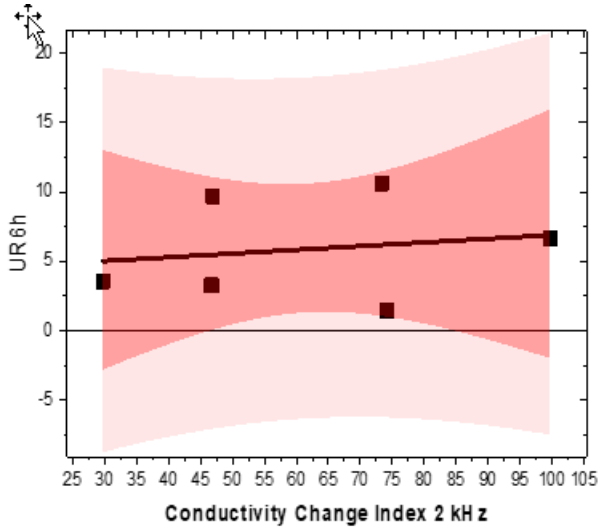

Figure 9a
Uptake ratio at 6 h versus Conductance change Index at 2 kHz after the last pulse

UR6h = 4.22 + 0.027*CCI(2)

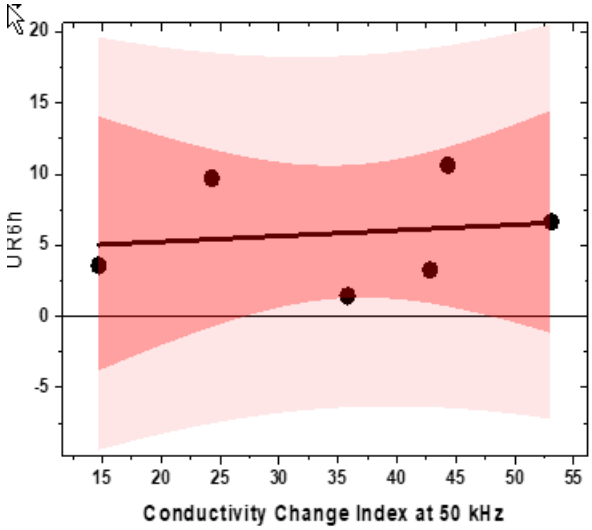

Figure 9b
Uptake ratio at 6 h versus Conductance change Index at 50 kHz after the last pulse

UR6h = 4.45 + 0.040*CCI(50)

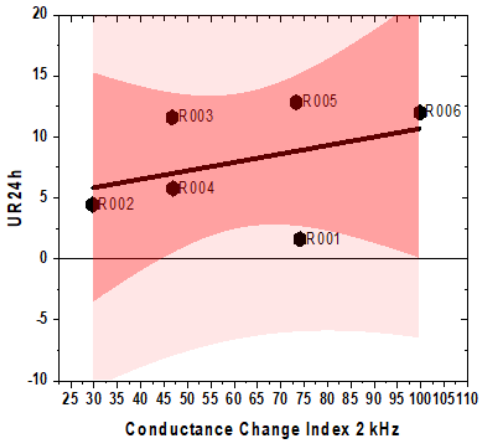

Figure 10a
Uptake ratio at 24 h versus Conductance change Index at 2 kHz after the last pulse
UR24h = 3.77 + 0.07*CCI(2)

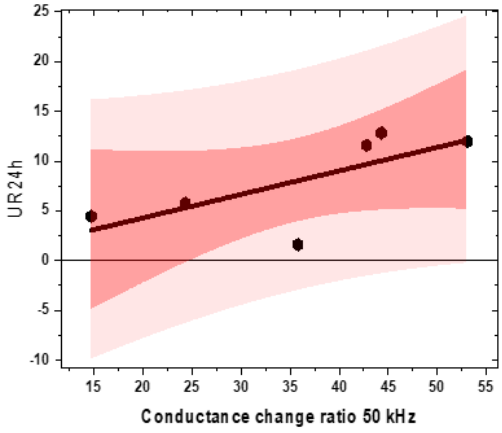

Figure 10
Uptake ratio at 24 h versus Conductance change Index at 50 kHz after the last pulse
UR24h = 0.44 + 0.24*CCI(50)

Uptake ratio at 6 and 24 h versus tissue Conductance change Index at 2 and 50 kHz after the last pulse

UR6h = 4.22 + 0.027*CCI(2); UR6h = 4.45 + 0.040*CCI(50)

UR24h = 3.77 + 0.07*CCI(2); UR24h = 0.44 + 0.24*CCI(50)

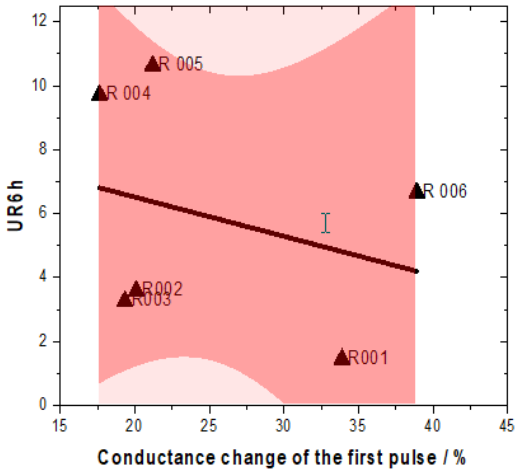

Figure 11a
Uptake ratio at 6 h versus conductance change of the first EP pulse
UR6h = 9.0 – 0.12·DC01Intercept

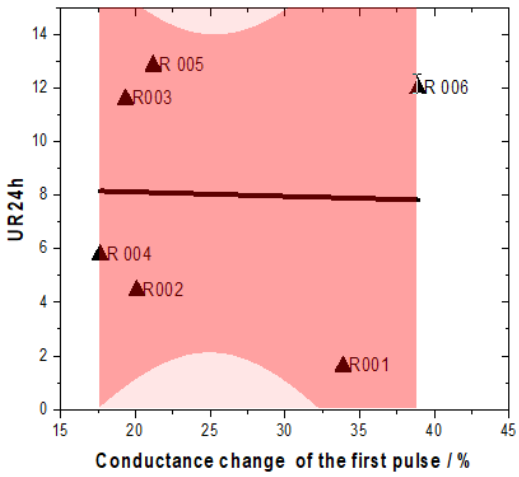

Figure 11b
Uptake ratio at 24 h versus conductance change of the first EP pulse
UR24h = 8.4 – 0.016·DC01

4 . Exploration of the phase angle in tissue before and after EP

Impedance Phase angle measurements

The ratio of the phase before and immediately after electroporation:

Tissue can be considered as a dielectric with losses, that is modelled as a parallel RC circuit with phase angle φ, that is derived from recorded data of the phase angle between the real and imaginary part of impedance. Since the tangent (tg) of the phase-angle φ, (in radians) is equal to the ratio between the imaginary and real part of the impedance $tg\varphi = imZ / reZ$ this is a more relevant measure than the direct phase in degrees.

Loss Change Index value immediately after electroporation LCI-End.

The loss angle δ of a capacitor is defined so that the ideal capacitor with zero losses has zero loss angle. This means that the loss angle δ = 90° - φ, where φ is the recorded phase angle. The loss tangent *tg*δ is also called the *dissipation factor* that is equivalent to the energy loss per cycle divided by the energy stored per cycle (rms or peakvalue). The impedance of the equivalent circuit $Z^* = |Z| \cdot \cos\varphi + j|Z| \cdot \sin\varphi$ and thus :

$$\tan\varphi = \frac{im[Z]}{re[Z]} = \frac{\varepsilon'}{\varepsilon''} \quad and \quad \tan\delta = \frac{re[Z]}{im[Z]} = \cot\varphi$$

The power loss in the circuit only takes place in the resistive part re[Z] if the capacitive part is considered as an ideal capacitor. The frequency dependence of the power loss is dependent on how the circuit is driven. With constant amplitude voltage U the power loss goes from zero level at very low frequencies to a defined value U^2/R at high frequencies. With constant amplitude current, the power level goes from a constant value at very low frequencies through a maximum at the frequency determined by the time constant $\tau = RC$ and to zero at high frequency. The "Loss Change Index" LCI at a specific frequency LCI is evaluated as follow:

$$Loss\ Change\ Index_{\omega} = \left(1 - \frac{cot\varphi_{before}}{cot\varphi_{after}}\right) = \left(1 - \frac{(\mathrm{Re}\,Z/\mathrm{Im}\,Z)_{before}}{(\mathrm{Re}\,Z/\mathrm{Im}\,Z)_{after}}\right)$$

The Loss Change Index is zero if there is no change in the phase angle and approach 1 as imZ_{after} goes to zero after heavy exposure.

Impedance measurements.

Bio-impedance measurements were performed at a frequency of 2-5-10-30-50 kHz. The electrical impedance was measured by applying a 1 mV pulse:

- Twice before the EP pulse
- After each EP pulse
- and after the last electroporation pulse in 1 s intervals.

In all experiments the applied electric field is given as applied voltage over the needle electrodes divided by their distance. The actual field strength in the tissue is, however, quite inhomogeneous in this setting.

Phase angle loss tangent *tan*δ measurements E=1000 V/cm, 100 µs, 6 pulses

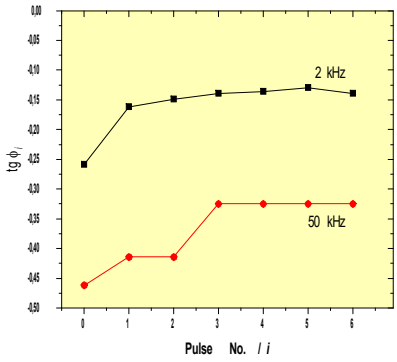

Figure 12
Tangent of phase angles recorded at 2 and 50 kHz after 6 consecutive pulses 1000 V/cm 100 µs. Rat R001

The Loss change index after each pulse is defined as

$$LCI(i) = \left(1 - \frac{cot\varphi_0}{cot\varphi_i}\right)$$

where

φ_0 is the phase angle of the tissue before any electroporation
φ_i is the phase angle of the tissue after the i^{th} electroporation pulse

LCI is an index that indicate the change of the dielectric properties of the tissue after each electroporation pulse.

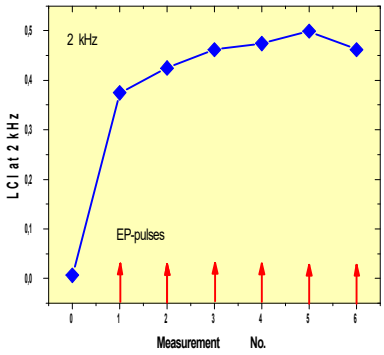

Figure 13
The Loss change index after each pulse defined as

$$LCI(i) = \left(1 - \frac{cot\varphi_0}{cot\varphi_i}\right)$$

Phase angle loss tangent *tan*δ measurements E=1000 V/cm, 100 µs, 12 pulses

The loss tangent *tan*δ is also called the *dissipation factor* that is equivalent to the energy loss per cycle divided by the energy stored per cycle (rms or peakvalue). The impedance of the equivalent circuit $Z^* = |Z| \cdot \cos\varphi + j|Z| \cdot \sin\varphi$ and thus :

$$\tan\varphi = \frac{im[Z]}{re[Z]} = \frac{\varepsilon'}{\varepsilon''} \quad and \quad \tan\delta = \frac{re[Z]}{im[Z]} = \cot\varphi$$

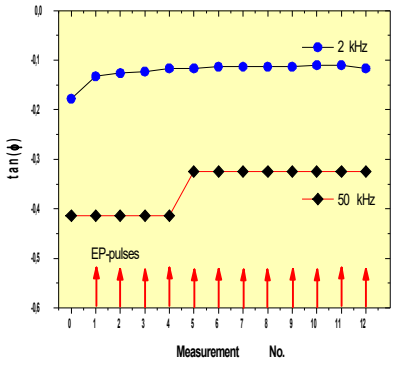

Figure 14
Tangent of phase-angle measured at 2 kHz and 50 kHz after each 12 Pulses 1000 V; 100 µs pulse

The Loss change index after each pulse is defined as

$$LCI(i) = \left(1 - \frac{cot\varphi_0}{cot\varphi_i}\right)$$

where

φ_0 is the phase angle of the tissue before any electroporation
φ_i is the phase angle of the tissue after the i^{th} electroporation pulse

LCI is an index that indicate the change of the dielectric properties of the tissue after each electroporation pulse.

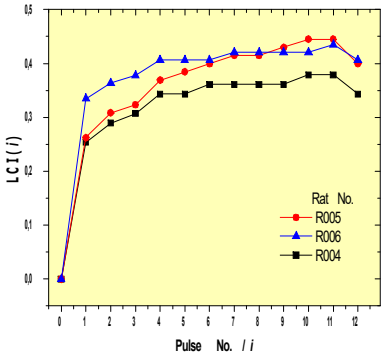

Figure 15
The Loss Change Index measured at 2 kHz after each 1000 V; 100 µs pulse

Table 6
Loss change Index in % after 12 pulses

Rat	2 kHz	50 kHz
R004	34,3	21,6
R005	39,9	36,2
R006	40,6	47,0

5. Specific absorbed energy and temperature increase

Specific absorbed energy from electric pulses and temperature increase

The specific absorbed energy W is calculated from the following expression

$$W = \frac{\sigma \cdot E^2}{\rho} \cdot t_p \cdot N \quad [J.kg^{-1}]$$

where

σ is the tissue conductivity for the tissue [S/m]
$\sigma = \sigma_{ini} \cdot G_{rel(i-1)}$
σ_{ini} is tissue conductivity 0.17 S/m
$G_{rel(i-1)}$ is relative conductivity recorded after each pulse i
ρ is the density of the tissue (muscle 1060 kg/m^3)
E is the electric field strength [V/m]
t_p is the pulse length [s]
N is the number of applied pulses

For the experimental case the cumulative absorbed power is derived from the following equation

$$W = \sum_{i=1}^{n} \frac{\sigma_{ini} \cdot G_{rel(i-1)} \cdot E^2 \cdot t_p}{\rho} \cdot N(i) \quad [\mathrm{J.kg^{-1}}]$$

where

σ_{ini} the tissue conductivity before EP-pulses
$G_{rel(i-1)}$ the relative conductance recorded between each pulse

The specific heat capacities c_p in the relevant temperature region from 20°C to 40°C, of muscles, skin and organs appear to be nearly independent of species and range mostly between 3.2 and 3.9 kJ.kg^{-1}.K^{-1}. Due to the variation in different measuring methods and biological variability of tissues no significant differences seems to occur between organs. The values of fat are distinctly lower; they range from 1 .6 to 3 .0 kJ.kg^{-1}.K^{-1}.

Due to the decrease in blood flow in the target volume treated with Electric pulses, the temperature increase is about $\Delta T = W/c_p$ degree.

Table 7
Estimated absorbed energy (J.kg^{-1}) per pulse, and corresponding sum of the 6 pulses.

No. Pulses	R001	R002	R003
0	0	0	0
1	227	196	195
2	247	211	211
3	251	213	221
4	265	214	228
5	280	214	236
6	279	208	235
Sum	1549	1256	1326
ΔT °C	0,45	0,37	0,39

Table 8
Estimated Absorbed power (J.kg^{-1}) per pulse and Corresponding sum of the 12 pulses and final Temperature increase ($\sigma_{ini} = 0{,}17\ S/m$).

No. Pulses	W /J.kg^{-1} R004	W /J.kg^{-1} R005	W /J.kg^{-1} R006
0	0	0	0
1	191	197	238
2	206	218	265
3	212	223	278
4	213	231	284
5	215	242	289
6	217	250	296
7	221	258	302
8	224	263	306
9	229	269	311
10	232	274	317
11	236	279	322
12	236	278	320
Sum W	2632	2983	3529
ΔT °C	0,77	0,87	1,03

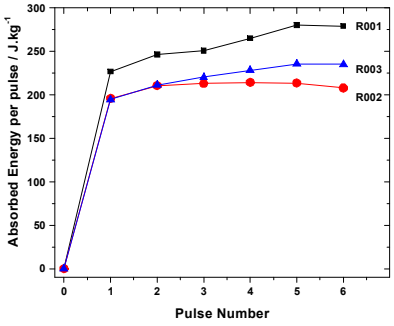

Figure 16a
Estimated Absorbed energy (J.kg^{-1}) per pulse

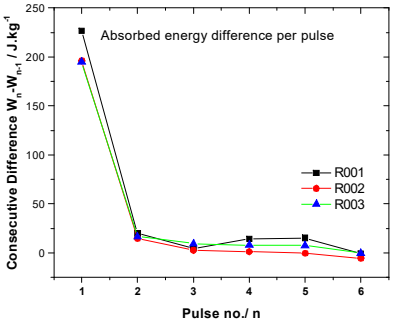

Figure 16b
Consecutive difference in Absorbed energy (J.kg^{-1}) per pulse

In the case of 12 pulses 1000V/cm 0.1 ms and an initial tissue conductivity of 0.17 S/m the temperature increase is about 0.7 – 1 degree °C.

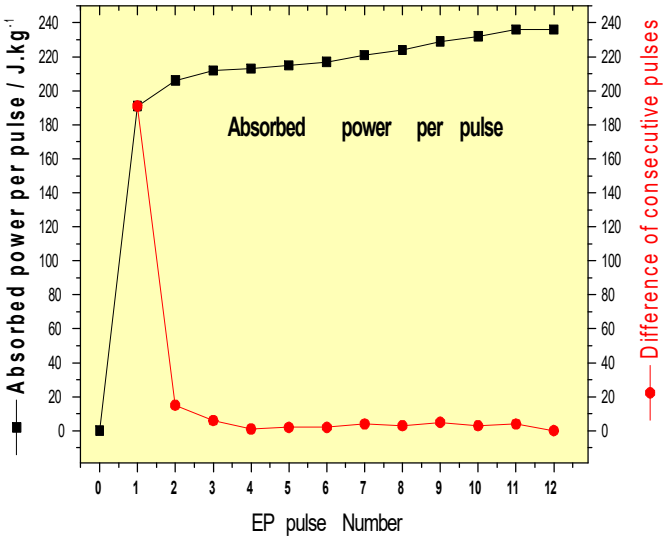

Figure 17 Estimated Absorbed power ($J.kg^{-1}$) per pulse with a corresponding sum of the 12 pulses 2632 $J.kg^{-1}$ (σ_{ini} = 0,17 S/m), and the difference of consecutive pulses (red curve)

The Current density (A/cm^2)

For the experimental case the average current density is derived from the following equation

$$J = \sum_{i=1}^{n} \sigma_{ini} \cdot G_{rel(i-1)} \cdot E \cdot 10^{-4} \cdot N(i) \quad [A.cm^{-2}]$$

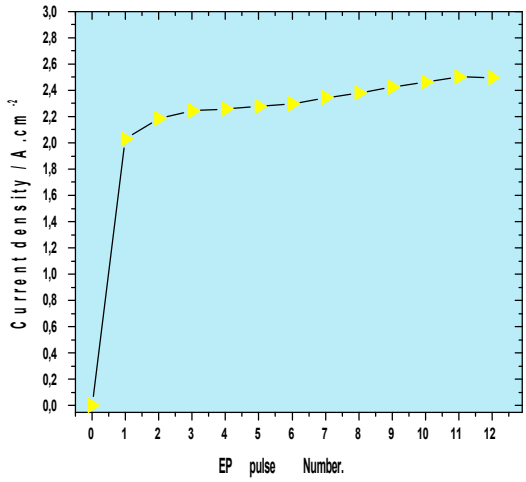

Figure 18
Derived current density after each of 12 consecutive voltage pulses of field strength amplitude 1000 V/cm and pulse-length 100 µs applied in 1 s interval. The conductance was recorded at 2 kHz between each pulse to estimate $G_{GG((GG)}$, and $G_{GG(}$ = 0.17 S/m.

The current density J (A.cm^{-2}) corresponds to the current I (A) recorded in each pulse with needle electrodes. The average corresponding to the values of **Figure 18** is 2.34 ± 0.13 A.cm^{-2}.

Table 9
Estimated Current density (As.cm^{-2}) per pulse and corresponding average of the 6 pulses

No. pulses	R001	R002	R003
1	2,40	2,08	2,06
2	2,61	2,23	2,24
3	2,66	2,26	2,34
4	2,81	2,27	2,42
5	2,97	2,27	2,50
6	2,96	2,20	2,49
Average±SD	2,7±0.2	2,22±0.07	2,34±0.15

Table 10
Estimated Current density (As.cm^{-2}) per pulse and corresponding average of the 12 pulses.

	J As.cm^{-2}		
No. Pulses	R004	R005	R006
0	0,00	0,00	0,00
1	2,03	2,09	2,52
2	2,18	2,32	2,81
3	2,24	2,37	2,94
4	2,26	2,45	3,01
5	2,28	2,56	3,07
6	2,30	2,65	3,14
7	2,34	2,73	3,20
8	2,38	2,79	3,25
9	2,42	2,85	3,30
10	2,46	2,90	3,37
11	2,50	2,95	3,41
12	2,50	2,95	3,40
Average±SD	2,32±0.13	2,63±0.27	3,12±0.26

6. Conductance relaxation

After the applied electro-permeabilization pulse the conductivity start to decrease and approach a plateau value. The fraction of the plateau value relative to the initial conductivity is a measure of the fraction of reversible electropermeabilized cells. This value is of importance for the long term transfer of exogenous substances to the cell and outflow of immunogenic substances from the cell. The relaxation curves for each rat was fitted to a single exponential decay

$$\sigma_{rlp} = A_0 + f_{rev} \cdot exp(-t/T1) \qquad \text{(eq. 6)}$$

where

f_{rev} is the fraction of reversible electroporation

$A_0 = 1 - f_{rev}$ is the fraction of irreversible electroporation

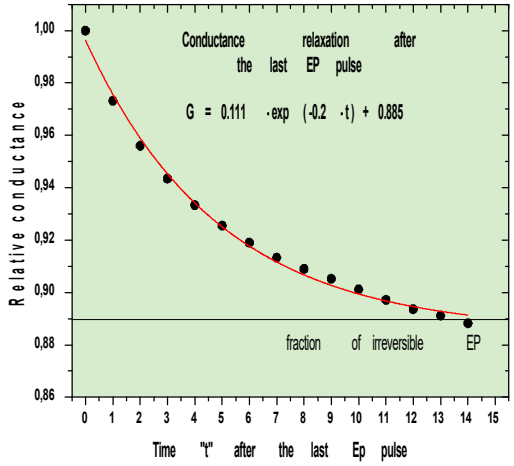

Figure 19 Conductance relaxation after 12 pulses 1000 V/cm and pulse-length 100 μs.

The mean relaxation curve of the conductivity relative to that after the last pulse G is fitted to an exponential equation

$$\mathbf{G = 0.885 + 0.111 \cdot \mathit{exp}(-0.2 \cdot t)} \qquad \text{(eq. 7)}$$

The relaxation rate constant is 0.2 s^{-1} corresponding to a mean relaxation time of 5 s the fraction of reversible electropermeabilized cells is 0.11 (11%)

Table 11
Mean relaxation time T1 (s) and the fraction of Reversible electropermeabilized tissue (f_{rev}) are given in the table

	Mean relaxation Time (s)		Fraction of Reversible EP	
Rat No.	T1	sd	F_{rev}	sd
R001	3,9	0,3	0,101	0,003
R002	4,0	0,3	0,120	0,003
R003	5,1	0,3	0,135	0,003

Table 12
Mean relaxation time T1 (s) and the fraction of reversible electropermeabilized tissue (f_{ev}).

Rat No.	T1	se	f_{rev}	se
R004	4,90	0,29	0,111	0,002
R005	5,61	0,44	0,133	0,004
R006	5,36	0,49	0,106	0,003

7. Conclusion

PLS modelling of the uptake ratios with all predictor variables resulted in the distribution of "Variable Importance in the Projection" (VIP) shown in **Figure 20.**

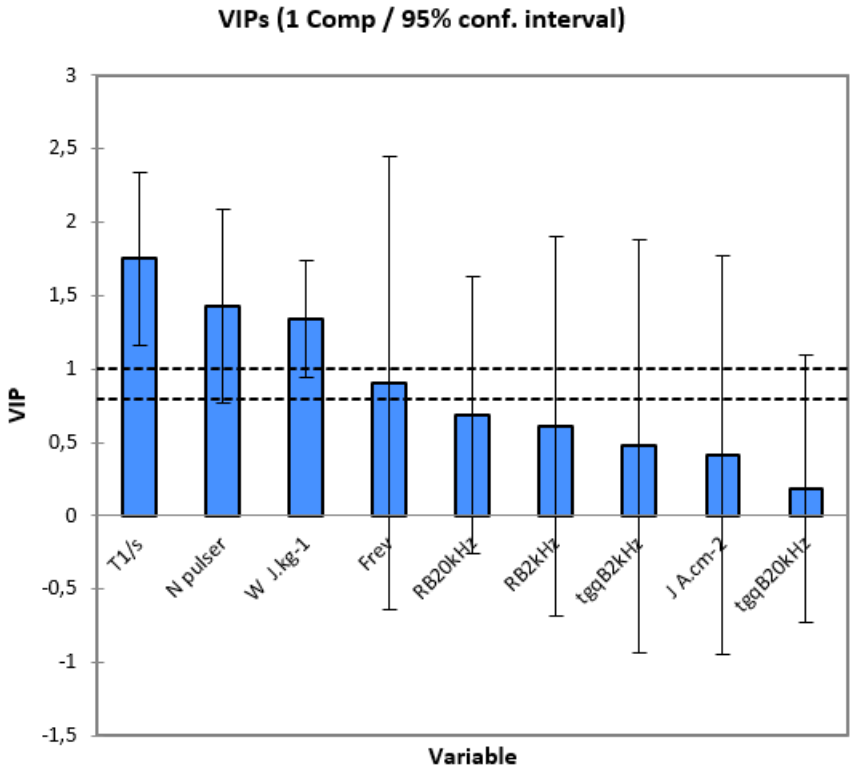

Figure 20 Distribution of "Variable Importance in the Projection" (VIP) when all predictor parameters were applied in the PLS model,

The model equations of the uptake ratios when the 2 most important predictor parameters were applied in the PLS model:

$$\mathbf{UR6h = -8{,}097+2{,}04*(T1/s)+1{,}88\cdot 10\text{-}3*(W\ \ J.kg^{-1})}$$

$$\mathbf{UR24h = -25{,}78+7{,}63*T1/s\text{-}1{,}36\cdot 10\text{-}3*W\ \ J.kg\text{-}1}$$

Such equations can be used to predict the outcome of the Electro Enhanced Chemotherapy treatment

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