

Analysis of damage-associated molecular pattern molecules due to electroporation of cells *in vitro*

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Background. Tumor cells can die via immunogenic cell death pathway, in which damage-associated molecular pattern molecules (DAMPs) are released from the cells. These molecules activate cells involved in the immune response. Both innate and adaptive immune response can be activated, causing a destruction of the remaining infected cells. Activation of immune response is also an important component of tumor treatment with electrochemotherapy (ECT) and irreversible electroporation (IRE). We thus explored, if and when specific DAMPs are released as a consequence of electroporation *in vitro*.

Materials and methods. In this *in vitro* study, 100 μ s long electric pulses were applied to a suspension of Chinese hamster ovary cells. The release of DAMPs – specifically: adenosine triphosphate (ATP), calreticulin, nucleic acids and uric acid was investigated at different time points after exposing the cells to electric pulses of different amplitudes. The release of DAMPs was statistically correlated with cell permeabilization and cell survival, e.g. reversible and irreversible electroporation.

Results. In general, the release of DAMPs increases with increasing pulse amplitude. Concentration of DAMPs depend on the time interval between exposure of the cells to pulses and the analysis. Concentrations of most DAMPs correlate strongly with cell death. However, we detected no uric acid in the investigated samples.

Conclusions. Release of DAMPs can serve as a marker for prediction of cell death. Since the stability of certain DAMPs is time dependent, this should be considered when designing protocols for detecting DAMPs after electric pulse treatment.

Key words: electroporation; pulsed electric field treatment; damage-associated molecular pattern molecules; immunogenic cell death; electrochemotherapy

Introduction

Electroporation or pulsed electric field (PEF) treatment can cause changes in membrane permeability, which allows molecules, that are otherwise membrane impermeable, to cross the plasma membrane. In reversible electroporation the damage to cell membrane is repaired, enabling the cell to reestablish its metabolism and survive. This type of electroporation is used in multiple therapies. Electrochemotherapy (ECT) is one of such widely used therapies in which the increased cell

membrane permeability enables chemotherapeutic drug to enter the cell and thus potentiates the cytotoxicity of the drug.^{1,2} In irreversible electroporation (IRE) the damage to the cells however is too severe for the cells to recover which leads to cell death. While the cells are destroyed, the integrity of tissue like vessels, nerves and extracellular matrix remains preserved^{3,4}, making this therapy very appealing for ablation of tumor and other tissues, otherwise unsuitable for surgical removal or thermal ablation such as radiofrequency ablation or cryo-ablation.^{5,6}

In ECT eight square 100 μ s electrical pulses, with an amplitude of 100-1000 V are usually used to induce a reversible membrane permeabilization. For IRE, more pulses (80-100 pulses) at higher amplitude (up to 3000 V) are required, to overwhelm the reparative capacity of the cells which leads to cell death.⁷ From morphological, biochemical, and functional perspectives, different cell death pathways/types can be activated.⁸ Historically, based on morphological changes, three different forms of cell death were defined: apoptosis (cell shrinkage, chromatin condensation, formation of apoptotic bodies); autophagy (cytoplasmic vacuolization); and necrosis (loss of plasma membrane integrity).^{8,9} Such classification is still employed, but in newer classification based on genetic, biochemical, pharmacological and functional differences, cell death is either accidental (uncontrollable death caused by disassembly of the plasma membrane) or regulated (activation of signal transduction). Depending on signaling pathways different types of regulated cell death are being characterized, e.g. intrinsic and extrinsic apoptosis, necroptosis, ferroptosis, pyroptosis, immunogenic cell death, lysosome-dependent cell death, mitochondrial permeability transition driven necrosis and many others gathered and described by Galluzi *et al.*¹⁰ In electroporation studies, cell death has been most extensively explored in the range of nanosecond pulse treatment, where the majority of studies confirmed cell death by apoptosis (intrinsic and extrinsic) and only few studies indicated necrosis.^{11,12} Both pathways were confirmed also in microsecond pulse treatment.¹³⁻⁹ Nevertheless, in recent studies new cell death types were also detected like pyroptosis²⁰, necroptosis^{20,21} and immunogenic cell death.²²⁻²⁹

In IRE^{18,30-34} and ECT with either bleomycin or cisplatin^{26,35-37} used for cancer treatment, involvement and importance of host immune response was demonstrated, counteracting tumor escape mechanisms.^{29,38,39} After these therapies, dying tumor cells can release specific molecules, which are being recognized by the cells of immune system. These molecules can activate the innate and adaptive immune response, leading to the destruction of the remaining tumor cells in the body⁴⁰ and inducing long-lasting protective antitumor immunity.⁴¹ Some studies even suggest that immunogenic effect of IRE is more pronounced than in other ablation therapies like radiofrequency ablation³¹ and cryoablation.³² Evidence suggests that administration of immune-stimulating molecules can even enhance the local effectiveness of ECT³⁵ and IRE^{29,42-44} allowing simultaneous treatment of distant tumors.

Our immune system consists of two complementary and closely collaborative systems, an innate (non-specific) and an adaptive (antigen-specific) system. Activation of immune system is essential for our survival, as it distinguishes and eliminates potentially harmful molecules, even the ones that derive from the host/our own tissues. Well known are the pathogen-associated molecules (PAMPs), which are present on microbes and are being recognized by cells of the innate immune system when they bind to pattern recognition receptors (PRRs). The same pathways are activated by the host's damage-associated molecular pattern molecules (DAMPs), which act as endogenous damage signal in case of cell death or response to stress, leading to inflammatory response.⁴⁵⁻⁴⁷ Release of DAMPs characterizes immunogenic cell death (ICD). Most of DAMPs are normally located intracellularly⁴⁸, where under normal physiological conditions have an important intracellular role. When a cell is damaged or dies, DAMPs are actively or passively exposed or released to extracellular space.⁴⁹⁻⁵¹ The release of DAMPs is often accompanied by cytokines, chemokines and other inflammatory mediators.⁵² In extracellular space DAMPs have a completely different function, as they are being recognized by pattern recognition receptors (PRRs), such as TLRs, NOD-like, PRRs and RAGE receptors on immune cells.^{50,53} Binding of DAMPs to these receptors stimulates innate immune response through promoting the release of pro-inflammatory mediators and recruiting immune cells (dendritic cells, macrophages, T cells and neutrophils). Usually, the exposure of different DAMPs depends on endoplasmic reticulum stress, followed by reactive oxygen species (ROS) production.⁴¹ Release of DAMPs correlates with the degree of trauma.⁵⁴ Some DAMPs can even be involved in tissue repair pathway.⁵⁵⁻⁵⁷ It depends on DAMPs and their triggered pathways, together with cytokines and growth factor to determine, if mild acute inflammation and wound healing^{56,58} or severe inflammation and fibrosis will follow.⁵⁹

Electroporation causes an increase in membrane permeability and allows molecules, for which the membrane is usually impermeable, including DAMPs, to cross it. ATP, one of the main DAMPs, was even used as an indicator of cell membrane permeabilization in the first electroporation studies.⁶⁰ In recent years reports on electroporation studies have started to emerge investigating the immunogenic cell death caused by electroporation. Studies detected DAMPs, like ATP, high-mobility group box 1 protein (HMGB1) release and calreti-

culin externalization, as they are the gold standard for predicting the ICD in cancer cells.⁴¹ So far mostly single (or a small subset of) DAMPs were studied. Most studies involved nanosecond pulse treatment²²⁻²⁵, whereas studies using microsecond^{26,29}, millisecond²⁸ and H-FIRE pulse treatments²⁷ are even more scarce. For now, different DAMPs were investigated at different intervals after electroporation ranging from 30 min to 72 hours, using different types of cancer cells.

Because in both ECT and IRE the immune system response is essential for successful and complete tumor eradication, we decided to explore if and when specific DAMPs are released in response to electroporation *in vitro*. The experiments were performed using 100 μ s long pulses, as they are most commonly used in ECT treatment and in IRE for soft tissue ablation.

Materials and methods

Cell preparation

Chinese hamster ovary (CHO-K1) from European Collection of Authenticated Cell Cultures were grown in culture flasks (TPP, Switzerland) filled with HAM F-12 growth medium (PAA, Austria) at 37°C with a humidified 5% CO₂. The growth medium was enriched with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany), L-glutamine (StemCell, Canada) and antibiotics penicillin/streptomycin (PAA, Austria) and gentamycin (Sigma-Aldrich, Germany). At 70% confluency, cells were detached with trypsin solution (10x trypsin-EDTA (PAA, Austria) 1:9 diluted in Hank's basal salt solution (StemCell, Canada), which was inactivated after 3 minutes by the growth medium. After 5 minutes of centrifugation at 180 g and 22°C supernatant was removed. Cell were mixed with the growth medium to obtain cell density at 2x10⁶ cells/ml.

Electric pulse generation

Laboratory prototype pulse generator (University of Ljubljana), based on H-bridge digital amplifier with 1 kV MOSFETs (DE275-102N06A, IXYS, USA), described in⁶¹ was used. Eight 100 μ s long monopolar electric pulses with repetition frequency 1 Hz and amplitude of 0–600 V (0–3 kV/cm; voltage to distance ratio) with increments of 100 V (and additional increments in the permeabilization assay) were applied between stainless steel 304 plate electrodes (d = 2 mm). Oscilloscope HDO6104A-

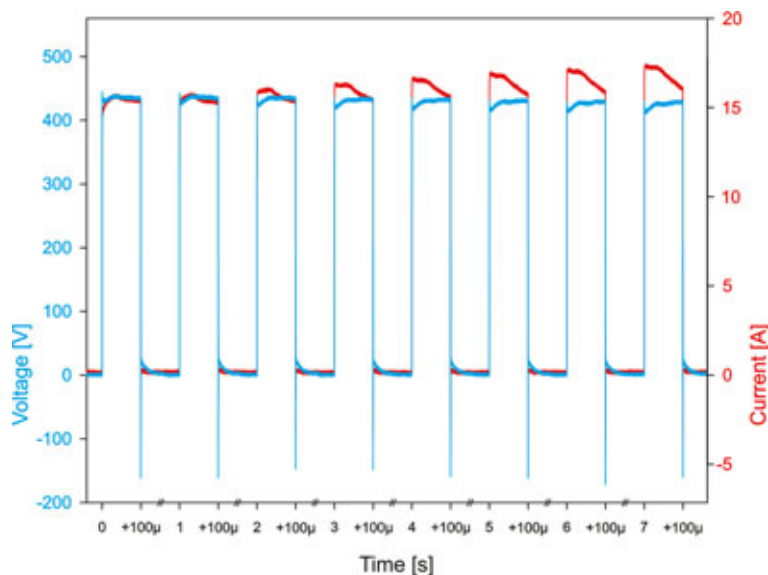


FIGURE 1. Application of 500 V pulses. Blue line shows voltage and red line shows current. Due to sequencing, all eight pulses are in one picture, separated by //.

MS, differential probe HVD3206A and the current probe CP031A, all from LeCroy, USA, were used to monitor the delivered pulses, i.e. voltage and current. The delivered voltage was approximately 10–15% lower than the value set on the pulse generator and the current was in the range of 3–21 A. When pulses with high amplitudes were applied (Figure 1), current decreased slightly during the pulse, presumably due to electrochemistry at electrode-electrolyte interface reducing the available interface area for ion exchange between the metal electrode and the electrolyte and possibly also due to ion depletion at the said interface.

Results

First, the permeabilization and the survival curves were obtained to determine experimental points for the studies on release of DAMPs. Permeabilization and survival curves are presented in all figures showing the concentration of various DAMPs to visualize how the presence of DAMPs is related to changes in permeabilization and cell viability. In figures the permeabilization and survival curves are shown only at pulse amplitudes tested for the presence of DAMPs; in steps of 50 V in the range of pulses where changes in permeabilization occur and in steps of 100 V above 200 V.

The concentration of ATP in supernatant was first measured with fluorescent method 30 minutes and

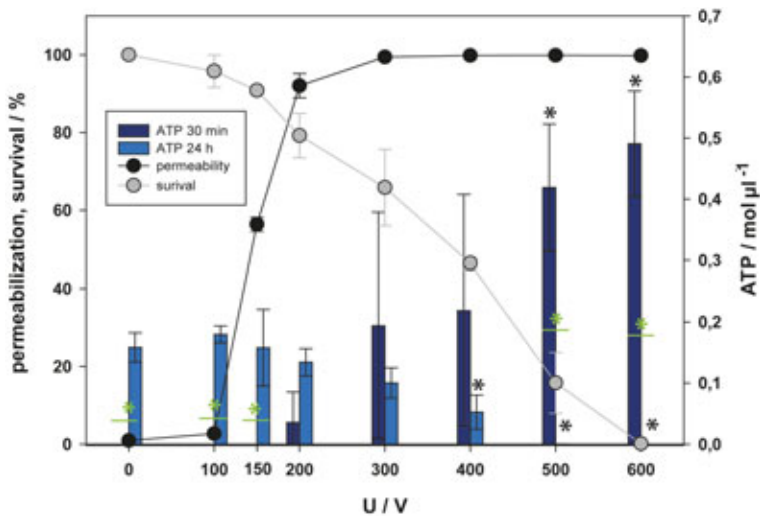


FIGURE 2. Release of adenosine triphosphate (ATP) as a function of electric pulse amplitude determined by fluorescent assay. Two-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

24 hours after electroporation (Figure 2). At 30 minutes the concentration of ATP in supernatant was detected at 200 V. However, statistical difference between the control and the treatment groups (obtained by one-way analysis of variance [ANOVA] followed by the post-hoc test) was only detected at 500 V and above. The concentration of ATP in su-

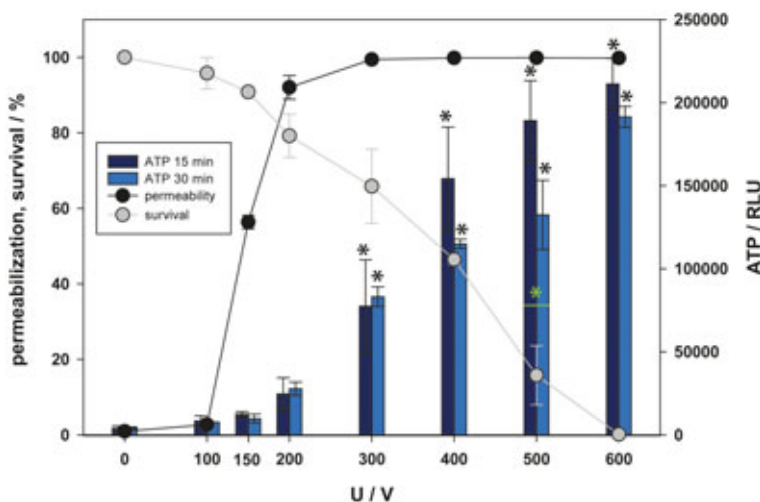


FIGURE 3. Release of adenosine triphosphate (ATP), as a function of electric pulse amplitude determined by luminescence assay. Two-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

pernatant grew with increasing pulse amplitude, which after 24 hours led to decreased cell viability; e.g. correlation between the cell survival and ATP concentration in supernatant detected after 30 min is quite strong and negative; $R = -0.864$. Also, weak correlation between cell permeabilization and ATP concentration in supernatant ($R = 0.594$) confirms, that ATP presence in supernatant is more strongly correlated with the irreversible than the reversible electroporation. It may indicate that strong ATP release from cells leads to cell death.

After 24 hours (Figure 2) the lowest ATP concentration was achieved at the pulse amplitude resulting in death of most cells (500, 600 V). The concentration of ATP at these points is statistically different to the results obtained 30 minutes after pulse treatment. After 24 hours the concentration of ATP had decreased with the lower viability, but statistical differences between the control and the treatment groups were present from 400 to 600 V. At 24 hours there is a positive statistical correlation between the cell survival and concentration of ATP in supernatant ($R = 0.888$), which is stronger than the correlation to permeabilization ($R = -0.695$).

Since no ATP was detected in supernatant within the range of reversible electroporation after 30 minutes using the fluorescent method (Figure 2), we also used a more sensitive luminescent method (Figure 3). Furthermore, since ATP analysis showed that 24 h after treatment ATP is not detected in all samples, we were also interested in how fast ATP was degraded. Scuderi *et al.* showed complete resealing of plasma membrane 10 minutes after pulse treatment using $8 \times 100 \mu\text{s}$ pulses.⁶³ Thus, another time point for ATP measurement was chosen, *i.e.* 15 minutes after (Figure 3). With more sensitive luminescent detection assay, ATP was detected in supernatant already at 100 V, however statistically significant difference to control was only detected at 300 V and higher. These results are more reliable due to higher assay sensitivity however even with this method statistically significant amount of ATP in supernatant is detected in the range of irreversible electroporation, as increased electric field/voltage kills more cells more ATP is present in the extracellular space. This is also confirmed by a strong correlation between the survival and the amount of ATP in supernatant, $R = -0.947$ for 15 min and $R = -0.964$ for 30 min, and much weaker correlation between the permeabilization and the amount of ATP ($R = 0.704$ for 15 min and $R = 0.728$ for 30 min). In our results, only one significant difference was found in detected ATP amount between 15 and 30 minutes after pulse treatment at 500 V. Since this

difference was not detected for all the experimental points (voltages), we believe that ATP in extracellular space is not degraded in this first 30 minutes after pulse treatment.

Calreticulin (CRT) is an endoplasmic reticulum protein which needs to be transferred to the outer leaflet of the plasma membrane in order to act as a DAMP. Externalization of calreticulin to outer membrane in an active process involving also its transport across the cell. Due to this active and time demanding process the externalization of calreticulin was investigated 4 and 24 hours (also used in previous studies^{23-25,28}) after pulse treatment (Figure 4) on viable cells (determined by propidium iodide [PI] staining). Calreticulin was first detected at 300 V and its fluorescence increased with increasing voltage of pulses. Furthermore, the lowest viability at 600 V with < 5% of viable cell has the strongest signal of calreticulin after 4 and 24 hours. This could indicate the amount of externalized calreticulin per viable cell increases with the level of stress (amplitude of applied electric pulses). Furthermore, analysis shows a strong correlation between survival determined by MTS test and externalization of calreticulin, as survival decreased, the detection of calreticulin increased ($R = -0.801$ for 4h and $R = -0.946$ for 24h) and weak correlation between permeabilization and externalization of calreticulin was observed ($R = 0.535$ for 4h and $R = 0.556$ for 24h). Since calreticulin was detected only in viable cells (determined by PI) additional information on viability was obtained, and results were normalized to control (0 V) for each investigated time point separately. Except for the results at 300 V, no statistically significant difference between 4 and 24 hours was detected at any other experimental point, suggesting that calreticulin can be detected 4 hours after pulse treatment and that expression of the protein remains stable for the next 20 hours.

Until now, nucleic acids (in the role of DAMPs) have not been investigated in relation to electroporation. Most of RNA (except fresh transcribed mRNA) is located in cytoplasm, while DNA is located in the cell nucleus. The concentration of RNA and DNA in supernatant has been detected 15, 30 minutes and 24 hours after electroporation like in ATP assay (Figure 5 and 6). Concentration of DNA/RNA started to rise from 400 V up (Figure 5 and 6). This happened at the same pulse amplitudes where after 24 hours cell viability was affected, indicating the amount of nucleic acid occurs in the range of cell death, *i.e.* irreversible electroporation. Exposure of cells to higher pulse amplitudes caused higher

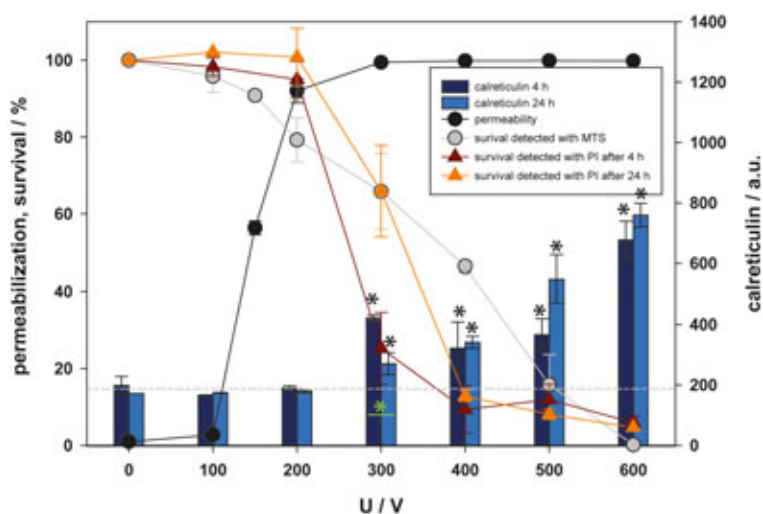


FIGURE 4. Externalization of calreticulin as a function of electric pulse amplitude. Two-time points after electroporation were assessed. Permeabilization and survival (MTS) curves are also presented. Survival detected by propidium iodide (PI) protocol is normalized to control and presented with red (4 hours after pulse treatment) and orange (24 hours after pulse treatment) line. Approximate baseline of calreticulin is presented with -----. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

release of nucleic acids, which after 24 h resulted in lower cell viability. This is confirmed also by a strong negative correlation between survival and

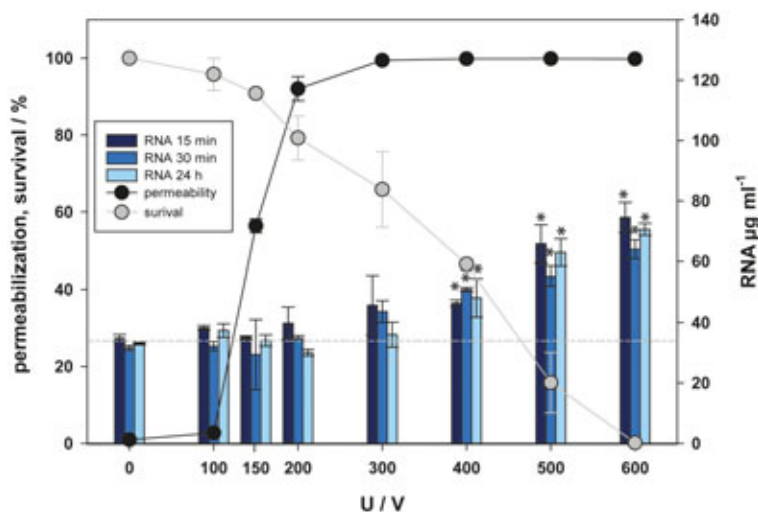


FIGURE 5. Release of RNA as a function of electric pulse amplitude. Three-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Approximate baseline of RNA is presented with -----. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$), respectively).

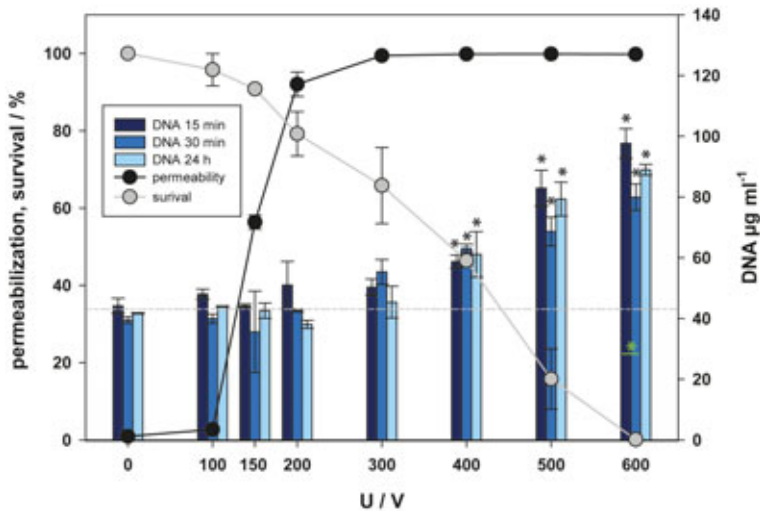


FIGURE 6. Release of DNA as a function of electric pulse amplitude. Three-time points after electroporation were assessed. Permeabilization and survival curves are also presented. Approximate baseline of DNA is presented with ----. Black and green asterisks (*) indicate statistically significant differences between the samples at different voltages and the corresponding control at 0 V (one-way analysis of variance [ANOVA] followed by Holm-Sidak post-hoc test, ($p < 0.05$) and within the pair of samples at different voltages (t-test, $p < 0.05$) respectively.

release of RNA ($R = -0.909$ for 15 min, $R = -0.909$ for 30 min, $R = -0.919$ for 24 h) and weak correlation between permeabilization and release of RNA ($R = 0.584$ for 15 min, $R = 0.696$ for 30 min, R is not significant for 24 h), respectively. Similarly, for DNA, a strong correlation between survival and release of DNA ($R = -0.935$ for 15 min, $R = -0.919$ for 30 min, $R = -0.928$ for 24 h) and a weak correlation between

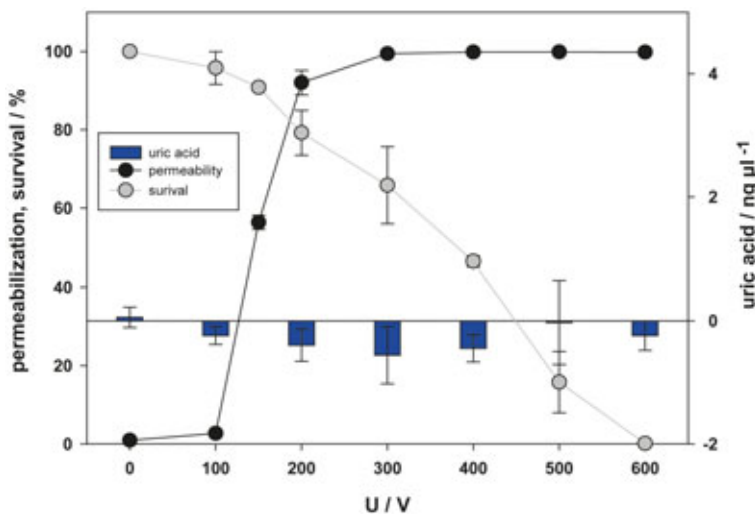


FIGURE 7. Release of uric acid as a function of electric pulse amplitude. Amount of uric acid was analysed 24 hours after pulse treatment in supernatant. Permeabilization and survival curves are also presented. No statistical difference was detected.

permeabilization and release of DNA ($R = 0.571$ for 15 min, $R = 0.689$ for 30 min, R is not significant for 24 h) was found. This correlation may indicate that loss of nucleic acids results in cell death.

Comparison of the concentration of RNA detected in supernatant 15, 30 minutes and 24 hours after pulse treatment did not show any significant differences. Comparison of the concentration of DNA detected in supernatant 15, 30 minutes and 24 hours after pulse treatment showed only significant differences between 15 and 30 minutes at 600 V, yet interestingly this was not the case between 15/30 minutes and 24 hours after pulse treatment. According to our results, the released nucleic acids *in vitro* are stable and are not degraded within 24 hours after pulse treatment.

We also tried to detect uric acid, another well know DAMP molecule. The release of uric acid in supernatant was analyzed 24 hours after pulse treatment (Figure 7). In our experiments we were however unable to detect any uric acid in supernatant after pulse treatment. Initial experiments were also performed after 30 minutes, but results were the same (data not shown).

Discussion

Besides the induced membrane permeabilization, followed by cell death, activation of the immune response seems to be an important component in effectiveness of ECT^{26,35-37} and IRE^{18,30-34} treatment *in vivo*. Activation of the immune system can be triggered by a special type of cell death, called immunogenic cell death (ICD) in which DAMPs are the key mediators.⁶⁴ Presence of DAMPs after pulse treatment has been detected in different types of cancer cells and normal tissues.²²⁻²⁹ However, only one study was performed with 100 µs pulses, which are predominantly used in ECT and IRE.²⁶ The authors investigated release of ATP, calreticulin and HMGB1 due to electroporation pulses alone, bleomycin alone and combination of electroporation pulses and bleomycin. Their study demonstrated the release of ATP and calreticulin after pulse treatment, but how this correlates to reversible and/or irreversible electroporation remained elusive. This question is addressed in our study, were release/detection of different DAMPs was correlated with permeabilization and survival curve *in vitro*.

Detected amounts of DAMPs were correlated to cell membrane permeabilization determined by PI assay immediately after pulse treatment and cell survival, analyzed 24 hours after treatment by

MTS test, *i.e.* to reversible and irreversible electroporation, respectively. It is generally believed that membrane permeabilization and cell survival after pulse treatment are causally related, *i.e.* in IRE cell death occurs due to membrane permeabilization and loss of cell homeostasis (in this study correlation coefficient between the two is -0.680) and in ECT increased accumulation of drug leads to increased cell cytotoxicity.

In ECT and IRE it was also demonstrated that the immune response plays an important role in achieving therapeutic effect.³³⁻³⁶ We have therefore determined different DAMPs at different times after exposing the cells to electric pulses and determined whether the concentrations of extracellular DAMPs were better correlated to cell death or to membrane permeabilization. While activation inflammatory response and activation of immune system is desired in cancer therapies, on the other hand it can be a wanted or an unwanted effect in gene therapy.^{68,69} In DNA vaccination therapies, changed permeability of cell membrane enhances the introduction of DNA vaccine inside of cell and the presence of DAMPs additionally activates inflammatory response, which leads to enhanced production of antibodies, thus enhancing the efficiency of vaccination.⁶⁵⁻⁶⁷ Nevertheless, in most cases of gene therapy, the immune response is unwanted, as it may destroy the transfected cells and prevent transgenic protein expression.^{68,69} By now many DAMPs have been identified and the number is still increasing. Most known are the HMGB1, nucleic acids, proteins like heat-shock proteins, S100 and calreticulin, purine metabolites like ATP and uric acid and saccharides. A list of known DAMPs and their receptors is given by Roh and Sohn.⁴⁸

ATP is a well-known molecule in biology and biochemistry for being a universal energy source in the cell and necessary for multiple cell processes and cell metabolism. Interestingly, first studies of electroporation and increase in plasma membrane permeability involved adenosine triphosphate ATP detection in electroporation buffer.⁷⁰ The released ATP is also considered a DAMP. In our study two types of ATP detection methods with different sensitivities were used.^{71,72} In a previous study performed by Calvet *et al.*²⁶ using the same pulses as in our study, the release of ATP was detected 30 minutes after the treatment. We were able to confirm their observations with the fluorescence and the luminescence method (Figures 2 and 3 respectively). Furthermore, the investigation of the effects at different pulse amplitudes showed that the release of ATP increases with increasing amplitude. This

was expected, as ATP release was previously used as a permeability marker after electroporation.⁷⁰ However, in our results statistical differences between the control and the treatment groups were not detected in the range where the permeabilization curve is ascending, but was detected only in the range of pulse amplitudes at which all cells were already permeabilized and many were dead. Since ANOVA analysis is less sensitive when large amount of samples with big differences between them are analyzed, additional ANOVA was performed, taking into account only the results from 0 to 300 V (e.g. where permeabilization changes from 0 to 100%). Now additional analysis showed that statistical differences between control and the treatment groups are present at 200 V and above in luminescent method, suggesting ATP release as possible membrane permeabilization detection method. In the fluorescence method, this statistical difference was obtained only at 300 V. Such difference in analysis and also a bigger ratio of ATP between the control and the treatment groups indicates that the luminescence method is more sensitive method than the fluorescence method. Taken into consideration ATP release at all investigated voltages (also the one leading to cell death after 24 hours) the release of ATP is more strongly correlated to cell death/irreversible electroporation ($R = 0.888$) than permeability/reversible electroporation ($R = -0.695$).

24 hours after pulse treatment (Figure 2) the highest amount of ATP was detected in the supernatant of control sample and the amount of ATP decreased with increasing pulse amplitude. This can be explained by homeostasis of ATP in living cells. In a living homeostatic cell most of the ATP is located intracellularly, however in considerably lower concentration ATP is also present in extracellular space.⁷³ When cells are damaged, considerable release of ATP molecule affects ATP pumps, causing depletion of intracellular K^+ and accumulation of intracellular Na^+ and Ca^{2+} and leading to cell death.⁷⁴ A previous study showed that electroporation pulses cause ATP depletion, which in 24 results in lower viability, presumably by affecting Ca^{2+} -ATPase.⁷⁵ In our study the effect on survival was also confirmed by very strong positive correlation between survival/irreversible electroporation and amount of ATP detected in supernatant ($R = 0.888$). Nevertheless, we need to consider, that some of the ATP detected in supernatant could be from the cells damaged due to cell handling during experiment. In extracellular space ATP is degraded by nucleotides like CD39 and CD37, which convert

ATP through ADP and AMP to adenosine⁷³, which explains why ATP was detected 30 minutes at very high voltages (500, 600 V), but was no longer detected after 24 hours (Figure 2). This can also explain why Calvet *et al.*²⁶, was unable to detect ATP 30 hours after pulse treatment alone, however it does not explain, why ATP was still detected when bleomycin alone or in combination with electroporation pulses was used. How fast ATP degrades in extracellular space, remains unknown. Our results do not indicate that ATP degrades within the first 30 minutes after pulse treatment, since no difference between 15 and 30 minutes after pulse treatment was detected. In a different study⁷⁶ the results for ATP 4 hours after pulse treatment was lower in samples exposed to pulse treatment than in the control. If this is taken into consideration together with our results, then ATP degradation *in vitro* occurs somewhere between 30 minutes and 4 hours after pulse treatment.

Calreticulin was another molecule of interest in our study. This highly conserved protein has major functions in lumen of the endoplasmic reticulum (ER). It is involved in correct folding of proteins that are produced in endoplasmic reticulum⁷⁷ and in regulation of calcium metabolism, as it affects Ca²⁺ capacity of the ER stores.⁷⁸ In the early phase of cell death, activated ER stress leads to translocation of calreticulin to cells surface through ER-Golgi pathway or lysosome exocytosis.^{79,80} Calreticulin, as DAMP, was investigated previously in electroporation studies.^{22-26,28} In Calvet's study²⁶, which used the same pulses as in our study (eight 100 μ s pulses), calreticulin was determined 30 hours after treatment using different treatments. Calreticulin was detected on the plasma membrane after electroporation pulses alone or in combination with bleomycin (ECT), yet no externalization was detected in cells treated with bleomycin alone. Since only calreticulin, exposed on the cell surface acts as a DAMP, only viable cells (determined by PI) were taken into analysis. The presence of calreticulin on the cell surface was previously detected already 4 hours after electroporation with millisecond pulses²⁸, thus we assumed 4 hours is sufficient time for calreticulin to transfer to cells surface. Additionally, calreticulin was detected also 24 hours after pulse treatment. In our study calreticulin was investigated 4 and 24 hours after treatment (Figure 4), which is after the resealing of cell membrane.⁶³ Even though calreticulin was detected on the surface of live cells, it was detected only in the range of irreversible electroporation. Additionally, calreticulin detection increased with decreasing

cell viability (less viable cells), implicating that bigger stress or in this case pulse amplitude causes more calreticulin molecules to be externalized to cell surface. Nevertheless, since it is believed that externalization of calreticulin occurs in early phase of cell death^{79,80}, it is possible that cell determined as viable would die within next hours.

In comparison to ATP, calreticulin is more stable. Only at 300 V the difference between 4 and 24 hours was statistically significant. Stability of externalized calreticulin was previously also confirmed in another *in vitro* study.²⁴ Nevertheless, *in vivo* study shows expression is the strongest between four and six hours, and diminishes 24 h after the treatment.²⁸

So far studies investigating DAMPs, released by the electroporation treatment, included ATP, calreticulin and HMGB1. In addition to ATP and calreticulin we also included other known DAMPs in our study, namely nucleic acids and uric acid, which so far have not been investigated as DAMPs after electroporation. Inside the cells nucleic acids are the source of genetic information. As DAMPs in extracellular space nucleic acids bind to TLR receptors. Bound DNA can even attract HMGB1 (a non-histone nuclear protein, which can be actively or passively released into extracellular space, where it acts as a DAMP⁸¹) and together they form complexes stimulating dendritic cells to produce type 1 interferon (non-specific immune response), which can lead to anti-DNA autoantibody production (specific immune response).⁸² Nucleic acids (RNA and DNA) can be detected in supernatant already within minutes after pulse treatment (Figure 5,6). Nevertheless, we need to consider – based on the control, 0 V in Figures 5 and 6, that some of the nucleic acids detected in supernatant could be from the cells damaged due to cell handling during experiment. Since RNA is more abundantly present in cells than DNA^{83,84}, the same was expected to be the case in the supernatant after pulse treatment. However, the amount of detected DNA in our samples was bigger than that of RNA. Since RNA is more prone to degradation than DNA⁸⁴, it is possible that some of the RNA was destroyed during the process of analysis. Nevertheless, the amount of released nucleic acids increases with increasing voltage of electric pulses to which the cells were exposed. Our results indicate that the release of nucleic acids (RNA and DNA) occurs in the range of irreversible electroporation; *i.e.* pulse amplitudes that lead to cell death as determined by MTS test at 24 h post treatment. This was confirmed also by very strong negative

correlation between the cell survival and the release for RNA and DNA.

Uric acid is a product of purine metabolism within the cell, like degradation of nucleic acids, and is released from injured and dying cells.⁸⁵ A molecule that is soluble inside the cell, accumulates in extracellular space, where it is transformed in insoluble crystal of monosodium urate, stimulating the maturation of dendritic cells and T-cell response.^{85,86} Here, the presence of uric acid after electroporation was investigated for the first time. Presence of uric acid in supernatant was investigated 24 hours after electroporation treatment (Figure 7). We expected uric acid to show a similar behavior in pulse parameter dependency as other DAMPs. However, we did not detect uric acid in supernatant after pulse treatment. Standard curve was obtained, therefore Uric Acid Assay Kit worked. Maybe uric acid production did not happen or uric acid was still inside of cells and not yet in supernatant as predicted. Furthermore, we found no existing data on CHO cells and uric acid in the literature, so maybe formation of uric acid in ovarian cells does not occur.

With respect to the results obtained, detection of DAMPs and its correlation to cell membrane permeabilization and cell survival seems to be more complex than initially thought. Even a DAMP like ATP, which can be released due to electroporation alone is better correlated to cell survival than membrane permeabilization (Tables 1, 2). A recent study performed by Ringel-Scaia *et al.*²⁷, in which multiple signaling pathways were analyzed, showed that the cell and cell population is a dynamic system which changes with time. Two hours after pulse treatment RNA analyses showed activation of immunosuppressive pathway, cell injury and apoptosis. With time these genes became less pronounced and after 24 hours change in gene expression indicated proinflammatory response, cell repair and necrosis/pyroptosis. This explains changes in DAMPs detection hours after pulse treatment, including the presence and absence of different DAMPs and its correlation with cell survival. Since statistical correlations between DAMP release and cell survival is much stronger than with membrane permeabilization, involvement of immune system in IRE can be explained. However, activation of immune system was demonstrated also in ECT treatments, where reversible electroporation is used. How can that be, if correlation between membrane permeabilization and released DAMPs is weak or does not even exist? Modeling⁸⁷ and *in vivo* experiments⁸⁸ show that application of

TABLE 1. Correlation (R) between survival and release of damage-associated molecular pattern molecules (DAMPs) after pulse treatment. Investigated time points for each molecule are presented in the bottom row. Correlation was evaluated with Pearson correlation coefficient and survival was analyzed via MTS assay 24 hours after pulse treatment

R vs. survival (MTS)						
PI	-0.680					
ATP	-0.947 (L)	-0.964 (L)/-0.864 (F)			0.888 (F)	
DNA	-0.935	-0.919			-0.928	
RNA	-0.909	-0.909			-0.919	
CRT				-0.801	-0.946	
uric acid					NS	
time points after EP	3 min	15 min	30 min	4 h	24 h	

ATP = adenosine triphosphate; CRT = calreticulin; (F) = fluorescence assay; (L) = luminescence assay; NS = no statistical significance; PI = propidium iodide

TABLE 2. Correlation (R) between permeabilization and release of damage-associated molecular pattern molecules (DAMPs) after pulse treatment. Investigated time points for each molecule are present in the bottom row. Correlation was evaluated with Pearson correlation coefficient and permeabilization was analyzed by propidium iodide (PI) assay 3 minutes after pulse treatment

R vs permeabilization (PI)						
MTS						-0.680
ATP	0.704	0.728 (L)/0.594 (F)			-0.695 (F)	
DNA	0.571	0.689			NS	
RNA	0.584	0.696			NS	
CRT				0.535	0.556	
uric acid					NS	
time points after EP	3 min	15 min	30 min	4 h	24 h	

ATP = adenosine triphosphate; CRT = calreticulin; (F) = fluorescence assay; (L) = luminescence assay; NS = no statistical significance

nominally reversible electroporation pulses such as those used for ECT of tumors still causes some cell death by means of irreversible electroporation in tissue close to the electrodes, due to inhomogeneous electric field distribution, which can thus lead to the release of DAMPs and activation of the immune system.

The aim of this study was to explore, if and when specific DAMPs are released as a consequence of electroporation and if the release of DAMPs can be correlated to reversible and/or irreversible electroporation. Even though detection of certain DAMPs remains uncertain, others show strong correlation to cell survival/irreversible elec-

trporation and much weaker correlation to membrane permeabilization/reversible electroporation. Release of DAMPs could perhaps serve as a predictor of cell death. In addition, it may indicate that the stability of certain DAMPs is questionable and thus their presence and detectability is time dependent. This needs to be taken into consideration when designing protocols to detect DAMPs after electroporation treatment. Finally, to obtain a better insight of DAMP release with respect to electroporation treatment other cell types including also cancer cell types should be investigated.

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