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Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy

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ABSTRACT

Electrochemotherapy is an effective local treatment of solid tumours which combines delivery of chemotherapeutic drug and electric pulses. Electric pulses increase permeability of plasma membrane transiently and reversibly, leading to increased transport of the drug into the cell. As all clonogenic cells in the tumour need to be eradicated for effective treatment, all cells have to be permeabilised, i.e. all cells in the tumour have to be exposed to appropriate electric pulses. Electric pulses are delivered to tissue by electric pulses generator via electrodes. In general there are two types of electrodes, plate electrodes and needle electrodes. The target tissue, i.e. tumour, is to be positioned well in-between the electrodes. The electrodes should thus fit the size of the tumour for good electric field distribution. Plate electrodes which are noninvasive are better suited for tumours on the surface on the skin, whereas needle electrodes which are used invasively with appropriate and sufficient depth of their insertion are more appropriate for treating tumours seeded deeper in the skin.

1. Cell in electric field and electrochemotherapy

Electrochemotherapy is an effective local treatment of solid tumours which combines delivery of corresponding drug and electric pulses.[1–10] Drugs with hindered transmembrane transport and having intracellular target are good candidates for electrochemotherapy. Bleomycin and cisplatin proved to be the best candidates so far.[11–13] Electric pulses with appropriate parameters, amplitude, duration, number, repetition frequency and shape, will increase permeability of plasma membrane transiently and reversibly, leading to increased transport of the drug into the cell. This increased transport will thus allow the drug to enter the cell in sufficient amount and reach its intracellular target, consequently killing the cell. As all clonogenic cells in the tumour need to be eradicated for effective treatment, all cells have to be permeabilised, i.e. all cells in the tumour have to be exposed to appropriate electric pulses. Effectiveness of electrochemotherapy thus depends on drug availability in the tumour and coverage of the whole area of the tumour by sufficiently high electric field/pulses.

When a cell is placed into electric field, its geometrical and material properties cause a transmembrane voltage to be induced and superimposed on the natural resting transmembrane voltage. The amplitude of the induced transmembrane voltage depends on the position on the membrane and is dictated by the following equation:

$$\Delta\Phi_m = \frac{3}{2} ER\cos\theta, \quad (1)$$

where $\Delta\Phi_m$ is the induced transmembrane voltage on the membrane, θ is the angle between the direction of the electric field E and radius vector R on the membrane[14] (see also Fig. 1).

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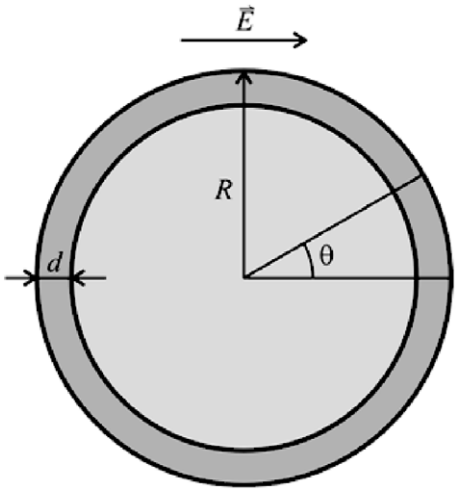

Fig. 1 – A schematic presentation of a cell having diameter 2R, membrane thickness *d*, where θ is the angle between the direction of the electric field *E* and radius vector *R* on the membrane.

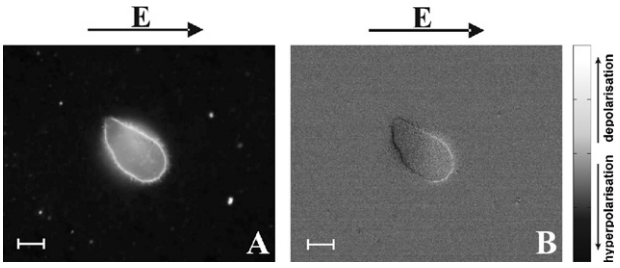

Fig. 2 – Induced transmembrane voltage (ITV) on an irregularly shaped CHO cell. Electric field *E* was directed from left to right. (a) The 8-bit fluorescence image of a cell stained with di-8-ANEPPS and acquired during the exposure to 40 V (100 V/cm), 100 ms rectangular pulse. The brightness of the image was automatically enhanced. Bar represents 10 μm. (b) Changes in fluorescence of cell obtained by subtracting the control image (not shown) from the image with pulse and shifting the greyscale range by 50%. The side of the cell coloured in white represents an increase in fluorescence (depolarisation), and the side of the cell coloured in black, a decrease in fluorescence (hyperpolarisation). The brightness of the image was automatically enhanced.

If the induced transmembrane voltage is sufficiently high,[15] the membrane permeability non-selectively increases and molecules which otherwise cannot cross the plasma membrane can now enter (or leave) the cell. The transport of small molecules like bleomycin and cisplatin across the membrane is predominantly diffusion-driven due to concentration gradient.[16] When electric pulses are applied, membrane permeability of cells in the tissue exposed to sufficiently high electric field will non-selectively increase. If the drug is present in the tumour (surrounding the tumour cells, but not being able to penetrate the cell through its membrane), this increased membrane permeability will allow entrance of the drug into the cells, increasing drug cytotoxicity.

Induced transmembrane voltage can easily be calculated for a spherical cell and it follows Eq. (1).[14] What we can see from this equation is that for a given cell the induced transmembrane voltage is proportional to the electric field; more precisely, it is proportional to the local electric field in which the cell is placed. The induced transmembrane voltage for non-spherical irregular shapes, such as cells are, can, however, be calculated by numerical methods or measured (Fig. 2).[17] It is important to note that if the induced voltage is not sufficiently high, no flow will occur as the membrane will not become permeabilised. Nevertheless, the flux of the drug occurs only through parts of the membrane where the induced transmembrane voltage exceeds a critical threshold (Fig. 3). Thus the flux of the drug through the membrane is established through areas where sufficiently high induced transmembrane voltage was induced, but also depends on pulse duration and number of pulses.

If pulse parameters are selected appropriately, the cell membrane will become transiently permeabilised, and will reseal afterwards, thus preserving cell viability. This is termed reversible permeabilisation. However, if the amplitude of pulses, their number is too large and/or duration is too long, the membrane will not reseal and the cell will lose its viability. This is termed irreversible permeabilisation.

The electric field in a tissue and electric current passing through the tissue are coexisting and are connected by Ohm's law (Eq. 2)

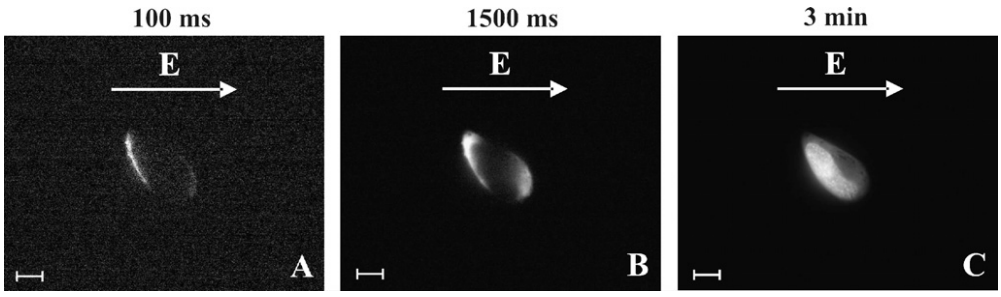

Fig. 3 – Electropermeabilisation of a CHO cell shown in Fig. 2. (A) Fluorescence of the cell 100 ms, (B) 1500 ms, and (C) 3 min after pulse delivery. The cell was exposed to a single 400 V (1000 V/cm) rectangular unipolar pulse (200 μs). The images were corrected for the background fluorescence and the brightness was automatically enhanced. Propidium Iodide (100 μM), which was here used as a marker of membrane permeabilisation, was added to suspension before the pulse was applied to visualize the permeabilised regions. Bar represents 10 μm.

$$j = \sigma \cdot E, \quad (2)$$

where j is current density, σ is tissue electric conductivity, and E the electric field. The corresponding integral values are electric current I, conductance G (which is the reverse of resistance R), and voltage U. The Ohm's law then takes the form of:

$$U = R \cdot I \quad \text{or} \quad I = G \cdot U. \quad (3)$$

Current passes through the tissue if voltage i.e. potential difference exists between two points in the tissue, and the current loop is closed. In practice, we generate the potential difference (voltage) on electrodes with an electric pulse generator. When both electrodes (one needs at least two electrodes to close the loop) are placed on/in the tissue (which is a conductive material where charge carriers are ions as in electrolyte solutions), the current loop is closed and the current passes through the tissue.

As the electric current passes through a biological tissue, it is distributed through different parts of the tissue, depending on their electrical conductivity. In general, better perfused tissues have higher conductivity. So blood is highly conductive, liver and muscles as well, whereas bone and fatty tissue have low conductivity. The current will flow easier and for the same voltage in higher proportion through more conductive tissues (e.g. muscles, liver). On the contrary, the electric field in these tissues will be lower than in tissues with low conductivity for the same current. Since the same voltage in higher conductive media will give higher currents and because conductivity of the tissue increases as it becomes permeabilised, rather high currents pass through the tissue during electrochemotherapy.[18,19]

Nevertheless, as the electric current takes the shortest and easiest path through the tissue, the current will be contained predominantly between the electrodes if they are close enough to each other. This property allows for relatively good control and containment of electric field distribution predominantly between the electrodes.[20]

Solid tumours usually have somewhat higher conductivity than the surrounding tissue due to its rich, irregular and fenestrated vasculature. This causes electric current to pass mainly through the tumour, but the electric field will be somewhat lower than in its surroundings. Nevertheless, when the tissue becomes permeabilised (i.e. plasma membrane becomes permeable) its conductivity also increases, which leads to electric field redistribution in the tumour and its surroundings. This phenomenon is most obvious in subcutaneous tumours.[21] Namely, the skin, serving as a natural barrier protecting internal tissues from exposure to chemical and physical trauma and bacteria, has an extremely low electric conductivity. When two electrodes are placed on the skin, practically the entire voltage drop rests on the skin (on its outermost layer, the *stratum corneum*, being the least conductive skin layer), where the electric field is by far the highest. The electric field, when applying high voltage pulses as in the case of electrochemotherapy, "breaks through" the skin, forming structures known as Local Transport Regions that make skin more conductive.[22] Now the current can pass through the skin more easily and the voltage drop and electric field in the skin are lowered (Fig. 4). Consequently, other skin layers and tissues under the skin are exposed to higher electric fields.

In conclusion, cells in tissues which are exposed to sufficiently high electric field will become permeabilised, rendering electrochemotherapy effective. If pulse parameters are selected correctly and sufficiently high (but not too high – see below) local field is assured and cells will become reversibly permeabilised, i.e. allowing for the uptake of cytotoxic drug and resealing of the membrane. This will allow the drug to exert its cytotoxic activity selectively to tumour cells and not to normal cells. If, however, the cells are exposed to too high an electric field (at given pulse parameters), cells will be killed nonselectively and instantly, *losing* selectivity and therapeutic index of electrochemotherapy, leading to localised tissue necrosis. Electric field associated *with* reversible permeabilisation is noted as E_{rev}, whereas the electric field associated *with* irreversible permeabilisation is noted as E_{irrev} in Figs. 6 and 7.

2. Selection and positioning of electrodes

In general there are two types of electrodes, plate electrodes and needle electrodes[23,24] (Fig. 5). Plate electrodes are noninvasive, usually parallel and separated by a distance d. The

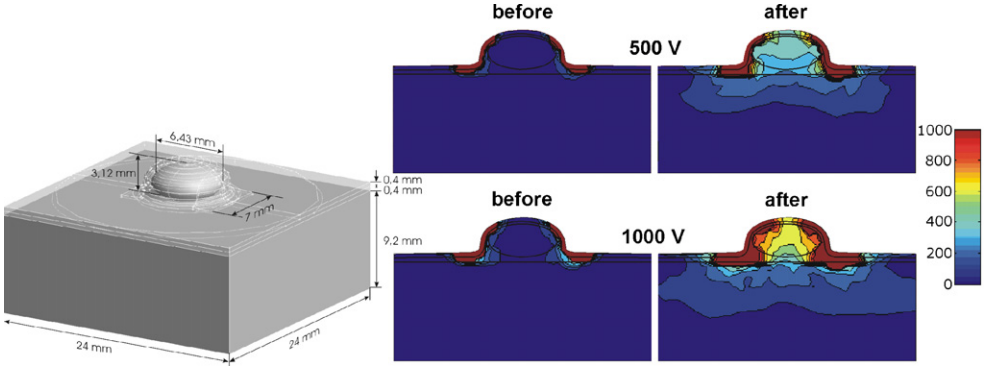

Fig. 4 – Modelled electric field distributions in the subcutaneous tumour and the surrounding tissues before and after tissue electropermeabilisation at 500 and 1000 V between two plate electrodes of 8 mm distance. The electric field distributions are shown in V/cm. The geometry of the numerical model is shown on the left. The electrodes are modelled as a boundary condition.

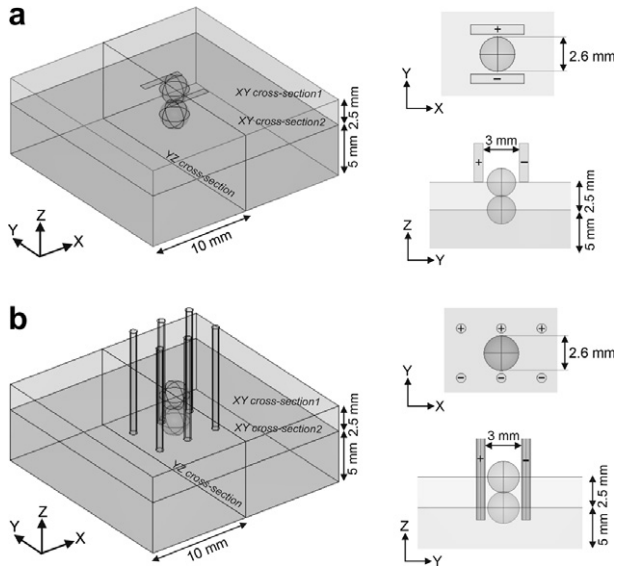

Fig. 5 – Geometry of tissue models with the given electrode configurations: (a) 3D model of cutaneous and subcutaneous tumour with two parallel plate electrode configuration and corresponding XY and YZ cross-sections across the target tissue i.e. tumour geometry and (b) 3D model of cutaneous and subcutaneous tumour with needle electrode configurations and corresponding XY and YZ cross-sections across the target tissue i.e. tumour geometry. Tumour was for demonstration purposes placed at two different positions in the model: as cutaneous and subcutaneous tumour. Each time only one tumour was considered in calculations and explanations.

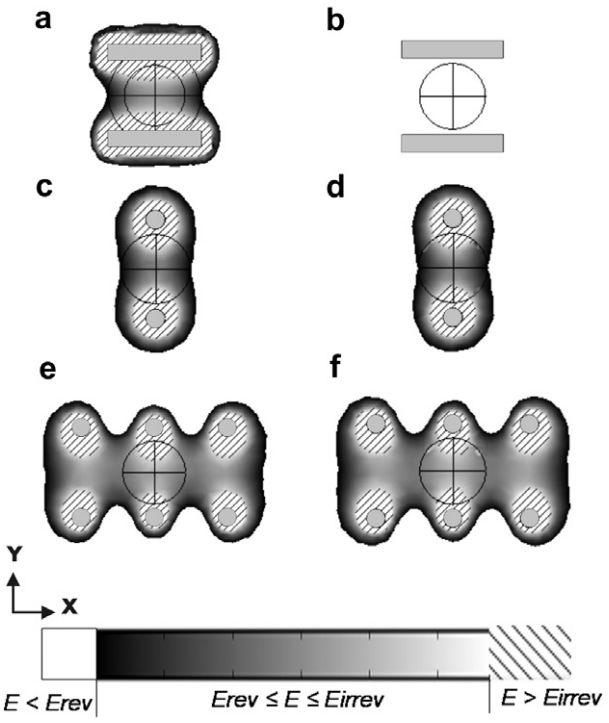

Fig. 6 – The comparison of electric field distribution for different electrode configurations shown in XY cross-section of cutaneous and subcutaneous 3D models from Fig. 5: (a) two parallel plate electrodes – cutaneous tumour including the electric field distribution within the gel layer surrounding the target tissue; (b) two parallel plate electrodes – subcutaneous tumour; (c) two parallel needle electrodes – cutaneous tumour; (d) two parallel needle electrodes – subcutaneous tumour; (e) six parallel needle electrodes – cutaneous tumour and (f) six parallel needle electrodes – subcutaneous tumour. In all cases the applied voltage U = 300 V. Black circle represents the target tissue and the patterned region represents part of tissue where $E \geqslant E_{irrev}$.

target tissue, i.e. tumour, is to be somehow placed in-between these two plates. The tumour is pinched or pushed between electrodes. The electrodes should fit the size of the tumour for good electric field distribution. Electrodes "too far" from the tumour will not allow for efficient electropermeabilisation. Either fixed distance electrodes (most often) or variable distance electrodes are used. In both cases it is important to assure good electrical contact between metal electrodes and tissue – this is usually assured by using conductive gel (see Figs. 6a and 7a) and exerting sufficient pressure to create and maintain good contact even in the case of tissue movement due to muscle contractions provoked by the electric pulses. Also, the applied voltage needs to be determined/calculated with respect to the distance between electrodes, their shape and tissue-electrode geometry, as well as tissue anatomy.

Needle electrodes are used invasively. In this way good electrical contact is assured. However, the electric field distribution is more inhomogeneous and depends on the electrode diameter, number of electrodes, distance between the electrodes and depth of their insertion (Figs. 6 and 7)[25,26]. In principle, thinner electrodes, larger distance and shallower insertion lead to more inhomogeneous electric field distribution. This causes extremely high current density and high electric fields in immediate vicinity of the needle electrode. But at even a short distance from the needle electrode the field amplitude is already very low – possibly too low to cause cell membrane permeabilisation. Furthermore, the effective electric field between the electrodes occurs at a lesser depth than needle electrode insertion. Thus needle electrodes generally need to be inserted deeper than the deepest part of the tumour.

For improving electric field homogeneity and local field distribution using needle electrodes at least two strategies can be proposed. More needle electrodes positioned in parallel rows improve the homogeneity, whereas multiple needle electrodes arranged into a matrix allow larger volumes of the target tissue to be treated.[25,27] Such an array of electrodes connected to an electroporator through a commutation/switching device can deliver well designed sequences of pulses that increase the probability for cell membrane permeabilisation. Usually electrodes are used in pairs and pulses are delivered in more than one direction to the same cells, which increases the probability of a cell to become permeabilised.[28] Also, improved coverage of tumour with a sufficiently high electric field can be achieved. The same can be done with plate electrodes.[29,30]

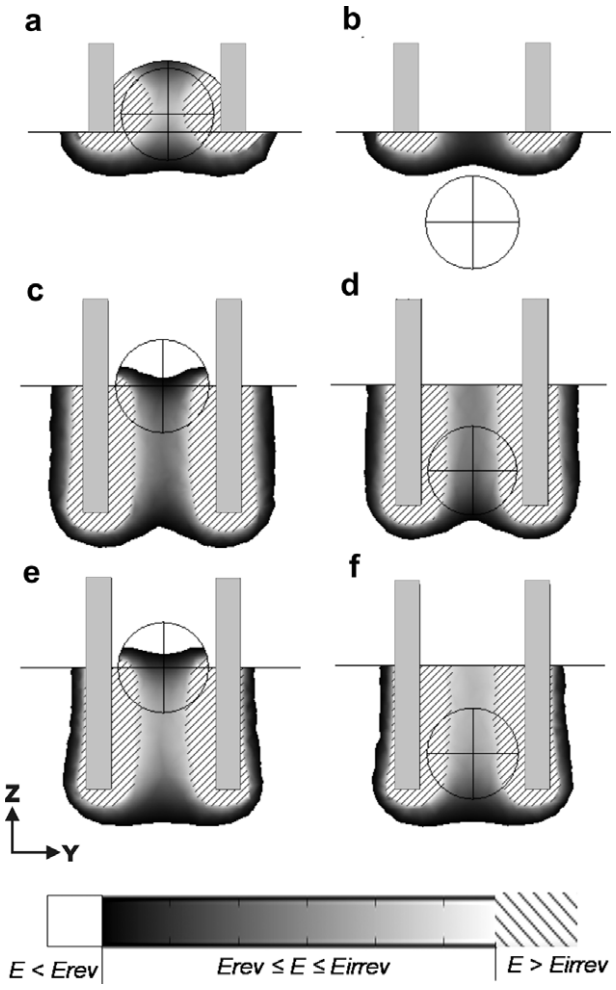

Fig. 7 – The comparison of electric field distribution for different electrode configurations shown in YZ cross-section of cutaneous and subcutaneous 3D models: (a) two parallel plate electrodes – cutaneous tumour including the electric field distribution within the gel layer surrounding the target tissue; (b) two parallel plate electrodes – subcutaneous tumour; (c) two parallel needle electrodes – cutaneous tumour; (d) two parallel needle electrodes – subcutaneous tumour; (e) six parallel needle electrodes – cutaneous tumour and (f) six parallel needle electrodes – subcutaneous tumour. In all cases the applied voltage U = 300 V. Black circle represents the target tissue and the patterned region represents part of tissue where $E \geqslant E_{irrev}$.

Adequate pulse amplitudes, set and delivered by a pulse generator, were initially and are all too often still determined empirically. Now they can be calculated by means of numerical models and tested.[15,26,30,31] The electric pulses voltage that needs to be applied depends on electrode type, number of electrodes and electrode geometry. Next necessary step is correct positioning of the electrodes into/onto the tumour, which will assure sufficiently high electric field in the whole tumour as to cause membrane permeabilisation of all clonogenic cells. In principle, after visualising the tumour, determining the size and position, the best suited electrode type has to be selected and plan has to be made as to where and how the electrodes need to be placed with respect to the tumour. Most of the time, however, physicians can be very rapidly trained to use the electrodes appropriately. Especially in treatment of cutaneous and subcutaneous tumours, a single training session is sufficient. Moreover, the electrical coverage of the vicinity of the tumour is recommended, particularly when the drug used is the bleomycin injected intravenously, due to the absence of toxic effects on the neighbouring normal tissue (see L.M. Mir, this issue).

3. Possible pitfalls and side effects of electrical nature

As in every treatment, there are number of possible mistakes that can be made. It is the procedures that have to be defined to minimize the risk of making mistakes,[32] which is why the Standard Operating Procedures for the Electrochemotherapy were prepared and reported in this issue. In addition, to be used in Europe, an electroporator has to be certified as a CE medical device, which assures safety of patients, physicians and members of medical staff performing the treatment. Besides the CE mark, the minimal requirement of a clinical electroporator, the equipment used to deliver the pulses *can* also include features allowing detecting potential mistakes of the operator. Indeed, after injecting the drug into the tumour or systemically, and allowing for distribution of the drug in the tumour, one selects and positions the electrodes and applies the appropriate electric pulses. It is of utmost importance to have a possibility to monitor and control the current and the voltage of the pulses being delivered.[24] This can either be achieved by using external oscilloscope, which can be difficult if not impossible in a clinical setting, or by using an electroporator that has such monitoring of pulses built in.

In addition, since high voltage electric pulses are delivered, they usually provoke muscle contraction. If, as it was often the case in the past, a 1 Hz repetition frequency is used, each single pulse leads to a twitch, which may result in losing electrical contact if insufficient pressure is exerted by the operator. This happens momentarily and is difficult to notice without proper monitoring of pulse delivery (as mentioned in the previous paragraph). Too high a pressure may on the other hand lead to large contact area, which requires high electric current – too high for the comfort of the patient and/or for the electroporator to deliver. Namely, some electroporators will, for security reasons, discontinue pulse delivery if the current exceeds a given level (e.g. 12 A). This kind of protection will prevent damage of the electroporator. Such discontinuation of pulse delivery may pass unnoticed and will result in lower or nonexistent electropermeabilisation, causing electrochemotherapy to fail. The undesired consequences of the twitch, caused by the first pulse in the train of "standard" eight electric pulses most often delivered to achieve a good cell permeabilisation, can also be completely avoided using a 5000 Hz pulse repetition frequency, instead of the classical 1 Hz frequency. When using this high pulse repetition frequency, the whole train of pulses is then delivered in 1.5 ms, that is before the twitch is provoked and the electrode displacement can occur. This high repetition frequency thus ensures the quality of the treatment and reduces patient discomfort. Moreover, in the

ESOPE study using the Cliniporator™ we have demonstrated good clinical results of electrochemotherapy, irrespective of pulse repetition frequency.

Finally, when plate electrodes are used, burn marks can appear on the skin where electrodes were in contact with it. This can be due to an insufficient electrical contact during pulse delivery. Good electrical contact can be assured by using conductive gel and exerting more pressure to the electrodes to avoid sparking and very local high current leaving burn marks on the skin, especially if the electrodes are placed perpendicularly to the skin, having small contact area. However, we have to be careful not to use too much conductive gel. Filling the gap between the electrodes with an excess of conductive gel may shunt the electrical path through the tissue, rendering electrochemotherapy inefficient. Hairy skin may cause sparking and give rise to unpleasant smell of burning hair. Thus it may be appropriate to shave and clean the skin above the tumour if possible.

In conclusion, the target tissue, i.e. tumour, is to be positioned well in-between the electrodes. The electrodes should fit the size of the tumour for good electric field distribution. Noninvasive plate electrodes are better suited for tumours on the surface on the skin, whereas needle electrodes, which are used invasively, with appropriate and sufficient depth of their insertion, are more appropriate for treating subcutaneous tumours seeded deeper in the skin.

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