INSTITUTE OF PHYSICS PUBLISHING PHYSIOLOGICAL MEASUREMENT

Physiol. Meas. **24** (2003) 75–90 PII: S0967-3334(03)54469-0

# Perturbation of blood flow as a mechanism of anti-tumour action of direct current electrotherapy

**Tomaž Jarm[1], Maja Čemažar[2], Fritz Steinberg[3,4], Christian Streffer[3,5], Gregor Serša[2] and Damijan Miklavčič[1]**

[1] Faculty of Electrical Engineering, University of Ljubljana, Trzaska 25, SI-1000 Ljubljana, Slovenia
[2] Institute of Oncology, Zaloska 2, SI-1000 Ljubljana, Slovenia
[3] Institute of Medical Radiobiology, University of Essen, Hufeland Str. 55, D-45122 Essen, Germany

E-mail: damijan@svarun.fe.uni-lj.si

Received 3 October 2002, in final form 27 November 2002
Published 9 January 2003
Online at stacks.iop.org/PM/24/75

**Abstract**

Anti-tumour effects of direct current electrotherapy are attributed to different mechanisms depending on the electrode configuration and on the parameters of electric current. The effects mostly arise from the electrochemical products of electrolysis. Direct toxicity of these products to tumour tissue is, however, not a plausible explanation for the observed tumour growth retardation in the case when the electrodes are placed into healthy tissue surrounding the tumour and not into the tumour itself. The hypothesis that the anti-tumour effectiveness of electrotherapy could result from disturbed blood flow in tumours was tested by the measurement of changes in blood perfusion and oxygenation in tumours with three different methods (*in vivo* tissue staining with Patent Blue Violet dye, polarographic oximetry, near-infrared spectroscopy). The effects induced by electrotherapy were evaluated in two experimental tumour models: Sa-1 fibrosarcoma in A/J mice and LPB fibrosarcoma in C57Bl/6 mice. We found that perfusion and oxygenation were significantly decreased after electrotherapy. Good agreement between the results of different methods was observed. The effect of electrotherapy on local perfusion of tumours is probably the prevalent mechanism of anti-tumour action for the particular type of electrotherapy used in the study. The importance of this effect should be considered for the optimization of electrotherapy protocols in experimental and clinical trials. The non-invasive technique of near-infrared spectroscopy proved to be a reliable method for detecting perfusion and oxygenation changes in small solid tumours.

[4] Present address: Institut für biowissenschaftliche Forschung, Universität Witten/Herdecke, Kruppstrasse 82–100, D-45145 Essen, Germany.
[5] Present address: Institute for Science and Ethics, University of Essen, D-45117 Essen, Germany.

  

Keywords: experimental tumours, direct current electrotherapy (DC-ET), electrochemical treatment, blood flow, oxygenation, Patent Blue Violet dye (PBV), polarographic oximetry ($pO_2$), near-infrared spectroscopy (NIRS)

## 1. Introduction

As described by various authors, electrotherapy with direct electric current (DC-ET) or electrochemical treatment (EChT) can be used effectively for local treatment of solid malignancies in experimental tumour models and in humans. For a review see, for example, the work of Watson (1991) and Nilsson *et al* (2000) and for some of the latest animal and clinical applications see the work of Wemyss-Holden *et al* (2002) and Fosh *et al* (2002). The most obvious mechanism of the anti-tumour effectiveness of DC-ET is the effect of various toxic chemicals produced at the electrodes by the electrochemical reactions due to electrolysis. However, the values of the treatment parameters used in different experiments vary tremendously, which indicates that the anti-tumour effect observed in different studies is most likely achieved through different mechanisms of action (Miklavčič *et al* 1993, 1994). The extent to which a particular mechanism contributes to anti-tumour action depends most likely on the values of DC-ET parameters associated with each type of DC-ET.

It is well known that both experimental and spontaneous tumours are in general characterized by insufficient blood supply and poor oxygenation in comparison to normal tissue in which tumours grow (Jain 1988, Vaupel 1979, 1990, Vaupel *et al* 1998, Raleigh *et al* 1996, Horsman 1998). This characteristic is mainly due to the structurally and functionally abnormal and deficient vascularization of tumours, which cannot meet the requirements of the rapidly multiplying tumour cells. Despite the large inter- and intra-tumoral differences in many aspects between tumours, the neovascularization of tumours in general is characterized by the lack of smooth muscles in vascular walls, the presence of non-functional shunts and dead ends, and by large inter-capillary distances. The resulting insufficient delivery of nutrients and oxygen to tumour cells bears important consequences on both tumour growth and on the outcome of different therapies (Horsman 1998, Vaupel *et al* 1998). For example, it has been demonstrated in experimental and clinical studies that the lack of oxygen in tumours correlates negatively with the outcome of radiation therapy (Höckel *et al* 1993). Lack of oxygen can also render ineffective certain drugs used in chemotherapy. On the other hand, the bioreductive drugs such as mitomycin C and tirapazamine, are more effective in a hypoxic environment (Brown 1999, Čemažar *et al* 2001). The hypoxic tumours seem ideal for the use of bioreductive drugs. However, effective drug delivery to tumours by blood flow can be prevented by deficient blood flow in tumours especially in comparison with normal tissues. Drug uptake by tumours is further obstructed by elevated interstitial fluid pressure in tumours in comparison with the surrounding normal tissue, which is another characteristic of tumours (Baxter and Jain 1989, Milosevic *et al* 1999, Podobnik and Miklavčič 2000). It follows from all this that the ability to modify local blood flow in tumours is important for tumour treatment. Many different approaches have therefore been used to enhance the anti-tumour effect of various therapies by either improving or impairing blood flow and oxygenation in tumours (Vaupel *et al* 1998, Chaplin *et al* 1998).

One of the proposed mechanisms of the anti-tumour effectiveness of electrotherapy by direct electric current (DC-ET) is also the effect of DC-ET on the blood flow and oxygenation of tumours (Griffin *et al* 1995). In the study presented here the electrodes were placed in the healthy subcutaneous tissue surrounding tumours in mice. We have already shown in

previous studies that such a treatment can reduce perfusion in solid tumours (Jarm *et al* 1997, 1998, 1999). In the present study we extended these studies by combining three different measurement methods to assess the effect of this type of DC-ET on blood flow, blood oxygenation and tissue oxygenation in two different experimental tumour models. Our goal was to provide further evidence that the anti-tumour effect of a particular type of DC-ET was at least partly, if not predominantly, induced by the effect of DC-ET on the blood supply of treated tumours.

## 2. Materials and methods

### *2.1. Tumours and animals*

Two experimental tumour models were used in our study: Sa-1 fibrosarcoma in A/J mice and LPB fibrosarcoma in C57Bl/6 mice. Mice were purchased from Rudjer Boskovic Institute, Zagreb, Croatia. Tumour cells for inoculation of solid tumours were obtained from the ascitic form of the tumour (Sa-1) or from *in vitro* cell culture (LPB and Sa-1). Solid subcutaneous tumours were inoculated dorsolaterally in the right flank of mice by the injection of a physiological saline solution containing approximately $5 \times 10^5$ (Sa-1) or $1 \times 10^6$ (LPB) viable tumour cells. Sa-1 and LPB tumours reached the size required for the experiments (volume between 50 and 100 $mm^3$) in 8 to 10 days and in 18 to 30 days respectively. Tumour volume was calculated using the ellipsoid formula $V = \pi abc/6$, where $a$, $b$ and $c$ were the three mutually orthogonal tumour diameters measured by a vernier caliper. The animals were randomly assigned to experimental groups. They were kept in standard animal colonies and fed and watered *ad libitum* throughout the experiment. Separate groups of animals were used for each measurement method. Five to nineteen mice per experimental group were used depending on the experimental method. The exact numbers of mice are given in tables 1–3.

Experiments on mice were conducted in accordance with the EC directive for experimental animal care and protection and were approved by the corresponding ethical committees in Germany and Slovenia. All invasive experimental procedures and measurements were conducted on anaesthetized mice. The mice were anaesthetized by inhalation anaesthetics isoflurane (measurements with near-infrared spectroscopy and Patent Blue staining) or enflurane ($pO_2$ measurements). While anaesthetized, the animals were kept on an automatically regulated heating pad to prevent hypothermia. Rectal temperature was kept at physiological 37–38 °C with the surface temperature of the heating pad never exceeding 39 °C. The ambient temperature was 22–24 °C.

### *2.2. Electrotherapy (DC-ET)*

Electrotherapy (DC-ET) consisted of low-level direct current (0.6 mA amplitude, 1 h duration) delivered continuously via two needle-shaped electrodes. The electrodes (platinum/iridium 90/10% alloy; diameter 1 mm; length 2 cm; rounded tips) were inserted in parallel through miniature punctures in the skin into normal subcutaneous tissue on two opposite sides of the tumour and at least 5 mm away from the tumour edge. At the start and at the end of DC-ET the current amplitude was gradually increased and decreased, respectively, over a period of approximately 1 min in order to minimize the transient excitation and to prevent muscle twitching in the mice. The control animals were treated in exactly the same way except that no current was applied to the inserted electrodes.

### *2.3. Tissue staining with patent blue-violet dye (PBV)*

*In vivo* tissue staining with patent blue-violet dye (PBV) is a simple and rapid method for qualitative estimation of tumour perfusion in experimental animals (Serša *et al* 1999). A volume of 0.1 ml of PBV dye (Byk Gulden, Konstanz, Germany) diluted in 0.1 ml of 0.9% NaCl was injected into the tail vein of a mouse either 4 or 24 h after completing the DC-ET or sham treatment. The dye was allowed to distribute around the body for 3 min. The mouse was then euthanized and the tumour removed and separated from the overlying skin. The tumour (a solid ellipsoid) was cut in half along its largest diameter. The percentage of stained versus non-stained tumour cross-section area was independently estimated by two persons. The average of both estimates was used as the result value for the particular tumour. The stained (blue) part of the tissue was considered perfused or well perfused as opposed to the non-stained tissue, which was considered non-perfused or poorly perfused.

### *2.4. Polarographic oximetry ($pO_2$)*

Polarographic oximetry is a well-known and established invasive method for the direct measurement of oxygen partial pressure in tissue ($pO_2$). The principle and use of this method are extensively documented (e.g. Vaupel *et al* 1991). In spite of some well-known drawbacks of this method, its implementation in the Eppendorf histograph is still a 'golden standard' method for $pO_2$ measurements in tumours.

In our study we used the Eppendorf $pO_2$ Histograph instrument (Eppendorf, Hamburg, Germany). The measurements were not performed immediately after the end of DC-ET because we wanted to avoid the effect of long-lasting anaesthesia. We knew from the previous studies that the decrease in perfusion due to DC-ET measured 4 h after treatment was the same as immediately after treatment. Therefore, we re-anaesthetized the mice 4 h after DC-ET and 20 min prior to $pO_2$ measurement. The reference wire Ag/AgCl electrode was inserted through a small incision under the skin on the mouse's back. After calibration the sensor electrode (encapsulated in a needle with a tip diameter of 0.3 mm) was inserted through a small puncture in the skin for about 2 mm into the tumour. After stabilization the probe was advanced automatically in a stepwise fashion through the tumour (the so-called 'pilgrim step' procedure). After each step the probe was kept still for 1.4 s, the measurement was taken, and then the probe was advanced to the next location. The procedure was repeated along several parallel measurement tracks, which were about 2 mm apart. The entry direction was always caudal–cranial with respect to the mouse orientation. On average about 50 measurements were taken from an individual tumour. After a completed set of measurements in one tumour the calibration procedure was repeated in order to compensate for the drift in the measured signal.

### *2.5. Near-infrared spectroscopy (NIRS)*

Near-infrared spectroscopy (NIRS) is a family of optical techniques used for non-invasive monitoring of different parameters of tissue blood perfusion and oxygenation. Since its introduction in 1977 (Jöbsis 1977) these techniques have undergone rapid development and have been used in various tissues in animals and humans. Application of NIRS is based on the relative transparency of tissues for NIR light in comparison with visible light and on differences in the absorption properties between different oxygenation-related tissue chromophores such as haemoglobin in blood, myoglobin in muscle cells and cytochrome aa3 in mitochondria (Delpy and Cope 1997).

In our study the parameters of tumour perfusion and oxygenation were measured by means of $NIRO_2X$-2 instrument (Keele University, Stoke-on-Trent, UK). In this instrument the NIR

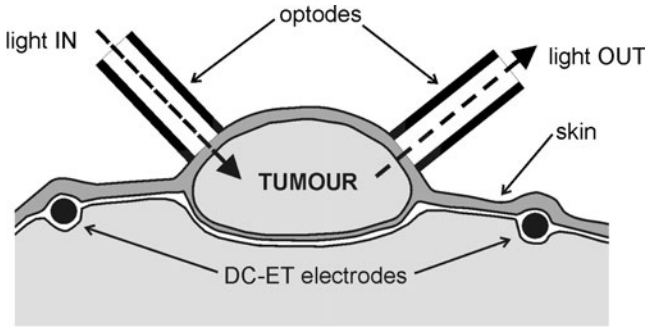


**Figure 1.** Experimental setup for NIRS measurements.

light is produced by laser diodes at wavelengths 775, 800, 845 and 904 nm. Two optodes are used to carry the light to and from the tissue. Changes in attenuation of NIR light are used to calculate the changes in the concentration of oxygenated and deoxygenated haemoglobin ($O_2$Hb and HHb respectively) using the modified Beer–Lambert law (Wickramasinghe *et al* 1992). In addition to $O_2$Hb and HHb, changes in total haemoglobin level (tHb = $O_2$Hb + HHb) reflecting blood volume changes and changes in differential haemoglobin level (dHb = $O_2$Hb − HHb) were evaluated. The differential haemoglobin level reflects the net blood oxygenation changes and is sometimes referred to as the oxygenation index (Thorniley *et al* 1997).

In figure 1 the experimental set-up for NIRS measurements is presented. The optodes (the inner diameter of the fibre bundle was 1.6 mm) were brought in contact with the surface of the skin above the tumour at the inter-optode angle and distance, approximately 120° and 8 mm respectively. Hair was previously removed from the tumour and a transparent gel was used to obtain good optical contact between the optodes and the skin. The optodes were held in place by two micromanipulators suspended from a horizontal bar above the mouse. NIRS measurements were taken continuously before, during and after DC-ET. DC-ET was started only after at least 15 min of stable NIRS readings. Similar measurements were performed in control tumours except that no DC-ET was applied during the measurement. Stable conditions were required throughout for valid measurements because the NIR$O_2$X-2 instrument can measure only changes from the initial baseline values. Therefore, every effort was made to keep the anaesthesia stable and to prevent movements of the animal during monitoring.

### *2.6. Histopathological examination*

Several control and DC-ET-treated tumours were excised after the end of NIRS measurement (20 min after the end of DC-ET) and fixed immediately in 10% buffered neutral formalin. One tissue block cut through the largest diameter of tumours was embedded in paraffin and stained with haematoxylin–eosin by the standard method. The tissue samples were examined microscopically for the effect of DC-ET.

### *2.7. Statistical analysis and data presentation*

The summarized data are graphically presented with the mean values and standard deviation bars. However, the statistical significance of the effects of DC-ET on tumour blood flow and oxygenation was evaluated by the non-parametric Mann–Whitney rank–sum test because

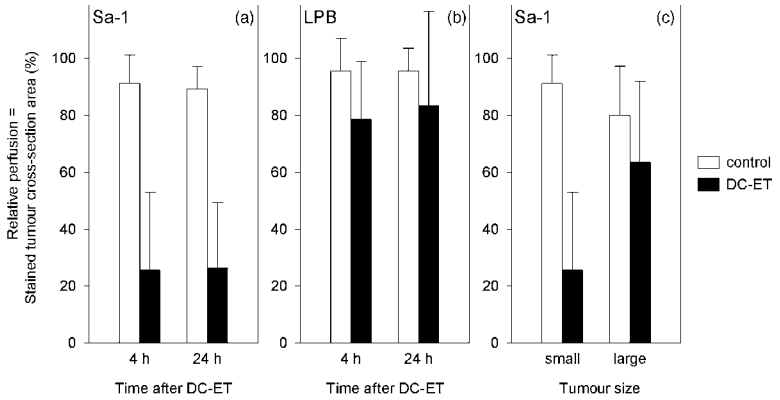


**Figure 2.** Relative blood perfusion assessed by means of PBV staining: (a) Sa-1 tumours 4 and 24 h after treatment; (b) LPB tumours 4 and 24 h after treatment; (c) small and large Sa-1 tumours 4 h after treatment. Mean values with SD bars are shown. Tumour volume was approximately 50 $mm^3$ and 150 $mm^3$ for 'small' and 'large' tumours respectively. Figures 2(a) and (b) belong to 'small' tumours. See also table 1.

**Table 1.** The effect of DC-ET on relative perfusion (% stained tumour cross-section area) evaluated by means of PBV staining method 4 and 24 h after the treatment in Sa-1 and LPB tumours.

| | | Control | | | | DC-ET | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Tumours | Time (h) | *n* | Mean | SD | Median | *n* | Mean | SD | Median | $p^b$ |
| Sa-1 | 4 | 8 | 91 | 10 | 95 | 8 | 26 | 27 | 18 | <0.001 |
| Sa-1 | 24 | 8 | 89 | 8 | 90 | 8 | 26 | 23 | 30 | <0.001 |
| LPB | 4 | 7 | 96 | 11 | 100 | 11 | 79 | 20 | 80 | 0.046 |
| LPB | 24 | 7 | 96 | 8 | 100 | 9 | 83 | 33 | 100 | 0.594 |
| Sa-1 large[a] | 4 | 5 | 80 | 17 | 90 | 13 | 64 | 29 | 60 | 0.401 |

[a] The average size of 'large' Sa-1 tumours was 151 ± 13 $mm^3$ and 122 ± 16 $mm^3$ in the control group and the DC-ET group respectively. The size of all other tumours was approximately 50 $mm^3$.
[b] Statistical significance between the median values of the DC-ET and the corresponding control group (Mann–Whitney rank–sum test).

in some cases either the normality test or the equal variance test failed when applied to experimental data. The exact *p*-value indicating the statistical significance of the observed differences is reported where applicable.

## 3. Results

### *3.1. Tissue staining with patent blue-violet dye (PBV)*

Relative perfusion of tumours was assessed 4 and 24 h after treatment with DC-ET or after sham treatment in control tumours. Figures 2(a) and (b) and table 1 present the results for Sa-1 and LPB tumours. DC-ET caused a decrease in blood perfusion in both tumour models. Four hours after treatment the average relative perfusion in control and DC-ET-treated Sa-1 tumours was 91% and 26% respectively. Practically the same average perfusion values were observed after 24 h in this tumour model. In both cases the effect of DC-ET was statistically significant. The effect of DC-ET on relative perfusion in LPB tumours was less pronounced than in Sa-1 tumours. The relative perfusion in DC-ET-treated LPB tumours was decreased in comparison with control tumours 4 h after treatment but the statistical significance of the

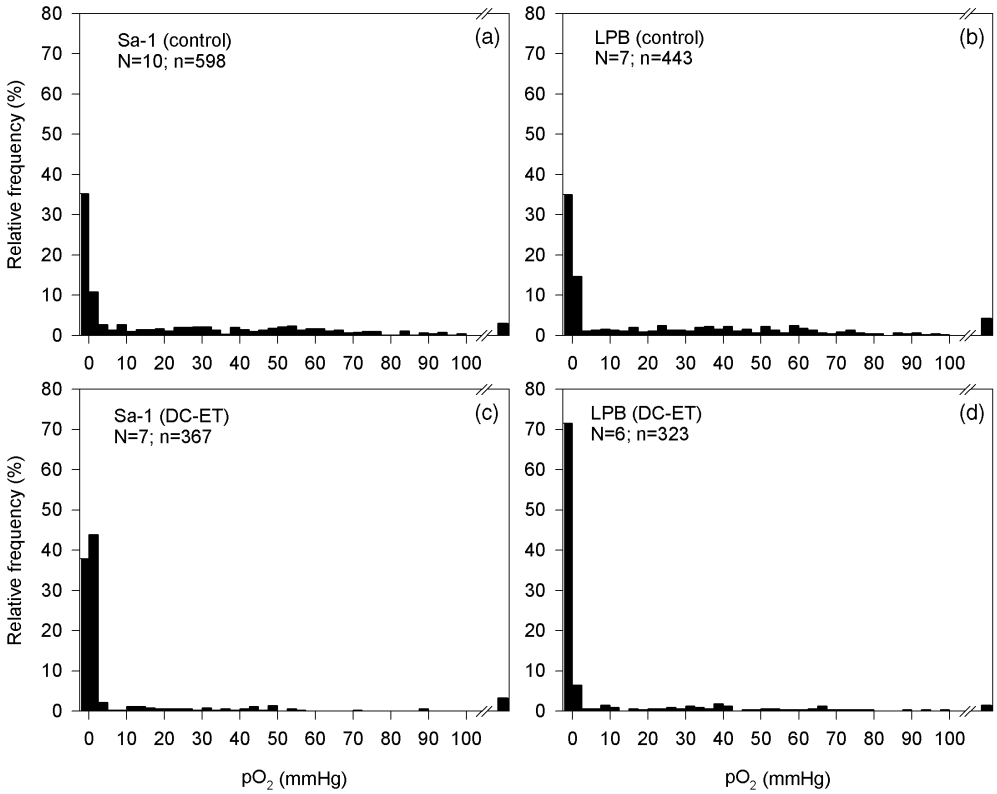


**Figure 3.** Histograms containing all $pO_2$ values measured in tumours 4 h after the end of treatment. ($N$ = number of tumours; $n$ = number of $pO_2$ measurement points): (a), (b) control tumours; (c), (d) DC-ET-treated tumours. See also table 2. The non-physiological $pO_2$ values above 100 mmHg presented in the right-most bars were measurement artifacts, which were excluded from further evaluation (not included in number $n$).

**Table 2.** The effect of DC-ET on oxygenation of Sa-1 and LPB tumours evaluated by polarographic oximetry 4 h after DC-ET.

| | | | Relative frequency of $pO_2$ values below | | | $pO_2$ (mmHg) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Tumours | $n$[a] | $N$[a] | $\leqslant$2.5 mmHg | $\leqslant$5.0 mmHg | $\leqslant$10.0 mmHg | Mean | SD | Median | $p$[b] |
| Sa-1 control | 10 | 598 | 46 | 49 | 53 | 24.9 | 34.0 | 7.2 | |
| Sa-1 DC-ET | 7 | 367 | 82 | 84 | 85 | 8.6 | 25.4 | 1.3 | <0.001 |
| LPB control | 7 | 443 | 50 | 51 | 54 | 24.8 | 34.2 | 3.5 | |
| LPB DC-ET | 6 | 323 | 78 | 79 | 81 | 9.6 | 23.8 | −0.4 | <0.001 |

[a] $N$: number of tumours; $n$: total number of $pO_2$ measurements.
[b] Statistical significance between the median values of the DC-ET and the corresponding control group (Mann–Whitney rank–sum test).

decrease was no longer observable 24 h after the treatment. Relative perfusion of control Sa-1 and LPB tumours was very similar.

The volume of tumours used to produce the results in figures 2(a) and (b) was within the 40–60 $mm^3$ range. In order to assess the potential impact of tumour volume on the DC-ET

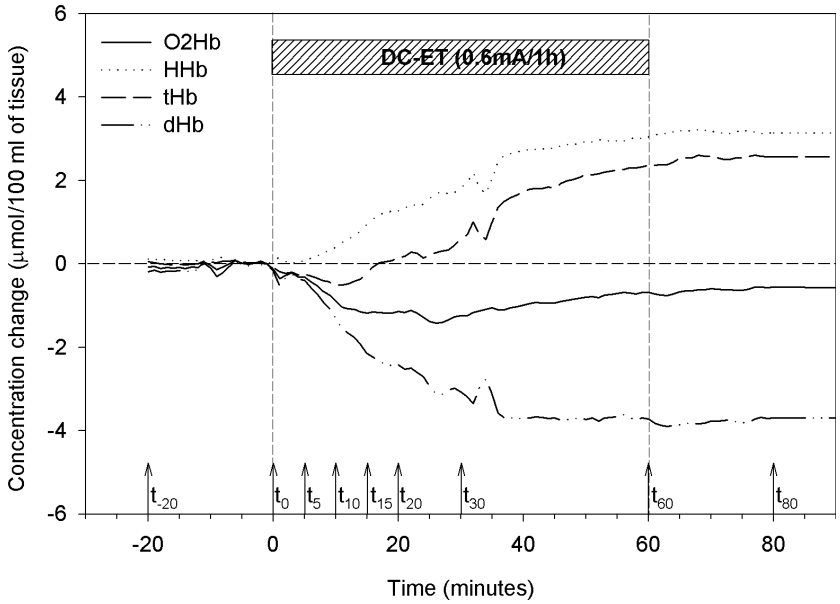


**Figure 4.** A typical response of a tumour to DC-ET as measured by means of NIRS. Also shown are the time intervals at which characteristic concentration changes were calculated with respect to the concentration baseline value at the beginning of DC-ET (time '$t_0$').

effect on perfusion we also measured the relative perfusion in 'large' Sa-1 tumours (volume approximately 150 $mm^3$). The comparison of 'small' and 'large' Sa-1 tumours 4 h after DC-ET is shown in figure 2(c) and table 1. It can be observed that the average perfusion in 'large' control tumours was lower than in 'small' control tumours. Furthermore, DC-ET was less effective in decreasing tumour blood flow in larger tumours than in smaller tumours.

### *3.2. Polarographic oximetry ($pO_2$)*

The partial pressure of oxygen was measured in tumours 4 h after completed DC-ET or sham treatment. Figures 3(a)–(d) present the pooled $pO_2$ data from all tumours. Additional information is contained in table 2. The histograms show relative frequencies of $pO_2$ values in a particular range with a resolution of 2.5 mmHg. It can be observed that both tumour models were very hypoxic prior to the application of DC-ET. The fraction of $pO_2$ values below 2.5 mmHg, the so-called hypoxic fraction, was 46% and 50% for Sa-1 and LPB control tumours respectively (table 2). Hypoxia was further increased after DC-ET as evidenced by the increase in hypoxic fraction to 82% in Sa-1 tumours and 78% in LPB tumours. The effect of DC-ET was statistically significant in both tumour models (table 2). The difference between Sa-1 and LPB tumours, however, was not statistically significant.

A considerable proportion of measured $pO_2$ values in all experimental groups was slightly negative within the $-1$ to 0 mmHg range. Even though negative $pO_2$ has no physical meaning, we did not attempt to correct or eliminate these measurements since the true $pO_2$ corresponding to these negative values was certainly close to zero and below 2.5 mmHg. Slightly negative $pO_2$ values could be the result of oxygen consumption by the electrode, poor accuracy of the measurement method near zero $pO_2$, and also inaccurate calibration. These are all well-known features of polarographic oximetry.

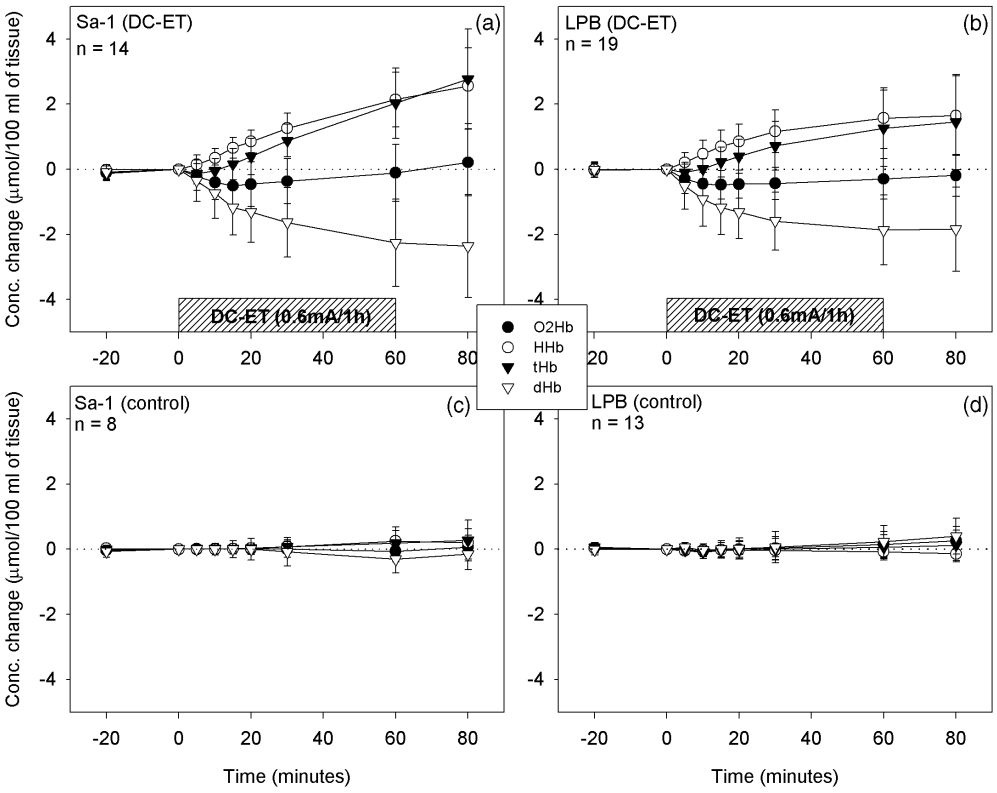


**Figure 5.** The effect of DC-ET on $O_2Hb$, HHb, tHb and dHb concentrations in Sa-1 (a) and LPB tumours (b) and comparison with sham-treated controls of Sa-1 (c) and LPB tumours (d). Mean values with SD bars are shown ($n$ = number of tumours) at characteristic time intervals before and after the beginning of treatment. See also table 3.

### *3.3. Near-infrared spectroscopy (NIRS)*

Approximately 20% of all NIRS measurements were excluded from further evaluation either because stable initial conditions prior to DC-ET could not be established or because the signals were corrupted during measurement by the movement artifacts or by the degradation of optical contact between the optodes and the tumour. The following description of the results therefore applies to valid NIRS measurements only (approximately 80% of all NIRS measurements).

Typical concentration changes of oxy-, deoxy-, total and differential haemoglobin ($O_2Hb$, HHb, tHb, dHb) recorded over a period of approximately 2 h are shown in figure 4 for one LPB tumour treated with DC-ET. The majority of all responses to DC-ET in both tumour models shared common characteristics, which can be observed here. A typical response of tumours to DC-ET was characterized by (a) a decrease of $O_2Hb$ to the lowest level within the first 5–20 min of DC-ET followed by a 'plateau' region of variable duration followed by a slow increase in the later stage of observation; (b) gradual increase of HHb over the whole observation period; (c) initial decrease in tHb followed by a steady increase afterwards; (d) decrease of dHb over the whole observation period. No comparable changes were detected in control tumours.

**Table 3.** Comparison of median concentration change ($\mu$mol/100 ml of tissue) of $O_2$HB, HHb, tHb and dHb between control tumours and tumours treated with DC-ET at four intervals after the beginning of DC-ET. See also figures 4 and 5. Boldface figures indicate statistically significant differences ($p < 0.05$).

| | | Sa-1 tumours (medians) | | | LPB tumours (medians) | | |
|---|---|---|---|---|---|---|---|
| Chromophore | Time[a] | Control ($n = 8$) | DC-ET ($n = 14$) | $p$[b] | Control ($n = 13$) | DC-ET ($n = 19$) | $p$[b] |
| $O_2$Hb | 10 min | 0.01 | −0.35 | **0.032** | −0.12 | −0.46 | **0.003** |
| | 20 min | −0.02 | −0.45 | **0.010** | −0.03 | −0.41 | **0.001** |
| | 30 min | 0.01 | −0.16 | 0.162 | −0.09 | −0.50 | **0.008** |
| | 60 min | −0.09 | −0.05 | 0.973 | 0.18 | −0.34 | **0.024** |
| HHb | 10 min | 0.02 | 0.29 | <**0.001** | −0.03 | 0.49 | <**0.001** |
| | 20 min | 0.07 | 0.79 | <**0.001** | −0.01 | 0.82 | <**0.001** |
| | 30 min | 0.15 | 1.25 | <**0.001** | −0.02 | 1.18 | <**0.001** |
| | 60 min | 0.33 | 2.29 | <**0.001** | −0.12 | 1.44 | <**0.001** |
| THb | 10 min | 0.00 | −0.10 | 0.125 | −0.11 | 0.01 | 0.220 |
| | 20 min | 0.05 | 0.31 | **0.007** | −0.01 | 0.28 | **0.017** |
| | 30 min | 0.10 | 0.79 | <**0.001** | 0.04 | 0.54 | **0.003** |
| | 60 min | 0.10 | 1.95 | <**0.001** | 0.04 | 0.99 | **0.002** |
| DHb | 10 min | −0.06 | −0.65 | **0.009** | −0.07 | −1.06 | <**0.001** |
| | 20 min | −0.22 | −1.13 | <**0.001** | −0.05 | −1.36 | <**0.001** |
| | 30 min | −0.41 | −1.29 | <**0.001** | 0.15 | −1.51 | <**0.001** |
| | 60 min | −0.66 | −1.90 | <**0.001** | 0.37 | −1.60 | <**0.001** |

[a] Time after the beginning of DC-ET ($t = 0$ min).
[b] Statistical significance between the median values of the DC-ET and the corresponding control group (Mann–Whitney rank–sum test).

It should be noted, however, that in spite of the common features outlined above, the amplitude and dynamics of changes in tumours during and after DC-ET varied considerably between tumours from almost no effect to an effect even more pronounced than the one shown in figure 4. For the purpose of statistical analysis of the effect of DC-ET on tumour perfusion we therefore calculated the absolute changes in the concentration of $O_2$Hb, HHb, tHb and dHb at several pre-defined intervals with respect to the absolute baseline concentration levels at the time $t = 0$ at the start of DC-ET (see figure 4). Values of concentration changes thus obtained from all DC-ET-treated and control tumours of both tumour models were averaged and are presented in figure 5. It can be seen from the averaged data that the two tumour models exhibited a similar response to DC-ET as outlined for an individual tumour in the previous paragraph. But the average effect of DC-ET appeared to be more pronounced in Sa-1 tumours than in LPB tumours. However, the difference between the two tumour models was not significant. From figures 5(c) and (d) it follows that there was no significant change in the concentration of the chromophores in control tumours during the observation period. Table 3 summarizes the statistical data for figure 5. In spite of great variability in the results from individual tumours treated with DC-ET, there were statistically significant differences between the DC-ET group and the control group of tumours for both tumour models. These differences were most clearly seen in deoxygenated and differential haemoglobin concentration changes (HHb, dHb) as early as 10 min after the start of DC-ET.

### *3.4. Histopathological examination*

In the Sa-1 control tumours focal geographic necrosis with pyknotic nuclei was observed, while the LPB tumours were, in general, not as necrotic at the same tumour size. Figure 6

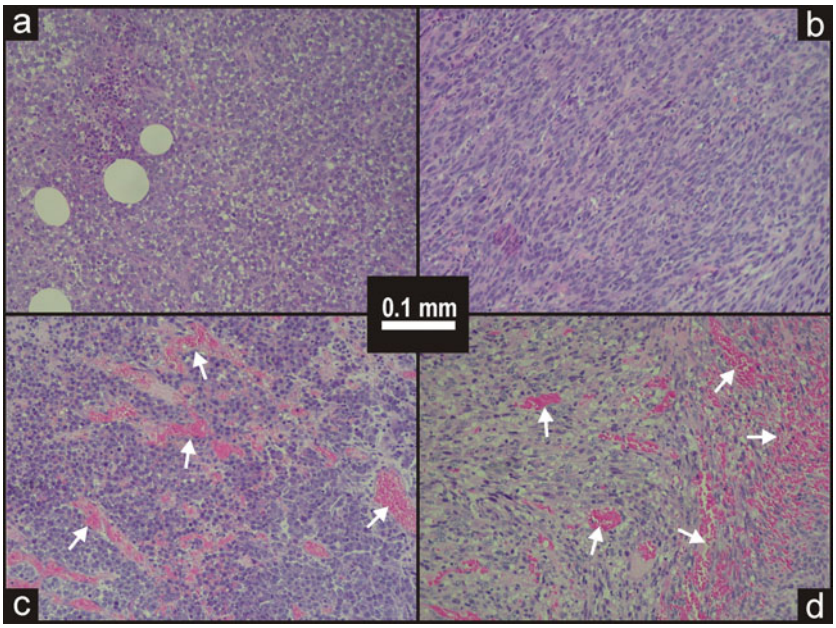


**Figure 6.** Histological evidence of extravasated red blood cells in DC-ET-treated tumours: (a) control Sa-1 tumour; (b) control LPB tumour; (c) DC-ET-treated Sa-1 tumour; (d) DC-ET-treated LPB tumour.

shows the most important difference between the control and the DC-ET-treated tumours. In both types of tumours DC-ET induced extensive local extravasation of blood cells. Distinct localized areas of accumulated erythrocytes were found throughout the majority of tumours treated with DC-ET (figure 6(c) and (d)) but were not observed in the control tumours (figure 6(a), (b)).

## 4. Discussion

The results of our study show that a 1 h application of DC-ET with direct current amplitude of 0.6 mA significantly decreased blood flow and oxygenation in Sa-1 and LPB tumours. In our previous studies, using the radioactive rubidium extraction technique and the PBV staining technique, we showed that DC-ET decreased relative perfusion of Sa-1 tumours by more than 50% and that the minimum perfusion was reached as early as 20 min after the start of DC-ET (Jarm *et al* 1998, 1999). Very similar dynamics of the decrease in perfusion were observed in the present study, where the concentration of $O_2$Hb decreased to the lowest level within 5–20 min of DC-ET in both Sa-1 and LPB tumours (figure 5). In all tumours the dHb signal (reflecting the net oxygenation of blood (Thorniley *et al* 1997)) started to decrease within the first 5 min of DC-ET. The decrease continued until the end of the observation period. This significant deoxygenation of blood in tumours was a result of decreased blood flow. In all tumours treated with DC-ET the tHb and the HHb levels were higher at the end of the observation period than at the beginning thus indicating that blood was accumulated in tumours due to DC-ET. In the majority of tumours $O_2$Hb also started to increase at a certain point during DC-ET after the initial rapid decrease, which further contributed to the increase of tHb. Averaged data in figures 5(a) and (b) indicate that accumulation of blood was more pronounced in Sa-1 than in LPB tumours even though the difference was not

statistically significant. Upon excision of tumours we observed macroscopically substantial quantities of pooled blood within most tumours treated with DC-ET and sometimes also in the immediate vicinity of these tumours. In general this effect was more pronounced in Sa-1 than in LPB tumours but it was completely absent in all control tumours, which is in agreement with the increased tHb level measured at the end of observation (figures 5(a)–(d)). Little or no macroscopically detected pooled blood, which was observed in some of DC-ET-treated tumours, correlated with the poor effect of DC-ET in the same tumours as evidenced by NIRS measurements. Large quantities of red blood cells were confirmed in the tumour tissue outside the blood vessels in the histological sections of the tumours treated with DC-ET for both tumour lines but not in control tumours (figure 6). From all this evidence it follows that at a certain point during the 1 h treatment with DC-ET blood started to leak from the tumour vessels, which are known to be abnormal in many aspects (Jain 1988, Vaupel 1979, 1990, Vaupel *et al* 1998, Raleigh *et al* 1996, Horsman 1998). The effect of blood accumulation in tumours found in our study most probably resulted from the tumour venous outflow being more severely impaired by DC-ET than the arterial inflow, as indicated by a steady increase of HHb and tHb and a relatively stable plateau level in $O_2$Hb after its initial decrease. The moment when the $O_2$Hb signal also started to increase in most tumours might indicate when extravasation of blood in tumours began, possibly as a result of some tumour vessels becoming leaky due to increased intravascular pressure.

Relative perfusion of tumours remained decreased and did not change further for at least 24 h following the treatment as demonstrated by the present PBV data (figures 2(a) and (b)), which are in good agreement with the previously published results (Jarm *et al* 1997, 1998, 1999). In these earlier studies it was found that perfusion of tumours improved during the following days but was still significantly poorer than in control tumours 3 days after DC-ET in Sa-1 tumours and 2 days after DC-ET in LPB tumours.

We used the well-established polarographic oximetry to obtain direct evidence that the decrease in blood flow caused a decrease in $pO_2$ in tumour tissue. In figures 3(a) and (b) and in table 2 it can be seen that Sa-1 and LPB tumours were very hypoxic even without the application of DC-ET and that the application of DC-ET further increased hypoxia as evidenced by the significantly decreased median $pO_2$ value and increased hypoxic fraction in both tumour models. This finding is in good agreement with the perfusion and blood oxygenation decrease assessed by means of the PBV staining technique and NIRS.

We used Sa-1 and LPB tumours because both tumour models were used extensively in our previous studies on DC-ET in tumours (Miklavčič *et al* 1993, 1994, Jarm *et al* 1997, 1998, 1999) and because the two tumour models are different in many aspects. The Sa-1 tumours grow approximately twice as rapidly as LPB tumours. The tumour doubling time at the size used in our study is approximately 2 and 4 days for Sa-1 and LPB tumours respectively (Jarm *et al* 1997, 1999). Solid Sa-1 tumours grow as flat soft disks, which start to ulcerate when they reach a size of approximately 200 $mm^3$. On the other hand, solid LPB tumours are almost spherical in shape, they are much tougher and do not ulcerate until they become much larger than the ulcerating Sa-1 tumours. However, these two tumours exhibit similar growth retardation after DC-ET. Growth delay induced by 1 h DC-ET with 0.6 mA current was in the 2–4 days range for both tumour models (Miklavčič *et al* 1993, Jarm *et al* 1997). In the present study the results of all the measurement methods demonstrate that DC-ET induced a similar decrease in perfusion and oxygenation in both tumour lines, even though the effect appeared more pronounced in Sa-1 than in LPB tumours. The observed difference in the decrease of relative perfusion between the two tumour models was statistically significant when assessed by means of the PBV staining method but not when assessed by means of NIRS. Polarographic oximetry showed no difference between the two tumour models.

Based on the results of the present and previous studies we suggest that tumour blood flow and subsequently tumour oxygenation were decreased as a result of vascular occlusion caused by DC-ET in normal subcutaneous tissue at the site of the insertion of the electrodes. When the electrode material is electrochemically insoluble (such as Pt/Ir alloy in our study), the main chemical reactions occurring at the electrodes during DC-ET include decomposition of water molecules and oxidation of chloride anions at the anode and decomposition of water at the cathode (Nilsson *et al* 2000). Electrolysis in tissue therefore produces extreme pH values and high concentrations of potentially toxic chemicals. The products of electrolysis can also combine with other chemicals present in the tissue, thus forming other cytotoxic products. However, due to various transport and buffering mechanisms in tissue, the presence of direct or indirect products of electrolysis is localized in the immediate vicinity of the electrodes and becomes undetectable very rapidly with increasing distance from the electrodes, which was demonstrated experimentally and predicted numerically (Miklavčič *et al* 1993, Cvirn *et al* 1994, Nilsson *et al* 1999, Nilsson and Fontes 2001, von Euler *et al* 1999, 2001). The effect of tissue destruction is therefore limited to the close proximity of the electrodes. In our study the electrodes were inserted in parallel under the skin on two opposite sides of the tumour (figure 1). The distance between the tumour edge and each electrode was at least 5 mm. Therefore, the products of electrolysis could not affect the tumours directly, and especially not so soon after the start of DC-ET as shown in our NIRS data. Macroscopic examination of the skin at the site of the insertion of the electrodes revealed lesions, which were localized to the area 1–2 mm away from the electrodes. This observation is in agreement with the results of von Euler *et al* (1999, 2001). They found cylindrically shaped destruction zones in the tissue surrounding the electrodes with the average radii of macroscopically detectable destruction zones of approximately 3 mm in rat mammary and liver tissues. They also found extreme pH values at the electrodes in rat mammary tissue. However, at a distance from the electrodes of approximately 4 mm (anode) and 6 mm (cathode) the pH values were already at a normal level. They found a strong correlation between the pH profiles and the span of the tissue destruction zones. It should be noted that in the studies by von Euler *et al* the 'dose' of DC-ET was considerably higher than in our study. In their study the current amplitude, duration and electric charge delivered were 1 mA, 85.3 min and 5 C (Coulomb) respectively. In our study the same parameters were 0.6 mA, 60 min and 2.1 C respectively. In one of our earlier studies we found no evidence of pH or temperature change in the tumours when the electrodes were placed in normal tissue on two opposite sides of the tumour. However, we found extreme pH values at the electrodes: pH $< 2$ at the anode and pH $> 10$ at the cathode (Miklavčič *et al* 1993). In our experiments with the PBV staining technique we observed that the effect of DC-ET on the relative perfusion seen in tumours was also present in the skin between the electrodes, which was also not stained by the dye. All the above-mentioned facts support the hypothesis that the effect of DC-ET on tumour perfusion and oxygenation was actually produced at the site of the insertion of the electrodes. Griffin *et al* (1995) proposed a similar explanation. They suggested that the secondary *peripheral ischemic necrosis* in the tissue not directly damaged by electrolysis was a result of the *primary central necrosis* and induced vascular thrombosis at the site of insertion of the electrodes. The importance of this effect should be considered for the optimization of DC-ET protocols in experimental and clinical trials.

We found high variability in both the dynamics and the amplitude of the response of individual tumours to DC-ET. The responses ranged from practically no effect to apparently complete shutdown of blood flow in some tumours. This high variability resulted in much larger standard deviation values for DC-ET-treated tumours in comparison with control tumours in figures 2 and 5. This is in agreement with the larger variability of tumour growth

data after DC-ET in comparison with control tumour growth observed in all our earlier studies. These facts indicate that the response of an individual tumour to DC-ET depended largely on whether the electrodes were inserted in such a way that the blood vessels supplying the tumour were included within the tissue destruction zone in the immediate vicinity of the electrodes and were thus directly affected by the products of electrolysis. This hypothesis, which was also proposed by Griffin *et al* (1995), can be used to explain the differences in response of LPB and Sa-1 tumours, as demonstrated by the results of PBV staining (figure 2). Both tumours were treated by DC-ET at approximately the same tumour size of about 50 $mm^3$. But due to the slower growth dynamics of the LPB tumours, these were on average at least twice as old as the Sa-1 tumours when DC-ET was applied. Even though this is certainly not the only physiological difference between the two tumour models, it means that the LPB tumours had more time to recruit a larger number of normal vessels to supply them and/or to probably grow a better developed neovasculature than the Sa-1 tumours. If this were the case, then it would be more likely for LPB than for Sa-1 tumours to have some of the supplying vessels not affected by DC-ET. Furthermore, when the Sa-1 tumours of different sizes are considered, it can be observed in figure 2(c) that the relative perfusion of larger (older) tumours was not decreased by the DC-ET to the same extent as in smaller tumours. This result points in the same direction as the difference between LPB and Sa-1 tumours of the same size.

Of the three measurement techniques used in our study, only NIRS provided insight into the dynamics of the changes induced by DC-ET. The nature of this method requires some caution in the interpretation of the results. Due to the specific measurement configuration (figure 1), a part of the measured signal might originate in the tissue surrounding the tumour. However, our Monte Carlo simulations of light transport for such a configuration and for a wide range of tested optical parameters showed that even for the worst-case combination of optical parameters the recorded NIRS signal largely originated from the tumour itself (Jarm 1999). Some of the increase in HHb, tHb and $O_2$Hb signals in the last stage of our measurements could also be contributed to the minute swelling of the tumour due to the demonstrated extravasation of blood, which could change the measurement geometry slightly. However, the results of NIRS measurements were in good agreement with the results of other methods and were also supported by macroscopic and microscopic observations.

## 5. Conclusions

The results of all applied measurement methods clearly indicate that the application of electrotherapy with low-level direct current induced severe reduction in blood flow (perfusion) of solid subcutaneous tumours. The observed effect was a result of occluded or damaged blood vessels due to electrolysis at the site of the insertion of the electrodes in the normal tissue surrounding the tumour. As a consequence, the oxygenation of blood in tumours decreased. During the later phase of the 1 h DC-ET some vessels inside the tumours became leaky, which resulted in pooling of blood inside the tumours. This was detected by the NIRS technique and was also observed macroscopically and confirmed microscopically. Polarographic oximetry revealed that Sa-1 and LPB tumours used in our study were very hypoxic even before the application of DC-ET but a further decrease in oxygenation was observed in tumours after DC-ET. The dynamics of gradual re-perfusion of tumours in the days following the treatment were in agreement with the re-growth dynamics of these tumours observed in previous studies. Our results and observations lead to a conclusion that the effect of electrotherapy (with electrodes placed outside the tumour) on perfusion of tumours was the major mechanism of anti-tumour action for the specific type of electrotherapy in the two tumour models used in our study.

## Acknowledgments

This research was supported through various grants by the Ministry of Education, Science and Sport of the Republic of Slovenia and by the European Commission's Tempus-Phare Programme.

## References

Baxter L T and Jain R K 1989 Transport of fluid and macromolecules in tumors 1. Role of interstitial fluid pressure and convection *Microvasc. Res.* **37** 77–104

Brown J M 1999 The hypoxic cell: a target for selective cancer therapy *Cancer Res.* **59** 5863–70

Čemažar M, Parkins C S, Holder A L, Kranjc S, Chaplin D J and Serša G 2001 Cytotoxicity of bioreductive drug tirapazamine is increased by application of electric pulses in Sa-1 tumours in mice *Anticancer Res.* **21** 1151–6

Chaplin D J, Hill S A, Bell K M and Tozer G M 1998 Modification of tumor blood flow: current status and future directions *Semin. Radiat. Oncol.* **8** 151–63

Cvirn P, Reščič J and Miklavčič D 1994 Tumour pH changes due to electrotherapy-experimental results and mathematical model *Electrotech. Rev.* **61** 37–42

Delpy D T and Cope M 1997 Quantification in tissue near-infrared spectroscopy *Phil. Trans. R. Soc. Lond., Biol. Sci.* **352** 649–59

Fosh B G, Finch J G, Lea M, Black C, Wong S, Wemyss-Holden S and Maddern G J 2002 Use of electrolysis as an adjunct to liver resection *Br. J. Surg.* **89** 999–1002

Griffin D T, Dodd N J F, Zhao S, Pullan B R and Moore J V 1995 Low-level direct electrical current therapy for hepatic metastases. I. Preclinical studies on normal liver *Br. J. Cancer* **72** 31–4

Höckel M, Vorndran B, Schlenger K, Baußmann E and Knapstein P G 1993 Tumor oxygenation: a new predictive parameter in locally advanced cancer of the uterine cervix *Gynecol. Oncol.* **51** 141–9

Horsman M R 1998 Measurement of tumor oxygenation *Int. J. Radiat. Oncol. Biol. Phys.* **42** 701–4

Jain R K 1988 Determinants of tumor blood flow: a review *Cancer Res.* **48** 2641–58

Jarm T 1999 The effects of electrotherapy on parameters of perfusion and oxygenation of tumours *PhD Thesis* (Ljubljana: University of Ljubljana, Faculty of Electrical Engineering)

Jarm T, An D, Belehradek J Jr, Mir L M, Serša G, Čemažar M, Kotnik T, Pušenjak J and Miklavčič D 1997 Study of blood perfusion with Patent Blue staining method in LPB fibrosarcoma tumors in immuno-competent and immuno-deficient mice after electrotherapy by direct current *Radiol. Oncol.* **31** 33–8

Jarm T, Čemažar M, Serša G and Miklavčič D 1998 Blood perfusion in a murine fibrosarcoma tumor model after direct current electrotherapy: a study with $^{86}$Rb extraction technique *Electro-Magnetobiol.* **17** 273–82

Jarm T, Wickramasinghe Y A B D, Deakin M, Cemazar M, Elder J, Rolfe P, Sersa G and Miklavčič D 1999 Blood perfusion of subcutaneous tumours in mice following the application of low-level direct electric current *Adv. Exp. Med Biol.* **471** 497–506

Jöbsis F F 1977 Noninvasive, infrared monitoring of cerebral and myocardial oxygen sufficiency and circulatory parameters *Science* **198** 1264–7

Miklavčič D, Fajgelj A and Serša G 1994 Tumour treatment by direct electric current: electrode material deposition *Bioelectroch. Bioener.* **35** 93–7

Miklavčič D, Serša G, Kryžanowski M, Novaković S, Bobanović F, Golouh R and Vodovnik L 1993 Tumor treatment by direct current—tumor temperature and pH, electrode material and configuration *Bioelectroch. Bioener.* **30** 209–20

Milosevic M F, Fyles A W and Hill R P 1999 The relationship between elevated interstitial fluid pressure and blood flow in tumors: a bioengineering analysis *Int. J. Radiat. Oncol. Biol. Phys.* **43** 1111–23

Nilsson E, Berendson J and Fontes E 1999 Electrochemical treatment of tumours: a simplified mathematical model *J. Electroanal. Chem.* **460** 88–99

Nilsson E and Fontes E 2001 Mathematical modelling of physicochemical reactions and transport processes occurring around a platinum cathode during the electrochemical treatment of tumours *Bioelectrochemistry* **53** 213–24

Nilsson E, von Euler H, Berendson J, Thorne A, Wersall P, Naslund I, Lagerstedt A S, Narfstrom K and Olsson J M 2000 Electrochemical treatment of tumours *Bioelectrochemistry* **51** 1–11

Podobnik B and Miklavčič D 2000 Influence of hydralazine on interstitial fluid pressure in experimental tumors—a preliminary study *Radiol. Oncol.* **34** 59–65

Raleigh J A, Dewhirst M W and Thrall D E 1996 Measuring tumor hypoxia *Semin. Radiat. Oncol.* **6** 37–45

Serša G, Čemažar M, Miklavčič D and Chaplin D J 1999 Tumor blood flow modifying effect of electrochemotherapy with bleomycin *Anticancer Res.* **19** 4017–22

Thorniley M S, Simpkin S, Balogun E, Khaw K, Shurey C, Burton K and Green C J 1997 Measurement of tissue viability in transplantation. *Phil. Trans. R. Soc. Lond., Biol. Sci.* **352** 685–96

Vaupel P 1979 Oxygen supply to malignant tumors *Tumor Blood Circulation: Angiogenesis, Vascular Morphology and Blood Flow of Experimental and Human Tumors* ed H-I Peterson (Boca Raton, FL: CRC Press) pp 143–68

Vaupel P 1990 Oxygenation of human tumors *Strahlenther. Onkol.* **166** 377–86

Vaupel P, Kelleher D K and Thews O 1998 Modulation of tumor oxygenation *Int. J. Radiat. Oncol. Biol. Phys.* **42** 843–8

Vaupel P, Schlenger K, Knoop C and Hockel M 1991 Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized $O_2$ tension measurements *Cancer Res.* **51** 3316–22

von Euler H, Nilsson E, Lagerstedt A-S and Olsson J M 1999 Development of a dose-planning method for electrochemical treatment of tumors: a study of mammary tissue in healthy female CD rats *Electro-Magnetobiol.* **18** 93–104

von Euler H, Nilsson E, Olsson J M and Lagerstedt A-S 2001 Electrochemical treatment (EChT) effects in rat mammary and liver tissue. *In vivo* optimizing of a dose-planning model for EChT of tumours *Bioelectrochemistry* **54** 117–24

Watson B W 1991 The treatment of tumours with direct electric current *Med. Sci. Res.* **19** 103–5

Wemyss-Holden S A, Dennison A R, Finch G J, Hall P de la M and Maddern G J 2002 Electrolytic ablation as an adjunct to liver resection: experimental studies of predictability and safety *Br. J. Surg.* **89** 579–85

Wickramasinghe Y A B D, Livera L N, Spencer S A, Rolfe P and Thorniley M S 1992 Plethysmographic validation of near infrared spectroscopic monitoring of cerebral blood volume *Arch. Dis. Child.* **67** 407–11