

## 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

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*Electrotherapy of tumors by low-level direct current can be used effectively to reduce tumor mass in experimental and clinical tumor models. The effects of such treatment on blood perfusion of tumors were studied on solid subcutaneous LPB fibrosarcoma model in immuno-competent C57Bl/6 mice and in immuno-deficient Swiss nude mice. Tumors were treated for one hour with electric current of amplitudes 0.6 mA and 1.0 mA delivered by Pt/Ir needle electrodes inserted subcutaneously on two opposite sides of the tumor. Study of tumor growth response to single-shot electrotherapy yielded highly significant growth retardation in C57Bl/6 mice but only insignificant effect on tumor growth in nude mice. Effect of electrotherapy on blood perfusion of tumors as one of the proposed mechanisms of antitumor action was evaluated by tissue staining method using Patent Blue Violet dye. Perfusion was estimated immediately after electrotherapy and 24 hours after the treatment. Perfusion of tumors in C57Bl/6 mice was only moderately decreased due to electrotherapy, whereas in nude mice there was practically no effect observed. The difference in growth response of the two models indicates that vascular occlusion which occurs due to the products of electrolysis at the site of insertion of the electrodes may not be the major cause for the observed tumor retardation in immuno-competent mice.*

**Key words:** fibrosarcoma electric stimulation therapy; regional blood flow; direct current electrotherapy, tumor growth retardation, blood perfusion

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### Introduction

It has been demonstrated in several studies that electrotherapy by low-level direct current can be applied successfully as a local treatment for solid

malignancies with minimum side effects for the host. The antitumor effectiveness has been achieved in various experimental animal tumor models<sup>1-6</sup> and in clinical trials.<sup>7,8</sup> In addition, electrotherapy can be used as an adjuvant treatment to other conventional therapies for it has been shown that it potentiates the effectiveness of certain biological response modifiers and anticancer drugs, e.g. tumor necrosis factor, interleukin-2 and bleomycin.<sup>9-11</sup> Regardless of the way of its application, either as a single treatment or in combination with other therapies, it

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is of great importance to understand the mechanisms of its antitumor action in order to be able to optimise existing and develop new treatment strategies.

Many attempts have been made to explain antitumor effectiveness of electrotherapy. It can hardly be expected that one single mechanism could be responsible for it since the parameters of treatment vary considerably from one author to the other. The differences appear in the type of tumor model, in electrical parameters (e.g. amplitude and shape of the signal), in electrode number and in material and the way of application.<sup>12</sup>

Several possible mechanisms that could be responsible for antitumor effectiveness of direct current electrotherapy have been proposed. When one or more of the electrodes are inserted into the tumor then the extreme pH changes measured in vicinity of the electrodes are believed to be responsible for cell killing. It has also been shown that with an appropriate configuration of multiple electrodes in the tumor it is possible to effectively eliminate most of the tumor mass<sup>13</sup>. Surprisingly enough it was found that when electrodes are inserted subcutaneously on two opposite sides outside the tumor so that the tumor is brought into so-called "field" configuration, then similar growth retardation is produced as in the case with one of the electrodes in the tumor. For the "field" configuration antitumor effectiveness of electrotherapy cannot be ascribed to pH changes and also not to temperature rise which were both not found in the tumor.<sup>12</sup> There was also no correlation found between tumor growth retardation and deposition of the electrode material<sup>14</sup>.

One of the possible mechanisms of antitumor action of sub-thermal low-level direct current electrotherapy for electrode configurations where tumor is not penetrated by stimulation electrodes is vascular occlusion at the site of insertion of the electrodes.<sup>15</sup> It was suggested that altering the blood supply to tumor tissue might lead to eradication of tumor mass. In one of our previous studies we have found that significant growth retardation of one particular tumor model was indeed accompanied by and correlated to large decrease of tumor blood perfusion as assessed by a tissue staining method.<sup>16</sup> In the present study an attempt was made to investigate this effect in LPB murine fibrosarcoma tumor in both immuno-competent and immuno-deficient animals.

## Materials and methods

### *Animals and tumors*

Female and male animals of C57Bl/6 strain were purchased from two sources, namely Rudjer Bošković Institute, Zagreb, Croatia and C.E.R.J animal facilities Laval, France. Nu/nu Swiss nude mice were bred at the animal facilities of the Institute Gustave-Roussy, Villejuif, France. Animals were housed in plastic cages in convenient colonies and kept at constant temperature of 24°C. They were watered and fed ad libitum. Additional standard measures were taken in order to prevent infection in nude mice. Animals in good condition and free of any infection, aged 8-12 weeks, were used in experiments. LPB fibrosarcoma cells syngeneic to C57Bl/6 mice were cultured in vitro. Solid subcutaneous tumors were initiated dorsolaterally in the right flank of mice by injection of  $0.8 \cdot 10^6$  and  $1.6 \cdot 10^6$  viable LPB cells in C57Bl/6 and nude mice respectively. Tumors of approximately 7 mm in diameter were obtained about 10 days later, when animals were randomly assigned to different experimental groups with 7-9 animals per group in each single experiment.

### *Electrotherapy*

Single-shot electrotherapy consisted of constant direct current which was applied for 60 minutes. Current amplitudes used were 0.6 mA and 1.0 mA. Selection of current level and duration was based on our previous studies.<sup>12</sup> Current was generated by a multichannel current source thus enabling simultaneous treatment of up to eight animals. Needle electrodes 1 mm in diameter and 20 mm in length made of Pt/Ir alloy (90/10%) with rounded tips were used to deliver the current. Electrodes were inserted subcutaneously through small punctures in the skin made by a sharp needle. Electrodes were inserted in parallel on two opposite sides of the tumor with the cathode on caudal side of the tumor. Distance between the electrodes was 20-22 mm and each electrode was placed 5-8 mm away from the tumor edge. During electrotherapy mice were anesthetised by intraperitoneal injection of 100 mg/kg of ketamine (Ketanast, Parke-Davis, Germany), 10 mg/kg of xylazine (Rompun, Bayer, Germany), and 0.1 mg/kg of atropine. Animals in control groups were treated in the same way except that no current was applied.

### Assessment of tumor growth

Tumor sizes were estimated before electrotherapy (day 0) and on the days following electrotherapy. Two largest perpendicular diameters were measured with vernier calliper and tumor volume was calculated using formula  $V = \pi ab^2/6$  ( $a$  and  $b$  being measured diameters). For each individual tumor its doubling time (DT) was calculated as a time needed for the tumor to double its initial size. Mean average tumor volumes and their standard errors were calculated and presented as growth curves. Mean average doubling times were also calculated for all experimental groups. Experiments were repeated three times in case of nude mice and twice for C57Bl/6 mice. Statistical significance of differences between experimental groups were evaluated using Student t-test and values of  $p$  less than 0.05 were considered as an indication of statistical significance.

### Assessment of tumor perfusion

Perfusion study was performed on separate animals from those used in tumor growth study. Saline solution (0.1 ml, 1.25%) of biological dye Patent Blue Violet (Byk Gulden, Switzerland) was injected in retroorbital sinus of animals. After the dye has been left to distribute evenly through tissues for approximately 3 minutes, animals were euthanised and tumors were carefully removed. Tumors were cut along their largest diameter and the percentage of stained versus non-stained cross-section area was visually estimated independently by two persons. The mean of both estimations was used as a relative measure of tumor perfusion. Solidly stained parts of the tissue were considered to be well perfused, absence of the dye was an indication of poor perfusion. Perfusion was assessed immediately after electrotherapy and 24 hours following the treatment. For each experimental group the mean value and standard error of the mean were calculated and presented. Student t-test was used to evaluate statistical significance of differences between experimental groups.

In our study Patent Blue Violet dye was used instead of a more widely used Evans Blue dye. The former yielded a much better color contrast between stained and non-stained parts of tissue than the latter. Furthermore, a study performed on fibrosarcoma SA-1 tumors in A/J mice has shown that both dyes produce essentially the same results.<sup>19</sup> This was confirmed in control tumors as well as in

tumors treated with electrotherapy. Therefore in our opinion the use of Patent blue instead of Evans Blue is entirely justifiable in perfusion studies such as ours.<sup>17,18</sup>

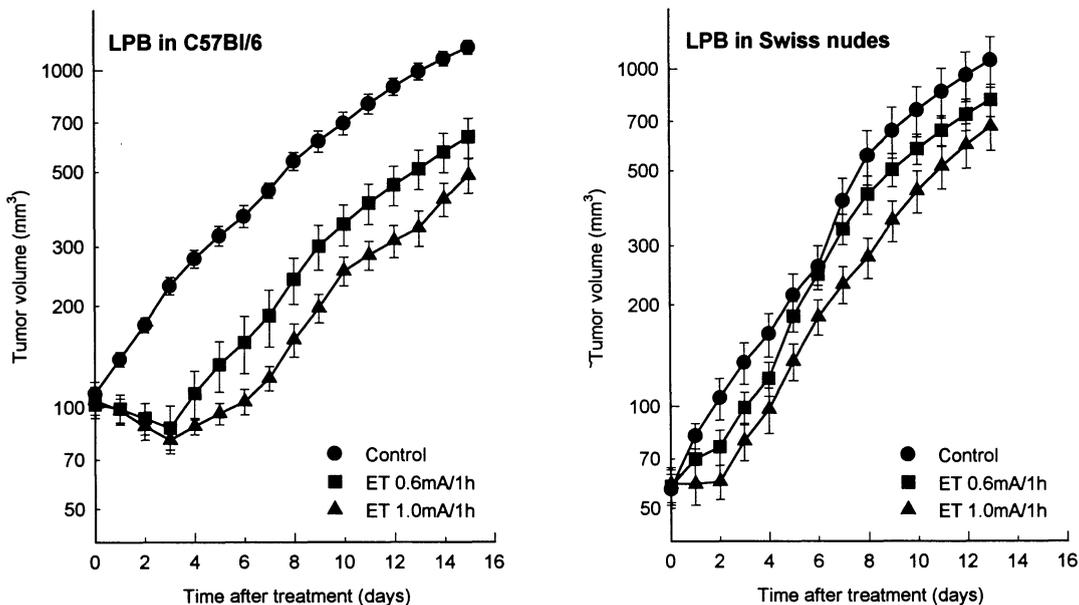
## Results

### Tumor growth

Application of the single-shot electrotherapy induced statistically significant tumor growth delay in immuno-competent C57Bl/6 mice. Typical growth of tumors in one of the experiments is given in Figure 1, where growth delay of treated tumors with respect to control tumors is clearly shown. It was also evident that larger dose (higher current level) yielded better antitumor effects. In Table 1 tumor doubling times (DT/days) are given for all experimental groups in two separate experiments. Tumors were arrested in their growth and their size was temporarily reduced. When regrowth occurred after several days, the growth rate was similar to that observed in control tumors. Apart from the observed fact that control LPB tumors in immuno-deficient mice grew faster than in C57Bl/6 mice it was also demonstrated that electrotherapy was much less effective in the case of nude mice, as shown in Figure 1 and presented in Table 1. Doubling time of tumours treated with 0.6 mA electrotherapy was not significantly different from doubling time of control tumors. Application of 1.0 mA however yielded significant growth delay but it was still much smaller than in immuno-competent mice.

### Tumor perfusion changes

In Table 2 perfusion of tumors as assessed by means of Patent Blue staining method is presented. Data of several experiments were pooled together. All control tumors in both strains of mice were practically completely stained, which indicates that tumors at that stage of development were well perfused. No reduction in staining of tumor tissue was observed in nude mice immediately after electrotherapy regardless of the current level applied. In C57Bl/6 mice reduced staining was observed immediately after treatment and this effect was maintained for at least 24 hours. As in the tumor growth study the effect was more pronounced for higher dose. There was also a large variability in staining among individual tumors treated with electrotherapy. On the other hand staining of control tumors was very uniform in both strains of mice.



**Figure 1.** Growth of LPB tumors in immuno-competent C57Bl/6 mice and in immuno-deficient Swiss nude mice following a single-shot one-hour electrotherapy with 0.6 mA and 1.0 mA. Data points represent the mean values for 7-8 animals with standard error bars.

**Table 1.** Effect of one hour electrotherapy (ET) with 0.6 mA or 1.0 mA on growth of LPB fibrosarcoma tumors in C57Bl/6 and Swiss nude mice. Tumor doubling times (DT) are given separately for each experiment and statistical significance of difference of DT in treatment groups with respect to corresponding control group is indicated by value of *p* as assessed by Student *t*-test.

Exp.	Treatment	C57Bl/6			Swiss nude		
		n	DT(days) mean±SD	<i>p</i>	n	DT(days) mean±SD	<i>p</i>
1	control	8	4.0±2.2		8	2.8±1.5	
	0.6mA/1h	8	6.3±2.9	0.096	8	3.4±1.0	0.362
	1.0mA/1h	8	11.7±5.4	0.002	8	4.5±1.2	0.025
2	control	8	2.8±0.7		7	1.8±0.8	
	0.6mA/1h	8	7.5±2.0	<0.001	7	2.5±1.0	0.174
	1.0mA/1h	8	10.4±3.1	<0.001	7	2.7±0.4	0.021
3	control				7	1.9±0.8	
	0.6mA/1h				6	2.3±0.8	0.388
	1.0mA/1h				7	3.2±1.2	0.034

**Table 2.** Percentage of stained tumor cross-section area as an indicator of quality of tumor perfusion for LPB fibrosarcoma in immuno-competent C57Bl/6 and immuno-deficient Swiss nude mice. Perfusion was estimated immediately after one hour electrotherapy (ET) for two current intensities (0.6 mA and 1.0 mA) and 24 hours after 0.6 mA ET. Value of *p* indicates statistical significance of difference between each particular treatment group and corresponding control group, as assessed by Student *t*-test.

Experimental group	C57Bl/6			Swiss nude		
	n	% stained (mean±SD)	<i>p</i>	n	% stained (mean±SD)	<i>p</i>
control	19	98±5		11	100±0	
0.6mA/1h (0h after ET)	27	80±30	0.013	7	100±0	1.000
0.6mA/1h (24h after ET)	8	81±35	0.044			
1.0mA/1h (0h after ET)	30	70±35	0.001	14	98±5	0.200

## Discussion

In this study it was demonstrated that low-level direct current electrotherapy induces significant retardation of LPB fibrosarcoma tumors growing in immuno-competent C57Bl/6 mice and that this effect is also dose dependent. This is in agreement with our previous work on this and other tumor models.<sup>12,14,20</sup> When LPB tumors were treated in immuno-deficient animals, electrotherapy appeared to be much less effective. In another tumor model of SA-1 fibrosarcoma growing in immuno-competent A/J mice the electrotherapy of the same type as in this study produced similar growth retardation as observed in C57Bl/6 mice. An extensive study on A/J mice by means of Patent Blue staining showed rapid decrease in perfusion during electrotherapy (data not shown).<sup>16,19</sup> Staining was decreased to the mean value of approximately 20% in treated tumors and even three days after treatment tumors were only partially reperfused (approximately 50% of stained tumor cross-section), which is very different from perfusion of 80% found in LPB tumors for the same treatment (0.6 mA for 60 minutes). That means that vascular occlusion due to electrotherapy in SA-1 tumors was much more expressed than in LPB tumors. Since the dynamics of deperfusion and reperfusion of SA-1 tumors was in good correlation with dynamics of growth retardation of that particular tumor model it was suggested that vascular occlusion occurring at the site of insertion of the electrodes might be the main factor of antitumor effectiveness. The presented study has raised some doubt to this hypothesis because the same extent of LPB tumor growth retardation was accompanied by a much less expressed occlusive effect. Furthermore, LPB tumors in immuno-deficient animals were significantly less affected by electrotherapy than the same type of tumors in immuno-competent animals, which indicates that host's immune response might play an important role in effectiveness of electrotherapy.<sup>20</sup> In our opinion, occlusion of supplying blood vessels outside the tumor inevitably occurs at the site of electrode insertion due to extreme pH changes that were measured in immediate vicinity of electrodes.<sup>12</sup> The extent of this occlusion is probably significantly tumor model-dependent, as indicated by the difference in Patent Blue staining between the two fibrosarcoma models. Some other factors beside interrupted blood supply are probably responsible for the observed tumor retardation and immune system of the host organism is one of them.

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