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Direct Current Ablation Destroys Multi-Stage Fibrosarcomas in Rats

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Abstract – ***Introduction:*** **Direct current (DC) ablation offers the potential for precise targeting of tumors and stimulation of the immune system, but has not achieved widespread use. This study was conducted to evaluate the impact on tumor size and subject survival of combining a main ablation treatment with a low-current pretreatment, to assess potential immune system activation, and to assess stimulation-related parameters.** ***Methods and Results:*** **Twenty-six female Fischer 344 rats were injected with methylcholanthrene-induced fibrosarcoma cells to create primary tumors and again in the contralateral flank when primary tumors reached 700 mm^3 (contralateral tumors were proxies for metastases). There were four treatment groups: two control animals received no treatment; six control plus placebo animals had electrodes implanted but received no stimulation; eight received high-current stimulation (80 coulombs (C) of charge at 20 milliamperes (mA), over 66 minutes); and eight received high-current treatment one day after a low-current pretreatment (10C, 10 mA, 16.6 min). Electrodes were inserted through the tumor base in a single plane, 4.0mm apart, alternating anode and cathode. Treatments commenced once primary tumors reached 700 mm^3. All control animals were sacrificed 55 days after primary tumor cell injection due to excessive tumor growth. Tumors disappeared from all 16 treated rats within eight days; retreatment was required in two animals. Pretreatment had no effect on tumor disappearance or survival.** ***Conclusion:*** **Direct current ablation provides highly effective tumor destruction in a rat model. Slower growth of contralateral tumors suggests a remote effect that may involve the immune system.**

Keywords - ***DC ablation; fibrosarcoma; electrode; rat; resistance; immune response***

I. INTRODUCTION

Recently there have been a number of reports presenting evidence of tumor cell destruction by localized delivery of direct current (DC) ablation therapy (also referred to as electrochemical therapy or electrolytic ablation) in both animal and human settings [1]-[8]. Despite these reports, DC ablation has not emerged as a viable cancer therapy outside of China, where its use has been relatively widespread [9].

The theoretical advantages of this approach to cancer therapy include precise targeting of tumor cell destruction since electrical stimulation is delivered via surgically placed electrodes, as well as the ability to treat multiple tumors simultaneously. In addition, the approach appears suitable for both superficial and deep tumors; in contrast to the recognized risk of blood vessel damage by radiofrequency ablation, Wemyss-Holden [10] has reported safe use of DC ablation near the hepatic vein in an animal study.

DC ablation is thought to produce cellular necrosis through the localized cytotoxic effects of the resulting pH shifts: chlorine and hydrogen ions are produced at the anode, and hydroxyl ions at the cathode [11], [12]. The pertinent reactions are given as [13]:

Anode: $H_2O - 2e^- \leftrightarrows \frac{1}{2} O_2 + 2H^+$

Cathode: $H_2O + e^- \leftrightarrows \frac{1}{2} H_2 + OH^-$

Chlorine evolves at the anode:
$2\ Cl^- \leftrightarrows Cl_2 + 2e^-$

This molecular chlorine reacts rapidly with water to produce hypochlorous acid:
$Cl^2\ (aq) + H_2O \leftrightarrows HClO + H^+ + Cl^-$

Hypochlorous acid has been reported by Chiang et al. to play a role in enhancing an immune system response [14] in the setting of ovarian carcinoma, offering another potential benefit of the DC ablation approach to cancer therapy.

At the least, further development of this approach to cancer therapy requires additional animal studies to establish fundamental guidelines for a number of stimulation parameters such as the size, shape and placement of electrodes; the characteristics of DC ablation therapy including voltage, current and treatment duration; targeted cancer types; and other variables. The work reported by Chou et al. [15] and Ren et al. [16] has formed an important start, and the present study was performed to extend and refine past work in identifying basic stimulation parameters.

Specifically, this study was conducted to evaluate the impact of combining a high-current treatment with a low-current pretreatment on tumor size and subject survival rate; and to gain a better understanding of the electrical parameters involved by measuring electrical resistance during the ablation procedure. The hypothesis regarding pretreatment was that it might serve to induce a beneficial immune response that could inhibit tumor growth.

The administration of the low-current pretreatment would also provide additional insights into the impact of multiple ablation treatments in the same area. Electrical resistance will be an important determinant of the required capabilities of a therapeutic stimulation device, and there is a scarcity of reported data on this topic.

The potential for DC ablation of tumors to stimulate the immune response, as measured by growth of a contralateral tumor after primary tumor treatment, was also evaluated.

II. METHODS

A. General

Twenty-six female Fischer 344 rats were injected with methylcholanthrene-induced fibrosarcoma cells to create primary tumors, and again in the contralateral flank when the primary tumors reached 700 mm^3 (contralateral tumors were proxies for metastases).

Following the injection of cancer cells, rats were randomly assigned to four groups:

1) Group 1 (controls) – two rats did not have electrodes implanted and received no treatment
2) Group 2 (controls with placebo treatment) – six rats had electrodes implanted for a period of ten minutes, but received no DC ablation therapy
3) Group 3 (treatment) – eight rats had electrodes implanted and received high-current treatment
4) Group 4 (pretreatment) – eight rats had electrodes implanted and received both a low-current pretreatment and a high-current treatment 24 hours later

The study was conducted in a manner generally compliant with Good Laboratory Practice (GLP) Standards. Table 1 summarizes the study design.

TABLE 1
SUMMARY OF STUDY DESIGN

Group	n	Primary and contralateral tumor cells injected	Electrodes implanted	Primary Tumor Pretreatment	Primary Tumor Treatment	Contralateral Tumor Pretreatment	Contralateral Tumor Treatment
1	2	**Yes**	No	No	No	No	No
2	8*	**Yes**	**Yes**	No	No	No	No
3	8	**Yes**	**Yes**	No	**Yes**	No	**Yes**
4	8	**Yes**	**Yes**	**Yes**	**Yes**	No	**Yes**

* Two rats from this group were transferred to Group 4 to replace two animals that died upon initial treatment of the secondary tumor.

B. Subjects

The animals were healthy young adult female (nulliparous and non-pregnant) Fischer 344 rats (Harlan, Inc., Indianapolis, IN). Animals were acclimated for five days before tumor cell injection and were housed individually in separate cages when not receiving the experimental treatments. Food and water were provided ad libitum, and there were no known contaminants in the food or water that might have interfered with the study. All Group 2, 3 and 4 rats were anesthetized by injection with a combination of ketamine (75 mg/kg) and acepromazine (2.5 mg/kg) before electrode implantation, and maintained under isoflurane anesthesia for the duration of treatment. Deviations to anesthesia protocol were made and are discussed later in this report. Control group animals (Groups 1 and 2) were sacrificed 55 days after tumor cell injection due to excessive tumor growth; all surviving treatment group animals (Groups 3 and 4) were sacrificed 121-143 days after the study began.

C. Cancer Model

Tumors were induced in all animals through subcutaneous injection in the right flank of 5 x 106 cells of methylcholanthrene-induced fibrosarcoma (MCA-R) obtained from one of the persons acknowledged below (Bull; original report in Grant et al. [17]). Tumor growth was monitored daily. Before any treatment began, a similar injection in the left flank was performed to produce a contralateral tumor once the primary tumor reached a size of approximately 700 mm^3. This size was also the starting point for treatment. (We use the term "primary" to refer to the first tumor induced, and "contralateral" to refer to the second tumor.) Creation of the contralateral tumor was intended to serve as a proxy for a metastasis.

D. Devices Used

For the high-current treatment, constant direct current was delivered via sterile platinum-iridium needle electrodes (supplied by OncoStim, Inc., Maple Grove, MN) implanted in the tumor. In the case of the Group 4 animals, the additional pretreatment was delivered via platinum-iridium point electrodes, which had a smaller surface area than the main treatment needle electrodes. Electrical power was provided by a custom-made Therapy Generator (OncoStim, Inc.). Voltage was measured using a digital multimeter (Model 22-816, Extech Instruments Corp., Waltham, MA).

E. Electrode Placement

For the high-current treatment, electrodes were inserted completely through the tumor near its base, 4.0 mm apart, in an alternating anode-cathode configuration, and in a single plane. Typically, four electrodes were used, although the number varied from two to six depending on tumor size and the 4.0mm spacing. During pretreatment in the case of group 4, two electrodes were inserted perpendicularly into the center of the tumor to a depth of approximately 5.0 mm, with a separation of 4.0 mm.

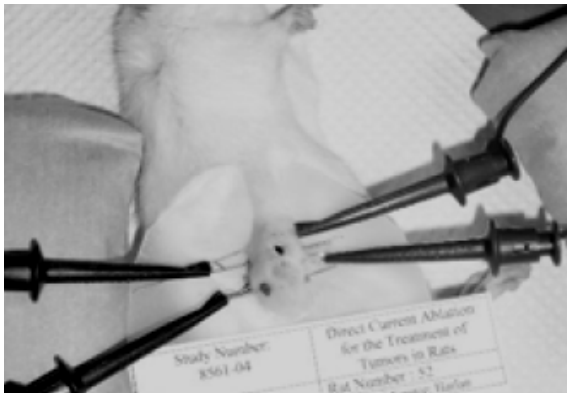

Fig. 1. A rat subject from group 4 during initial tumor treatment that commenced 28 days after tumor cell injection.

F. DC Ablation Therapy

Group 1 control rats had no electrodes implanted and did not receive treatment.

Group 2 control, plus placebo rats had electrodes implanted to act as a placebo, but received no direct current stimulation.

Group 3 rats received high-current treatment one day after the contralateral tumor cell injection, which occurred when the primary tumor reached a size of approximately 700 mm^3. The high-current treatment consisted of direct current applied to the tumor via the implanted electrodes in such a fashion as to deliver a total charge of 80 coulombs (C), at a current level of 20 milliamperes (mA), over a period of 66 minutes. In all cases, the current was gradually increased from 0 mA to the prescribed level. Following DC ablation therapy, the electrodes were removed and each animal was returned to her cage.

Group 4 rats received low-current pretreatment one day after the contralateral tumor cell injection, which occurred when the primary tumor reached a size of approximately 700 mm^3. The low-current pretreatment consisted of a total charge delivery of 10 C, at a current level of 10 mA, over a period of 16.6 minutes. The primary goal of the pretreatment was to stimulate a possible immune response by current application; not necessarily to treat the tissue. High-current treatment of primary tumors in Group 4 rats was performed in the same manner as Group 3 rats; in this case, one day after the low-current pretreatment. At this point, swelling from the pretreatment therapy (presumably caused by the insertion and manipulation of the needle electrodes) made tumor measurement and electrode placement difficult.

During the low-current pretreatment of the Group 4 rats, two animals died, most probably due to complications with injection of ketamine and acepromazine anesthesia. As a result, a slight deviation in protocol was made; Group 4 rats were anesthetized by isoflurane gas only.

Contralateral tumors were treated using the same parameters as were used for the treatment of primary tumors: 60 C, 20 mA, over 66 minutes. The initial treatment of contralateral tumors was performed about seven weeks after primary tumor cell injection (three weeks after contralateral tumor cell injection), irrespective of contralateral tumor size. In the case of Group 4 rats (the pretreatment group), no pretreatment was initiated for the contralateral tumors; Group 3 and 4 contralateral tumors, where present, were treated identically.

Following each animal's initial treatment, retreatment using the same parameters and procedure was performed on both primary and contralateral tumors, whenever a tumor grew back to 700 mm^3. This data can be seen in Fig. 3, 4.

G. Data Collection and Analysis

The weight, temperature, visual appearance and behavior of all animals were monitored daily. Tumor growth was monitored daily using calipers, and the estimated volume was calculated according to the following ellipsoidal formula and recorded:

$$4/3\ \pi\ (r1 \bullet r2 \bullet r3)$$

Where r1, r2 and r3 are the radii of the tumor in three dimensions.

During ablation, the voltage between the anode and cathode was measured at the start of treatment and then every five to ten minutes. Resistance was calculated based on the ratio of the measured voltage to the constant current being supplied (10 mA for low-current pretreatment, 20 mA for high-current treatment).

The major outcome measures were tumor size and animal survival. Differences in tumor size between the groups were analyzed using two-tailed t-tests, and a significance level of $p < 0.05$ was used throughout. The presence of an immune response was evaluated indirectly by observing the growth of the contralateral tumor following treatment of the primary tumor. Descriptive statistics (mean and maximum) documenting electrical resistance were calculated from measurements taken during treatments.

III. RESULTS

A. General

Tumors in all four groups grew aggressively. DC ablation was successfully applied to Group 3 and 4 rats, and was shown to provide a promising treatment modality for aggressive cancerous tumors, as measured by drastic decrease in tumor volume.

Group 1 rats were the naïve control animals and were not treated. These rats were sacrificed on Day 55 of the study due to tumor size.

Group 2 rats were the sham treated animals, where electrodes were placed on the base of the tumor but no DC ablation was performed. The average tumor volume for the group was 697 mm^3. These animals were sacrificed on Day 55 of the study due to tumor size.

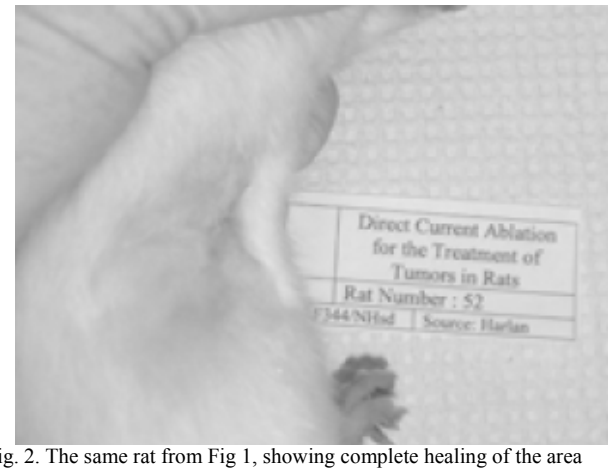

Fig. 2. The same rat from Fig 1, showing complete healing of the area where the tumor had been, about 10 weeks post-treatment.

All animals exhibited a normal rate of development until the commencement of DC ablation therapy. Group 1 and 2 rats did not exhibit any weight loss during the study, while Group 3 and 4 animals lost weight following stimulation, but recovered within 10-15 days.

Immediately after treatment, all treated animals (Groups 3 and 4) presented an area of laceration where the tumor was previously attached. These wounds were observed for approximately 15 days until scarring was present. A

decrease in motility was also observed in the animals after treatment until the wounds healed. Several rats were observed trying to gnaw off their tumors post-treatment. At the end of the study, scars were visible on the areas where the tumors had been attached, but fur had grown back to cover the area. See Fig. 2.

B. Primary Tumor Growth

Primary tumors developed in all animals from all four groups. DC ablation was found to induce necrotic zones around the electrodes and, because electrode placement spanned the entire base diameter of each tumor, all treated animals (Groups 3 and 4), were successfully treated as measured by tumor volume obliteration. The sizes of primary tumors and the dates of treatment deliveries are shown in Table 2.

TABLE 2
PRIMARY TUMOR VOLUMES OF GROUP 3 & 4 RATS

Group	ID #	Day of First Treatment	Days Post Tumor Cell Injection: 27	36	40	49	53	55	63	90	121-143
			Primary Tumor Volume (cm^3)								
3	41	27	659	0	0	0	0	0	0	0	S
3	46	27	624	0	0	0	0	0	0	0	S
3	51	27	721	0	0	0	0	0	0	0	S
3	56	27	878	0	0	0	0	0	269 R	0	S
3	65	27	608	0	0	0	0	0	0	0	S
3	68	27	477	0	0	0	0	0	0	0	S
3	69	27	772	0	0	0	0	0	0	0	S
3	73	27	1089	0	0	0	0	0	0	0	S
4	43	28	723	0	0	0	0	0	0	0	S
4	44	28	876	0	0	0	0	0	0	0	S
4	49	28	559	0	0	0	0	0	0	1574 R	S
4	50	28	912	0	0	0	0	0	0	0	S
4	52	28	623	0	0	0	0	0	0	0	S
4	58	28	679	0	0	0 D					
4	59	28	799	0	0	0 D					
4	66	28	633	0	0	0	0	0	0	0	S

Primary tumor volumes of Group 3 & 4 rats as measured before and after DC ablation treatment. While tumors from control groups 1 & 2 continued to grow, this table demonstrates that DC ablation successfully treated methylcholanthrene-induced Fibrosarcomas.

Note: D = Death (possibly from hyperthermia or anesthesia issues); R = Retreatment; S = Sacrifice

Tumors disappeared from all 16 treated rats within a maximum of eight days. Retreatment was required in two animals (#56, and #49) after apparent disappearance of the original primary tumor (see Fig.3). One Group 4 rat was retreated approximately two months after the initial primary tumor disappeared, and the retreated tumor disappeared within two months; one Group 3 rat was retreated about one month following initial primary tumor disappearance, with the retreated tumor disappearing within one month.

Differences in mean tumor size between treatment and control groups were highly significant: $p < 0.0018$ at week five and $p < 0.001$ at week eight.

C. Pretreatment

Pretreatment was successfully applied to the primary tumors within the Group 4 rats. Pretreatment was not applied to the contralateral tumors. Pretreatment appeared to have no effect on tumor disappearance or subject survival.

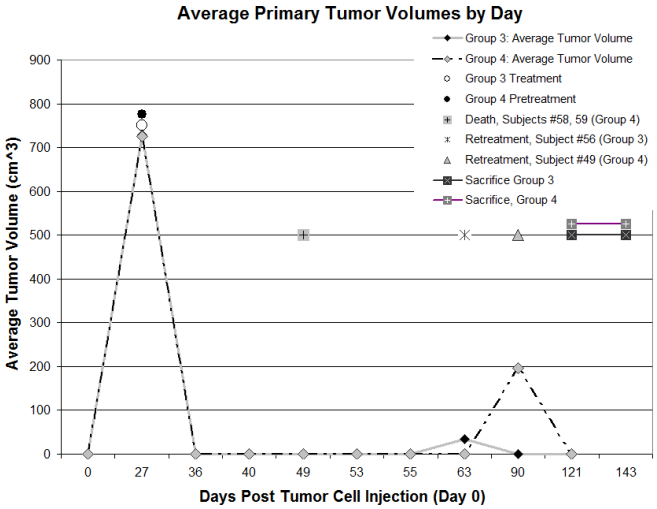

Fig. 3. Average volumes of Group 3 & 4 primary tumors over the course of the study. Methylcholanthrene-induced Fibrosarcoma cells were injected on day 0. Group 3 animals were treated on day 27; Group 4 animals were pretreated on day 27 with the high-dose treatment following one day later. Death, retreatment, and sacrifice events are also indicated.

D. Contralateral Tumor Growth

Overall, Group 3 (high-current treatment) rats developed significantly smaller contralateral tumors than Group 4 (pretreatment) or Group 1 and 2 (control) animals. This pattern was particularly apparent in the second week of tumor growth, during which the contralateral tumors of Group 3 rats were smaller than those of controls, and even smaller than those of Group 4 animals.

TABLE 3
CONTRALATERAL TUMOR VOLUMES OF GROUP 3 & 4 RATS

Group	ID #	Day of First Treatment	Days Post Tumor Cell Injection: 14	23	27	29	37	64	92-114
			Contralateral Tumor Volume (cm^3)						
3	41	23	140	353	0	0	0	0	S
3	46	23	218	332	0	0	0	0	S
3	51	23	157	312	0	0	0	0	S
3	56	23	223	507	1453	1456 R	0	0	S
3	65	NA	0	0	0	0	0	0	S
3	68	23	81	297	0	0	0	0	S
3	69	24	206	371	0	0	0	0	S
3	73	NA	0	0	0	0	0	0	S
4	43	23	265	933	0	0	0	0	S
4	44	NA	114	0	0	0	0	0	S
4	49	23	83	458	0	0	0	0	S
4	50	23	236	577	0	0	0	0	S
4	52	23	274	537	0	0	0	0	S
4	58	23	383	1144 D					
4	59	23	0	130 D					
4	66	23	137	1098	0	0	0	0	S

The average size of the contralateral tumors was smaller than that of the primary tumors. This is suspected to be due to the role of the immune system following treatment of the primary tumor. This was evaluated by measuring and comparing contralateral tumor growth to primary tumor growth.

Note: D = Death (possibly from hyperthermia or anesthesia issues); R = Retreatment; S = Sacrifice

Specifically, of the 16 animals in the two treatment groups: contralateral tumors disappeared within four days of treatment in ten subjects; contralateral tumors failed to develop in three animals (one in Group 4 and two in Group 3); one Group 3 rat required retreatment one week after initial treatment, with the tumor disappearing about a week later; and two Group 4 animals died upon initial contralateral

tumor treatment. This could be due to hyperthermia, anesthetic overdose, or stress due to excessive handling. (See Table 3 and Fig.4).

At eight weeks (the point at which control animals were sacrificed), mean contralateral tumor size was significantly smaller ($p < 0.003$) in the treatment group.

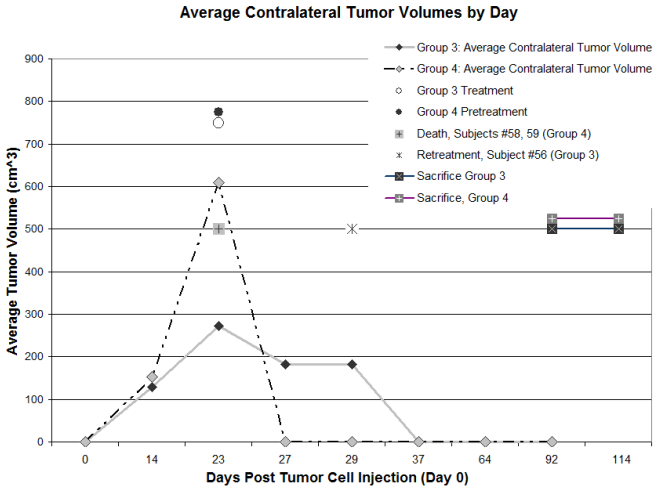

Fig. 4. Average volumes of Group 3 & 4 contralateral tumors over the course of the study. Death, retreatment, and sacrifice events are also indicated.

E. Resistance

In general, electrical resistance had an initial increase upon treatment commencement, but later declined with increasing exposure to stimulation. Electrical resistance during pretreatment of Group 4 animals was much higher than during high-current treatment. This may be due to the smaller electrode surface area of the pretreatment electrodes.

F. Survival

All Group 3 animals survived the treatment; however, there was some mortality in Group 4 animals: two rats died immediately following pretreatment (to compensate for the loss, two rats from Group 2 that had not yet received the placebo treatment were transferred to Group 4); and two rats (subjects #58 and #59) died following initial treatment of the contralateral tumor (one at the end of treatment and one about ten minutes after treatment). This can be seen in *Tables 1 and 2*.

IV. DISCUSSION

In this study, DC ablation (80 C of charge, at a constant current of 20 mA, over a period of 66 minutes, delivered via multiple electrodes implanted so as to "skewer" the base of the tumor) was essentially 100% effective in eliminating MCA-R tumors in rats. The treatment presumably created a necrotic zone, and the tumors subsequently disappeared.

With a total of 26 animals in this study, four died; not necessarily due to DC ablation treatment. Four animals from Group 4 (pretreatment plus treatment) died—two of which died upon pretreatment (these were replaced by two animals from Group 2); and the other two upon initial high-current treatment of the contralateral tumor. Based on the lack of other mortality or significant morbidity associated with treatment, and the fact that these deaths occurred in close temporal proximity, we attribute these deaths to variations in operative technique, possibly related to hyperthermia, anesthetic overdose, or animal stress due to excessive handling.

Low-current pretreatment did not appear to be beneficial in terms of primary tumor disappearance or survival rate. In fact, judging by the apparent impact on contralateral tumor growth, it appears that the low-current pretreatment was actually associated with more rapid tumor growth. While this seems unlikely, it is possible that pretreatment stimulated contralateral tumor growth. However, the more rapid growth of contralateral tumors in pretreated animals might have been related to a delay in high-current treatment, since Group 3 rats received their primary high-current treatment when their tumors reached 700 mm^3, while Group 4 animals received the pretreatment at that point, with primary high-current treatment coming one day later. Pretreatment did however, appear to alter electrical resistance in such a way as to reduce the variability in resistance for subsequent treatments, and thus may provide insights necessary to continue refining this therapy.

Finally, with the absence of contralateral tumor development in three treated animals (two in Group 3 and one in Group 4), this study provided some evidence consistent with the possibility of immune system activation following DC ablation. This potential is also supported by the generally slower growth of contralateral tumors than primary tumors in this study. Although identification and analysis of immunologic mechanisms was beyond the scope of this study, the production of chlorine and hypochlorous acid by DC ablation may be important. Berendson et al. have identified chlorine as an important by-product of DC stimulation [12], and Chiang et al. have recently shown that ovarian cancer cells killed by oxidation with hypochlorous acid are taken up by dendritic cells stimulated by tumor-specific T cell responses [14]. This area in particular merits further study.

We relied heavily on the work of Chou et al. [15] and Ren et al. [16] in designing this study, particularly in the areas of electrode placement and dosage, although both of these studies employed constant-voltage stimulation. In a group of 24 female Fisher 344 rats with MCA-R tumors, and using multiple treatments for recurrent tumors, Chou et al. achieved 75% six-month survival. The ablation procedure employed six or seven electrodes inserted at the base of the tumor, alternating anode and cathode. A mean charge of 147.2 C was delivered over a mean of 120.21 minutes, at a mean current of 21.69 mA. While the authors did not provide specific information on inter-electrode spacing, they concluded that the effectiveness of the procedure is dependent on electrode spacing and dosage.

Similarly, in a group of 120 female Fisher 344 rats injected with MTF-7 rat breast cancer cells, Ren et al. [16] reached tumor control rates above 70% in animals treated with 80 and 100 C. Tumor control rates increased with increasing dosage, although no significant change in tumor

control was associated with differences in electrode spacing. This study employed electrodes inserted at the base of the tumor, at separations of 3.0, 5.0 and 10.0 mm, and in alternating anode-cathode order. A constant voltage (8.0V) was delivered until target levels of 0, 40, 60, 80 and 100 C were delivered.

Our study builds on the success of past approaches. Taken together, these investigations may provide guidance for further research in this area in terms of electrode placement and dose ranges that appear most effective.

We are unfamiliar with any other studies that report the pattern of electrical resistance during DC ablation. This information may be useful in the design of therapeutic stimulation devices to deliver DC ablation therapy. To better understand resistance, further study should be conducted.

V. CONCLUSION

Direct current ablation via electrodes implanted so as to skewer the deepest boundaries of the induced fibrosarcoma provides highly effective tumor destruction in the rat model. This benefit was seen with a single DC ablation treatment. In addition, there was evidence that the treatment was associated with a remote effect that may involve the immune system. Low-energy pretreatment was not beneficial in terms of primary tumor disappearance or animal survival. Further study of DC ablation as a therapeutic modality for tumors should be carried out to better understand the properties that will most optimally deliver therapy.

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