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Treatment of hepatocellular carcinoma in a rat model using electrochemotherapy

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Abstract

The effectiveness of antineoplastic agents has been augmented by applying pulsed electric fields directly to tumours after the administration of the drug. This treatment, known as electrochemotherapy (ECT), has been successful for cutaneous malignancies in animal models and in recent clinical trials. This study was aimed at investigating the applicability of ECT in a surgical setting for hepatocellular carcinomas induced in the livers of rats. Established tumours were injected with bleomycin, and electric pulses were then administered locally. Animals were followed based on tumour volumes and histological samples. Dose response data were obtained for both electric field intensity and bleomycin. Complete response rates for animals treated with electrochemotherapy ranged from 26.67% to 93.33 and were durable. In contrast, tumours that received no treatment, pulses only or drug only responded minimally. This supports the feasibility of using a ECT as a modality for treating hepatocellular carcinoma.

Keywords: Electrochemotherapy; Electroporation; Electropermeabilisation; Bleomycin; Hepatocellular carcinoma; Chemotherapy; Liver neoplasm

1. Introduction

Hepatocellular carcinoma is estimated to cause over 100 000 deaths per year worldwide [1]. Over 13 600 cases are diagnosed annually in the United States [2]; however, the disease is most prevalent in Southeast Asia, Southern Africa and China [3,4]. This disease is typically multifocal and associated with chronic liver disease resulting from hepatitis viruses and/or alcoholism. Eighty per cent of the patients typically present with cirrhosis [3,5] which limits hepatic reserve and the ability to undergo resection. These characteristics of the disease limit resection to approximately 20% of all patients [6].

A relatively new type of treatment has recently been applied in preclinical studies for the treatment of tumours in the liver. This type of treatment combines the use of non-cytotoxic electric pulses to facilitate the entry of chemotherapeutic agents that do not readily pass through the cell membrane into cancer cells. This treatment is called electrochemotherapy (ECT). Molecule entry is facilitated by a process known as electroporation which is a direct result of electrical treatment. When a cell has been electroporated, the barrier properties of the plasma membrane are temporarily diminished which allows non-permeant molecules to pass through the cell membrane [7,8]. ECT is typically administered by injecting a drug, systemically or locally, and then applying pulses to the tumour after allowing time for the drug to disseminate throughout the treatment site. ECT can be used intraoperatively and requires only a few minutes to perform.

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Hepatocellular carcinomas in a rat model have been treated with ECT using bleomycin as the drug. One exploratory study resulted in 69% complete responses by using ECT intraoperatively [9]; a 15.4% partial response was also achieved. A subsequent study investigated the use of other chemotherapeutic agents in the same animal model. The results indicated that bleomycin was more effective than 5-fluorouracil, doxorubicin and paclitaxel when used as part of an ECT protocol [10]. A similar study treated VX2 carcinomas induced in rabbit livers with ECT using bleomycin as the drug. Durable complete response rates of 30–50% resulted [11]. Colorectal carcinoma metastases to the liver have also been treated with ECT in a rat model with 22% complete and 78% partial response rates [12].

The initial studies that applied ECT for the treatment of liver malignancies followed development of this combination treatment from preclinical studies to the clinic for several other types of cancer. These studies have resulted in impressive responses for melanoma [13–15], sarcoma [10,16–18] and breast cancer [19] in animal models; response rates ranging from 70 to 100% were reported. Clinical trials for melanoma, basal cell carcinoma, and squamous cell carcinoma [20–26] have been conducted. Complete durable response rates of 89–100% based on individual tumours were reported for melanoma [21,24,25]. Both bleomycin and cisplatin have been delivered clinically with electric pulses in melanoma ECT trials. Similarly, a complete response rate of 94% has been reported in a basal cell carcinoma [21]. Complete response rates ranging from 42.8 to 57% have been reported for head and neck squamous cell carcinoma [22,26].

Preliminary animal studies indicate that ECT has potential as an intraoperative means for treating hepatocellular carcinomas. In addition, the effects of ECT on normal liver tissue was the main focus of a recent study in this issue of the *European Journal of Cancer*, pp. 414–421 [27]. The histological study established that several bleomycin doses and a range of electric fields have negligible effects on normal liver in a rat model. The tumour treatment data mentioned above combined with the results of the histological study motivated a larger scale and more detailed examination of the antitumour effects of ECT for primary hepatocellular carcinoma.

2. Materials and methods

2.1. Cell line and culture method

N1S1 rat hepatocellular carcinoma cells (ATCC CRL-1604; American Type Culture Collection, Rockville, MD, USA) were grown in Swimms S-77 medium supplemented with 4 mM L-glutamine, 0.01% Pluronic F68, 9% fetal bovine serum, and 90 μg/ml gentamycin. Cells were maintained in a humidified atmosphere that contained 5% CO_2. Cells used in this study were greater than 95% viable based on the trypan blue exclusion dye method.

2.2. Animals and anaesthesia

Male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) 7–8 weeks old (250 g) were used for this study. Animals were housed and cared for according to National Institutes of Health (NIH) guidelines. All methods for this study were approved by the University of South Florida Institutional Animal Care and Use Committee (IACUC). Procedures were conducted with animals that were under general anaesthesia using 3% isoflurane (Mallinckrodt Veterinary, Mundelein, IL, USA) in oxygen.

2.3. Tumour induction

The right median lobe of male Sprague Dawley rats was surgically exposed and injected with 1.5×10^6 viable N1S1 cells suspended in 0.05 ml of saline. Animals were then closed using surgical staples. Tumours were allowed to grow for 8 days to a size of approximately 100 mm^3.

2.4. Tumour treatment

ECT was performed by injecting bleomycin (Bristol-Myers Squibb, Princeton, NJ, USA) directly into the tumours. Hepatomas received a 100 μl injection of the drug dissolved in saline. Bleomycin doses were an experimental variable for hepatoma treatment. Tumours that did not receive bleomycin were injected with a 100 μl dose of saline as a sham treatment.

Electric pulses were administered 2 min after the intratumour injection of bleomycin or saline. Electric pulses were administered by inserting a circular array of six needle electrodes (1 cm diameter, Genetronics, Inc., San Diego, CA, USA) to an appropriate depth (3–5 mm) into the tissue immediately surrounding tumours so that the neoplasm was contained within the volume delineated by the needles. Six 99 μs electric pulses were delivered via the inserted needle electrodes in a manner that rotated the applied field around the treatment site [9,28] using a DC generator (BTX T820) and mechanical switch. The nominal applied field strengths (voltage to electrode spacing ratio) of the pulses were experimental variables that ranged from 500 to 1500 V/cm. The voltage of each applied pulse was monitored using a digital storage oscilloscope (PM3394A, Fluke Corporation, Palatine, IL, USA). The electrode was inserted into the tumour-bearing livers of animals that were not scheduled to receive pulses as a sham electrical treatment. Insertion was conducted in a manner identical to those tumours that received pulses.

2.5. Posttreatment monitoring

Animals were surgically explored at days 14, 28, 56 and 100 after treatment. Tumour volumes were determined on these days based on three mutually orthogonal tumour dimensions (a, b and c) using the formula $V = abc\Pi/6$. Measurements were made using a digital Vernier caliper. Responses to ECT treatment were determined based on tumour volume reduction relative to the tumour size that was measured immediately prior to treatment. A complete response (CR) was defined as no visible tumour evident. Greater than 50% reduction in tumour volume was considered a partial response (PR), and less than 50% reduction in tumour volume was defined as stable disease (SD). Tumours with increased volumes were categorised as progressive disease (PD). An animal was considered cured for the purposes of this study if there was no tumour present at 100 days post-treatment.

2.6. Liver function

Blood (1 ml) was drawn from anaesthetised animals through the external jugular vein using a 1 ml syringe and 0.75 inch 28-gauge needle. Samples were immediately placed into vacutainer tubes (366387; Becton Dickinson, Franklin Lakes, NJ, USA). Blood draws were conducted at the same time of day for all animals in the study. Standard clinical laboratory methods were used to analyse plasma for albumin, total protein, lactate dehydrogenase, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase.

2.7. Histology

Treated liver lobes were harvested at 3, 7, 10, 14 and 21 days after treatment. The resulting specimens were submitted entire for histological examination. Each specimen was fixed in 10% neutral buffered formalin for less than 24 h, and sections were cut to represent the longest diameter of the tumour. Tissue samples were processed into paraffin blocks and 4 μm sections were then stained with haematoxylin and eosin using standard histological techniques (Richard-Allen Scientific, Kalamazoo, MI, USA).

Sections were coded and submitted for examination by a pathologist. The treatment sites were examined and graded for five different parameters: necrosis, apoptosis, viable tumour size, inflammation and giant cell reaction. Necrosis was expressed as a percentage of the entire tumour section; apoptosis was graded in the same manner. Both of these parameters were graded based on measurements of necrotic or apoptotic areas which were then summed and respectively expressed as a percentage of the tumour section area. Viable tumour size was expressed by measuring the longest tumour dimension in each specimen. Inflammation was subjectively scored as none, minimal and present. Giant cell reaction was scored as none, few ($\leqslant 10$), and many (> 10) per tumour section.

3. Results

3.1. Effect of changing the electric field on tumour response

Tumour-bearing animals were randomised into eight different treatment groups with five or six animals in each group in order to determine the effect of different electric field strengths on tumour response. For this phase of the study, tumours in five of the eight groups were treated with a complete ECT protocol which consisted of injection of 0.5 units of intratumour bleomycin followed by electrical treatment. A 0.5 unit bleomycin dose was used as it has been proven to be successful in preliminary ECT studies using this model [9,10] and in other tumour types [29]. Tumours within each group were treated with six 99 μs pulses that had the same field strength; however, the magnitude of the field used for each group was changed. The five groups received either 500, 750, 1000, 1250 or 1500 V/cm pulses. The three remaining groups were designated as control groups and were treated as follows: no treatment, bleomycin injection alone and treatment with 1500 V/cm field alone. This experimental design was repeated three times so that the total number of animals in each treatment group was greater than or equal to 15.

The responses from treating established tumours with 0.5 units of bleomycin followed by a range of different field strengths are shown in Table 1. The data indicate the complete response rates at days 14 and 28. On each of these follow-up days, the complete response rate increased with the applied field. The cure rate, also indicated on the table, followed a similar trend. It ranged from 33.33% for tumours treated with 500 V/cm pulses to 86.67% for 1500 V/cm pulses. In contrast, animals treated with 0.5 units of bleomycin alone or 1500 V/cm pulses alone had complete response rates of 13.33 and 0%, respectively, at day 100. One of the 15 animals that received no treatment responded completely at day 100.

3.2. Effect of changing the bleomycin dose on tumour response

Tumours were treated with a range of different intratumour bleomycin doses as part of a separate experimental scheme that was similar to the one described above. Animals were randomised into nine different groups. Six groups were treated with bleomycin doses of

Table 1
Responses of hepatocellular carcinomas to electrochemotherapy using 0.5 units bleomycin followed by pulses with a range of field strengths

Treatment[a]	Bleomycin dose (U)/ field strength (V/cm)	Initial mean tumour volume (mm^3)	*n*	Day 14					Day 28					Day 100	
				%PD[b]	%SD[c]	%PR[d]	%CR[e]	*n*	%PD[b]	%SD[c]	%PR[d]	%CR[e]	*n*	%Cure[f]	*n*
D−E−	−/−	111.13	15	100	0	0	0	15	93.33	0	0	6.67	15[g]	6.67	15
D+E−	0.5/−	97.37	15	86.67	6.67	0	6.67	15	86.67	0	0	13.33	15	13.33	15
D-E+	−/1500	59.59	15	100	0	0	0	15	100	0	0	0	15[h]	0	15
D+E+	0.5/500	108.63	15	60.00	6.67	6.67	26.67	15	73.33	0	0	26.67	15	33.33	15
D+E+	0.5/750	93.2	15	40.00	13.33	13.33	33.33	15	46.67	0	20.00	33.33	15	60.00	15
D+E+	0.5/1000	92.12	15	6.67	6.67	20.00	66.67	15	42.86	0	0	57.14	14[i]	57.14	14
D+E+	0.5/1250	95.97	16	6.25	5.88	25.00	62.5	16	33.33	0	0	66.67	15[j]	73.33	15
D+E+	0.5/1500	82.44	15	20.00	6.67	13.33	60.00	15	26.67	0	0	73.33	15	86.67	15

[a] Each D+ animal received 0.5 units of bleomycin; each E+ animal received six 99 μs pulses at the nominal electric field strength noted (V/cm).
[b] PD, progressive disease = tumour increasing in size compared with day 0.
[c] SD, stable disease = tumour decreased less than 50% in size compared with day 0.
[d] PR, partial response = tumour decreased in size more than 50% compared with day 0.
[e] CR, complete response, no tumour present.
[f] Cure = no evidence of tumour 100 days after treatment.
[g] One PD animal died on day 28. Data reflect inclusion of the animal in day 28 and cure determinations as a PD.
[h] One PD animal died on day 20. Data reflect inclusion of the animal in day 28 and cure determinations as a PD.
[i] One PR animal died on day 16. Data reflect exclusion of the animal from day 28 and cure.
[j] One PR animal died on day 20. Data reflect exclusion of the animal from day 28 and cure.

0.25, 0.5, 0.75, 1.0, 1.25 or 1.5 units/tumor followed by 1250 V/cm electric pulses. Fields of this magnitude were selected because they showed very high complete response and cure rates when used with a 0.5 unit dose of bleomycin (Table 1). Previous research [27] also indicated that 1250 V/cm pulses combined with bleomycin had negligible adverse histological effects on normal liver in this same model; however, 1500 V/cm and higher fields resulted in detectable necrosis 14 days after treatment. The three other groups in this phase of the research were designated as control groups and received no treatment, 1.5 units of bleomycin alone, or 1250 V/cm pulses alone.

Tumour responses for the six different bleomycin dose groups that received 1250 V/cm pulses are shown in Table 2. Day 14 and 28 complete response data for those animals treated with the drug and pulses ranged from 53.33 to 93.33% with an increasing complete response trend as the drug dose was increased. Cure rates for this phase of the experimental work were approximately 80% (range 73.33–86.67%)for all groups of animals treated with 0.5 units of bleomycin and above. The variation in these groups was 1 out of 15 animals.

3.3. Histological and antitumour effects of optimal ECT treatment parameters

Optimised ECT treatment parameters were identified and used to extend the investigation of ECT antitumour effects. In addition, post-treatment histological events and liver function were examined. Animals were randomised into three designated control groups and one ECT treatment group with five to six animals in each group. The randomised animals received no treatment, 1250 V/cm pulses only, 1.0 unit of bleomycin only, or 1.0 unit of bleomycin followed by 1250 V/cm pulses. Four replicates of this experiment were performed so that, upon pooling the data, at least 23 animals were treated within each group. Although the cure rates for bleomycin doses ranging from 0.5 to 1.5 units combined with 1250 V/cm pulses were very similar, a 1.0 unit dose of the drug was selected because it provided a response that was far more rapid than 0.5 units (see Table 2). For example, the day 14 complete response rate for 0.5 units was 46.67% compared with 73.33% for the 1.0 unit dose.

Table 3 shows the responses of tumours treated with the optimised ECT protocol. Day 14 and 28 complete responses for the animals treated with bleomycin and pulses were 79.17 and 87.50%, respectively. Objective responses at these same time points were nearly identical. The cure rate was 100%.

A final experiment was conducted using a 1.0 unit dose of bleomycin followed by 1250 V/cm pulses. The same groups as indicated in Table 3 were treated using 15 animals per group. Three animals from each treatment group were humanely euthanised at 3, 7, 10, 14 and 21 days after treatment. The tumour and surrounding liver tissue were excised and histologically examined. Fig. 1 shows the mean percentage of necrosis and/or apoptosis in tumour sections for each group at each time point. The percentage of necrotic tumour in each sample that was treated with the drug and pulses was 60% on day 3, 81.7% on day 7, 90% on day 10, 95% on day 14 and 100% for day 21. When apoptosis

Table 2
Responses of hepatocellular carcinomas to electrochemotherapy using a range of bleomycin doses followed by 1250 V/cm pulses

Treatment[a]	Bleomycin dose (U)/ field strength (V/cm)[a]	Initial mean tumour volume (mm^3)	n	Day 14					Day 28					Day 100	
				%PD[b]	%SD[c]	%PR[e]	%CR[d]	n	%PD[b]	%SD[c]	%PR[d]	%CR[e]	n	%Cure[f]	n
D−E−	−/−	114.70	14	100	0	0	0	13	100	0	0	0	13	15.38	13
D+E−	1.5/−	114.22	15	100	0	0	0	15	100	0	0	0	15[g]	26.67	15
D−E+	−/1250	118.54	15	93.33	6.67	0	0	15	86.67	6.67	0	6.67	15[h]	40.00	15
D+E+	0.25/1250	124.95	15	20.00	26.67	0	53.33	15	33.33	6.67	6.67	53.33	15	66.67	15
D+E+	0.50/1250	120.75	15	33.33	6.67	13.13	46.67	15	33.33	0	0	66.67	15[i]	86.67	15
D+E+	0.75/1250	136.61	15	20.00	13.33	6.67	60.00	15	13.33	6.67	0	80.00	15[j]	73.33	15
D+E+	1.00/1250	127.24	15	20.00	6.67	0	73.33	15	20.00	6.67	0	73.33	15	80.00	15
D+E+	1.25/1250	113.02	15	13.33	20.00	0	66.67	15	13.33	0	0	86.67	15	80.00	15
D+E+	1.50/1250	105.51	15	0	6.67	0	93.33	15	0	6.67	0	93.33	15	80.00	15

[a] Each D+ animal received the bleomycin dose noted; each E+ animal received six 99 μs pulses at a nominal electric field strength of 1250 V/cm.
[b] PD, progressive disease = tumour increased in size compared with day 0.
[c] SD, stable disease = tumour decreased less than 50% compared with day 0.
[d] PR, partial response = tumour decreased in size more than 50% compared with day 0.
[e] CR, complete response = no tumour present.
[f] Cure = no evidence of tumour 100 days after treatment.
[g] One PD animal with a large tumour died on day 19. Data reflect inclusion of the animal in day 28 and cure determinations as a PD.
[h] One PD animal with a large tumour died before day 28. Data reflect inclusion of the animal in day 28 and cure determinations as a PD.
[i] One PD animal was euthanised on day 19 due to paralysis. Data reflect inclusion of the animal in day 28 and cure determinations as a PD.
[j] One PD animal died on day 22. Had tumour and metastatic tumours. Data reflect inclusion of the animal in day 28 and cure determinations as a PD.

and necrosis were considered together, it became evident that 3 days after treatment almost all of the tumours had been eliminated. In these samples only a few residual tumour cells could be histologically identified. Animals in the three control groups had considerably lower levels of necrosis than the group that received ECT. This was especially true for post-treatment times of up to 14 days with the exception of one highly necrotic tumour from the day 3 samples that were treated with drug alone. This tumour increased the mean necrosis to approximately 35%. Tumours in the control groups occasionally had inflammation present, and giant cells were few. In contrast, inflammation was present in all samples that received drug and pulses, and the number of giant cells was many in all day 7, 10, 14 and 21 samples.

The size of viable tumours in animals that received no treatment, pulses alone, or drug alone increased with increasing time during the 21 day histological portion of this experiment. It was also noted that the percentage of apoptotic cells in these tumours ranged from about 10 to 40% for all time points up to and including day 14 and was not dependent on the treatment received or the absence of treatment. In contrast, viable tumour size from the group of animals that received the optimised ECT protocol was markedly reduced beginning at day

Table 3
Responses of hepatocellular carcinomas to optimised electrochemotherapy

Treatment[a]	Bleomycin dose (U)/ field strength (V/cm)[a]	Initial mean tumour volume (mm^3)	n	Day 14					Day 28					Day 100	
				%PD[b]	%SD[c]	%PR[d]	%CR[e]	n	%PD[b]	%SD[c]	%PR[d]	%CR[e]	n	%Cure[f]	n
D−E−	−/−	98.09	24	95.83	0	4.17	0	24	95.83	0	4.17	0	24	8.33	24
D+E−	1.0/0	110.04	24	83.33	4.17	4.17	8.33	24	65.25	8.70	4.35	21.74	23[g]	26.10	23
D−E+	−/1250	103.86	23	86.96	0	8.70	4.17	23	91.30	0	0	8.70	23	8.70	23
D+E+	1.0/1250	104.46	24	8.33	4.17	8.33	79.17	24	12.50	0	0	87.50	24	100.00	24

[a] Each D+ animal received a 1.0 unit bleomycin dose; each E+ animal received six 99 μs pulses at a nominal electric field strength of 1250 V/cm.
[b] PD, progressive disease = tumour increased in size compared with day 0.
[c] SD, stable disease = tumour decreased less than 50% in size compared with day 0.
[d] PR, partial response = tumour decreased in size more than 50% compared with day 0.
[e] CR, complete response = no tumour present.
[f] Cure = no evidence of tumour 100 days after treatment.
[g] One PR animal died on day 17. Data reflect exclusion of the animal from day 28 and cure determinations.

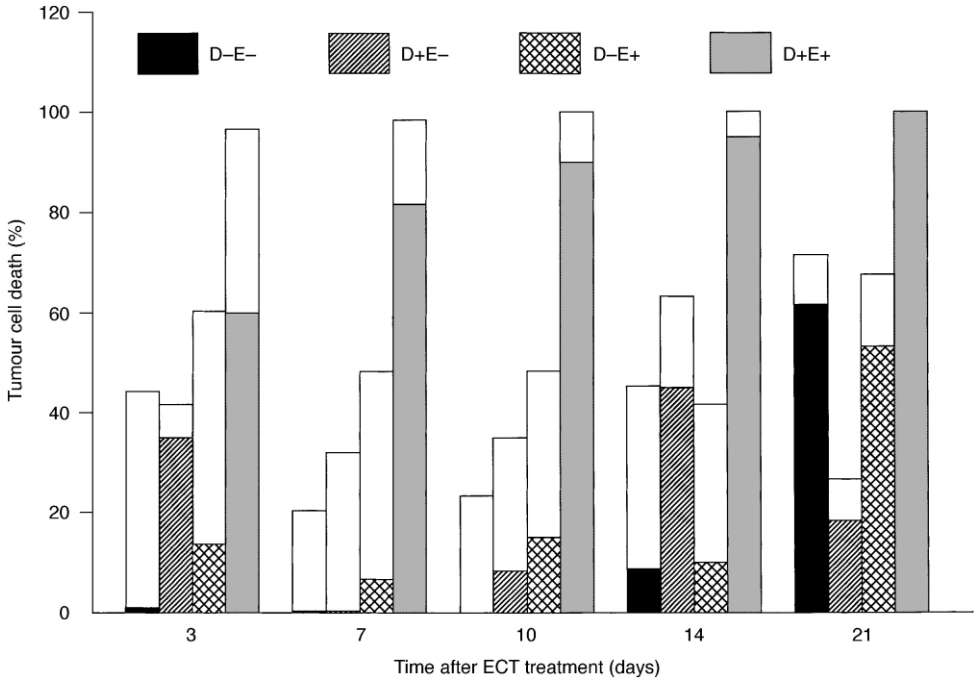

Fig. 1. Tumour cell death in hepatocellular carcinomas as a function of time after ECT with a 1.0 unit dose of bleomycin followed by 1250 V/cm electric pulses. Lower bars represent the mean necrosis present in three tumours for a particular treatment. Solid bars represent no treatment (D−E−), thatched bars represent bleomycin administration only (D+E−), cross-patterned bars indicate electrical treatment only (D−E+), and shaded bars represent animals treated with both drug and electric pulses (D+E+). Each unshaded bar represents the mean apoptosis present in the same three samples, i.e. this proportion decreases with time. Standard deviations for the D+E+ necrosis and apoptosis data ranged from 15 to 33% of the respective means for days 3, 7 and 10. These standard deviations were 5–10% of their respective means for day 14 data. All D+E+ samples were 100% necrotic at day 21. In contrast, the majority of the standard deviations from the D−E−, D−E+, and D+E+ treatment groups fell within the range of 35 to 150% of their means.

3. Fig. 2 shows examples of the necrosis and apoptosis in these tumours and provides details about the criteria used to evaluate these effects.

Blood was drawn from three animals from each group on days 0, 1, 3, 7, 10, 14 and 21 of experiment. The same three animals were sampled at each time point. No changes in albumin, total protein, alkaline phosphatase, lactate dehydrogenase and total bilirubin were observed. However, there were transient increases in aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase at day 1 post-treatment in groups treated with drug alone, pulses alone and drug followed by pulses. Enzyme levels were compared with the group that did not receive treatment; these animals were surgically explored to obtain tumour measurements, an intratumour saline injection was administered as a sham drug dose, and the electrode was inserted with no pulses applied. This comparison group had moderately elevated enzyme levels compared with baseline. However, the treatment groups had enzyme levels that were 2–3 times greater than the untreated group; thus, bleomycin administration and/or electric pulses resulted in 2–3-fold increases. All groups returned to baseline levels by day 7 and remained there for the duration of the experiment. Similar transient increases in these enzyme levels were noted when normal liver tissue was treated with ECT, bleomycin or electric pulses [27].

4. Discussion

This study demonstrates the efficacy of ECT for hepatocellular carcinoma. Animals treated with a 0.5 unit intratumour bleomycin dose followed by fields ranging from 500 to 1500 V/cm had complete response rates that ranged from 26.67 to 73.33% in a field strength-dependent manner. These complete response rates were durable; cure rates were similar to day 28 complete responses. Thus, efficacy is strongly dependent on the field strength chosen for treatment.

Complete ECT protocols for hepatocellular carcinomas with different drug doses followed by 1250 V/cm electric pulses produced very high complete response rates that were durable. Cure rates were approximately 80% over the range of 0.5 to 1.5 units of bleomycin per tumour which indicated that the cure rate was not dependent on the drug dose in this range. However, faster responses were obtained for tumours that were treated with higher, 1.0–1.5 units of drug.

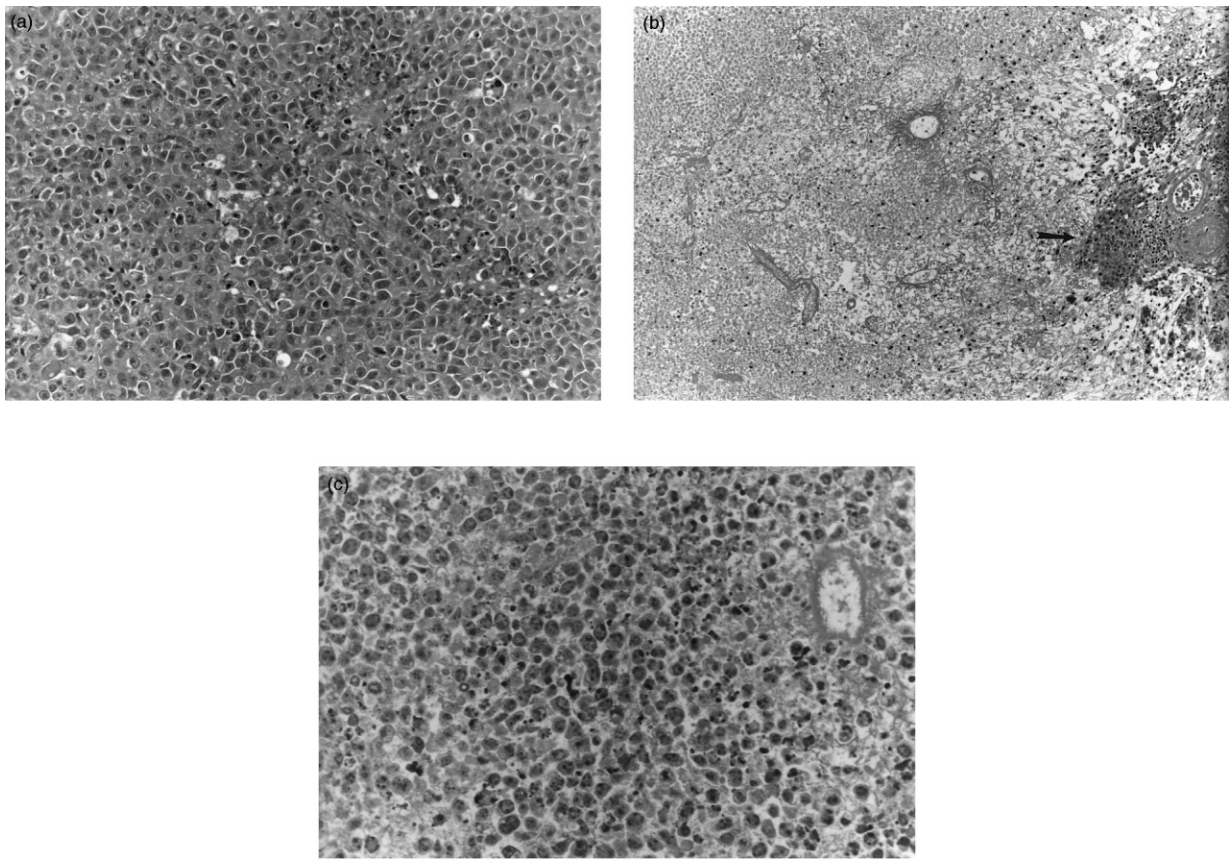

Fig. 2. Histological appearance of tumours treated with 1.0 unit of bleomycin followed by 1250 V/cm pulses. Tumours were excised and processed. (a) Untreated specimen showing mostly viable tumour and minimal apoptosis and is representative of all time points. This specimen was also representative of observations from samples treated with drug only and pulses only. (b) Specimen treated with bleomycin followed by pulses showing extensive coagulative necrosis 7 days post-treatment with only a small focus of residual viable tumour (see arrow) around a blood vessel. (c) Apoptosis, when present, was characterised by cells undergoing cytoplasmic blebbing, pyknosis, and karyorrhexis.

A 1.0 unit bleomycin dose followed by 1250 V/cm pulses was identified as the optimal antitumour therapy. Higher drug doses yielded nearly identical antitumour effects; however, 1250 V/cm and 1.0 unit of bleomycin were determined in a previous study [28] to have negligible effects on normal liver tissue when each was administered alone. In addition, this same study concluded that this field strength combined with a similar bleomycin dose had negligible adverse histological effects on normal liver in this model. At day 100 after optimised ECT, all of the tumours underwent complete regression when these optimal parameters were used for tumour treatment. This result is slightly better than the result obtained with the same conditions during optimisation experiments, but was in agreement with the histological findings which indicated that 98% of the ECT treated tumour cells died by either necrosis or apoptosis at 1 week post-treatment. Longer follow-up revealed that 100% of the tumour cells had been eliminated from day 10 onward; this death was largely observed as necrosis. These data confirmed the complete responses and also indicated that a rapid cytoreductive effect is a result of ECT. In contrast, animals that received only one treatment component or no treatment had tumours that increased in size. Thus, both drug and electric field components are required for efficacious treatment.

Several tumours in this study regressed without treatment. This regression was primarily noted to take place between days 28 and 100. Tumour immunogenicity is one likely explanation for this effect. A higher number of tumours that received only electrical treatment also regressed in this same time frame. Although the electric fields used in this study were not intense enough to cause measurable tumour destruction, it is possible that enough tumour cells were irreversibly electroporated (lysed) to enhance the immune response by means of antigen release. When combining all data from Tables 2 and 3, electrical treatment alone (1250 V/cm) resulted in an additional 13% of the animals being cured relative to the day 28 complete responses. Similarly, an additional 10% of the tumours treated with 1.0 unit of bleomycin

and 1250 V/cm were cures relative to the day 28 CR rate. The histological portion of this study indicated that the effects of ECT are rapid. Therefore, this delayed response should be evaluated in future studies to distinguish if a different mechanism, such as the immune system, is responsible. Although this study was not designed to investigate immunological effects of ECT, other studies have focused on this topic and found that the ECT does increase immune response to tumours which, in turn, can be exploited with immunotherapy to increase response rates [30,31].

All of the animals in this study tolerated the treatment well. No adverse events occurred during the administration of ECT to the animals. Liver dysfunction was minimal and temporary after ECT treatment. Levels of aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase were elevated 1 day post-treatment when ECT was administered using the optimal conditions. However, a similar degree of elevation was present in animals that received partial treatment in the form of drug or pulses. These enzymes reverted back to normal levels by 7 days post-treatment and remained there. Albumin, total bilirubin, total protein, lactate dehydrogenase and alkaline phosphatase levels were unaltered in animals that received a partial or complete ECT treatment.

In conclusion, tumour responses and histological data from this study demonstrate that electrochemotherapy is effective for hepatocellular carcinoma and can be performed during surgery using conditions that have previously been shown not to adversely effect normal liver. The high complete response data obtained in this study are in good agreement with data from other ECT studies, clinical and animal, that focused on cutaneous/subcutaneous tumours. Response rates in this study were considerably higher than those in other studies that have used ECT intraoperatively. One factor in this was probably the extensive characterisation of the bleomycin doses and field strengths necessary to obtain these high response rates. This study also reinforces the observation that bleomycin, a highly cytotoxic but non-permeant molecule, is a very effective anticancer agent when combined with electroporation *in vivo*. Thus, an optimal ECT protocol is an efficient means for circumventing the usual resistance to bleomycin entry imposed by the cell membrane.

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