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Effects of electrochemotherapy with bleomycin on normal liver tissue in a rat model

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Abstract

Preliminary studies that used electric pulses *in vivo* to facilitate entry of chemotherapeutic agents into tumour cells resulted in a 69% complete response rate for hepatocellular carcinoma in rats. This success motivated a focused investigation to define the adverse effects of this treatment on normal liver tissue. Bleomycin doses ranging from 0.5 to 2.5 U and electric fields from 500 to 2250 V/cm were investigated. Electrical treatment was administered using an array of six needles arranged in a circular pattern. Necrosis and four other histological parameters were examined 14 and 56 days after treatment. Results indicated that treatment effects were localised to the volume of treated tissue. These parameters, at both time points, were not significantly altered for liver tissue that was treated with all drug doses and electric fields of 1250 V/cm and below. Only the combination of more intense electric pulses with bleomycin produced adverse histological events in the form of localised liver necrosis at day 14. These effects were not visible at day 56. Liver function was normal through all of the treatment except for an elevation of several enzymes 1 day post-treatment.

Keywords: Electrochemotherapy; Bleomycin; Electroporation; Electropermeabilisation; Liver

1. Introduction

The combination of a chemotherapeutic agent coupled with non-cytotoxic pulsed electric fields that permeabilise cell membranes has been termed electrochemotherapy (ECT). This combination therapy has been investigated as an antitumour treatment in subcutaneous tumour models for melanoma [1–3], sarcoma [4–6] and breast cancer [7]. All of these studies have shown very rapid tumour regression with complete response rates ranging from 70 to 100%. Electrochemotherapy has also been reported to preserve tissue function [5]. It has been established in preclinical studies that the treatment can be administered several times [8] for highly aggressive tumours or for large masses [5]. A number of different single chemotherapeutic agents have been tested as part of ECT protocols. These studies have revealed that bleomycin is highly effective when combined with electric pulses.

The underlying mechanism of electrochemotherapy is physical in nature. Pulsed electric fields applied to cells induce membrane potentials that are sufficient to cause a temporary breakdown of plasma membranes [9]. This allows exogenous molecules that do not normally pass through the membrane to have access to the cell interior for a time that is in the order of minutes [10]. Thus, electrochemotherapy is typically carried out by first administering a drug directly to the tumour [2,11], systemically [1,4] or intramuscularly [3,7]. Electric pulses are typically delivered directly to the tumour after the

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drug has reached and/or disseminated throughout the tumour.

Animal model results have led to clinical trials for cutaneous malignancies. Trials for melanoma, basal cell carcinoma and head and neck squamous cell carcinoma have been successfully completed [12–19]. Non-cutaneous head and neck cancers have also been treated in a recent clinical trial [20]. Complete durable response rates of 89–100% are attainable for melanoma using optimised protocols [13,16,17,19]. Both bleomycin and cisplatin have been used in melanoma ECT trials. Similarly, a complete response rate of 94% has been reported in a basal cell carcinoma [13]. Complete response rates ranging from 42.8 to 57% have been reported for cutaneous head and neck squamous cell carcinomas [14,18]. Treatment of non-cutaneous head and neck cancer resulted in a 50% complete response rate [20].

The successful clinical ECT studies suggest that there are a number of other malignancies that could be amenable to treatment with ECT. One of these malignancies is hepatocellular carcinoma. Over 13 600 cases are diagnosed annually in the United States (US) [21]. On a global scale, this disease is estimated to cause over 100 000 deaths per year worldwide [22]. Hepatocellular carcinoma is particularly prevalent in Southeast Asia and Southern Africa [23].

Hepatocellular carcinoma is commonly associated with chronic liver disease resulting from hepatitis viruses and alcoholism. Thus, 80% of the patients typically present with cirrhosis [24] which limits the hepatic reserve and ability to undergo resection. The multifocal nature of this disease coupled with the associated cirrhosis limits resection to approximately 20% of all patients [25].

Recently, a study that treated rat hepatocellular carcinomas with ECT was reported [26]. The intraoperative animal study used bleomycin followed by electric pulses and resulted in 69% complete and 15.4% partial responses. Subsequent studies in the same animal model indicated that ECT with bleomycin was more effective than with 5-fluorouracil, doxorubicin and paclitaxel [5]. Similarly, VX2 carcinomas induced in rabbit livers have been treated with standard electrochemotherapy using bleomycin which resulted in 30–50% durable complete responses [27]. Treatment of colorectal metastases in the liver using electric pulses combined with bleomycin resulted in a 22% complete response rate and a 78% partial response rate [28] in a rat model.

Preliminary animal studies indicate that ECT has potential as an intraoperative means to treat hepatocellular carcinoma. However, before further investigation of the effects of ECT on hepatocellular carcinoma it was necessary to elucidate the effects of this treatment on normal liver tissue. This is an important consideration for patients that have hepatocellular carcinoma. It is also critical to determine the effect of ECT on normal liver as a fraction of patients with this disease have a compromised hepatic reserve due to cirrhosis. Therefore, a study was initiated to determine the long-term cytopathic effects of ECT with bleomycin on normal liver tissue and on its function.

2. Materials and methods

2.1. Animals and anaesthesia

Seven- to eight-week old male Sprague–Dawley rats (weighing approximately 250 g) were used in this study (Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA). Animals were housed and cared for according to National Institute of Health (NIH) guidelines. The methods for this study were approved by the University of South Florida Institutional Animal Care and Use Committee (IACUC). All procedures were conducted with animals that were under general anaesthesia using 3% isoflurane (Mallinckrodt Veterinary, Mundelein, IL, USA).

2.2. Electrode and electrical treatment

An array of needles was used to deliver electric pulses for this study [29]. The array was comprised of six 28-gauge needles that were equispaced around the circumference of a 1 cm diameter circle (Genetronics, Inc., San Diego, CA, USA). The needles were inserted into the normal liver tissue to a depth of 5 mm. Pulses were generated by a commercially available electroporation power source (T820, Genetronics, Inc.). Each electrical treatment consisted of six 100 μs rectangular direct current pulses that were initiated at a rate of 1 pulse per s. Pulses were routed through a mechanical switch (Genetronics, Inc.) that rotated the applied field around the treatment site [29]. The magnitude of the applied electric field was an experimental variable.

2.3. Treatment of normal liver tissue

The median hepatic lobe was used for all aspects of this study. An open laparotomy was first performed to expose the liver. Then a 100 μl injection of either bleomycin or normal saline was administered at a single location in the central region of the lobe. Bleomycin dose was an experimental variable; however, the volume of injection was a constant. This injection method simulated the direct intratumour injection that has been used successfully with ECT for the treatment of hepatocellular carcinomas [26], colorectal metastases in the liver [28] and for cutaneous tumours [11,13]. The electrode was inserted 2 min after injection. Pulses were then immediately administered for animals scheduled to receive electrical treatment; the electrode was inserted

for an appropriate time as a sham electrical treatment. Rats were closed using surgical staples immediately after treatment.

2.4. Blood sampling and liver function assays

Blood (1 ml) was drawn from three anaesthetised animals through the external jugular vein and immediately placed into vacutainer tubes (366387; Becton Dickinson, Franklin Lakes, NJ, USA). Blood draws were conducted at the same time of day and for the same three animals in each treatment group. 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.

2.5. Histological analysis

Three liver lobes were harvested at either 14 and 56 days after treatment. The injection and/or treatment site on each lobe was inked before harvesting based on landmark measurements. Then, entire specimens were submitted for histological examination. Each specimen was fixed in 10% neutral buffered formalin for not more than 24 h. Each lobe was then cut in half longitudinally and was submitted for histology. One half of each specimen was cut cross-sectionally, and the other half was sectioned longitudinally. This method allowed visualisation of the grossly identifiable, but normally small needle-marks resulting from electrode insertion and electrical treatment. At least three sections were cut from each specimen half. All sections were stained with haematoxylin and eosin (Richard-Allen Scientific, Kalamaazoo, MI, USA).

Sections were coded and submitted for microscopic examination by a pathologist. The treatment sites were examined and graded for six different parameters. Necrosis was graded on a scale from 1 to 3 based on the size of the necrotic area within the volume of tissue treated as follows: grade 1 = 0–0.5 mm^2; grade 2 = 0.6–1.0 mm^2; and grade 3 = > 1.0 mm^2. Measurements from multiple areas of necrosis were summed to obtain an overall score for the specimen. Scarring was graded from 0 to 3 which corresponded to none, mild, moderate and severe. Acute and/or chronic inflammation was stratified into a numeric scale where: 0 = none, 1 = mild, 2 = moderate and 3 = severe. The presence or absence of giant cell reaction was described in the following manner: 0 = none, 1 = rare, 2 = greater than 10 per mm^2 of treatment area and 3 = greater than 30 per mm^2 of treatment area. The presence or absence of biliary proliferation within the treatment site was also determined. Changes in vessels were noted as: none, vascular proliferation, congestion if vascular dilation was noted, and granulation (type of tissue was noted). In addition, the tissue adjacent to the treatment site was examined for each sample to detect extension of the above changes to the hepatic parenchyma outside the treated area. Mean numeric values of necrosis, scarring, inflammation and giant cell reaction were computed from the sections of each treated sample. Mean values of these parameters were then computed for each group of identically treated samples.

3. Results

The results from this normal liver tissue study were stratified based on four types of experiments. The first two experiments each employed eight groups of animals to determine the impact of the drug alone and the electric fields alone on normal liver tissue. The third and fourth experiments had five and eight groups of animals, respectively. These latter two ECT experiments focused on a bleomycin dose range with a fixed electric field and a fixed bleomycin dose followed by an electric field range. In all four experiments, histological specimens and liver function were examined. All determinations were made based on treated liver tissue or blood from three identically treated animals.

3.1. Histological effects of ECT on liver tissue

Eight animal groups were employed for the experiment that examined the effects of different bleomycin doses. The normal liver tissue from five groups in this set were treated with 0.5, 1.0, 1.5, 2.0 or 2.5 U of bleomycin only. The remaining three animal groups were established as separate control groups and were subjected to no treatment, surgical exposure of the liver only or surgical exposure of the liver followed by an injection of normal saline.

Histological analysis of the treatment sites 14 and 56 days after treatment revealed minimal or no differences between the control groups and those treated with the different bleomycin doses. Minimal levels of necrosis were detected in the specimens subjected to no treatment and 1.5 U of bleomycin at day 14 (see Fig. 1a). Tissue adjacent to the injection site was not affected.

Eight animal groups were also used for an experiment examining the effects of electric fields alone on liver. Each group of animals was subjected to 100 μs pulses following a sham saline injection. The liver of each animal in these groups received pulses at a nominal electric field strength of either 500, 750, 1000, 1250, 1500, 1750, 2000 or 2250 V/cm. The field strengths selected included values that have been used previously in ECT clinical trials [30] and other animal model studies [31]. This range also included fields that were higher and lower than values used in the previous studies.

Histological analysis of the treated tissue at days 14 and 56 indicated no significant differences in any of the histological parameters examined. Two experimental groups, 1500 and 1750 V/cm, did exhibit slightly elevated necrosis on day 14 as shown in Fig. 1b. This necrosis was not detected on day 56. It was also noted that there was a stasis of blood flow to the treatment site immediately following pulsation. Stasis was observed to cover a broader area as the field strength was increased. In all cases, it appeared that blood flow began to return to the treatment site during the several minutes of time required to close each animal. No significant histological effects could be attributed to this stasis at the time points examined (Fig. 1b).

As indicated above, two additional experiments were conducted using complete ECT protocols. Five groups of animals were used for the experiment that used a range of drug doses followed by pulses with a fixed field strength. One group was treated with each of the following bleomycin doses: 0.5, 1.0, 1.5, 2.0 and 2.5 U per animal. Drug administration was followed by 1000 V/cm pulses for all animals in these five groups. This pulse magnitude was selected as it resulted in strong antitumour effects for hepatocellular carcinomas in this same animal [5,26]. Even though a 5-fold increase in drug dose was used, none of the parameters evaluated indicated any notable trend or increase relative to the lowest dose used and/or the controls. There was a modest/detectable amount of necrosis at day 14. This information is reflected in Fig. 1c. Necrosis was not detectable at day 56 in any of the samples.

The final experiment of the study used a 0.5 unit bleomycin dose coupled with a range of electric field strengths in order to treat eight different experimental groups. In this ECT protocol, groups received either 500, 750, 1000, 1250, 1500, 1750, 2000 or 2250 V/cm pulses. The 0.5 U bleomycin dose was selected because it was used successfully to treat hepatocellular carcinomas that were on the order of 100 mm^3 [5,26]. With the exception of necrosis, none of the histological parameters revealed significant differences among these groups (Fig. 1d) (and data not shown). Field strengths

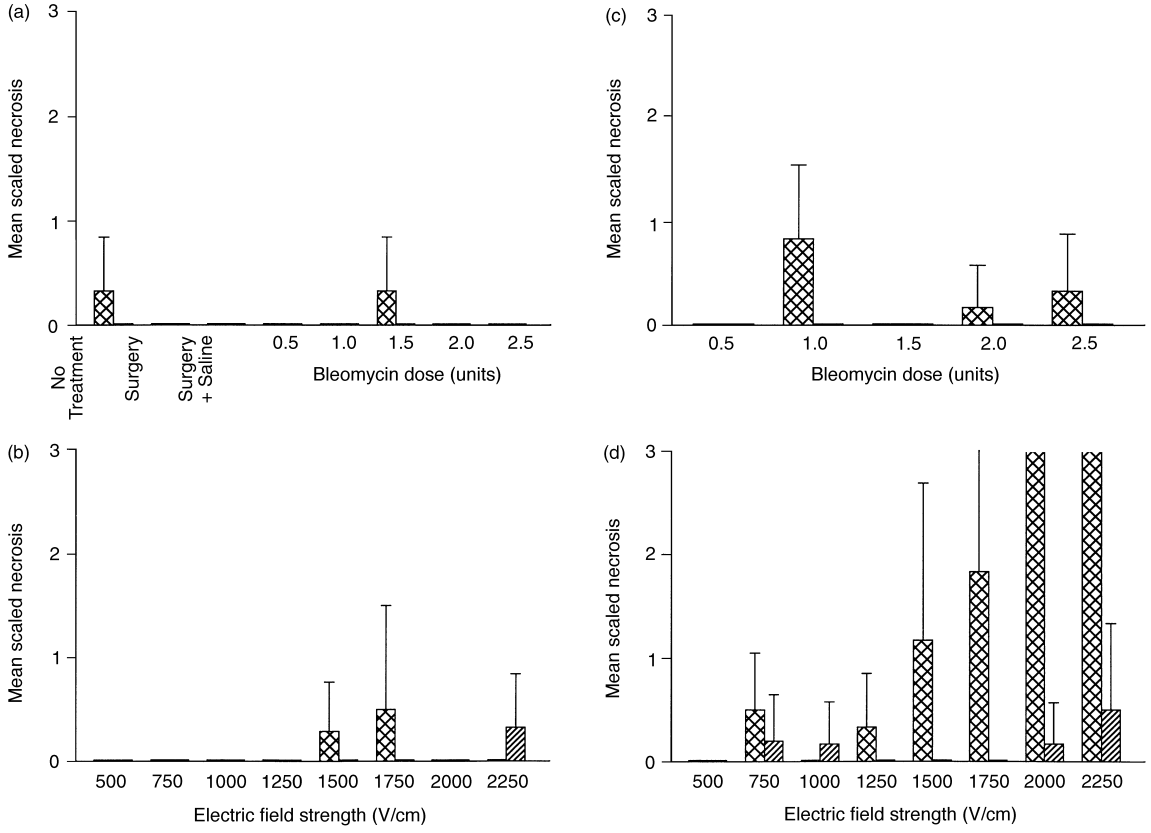

Fig. 1. Mean scaled necrosis detected in normal liver tissue 14 and 56 days after treatment with electrochemotherapy. Thatched bars indicate day 14 results and shaded bars indicate day 56 results. Each bar represents the mean from three different hepatic lobes. (a) Treatment of normal liver with bleomycin alone. Bleomycin doses and treatment conditions for three control groups are noted on the *x*-axis. (b) Treatment of normal liver with electric fields alone. The range of field strengths tested is noted on the *x*-axis. (d) Treatment with a range of bleomycin doses followed by 1000 V/cm pulses. Drug doses are indicated on the *x*-axis. (d) Treatment consisting of a 0.5 U dose of bleomycin followed by a pulses with a range of field strengths. The *x*-axis indicates the field conditions.

between 500 and 1250 V/cm showed minimal necrosis at day 14. The amount of necrosis increased for the >1500 V/cm groups.

Animals treated with bleomycin followed by 2000 and 2250 V/cm pulses showed necrosis at day 14 that was above the maximum value on the scale used for this study. These specimens were examined again. Measured necrotic areas were summed for each section and then expressed as a percentage of the total treated area. Data from multiple sections treated alike were expressed as a mean. These data indicated that samples treated with 2000 and 2250 V/cm had areas of necrosis that involved 20–30% of the treatment area. However, like the samples treated with lower field strengths, necrosis was detected only at very low levels on day 56. Resolution of necrosis by day 56 indicated that the side effects of ECT using these field strengths are relatively short in duration.

The microscopic appearance of samples in this final experiment of the study is illustrated in Fig. 2a–c. Necrotic areas corresponding to the needle tracks from the electrode were observed. The size of the necrotic areas increased with field strength starting at 1500 V/cm. Large areas of necrosis within the centre of the treatment site, as well as near the needle tracks, were observed for field strengths of 2000 and 2250 V/cm. Necrosis was usually associated with granulation tissue and giant cell reaction at 14 days (Fig. 3) after treatment. Within 56 days after treatment, these areas showed fibrosis and hepatocyte regeneration.

3.2. *Effect of ECT on liver function*

Plasma samples from all animals within the study were taken and analysed at days 0, 1, 7 and then once per week until a time of 56 days posttreatment. Analysis results for albumin, total protein, lactate dehydrogenase, total the bilirubin and alkaline phosphatase were not significantly different among the untreated groups and the groups subjected to bleomycin, pulses or ECT treatments. However, there were changes in the levels of aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transferase as a result of the treatment.

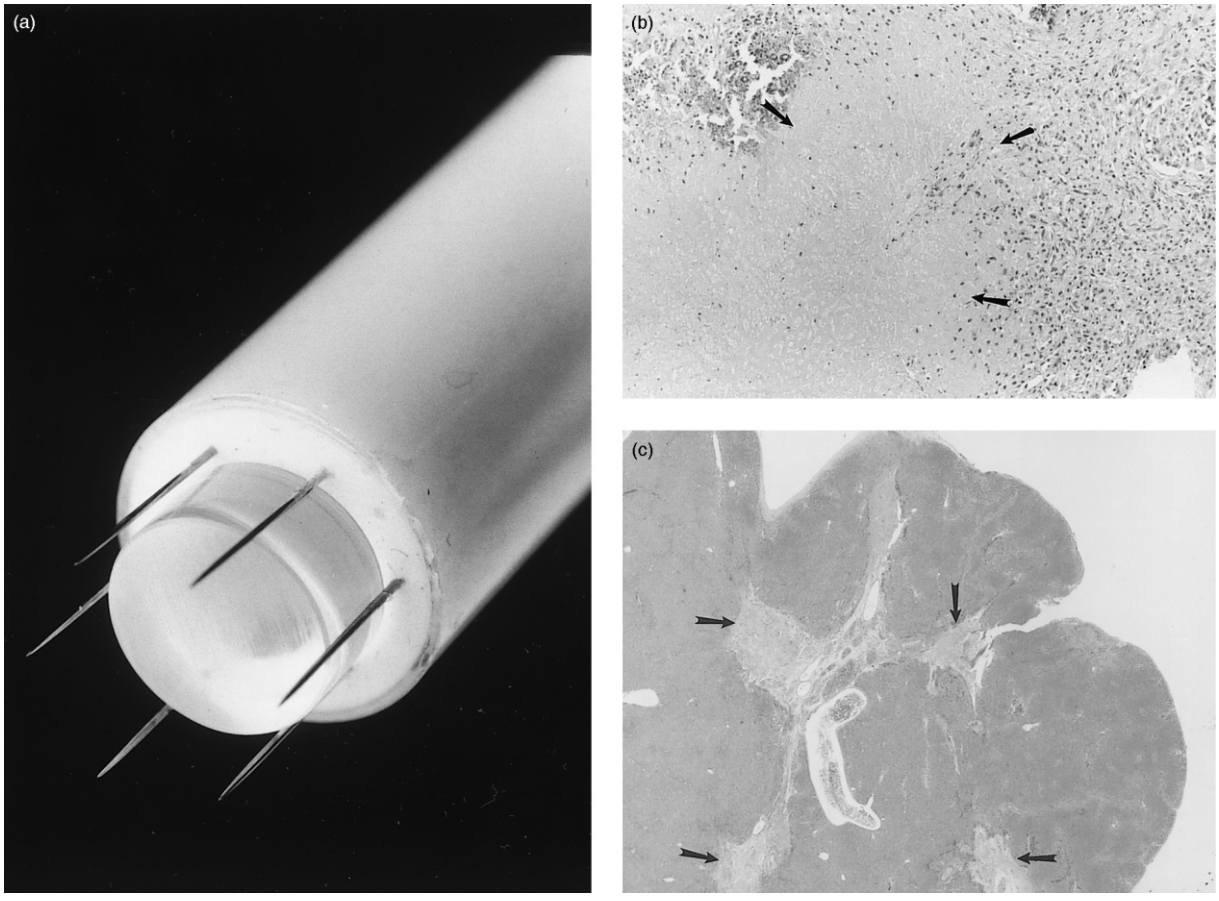

Fig. 2. Necrosis in samples of normal liver tissue treated with bleomycin followed by high electric fields: (a) electrode inserted into normal liver tissue for administering electric fields; (b) hepatocyte necrosis walled-off with granulation tissue and microcalcifications resulting from 0.5 U of bleomycin followed by 2000 V/cm pulses 14 days after treatment (see arrows); (c) these areas were visible even on low power examination (see arrows).

Fig. 4 illustrates the changes in plasma levels of aspartate aminotransferase as a function of time. The plot summarises data from all experiments by showing traces for the maximum doses of bleomycin and electric fields from each of the experiments described above. Aspartate aminotransferase levels were higher at day 1 compared with the pretreatment determinations (day 0). Levels of this enzyme returned back to baseline levels by day 7 as indicated in the figure. Control animals that were not manipulated in any manner did not have increased enzyme levels. Alanine aminotransferase and γ-glutamyl transferase levels paralleled the results obtained for aspartate aminotransferase.

Increased enzyme levels are a common result after surgery and procedures that manipulate the liver; thus, transient initial increases were expected. The magnitude of the peak enzyme levels was dependent on the extent of treatment. For example, surgery alone and the maximum dose of bleomycin (Fig. 4) had approximately equal peak aspartate aminotransferase levels above baseline. The ECT treatment protocol that employed a range of bleomycin doses followed by 1000 V/cm pulses resulted in peak aspartate aminotransferase levels that were above those for treatment with bleomycin alone. The magnitude of the peaks for samples treated with bleomycin and 1000 V/cm pulses was proportional to the drug dose; the maximum peak occurred for a 2.5 unit dose of bleomycin as indicated in Fig. 4. Similarly, treatment with a 0.5 U bleomycin dose followed by a range of field strengths resulted in peak aspartate aminotransferase levels that were also elevated relative to treatment with bleomycin alone. This increase depended on field strength; 2250 V/cm pulses combined with 0.5 U of bleomycin produced the highest peak as shown in Fig. 4. Alanine aminotransferase and γ-glutamyl transferase levels were also dependent on the field strength and followed a similar pattern of elevation 1 day after treatment. These enzymes returned to baseline levels by day 7 posttreatment and remained there until the end of the study.

4. Discussion

Published electrochemotherapy studies primarily focus on antitumour effects which are very important

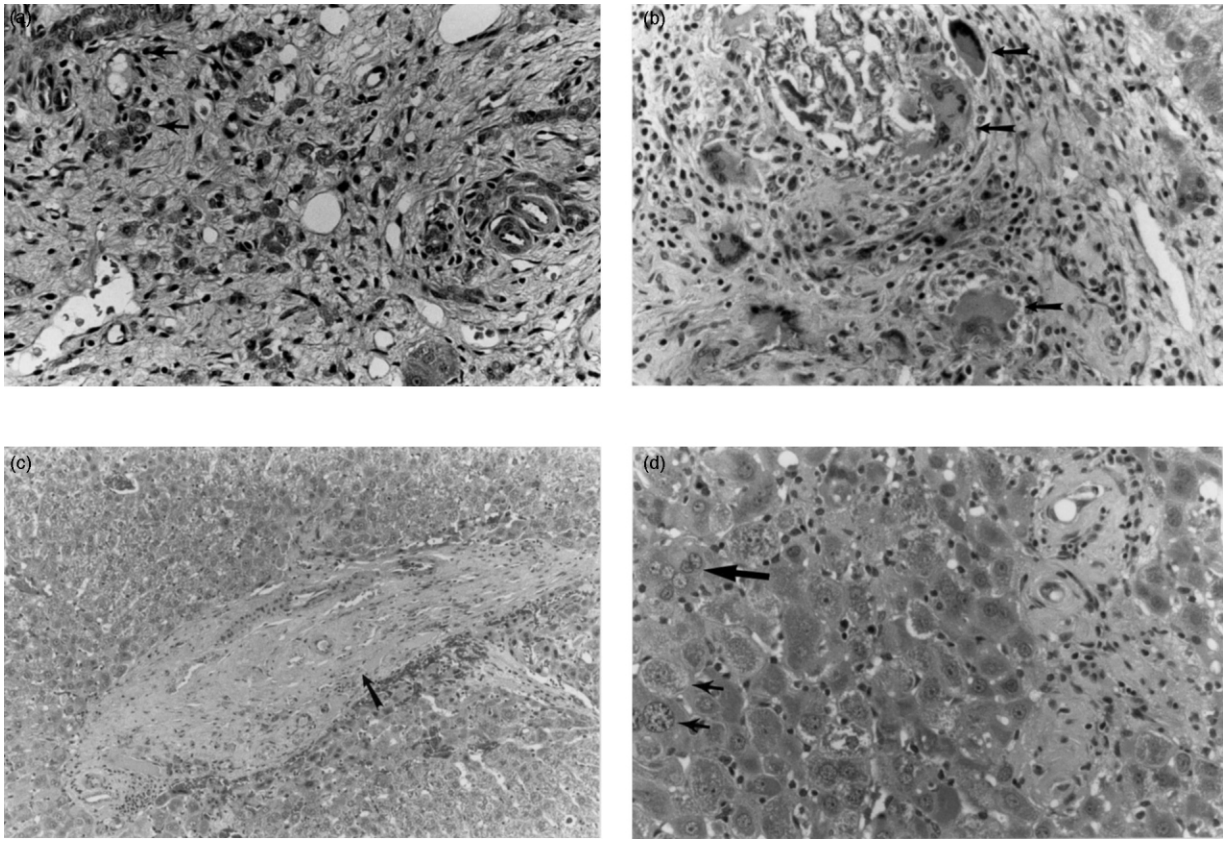

Fig. 3. Healing process after electrochemotherapy (ECT). (a) At day 14, areas of necrosis were identified. The liver parenchyma adjacent to these areas had been replaced by granulation-type tissue characterised by new blood vessel formation, fibroblasts and bile duct proliferation (see arrows). (b) Multinucleated giant cells were also present (see arrows). (c) After 56 days, the treatment site was characterised by fibrosis in the regions affected by the treatment (see arrow). (d) The liver parenchyma adjacent to these areas revealed binucleated hepatocytes (see larger arrow), and hepatocytes with vesicular, large nuclei indicating regeneration (see smaller arrows).

for establishing efficacy for this new therapeutic modality. Most studies have reported that ECT is safe and well tolerated. Some of these studies have made these same observations in models for tumours of the liver [26–28]. However, comprehensive information about the effects of electrochemotherapy on normal tissues have not been the primary focus of any published studies to date. Thus, detailed knowledge about the safety of this type of treatment is currently very limited. This investigation focused on characterising the histological effects resulting from electrochemotherapy treatment of normal liver 14 and 56 days after treatment as well as the effects on liver function posttreatment.

Results from this study clearly establish that the two treatment components of bleomycin-based ECT have very minimal effects on normal liver tissue in the model used. Dose response data for both the electric fields and bleomycin reflect that neither treatment component alone adversely affects normal liver tissue. Typical field strengths used for ECT range from 1000 to 1500 V/cm [1,2]. Bleomycin doses that have been used to treat tumours are approximately 0.25–1.0 [2,5,26]. Even when electric fields and drug doses outside these ranges were used, no significant histological effects were observed from either the drug or electrical treatment components alone.

Histological examination of complete ECT protocol samples within this study clearly indicate that normal liver tissue damage is negligible for fields of 1250 V/cm and below. Fields of 1500 and 1750 V/cm resulted in minor necrosis 14 days after treatment when used in combination with 0.5 U of bleomycin. This damage was localised primarily to liver areas that were immediately adjacent to the needles used to apply the fields. These two treatment conditions resulted in mean scaled necrosis values of 1.3 and 1.75, respectively. These values correspond to less than 1.5% of the treatment area. Fields of 2000 and 2250 V/cm, when used with the same 0.5 U bleomycin dose, resulted in damage to normal liver tissue that occupied 20–30% of the treatment sites which constituted a more significant adverse effect than that caused by the lower field strengths. Necrosis that was induced by ECT was completely resolved by day 56.

Results from normal liver that was treated with a complete ECT protocol consisting of 0.5 U of bleomycin with a range of field strengths are most relevant to the treatment of hepatic malignancies. This is true because ECT protocols are typically optimised to a particular tumour type by changing the electric field of the applied pulses. This strategy is employed because electroporation is known to be a threshold phenomenon. Thus, fields that are moderately above this threshold are identified in order to produce an effective treatment. However, tissue damage/cell lysis occurs when field strengths are significantly increased above this threshold. Thus, this study defined the limits of safe ECT treatment with respect to normal liver tissue. Based on preclinical studies for treating tumours in the liver with ECT, high response rates can be achieved using conditions that fall within the safe treatment parameters defined by this study. Future research will focus on such treatments.

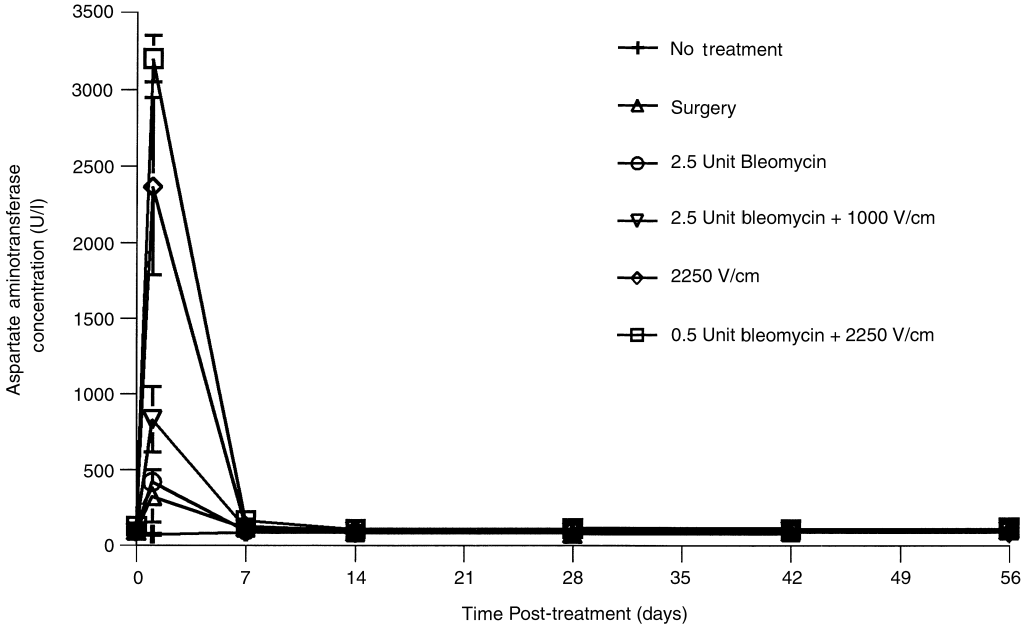

Fig. 4. Aspartate aminotransferase serum levels for selected treatment groups over a time course of 56 days after treatment. Each point on the plot represents the mean from three identically treated animals.

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