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Combined electric field and ultrasound therapy as a novel anti-tumour treatment

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Abstract

The permeabilising effects of electric pulses on cell membranes and the use of ultrasound energy of various intensities, for both thermal effects and enhancement of drug and gene delivery, have led to extensive research into the potential applications of these systems in the development of novel anti-cancer treatments. In the present study we have demonstrated for the first time that the application of brief electric pulses 'sensitises' tumour cells to the effects of low intensity ultrasound. The studies were conducted in human tumours established in athymic nude mice and in many instances resulted in the reduction of tumour mass. The combined electric field and ultrasound approach (CEFUS) was applied *in vivo* to a murine colon adenocarcinoma (C26) and a human oesophageal adenocarcinoma (OE19). The experiments performed demonstrated the anti-tumour effects of the combined therapy. Varying the electrosensitisation parameters used (voltage, waveform, electrode type) contributed to optimise the procedure. Exponential electric pulses with a peak of 1000 V/cm were initially used, but square wave pulses (1000 V/cm, 1 ms, ×2, 1 Hz) were found to be just as effective. All ultrasound application parameters were kept constant during the study. The growth rate of C26 tumours treated with CEFUS was significantly reduced with respect to untreated controls at day 7 (96% of average initial tumour volume in CEFUS group *versus* 615% for controls, $P < 0.05$). Similar reduction was observed in OE19 tumours treated with CEFUS by day 4 (82% *versus* 232%, $P < 0.032$). Our preliminary data suggest that this novel technology could potentially be of wide application in clinical practice for the treatment of solid tumours and is worth further investigation.
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1. Introduction

Cancer remains a leading cause of morbidity and mortality despite knowledge of its molecular basis, detection and treatment. Many cancers evade the curative endeavours of conventional therapies like surgical resection, chemotherapy and radiotherapy. Many are inoperable, metastatic at first presentation, fail to respond to treatment or following successful initial treatment may subsequently recur. The development of alternative therapies for such cancers is clearly an imperative.

In the case of cancers responsive to conventional treatments, it is accepted that surgical intervention and less invasive oncology choices are limited in their ability to effectively localise treatment to cancerous tissues without causing disruption to non-target physiological functions, tissues and organs [1,2]. It is highly desirable

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if different types of treatment are developed as adjuncts or alternatives to currently available therapies. New therapies can then be applied more locally (involving minimally invasive techniques exerting fewer and less severe side effects), and be used to treat a greater range of cancers than is currently considered possible.

The application of ultrasound, electric pulses and liposomes to safely increase the efficacy of gene and drug delivery to cells has been reported. Discovery of the permeabilising effects of exposure to high intensity electric fields of short duration on mammalian cell membranes has resulted in the emergence of therapeutic strategies such as electrochemotherapy and electrogenetherapy, wherein the delivery of macromolecules to the cytoplasm is greatly enhanced by transient pores electrically induced in the cell membrane [3–5]. Furthermore, the recent literature contains a number of reports on the use of high intensity focused ultrasound (HIFU) as an effective means of ablating a wide histological variety of human carcinomas [6–8]. While initial reports on this technique have largely been encouraging, showing the technique to be an effective and relatively safe treatment, several complications have been observed following treatment of certain tumour types using this modality. Nonetheless, it has been demonstrated to produce highly anatomically accurate and externally controllable thermal ablation in an extensive and diverse array of tumours and carries the advantages of being minimally invasive and potentially repeatable if necessary. However, the technology is not currently widely available and its great expense is an obstacle to its widespread proliferation.

The application of ultrasound at lower energy frequencies (<1 MHz) has also been shown to disrupt the lipid packing of the stratum corneum by cavitation. Shock waves of collapsing vacuum cavities increase free volume space in bimolecular leaflets and thus enhance drug penetration into the tissue [9,10]. Recent *in vitro* work has suggested that application of low voltage electric pulses to tumours, prior to treatment with low frequency ultrasound, sensitises the cells to the ultrasound effects and may render them apoptotic [11].

In this study, we demonstrate that a combination of an electric field and ultrasound (CEFUS) can be applied to solid tumours *in vivo* to exert an anti-tumour effect on moderately large tumours and reverse the growth of smaller tumours in mice. Specifically, this study substantiates the effects of CEFUS on solid mass tumours and reports on optimisation of the technique *in vivo*.

It is hoped that by demonstrating a synergistic effect of the two modalities and by developing the combination using optimal conditions for both, wherein a lesser amount of energy would be imparted from the individual components than when either is used alone, that a widely clinically applicable anti-cancer therapy may emerge for the treatment of recurrent or inoperable tumours. This should exhibit a narrower side effect profile than the individual components used in isolation, yet should be at least as effective, safe and cheap and would similarly carry the advantage of being safe to repeat in the case of incomplete tumour ablation or of local recurrence following successful initial treatment. It is envisaged that refinement of the technique using parameters ascertained during further experimental investigation should produce significant improvements in response to the treatment such that this may become a viable and beneficial treatment option in human cancer.

2. Materials and method

2.1. Cell tissue culture

The cell lines used were a human oesophageal adenocarcinoma – OE19 and a murine colonic adenocarcinoma – C26. Tumour cell lines were obtained from the American Type Culture Collection (ATCC – Manassas, VA, USA) and grown in tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2, in RPMI (Roswell Park Memorial Institute – GIBCO, Invitrogen Corp., Paisley, Scotland) or OE19 and in DMEM (Dulbecco's Minimal Essential Medium – GIBCO, Invitrogen Corp., Paisley, Scotland) for C26. The tissue culture media were supplemented with 10% iron-supplemented donor calf serum, 50 μg/ml gentamycin, 300 μg/ml L-glutamine and 10 mM HEPES (1-piperazineethane sulfonic acid, 4-(2-hydroxyethyl) monosodium salt), pH 7.4. Tumour cells were dislodged from the surface of the flasks by first removing the tissue culture medium and washing the cells with an equal volume of phosphate buffered saline (PBS). Cells were then treated with 0.05% w/v trypsin + 0.02% w/v EDTA (ethylenediaminetetraacetic acid) in PBS and incubated for 2 min until cells became rounded and readily dislodged from the flask surface. Tissue culture medium was added to the harvested cells, and the cell suspensions were centrifuged at 180*g* for 5 min. The cell pellets were then resuspended in the appropriate tissue culture medium and cell densities of the resulting suspensions were determined by visual count using a haemocytometer and viable cell counts were conducted using Trypan Blue Dye Exclusion (Sigma).

2.2. Tumour induction

Mice were obtained from Harlan Laboratories (Oxfordshire, England). They were kept at a constant room temperature (22 °C) with natural day/night light cycle in a conventional animal colony. Standard laboratory food and water were provided ad libitum. Before experiments, the mice were afforded an adaptation period of at least 14 days. Mice of both sexes in good condition,

without fungal or other infections, weighing 16–22 g and of 6–8 weeks of age were included in experiments. For tumour induction, 2×10^6 tumour cells, suspended in 100 μl PBS, were injected subcutaneously into the flank of female BALB/c (C26) and athymic male BALB/c HsdOla:MF1-nu mice (OE19).

The viability of the cells was over 95% as determined by Trypan blue dye exclusion test. When the tumours reached approximately 100 mm^3 in volume, the mice were randomly divided into experimental groups and subjected to specific experimental protocols. The experiments were performed with groups of equal numbers of mice ($n = 6$ except for the experiment on comparison of intratumoral with parallel subcutaneous needle electrodes, $n = 11$).

2.3. In vivo treatments

All *in vivo* experiments were approved by the ethics committee of University College Cork. All tumours were measured with digital callipers using the formula for tumour volume, $V = ab^2\Pi/6$ where a is the longest diameter of the tumour and b is the longest diameter perpendicular to diameter a [12]. Mice were standardised into four groups on the basis of tumour volume such that the average tumour volume in each of the four groups was equivalent. The four groups were: control, electric field alone, ultrasound alone and CEFUS. Mice were anaesthetised using halothane. Subsequent treatment was determined by the group category of each mouse.

Control. For the parallel needle array, two 21 gauge stainless steel needles were tunnelled subcutaneously on either side of the tumour, parallel to its longest diameter; for the intratumoral needle array, needles were inserted into the tumour. No current was passed. The ultrasound probe was held against the tumour but no ultrasound was delivered.

Electric field. The needle electrodes were positioned as above. Exponential electric pulses were delivered to the tumour using a BTX 630 electroporator (BTX, MA, USA). Two pulses of 1000 V/cm, lasting 600 μs, were given 5 s apart. Square wave electric pulses were generated by an electropulsator BTX ECM 2001 (BTX, MA, USA). Two pulses of 1000 V/cm, lasting 1 ms were given 1 s apart.

Ultrasound. A 1 MHz ultrasound transducer with a surface area of 5 cm^2 (Rich-Mar Corporation, Inola, OK, USA) was held against the tumour. Contact with tissue was maintained using an ultrasound contact gel (BCF Technology Ltd., Livingston, Scotland). Ultrasound was irradiated for 2 min at an intensity of 3.5 W/cm^2 at a 35% duty cycle.

CEFUS. Electric field treatment was carried out as described above and was followed after 20 s by 2 min of ultrasound also as described previously.

2.4. Tumour monitoring

Tumours were measured every 48 h using a digital calliper. Tumour volume, V, was calculated using the standard formula and tumour growth curves were constructed. The mice were humanely euthanised in instances where the tumour reached 2 cm in diameter.

2.5. Histological analysis

Tissues were fixed in 10% v/v buffered formalin and embedded in paraffin; serial sections were cut at 5 μm. The sections were mounted on slides and stained with Haematoxylin and Eosin using standard procedures. Images were collected using a microscope (Nikon TE2000-S) with 20× or 40× objective (×200 or ×400 magnification respectively) and a digital camera (Finepix model 2600, Fuji, Japan).

2.6. Apoptosis detection

The TUNEL (transferase-mediated dUTP nick end-labelling) method was used to demonstrate apoptosis on paraffin block sectioned C26 tumour samples after CEFUS treatment using the In Situ Cell death Detection Kit (Roche, Germany). Tissue was harvested from tumours at timepoints after treatment, stored fixed in 10% v/v buffered formalin and embedded in paraffin; serial sections were cut at 5 μm. Sections were de-paraffinised, rehydrated and made permeable by incubating sections with Proteinase K (2 μg/ml) (Roche, Germany) for 15 min at 37 °C. Labelling buffer was omitted from negative controls and positive controls were obtained by digestion of the sections with deoxyribonuclease I for 10 min at room temperature before the detection procedure.

2.7. Statistical analysis

Experimental groups contained at least six subjects per group and results were shown as mean ± SD. We tested the significance of the differences between the individual groups using the two-tailed Student's t-test for paired values. Differences with a P value less than 0.05 were considered significant. For statistical comparison of several groups we used the one-way ANOVA.

3. Results

3.1. Combined electric field and ultrasound therapy regresses tumour growth in mice

The combined electric field and ultrasound (CEFUS) therapy was evaluated initially in athymic nude mice bearing OE19 tumours. These were standardised into

four groups containing six mice per group having approximately equivalent average tumour volumes (72–84 mm^3) and treated either with an electric pulse (using needle electrodes tunnelled subcutaneously parallel to the tumour), ultrasound alone, CEFUS, or were left as untreated controls (Fig. 1). The tumour volume was calculated at 48 h intervals and within 4 days of starting treatment, the CEFUS group was found to have statistically significantly suppressed tumour growth (82% of average initial tumour volume, standard error of the mean, SEM = 0.0273) with respect to the untreated control group (232% of average initial tumour volume, SEM = 0.0294, $P < 0.032$). Neither administration of ultrasound alone ($P = 0.67$), nor exposure to electric field alone ($P = 0.94$) had any significant effect on subsequent tumour growth with respect to the untreated tumours. At 10 days post-treatment, two of the six treated tumours in the CEFUS treated group had completely regressed while a third tumour was at 17% of its original volume. All tumours in the other three groups were larger than when treated 10 days earlier. There was no evidence of tumour growth in the two mice with complete regressions for a further 3 weeks, at which time the tumours reappeared at the same site. No adverse results were noted from the treatment.

3.2. Efficacy of intratumoral needle versus parallel subcutaneous needle electrodes

To investigate the different electric fields generated by intratumoral needles and two parallel subcutaneous needle electrodes, we assessed the anti-cancer effect of these two electrode arrays on the overall efficacy of treating athymic nude mice with human oesophageal OE19. At the start of the experiment, tumour volumes were calculated and three groups of eleven mice were established, with each group having an equivalent average tumour volume (99–104 mm^3). The group with untreated tumours were used as controls while the remaining two groups were treated with the CEFUS: one with electric fields delivered by an array of intratumoural needles, the other with parallel subcutaneous needle electrodes. Growth curves constructed for the three groups showed that both CEFUS regimens had caused significant regression in the average tumour volume by day 6 (Fig. 2 and Table 1). The average tumour volume in the control group was 111% on day 6 (SEM = 0.0139), compared to 59% (SEM = 0.0096, $P < 0.07$) for the CEFUS parallel needle group and 49% (SEM = 0.0138, $P < 0.06$) for the CEFUS intratumoral needle group. After 10 days, there were nine regressions out of 11 in the intratumoral needle group. Of these, four were complete regressions of which two recurred within 3 weeks, the average tumour volume in this group was 67% of initial average tumour volume (SEM = 0.0178), compared to 144% for the controls (SEM = 0.0359, $P = 0.07$). There were nine regressions out of 11 in the parallel needle group, of which two were complete regressions and were still tumour free 31 days after treatment. The average tumour volume at day 10 in this group was 85% of initial volume (SEM = 0.0211, $P = 0.184$). The difference between the two treated groups did not approach statistical significance at any point ($P = 0.57$ at 6 days, $P = 0.52$ at 10 days). Table 1 shows the number of mice in each group that were found to be tumour free at various timepoints following treatment. In both treatment groups, the increase in volume of those tumours that did not respond to treatment or which grew back following

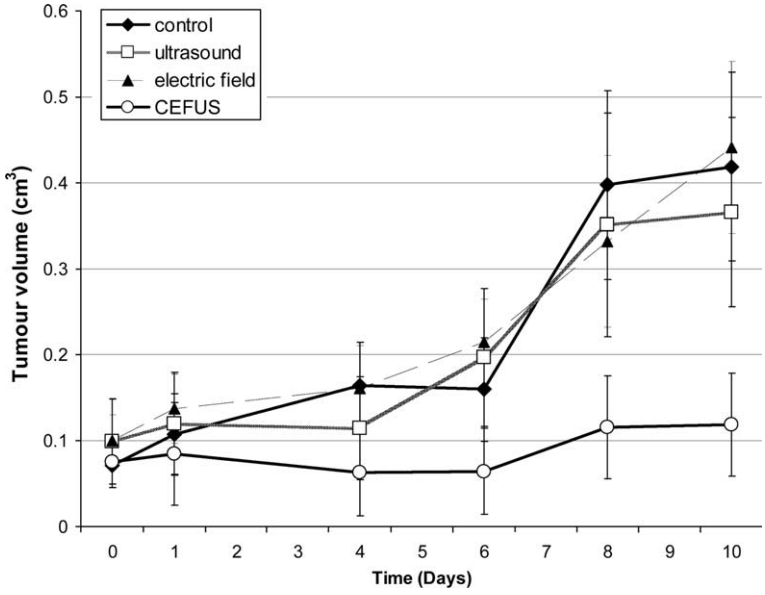

Fig. 1. Tumour growth curves for four groups of athymic nude mice ($n = 6$) bearing tumours of the human oesophageal adenocarcinoma line OE19. The groups were treated with either CEFUS, ultrasound alone, electric field alone or were left as untreated controls.

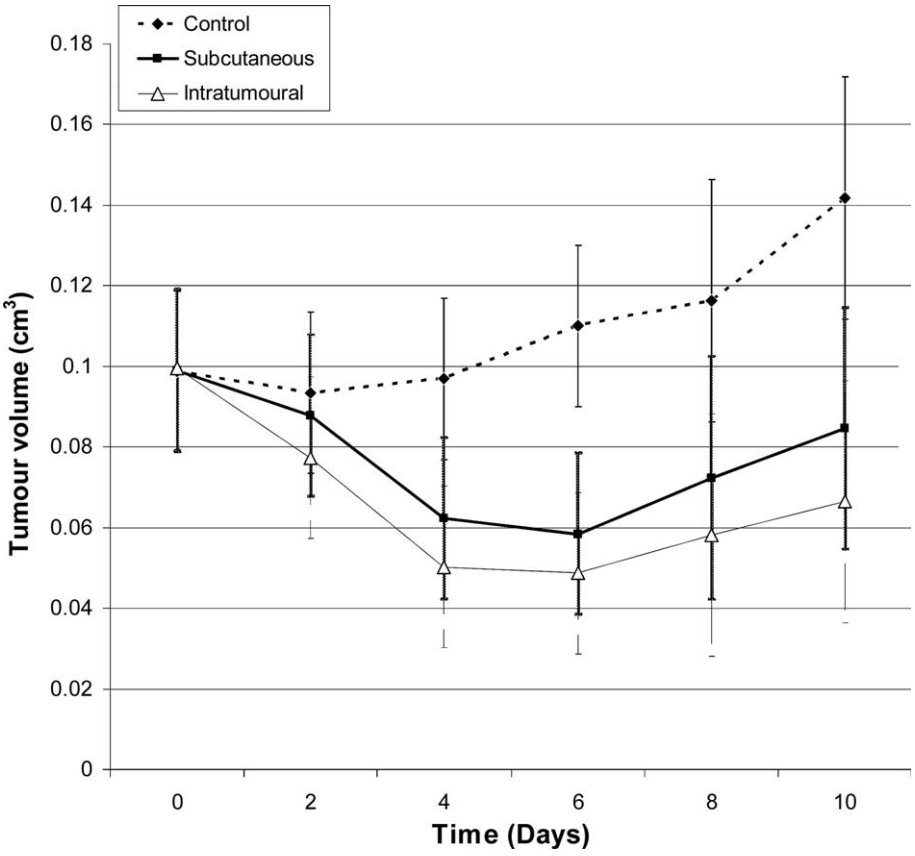

Fig. 2. Tumour growth curves for three groups of athymic nude mice ($n = 11$) bearing tumours of the human oesophageal adenocarcinoma line OE19. Two of the groups received CEFUS treatment. The electric field was delivered to one group using intratumoural needle electrodes and to the other group using subcutaneous parallel needle electrodes.

Table 1
Number of mice in each group found to be tumour-free at various timepoints following treatment

Treatment group	Day										
	0	2	4	6	8	10	12	14	20	31	35
Control ($n = 11$)	0	0	0	0	0	0	0	0	0	0	0
CEFUS: parallel needle array ($n = 11$)	0	0	0	2	1	2	3	3	2	2	2
CEFUS: intratumoural array ($n = 11$)	0	2	4	5	4	4	3	3	3	2	2

Table was compiled from data in Fig. 2.

an initial response gave a growth curve similar to that of the untreated tumours within 3 weeks.

3.3. Optimising parameters in CEFUS for increased anti-tumour effect

To examine the effect or contribution of the electric field and pulse parameters on the overall efficacy of CEFUS, we compared the effect of exponential and square wave pulses delivered to the tumour tissue via either intratumoral needles or with the two parallel subcutaneous needle electrodes. The parameters were assessed using an aggressive murine colon carcinoma cell line, C26, in BalbC mice. Tumour cells were injected subcutaneously on the flank and after the tumour volume reached approximately 0.2 cm^3, the mice were standardised into 10 groups of six mice with equivalent tumour volumes. The groups received the following treatments: (i) untreated, (ii) ultrasound alone, (iii) square wave pulse intratumoral needle electrodes, (iv) exponential pulse with parallel needle electrodes, (v) square wave pulse with parallel needle electrodes, (vi) exponential pulse with intratumoral needle electrodes and (vii)–(ix) CEFUS, with the electric field component of each treatment corresponding to those of groups iii–vi in combination with ultrasound (3.5 W/cm^2, 35% duty cycle at 1 MHz). Mice in the first six groups which did not receive CEFUS and which did not subsequently exhibit reduced growth rates after treatment were euthanised at day 7 due to growth of their tumours and in order to

comply with ethical guidelines. As there were no significant differences between mice not receiving CEFUS in the first six groups, a representative tumour growth curve is represented in Fig. 3, control plot. The growth of tumours in all four CEFUS treated groups was significantly retarded with respect to untreated control ($P < 0.009$ at day 4 for groups ix SEM = 0.0675 and x SEM = 0.0287; $P < 0.05$ at day 4 for groups vii SEM = 0.0733 and viii SEM = 0.0616). There was a significant difference in the four CEFUS treated groups between those tumours which were treated with intratumoral needle electrodes and those treated with parallel needles ($P < 0.0006$ for the exponential pulses group viii *versus* group x; $P < 0.07$ for the square wave pulses group vii *versus* group ix). The mice in these four groups were sacrificed at day 11 as the tumours in group vii–ix approached the ethically allowed limit and clear statistical difference between CEFUS and the control groups had been demonstrated. The results show that the intratumoural is superior over parallel subcutaneous needles (Fig. 3).

3.4. Histological analysis

The TUNEL assay to detect apoptosis was performed on sections of C26 tumours which were treated either with CEFUS, ultrasound alone, electric field alone or left as untreated controls. Mice from each of these groups were culled at 24, 48 and 96 h and their tumours removed, sectioned and analysed. It was seen in each of the CEFUS treated tumours from as early as 24 h that several disparate areas of apoptotic cell death were present, whereas no tumour from any of the other three groups exhibited evidence of cell damage at any point (Fig. 4).

In order to determine the effect of CEFUS on the healthy tissue surrounding treated tumour tissue, haematoxylin and eosin (H&E) staining and the TUNEL assay were performed on the thigh muscles of BALB/c mice which had been exposed *in vivo* to the CEFUS protocol, ultrasound alone, electric field alone or untreated controls. Mice from each group were sacrificed at 12, 24 and 48 h. The thigh muscles were removed at necropsy and sectioned. Analysis of normal tissue sections that had been treated with CEFUS showed that, in contrast to tumour tissue, no apoptosis had been induced in muscle tissue in any of the specimens examined (Fig. 5). H&E histology revealed that there was some fraying of muscle fibres in those muscles that had undergone either CEFUS or electric field treatment 24 h previously. Where present, this was associated with a mild inflammatory reaction; however, this was not present in all sections from these muscles (Fig. 6).

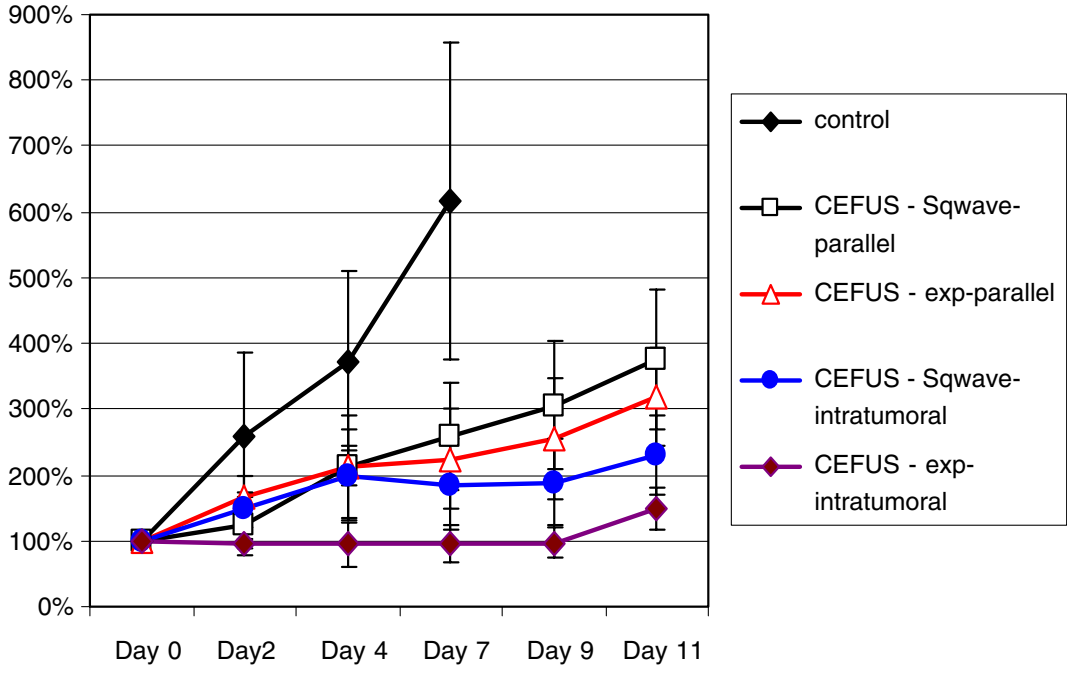

Fig. 3. Comparison of exponential/square wave pulses using parallel/intratumoral needle electrodes to deliver the electric field component of CEFUS. The ultrasound parameters used were 3.5 W/cm^2 intensity, 35% duty cycle at a frequency of 1 MHz for 2 min. Control plot is a representative curve from samples that were: untreated, ultrasound alone, square wave pulse intratumoral needle electrodes, exponential pulse with parallel needle electrodes, square wave pulse with parallel needle electrodes and exponential pulse with intratumoral needle electrodes. There were no significant differences between the individual control treatment conditions.

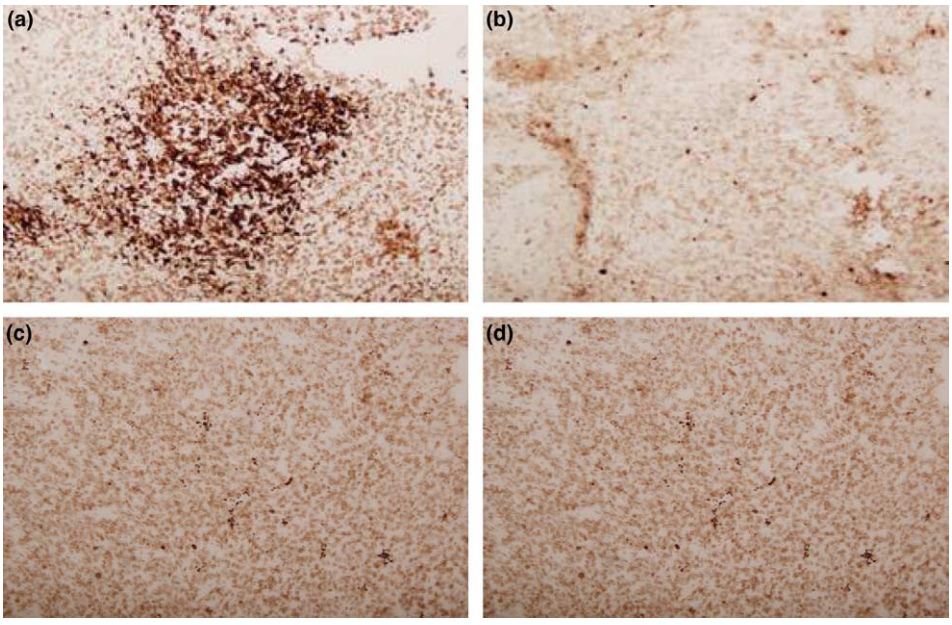

Fig. 4. Focal areas of apoptosis induced in C26 tumour tissue after (a) 24 h after CEFUS treatment. (b) No evidence of apoptosis is seen in the untreated control (c) or in tumours treated by ultrasound (d) or by electric field alone after 24 h.

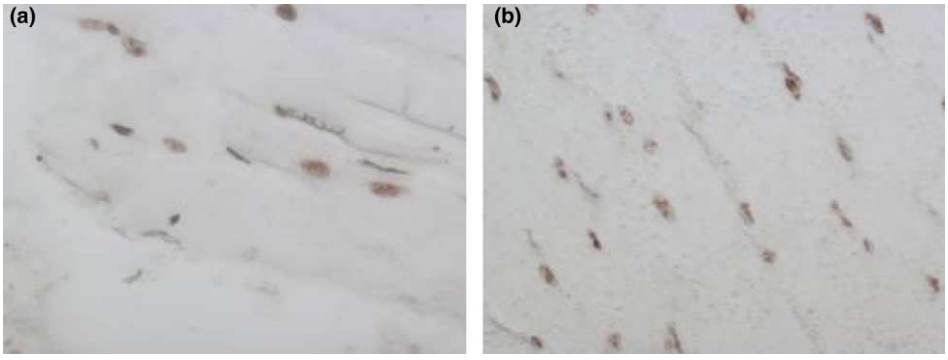

Fig. 5. Comparison of mouse thigh tissue following application of CEFUS. (a) Untreated control and (b) CEFUS treated sample. No evidence of apoptosis was detected in the muscle tissue at any time point up to 96 h after exposure to the treatment protocol.

4. Discussion

The treatment of localised cancer ideally should produce complete, irreversible tumour cell death without damage to the surrounding normal tissue. We report for the first time on the combination of electric pulses and low intensity ultrasound as a local treatment for subcutaneously implanted tumours of human tumour cell lines in athymic nude mice and demonstrate its potential for arresting the growth of many tumours and eliminating smaller tumours.

The manipulation and development of physical modalities such as electric fields and acoustic energies have been investigated extensively in various areas of cancer research during the last few decades. Much of this study has concentrated on improving the efficacy of established anti-cancer chemotherapy and emerging gene therapies. The combination of electric pulses and ultrasound, previously termed electro-sonoporation, has been investigated as a technique for enhanced *in vivo* gene transfer into muscle [13] using ultrasound of longer duration (5–10 min) and lower output intensity (2 W/cm^2) than in the current study. The combination was found to significantly enhance expression levels of luciferase and mouse IL-2 at higher voltages compared to the upregulation of gene expression achieved by electroporation alone. However, six pulses of 100 ms were used, and at the higher voltages, substantial damage to muscle fibres was noted. This combination was not used directly as an anti-tumour therapy.

In the absence of electric pulses, low intensity ultrasound (0.4 W/cm^2 in combination with echo contrast microbubble solutions has been demonstrated to enhance transfer of plasmid DNA in skeletal muscle [14]. Various types of acoustic energy have been successfully used to demonstrate enhanced *in vitro* gene transfer [15,16], which is also significantly increased by irradiating subcutaneous tumours in mice with higher intensity

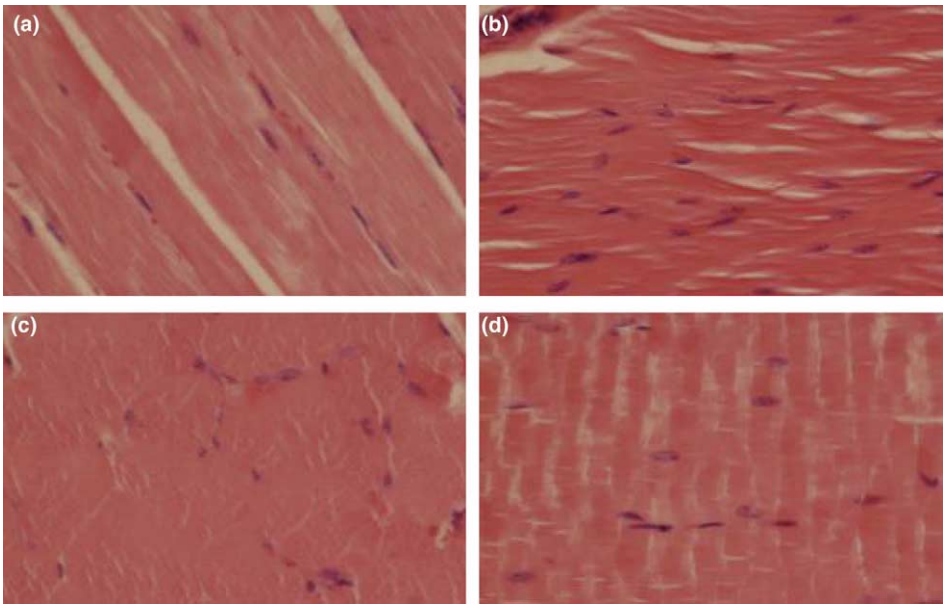

Fig. 6. Haematoxylin and eosin stained mouse thigh muscle: (a) control tissue or (b) tissue exposed 48 h previously to electric pulses or (c) ultrasound (d) or the CEFUS protocol.

(20 W/cm^2) focused ultrasound [17]. Electron microscopic examination of the effect of low intensity (0.4 W/cm^2) ultrasound on cancer cells in the presence of a photosensitive cytotoxic drug [18] has revealed the development of multiple surface pores, and subsequent progression to cell death. In contrast, cells which were exposed to this low level of ultrasound in the absence of the drug, showed only minor disruptions of the cell surface, suggesting that the cell killing was due to temporary pore formation induced on the cell surface, similar to the permeabilising effect of electric pulses. Low intensity ultrasound has also been demonstrated in several studies to enhance the cytotoxic effect of a chemotherapeutic agent *in vitro* [19–21], which has also been proposed to be due to a temporary membrane permeability induced by the ultrasound.

The recent literature contains a number of reports on the early experience of extracorporeal high intensity focused ultrasound (HIFU) in producing thermal ablation in a wide and diverse array of solid carcinomas [6–8,22]. Although reported to be largely successful, some serious adverse effects of the therapy including cutaneous burns, nerve fibre damage, pathological fracture and tumour abscess formation have been observed, and the generation of temperatures of up to 90 °C at the focal area of treatment in less than 10 s causing instant cell death and vascular obliteration in normal and healthy tissue [23,24]. These side effects have the potential to induce systemic toxicity, which would be exacerbated by the creation of a substantial area of necrosis and the attendant metabolic consequences of tumour lysis.

The exposure of tissue to high intensity electrical pulses of very short duration can induce reversible (electropermeabilisation) and irreversible structural changes in the cell membrane, thereby facilitating and enhancing the uptake of normally impermeant substances into the cytosol. Several pulse shapes have been employed during research into electropermeabilisation of cell membranes, with the early focus being on the use of exponentially decaying pulse waves. Such exponential pulses were used in the initial experiments carried out within this study. However, for precise calculation of the electrical field generated and energy delivered during a high voltage pulse, a more defined square wave pulse is preferred. From the clinical perspective, square wave pulses are more easily defined, quantified and controlled in terms of the total energy delivered and are as such better for use in the clinical setting. In this study, the square wave pulses employed reproduced the results observed with the exponential pulse, however, it is important to note that the total pulse length applied with the square wave was less than half of that delivered by the exponential (data not shown, validated by oscilloscope measurement), indicating that the voltage of the electric field delivered is more significant in generating the CEFUS effect than the pulse length. Further studies are required to assess if the total power (Joules) delivered during electrosensitisation plays a significant role in the CEFUS effect.

The discovery of the phenomenon of electropermeabilisation gave rise to a number of experimental and clinical therapies that took advantage of the induction of transient porosity of the cell membrane. Although the exact molecular mechanism behind this phenomenon of transient reversible electrical breakdown of the cell membrane has yet to be fully elucidated, at the level

of the entire cell, the consequences of cell exposure to electric pulses is understood. The most commonly accepted theory to explain the permeabilisation that occurs is electroporation, whereby transient pores form in the cell membrane and reseal in a matter of seconds to minutes. This physical phenomenon has been harnessed to allow temporary access to the cytosol and the most successful clinical application of this undertaken to date has been the delivery of chemotherapeutic agents, or electrochemotherapy. A great deal of work has been done on optimisation of electrochemotherapy using bleomycin and cisplatin, both anti-cancer drugs with a high intrinsic cytotoxicity but which do not readily cross the cell membrane. Passage of these molecules to the cytosol is increased hundreds to thousand fold by electroporation and consequently their cytotoxicity is similarly potentiated [25]. Several reports in the literature describe the successful clinical application of this therapy [26–30]. However, although less than with systemic chemotherapy, the attendant risk of the side effects of the chemotherapeutic agent remains. In addition, the combination of electrochemotherapy with immunogene therapy has shown potential. The combination of electrochemotherapy and cytokine plasmid delivery by electroporation into mouse melanomas has been shown to prevent tumour recurrence and induce long-term antitumour immunity [31].

This paper reports on a modality for cancer treatment *in vivo* that does not require the use of chemotherapeutic drugs and can downstage human oesophageal and murine colon tumours in mouse models via very brief electric pulses. The short application of low intensity ultrasound in the present study suggests that delivery of electric pulses to cancers temporarily sensitises the tumour tissue to the application of low intensity ultrasound. Full tumour regressions were noted in a human oesophageal tumour model, with remission lasting for up to 30 days after the initial treatment. Significant retardation of growth was achieved in an aggressive murine colon adenocarcinoma, although with this cell line the effect was more short-lived. We anticipate that the effect observed should be enhanced and prolonged by further optimisation of the technology. The antitumour effect mediated by the combination of ultrasound and electric pulses is not observed when the modalities are used individually ([11] and unpublished data). Furthermore, the order of application affects the degree and consistency of tumour regression observed, with the optimal response occurring when ultrasound is applied after the electric pulses. ([11] and unpublished data) The ultrasound energy employed during this study (3.5 W/cm^2, 35% duty cycle) is slightly greater than the standard for physiotherapeutic application of ultrasound. No adverse effects were noted in any of the mice treated either with the ultrasound alone or with the full CEFUS protocol.

To further develop this therapy, it is necessary to understand the detailed interaction mechanism between the electric pulses and ultrasound waves on cells both *in vitro* and *in vivo* and their effects on both the cell membrane and intracellular organelles. At present, neither mechanism is fully understood. However, any heating effects associated with the electric fields and ultrasound intensity used within these experiments can be considered to have negligible clinical significance [21,32]. Ultrasound or electric pulses alone were found to have a minimal effect on tumour growth *in vivo*, with full regression only noted when both were used in combination with the electric pulses preceding the ultrasound. This suggests there may be a stress response immobilised by exposure to electric pulses that sensitises cells to the ultrasound application and inhibits further growth. It is envisaged that further refinement and optimisation of this technique may lead to development of a clinical cancer therapy that would be effective, safe, cheap, and repeatable. It is conceivable that this protocol could be developed for application as an endoscopic or laparoscopic modality and could be applicable in the setting of recurrent or inoperable malignant disease.

Conflict of interest statement

None declared.

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