Electrochemotherapy – Evidence for Cell-type Selectivity *In Vitro*

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Abstract. *Aim: Electrochemotherapy (ECT) is a new cancer treatment modality that uses electroporation to potentiate chemotherapeutic agents, especially bleomycin. ECT causes both a direct toxic effect and an anti-vascular effect. The aim of the present study was to investigate a possible selective effect of ECT on the survival of fibroblasts, endothelial cells (HUVEC) and two squamous cell carcinoma cell lines (CAL-27 and SCC-4). Materials and Methods: Cells were electroporated using two bleomycin concentrations. The survival rate was assessed 1, 2, 3 and 4 days after treatment, by two different assays. Results: The survival rate of the fibroblasts was statistically significantly higher than the other cell lines at day 4. The HUVEC survival rate was statistically significantly lower than the other cell types at day 1 after electroporation-alone. Conclusion: A selective survival effect after ECT was observed in vitro, supporting the anti-vascular effect seen in vivo.*

Electrochemotherapy (ECT) is a relatively new cancer treatment modality. It is a local chemotherapy treatment that uses the phenomenon of electroporation (EP) to achieve a high intracellular accumulation of hydrophilic chemotherapeutic agents. EP is the observed increase in cell membrane permeability when the cell is exposed to an electric field (1). Out of the agents tried, bleomycin (BLM) a large polar molecule appears to be the agent most enhanced by EP. *In vitro*, the cytotoxic effect of BLM can be enhanced several hundred times by EP (2, 3). The cytotoxic effect of the smaller polar molecule cisplatin is also enhanced by EP, but to a lesser degree than BLM (2.3-times) (3). On the other hand, EP does not enhance the effect of non-polar molecules like daunorubicin and doxorubicin at all (3). The two major mechanisms for the anti-tumoral effect of ECT are the cytotoxic effect on cancer cells themselves and a vascular effect that decreases the blood flow to the tumor. These effects are seen both with ECT and EP alone *in vivo* (4-6). With EP alone the reduced tumor blood flow is reversible and restored within 48 h whereas with ECT with BLM there is no recovery of tumor blood flow (5, 6). This so called "vascular lock" effect retains the chemotherapeutic agent in the treatment area thereby increasing the treatment effect further (6). Furthermore, the type of cell death that is mediated is dependent on the number of intracellular BLM molecules. A few hundred to few thousand molecules lead to a slow mitotic cell death and more internalized molecules lead to a faster pseudoapoptotic cell death (7). The efficacy of ECT in clinical treatment of different types of cancer has been previously reported (8-11). We have previously reported the 2-year outcome in patients with skin, oral cavity and oropharyngeal cancer treated with ECT (12-14). In summary, the local control rate was excellent and the functional outcome was very good for skin and tongue cancer patients, but poor for patients with floor of mouth, buccal and tongue base cancer (12-14). ECT can also be both an organ- and function-sparing modality (12). Treatment of head and neck cancer often results in significant morbidity, which can affect the ability to swallow, speak and may bring unwelcome changes in physical appearance. For these reasons treatment modalities that have a selective effect on tumor tissue are required. Selective treatment techniques could spare normal tissues and -more importantly- function. In the present study the aim was to investigate possible selective survival effects after ECT in four different cell types *in vitro*, normal and malignant, with EP parameters as close as possible to those used in clinical practice. Under the null hypotheses the survival means would be equal in all cell groups. If rejected there would be evidence suggesting a selective effect of ECT in the investigated cells.

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Materials and Methods

Cells. Four different human cell types were chosen for the investigation, endothelial cells, fibroblasts (FB), and two different squamous cell carcinoma (SCC) cell lines. Endothelial cells were selected in order to investigate a possible endothelial basis for the anti-vascular effects of ECT seen *in vivo* (5, 6) and fibroblasts because of their important role in wound healing after treatment. SCC was chosen because it is the most common type of head and neck cancer. Normal human fibroblasts were isolated by explanting pieces of dermis obtained from elective abdominal or chest surgery. The tissue was removed using standard surgical procedures. The fibroblasts (FB) were sub-cultured in Dulbecco's modified Eagle's medium (DMEM) from Invitrogen (Paisley, Renfrewshire, UK) supplemented with 10% fetal bovine serum (FBS) and 1 mg/ml gentamicin from Invitrogen (Paisley, Renfrewshire, UK). Human umbilical vein endothelial cells (HUVEC) were prepared from umbilical cords of healthy infants, as previously described (15) with minor modifications. Briefly, the umbilical cord vein was flushed with cord buffer (0.137 M NaCl, 4 mM KCl, 0.52 mM $Na_2HPO_4 \times 2H_2O$, 0.15 mM KH_2PO_4 and 11 mM glucose). The vein was incubated with 0.2 % collagenase type I approximately 200 units/mg from Invitrogen (Paisley, Renfrewshire, UK) in cord buffer at 37˚C for 15 min. Endothelial cells were collected by flushing the vein with Medium 199 with Earle's Salt and GlutaMAX™ from Invitrogen (Paisley, Renfrewshire, UK) containing 25% heat-inactivated fetal bovine serum (Invitrogen), 20 units/ml heparin (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and antibiotics (penicillin G, streptomycin and amphotericin B, Invitrogen) (growth medium). Cells were recovered by centrifugation at 140 × *g* for 10 min. The cells were washed with growth medium and centrifuged again. Cells from one vein were seeded in a 25 cm^2 tissue culture flask in growth medium. The medium was changed to EGM-2 Bulletkit™ medium (Cambrex, East Rutherford, NJ, USA) after 24 h and expanded by passaging 1:3 approximately twice-a-week.

Two human tongue squamous cell carcinoma cell lines CAL 27 (CRL-2095) and SCC-4 (CRL-1624) were obtained from American Type Culture Collection (Manassas, VA, USA) (16-19). CAL 27 was sub-cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and gentamycin (1 mg/ml), all from Invitrogen. SCC-4 was sub-cultured in DMEM/Ham's F12 (1:1) supplemented with 10% FBS, hydrocortisone (0, 4 μg/ml) and gentamicin (1 mg/ml). All cells were stored in liquid nitrogen vapour temperature in 50% FBS, 40% DMEM, and 10% dimethylsulfoxide (VWR, Stockholm, Sweden). The cells were removed from culture plastic surface by incubation in 0.25% trypsin and 1 mM EDTA (Invitrogen) at 37 1 ˚C for 5 min. The cells were suspended in medium containing serum to inactivate the trypsin and centrifuged. The cells were resuspended in fresh medium and plated in tissue culture flasks or in multi-well plates.

Bleomycin. Fifteen mg bleomycin (Baxter, Halle, Germany), equal to 15,000 IU were dissolved in 1.5 ml sterile 0.9% sodium chloride to a stock solution of 10 mg/ml (10,000 IU/ml).

Electrochemotherapy protocol. For each cell type, 10^5 cells in 200 μL of serum-free cell culture medium were added to electroporation cuvettes with a 2 mm electrode gap (Bio-Rad, Hercules, CA, USA). BLM was then added to final concentrations of 1, 10, 100 and 1,000 μg/ml. Electroporation was performed using the Gene Pulser Xcell™ (Bio-Rad, Hercules, CA, USA). Eight square-wave pulses, each with a duration of 0.1 ms and a 0.1 s interval between pulses were used, corresponding to clinical ECT parameters (8). The electrical field strengths were 500, 1,000, 1,200 and 1,500 V/cm. 1.8 ml of cell culture medium was then added to the cuvette. After mixing, the electroporated cells were seeded into a black 96-well plate in quadruplicates. To separate the effect of electroporation itself from the effect of ECT we also exposed each cell type to EP only. As controls, quadruplicates of each cell-type were added to the cuvette and mixed without BLM and then seeded in a 96-well plate without being electroporated. To account for the background fluorescence (blank) we also added wells with only cell culture medium.

Assessment of cell survival. The survival of all cell types was assessed at 24 (day 1), 48 (day 2), 72 (day 3) and 96 h (day 4) after EP/ECT using both the protease-based MultiTox-Fluor™ cytotoxicity assessment assay (Promega, Madison, WI, USA) and a crystal violet assay (Sigma-Aldrich). All fluorometric readings were done using the FLUOstar Optima™ microplate reader (BMG Labtech, Ortenberg, Germany). The fluorescence values of the blanks were subtracted from the data.

Statistical analysis. To accept or reject the null hypotheses we were only concerned with the survival data after 96 h. In order to compare the between-group survival independently from cell-type-specific factors, for example different proliferation rates, the survival data was normalized (% of control group from the same cell-type) prior to statistical analysis. The data were tested for normality and equal variance using the Shapiro-Wilk and Leverne's tests respectively. Welch F-test was used to establish if there were any statistically significant within-group differences. In those cases we performed the Games-Howell post-hoc test for between-group comparisons. α=0.01 was chosen as the level of statistical significance. SPSS (Armonk, NY, USA) software was used in all statistical tests. To measure the effect size of the statistically significant survival differences Cohen's d formula with pairwise pooled standard deviations to account for unequal variances in the different cell groups was used.

Results

Determination of optimal BLM dose and electrical field strength. Due to drastic differences in conditions between the clinical and *in vitro* settings the optimal BLM dose and electrical field strength could not simply be extrapolated from clinical ECT. So initially, the FB cell survival with different doses of BLM and electrical field strengths was investigated in a pilot experiment. The goal was to have a statistically significant effect of ECT on cell survival, compared to exposition to BLM-alone, and at the same time have enough FB cells left alive to make comparisons to the other cell types. We also wanted two different BLM concentrations to investigate if there existed dose-dependent differences in survival, possibly related to the two different cell deaths mediated by ECT. The initial intention was to end the experiment after three days; therefore the survival assessment was done after 72 h. The FB cells were exposed to four different BLM concentrations (1, 10, 100 and 1,000 μg/ml)

Table I. *Investigation of the optimal BLM concentrations and electrical field strengths. Welch F-test with the Games-Howell post-hoc test for FB cells treated with BLM alone (0 V/cm) compared to cells treated with BLM and EP with three different field strengths (500, 1000, 1500 V/cm). The tests were done with four different BLM concentrations (1, 10, 100, 1000 μg/ml). In all cases, except for 1 μg/ml, the Welch F-test showed significant within-group differences. The p-values are from the Games-Howell test for the between group comparisons in these cases. The data was based on four independent experiments.*

Electrical fields (V/cm)	Bleomycin concentrations (μg/ml)			
	1	10	100	1,000
0-500	NS	p=0.07	p=0.009	p<0.001
0-1,000	NS	p=0.009	p=0.001	p<0.001
0–1,500	NS	p=0.01	p=0.006	p<0.001

NS, Not significant.

Table II. *Pairwise cell type comparison of survival four days after ECT using the Games-Howell post-hoc test for all significant results from a Welch F-test for low and high doses of bleomycin (ECT L and ECT-H). Cohen's d value for these statistically significant differences shows large differences. Data were based on four independent experiments.*

Cell type	ECT L		ECT H	
	Games-Howell	Cohen's d	Games-Howell	Cohen's d
FB - HUVEC	p<0.001	21.8	p<0.001	34.8
FB - CAL-27	p<0.001	24.5	p<0.001	42.6
FB - SSC-4	p<0.001	21.8	p<0.001	18.8
HUVEC - CAL-27	p=0.20	NS	p=0.88	NS
HUVEC - SCC-4	p=0.28	NS	p=0.09	NS
CAL-27 - SCC-4	p=0.02	NS	p=0.08	NS

Table III. *Pairwise cell type comparison of survival one and two days after electroporation alone using the Games-Howell post-hoc test for the significant results from a Welch F-test (EP day 1 and day 2). Cohen's d value for these statistically significant differences showing large differences. The data was based on four independent experiments.*

Cell type	EP day 1		EP day 2	
	Games-Howell	Cohen's d	Games-Howell	Cohen's d
HUVEC – FB	p=0.001	13.4	p<0.001	19.8
HUVEC -CAL-27	*p=0.001*	*11.2*	*p=0.001*	*14.7*
HUVEC -SCC4	p=0.007	9.4	p=0.016	NS

NS, Not significant.

and four different electric field strengths (0, 500, 1,000 and 1,500 V/cm). Untreated cells were used as controls. The other EP parameters were kept the same as in clinical ECT (8 square waves, 0.1 ms duration). The survival data with both fluorometric methods 72 h after treatment are shown in Figure 1. Both methods showed good agreement except for the combination of the highest BLM concentration (1,000 μg /ml) and the strongest electrical field (1,500 V/cm) (Figures 1A and B). We used the Multitox-Fluor live cell data for statistical analysis. The data were consistent with a normal distribution (Spiro-Wilks test) but did not have equal variances (Leverne's test) therefore; Welch F-test with the Games-Howell post hoc test was chosen. Welch test for all concentrations, except 1 μg/ml, was statistically significant (Table IB). For these reasons the 1 and 1,000 μg/ml BLM doses were excluded and the 10 and 100 μg/ml doses chosen for the subsequent investigation. The test also showed no statistically significant survival differences between cells exposed to the 10 μg/ml dose alone and cells exposed to a combination of the 10 μg/ml BLM dose and EP with 500 V/cm (p=0.071) (Table I). Hence, 500 V/cm was excluded and since there were enough FB cells left alive with both the 1,000 and 1,500 V/cm field strengths (between 20 and 40% relative untreated control cells) for subsequent statistical comparison with the other cell types a field strength in-between, 1,200 V/cm, was chosen for the investigation. This is a field strength often used in clinical ECT (8).

Survival after ECT. The relative survival (% of control group) for all four cell types (FB, HUVEC, CAL-27 and SCC-4) following EP-alone, low-dose and high-dose ECT with both fluorometric methods used is shown in Figures 2 to 4. Generally, the Multitox-Fluor Cytotoxicity and the crystal violet assays again showed a good agreement throughout. The data were analysed using the Shapiro-Wilk and Leverne's tests for normality and equal variance, respectively. The data were normally distributed but the variances were not equal, therefore the Welch F-test with the Games-Howell post hoc test was used. The survival after ECT with the low BLM concentration is shown in Figure 3; there was a trend of decreased survival in all cell-types up to and including day 3 with both assessment methods (Figures 3A and 3B). At day 4 there was an increase in viable FB cells alone and the survival (mean value 56.7% of the untreated control FB cells) was significantly greater than the survival at day 4 for CAL-27 (1.9 %), SCC-4 (5.4%) and HUVEC (3.7%). There were no significant differences in survival between CAL-27, SCC-4 and the HUVEC cells. After ECT with the high BLM concentration there was no trend of increase in viable cells as seen with the low dose ECT (Figures 4A and B). There was, however, still a significant difference in survival at day 4 for FB (27.8 %) compared to the other cell types. Again, there were no

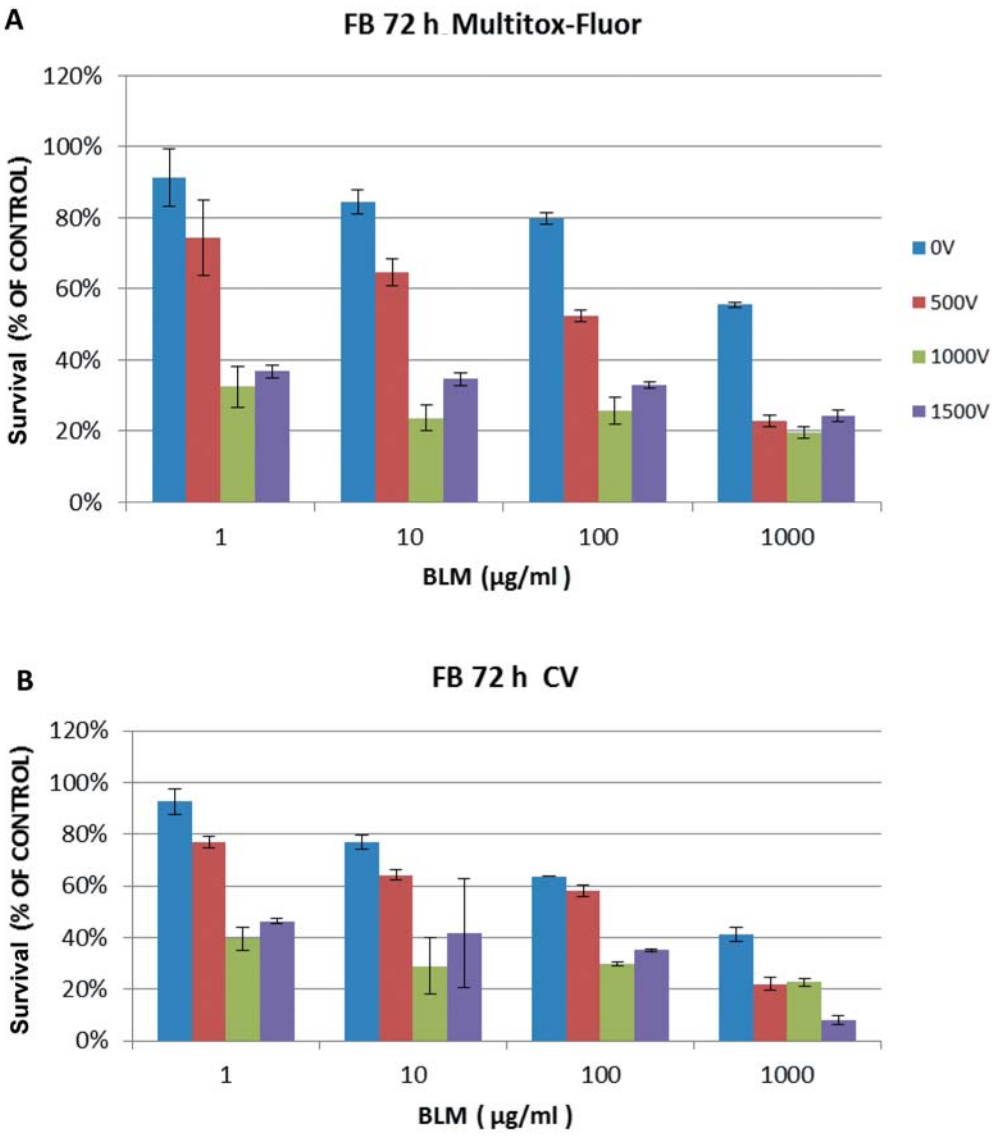

Figure 1. *Investigation of the optimal BLM concentrations and electrical field strengths. Mean relative survival±SEM for fibroblasts (FB) 72 h after ECT treatment with 4 different BLM concentrations (1, 10, 100, 1,000 µg/ml) and four different electric field strengths (0, 500, 1,000, 1,500 V/cm) measured with the Multitox-Fluor assay (A) and the crystal violet (CV) staining assay (B). Data are from four independent experiments.*

significant differences in survival between the CAL-27, SCC-4 and the HUVEC cells. We can, therefore, reject the null hypothesis and conclude that ECT appears to have a selective effect on the survival of the fibroblasts relative both SCC cell types and both doses of BLM. This selective effect seems to be quite large with Cohen's d ≥18.7 for all pairwise viability comparisons (Table II) (Cohen's d >0.8 is considered a large effect). Moreover, we can also accept the null hypothesis for the HUVEC cells and both SCC cell types and conclude that ECT appears to have no selective effect on the survival in these cells for both doses of BLM.

Survival after EP alone. Interestingly the survival of the HUVEC cells after EP alone showed a drastic decrease day 1 and 2 with both the Multitox-Fluor and the crystal violet assays (Figure 2A and 2B). When compared to the other cell types the mean survival differences were significant except for the SCC-4 cells at day 2 (p=0.016). There were no statistically significant viability differences between CAL-27, SSC-4 and FB at day 1 and 2 (Table III). The HUVEC cell viability then subsequently increased and approached the other cell types. At day 4 after EP there were no significant differences between any of the cell types at the p=0.01 level.

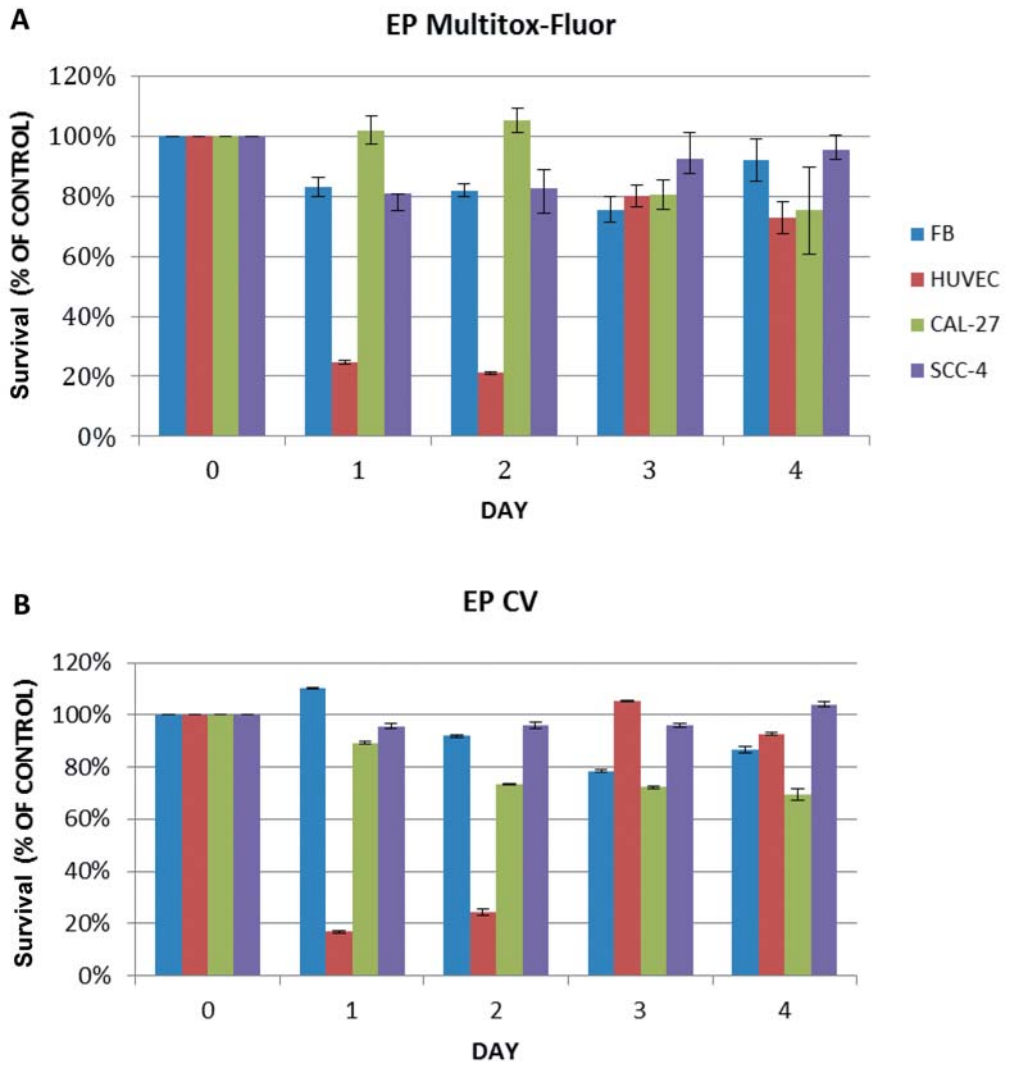

Figure 2. *Mean relative survival±SEM for all cell types day 1-4 after EP measured with the MultiTox-Fluor assay (A) and the crystal violet (CV) staining assay (B). Data are from four independent experiments.*

This selective effect of EP on the HUVEC cells was also quite large with Cohen's d ≥9.4.

Discussion

ECT is a new treatment modality and its future role in the treatment of head and neck cancer has not yet been established. However, apart from the efficacy and safety aspects of ECT, which have been well-established, there is an intriguing possibility that sparing of non-tumor tissue is a feature of ECT. As mentioned above, we have some reason to believe that curative ECT can be both organ and function-sparing in certain cases (12). However, in other-cases it appears to be just as mutilating as surgery (floor of the mouth and bucca) (14). Because of this unpredictability clinical ECT, with the current approaches, has its limitations. The aim of the present study was, from a clinical perspective, to investigate possible selective effects when comparing the survival of human fibroblasts, endothelial cells and two squamous cell carcinoma cell lines (CAL-27 and SCC) after ECT with BLM. The increased potency of especially BLM by electroporation is well known and is again confirmed in this study (Figure 1, Table IA) (2, 3). In this study we also showed selective survival effects of ECT on SCC and endothelial cells compared to fibroblasts. The survival differences after ECT when the fibroblasts were compared to the other cell lines were statistically significant and the effect size was large. Moreover, the fibroblast viability also increased from day 3 to

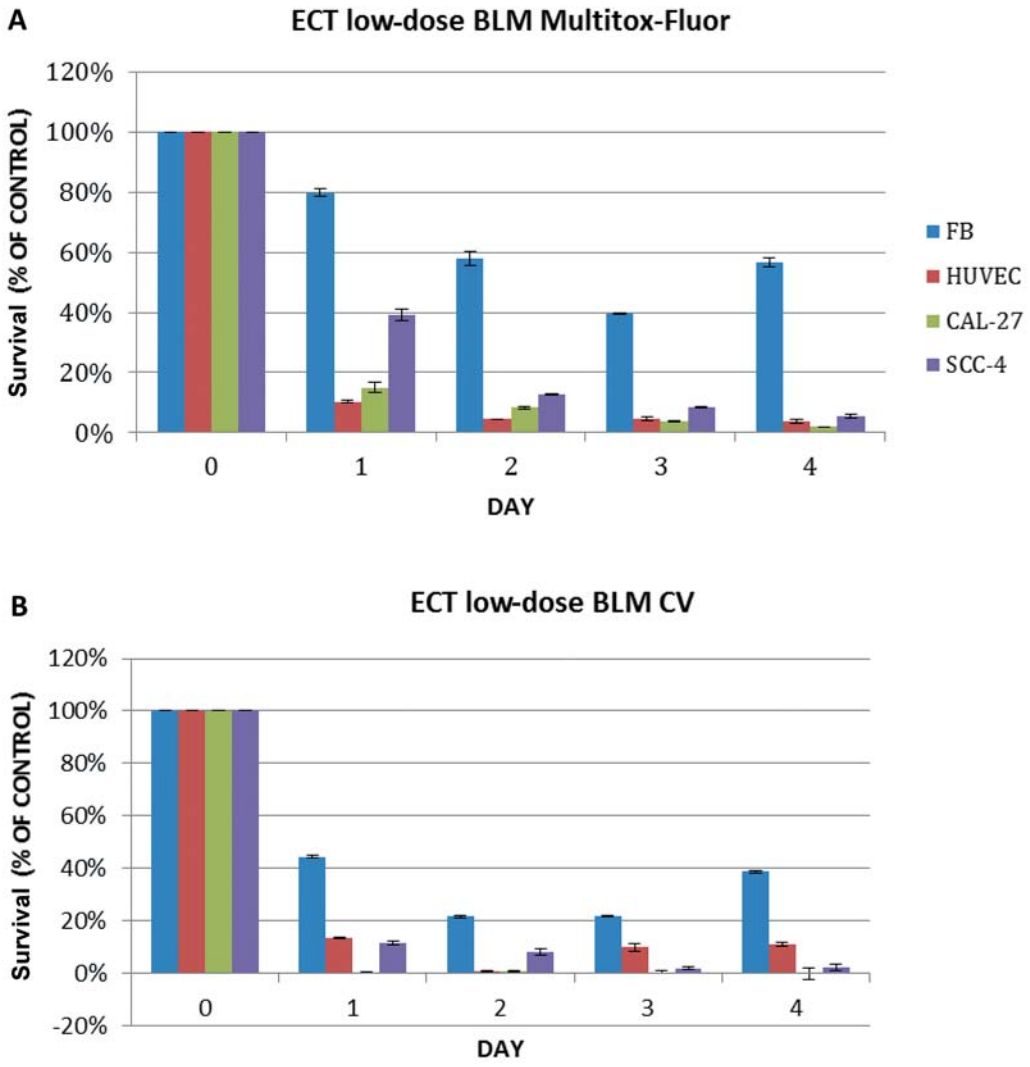

Figure 3. *Mean relative survival±SEM for all cell types from day 1-4 after ECT with the 10 μg/ml BLM concentration measured with the MultiTox-Fluor assay (A) and the crystal violet (CV) staining assay (B). Data are from four independent experiments.*

day 4 with the 10 μg/ml bleomycin concentration (Figure 3, Table II). These results are in agreement with recently published results where human gingival fibroblasts showed an increase in viability 72 h after ECT with a combination of 5-fluorouracil and cisplatin (20). Because ECT can mediate two types of cell death there is always the possibility that the time frame of the experiment was too short to detect the slow mitotic cell death that a lower dose of BLM can mediate (7). But because of increased viability seen for the fibroblasts between day 3 and 4, requiring intact mitotic capabilities, this seems unlikely. No significant selective effect was seen when comparing the survival of human umbilical vein endothelial cells and the squamous cell carcinoma cell lines (Figures 2 and 3, Table II). These results suggest that endothelial cells in the vasculature are vulnerable to ECT and could possibly be involved in the irreversible anti-vascular effects of ECT with BLM seen *in vivo* (4, 5). *In vitro* studies investigating the effects of ECT on endothelial cells have shown a very high sensitivity of HMEC-1 cells to ECT with BLM but only a moderate sensitivity to ECT with cisplatin (4, 21). In addition histological changes in endothelial cells consistent with apoptosis have been reported after ECT with BLM (22). We also found a significant reversible decrease in HUVEC cell survival from EP alone day 1 and 2 (Figure 2, Table III). This result is in agreement with the reversible anti-vascular effect seen *in vivo* with EP alone (4, 6); an effect that can be due to the structural changes in the cytoskeleton seen in endothelial cells after ECT (23). More research is required in order to

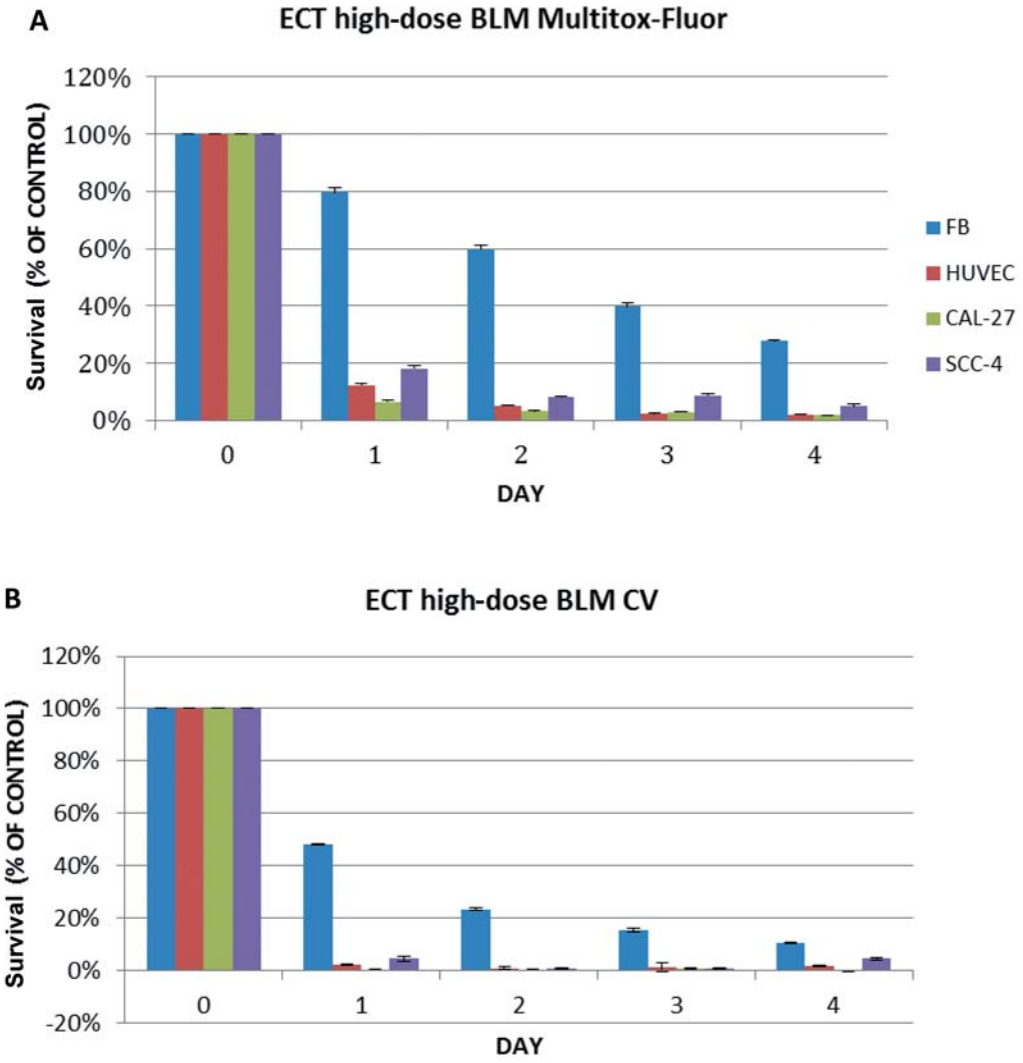

Figure 4. *Mean relative survival±SEM for all cell types for day 1-4 after ECT with the 100 μg/ml BLM concentration measured with the MultiTox-Fluor assay (A) and the crystal violet (CV) staining assay (B). Data are from four independent experiments.*

confirm cell-type specificity of ECT, using other cell-types, as well as other chemotherapeutic agents. Hopefully, this can help develop clinical ECT into an even more promising treatment alternative.

Acknowledgements

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