Human Reproduction Vol.16, No.2 pp. 340–348, 2001

A parametric study of freezing injury in ELT-3 uterine leiomyoma tumour cells

John C.Bischof[1,2,3,5], Walid M.Fahssi[1], David Smith[1], Theodore Nagel[4] and David J.Swanlund[1]

[1]Department of Mechanical Engineering, [2]Department of Biomedical Engineering, [3]Department of Urologic Surgery, and [4]Department of Obstetrics and Gynecology, University of Minnesota, Minneapolis, MN 55455, USA

[5]To whom correspondence should be addressed at: Department of Mechanical Engineering, University of Minnesota, Minneapolis, MN 55455, USA. E-mail: bischof@maroon.tc.umn.edu

Cellular level freeze injury was investigated after controlled freezing of an Eker rat uterine fibroid cell line in both the presence and absence of oestradiol. The connection between thermal history and cell injury in single ELT-3 cells in suspension (without oestradiol) was studied through a two-level, four-parameter (2^4) experiment with membrane dye exclusion as the end-point. The four parameters considered were cooling rate (CR), end temperature (ET), hold time (HT) and thawing rate (TR). A high and low value of each parameter was selected as follows: CR, 5–25°C/min; ET, –20 to –30°C; HT, 0–5 min; TR 20–200°C/min. The greatest parameter effect on freeze injury in this range was ET followed by HT, then TR and finally CR. In addition, significant parameter interactions and curvature were found. Additional CR results outside the original parameter range showed a reduction in survival at both 1 and 50°C/min suggestive of an inverted *U*-shaped survival curve. These results show that this tumour system is susceptible to cryoinjury, particularly at temperatures below –30°C with HT of >5 min and slow thawing. In addition, the presence of oestradiol was found to increase the susceptibility of these cells to cryoinjury.

Key words: cryosurgery/leiomyoma/oestradiol/thermal history/tumour

Introduction

Leiomyomata (fibroids) are the most common indication for hysterectomy in premenopausal women (Carlson *et al*., 1993; Wilcox *et al*., 1994). Pathologically, uterine leiomyomata are almost uniformly benign tumours. However, they are responsible for significant morbidity and even mortality. Several alternative treatments to hysterectomy include myomectomy and hormonal therapy with gonadotrophin-releasing hormone (GnRH) analogues which depress circulating oestradiol concentrations by creating a temporary state of hypogonadotrophic hypogonadism. Traditional myomectomy via laparotomy entails considerable morbidity and is commonly associated with adhesion formation which may impair fertility and, on occasion, may even result in bowel obstruction. Hysteroscopic myomectomy, a technique especially suited to treatment of intracavitary myomata, is reported to control excessive bleeding in 75–90% of the patients, although it is necessary to repeat the procedure in a significant number of women; perioperative complications were limited to 5% of the cases (Malone, 1969; Berkeley *et al*., 1983). Although GnRH analogues may be useful in premenopausal women to control symptoms, the regrowth of leiomyomata after the cessation of treatment limits their use (Matta *et al*., 1989; Friedman *et al*., 1991). In the interest of pursuing alternative means of controlling uterine leiomyomata, freezing (or cryosurgery) shows some promise.

The current applications of cryosurgery have been reviewed recently (Gage and Baust, 1998). For visceral deep-seated soft tissue tumours, cryotherapy can be used through laparoscopy or percutaneously by ultrasound guidance. This approach has been used in liver (Onik *et al*., 1993a; Korpan, 1997), prostate (Onik *et al*., 1993b, 1995), and in initial clinical trials in kidney (Uchida *et al*., 1995; Delworth *et al*., 1996). Cryotherapy has also been used on uterine tissue in the past (Cahan, 1964; Boonstra *et al*., 1990), and is currently being re-evaluated for the treatment of both endometrial disorders and leiomyomata (Rutherford *et al*., 1998; Zreik *et al*., 1998). A recent pilot clinical study suggests that cryomyolysis, or cryosurgery of uterine leiomyomata, may be able to reduce uterine fibroids, thus making it a potential treatment or management option for this disease (Zreik *et al*., 1998). However, knowledge of the precise conditions (and mechanisms) whereby freezing destroys tumour cells and tissues remains an area of active research.

Based on available empirical survival results, it is clear that the thermal dose necessary to destroy tumour cells varies tremendously from cell type to cell type (Gage and Baust, 1998; Smith *et al*., 1999). At the cellular level, mechanisms which are known to affect cell viability are dependent on the thermal history a cell experiences, i.e. the cooling rate (CR),

minimum or end temperature (ET), time held at the end temperature (HT), and thawing rate (TR) (Mazur, 1984; Gage and Baust, 1998). Each of these parameters varies with distance from the cryoprobe in an iceball during cryosurgery. CR-dependent biophysical events in frozen cells [intracellular ice formation (IIF) and severe cell dehydration] have been correlated with cell injury in a variety of cell types (Mazur, 1984), and an ET-dependent injury effect has been observed in Walker carcinoma cells (Jacob *et al*., 1985), human prostate cancer cells (Tatsutani *et al*., 1996) and in a rat prostate cancer line (Bischof *et al*., 1997; Smith *et al*., 1999). Furthermore, HT and TR were found to be correlated with cell death in cryopreserved cell systems, due at least in part to recrystallization (Mazur, 1984). The following study was designed to establish the thermal conditions required to reproducibly destroy uterine leiomyomata cells.

In this study, a two-level, four-parameter (2^4) set of experiments (Box *et al*., 1978) was performed to explore the connection between thermal history and post-thaw viability in cultured uterine leiomyoma cells from the ELT-3 cell line. The ELT-3 and several other leiomyoma cell lines were established and characterized in the laboratory of Dr Cheryl Walker from spontaneously growing leiomyoma tumours in an Eker rat line (Everitt *et al*., 1995). The four parameters of this study were: CR, ET, HT and TR; together these four parameters define the subzero portion of a freeze–thaw process. The thermal parameters which most significantly affect survival outcome were determined through calculation of individual parameter effect values (*E*), according to the experimental design guidelines (Box *et al*., 1978). In addition, any synergy between two parameters in determining survival outcome was revealed by calculation of the interaction value for those parameters (*I*), and the degree of non-linearity in the dependence of survival on parameter variations was assessed based on calculation of the survival curvature (*C*) in the parameter ranges studied. The *E*, *I* and *C* values were used to establish thermal dose thresholds and, where possible, to assist in uncovering cellular injury mechanisms which potentially could contribute to tissue destruction after cryosurgery of a leiomyoma tumour. Additional studies outside the parameter range of the factorial design were performed to assess the broader effects of cooling rate at 1–50°C/min, as well as the presence of the hormone oestradiol on cryoinjury.

Theoretical background

The two-level parametric experimental design was developed to understand systems or processes in which multiple parameters affect the quality of the system response or product (Box *et al*., 1978). The intent of this experimental approach is not to determine precisely a system's dependence on any one parameter, but rather to rank the individual and interactive effects of all possibly important parameters on system response, based on results from as few experiments as possible. The experiments conducted for this work were designed to test the relative individual and interactive effects of the four thermal parameters described above (CR, ET, HT and TR) on ELT-3 cell survival outcome *in vitro*. A high and a low value of each parameter was chosen which define a range of parameter values possible in a typical cryosurgery. These four parameter ranges define a four-dimensional 'parameter space' shown in Figure 1; each point in this space represents a set of freeze–thaw conditions to which ELT-3 cells could potentially be exposed. Because only three dimensions can be depicted, variation in the fourth parameter (HT in Figure 1) is shown as two separate three-dimensional spaces, one for each of the two values that HT can take in the experimental matrix. The central premise of statistically-designed experiments is that the general trends in this variation in ELT-3 survival *inside* the parameter space can be captured by survival measurements at the *corners* of the parameter space. These trends include the relative effect (*E*) of each parameter on ELT-3 survival, as well as the degree of interaction (*I*) between parameters in their effect on ELT-3 survival. ELT-3 survival was measured after exposure to freeze–thaw conditions described by each of these corner points, and these survival values were used to calculate all effect and interaction values. Further general examples of the use of statistical design of experiments can be found in Box *et al*. (Box *et al*., 1978). In addition, the specific experimental design used here is similar to that of a recent study on a rat prostate cancer cell line with the important note that both the thermal parameter range and cell type are different (Smith *et al*., 1999).

Materials and methods

ELT-3 cell culture

ELT-3 cells were cultured *in vitro*. Briefly, cells were grown as monolayers in 25 cm^2 T flasks under 10 ml of Dulbecco's modified Eagle's medium (DMEM)-F12 growth media, containing 1.6×10^{-6} mol/l ferrous sulphate (Sigma Chemical Co, St Louis, MO, USA), 1.2×10^{-5} IU/ml vasopressin (Sigma), 1.0×10^{-9} mol/l triiodothyronine (Sigma), 0.025 mg/ml insulin (Sigma), 1.0×10^{-8} mol/l cholesterol (Sigma), and penicillin/streptomycin supplemented with 10% fetal bovine serum (FBS), in a 37°C, 5% CO_2/95% air atmosphere. Cells were separated from the flask by immersion in 0.05% trypsin and 0.53 mmol/l EDTA, then suspended in 10% FBS-supplemented media such that the final trypsin concentration was $<0.005\%$. Characterization experiments were performed to ensure that 0.005% trypsin in the presence of 10% FBS did not affect the cryosensitivity of ELT-3 cells. After separation from the culture surface, the cell solution was pelleted by centrifugation and the excess medium removed, so that the total suspension volume was <500 µl. The cell suspension was held in a 1.5 ml microcentrifuge tube on ice (+2°C) in preparation for experiments.

A supplemental study to assess whether cryoinjury is enhanced or depressed in the presence of a mitogenic hormone was also performed. ELT-3 cells were cultured in the presence of 10^{-8} mol/l water soluble oestradiol-17β (Sigma). This concentration of oestradiol was selected on the basis of a previous study assessing the impact of the drug Tamoxifen on ELT-3 cell growth in the presence and absence of oestradiol (Howe *et al*., 1995). It should be noted that the selected concentration of hormone is several hundred times the peak circulating values found during pro-oestrus in Sprague–Dawley rats (Butcher *et al*., 1975). Cells were cultured in the presence of hormone for at least 1 week and split continuously every other day in order to ensure dense, log-phase populations.

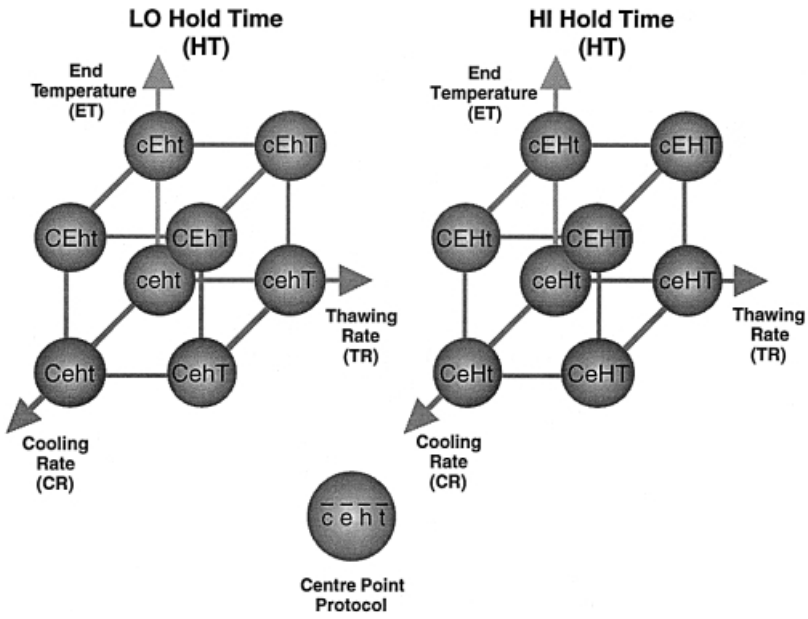

Figure 1. Diagram of the experimental matrix for ELT-3 cell survival over high and low values of four thermal parameters: cooling rate (CR), end temperature (ET), hold time (HT) and thawing rate (TR). In each node of the matrix, the level of each parameter is indicated using the first letter of each parameter designation (c, e, h and t); high and low values of each parameter are designated by upper and lower case letters respectively. The overscore bar on each parameter in the centre point protocol indicates the average of the high and low values of that parameter. Reproduced from Smith *et al*. (1999) with permission of Academic Press. Copyright © 1999 Academic Press. All rights reserved.

Table I. High and low values of each parameter used to define the experimental matrix of freeze–thaw protocols. The matrix is defined graphically in Figure 1 as the 16 possible combinations of these parameter values

Parameter	Low value	High value
Cooling rate	5°C/min	25°C/min
End temperature	–30°C	–20°C
Hold time	0	5 min
Thawing rate	20°C/min	~200°C/min

Experimental design

The experimental matrix for determining how cell survival is dependent on thermal history was designed according to the principles of two-level parametric experimental design (see above). The theory of experimental factorial design is explained in more detail elsewhere (Box *et al*., 1978). The adaptation of this design for the study of cryoinjury on single cells used here was first described in 1999 (Smith *et al*., 1999). The four parameters used in the study to describe a freeze–thaw included: CR, ET, HT and TR. High and low values of each parameter were chosen to be within the range of each parameter typically experienced by cryotreated tissue in cryosurgery (Table I). Survival tests were performed for every combination of parameter values; this results in $2^4 = 16$ experimental protocols, each of which is one point in the matrix as shown in Figure 1. To assess non-linearity in the survival dependence on thermal history, an additional protocol (the centre point protocol) defined by the midpoint of each of the four parameter ranges was included, for a total of 17 freeze–thaw protocols in the parametric experimental design. Additional protocols were used to assess CR dependence over a broader CR range than that defined in Table I for the minimally injurious values of the other parameters (i.e. high ET, low HT and high TR). The additional CR tested were 1, 10, 20 and 50°C/min to –20°C. Each protocol was performed three times on separate cell suspensions, so that an average survival value and SD could be calculated for each protocol in the matrix (Figure 1). By measuring cell survival in cells exposed to each of the 17 protocols in the parametric experiment design, survival dependence on each thermal parameter can be assessed.

Freeze–thaw protocols

All freezing protocols were carried out by directional solidification. The directional solidification process for cell suspensions is described in previous studies (Bischof *et al*., 1997; Smith *et al*., 1999). Briefly, ELT-3 cells in a 30 µl suspension on a glass microslide were passed at a constant velocity between two precisely controlled thermal reservoirs, the first held at suprazero temperature and the second at subzero temperature. In the present study, the glass microslides used to carry the cells had a rectangular groove, or well, milled perpendicular to the direction of microslide travel to carry the cell suspension. The well was 3.2 mm wide (in the direction of travel) and of 1 mm depth (into the microslide surface). The presence of the well reduced spreading of the suspension over the microslide surface, which minimized differences in HT across the width of the cell suspension during directional solidification, and allowed reproducible recovery of 10 µl of suspension after freezing and thawing when a 30 µl sample was loaded initially. High TR (~200°C/min) were achieved by removing the glass microslide from the directional solidification device and quickly bringing the bottom of the microslide into contact with an aluminum block held at 37°C. Although the CR and ET achieved in the cell suspension can be set nominally through setting the two reservoir temperatures and microslide velocity, the actual CR and ET achieved in the sample can be somewhat different from these nominal settings, particularly at high rates of cooling (>50°C/min) as previously described (Kourosh *et al*., 1990; Smith *et al*., 1999).

After the freeze–thaw process, the viability of cell suspensions was

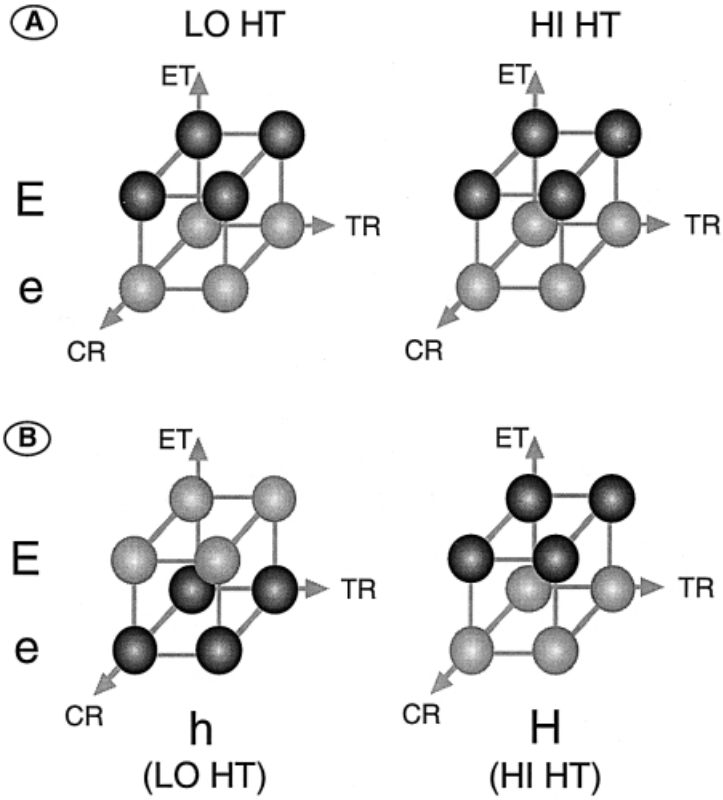

Figure 2. Parameter effects and interactions between the parameters in Figure 1 are calculated as the difference between two averages of eight experimental survival values: 1/8 Σ ● values – 1/8 Σ ○ values. Calculation of the end temperature effect (E_{ET}) is shown in (**A**) and the end temperature-hold time interaction ($I_{ET–HT}$) is shown in (**B**). Reproduced from Smith *et al.* (1999) with permission of Academic Press. Copyright © 1999 Academic Press. All rights reserved.

measured using calcein AM and propidium iodide as previously described (Smith *et al.*, 1999). A 10 µl sample of cell suspension was removed from the microslide and incubated with 2 µmol/l calcein AM and 3 µmol/l propidium iodide for 15–30 min at 37°C. After incubation, cells were scored as either live or dead under a fluorescent microscope. Three separate freeze–thaw experiments were performed for each experimental protocol. Viability measurements on cells taken directly from the stock suspension without freezing were determined before and after every three freeze–thaw experiments as a control; all experimental viability values were normalized to the average control viability. Control viabilities were all >95%. The viability of cell suspensions frozen *in vitro* and assessed immediately post-thaw likely underpredict the true cryosurgical injury for the same thermal history experienced *in vivo* (Bischof *et al.*, 1997), but these short-term viability measurements do serve as conservative estimates of possible in-vivo cryodestruction.

Statistical analysis

Once the survival data were collected, the parameter effects, interactions and curvature were calculated using the formulation originally described by Box (Box *et al.*, 1978) and recently applied to cryoinjury in AT-1 cell suspensions (Smith *et al.*, 1999). Each parameter effect was calculated from the survival results as the average difference in survival percentage caused by change in that parameter. This difference was calculated by averaging the survival values (0–100%) for the eight protocols in which the high value of the parameter was used, and subtracting the average survival for the eight protocols in which the low parameter value was used. Calculation of the ET effect is shown in equation 1 as an example (using the nomenclature of Figure 1) and shown pictorially in Figure 2A.

$$E_{ET} = 1/8\,(cEht + CEht + cEHt + cEhT + CEHt + CEhT + cEHT + CEHT) - 1/8\,(ceht + Ceht + ceHt + cehT + CeHt + CehT + ceHT + CeHT)$$

[equation 1]

E_{ET} is the ET effect value, and each four-letter combination of *c, e, h* and *t* represents one of the 16 average survival measurements in the experimental matrix. The capital or lowercase letters represent the high or low value for a parameter respectively. For example, *cEHt* represents the average survival for cells exposed to the low CR, high ET, high HT and low TR, according to the parameter values in Table I.

The magnitude of the minimum significant parameter effect can be calculated using the Student's *t*-distribution and the pooled SD for the 17 protocols used as part of the matrix for the two-level experiment (Moffat, 1985). This minimum value represents the average difference in survival between two protocols caused solely by experimental uncertainty in the survival measurements. The magnitude of any parameter effect must be greater than this minimum value to be considered significant, and the relative magnitudes of parameter effects indicated which effects most significantly affect survival in the parameter ranges studied (Table I). The minimum significant parameter effect was calculated using the following formula (Moffat, 1985):

$$\mu_E = ts\sqrt{\frac{2}{mk}}$$

[equation 2]

The parameter *t* is the *t* statistic for the chosen confidence interval from the Student's *t*-distribution. For the present set of experiments, the number of degrees of freedom for the parametric design is the

total number of data points collected minus the total number of combinations of parameters. The total number of data points was 17 protocols, at three trials per protocol, or 51. The total number of parameter combinations for which survival was measured was 17; 16 combinations of extreme parameter values plus the additional survival measurement for the centre point protocol. The number of degrees of freedom was thus 51 – 17 = 34. For a 99% confidence level with 34 degrees of freedom, $t = 2.72$ (Moore and McCabe, 1993). The parameter s is the pooled SD in all 17 protocols. The parameter m is the number of parameter levels (two) raised to the power of the number of parameters minus 1, or $m = 2^3 = 8$. The parameter k is the number of separate trials for each protocol in the matrix; in this experimental design, $k = 3$. The value of μ_E computed from the survival results for this study was 3.45%.

The parameter interactions were also calculated using the formulation of Box *et al*. (1978) which has recently been applied to cryoinjury in cell systems (Smith *et al*., 1999). The interaction between two parameters was calculated from the survival results as the average change in the parameter effect for one of the parameters caused by increase of the other parameter. This calculation was carried out by averaging the survival values for the eight protocols in which the same level of each parameter was used (i.e. both high values or both low values) and subtracting the average survival for the eight protocols in which the high value of one parameter and the low value of the other parameter was used. The ET–HT interaction calculation is shown in equation 3 as an example (using the nomenclature of Figure 1) and shown pictorially in Figure 2B.

$$I_{ET-HT} = 1/8\,(cEHt + CEHt + cEHT + CEHT + ceht + Ceht + cehT + CehT) - 1/8\,(cEht + CEht + cEhT + CEhT + ceHt + CeHt + ceHT + CeHT)$$

[equation 3]

I_{ET-HT} is the ET–HT interaction value, and the other symbols are described above for equation 1. The relative magnitudes of parameter interactions indicate which ones most significantly affect survival in the parameter ranges studied (Table I). The magnitude of the minimum significant parameter interaction μ_I is equal to μ_E, because in both cases the calculated quantity is computed from the same 16 survival values (Moffat, 1985). The magnitude of any parameter interaction must be greater than this minimum value to be considered significant.

The non-linearity of survival dependence on thermal history was assessed by calculation of the curvature in the results. The curvature (C) is the extent to which cell survival variation departs from linear dependence on the parameters (Moffat, 1985). It was calculated from the survival results as the difference in survival percentage between a linear interpolation of the experimental data and actual survival measurement at a point in the parameter space midway between the high and low levels of each parameter. The linear interpolation of the experimental data at the centre point in the parameter space was simply the average survival for all 16 protocols in the experimental matrix. The curvature was calculated by taking the average survival for the 1 protocols and subtracting the survival measured for the centre point protocol:

$$C = 1/16\,(cEHt + CEHt + cEHT + CEHT + ceht + Ceht + cehT + CehT + cEht + CEht + cEhT + CEhT + ceHt + CeHt + ceHT + CeHT) - \overline{cehT}$$

[equation 4]

C is the value of the curvature, and the other symbols are described above for equation 1. The survival value $\overline{cehT}$ is the measured survival for the centre point protocol. The magnitude of the minimum significant curvature can be calculated using the Student's t-distribution. The minimum significant curvature μ_C was calculated using the following formula (Moffat, 1985):

$$\mu_C = ts\sqrt{\frac{1}{mk} + \frac{1}{C}}$$

[equation 5]

The parameters t, s, m and k are defined above. The parameter c is the number of separate trials performed for the centre point protocol; in this experiment, $c = 3$. μ_C is not equal to μ_E or μ_I because the curvature calculation includes an additional survival value, the centre point survival. The value of μ_C computed from the survival results for this study was 7.3%.

Results

The survival results for the two-level parametric experiment on ELT-3 cells are summarized in Figure 3. The results are presented in the graphical parameter space first introduced in Figure 1. The results showed a large variation in survival, from a maximum of 84.1% to a minimum near 0%, indicating that cell survival was sufficiently sensitive to the parameter changes used in this study to show significant trends. These trends were quantified through the parameter effect and interaction calculations, presented below.

The parameter effect values (E), calculated as described above, are shown in Table II. The parameter effect values were interpreted as the average difference in survival percentage due to that effect. For example, the CR effect of –0.3% implies that the average survival decreases by –0.3% if the CR is increased from 5–25°C/min. The larger the magnitude (absolute value) of the effect value, the larger the relative importance in determining cell survival after a freeze–thaw protocol. The sign of a parameter effect value indicates whether survival depends directly (positive) or inversely (negative) on that parameter. The minimum significant value of parameter effects μ_E was 3.45%. This means that experimental uncertainty alone could account for a 3.45% difference in survival between any two protocols, so only parameter effects with values greater than this will have statistically significant survival dependence ($P \leqslant 0.01$). Of the four thermal parameters, only ET, HT and TR appeared to have an effect on survival; the CR effect was below the minimum significant value. Of these three parameters, TR and HT appeared to be less important than ET, both having a magnitude roughly 2/3 that of the ET effect. Based on the results in Table II, reduction in ET from –20 to –30°C reduced survival values an average of 32.8 percentage points, an increase in HT from 0–5 min reduced survival values an average of 21.4 percentage points, and a decrease in TR from 200 to 20°C/min decreased survival by 21.0 percentage points. The parameter-effect results clearly indicated that, for the parameter ranges studied, ET was the most important parameter followed by HT and TR as the parameters that affected survival outcome most.

The parameter interaction results (I) shown in Table III also reflect the importance of ET, HT and TR in determining ELT-3 cell survival in the freeze–thaw experiments. The values of each interaction between two parameters were interpreted as the average difference in one parameter effect due to change

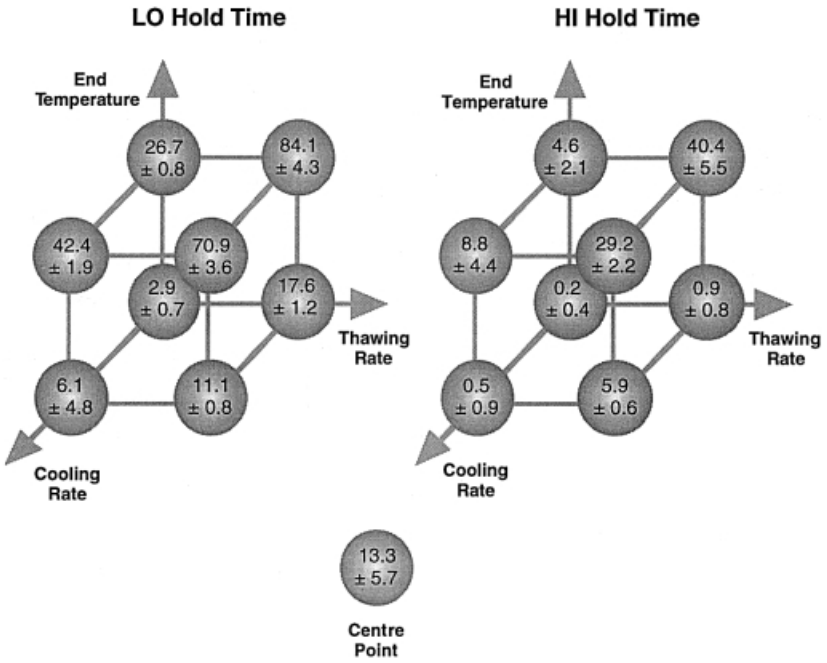

Figure 3. Survival of ELT-3 cells exposed to the experimental matrix of freeze–thaw protocols defined in the text. Survival is expressed as percentage of control survival, where control survival was measured in suspended cells not exposed to a freeze–thaw protocol. The average and SD of three measurements are reported for each point in the matrix. Survival was assayed using the fluorescent probes calcein AM and propidium iodide.

Table II. Parameter effects calculated from the survival results in Figure 3, using the calculation procedure described in the text

Cooling rate	End temperature	Hold time	Thawing rate
–0.3	32.8	–21.4	21.0

Table III. Parameter interactions calculated from the survival results in Figure 3, using the calculation procedure described in the text

Cooling rate end temperature	Cooling rate hold time	Cooling rate thawing rate
–0.8	–0.1	–6.2
End temperature hold time	**End temperature thawing rate**	**Hold time thawing rate**
–13.9	14.6	–5.5

in the other parameter. For example, the ET–HT interaction value of –13.9% implied that the average change in survival caused by reducing the ET was further reduced by 13.9% when the HT was increased. The sign of each parameter interaction value indicates whether the change in survival due to one parameter depends directly (positive) or inversely (negative) on the other parameter. The minimum significant value of parameter interactions μ_I was equal to μ_E, 3.5%. Of the six possible parameter interactions, two had magnitudes below μ_I. They both involved cooling rate: CR–ET and CR–HT. Of the four remaining interactions, the ET–HT and ET–TR interactions were roughly two to three times greater in magnitude than the HT–TR or CR–TR interactions. The parameter interaction results in Table III, combined with the parameter effect results in Table II, show that predominantly ET (and secondarily HT and TR) determined ELT-3 cell survival after a freeze–thaw protocol.

The curvature in the survival results (C) indicated a strongly non-linear dependence on the thermal parameters. The curvature value calculated from the average survival for the 16 parameter extrema data points and the centre point survival was –29.6%, which was much greater in magnitude than the calculated value of μ_C, 7.5% (equation 5). This indicated that survival of cells exposed to a thermal history defined by the midpoint of each parameter range was nearly 30 percentage points higher than the survival predicted for this thermal history by a linear interpolation of the other survival results. This suggested that survival depended non-linearly on the four thermal parameters. One cannot determine from this result whether this behaviour is due primarily to one or more individual parameters, or due to the combined effect of all four. Still, the fact that some threshold in one or more parameters must be reached to achieve significant cellular destruction may have implications regarding possible mechanisms of injury.

Tissues during cryosurgery will often experience cooling rates which vary from 50–100°C/min at the probe to as low as 1–5°C/min, or even less, at the advancing ice front boundary. Thus, additional CR studies beyond the 5–25°C/min selected in the factorial design were also performed and are shown in Figure 4. In order to isolate the specific effects of CR, studies were conducted between 1 and 50°C/min for mild values of the other three parameters (i.e. high ET, low HT and high TR). The survival values ± SD for cells cooled at 1, 5, 10, 20, 25 and 50°C/min to –20°C were 14.6 ± 6.6%, 82.3 ±

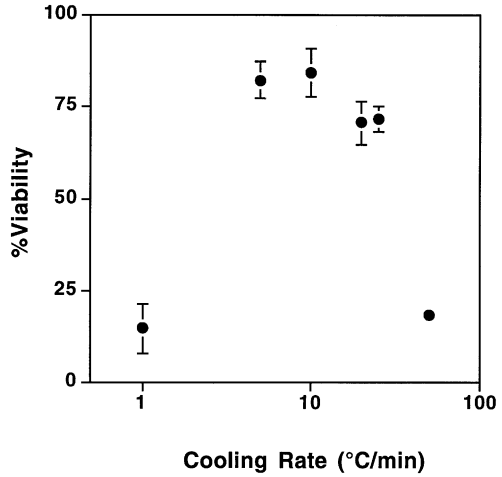

Figure 4. ELT-3 survival as a function of cooling rate. Cooling rates in the range of 1–50°C/min are given on the *x* axis, while survival is plotted on the *y* axis. The end-temperature for all cases was –20°C, 0 hold time, and 200°C/min thawing rate.

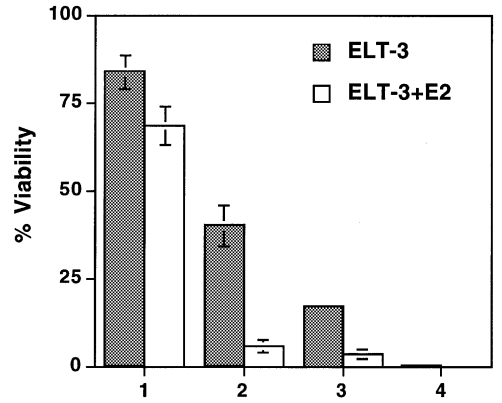

Figure 5. ELT-3 survival as a function of end-temperature (ET), hold time (HT) and the presence of oestradiol (E2). Four experiments, one for each combination of ET and HT were performed in the presence of oestradiol and compared with the results already available from Figure 3 in the absence of oestradiol. The four cases were: 1: high ET and low HT; 2: high ET and high HT; 3: low ET and low HT; 4: low ET and high HT. A low CR and a high TR were fixed in all protocols. High and low values of the thermal parameters are selected from Table I.

5.1, 84.4 ± 6.6, 70.6 ± 5.9, 71.8 ± 3.4 and 18.33 ± 2.3% respectively. The results at 5 and 25°C/min were taken directly from Figure 3. These results suggest an 'inverted *U*' survival curve for ELT-3 cells as typically seen in mammalian cells (Mazur, 1970, 1984). Preliminary observations of intracellular ice formation (IIF) in ELT-3 cells ($n = 20$) cooled to –20°C, showed that IIF began to form only at CR ⩾25°C/min (data not shown). This IIF measurement was taken using standard cryomicroscopy techniques, described in detail in other work (Toner *et al.*, 1992; Bischof *et al.*, 1997).

The effects of the presence of the steroid hormone oestradiol-17β on cryoinjury in the ELT-3 cells was also investigated. Figure 5 shows a comparison of freezing in the presence and absence of the hormone (10^{-8} mol/l) for four selected protocols which give high and low survival in the absence of hormone as seen in Figure 3. A low CR and a high TR were fixed in these experiments, while allowing the ET and the HT to vary. As can be seen in Figure 5, the high ET, low HT experiment lead to the highest survival of 84 ± 4 and 68.5 ± 5.5 in the absence and presence of oestradiol respectively. In the second experiment a high ET and a high HT showed a drop in viability to 40.3 ± 5.5 and 6.11 ± 1.7 in the absence and presence of oestradiol respectively. The third experiment used a low ET and a low HT showing a further drop to 17.6 ± 1.2 and 3.8 ± 1.5 in the absence and presence of oestradiol respectively. Finally, the last experiment used a low ET and a high HT resulting in a further drop to 0.85 ± 0.78 and 0 in the absence and presence of oestradiol respectively. Thus, under the conditions tested, the presence of oestradiol always contributed to an increase in cryoinjury.

Discussion

Tumour cell cryosensitivity

The survival trends determined for ELT-3 cells are consistent with those observed in other tumour cell lines; however, the thermal thresholds which induce significant survival changes show that ELT-3 cells are relatively cryosensitive. Although documentation of the entire thermal protocol is not always given, the determination of a lethal ET for tumour systems has long been an area of study in cryobiology. Some of the reported measurements are: mouse mammary tumour 39% survival at –79°C (Ludwin, 1951), 0% survival at –40°C in Walker carcinoma cells (Jacob *et al.*, 1985) and, with single freezes, ~50% survival at –20°C and ~20% survival at –30°C in ND-1 human primary prostate adenocarcinoma cells (Tatsutani *et al.*, 1996). The CR dependence of survival is also cell-type dependent; Tatsutani *et al.* found a significant decrease in ND-1 cell survival when CR was increased from 5 to 25°C/min, while another study found no statistically significant difference in the survival of HeLa cells cooled to –20°C at rates of 5, 20 and 30°C/min (McGrath *et al.*, 1975). The combined results of the present study and other tumour cell studies show that survival CR dependence can vary from tumour cell line to line, but the CR which yields maximal survival for tumour cells in general appears to be in the range 5–15°C/min, a rate which is common in many cryosurgical procedures. HT and TR effects on tumour cell survival have not been studied as extensively. A further study did consider HT of 0–30 min in their work, and although they concluded there was no HT dependence in their survival results, there was a statistically significant survival decrease between 0 min and 30 min HT for cells cooled to –15°C (Jacob *et al.*, 1985). In order to evaluate the effects of all thermal parameters simultaneously on a given cell type, a factorial design approach was recently developed.

Results of the factorial design approach for thermal sensitivity in Dunning rat AT-1 prostate tumour cells show similar survival trends over a much broader parameter range than in the present uterine leiomyoma study (Smith *et al.*, 1999). Due to the lack of thermal sensitivity of the AT-1 line, the parameter space of interest was much larger: CR, 5–50°C/min; ET, –20 to –80°C; HT, 0–15 min; TR 20–200°C/min. Clearly the thresholds of injury in the AT-1 prostate cancer model were

very different. In particular, AT-1 cells survived freezing to –80°C under various conditions, whereas the ELT-3 cells only survive to roughly –30°C. In addition, AT-1 cell survival began dropping after 15 min of HT at –20°C whereas similar survival drops occurred within ELT-3 cells after only 5 min. When the specific parameter effects and interactions were investigated, the AT-1 study showed that the most important thermal parameter was ET, followed closely by HT and to a lesser extent TR. The interactions between any of these three effects were significant, as they are for ELT-3 cells as well. Again, in the AT-1 study as here, CR was found to play a statistically small to negligible role within the parameter space tested. It should be noted that expanded cooling rates were investigated in both the AT-1 (0.6–90°C/min) and ELT-3 (1–50°C/min) studies. The expanded CR range led to a drop from 75–85% to 40% in survival for the AT-1 cells at 0.6°C/min, while the survival drop in ELT-3 cells was from between 70–80% to 15–20% at both 1 and 50°C/min when all other parameters were held at mild values. A comparison between the AT-1 and ELT-3 studies suggests that the trends in thermal parameter sensitivity between two cell types were similar, however the parameter thresholds necessary to induce significant survival reduction were dramatically different. It should also be noted that in addition to sharing similarities with single-cell studies, the general trends in ELT-3 cell survival are similar to those found in an experimental study of in-vivo skin cryosurgery in dogs (Gage *et al*., 1985).

Finally, since the growth of many tumours including leiomyomata are hormonally dependent, and cryosurgical patients may be in varying stages of the menstrual cycle, it is important to investigate the relative effects of hormonal presence on cryosensitivity. It is perhaps not surprising that when oestradiol is present, under all conditions tested, the cryosensitivity of the cell line is increased as shown in Figure 5. This is perhaps an indication that a proliferating cell is more sensitive to thermal insult. It is well known that heat shock is tolerated poorly by cells in mid- to late S phase when the cell is fully committed to mitosis (Walsh *et al*., 1991). Perhaps the same or a similar phenomenon is acting in the oestradiol-treated ELT-3 cells, which are reproducing more actively than cultures in the absence of hormone.

Cryosurgical implications

By far the most widely used approach in cryosurgery consists of an attempt to reach a lethal ET within the entire tumour by allowing the ice volume to grow beyond the tumour boundary (Gage, 1992). This critical isotherm approach is based on the clinical and experimental evidence reviewed above and elsewhere which supports the importance of ET in determining survival outcome at the cellular level. The ELT-3 cell-survival results support the importance of ET. The results also indicate that this destruction will occur in cells cooled to below –30°C, a temperature above the criteria usually used in designing cryosurgical protocols (–40 to –60°C) (Gage, 1992). In addition to the end-temperature effect, increased HT and slow thaw rate also have a positive effect in lowering cell survival in the ELT-3 system. This approach is generally recommended for any tumour in reviews on the subject of cryosurgery, however not all tumour cells show the same cryosenstivity (Gage, 1992; Gage and Baust, 1998). Our recent report on cryosensitivity in AT-1 prostate tumour cells using the same technique showed that these cells can significantly withstand cooling protocols to –60°C and below (Smith *et al*., 1999). It is thus very important to verify the cryosensitivity of the specific tumour type one hopes to treat with cryosurgery.

In vivo, additional injury mechanisms may assist in defining the injury event. First, the cells are embedded in an extracellular matrix and may have a different response to freezing than when cultured and trypsinized prior to experimentation in suspension. Secondly, the microvasculature of the tissue usually contributes to injury by occluding after a freeze–thaw cycle (Gage and Baust, 1998; Hoffmann *et al*., 1999). And finally, there is the possibility that an immunological reaction to the tissue is activated by the freeze–thaw process which accentuates its further destruction post-freeze. Unfortunately, these in-vivo effects have not been quantified in most tissues. Further understanding of these additional injury mechanisms in both animal and human tissue is currently being pursued in several laboratories (including ours). This information, combined with a knowledge of cellular injury due to the thermal parameters as reported here, will help to insure a good clinical outcome if uterine leiomyomata are treated with cryosurgery.

Acknowledgements

The ELT-3 cell line was obtained courtesy of Cheryl Walker, of the MD Anderson Cancer Center, Smithville, TX, USA. This work was supported by grant# NSF BES–9703326 and a gift from Candela Corporation.

References

Berkeley, A.S., DeCherney, A.H. and Polan, M.L. (1983) Abdominal myomectomy and subsequent fertility. *Surg. Gynecol. Obstet.*, **156**, 319–322.

Bischof, J.C., Smith, D.J., Pazhayannur, P.V. *et al*. (1997) Cryosurgery of Dunning AT-1 rat prostate tumor: thermal, biophysical and viability response at the cellular and tissue level. *Cryobiology*, **34**, 42–69.

Boonstra, H., Koudstaal, J., Oosterhuis, J.W. *et al*. (1990) Analysis of cryolesions in the uterine cervix: application techniques, extension, and failures. *Obstet. Gynecol.*, **75**, 232–239.

Box, G.E.P., Hunter, W.G. and Hunter, J.S. (1978) *Statistics for Experimenters.* Wiley, New York, USA.

Butcher, R., Collins, W. and Fugo, N. (1975) Plasma concentration of LH, FSH, Prolactin, Progesterone and Estradiol-17β throughout the 4-day estrus cycle of the rat. *Endocrinology*, **94**, 1704–1708.

Cahan, W.G. (1964) Cryosurgery of the uterus: Description of technique and potential application. *Am. J. Obstet. Gynecol.*, **88**, 410–414.

Carlson, K.J., Nichols, D.H. and Schiff, I. (1993) Indications for hysterectomies. *N. Engl J. Med.*, **328**, 856–860.

Delworth, M.G., Posters, L.L., Fornage, B.D. and von Eschenback, A.C. (1996) Cryotherapy for renal cell carcinoma and angiomyolipoma. *J. Urol.*, **155**, 252–254.

Everitt, J.I., Wolf, D.C., Howe, S.R. *et al*. (1995) Rodent model of reproductive tract leiomyomata: clinical and pathological features. *Am. J. Pathol.*, **146**, 1556–1567.

Friedman, A.J., Hoffman, D. I., Comite, F. *et al*. (1991) Treatment of leiomyomata uteri with leuprolide acetate depot: a double-blind, placebo-controlled, multicenter study. *Obstet. Gynecol.*, **77**, 720–725.

Gage, A.A. (1992) Cryosurgery in the treatment of cancer. *Surg. Gynecol. Obstet.*, **174**, 73–92.

Gage, A.A. and Baust, J. (1998) Mechanisms of tissue injury in cryosurgery. *Cryobiology*, **37**, 171–186.

Gage, A.A., Guest, K., Montes, M. *et al*. (1985) Effect of varying freezing and thawing rates in experimental cryosurgery. *Cryobiology*, **22**, 175–182.

Hoffmann, N., Swanlund, D.J. and Bischof, J.C. (1999) Observation of vascular injury after freezing: investigating the response of normal skin and subcutaneous tumor tissue to cryosurgery in the Dorsal Skin Flap Chamber. *ASME BED-44/HTD-363*, 39–42.

Howe, S., Gottardis, M., Everitt, J. and Walker, C. (1995) Estrogen stimulation and tamoxifen inhibition of leiomyoma cell growth *in vitro* and *in vivo*. *Endocrinology*, **136**, 4996–5003.

Jacob, G., Kurzer, M.N. and Fuller, B.J. (1985) An assessment of tumor cell viability after *in vitro* freezing. *Cryobiology*, **22**, 417–426.

Korpan, N.N. (1997) Hepatic cryosurgery for liver metastases. Long-term follow-up. *Ann. Surg.*, **225**, 193–201.

Kourosh, S., Crawford, M.E. and Diller, K.R. (1990) Microscopic study of coupled heat and mass transport during unidirectional solidification of binary solutions—I. thermal analysis. *Int. J. Heat Mass Transfer*, **33**, 29–38.

Ludwin, I. (1951) Survival of tumor material after freezing at –79°C. *Biodynamica*, **7**, 53–55.

Malone, L.J. (1969) Myomectomy: recurrence after removal of solitary and multiple myomas. *Obstet. Gynecol.*, **34**, 200–203.

Matta, W.H.M., Shaw, R.W. and Nye, M. (1989) Long-term follow-up of patients with uterine fibroids after treatment with the LHRH agonist buserelin. *Br. J. Obstet. Gynaecol.*, **96**, 200–206.

Mazur, P. (1970) Cryobiology: the freezing of biological systems. *Science*, **168**, 939–949.

Mazur, P. (1984) Freezing of living cells: mechanisms and implications. *Am. J. Physiol.*, **143**, C125–C142.

McGrath, J.J., Cravalho, E.G. and Huggins, C.E. (1975) An experimental comparison of intracellular ice formation and freeze–thaw survival of HeLa S-3 cells. *Cryobiology*, **12**, 540–550.

Moffat, R.J. (1985) *Planning Experimental Programs – Lecture Notes*. Stanford University, Stanford, CA, USA.

Moore, D.S. and McCabe, G.P. (1993) *Introduction to the Practice of Statistics*. W.H.Freeman and Co, New York, USA.

Onik, G.M., Atkinson, D., Zemel, R. and Weaver, M.L. (1993a) Cryosurgery of liver cancer. *Semin. Surg. Oncol.*, **9**, 309–317.

Onik, G.M., Cohen, J.K., Reyes, G.D. *et al*. (1993b) Transrectal ultrasound-guided percutaneous radical cryosurgical ablation of the prostate. *Cancer*, **72**, 1291–1299.

Onik, G.M., Rubinsky, B., Watson, G. and Ablin, R.J. (1995) *Percutaneous Prostate Cryoablation*. Quality Medical Publishing Inc, St Louis, USA.

Rutherford, T., Zreik, T., Troiano, R. *et al*. (1998) Endometrial cryoablation, a minimally invasive procedure for abnormal uterine bleeding. *J. Am. Assoc. Gynecol. Laparosc.*, **5**, 23–28.

Smith, D.J., Fahssi, W.M., Swanlund, D.J. and Bischof, J.C. (1999) A parametric study of freezing injury in AT-1 rat prostate tumor cells. *Cryobiology*, **39**, 13–28.

Tatsutani, K., Rubinsky, B., Onik, G. and Dahiya, R. (1996) Effect of thermal variables on frozen human primary prostatic adenocarcinoma cells. *Adult Urol.*, **48**, 441–447.

Toner, M., Tompkins, R.G., Cravalho, E.G. and Yarmush, M.L. (1992) Transport phenomena during freezing of isolated hepatocytes. *Am. Inst. Chem. Eng. J.*, **38**, 1512–1522.

Uchida, M., Imaide, Y., Sugimoto, K. *et al*. (1995) Percutaneous cryosurgery for renal tumours. *Br. J. Urol.*, **75**, 132–136.

Walsh, D., Li, K., Crowther, C. *et al*. (1991) Thermotolerance and heat shock response during early development of the mammalian embryo. In Hightower, L. and Nover, L. (ed.), *Heat Shock and Development*. Springer-Verlag, Berlin, pp. 58–69.

Wilcox, L., Koonin, L., Pokras, R., *et al*. (1994) Hysterectomy in the United States, 1988–1990. *Obstet. Gynecol.*, **83**, 549–555.

Zreik, T., Rutherford, T., Palter, S., *et al*. (1998) Cryomyolysis, a new procedure for the conservative treatment of uterine fibroids. *J. Am. Assoc. Gynecol. Laparosc.*, **5**, 33–38.

Received on January 12, 2000; accepted on October 18, 2000