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Journal

Breast Cancer Research and Treatment, 53(2)

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Publication Date

1999

DOI

10.1023/a:1006182618414

Peer reviewed

Report

Effect of thermal variables on human breast cancer in cryosurgery

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Key words: breast cancer, cryosurgery, cellular freezing injury, freezing damage, surgical oncology

Summary

There is a growing interest in the use of cryosurgery to treat breast cancer, following recent breakthroughs in non-invasive imaging and in cryotechnology, as well as the recent success of cryosurgery in treating various types of cancer. However, since haphazard freezing does not guarantee tissue destruction, in order to apply this technique effectively it is essential to determine the thermal parameters that produce complete destruction of malignant tissue. This study seeks to quantitatively identify the relationship between thermal variables and the degree of freezing damage to human breast cancer cells. In order to do this, human breast cancer and normal cells were frozen with controlled thermal parameters using a directional solidification apparatus. Cell viability was determined after thawing using trypan blue, and correlated to the thermal variables used during freezing. Cellular damage is observed to increase with increasing cooling rates, due to the higher probability of intracellular ice formation. A double freeze thaw cycle significantly increases the extent of cell damage, and is sufficient to ensure complete cell destruction at final freezing temperatures of -40°C for a $25^{\circ}\text{C}/\text{min}$ cooling rate, and -20°C for a $50^{\circ}\text{C}/\text{min}$ cooling rate. The correlations between cell death and thermal parameters are qualitatively identical for all the cell types in this study, although there is some variation from one cell type to another in the overall susceptibility to freezing damage. The correlations established in this study can be used to design systematic and optimal breast cryosurgery protocols.

Introduction

The high incidence of breast cancer coupled with a re-examination of the comparative efficacy of radical mastectomy has encouraged a search for alternative methods of treating the disease. Alternative treatment options include lumpectomy, radiation therapy, cytotoxic chemotherapy, and endocrine manipulation therapy singly or in combination [1, 2]. Cryosurgery, the *in situ* destruction of cancerous tissues by localized freezing, could potentially become an effective technique for treating breast tumors and may have advantages over other surgical techniques by providing the physician with a treatment option that could produce excellent medical results with less distress and disfiguration at a lower cost.

Cryosurgery is a procedure that uses freezing to destroy undesirable tissue. Freezing destroys tumors locally without requiring resection, with an effectiveness comparable to resection [3]. The technique is

minimally invasive, usually requiring the insertion of one or more thin, cylindrical, cryosurgical probes into the tumor. The probes are cooled internally with a cryogen and are insulated except at the tip. The uninsulated tip is inserted in the tumors and as heat is removed the undesirable tissue is frozen from the probe surface outward. When the desired amount of tissue has been frozen, the flow of the refrigerant is stopped and the tissue is allowed to thaw. After cryosurgery the frozen tissue is left *in situ* to be re-absorbed by the immune system, over time. The focal nature of the freezing process permits the procedure to be confined to the region of diseased tissue, thereby sparing surrounding healthy tissue. Since the freezing interface is sharp and propagates slowly (on the order of mm/min), the process is precise and easily controllable. Relatively large treatment volumes can be achieved with small probes, for example a 3 mm diameter probe can produce a 3.5 cm ice ball. When the shape of the pathological tissue is large and complex, several probes can be used simulta-

neously to generate a frozen region of the desired shape (prostate and liver cryosurgery are currently performed with five 3 mm diameter probes [4, 5]). Multiple sites can be treated separately or together. Because the only physical invasion of the tissue is the insertion of the cryoprobes, the procedure has reduced complications and patient morbidity. Cryosurgery is not dose limited so retreatment is possible if the disease recurs. Finally, if resection of the diseased tissue is desired, freezing the tumor first can reduce the spread of viable tumor cells to adjacent tissues during resection. Cryolumpectomy (freezing the breast tumor before resecting it) has been attempted in the past with success [6, 7].

Until recently a major impediment to the extensive use of cryosurgery on internal tissues has been the inability to observe the frozen region deep in the body and the consequent complications of over- or under-freezing. However, recent breakthroughs in non-invasive imaging technology have made major advances possible in cryosurgery in general and prostate and liver cryosurgery in particular. We have shown that intraoperative ultrasound could image the progress of freezing during cryosurgery by virtue of the fact that the frozen/unfrozen interface is associated with a change in acoustic impedance that reflects ultrasound waves [8–10]. Research over the past ten years has given significant impetus to the clinical use of ultrasound monitored cryosurgery, first in the liver [4, 8], then in the prostate [5, 11], as witnessed by the fact that prostate cryosurgery is now almost universally carried out under transrectal ultrasound guidance [5, 12]. Cryosurgery coupled with advanced medical imaging technology is gaining acceptance as a first-line therapy for prostate, liver, and other cancers on the strength of its attributes [13]. Recently magnetic resonance imaging (MRI) was introduced as a means to monitor cryosurgery [14]. This technique, which images the process of freezing in three dimensions, can monitor the freezing interface location with a resolution of 200 μm [15], yields precise data on the temperature distribution within the frozen region [16], and can control its shape through MRI feedback [17]. We know of no other chemical, thermal, or surgical method of affecting tissue that can be monitored, in real time and in three dimensions, with an accuracy comparable to cryosurgery.

If the success of cryosurgery in other tissues such as liver [4] and prostate [5] is any indication, freezing could be also used effectively for treating breast tumors. There are no obvious significant impediments in applying cryosurgical techniques to treat breast cancer. The tumors are relatively accessible with a minimum of surgical intervention. Depending on the shape, size,

and location of the tumors, ultrasound transducers would have clear access to the tissues and should be able to image the cryosurgical process. Results comparable with those seen in the prostate and the liver should be expected. Furthermore, the minimal degree of disfigurement as a result of the procedure should be advantageous to the patients psychological well being. Clearly, the relative simplicity of the technique coupled with the availability of new cryosurgical systems in hundreds of centers and the success of the procedure in other tissue is bound to induce physicians to attempt treatment of breast cancer with cryosurgery. Such an attempt has been recently reported [18]. We anticipate that physicians who have access to cryosurgical instrumentation will continue their attempts to use cryosurgery to treat breast cancer, on the strength of its attributes. However, using cryosurgery in the breast is of concern because standardized treatment protocols are currently lacking. While we believe that cryosurgery can be used to treat breast tumors, it has been firmly established that haphazard freezing is not guaranteed to destroy tissue [19]. Freezing has a paradoxical effect that is well-known to researchers in cryobiology: it can both preserve and destroy cells. Whether cells are preserved or destroyed depends on the thermal history they experience during freezing and thawing. The thermal history can be defined in terms of several thermal variables. Extensive studies over the last 50 years have shown that these thermal variables include: cooling rate, temperature gradient, freezing interface velocity, final freezing temperature, holding time at final temperature, warming rate, and single versus multiple freeze/thaw cycles [20–26]. The destruction of frozen cells can be correlated to particular values of these thermal variables. Optimal cryosurgical protocols must use appropriate thermal parameters to ensure tissue destruction, or the treatment may fail. However, the particular values of the thermal parameters required to achieve breast tissue destruction are not known. Determining these values in breast cells is the primary goal of this study. With the values determined in this study it should be possible to establish standardized treatment protocols for breast cryosurgery.

Materials and methods

Studies to evaluate quantitatively the effect of two important thermal variables, the cooling rate and the end temperature to which the cells are frozen, on the degree of damage caused by freezing were conducted on cultured human breast cells. The experiments were per-

formed with several cell lines obtained from the American Type Culture Collection (Rockville, MD): MCF-7 (breast cancer cell line estrogen receptor/progesterone receptor (ER/PR) positive; MDA-468 (breast cancer cell line ER/PR negative); and HBL-100 (normal breast cell line ER/PR negative). The HBL-100 was grown in McCoy's Modified Medium, the MDA-468 was grown in DME-H21 Medium, and the MCF-7 was grown in MEM Eagle's Medium (St. Louis, MO). Each of these was also supplemented with 10% fetal bovine serum, and 1% penicillin (100 units/ml) and streptomycin phosphate (100 μ g/ml). The cells were cultured in 75 cm³ flasks incubated at 37°C in a humidified 5% CO₂ atmosphere. Once the cells reached confluence, the growth medium was aspirated and 3 ml of 0.05% trypsin was added to detach the cells from the flask. The trypsin suspension was centrifuged to allow aspiration of the trypsin, and the cells were resuspended in buffered physiological saline. The suspension was placed onto slides with specially designed 50 μ l silicon wells to contain the suspension. The samples within the slides were frozen and thawed under controlled thermal conditions, with a directional solidification stage. The viability of the cells was assessed after thawing.

The directional solidification stage is an apparatus that was designed for systematic studies of the effects of thermal parameters on frozen cell and tissue survival [27]. The apparatus is shown in a schematic form in Figure 1. It consists of two copper blocks whose temperature can be controlled with a temperature-control system [27]. The blocks sit adjacent to one another on a microscope stage and are separated by a narrow gap in the optical path of the microscope. In typical experiments, the copper blocks are set to desired temperatures, one above and the other below the freezing temperature. The microslide with the samples is placed

on the two constant temperature blocks, with the samples first on the high temperature block. The system is designed in such a way that the microslide and the sample take the temperature of the blocks, where they touch the blocks, and take a linear temperature distribution from a temperature above freezing to a temperature below freezing in the gap between the blocks. To initiate freezing the microslide with the sample is translated at a constant velocity across the gap by a stepping motor, from the base maintained at a temperature above freezing towards the base maintained at a temperature below freezing. This controls the temperature gradient during freezing, the temperature range in which the samples are frozen, the freezing front velocity and the cooling rates during freezing. Once the sample has reached the low temperature copper block it is held at that temperature for a prescribed period of time. Warming is accomplished by translating the microslide with the sample from the cold base back to the warm base with a predetermined velocity thus controlling the warming rate. When thin samples are used, it is possible to view their freezing and thawing through the microscope. Here, the directional solidification stage was used only to freeze and thaw sample under controlled thermal conditions, without imaging.

In this study the high temperature base was maintained at 37°C and the cold side of the stage was brought in different experiments to various temperatures between -5°C and -40°C. The velocity of the microslide translation from the high temperature base to the low temperature base was varied to achieve cooling rates of 5°C/min, 25°C/min, and 50°C/min. These cooling rates are typical of cooling rates in the frozen tissue close to the outer rim of the frozen lesion. After freezing, the cells were kept on the low temperature stage for 5 min and rapidly brought back to the warm side of the stage, to mimic the rapid thawing of the frozen tissue on the outer rim of the frozen cryosurgery lesion. Two sets of experiments were performed, one in which the cells were exposed to only one freeze thaw cycle and another in which the cells were exposed to two identical freeze thaw cycles. At least three experiments were performed for each experimental condition.

The viability of the thawed cells and of unfrozen sham operated controls was evaluated with trypan blue exclusion dye. In order to assess membrane integrity, the cell suspension was mixed with equal volumes of a trypan blue solution (0.3%). A hemocytometer was used to facilitate a count of viable (non-stained) versus non-viable (stained) cells. In every experiment the count exceeded a total of 300 cells.

Figure 1. Schematic of a directional solidification stage.

Figure 2. Percentage of cells destroyed by freezing with a cooling rate of 5°C/min as a function of the temperature to which they were frozen: (a) single freeze/thaw cycle for MCF-7, MDA-468, and HBL-100; (b) double freeze/thaw cycle for MCF-7, MDA-468, and HBL-100.

Results

Figures 2–4 show the percentage of cells destroyed by freezing as a function of the final temperature to which the cells were frozen, at cooling rates of 5°C/min, 25°C/min, and 50°C/min, respectively. Figures (a) are for one-cycle freezing and Figures (b) are for two-cycle freezing. Results are shown for MCF-7, MDA-468, and HBL-100, and the cell type is indicated in each figure. In these plots each data point represents the result of an individual experiment, and the results are normalized with respect to unfrozen controls. A minimum of three experiments was performed for each set of thermal conditions, and where fewer points appear, data points have become superimposed. This is seen in particular at low final freezing temperatures which lead most reliably to complete cell destruction. It is clearly evident from

these results that the level of breast cell destruction is dependent on the thermal conditions of the freeze.

As seen in Figure 2a, for single-cycle freezing at the lowest cooling rate in our study (5°C/min), cell death increases in an approximately linear fashion with decreasing final freezing temperatures. This gradual rise in cell death is observed for all three cell types, although the MCF-7 cell line appears to be slightly more susceptible to freezing damage, registering higher death percentages at the higher final freezing temperatures, and then a tapering of the cell destruction curve after –20°C. For this cooling rate, however, cell destruction in all three cell lines is incomplete even at the lowest final temperature in our experimental range (–40°C).

Figure 2b presents data for cells subjected to a double freeze thaw cycle with a cooling rate of 5°C/min. A comparison of Figures 2a and 2b reveals the relative

Figure 3. Percentage of cells destroyed by freezing with a cooling rate of 25°C/min as a function of the temperature to which they were frozen: (a) single freeze/thaw cycle for MCF-7, MDA-468, and HBL-100; (b) double freeze/thaw cycle for MCF-7, MDA-468, and HBL-100.

effects of single- and double-cycle freezing. In the range of higher final freezing temperatures from about -5°C to -20°C , the double-cycle freeze causes an apparent increase in the extent of cell damage. Again, the MCF-7 cell line appears to be somewhat more vulnerable to freezing damage than the other two cell types. However, for each cell-type the maximum cell destruction, correlating to the final temperature of -40°C , does not change significantly from that of Figure 2a. This suggests that although a double freeze/thaw cycle improves overall cell destruction at some final temperatures, it is still insufficient to guarantee total cellular destruction at the $5^{\circ}\text{C}/\text{min}$ cooling rate and within the range of final temperatures in our study.

Figures 3a and 3b show results for freezing damage to MCF-7, MDA-468, and HBL-100 cell lines at a cooling rate of $25^{\circ}\text{C}/\text{min}$. A comparison of Figures 2 and 3 demonstrates that the five-fold increase in cooling

rate produces a visible increase in cell destruction over the entire range of final freezing temperatures. At the high cooling rate, cell death rises more steeply within the temperature range of -5°C to -20°C . Within the temperature range -20°C to -40°C , the cell death curves level out to slightly higher final values than the maximum cell destruction observed at the lower cooling rate. Inspection of Figure 3b reveals that a double freeze/thaw cycle again serves to promote cellular destruction. The susceptibility of MCF-7 to freezing damage is especially pronounced in Figure 3b, where nearly complete cellular destruction is obtained as early as -30°C . For the other two cell lines, a final temperature of -40°C must be reached to obtain complete cellular destruction with a double freeze/thaw cycle.

In Figures 4a and 4b, the cooling rate is further increased to $50^{\circ}\text{C}/\text{min}$. This produces a significant increase in cell death, and total cell destruction is

Figure 4. Percentage of cells destroyed by freezing with a cooling rate of 50°C/min as a function of the temperature to which they were frozen: (a) single freeze/thaw cycle for MCF-7, MDA-468, and HBL-100; (b) double freeze/thaw cycle for MCF-7, MDA-468, and HBL-100.

achieved as early as -15°C to -30°C , depending on cell-type and the number of freezing cycles. In Figure 4a for single cycle freezing, cell destruction curves have a steep slope within the range of -5°C to -20°C , and complete cellular destruction is observed at a final freezing temperature of -30°C for all three cell types. For double-cycle freezing, Figure 4b shows that cell death jumps to 100% by final freezing temperatures as high as -15°C for MCF-7, and -20°C for MDA-468 and HBL-100.

Discussion

The results of this study demonstrated that freezing damage to breast cancer cells increases with decreasing final temperature and increasing cooling rate. The generally accepted 'two factor' theory of Mazur [24,

25] can be used to explain these results. This theory is based on past research on the effects of freezing on biological materials [19]. Mazur proposed that during freezing of cells in a suspension, ice forms first in the extracellular fluid while the intracellular fluid remains unfrozen and supercooled. The ice that forms in the extracellular space rejects solutes into the remaining unfrozen solution, and thus the osmolality of the extracellular fluid increases. Water flows out of the cell to equilibrate this osmotic difference, causing the cell to undergo damaging excursions in volume and intracellular solute concentration. The degree of this osmotic damage can be related to the amount of time the cell is exposed to the damaging condition. At low cooling rates this mechanism of damage dominates. As the cooling rate increases the intracellular supercooling becomes greater, and this thermodynamic instability results in the formation of intracellular ice. This is the

second mechanism of damage as intracellular ice is lethal to the cells.

For every cooling rate and cell type in our study, cell death was observed to increase with decreasing final temperature. This relationship is consistent with both mechanisms of damage predicted by Mazur's two factor theory. At slow cooling rates as the target temperature is lowered and the extracellular solution freezes, the cells are exposed to the hypertonic conditions for greater periods of time, inducing greater osmotic damage. Intracellular ice formation, the mechanism of damage predicted at higher cooling rates, has been found to occur within a narrow range of subzero temperatures [20, 21, 26]. Thus, this type of damage is only induced when the final temperature is lowered below some threshold value.

A double freeze thaw cycle was shown to produce significant increases in cell destruction for every set of thermal parameters investigated. This finding is also in agreement with the two factor theory. At high cooling rates a double freeze thaw cycle should increase the statistical probability of intracellular ice formation and cell damage. At lower cooling rates the effect of a double freeze thaw cycle is to increase the amount of time cells are exposed to the hypertonic conditions, and thus increase the degree of osmotic injury.

Finally, our data indicates that higher cooling rates induce greater cellular destruction. This is most likely due to an increased contribution by the high cooling rate injury mechanism, since the probability of intracellular ice formation increases with increasing cooling rates. The practical implication of this outcome is that for the high freezing rate, complete cellular destruction is achieved at a significantly higher temperature of -20°C vs. -40°C or lower.

It should be emphasized that while most of the studies on the effects of cooling rates on cell damage were done with cells frozen in suspension, it has been shown both mathematically and experimentally that cells frozen in tissue experience the same mechanisms of damage with similar thermal parameters as cells frozen in cellular suspensions [28]. Furthermore, it has been shown that in tissue, cells experience additional modes of damage related to the destruction of the structural integrity of the tissue and vasculature. Consequently, cells that survive freezing's primary insult will die due to this structural damage and ischemia [28]. It is these secondary effects that have made it impractical at this time to preserve whole tissues and organs by freezing. Studies, therefore, on the survival of cells frozen in suspension should provide a more stringent upper limit for the survival of cells frozen

in tissue, which most likely will experience greater damage. Development of freezing protocols based on the data obtained in these experiments therefore should provide some measure of additional safety margin in obtaining complete tissue destruction.

The data presented has clinical implications for the application and technique of percutaneous breast cancer cryosurgery. Since the data clearly shows that the final freezing temperature and the cooling rate have definite target values, it is an obvious conclusion that thermocouple monitoring in the region of the known tumor is desirable.

The data from our experiments also shows that higher freezing rates, $25^{\circ}\text{C}/\text{min}$ and $50^{\circ}\text{C}/\text{min}$., produce complete cellular destruction reliably at a higher overall temperature than slower freezing rates. This rapid freezing rate is certainly attainable clinically. A drop of temperature from 0°C to -25°C in 1 min is well within the realm of freeze rates during cryosurgery. Available data from conventional cryosurgical systems show that $25^{\circ}\text{C}/\text{min}$ can be achieved in an 8–10 mm diameter around a single cryoprobe. However, it should be appreciated that as the iceball tends to reach equilibrium and attain its maximal size, the freezing rates at the periphery of the iceball slow significantly. Therefore, in clinical practice to expose the cancer to the highest cooling rates possible, an effort should be made to place the cryosurgical probes directly into the abnormal tissue.

The data presented in this paper has implications for the development of cryosurgical equipment as well. Based on the data, a faster freeze rate is obviously desirable. Furthermore, because most of the damage occurs in the high subzero temperature range, it is more important to freeze with a higher cooling rate, above $25^{\circ}\text{C}/\text{min}$, than to reach a lower cryogenic temperature. Use of a system which exposes the tissue to faster freezing rates may over time, therefore, produce more reliable results. In addition, since freezing rates are greater the closer the tissue is to the cryosurgical probe, [29–31] a further improvement in results could be achieved by perhaps increasing the number of probes in the volume of tissue to be treated. This approach would have to be balanced against the increased tissue trauma of placing the additional probes.

Lastly, the data shows that a double freeze thaw cycle is mandatory for reliable cryosurgical treatments. While this clinical approach has been advocated in the past [3] it has not been uniformly adopted. The data presented now provides a firm basis upon which to affirm the need for a double freeze thaw cycle in breast cryosurgery. The results indicate that the qualitative

results are similar for all breast cell lines we have tested, although the MCF-7 appears to have greater overall susceptibility to freezing damage. Further work to be carried out in this area includes extrapolating the behavior of cells frozen *in vitro* to their behavior when frozen *in vivo* in tissue.

In summary, this study provides quantitative information on the relationship between thermal variables and frozen cell destruction. This data can provide a basis for the development of protocols to perform cryosurgery more precisely and with greater control over the outcome of the procedure. If the thermal conditions in the tissue can be monitored during cryosurgery, the correlations established in this paper can be used to predict the corresponding degree of tissue damage. Further research in this area to expand the range of parameters studied in this paper is warranted in order to provide a better foundation for the reliable application of cryosurgery in breast cancer.

Acknowledgements

This research was supported by the California Breast Cancer Research Program.

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