Liver and Pancreatic Sciences

Research Article

Dual thermal ablation of pancreatic cancer cells as an improved combinatorial treatment strategy

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Abstract

Pancreatic cancer (PaCa) is one of the leading causes of cancer related deaths in the world today. It is estimated that 95% of patients diagnosed with PaCa will die because of limited treatment options. As standard treatments have not yielded an improvement in patient outcome, alternative approaches, such as thermal ablation, may offer a new treatment path. We previously reported the lethal temperatures necessary for PaCa ablation for both freeze and heat based treatments. In this study, we investigated the response of PaCa cells *in vitro* to a combination treatment of heating and freezing, dual thermal ablation (DTA), in an effort to further improve ablation efficacy.

PaCa cell lines, PANC-1 and BxPC-3, were subjected to heating (45 to 50°C), freezing (-10 to -20°C), or DTA exposure. Post-exposure viability was assessed over a 7-day recovery interval. Modes of cell death were analyzed using fluorescence microscopy and flow cytometry.

Our results indicated that in comparison to single thermal modalities, DTA resulted in greater cell destruction at a more rapid rate. Specifically, -15°C had a moderate impact on day one cell viability (PANC-1=80%; BxPC-3=21%) and 50°C (PANC-1=74%; BxPC-3=18%) caused a slow decline in cell number post-treatment. The combination of these treatments resulted in an increased cell death one-day post-treatment (PANC-1=28%; BxPC-3=5%) and achieved complete cell destruction within three days. Overall, these data suggest that a combination of heat and freeze ablation, DTA, may provide for an improved treatment strategy for PaCa.

Introduction

Pancreatic Cancer (PaCa) is one of the deadliest cancers in the world today. It is estimated that 1 in 67 individuals will be diagnosed with PaCa in their lifetime [1,2]. Based on the latest statistics from the National Cancer Institute, over 53,000 individuals are diagnosed with and over 43,000 die annually from PaCa in the US alone [1,2]. Beyond the standard cancer treatment options, chemotherapy and radiation, there has been minimal progress made in improving PaCa patient survival. One of the latest attempts is the development of FOLFIRINOX, a combination of five chemotherapeutic agents, which has shown some progress but is associated with toxic side effects if not managed properly [3-8]. With the need to develop more effective treatment options, alternative approaches, such as thermal therapy, may offer a viable strategy [9].

Cryotherapy is a minimally invasive technique that utilizes a cryogen, such as argon gas or liquid nitrogen (LN_2), to freeze a tissue, reaching temperatures well below -100°C nearest the cryoprobe [10-12]. This ultra-cold environment causes multiple physical and molecular stressors, such as ice rupture, osmotic imbalances, dehydration, oxygen deprivation, and ischemia/reperfusion injury, among others [10-15]. The primary advantage of cryotherapy over radiation and chemotherapy is that cryoablation can be directly applied to the diseased tissue. Further, unlike other treatment strategies, molecular drug resistance can be overcome with thermal injury [10]. Although cryoablation is not a mainstream thermal technique for PaCa, it is a primary treatment option in other cancers [6,16-22]. In 2008, the American Urological Association published a best practice statement on prostate cancer, recommending cryosurgery as a primary treatment option [23]. Furthermore, the AUA has deemed it effective for the treatment of renal cancer [21,23]. As our understanding of cryotherapy has improved, so has the technology required to perform this technique, offering enhanced performance and improved patient outcome.

Another strategy for cancer ablation is that of heat based therapies, such as radio frequency ablation (RFA) and high-frequency ultrasound (HIFU). Heat based treatments subject diseased tissue to hyperthermia, inducing coagulative necrosis, protein denaturation, cytoskeletal breakdown, and severe connective tissue damage [15, 24-28]. Solid tumors in the liver and kidneys have been effectively treated using hyperthermia, yet numerous side effects have been reported [21,24,25,28,29,30]. Despite limited use of heat ablation therapy, we hypothesize that based on success in other tissues, hyperthermia would provide for the effective ablation of PaCa [9,19,31,32].

Thermal therapies are normally utilized in a context where one technique is applied to treat a given tumor based on the patient's specific needs. One advantage of thermal therapies over conventional

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treatments is that thermal techniques overcome the "hallmarks of cancer" which challenges other treatment regimens, such as chemotherapy efficacy [10,33,34]. Given that heat and cryoablation operate on opposite ends of the thermal spectrum, it has been suggested that they may be utilized in combination as a single treatment regime [35]. Similar to FOLFIRINOX as a combination chemotherapy treatment regime, we propose that a combinatorial thermal strategy may improve cancer ablation. It has been suggested by Shafirstein *et al.* that such a combination could result in improved tumor ablation [36]. In contrast to the thermal model utilized by Shafirstein *et al.* where breast cancer was frozen prior to a rapid heating, we investigated first heating the target tissue followed by freezing to achieve cancer ablation. We hypothesize that the combined exposure of heating and freezing on PaCa would act synergistically, increasing the amount of cellular damage, resulting in rapid and complete PaCa destruction. Furthermore, we hypothesize that by applying heat followed by freezing, the collateral damage associated with heating would be reduced by providing a heat sink immediately following heat exposure. Our results suggest that DTA may serve as a more effective means of targeting PaCa versus a mono thermal therapy approach.

Material and methods

Cell culture: PANC-1 (CRL-1469) and BxPC-3 (CRL-1687) cells lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Dubecco's Modified Eagle's Medium was used to culture PANC-1 cells and RPMI-1640 medium was used for BxPC-3 (Caisson Laboratories, Inc, Logan City, UT). Cell culture media was supplemented using 10% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 1% Penicillin/Streptomycin (Corning, Inc, Corning, NY). Cells were seeded into Costar 96 stripwell plates (Corning) at a cell density of approximately $3.75x10^4$cells/cm^2 with appropriate medium 24 hours prior to experimentation.

Thermal exposures: Culture medium was exchanged with 75µl/well fresh media 30 minutes prior to experimentation. Eight-well strips were placed into pre-cooled or heated aluminum blocks in a temperature controlled bath set to reach target temperatures. For freezing experiments, samples underwent a 5-minute exposure in baths set to achieve temperatures of -10, -15 and -20°C. When sample temperature approached -2°C, ice nucleation was initiated with LN_2 vapor to prevent supercooling of samples. Following freezing, samples were placed at room temperature (RT) for 10 minutes to passively thaw before being returned to standard culture conditions. For heating experiments, samples underwent a 5-minute exposure in baths set to achieve temperatures of 45, 48 and 50°C. After the heat exposure, samples were removed and placed at RT for 1 minute before returning to culture conditions. Thermal profiles of samples under each condition were recorded using a T-Type Thermocouple (Omega Engineering, Stamford, CT) at one second intervals during exposure.

Double exposure: Freezing exposure was performed using temperatures of -10, -15 and -20°C. Samples were subjected to a 5-minute exposure, passively thawed at RT for 5 minutes, and subsequently refrozen at the same temperature for an additional 5 minutes. After the second exposure, samples were passively thawed for 10 minutes at RT before returning to standard culture conditions. Heating exposure used temperatures of 45, 48 and 50°C. Samples were heated for 5 minutes as described, placed at RT for one minute, and then exposed to a second heat exposure to the same temperatures for an additional 5 minutes. Following second exposure, samples were placed at RT for one minute before returning to culture conditions.

DTA exposure: Heating exposure was performed first, utilizing temperatures of 45, 48 and 50°C, for 5 minutes and then placed at RT for one minute. Immediately following RT hold, samples were exposed to temperatures of -10, -15 or -20°C for 5 minutes. Samples were seeded as temperature approached -2°C with LN_2 to prevent supercooling. After the freeze, samples were passively thawed at RT for 10 minutes and returned to 37°C culture for recovery. Thermal profiles were recorded using a T-Type Thermocouple (Omega Engineering) at one second intervals during DTA exposure.

Cell viability assay: Viability of PANC-1 and BxPC-3 samples were assessed using the alamarBlue metabolic activity assay. AlamarBlue stock solution (Invitrogen, Carlsbad, CA) was reconstituted at 1:20 in Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} (Mediatech, Inc, Herndon, VA). Culture medium was then aspirated and 100µl/well alamarBlue solution was added to each well and incubated at 37°C for 60 minutes. Immediately following incubation, samples were read using a Tecan SPECTRAFluor Plus (Tecan, Austria) at excitation/emission of 530nm and 590nm. Sample raw fluorescence units (RFUs) were converted to percent viability based on pre-treatment control RFUs. Viability assessment was conducted over 7 days on days 1, 3, 5 and 7 following thermal exposure.

Apoptosis/Necrosis Assay: Relative levels of apoptosis and necrosis were assessed using microfluidic flow cytometry. At 1, 4, 8 and 24 hours following thermal exposures, samples were incubated with propidium iodide [3.75µM] and YO-PRO-1 [0.35µM] (Invitrogen) for 15 minutes at 37°C. Cells were then lifted from Costar plate and analyzed using a Guava EasyCyte Plus flow cytometer (Millipore, Billerica, MA). Analytical gates were set using unstained, non-fluorescence controls and a positive control sample exposed to Camptothecin [0.2mM] to induce apoptosis for flow cytometer analysis. 5000 events per condition were analyzed, in triplicate, and live, apoptotic, necrotic, and secondary necrotic cell populations were quantified as a percent of the analyzed populations.

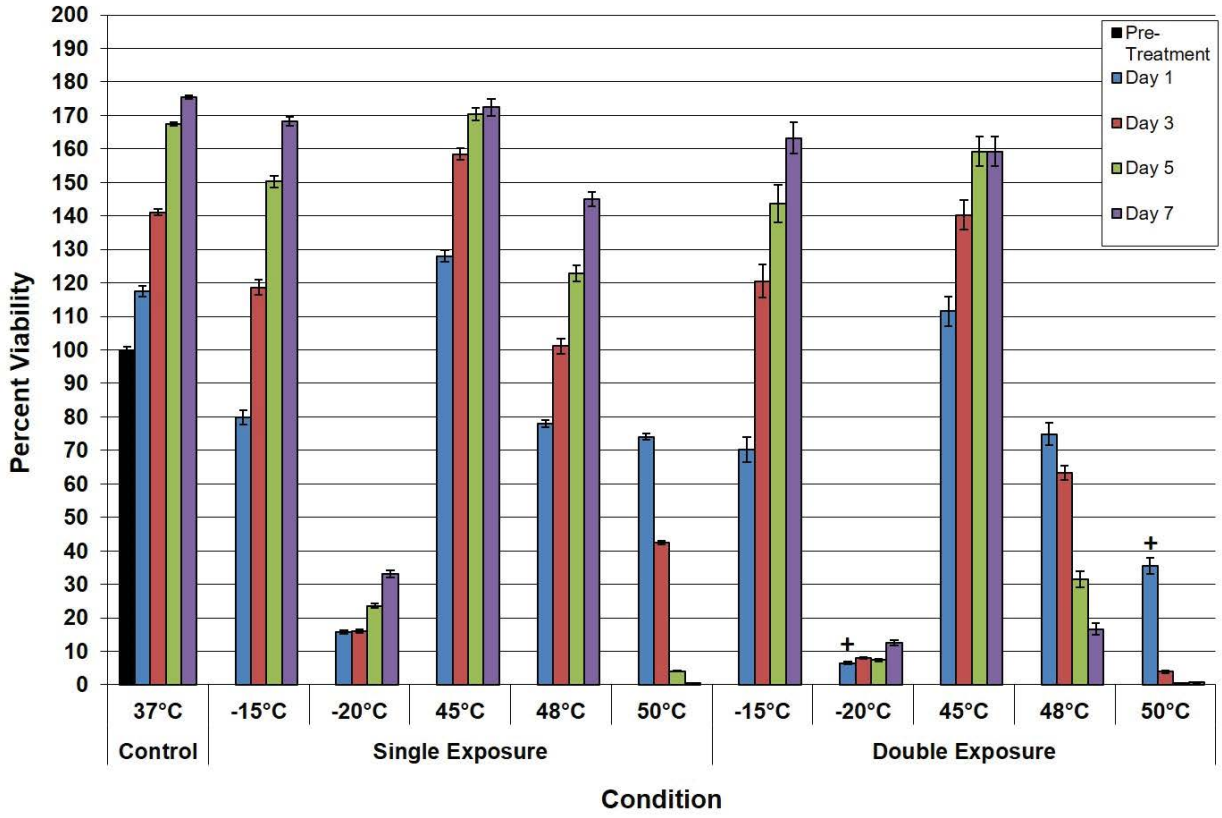
Fluorescence Microscopy: PANC-1 and BxPC-3 cells were probed with propidium iodide [3.75µM] and calcein-AM [2.5µM] (EMD Chemicals, Millipore) for 30 minutes at 37°C at 1, 4, 8 and 24 hours following thermal exposures. Samples were then visualized using a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at 20X magnification. Fluorescence micrographs were complemented with phase microscopy to permit visualization of changes in cell morphology. Fluorescence images were correlated with flow cytometry and metabolic activity data to verify the levels of cell death.

Statistical Analysis: Statistical significance was determined using single factor ANOVA and t-test. Standard error was used to represent experimental variability. All experiments were repeated a minimum of three times (N=3) with an inter-experimental replicate of n=7. Statistical significance is denoted by $p<0.01$ unless stated otherwise.

Results

PaCa assessment to freezing exposure

To determine the effect of freezing on PANC-1 and BxPC-3 cells, samples were subjected to temperatures of -10, -15, and -20°C and sample viability and post-treatment recovery were assessed. Cell lines yielded minimal cell death following exposure to -10°C. When -15°C was utilized, a decrease in viability was observed. PANC-1 samples showed a slight decrease in cell viability on day one (80%(±2)), but recovered to pre-freeze control levels by day 3 (Figure 1A). BxPC-3 samples exhibited a larger decrease in cell viability compared to


Percent Viability
Pre-Treatment
Day 1
Day 3
Day 5
Day 7
37°C
-15°C
-20°C
45°C
48°C
50°C
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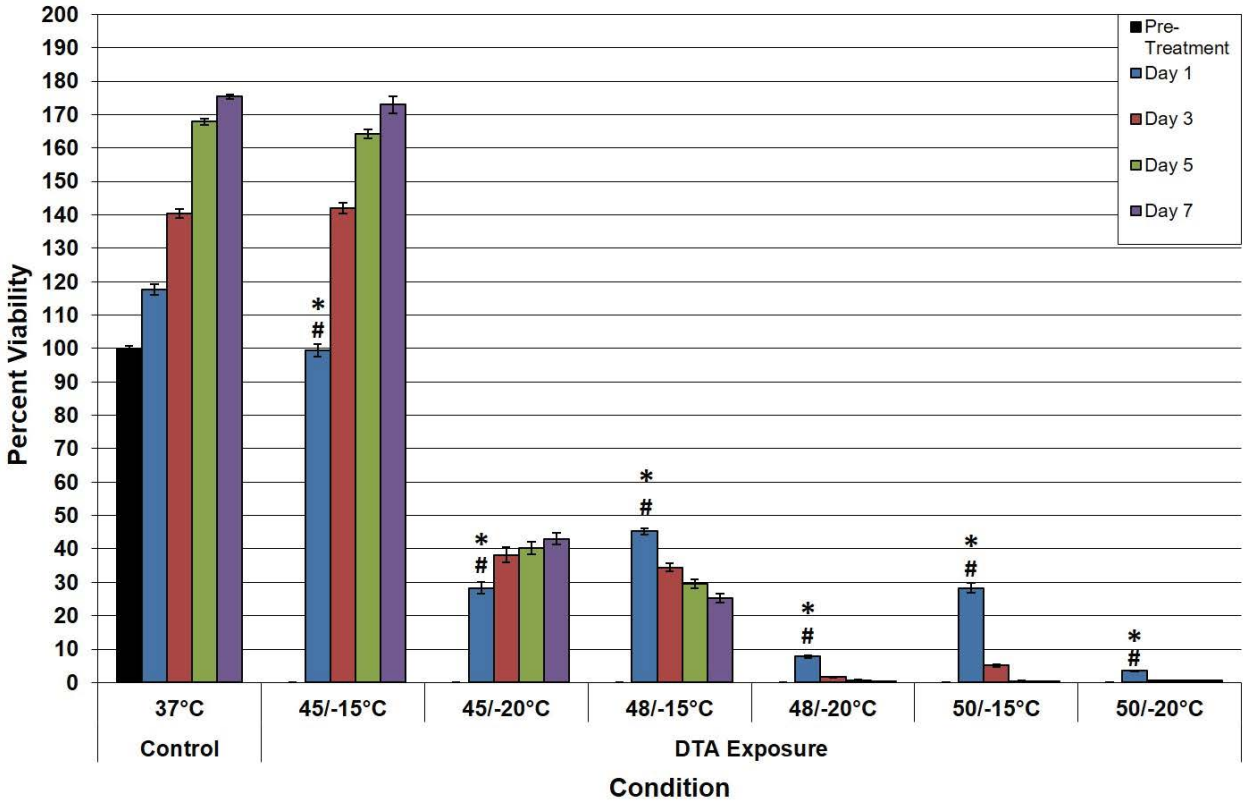
-20°C
45°C
48°C
50°C
Control
Single Exposure
Double Exposure
Condition


Percent Viability
Pre-Treatment
Day 1
Day 3
Day 5
Day 7
37°C
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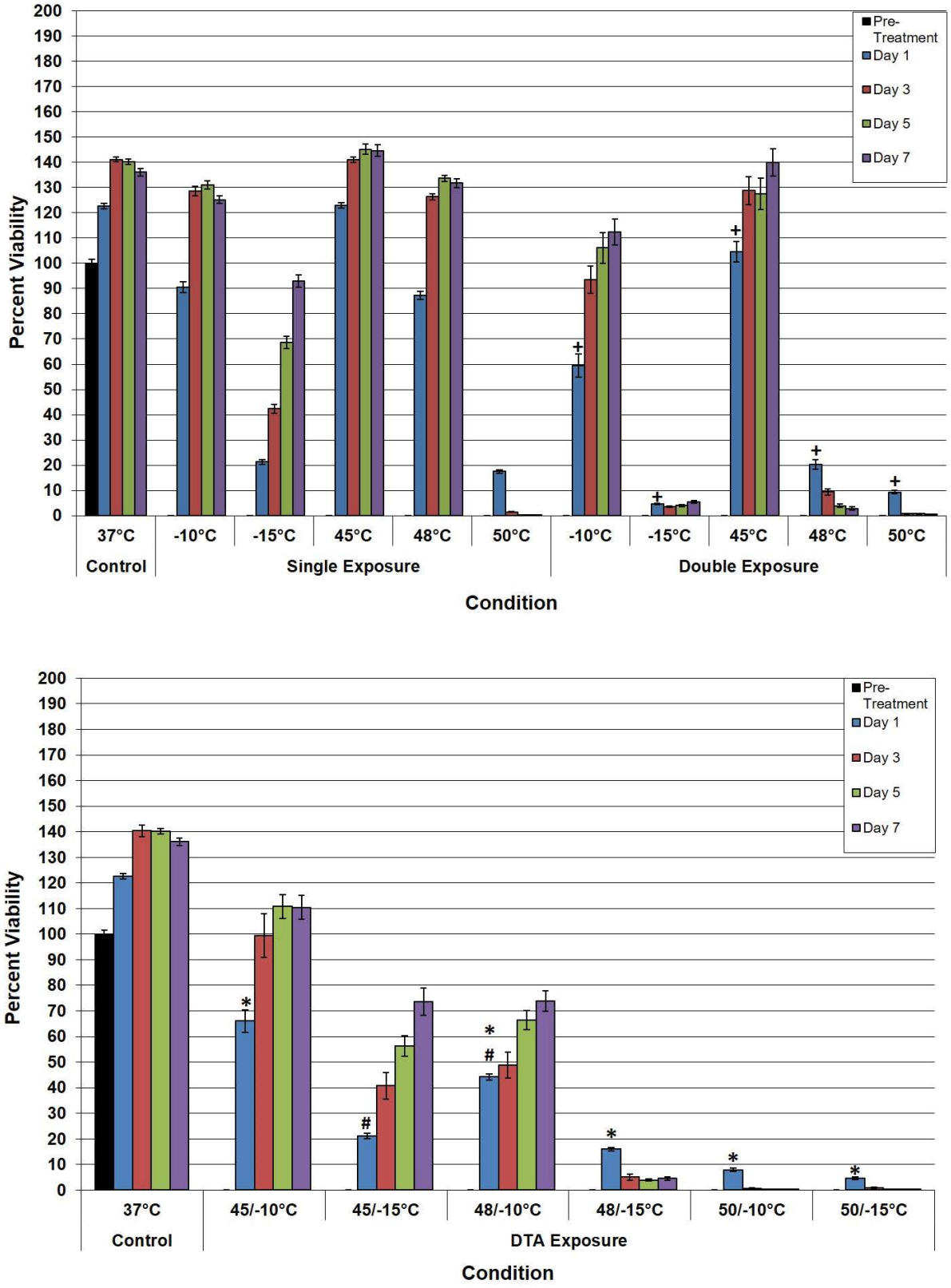


Figure 1. Assessment of post-treatment viability and recovery of PaCa cells following a freezing, heating, or DTA exposure. PANC-1 (A and B) and BxPC-3 (C and D) cells were subjected to a brief freezing or heating exposure versus DTA and survival was assessed over seven days post-treatment. Data suggest that select milder temperatures (48°C, -15°C, -20°C) previously used in a single thermal regime become capable of increased cell death, and in some cases complete lethality, when used in a combination DTA capacity. (+$p<0.01$ comparing single to double exposure; *$p<0.01$ comparing single to DTA exposure; #$p<0.01$ comparing double to DTA exposure).

controls (21%(±1) (Figure 1B). At -20°C, viability further declined in both cell lines (PANC-1=15%(±1); BxPC-3=2%(±0.1) [37]) with reduced recovery observed (Figure 1). As previously reported, following exposure to -25°C, minimal survival (<2%) and no repopulation was observed in both cells lines [37]. Assessment of cell proliferation and recovery post-treatment indicated that following exposure to temperatures of -15°C or warmer, both PANC-1 and BxPC-3 cells were capable of repopulating. However, at temperatures at or below -20°C, repopulation was either suppressed or did not occur.

Assessment of PaCa exposure to heating

The impact of heating samples to 45, 48 and 50°C was assessed. PANC-1 and BxPC-3 samples subjected to 45°C showed no substantial change in viability or proliferation compared to non-treated controls over the assessment period. In contrast, samples exposed to 50°C revealed a significant decline in viability one-day post-treatment in both PANC-1 (74%(±1)) and BxPC-3 (18%(±1)) samples ($p<0.01$) (Figure 1A, 1C). Further, over the assessment interval, sample viability continued to decline to 0% by day 3 for BxPC-3 and by day 5 for PANC-1 samples. The intermediate temperature, 48°C, yielded a slight drop in day 1 viability in both PANC-1 (78%(±1)) and BxPC-3 (87%(±2)) cells (Figure 1A, 1C) and sample recovery was observed during the post-treatment assessment interval. After heating to 48°C or 50°C, both cell lines displayed rounded morphology and loose adherence when observed via phase microscopy. Samples heated to 48°C regained proper morphology during the recovery interval (Figure 2); however, 50°C samples did not (Figure 2).

Impact of dual thermal ablation

Previous reports have demonstrated that repeated exposure of cancer cells to thermal treatment results in increased cell death [18,38]. As such, thermal ablation is typically applied as a monotherapy in a repeat exposure protocol. While repeat exposure of a mono thermal treatment (freezing or heating) is standard practice for many cancers, there are only a few reports investigating the use of heating and freezing in combination as a therapeutic protocol to ablate cancerous tumors [35,36]). As such, a DTA protocol (heat then freeze) was investigated as a potential treatment path for PaCa. The combination of 45°C/Freezing revealed minimal differences from freezing alone. When 48°C and 50°C were utilized in combination with freezing, a noticeable impact was observed. The 48°C/Freezing regime resulted in a decrease in day one cell viability compared to either treatment alone. For example, compared to PANC-1 samples subjected to -15°C (80%(±1)), -20°C (15%(±1)), or 48°C (78%(±1)) alone for 5 minutes, the 48°C/freezing combination in a DTA protocol yielded day one viabilities of 45%(±1) (48/-15°C) and 7%(±0.3) (48/-20°C) (Figure 1B). The combination of 48°C and freezing also resulted in a continued decline over the seven-day assessment differing from the recovery observed following single exposure temperatures. In BxPC-3 samples, freeze or heat mono exposure yielded mild decreases in viability (-10°C (90%(±2)), -15°C (21%(±1)) and 48°C (87%(±2))) followed by recovery whereas the heat then freeze combination caused significantly greater cell death compared to heat or freeze alone (48/-10°C=44%(±1); 48/-15°C=16%(±1)) ($p<0.01$) (Figure 1D). Interestingly, in the 48/-15°C condition, sample viability was found to continue to decline over the assessment interval whereas in the 48/-10°C condition, BxPC-3 samples retained their proliferative capacity and recovered over the assessment interval. When PaCa samples were subjected to DTA utilizing 50°C, in all cases, a significant decrease in viability was observed compared to single exposures ($p<0.01$). PANC-1 samples exposed to 50/-15°C were found to have greater cell death than in either 50°C or -15°C alone ($p<0.01$). Combination treatment with 50/-15°C resulted in 28%(±2) viability on day one and declined to 0% by day five (Figure 1B). When PANC-1 samples were subjected to 50°C/-20°C conditions, day 1 viability was <4% and reached 0% by day 3 (Figure 1B). Comparison of the effects of DTA on BxPC-3 samples versus PANC-1 samples suggested that while BxPC-3 samples were impacted, there was less of a synergistic effect. For example, samples subjected to 50/-15°C combination yielded 5%(±0.5) versus 21%(±1) in -15°C alone and 18%(±1) in the 50°C only condition on day one (Figure 1C and 1D). Although there was a more rapid decline, it was not deemed a synergistic impact due to DTA.

Comparison of DTA to single exposures

The impact of DTA on PaCa cells depends on several factors, most importantly, exposure temperatures. When heating to 45°C was applied in tandem with freezing temperatures (-10 to -20°C), no substantial decline in viability was observed in either PANC-1 or BxPC-3 samples (Figure 1). This suggested that 45°C was not an effective temperature for thermal ablation either alone or in combination with freezing in PaCa. The application of 48°C resulted in a more potent effect. Whereas previously, cells exposed to 48°C alone showed minimal decline in day one viability, when applied in conjunction with moderate freezing temperatures, a significant decline in viability was observed on day seven compared to single exposures ($p<0.01$) (Figure 1). This was most noticeable in the combination of 48°C and freezing to -15°C or colder. This finding was noteworthy as the application of 48°C or -15°C alone resulted in minimal cell death, yet the combination yielded an increase in cell death ($p<0.01$). Combination exposure with 50°C yielded complete cell death with all freezing conditions within the 7-day assessment period (Figure 1). This was pertinent as while the use of 50°C alone yielded complete cell death on its own over several days, when combined with freezing, a reduced time to achieve complete cell death as well as significantly decreased viability levels on day one were noted in comparison to single exposures ($p<0.01$). This impact was not significant compared to double 50°C exposure, particularly in BxPC-3 cells ($p=0.4$). Observation of PaCa cells via fluorescence microscopy further corroborated this finding. In freeze only conditions, it was observed that despite morphological changes and increases in propidium iodide (PI) positive (dead) fluorescing cells over time (Figure 2), PaCa cells continued to proliferate, corroborating the metabolic activity data. In heat only conditions, cell morphology became rounded within 1 to 4 hours and some loss of cell adherence was observed. This did not lead to cell death, however, as live (Calcein-AM positive) fluorescing cells predominated both PaCa cell lines throughout the 24-hour assessment period (Figure 2). It was not until DTA was performed that a substantial increase in PI-positive fluorescence was observed in these conditions throughout the assessment period. In PANC-1, PI-positive cells were observed as early as 1 hour and the cell death population increased through 24 hours in 48/-20°C, 50/-15°C and 50/-20°C conditions (Figure 2A). In 48/-15°C, although there was not a considerable amount of cell death observed at 1 hour, the number of PI-positive reporting cells at 24 hours was greater than was observed in either 48°C or -15°C conditions alone at that time. In BxPC-3 cells, with the exception of 48/-10°C, which did not show an increase in PI-positive staining within the micrographs, all DTA conditions resulted in an overall increase in cell death over the initial 24 hours (Figure 2B). These fluorescence micrographs corroborate our metabolic activity data and support the notion that cell death is increasing and occurring more rapidly in DTA conditions.

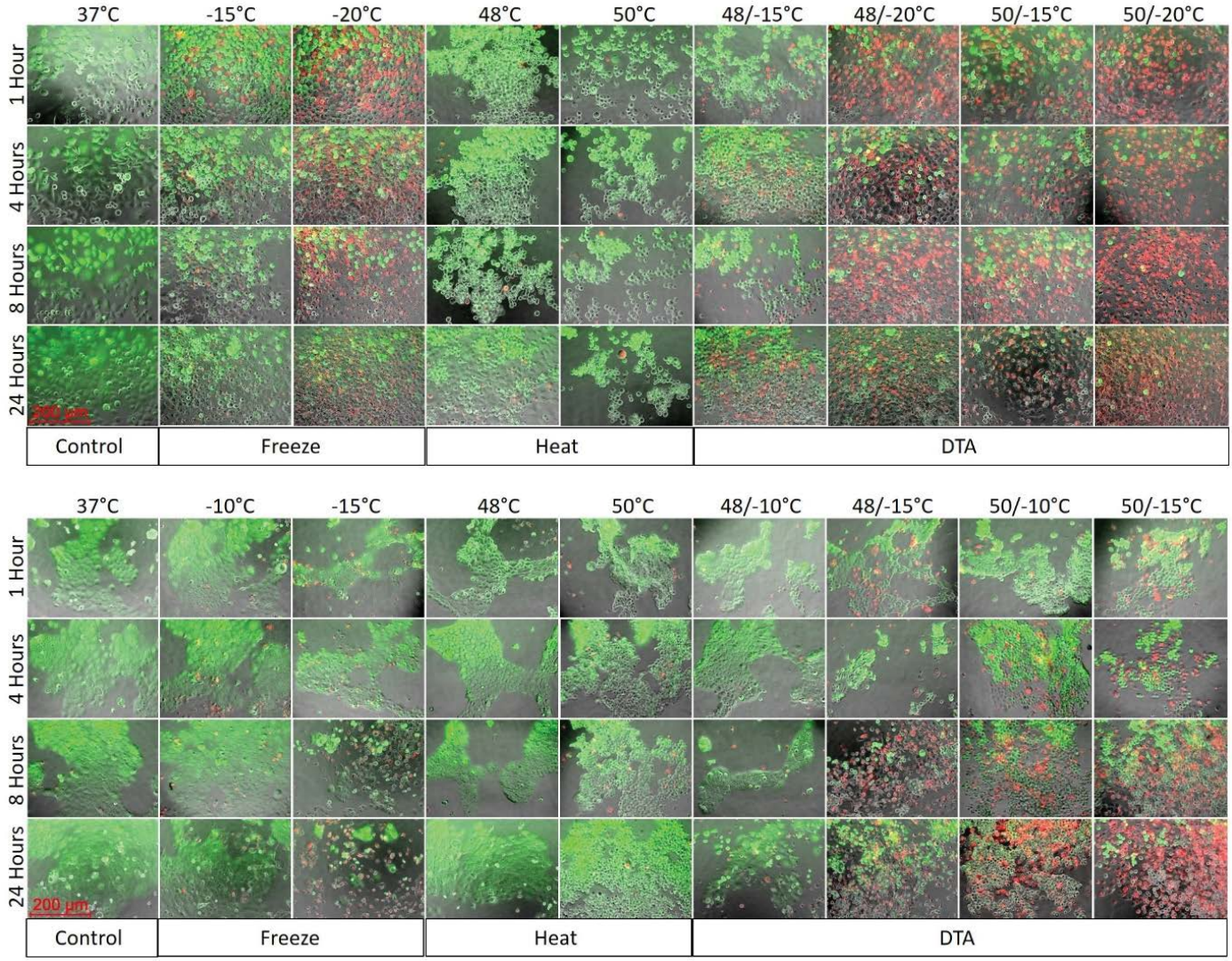


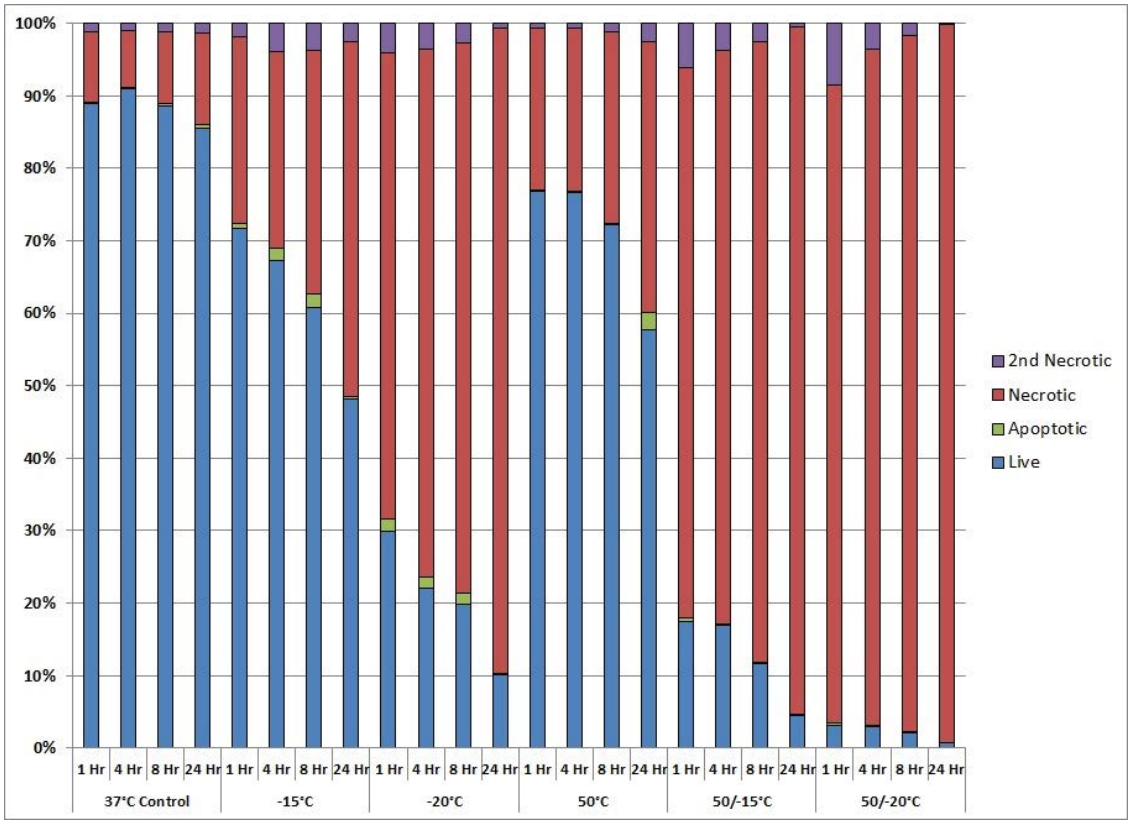
Figure 2. Fluorescence micrographs of PaCa cells following thermal exposures. Following thermal exposures, PANC-1 (A) and BxPC-3 (B) cells were stained with Calcein-AM (green) and propidium iodide (red) at 1, 4, 8 and 24 hours and visualized under 20X magnification. Green fluorescence indicates live cells whereas red fluorescence indicates dead cells. Phase contrast indicates changes in cell morphology over time. Fluorescence images illustrate an increase in cell death in DTA conditions versus their single exposure counterparts. These findings appear to correlate with post-treatment viability results observed from flow cytometry and alamarBlue viability analysis.

Comparison of DTA to double exposures

In order to fully assess the impact of freezing or heating to DTA, we also examined a double heat and a double freeze regimen for PaCa cell ablation, as a double heat or freeze exposure is often applied clinically [10-12,39,40]. When examining the impact of double freezing, an increase in cell death was observed compared to the single freeze condition. While an increase was observed, it was not as prominent as was observed in the DTA combination. In PANC-1 samples, double -20°C exposure reduced day one viability by more than half (6%(±0.4)) which plateaued over the assessment period (Figure 1A). In comparison, the combination of 48/-20°C or 50/-20°C resulted in 7%(±0.3) and 3%(±0.1) viability at day one, yet cell death continued over the seven-day assessment interval, resulting in complete sample destruction (Figure 1A). In the more thermally sensitive BxPC-3 samples, double -15°C resulted in a severe decline in viability (5%(±0.3)) at day one which plateaued over the seven-day assessment period (Figure 1C). In comparing double freeze to DTA, the double freeze was found to have a similar overall impact on cell viability as DTA over the seven-day period. A similar trend was also observed in the double heat exposure conditions for both PaCa samples (Figure 1). At temperatures of -10°C in BxPC-3 cells or -15°C in PANC-1 cells, despite the initial decrease in day one viability, cells were still capable of recovery during the seven-day assessment interval following double exposure.

Investigation of the modes of cell death following thermal exposures

Following establishment of enhanced cell death in PANC-1 samples using DTA, studies were conducted to assess the modes of cell death in DTA samples compared to heating or freezing only. Flow cytometry was performed at 1, 4, 8 and 24 hours post-exposure using YO-PRO-1 and propidium iodide to assess the relative levels of apoptotic and necrotic populations, respectively, compared to live cells. Post-treatment assessment indicated that necrosis was the primary form of cell death in all samples (Figure 3). The necrotic population was found to increase from 1 to 24 hours in all experimental conditions. Analysis of apoptosis revealed varying levels in freeze samples. A small



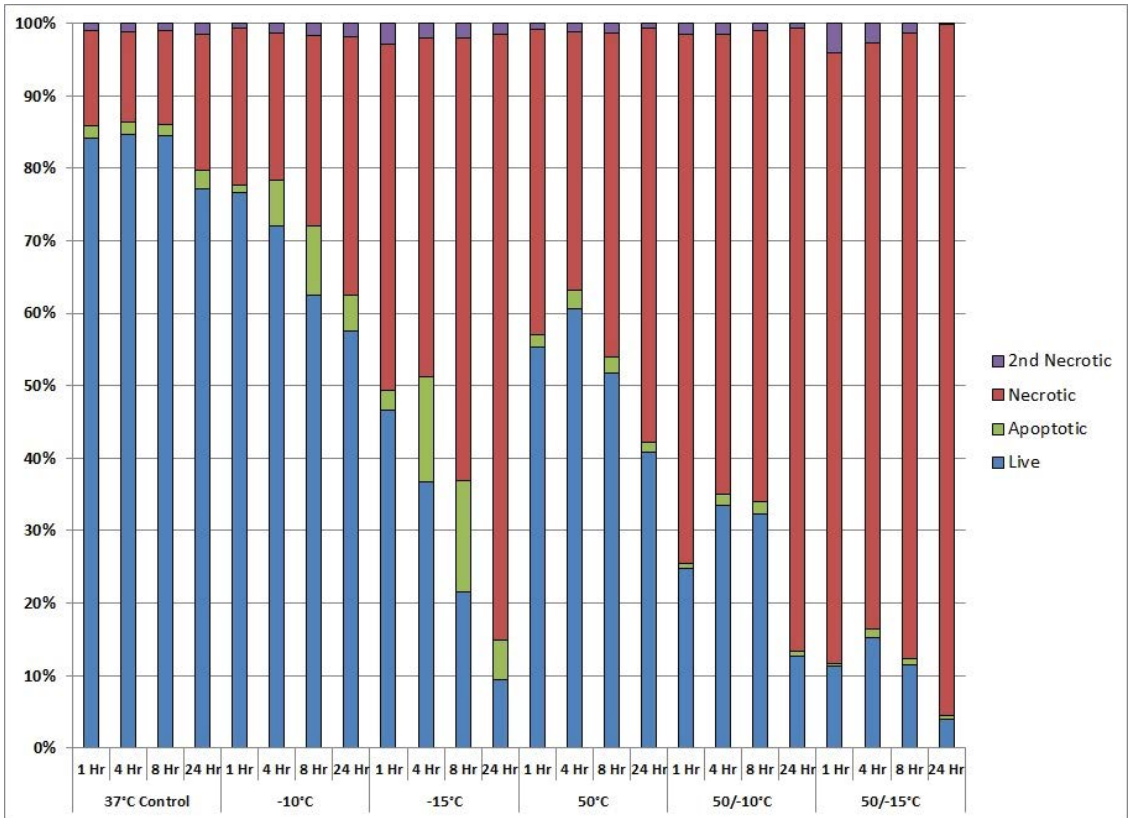


Figure 3. Assessment of cell death subpopulations following thermal treatments. Living, apoptotic, necrotic and secondary necrotic subpopulations were analyzed as a percentage of the whole sample. PANC-1 (A) and BxPC-3 (B) cells were subjected to selected thermal temperatures and conditions and assessed using microfluidic flow cytometry. Samples were stained with YO-PRO-1 and propidium iodide to detect the levels of apoptosis and necrosis versus live cells at 1, 4, 8 and 24 hours post-thermal exposure. Data illustrate necrosis as the predominant mode of cell death, with apoptosis contributing to a lesser degree following freezing. When DTA was performed, apoptosis levels diminished and necrosis levels increased further.

apoptotic population was observed in BxPC-3 samples at 4 and 8 hours following freezing (8% and 15%, respectively) which dissipated by 24 hours (Figure 3B). Specifically, BxPC-3 samples exposed to -10°C or -15°C resulted in the largest apoptotic population observed during the assessment interval. PANC-1 samples revealed primarily necrotic cell death at each timepoint investigated (Figure 3A). When DTA was applied, minimal apoptosis was observed and a substantial increase in necrosis was found in both cell types (Figure 3). Based on these data, we hypothesize that DTA results in increased stress, shifting cell death towards necrosis.

Discussion

Thermal ablation techniques are now recognized for their potential in the treatment of various diseases including solid tumor carcinomas. While cryotherapy and heat ablation are commonly utilized in a clinical setting, limited studies have examined the combination of these techniques to ablate solid tumors. Although few studies have examined DTA, several have reported on the combination of cryoablation or heat thermal therapy with other methods, including radiation, chemotherapy (e.g. FOLFIRINOX) and excision, yielding improved outcome [15, 41-45]. As such, we examined the combination of heat and freezing on PaCa. We hypothesized that the application of a dual thermal treatment regime would provide for more effective ablation than mono thermal treatments. Our study utilized two model PaCa cell lines, PANC-1 and BxPC-3, to determine the impact of a dual thermal protocol in an *in vitro* setting. The objective of this study was to apply two minimally lethal temperatures to induce increased levels of cell death than when applied as a monotherapy.

Studies using PaCa cells previously revealed that temperatures below -20 or -25°C were necessary for complete ablation of BxPC-3 and PANC-1 samples, respectively [37]. When PANC-1 cells were exposed to -15°C there was an initial drop in viability; however, samples recovered over the seven-day assessment interval. This was also observed in BxPC-3 samples subjected to -10°C. Exposure to -15°C in BxPC-3 or -20°C in PANC-1 resulted in a decrease in viability to <25% on day one, yet both cell lines were able to recover throughout the assessment interval. In order to determine if DTA could generate an improved result over mild freezing, we compared DTA to a double freezing exposure. A moderate temperature, -15°C, was selected to examine if the combination of heating and freezing would increase cell death compared to the double exposure alone. When PANC-1 samples were subjected to a double -15°C exposure, a decline in day one viability to 70%(±4) was observed, yet the surviving cells were able to proliferate over the assessment period, returning to control levels by day five. In BxPC-3 samples, day 1 viability decreased to <10% and were found to plateau over the assessment period. In comparison, the combination of 48/-15°C or 50/-15°C was found to be more effective than the double -15°C exposure in PANC-1 samples. Interestingly, this was not the case with BxPC-3 samples where the double exposure to -15°C was more effective than the 48/-15°C exposure. DTA using 50/-15°C in BxPC-3, however, yielded 0% viability by day three.

When samples were exposed to 50°C, while samples were ultimately ablated, it took several days to achieve complete cell death. Although complete ablation was observed *in vitro*, the time to cell death may result in a different outcome *in vivo*. As such, we elected to examine the impact of a double heat exposure in comparison with a combination of heating with freezing. When heating was combined with freezing, the time to complete ablation was reduced significantly compared to either alone as a monotherapy ($p<0.01$). This was also observed in double heat exposure to 48°C, but not 45°C exposure. This suggests that in line with previous studies from Gage and Klossner, a repeat thermal exposure should cause a greater amount of damage and reduce the likelihood of disease recurrence in a clinical scenario [18,38].

Investigation into the combination of heating and freezing (DTA) with PANC-1 samples revealed a substantial drop in day one viability when 50°C/-15°C exposure was applied. In comparison to 50°C (74%(±1)) or -15°C (80%(±2)) viability on day one, the combination resulted in viability to 28%(±1) with a continued decline to 5%(±0.4) on day 3 and 0% by day 5 post-treatment. In BxPC-3 samples, a 5%(±0.5) viability on day 1 in the 50°C/-15°C condition was observed which decreased to 0% by day 3. Interestingly, the use of 45°C in a single, double, or combinatorial setting was ineffective in ablating PaCa samples. In fact, the data suggest that pre-treatment with 45°C may contribute to a slight protective effect against freezing (Figure 1). DTA treatment utilizing the intermediate temperature of 48°C yielded the most substantial impact on cell death in all conditions tested. Most notable was the decrease in day one viability following 48/-15°C exposure in PANC-1 samples which yielded 45%(±1) viability in comparison to 78%(±1) or 80%(±2) in 48°C or -15°C exposures alone, respectively (Figure 1A and 1B). When 48°C was combined with -20°C, cell survival decreased to 8%(±0.3) overall and continued to decline to 0% by day five, which differed from either temperature when applied as a monotherapy. When 48°C was combined with -10°C in BxPC-3 samples, although there was a significant difference from single exposures of -10°C (90%(±2)) or 48°C (87%(±2)) to 44%(±1) in 48/-10°C ($p<0.01$), samples retained their ability to proliferate (Figure 1C and 1D). While the combination of 48/-15°C was found to have a similar impact in BxPC-3 samples compared to freeze alone (16%(±1) versus 21%(±1)), the combination exposure led to the inability of BxPC-3 samples to recover. This suggests that in the case of more thermally sensitive cells, the use of DTA has a similar effect and does not compromise outcome. Importantly, in cases where the cells are not as thermally sensitive, such as PANC-1 cells, a combinatorial thermal strategy may reduce the likelihood of cell survival as well as increase the rate of destruction.

In considering the order in which DTA is performed, several factors suggest a protocol of heat then freeze would be beneficial. When hyperthermia techniques are performed, it is difficult to determine the margin of the cytotoxic effects of heat ablation and overheating may occur [40,46-47]. This can generate a residual heat load in the tissue that remains for an extended period of time and slowly dissipates into the tissue, resulting in continued damage, lesion expansion, thrombus formation, strictures, and other complications [40,46-50]. If a double heat exposure is applied, this effect intensifies, further damaging the surrounding tissues. This continued destruction, while positive in diseased tissue, is difficult to monitor and control and may result in a high degree of heat based complications, such as hematoma and neoplasia [48-52]. The addition of the freezing step after heating could theoretically remove residual heat load remaining following hyperthermia, potentially reducing the risk of collateral damage and unwanted complications to surrounding tissue. This advantage combined with the ability to monitor ice ball size during cryotherapy using ultrasound would allow for greater control of the ablation zone [53]. As such, this combination treatment may not only destroy target tumors following exposure to less extreme temperatures, but may also aid in reducing unintended overtreatment of surrounding healthy tissues.

When examining the forms of cell death associated with DTA, the combination regime was found to reduce the overall level of apoptosis while increasing the level of necrosis. Comparison of heating versus freezing from our previous study [37] indicated that apoptosis was

more prominent following freeze exposure and almost completely absent after heating. Even in the case of BxPC-3 samples that possessed an apoptotic peak, once DTA was performed, the levels of apoptosis declined dramatically. We hypothesize that this shift towards greater necrotic cell death is due to the increased potency of the DTA combination. To this end, further investigation into the molecular mechanisms regulating cell death under these conditions is necessary to determining how DTA specifically increases cell death.

In conclusion, the data suggest that DTA, heating then freezing, at moderate temperatures offers an effective paradigm for targeting PaCa. In the case of PANC-1 samples, it was observed that combination of 48°C or 50°C with -20°C was sufficient to reduce cell viability to <10% day one post-treatment with no observed recovery throughout the remainder of the assessment interval. The combination of 50°C and -15°C was also found to be effective, with day one viability of BxPC-3 at 5% and PANC-1 at 28%, declining to 0% by days 3 and 5, respectively. The studies also revealed that the temperature used in each aspect of the combination directly impacted the outcome. One aspect that remains to be addressed is the best order of exposures. To this end, future studies are needed to determine if heating then freezing is mechanistically more effective, or comparable, to freezing followed by subsequent heating. Furthermore, investigation into the molecular mechanisms responsible for the observed increase in cell death in PaCa following DTA are warranted. In summary, the results presented herein suggest that the use of a DTA regimen may provide an effective option for treatment of PaCa as well as other cancers.

Conflict of interest

KWB is a consultant for CPSI Biotech. JMB, KKS and RVB are employees of CPSI Biotech. JGB has no competing interests.

Authors' contributions

KWB, JMB and KKS contributed to experimental design, experimentation and data analysis for this study. RVB and JGB conducted data and experimental design review and assisted in data interpretation. KWB and JMB prepared the draft manuscript. KKS, RVB and JGB provided review and revision input for the manuscript. All authors read and approved the final manuscript.

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