British Journal of Cancer Research


Research Article

Investigation of Liver Cancer Cell Response to Cryoablation and Adjunctive Based Cryo/Chemotherapy

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Received: April 30, 2020; Accepted: May 26, 2020; Published: May 28, 2020

Abstract

As the annual incidence of liver cancer continues to grow worldwide so does the need for new strategies for treatment. While numerous treatment options exist, many if not all remain suboptimal. As such many treatments are being reexamined as a monotherapy or in combination with others in an attempt to identify improved efficacy. Cryoablation is one such treatment being reevaluated. While various studies have shown cryoablation to be an effective treatment option for liver cancer, a lack of basic information pertaining to dosing (minimal lethal temperature) necessary to destroy liver cancer as well as technological issues with overcoming the high heat load within the liver has limited its use as a primary therapeutic option. Additionally, there is limited information on the potential of combining freezing with other treatments, such as chemotherapy, to improve outcome. In an effort to elucidate the effects of freezing on liver cancer, a human liver cancer cell line (C3A cells) was evaluated in vitro. C3A cells were exposed to a range of freezing temperatures from -10 to -25°C and compared to non-frozen controls. The data show that a single 5 minute freeze to -10°C did not affect cell viability, whereas -15°C and -20°C results in a significant reduction in viability 1 day post freeze to 58% and 11%, respectively. These populations, however, were able to recover when returned to culture medium at normothermic temperatures. A complete loss of cell viability was found following a single freeze at -25°C. Application of a repeat (double) freeze resulted in a reduction of survival following freezing to -15°C to 14% and complete cell destruction at -20°C. In addition to freezing alone, studies investigating the impact of adjunctive low dose gemcitabine and oxaliplatin pre-treatment (48 hours) in combination with freezing were conducted. The combination of 100nM gemcitabine pre-treatment and a single freeze at -15°C resulted in complete cell death. Complete cell death was also found following the combination of 1.5µM oxaliplatin pre-treatment and a single -15°C freeze. This suggests that the use of either low dose gemcitabine or oxaliplatin may be synergistically effective when combined with freezing. In summary, these in vitro results suggest that freezing to temperatures in the range of -20 to 25°C results in a high degree of liver cancer cell destruction. Further, the data support a potential combinatorial chemo/cryo therapeutic strategy for the treatment of liver cancer.

Keywords: ablation, cryosurgery, freeze dose, liver cancer, gemcitabine, oxaliplatin

Introduction

The incidence of liver cancer has more than tripled in the last 40 years. The American Cancer Society estimates 42,810 new cases will be diagnosed and 30,000 will die from liver cancer in 2020 in the US alone [1]. Current treatment depends on staging, with early stage tumors defined as potentially resectable. In cases where surgery is not possible due to tumor size, proximity to blood vessels, or other factors chemotherapy, targeted molecule therapy, and immunotherapy via systemic or intrahepatic infusion are often employed. Thermal ablation techniques, such as radiofrequency (RFA) or cryoablation (CA), are also often utilized. For advanced metastatic disease, treatment with small molecules like sorafenib or Lenvatinib can slow disease progression but are not curative [2].

Liver cancer is now being treated using thermal ablation (RFA, HiFu and CA). The advantages of the various ablative treatment approaches over surgical excision are similar given that they are relatively non-invasive and can be performed percutaneously or laparoscopically. Each has been shown to be
successful for the treatment of primary hepatic tumors as well as liver metastases such as those originating from the colon. Wang et al.[3] have reported RFA to be technically successful in 127 patients with no severe treatment-related complications with 1, 2 and 3-year survival rates after RFA of 83%, 56% and 44%, respectively, for patients with nonresectable hepatic lesions.

CA has also shown promise in the ablation of a host of solid tumors including prostate, breast, kidney, and liver [4-11]. CA has been successfully utilized to treat both primary liver neoplasms as well as metastases from other sites, most commonly colon, breast, lung, or pancreas. CA has also been extensively utilized for nonresectable primary and secondary (e.g. colon, gastric) cancers [12-15]. Studies have shown that CA is most effective in liver cancer for tumors ≤4 cm [9]. Orlacchio et al.[16] reported all patients subjected to cryosurgery of small hepatocellular carcinomas survived without short or long term complications. In a study by Paganini et al.[17] on hepatic colorectal metastases, none of the cryoablated intra-hepatic tumors recurred at the CA site and median survival of the patients was 94.2 months, leading to the conclusion that CA improves survival as compared to patients receiving chemotherapy alone. A similar positive outcome has been reported by Osada et al. [18]. Yet, not all reports are equally positive. For instance, Bageau et al.[19] reported that among 31 patients with resectable liver colorectal metastases, 7 patients developed recurrence at the CA site. Kim et al.[20] has shown local recurrence of primary hepatic tumors near large blood vessels where inadequate freezing may be responsible for failure [21-23].

CA for liver tumors is typically performed percutaneously whereby a cryoprobe is inserted and heat is extracted from the tissue through circulation of a cryogen such as argon, CO₂, or nitrogen in the probe. The resultant ice formation can be visualized through ultrasound, CT, or MRI and is completely destructive to the tissue closest to the cryoprobe where temperatures are coldest [24, 25]. As the distance from the cryoprobe increases so do temperatures thereby resulting in a thermal gradient within the frozen tissue mass. As a result, differential responses across the treatment zone are evident due to differences in the rate of cooling and final temperature experienced based on distance from the cryoprobe. Cells close to the cryoprobe tip experience rapid cooling rates, ultralow temperatures, and an increased probability of intracellular ice. Cells more distant from the probe primarily experience extracellular ice, whereas cells even more distal experience only hypothermia. This results in the activation of multiple modes of cell death (ice rupture, necrosis and apoptosis) within a cryogenic lesion [26-28]. Further, this temperature gradient results in warmer temperatures in the periphery of the cryogenic lesion which may be insufficient to yield complete cell death and can result in tumor recurrence [29]. The extent of the “cryolesion” is dependent on a number of factors, including cryogen-type, tip contact, cooling rate, tip temperature, duration of freeze, repetition of the freeze-thaw cycle, tissue vascularility, size of the target tumor, and importantly, cell sensitivity to cooling temperature [30, 31]. Different cancer cell types have inherent variability in the minimum lethal temperature necessary for complete ablation. For instance, prostate cancer requires temperatures of >40 °C or below [30, 32, 33], while renal cancer -25 °C or below [34].

Given reports detailing both the success and failure of CA for the treatment of liver cancer combined with the complex destructive environment and differential response of cancers to ablation, in this study we investigated the freeze response of liver cancer in vitro using the C3A cell line (derivative of HepG2, a hepatocellular carcinoma (HCC)) in an effort to identify the minimum lethal temperature for liver cancer. In addition to identifying the minimal lethal temperature, we also investigated the effect of the combination of CA and chemotherapy for improving cancer kill. The use of adjuvants in combination with CA to sensitize cells in the ice ball periphery prior to or during a freezing event is an area of growing interest [32, 35-51]. Given that cells within a frozen mass experience different rates of cooling and nadir temperatures based on proximity to a cryoprobe, maximizing the volume of complete cell death is imperative to procedure success. There are a number of small molecules and cytotoxic agents used in the treatment of liver cancer. The first line gold standard is sorafenib. Yet acquired sorafenib resistance often becomes a limiting issue [52]. Other chemotherapeutic regimes often include gemcitabine and/or oxaliplatin, which have shown efficacy in liver and bile duct carcinomas [52, 53]. In later stages these agents are often administered systemically at doses of 1,000 mg/m² and 100 mg/m², respectively. At these doses, the well-known side effects of chemotherapy can impede patient quality of life. Chemotherapy administered via intrahepatic infusion allows for targeting of the tumor(s) while minimizing systemic exposure and related side effects and is usually reserved for early to mid-stage disease [54, 55]. The combination of CA with either systemic or infusion based chemotherapy may allow for reduced levels of drug needed to achieve complete cancer destruction within a frozen mass, thus enabling the possibility of a curative response. As such, in this study we also investigated the impact of the pre-treatment of C3A cells with sub clinical doses of gemcitabine or oxaliplatin followed by freezing. We hypothesized that the use of a low-dose adjunctive agent prior to freezing would increase cell destruction at typically non-lethal temperatures while reducing the overall negative effects associated with typical clinical chemotherapy doses.

**Materials and Methods**

**Cell culture**

Liver cancer cells C3A (derivative of HepG2, ATCC- CRL 10741) were cultured in T-75 flasks (Cell Treat, Shirley, MA, USA) with EMEM (ATCC 30-2003) and supplemented with 10% FBS (Peak Serum, Wellington, CO, USA) and 1% penicillin/streptomycin (Lonza, Walkersville, MD, USA). Cells were Lifted using TrypLE Express (Gibco/Life Technologies, Grand Island, NY, USA), centrifuged and plated into Costar strip well plates (Corning, Tewksbury, MA, USA) at 15,000 cells per well and cultured for 24h prior to drug exposure and 48h prior to freezing. Cell Identity: The cell line used in this study were purchased directly from ATCC. This cell line is not listed in the database of commonly misidentified cell lines maintained by the International Cell Line Authentication Committee (Version 10. March 25, 2020).

**Adjuvant chemotherapy treatment**

Gemcitabine (Sigma Aldrich #G-6423, St. Louis, MO,
USA) was prepared fresh in sterile water prior to each use and diluted to final concentrations of 10, 100, 500, 1,000, and 5,000 nM in media. Samples were exposed to a single application of gemcitabine for 48 hours prior to freezing.

Oxaliplatin (Sigma Aldrich #O-9512) was prepared as a 5mM stock solution in sterile water and stored at -20 °C. Stock solutions were thawed and diluted to final concentrations of 0.88, 1.5, 3, 8.8, and 10 µM in media. Samples were exposed to a single application of oxaliplatin for 48 hours prior to freezing.

**Freezing protocol**

Samples in Costar 8-well strips (75 µL medium/well) were exposed to freezing temperatures of -10 °C, -15 °C, -20 °C or -25 °C in a refrigerated circulating bath (Neslab/Thermo Scientific, Waltham, MA) for 5 minutes. Prior to freezing (30 minutes), culture medium was aspirated and replaced with 75µL per well of fresh culture medium. Strips were placed into aluminum blocks, containing a thin coating of ethanol to facilitate complete thermal contact and heat exchange with each well. Ice nucleation was initiated at -2 °C using liquid nitrogen vapor to prevent supercooling. Sample temperature was recorded at 1 second intervals using a type T thermocouple (Omega HH806AU, Omega, Stamford CT). For single freeze conditions, samples were held for a total time of 5 minutes in the freezing bath, passively thawed at room temperature for 10 minutes under a laminar flow hood and then placed at 37 °C for recovery and assessment. For repeat (double) freeze conditions, samples were held for 5 minutes, passively thawed for 10 minutes, and then frozen again for an additional 5 minutes (5/10/5 protocol). Following the second freeze interval, samples were passively thawed at room temperature for 10 minutes and then placed into 37 °C for recovery and assessment.

**Viability assessment**

The metabolic activity indicator alamarBlue (Invitrogen, Carlsbad CA) was utilized to assess cell viability. Stock alamarBlue was diluted 1:20 in Hank’s Balanced Salt Solution (HBSS, Corning/Mediatech) and applied to samples for 60 min (±1 min) at 37°C. Raw fluorescent units were obtained using a TECAN Infinite plate reader (excitation 530 nm and emission 590 nm, TECAN Austria GmBH, Grodig, Austria) and analyzed using Microsoft Excel. Raw fluorescence units were converted to percent survival = 0.4% (±0.1), D7 = 0.4% (±0.1).

**Results**

**Freeze response of C3A liver cancer cells**

In order to identify the minimal lethal temperature for liver cancer, C3A cells were exposed to a single 5 minute freeze at -10 °C, -15 °C, -20 °C or -25 °C, thawed, allowed to recover in culture and assessed for initial cell viability (24 hours) and recovery over a 7 day period (Figure 1). Following a single 5 minute freeze to -10 °C, C3A viability was similar to non-frozen controls at 99.0% (±1.2) vs. 100% (±1.0) (p= 0.6). Exposure to -15 °C yielded a decrease in viability to 58.9% (±2.6) 1 day post-freeze, which was significantly different than both the -10 °C and non-frozen control samples (p≤ 0.01). The surviving cells were found to recover reaching 74.6% (±3.6) by day 7. Exposure to -20 °C resulted in a further reduction in C3A viability to 11.0% (±0.7) 1 day post freeze. A low but significant level of regrowth was observed over the 7 day post-thaw analysis interval (D7 = 17.2% (±2.1) vs. D1 = 11% (±0.7); P<0.01). When C3A cells were exposed to -25 °C, complete cell death was observed at day 1 and no regrowth was noted over the 7 day recovery period (D1 survival = 0.4% (±0.1), D7 = 0.4% (±0.1).

With the identification of -25 °C as lethal for C3A cells, studies were conducted to assess the impact of a repeat (double) freeze exposure on cell viability and recovery. To this end, samples were exposed to repeat freezing at -15 °C and -20 °C (Figure 2) Repeat freeze exposure (double 5 minute freezes) to -15 °C resulted in a significant increase in cell survival followed by a low level of recovery over the 7 day assessment interval.
ples were found to recover to ~22% over the 7 day recovery interval. While less than 10%, this recovery was significant compared to day 1 survival (D1 vs. D7 P< 0.01). Repeat exposure to -20 °C resulted in complete cell destruction with no recovery over the 7 day assessment interval (D1 = 0.6% (±0.1); D7= 0.6% (±0.1)). The results from the double freeze experiments suggested that the repeat exposure results in an elevation of the minimal lethal temperature to -20 °C.

Analysis of samples post freeze using brightfield microscopy revealed that cellular morphology became increasingly rounded as the severity of the freeze increased. Only minor changes were observed on day 1 following a single -10°C freeze while a single -15°C freeze proved to be an intermediate insult. A single -20°C or repeat -15°C yielded significantly greater cell loss. Very few cellular structures appeared viable in the repeat -20°C condition (-25°C not shown).

Assessment of the combination of gemcitabine and freezing
With the identification of the minimal lethal temperature for C3A cell destruction (-25 °C) and the elevation of this temperature to -20 °C when a repeat freeze was applied, we explored combining low dose chemotherapy agent pretreatment followed by freezing to further increase cell death at a non-lethal temperature. Given the significant decrease in viability observed following exposure to -15 °C with subsequent repopulation over the 7 day recovery interval following either a single or double freeze, we chose to investigate the combination of gemcitabine and freezing at -15 °C.

Initial studies focused on gemcitabine dose response at 37 °C to evaluate the impact of 48 hour exposure to low (subclinical) concentrations of 10, 100, 500, 1,000, and 5,000 nM on cell viability (Figure 4). These concentrations equate to 0.11, 0.56, 1.11, 5.57, and 55.5 mg/m², respectively, all of which were significantly lower than the clinical dose (800-1000 mg/m²) when applied as a single agent, but are in line with published in vitro concentrations [56-58]. Sample assessment following a 48 hour treatment revealed that sample viability decreased to 76% (±2.0), 81% (±1.4), 76% (±1.8), 70% (±2.0), and 56% (±2.8) of non-treated controls for 10, 100, 500, 1,000, and 5,000 nM gemcitabine, respectively (Figure 4). In all conditions, sample viability was found to decrease over the 7-day assessment period, yielding 22% (±1.4), 28% (±1.2), 21% (±1.3), 17% (±1.1), and 12% (±1.0) survival by day 7, respectively. All treatment conditions were found to be statistically significant compared to non-treated controls.

Assessment of low dose gemcitabine pretreatment in combination with freezing on liver cancer cell survival. C3A cells were subjected to 48 hours pretreatment with low dose (100, 500, or 1000nM) gemcitabine followed by a 5 minute freeze at -15°C. Data suggest that the combination of gemcitabine pretreatment and -15°C freezing results in complete liver cancer cell death over the 7 day assessment period.

Figure 3. Brightfield micrographs of C3A samples following freezing. Cellular morphology became increasingly rounded as the severity of the freeze increased. Only minor changes were observed on day 1 following a single -10°C freeze while a single -15°C freeze proved to be an intermediate insult. A single -20°C or repeat -15°C yielded significantly greater cell loss. Very few cellular structures appeared viable in the repeat -20°C condition (-25°C not shown).

Figure 4. Assessment of low dose gemcitabine treatment on liver cancer cell survival. C3A cells were subjected to 48 hours pretreatment with sub-clinical doses (10, 100, 500, 1000, or 5000nM) gemcitabine at 37°C. Cell viability declined steadily in all samples over seven days post-treatment, but complete ablation was not observed.

Figure 5. Effect of adjunctive low dose gemcitabine pretreatment in combination with freezing on liver cancer cell survival. C3A cells were subjected to 48 hours pretreatment with low dose (100, 500, or 1000nM) gemcitabine followed by a 5 minute freeze at -15°C. Data suggest that the combination of gemcitabine pretreatment and -15°C freezing results in complete liver cancer cell death over the 7 day assessment period.

Figure 6. Assessment of low dose oxaliplatin treatment on liver cancer cell survival. C3A cells were subjected to 48 hours pretreatment with sub-clinical (0.88, 1.5, or 3 µM) and clinical (8.8 or 10µM) oxaliplatin at 37°C. Cell viability declined steadily in all samples over seven days post-treatment, but complete ablation was not observed.
conditions were found to be significantly different compared to untreated controls (p<0.01). Additionally, the 1,000 nM and 5,000 nM pretreatment samples were also found to be significantly different from the 10, 100, and 500 nM pretreatment samples (p≤0.01).

With similar mild levels of cell death observed following exposure to gemcitabine, the 100, 500, and 1,000 nM concentrations were selected for freeze response combination studies as they represented a broad low-dose concentration range. For combination studies, samples were pretreated with gemcitabine for 48h, exposed to a single 5 minute freeze to -15 °C, then allowed to recover and assessed for 7 days (Figure 5). C3A cell exposure to 100, 500, and 1,000 nM gemcitabine for 48 hours followed by a single freeze at -15 °C resulted in increased cell death compared to -15 °C freeze (-15) or gemcitabine alone samples (G/-15: 11% (±1.1), 8% (±0.6), and 7% (±0.7), respectively vs. -15: 60% (±2.3); P<0.01) (Figure 5). While a significant decrease in day 1 post-freeze viability was noted, importantly sample viability continued to decrease to 2% (±0.3), 1% (±0.1), and 1% (±0.1) in combination samples by day 7, respectively. This differed significantly from both -15 °C and gemcitabine alone conditions (p<0.01).

Impact of the combination of oxaliplatin and freezing

In an effort to further explore the potential benefit of CA in combination with low-dose chemotherapy, studies were expanded to investigate the combination oxaliplatin and freezing. As with gemcitabine studies, a dose response study was conducted to evaluate the level of cell death following 48 hour exposure to 0.88, 1.5, 3, 8.8, and 10 µM oxaliplatin under normothermic conditions (Figure 6). These concentrations equate to 13, 22, 45, 130, and 150 mg/m2, respectively. Typical clinical dose for oxaliplatin is 130 mg/m2 or 8.8 µM. Assessment upon drug removal revealed that sample viability decreased to 73% (±0.8), 81% (±1.4), 81% (±1.4), 74% (±1.1), and 62% (±1.5) of non-treated controls following 48 hours of treatment with 0.88, 1.5, 3, 8.8, and 10 µM, respectively. In all conditions, sample viability was found to decrease over the 7-day assessment period, yielding 23% (±0.7), 25% (±1.2), 22% (±1.0), 15% (±0.6), and 13% (±0.6) survival by day 7, respectively (Figure 6). All treatment conditions were found to be significantly different compared to untreated controls (p<0.01). Based on the dose response studies, oxaliplatin concentrations of 1.5, 3, and 8.8 µM were selected for combination studies (Figure 7). Samples were pretreated with the indicated concentration of oxaliplatin for 48h prior to a single 5 minute freeze to -15 °C and then allowed to recover and assessed daily. C3A cell exposure to 1.5, 3, and 8.8 µM oxaliplatin for 48 hours followed by a single freeze at -15 °C resulted in an increase in cell death compared to the -15 °C freeze (-15) or Oxaliplatin alone samples (O/-15: 26% (±1.7), 26% (±1.6), and 16% (±1.2), respectively vs. -15: 60% (±2.3); p<0.01) (Figure 5). All combination samples were found to be significantly different from both the 1.5 and 3 µM samples (p<0.01). As with the gemcitabine combination, oxaliplatin combination sample viability continued to decrease to 2% (±0.2), 2% (±0.1), and 1% (±0.2) by day 7, respectively, which differed from that of freeze and oxaliplatin alone samples.

Discussion

This study investigated the survival of a hepatic cancer cell line (C3A) following freezing in an effort to identify the minimal lethal temperature (dose) necessary for complete cell destruction. Studies also examined the impact of double freeze exposure. These studies were conducted as CA is often applied in a repeat (double) freeze procedure for the treatment of many cancers including prostate, renal and liver [26, 30, 59-62]. Identification of the minimal lethal temperature could aid in the application of CA to treat liver cancer moving forward enabling enhanced outcome and precision while reducing the risk of negative side effects associated with over freezing. Investigations into the impact of the combination of low-dose (sub-clinical) gemcitabine and oxaliplatin pre-treatment and mild freezing (-15 °C) were also conducted as studies have suggested the benefit of adjunctive drug/freezing in enhancing cancer kill (elevating the minimal lethal temperature) under conditions which when applied as a monotherapy (freeze or drug alone) are non-lethal [26, 36, 38, 39, 41-51, 63].

Initial freeze response studies examined C3A cell survival following exposure to temperatures ranging from -10 °C to -25 °C. Studies revealed complete destruction was attained following a single 5 minute freeze at -25 °C whereas exposure to -10 °C resulted in no cell death (Figure 1). A single freeze to -15 °C or -20 °C resulted in a significant level of cell death at 1 day post-freeze. Samples, however, were observed to recover over the 7 days assessment interval. As CA is often applied clinically using a double freeze protocol, studies applying a repeat (double) 5 min freeze protocol with an intermediate 10 min thaw (5/10/5), were conducted. Application of a double freeze resulted in an increase in the minimal lethal temperature to -20 °C as well as a significant increase in cell death and reduction in sample repopulation following exposure to -15 °C (Figure 2). This 5 °C elevation in the minimal lethal temperature was significant as it represented a ~25% increase in the destructive volume over a single freeze when correlated with the isothermal distribution within an ice ball created by a argon-based JT cryosystem [31, 64, 65].
Studies continue to document the benefit of adjunctive treatment combining CA with chemotherapy, nutraceuticals or other agents in a number of cancers including prostate, breast, lung, and liver, among others [36, 38, 39, 41-51, 63]. These and other studies have demonstrated that combinatorial approaches can increase the minimal lethal temperature necessary to kill cancer cells while offering the potential of reducing the overall negative side effects associated with traditional systemic chemotherapy. While the combination of CA and low-dose chemotherapy remains in the investigational stages, published reports on the adjunctive application of RFA and chemotherapy lend support to its potential. Morimoto, et al. have shown that intrahepatic infusion, also called trans-arterial chemoembolism (TACE), in combination with RFA as a multidisciplinary (ablation and chemotherapy) approach to treat HCC results in a three year tumor progression rate of 6% in the TACE-RFA group versus 39% in the RFA group alone [66]. Even in intermediate stage HCC, TACE approaches are recommended and show survival benefit over supportive care measures [55]. As studies have shown that the one and three year survival rates following percutaneous image-guided treatment of HCC are similar between RFA and CA [67], the combination of CA and TACE using gemcitabine and/or oxaliplatin may show a similar benefit. Importantly, given the dual blood supply from the hepatic artery (HA) and portal vein, the liver provides a unique opportunity for local agent administration. As tumors are often perfused by the HA while the rest of the liver is supplied by the portal vein this offers the potential of local introduction of chemotherapy agents to tumors followed by the application of CA thereby offering the potential to further reduce toxic side effects on non-targeted tissue. Based on these reports and the current usage of gemcitabine and oxaliplatin to treat liver cancer, we investigated the potential of combining low dose sub-clinical agent pretreatment with freezing. Pretreatment with 500 or 1,000 nM gemcitabine (1.11 or 5.57 mg/m2) in combination with a single freezing event was found to increase the minimal lethal temperature to -15 °C, as illustrated by a significant increase in cell death on day 1 and complete cell death by day 7 (Figure 5). Similarly, pretreatment with 1.5 or 3 µM oxaliplatin (~1/6 and 1/3 the current clinical dose of 8.8 µM) in combination with freezing to -15 °C resulted in a significant increase in cell death on day 1 post-freeze and near complete cell death at day 7 (Figure 7). The combination of 8.8 µM (130 mg/m2) oxaliplatin (clinical dose) pretreatment and freezing to -15 °C resulted in a similar response to 1.5 or 3 µM pre-treatment.

Although treatment of C3A cells with gemcitabine or oxaliplatin at 37°C resulted in a gradual decline in viability over the 7 day assessment period at each concentration evaluated, combination of drug pretreatment and freezing significantly accelerated the rate of decline and under select conditions (doses), complete cell death was achieved following the combination of low-dose agent pre-exposure and a single freeze at -15 °C. This is compared to -25 °C necessary for complete cell destruction for freezing alone whereas in the drug alone condition, complete cell death was not attained. Assuming an elliptical ice ball formed on a 3cm long freeze zone cryoprobe, this 10 °C shift in the minimal lethal temperature to -15 °C was significant as it represents an increase in ablative volume of an ice ball from <40% within -25 °C to ~62% within the -15 °C isotherm boundary, a ~70% increase in ablative volume, based on the reported isothermal distribution within argon-based JT ice ball [31, 64, 65]. This increase in destruction could decrease the risk of cancer survival and recurrence in the periphery of a frozen mass thereby increasing the likelihood of a curative outcome. Further, this could also have the benefit of expanding inclusion criteria to patients with tumors located in close proximity to vital structures where application of ultracold temperatures necessary for assured cancer destruction is challenging. A 2016 report on long term assessment of percutaneous CA of the liver detailed the safety and efficacy of the procedure, with low local recurrence rates even for tumors larger than 3cm or located near critical structures [10]. However, the average tumor size was 2.8cm and the average frozen mass was 5.2cm, indicating the necessity of a large volume of over-freeze to assure tumor destruction. Assuming a spherical ice ball, this suggests an average 73.62 cm3 volume of tissue was frozen to target a 11.49 cm3 mass. The application of adjuvant procedures, such as described herein, may allow for a reduction in the overall frozen tissue volume to achieve a similar or better outcome.

While promising, this study is not without limitations. The primary limitation is that this study was conducted on an in vitro cell model. As such these findings need to be further explored in vivo. Further, the in vitro nature of this study provides a near optimal environment for cell recovery following treatment. In vivo, following freezing and thawing, additional stressors including, prolonged tissue ischemia, inflammatory response, activation of apoptosis, etc., provide additional destructive events thereby increasing the level of cell death. These factors may further increase the minimal lethal temperature. Another limitation is that the freeze interval was limited to 5 minutes. Clinically, 10 minute freeze procedures have been traditionally utilized. Studies have suggested that attainment of the minimal lethal temperature for 30 seconds to 1 minute can achieve cell death and that longer holds do not yield increased survival [30, 31, 40]. As such shorter freeze intervals are possible if target temperatures are attained. Given this, combined with the push to improve outcome while reducing procedure times (increased efficiency) we elected to investigate shorter freeze times. Overall while offering potential benefits, these in vitro findings need to be examined in vivo prior to clinical utilization.

Conclusions

Although CA has been utilized for a number of years for the treatment of HCC [61, 67-69], identification of minimum lethal temperature (ablative dose) has not been reported. This information could play an important role in procedural planning to further improve therapeutic efficiency and patient outcomes. Our findings suggest that the minimal lethal temperature for liver cancer following a single freeze episode is -25 °C. Application of a repeat, double freeze protocol was found to increase the minimal lethal temperature to -20 °C. Combination studies demonstrated that pretreatment with sub-clinical doses of gemcitabine or oxaliplatin followed by single freeze to -15 °C resulted in complete cell destruction. The data suggest that this combination resulted in a shift of the minimal lethal temperature for liver cancer from -25 °C to the -15 °C range. Extrapolating these in vitro findings to an in vivo scenario, the data suggest that both freezing alone and in combination with low-dose gemcitabine or oxaliplatin has
the potential to improve outcome while reducing comorbidities associated with freezing and/or chemotherapy and may provide an improved path for the treatment of liver cancer.

Funding
This study was supported in part by funding from the National Institutes of Health 5R44CA183265-03 awarded to CPSI Biotech.

Conflicts of Interests
JMB, KLS, KKS, and RVB are employees of CPSI Biotech. JGB has no financial competing interests. JMB and JGB are related.

Authors’ Contributions
KLS, JMB and KKS performed all experimental design, experimentation, and data analysis for this study. RVB and JGB conducted data and experimental design review and assisted in data interpretation. JMB and KLS prepared the draft manuscript. JMB, KKS, RVB, and JGB provided review and revision input for the manuscript. All authors read and approved the final manuscript.

Availability of Data and Material
The data that support the findings of this study are available from CPSI Biotech but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of CPSI Biotech.

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