Dose Escalation of Vitamin D3 Yields Similar Cryosurgical Outcome to Single Dose Exposure in a Prostate Cancer Model

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Abstract
Vitamin D3 (VD3) is an effective adjunctive agent, enhancing the destructive effects of freezing in prostate cancer cryoablation studies. We investigated whether dose escalation of VD3 over several weeks, to model the increase in physiological VD3 levels if an oral supplement were prescribed, would be as or more effective than a single treatment 1 to 2 days prior to freezing. PC-3 cells in log phase growth to model aggressive, highly metabolically active prostate cancer were exposed to a gradually increasing dose of VD3 to a final dose of 80 nM over a 4-week period, maintained for 2 weeks at 80 nM, and then exposed to mild sublethal freezing temperatures. Results demonstrate that both acute 24-hour exposure to 80 nM VD3 and dose escalation resulted in enhanced cell death following freezing at −15°C or colder, with no significant differences between the 2 exposure regimes. Apoptotic analysis within the initial 24-hour period postfreeze revealed that VD3 treatment induced both caspase 8- and 9-mediated cell death, most notably in caspase 8 at 8-hour postfreeze. These results indicate that both the intrinsic and extrinsic apoptotic pathways are involved in VD3 sensitization prior to freezing. Additionally, both acute and gradual dose escalation regimes of VD3 exposure increase prostate cancer cell sensitivity to mild freezing. Importantly, this study expands upon previous reports and suggests that the combination of VD3 and freezing may offer an effective treatment for both slow growth and highly aggressive prostate cancers.

Keywords
prostate cancer, vitamin D3, cryotherapy, apoptosis, cell death, freezing

Introduction
The development of adjunctive strategies for cryoablation is of high interest due to the fact that simply freezing a given tumor may not completely destroy all cancerous cells. Reports have demonstrated that depending on the cancer type, cells can survive temperatures as low as −40°C. For instance, renal and liver cancer is destroyed at temperatures of −20°C or lower, whereas prostate cancer ranges from −25°C to −40°C depending on the molecular disposition of the particular cancer (ie, androgen-sensitive vs androgen-insensitive prostate cancer, respectively), as well as exposure intervals. Given this differential cancer specific response, it is critical to attain a specific target temperature (minimum lethal temperature) throughout a tumor to assure complete destruction. Due to the thermal gradients created within a frozen tissue mass, the necessity to freeze beyond the edge of a given tumor is often required. This positive freeze margin often results in the damage of nontargeted tissues, thus creating unwanted...
comorbidities. For instance, in prostate cryoablation, attainment of $-40^\circ C$ is often recommended to assure complete destruction.\textsuperscript{7-10} Attainment of $-40^\circ C$ at the tumor edge often requires the application of a positive freeze margin.\textsuperscript{1,11-14} With the application of a positive freeze margin in the prostate, physicians must be cognizant not to freeze the rectal wall or neurovascular bundle in an effort to avoid comorbidities. Given the balance between attaining a targeted minimal lethal temperature with a reduction of collateral damage associated with the positive freeze margin, the development of adjunctive strategies which can increase prostate cancer sensitivity to milder subfreezing temperatures is of great interest.

The objective of these strategies is to elevate the minimum lethal temperature through the use of low-dose, minimally toxic, adjunctive anticancer agents, thereby eliminating the need for a positive freeze margin while deploying a highly effective focal treatment strategy. Given the involvement of apoptotic cell death in freezing,\textsuperscript{4} agents that enhance apoptosis are of great interest. Combinatorial agents that have been studied range from cytotoxic chemotherapeutic agents\textsuperscript{1,17,18} to nutraceuticals,\textsuperscript{17,18} with the latter being the most attractive option due to typically decreased side effects. One such agent is calcitriol, the active metabolite of vitamin D$_3$ (VD$_3$).

Vitamin D$_3$ has been reported to have positive anticancer properties in numerous in vitro and in vivo studies.\textsuperscript{1,17,19-24} Clinical studies investigating the correlation between VD$_3$ status and an individual’s cancer risk have yielded conflicting results, particularly breast, colon, and prostate.\textsuperscript{25} Associations between vitamin D status, including single nucleotide polymorphisms in vitamin D binding protein and prostate cancer risk, have been reported.\textsuperscript{26-29} Although some clinical studies have found no significant differences between mortality rate and VD$_3$ supplementation, many preclinical studies have shown the benefit that supplementation has on cancer prevention and treatment.\textsuperscript{30} One reason for this may be the body’s ability to retain tight homeostatic control over 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ conversion (activation).\textsuperscript{31} As such, the measurable level of active 1,25(OH)$_2$D$_3$ has not been reported to be highly variable between individuals regardless of their 25(OH)D$_3$ serum status.

For instance, a study of patients diagnosed with low-risk prostate cancer who took 4000 IU per day of VD$_3$ revealed a potential reduction in cancer progression after 1 year as evidenced by a decrease in the number of positive cores or Gleason score (55%).\textsuperscript{21} The level of 25(OH) D$_3$ in these patients was significantly higher at 1 year of supplementation (66.2 $\pm$ 14.5 vs 32.8 $\pm$ 13.3 at baseline). Interestingly, this study also noted that levels of 1,25(OH)$_2$D$_3$ were also significantly increased with this dosage,\textsuperscript{21} suggesting a continued role in the monitoring of calcitriol levels as more is learned about its significance.

One highly studied effect of VD$_3$ is the ability to inhibit cell proliferation\textsuperscript{22,23} and induce apoptosis.\textsuperscript{24,32} This has made VD$_3$ an attractive adjunctive agent for cancer therapies. Studies have shown that the use of VD$_3$ in conjunction with various chemotherapeutic agents, radiation, and even cryoablation can yield improved cancer destruction.\textsuperscript{19,33-35} Studies have demonstrated that exposure of prostate cancer cells to high-dose (50-80 nM) VD$_3$ in conjunction with mild freezing in the $-15^\circ C$ to $-20^\circ C$ range results in a significant increase in cell death.\textsuperscript{17,19,20} While effective, these studies focused on acute short-term (2 day) exposure of samples to VD$_3$ followed by freezing. Further, these studies focused on plateau phase, highly confluent monolayers which correspond to slow growth, low metabolically active (low Ki67) prostate cancer models. This is significant given that the measurement of Ki67 antigen expression is widely used to determine growth fraction, and thus a logarithmically growing population of PC-3 cells express a higher percentage of Ki-67 positive cells than one that has reached high confluence and subsequent growth inhibition/plateau (unpublished results). A recent meta-analysis by Berlin et al strongly suggests the use of Ki67 as a prognostic marker for predicting overall poor prognosis in prostate cancer, as high Ki67 was consistently correlated with negative clinical outcomes. Low Ki67 was strongly correlated with better outcomes including disease free survival, lower rates of distant metastasis, and biochemical failure free survival among others.\textsuperscript{36}

Given the importance of improved treatment studies for aggressive, highly active prostate cancers,\textsuperscript{37,38} we investigated the combination of VD$_3$ exposure and freezing using a log-phase growth androgen-insensitive in vitro prostate cancer model. Studies were designed to compare the impact of gradual dose escalation of VD$_3$ levels, such as would be attained via an oral dose regimen versus an acute high-dose exposure (utilized in previous studies). These studies expand upon our previous work to include a modified treatment to model clinical application, evaluation of an aggressive log-phase growth prostate cancer model, as well as a more in-depth analysis of the apoptotic pathways involved in VD$_3$-mediated cell death.

**Materials and Methods**

**Cell Culture**

PC-3 (ATCC, CRL-1435) were cultured in T-75 flasks (Cell Treat, Shirley, Massachusetts) in Roswell Park Memorial Institute (RPMI) 1640 medium (Caisson, North Logan, Utah) supplemented with 10% fetal bovine serum (Peak Serum, Colorado) and 1% Penicillin/Streptomycin (Corning/Mediatech, Manassas, Virginia). Cells were lifted using TrypLE Express (Gibco/Life Technologies, Grand Island, New York), centrifuged, and plated into Costar stripwell plates (Corning, Tewksbury, Massachusetts) at 3.2 $\times$ 10$^3$ cells/cm$^2$ for 24 hours prior to VD$_3$ treatment or 48 hours prior to freezing.

**Vitamin D$_3$ Treatment**

Calcitriol (1,25(OH)$_2$D$_3$; Calbiochem/EMD Millipore, Billerica, Massachusetts) was reconstituted in ethanol to a (100 $\mu$M) stock solution and stored at $-80^\circ C$. Stock solutions were diluted to final working concentrations in media immediately prior to application. For single applications, cells were exposed to 80 nM for 24 hours prior to freezing and returned to normal media for the freeze and recovery.
**Dose Escalation**

PC-3 cultures in T-75 flasks were exposed to gradually increasing concentrations of calcitriol during the course of treatment. Beginning at 5 nM, exposure was increased by 5 nM every 2 days until the final concentration of 80 nM was achieved. Dose-escalated cultures were maintained at 80 nM for 2 weeks prior to experimentation with media replenished with fresh calcitriol every 2 days. At 1-week dose-escalated, a subculture was performed and a “recovery” condition established. This culture was returned to normal media conditions for 1 week prior to experimentation.

**Freezing Protocol**

PC-3 samples in log-phase growth in Costar 8-well strips (75 μL medium/well) were exposed to freezing temperatures of $-10^\circ\text{C}$, $-15^\circ\text{C}$, $-20^\circ\text{C}$, or $-25^\circ\text{C}$ in a refrigerated circulating bath (Neslab/Thermo Scientific, Waltham, Massachusetts) for 10 minutes. Ice nucleation was initiated at $-2^\circ\text{C}$ using liquid nitrogen vapor to prevent supercooling. Sample temperature was recorded using a type T thermocouple (Omega HH806AU; Omega, Stamford, Connecticut). Samples were thawed passively at room temperature for 10 minutes before recovery incubation at $37^\circ\text{C}$.

**Viability Assessment**

The metabolic activity indicator alamarBlue (Invitrogen, Carlsbad, California) was utilized to assess cell viability diluted 1:20 in Hank balanced salt solution (Corning/Mediatech) and applied to samples for 60 minutes ($\pm$ 1 minutes) at $37^\circ\text{C}$. Raw fluorescent units were obtained using a TECAN SpectraFluor Plus 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 percentages based upon prefreeze control values. A minimum of 3 experimental repeats with an intraexperimental repeat of 7 wells was performed in each condition ($n \geq 21$). Assessments were repeated on alternating days for at least 5 days of recovery.

**Caspase Inhibition Assays**

Caspase 8 inhibitor II or Caspase 9 Inhibitor I (Calbiochem, #218759 and 218761) were diluted to 10 μM in culture medium and applied 30 minutes prior to freezing. Inhibitors remained on cultures for 24 hours postfreeze.

**Immunofluorescence**

Samples were fixed in Costar strips with ice-cold 100% methanol for 15 minutes at $-20^\circ\text{C}$, followed by 3 × 5-minute washes in 1× phosphate-buffered serum (PBS; Corning). Samples were blocked in PBS containing 5% normal goat serum (Sigma-Aldrich, St. Louis, Missouri) and 0.03% Triton X-100 (VWR, Radnor, Pennsylvania) for 60 minutes at room temperature. Immunostaining with primary antibodies (cleaved Caspase 3, CST#9664; cleaved Caspase 8, CST#9496; cleaved Caspase 9, CST#20750) at 4°C overnight was followed by secondary antibody binding (goat anti-rabbit IgG Alexa Fluor 594 conjugate, CST #8889) for 60 minutes at room temperature. Both primary and secondary antibodies were diluted in PBS containing 1% bovine serum albumin (VWR) and 0.03% Triton X-100 (VWR). Samples were then rinsed in 1× PBS, counterstained with 1 μg/ml Hoechst 33342 (Molecular Probes), and imaged using a Zeiss Axiovert 200 (Zeiss, Thornwood New York). Three images per well at 10× magnification were obtained and ImageJ software (National Institutes of Health, Bethesda, MD) analysis was performed to quantitate positive staining. All experiments were performed at least in triplicate.

**Results**

**Baseline Viability of Treatment Regimes in Combination With Freezing**

Sample viability was assessed at 24 hours following a single 10-minute exposure to a range of sublethal temperatures associated with the periphery of a cryosurgical iceball. At temperatures associated with those near the iceball edge ($-10^\circ\text{C}$ to $-15^\circ\text{C}$), minimal cell death was observed in freeze alone samples (128.1% ± 9.3% of prefreeze control at $-10^\circ\text{C}$ and 103.8% ± 6.4% at $-15^\circ\text{C}$, compared to 139.5% ± 5.8% in nonfrozen samples, Figure 1). Following exposure to $-20^\circ\text{C}$ and $-25^\circ\text{C}$, viability was found to decrease to 16.6% ($\pm$ 4.0%) and 3.2% ($\pm$ 0.6%), respectively. Vitamin D$_3$ pretreatment, either a single 24-hour exposure or the dose escalation regimen, significantly increased cell death following freeze exposure compared to nontreated samples frozen to $-15^\circ\text{C}$, $-20^\circ\text{C}$, and $-25^\circ\text{C}$. Specifically, 24-hour VD$_3$ pretreatment decreased viability by 46.4%, 75.4%, and 66.3% following freezing to $-15^\circ\text{C}$, $-20^\circ\text{C}$, and $-25^\circ\text{C}$, respectively. Dose-escalated samples exposed to the same temperatures decreased by 49.7%, 73.9%, and 81.2%, respectively.

Comparison of VD$_3$ single exposure or dose-escalated samples revealed no significant difference in the level of cell death following freezing. Both 24-hour VD$_3$ exposure and dose-escalated VD$_3$ samples exposed to $-15^\circ\text{C}$ resulted in similar levels of posttreatment viability, both significantly lower than $-15^\circ\text{C}$ alone samples (55.6% ± 9.8% vs 52.5% ± 8.6%, $P = .07$ compared to 103.8% ± 6.4%; Figure 1). Similar results were seen with samples exposed to $-20^\circ\text{C}$ (41.1% ± 2.2% vs 4.3% ± 2.1%, $P = .9$ compared to 16.6% ± 4.0%). While similar increases in cell death were observed, a trend toward increased cell death was observed in dose-escalated samples compared to single treatment in the $-25^\circ\text{C}$ samples (0.61% ± 0.4% vs 1.1% ± 0.5%, $P = .001$). Interestingly, even under ideal recovery conditions created in vitro, sample exposure to VD$_3$ dose escalation followed by freezing to $-25^\circ\text{C}$ demonstrated a much lower level of repopulation than that of either freeze alone or single VD$_3$ exposure + $-25^\circ\text{C}$ samples.

Analysis of the VD$_3$ dose-escalated recovery samples (2 weeks of culture without VD$_3$ prior to freezing) revealed
no significant difference in cell death from that of untreated freeze alone samples at any temperature (Figure 1 and Table 1).

Membrane-Mediated Cell Death Is Increased in VD₃-Treated Cells

Given the significant increase in cell death observed with VD₃ pretreatment, a series of investigations were conducted to determine (1) the level of apoptotic involvement and (2) if either mitochondrial or membrane-mediated apoptosis was most prominently involved. To this end, samples were frozen to −15°C with and without VD₃ dose escalation and then assessed for caspase 8 (membrane), caspase 9 (mitochondrial), and caspase 3 (downstream execution) activity (caspase cleavage) at 2, 8, and 24 hours following freezing and compared to nonfrozen matched controls. Immunofluorescent staining revealed minimal changes in VD₃ dose escalation nonfrozen control samples; however, in VD₃ exposed/−15°C samples elevated levels of all active caspases were found following freezing.

Within 2 hours following −15°C freezing in VD₃ samples, 42% of cells stained positive for cleaved caspase 3, which increased slightly at 8 hours (49%) before decreasing to 34% at 24-hour postfreeze. This represented an approximated doubling in caspase 3 activity in the VD₃ dose-escalated samples from that of −15°C freeze alone samples at all 3 time points (Table 2). Analysis of the levels of 2 initiator caspases revealed that activated caspase 8 was detected in 32% of cells at 2 hours postfreeze, peaked at 8 hours (42%), and decreased to 17% by 24 hours. The 32% caspase 8 staining at 2-hour postfreeze was a 166% increase in activity compared to non-VD₃ exposed freeze samples (32% vs 12%, respectively). Similarly, at 8- and 24-hour VD₃/−15°C sample caspase 8 levels were significantly elevated compared to either VD₃ exposure or freeze alone samples (42% vs 15% at 8 hours and 17% vs 3% at 24 hours).

Analysis of caspase 9 activity in dose-escalated samples revealed that caspase 9 cleavage products were observed in 27% of cells at 2 hours postfreeze, increasing to 30% at 8 hours followed by a decrease to 12% by 24 hours. When comparing caspase 9 levels in VD₃/−15°C samples to −15°C samples, both the 2- and 8-hour time points were found to be elevated in the VD₃ exposed conditions. By 24 hours there were no

### Table 1. Assessment of the Percent Change in 1-Day Postfreeze Viability Following VD₃ Exposure in Comparison to Matched Freeze Only Samples.

|                        | 37°C | −10°C | −15°C | −20°C | −25°C |
|------------------------|------|-------|-------|-------|-------|
| Dose escalated         | −4.19| 2.55  | −54.31| −73.39| −82.79|
| 24-hour Vitamin D₃     | −3.42| 8.73  | −42.10| −75.42| −62.45|
| Recovery from escalation| −10.53| 3.52  | −0.65 | −0.65 | −3.93 |

![Figure 1. Viability of human prostate cancer cells following treatment with vitamin D₃ and freezing. Cells were either untreated or treated with 80 nM calcitriol for 24 hours, dose escalated to 80 nM, or recovery from dose escalation and exposed to freezing temperatures of −10°C, −15°C, −20°C, or −25°C. Error bars represent standard deviation and each condition is compared to the prefreeze value of the individual 37°C control sample.](image-url)
differences in caspase 9 levels between freeze alone and VD3/C015/C14C samples (11% vs 13%).

In addition to non-VD3 exposed and VD3/C015/C14C samples, VD3 exposure recovery samples were analyzed for caspase 3, 8, and 9 activities. Analysis of the recovery samples revealed a slight decrease in overall caspase activity at all time points postthaw in comparison to both the VD3/C015/C14C and /C015/C14C alone samples (Figure 2 and Figure 3, Table 2).

Caspase Inhibition Improves Cell Viability Postfreeze

With the observed increase in caspase activity in VD3 pre-treated/frozen samples, studies were conducted to determine if the increase in caspase 8 and 9 activity resulted in the observed increase in cell death or if it was simply a nonlethal cellular response. To test this, samples with or without VD3 exposure were frozen to −15°C or −20°C with either a caspase 8 or 9 inhibitor. Addition of either inhibitor had minimal effect on nonfrozen and VD3-treated control samples (Figure 4A).

Addition of 10 μM caspase 8 inhibitor prior to freezing to −15°C resulted in an increase in postfreeze viability at 24 hours by 14.3% (105.7% ± 5.5% vs 92.5% ± 12.6%, Figure 4B). When caspase 8 inhibitor was applied to VD3 dose-escalated samples frozen to −15°C, postfreeze viability increased significantly (71%) compared to nontreated dose-escalated samples (72.0% ± 7.2% vs 42.0% ± 5.5%; Figure 4B). Similar outcomes were observed in samples frozen to −20°C, where

### Table 2. Quantification of the Percent Positively Labeled Cells in Immunofluorescent Micrographs for VD3-Treated Samples Following Freezing to −15°C.

|                | 2-Hour PF  | 8-Hour PF | 24-Hour PF |
|----------------|------------|-----------|------------|
|                | cl C3      | cl C8     | cl C9      | cl C3      | cl C8     | cl C9      | cl C3      | cl C8     | cl C9      |
| Ctrl 37°C      | 0.73 (0.1) | 1.04 (0.5)| 1.05 (0.2) | 0.43 (0.1) | 0.52 (0.3)| 0.56 (0.1)| 0.34 (0.2) | 0.28 (0.2)| 0.31 (0.2) |
| Ctrl −15°C     | 24.26 (2.1)| 11.5 (5.4)| 19.7 (3.2) | 26.47 (9.2)| 15.27 (5.6)| 25.34 (10.5)| 18.13 (4.9)| 2.50 (2.4)| 13.65 (5.2) |
| ESC 37°C       | 1.37 (0.3) | 1.44 (0.8) | 1.52 (0.1) | 0.82 (0.2) | 0.9 (0.6)  | 0.91 (0.3) | 0.97 (0.6) | 0.73 (0.7) | 0.86 (0.7) |
| ESC −15°C      | 41.71 (8.5)| 31.5 (17.8)| 26.96 (6.2)| 48.74 (15.8)| 41.79 (19.2)| 29.97 (18.9)| 33.72 (14.0)| 16.95 (6.0)| 11.75 (2.7) |
| Recovery 37°C  | 0.87 (0.4) | 0.8 (0.5)  | 0.76 (0.3) | 0.61 (0.2) | 0.40 (0.3) | 0.62 (0.03)| 0.51 (0.2) | 0.40 (0.2) | 0.53 (0.2) |
| Recovery −15°C | 19.54 (7.6)| 9.37 (9.9) | 17.88 (7.2)| 24.2 (10.7)| 12.26 (11.5)| 21.09 (6.9)| 13.18 (5.1)| 1.73 (2.8) | 11.0 (6.3) |

Abbreviations: cl C, cleaved caspase; VD3, vitamin D3; PF, postfreeze; ESC, dose escalated.

*Standard deviation is represented.

Figure 2. Immunofluorescence micrographs of dose-escalated VD3 samples following freezing to −15°C. Immunofluorescence was performed on methanol fixed samples using caspase 3, 8, or 9 as primary antibody followed by secondary antibody staining with an AlexaFluor 594 conjugate. Samples were counterstained with Hoechst 33342 for DNA visualization and fluorescence images were obtained at 2, 8, and 24 hours postfreeze at 10x magnification.
**Figure 4.** Assessment of the effect of caspase inhibition on postfreeze viability in VD₃-treated and/or frozen PC-3 samples. Metabolic activity of untreated or VD₃ dose-escalated PC-3 cells was obtained in response to caspase inhibition and freezing to −15°C and −20°C. A, Normothermic controls; B, −15°C; C, −20°C.

**Figure 3.** Time course graphical analysis of positively labeled caspase 3, 8, and 9 following VD₃ treatment and/or freezing to −15°C. Number of cells staining positive for each antibody were divided by the total number of Hoechst staining cells to determine the percentage of positively staining cells in each population. Three separate images per well were obtained and data from 3 separate immunofluorescence experiments were combined.
postfreeze viability increased by 73.4% in non-VD$_3$-treated samples (25.8% ± 8.5% vs 14.9% ± 4.3%, Figure 4C). Caspase inhibition in VD$_3$ dose-escalated samples frozen to −20°C also yielded a significant increase (300%) compared to noninhibited VD$_3$ dose-escalated samples (16% ± 4.3% vs 4% ± 0.8%, Figure 4C).

Analysis of the effect of caspase 9 inhibition on various samples revealed a similar yet lower impact as caspase 8 inhibition following both −15°C and −20°C exposure in both the untreated and VD$_3$ dose-escalated samples (VD$_3$ dose escalation/−15°C: 56.7% ± 8.6% vs 42% ± 5.5%; VD$_3$ dose esc/−20°C: 8.7% ± 1.4% vs 4% ± 0.8%; Figure 4 B and C).

**Discussion**

This study investigated the impact of a dose escalation regime of VD$_3$ on the cryosensitization of prostate cancer cells in vitro. This study was designed to model the bioavailability of VD$_3$ in vivo following a 1-month prescription of a moderately high oral dose. The effects of the dose escalation regime were compared to that of a single acute high-dose exposure as well as non-VD$_3$-treated samples. The impact of VD$_3$ exposure was assessed in conjunction with freezing to −15°C and −20°C. These temperatures were selected as they represent nonlethal subfreezing temperatures for prostate cancer which are associated with the periphery of the iceball. This thermal region (≥−20°C) is of interest, given that in prostate cancer cryosurgery, concerns of potential disease recurrence are associated with incomplete death if a temperature of −40°C isn’t attained at the tumor edge. As such, efforts to increase cell death within the periphery thereby increasing the critical isotherm for prostate cancer from the reported −40°C to −20°C or warmer are of great interest. This would reduce the necessity for tissue overfreezing and associated collateral damage.

Given the substantial benefit of VD$_3$ reported in earlier studies coupled with similar outcomes reported in a murine in vivo model, we elected to expand our line of investigation to determine the impact of gradual dose escalation of VD$_3$ levels, as would be associated with an oral dose prescription model, on prostate cancer cell response to freezing. Further, with the growing interest in the use of adjunctive therapies to treat aggressive fast growing (elevated Gleason and Ki-67) prostate cancer, we utilized a log-phase growth in vitro model to determine if VD$_3$ sensitization in conjunction with freezing at sublethal temperatures had a similar impact on fast growing highly metabolically active samples as were found in slow growth models.

Another interesting aspect is as prostate cancer progresses from an androgen-sensitive (early stage) to androgen-insensitive state, the ability of VD$_3$ to halt cell cycle progression is lost, thereby negating many of its antiproliferative/anticancer benefits. This shift in androgen responsiveness has also been reported to be associated with increased prostate cancer resistance to a number of treatment regimes including various chemotherapeutic agents, radiation, and even impact cancer cell tolerance to cold ablation. While there is some debate within the literature on the extent of the antiproliferative responsiveness of PC-3 cells to calcitriol, many studies report little to negligible proliferation inhibition. These studies are in agreement with our observation of minimal impact of VD$_3$ on actively dividing log phase PC-3 cells. While the overall impact of VD$_3$ on androgen-insensitive prostate cancer is altered, the ability of VD$_3$ pretreatment to increase cell sensitivity to freezing injury does not seem to be impacted. In this study, we used the active form of VD$_3$, calcitriol (1,25(OH)$_2$D$_3$) due to the lack of adequate hydroxylase activity in vitro. We chose the active metabolite to model a regime of gradually rising VD$_3$ availability and conversion, which would result from an oral dose prescription. Due to the short half-life of 1,25(OH)$_2$D$_3$, it is rarely used as a reliable measurement of VD$_3$ status in vivo. More typically, the precursor metabolite 25(OH)D$_3$ is measured as serum levels; however, studies suggest that there is not a linear conversion.

The data presented herein suggest that VD$_3$ pretreatment, whether applied as a short acute dose or in a dose escalation model, significantly improves cell death following freezing to temperatures associated with the iceball periphery. Further, the benefit of VD$_3$ pretreatment in conjunction with cryoablation was also found in an actively dividing, highly metabolically active androgen-insensitive prostate cancer model.

To this end, the combination of VD$_3$ exposure coupled with −25°C freezing resulted in near complete cell destruction 1 day following freezing with minimal culture recovery (under ideal recovery conditions). This was significantly different from −25°C freeze alone samples which demonstrated substantial repopulation following freezing. Similar outcomes were observed following VD$_3$ pretreatment combined with freezing to −20°C. Following exposure to −15°C, VD$_3$-treated samples yielded a significant decrease in viability; however, they were able to repopulate. This differed from previous reports where near complete ablation was observed following exposure to −15°C and 48-hour VD$_3$ treatment. The differences in the critical isotherm following VD$_3$ pretreatment in this study (−20°C to −25°C range) and previously reported −15°C are believed to be a result of the log phase, highly metabolic nature of the current model versus the plateau phase, low metabolic activity utilized in previous studies. Further, in this study, we utilized the androgen-insensitive PC-3 prostate cancer cell line (late stage, aggressive cancer) versus the LNCaP-HP (late-stage slow growth cancer) cell line. While different, both these studies illustrate the benefits of VD$_3$ pretreatment in combination with cryoablation which results in a shift in the minimal lethal isotherm for late-stage androgen-insensitive prostate cancer from the previously established −40°C to the −20°C (± 5°C) region, regardless of the growth fraction of the cancer.

While the benefit of the combination of VD$_3$ pretreatment and freezing was found to be significant, it was also found that once VD$_3$ was removed (within 2 weeks of recovery), this positive effect was negated thereby suggesting that active exposure to VD$_3$ immediately prior to freezing is necessary. Further, unpublished observations indicate a pretreatment interval of 24 hours or longer is required to yield the positive synergistic effect.
Previous studies from our laboratory have shown the involvement of both extrinsic and intrinsic apoptotic signaling pathways in freezing-induced cell death. Specifically, membrane-mediated cell death was most prominent in severe freeze insults ($\leq -30^\circ$C), and the mitochondrial pathway in a milder freeze event ($-15^\circ$C). Further, in vitro studies on plateau phase slow growth LNCaP-HP prostate cancer cells have suggested that VD$_3$ pretreatment results in an increase in apoptotic cell death via the mitochondrial-mediated caspase 9 pathway following exposure to $-15^\circ$C. With the observed continued benefit of VD$_3$ pretreatment in our rapidly dividing aggressive prostate cancer model, yet an observed shift in the minimum lethal isotherm for $-15^\circ$C to the $-20^\circ$C to $-25^\circ$C range, we investigated if this was due to an alteration in the apoptotic response following combination treatment. To this end, our results indicate that VD$_3$ pretreatment in the actively dividing log-phase growth prostate cancer model result in increased activity in both the mitochondrial and membrane-mediated apoptotic pathways. Inhibition studies suggested that in the highly active model, the extrinsic membrane-mediated apoptotic induction pathway (caspase 8) played a greater role in increasing cell death following the combination of VD$_3$ pretreatment and freezing. These results suggest that while the typical effect of VD$_3$ on halting cell cycle activity is negated in aggressive androgen-insensitive PC-3 cells, at the molecular level the impact of VD$_3$ pretreatment still results in the upregulation of the apoptotic cell death machinery which when combined with a sublethal freeze insult yields an increase in cell death. Further, these results clearly establish a role for membrane-mediated cell death in VD$_3$-induced cryosensitization, which previously has been seen in more severe freezing insults. The 72% improvement in viability following inhibition of caspase 8 in dose-escalated cells further confirmed the large role of the extrinsic cell death pathway.

The overall findings of this study support the conclusion that VD$_3$ pretreatment sensitizes prostate cancer cells via priming of the apoptotic pathway, thereby making the cell more susceptible to freezing injury. The data suggest that the combination of VD$_3$ pretreatment and mild freezing increases the minimal lethal temperature from $-40^\circ$C to the $-20^\circ$C to $-25^\circ$C range when applied to aggressive, rapidly dividing, highly metabolically active prostate cancer. As described, similar results have also been reported in slow growth cancer models as well as in an in vivo murine study. In the murine study, the increase in cell death as measured by % necrotic tissue was found to be 25% in the VD$_3$ freeze combination over the freeze alone condition. This increase correlates with the reported differential between the volume of tissue found within the $-20^\circ$C and $-40^\circ$C isotherms following a given freeze interval. This suggests that the lethal isotherm in the Kimura study was around $-20^\circ$C following VD$_3$ sensitization combined with freezing, which is in agreement with the findings of this study.

While the current findings support the benefit of the combination of VD$_3$ and freezing, there are several limitations. Firstly, the study was conducted in an in vitro prostate cancer cell model. The in vitro nature of the model provides ideal culture conditions for cell survival and recovery and thus the results presented herein may not directly reflect a clinical scenario. As such, future studies in vivo are necessary to elucidate the clinical potential of this approach.

Another limitation is the use of the active form of VD$_3$, calcitriol, and final concentrations utilized may be higher than levels attained in vivo via an oral dose regimen. To overcome this, an intratumoral injection (ITIJ) of calcitriol prior to cryoablation, as used in the Kimura study, may provide for a more effective means of delivering high doses of active VD$_3$ than a systemic dose escalation approach. To this end, the results of this study suggest that the duration of VD$_3$ pretreatment does not significantly affect postfreeze outcomes. Accordingly, a patient could potentially receive a dose of VD$_3$ 1 to 2 days before a cryosurgical procedure to significantly improve treatment success. However, little research exists into the distribution of calcitriol or other drugs following ITIJ, but studies in rats indicate successes in ITIJ of paclitaxel and the $\beta$-emitting radionuclide (166)Ho, and zinc-acetate in models of prostate cancer. Intratumoral injection of calcitriol has been utilized as a treatment in Japan for hyperparathyroidism, and a phase I/II clinical trial was conducted using ITIJ of inactivated sendai virus particles (Hemagglutinating virus of Japan Envelope, or HVJ-E) for prostate cancer treatment. Though promising, further studies are needed to determine the efficacy of calcitriol ITIJ in human prostate cancer treatment.

In conclusion, our findings suggest that VD$_3$ pretreatment in combination with cryoablation results in a significant increase in the level of cell death. The data suggest that both acute (1 day) and dose escalation–based exposure of actively dividing androgen insensitive prostate cancer cells to elevated VD$_3$ levels provided benefit. The data suggest that the combination results in a shift of the minimum lethal isotherm for aggressive prostate cancer from $-40^\circ$C to the $-20^\circ$C to $-25^\circ$C range. Extrapolating these in vitro findings to an in vivo scenario, the data suggest the strategy of VD$_3$ pretreatment may increase the lethality of the iceball. If correct, this could be critical as in a typical cryosurgical procedure, the volume of iceball between the $-20^\circ$C and $-40^\circ$C isotherms represents $\sim 20\%$ of the frozen mass. Importantly, an increase in the lethal isotherm to $-20^\circ$C would represent an overall doubling of the ablation volume within the frozen mass ($12.67$ cm$^3$ vs $6.4$ cm$^3$, respectively) following a standard 10/5/10 minutes double freeze procedure using an argon-based cryosurgical device. This in turn has the potential to improve outcome while reducing comorbidities associated with overfreezing (positive freeze margins) to assure cancer destruction. In combination with previous in vitro and in vivo reports, these data suggest VD$_3$ sensitization combined with freezing may provide an improved path for the treatment of prostate cancer at any stage.

**Declaration of Conflicting Interests**

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