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Vitamin D₃ cryosensitization increases prostate cancer susceptibility to cryoablation via mitochondrial-mediated apoptosis and necrosis

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Abstract

- To investigate the effect and molecular mechanisms of action of Vitamin D₃ (VD₃) as a neo-adjuvant agent before cryosurgery in an effort to increase treatment efficacy for prostate cancer (CaP).
- To eliminate the potential for disease recurrence that exists at the periphery of the freeze lesion, where temperatures may be insufficient to destroy both androgen-sensitive (AS) and androgen-insensitive (AI) CaP.
- Human CaP cells, LNCaP, were each genetically altered to express the AS and AI phenotypes and subjected to VD₃ treatment and freezing in an *in vitro* and tissue-engineered model.
- Cell viability, caspase inhibitor and western blot studies were used to determine the basis of the different responses of AI and AS cells to VD₃ cryosensitization.
- VD₃ was found to be a highly effective cryosensitizer, resulting in a >50% overall increase in cell death after -15°C freezing.
- Fluorescence microscopy, western blot analysis and caspase protease assays confirmed that the increased activation of apoptosis was modulated through a mitochondrial-mediated pathway.
- Caspase inhibition studies showed that apoptosis played an integral role in cell death, with VD₃ cryosensitization-induced apoptotic events responsible for > 30% of the overall cell death after -15°C freezing.
- The present study suggests that the use of VD₃ as a cryosensitizer increases cryoablation efficacy through the increased activity of apoptosis as well as through necrosis.
- The data show that through VD₃ treatment the overall level of AI CaP cell tolerance to freezing is reduced to a level similar to that of AS CaP.

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- VD₃ pre-treatment in conjunction with cryoablation may increase treatment efficacy and reduce disease recurrence for CaP patients.

Keywords

cryosurgery; apoptosis; prostate cancer; vitamin D; adjunctive; cryosensitization; cryoablation

Introduction

In 2009 \approx 250 000 new cases of prostate cancer (CaP) were diagnosed and nearly 40 000 deaths from CaP were reported in the USA [1, 2]. While the outlook for patients with early-stage CaP has markedly improved, the prognosis for advanced stage disease remains poor. Cryosurgery is now one of the many tools available for the treatment of CaP [3--8]. In addition to being highly effective, cryoablation reduces hospitalization time, postoperative morbidity, the interval before return to daily activities, and overall treatment cost compared with some conventional treatments [9--15]. In 2008, the AUA released a best practice statement recommending cryoablation of the prostate as both a primary and salvage therapy [16]. That same year, Cohen *et al.* [17] published the first 10-year study to report cryoablation efficacy equivalent to that of other therapies. Although cryoablation is highly effective, there remains a degree of concern about potential cell survival at the periphery of the frozen volume of tissue where lethal temperatures are not achieved, leading to possible disease recurrence. In addition, there is the need to avoid injury to adjacent anatomical structures which may limit the aggressiveness of freezing. Strategies to improve primary and salvage cryosurgical options are therefore necessary to improve this form of CaP treatment and prevent recurrence [11, 13].

To improve efficacy, research has focused on the freeze zone periphery and the use of agents to sensitize cells. Some studies have reported the synergistic effects of administering low-dose 5-fluorouracil, Taxotere[®], or cisplatin before cryotherapy, which results in improved cell ablation for the treatment of CaP in *in vitro* and *in vivo* models [18--26]. These studies have shown that sensitizing cell populations before freezing achieves enhanced cryoablative efficacy through the induction of apoptosis and secondary necrosis. The aim of this approach is to increase the volume of tissue destroyed by bringing the temperature that is lethal to cells closer to 0°C. Despite the improved performance, cells treated with chemotherapy-based sensitizers cause problematic patient toxicity, side effects and drug resistance, especially for hormone-refractory tumours [27].

Among the agents that have been considered to increase cell sensitivity to freezing is cholecalciferol, or vitamin D₃ (VD₃), which is thought to have a beneficial effect attributable to the induction of apoptosis, regulation of cell growth and antiangiogenesis [3, 7]. These properties have been recognized as having potential for use in breast, pancreatic and ovarian cancer, as well as in CaP therapy [21, 28--32]. Kimura *et al.* [33] recently reported on the benefit of VD₃ cryosensitization in a murine CaP model. This report, along with the range of cellular effects of VD₃, supports the potential of VD₃ to increase the efficacy of cryotherapy, thereby reducing associated morbidity and risk of recurrence.

The ability of VD₃ to inhibit growth factor signalling pathways is believed to underlie the potential of this agent as a cryosensitizer. VD₃ inhibits the mitochondrial protein Bcl-2 thereby activating the apoptotic caspase cascade [29]. The Bcl-2 family of proteins is responsible for maintaining mitochondrial membrane potential via the mitochondrial transition pore. A reduction in Bcl-2 can result in the opening of the pore, the release of cytochrome c and the activation of apoptosis. Agents that reduce Bcl-2 levels increase cell susceptibility to apoptotic induction, thereby increasing treatment efficacy [34--36]. We

therefore hypothesized that VD₃ activates mitochondrial-based apoptosis, resulting in increased cell death (apoptotic and necrotic) in response to a mild freeze insult, such as that experienced at the periphery a cryogenic lesion. As such, we investigated the use of VD₃ as a cryosensitizer to increase treatment efficacy for late [androgen-insensitive (AI)] and early stage [androgen-sensitive (AS)] CaP.

Materials and Methods

Cell culture

The human CaP cell line, LNCaP, was obtained from the American Type Culture Collection (Manassas, VA, USA). The AI LNCaP high passage (HP) cell line was derived by repeated culture (> 60 passages) of the AS LNCaP low passage (LP) cell line in low-hormone medium (RPMI-1640, supplemented with 10% charcoal stripped serum [Biomed, Foster City, CA, USA] and 1% Penicillin-Streptomycin [Life Technologies, Carlsbad, CA, USA]) as previously described [37]. Cultures were maintained at 37°C, 5% CO₂/95% air in RPMI-1640 growth medium (Caisson Laboratories, Inc., North Logan, UT, USA) supplemented with 10% FCS (Atlanta Biologicals, Lawrenceville, GA, USA) and 1% Penicillin-Streptomycin (Mediatech, Manassas, VA, USA). Cultures were grown in Falcon 75 cm² T-flasks with medium exchange every 3 days. Subcultures were prepared in Costar 96-well, strip plates at 18 000 cells/well, and experiments were performed 2 days after subculture.

For tissue-engineered prostate cell (pTEM) studies, rat tail type I collagen solution (BD Bioscience, Bedford, MA, USA) was used to form gel matrices. Cells, 2.5×10^6 cells/mL, were suspended in the collagen solution before gel solidification in 35-mm Petri dishes. Matrices were cultured 24 h before freezing, and media were replenished each day.

Freezing protocol

Strip wells were placed into an aluminum block in a cooling bath pre-set at -15°C. The sample temperature was monitored at regular intervals using a type-T thermocouple. Ice nucleation was initiated by contact with a cold metallic probe when the sample temperature reached -2°C ($\pm 1^\circ\text{C}$). After nucleation, samples were held for 12 min (15 min total) to allow for thermal equilibration. Samples were thawed at room temperature and then returned to normothermic culture. Where indicated, cells were treated as follows: (1) frozen to -15°C alone; (2) exposed to 50nM VD₃ (1,25-dihydroxycholecalciferol; Calbiochem, San Diego, CA, USA) for 2 days before freezing; or (3) exposed to caspase 3,8 or 9 inhibitors (Calbiochem) immediately before freezing.

The pTEMs were frozen using a cryosurgical system (Galil) with a 1.6mm needle cryoprobe. Briefly, a cryoprobe was placed into the centre of the model and a single or double freeze cycle was initiated, each cycle consisting of a 10-min freeze followed by 20 min of thawing at 37°C. The temperature profiles were recorded with an array of type T thermocouples extending radially from the probe tip (Omega TempScan 1100, Omega, Stamford, CT, USA). Once thawed, samples were returned to culture for further assessment.

Cell viability

Cell viability was assessed using the alamarBlue™ assay (Invitrogen, Carlsbad, CA, USA) in HBSS (1:20 dilution) every other day following the freezing insult. Samples were exposed to alamarBlue™ for 1 h at 37°C and then analysed using a Tecan SPECTRAFluorPlus plate reader (TECAN GmbH, Grödig Austria) with an excitation of 530nm and emission of 590nm. Subsequently, cell culture media were replenished and returned to normal culture.

Fluorescence imaging

To determine the mode of cell death, samples were frozen then assessed via triple labelling using the fluorescent probes (Molecular Probes, Invitrogen) Hoechst (blue fluorescence, 0.06 $\mu\text{g}/\mu\text{l}$), propidium iodide (red fluorescence, 0.007 $\mu\text{g}/\mu\text{l}$), and YO-PRO-1 (green fluorescence, 0.8 μM) to detect living, necrotic (freeze-ruptured), and apoptotic cells, respectively. Probes were added to each sample and incubated in the dark for 20 min. Samples were then visualized via fluorescence microscopy using a Zeiss Axiovert 200M microscope (Carl Zeiss,) at 240 \times magnification and quantified via automated cell counts using the Axiovision software (Carl Zeiss).

Western blot

Samples were cultured in Petri dishes and frozen. Cell lysates were extracted on ice at 1, 3, 6, 12, and 24 h after thawing using ice-cold radio-immunoprecipitation assay cell lysis buffer with phosphatase inhibitor (sodium fluoride 1mM, sodium orthovanadate 1mM, sodium pyrophosphate 1mM), leupeptin (1 $\mu\text{g}/\text{ml}$), PMSF (1mM), and 1 \times Halt Protease Cocktail Inhibitor (Pierce, Rockford, IL, USA). Samples were homogenized by vortex mixing and centrifuged at 16 000 $\times g$ for 15 min at 4 $^{\circ}\text{C}$. Protein concentrations were determined using the BCA (Pierce) and quantified using a Tecan spectrophotometer (TECAN GmbH). Equal amounts of protein (25 μg) for each sample were separated on a 10% SDS-PAGE gel (Bio-Rad, USA). Proteins were transferred to PVDF membranes (Bio-Rad), blocked with 3% BSA 0.05% Tween-20 solution, and incubated at 4 $^{\circ}\text{C}$ overnight in the presence of 1 $\mu\text{g}/\text{ml}$ of each antibody (β -tubulin [BD Pharmingen,], pro-caspase-3 [Cell Signaling (CST)], pro-caspase-8 [CST], or pro-caspase-9 [CST]). Membranes were then washed three times with 0.05% Tween-20 in PBS and exposed with HRP-conjugated secondary antibodies.

Membranes were visualized using a Fujifilm LAS-3000 luminescent image analyser. All protein levels were compared with tubulin (loading control) and quantitative assessment was conducted via densometric analysis using the Fuji software.

Caspase activity assays

Cell samples for caspase activity assays were frozen at -15 $^{\circ}\text{C}$ for 15 min. Cell lysates were collected on ice at 1, 3, 6, 12, and 24 h after thawing using ice-cold radio-immunoprecipitation assay cell lysis buffer without protease or phosphatase inhibitors. Protein concentrations were quantified and 50 μg of protein were assessed for caspase activity using the BD ApoAlertTM Caspase Fluorescent Assay Kits for Caspase-3, -8, and -9 (BD Pharmingen). Sample fluorescence (caspase activity) was quantified using the Tecan spectrophotometer and converted to fold-change in activity based on non-frozen controls.

Data analysis

Fluorescence units were converted to percent survival based on non-frozen controls (37 $^{\circ}\text{C}$). Viability experiments were repeated a minimum of three times with an intra-experiment repeat of 8. Western Blot, fluorescence imaging and protease activity assays were conducted on a minimum of three separate experiments. Standard error calculations were performed and statistical significance was determined by single-factor ANOVA.

Results

VD₃ pretreatment increases cell sensitivity to freezing

Cryochemo approaches have been shown to increase cell death, but at higher temperatures (such as -15 $^{\circ}\text{C}$) there is substantial cell survival. The use of VD₃ as a neo-adjunctive agent

was investigated to determine whether improved CaP ablation could be achieved. Accordingly, AS and AI samples (LNCaP LP and LNCaP HP) were treated with VD₃ for 2 days before freezing (Fig. 1). In AS samples, a combination of VD₃ cryosensitization and freezing resulted in increased cell death compared with freezing alone. With the combination treatment at 1-day after freezing, AS samples showed an increase in cell death compared with freezing alone and total cell loss by 3 days after freezing to -15°C. In the AI sample, the combination of VD₃ cryosensitization and freezing to -15°C had better efficacy compared with either treatment alone, with a 30% overall reduction in cell viability after freezing that was 2.2 times greater than freezing alone ($P < 0.01$; Fig. 1). Interestingly, the cell viability in the AI sample continued to decline during recovery after the combination treatment and consequently the cells were unable to re-populate. These data suggest that cryosensitization using VD₃ in combination with cryotherapy achieved complete ablation for both AS and AI cells after freezing to -15°C.

VD₃ treatment increased freeze-induced apoptosis and necrosis levels

The enhanced cell death resulting from VD₃ cryosensitization for both AS and AI CaP cells prompted our investigation into the mechanism of cell death. Total levels of apoptotic and necrotic cell death were evaluated in the various conditions using fluorescence microscopy (Fig. 2). AS samples (Fig. 2A) showed little apoptosis and necrosis in controls (unfrozen), but after freezing at -15°C, apoptosis was found to increase, peaking after 3 h and remaining high up to 24 h after freezing. VD₃-treated AS samples had higher levels of apoptosis and necrosis in controls (unfrozen) compared with untreated controls. The combination of VD₃ cryosensitization and freezing yielded a slight increase in apoptosis and necrosis compared with freezing alone in AS samples at all time points evaluated (Fig. 2B). Quantitative analysis of the 3-h time point showed the greatest difference between freezing and freezing with VD₃. After freezing alone, AS samples showed 22.1 (± 1.2) % viability, 58.2 (± 0.8) % necrosis, and 19.7 (± 1.5) % apoptosis, and after the combination of VD₃ and freezing the AS samples showed 14.2 (± 1.1) % viability, 60.2 (± 0.7) % necrosis, and 25.6 (± 0.9) % apoptosis. Compared with AS samples, the AI (Fig. 2C) samples subjected to freezing alone showed less overall apoptosis and necrosis. After freezing at -15°C, apoptosis peaked after 6 h and rapidly declined by 24 h. VD₃-treated AI samples had higher levels of apoptosis and necrosis vs freezing alone and vs AS samples subjected to the combination treatment ($P < 0.01$; Fig. 2D). Post-freezing analysis showed higher levels of apoptosis and necrosis in the combination-treated AI samples, with peak levels occurring at 3 h after freezing. This represented an acceleration in cell death progression compared with freezing alone samples. Quantitative analysis of freezing alone AI samples at 3 h after freezing showed 63.7 (± 1.4) % viability, 23.4 (± 0.6) % necrosis and 12.9 (± 1.1) % apoptosis, whereas samples treated with VD₃ showed 27.3 (± 0.6) % viability, 50.6 (± 1.2) % necrosis, and 22.1 (± 1.4) % apoptosis (Fig. 2C vs 2D). These data indicated that VD₃ treatment before freezing significantly increased both necrotic and apoptotic cell death, thereby reducing overall cell survival by more than half (27.3% vs 63.7%, respectively, [$P < 0.01$]). Interestingly, the AI samples showed a greater increase in apoptosis and necrosis (and thus cryosensitization) than the AS samples.

VD₃ increases mitochondrial-mediated apoptosis

In the present study, we investigated the involvement of apoptosis in CaP cellular response to VD₃ cryosensitization and freezing. Western blot and protease activity analyses were performed to assess the alterations and activations in the apoptotic proteins caspase-3, -8 and -9 after freezing and VD₃ cryosensitization (Figs 3 and 4). Analysis after freezing showed that the AS samples maintained higher levels of pro-caspase-3 and -8, compared with the AI samples (Figs 3A and B). This suggested a greater overall potential for apoptotic involvement in AS systems. AS samples had substantial decreases in pro-caspase-3, -8, and

-9 levels by 3 h after freezing, indicating protein cleavage to an active form (Fig. 3A). Interestingly, compared with AS samples, AI samples had lower pro-caspase levels, and revealed little cleavage after freezing (Figs 3A vs 3B).

Compared with the freezing alone treatment, both AS and AI VD₃-treated and frozen samples showed significantly lower levels of caspase proteins (Figs 3C and D). AS samples treated with VD₃ and frozen had lower levels of pro-caspase-8 compared with AS samples frozen alone (Fig. 3C vs A). Similarly, AI VD₃-treated and frozen samples showed lower levels of pro-caspase-8 compared with AI samples frozen alone (Fig. 3D). Analysis after VD₃ cryosensitization and freezing showed that caspase-9 levels were significantly different from frozen alone samples. There were similar levels of pro-caspase-9 in VD₃-treated and untreated (unfrozen) controls (Fig. 3D), however, after the combination of VD₃ and freezing a significant decrease in pro-caspase-9 expression levels in both AS and AI samples was noted (Figs 3C and D vs 3A and B).

We further analysed alterations in pro-caspase levels to determine the extent of their proteolytic activity and involvement in the freezing responses after VD₃ cryosensitization in AS and AI samples (Fig. 4). Analysis of caspase 3 activity showed that in frozen-only AS samples there was an overall increase in protease activity compared with AI samples, which peaked at 3 to 6 h after freezing, suggesting that freezing rapidly induced apoptotic death cascades (Fig. 4A). Conversely, frozen-only AI samples showed lower overall levels of caspase-3 activity, peaking at 6 h, indicating delayed activation. Freezing at -15°C resulted in peak caspase-3 activity in AS samples 2.8 times greater than in AI samples (3.25 vs. 1.2, respectively). In AI samples, the combination of VD₃-treatment and freezing resulted in a significant increase ($P < 0.01$) in caspase-3 activity compared with freezing alone (1.2 vs. 2.75, respectively; Fig. 4A). In sharp contrast to freezing-alone, there was an increase in caspase-3 activity in VD₃-treated AI samples throughout the 24-h interval after freezing (twofold increase, $P < 0.001$). In VD₃-treated samples, the peak caspase-3 activity in AS and AI samples was similar, indicating that VD₃ treatment induced similar post-freezing responses. These data suggest that VD₃ treatment before freezing increased the caspase-3 activity level and duration in AI cells, resulting in the greater level of cell death observed after freezing.

Assessment of caspase-8 activity in VD₃-treated and frozen samples showed activity levels similar to freezing alone (Fig. 4B). AS samples showed greater caspase-8 activity after both freezing alone and VD₃ treatment than did AI samples (7.1 fold increase, $P < 0.01$). Overall caspase-8 activity after VD₃ cryosensitization did not significantly differ in either the AS or AI samples compared with freezing alone ($P \neq NA$ for both treatments). These data suggested that VD₃ cryosensitization had little effect on caspase-8 activity.

Analysis of caspase-9 activity showed significant differences between frozen alone and VD₃- treated samples (Fig. 4C). AS samples treated with VD₃ had increased levels of caspase-9 activity, peaking at 3 h after freezing. Although overall caspase-9 activity was lower in the AI samples, the level of activity was markedly higher in VD₃-treated samples than in the AI frozen-alone samples (22 vs. 10, respectively; $P < 0.01$). Interestingly, AS samples treated with VD₃ and frozen were found to have only a 1.7 fold difference (increase) in caspase-9 activity compared with the VD₃-treated and frozen AI samples (31 vs. 22, respectively). The higher caspase-9 activity for the AI samples indicated that VD₃ treatment before freezing was able to initiate a greater mitochondrial-mediated apoptotic response in the AI samples than freezing only.

Caspase inhibition corroborates a mitochondrial-mediated apoptotic mechanism

After increased caspase activity as a result of VD₃ cryosensitization had been identified, studies were conducted to assess the overall influence of caspase activation in the observed cell death. Accordingly, AS (Fig. 5A) and AI (Fig. 5B) samples were treated with VD₃ and caspase inhibitors (caspase-8, caspase-9, or pan-caspase) before freezing. It was hypothesized that the modulation of caspase activity during cryosensitization would negate the beneficial effects of VD₃ pretreatment.

The AS samples showed greater cell viability after freezing for all caspase inhibitors tested (Fig. 5A). Compared with freezing alone, viability after freezing was increased by 14.4, 24.4, and 45.1% using caspase-8, -9, or pan-caspase inhibitors, respectively ($P < 0.01$ for each vs freezing alone). These data showed that caspase activity played a substantial role in cell death associated with freezing to -15°C in the AS samples. Cryosensitization with VD₃ reduced viability after freezing in the AS samples by 2.6 times compared with freezing alone ($P < 0.01$). Caspase-8 inhibition with VD₃ had little effect on viability after freezing, compared with VD₃-treatment combined with freezing. The addition of caspase-9 and pan-caspase inhibitors, however, increased cell viability after freezing in the AS samples by 1.7- and 2.3 fold, respectively ($P < 0.01$ for each compared with freezing alone).

Results for caspase inhibition in AI samples were similar to those in the AS samples (Fig. 5B). The inclusion of a pan-caspase inhibitor in frozen-alone samples resulted in a 19.5% increase in cell viability ($P < 0.01$). Interestingly, caspase-8 and -9 inhibition in AI samples had no protective effect after freezing. As previously described, VD₃-treatment combined with freezing of AI samples resulted in a 2.2 fold reduction in cell viability compared with freezing alone (21% vs. 48%, respectively). Caspase-8 inhibition with VD₃-treatment combined with freezing resulted in no change in cell viability in the AI samples after freezing compared with non-inhibitor VD₃-treatment combined with freezing. Compared with VD₃-treatment combined with freezing, however, caspase-9 and pan-caspase inhibition significantly increased cell viability by 2.1 times (46 vs. 21%) and 2.4 times (52 vs. 21%), respectively ($P < 0.01$). The results from the caspase inhibition studies confirmed the involvement of the apoptotic cascade after freezing as well as the ability of VD₃ cryosensitization to increase the level of mitochondrial-based apoptosis and, thereby, the overall level of cell death.

VD₃ cryosensitization enhances cell death in a tissue-engineered model

To further assess the impact of VD₃ cryosensitization, an *in vivo*-like tissue-engineered prostate model (pTEM)[38] was used. The pTEM, containing AS (Fig. 6A) and AI CaP cells (Fig. 6B), was frozen using a centrally placed cryoprobe and the zones of live (green) vs dead (red) cells were assessed 24 h after thawing. As in the *in vitro* studies, AI pTEM showed increased resistance to freezing alone, with extensive cell survival observed out to the -30°C and -40°C isotherm (Fig. 6B). In contrast, AS samples showed survival only to the 20°C isotherm (Fig. 6A). VD₃ cryosensitization and freezing achieved two effects. First, the treatment reduced the density of surviving cells in both AS and AI samples. Second, the cell survival margin was reduced by half. VD₃ cryosensitization and freezing of AI samples decreased the critical isotherm to about -15°C (Fig. 6B). These data showed that VD₃ cryosensitization was able to elevate the lethal (critical) isotherm in both the AS and AI cell populations after a single freeze event.

Studies evaluating the effect of the combination after a double freeze event, which is often used clinically, showed an even further increase in cell destruction through VD₃ cryosensitization (Figs 6C and D). Overall, the double freeze cycle (untreated and VD₃-treated) showed increased cell death compared with the single freeze cycle (Figs 6C & D vs.

A & B). Similar to a single freeze, VD₃ with a double freeze cycle reduced cell survival by 50% in both the AS and AI samples. Double-freeze-alone AI samples showed cell survival up to between -20°C and -25°C (Fig. 6D). The -20 to -25°C critical isotherm was increased from that of -35 to -40°C after a single freeze (Figs 6B vs D). The incorporation of VD₃ cryosensitization and a double freeze cycle resulted in a further increase in the critical lethal isotherm to the -10°C region. The data from the pTEM studies for single and double freeze cycles correlated well with the *in vitro* data, suggesting the possible use of VD₃ as a highly effective cryosensitization agent, when used in combination with freezing.

Discussion

Cryosurgery is an effective treatment option for both early and advanced CaP [11, 39]. Despite successes in disease treatment, cryosurgery still results in low, yet noteworthy, recurrence rates, for advanced stage disease [11, 13]. Furthermore, the progression of CaP to an AI, treatment-resistant form [28] remains a therapeutic challenge. The identification of VD₃ as a cryosensitizer with antineoplastic and antimetastatic properties that 'cross-talk' with androgen signalling makes it a promising candidate for the treatment of both AS and AI CaP. Interest in VD₃ is attributable to observations that men have higher rates of CaP occurrence when they have low levels of VD₃ [40-43]. These observations led to the concept that the administration of this agent might prevent or treat CaP. In this study, we investigated the effects of VD₃ cryosensitization on cell death after cryoablation. The data showed that neo-adjunctive use of VD₃ in cryotherapy was able to enhance delayed onset cell death (apoptotic and necrotic) in both AS and AI CaP models.

The increased ablative capacity of VD₃ cryosensitization prompted the investigation into the cell death pathways activated, resulting in enhanced CaP destruction. Research has shown that VD₃ can regulate apoptotic processes [29, 44], but its role in the freeze response of AS and AI CaP is unknown. Fluorescence microscopy revealed that levels of apoptosis and necrosis were significantly higher for AI cells treated with VD₃ before freezing, while AS cells showed minimal changes (Fig. 2). Western blot analysis showed that pro-caspase levels were reduced after freezing with VD₃ treatment compared with freezing alone (Fig. 3). Caspase activity analysis showed increases in proteolytic caspase activity after thawing (Fig. 4). Caspase-3 analysis revealed that VD₃-treated AS cells had a lower relative increase in activity than AI cells. Furthermore, AI samples showed increased caspase-3 activation during the 24-h period after freezing, which contrasted with that of frozen-alone samples, which decreased during the 24-h period. This increased activity correlated with viability studies showing that AI cells had significantly greater reductions in viability after freezing compared with AS cells after VD₃ treatment. Importantly, VD₃-treated and frozen AI samples showed caspase-3 activity similar to that in AS samples, indicating that VD₃ treatment achieved a similar total level of apoptosis in AS and AI samples. VD₃ had little effect on caspase-8 activity for AS and AI samples, suggesting little involvement of the extrinsic apoptotic pathway. The increases in caspase-9 activity for both AS and AI samples indicated that VD₃ cryosensitization is mediated through mitochondrial cell death pathways. Final corroboration of this VD₃ mitochondrial-mediated apoptotic mechanism was provided in caspase inhibition studies. These data showed that caspase-9 inhibition, but not caspase-8, was able to reverse the effects of VD₃ cryosensitization (Fig. 6). These results further confirmed that VD₃ cryosensitization activated a mitochondrial-mediated apoptotic response vs an extrinsic pathway.

Several reports have detailed the advantages of using neo-adjunctive, low-dose chemotherapeutic agents to weaken prostate tumours before cryosurgery. These studies have shown a beneficial effect of this sensitization strategy greater than that of either treatment alone [18-20, 45]. This study supports the hypothesis that VD₃ has potential for use as an

effective neo-adjunctive agent before cryoablation. The data also suggest that VD₃ may increase treatment efficacy for AI CaP compared with traditional agents. Importantly, the mechanism of VD₃ cryosensitization of CaP (AS and AI) is shown to be linked to increased mitochondrial-mediated apoptosis and secondary necrosis. These data provide the direction for further investigation into VD₃ cryosensitization therapy to increase the efficacy of CaP treatment.

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Abbreviations

| | |
|-----------------------|----------------------------------|
| CaP | prostate cancer |
| AS | androgen-sensitive |
| AI | androgen-insensitive |
| VD₃ | vitamin D ₃ |
| LNCaP | human prostate cancer cell line |
| HP | high passage |
| LP | low passage |
| pTEM | tissue-engineered prostate cells |

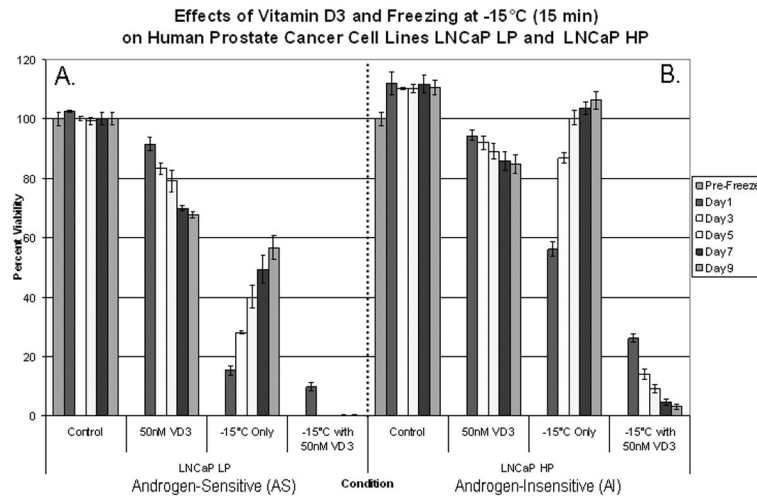


Fig. 1. AS samples (A) treated with VD₃ showed continuous cell death over the 9-day assessment period, while VD₃ and freezing combined treatments achieved total cell ablation with lack of re-growth. Similarly, VD₃-treated AI samples (B) showed continuous cell death over the 9-day assessment period. The combination of VD₃ and freezing treatment achieved total cell ablation ($P < 0.05$) with lack of re-growth for AI samples.

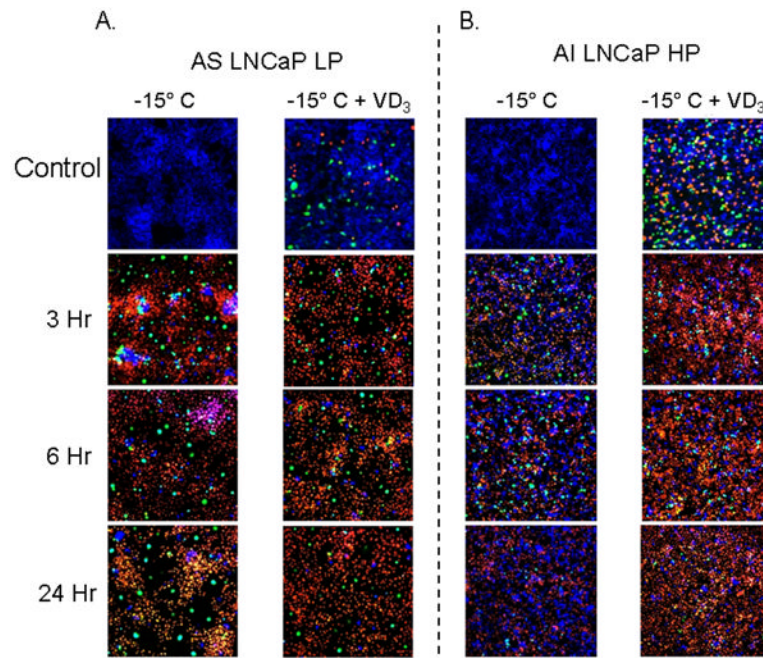


Fig. 2. Levels of necrotic and apoptotic cell death were evaluated in AS (A) and AI (B) cells after freezing or VD₃ cryosensitization. Samples were frozen at -15°C and triple-probe fluorescent micrographs were taken after 3h, 6h, and 24h using Hoechst (blue-viable), propidium iodide (red- necrotic), and YO-PRO-1 (green-apoptotic). Compared with freezing alone, AS samples (LNCaP LP) treated with VD₃ showed only slight increases in necrotic and apoptotic cells. VD₃ treated AI samples exhibited significant increases ($P<0.05$) in necrotic and apoptotic cell death at the 3h and 6h time points, indicating that AI cell lines experienced greater activation of apoptotic cascades resulting from VD₃ treatment before freezing.

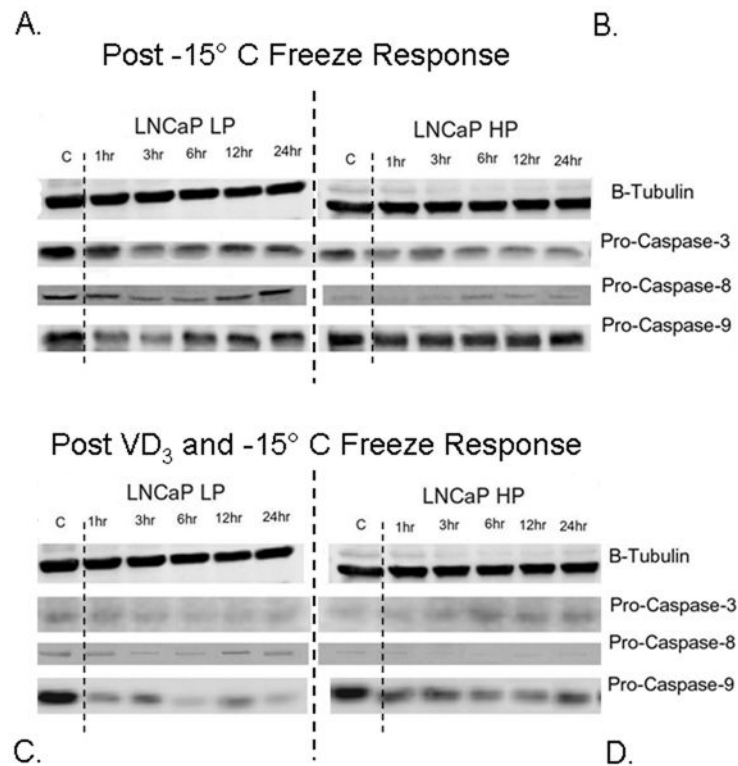


Fig. 3.

Levels of pro-caspase-3, pro-caspase-8, and pro-caspase-9 levels were assessed via western blots after freezing alone (A and B) or VD₃ and freeze treatment combination (C and D) in AS and AI samples for a 24-h period after freezing. The AS cells (A) exhibited greater control levels of each caspase than AI samples (B). Compared to freezing alone, levels of pro-caspase-3 and pro-caspase-8 after the combination of VD₃ and freezing were found to be reduced in all cell lines evaluated, indicating increased caspase activation (C and D). AI samples exhibited decreases in pro-caspase expression (especially pro-caspase-9) which was similar to AS samples (D and A vs B) indicating that VD₃ was able to induce similar apoptotic effects in response to freezing in all samples tested.

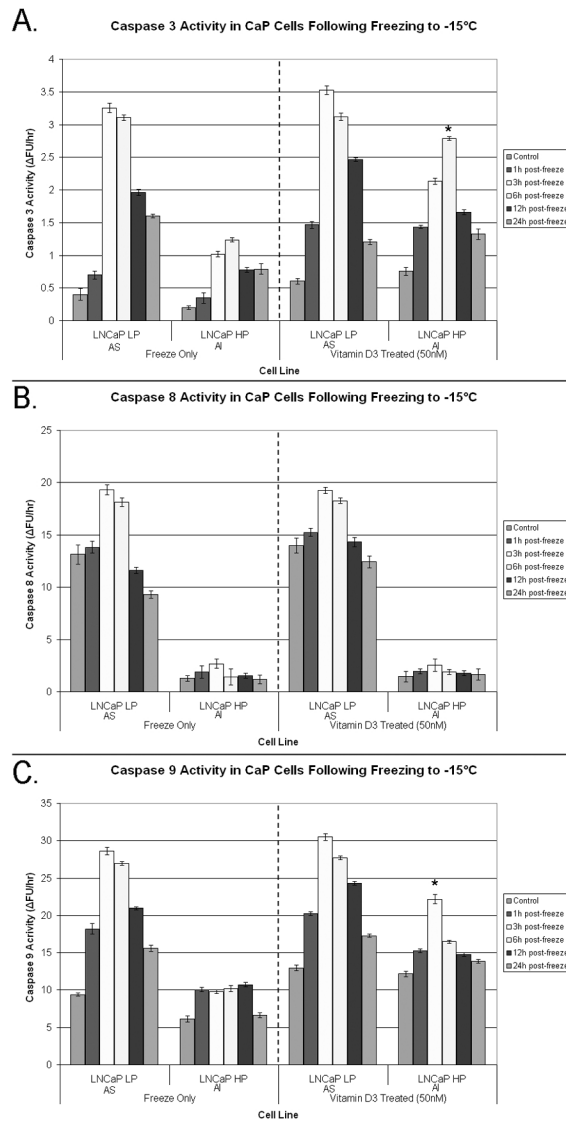


Fig. 4. Caspase-3 (A), caspase-8 (B), and caspase-9 (C) activity was determined for AS and AI samples treated with VD₃ before freezing. Samples were frozen and cell lysates were collected 24 h after freezing. Compared with freezing alone, VD₃-treated AS exhibited slightly higher ($P < 0.05$) levels of caspase-3 (A) and caspase-9 (C) activity that peaked 3 h after freezing. Caspase-8 (B) activity was not significantly different from freeze alone. Interestingly, VD₃ treated AI samples exhibited significantly ($P < 0.05$) greater levels of caspase-3 and caspase-9 activity (compared with frozen alone samples) that peaked after 3--6 h, while caspase-8 activity was not statistically different from that in frozen alone samples. The data indicated that VD₃ treatment induced greater cell death responses in AI cells that primarily resulted in increased caspase-9 (and thus caspase-3) activity.

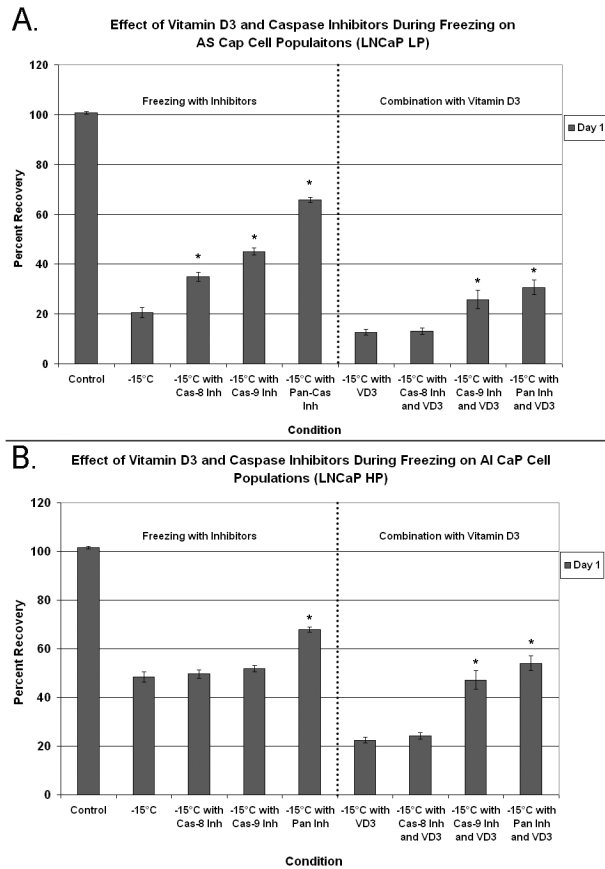


Fig. 5. The effect of apoptosis in VD_3 cryosensitization assessed using caspase inhibition in combination with VD_3 and freezing in AS (A) and AI (B) samples.

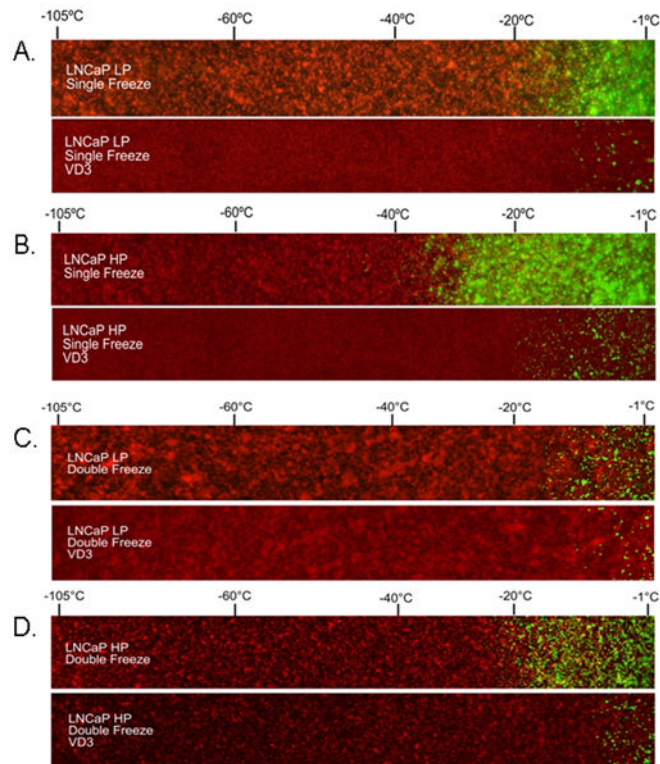


Fig. 6.

Tissue-engineered matrices containing untreated or VD₃-treated AS and AI HP cells were frozen using a single freezing cycle (A and B) or a double freezing cycle (C and D). Matrices were frozen for a single or double freeze followed by return to 37°C. Twenty-four hours after thawing, matrices were probed with calcein AM (green, live cells) and propidium iodide (red, dead cells). A 50× panoramic series of fluorescent micrographs was taken extending from the centre near the cryoprobe tip (left of images) to the periphery of the ice sphere (right of image).