The Vitamin C:Vitamin K3 System – Enhancers and Inhibitors of the Anticancer Effect

Davis W. Lamson, MS, ND; Yu-Huan Gu, PhD; Steven M. Plaza, ND, LAc; Matthew S. Brignall, ND; Cathy A. Brinton, ND; Angela E. Sadlon, ND

Abstract

The oxidizing anticancer system of vitamin C and vitamin K_3 (VC:VK_3, producing hydrogen peroxide via superoxide) was combined individually with melatonin, curcumin, quercetin, or cholecalciferol (VD_3) to determine interactions. Substrates were LNCaP and PC-3 prostate cancer cell lines. Three of the tested antioxidants displayed differences in cell line cytotoxicity. Melatonin combined with VC:VK_3 quenched the oxidizing effect, while VC:VK_3 applied 24 hours after melatonin showed no quenching. With increasing curcumin concentrations, an apparent combined effect of VC:VK_3 and curcumin occurred in LNCaP cells, but not PC-3 cells. Quercetin alone was cytotoxic on both cell lines, but demonstrated an additional 50-percent cytotoxicity on PC-3 cells when combined with VC:VK_3. VD_3 was effective against both cell lines, with more effect on PC-3. This effect was negated on LNCaP cells with the addition of VC:VK_3. In conclusion, a natural antioxidant can enhance or decrease the cytotoxicity of an oxidizing anticancer system *in vitro,* but generalizations about antioxidants cannot be made. *(Altern Med Rev* 2010;15(4):345-351)

Davis W. Lamson, MS, ND – Adjunct faculty in oncology, Bastyr University, Kenmore, WA; Private practice, Tahoma Clinic, Renton, WA; Product consultant, Thorne Research; Contributing editor, *Alternative Medicine Review.*
Email: davisl@seanet.com

Yu-Huan Gu, PhD – Research department, Bastyr University, Kenmore, WA.

Steven M. Plaza, ND, LAc – Adjunct faculty in oncology, Bastyr University, Kenmore, WA. Private practice, Northwest Center for Natural Medicine, Olympia WA; Product consultant, Thorne Research.

Matthew S. Brignall, ND – Adjunct clinical faculty, Bastyr University, Kenmore, WA; Science Director for SaluGenecists, Inc.

Cathy A. Brinton, ND – Adjunct faculty in anatomy, Bastyr University, Kenmore, WA. Private practice, Seattle, WA.

Angela E. Sadlon, ND – Holder of the Thorne Post-Doctoral Fellowship 2009-2010; Research associate to Dr. Davis Lamson

Introduction

While there are many reports on the anticancer effects of various natural agents, there is little information available on results of combining these agents. A number of such materials are classified as antioxidants and there has been a longstanding concern whether these compounds would decrease the effectiveness of standard chemotherapies, especially those regarded as oxidative. Many publications have been devoted to both sides of this topic. Research has demonstrated the anticancer activity of vitamin C (VC) for some decades. The necessary concentration of VC for a cytotoxic effect on malignant cells in humans requires intravenous administration.[1] The mechanism involves generation of hydrogen peroxide (H_2O_2) from ascorbic acid. The researchers present arguments for the requirement of extracellular generation of H_2O_2[1] and mentioned the high membrane permeability of H_2O_2.[2,3] Other researchers studying the combined effect of the VC and vitamin K_3 (VC:VK_3) system concentrate on the effect of intracellular H_2O_2 (via superoxide).[4] Figure 1 illustrates this interaction. A number of human cancers demonstrate low levels of intracellular antioxidant enzymes (catalase, glutathione peroxidase) and smaller antioxidant molecules (glutathione, vitamin E, vitamin C, and vitamin A). Such cancer cells are unable to detoxify large quantities of H_2O_2, whereas non-transformed (normal) cells can compensate.[5-7]

The VC:VK_3 combination generates H_2O_2 efficiently by redox cycling,[8,9] such that a high level of VC by the intravenous route may not be necessary for cancer cell death. Since the VC:VK_3 combination increases the cytotoxicity by six- to seven-fold over individual vitamin use, the oral route might suffice.[9] Research on this concept proceeded through the usual route from *in vitro*, to *in vivo*, to human trial. The VC:VK_3 system has performed positively *in vitro* for prostate cancer,[10] breast cancer,[11] ovarian cancer,[12] bladder cancer,[13] hepatocarcinoma,[14] and some leukemias.[8,15] Similar anticancer results have been cited for animal studies.[4] Two human trials with oral VC:VK_3 on patients with advanced prostate cancer showed modest benefit.[16,17]

To explore the outcome of combining antioxidants with an oxidative anticancer therapy, VC:VK_3 was combined with each of the following known anticancer antioxidants: melatonin, curcumin,

Key words: prostate cancer, vitamin C, K_3, antioxidants, LNCaP, PC-3, anticancer, ascorbate, ascorbic acid, vitamin K

quercetin, and cholecalciferol (VD_3). The targets were androgen-dependent (LNCaP) and -independent (PC-3) prostate cancer cells. The experimental procedures and results are detailed below along with discussion of results.

Materials and Methods

Cell Lines

LNCaP and PC-3 prostate cancer cell lines were obtained from American Type Culture Collection. The cells were grown in RPMI 1640 medium supplemented with 10-percent fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin (10%). Cells were cultured at 37°C in a humidified atmosphere containing five-percent CO_2 and were maintained by subculturing cells twice weekly.

Test Solutions

Sodium ascorbate, VK_3, curcumin, melatonin, VD_3, and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO) and stored at appropriate temperatures as indicated by the manufacturer. A 100 mmol/L solution of sodium ascorbate was prepared with 1x phosphate buffer solution (PBS) and 100 mmol/L solution of VK_3 was made with dimethylsulfoxide (DMSO). Solutions (10 mmol/L each) of curcumin, melatonin, quercetin, and VD_3 were made in 50-percent ethanol (EtOH) (curcumin) or DMSO (melatonin, quercetin, and VD_3). All solutions were stored at 4°C, except the curcumin solution, which was stored at -20°C.

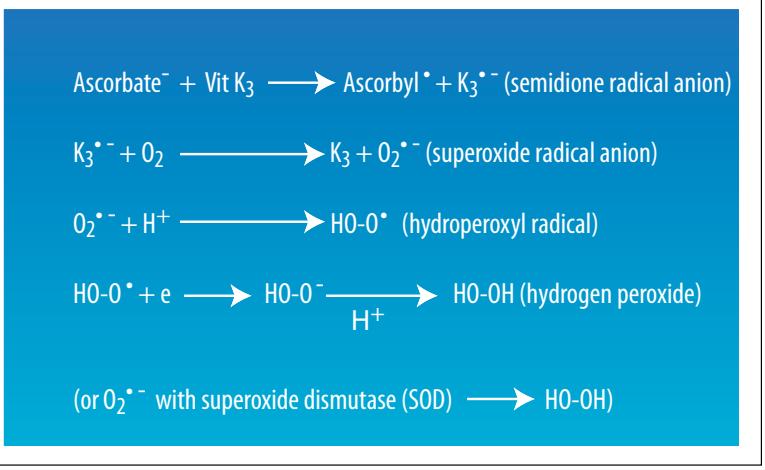
VC:VK_3 (100:1) Antitumor Activity Assay

Tumor cell cytotoxicity was evaluated following two-, three-, or five-day vitamin exposure using trypan blue exclusion assay and CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay as described previously.[18] About $1x10^6$ LNCaP or $1x10^5$ PC-3 cells were seeded onto six-well cell culture clusters with 2.5 mL of RPMI-1640 pre-warmed medium. After 24-hour incubation to allow cells to adhere, cells were treated with VC:VK_3 at a concentration of 250 µM:2.5 µM, 500 µM:5 µM, or the same volume of PBS for vehicle controls. All plates were continuously incubated at 37°C in a humidified atmosphere containing five-percent CO_2 for two, three, or five days. At the end of treatment, cells were harvested and resuspended into culture medium. Cell viability was determined using two methods: trypan blue exclusion and CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega; Madison, WI).

For trypan blue exclusion, 0.5 mL of a cell suspension ($1-5x10^5$/mL) was mixed with 0.4-percent trypan blue stain (0.1 mL, Sigma).

Two samples were counted for each suspension and three replicates were performed for each treatment. Viable cells were measured over time using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's protocol. This assay measures the bio-reduction by intracellular dehydrogenases of the tetrazolium compound MTS in the presence of the electron coupling reagent phenazine methosulfate. MTS and phenazine methosulfate were added to the cell suspensions and the mixture was incubated for three hours at 37°C. Absorbance was measured at 490 nm using a microplate reader (Bio-Tek, Inc.) and was directly proportional to the number of viable cells in the cultures. Percentage cytotoxicity was calculated from the loss of cell viability in cultures.

Figure 1. Hydrogen Peroxide Production by the Vitamin C:Vitamin K_3 System



Interaction of the VC:VK_3 System and Natural Anticancer Agents

Combination effects of VC:VK_3 and four natural substances (melatonin, curcumin, quercetin, and VD_3) on LNCaP and PC-3 cells were examined by using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay solution as previously described.[18] Prostate tumor cells were seeded onto the well of a 96-well cell culture plate (5,000~8,000 cells/100 µL/well) and the plate was incubated for 24-hours (37°C, 5% CO_2). Specific individual

solutions of melatonin, curcumin, quercetin, and VD_3 were premixed with fresh culture medium (100 µL) in different concentrations prior to introduction to the cell culture. A VC:VK_3 solution at 250 µM:2.5 µM was also added to the culture medium and the mixed medium (100 µL) containing a specific natural substance plus VC:VK_3 was added to each well, bringing the total volume of liquid to 200 µL per well (pH 7.5). The cell culture wells that only contained VC:VK_3 (250 µM:2.5 µM) or a natural substance (100 µM) were included in the 96-well plates for comparison. The cell culture wells without testing chemicals were performed as normal cell growth controls. Each combination of VC:VK_3 plus a test substance was examined for a cytotoxic effect on LNCaP and PC-3 cells in triplicate after three-day exposure. Plates were incubated at 37°C (5% CO_2) for 24 hours. Cell viability was determined with the MTS dye uptake assay described previously.

Results

VC:VK_3 on Androgen-dependent and -independent Prostate Cancer Cell Lines

The percentage of viable prostate cells after treatment of VC:VK_3 for three days was analyzed at two concentrations. Across the experiments performed, VC:VK_3 inhibited LNCaP viability by 15-35 percent (250 µM:2.5 µM) or 50 percent (500 µM:5 µM). PC-3 cells were inhibited by 21-47 percent (250 µM:2.5 µM) and 51 percent (500 µM:5 µM).

Melatonin Anticancer Effects: Alone and Combined with VC:VK_3 (Table 1)

LNCaP cell viability decreased 16 percent after a three-day exposure to melatonin (100 µM). Melatonin had no effect on PC-3 cells after three days of exposure. When melatonin (100 µM) was combined with VC:VK_3 (250 µM:2.5 µM), there was no cytotoxic effect on LNCaP or PC-3 cells. When LNCaP or PC-3 cells were exposed to melatonin (100 µM), with VC:VK_3 added 24 hours later, the results after three days were comparable to VC:VK_3 alone.

Table 1. Effect of VC:VK_3 (250 µM:2.5 µM) and Melatonin (100 µM) on LNCaP and PC-3 Cells

Cell Line	Cell Viability (%) Following Three-day Exposure of:			
	VC:VK_3	Melatonin	VC:VK_3 + Melatonin	Melatonin only; then VC:VK_3 in 24 h (VC:VK_3 was for 3 days)
LNCaP	66	84	99	62
PC-3	74	104	110	68

Curcumin Anticancer Effects: Alone and Combined with VC:VK_3 (Table 2)

LNCaP and PC-3 cells exposed to curcumin (100 µM) decreased cell viability by 55- and 58 percent, respectively. Curcumin (25, 50, and 100 µM) in combination with VC:VK_3 (250 µM:2.5 µM) after a three-day exposure produced cytotoxic effects of 32-65 percent (LNCaP) and 24-55 percent (PC-3) . The efficacy of curcumin plus VC:VK_3 increased as the curcumin dose increased.

Table 2. Effect of VC:VK_3 (250 µM:2.5 µM) and Curcumin (25, 50 and 100 µM) on LNCaP and PC-3 Cells

Cell Line	Cell Viability (%) Following Three-day Exposure of:				
	VC:VK_3	Curcumin (100 µM)	VC:VK_3 + Curcumin (25, 50, and 100 µM, respectively)		
LNCaP	65	45	68	57	34.5
PC-3	75	41.5	76	70	45.5

Quercetin Anticancer Effects: Alone and Combined with VC:VK_3 (Table 3)

Quercetin (100 µM) decreased cell viability of LNCaP cells by 75 percent and PC-3 cells by 52 percent after a three-day exposure. In combination with VC:VK_3 (250 µM:2.5 µM), quercetin greatly lowered the cell viability of LNCaP by 81 percent and PC-3 by 73 percent.

Vitamin D_3 Anticancer Effects: Alone and Combined with VC:VK_3 (Table 4)

VD_3 (100 µM) decreased cell viability of LNCaP by 58 percent and PC-3 by 99.5 percent after a three-day exposure. VC:VK_3 (250 µM:2.5 µM) plus VD_3 had only an 11-percent cytotoxic effect on LNCaP cells, but a 99-percent cytotoxic effect on PC-3 cells.

Discussion

Abundant research has been published on the anticancer properties of melatonin,[19-21] curcumin,[22,23] quercetin,[24,25] and VD_3.[26,27] There was considerable variability in the effect of VC:VK_3 on the two prostate cancer cell lines. Addition of the antioxidant compounds both increased and decreased the tumoricidal effects of VC:VK_3.

In the melatonin experiment, simultaneous application of melatonin and VC:VK_3 resulted in complete elimination of any effect of VC:VK_3 on prostate cancer cells. This seems reasonable from the evidence that melatonin quenches all the major reactive oxygen species effectively.[28] In one study evaluating the direct interaction of melatonin with H_2O_2, melatonin was incubated with H_2O_2 for various lengths of time before applying to human lymphocytes for measurement of DNA damage. The DNA damaging effect of H_2O_2 declined the longer it was left in contact with melatonin and was much less when time-dependently combined with melatonin.[29] In the present experiment, when melatonin was applied 24 hours before VC:VK_3, cell viability was similar to that produced by VC:VK_3 alone. This result is understandable in view of the short half-life of melatonin (32-40 minutes)[30] and may indicate that melatonin taken at bedtime may not affect oxidizing therapy the following day.

Curcumin at 100 µM lowered the viability of both prostate cancer cell lines below that of VC:VK_3 alone. When the two agents were used in combination, there was no effect of curcumin (25 µM) beyond that of VC:VK_3. As the concentration of curcumin increased to 50 µM, then 100 µM, in combination with VC:VK_3, the cytotoxicity incrementally increased to approximately that of 100 µM curcumin alone. Unfortunately, since curcumin was not studied alone at 25 and 50 µM, it is not possible to say whether VC:VK_3 retarded the effect of curcumin. Although the activity of curcumin at 100 µM was greater than that of VC:VK_3, the effect of increasing concentrations of curcumin in the combination was non-linear; the meaning is uncertain.

Table 3. Effect of VC:VK_3 (250 µM:2.5 µM) and Quercetin (100 µM) on LNCaP and PC-3 Cells

Cell Line	Cell Viability (%) Following Three-day Exposure of:		
	VC:VK_3	Quercetin	VC:VK_3 + Quercetin
LNCaP	85	25	19
PC-3	53	48	27

Table 4. Effect of VC:VK_3 (250 µM:2.5 µM) and Vitamin D_3 (100 µM) on LNCaP and PC-3 Cells

Cell Line	Cell Viability (%) Following Three-day Exposure of:		
	VC:VK_3	Vitamin D_3	VC:VK_3 + Vitamin D_3
LNCaP	85	42	89
PC-3	53	0.5	1

Quercetin had a greater cytotoxic effect on LNCaP cells, whereas VC:VK_3 alone had more effect on PC-3 cells, indicating different susceptibilities of the two cell lines (Table 3). In combination with VC:VK_3, quercetin appears to further increase cytotoxicity on PC-3, in an additive manner. The small effect on LNCaP using the combination may be within experimental variability. The lower cell viability of combined quercetin and VC:VK_3 over that of the agents alone indicates that there is no decrease of the cytotoxic effect of VC:VK_3 by quercetin.

Vitamin D_3 resulted in a substantial reduction in cell viability on LNCaP cells and an almost complete elimination of PC-3 cells. The effect of VD_3 on different prostate cell lines is known to differ. Since cholecalciferol-25-hydroxylase (25-OHase) and 25-hydroxyvitamin D-1-alpha-hydroxylase (1α-OHase) are present and functioning within normal prostate cells, VD_3 can metabolize to calcitriol intracellularly (Figure 2).[31,32] Further, calcifediol (25-hydroxyvitamin D [25-OHD_3]) was shown to have an antiproliferative effect similar to calcitriol in normal prostate cells.[33,34] While normal prostate cells can absorb calcitriol and 25-OHD_3,

Figure 2. Vitamin D Metabolism with Enzymes

absorption of VD_3 and 25-OHD_3 will generate calcitriol internally. Calcitriol has been shown to control or decrease cell proliferation in both normal and LNCaP cells, but with little effect in PC-3 cells.[33-35]

LNCaP cells contain the necessary 25-OHase to synthesize 25-OHD_3 from VD_3.[32,36] Studies have shown that 1α-OHase is expressed only at very low levels, making conversion to calcitriol undetectable.[31,37] In one study, concentrations of 25-OHD_3 at 10 nM had no effect on LNCaP proliferation.[34] However, a 2010 study showed that a 500 nM concentration of 25-OHD_3 (but not 100 nM) inhibited LNCaP cell growth by approximately 60 percent, even when 1α-OHase was blocked.[38] So, it appears that 25-OHD_3 can be effective on LNCaP cells with high enough concentration. In PC-3 cells, no presence of messenger RNA for 25-OHase conversion of VD_3 to 25-OHD_3 was found among the RNAs examined.[32] There is evidence of 25-OHD_3 conversion to low amounts of calcitriol[31] and of decreased amounts of the vitamin D receptor (for calcitriol and weaker for 25-OHD_3 binding)[33,39] within PC-3 cells. No reports were found for production of 25-OHD_3 or its direct effect on PC-3 cells. So, the effect of VD_3 shown here on PC-3 cells is undefined and not shown elsewhere in the literature.

In the experiment reported here, the combination of VC:VK_3 with VD_3 decreased the cytotoxic effect of VD_3 on LNCaP cells, comparable to that of VC:VK_3 alone. There was no detectable effect of VC:VK_3 on PC-3 cells in the experiment. The reason for this difference in effect on the two cell lines is unclear. Because of the extreme effect of VD_3 on PC-3 cells, it is impossible to understand the added effect of VC:VK_3. Rerunning the experiment with lower concentrations of VD_3 might further the interpretation.

A single experiment was conducted (results not shown) applying VC:VK_3 (250:2.5 μM) twice in two days to both cell lines (changing the medium daily). Viable cells were reduced 15-percent more than after a single application. Application of VC:VK_3 to the PC-3 cell line, three times in five days, resulted in 45-percent cell viability compared to the untreated control. Thus, multiple applications of VC:VK_3 appear to yield further decreased viability.

A previous review by this research group concluded that the outcomes of simultaneous treatments with oxidative cancer therapies and nutritional antioxidants are unpredictable and need to be assessed through specific experimental protocols.[40,41] The present preliminary *in vitro* experiment supports our previous conclusion.

Future studies with these combinations are needed to confirm the observations of enhancing or inhibiting a known oxidizing anticancer system. Further research on this concept with other combinations could reveal more powerful anticancer strategies.

Summary

The effect of the VC:VK_3 H_2O_2-producing system (via superoxide) on prostate cancer cell lines was examined for additional effects after inclusion of antioxidants with known anticancer capability. The VC:VK_3 combination has been previously explored with these cells. The demonstration that different antioxidants provide varying results with VC:VK_3 makes it clear that all antioxidants do not behave the same in an oxidizing anticancer system. Of note, some of the chosen antioxidants alone displayed quite different anticancer effects on the androgen-dependent versus -independent prostate cancer cell lines.

While melatonin quenches the oxidizing and cell killing effect of VC:VK_3 when applied concurrently, it did not affect cell viability when VC:VK_3 was applied 24 hours after melatonin. The lowest concentration of curcumin with VC:VK_3 provided about the same effect as VC:VK_3 alone. As the concentration of curcumin increased, it appeared that a combined effect of VC:VK_3 and curcumin

occurred on LNCaP cells; VC:VK_3 seemed to furnish no advantage over curcumin alone on the PC-3 cells. While quercetin alone was effective on both cell lines, an additional 50-percent reduction occurred in cell viability when both agents were used together on PC-3 cells. VD_3 was effective against both cell lines, but the effect was negated on LNCaP cells when combined with VC:VK_3. The VD_3/VC:VK_3 combination effect on PC-3 cells is unclear for reasons discussed above. The results demonstrate that a natural antioxidant can enhance the cell killing effect of an oxidizing anticancer system *in vitro*. The opposite was true for melatonin only when applied concurrently, but generalizations about antioxidants cannot be made.

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