



*Conference Proceedings Paper – 1st International Electronic Conference on Molecular Science:
Cell Signaling, Survival and Growth*

Vitamin C Impact on Genistein-Induced Cell Death in Prostate Cancer

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Received: 20 September 2015 / Published: 15 October 2015

Abstract: Prostate cancer is the second leading cause of cancer-related deaths in America. An estimated 220,800 new cases and 27,540 cancer-related deaths are expected in the year 2015. The purpose of this study was to investigate the impact of vitamin C on genistein-induced apoptosis in LNCaP cells and the potential pathways involved, using cell-based assays including: MTT assay to determine the effect of the various treatments (Gn, Vit C and Gn+Vit C combination) on LNCaP; Nitroblue tetrazolium assay (NBT) to assess treatment-induced intracellular ROS levels; Fluorescence microscopy to determine the mode of treatment-induced cell death. Briefly, LNCaP cells were exposed to varying concentrations of genistein (Gn₁₀₋₇₀ μ M) and vitamin C (C₁₀₋₇₀ μ M) as single treatments, and Gn-VitC combination. For Gn-VitC combination the IC₅₀ (40 μ M) of the Vit C was used with each concentration of the genistein. Vitamin C significantly augmented the effects of genistein. The combination treatment showed the most dramatic effect, causing mostly apoptosis. The chi-square test shows that the p-value < 0.01 (which was the significant level). The null hypothesis that combination treatment will not cause more cells to die by apoptosis as compared to the single treatment of genistein was rejected.

Keywords: Genistein; Vitamin C; proliferation; antioxidant; treatment-induced; dose-dependent; apoptosis; necrosis,

1. Introduction

The most commonly diagnosed type of cancer among men in 2012 was prostate cancer (PCa) accounting for 29% of all new cancer cases. PCa ranks second to lung cancer in cancer-related deaths and this accounts for 9% of all male cancer deaths in 2012. [1]. Despite the large investments in cancer researches and the wealth of knowledge and discoveries in cancer biology and genetics, prostate cancer is still at best, minimally controlled by modern medicine [2,3]. The age adjusted mortality rate for cancer is about the same in the 21st century as it was 50 years ago. Prostate cancer is the most common non-cutaneous cancer and the second cause of cancer-related deaths in men of the United State of America, with nearly 1 out of every 7 men expected to be diagnosed with prostate cancer during their lifetime. An estimated 220,800 new cases and 27,540 cancer-related deaths are expected in the year 2015 [4]. Significant achievements in cancer treatment have been accomplished due to aggressive pursuit of research in therapeutic regimens. However, although the locally confined disease is treatable, the metastasized prostate cancer still create therapeutic challenges; and consequently prognosis still remains poor. In addition to surgery, the standard chemotherapy has been the most effective treatment regiments. But such therapies have their limitations in terms of safety, tolerability, and efficacy, partly due to incomplete elimination of the metastasized cancer and the serious side-effects resulting from treatment induce toxicity [5]. Development of alternative strategic therapeutic regimen has been the on-going focus of major research endeavors. Indeed, current approach in therapeutic research is focusing on attacking cancer cells at the molecular level in pursuit of signal transduction inhibitors, apoptosis-inducing molecules, and growth inhibitors since the 1990s [6]. This means identifying specific cancer-associated biomarkers/proteins, growth promoting molecules, and signaling pathways within the cells that could be specific targets for therapeutic agents.

DHA is taken up into cells by glucose transporters [7,8]. Inside the cell, it is reduced to ascorbic acid [7,8] and decreases intracellular ROS levels, thus acting initially as an antioxidant [9–11]. Ascorbate is considered to be an important antioxidant in extracellular fluid [12]; it also guards against aqueous radicals in blood [13] and protects plasma lipids from peroxidative damage caused by peroxy radicals [14]. Conversely, ascorbate also accelerates oxidative metabolism by preventing the use of pyruvate for glycolysis [15]. This property helps to inhibit the proliferation of tumor cells, but not normal cells [16,17]. In a great number of malignant cancer cell lines, it is quite interesting that the cytotoxic effect of ascorbate is correlated with its prooxidant activity [18–22].

High-dose intravenous (IV) vitamin C has been administered by physicians for many decades as a complementary and alternative therapy for cancer patients [23] Vitamin C administered intravenously bypasses the regulated intestinal uptake mechanism and results in significantly higher plasma concentrations than are obtained through oral intake [24] When intake of vitamin C is below a critical amount (10 mg/d) for prolonged periods, failure of wounds to heal, petechial hemorrhages, follicular hyperkeratosis, bleeding gums, and related abnormalities ensue in a condition known as scurvy [25]. It is proposed that at these high-doses vitamin C acts as a pro-drug via metal ion-dependent generation of cytotoxic hydrogen peroxide [26] however, other potential anti-cancer mechanisms, such as regulation of epigenetic marks and transcription factors, are also possible [27]. The increased use of dietary supplements for prostate cancer is occurring despite data showing consumption of some supplements

actively promoted for anti-prostate cancer activity actually increased the risk of prostate cancer (Mercadante S et al., 2011).

The role of ascorbate (vitamin C) in cancer treatment is a subject with a controversial history [28,29]. The core of this controversy is the lack of reproducibility of the therapeutic effects of ascorbate on cancer patients [30], a problem compounded by uncertainties associated with deficiencies of independent pathologic confirmation and failure to include appropriate placebo groups in clinical studies [31–34]. On the basis of an initial study of the antitumor effects of ascorbate in 50 patients with advanced cancer, Cameron and colleagues concluded that high-dose ascorbate improved treatment outcome [35], showing that the long-term survival of cancer patients who received high-dose ascorbate supplements was 20 times greater than that of patient in the control group [36]. However, Moertel and Mayo concluded that there was no significant difference in survival between ascorbate-treated and -untreated groups [32,33].

Conner and colleagues reported that all antineoplastic drugs tested produced mitochondrial dysfunction, including loss of mitochondrial membrane potential and an increase in ROS levels, and showed that this phenomenon was inhibited by vitamin C. They postulated that vitamin C acts as an antioxidant to protect cells against mitochondrial dysfunction induced by antineoplastic agents, and thus antagonizes the cytotoxic effects of antineoplastic drugs [37]. In a similar vein, Blair cautioned that because vitamin C/d (200 mg) induced decomposition of lipid hydroperoxides to endogenous genotoxins, it might be counterproductive in cancer treatment [38,39].

Patients undergoing chemotherapy commonly experience fatigue, with more than 75% of patients reporting feelings of debilitating tiredness or loss of energy [40]. Fatigue has a constant presence following chemotherapy, increases incrementally with consecutive cycles of chemotherapy [41], and may persist for years after treatment completion [42]. Regarding genistein alone, one large study reported that genistein caused significant gastrointestinal side effects in almost 20% of participants [43].

Although cancer-related pain can be managed with opioids [44], no effective therapy for fatigue has yet been identified. However, more recent studies on the therapeutic effects of vitamin C have provided a clearer understanding of its effect in cancer treatment. One hypothesis was that ascorbate exerts an antitumor effect by increasing collagen synthesis [45,46] A high dose of ascorbate induced a decrease in mitochondrial membrane potential and a release of cytochrome c from mitochondria to cytosol, which acted to promote apoptosis. A low dose of ascorbate induced cell-cycle arrest of cancer cells [47,48]. Thus, the effect of ascorbate on cancer cells was mediated by an increase in intracellular ROS levels.

Levine and colleagues have also reported anticancer activities of ascorbate that were attributable to its pro-oxidant properties, showing that ascorbate acts as a pro-oxidant and decreases tumor growth in mice [49]. They also showed that ascorbate produced hydrogen peroxide-dependent cytotoxicity in various cancer cells without affecting normal cells. More importantly, Levine suggested that ascorbate-induced formation of hydrogen peroxide preferentially occurs in extracellular fluid compared with blood [50]. These studies provide a mechanistic basis for applying ascorbate as a pro-oxidant therapeutic agent for cancer treatment. In this study, the additive effect of vitamin C on genistein chemo-preventive action was assessed using MTT assay and fluorescence assay. The proportion of percentage apoptosis to percentage necrosis was determine for each treatment group. The action of ascorbate in cancer cells has also been more clearly defined by in vitro studies. In this review, we summarize these new findings and discuss the biological mechanism of action of ascorbate in cancer therapy in addition to genistein.

2. Results and Discussion

To determine the impact of Vitamin C supplement on genistein-induced apoptosis in combination treatment and singly treated, LNCaP prostate cell lines, was subjected to genistein and/or vitamin C treatment. Treated cells were analyzed with various bioassays MTT, NBT, and AO/ EB fluorescence microscopy, as described in the experimental section. The MTT assay revealed a post-treatment dose-dependent survival rate in all the treatment regimens. The number of life cells declined significantly with increasing concentration of the drugs, and with non-significant inter-treatment differences ($p > 0.01$) (Figure 2). The highest cell death occurred in the Gn-Vit C combination treatment and the lowest in cells treated with Vit C alone.

This study was designed to investigate the therapeutic potential of genistein and/or vitamin C on LNCaP cells. The result obtained clearly demonstrate that both genistein and vitamin C in single and combination treatments, induced growth arrest in LNCaP cells. This study suggest that the combination treatments are more effective than single treatments. Prostate cancer is known to be an age dependent disease that can be caused by increased oxidative stress in older men [53]. MTT assay is validated to assess the response of cancer cells including prostate cancer cells, to both radiotherapy and chemotherapeutic agents [54,55]. In all the treatment groups, the MTT assay indicated a dose-dependent response of the cells to the various treatment regimens, in conformity with results of previous studies [51–57]. The NBT assay results also revealed a dose-dependent decline in ROS production in all the three treatment regimens. Cancer cells produce high levels of ROS to survive oxidative stress conditions/environment [57–59]. However, for cellular proliferation, these cells must maintain a balanced redox signaling system [51]. An upregulation (overproduction) or down-regulation (underproduction) of ROS beyond the physiologic state may result in pathologic oxidative stress and consequent cell death/ growth inhibition [58–60].

Genistein has been demonstrated to induce apoptosis cell death in many human cancer cells, including LNCaP and PC3 prostate cancer cells [32,34,41]. The fluorescence microscopy study was done to assess the level of apoptosis cell death relative to concentration of drug in each treatment group. The results indicated that significant dose-dependent treatment-induced apoptosis occurred in LNCaP cancer cells; with significant differences ($p < 0.01$) between the treatment groups. This result is consistent with previously reported data on genistein-induced apoptosis in human cancer cells [34,41]. The combination-treatment studies demonstrate the potential therapeutic impacts of vitamin C and genistein on LNCaP prostate cancer cells. The highest percentage apoptosis induced in the cells was produced in the genistein-vitamin C combined treated cell group, followed by the 10uM concentration of genistein treated cells, and the least percentage of apoptosis was found in the 70uM genistein treatment cell group. These results are in conformity with previous studies [61]. Furthermore, adding vitamin C as in the combination treatment, significantly augmented the genistein induced apoptotic cell death.

$$\% \text{ Cell Survival Rate (CSR)} = (AT - AB) / (AC - AB) \times 100 \quad (1)$$

where AC is the absorbance of the control (mean value), AT is the absorbance of the treated cells (mean value), and AB is the absorbance of the blank (mean value). The means of the absorbance were graphed against the mean concentrations of the drugs/reagents. All the microtiter plates were micro-

photographed under inverted microscope (200×) using digital camera (Nikon: Coolpix VR & ISO 2000, Japan), for histomorphological studies.

2.1. MTT assay

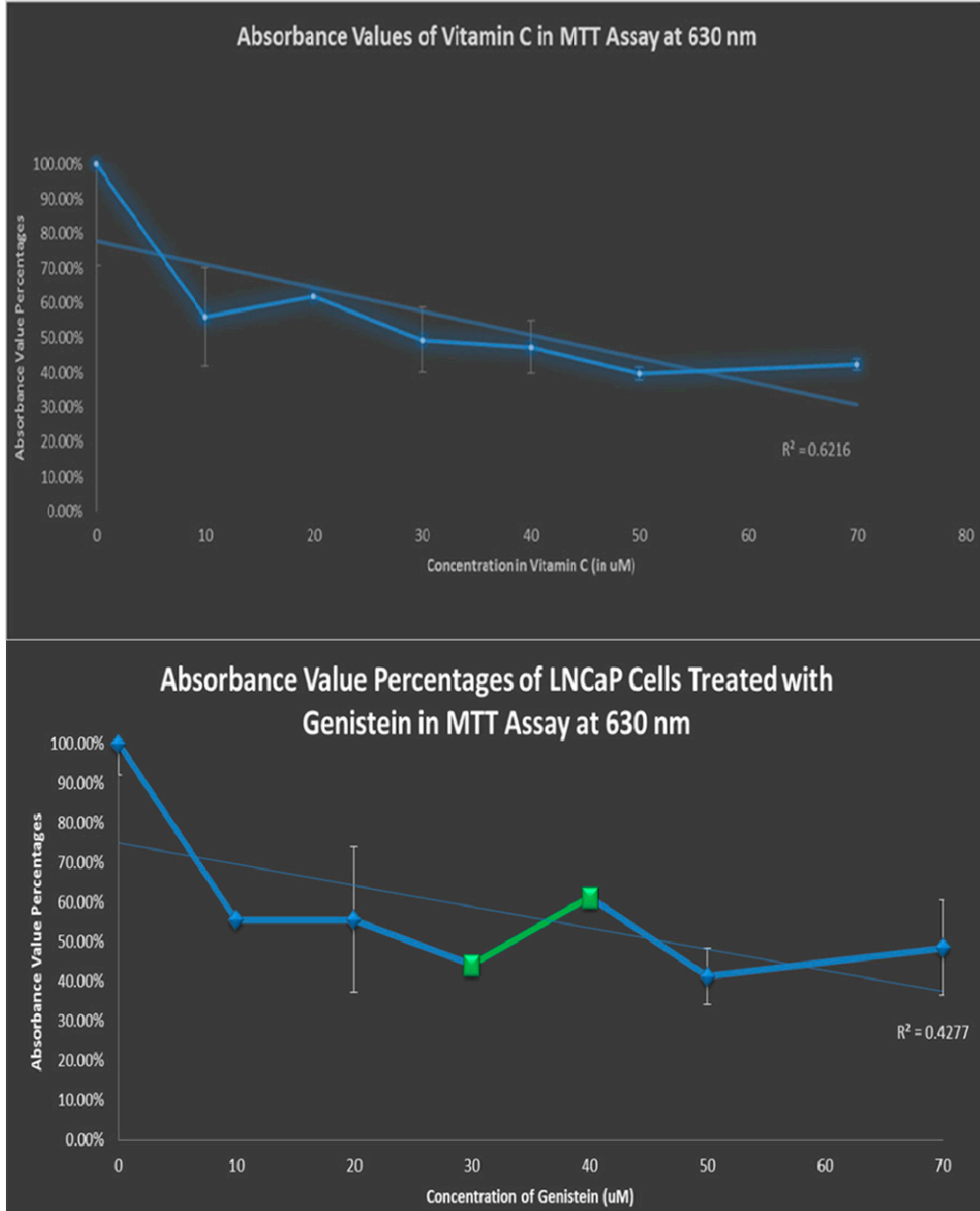


Figure 1. IC-50 of vitamin C = 40 μ m; IC-50 of genistein = 28 μ m; Higher concentration of vitamin C is needed to kill 50% of LNCaP cells.

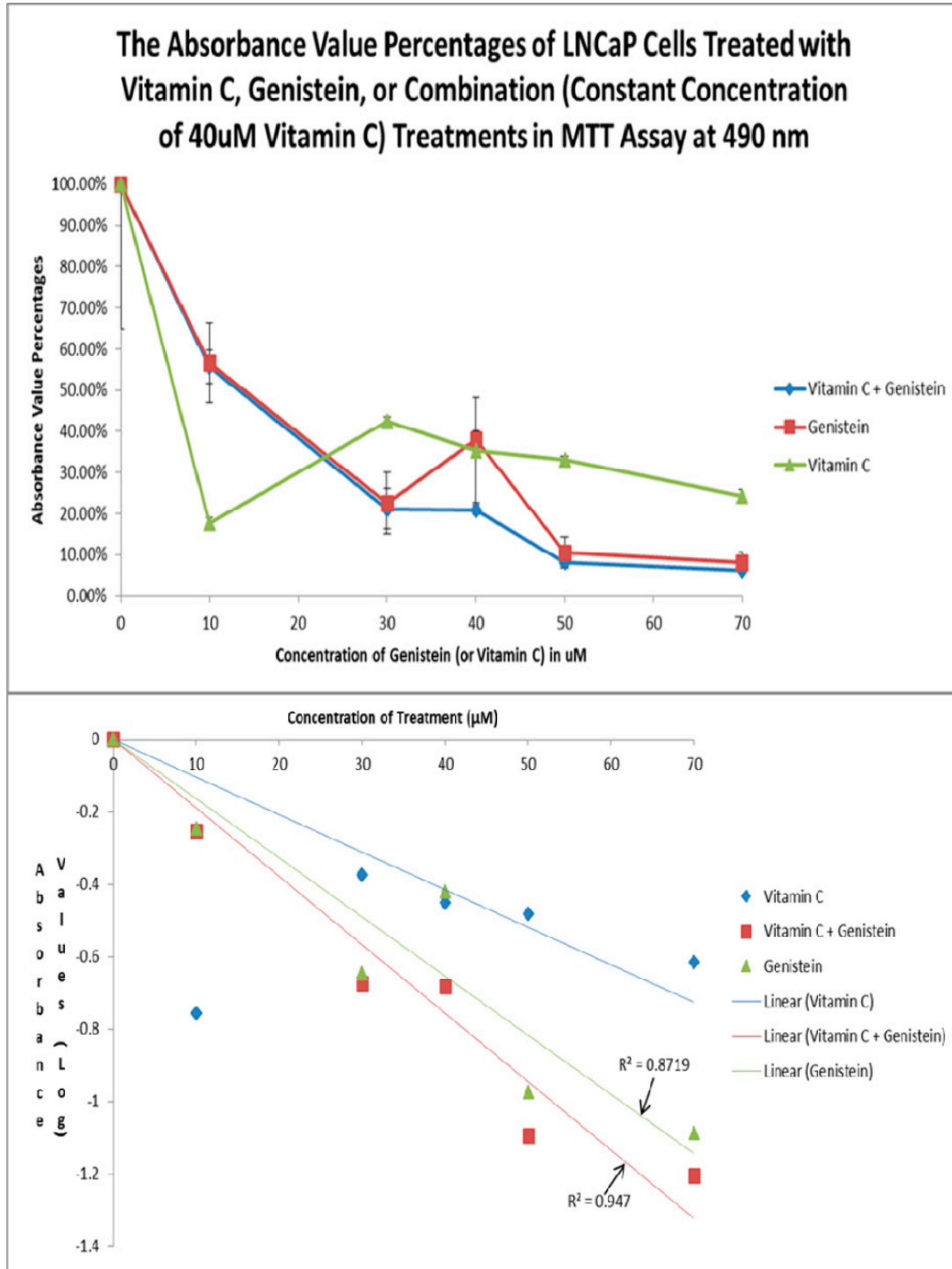


Figure 2. Level of significance = 0.01 and p value of student t -test = 0.11. Therefore $p > 0.01$.

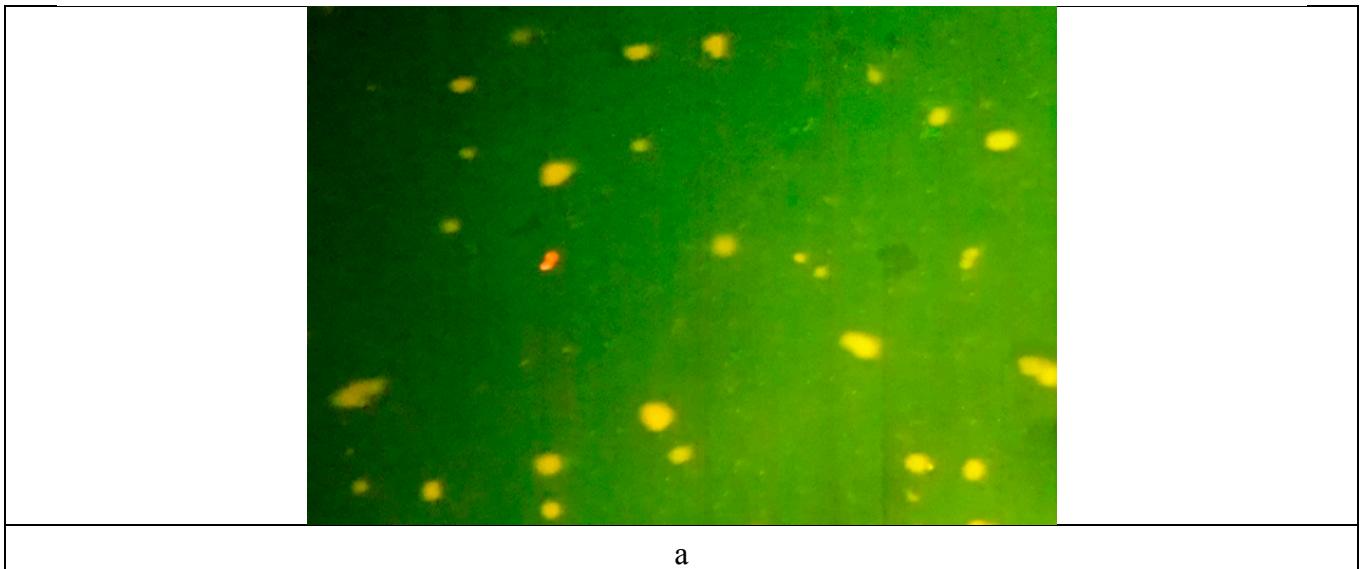
2.1.1. Florescence assay

Treatment-induced apoptotic cell death in LNCaP cells. Cells were cultured and treated as described previously; then prepared and analyzed under fluorescence microscopy. The results/data points were from two independent experiments performed in triplicates. The bar chart in fig.1 shows the relative percentages of apoptotic, necrotic and living cells. The percentage apoptosis at each concentration point are statistically different ($p < 0.01$) between the three different treatment regimens.

The ratio of percentage apoptosis to percentage necrosis is a one digit figure for all the single treatments of genistein while ratio of percentage apoptosis to percentage necrosis is a two digit figure for the combination treatments.

Table 1. Ratio of apoptosis to necrosis in each treatment groups of the LNCaP cells.

Ratio of Apoptosis to Necrosis in each treatment			
Treatment	% Apoptosis	% Necrosis	% Apoptosis/ %Necrosis
Control	0	0	0
10uM of genistein	48	17	2.8= 3
70uM of genistein	43	40	1.1= 1
10uM of genistein + 40uM of vitamin C	48	2	24.8 =25
30uM of genistein + 40uM of vitamin C	48	4	12
70uM of genistein + 40uM of vitamin C	62	6	10.3= 10



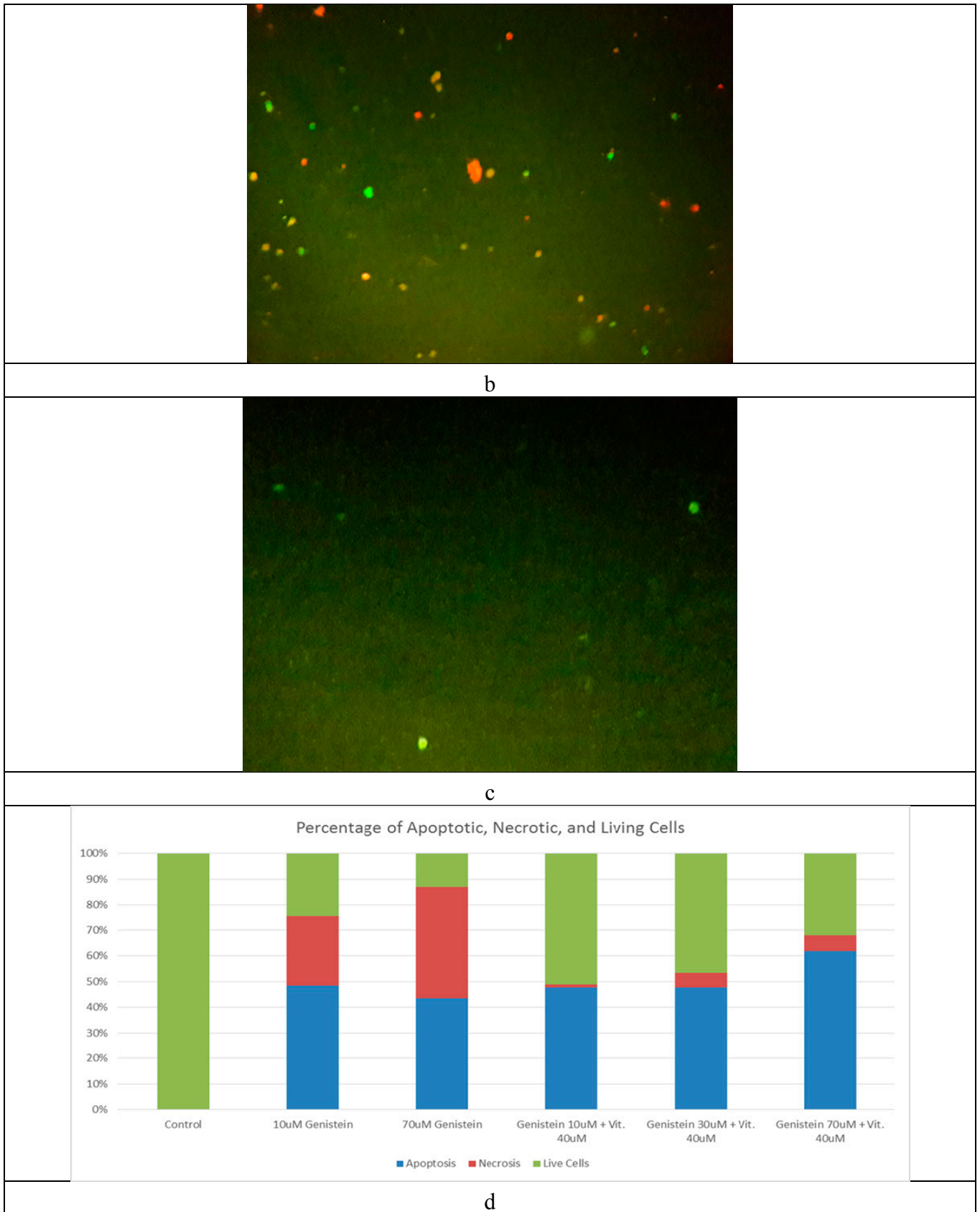


Fig 3. (a) Microphotograph picture of florescence assay-Genistein 70uM + Vitamin C 40uM; (b) Microphotograph picture of florescence assay-70uM gensitein single treatment; (c) Microphotograph picture of florescence assay-Control; (d) Percentage apoptosis, necrosis and living cells in all the treatment groups.

3. Experimental Section

3.1. Drugs (Micronutrients)

Genistein (4', 5' 7- trihydroisoflavone) was purchased from Indoline Chemical Co., Summerville, NJ, USA. It was dissolved in DMSO (Dimethylsulfoxide) to make a 80 μ M stock solution. The final concentration of the DMSO solvent was 0.5%. Aliquots in concentrations of 0, 10, 30, 40, 50 and 70 μ M (G₀-G₇₀) were made based on previous studies done in the laboratory [51,52] and stored at 3 °C until used. Vitamin C supplement (Swanson, ND, USA) containing 100% ascorbic acid micronutrient was dissolved in distilled water to make a 100 μ M stock solution. Aliquots in concentration of 0, 10, 30, 40, 50, 70 μ M (VtC₀₋₇₀) from preliminary findings and was stored at 3 °C until used.

3.2. Bioassays and staining reagents

MTT (3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyletrazolium bromide) assay, Nitroblue Tetrazolium (NBT) assay, and Acridine orange/Ethidium bromide (AO/EB) were purchased from Sigma Aldrich, MO, USA. LNCaP prostate cancer cell lines were purchased from American Type Culture Collection-Manassas, VA. The cell lines were grown in RPMI 1640 complete medium (ATCC, VA) and maintained as monolayers in 75m² tissue culture flasks (Sigma Scientific, St. Louis, MO, USA).

3.3. Cultivation of LNCaP cells

Briefly, LNCaP cells were plated in triplicates into 96-well microtiter plates (MTP) at a concentration of 1.0×10^4 cells per well. The plates were incubated in humidified CO₂ incubator at 37 °C and 5% CO₂ for 48 h to attain 80%–90% confluence. The cells were then subjected to the following treatments.

3.4. Treatment of LNCaP cells

The cells were treated with: a. varying concentrations of genistein (Gn₀₋₇₀); b. varying concentrations of vitamin C (VtC₀₋₇₀); c. combination of Gn and Vit C (Gn₀₋₅₀ + VtC₄₀).

From our preliminary study, the IC₅₀ (i.e. the concentration of Vit C at which 50% of the cells were killed or inhibited) was observed to be 40 μ M. Thus in the present study, Vit C at concentration of 40 μ M (IC₅₀) was used in the VtC-Gn combination treatment of LNCaP. The plates/cells were then incubated for 24 to 48 h at 37 °C and 5% CO₂.

3.5. MTT assay

To determine the anti-proliferative or growth inhibitory effects of genistein and/or vitamin C treatments on each prostate cancer cell line, MTT assay was used. MTT (3-[4, 5-dimethylthiazolyl-2]-2,5-diphenyletrazolium bromide) is a tetrazolium dye used to determine percentage cell survival rate (% CSR) or differential growth inhibition rate (DGIR). The MTT dye detects metabolic activity through preferential conversion of viable cells into purple colored formazan. The enzyme mitochondria dehydrogenase is only present in the active mitochondria of living cells, and is responsible for cleaving the tetrazolium substrate into insoluble formazan. The color intensity generated is directly proportional

to the number of viable (metabolically active cells) and quantitatively determined by optical absorbance from individual wells. Briefly, LNCaP cells were plated onto 96 well plate each at a density of 1.0×10^4 cells per well. The cells were incubated with graded doses of genistein (Gn0-70) for 24 h. In the combination treatment group, cells were treated with genistein (Gn0-70) and vitamin C (40 μ M). 100 μ L of MTT (2.5 mg/mL in PBS-phosphate buffered saline) (Sigma Scientific Chemical Co., St Louis, MO, USA) solution was added to each well and incubated for 4 h at 37 °C and 5% CO₂ to allow for the reduction of MTT into formazan by viable cells. The insoluble formazan crystals formed were solubilized or lysed to release the formazan product by adding 100 μ L of lysing solution (DMSO) to each pellet. Cells from each well were harvested (by vigorous aspiration with the micropipette), transferred to a labeled microcentrifuge tube and centrifuged for 10 minutes at 5000rpm. Any unincorporated MTT dye was removed by discarding the supernatant. The contents of each tube were transferred into different wells of a 96-well microtiter plate. The absorbance values were measured at 490nm with an automated microplate reader (BioTek, Vermont, USA). Relative numbers of live cells could therefore be determined based on the optical absorbance (optical density, OD) of the sample. The values of the blank wells were subtracted from each well of treated and control cells; and the mean percentage of post treatment viable cells relative to the controls was calculated as shown earlier.

3.6. Nitroblue Tetrazolium NBT/ROS assay

Cells have many defense mechanisms against effects of reactive oxygen species (ROS). In this assay, superoxide ions (O₂⁻) and/or hydrogen peroxides generated by X-rays and/ or graded genistein and or vitamin C treatments reduced or converted NBT to NBT diformazan. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation. In this experiment, the treated LNCaP cells, as previously described above (with Gn and / or Vit C) were subjected to NBT assay. The extent of treatment-induced reduction in the appearance of NBT diformazan is a measure of SOD activity present in the cancer cells. Since the absorbance at 490nm is proportional to the amount of superoxide anion (ROS) formed, the SOD enzyme as an inhibition activity can be quantified by measuring the decrease in the absorbance at 490nm, whereas an increase in absorbance reflects treatment-induced elevation of intracellular ROS produced by the cancer cells as demonstrated by Oseni et al. [51].

Briefly, 40 μ L NBT solution (1mg/mL in HBSS medium) was added to the adherent cells in each well in the appropriate MTP, and incubated for 4 hours at 37°C and 5%CO₂. The cells were trypsinized, pipetted into individual micro-centrifuge tubes, and diluted with equal volumes of PBS. The cell suspensions were centrifuged to collect the pellets. The pellets were washed three times with DMSO to lyse the cell membranes. The supernatants were pooled for absorbance reading on microplate reader at 490nm, to determine ROS production levels. The results were expressed as percentage of ROS (or SOD inhibition), relative the control. The means of the absorbance were graphed against the mean concentrations of the drugs/reagents.

3.7. Fluorescence microscopy: Differential cell death detection

Acridine orange and Ethidium bromide dyes have different fluorescence emission spectra. Therefore a cocktail solution of the two markers can be used to differentiate between viable, apoptotic and necrotic cells. Acridine orange permeates both viable and non-viable cells, causing the nuclei to emit green fluorescence. Ethidium bromide permeates dead cells (with compromised cell membrane integrity) and selectively stains the nuclei (nonviable) cells to produce red fluorescence. Cells that emit orange/brown colors are indicative of apoptosis, while necrotic cells emit red fluorescence. Briefly, cell suspensions from each well of the treated MTP were transferred into identical micro-centrifuge tubes for centrifugation. The supernatants were discarded and the cell pellets were re-suspended with PBS to constitute final cell suspensions for further analysis. Then a cocktail of Ethidium bromide (25 μ l) and Acridine orange (75 μ l) was prepared for staining the cells. For the fluorescence microscopy, 3 μ L of the cocktail solution was added to 50 μ l each cell suspension (final dye-cell suspension). To examine the cells, 10-20 μ l of each dye cell suspension was transferred onto a microscope slide, covered with a cover slip and analyzed under a fluorescent microscope with a band-pass filter (200X). Detection of apoptosis was based on morphological and fluorescent characteristics of stained cells. Viable cells were indicated by bright green color, apoptotic cells

3.8. Statistical analysis

Data were expressed as means + standard deviation (SD) from two different triplicate experiments to confirm similar result. The significance of the statistical difference in the mean differences between various experimental and control groups was determined using student's t-test, linear regression and chi-square test. P value of ≤ 0.01 was considered statistically significant.

4. Conclusions

P value of chi-square test = 0.0003 and the level of significance is 0.01. The null hypothesis that combination treatment will not cause more cells to die by apoptosis was rejected. Fluorescence assay shows different number of apoptotic death at different combination concentrations. Decrease in absorbance reflects a decreased level of intracellular ROS. The order of effectiveness of treatment is (Gen 10 μ M + Vit C 40 μ M) > (Gen 30 μ M + Vit C 40 μ M) >(Gen 70 μ M + Vit C 40 μ M) > > 10 μ M genistein only > 70 μ M genistein only. The concentration (Gen 10 μ M + Vit C 40 μ M) is the most effective while 70 μ M genistein only is the least effective.

Acknowledgments

We acknowledge the Department of Biological Sciences of Florida Atlantic University for the provision of laboratory space. The technical support of the many research students in our lab is highly appreciated.

Author Contributions

All authors contributed extensively.

Conflicts of Interest

The authors declare no conflict of interest.

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