IMPACT OF VITAMIN C ON GENISTEIN INDUCED APOPTOSIS ON PROSTATE CANCER

by

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ABSTRACT

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This study determined the impact of vitamin C dose on genistein-induced apoptosis in LNCaP cancer cells at various treatment regimens in vitro. Although the linear regression of viability assay (MTT) indicated a p-value = 0.11; NBT assay reveal a declining SOD activity during cell death. Apoptosis induction was the main mode of treatment induced cell death. The overall data showed the trend of treatment efficacy as; (Gen 10uM + Vit C 40uM) > (Gen 30uM + Vit C 40uM) > (Gen 70uM + Vit C 40uM) >10uM genistein > 70uM genistein. The chi-square test for comparing necrosis, apoptosis and life cells showed that Vitamin C could impact genistein-induced apoptosis in LNCaP cells (p = 0.0003). This study forms the basis for in vivo studies of the impact of vitamin C on genistein-induced apoptosis in LNCaP prostate cancer cells.

DEDICATION

This manuscript is dedicated to my wife, parents and siblings, for their understanding and support during my research endeavors.

IMPACT OF VITAMIN C ON GENISTEIN INDUCED APOPTOSIS IN PROSTATE CANCER

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INTRODUCTION

Prostate Cancer

Prostate Cancer (PCa) is the most common non-skin malignancy in men and is a rising health problem worldwide (Bill-Axelson, et al., 2011). Prostate cancer is one of the most significant pathology in the field of Urology. In Europe it is the most common neoplasm with an incidence of 214 cases per 1000 inhabitants (Bozzini G. et al., 2013) and is projected to increase at even higher rates across the world due in part to increase in longevity, with more men living to the age when prostate cancer is more likely to develop (Lalitha et al., 2012; Verim et al., 2013).PCa broadly consists of two forms; the first latent organ-confined, which is slow growing and generally leads to mortality from other causes such as old age instead of PCa directly (Harris et al., 2009). In approximately 20% of cases though the PCa is aggressive and metastatic, which is lethal and requires aggressive therapy (Harris et al., 2009). The main risk factors are family history, age and ethnicity (Hsing and Devesa, 2001). A substantial number of clinicians perceived that there was a link between Lower Urinary Tracts Symptoms and PCa and recommend screening for early cancer in men with urinary symptoms (Martin et al., 2008; Hoffman, 2011; Belbase N.P. et al., 2013). The screening programs for PCa in healthy adult men are however, not completely without fault.

The detection of latent asymptomatic disease in very old men (>75 years) is an important concern, with regard to increasing costs, over-diagnosis, overtreatment and reduction in quality of life (Verim et al., 2013).

Epidemiology of Prostate Cancer. 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. (Siegel et. al, 2012). In 2009, there were;192,280 cases of prostate cancer reported and 27,360 related deaths in the United States(ACS, 2009).Worldwide, incidence rates increased dramatically through the early 1990s.(Hsing et. al, 2000). Between 1986 and 1992 there was another sharp rise in incidence largely due to increasing use of prostate specific antigen (PSA)as a diagnostic measure (Potosky et. al, 1995).

Prostate cancer can be a seus disease, but most men diagnosed with prostate cancer do not die from it. More than 2.9 million men in the United States who have been diagnosed with prostate cancer are still alive today (ACS, 2015).According to the most recent data, when including all stages of prostate cancer: The relative 5-year survival rate is almost 100%.The relative 10-year survival rate is 99%.The 15-year relative survival rate is 94% (ACS, 2015). Keep in mind that just as 5-year survival rates are based on patients diagnosed and first treated more than 5 years ago, 10-year survival rates are based on patients diagnosed more than 10 years ago (and 15-year survival rates are based on patients diagnosed at least 15 years ago).

During the mid-1990s, incidence rates in the United States declined, but has begun to slowly rise again(Ries et. al, 2003).Incidence rates in Asian countries are

generally low, but in recent years have risen proportionately more than in western countries. Part of the increase in incidence has been attributed to increased westernization. (Hsing et. al, 2000) Prostate cancer incidence data from Africa are sparse, with only 4 registries from 1994 included in the 2003 IARC report, which showed incidence rates ranging from 5 to 37 per 100,000 person-years (Parkin et. al, 2003).Metastatic PCa is not curable and continues to be the major cause of cancer deaths. (Mehlen & Puisieux, 2006).Palliation can be achieved by hormone deprivation therapy. However after an excellent initial response, in approximately 2 to 3 years most of these PCas will relapse to the castration resistant form of the disease (Attar et. al, 2009) with death usually occurring within several years (Nelson et. al, 2008).

Risk factors for prostate cancer

Ethnicity, advanced age and family history are well known risk factors for prostate cancer. (Hsing and Chokkalingam, 2006). Currently, prostate-specific antigen (PSA) is the putative biomarker for PCa screening. Two consecutive rises in PSA value over 0.5 ng/mL or one single value $\geq$ 4 ng/mL are indications for biopsy (Mistry & Cable, 2003).However, although PSA testing has high sensitivity, its specificity is rather low, causing clinicians to have doubts with regard to biopsying; since increased false-positive rates, mis-diagnosis and overtreatment have been reported to be associated with PSA testing (Nash & Melezinek, 2000; Welch & Albertsen, 2009).

Evidence suggests that the presence of inflammatory factors and cytokines at the tumor site results in tumor cell survival, proliferation, invasion and metastasis(Germano, et. al, 2008).Elevated levels of IL-6 in men with local PC and

advanced disease made IL-6 a candidate biomarker for PC development and progression(Lee et. al, 2003).

Demographic. Over 80% of prostate tumors in the U.S. are diagnosed in men over age 65 (Parkin, et. al, 1999).

Racial /ethnic variation : it is unlikely that differences in detection (i.e., screening) account for all of the variability in prostate cancer risk between populations. Adjustment of incidence rates for the prevalence of latent disease at autopsy, and proportion of localized tumors among all prostate cancers, revealed that Japanese men still experience a markedly lower incidence than Americans; indicating that the large international variation cannot be explained by differences in detection rate alone(Shimizu, et al., 1991).

Physical activity may decrease levels of total and free testosterone, reduce obesity, and enhance immune function (Lee, et. al, 2001) all of which may offer protection against prostate cancer. However, results from numerous epidemiologic studies are equivocal in part, due to challenges in classifying physical activity and/or identifying the age/time periods during which such activity may be most protective (Lee, et. al, 2001 ; Lee, I. M, 2003).

Occupation: There is a large body of literature on prostate cancer and occupation, and one consistent result from these studies is that farmers and other agricultural workers have a 7-12% increased risk (van der Gulden, et. al, 1995).While this excess could reflect lifestyle factors such as increased intake of meat and fats, exposures to chemicals may also play a role. Chemicals commonly encountered in

agriculture include fertilizers, solvents, pesticides, and herbicides, which have a wide variety of poorly characterized effects (Alavanja, et al., 2003).

Diet: Association of polyunsaturated fat with incidence of cancer is less consistent (Kolonel, 2001; Kolonel, et. al, 1999). However, it is unclear whether the excess risk is due to the high-fat content, mutagens such as heterocyclic amines that are induced during high-temperature cooking, animal proteins, or other unidentified factors (Norrish et. al., 1999).

Fatty fish are rich in tumor-inhibitory marine fatty acids, such as omega-3. However, a recent review of 17 studies, including 8 prospective studies, found suggestive but inconsistent results, possibly due to inadequate assessment of fish intake or lack of information on specific marine fatty acids, particularly the two omega-3 polyunsaturated fatty acids, eicosapentaenoic and docosahexaenoic acids (Terry et. al, 2003).In two large prospective studies, higher intake of fish was associated with a lower risk of total prostate cancer and metastatic prostate cancer (Terry et. al, 2001). Abundant in fatty fish, omega-3 fatty acids are known antagonists of arachidonic acid and suppress the production of pro-inflammatory cytokines (Calder, 2002).

A recent review concluded that there is modest evidence that intake of cruciferous vegetables, including broccoli, cabbage, cauliflower, and Brussels sprouts, is inversely associated with prostate cancer risk, possibly due to their content of isothiocyanates (Kristal, et. al, 2002). In addition, intake of allium vegetables, including onions, garlic, and chives, were inversely associated with prostate cancer in a case-control study in China (Hsing et al., 2002).

Molecular data show that selenium prevents clonal expansion of tumors by causing cell cycle arrest, promoting apoptosis, and modulating p53- dependent DNA repair mechanisms. Secondary analyses of clinical trial data have also shown that vitamin E supplementation is associated with a reduced risk of prostate cancer (Pak, et. al, 2002; Klein, et. al., 2001).

Obesity: Epidemiologic studies data suggest that obesity is related to aggressive prostate tumors and that, abdominal obesity may be associated with an increased risk of prostate cancer even in relatively lean men (Hsing, et. al, 2000; Hubbard et al., 2004). In addition, higher serum levels of insulin have been linked to an increased risk of prostate cancer (Hsing, et. al, 2001).

Smoking: Although prostate cancer has not been traditionally included among the smoking-related malignancies (Nomura & Kolonel, 1991; Pienta & Esper ,1993), the results of two reported cohort studies have drawn attention to the possibility that cigarette smoking may be a preventable cause of mortality from prostate cancer(Hsing et. al, 1991 ; Hsing, et. al., 1990).

Sexual frequency: Recent studies have indicated that increased sexual frequency may be associated with an increased risk of prostate cancer, because it may serve as an indicator for either a greater opportunity of infection or higher androgenic activity (Dennis & Dawson, 2002; Fernandez, et. al, 2005 ; Strickler & Goedert, 2001).

Sexually Transmitted Diseases. A large, population-based study showed 2-3-fold prostate cancer risks associated with STDs, particularly syphilis and recurrent gonorrhea infections(Hayes, et al., 2000).Other studies reported associations of human papillomavirus-16, -18, and -33 serology with an increased risk of prostate cancer(Adami et. al, 2003 ; Rosenblatt et. al, 2003), while a study of a human immunodeficiency virus (HIV)-infected population found that duration of HIV infection was associated with increased prostate cancer risk (Crum et. al, 2004). Meta-analysis of 17 studies concluded that a higher number of sexual partners is associated with increased prostate cancer risk, possibly through increased opportunity for sexually transmitted infections (Dennis & Dawson, 2002).

Chronic Inflammation: Evidence for a role of chronic inflammation in prostate cancer is beginning to emerge (Platz &De Marzo, 2004), but an association of prostate cancer with chronic inflammation of the prostate (chronic prostatitis) has long been suspected. Meta-analysis of 11 studies of prostatitis and prostate cancer reported an overall relative risk of 1.6. (Dennis & Dawson, 2002). Inflammation is frequently found in prostate biopsy specimens (Di Silverio, et. al, 2003; Nickel, et. al, 2001).

Results from pathologic and molecular surveys suggest that the earliest stages of prostate cancer may develop in lesions generally associated with chronic inflammation (De Marzo, et. al, 1999; De Marzo, et. al, 2003). De Marzo et. al, (1999) showed that almost all forms of focal prostatic glandular atrophy are proliferative, and that such proliferative inflammatory atrophy (PIA) lesions often contain inflammatory infiltrates and are frequently found adjacent to or near high-grade prostatic intraepithelial

neoplasia (PIN); a precursor of prostate cancer (De Marzo et. al, 1999 ; DeMarzo, et. al, 2003).

Vasectomy: Several, but not all studies investigating the association between vasectomy and prostate cancer risk suggest a modest positive association (Dennis, et. al, 2002). However, the role of vasectomy remains controversial, since most studies are unable to exclude the possible effect of detection bias; that is, men undergoing vasectomies are more likely to have prostate cancer detected than men who do not. Vasectomy is linked to elevations in anti-spermatozoa antibodies, reduced hormone concentrations in the semen, and reduced prostatic secretion (Bernal-Delgado, et. al, 1998).

Diseases: Utilizing self-rated health (SRH) trajectories to elucidate disease progression and inform intervention efforts might ameliorate well-being decline associated with a cancer diagnosis (Foraker et. al, 2011). The majority of studies utilize lengthy instruments to capture the multi-dimensional nature of cancer patients' health-related quality of life (HRQoL) (Reeve et. al, 2009 ; Staren, et. al, 2011 ; Osthus, et. al, 2011 ; Reeve et. al, 2012 ; Gray et. al., 2011). While a few recent studies measure general well-being across the cancer continuum(Foraker et. al, 2011 ; Reeve, et. al, 2009 ; Reeve et. al, 2012) most studies are limited to highly selected patient populations, have short follow-up time, or assess measurements after the diagnosis has occurred (Staren et. al, 2011 ; Osthus et. al, 2011; Gray et. al, 2011).

Benign prostatic hyperplasia: in most epidemiologic studies, it has been difficult to completely rule out the presence of BPH in control populations, since the prevalence of BPH is very common in elderly men. Due in part to these limitations, the epidemiologic evidence for BPH as a risk factor for prostate cancer remains weak and inconsistent (Guess, H. A, 2001), with the largest study to date (over 85,000 BPH patients) showing only a marginally elevated age-adjusted risk of prostate cancer among BPH patients versus the general population (<2% in 10 years) (Chokkalingam, et. al, 2003).

Genetic Factors

Family history of cancer : Numerous studies have consistently reported familial aggregation of prostate cancer, showing a 2- to 3-fold increased risk of prostate cancer among men who have a first-degree male relative (father, brother, son) with a history of prostate cancer (Stanford & Ostrander, 2001). High-penetrance markers: Segregation and linkage analyses have shown that certain early-onset prostate cancers may be inherited in an autosomal dominant fashion (Carter et. al, 1993) and it is estimated that such hereditary prostate cancers (HPCs) due to highly penetrant genes may account for about 10% of all prostate cancer cases (Lichtenstein et. al, 2000). Common low-penetrance markers: Review of several of these markers and genes can be found elsewhere. It is important to note that, as with any other epidemiologic exposure, replication of findings is critical to establishing causality (Schaid, 2014).

Androgen biosynthesis and metabolism pathway**:** Because most prostate cancer is an androgen-dependent tumor, it is likely that markers in genes whose products are

involved in androgen biosynthesis and metabolism may be associated with prostate disease. This is supported by evidence that there is racial/ethnic variation in polymorphisms of genes involved in the androgen pathways (Hsing et. al., 2002; Ross et. al, 1998).

Growth factor and non-androgenic hormone pathways **:** Due to serological evidence linking them to prostate cancer, a number of studies have explored the prostate cancer risk associated with polymorphic markers in genes involved in the insulin and insulin-like growth factor (IGF) signaling pathways. Two of three early studies of the insulin gene (INS) have shown promising results (Claeys et. al, 2005; Ho et. al, 2003; Neuhausen et. al, 2005).

DNA repair pathway**:** Genes in the DNA repair pathway prevent disruptions in DNA integrity which may otherwise lead to gene rearrangements, translocations, amplifications, and deletions, contributing to cancer initiation (Berwick &Vineis, 2000). Initial reports of markers in genes encoding DNA repair enzymes, including the X-ray repair cross-complementing groups 1 and 3 (XRCC1 and XRCC3), human 8-oxoguanine glycosylase I (hOGG1), xeroderma pigmentosum group D (XPD), methylguanine DNA methyltransferase (MGMT, also known as alkylguanine DNA alkyltransferase, or AGT), and ataxia telangiectasia mutated protein (ATM, involved in DNA damage signalling)) show promising results to detect cancer (Angele et. al, 2004; Xu et. al, 2002).

Chronic inflammation pathway**:** Several lines of evidence point to a role of inflammation in prostate cancer etiology.Initial studies show positive results for

transforming growth factor-beta (TGF-beta) and COX-2 (Ewart-Toland et. al, 2004) and negative results for tumor necrosis factor-alpha-308 (TNF-alpha-308) (McCarron et. al, 2002 ; Wu et. al, 2004).

Angiogenesis pathways**:** The need for increased vasculature to support cancer growth forms an area of research that is currently gaining momentum. Genetic investigations of angiogenesis in prostate cancer have involved the vascular endothelial growth factor (VEGF) gene as well as the genes for IL-8 and IL-10, and thus far have shown positive results (McCarron, et. al, 2002).

Other Factors. Several other risk factors, such as, smoking, use of alcohol, diabetes, and liver cirrhosis, have been investigated, but their roles in prostate cancer are weak or unclear based on data in the literature. (Dennis and Hayes, 2001 ; Giovannucci, 2001 ; Hickey, 2001).

TREATMENT OF PROSTATE CANCER

Significant success 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 poses therapeutic challenges; and consequently prognosis still remains poor. Vitamin C as an anti-cancer therapy adjuvant may reduce necrosis, inhibit cancer cell growth, lessen chemo-prevention side effects such as nausea, fatigue, pain, and depression in-vivo and in-vitro (Fritz H, et. Al, 2014 ; James Kumi-Diaka et. al, 2015).In order to reduce this side effect, vitamin C was used as an adjuvant therapy with genistein in treatment of prostate cancer. Numerous studies have found a link between vitamin C intake and the incidence of several different cancers, especially non-hormonal cancers (Head, 1998)Also, having a high plasma level of vitamin C cuts your risk of dying from cancer (Loria et. al, 2000).

GENISTEIN ISOFLAVONE : STRUCTURE AND EPIDEMIOLOGY

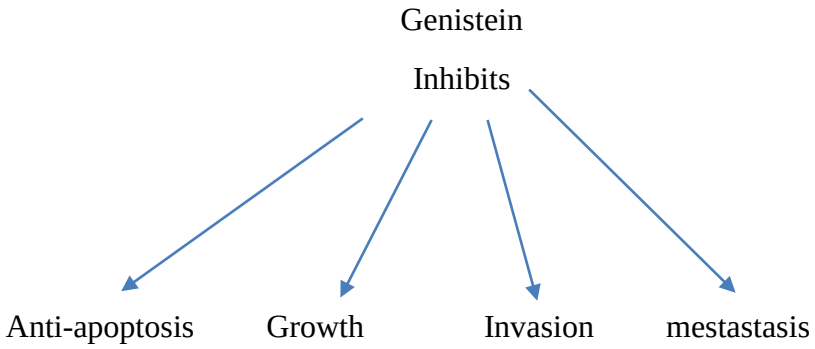

Figure 1.Machanism of action of genistein on LNCaP cells. Genistein modulates a critical transforming growth factor-b signaling pathway in human prostate cancer. A schematic diagram of this signaling network and relevant phenotypes associated with genistein treatment in mice and men is shown in Figure 1.

Development of chemotherapeutic agents with low patient toxicity is currently being investigated by many scientists. Many of these agents are derived from natural plant products, for example genistein. Genistein is a phyto estrogenic isoflavonoid that has multiple effects in a wide variety of cancers without any visible toxicity to normal cells (Li & Sarkar, 2002).

The effects of genistein on the regulation of several miRNAs have been reported (Zaman et. al, 2012; Chen et. al, 2011; Chiyomaru, et. al, 2012).miRNAs play an important part in many biological processes, such as development, differentiation, proliferation, apoptosis, angiogenesis and metabolism. In addition, they are key regulators in many diseases including cancer (Ryan et. al, 2010). miRNAs may function as oncogenes or tumor suppressor genes (Croce, 2009 ; Zhang et. al, 2007).

Genistein (4′,5,7-Trihydroxyisoflavone), a major isoflavone constituent of soybeans and soy products, has been shown to exhibit potent anticancer effects on PCa. (De Souza et. al, 2010; Pavese et. al, 2010). Furthermore, consumption of soy products leads to increased blood concentrations of genistein (Adlercreutz et. al, 1993).Several studies associated dietary soy consumption with a lower incidence of mortality from cancer, and from PCa in particular, compared with studies in individuals who consume low-soy, meat-based diets(Adlercreutz, 1990 ; Yan & Spitznagel, 2009).Epidemiological evidence indicate that the incidence and mortality rates of PCa are considerably lower in Asia compared to the United States. (Mc Cracken et. al, 2007).The mean serum concentration of genistein in Asian men was higher than that of the US population. (McCracken et. al, 2007); and several studies have demonstrated that isoflavone intake was associated with a reduction in PCa risk (Kurahashi, et. al, 2007; Yan & Spitznagel, 2009).

Structural Characteristic and Synthesis of Genistein

Genistein was originally isolated by Perkin and Newbury in 1899 from Dyer's Broom (Genista tinctoria) (Perkin & Newbury, 1899). This naturally derived compound is a member of the isoflavone branch of the flavonoid family of small molecules, which includes over 5,000 compounds (Andersen & Markham, 2006)

Mechanism of Action of Genistein

Genistein's structural characteristics also impart this compound with the ability to act as a weak estrogen mimic, leading to its classification as a phytoestrogen (i.e., an estrogen-like compound derived from a plant source) (Matsumura et. al, 2005). In particular, compared to 17-β-estradiol, the predominant sex hormone present in females,

genistein shares both a near identical molecular weight as well as a similar hydroxylation pattern, with two key phenolic groups at C7 and C4′(Dixon & Ferreira , 2002). Importantly, the C7 hydroxyl group is needed for genistein to bind to the estrogen receptor (ER), as it mimics the A ring of the steroidal estrogen core.

Furthermore, the distance (~11.5 Å) between the C7 and C4′ phenolic groups allows for optimal binding of genistein to the ER, as they are in very similar positions to the key hydroxyl groups on the estradiol core.(Andersen & Markham , 2006). Both the C4′ and C7 phenolic groups have been shown in a crystal structure to form key contacts with ERβ, with the C4′ phenol binding to Glu305 and Arg306 and the C7 phenol to His475 (Pike et. al, 1999). Because of these structural characteristics, genistein can bind to both α and β isoforms of the ER (Kuiper et. al, 1998; Mueller, et. al, 2004), although it binds to ERβ with 20-fold higher affinity than ERα (Kuiper et. al, 1997). Genistein's ability to both bind and stimulate the ER has led to several studies on its effects in postmenopausal women, with results suggesting that genistein could restore bone mass and relieve menopausal symptoms such as hot flashes and vaginitis (Andersen & Markham, 2006).

Genistein alters cellular detachment and cell flattening: Genistein has multiple molecular targets including receptors, enzymes, and signaling pathways. (De Souza et. al, 2010).Changes in cellular adhesion are a critical step in the metastatic cascade. For a cell to move to another location, it must detach from the extracellular matrix (ECM), to which the primary tumor is attached. Previous studies showed that genistein inhibits human PCa cell detachment in a dose-dependent fashion, both in vitro (Bergan et. al,1996 ; Kyle E, et al,1997) and in vivo (Lakshman et. al, 2008). Genistein inhibits cell detachment in vitro

at concentrations as low as 1 mmol/L in PC3-M, PC3, and DU-145 human PCa cell lines after 3 three days and as low as 10 nmol/L in PC3-M cells. The ability of genistein to increase cell adhesion directly counteracts cell detachment and thereby inhibits an initial step in the metastatic cascade. In addition to these in vitro findings, it was also shown that genistein affects cellular adhesion in vivo (Lakshman et. al, 2008).

Genistein decreases production of proteases: In addition to detaching from the ECM, cancer cells must degrade the surrounding tissue to move outside the primary organ. This is accomplished primarily via increased production of matrix metallo proteinases, a family of zinc endopeptidases whose normal physiologic function is tissue remodeling and embryonic development (Vihinen et. al, 2005).

Increased expression of MMP-2 in prostate tissue is associated with the future development of metastatic disease. MMP-2 is critical for the degradation of collagen IV (Vihinen et. al, 2005) .Specific inhibition of MMP-2 mRNA expression by genistein was confirmed (Kumi-Diaka et. al, 2006).

Genistein inhibits PCa cellular invasion: The most robust measure of a cell's ability to drive metastasis in vitro is its ability to invade, as measured in an in vitro invasion assay. In this assay, a cell must both degrade a protein layer and move from one location to another. It was first reported in 2005 that genistein can directly inhibit cellular invasion in a Boyden chamber assay in the panel of cell lines (Huang et. al, 2005).

Genistein decreases metastatic formation in vivo: It is also known that genistein regulates the formation of metastasis in a murine model of PCa (Helenowski et al., 2008). In the transgenic adenocarcinoma of the mouse prostate- inbred mouse strain murine

model (TRAMP-FVB murine model), treatment with 250 mg genistein/kg feed pellets significantly decreased pelvic lymph node metastasis (El Touny & Banerjee, 2009). By using a Simian virus 40 transgenic rat model of treatment with 250 mg genistein/kg feed pellets, cell proliferation decreased and apoptosis increased, resulting in an overall increase in survival rate of treated rats (Harper et. al, 2009).

Dietary consumption and epidemiology of genistein: The primary chemical form present in soybeans and non-fermented soy products is the C7-glycosylated form, also known as genistin (Fukutake et. al, 1996). After ingestion of genistin, hydrolysis within the intestine yields the aglycone, genistein, and the free sugar (Rowland et. al, 2003). Rodent-based pharmacologic studies demonstrate that, after absorption from the intestine, genistein undergoes first-pass metabolism in the liver, as well as enterohepatic circulation (Sfakianos et. al, 1997).

VITAMIN C

The ability of ascorbic acid to act as a pro-oxidant has been well documented (Halliwell & Gutteridge, 1989).Vitamin C, also known as ascorbic acid is, one of the known potent antioxidants in humans. Due to the lack of enzyme gulonolactone oxidase, vitamin C must be obtained from food such as citrus fruits and vegetables (Donkena et. al, 2010; ACS, 2013). The importance of this vitamin was first revealed by studies conducted by Linus Paulin in the 1970s (Fritz, 2014). Vitamin C has been demonstrated to induce apoptosis in cancer cells by creating oxidative stress via upregulation of reactive oxygen species (ROS) release (Park, 2013). Using high dosage of vitamin C in vivo as an anti-cancer therapy adjuvant, reduced cancer cell growth and lessen chemo-therapy side effects such as nausea, fatigue, pain and depression in human (Fritz, 2014).

The Physician Health Study (Gaziano et. al, 2009) illustrated that vitamin C showed neither health benefits nor safety issues.In fact Combs, 2008 reported that increased vitamin C intake had adverse effects, e.g., kidney stones and iron-related disorders. In contrast to studies that indicate possible adverse effects (Brent & Oakley, 2006), the Institute of Medicine (IOM, 1998) report illustrated that the use of folic acid can reduce the incidence of malformation, i.e., neural tube defects and spina bifida. A cancer prevention trial, which looked at the recurrence of cancers in those who had a history of cancer, reported that the death rate was 30% in those assigned to vitamin E, vitamin C supplementation has a lower percentage and follow-up for 6.5 years(Bairati et. al, 2006).

Studies suggest that dietary supplements are used by up to 81% of cancer survivors, and that 14% to 32% begin using supplements following diagnosis (Velicer & Ulrich, 2008) Increasing one's intake of antioxidants, which are involved in repairing cellular oxidative damage, theoretically should lower cancer risk, but there is little evidence to suggest that supplementation is efficacious, or even safe (Rock et. al,2012)Of particular concern to clinicians and patients is the use of antioxidant supplements during the course of chemotherapy and/or radiation therapy, as antioxidants consumed at doses common in nutritional supplements could potentially interfere with the effectiveness of these treatments (Lawenda et. al, 2008).

In order to reduce cancer risk, the American Cancer Society (ACS) recommends that antioxidants be consumed through food sources rather than as dietary supplements. "The best advice," the ACS guidelines state, "is to consume at least 2.5 cups of a variety of colorful fruits and vegetables each day (Kushi et. al, 2012) Many clinicians are concerned that high-dose antioxidant supplements could decrease the effectiveness of chemotherapy or radiation by protecting cancer cells as well as healthy cells from oxidative damage associated with these treatments. Others argue that antioxidant supplementation could selectively protect healthy cells from such damage, thus minimizing toxicity and adverse side effects of the treatment regimen (Lawenda et. al, 2008).

Although findings vary among existing randomized controlled trials of antioxidant use during cancer treatment, many are of relatively low quality and/or limited statistical power. Moreover, it is difficult to draw conclusions due to variations in malignancy type, antioxidant type and dose, and treatment regimen, among other factors (

Lawenda et. al, 2008).The ACS further states that “it is prudent for cancer survivors currently receiving chemotherapy or radiation therapy to limit the usage of supplements to nutrients for which a deficiency has been demonstrated, and avoid dietary supplements exceeding 100% of the Daily Value for antioxidant vitamins (Rock et. al, 2012).Cancer survivors are at an increased risk of secondary cancers, and the ACS advises that such patients “should be encouraged to consume a variety of antioxidant-rich foods each day (Rock et. al, 2012).

A 2006 systematic review of randomized controlled trials in patients with cancer or pervasive lesions found no connection between antioxidant supplementation and all-cause mortality(Davies et. al, 2006) Another study followed 77,719 residents of Washington State over 10 years and found no cause and effect association between supplementation with vitamin E or C and cancer mortality (Pocobelli, et. al, 2009).Based on existing evidence, patients with cancer should minimize supplement use, especially in doses higher than the recommended daily value, except to meet nutritional needs or as evidence-based therapy for a chronic condition such as osteoporosis (Rock et. al, 2012 ; Kushi et. al, 2012).Therefore, there are conflicting reports in the literature, which has served as our motivation to organize this study.

Clinical Approach

(PSA) and digital rectal examination (DRE) are commonly used for the initial evaluation of PCa risk, whiles systematic transrectal ultrasound (TRUS)-guided prostate biopsy remains the most common means of PCa diagnosis. In the current era with the widespread use of PSA, many patients undergo systematic TRUS-guided biopsy (SB), resulting in the detection of clinically insignificant cancers (Catto et. al, 2011).

Prostate Specific Antigen (PSA). The Prostate, Lung, Colorectal and Ovarian (PLCO Trial) of cancer screening in the US with 76,698 men aged between 55 and 74 years which after 13 years of follow-up found no evidence of mortality benefit for annual PSA screening compared to usual care which included opportunistic screening(Andriole et. al, 2012), A systematic review from the Cochrane database of five trials with 341,342 participants in 2013 did not find any significant decrease in prostate cancer-specific mortality in a meta-analysis of the five randomized controlled trials. There were significant treatment related harms. Men who have a radical prostatectomy had an 11% increased risk of urinary incontinence and a 37% increased risk of erectile dysfunction (Ilic et. al, 2013). The American Urological Association in 2013 recommend no screening of men under 40 years; no screening of men aged 40 to 54 at average risk; shared decision making for men 55-69 years for PSA analysis with no routine screening of other age groups. No screening of men older than 70 years or in men with a life

expectancy of less than 10-15 years. The screening interval should be two years or more (American Urological Association, 2013).

Imaging or Systemic Transrectal Ultrasound (TRUS). TRUS is an imaging technique that uses harmless sound waves and their echoes to "map" the prostate. Radiologist use TRUS to guide their biopsy needles through the perineum into the prostate (directed biopsies). This procedure detects about 68% of cancers in the prostate peripheral zone and 8% from the central zone (McNeal et. al, 1988).Technologic advances in ultrasound and biopsy since the 1940s have made TRUS an integral tool in detecting early stages of PCa (Applewhite et. al, 2001).

Staging Approach

Prediction tools for PCa have been developed to assist in the accurate diagnosis and treatment of the disease, and address a wide variety outcomes; e.g. the Partin tables (Makarov et. al, 2007 ; Partin et. al, 1997 ; Partin et. al, 2001 ; Partin et. al, 1993) Partin nomogram (Huang et. al, 2011) Kattan and Stephenson nomograms (Smaletz et. al, 2002 ; Stephenson et. al, 2005 ; Stephenson et. al, 2006) D'Amico risk classification (D'Amico AV, et al 2002) CAPRA score (Cooperberg et. al, 2005) and many others (Haese et. al, 2003 ; Veltri et. al, 2002 ; Veltri et. al, 2001). For the prediction of stage at radical prostatectomy (RP), the Partin tables not only represent the most common prediction tool used by clinicians, but have also undergone extensive validation in a number of cohorts (Karakiewicz et. al, 2008 ; Augustin et. al, 2010 ; Bhojani et. al, 2009 ; Bhojani and Salomon et. al, 2009 ; Fanning et. al, 2009 ; Xiao et. al, 2011 ; Yu et. al, 2010).The Partin table uses clinical stage based on digital rectal exam (DRE), Gleason score (GS) of the prostate needle biopsy (Gleason et. al, 1966 ; Epstein et. al, 2005 ;

Epstein et. al, ; Albertsen et. al, 2005 ; Albertsen, 2007), and serum prostate specific antigen (PSA) to predict stage at RP. PCa stage indicates the extent or location of the cancer, and can be categorized as; organ confined (OC), extracapsular extension (ECE), seminal vesicle invasion (SVI) and /or lymph node involvement (LNI). Nonorgan confined (NOC) disease represents any stage which extends beyond the prostate organ, i.e. ECE, SVI or LNI.

TREATMENT OPTIONS OF PROSTATE CANCER

There are dilemmas associated with the diagnosis and prognosis of PCa which has lead to the over diagnosis and over treatment of the disease (Oon et. al, 2011). However, new treatments such as active surveillance are being introduced to overcome these issues (Berglund et. al, 2008 ; Carter et. al, 2011 ; Etzioni et. al, 2002 ; Tosoian et. al, 2011).The treatment of prostate cancer has extended in recent years from use predominantly in symptomatic metastatic disease to asymptomatic metastatic disease, primary therapy in localised disease when men are considered unfit for surgery or radiotherapy, adjunct treatment in high risk disease treated with radiotherapy and salvage therapy following a biochemical relapse after surgery or radiotherapy for presumed localised disease (Abrahamsson, 2010; Engel et. al, 2010; Roach et. al, 2008; Studer et. al, 2008).

Chemotherapy

In the past, some phase II trials of PC patients with Neuroendocrine differentiation (NED) have evaluated, at various tumor stages, the efficacy of single drug, as oral estramustine (Zaky Ahel et. al, 2001) or the combination of different cytotoxic drugs, for example, the association of cisplatin, etoposide and doxorubicin (Papandreou et. al, 2002) or the association of cisplatin and docetaxel (Culine et. al, 2007).

Somatostatin Analogs

It was shown that probable anti-tumoral effect of somatostatin analogs might be for the inhibition of angiogenesis and proliferation and for the promotion of apoptosis, linking to somatostatin receptors (SSTR) expressed on secretory (Neuroendocrine) NE, stromal and endothelial prostatic cells (Hansson et. al, 2002 ; Msaouel et. al, 2009).

Radiolabelled Somatostatin Analogs. Treatment with radiolabelled somatostatin analogs is a promising new tool in the management of patients with inoperable or metastasized Neuroendocrine tumors (NET), particularly pancreatic NET, Thus, the use of radiolabelled somatostatin analogs might become an alternative effective therapeutic approach for neuroendocrine prostate cancers (NEPC), especially in patients with metastasized or inoperable tumors (Sansovini et. al, 2013).

Bombesin and Serotonin Antagonists

Some in vitro studies have shown that antibodies against bombesin /GRP and serotonin inhibitors are able to inhibit prostate cell line growth. Therefore, bombesin and serotonin antagonists could become an effective treatment option in the future for NEPC blocking the ability of NE cell secretion and their mitogenic properties (Stangelberger et. al, 2005; Abdul, 1994).

Other therapeutic strategies: Src inhibitor AZD0530, Aurora kinase inhibitors, Suppressors of relaxin receptor RXFP1. mTOR inhibitors, Zoledronic acid, Anti-CSC therapies, EMT blockade, MicroRNA blockade.

APOPTOSIS

Multicellular organisms normally eliminate damaged cells effectively through apoptosis, a controlled cellular mechanism resulting in cell death. The concept of physiological cell death was developed by Kerr et al, (1972) with the publication of a seminal paper on apoptosis. The term apoptosis is derived from the Greek word describing the falling off of petals from a flower or leaves from a tree. It has become clear that apoptosis is a highly conserved mechanism that has evolved to maintain cell numbers and cellular positioning within tissues comprised of different cell compartments (Fadeel & Orrenius, 2005; Khan et. al, 2007).

Characteristic apoptotic features include cell shrinkage, membrane blebbing, chromatin condensation, and formation of a DNA ladder with multiple fragments caused by inter-nucleosomal DNA cleavage finally ending with the engulfment by macrophages or neighboring cells, thereby avoiding an inflammatory response in surrounding tissues (Savill & Fadok, 2000). For the treatment of advanced metastatic PCa and the appearance of therapeutic resistance of prostate tumors, the challenges in the implementation of effective therapeutic strategies involve functional significance of anti-apoptotic pathways (Reynolds & Kyprianou, 2006).

HYPOTHESIS

Vitamin C will impact genistein-induced apoptosis in LNCaP prostate cancer cells.

OBJECTIVE

To determine the potential therapeutic additive effect between genistein and vitamin C.

METHOD

Chemicals and Phytochemicals

Genistein (4', 5' 7- trihydroisoflavone) (Indoline Chemical Co., Summerville, NJ, U.S.A.) will be dissolved in DMSO (Dimethylsulfoxide) solvent as 10, 20, 30, 50, 70, 80, 100 ug/ml solutions and frozen at -37°C obtaining a 100µM stock solution; with the final concentration of solvent not exceeding 0.5%. Previous studies demonstrated that up to 1% DMSO solvent in the final sample will not alter the assay result (Hormann et al., 2012). Stock solution will then be diluted with RPMI-media to produce aliquots in concentrations of 0, 10, 20, 30,40, 50, 70,µM (G_0-G_{70}).This is similar to previous studies, RPMI-media to produce aliquots in concentrations of 0, 10, 20, 30, 40,50 and 70µM (G_0-G_{70}) (Kumi-Diaka, 2002). These will be frozen and stored at 4°C until needed. Vitamin C (Ascorbic acid and Bioflavonoids) at the concentrations of 40uM IC50 was used as combine treatment with genistein.

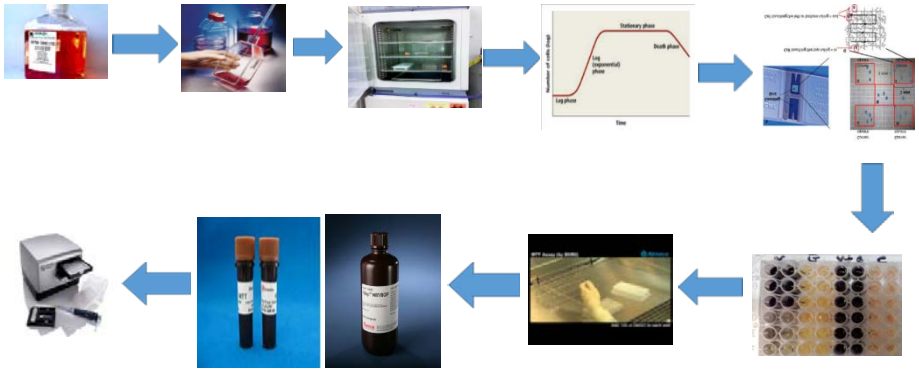

Figure 2. Flow chart of Materials and method

LNCaP cells were cultured in $25m^2$ culture flask and placed in the incubator at 37^oC and 5% CO_2 to grow. After the cells reached their log phase, trypan blue assay was carried out.Based on the result from the trypan blue assay, $1x10^4$ cells were seeded in each well of the 96-well micrtiter plate and the microtiter plate placed in the incubator.After 24 hours the cells adhere to the surface of the plates and are now at 80% confluence on the 4^{th} day of cell culture.On the 5^{th} day, the LNCaP cells were assayed using the NBT solution to access Superoxide Dismutase activities going on in the cells and MTT solution to access the anti-proliferative effect of the three treatment groups.

Cell Cultures and Culture Conditions

In order to determine the chemo-sensitivity LNCaP Cell line to single and combine treatment with genistein and /or vitamin C, cells were incubated at 37°C and 5% CO_2 for 24-48 hours to reach 80% confluence. Human prostate cancer LNCaP cell line will be obtained from the American Type Culture Collection (Manassas, VA).Cell line will be grown in RPMI 1640 containing 15mM HEPES and maintained as monolayers in $25m^2$ tissue culture flasks (Sigma Scientific, St. Louis, MO, USA).

They will also be supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 10% fetal bovine serum, and 100µg/ml L-glutamine (Sigma Scientific, St. Louis, MO, USA).

Trypan Blue Exclusion Assay

Trypan blue exclusion assay was carried out on cells before and after treatments. This assay usually performs two major functions; can be used to assess cell viability and also estimate the average number of cells per ml in the culture. The Trypan blue is a ~960 Daltons molecule that has impermeable cell membrane and therefore only cells with

compromised membranes can pass through it. Upon entry it binds to intracellular proteins thereby rendering the cells bluish in color. It therefore allows for direct identification and enumeration of live (unstained) and dead (blue) cells in a given population of cells. It is a fast and straight-forward test that can estimate cell death, but fail to differentiate between necrotic and apoptotic cells, since both will pick up the dye.

In summary, a cocktail of 0.01ml trypan blue solution with 0.01 of cell culture suspension will be created and allowed to incubate at 25°C (room temperature) for 2 minutes. A drop of this cell mixture will be loaded into both chamber of a hemocytometer and cells will be counted in duplicates under the light microscope (Olympus BH2) at 200X. Before treatment with the phytochemicals, a trypan blue exclusion assay will be performed on cell culture to assess viability and number of cells/ml of the culture. The calculated number of cells per ml of culture is used to determine the volume of cell suspension to be distributed into each well of the micropipette plate. An average of 1.0 $\times 10^4$ cells per well in 48-well microtiter plates (MTP) will be used for subsequent experiments. The percentage post-treatment live cells was estimated as:

% of live (Recovery rate) = Live cells counted /Total cells counted x 100%

Equation 1 Percentage of live cells

% cell viability (%) = Number of viable cells(unstained cells)/Total number of cells X100.

Equation 2 Percentage cell viability

MTT Assay

In order to determine the anti-proliferative or growth inhibitory effects of genistein and vitamin C treatments on 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 each wells.

Concisely, LNCaP cells will be plated onto 96 well plate each at a density of 1.0 $\times 10^4$ cells per well. The cells will be treated with graded doses of Genistein ($Gn_{0\text{-}70}$ and /or vitamin C ($VC_{10\text{-}70}$) for 24 hours.20µL of MTT (2.5 mg/ml in PBS-phosphate buffered saline) (Sigma Scientific Chemical Co., St Louis, MO, USA) solution will be added to each well and incubated for 4 hours at 37°C and 5% CO_2 to allow for the reduction of MTT into formazan by viable cells. The insoluble formazan crystals formed will be solubilized or lysed to release the formazan product by adding 100µL of lysing solution (DMSO) to each pellet. Cells from each well will be harvested (by vigorous aspiration with the micropipette), transferred to a labeled microcentrifuge tube and centrifuged for 10 minutes at 5000rpm. Any unincorporated MTT dye will be removed by discarding the supernatant. The contents of each tube will be transferred into different wells of a 96-well microtiter plate. The absorbance values will be measured at 490nm and

630nm 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 will be calculated as shown:

% Cell Survival Rate (CSR) = ((AT – AB))/((AC-AB))× 100

Equation 3 Percentage cell survival

Where AC = absorbance of the control (mean value), AT = absorbance of the treated cells (mean value), and AB= absorbance of the blank (mean value). For histomorphological studies, the various cells will be treated as described, and microphotographed directly under inverted microscope using a digital camera (Nikon: Coolpix VR & ISO 2000, Japan).

IC-50 of vitamin C =40uM, IC-50 of genistein = 28uM, Higher concentration of vitamin C is needed to kill 50% of LNCaP cells.

Nitroblue Tetrazolium (Reactive Oxygen Species) Assay

Cells have a variety of defense mechanisms to ameliorate the harmful effects of reactive oxygen species (ROS). Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2), while the catalase and peroxidases convert H_2O_2 to water and as such is one of the most important anti-oxidative enzymes that provides important defense against the toxicity of the superoxide radicals (Dominguez-Rodriguez et al., 2001; Zhao et al., 2001). In this assay, superoxide ions (O_2^-) and/or hydrogen peroxides generated by X-rays and/ or

graded genistein treatments reduce or convert NBT to NBT diformazan. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT diformazan is a measure of SOD activity present in the LNCaP 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 (optical density) reflects an increased level of intracellular ROS produced by the LNCaP cells.

Concisely, LNCaP cells was seeded at 1.0×10^4 cells per well in 96-well microplates and allowed to attach for 24 hours at 37°C and 5% CO_2. Relevant plates was treated as discussed previously. NBT (1mg/mL) in HBSS medium was added to the wells 24 hours after treatment, and incubated for 4 hours at 37°C in the dark. Following incubation, cells was trypsinized and counted. Finally, samples was diluted to equal cell quantities in PBS buffer and centrifuged. Pellets collected was washed four times in DMSO, to lyse the cell membrane and the supernatant was collected. SOD/ROS production levels were quantified using an absorbance microplate reader at 490nm. The results will be expressed as relative percentage of SOD or ROS (SOD inhibition).

Cell Death Detection Assay

Fluorescence Assay using Acridine Orange/ Ethidium Bromide. It is based on the differential staining of viable/apoptotic cells in mixture of acridine orange/ethidium bromide. The Acridine orange/Ethidium bromide fluorescence assay was used to differentiate between living, apoptotic and necrotic cells. The two dyes function simultaneously to emit different fluorescent spectra. Acridine orange permeates both

viable and non-viable cells, causing the nuclei to emit green fluorescence. The assay becomes differential with the use of Ethidium bromide, since absorption is based on the lack of cell membrane integrity; therefore it selectively stains the nuclei of dead (non-living) cells to produce red fluorescence. Cells that emit orange/brown colored fluorescence are indicative of apoptosis, while necrotic cells emit red fluorescence. This assay can be used for the qualitative and quantitative assessment of cell death.

In summary, Ethidium bromide (25µl) and Acridine orange (75µl) reagents will be combined in a cocktail; of which 1µL will be added to 25µl cell suspension, previously washed (to remove phytochemical treatment and background artifacts) and re-suspended in PBS buffer (25µl)). This will thereafter be incubated at room temperature in a dark cabinet for 2 minutes. After, 10µl of cocktail will be transferred onto a microscope slide, covered with a cover slip and analyzed under a fluorescent microscope with a band-pass filter (Excitation range from 450-490).Detection of apoptosis will be based on morphological and fluorescent characteristics of stained cells. Viable cells are indicated by bright green color, apoptotic cells by yellow/orange/brown, and necrotic cells by red. Quantitative assessment of Cell death was made by counting a minimum of 50 cells in 7-9 fields of view per slide (James Kumi Diaka et al., 2015).For each relevant treatment regimen, fluorescence microphotographed pictures will be taken directly from under the fluorescence microscope using a digital camera (Nikon: Coolpix VR & ISO 2000, Japan).

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.

RESULT

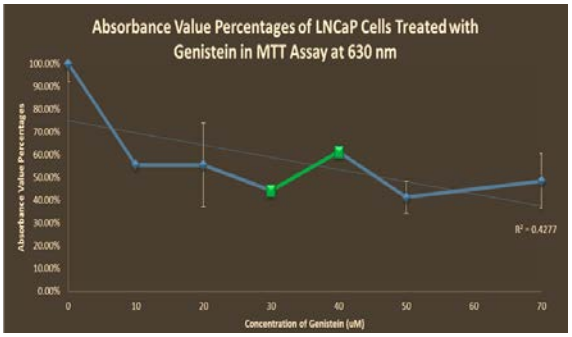

Figure 3. MTT Assay graph for genistein single treatment. The cells were treated with varying concentrations of ($Gn_0 - Gn_{70}$) for 24hrs. The linear regression analysis for each treatment showed that there was a negative correlation percent viability and treatment concentrations ($P < 0.05$). Data are the mean ± SD of two independent experiment performed in triplicate. Bar= SD.

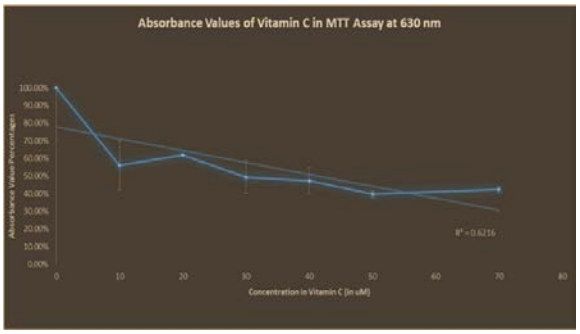

Figure 4. MTT Assay graph for vitamin C single treatment. Growth and viability of LNCaP cells was assessed using the MTT assay. The cells were treated with varying concentrations of ($VitC_{0-70}$). The linear regression analysis for each treatment showed that there was a negative correlation percent viability and treatment concentrations ($P < 0.05$). Data are the mean ± SD of two independent experiments performed in triplicate. Bar= SD.

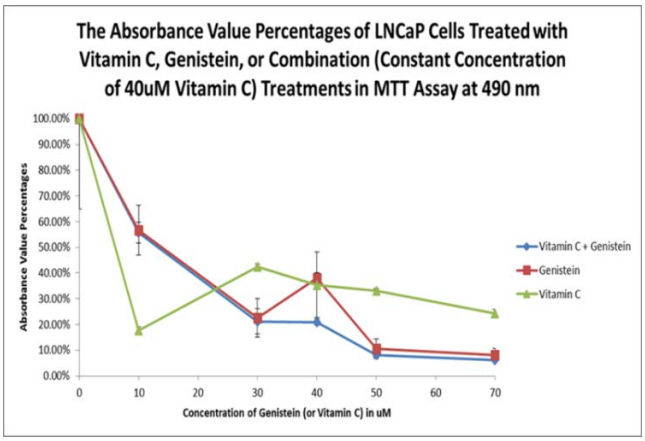

Figure 5. MTT Assay graph for the three treatment groups.Growth and viability of LNCaP cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein (Gn_{0-70}), Vitamin C ($Vit\ C_{0-70}$) and genistein-vitamin C combination for 24hrs as described in the methods. Data are the mean ± SD of two independent experiments performed in triplicate. Bar=Standard Deviation.

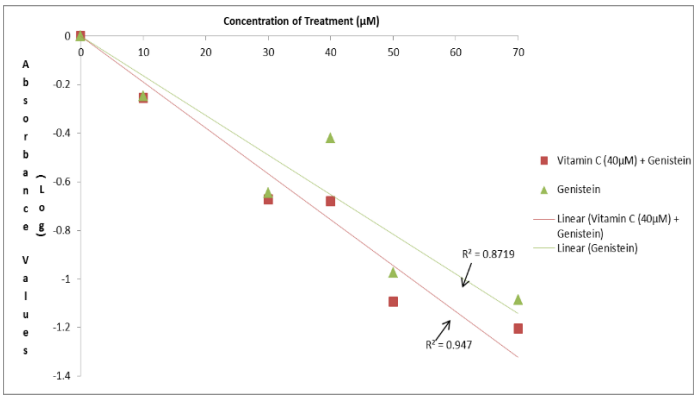

Figure 6. Linear regression graph of the MTT results for treatment groups (

vitamin C, genistein and genistein-vitamin C combination). The linear regression indicate the slope for single and combination treatment groups. The p-value of the linear regression performed = 0.11.The single treatment (Gn_{0-70uM}) and combination treatment of genistein (Gn_{0-70uM} + Vitamin 40uM) impacted the same level of anti-proliferative effect on LNCaP prostate cancer cells.

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 data points were from the best and consistent independent experiment performed in triplicates. The bar chart in fig.10 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. This is shown in Table 1

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

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

Note: % apoptosis to % necrosis is a method for comparing the effectiveness of single treatment of genistein and combination treatment(genistein + vitamin C) in impacting apoptosis on LNCaP prostate cancer cells. The number of apoptosis, necrosis and living cells was counted from the microphotograph picture after florescence assay.50 cells was counted in three different fields and the average was calculated. The average was converted to percentage and the ratio of apoptosis to necrosis was calculated for the genistein treatment and the genistein-vitamin C combination treatments.

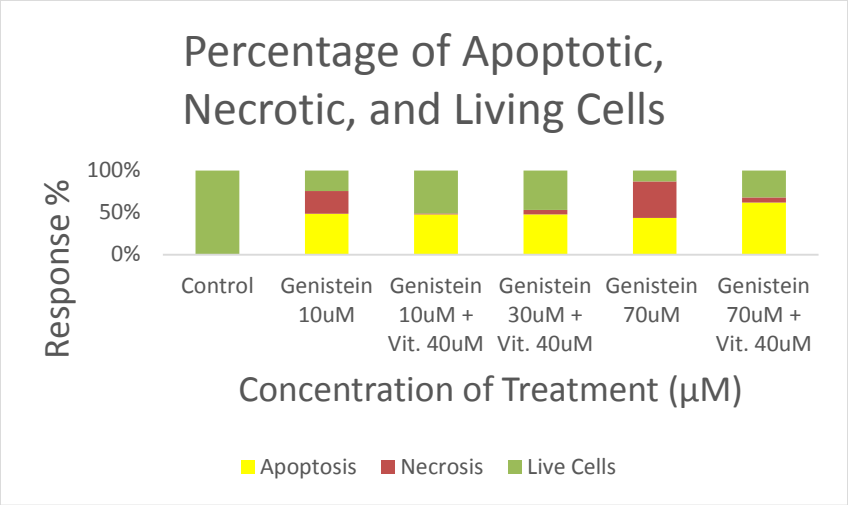

Figure 7. Percentage apoptosis, necrosis and living cells in all the treatment groups.Apoptosis in LNCaP prostate cancer cells were assessed using the EtBr/AcrO florescence assay. The cells were exposed to varying concentrations of genistein ($Gn_{0\text{-}70uM}$) for 24hrs at 37^{o}C, 5% CO_2. Data are the mean ±SD(Standard Deviation) of two independent experiments performed in triplicate. Bar=SD. The p-value of the chi-square for comparing mode of cell death was = 0.0003.

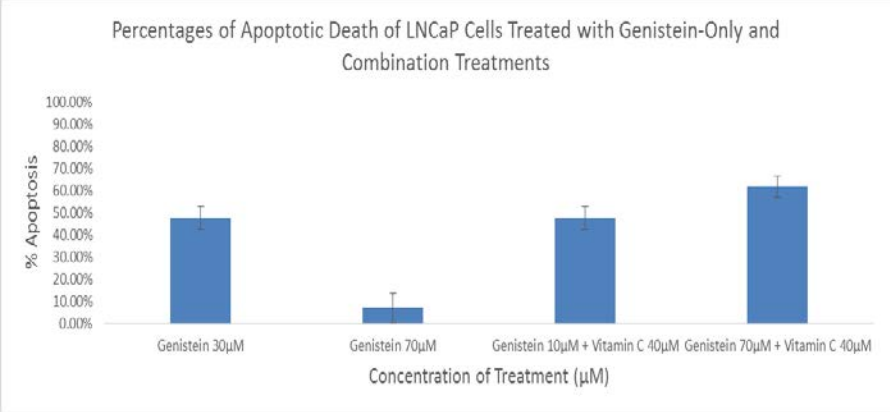

Figure 8. Percentage of Apoptosis in single and combination treatment. Percentage of Apoptosis in single and combination treatment.P-value of chi-square test comparing the single treatment of 70uM genistein with the combination treatment of Genistein 70uM + Vitamin C 40uM = 0.0164, at significance level of 0.05.This result further indicate that there is significant difference in the apoptotic induction in the single treatment and combination treatment.

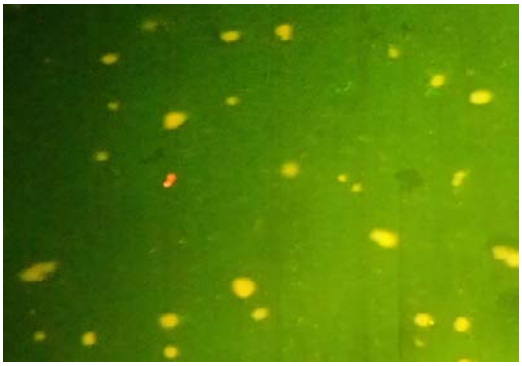

Figure 9. Microphotograph picture of florescence assay-combination 70uM + Vitamin C 40uM.

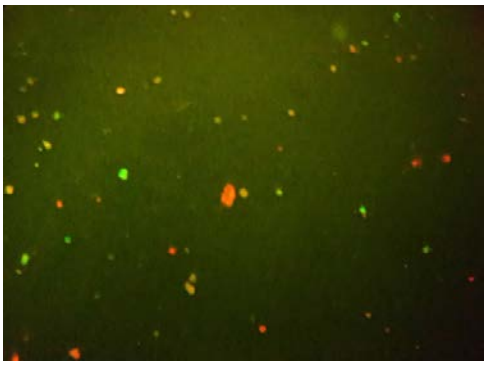

Figure 10. Microphotograph picture of florescence assay gensitein single treatment (70uM).

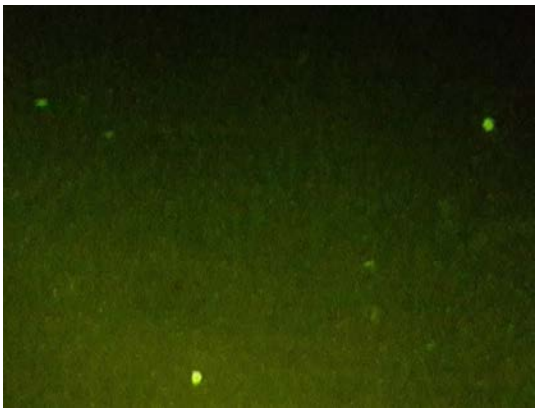

Figure 11. Microphotograph picture of florescence assay-Control.

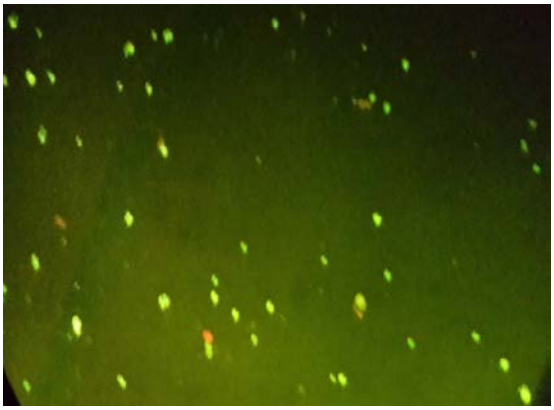

Figure 12. Mirophotograph picture of Florescence assay-combination (10uM + Vitamin C 40uM).

Apoptosis is involved in genistein-induced Chemo-enhancement of LNCaP cell line. In order to confirm that apoptosis was induced by genistein and /or vitamin C at IC 50 concentrations of 40µM and 25µM respectively LNCaP cells were analyzed in the presence of acridine orange/ethidium bromide staining (AO/EB staining). As a control, LNCaP cells were cultured in RPMI 1640 growth media and stained with AO/EB. Combination treatment of Genistein and vitamin C at all concentrations induced

apoptosis after 24 hours of incubation. Cells stained stained green represent viable cells, whereas yellow stained cells denote early apoptotic cells, and reddish cells denote necrotic cells.

Genistein and/or vitamin C alters the Intracellular levels of ROS and SOD in LNCaP cells: Intracellular % ROS levels were analyzed using Nitroblue Tetrazolium (NBT)-ROS assay based on the mechanism of SOD inhibition. NBT reduction is used as an indicator of superoxide ion (free radical) production. When compared with the control treatment, all treatment regimens had decreased ROS levels.This can be observed in figure 13 and 14.

NBT Assay

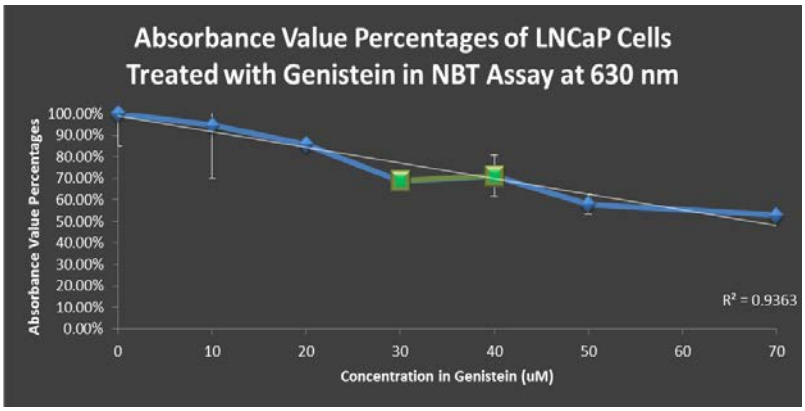

Figure 13. NBT Assay graph of genistein treated LNCaP cells. Concisely, LNCaP cells was be seeded at 1.0×10^4 cells per well in 96-well microplates and allowed to attach for 24 hours at 37°C and 5% CO_2. NBT (1mg/mL) in HBSS medium was added to the wells 24 hours after treatment, and incubated for 4 hours at 37°C in the dark. After incubation, cells was trypsinized and counted.

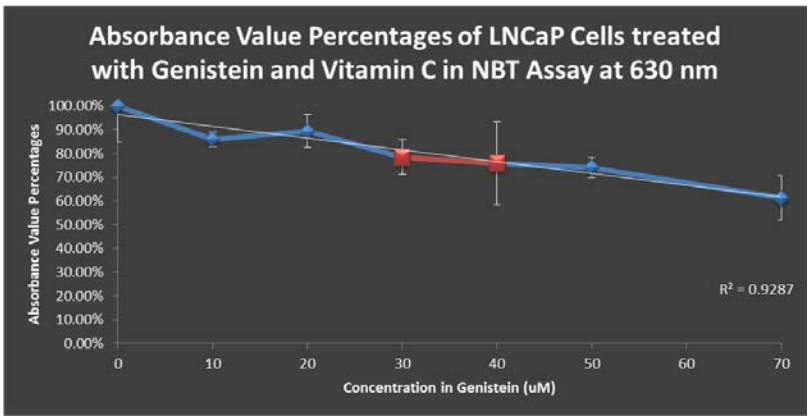

Figure 14. Absorbance value of LNCaP cells treated with combination treatment. Concisely, LNCaP cells was be seeded at 1.0×10^4 cells per well in 96-well microplates and allowed to attach for 24 hours at 37°C and 5% CO_2. NBT (1mg/mL) in HBSS medium was added to the wells 24 hours after treatment, and incubated for 4 hours at 37°C in the dark. After incubation, cells was trypsinized and counted.

DISCUSSION

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 and apoptosis in LNCaP cells. This study suggest that the combination treatments are more effective than single treatments in apoptosis induction. Morphological changes induced by the treatments in the cells (denote cell differentiation, inhibition and cell death) were consistent with previously observed results (Kumi Diaka & Butler, 2000).Prostate cancer is known to be an age dependent disease that can be caused by increased oxidative stress in older men (Ferlay et. al, 2013). Cancer cells are known to produce high levels of ROS and survive under physiologic oxidative stress (Finkel & Holbrook, 2000). For these cancer cells to survive, they must maintain a balanced redox signals for cellular proliferation (Martin & Barrett, 2002). However, any pathologic change in the redox signaling system, such as overproduction or underproduction of ROS beyond the physiologic state may results in a significant increase in pathologic oxidative stress, which leads to growth inhibition and/or cell death (Finkel & Holbrook, 2000).

An apparent chemo-preventive solution, involving the sufficient consumption of dietary supplements, including phytochemicals to reverse or inhibit the process of carcinogenesis (Tsuda et. al, 2004) have shown great potential in preventing the occurrence of cancer and other chronic diseases that result from oxidative stress induced by free radicals (Abdulla & Gruber, 2000). Thus, chemo-preventive interventions with

combination therapies-drugs, diets or low dose radiation-that slow down or inhibit the growth and progression of these small tumors are potentially very effective in reducing the burden of prostate cancers in the population, particularly if these treatments also prevent the de novo development of new prostatic malignancies (Kelloff et. al, 1999).Hence the reason for combination treatement(genistein + vitamin C) used in this study.

MTT assay has been used by many researchers to measure chemo-sensitivity in human tumor cell lines (Kumi-Diaka et. al, 2004). Like most flavonoids, Genistein is known to exhibit dual redox activity at different treatment conditions. Genistein have been demonstrated in prostate cancer cells to have anti-cancer activities in both low and high dose concentrations against both androgen-dependent (LNCaP) and androgen-independent (PC3) human prostate carcinoma cell lines (Hormann et. al, 2012; Kumi-Diaka et. al, 2004). At high or low dose concentrations, they are known to exhibit pro-oxidant and antioxidant activities respectively. Both mechanisms have been indicated to increase the antitumor activity of genistein (Hormann et. al, 2012). In the current study, at low concentration (10μM) of genistein, we observed reductions in the intracellular ROS generation and increase in SOD. Interestingly, this is consistent with most published reports on anti-cancer properties of genistein and other polyphenolic phytochemical compounds in modifying ROS production and activity (Hormann et. al, 2012).

The rapid development in cell and molecular biology has increased the understanding of programmed cell death (apoptosis) (Hu & John, 2003). Elimination of cells by apoptosis can potentially result in a decrease in damaged cells or transformed cells; resulting in a protective effect (Evan & Vousden, 2001). This observation is

consistent with the results of our study. The overall data indicate that LNCaP cells are susceptible to death by apoptosis in response to genistein treatment and combination of genistein + vitamin C. Most of the cellular deaths observed in the combination treatment groups are apoptosis, as revealed by the acridine orange/ ethidium bromide fluorescence assay(chi- square test $p < 0.05$ and comparing the % apoptosis to % necrosis death in all treatment groups).This is consistent with previous studies on genistein (Hormann et. al, 2012; Kumi-Diaka et. al, 2004). The data also demonstrated the additive or potential benefit of genistein and vitamin C combination to growth inhibition and ultimately cell death by apoptosis.

Fluorescence assay shows different percentage of apoptotic death at different combination concentrations. Apoptosis induction was the main mode of treatment induced cell death. The overall data showed the magnitude of treatment efficacy as; (Gen 10uM + Vit C 40uM) > (Gen 30uM + Vit C 40uM) > (Gen 70uM + Vit C 40uM) >10uM genistein > 70uM genistein. The chi-square test for comparing necrosis, apoptosis and life cells showed that the p value= 0.0003; Vitamin C could impact genistein-induced apoptosis in LNCaP cells.

CONCLUSION

Combination treatment(Gn-Vit C) was more effective in apoptosis induction. This result indicate the potential clinical significance of vitamin C supplementation on genistein action as a chemo-preventive drug. In accordance with (Tsuda H, et al. , 2004). This study forms potential basis for in vivo studies of the impact of vitamin C on genistein-induced apoptosis in LNCaP cells.

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