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Therapeutic efficacy of Genistein-Cytoreg® combination in breast cancer cells

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ABSTRACT:

Background: In spite the heavy investments in therapeutic research breast cancer still impacts the lives of women globally. The projected incidence of new cases of in situ breast cancer in the USA for 2011 is 57,650, with estimated 39,520 deaths. The phytoestrogen, genistein and the synthetic compound, Cytoreg® have been shown to inhibit growth and proliferation in many cancer cell lines.

Purpose of the Study: In this study, we investigated the therapeutic efficacy of Cytoreg®-genistein combination on growth inhibition in the MCF-7 human breast cancer cells.

Method: MCF-7 cells were treated with genistein and Cytoreg® single and combination treatments for 24-48hrs; and post treatment chemosensitivity assessed, using: Trypan Blue exclusion and MTT assays for cell viability, Ethidium bromide/Acridine orange to assess apoptosis induction, and FAM Poly-Caspase binding assay for mechanism of action.

Results: The overall data indicated dose- and time- dependent cell death in the MCF-cells and apoptosis as the major means of treatment-induced growth inhibition with all the treatment regimens.

Conclusion: Comparatively, the genistein-Cytoreg® combination treatment was significantly more efficacious in growth inhibition in the MCF cells than either genistein or Cytoreg® alone. Genistein seems to act additively with Cytoreg® in combination treatment-induced apoptosis in

MCF-7 cells. The normal human breast epithelial cells were not significantly inhibited by either single or the combination treatments.

Key words: Cytoreg®, Genistein, Combination treatment, MCF- cancer cells, apoptosis

INTRODUCTION:

Breast cancer is the most frequently diagnosed cancer in women and is three times more common than all gynecologic malignancies combined. An estimated 230,480 women will be diagnosed with invasive breast cancer in 2011 with an estimated 39,520 deaths [1, 9]. The factors predicting the development of breast cancer are poorly understood and it is difficult to determine why one individual is more susceptible to developing breast cancer than another [2]. Known risk factors in breast cancer include: environmental effects, lifestyle, race/ethnicity, age, medical, and genetic predisposition [22, 30]. Standard treatment regimens include chemotherapy, radiation therapy and surgery, the choice of which depends on the stage of progression of the disease. However, these treatments are flawed with mild to severe and often fatal, side effects due to cytotoxicity. Currently attention is focused on phytochemicals as potential monotherapy or adjuvant to chemotherapy and/or radiation therapy [3]. Two therapeutic agents currently under study are genestein isoflavone, a phytochemical, and the chemical, Cytoreg®.

Genistein is an isoflavone found most commonly in soy products and displays structural similarities to estrogen. Isoflavones benefit humans in four ways: as estrogens and anti-estrogens, as cancer-enzyme inhibitors, as antioxidants, and as immune system enhancers or stimulants [28]. The mechanism by which genistein induces cell proliferation is still unclear; however, studies involving genistein have shown that phytochemicals can act in conjunction with other agents to inhibit the growth of cancer cells [5, 6, 10]. Genistein can bind to estrogen receptors, regulate gene expression and display both estrogen agonist and antagonist properties [28]. This ability may allow genistein to control cell growth, thus preventing growth and proliferation of hormone dependent tumors [2, 5]. Several mechanisms of action which may contribute to its anticancer properties include: apoptosis upregulation, angiogenesis inhibition, DNA topoisomerase II inhibition and protein tyrosine kinase inhibition [11]. Existing data shows that genistein also exhibits a concentration dependent bi-phasic effect of ER-□ positive/estrogen-dependent cells such as MCF-7 [7, 17].

Cytoreg® is a novel, synthetic, anti-tumor, pro-apoptotic, therapeutic agent that is pharmacologically active and demonstrates a low pH (pH < 1.0) of which hydrofluoric (HF), hydrochloric (HCL) and sulfuric acids (H_2SO_4) are active principles [8, 13, 27]. Cytoreg® also acts as a cellular regulator and antioxidant agent [8, 11, 13]. Greater penetration and increased regulation of cellular activity is achieved because Cytoreg® has a low molecular weight and high oxidative activity with consequent, greater efficacy [8, 13, 27].

Cytoreg® functions to stimulate the immune system by the presence of the fluoride ion, which induces the production of IL-1, IL-6, TNF-α, macrophages and granulocytes [8, 13, 27]. One desirable characteristic is its ability to cross the cell membrane of both quiescent and proliferating cells. Presently, most therapeutic regimens are able to treat proliferating cells only.

Existing data shows a dose- and time- dependent growth inhibition in MCF-7 breast cancer cells with significant differences ($P<0.05$) in chemosensitivity between the different breast cancer cell lines [8, 11, 13]. Furthermore, both hormone sensitive and hormone independent tumors display susceptibility to Cytoreg®-induced growth inhibition, implying that the action of Cytoreg® does not appear to be directly related to hormone regulation activities [8, 13].

Previous studies involving genistein and Cytoreg® combination have shown that the presence of Cytoreg® in the combination appears to enhance the therapeutic efficacy of genistein in an additive manner in PC3 and LNCaP cell lines [17, 25]. Genistein-arrested cancer cell growth occurs at the G2/M phase of the cell cycle [5, 11]. Cytoreg® enters both proliferating and non-proliferating cells, disrupting the mitochondrial transmembrane, leading to the release of cytochrome c and the initiation of apoptosis. The modus operandi of both treatments differ and this difference allows them to achieve a higher therapeutic efficacy when used in combination [8, 10, 12]. The present study is in conformity with the previous observation.

MATERIALS AND METHODS:

Materials

Cell lines and culture medium: Normal Human Breast Epithelial Cells (NHBC), and MCF-7 human, breast, adenocarcinoma cell line (ATCC, Manassas, Virginia USA) were utilized in this study. The cells were maintained in RPMI-1640 medium (Sigma-Aldrich Chemical Co., St Louis MO USA), supplemented with 10% fetal bovine serum (FBS) and100 IU/ml of penicillin and 100µg/ml of streptomycin (Sigma Aldrich). MCF-7 cells are well-characterized oestrogen receptor positive (ER+) cells, and therefore are useful in vitro model for hormone-dependent breast cancer studies. MCF-7 cells are also HER2/*neu* positive, positive for cytokeratin and negative for desmin, endothelin, GFAP (Glial Fibrillary Acidic Protein), neurofilament and vimentin; NHBC (ER+) normal human breast cancer cells.

Reagents and Bioassays: Dimethylsulfoxide (DMSO); MTT (3-(4, 5-dimethyl-thiozol-2-ayl)-2, 5-diphenyl tetrazolium bromide); Ethidium Bromide (EtBr), Acridine Orange (AcrO) were purchased from Sigma Aldrich Chemical Co.; and FAM Poly-caspase (Invitrogen).

Test agents:

i) ***Genistein isoflavone*** (4', 5', 7-trihydroisoflavone) (Indofine Chemical Co, Summerville, NJ, USA). Genistein isoflavone was constituted in DMSO solvent to make a stock solution of 10,000µM, from which aliquots of working concentrations of 10, 20, 30, 40, 50 and 60µM (G_{10-60}) were made.

ii) ***Cytoreg*®** (complement of Cytorex Biosciences Inc.) was supplied as a stock solution. A series of dilutions were prepared at six cytotoxic dose ranges of 0.0005, 0.001, 0.002, 0.005, 0.01, and 0.02µg/ml ($Cyt_{.0005 - 0.02}$) for single treatments. Combination treatments consisted of varying concentrations of genistein and Cytoreg® as shown in Table 1.

Table 1 Chart of combination treatment ($G_{10}/Cyt_{0.0005}$–$G_{60}/Cyt_{0.02}$)

Combinations	1	2	3	4	5	6
Genistein μM	10	20	30	40	50	60
Cytoreg® μg/ml	0.0005	0.001	0.002	0.005	0.01	0.02

The combination treatment was chosen based on preliminary studies of several combinations to choose the most efficacious and rational EC50.

Methods:

Cell culture and treatment

MCF-7 and NHBC cells were separately cultured in 75cm^3 flasks at 37°C, 5% CO_2, and 89% humidity, to achieve 80 - 90% confluence. The cells were harvested, centrifuged and reconstituted into suspension with fresh RPMI 1640 media. Then 1.5 x 10^3 cells (MCF-7 or NHBC) in 100□L were dispensed into each well of 96-well microtiter plate (MTP) and cultured for two days to allow adherence and 80-90% confluence. The supernatants were discarded and adhered cells were directly exposed to varying concentrations of the treatment agents as follows: i) various concentrations of genistein (Gn_{10-60}) in triplicates; ii) various concentrations/dilutions of Cytoreg® ($Cyt_{0.0005\ -0.02}$) in triplicates, and iii) various concentrations of the Genistein-Cytoreg® combination (Gn_{10-60}+$Cyt_{.0005-.02}$) as in Table1, in triplicates. The MTPs were incubated for 24 to 48 hr. At each time point, the cells were processed with the various bioassays as below:

The rationale for choosing MCF-7 cells: We have repeated these experiments in other breast cancer cell lines (GI-101 and MDA-MB-435S), and the results were similar. MCF-7 expresses the estrogen receptor and is hormone responsive. It is one of the classical cell line models used in breast cancer research.

Cell proliferation Assay: Post-treatment determination of cell proliferation was performed using standard MTT tetrazolium ELISA calorimetric assay as previously described [4, 11]. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay, assesses the metabolic status of cells.

The validity and consistency of the result was assessed using the standard Trypan Blue exclusion – hemocytometer counting assay [11]. For morphological studies, the cells were treated as described, and micro-photographed directly under inverted microscope using a digital camera (Nikon: Coolpix VR & iso 2000, Japan).

Ethidium Bromide/Acridine Orange assay: AcrO/EtBr assay was used to determine the presence of apoptosis. Briefly the cells were cultured and treated as described previously [29]. The cells were harvested, centrifuged and resuspended in Dulbecco's phosphate buffered saline for EtBr-AcrO staining [29]. A fresh cocktail of EtBr (10μl) and AcrO (75μl) was prepared

according to the manufacturer's protocol. About 1.5µl of EtBr-AcrO cocktail was added to 25µl of cell suspension and the mixture incubated in the dark, at room temperature for 2 minutes. 10µl of the stained cells were transferred onto microscopic slides and examined under fluorescence microscope for apoptosis, necrotic and/or live cells based on morphologic and fluorescence emission characteristics. Fifty to sixty cells in four different fields per slide were counted and quantitative analysis done for percentage of apoptosis, necrosis and viable cells. Apoptosis cell death was confirmed in parallel experiments using propidium iodide PI nuclear stain [16, 17, 23, 31].

FAM Poly-caspase binding assay: Treatment-induced caspase activation was determined using the FAM Poly-caspase binding assay (FAM Poly-caspase; Invitrogen), which utilizes FAM-VAD-FMK to measure caspase activation in situ. Briefly the cells were cultured and treated as described previously then the caspase assay was carried out according to the manufacturer's protocol. The cells were then examined under fluorescent microscope using a band-pass filter (excitation 490nm, emission>520nm). Quantitative analysis of apoptosis was determined by counting all cells in 4-5 fields per slide, and recording the percentage of viable, apoptotic and necrotic cells based on fluorescent characteristics.

Statistical analysis: All experiments were performed in triplicates, and repeated twice to ensure comparative results. Linear regression analysis was used to identify trends within treatments. Differences between treatments were assessed by ANOVA and significant differences were investigated with post-hoc analysis. Alpha was set at <0.05 for all analyses.

RESULTS:

The effects of genistein and Cytoreg® single treatments on MCF-7 cell growth and proliferation:

MCF-7 cells were treated with various concentrations of genistein or Cytoreg® for up to 48 hr. NHBC were treated for comparison. Both genistein and Cytoreg® had no significant inhibitory effects on NHBC cells at the dose ranges used in this experiment. Slight growth inhibition was manifested at 60µg/ml Cytoreg®.

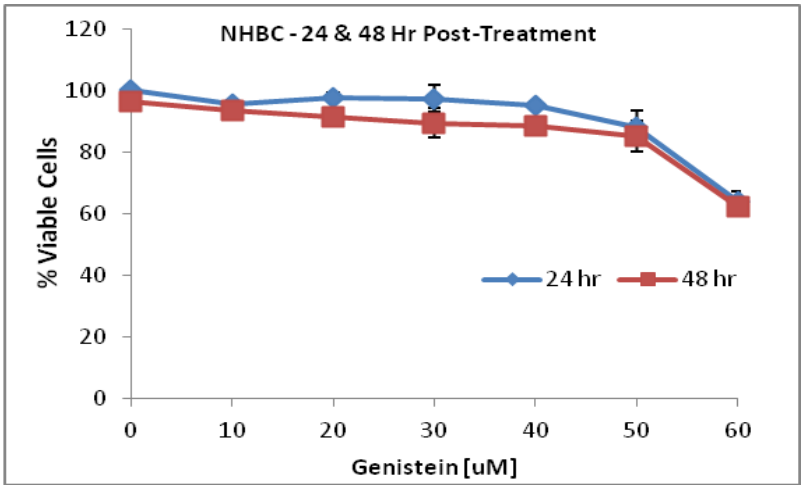

Figure 1. MTT assay was performed to assess the viability and chemosensitivity of normal cells. Cells were exposed to varying concentrations of genistein (Gn_0 – Gn_{60}) for 24 - 48hr, incubated at 37°C, 5% CO_2, and 89% humidity, as previously described. Similar results were observed for Cytoreg® ($Cyt_{0.0005}$ – $Cyt_{0.02}$). Percentage viability between the dosage levels were significantly different ($P<0.05$). Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

A significant positive correlation was observed between cell growth inhibition and dosage of genistein and Cytoreg® ($r^2 = 0.981$; $P< 0.01$). Antiproliferative and cytotoxic effects were observed in the MCF-7 cells at EC_{50} concentrations of 25-30µg/ml and 0.0005-0.005µg/ml respectively for genistein and Cytoreg®, as revealed by the MTT- assay and confirmed by the TB exclusion assay **[Figure 2A-C]**. The MTT assay revealed a dose- and time- dependent relationship between dosage and growth inhibition in both genistein- and Cytoreg® - treated cells; consistent with the data obtained from the Trypan blue exclusion assay (data not shown). In general, at any comparative dosage/concentration, Cytoreg®-induced growth inhibition was significantly higher ($P<0.05$) than genistein-induced growth inhibition as indicated by the MTT assay.

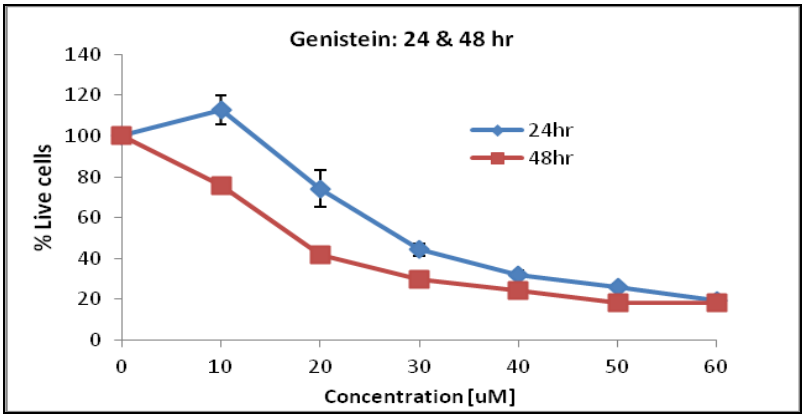

Fig 2A

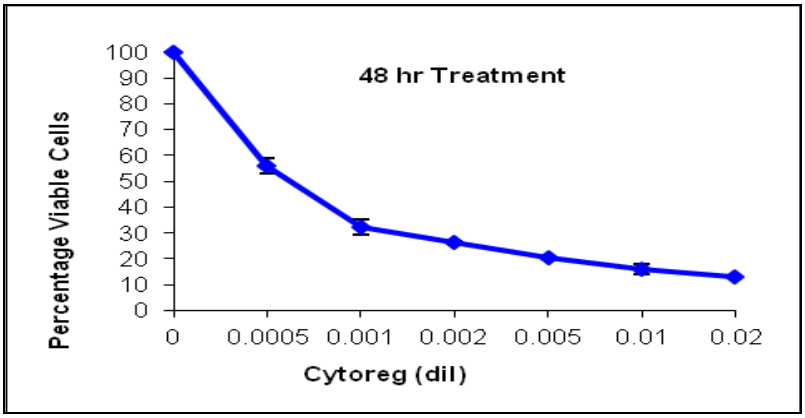

Fig 2B

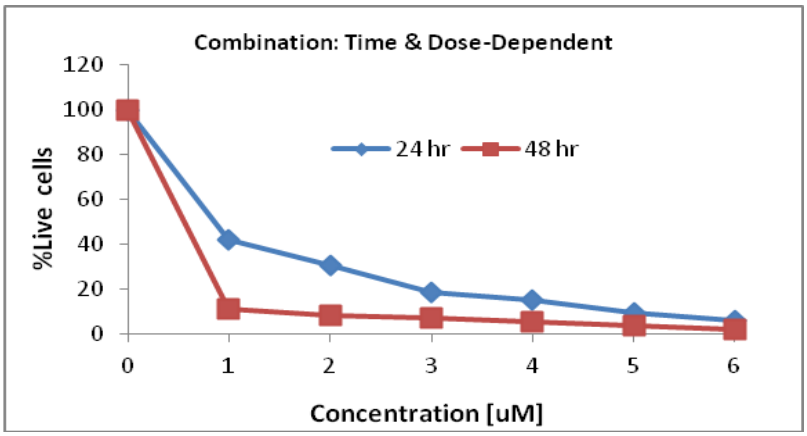

Fig 2C

Figure 2A-C. Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein (Gn_0 – Gn_{60}), Cytoreg® ($Cyt_{0.0005}$ – $Cyt_{0.02}$) and genistein-Cytoreg® combination for 24 - 48hrs; then incubated at 37°C, 5% CO_2, and 89% humidity, as previously described in the experiment. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

The effects of genistein-Cytoreg® combination on MCF-7 cell growth and proliferation: MCF-7 cells were treated with varying concentrations of genistein-Cytoreg® combination (Gn_{10-60}+$Cyt_{.0005-.02}$) for up to 48 hr. The results showed greater growth inhibitory effect ($P < 0.05$) in MCF-7 cells than either of the single treatments with genistein or Cytoreg® at corresponding doses **[Figures 3]**.

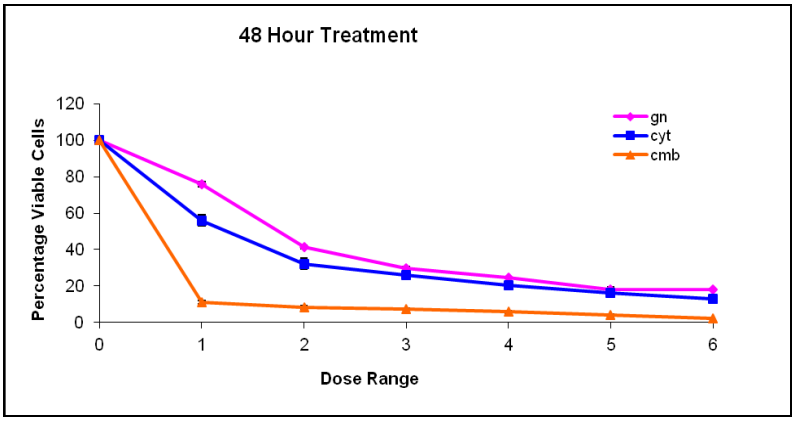

Figure 3. Growth and viability of MCF-7 cells was assessed using the MTT assay. The cells were treated with varying concentrations of genistein (Gn_0 – Gn_{60}), Cytoreg® ($Cyt_{0.0005}$ – $Cyt_{0.02}$) and genistein-Cytoreg® combination for 48hrs; then incubated at 37°C, 5% CO_2, and 89% humidity, as previously described in the experiment. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

Treatment-induced apoptosis in MCF-7 cells: To determine whether any of the treatment regimen induced morphological features of apoptosis in the cells, we stained the cells with EtBr-AcrO after treatment of the MCF-7 cells for 24 and 48 hrs as previously described. The results

indicated that both single and combination treatments induced apoptosis as the main mode of growth inhibition in the cells **[Figures 4 and 5]**. At 48 hr exposure, the combination (Gn_{10-60}+$Cyt_{.0005-.02}$) treatment induced the greatest apoptosis cell death at lowest EC_{50} relative to the single treatments ($P<0.05$) (data not shown). Figures were not shown for Cytorex, as the pattern was similar to that of genistein.

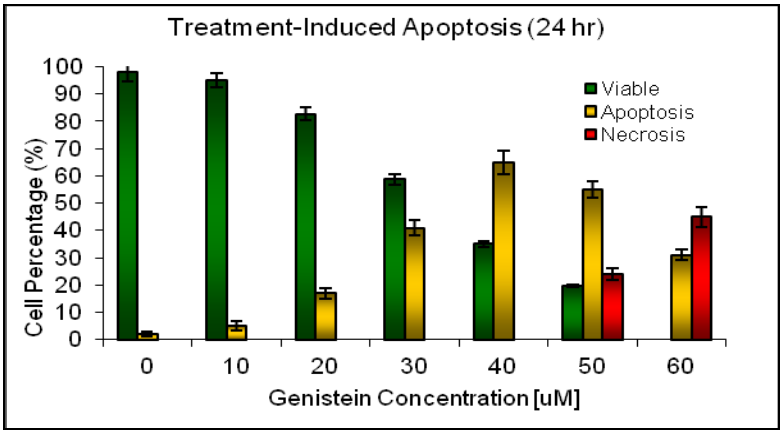

Figure 4. Treatment-induced apoptosis was assessed morphologically using EtBr-AcrO to stain the cells. The cells were treated as previously mentioned.

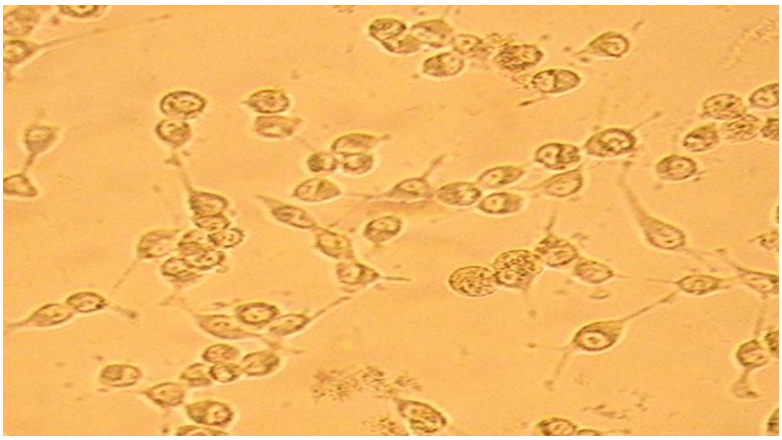

Figure 5A Apoptosis in Cytoreg®-treated cells at 24 hours after treatment

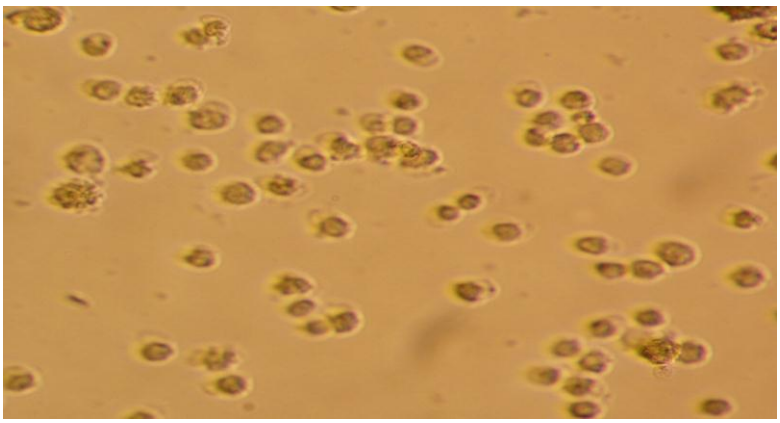

Figure 5B Apoptosis in Combination-treated cells at 24 hours after treatment.

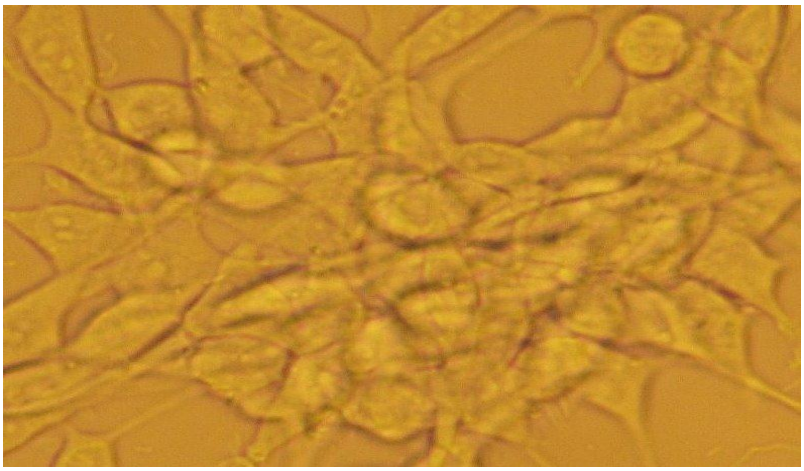

Figure 5C Normal growth pattern of untreated cells

Furthermore, Cytoreg® induced morphological changes in the MC-7 cells consistent with autophagic features **[Figure 6]**. This was not observed in genistein-exposed cells at any dose level.

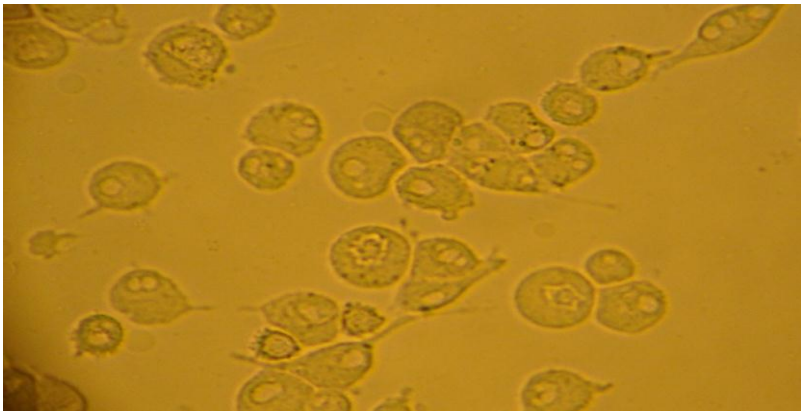

Figure 6 Autophagy and karyorrhexis demonstrated in Cytoreg®-treated cells.

The effects of treatment on caspase protease expression in MCF-7 cells: Caspase expression was determined using FAM Poly-caspase binding assay. The data revealed a dose - dependent response to caspase expression within the cells **[Figure 7].** There was a strong correlation between caspase activity and increased apoptosis induction (r^2=0.981; P<0.05) in both single and combination treatments; implicating caspase protease in the apoptosis induction pathway. In general, the correlation between caspase expression and treatment dose (r^2=0.992; P<0.05) was greatest in the combination treatment relative to the single genistein or Cytoreg® single treatment. Treatment-induced caspase expression was significantly greater (P < 0.01) in the combination treatment than in either single treatment. Whilst genistein and Cytoreg® single treatments showed greatest percentage caspase activity at the higher therapeutic dosages (higher EC_{50}), the combination treatment showed greatest caspase activity at the lower cytotoxic dose ranges (lower EC_{50}); implying that combination treatment was more efficacious and potentially less cytotoxic than single treatments. Graph was not shown for Cytorex as the pattern was similar to genistein.

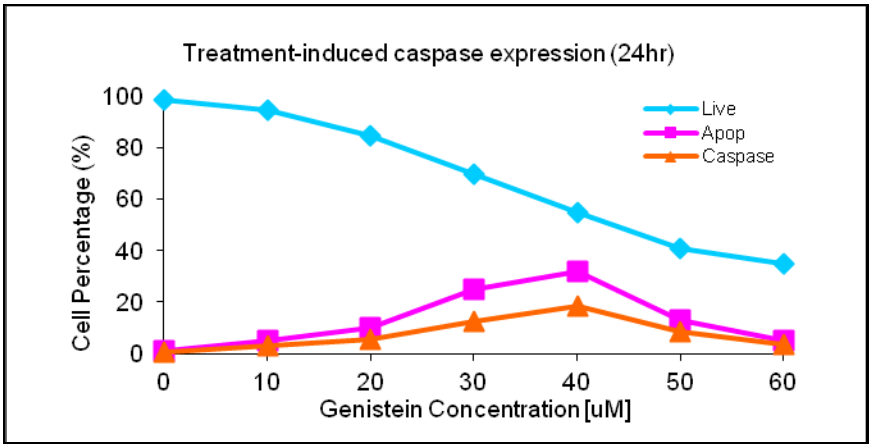

Figure 7. The correlation between apoptosis and caspase activation in MCF-7 cell was assessed using the FAM poly-caspase binding assay. The cells were exposed to varying concentrations of genistein (Gn_0 – Gn_{60}) for 48hr at 37^0C, 5% CO_2 and 89% humidity, as previously described. The pattern of response to Cytoreg® was similar to that of genistein. Data are the mean ± SEM (Standard Error of the Mean) of two independent experiments performed in triplicate. Bar = SEM.

DISCUSSION:

Searching for treatments with the least cytotoxicity and minimal side effects is at the forefront of therapeutic investigations. Current ongoing investigations are targeting phytochemicals and other natural compounds as alternative therapeutic treatments or adjuvant to standard chemotherapy. One such phytochemical with known anti-cancer properties is genistein isoflavone, the main nutrient in soybeans and soy products [5, 12, 18]. The purpose of this study was to investigate if the combination of genistein with Cytoreg®, a synthetic compound, would significantly enhance the anti-tumor activity of genistein in MCF-7 breast cancer cells through apoptosis induction. Our results/data indicated that both genistein and Cytoreg® were cytotoxic to MCF-7 cells in a dose- and time-dependent manner as revealed by MTT stain and confirmed with TB exclusion assay. These findings are consistent with previous observations where both compounds were cytotoxic in PC3 prostate cancer cells in a dose- and time- dependent manner [8]. The data also indicated that the combination of the two was significantly more cytotoxic to the cells at a lower EC_{50} than the single compounds working alone; implying that combination treatment is potentially more efficacious than single treatments.

The AcrO/EtBr analysis revealed treatment-induced apoptosis was the main mode of cell death/growth inhibition in MCF-7 cells. This observation was in conformity with previous studies [19, 29]. The combination treatment yielded the highest percentage of apoptosis at lowest EC_{50}, and consequently the highest growth inhibition in the cell [19, 25]. This implies a greater therapeutic/phyto-preventive potential of the combination treatment over the single treatments [19].

Genistein has the ability to mimic 17-β-estrodiol partly due to structural similarities; and can regulate gene expression by binding to estrogen receptors [4, 7]. This characteristic partially accounts for its ability to influence cell growth, thus inhibiting growth and proliferation of hormone dependent carcinomas [9]. The MCF-7 cell line is an HER2/*neu* positive, estrogen-

dependent human breast cancer cell line. Genistein demonstrates increased effectiveness against MCF-7 cells whereby when genistein-induced apoptosis occurs; this triggers a dose-dependent increase in BRCA1 and BRCA2 protein levels in MCF-7 cells [20]. It appears that the addition of Cytoreg® to genistein additively enhanced/increased the effectiveness of genistein in the MCF-7 cells.

Cytoreg® induced other morphologic features/changes in the MCF-7 cells consistent with autophagy, a form of cellular degradation that leads to lysis of cellular components which in turn may lead to karyorrhexis [22]. Genistein-treated cells did not show any evidence of autophagy at any dosage level used in this study. This observation implies that one of the mechanisms by which Cytoreg®-induced cell death occurs, may be initiation of autophagy, leading to apoptosis and/or necrosis in the cells. We are hypothesizing that autophagy may release apoptotic inducing factors/proteins with subsequent induction of apoptosis; bypassing the caspase-protease signaling pathway. The alternate possibility is that such released factors could cause releasing of caspase protease cascade, leading to induced apoptosis. In previous studies in our laboratory, blocking of caspase did not totally abrogate Cytoreg®-induced apoptosis in the cells [8].

In the present investigation, caspase protease activation/expression was the major signaling pathway in treatment-induced apoptosis in the cells [20]. Previous studies reported caspase 3 expression in genistein- and Cytoreg®- induced apoptosis in PC3 prostate cancer and testicular cells [23, 24, 25]. Caspase protease activation was confirmed with data obtained from the results. A strong, positive correlation ($r^2 = 0.981$; $P<0.05$) between caspase expression and apoptosis induction was identified. Of the three treatment groups, combination treatment showed the most significant apoptotic response with respect to caspase activity. The combination treatment group was more sensitive to caspase expression than either single treatment group. Significant apoptosis induction was manifested in the former group at lower caspase expression levels. However, the overall results indicated caspase protease as the major pathway in apoptosis induction in all treatment groups. These results were in agreement with findings in previous studies [25, 26].

CONCLUSION: Both genistein and Cytoreg® induced growth inhibition through apoptotic cell death in MCF-7 cells with evidence of autophagy in Cytoreg® - exposed cells. The apoptotic cell death was mostly caspase-dependent. The genistein-cytoreg® combination was significantly more efficacious at lower EC50 (lower cytotoxic level) than either compound alone; implying a greater therapeutic potential of the combination.

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List of Abbreviations: AcrO - Acridine Orange; DMSO – Dimethylsulfoxide; ER-α positive – estrogen-dependent cells; EtBr - Ethidium Bromide; FBS - fetal bovine serum; GFAP - Glial

Fibrillary Acidic Protein; HF - Hydrofluoric Acid; MCF-7 - Michigan Cancer Foundation – 7 - human breast cancer cell line; MTP - microtiter plate; MTT - (3-(4, 5-dimethyl-thiozol-2-ayl)-2, 5-diphenyl tetrazolium bromide); NHBC - Normal Human Breast Epithelial Cells; TB exclusion assay – Trypan Blue exclusion assay

Competing interests: We the authors, declare that we have no existing competing financial interests with respect to this research.

Authors' contributions: JK-D conceived the idea, designed the experiment and contributed to the execution of the experiment, analysis of the data and writing of the manuscript. MMJ contributed to the culturing and bioassays, writing of the manuscript and analysis of data. KM contributed to the culturing and bioassays, and drafting of the manuscript. VH contributed to the culturing and proof read the drafted manuscript for submission. ZR, GBS and MH contributed to the design, implementation and drafting of the manuscript.

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