Evaluation the Effect of Natural Compounds: Vitamin C, Green Tea, and their Combination on Progression of Mg-63 Osteosarcoma Cell Line Cells. (An In Vitro Study)

Hiam Rifaat Hussein Mohammed, Amr Helmy Moustafa El Bolok, Sherif Farouq Elgayar, Maii Ibrahim Ali Sholqamy

Introduction

Cancer is considered one of the most common causes of death all over the world according to the recorded global statistics [1], [2]. Osteosarcoma (OS) is a malignant bone tumor of unknown etiology; it has many different histological subtypes that reflect presence of pleomorphic malignant cells which considered the source of osteoid [3]. Recurrence of cancer is based mainly on migration and metastasis of cancer cells. One third of cases of OS develop recurrency, mostly osteolytic bone metastases [4]. There are many different common treatment options of cancer: Surgery, radiation, and chemotherapy [5]. To avoid the adverse effects of these methods, recent studies have been preferred to use natural products and herbal plant extracts that have anticancer properties [6]. Vitamin C is naturally occurred in many different types of food and it also known as ascorbic acid, previously, many different studies assured that Vitamin C has anticancer effect on cancer cells resulting in cell death of tumor and also it possesses antioxidant capacity [7], [8]. Green tea polyphenols (GTPs) are used in the treatment of cancers as GTPs consist of 80–90% catechins [9], [10]. Green tea catechins (GTCs) have anticancer properties in many cancer cells as stomach, small intestine, lung, prostate, and breast because they can regulate deoxyribonucleic acid (DNA) methylation, histone modification, cell cycle regulation, apoptosis, invasion, and angiogenesis [11], [12]. GTPs consists of four main polyphenolic flavonoids constituents (epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG)). [13] Interaction therapy between drugs is used in cancer prevention and treatment. In interaction therapy, the combination may be synergistic, additive, antagonist, or potentiation interaction of green tea extract and Vitamin C is additive type in which antioxidant property of tea is improved [14]. In our study, we compare the role of natural materials: Vitamin C, green tea, and their combination on Mg-63 cells by studying the effect of different types of food and it also known as ascorbic acid, previously, many different studies assured that...
these natural drugs on cell migration, free radicals, and induction of cell death.

Material and Methods

**SRB cytotoxicity assay**

It is sulforhodamine B (SRB) colorimetric assay for cytotoxicity screening, used for cell density determination, based on the measurement of cellular protein content. It is one of the most important methods for *in vitro* cytotoxicity.

Mg-63 OS cell line was obtained from Nawah Scientific Inc, (Mokatam, Cairo, Egypt). Cells were maintained in DMEM media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin, 10% of heat-inactivated fetal bovine serum in humidified, and 5% (v/v) CO\textsubscript{2} atmosphere at 37°C [15], [16].

**Antioxidant test**

Initial screening step, solution of the provided samples was prepared in concentrations of 1000 and 100 μg/mL in dimethyle sulfoxide to identify a range within which the inhibitory concentration 50 (IC\textsubscript{50}) lies. For IC\textsubscript{50} determination of free radicals in Mg-63 control cells and treated cells, extracts that exceeded 50% inhibition in any of the initial screening step concentrations were serially diluted to provide five concentrations. For trolox standard preparation, a stock solution of 100 μM concentration of trolox was prepared in methanol from which seven concentrations were prepared including 50, 40, 30, 20, 15, 10, and 5 μM. DPPH assay was carried out according to the method of Boly et al., 2016. (1) Briefly; 100 μL of freshly prepared DPPH reagent (0.1% in methanol) were added to 100 μL of the sample in 96 wells plate (n = 6), the reaction was incubated at room temperature for 30 min in dark. At the end of incubation time, the resulting reduction in DPPH color intensity was measured at 540 nm. Data are represented as means ±. (Figure 1) [17], [18].

**Flow cytometric analysis**

**Cell cycle analysis**

After treatment Mg-63 with Vitamin C, green tea, and combination for 72 h, cells (105 cells) are collected by trypsinization and washed twice with ice-cold phosphate buffer saline [PBS], pH 7.4. Cells are re-suspended in 2 mL of 60% ice-cold ethanol and incubated at 4°C for 1 h for fixation. Fixed cells are washed twice again with PBS (pH 7.4) and re-suspended in 1 mL of PBS containing 50 μg/mL RNAase A (ribonuclease) and 10 μg/mL propidium iodide (PI). After 20 min of incubation in dark at 37°C, cells are analyzed for DNA contents using flow cytometry analysis [19], [20], [21], [22], [23].

**Apoptosis assay**

Apoptosis and necrosis cell populations are determined using annexin fluorescein isothiocyanate (V-FITC) apoptosis detection kit coupled with two fluorescent channels flow cytometry. After treatment with test compounds for 48/72 h, cells (105 cells) are collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Then, cells are incubated in dark with 0.5 ml of annexin V-FITC/PI solution for 30 min in dark at room temperature according to the manufacturer protocol. After staining, cells are injected through ACEA Novocyte™ flow cytometer and analyzed for FITC and PI fluorescent signals using FL1 and FL2 signal detector, respectively, (λ ex/em 488/530 nm for FITC and λ ex/em 535/617 nm for PI). For each sample, 12,000 events are acquired and positive FITC and/or PI cells are quantified by quadrant analysis [19], [20], [21], [22], [23].

**Wound healing test**

Cells were plated at density 3 × 105/well onto a coated 6-well plate for scratch wound assay and cultured overnight. On the next day, scratches were introduced into the confluent monolayer, and the plate was washed thoroughly with PBS. Control wells were replenished with fresh medium while drug wells were treated with fresh media containing drug. Images were taken using an inverted microscope at the indicated time intervals. The plate was incubated at 37°C and 5% CO\textsubscript{2} in-between time points [24], [25], [26], [27], [28], [29]. Wound width and migration rate were calculated.

Results

**SRB cytotoxicity assay**

Data obtained from SRB assay revealed that the half-maximal IC\textsubscript{50} of Vitamin C is 5.5 μg/ml, green
tea is 42.03 μg/ml, and their combination is 4.6 μg/ml on Mg-63 treated cells (Figure 2).

Table 1: IC\textsubscript{50} of free radicals in Mg-63 treated cells with Vitamin C, green tea, and their combination compared to trolox

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC\textsubscript{50} (Mean ± SE)</th>
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</thead>
<tbody>
<tr>
<td>Vitamin C (μg/mL)</td>
<td>6.11 ± 1.04</td>
</tr>
<tr>
<td>Green tea (μg/mL)</td>
<td>22.50 ± 1.02</td>
</tr>
<tr>
<td>Combination (μg/mL)</td>
<td>6.66 ± 1.07</td>
</tr>
<tr>
<td>Trolox (μg/mL)</td>
<td>5.61 ± 0.87</td>
</tr>
</tbody>
</table>

**Antioxidant results**

Table 1 shows IC\textsubscript{50} of free radicals in Mg-63 treated cells with Vitamin C, green tea, and their combination compared to trolox. Vitamin C has the most antioxidant property on Mg-63 treated cells followed by their combination with green tea and the last one is green tea. IC\textsubscript{50} of free radicals of Vitamin C treated cells is 6.11 μg/mL, 22.50 μg/mL for green tea treated cells, and 6.66 μg/mL for compounds combination treated cells.

**Flow cytometry results**

**Cell cycle analysis**

The IC\textsubscript{50} concentration of Vitamin C, green tea, and compound combination produced pre-G1 apoptosis. As the percentages of cells in pre-G1 are increased when compared to the control cells. There is cell cycle arrest at G2/M phases in green tea treated cells when compared to control cells. On the other hand, in Vitamin C and drug combination treated cells, the percentages of cells in G2/M phases decreased(Figure 3), when compared to the control cells as shown in Table 2 and Figure 4a-d.

Table 2: Cells distribution in cell cycle phases of control and treated cells of Mg-63 cells

<table>
<thead>
<tr>
<th>Frequency</th>
<th>G1</th>
<th>S</th>
<th>G2/Sub-G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>84.19</td>
<td>5.20</td>
<td>8.80</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>60.07</td>
<td>9.98</td>
<td>6.15</td>
</tr>
<tr>
<td>Green Tea</td>
<td>74.02</td>
<td>10.35</td>
<td>10.16</td>
</tr>
<tr>
<td>Drug combination</td>
<td>58.08</td>
<td>11.38</td>
<td>2.80</td>
</tr>
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**Assessment of apoptosis**

The IC\textsubscript{50} concentration of Vitamin C, green tea, and drug combination increased cell death of...
Mg-63 cells when compared to control untreated Mg-63 cells. The highest fraction of control cells is living cells (in the lower right quadrant) with only few necrotic and apoptotic cells. The living cells of treated Mg-63 cells are decreased with an increasing in apoptotic and necrotic cells (apoptotic cells in the upper and lower left quadrant and necrotic cells in the upper right quadrant). Figure 5 and Table 3 showing that the total percentage of apoptotic and necrotic cells is highest in Vitamin C, followed by drug combination and finally green tea treated Mg-63 cells.

Table 3: Effect of Mg-63 treated cells with Vitamin C, green tea, and their combination on apoptosis and necrosis

<table>
<thead>
<tr>
<th>Sample data</th>
<th>% Normal intact cells</th>
<th>% Early Apoptosis Q2-3</th>
<th>% Late Apoptosis Q2-4</th>
<th>% Necrosis Q2-2</th>
<th>% Total Apoptosis and Necrosis Q2-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>98.67%</td>
<td>0.19</td>
<td>0.48</td>
<td>0.66</td>
<td>1.33</td>
</tr>
<tr>
<td>Vitamin C treated cells</td>
<td>95.35%</td>
<td>0.23</td>
<td>1.55</td>
<td>2.87</td>
<td>4.65</td>
</tr>
<tr>
<td>Green tea treated cells</td>
<td>98.33%</td>
<td>0.33</td>
<td>0.91</td>
<td>0.44</td>
<td>1.68</td>
</tr>
<tr>
<td>Compounds combination treated cells</td>
<td>95.45%</td>
<td>0.40</td>
<td>1.63</td>
<td>2.52</td>
<td>4.55</td>
</tr>
</tbody>
</table>

**Wound healing assay**

The wound width decreases as cell migration is induced. The Vitamin C treated cells showed the widest wound when compared to green tea and compounds combination treated cells (Figure 4 and Table 4). Meanwhile, the migration rate of Vitamin C treated cells was least when compared to other groups (Figure 6).

**Discussion**

In the present research, we establish that there is a cytotoxic effect of natural compounds: Vitamin C, green tea, and their combination on Mg-63 OS cell line, but in different levels. The most cytotoxic effect was occurred in Vitamin C treated cells, followed by drug combination and finally green tea treated cells. This different effect based on the structure of each compound and their properties. The cytotoxic effects were in a concentration dependent manner as the mean viability percentage of Vitamin C, green tea, and their combination treated cells in relation to control cells were increased with decrease concentrations of the three drugs (Figure 2). These results are in agreement with (Ngo et al., 2019) [30] who said that the high concentrations of Vitamin C have antitumor, anti-proliferative effects, and produced apoptosis on different OS cell lines as Mg-63 and G292 cell lines. In addition, (Valenti et al., 2014) [4] reported that depending on past researches Vitamin C in the high concentrations suppressed growth of Mg-63 cells results in apoptosis but in the low concentrations resulted in differentiation of the same cell lines. (Ni et al., 2018) [31] who studied the cytotoxicity effect of EGCG in colon adenocarcinoma (COLO205) cells were exposed to EGCG (5, 10, 20, 40, and 80 μg/mL) for 24 h. The results showed that cells number dropped when treated with 40 μg/mL EGCG also demonstrated that EGCG induced genetic abnormalities in COLO205 cells and apoptosis, and inhibition of mitosis. These results suggested that EGCG affected cancer cells genetically and cytologically.

Regarding to antioxidant DPPH test, trolox is a soluble form of Vitamin E and is used as a standard antioxidant against which the antioxidant capacity of compounds is compared. The data revealed that the Vitamin C, green tea, and combination of both compounds have antioxidant properties on Mg-63 cells. These results are in agreement with (Cimmino et al., 2018) [32] studies of who reported that Vitamin C is considered as a natural antioxidant agent and (Intra and Kuo, 2007) [33]; who stated that the polyphenols in tea are responsible for its antioxidant properties mainly EGCG. Our results showed that the combination between green tea and Vitamin C is more potent as antioxidant than green tea on treated cells. This result is supported by a study of (Majchrzak et al. 2004) [14] who reported that the antioxidant properties of tea are induced when is combined with Vitamin C.

Our flow cytometry results revealed that Vitamin C, green tea, and combination affected cell cycle and apoptosis of Mg-63 cells. The combination treated cells showed higher ability to arrest cell cycle at G0/G1 phase when compared to Vitamin C and green tea treated cells. This is supported by apoptotic assessment which revealed that there are 2.03% of cells.

(Zhou et al., 2020) [34] reported that the Vitamin C can cause G0/G1 cell cycle arrest and mitochondrial caspase-dependent apoptosis in oral squamous cell carcinoma cell line, GTCs especially EGCG has ability to arrest cell cycle at G0/G1 phase of prostate cell line with apoptosis induction (Gupta et al., 2000) [35] and (Umeda et al., 2008) [36] revealed that green tea has can arrest cell cycle at G2/M phase, which is in agreement with our result, as the only green tea treated cells showed accumulation of cells at G2/M phase when compared to control cells. (Gupta et al. 2003) [37] demonstrated that EGCG causes cell cycle arrest resulting in apoptosis, this potential effect of EGCG through using it alone or in combination with other.

Wound healing assays revealed that the migration rate of MG-63 cells is influenced by Vitamin
After 72 h, the wound width was widest in Vitamin C treated cells when compared to green tea and compounds combination treated cells. Meanwhile, the Vitamin C treated cells showed that least migratory property followed by combination treated cells and finally green tea treated cells when compared to control untreated cells. Our results are in agreement with (Zhou et al., 2020) [35] who reported that the Vitamin C inhibits the migration of oral squamous cell carcinoma CAL27 cells, this is may be due to ability of Vitamin C to inhibit osteopontin (Valenti et al., 2014) [4]. EGCG of GTCs inhibits migration and metastasis of lung metastasis of melanoma cells, this is explained as EGCG downregulate epithelium-mechanism transition and matrix metalloproteinase (Liu et al. 2017) [34], [38]. Further studies are needed to establish the effect of Vitamin C, green tea, and their combination on cancer cells and order to confirm the effect of these natural compounds on progression of tumor cells.
Conclusions

Vitamin C, green tea, and their combination have a cytotoxic effect on Mg-63 cell line. They also arrest cell cycle with induction of apoptosis and necrosis. These drugs possess antioxidant capacity. Vitamin C, green tea, and their combination decrease metastasis of MG-63 cells as they have property to inhibit cell migration. The combination between Vitamin C and green tea has highest effect on cell cycle arrest and apoptosis. While, Vitamin C has more antioxidant property than green tea and their combination on MG-63 cells. Furthermore, Vitamin C inhibits migration of MG-63 cells more than green tea and their combination.

References

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