Research has enabled the discovery of many plants with bioactive compounds. Currently, plants are the major sources of these compounds and more to be discovered, which have potential antioxidant effects. These antioxidants could reduce the incidences of many disorders.

Inhibition of their activities are not just beneficial to the cell, but as oxidative stress [42]. Thus the scavenging of ROS and/or the activities of ROS, serious pathological aberrations may occur when the body has efficient antioxidant systems in place to counter the oxygen species (ROS) are implicated in many disorders such as diabetes, ageing, neurodegradative disorders, etc [41]. Though the body has efficient antioxidant systems in place to counter the activities of ROS, serious pathological aberrations may occur when these highly reactive species overwhelm the activities of the so-called antioxidant mechanisms - a condition commonly known as oxidative stress [42]. Thus the scavenging of ROS and/or the inhibition of their activities are not just beneficial to the cell, but could reduce the incidences of many disorders.

Several compounds of natural origin have been studied (many more to be discovered) which have potential antioxidant effects. Currently, plants are the major sources of these compounds and research has enabled the discovery of many plants with bioactive potentials. One of plants of significant attention for its bioactive potential is Garcinia kola (Clusiaceae). It is a medium-sized tree whose seeds (commonly called bitter kola) are highly valuable and consumed as an aphrodisiac and antidote for bronchitis, diarrhea, laryngitis, liver diseases, cough etc. [15,18,24]. The extract of the seeds has been reported to possess various bioactivities which include antimicrobial, antiparasitic, antinephrotic [1,6,19,31]. One of the substances isolated from Garcinia kola seeds is kolaviron (actually a biflavonoid complex of three compounds) [17]. Kolaviron has offered protection against chemical-induced hepatotoxicity and oxidative stress in animal models [8,17,18]. It has also been reported that the biflavonoid complex protects chemical-induced testicular damage [2,16]. Other reported activities of kolaviron are hypoglycaemic, hypolipidaemic, antiulcerogenic, potential is [1,17,35,38]. Kolaviron has offered protection against chemical-induced hepato-

1. Introduction

It has been widely reported that the activities of reactive oxygen species (ROS) are implicated in many disorders such as diabetes, ageing, neurodegradative disorders, etc [41]. Though the body has efficient antioxidant systems in place to counter the activities of ROS, serious pathological aberrations may occur when these highly reactive species overwhelm the activities of the so-called antioxidant mechanisms - a condition commonly known as oxidative stress [42]. Thus the scavenging of ROS and/or the inhibition of their activities are not just beneficial to the cell, but could reduce the incidences of many disorders.

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Selenium is an essential trace element obtained from dietary sources which include cereals, grains and vegetables [47]. Crabs, fish and shellfishes are also moderately good sources [33]. The trace element is an antioxidant whose activity is thought to be...
mediated via selenoproteins many of which are powerful antioxidant enzymes. It protect tissues against free radicals thus deficiency of the trace element makes organisms susceptible to oxidative stress mediated injuries such as cancer and heart diseases [9,47,48].

Since selenium and kolaviron are potent antioxidants, it is hypothesized that their combined action could result in a stronger biological effect than their individual responses. This work presents the effect of these antioxidants on hydrogen peroxide-induced alterations in the human monocytic cell line U937 and myoblast H9c2 cells.

2. Materials and method

2.1. Chemicals and reagents

Selenium (as sodium selenite), fetal calf serum (heat inactivated), L-glutamine, phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Dulbecco’s modified Eagle’s medium (DMEM) and RPMI-1640 were purchased from Sigma-Aldrich (USA). The myoblast cell line H9c2 and macrophage cell line U937 were obtained from the European Collection of Cell Cultures. All antibodies and biotylated cytokines were products from Pharmin (US). All other chemicals and reagents were of the highest purity and commercially available. Buffers and solutions were prepared using Milli-Q (18 mΩ cm⁻¹) water and stored at room temperature.

2.2. Extraction of kolaviron

Garcinia kola seeds were obtained fresh from the Obunagha forest, Bayelsa State, Nigeria. The seed coats were removed and the seeds were cut to smaller bits and sun-dried. The dried seeds were later ground using a waring blender. Kolaviron was isolated from the resulting powder according to Iwu [25]. Briefly, 4 kg of the powdered seeds were extracted with light petroleum ether (bp 40–60 °C) in a soxhlet for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6 x 300 ml). The ethylacetate fraction was concentrated to give a yellow solid known as kolaviron. Percent recovery was 5.6.

2.3. Preliminary cell culture

Macrophage U937 cells were grown in RPMI-1640 supplemented with fetal calf serum (heat inactivated), penicillin (100 U/mL), streptomycin (100 mg/mL) and L-glutamine (2 mM) while the cell line H9c2 was grown in DMEM supplemented with heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 mg/mL) and L-glutamine (2 mM). Cells were kept in an incubator at a temperature of 37 °C in an atmosphere of 5% CO₂.

2.4. Cell viability

Both cell lines were seeded at 5 x 10⁴ cells/ml before being exposed to 100 μM hydrogen peroxide (H₂O₂) (in PBS) for 1 h. For the evaluation of the protective effects of selenium and kolaviron, each culture (in complete medium) was supplemented with either selenium (2.5 μM sodium selenite) or kolaviron (25 μg/mL) or both for 24 h before exposure to H₂O₂. Viable cells were determined 1 h after H₂O₂ exposure. For U937, viable cells were determined via Trypan blue dye exclusion while for H9c2 cells, cellular damage was determined via MTT reduction as reported by Zhou et al. [50] with a slight modification. Briefly, 10 μL of MTT solution was added to each cell culture and incubated for 2 h at 37 °C. MTT was removed followed by the addition of 250 μL of DMSO. Absorbance was measured using a microplate reader at 570 nm.

2.5. Assessment of oxidative stress

The production of oxidative stress by H₂O₂ in H9c2 cells was analyzed based on the oxidation of 2',7'-dichlorodihydrofluorescein by intracellular peroxides as reported [28]. Briefly, the myoblast cell line H9c2 was incubated with sodium selenite and kolaviron for 24 h (as described above). Cells were washed with PBS and incubated in Hank’s balanced Salt Solution (HBSS) containing 50 μM DCHF-DA/L for 30 min at 37 °C. Cells were washed with PBS and incubated with H₂O₂ for 45 min. Fluorescence of cells of each well was measured at excitation and emission wavelength at 485 nm and 530 nm respectively. Antioxidant activity was expressed as percent inhibition of intracellular ROS following H₂O₂ exposure.

2.6. Assay for nitric oxide and cytokine production

In this case the U937 cells were seeded into culture plates and treated with PMA (100 ng/mL) for 48 h in order to transform them to the macrophage form [37]. Supernatants were removed from cells and incubated with selenium and kolaviron for 24 h. Hydrogen peroxide (100 μM) was added and the supernatant of each cell culture analyzed for nitric oxide production and the pro-inflammatory cytokines 1 h later. Nitric oxide production was determined according to Hwang et al. [23] as modified [22] while the secretion of the cytokines tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) was measured by cytokine-capture ELISA as described [37]. The production of these pro-inflammatory mediators was expressed as percent inhibition following H₂O₂ exposure.

2.7. Statistical analysis

Representative values for various experiments were expressed as mean ± SEM. Where applicable, data were analyzed using ANOVA followed by Duncan’s multiple range test. Confidence exhibited at p < 0.05 was considered statistically significant.

3. Results

3.1. Effect of selenium and kolaviron on cell viability

In order to investigate the effect of selenium and kolaviron on H₂O₂-induced cell death, viability of U937 and H9c2 cells was measured by the trypan blue dye and MTT reduction assays. (Figs. 1 and 2). Treatment of cells with H₂O₂ alone resulted in 19% and 31% viability in U937 cells and H9c2 cells respectively. However, supplementation of cell culture with selenium and kolaviron before incubation with H₂O₂ significantly increased viable cell count closer to control levels. However, selenium had no significant effect on H₂O₂-induced reduction in viability of H9c2 cells. In both cell lines, combined supplementation with both selenium and kolaviron significantly (p < 0.05) enhanced viable cell count when compared to their separate effects.
3.2. Inhibition of ROS production

The production of reactive oxygen species in H9c2 cells was assessed by DCHF assay. In order to measure ROS production in cells, H9c2 cells were supplemented with selenium and kolaviron before incubating with H₂O₂. The result revealed that both selenium and kolaviron reduced the production of ROS by H₂O₂ but the combined supplementation with selenium and kolaviron produced a greater reduction than did selenium or kolaviron (p < 0.05, Fig. 3).

3.3. Effect on nitric oxide production

The cell line U937 was transformed using PMA, and pre-treated with selenium/kolaviron before incubated with H₂O₂. The results reveal both selenium and kolaviron reduced H₂O₂ mediated release of nitric oxide (Fig. 4) however the combined pre-incubation with both selenium and kolaviron was better at reducing the H₂O₂ mediated release of nitric oxide than their individual abilities (p < 0.05).

3.4. Inhibition of cytokine production

The ability of selenium and kolaviron to inhibit cytokine production is shown in Figs. 5–7. Treatment of transformed U937 cells with H₂O₂ alone resulted in significant production of the pro-inflammatory cytokines TNF-α, IL-1 and IL-6 over control (p < 0.05). But supplementation of the cells with either selenium or kolaviron before incubation with H₂O₂ significantly reduced the production of the cytokines (p < 0.05). Kolaviron produced greater reduction in IL-6 levels than selenium (p < 0.05). However, the combined pre-treatment of the U937-derived macrophages with...
Se and kolaviron produced greater reductions in the pro-inflammatory cytokines.

4. Discussion

Cells, organs and tissues are continuously exposed to free radicals and reactive oxygen species either as a consequence of normal redox processes or the intake of xenobiotics. Serious pathological consequences may occur from these unavoidable circumstances if the endogenous antioxidants/antioxidant systems are unable to deal with these reactive species. The fallout of these imbalances could be cancer, cardiovascular disorders, ageing, diabetes etc [41].

Hydrogen peroxide is a non-radical reactive oxygen species produced by cells in the course of active metabolism. It reacts with various cellular targets including transition metal ions to generate the hydroxyl radical which is considered as the most reactive of all cell damaging free radicals [12]. Although the precise mechanism of H$_2$O$_2$-mediated oxidative damage is unknown, it could cause apoptosis or necrosis [21,43].

Hydrogen peroxide and other products it generates following its oxidative activity have been reported to inhibit the mitochondrial Na$^+/Ca^{2+}$ antiporter thereby blocking the efflux of Ca$^{2+}$ from the mitochondrion [32]. In various studies involving cell lines, H$_2$O$_2$ has been shown to cause cell death for as low as 50 µM [3] though observed toxicity may depend on the cell line involved. Sonoda et al. [44] reported that cell viability of the human glioblastoma cell line reduced by less than 12% upon treatment with 1 mM H$_2$O$_2$ for 4 h. It has also been reported that H$_2$O$_2$ concentrations of up to 600 µM induced apoptosis in the retinal pigment epithelial cell line, though necrosis was found to occur at higher concentrations [27]. However in hepatic stellate cell lines, 35% of cells became necrotic when incubated with 200 µM H$_2$O$_2$ for 3 h [13]. This necessitated the use of 100 µM H$_2$O$_2$ was used for this study.

Hydrogen peroxide reduced the proliferation of both U937 and H9c2 cell lines as evidenced from the trypan blue exclusion and the MTT assays respectively. However, pre-treatment with kolaviron significantly protected cells from H$_2$O$_2$-induced alterations. Though Se did not significantly protect the H9c2 cells, the combined treatment with Se and kolaviron produced a significantly (p < 0.05) higher protection than treatment with kolaviron alone. It has been reported that one of the mechanisms via which flavonoids (e.g. kolaviron) protect cells is by preventing DNA damage (which is induced by H$_2$O$_2$) [36]. This may be responsible for the ability of kolaviron to reduce H$_2$O$_2$-mediated alterations though it remains to be further investigated.

Interest on the benefits of flavonoids has grown over the years and several modes of action have been proposed concerning their ability to protect cells. It has been reported that they induce the expression of detoxifying enzymes such as glutathione S-transferases, epoxy hydrolase, UDP-glucuronosyl transferase, γ-glutamyl cysteine synthetase etc which contribute significantly to cellular protection against redox cycling and oxidative stress [30]. Flavonoids also modulate intracellular signaling pathways that could contribute to the protection of the cell [4]. Another reported mechanism is the inhibition of mitochondrial dysfunction [39].

Several selenoproteins have been shown to possess antioxidant functions such that human disorders associated with selenium deficiency could be attributed to increased oxidative stress and...
alterations in redox cycling [47]. Selenium deficiency is often manifested due to limited expression of selenoproteins which are involved in antioxidation and redox cycling and the implications could even be cancer and precancerous lesions [47]. Thus the protective role of selenium on cell death has been ascribed to antioxidation of selenoproteins [46]. It has also been reported that selenium reduces gene mutations and other chromosomal damages which may be implicated in cancer [7].

In order to establish a model of oxidative stress, it was necessary to investigate the production of reactive oxygen species (ROS). Both selenium and kolaviron reduced $H_2O_2$-mediated generation of ROS by the DCHF assay though combined pre-treatment of H9c2 cells with kolaviron and selenium was better at reducing ROS production. Flavonoids are known to reduce ROS production probably by direct scavenging of free radicals or by single electron transfer mechanisms [49]. Other mechanisms by which flavonoids reduce oxidative stress are by terminating chain radical reactions, inhibition of superoxide driven Fenton reaction and the upregulation of innate antioxidant defenses [10,29].

In the study, hydrogen peroxide significantly enhanced the release of nitric oxide and the pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-2 (IL-2) and tumour necrosis factor-alpha (TNF-α) from the transformed U937 cells (otherwise known as U937-derived macrophages). Though the monocyte form could also release the cytokines, treatment with phorbol esters enhances their transformation to the macrophage form and enables them to maintain cell numbers [11]. The release of the cytokines and nitric oxide due to excessive macrophage activation has been implicated in systemic inflammatory responses, organ failure, septic shock and hypotension [11].

Oxidative stress increases the level of intracellular nitric oxide via the upregulation of inducible nitric oxide synthase (iNOS) which catalyses the conversion of arginine to nitric oxide and citrulline [40]. The upregulation of iNOS contributes to the pathophysiology of inflammatory diseases and septic shock. Cell and tissue damage could occur due to the overproduction of nitric oxide since it interacts with superoxide radical to form peroxynitrates and other powerful tissue oxidizing agents [20]. Both selenium and kolaviron reduced $H_2O_2$-mediated release of nitric oxide. Nitric oxide, peroxynitrates and similar oxides deaminate DNA and impair DNA repair systems thus mutagenic [26,45]. Though the role of Se mediated decrease in nitric oxide is quite not clear, it has been reported that kolaviron suppresses the expression of iNOS [18].

Selenium deficiency could favour the formation of pro-inflammatory compounds that could predispose individuals to various disorders such as cancer and heart disease [5]. In order words, selenium can reduce inflammatory responses by reducing the secretion of pro-inflammatory cytokines. This could justify the effect of selenium on hydrogen peroxide-induced activation of U937 macrophages. Increased selenium levels also impede the transactivation of genes that encode pro-inflammatory cytokines thus could cause a decrease in cytokine production. This could be an underlying mechanism by which selenium exerts its anti-inflammatory action [14,34].

5. Conclusion

The study shows that both Se and kolaviron reduced $H_2O_2$-mediated alterations in raw U937 cells, U937-derived macrophages and myoblast H9c2 cells. In some instances, kolaviron seems to show higher response than Se however this trend may vary with change in concentrations. Though the combined pre-treatment of the cells with Se and kolaviron seem to have higher responses than their individual response (in most instances), it may not be synergistic nor additive. Similar experiments using different concentrations of the antioxidants are in progress. The combined action of Se and kolaviron could be exploited pharmacologically. It is probable that the inhibition of excessive macrophage activation may protect against cell death.

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