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Diosgenin induces genotoxic and mutagenic effects on HepG2 cells

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ABSTRACT

Yam roots and other plants from *Dioscorea* genus have cultural, nutritional and economic importance to tropical and subtropical regions and have a great amount of diosgenin in its composition. In the present study the cytotoxic, genotoxic and mutagenic potential of diosgenin on HepG2 cells was investigated. Cytotoxicity was assessed using MTT and clonogenic assay. Genotoxic and mutagenic effects were performed using single cell gel electrophoresis and cytokinesis-block micronucleus assay, respectively. A reduction on cell viability was observed due to diosgenin treatment at concentrations higher than 30 μM. A genotoxic effect was shown by comet assay and CBMN. Besides, an increase in micronucleus frequency along with a significant cytostatic effect were observed. Diosgenin elicited DNA damage on HepG2 cells which could not be efficiently repaired contributing to the mutagenic effect observed. Those results suggest that diosgenin deleterious effect could take place through genetic instability, fact that affects the normal cell cycle, leading to cell's death.

1. Introduction

Plants belonging to *Dioscoreaceae* family have cultural, medicinal, economic and nutritional importance in tropical and subtropical regions (Rakotobe et al., 2010). They are used in Traditional Chinese Medicine, and have abundant content of diosgenin (Li et al., 2015), which is the sapogenin (aglycone moiety) originated from hydrolysis of the steroidal saponin dioscin (Patel et al., 2012) (Fig. 1).

Beneficial therapeutic properties of the use of diosgenin have already been reported in literature. Studies demonstrated that diosgenin presents therapeutic efficacy against skin aging (Tada et al., 2009), in the control of hypercholesterolemia (Son et al., 2007), along with preventing bone loss (Yen et al., 2005). Besides, it also presents other beneficial effects such as myocardium protection of patients with arrhythmias (Badalzadeh et al., 2014), control of diabetes *mellitus* type II (Ghosh et al., 2014), and antioxidant (Gong et al., 2010), antitumoral (Liu et al., 2005), analgesic and anti-inflammatory effects (Mbiantcha et al., 2010).

Besides its traditional use, diosgenin is of great importance in pharmaceutical industry since it has been used as the main precursor in the synthesis of semi-synthetic steroidal hormones such as sex hormones, oral contraceptives and adrenal hormones, due to their similar structure (Dias et al., 2007). Nevertheless, studies aimed to provide information about the safety of using this compound is very scarce yet (Raju and Rao, 2012).

Additionally, an important point to be taken into account is the suitability of the biological model applied in the toxicological assessment, since many xenobiotics need to be metabolized to become active to reach specific cellular targets. Therefore, the use of cell lines containing endogenous metabolizing enzymes broadens the scopes of the biological model studied. In this context the use of Human hepatoma cell line (HepG2) that contains several metabolic enzymes of phases I and II, helps to provide a suitable model for assessing xenobiotics that need previous bioactivation (Knasmüller et al., 2004). Until now there are no reports of studies assessing diosgenin genotoxic or mutagenic effects in this type of cell lines.

Toxicological approaches can be carried out using a variety of assays. Among them, the utilization of cytotoxic, genotoxic and mutagenic tests are useful tools that have been applied to provide toxicological information about new xenobiotics in relation to their potential adverse effects for being used as therapeutic drugs.

Due to the lack of information about the safety of diosgenin, this work aimed to access the cytotoxic effect of diosgenin and, mainly, evaluate its genotoxic and mutagenic potential through *in vitro*

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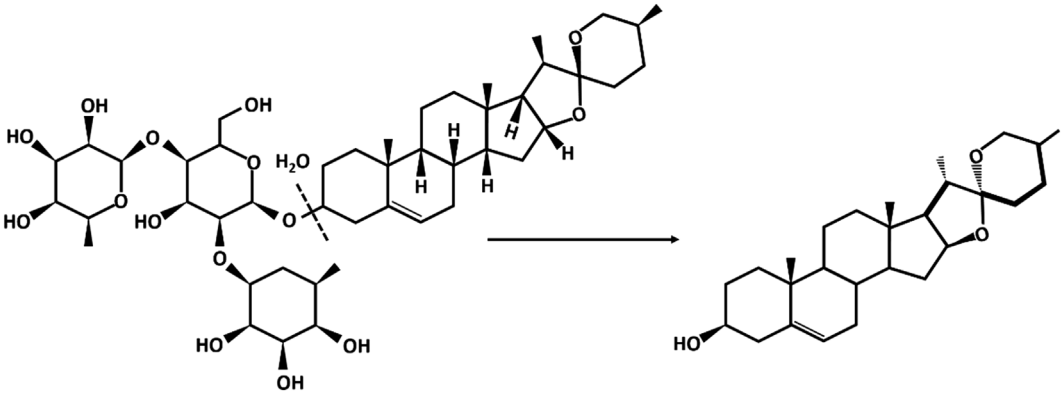

Fig. 1. Hydrolysis of dioscin, releasing diosgenin structure.

bioassays as MTT, clonogenic assay, comet assay and CBMN respectively using HepG2 cells.

2. Material and methods

2.1. Reagents and compounds

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cytochalasin B, cyclophosphamide, Giemsa dye, silver nitrate and SYBR Green were purchased from Sigma-Aldrich (St. Louis, MO, EUA). Normal-melting point (NMP) agarose, low-melting point (LMP) agarose, Dulbecco's modified Eagle's medium (DMEM), streptomycin/penicillin, fetal bovine serum and trypsin were acquired from Life Technologies (Carlsbad, CA, EUA). Diosgenin was purchased from Sigma-Aldrich (St. Louis, MO, EUA). Dry powder diosgenin was suspended in methanol before used. The final methanol concentration in culture was less than 1%.

2.2. Cell line

Human hepatoma (HepG2) cell line was obtained from American Type of Culture Collection (ATCC, Rockville, MD). The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% of fetal bovine serum, 1% streptomycin/penicillin and 2% L-glutamine at 37 °C and 5% CO_2.

2.3. Cell viability

Cytotoxicity of diosgenin was determined by MTT test, which is based in the mitochondrial enzymatic conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) directly related with cell viability (Mosmann, 1983), and was performed as previously described by Mosmann (1983) with minor modifications. Briefly, cells were seeded in 96-well flat bottom culture plates in a density of 10^4 cells per well and incubated at 37 °C for 24 h and 5% CO_2. After incubation, cells were treated with concentrations of diosgenin from 0 μM (negative control) to 100 μM for 24 h. MTT was added to each well at a final concentration of 1 mg/mL and incubated for 4 h. Then the medium containing MTT was removed and ethanol 100% was used to dissolve the formazan. Absorbance was determined at 570 nm in a microplate reader.

2.4. Clonogenic survival determination

It was performed the clonogenic assay in order to evaluate cytotoxicity through cell survival and proliferation after a treatment that can promote DNA damage, apoptosis or other mechanisms, interfering in cellular cycle (Franken et al., 2006). The assay was performed as previously described by Franken et al. (2006) with minor modifications. Cells (3×10^3) were plated and let to grow at 37 °C for 24 h, and then they were exposed to the cytotoxic concentration range of diosgenin observed by MTT test. After 14 days of incubation, cells were washed twice with phosphate saline buffer (PBS), fixed with methanol and acetic acid (v/v = 3:1) for 30 min, then stained with Giemsa for 15 min, and, finally, washed with distilled water and dried at room temperature. Experiments were performed in triplicates and repeated 3 times. Colonies containing more than 50 cells (> 50 μm) were visually counted in randomly chosen 1 mm × 1 mm grid as previously described (Das et al., 2009).

2.5. Single cell gel electrophoresis (comet assay)

Comet assay, also known as single cell gel electrophoresis, is a test extensively used to assess DNA damage caused by different compounds. This is an inexpensive, fast and simple assay, used in different types of biological samples (Fikrová et al., 2011), that gives useful information about genotoxic effect (Azqueta and Collins, 2013). It is based on the principle that undamaged DNA retains association with nuclear proteins, and damaged DNA loses it. When an electric field is applied, smaller damaged DNA fragments can move away from nucleoid structure and are visualized as comets in an agarose gel. This assay was performed as previously described by Hartmann et al. (2003) with minor modifications. Cells (10^5 cells/well) were seeded in 6-well plates and incubated during 24 h, and then cells were exposed to different concentrations of diosgenin, 0 μM (negative control), 20 μM, 40 μM and 60 μM, during 24 h. Positive control was performed using a 30 μM solution of H_2O_2. Then, cell suspension were mixed with 100 μL of 1% (w/v) low-melting agarose and loaded onto the frosted slides precoated with 1.5% (w/v) normal melting agarose gel. After agarose solidification, the slides were submersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl with 10% DMSO e 1% Triton X-100 freshly added; pH 10.0) for 3 h. In this step cellular and nuclear membrane were lysed and proteins removed, leaving only a nucleoid in each cell. Then, the slides were transferred to the electrophoresis tank and submersed in electrophoresis buffer (10 M NaOH and 200 mM EDTA; pH 13.0, 30 min, 4 °C) for 20 min to allow DNA to unwinding and the expression of alkali-labile sites. Electrophoresis was conducted for 30 min at 30 V and 400 mA. After electrophoresis, the slides were dipped in neutralization buffer (0.4 M de Tris-HCl buffer; pH 7.5) twice for 5 min and washed with ethanol 100% three times before staining. Cells were fixed and then stained with silver nitrate, according to Nadin et al. (2001) with minor modifications. First, the material was fixed in fixative solution (15% trichloroacetic acid, 5% zinc sulfate heptahydrate and 5% glycerol, 10 min) and placed to dry at room temperature until the following day. For staining, slides were rehydrated in distilled water for 5 min. Then, they were stained in the dark at 37 °C by mixing 32 mL of solution A (5% sodium carbonate) and 68 mL of solution B (0.02% ammonium nitrate, 0.02% silver nitrate, 0.1% tungstosilicic