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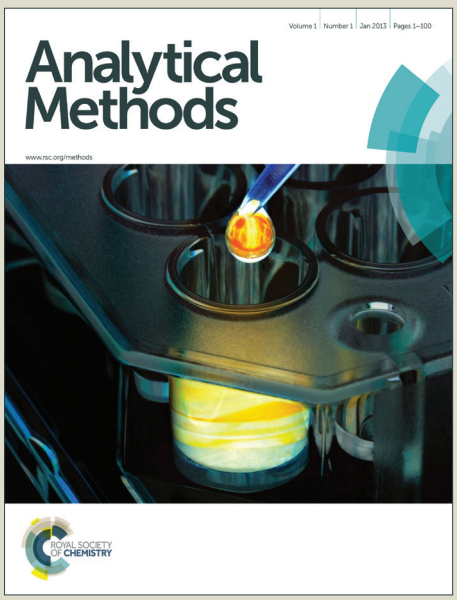

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Genomodifier capacity assay: a non-cell test using dsDNA molecules to evaluate the genotoxic/genoprotective properties of chemical compounds

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We describe here an ultrasensitive and fast protocol called a GEMO Assay (Genomodifier capacity assay). This non-cell method was developed to identify chemicals with genomodifier (genotoxic and/or genoprotective) capacity. The assay is performed in a black 96-well plate using calf thymus dsDNA exposed to different concentrations of chemicals tested (CT) for 30 minutes with and without the addition of a prooxidant substance that causes dsDNA damage (H_2O_2, 3M). Furthermore, PicoGreen®, a highly sensitive dsDNA dye is added and the dsDNA fluorescence. Chemicals that cause a break in dsDNA are identified by a decrease in fluorescence in comparison with the fluorescence observed in an untreated dsDNA (control group) indicating a genotoxic capacity. On the contrary, attenuation of dsDNA degradation caused by H_2O_2 exposition indicates CT genoprotective capacity. The GEMO Assay was validated by comparing peripheral blood mononuclear cells (PBMCs) and an HT29 colorectal cell line exposed to similar conditions where the effect on dsDNA was also evaluated by a DNA Alkaline Comet Assay. Vitamin C was used as CT and other variables were also evaluated to confirm the cytotoxic action of H_2O_2. The results showed a strong negative correlation between the GEMO Assay and the Comet Assay performed in PBMCs (r^2 = -0.828; $p<0.0001$) since higher dsDNA fluorescence measured by the GEMO Assay was associated with lower index damage measured by the DNA Alkaline Comet Assay. Therefore, the GEMO Assay could be useful to early screening of genoprotective and genotoxic effects of chemicals and plant extracts without interfering cell biological variables.

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Introduction

The relevance of genotoxicity analysis

Mammalian cells under normal growth conditions are subject to several thousand DNA injuries per day which include base loss, base alterations, and strand breakage. Therefore, the generation of DNA damage (genotoxicity) could be considered the main event that causes several human morbidities with an emphasis on cancer due to environmental, occupational or pharmacological variables.[1]

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The harmful effects of genotoxic compounds are related to the ability of these substances to alter cell signaling pathways, cell cycles, strength promoting apoptosis, inhibit DNA repair, alter methylation processes (epigenetic effect) and increase the oxidative stress that occurs in arsenic,[2] heavy metals[3] and mercury[4] exposure.

For this reason, toxicological screening of chemical compounds and drugs with pharmacological interests include analysis of genotoxic and/or genoprotective effects of these substances. The regulatory battery of drugs includes several genotoxicity tests.

Several *in vitro* and *in vivo* assays for genotoxicity have been developed that, with a range of endpoints, detect DNA damage or its biological consequences in prokaryotic (AMES test) or eukaryotic (e.g. mammalian, avian or yeast) cells. These assays are used to evaluate the safety of environmental chemicals and consumer products and to explore the action or mechanism of known or suspected carcinogens.[5]

Apparently, a cell culture approach is the best way to evaluate the genotoxicity or genoprotective effects of chemical compounds. In fact, despite some disadvantages at the level of genetic stability, cell lines are often preferred by some laboratories based on ease of handling frozen stocks of cells, lack of variation that can occur in human lymphocyte donors and the existence of large historical databases. However, the design of the protocol is crucial in the generation of accurate results and assessment of the genotoxic potential of the test substance. Therefore, the choice of the cellular system, treatment duration, the use of a cytokinesis blocker, the class of the test compound or the addition of metabolic components may significantly influence the test outcome.[6]

Despite the relevance in the use of these biological models, genetic variations related to cellular and body metabolism present in tissues and cells may produce under or overestimated toxicological or pharmacological results.

For example, several studies have suggested that genetic variations present in the human superoxide dismutase manganese dependent gene (Ala16Val-SOD2) are associated with the susceptibility of some neoplasia like prostate, breast and lung cancers.[7,8,9,10] However, this association is also influenced by environmental factors such as diet, smoking habits and occupational exposition.[11,12,1314,15,16,17,18,19] Based on these results, *in vitro* investigations considering the Ala16Val-SOD2 polymorphism have been performed showing the effect of the toxicological response results.[20,21,22] As in general toxicology *in vitro* tests, using a small number of samples from donors of this genetic variation may be an important intervening factor in the results obtained.

Another concern involving initial genotoxicity screening of chemical compounds, potentially toxic or with pharmacological interest, is the time and cost of the investigations involving DNA damage. The vast numbers of biologically uncharacterized environmental, industrial, and novel pharmaceutical chemicals do not allow the testing of each for genotoxicity in the standard resource-intense battery. The high economic costs of these tests sometimes limit the analysis to a few compounds in the early genotoxicity testing.[23] In methodological terms, the genotoxicity screening of chemical compounds using biological models always involves some type of variation related to genetic and experimental conditions where *in vitro* and *in vivo* assays are performed.

Primary *in vitro* analysis indicates potential genotoxic effects of some chemical compounds or plant extracts with toxicological and/or pharmacological interest

without interference of cellular and physiological metabolic variables like DNA repair. Oxidative, absorptive and detoxification metabolisms can be a useful tool for toxicological and pharmacological studies.

Due to the environmental and health importance of detecting genotoxic effects of chemical compounds, different *in vivo* and *in vitro* assays have been developed including, the DNA comet assay developed by Ostling and Johanson in 1984[24] and further modified by Singh et al.[25] by the inclusion of unwinding DNA under alkaline conditions (pH > 13). Several versions of the Comet Assay are currently in use, but there are some general steps which apply to all versions. After obtaining a suspension of the cells, the basic steps in the assay include the preparation of microscope slides layered with cells embedded in an agarose gel, lysis of the cells to liberate the DNA, DNA unwinding, electrophoresis, neutralization of the alkaline DNA staining, and scoring. Unwinding of the DNA and electrophoresis at a neutral pH predominantly facilitates the detection of double-strand breaks and cross-links; unwinding and electrophoresis at pH 12.1–12.4 facilitates the detection of single and double-strand breaks, incomplete excision repair sites and cross-links[26].

Comet Assay is a method with great potential to evaluate the DNA damage status from *in vitro* and *in vivo* protocols, its use for the initial screening of genoprotective/genotoxic effects of environmental or pharmacological compounds is not realistic.

Although the genotoxicity tests are well established and consistent results are produced from *in vivo* models using rodent cancer bioassays as the "gold standard" to determine the carcinogenic potential of a chemical, this assay uses more than 800 mice and rats and the histopathological examination of more than 40 tissue samples. However, this assay is extremely costly and time consuming, and for this reason its use is limited to free chemicals[27]. The cost and time limitation of this test corroborate the need for the development of fast and quick tests that preliminarily investigate the potential genotoxic and genoprotective capacity of some determined chemical. This test could help the researcher identify the potential chemical that presents an effect on DNA as well as to identify the grade-concentration at which these effects are detected.

Therefore, we offer a description here of a fast and inexpensive non-cell *in vitro* fluorimetric assay that uses pure double-stranded DNA molecules (dsDNA) to detect the genotoxic and/or genoprotective capacity of a specific single chemical compound or of plant extracts called "Genomodifier capacity assay" (GEMO assay). The name of the assay is based on the fact that some substances have genotoxic and/or genoprotective properties (Genomodifier substances) that need to be identified to evaluate their toxicological or pharmacological potential.

The concept of the Genomodifier Capacity test (GEMO Assay)

The development of the GEMO Assay was based on the DPPH (1, 1-Diphenyl –2-picrylhydrazyl) method used to quickly estimate the antioxidant capacity of some substances or extracts. DPPH is a well-known synthetic radical and a scavenger of other radicals. In this non-cell assay, the rate of the reduction of a chemical reaction upon the addition of DPPH is used as an indicator of the radical nature of that reaction. Because of a strong absorption band centered at about 520 nm, the DPPH radical has a deep violet color in the solution, and becomes colorless or pale yellow when neutralized. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 520 nm or in the EPR signal of the DPPH.[28]

The assay described here uses pure dsDNA (calf thymus DNA) and a highly specific dsDNA dye (PicoGreen®) as the basic reagents. PicoGreen® dye is an ultrasensitive fluorescent reagent that allows quantification of dsDNA in the solution and can detect minute concentrations of DNA, up to 25 pg / mL.[29] The fluorescence determined by a specific fluorochrome dye (PicoGreen®) is used to estimate if the compound-test presents some level of interference in

dsDNA molecules that indicate genotoxic or genoprotective effects. The PicoGreen® dye makes a very stable complex with dsDNA in alkaline conditions instead of ssDNA (single-strand DNA), proteins, SDS, and urea. The PicoGreen® characteristic selectivity is used to follow DNA denaturation with decreasing fluorimetric signal intensity proportionate to the production of ssDNA and mononucleotide content when dsDNA is attacked by some chemical molecule or environmental variable at higher temperatures.[30]

Therefore, the GEMO Assay is constituted for two chemical reactions to access the genotoxic and genoprotective capacity of some specific chemical or extract (chemical-test, CT). The first reaction is based on the follow equation: F= dsDNA + CT where F= fluorescence at 480 nm excitation and 520 nm emission determined from a known dsDNA concentration exposed to some chemical-test (CT) that can be pure molecules or extracts. Molecules and extracts that cause a break in dsDNA are identified by decreasing the fluorescence in comparison to the fluorescence observed in the untreated dsDNA (control group). The second reaction that analyzes the genoprotective capacity of some molecule or extract is based on the following equation: F= dsDNA+ GS+ CT where F= fluorescence at 480 nm excitation and 520 nm emission is determined from a known dsDNA concentration exposed to some CT in the presence of a genotoxic standard molecule (GS). If the CT has a protective effect, the dsDNA degradation promoted by the GS molecule will be prevented and the fluorescence will increase when compared to a dsDNA treated just with GS. The genoprotective capacity can be complete (if the fluorescence is similar to the control group) or partial (if the fluorescence is higher than the GS treatment and lower than the control group).

When establishing which category of chemical molecules will be tested by the GEMO Assay, it is important to consider that several types of molecules have genotoxic action. It is important to discriminate between genotoxic carcinogens and non-genotoxic chemicals because their mechanisms of action are quite distinct. Their dose-response curves, reversibility, and organ and species-specificity are also quite distinct. Thus, the mode of action of the agents involved in mutagenesis related to cancer causation and development needs careful analysis. There are a large number of molecules in nature chemically classified as antioxidants that present potent antigenotoxic effects. This is the case of polyphenols as well as some vitamins habitually ingested from our diet or by supplementation and/or use of phytotherapic compounds. Polyphenols have several anticancer effects such as blocking carcinogenesis initiation by inactivation of exogenous or endogenous genotoxic molecules including reactive oxygen species (ROS).[31]

On the other hand, some polyphenols and other antioxidant vitamins with genoprotective effects can also present carcinogenic/genotoxic effects. The idea of hormesis, a biphasic dose-response relationship in which a chemical exerts the opposite effects dependent on the dose, is very important in the field of carcinogenesis.[32] Many antioxidants present in plants have been shown able to prevent free radical-related diseases by counteracting cell oxidative stress. However, the *in vivo* beneficial effects are not so evident. This occurs because several plant antioxidants exhibit hormetic properties by acting as 'low-dose stressors' that may prepare cells to resist more severe stress from the activation of cell signaling pathways, but high doses are cytotoxic.[33]

For this reason, it is important to consider the doses at which genoprotective and genotoxicity effects occur. The GEMO Assay was initially developed to test the antioxidant nature of chemical compounds that can revert oxidative damage which causes dsDNA breaks (genotoxicity).

To develop the GEMO Assay, a complementary test was performed using a well-known antioxidant molecule (vitamin C) that previous studies described as being dose-dependent antioxidant, antimutagenic and anticarcinogenic properties. The choice to use vitamin C in the ascorbic acid form in an experiment involving the GEMO Assay was based on the large body of evidence that described the ability of vitamin C to affect genetic damage from studies that investigated its action on the formation of DNA adducts,

DNA strand breakage (using the Comet Assay), oxidative damage measured as levels of 8-oxo-7,8-dihydroxy-2'-deoxyguanosine (8-oxodG), cytogenetic analysis of chromosomal aberrations and micronuclei, and the induction of DNA repair proteins.[34]

The GEMO Assay was also validated by comparing DNA damage evaluated for a DNA Alkaline Comet Assay with the same prooxidant and methodological conditions using two cell types: peripheral blood mononuclear cells, (PBMCs) and colon carcinoma cell line (HT29). The HT29 cells were isolated from a primary tumor in a 44 year old Caucasian female and formed a well-differentiated adenocarcinoma colorectal consistent with the primary grade I colony. A previous study showed the antiproliferative effects of ascorbic acid associated with the inhibition of genes necessary to cycle the progression in these cells.[35]

Description of Methods

GEMO Assay: general conditions

The GEMO Assay consists of a fast, inexpensive fluorimetric method for the screening the direct genotoxicity/antigenotoxicity effects of one determined chemical or extract without cell metabolic (mainly DNA repair and antioxidant systems) and structural (histone proteins and others) interferences. The assay includes a standardized prooxidant that is used to compare the effects of dsDNA damage on the compound-test that is evaluated with and without the addition of this prooxidant. The standard prooxidant chosen to perform the GEMO Assay was the H_2O_2. The Fenton reaction [(H_2O_2)+ $FeSO_4.7H_2O$) was also tested as a prooxidant condition, however the reaction presented higher instability, producing many variable results. Therefore, the dsDNA exposition to H_2O_2 at 3 M concentration for 30 minutes was chosen as the better prooxidant condition. After this treatment, the PicoGreen® dye (1:200 TE) was added to the wells and the fluorescence was read after five minutes at room temperature.

The assay was performed in a black, 96-well plate and used Quant-iT™ PicoGreen® dsDNA Reagent DNA from calf thymus purchased from Invitrogen (Eugene, OR, USA) diluted in a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) with reagents of the highest purity/grade purchased from Sigma-Aldrich (St Louis, MO, USA). The fluorimetric analyses are measured by SpectraMax M2/M2e Multi-mode Plate Reader, (Molecular Devices Corporation, Sunnyvale, CA, USA) at an excitation of 480 nm and an emission of 520 nm recorded at room temperature. To improve the experiment by avoiding oxidative light effects on the reaction, the incubation periods of H_2O_2 and PicoGreen® must be conducted in darkness. Since the fluorimeter equipment is highly sensitive, and to avoid a misinterpretation of the data obtained, it is recommended that any compound or extract tested by GEMO Assay must be performed in three independent repetitions with each treatment replicated in eight wells.

Also, since the Quant-iT™ PicoGreen® dsDNA reagent used in the GEMO Assay is able to quantitate lower dsDNA concentrations (~25 pg/mL) using a standard spectroflurometer, differences among fluorescence levels observed between dsDNA controls and dsDNA exposed to some genotoxic molecules can indicate dsDNA degradation. After 30 minutes of H_2O_2 incubation in the GEMO Assay, approximately $\geq$ 55% of dsDNA is degraded. Therefore, the H_2O_2 is used as a genotoxic standard molecule in the GEMO Assay.

Furthermore, the standardization of genotoxic molecules used in the test allowed the GEMO Assay to be organized into two complementary parts. The first part evaluates if the chemical test presents genotoxic capacity. In this case, the dsDNA is exposed to different concentrations of the chemical test and the PicoGreen® fluorescence is compared with these concentrations and a non-treated dsDNA sample (negative control). The second part evaluates the genoprotective capacity of the compound-test. To analyze this potential effect, the dsDNA is exposed to a genotoxic molecule that causes a break in the dsDNA, producing a

single-strand DNA (ssDNA) and/or nucleotides that were not detected by PicoGreen® dye. This effect causes a decrease in the dsDNA fluorescence when compared with the dsDNA control group. The analysis is also performed after 30 minutes of genotoxic exposition with and without the presence of a compound-test. Since the genotoxic substance causes a decrease in dsDNA fluorescence if the compound-test is present, some genoprotective capacity will be observed at elevated fluorescence levels.

To permit data reproduction of each repetition, the results must be presented as a percentage of the negative control group considered as 100% of dsDNA concentrations measured by fluorescence. The following equation is used to determine the mean percentage of the control sample: control sample = (fluorescence of each treatment x 100)/fluorescence of the non-treated sample. The results are presented as mean ± standard error (SE) and are compared by an analysis of variance followed by a *post hoc* test, preferentially Tukey test.

In the first part of GEMO Assay, it is possible to observe if the compound-test presents: (1) genoprotective capacity (dsDNA fluorescence higher than 100% when compared to a control group); (2) no genomodification capacity (dsDNA fluorescence similar to an untreated control group); (3) moderate genotoxicity (dsDNA fluorescence lower than 100% yet higher or equal to 50% when compared with a control group) or (4) higher toxicity (dsDNA fluorescence lower than 50% when compared with a control group).

In the second part of the GEMO Assay, where the compound-test is added with a genotoxic substance (H_2O_2), it is possible to observe: (1) higher genoprotective capacity (dsDNA fluorescence higher than 100% when compared with a control group); (2) genoprotective capacity (dsDNA fluorescence similar to a control group); (3) partial genoprotective capacity (dsDNA fluorescence lower than 100% yet higher or equal to 50% when compared with a control group), and (4) no genoprotective capacity (dsDNA fluorescence similar to the group treated just with H_2O_2, a positive control). In fact, only one compound-test can present all categories of the GEMO Assay dependent of its concentrations. However, the detection of this category permits a quick identification of the concentration zone that is potentially safe in terms of the effects on dsDNA and the concentration zone that presents genotoxicity indication.

To demonstrate the GEMO Assay's applicability, we treated the dsDNA with vitamin C (Sigma-Aldrich, St Louis, MO, USA), with and without the addition of H_2O_2. We choose vitamin C to perform the GEMO Assay because this antioxidant, genoprotective and antitumoral activity are well characterized.[36] Vitamin C was also used in the validation tests using cell systems exposed in the same genotoxic conditions of GEMO Assay.

Assay Standardization

For the GEMO Assay standardization, the adopted analytics conditions are showed in the tables 1 and 2. The schematic of experiment is represented in the Figure 1.

TABLE 1 – Reagents used for standardization and development of GEMO Assay

Buffer	TE (10 mM Tris-HCl e 1 mM EDTA pH 7,5)
Sample	dsDNA Calf Thymus (1 µg/mL)
Prooxidant	H_2O_2 (3M)
Compound-test	Vitamin C (0,1; 1 e 10 µg/mL)
Specific dye for dsDNA	Quant-iT™ PicoGreen® (1:200)

TABLE 2 – Optimized conditions for the GEMO Assay standardization

Temperature	Room temperature
Time of Incubation	30 minutes
Luminosity	Darkness
Kind of material	Black plate of 96 wells
Wave length	Emission: 520 nm/ Excitation: 480 nm

Legend	Calf Thymus DNA 1µg/mL	TE Buffer 1x	H_2O_2 3M	Compound test
Negative Control	20 µL	180 µL	------	------
Positive Control genotoxic	20 µL	130 µL	50 µL	------
Several concentrations of compost test	20 µL	130 µL	------	50 µL
Several concentrations of compost test + H_2O_2 3M	20 µL	80 µL	50 µL	50 µL

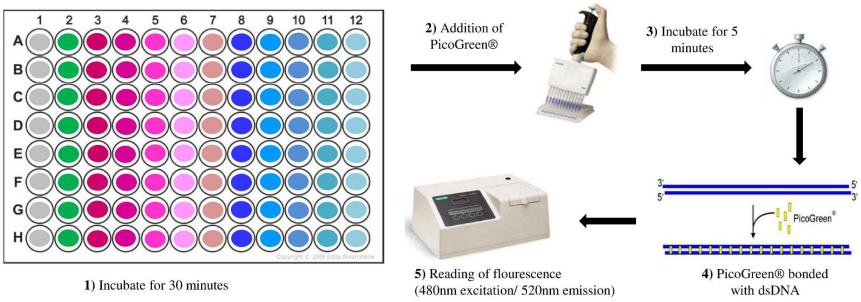

Figure 1. *GEMO Assay Standardization.*

Selectivity, precision and stability

Selectivity is the capacity of a method to measure a compound in presence of the others reagents. Test GEMO's selectivity is showed in the Figure 2, the same results were found in three repetitions and the mean is showed in the graphic. Analyses of potential interference of the TE buffer and H_2O_2 on PicoGreen® dye fluorescence were tested. The control group presented 100% of fluorescence. This value decreased to 50% of fluorescence when H2O2 was present, indicating dsDNA degradation. The results did not show any significant influence of TE and H_2O_2 on the fluorescence excitation at 480 nm and emission at 520 nm. As seen in Figure 2, the control group presented 1521.2 ± 154.8 of fluorescence. This value decreased to 643.3 ± 124.4 of fluorescence when H_2O_2 was present, indicating dsDNA degradation. Analysis of potential interference of the TE buffer and H_2O_2 on PicoGreen® dye fluorescence was also tested; the results did not show any significant influence of TE and H_2O_2 on the fluorescence excitation at 480 nm and emission at 520 nm.

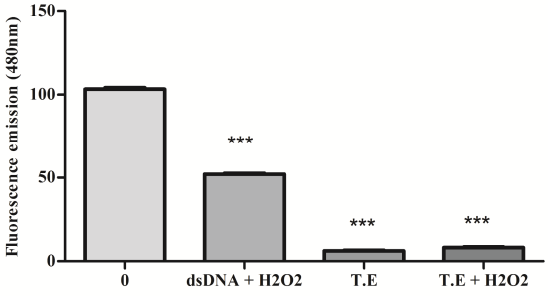

Figure 2. *The fluorescence decreased when H2O2 was present, indicating dsDNA degradation. Analysis of the potential interference of the TE buffer and H2O2 on PicoGreen® dye fluorescence did not show any significant influence of TE and H2O2 on the fluorescence. *** p<0,001, n=3.*

The intra- (Figure 3) and inter-day (Figure 4) precision and accuracy of the method were evaluated on three

different days. It was found to intra- and inter-day repeatability of the method, indicating its accuracy. Figure 3 shows the results found on intra-day analyses.

The first equation: F= dsDNA + CT of the GEMO Assay is demonstrated in the Figure 3 A, where F= fluorescence at 480 nm excitation and 520 nm emission determined from a known dsDNA concentration exposed to chemical-test (Vitamin C). Molecules and extracts that cause a break in dsDNA by a decrease in fluorescence in comparison to the fluorescence observed in the untreated dsDNA (control group), indicating a genotoxic capacity. The fluorescence in treated groups did not decrease, therefore the Vitamin C did not show genotoxic capacity. The second reaction that analyzes the genoprotective capacity of chemical-test (Vitamin C) is showed in the Figure 3 B, the following equation: F= dsDNA+ GS+ CT where F= fluorescence at 480 nm excitation and 520 nm emission is determined from a known dsDNA concentration exposed to Vitamin C in the presence of H_2O_2. The genoprotective capacity was complete in the concentration of 0,1 and 1 ug/mL of Vitamin C and it was partial in the concentration of 10 ug/mL.

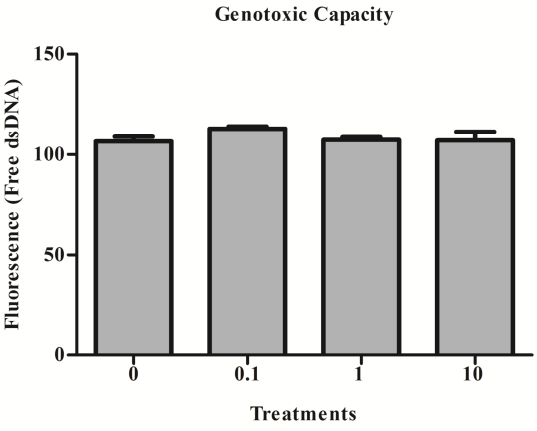

Figure 3 A. *dsDNA exposed to different concentrations of Vitamin C. The results were compared with the negative control (dsDNA). n=3.*

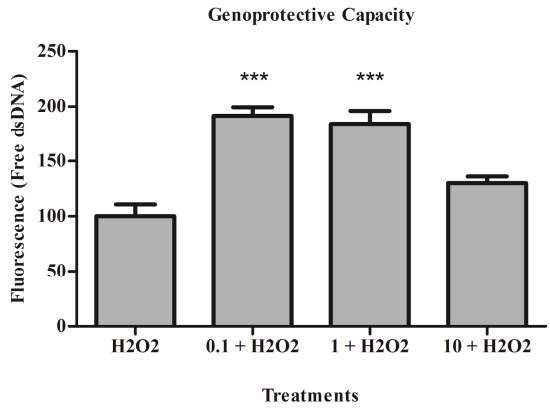

Figure 3 B. *dsDNA treated to different concentration of vitamin C and H2O2. The results were compared with the positive control (H2O2). p<0,001, n=3.*

The reproducibility of the GEMO Assay was evaluated by comparing data assessed by three independent experiments that followed similar laboratorial conditions (inter-day precision and accuracy showed in the figure 4). The dsDNA degradation caused by H_2O_2 showed a similar pattern in the three experiments as well as the results found to CT (Vitamin C). The Pearson correlation was high and significant (p< 0.0001) among the three experiments: r2first x second= 0.92; r2first x third=0.84; r2second x third=0.916.

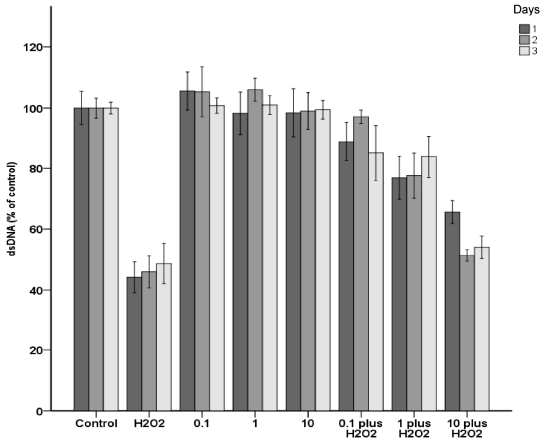

Figure 4. *Inter-day precision and accuracy of GEMO Assay, using different Vitamin C concentrations isolated and associated with H_2O_2.*

The stability of the dsDNA diluted in buffer TE used in the GEMO Assay is showed in Figure 5. The fluorescence was obtained in different times, 0, 30, 60, 90 and 120 minutes, after the results were compared with the fluorescence in the zero time. The results showed that the fluorescence remained the same in all the periods. So, the buffer TE allows that the dsDNA keeping stability during the period necessary of the GEMO Assay (30 minutes).

Comparison data from the GEMO Assay and the Alkaline DNA Assay

Although the GEMO Assay is an easy, fast and direct test using pure dsDNA, it is necessary to validate its applicability by a comparison with the alkaline Comet DNA assay, a traditional genotoxic test. To perform this comparison test, PBMCs and HT29 cells were cultured in controlled conditions.

First, PBMCs were obtained from peripheral blood samples collected from three to four healthy adult volunteers after 12 hours of overnight fasting, via venipuncture using top Vacutainer (BD Diagnostics, Plymouth, UK) tubes with heparin. Blood specimens (5 ml) were routinely centrifuged within 1 hour of collection for 15 minutes at 2500g using histopaque-1077® (Sigma-Aldrich, St. Louis, MO, USA) density gradient to obtain PBMC samples. The cells were then transferred to culture media containing 5 mL RPMI 1640 with 10% fetal bovine serum, 1% penicillin and streptomycin and phytohemaglutinin. The cells were cultured at an initial density of 2 x 10^5 cells for 72 hours at 37°C in a humidified atmosphere of 5% CO_2[37]. The HT29 cells, a human colon adenocarcinoma cell line (ATCC), were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/L, InvitrogenLife Technologies, Karlsruhe, Germany). Cell culture medium was also supplemented with 10% fetal calf serum, 1% penicillin/streptomycin (Invitrogen, USA) and cultured at 37˚C in a water-saturated atmosphere containing 5% CO_2.[38]

Both cells types were also counted, centrifuged for 10 minutes at 2000g and transferred to a new culture media with and without H_2O_2 (3M) and different vitamin C concentrations. The exposition was also performed for 30 minutes. Next, each cell sample treatment was centrifuged at 2000g for 10 minutes and cells were isolated from the supernatant culture medium. The cells were used to evaluate the genotoxic damage by the Alkaline Comet Assay; the viability was also evaluated by MTT Assay, a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. [39] The cell viability was also determined by cell-free dsDNA assay using PicoGreen® dye measured in the supernatant medium.[40]

The Alkaline Comet Assay was performed as described by Singh et al. (1995) in accordance with the general guidelines for use of the Comet Assay.[41,42,43] One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analyzed under blind conditions by at least two different individuals.

Complementary cytotoxic and biochemical test

Despite the GEMO Assay being a genotoxic/genoprotective assay and its validation being dependent on a comparison of a traditional genotoxic test like the Alkaline Comet Assay, we performed a complementary investigation on PBMCs to observe if the prooxidant conditions used in the dsDNA pure molecule represent cytotoxic and oxidative stress to the cell systems.

The cytotoxicity was assessed using MTT reduction assays. The MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a 5 mg/ml phosphate buffer (PBS, 0,01M; pH 7.4), added into a 96-well plate containing the

sample treatments and incubated for 4 hours. The supernatant was then removed from the wells; next, the cells were resuspended in DMSO (dimethylsulfoxide) (200 μL). The absorbance at 560 nm was read in the fluorimeter.

The cell-free dsDNA[44] (that indicates apoptotic cells) was determined by using the PicoGreen® dye in conditions similar to those used in the GEMO Assay. The genotoxicity and cytotoxicity was analyzed and compared among treatment groups of both cell lines through an analysis of the variance followed by a Tukey *post hoc* test.

Intracellular ROS production exposed to H_2O_2 plus vitamin C was detected in PBMCs using the non-fluorescent cell-permeating compound 2'-7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is hydrolysed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cellular oxidants. After the H_2O_2 and vitamin C exposure, the cells were treated with DCFH-DA (10 mol/l) for 60 minutes at 37° C. The fluorescence was measured at an excitation of 488 nm and an emission of 525 nm. The calibration curve was performed with standard DCF (0–1 mmol) and the level of ROS production was calculated as nmol DCF formed/mg protein.[45,46] Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS).[47]

Statistical analysis

All analyses were carried out using the statistical package for social studies (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA). The mean values among different dsDNA treatments with and without vitamin C supplementation were compared using an analysis of variance followed by a *post hoc* Tukey test. The Pearson correlation was calculated to compare the results obtained by a GEMO Assay test in three different experiments as well as to compare the DNA damage investigated by a GEMO Assay and the Alkaline DNA Comet Assay in cells submitted to the same prooxidant conditions. All p values were two-tailed. The alpha value was considered to be statistically significant was $p = 0.05$.

Results and Discussion

General conditions of GEMO Assay

From a pilot test, H_2O_2 was chosen to be a better prooxidant standard molecule to use in the GEMO Assay. Initially, it was used to evaluate the Fenton reaction (H_2O_2 + $FeSO_4.7H_2O$) for the prooxidant standard reaction (data not shown). However, the results were highly variable, most likely related to the instability of the chemical reaction. On the other hand, H_2O_2 in a high concentration presented an important effect on dsDNA degradation. The best pro-oxidant conditions were the exposition of dsDNA to H_2O_2 (3M) during 30 minutes in darkness at room temperature to avoid any influence of light on the chemical reaction.

H_2O_2 is a molecule involved in several signaling cell pathways. However, when found (or used) in higher levels produced by different insults such as UV, X and γ radiation, pollutants, poisons, or endogenous disequilibria can produce different types of DNA damage.[48] There is consistent evidence that H_2O_2 causes genomic damage by indirect action such as higher order chromatin degradation, enzymatic excision of chromatin loops and their oligomers at matrix-attachment regions. However, the hydroxyl radical, generated through the Fenton or Haber-Weiss reaction, is more reactive than either superoxide or H_2O_2 and causes direct damage to DNA and other macromolecules resulting in DNA strand breaks and mutations.[49,50] When we performed preliminary tests to develop the GEMO Assay, we analyzed the possibility of using the Fenton reaction to generate dsDNA damage. However, a greater instability occurred in the reaction between H_2O_2 and $FeSO_4.7H_2O$ producing highly variables results with lower precision and reproducibility.[51]

Despite the direct action of H_2O_2 on DNA damage being seen as controversial, some studies performed by Driessens et al.[52] investigated whether the high levels of H_2O_2

produced in the thyroid to oxidize iodide could induce genotoxicity and if they showed DNA damage. It would be difficult to compare these data with our results. The majority of our investigations used biological systems to test the genotoxic compounds; these systems present several structural and metabolic pathways and the GEMO protocol indicates H_2O_2 damage action to dsDNA. Perhaps, in the GEMO Assay, the H_2O_2 effect on dsDNA damage is associated with a higher concentration of this molecule (3M).

GEMO Assay evaluation of vitamin C genotoxic and genoprotective capacity

After the standardization of GEMO conditions, a test using vitamin C as the compound-test was performed. The results obtained in the GEMO Assay are presented in the Figure 6. As expected, the assay showed no genotoxic effect from different vitamin C concentrations on dsDNA since the fluorescence was similar to that which was observed in the untreated dsDNA sample (Fig. 6 A). Conversely, vitamin C in lower doses tested (0.1 to 1 µg/mL) protected the dsDNA from genotoxic effects caused by H_2O_2. Higher vitamin C concentrations did not reverse the DNA damage caused by exposition to H_2O_2 (Fig. 6 B). The H_2O_2 alone and with several vitamin C concentrations showed higher CV (>10< 18%) than dsDNA control (<10%) which was only treated with different vitamin C concentrations. These differences between CV indicate some level of instability in the H_2O_2 reaction with dsDNA as well as vitamin C.

Comparison between the GEMO Assay and the Alkaline DNA Comet Assay

The Comet Assay has been developed as a means of detecting cellular DNA damage; it is generally used in a variety of fields, such as biological monitoring and genetic toxicology. The distance migrated by cellular DNA during electrophoresis directly reflects the extent of DNA damage present.[53] Therefore, we used this method to measure DNA damage in lymphocytes as well as HT29 colon cancer cells exposed to the same methodological conditions used in the GEMO Assay.

The index damage results for both cell lines are presented in Figure 7. The PBMCs exposed to H_2O_2 showed higher DNA damage when compared with the control group. The vitamin C alone presented similar index damage to the control group as well as protected against H_2O_2 damage, although this protection was partial when compared with an untreated control group ($p<0.0001$). A Pearson correlation was performed between the GEMO Assay considering the dsDNA percentage of fluorescence control and the Comet alkaline results considering the index damage. The results showed a high negative correlation between both tests $r^2 = -0.828$ ($p<0.0001$). Higher dsDNA fluorescence measured by GEMO Assay is associated with lower index damage measured by an Alkaline DNA Assay (Figure 7 A).

However, when the effect of H_2O_2 and vitamin C on the HT29 cancer line was analyzed (Figure 7 B), untreated control cells presented similar index damage observed in the H2O2 exposition. Vitamin C at 1 and 10 µg/mL exposition increased the index damage. The presence of H_2O_2 decreased this damage to values similar to the control groups and H_2O_2 treatments. Contrary to the results found in PBMCs, no significant correlation was found between GEMO Assays and the Alkaline DNA Assay performed in HT29 cells (r2= 0.105, =0.624). The differences between the results obtained from PBMCs and HT29 probably reflect the differences between the biology of normal and cancer cells and antitumoral vitamin C activity.[54]

Complementary tests were performed to confirm the prooxidant and toxic conditions of the experiment (Table 3). The cell viability was significantly affected by a high H_2O_2 concentration when compared with the control group. The vitamin C treatment did not affect the PBMCs viability, and when H_2O_2 was present, the cytotoxicity partially reverted. As expected, the treatment with H_2O_2 generated higher levels of ROS when compared with the control group. The ROS levels were similar in cells exposed to vitamin C whereas cells

exposed to vitamin C plus H_2O_2 presented intermediary ROS levels when compared with the control group and H_2O_2 cell treatments. The lipoperoxidation was also significantly affected by vitamin C with and without H_2O_2 exposition. The vitamin C concentrations caused an increase in the TBARS levels without H_2O_2 exposition. However, the cells exposed to vitamin C plus H_2O_2 presented partial reversion of lipoperoxidation when compared to the cells treated just with H_2O_2. The whole of these results confirms the toxic conditions created by the conditions used in the GEMO Assay suggesting that this test conveys some real conditions found when the biological systems are exposed to prooxidant and antioxidant molecules that affect the DNA damage.

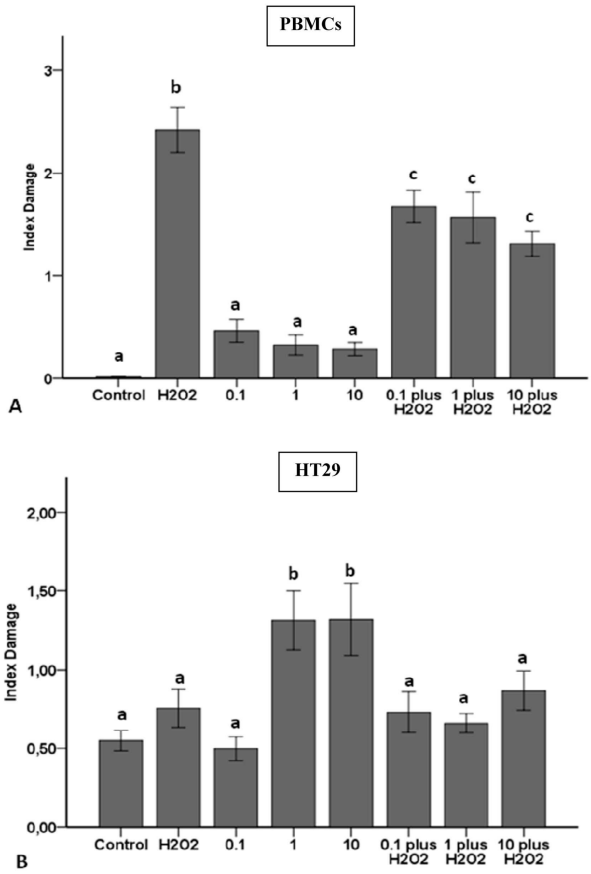

Figure 7. *The index damage results for both cell lines. The PBMCs exposed to H_2O_2 showed higher DNA damage when compared with the control group. The vitamin C isolated presented similar index damage to the control group as well as protected against H_2O_2 damage (Figure 7 A). Untreated control cells HT29 presented similar index damage observed in the H_2O_2 exposition. Vitamin C at 1 and 10 µg/mL exposition increased the index damage. The presence of H_2O_2 decreased this damage to values similar to the control groups and H_2O_2 treatments (Figure 7 B).*

Table 3 Complementary tests of pro-oxidant conditions of the GEMO Assay test using PBMCs samples

Treatments	MTT	ROS	TBARS
	Mean ± SE	Mean ± SE	Mean ± SE
Control	99.9 ± 1.1^a	102.9 ± 1.3^a	100.2 ± 0.4^a
H_2O_2	4.2 ± 0.6^b	$371{,}9 \pm 2.5^b$	345.2 ± 1.2^b
0.1	88.2 ± 2.3^a	97.1 ± 1.4^a	121.7 ± 1.1^c
1	86.3 ± 1.3a	90.4 ± 1.5^c	118.1 ± 1.6^c
10	86.9 ± 1.5^a	108 ± 2.2^a	119.3 ± 1.9^c
0.1 plus H_2O_2	13.7 ± 1.9^c	144.6 ± 1.8^d	178.4 ± 1.8^d
1 plus H_2O_2	15.5 ± 1.7^c	160.7 ± 2.3^e	190.1 ± 2.1^e
10 plus H_2O_2	7.8 ± 0.8^b	171.6 ± 1.8^e	197.3 ± 2.0^e

SE= standard error. The results are expressed as % of control dsDNA group. Different letters indicating significant statistic differences among the treatments compared by analysis of variance followed by *post hoc* Tukey test at p=0.05 significance.

From these results, the GEMO Assay could be a complementary test to the screening of new chemicals or unknown plant extracts to detect the dose-range that presents genoprotective and/or genotoxicity capacity using a dsDNA pure molecule. From the results obtained using this fast and inexpensive assay, it is possible to identify the range of concentrations that can potentially be used to realize additional tests using biological systems (cells and animals). The GEMO Assay does present limitations intrinsic to non-cell *in vitro* tests such as: (1) the effect of cellular protective mechanisms against prooxidants with potential carcinogenic proprieties is not evaluated; (2) the interactions between the prooxidant and other molecules present in the extra and intra-cellular environment that can attenuate or increase the mutagenic effect is also not evaluated, and (3) the test is limited to molecules that have some effect on H_2O_2 that does not represent a "universal" prooxidant. However, the use of other prooxidant compounds or with other chemical properties that cause mutagenesis can be used as a substitute for H_2O_2 used to develop the GEMO Assay.

Conclusions

Due to the necessity of the identification of chemicals with genoprotective and genotoxic effects and that the contemporary Eukaryotic Assay involves more complex and expensive tests, the fluorimetric GEMO Assay was developed. This test permits a rapid assessment of a CT effect on a dsDNA molecule without interfering variables to estimate if this compound does or does not have Genomodifier capacity (genotoxic and genoprotective) and the range of concentrations that these properties occurs. The GEMO Assay can be used for detecting dsDNA damage alterations caused by pesticides that we are exposed daily through food, mainly found in fruits. Furthermore, the GEMO Assay can be useful to investigate the interaction between dsDNA and several drugs that are used in the pharmacology treatments.

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