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Original Article

Bisphenol-A induced cyto-genotoxicity on retinal pigment epithelial cells is differentially modulated by a multi-supplement containing guarana, selenium, and L-carnitine

A citogenotoxicidade induzida pelo bisfenol-A em células epiteliais pigmentares da retina é modulada diferencialmente por um multissuplemento contendo guaraná, selênio e L-carnitina

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Abstract

Bisphenol A (BPA) may adversely affect human health by inducing oxidative stress and irreversible damage to cells. Bioactive compounds found in some functional foods, individually or in combination, can attenuate the negative effects of BPA exposure; an example is the multi-supplement containing guarana (Gua), selenium (Se), and L-carnitine (LC) — GSC— which has already demonstrated antioxidant, genoprotective, and immunomodulatory activities. This study aimed to determine the effect of GSC and its constituents on oxidative and genotoxic alterations triggered by BPA exposure in the retinal epithelial cell line. The cells exposed to BPA (0.001, 0.01, 0.1, 1, 3, and 10 µM) to determine the lowest concentration required to induce cyto-genotoxicity. ARPE-19 cells were then concomitantly exposed to the selected BPA concentration, GSC, and its components (Gua, 1.07 mg/mL; Se, 0.178 µg/mL; and LC, 1.43 mg/mL). Flow cytometry, biochemical assays, qRT-PCR, genotoxicity, apoptosis, and cellular proliferation. Based on our results, 10 µM of BPA could induce cyto-genotoxic and oxidative alterations. BPA did not alter the Bcl-2/BAX expression ratio but induced Casp3 and Casp8 overexpression, suggesting that apoptosis was induced mainly via the extrinsic pathway. GSC partially reversed the alterations triggered by BPA in ARPE-19 cells. However, Se had unexpected negative effects on ARPE-19 cells. The multi-supplement GSC may attenuate changes in oxidative and genotoxic markers related to exposure of ARPE-19 cells to BPA. our results revealed that the antioxidant, anti-apoptotic, and genoprotective properties of GSC were not universally shared by its individual, once Se did not exhibit any positive impact.

Keywords: food supplement, apoptosis, ARPE-19 cells, bisphenol-A.

Resumo

O Bisfenol A (BPA) pode afetar negativamente a saúde humana, induzindo estresse oxidativo e danos irreversíveis às células. Os compostos bioativos encontrados em alguns alimentos funcionais, individualmente ou em combinação, podem atenuar os efeitos negativos da exposição ao BPA; um exemplo é o multissuplemento contendo guaraná (Gua), selênio (Se) e L-carnitina (LC) —GSC— que já demonstrou atividades antioxidantes, genoprotetoras e imunomoduladoras. Este estudo teve como objetivo determinar o efeito do GSC e seus constituintes nas alterações oxidativas e genotóxicas desencadeadas pela exposição ao BPA na linhagem celular epitelial da retina (ARPE-19). Células foram tratadas em diferentes concentrações de BPA (0,001, 0,01, 0,1, 1, 3 e 10 µM) a fim de determinar a concentração mais baixa necessária para induzir citogenotoxicidade. As células ARPE-19 foram então expostas concomitantemente à concentração selecionada de BPA, o GSC e seus componentes (Gua, 1,07 mg/mL; Se, 0,178 µg/mL; e LC, 1,43 mg/mL). Ensaios de citometria de fluxo, bioquímicos, qRT-PCR, genotoxicidade, apoptose e proliferação celular foram executados. Com base em nossos resultados, 10 µM de BPA foi capaz de induzir alterações citogenotóxicas e oxidativas. O BPA não alterou a razão de expressão de Bcl-2/BAX, mas induziu a superexpressão de Casp3 e Casp8, sugerindo que a apoptose foi desencadeada principalmente pela via extrínseca. O GSC reverteu parcialmente as alterações desencadeadas pelo BPA nas células ARPE-19. No entanto, o Se teve efeitos negativos inesperados nas células ARPE-19. O GSC multissuplemento pode atenuar alterações nos marcadores oxidativos e genotóxicos relacionados à exposição das células ARPE-19 ao BPA. Nossos resultados revelaram que as propriedades antioxidantes, antiapoptóticas e genoprotetoras do GSC não foram universalmente compartilhadas pelo seu indivíduo, uma vez que o Se não apresentou nenhum impacto positivo.

Palavras-chave: suplemento alimentar, apoptose, células ARPE-19, bisfenol-A.

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1. Introduction

Bisphenol A (BPA), the plastic monomer chemically named 4,4-isopropylidene diphenol (2,2-bis(4-hydroxyphenyl)-propane), is one of the highest-volume chemicals produced worldwide. It is used to manufacture polycarbonate plastics, epoxy resins, food packaging, and household items. Based on epidemiological and experimental investigations, BPA can adversely affect human health because it is an estrogen-like hormone (Abraham and Chakraborty, 2020; Tarafdar et al., 2022). BPA is associated with the risk of carcinogenesis, reproductive toxicity, infertility, and abnormal nervous system development (Murata and Kang, 2018). BPA and its analogs can affect eye function given their ability to accumulate in the aqueous humor (Flieger et al., 2022) exhibiting toxicity at certain stages of neural retinal development (Li et al., 2021).

Some organs, such as the eyes, can be more susceptible to the negative effects of BPA. Experimental studies, such as those conducted by Chiang et al. (2022), have revealed that BPA triggers oxidative stress and apoptotic events in retinal pigment epithelial cells (ARPE-19), which were used as an *in vitro* model of age-related macular degeneration (AMD). AMD affects millions of people and is a leading cause of blindness globally. Oxidative stress is considered a major contributor to the emergence and progression of AMD because it acts directly on retinal pigment epithelial cells by accumulating cellular waste (such as lipofuscin and macromolecule oxidation products) and impairing autophagic processes (Zhang et al., 2020). These events contribute to establishing chronic inflammatory states and vascular alterations related to AMD pathology (Tan et al., 2020).

The effects of BPA can be attenuated by some plant extracts or bioactive molecules that have antioxidant, genoprotective, and anti-inflammatory activities (Nie and Wang, 2022). However, the mechanisms underlying the protective effects of these bioactive compounds against the adverse effects of BPA exposure require clarification. To determine whether different dietary components with well-known antioxidant and genoprotective activities have similar effects against the alterations caused by BPA, *in vitro* experiments were performed using ARPE-19 cells, a commercially available retinal pigment epithelial cell line (Chiang et al., 2022).

Studies performed by our research team revealed the *in vitro* and *in vivo* antioxidant and immunomodulatory actions of a multi-supplement named GSC, which contains guarana (Gua) (*Paullinia cupana*) extract, selenium (Se), and L-carnitine (LC) (Teixeira et al., 2021). Gua is a natural plant native to the Amazon rainforest in Brazil and has been cultivated since the pre-Columbian period. The seeds are the most crucial part of the Gua plant; they are used to make Gua powder or extracts, which have gained popularity for their potential health benefits. Epidemiological and experimental evidence showed that Gua has antioxidant, anti-fatigue, anti-inflammatory, genoprotective, and other biological effects (Torres et al., 2022). Besides caffeine, Gua also contains other beneficial compounds like theobromine and theophylline, contributing to its stimulatory effects. Given its high caffeine concentration, Gua is widely used worldwide as a raw material in the energy drink industry (Abalo, 2021).

The Se is a trace mineral that is considered an essential nutrient. Hence, it must be supplemented through the diet as it is not produced by the body. The foods richest in Se are nuts and seeds (especially Brazil nuts, *Bertholetia excelsa*), seafood, meat and poultry, grains, dairy products, and some vegetables. In addition to natural dietary sources, Se supplements are available as Se-enriched yeast or synthetic compounds like Se methionine and chelated Se produced with sodium selenate, hydrolyzed rice, and soy protein. These supplements are commonly used to address Se deficiencies or as a preventive measure in regions with low Se levels in the soil and diet (Rayman, 2020; Pecoraro et al., 2022).

LC is a water-soluble compound obtained mainly from red meat, poultry, fish, dairy products, and some vegetables. LC supplements can be produced in association with other molecules, such as LC tartrate, which is obtained from LC and tartaric acid. LC supplementation has been associated with the attenuation of neuroinflammatory states (Traina, 2016).

A previous study described that Gua exerted cytoprotective and genoprotective effects *in vitro* in ARPE-19 cells exposed to paraquat, which increased cellular superoxide anion levels (Bonadiman et al., 2017). Therefore, we investigated the potential mitigating effects of GSC on ARPE-19 cells exposed to BPA. In addition, we examined the impact of each component (Gua, LC, and Se) on ARPE-19 cells exposed to BPA to determine whether they exhibit similar, synergistic, or antagonistic effects. A previous investigation showed that treating ARPE-19 cells with Se nanoparticles attenuated the cytotoxic effects caused by hypoxia (Özkaya et al., 2021). Another study found that selenomethionine (SeMet) induced antioxidant effects mediated by the cystine/glutamate exchanger SLC7A11 in ARPE-19 cells (Ananth et al., 2020). Additionally, one study reported that LC attenuated epithelial-mesenchymal transition (EMT), which is an important underlying mechanism of proliferative vitreoretinopathy (PVR) in ARPE-19 cells (Li et al., 2021).

These results indicate that GSC or its constituents could attenuate the cytotoxicity induced by BPA in ARPE-19 cells. This hypothesis was tested by analyzing the protective effects of GSC on cytotoxicity, apoptosis induction, cell-proliferation rate modulation, and oxidative marker and DNA damage levels as assessed by a micronucleus assay.

2. Material and Methods

2.1. Experimental design and treatments

An *in vitro* protocol was employed to identify the lowest concentration of BPA that can trigger changes in the viability, proliferation, and expression of oxidative metabolism markers in ARPE-19 cells. Subsequent experiments used BPA concentrations of 0.001, 0.01, 0.1, 1, 3, and 10 µM, corresponding to 0.000228, 0.00228, 0.028, 0.228, 0.68, and 2.28 µg/mL, respectively.

The beneficial effects of GSC and its components in BPA-exposed ARPE-19 cells were determined. The second protocol evaluated the modulatory effects of GSC and its components in the BPA-induced changes in the rate of apoptosis, cell cycle, genotoxicity, and apoptosis-related gene expression.

The tested multi-supplement was developed by the research group, with the aim of being used as an adjuvant to pharmacological treatment in multiple sclerosis. Its conception was based on the premises suggested by studies that evaluated the effects of isolated food supplements in Multiple Sclerosis (Marx et al., 2020) and by studies that have already created a mixture of phytochemicals and nutraceuticals for use in other clinical conditions and obtained potential benefits (Bulku et al., 2010; Ray et al., 2006). The choice of specific combinations of guarana, selenium and L-carnitine was based on an extensive literature review and pilot tests. The GSC multi-supplement was formulated by the researchers and prepared in the form of capsules, in a compounding pharmacy. For the formulation, the maximum daily intake doses of each GSC component, recommended by Brazilian Health Regulatory Agency (ANVISA), were considered: 500 mg of guaraná dry extract, 320 µg of selenium and 2000 mg of L-carnitine. Based on these values and considering a 70 kg adult as a reference (mg GSC/kg), concentrations equivalent to doses 1 to 50 times higher than the maximum recommended doses were calculated. Please note that the multi-supplement is not commercially available.

GSC concentration that exhibited antioxidant and anti-inflammatory effects, as previously described by Teixeira et al. (2021), was considered as a reference. A complementary study performed by Teixeira et al. (2023) in patients with multiple sclerosis supplemented with GSC for 12 week showed that GSC did not induce any type of adverse effect indicative of toxicity. Rodents treated with the components of GSC showed that Gua had low toxicity (oral lethal dose to kill 50%, LD_{50} = 825 g/Kg) (Antonelli-Ushirobira et al., 2010). In contrast, sodium selenite had an LD_{50} = 8.08-12.11 mg/Kg and LC (Jacevic et al., 2011). On the other hand, LC is regarded as safe as studies in rodents have confirmed no acute toxicity.

Therefore, in this study, we tested a GSC formulation containing 1.07 mg/mL Gua, 0.178 µg/mL Se, and 1.43 mg/mL LC. A pilot experiment evaluating the potential cytotoxicity of GSC on ARPE-19 cells using half and twice the concentration of GSC used here showed no cytotoxic effect. The cells' exposure time to BPA and its components varied according to the protocols employed, with all *in vitro* assays conducted with at least three replicates.

Since commercially acquired cell lines were used, ethics approval was not required.

2.2. Chemicals, reagents, and solutions

Analytical grade chemicals and reagents, which were mainly obtained from Sigma-Aldrich®, Inc. (St. Louis, MO, USA), were used in this study. Cell culture plasticware and reagents were purchased from Gibco Life Technologies®, Inc. (Grand Island, New York, USA) and Invitrogen Life Technologies® (São Paulo, Brazil). A BPA stock solution (1000-10000×) in dimethyl sulfoxide (DMSO) was prepared before 24 h of use to avoid chemical degradation of the substance. The stock solution was stored at –4 °C until further use.

Working solutions were prepared by diluting the BPA stock solutions with the culture medium. The final DMSO concentration was 0.1%, owing to the limited solubility of BPA. GSC, Gua powder extract, Se, and LC tartrate were purchased from SM Pharmaceutical Enterprises Ltd. (São Paulo, SP, Brazil) and prepared as described by Teixeira et al. (2021, 2023). The Gua supplement was manufactured as a powder dried by seed spraying with 7.79% caffeine and 1.02% tannins. Commercial grade GSC and its components were formulated by a manipulation pharmacy (Nova Derme, Santa Maria, RS, Brazil) with raw material authorized by the National Health Surveillance Agency (ANVISA) of the Ministry of Health of Brazil as safe for human consumption.

All GSC constituents did not contain heavy metals (lead, copper, antimony) or microorganisms. At the research team's request, a manipulation pharmacy prepared the multi-supplement and encapsulated it. The capsules were then sent to the laboratory, opened under aseptic conditions, and dissolved in phosphate-buffered saline (PBS, pH 7.4) before the experiments.

The multi-supplement preparations were diluted in the cell culture medium Dulbecco's Modified Eagle Medium/Hams Nutrient Mixture F-12 (DMEM/F-12). The qRT-PCR reagents were obtained from Promega Corporation (Madison, Wisconsin, USA). The primers were synthesized by Merck KGaAm (Darmstadt, Germany) and a cDNA synthesis kit was obtained from Bio-Rad Laboratories (Hercules, CA-USA). The flow cytometry results were analyzed using FlowJo vX.0.7 software (Tree Star, Inc., Ashland, OR, USA). The samples were stored at –80 °C and analyzed within 20 days of treatment and collection.

Protocols were performed with at least five replicates in 96-well plates and in independent triplicates. As it is impossible to obtain a sample of cells to perform all laboratory analyses simultaneously, the protocols of exposure to BPA, GSC, and its components were repeated in different contexts.

2.3. Cell culture conditions

The immortalized cell line ARPE-19 (ATCC® CRL-2502) was acquired from the Rio de Janeiro Cell Bank (BCRJ, https://bcrj.org.br/). This nonprofit, nongovernmental organization acquired ARPE-19 cells from the American Type Culture Collection (ATCC, Manassas, VA, USA). ARPE-19 cells were cultured under standardized conditions at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM/F-12 supplemented with 15% fetal bovine serum (FBS), antibiotics (1% penicillin/streptomycin), and 1% amphotericin B (antifungal). Finally, the cells were cultured in bottles and subcultured for a maximum confluence of 80%.

2.4. Cell viability assays

Cellular viability was assessed after 24 h of incubation using spectrophotometric 3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyltetrazolium bromide (MTT) and adenosine triphosphate (ATP) bioluminescence assays. The MTT assay is a widely used colorimetric assay that measures cell viability and proliferation based on the conversion of the yellow, water-soluble MTT dye into a purple formazan product by metabolically active cells. Mitochondrial dehydrogenases in viable cells reduced MTT into formazan crystals, which are insoluble in water and accumulate inside the cells. The quantification of cell viability using the MTT assay was performed as described by Teixeira et al. (2021). The ATP bioluminescence assay was performed using the firefly luciferin-luciferase system. The resulting luminescence intensity indicates the number of cells with ruptured membranes or increased permeability, which occurs in dead or dying cells. An ATP Assay Kit (Sigma-Aldrich®) was used to perform the assay according to the manufacturer's instructions. A cell density of 1×10^5 cells/mL was used in the cell viability experiments.

2.5. Oxidative marker assays

The levels of the following four oxidative markers were spectrophotometrically analyzed: nitric oxide (NO), superoxide anion (SA), lipoperoxidation (LPX), and protein carbonylation (PCarb). A cell density of 1×10^6 cells/mL was used in the experiments. NO levels were indirectly estimated by quantifying nitrate levels using the Griess reagent (Noh et al., 2015). SA was quantified by analyzing the formazan concentration triggered by the nitroblue tetrazolium (NBT) reaction (Morabito et al., 2010). LPX was measured by quantifying its products, mainly thiobarbituric acid reactive substances (TBARS), of which malondialdehyde was the primary product (Jentzsch et al., 1996). PCarb was measured by determining the presence of carbonyl groups according to their reaction with dinitrophenylhydrazine, indicating protein oxidation and oxidative stress (Levine et al., 1994). All spectrophotometric analyses were performed within 30 days of performing the experimental protocols.

2.6. Effects of BPA exposure on cell proliferation

The proliferation of ARPE-19 cells exposed to different BPA concentrations was evaluated daily for 7 days using a clonogenic assay described previously (Cubillos-Rojas et al., 2014) with some modifications. A total of 1×10^3 cells/well were seeded in triplicate in 6-well plates. Each day, the cells were fixed and stained with crystal violet, and the monolayer filling pattern and morphology of ARPE-19 cells were analyzed using ImageJ software. The effect of BPA on ARPE-19 cell proliferation in 72h cultures was measured using the MTT assay, using the same protocol described previously.

2.7. Flow cytometry analysis of apoptosis and the cell cycle

The effects of GSC and its components on the apoptosis rate and cell cycle progression of ARPE-19 cells exposed to BPA were assessed using flow cytometry. The quantification of apoptotic events in ARPE-19 cells was performed using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit according to the manufacturer's instructions. Briefly, 1×10^6 cells were seeded per well in 6-well plates containing 2 mL of the different treatment solutions in DMEM/F12 and incubated for 24h. The cells were then trypsinized and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL; 100 µL of the cell suspension was transferred to a new tube. The resuspended cells were gently vortexed, stained with 5 µL of Annexin-V-FITC and 5 µL of PI, and incubated at room temperature (± 24 °C) in dark for 15 min. Fluorescence readings were captured after adding 400 µL of 1× binding buffer to the samples.

To quantify the proportion of cells in each stage of the cell cycle, the cells were centrifuged and washed with PBS, pH 7.4. The cells were then suspended in 500 µL of a solution containing PI dissolved in PBS (50 µg/mL PI from 50 mg/mL stock solution), 2.5 mg/mL, 0.1 mg/mL RNase A, and 0.05% Triton X-100, and incubated for 40 min at 37 °C. Afterward, the cells were washed with 3 mL of PBS, suspended in 500 µL PBS, and flow cytometry analysis was performed. The cells were sorted based on their forward scatter/side scatter (FSC/SSC) patterns captured using a BD FACSVerse™ system. Data acquisition and cell content analyses were performed using FlowJo vX.0.7 software (Tree Star, Inc., Ashland, OR, USA).

2.8. Micronucleus (MN) assay

DNA damage was quantified using a protocol described by the Organization for Economic Co-operation and Development (OECD, No. 487, 2016) with slight modifications (OECD, 2016). The main modification was performed to avoid monolayer breakage or cell release before analysis. Cell rupture releases many nuclei from the cytoplasm, making analysis difficult. A previous cell proliferation analysis revealed that the cells had a high proliferative rate over 72 h, a mandatory condition for MN analysis. A lower initial cell concentration was used to avoid overlap. Therefore, ARPE-19 cells were transferred to 24-well plates (1×10^2 cells/well) and treated with BPA for 72 h.

After 72 h, the spent culture medium was removed and the cells were washed with PBS. DAPI (5 mg/mL diluted in deionized water) was then added to each well. A panoptic staining kit was used according to the manufacturer's instructions to evaluate nuclear and cellular morphologies. Microscopy analysis was performed at 200× magnification using a fluorescence or light microscope, and alterations in 1000 cells per treatment were scored using the established criteria for MN evaluation (Thomas et al., 2009). Digimizer Image Analysis Software (Ostend-Belgium) was used to investigate the presence of MNs and other alterations.

2.9. Gene expression analysis using qRT-PCR

Additionally, we determined the expression levels of genes associated with apoptosis triggered by BPA upon interaction with GSC and its components. Cells could undergo the following two apoptotic mechanisms: the intrinsic and extrinsic pathways. Gene expression analysis was performed as previously described by Barbisan et al. (2014). The initial cell concentration used in this experiment was 1×10^6 cells/mL. For RNA extraction, TRIzol reagent was used according to the manufacturer's instructions. Prior to the analysis, the extracted RNA was quantified using a

Thermo Scientific NanoDrop™ 1000 spectrophotometer at a wavelength of 532 nm. Reverse transcription was performed by adding RNA samples (1 µg/mL) to 0.2 µL of DNase (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37 °C for 5 min, and then heating the sample at 65 °C for 10 min. cDNA was generated using 1 µL of reverse transcriptase and 4 µL of Master Mix. The following cycling conditions were employed: 25 °C for 5 min, 42 °C for 30 min, and 85 °C for 5 min, followed by incubation at 5 °C for 60 min. qRT-PCR was performed using a 20 µL reaction volume comprising 1 µL of cDNA and 19 µL of GoTaq qPCR Master Mix. The following cycling conditions were employed for qRT-PCR: 95 °C for 3 min, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s, followed by a melting curve analysis at 0.1 °C/min from 40 to 90 °C. β-actin was used as the housekeeping gene. Relative expression was calculated using the comparative Ct method and expressed as the fold change relative to the control. The primer characteristics are listed in Table 1.

2.10. Statistical analysis

The data were analyzed using GraphPad Prism software (version 6.0, 2015) and are presented as a percentage of the control based on data obtained from at least three replicates. Quantitative variables were compared using a one-way analysis of variance followed by Tukey's *post hoc* test or a two-way analysis of variance followed by Bonferroni's *post hoc* test. The chi-square test was used to compare the frequency of MN between treatments, and the data are presented as the relative frequency of MN for every 1000 nuclei analyzed. This statistical procedure followed the OECD report nº. 487 (2016). A *p*-value less than 0.05 was considered to indicate statistical significance.

3. Results

Initially, the acute cytotoxic effect of BPA on 24-h ARPE-19 cell cultures was evaluated after exposure to low BPA concentrations (0.001, 0.01, and 0.1 µM). In both assays, no differences in cell viability were observed compared to the control. However, the results of the two tests were inconsistent at the three highest concentrations of BPA tested (1, 3, and 10 µM).

The MTT assay (Figure 1A) indicated a significant increase in cell viability for cultures exposed to 1 and 3 µM of BPA compared to controls. Cells exposed to 10 µM of BPA showed similar viability to unexposed cells. However, results from the ATP assay (Figure 1B) indicated similar viability in ARPE-19 cells exposed to BPA at concentrations up to 3 µM, with a significant decrease in viability observed only after exposure to 10 µM of BPA. However, it is important to highlight that the decrease in cell viability observed in these experiments was less than 10%. Since both protocols assess cell viability by analyzing cellular metabolic activity, the combined results indicated that BPA did not induce considerable cytotoxicity in ARPE-19 cells.

However, depending on the concentration, BPA differentially modulated the levels of oxidative markers. In BPA-exposed cells, NO levels remained significantly higher than those in control cells after 6, 24, and 72h of culture (Figure 1C). Conversely, exposure to BPA at all tested concentrations significantly decreased the SA levels (Figure 1D). All concentrations of BPA resulted in higher levels of LPX than those in the controls (Figure 1E). At 24 hours, ARPE-19 cells had higher PCarb levels than the controls (Figure 1F).

We then evaluated whether BPA induced changes in the proliferation of ARPE-19 cells. Up to day 3, the proliferation rate was similar between the cells with and without BPA exposure (Figure 2A, 2B). After this period, all BPA concentrations decreased cell proliferation compared to the control. The highest concentration of BPA (10 µM) affected the lowest growth rate based on the confluence (%) of the 7d cultures divided by the confluence of the 24h cultures (Figure 2C).

The potential genotoxic effect of BPA was evaluated through a qualitative and quantitative analysis of MN. The main alterations in the DNA and morphology of ARPE-19 cells caused by exposure to different concentrations of BPA (Figure 3) were MN, dark condensations potentially due to fragments of genetic materials, rough nuclear edges, and large and irregular nuclei potentially from non-disjunction during cell division. The amount of MN per 1000 nuclei was four times greater in ARPE-19 cells exposed to 3 and 10 µM of BPA than that in the controls and cells exposed to 1 µM of BPA (Figure 2D).

Collectively, these results indicated that a BPA concentration of 3 µM could induce oxidative and genotoxic alterations on ARPE-19 cells, although it did not show considerable cytotoxicity and alterations in cell proliferation. Therefore, this concentration was used to determine the extent of attenuation of the negative effects of BPA on ARPE-19 cells by GSC and its components.

First, we postulated that oxidative alterations and increased MN frequency in the 72h cultures decrease cell proliferation via apoptosis. We found that treatment with 3 µM BPA significantly increased the proportion of

Table 1. Gene primers.

Gene	Genbank	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
β-Actin	Gene ID: 60	TGTGGATCAGCAAGCAGGAGTA	TGCGCAAGTTAGGTTTTGTCA
Bax	Gene ID: 596	CCCTTTTCTACTTTGCCAGCAA	CCCGGAGGAAGTCCAATGT
Bcl-2	Gene ID: 581	GAGGATTGTGGCCTTCTTTGAGT	AGTCATCCACAGGGCGATGT
Casp3	Gene ID: 836	TTTGAGCCTGAGCAGAGACATG	TACCAGTGCGTATGGAGAAATGG
Casp8	Gene ID: 841	AAGGAGCTGCTCTTCCGAATT	CCCTGCCTGGTGTCTGAAGT

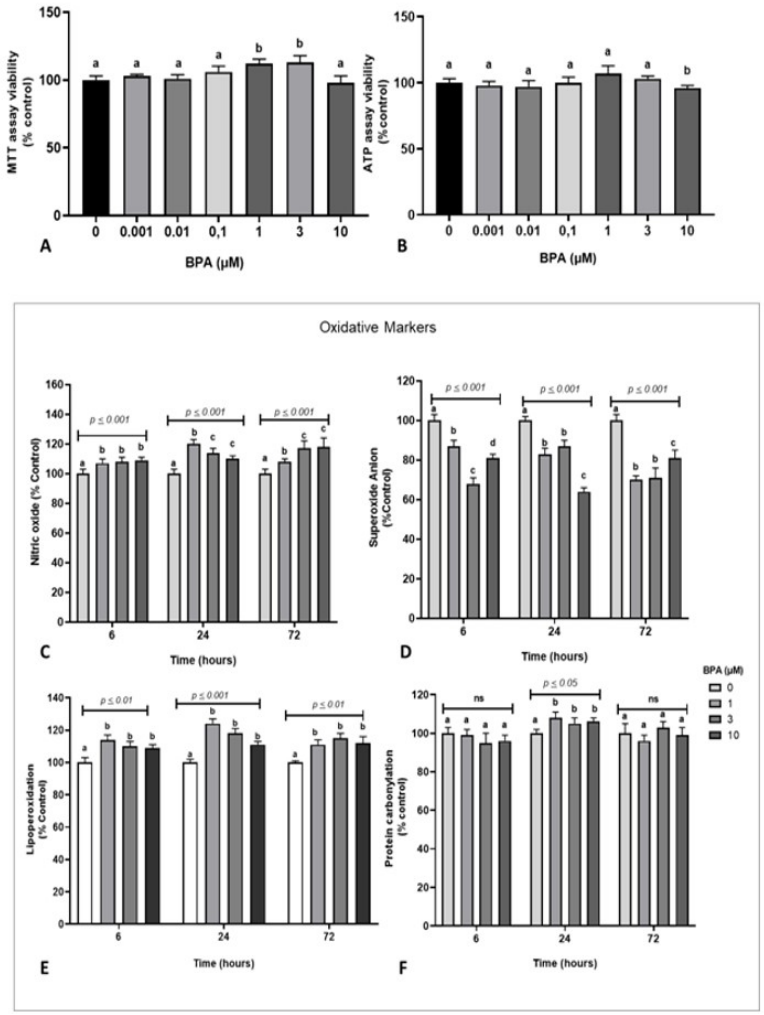

Figure 1. Impact of BPA on ARPE-19 cell viability as determined using the MTT (A) and ATP (B) assays; (C-F) Modulation of oxidative markers in ARPE-19 cells exposed to BPA at different concentrations. Oxidative marker levels were quantified using spectrophotometric assays. A two-way analysis of variance followed by the Bonferroni *post hoc* test was performed. Different letters represent statistically significant differences between treatments at $p < 0.05$.

apoptotic cells compared to that in the control (Figure 4A-C). Exposure to GSC significantly decreased the frequency of apoptosis compared to control cultures (Figure 4D-F). Although the three components of GSC significantly decreased the apoptosis frequency in combination with BPA (Figure 4D, 4E), cells exposed to Se only had a higher apoptosis rate than control cells (Figure 4J-4M).

Next, we investigated whether the decrease in the proliferation rate of ARPE-19 cells after exposure to 3 µM BPA involved changes in the cell cycle (Figure 5). All treatments significantly decreased the frequency (%) of S+G2 cells compared to that in the control. However, cells exposed to GSC and GSC+BPA showed a higher frequency than the other treatments, followed by cells exposed to SE+BPA, LC, and LC+BPA. The cells exposed to BPA, Gua, Gua+BPA, and Se showed the lowest frequency of cells in the S+G2 phase.

The effects of GSC and its individual components on the modulation of oxidative markers and MN frequency, with or without BPA exposure, were analyzed. The data are summarized in Table 2. NO levels were significantly increased in cells exposed to Se+Gua and Se than in the control and other treatments. Exposure to Gua+BPA also significantly increased NO levels compared to those in the control. All other treatments had decreased NO levels compared to the control.

Exposure to Se and Se+BPA significantly increased SA levels than the control and other treatments, similar to cells exposed to BPA only. All other treatments had decreased AS levels compared to the untreated controls. Considering LPX levels, some treatments showed an oxidative effect since they increased the levels of these molecules (Se, Se+Gua > Gua+BPA > BPA). However, exposure to GSC and its individual components showed an antioxidant effect by decreasing LPX levels. PCarb levels in cells exposed to BPA, Gua, Se+BPA, LC, and LC+BPA were similar to the control. While exposure to Se increased PCarb levels (indicating an

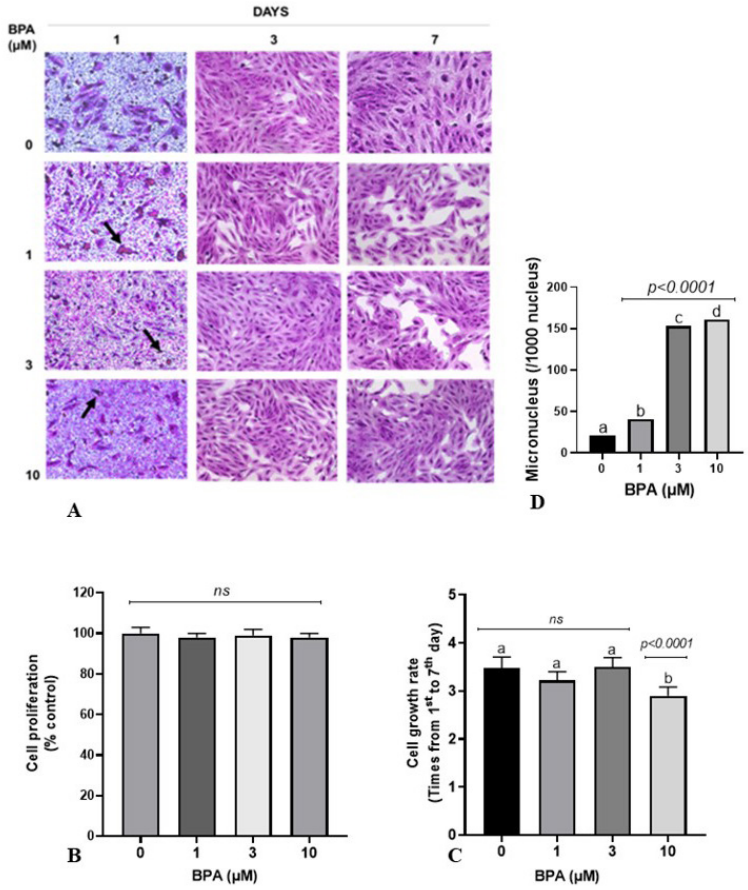

Figure 2. Effect of bisphenol A (BPA) on the proliferation and micronucleus (MN) frequencies in ARPE-19 cells. (A) Microscopy images of the cell cultures (200× magnification) exposed to different concentrations of BPA after 1, 3, and 7 days. Arrows indicate the deposition of residues or dead cells in cultures; (B) Cell proliferation rate of the 72h cultures as determined using the MTT assay; (C) Cell growth rate based on the clonogenic assay and cell doubling rate after 7 d compared to that on the first day of culture; (D) Number of micronuclei (per 1,000 nuclei) was quantified in 72h ARPE-19 cell cultures stained with DAPI. Statistical comparisons of the MTT and clonogenic assay data were performed using a one-way analysis of variance followed by Tukey's *post hoc* test. MN frequency was statistically evaluated using the chi-square test. Different letters represent statistically significant differences between treatments $p < 0.05$.

Table 2. Interaction effect of BPA, the GSC multi-supplement, or its individual components on the oxidative stress markers and micronucleus (MN) frequency of ARPE-19 cells at 72 h.

	Oxidative markers (% of control) and MN (%)				
	Media ± SD				
	NO	SA	LPX	PCarb	MN
Control	100.0 ± 2^{a}	100.0 ± 1.9^{a}	100.0 ± 3.1^{a}	100.0 ± 3.9^{a}	1.7 ± 0.5^{a}
BPA	111.2 ± 3.5^{b}	108.2 ± 1.6^{b}	118.4 ± 3.6^{b}	103.6 ± 1.5^{a}	5.2 ± 0.9^{b}
GSC	96.1 ± 2.9^{b}	91.4 ± 2.7^{b}	96.4 ± 2.9^{a}	94.9 ± 3.9^{b}	2.1 ± 0.3^{a}
GSC+BPA	97.5 ± 1.2^{a}	85.5 ± 3.3^{b}	99.7 ± 1.5^{a}	91.3 ± 1.2^{b}	2.4 ± 0.6^{a}
Gua	91 ± 4.1^{c}	98.2 ± 7.5^{a}	91.2 ± 4.1^{b}	105.6 ± 3.4^{a}	1.2 ± 0.3^{a}
Gua+BPA	145.2 ± 4.7^{d}	89.9 ± 2.5^{b}	145.2 ± 3.6^{d}	90.9 ± 1.0^{b}	2.4 ± 0.2^{a}
Se	303.2 ± 3.7^{e}	138.2 ± 2.5^{c}	323.6 ± 5.3^{e}	116.7 ± 3.27^{c}	5.1 ± 0.5^{b}
Se+BPA	304 ± 4.5^{e}	134.5 ± 3.6^{c}	297.1 ± 4.6^{f}	93.5 ± 2.67^{a}	8.5 ± 0.9^{c}
LC	94.3 ± 3.6^{c}	88.2 ± 2.8^{b}	94.9 ± 2.0^{b}	92.7 ± 2.0^{a}	2.2 ± 0.7^{a}
LC+BPA	94.7 ± 8.1^{c}	95.9 ± 3.6^{b}	92.2 ± 8.1^{b}	92.7 ± 1.8^{a}	1.4 ± 0.4^{a}

BPA = Bisphenol A; Gua = guarana (1.07 mg/mL); Se = selenium (0.178 μg/mL); LC = L-carnitine (1.43 mg/mL); NO = nitric oxide; SA = superoxide anion; LPX = lipoperoxidation; PCar = protein carbonylation; MN = micronucleus frequency determined based on at least 10 replicates. A total of 1000 scores were analyzed for each treatment. Each marker was compared between treatments using a two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test. Significant differences at $p < 0.05$ are indicated by different letters for each of the investigated markers.

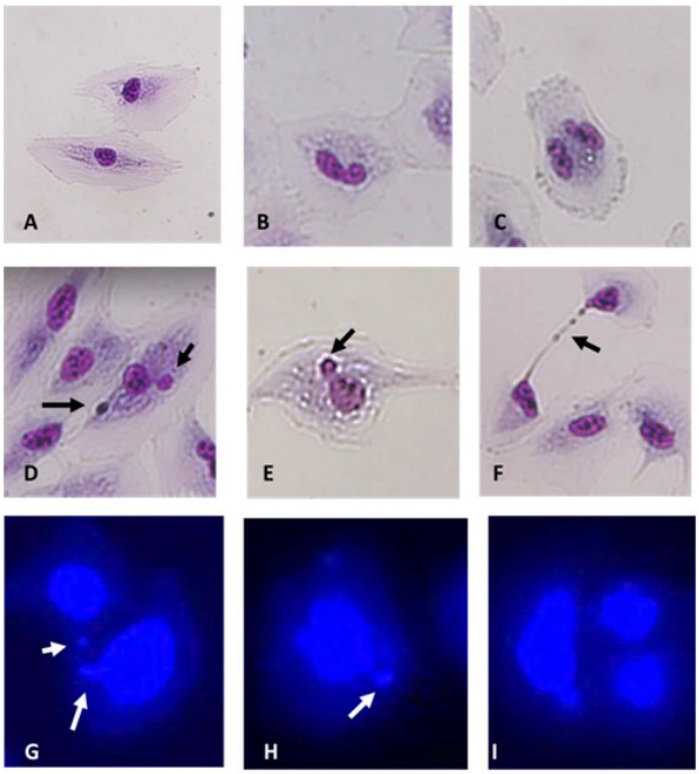

Figure 3. Representative microphotographs of ARPE-19 cells stained with Panopticon, in which nuclear and genetic materials are denoted by a purple color (200× magnification). (A) ARPE-19 cells with eccentric nuclei and poorly stained cytoplasm; (B, C) Binucleated cells undergoing mitotic division; (D) Cells with micronuclei (MN) and dark condensations may be fragments of genetic material (arrow); (E) Cells with visible MN (arrow); (F) Two cells with nuclear bridges (arrow); (G) Nuclei stained with DAPI showing the nuclear button (arrow) and MN (arrow); (H) Cells with MN and rough nuclear edges; (I) Cells with a large and irregular nucleus potentially originating from non-disjunction associated with a binucleated cell in cell division, which also presents nuclei with irregular edges.

oxidative effect), exposure to GSC, GSC+BPA, and Gua+BPA presented an antioxidant effect through a decrease in the levels of this marker. A genotoxic effect via increased MN frequency was observed in cultures exposed to Se+BPA, Se, and BPA. The other treatments showed an MN frequency similar to the control.

The effects of the GSC multi-supplement and its individual components on the expression of four genes related to apoptosis were evaluated (Figure 6A). To facilitate the interpretation of the results, the following three possibilities were considered: gene expression similar to the control (1 ± 0.2), upregulation (> 1.2), and downregulation (< 0.8). As apoptosis can be induced by two pathways (intrinsic regulation by the mitochondria and extrinsic regulation by death receptors), we also analyzed the Bcl-2/BAX expression ratio (Figure 6B).

A decreased Bcl-2/BAX expression ratio, indicated higher BAX gene expression, suggesting the induction of apoptosis via the intrinsic pathway. Overall, BPA did not alter the Bcl-2/BAX ratio but induced the overexpression of the Casp3 and Casp8 genes, suggesting that apoptosis was induced mainly via the extrinsic pathway. GSC treatment increased the Bcl-2/BAX ratio, exhibiting gene expression-like control of Casp3 and Casp8 genes, indicating no modulation of the apoptotic pathways. However, GSC did not modify the effect of BPA on ARPE-19 cells as the Casp3 and Casp8 genes were significantly overexpressed.

Regarding the effects of the individual GSC components, Gua increased the Bcl-2/BAX ratio and slightly increased Casp8 gene expression. In contrast, Gua reversed the BPA-induced overexpression of Casp3 and Casp8. This observation was corroborated by an increased Bcl-2/BAX ratio in the cells exposed to Gua+BPA. Cells exposed only to LC overexpressed Casp8, whereas cells concomitantly exposed to LC+BPA overexpressed Casp3. Exposure to Se with or without BPA induced the overexpression of Casp3, although an increased Bcl-2/BAX ratio was also observed.

4. Discussion

Adult cross-sectional epidemiological and pre-clinical *in vitro* and *in vivo* studies suggest that BPA, an endocrine-disrupting pollutant, induces oxidative stress and that persistent exposure may cause serious health problems (Santoro et al., 2019). A prior study conducted by Chiang et al. (2022) suggested that, in addition to its negative effects on reproductive cells and tissues, BPA can impact other tissues, including the human retinal pigment epithelium using ARPE-19 cells as *in vitro* experimental

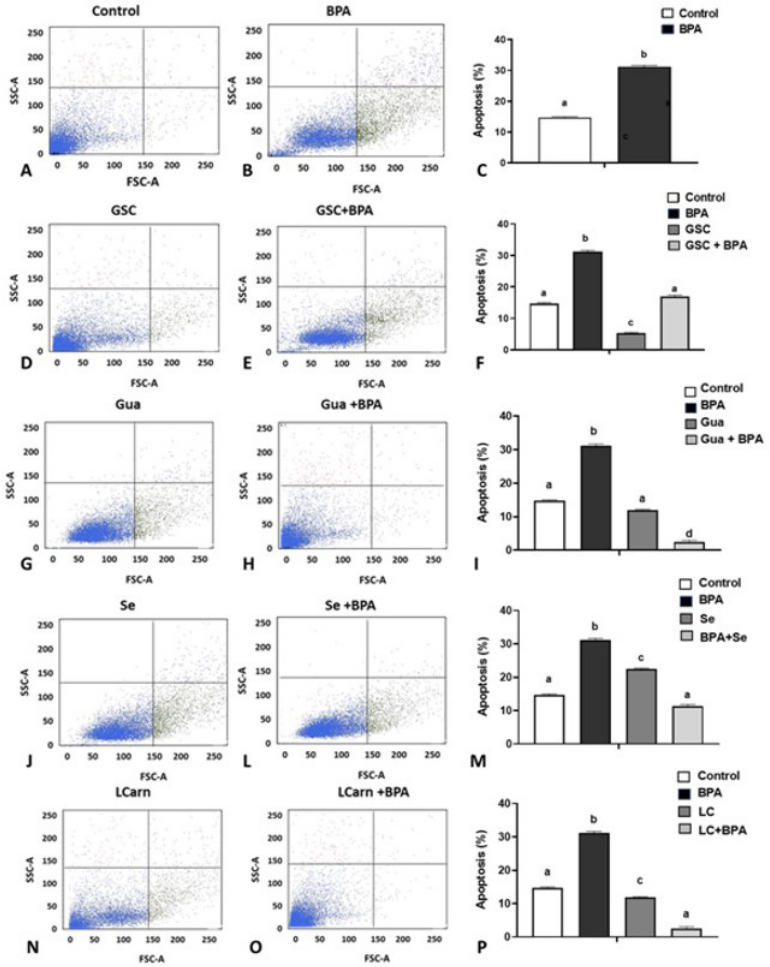

Figure 4. Effect of 3 µM bisphenol A (BPA), GSC, or its individual components (Gua = guarana, Se = selenium, LC = L-carnitine) on the apoptosis rate of ARPE-19 cells cultured for 72h. Cells were stained using an Annexin-V/Propidium Iodide kit and apoptosis was evaluated by flow cytometry. Annexin-V was used to label dead and apoptotic cells. Two representations of flow cytometry and a comparative graph are presented for each treatment: (A-C) controls and BPA; (D-F) GSC and GSC+BPA; (G-I) Gua and Gua+BPA; (J-M) Se and Se+BPA; and (N-P) LC and LC+BPA. As the statistical analysis was bivariate (two-way analysis of variance followed by Tukey's *post hoc* test), the data for each type of treatment are presented separately to facilitate the interpretation of the interaction effect between BPA and GSC or its individual components. Different letters represent statistically significant differences between treatments at $p < 0.05$.

model. Their results showed that BPA exposure induced ROS generation and cytotoxicity on ARPE-19 cells, which was reduced when the antioxidant molecule - N-acetyl-L-cysteine - was added to the culture medium.

Indeed, considering the importance of ocular function for autonomy and quality of life, especially in elderly population, assessment of whether dietary components, with antioxidant properties, could attenuate the negative effects induced by BPA on ARPE cells is highly relevant. Based on previous evidence from *in vitro*, *in vivo*, and human studies that GSC presents antioxidant and anti-inflammatory effects (Teixeira et al., 2021, 2023), we postulated that it could also attenuate the negative effects of BPA on ARPE-19 cells. To test this hypothesis, we exposed ARPE-19 cells to BPA with or without the addition of GSC or its individual components. BPA exposure triggered alterations in some oxidative markers, but these effects were mitigated by the exposure to GSC (particularly Gua and LC). However, exposure to Se or Se+BPA exacerbated the oxidative stress induced by BPA. These findings suggest that the relationships between BPA and the antioxidant components of GSC, Gua, Se, and LC are not universally applicable to ARPE-19 cells.

Initially, it is essential to emphasize the need for selecting a representative concentration of BPA to induce oxidative stress and genotoxicity in ARPE-19 cells. This is because *in vitro* protocols may elicit variations in cytotoxic response depending on the culture conditions, even when laboratories follow the guidelines outlined in the Guidance Document on Good *In Vitro* Method Practices (GIVIMP) published by the OECD (2016) (Eskes et al., 2017). Furthermore, there are no previous studies involving genotoxicity analysis through the MN assay in ARPE-19 cells.

The MN assay is a widely used genotoxicity test in toxicology and regulatory sciences to evaluate the

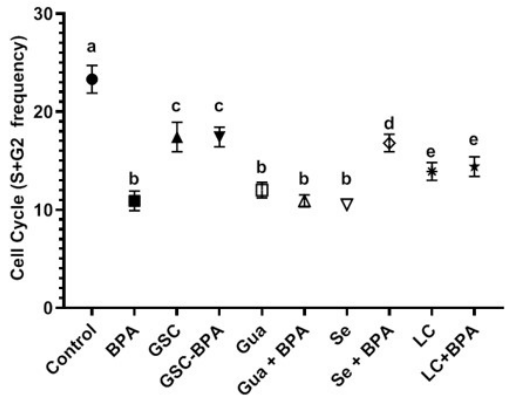

Figure 5. Effect of treatment with the GSC multi-supplement and its individual components (Gua = guarana, Se = selenium, LC = L-carnitine) with or without bisphenol A (BPA) on the frequency (%) of ARPE-19 cells in the S+G2 phase of the cell cycle at 72h. Statistical comparison was performed using two-way ANOVA followed by a *post hoc* Bonferroni test. Different letters represent statistically significant differences between treatments at $p < 0.05$.

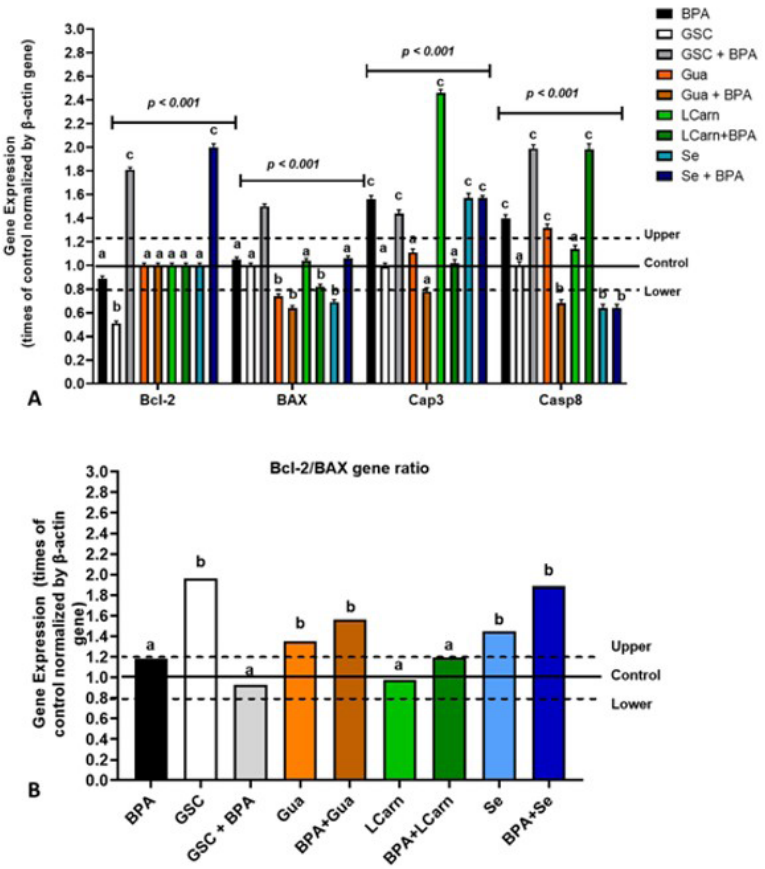

Figure 6. Differential expression of apoptotic genes (Bcl-2, BAX, Casp3, and Casp8) in ARPE-19 cells exposed to BPA, the GSC multi-supplement, and its individual components (Gua = guarana, Se = selenium, LC = L-carnitine). (A) Comparison of gene expression levels of Bcl-2, BAX, Casp3, and Casp8. Values over 1.2 indicate upregulation and those below 0.8 indicate downregulation relative to the control. The solid line represents the value of 1 in control cells, whereas the dotted lines above and below represent the upper and lower limits of gene expression variation. Treatments with values between the two dotted lines were considered to have similar gene expression to the control; (B) Bcl-2/BAX ratio gene expression among ARPE-19 cells exposed to BPA (3 µM) with or without GSC supplementation or its individual components (LC, Gua, and Se). Differences in gene expression were analyzed using two-way ANOVA followed by a Bonferroni *post hoc* test. Different letters indicate significant differences. Gene expression values were normalized to that of β-actin. Values are expressed in relation to the control, which had a reference value of 1.

potential of chemical substances to induce DNA damage and chromosomal abnormalities. This assay is based on detecting micronuclei, small fragments of chromosomes that are not incorporated into the nucleus during cell division. MN can arise from a variety of DNA-damaging events, such as chromosomal breakage, mitotic spindle disruption, or aneuploidy. Due to its relevance in detecting genotoxic states, the OECD has developed guidelines and recommendations for this assay, providing standardized procedures and best practices for its implementation. The OECD Test Guideline 487 (TG 487) specifically addresses *in vitro* MN tests. The relevance of the MN assay lies in its ability to assess the potential genotoxicity of various substances, including chemicals used in pharmaceuticals, cosmetics, pesticides, and industrial products. This assay is crucial in the safety assessment of these substances to protect human health and the environment. By evaluating the occurrence of MN in cells exposed to a test substance, researchers can identify compounds that may cause genetic damage and chromosomal aberrations, which could lead to adverse health effects, including cancer and birth defects. Therefore, the OECD guidelines ensure that the MN assay is conducted using standardized protocols, enhancing the reliability, reproducibility, and comparability of results across different laboratories and studies (OECD, 2016).

For this reason, we paid special attention in selecting the concentration of BPA to evaluate the antioxidant effects of GSC and its individual components. Our goal was to ensure that the concentration was not too low to prevent oxidative changes or too high to cause acute cytotoxicity, leading to the death of ARPE-19 cells within 24h. By carefully choosing an appropriate concentration range, we balanced the induction of oxidative stress and maintenance of cell viability for an adequate experimental duration. This approach allowed us to effectively assess the potential protective effects of GSC and its individual components against the oxidative impact of BPA on ARPE-19 cells.

The results from two viability assays did not demonstrate acute cytotoxicity (24h cultures) in ARPE-19 cells exposed to BPA in the 0.001 to 3 µM concentration range. However, the findings from cells exposed to 10 µM of BPA were contradictory. Although the MTT assay showed no significant decrease in ARPE-19 cell viability, the ATP assay revealed a significant reduction in viability compared to the controls.

Although the MTT assay is widely used for cytotoxicity testing in *in vitro* models, it has some limitations due to potential interference from certain molecules, such as polyphenols (Bruggisser et al., 2002). In our study, in addition to the maximum concentration of BPA (10 µM) that did not reduce cell viability, concentrations of 1 and 3 µM showed a significant increase in viable cells compared to that in the control, albeit small (<10%). These results differed from those published by Chiang et al. (2022), who observed a cytotoxic effect when ARPE-19 cells were exposed to 10 µM BPA. As this was the lowest concentration tested by the authors, and we found this inconsistency regarding BPA cytotoxicity, we cannot confirm whether BPA might have affected the MTT assay results. As a result, we excluded this concentration from the protocols used to test the antioxidant and genoprotective effects of GSC and its components. This ensured that any observed effects were not compromised by confounding factors related to the MTT assay, maintaining the integrity and reliability of our experimental approach.

This decision was reinforced by analyzing the effect of the three highest concentrations of BPA (1, 3, and 10 µM) on the levels of four oxidative markers. We observed an increase in NO and LPX levels compared to those in the controls in the 24, 48, and 72h cultures. PCarb levels were also higher in 48-h cultures treated with BPA only, suggesting a transient oxidative effect on cellular proteins. This was possibly regulated by the endogenous antioxidant components of ARPE-19 cells.

One intriguing result that requires further discussion is the decrease in SA levels in cells exposed to BPA compared to the control. In the study by Chiang et al. (2022), they observed a reduction in the concentrations of the enzymes - superoxide dismutase (SOD) and catalase - in ARPE-19 cells exposed to BPA. They also noted an increase in lipid peroxidation (MDA) levels in the cells; however, they did not quantify the levels of NO. The SA has a high affinity for NO, and the reaction of these two molecules generates a reactive nitrogen species (peroxynitrite) that leads to extensive lipid peroxidation of membranes. Since the levels of superoxide were significantly reduced in ARPE-19 cells exposed to BPA, it is possible that BPA induces a substantial increase in the concentration of NO, with a large portion of it reacting with the SA, consequently elevating lipid peroxidation levels. This condition could potentially explain why Chiang et al. (2022) observed a decrease in the expression of SOD. The interplay between the SA and NO may be a crucial factor contributing to the observed alterations in oxidative stress markers and antioxidant enzyme levels in response to BPA exposure. Further investigation into the relationship between these reactive species is warranted to fully comprehend the mechanisms underlying BPA-induced oxidative stress in ARPE-19 cells.

This assumption is supported by previous studies. One study described an increase in the expression of inducible nitric oxide synthase (iNOS) and NO levels in the testicles of mice exposed to BPA (Chouhan et al., 2015). The authors suggested that the modulatory effect of BPA on NO metabolism could contribute to a decline in male steroidogenesis. Other investigations also described an increase in iNOS and/or NO levels in chicken embryo kidney (CEK) cells (Chen et al., 2022) and in the human hepatoma Hep3B cell line (Nakamura et al., 2018). A study by Wang et al. (2019) described that BPA induced neurotoxicity in glutamatergic neurons derived from human embryonic stem cells due to increased iNOS and nNOS enzymatic activity. Another *in vitro* investigation revealed that BPA induced cytotoxic NO and caspase-8 levels in SHSY-5Y neuron-like cells (Ayazgök and Tüylü Küçükkilinç, 2019). The results of these studies confirm the possibility of BPA inducing elevated NO levels, leading to potential interactions with SA and subsequent alterations in the levels of oxidative stress markers and enzymes.

Although the rate of cell proliferation was not impacted by exposure to BPA, the DNA quality of cells decreased, as shown in the results of the MN assay. We were unable to identify previous studies involving MN analysis in

ARPE-19 cells, indicating that this approach in this type of *in vitro* model is novel and can serve as a reference for future studies that evaluate the genotoxicity of substances against retinal epithelial cells. Our results showed that the MN frequency in cells exposed to BPA at a concentration of 3 µM was approximately four times greater than that of cells exposed to a concentration of 1 µM. Along with the results of the other analyses on cell viability and oxidative marker levels, the best concentration of BPA to test the cytoprotective effect of GSC and its components is 3 µM.

Our results confirmed that BPA can increase the rate of apoptosis and decrease cell proliferation in 72h cultures of ARPE-19 cells. However, apoptosis rates were reversed in ARPE-19 cells exposed to GSC and its individual components, indicating a cytoprotective effect; in contrast, Se-exposed cells showed a higher apoptosis rate than the controls. Se is an essential trace mineral that plays a crucial role in various biological processes. However, Se supplementation has both risks and benefits, depending on the intake levels. While Se is beneficial in small amounts, excessive intake can lead to toxicity (selenosis). In humans, the recommended daily intake of Se varies depending on age, sex, and life stage. For most adults, the recommended daily intake is approximately 55-70 µg/day.

Se is usually attainable through a balanced diet that includes Se-rich foods, such as fish, shellfish, nuts, seeds, and whole grains (Gore et al., 2010). Although we tested a Se concentration lower than the recommended dose (40 µM) in this study, it is possible that this concentration is toxic to ARPE-19 cells, considering that there is no clearance mechanism in cell cultures and exposure to Se at this concentration was continuous.

Another possible explanation for these results lies in the chemical formulation of Se used in this study. Supplements are found in different formulations to facilitate their absorption by the body. Some of the more common types of Se formulations used in supplements include selenomethionines, selenide and selenate, selenium yeast, and other special formulations. In Brazil, the most common commercial form of Se is 0.5% chelated selenium bis-glycinate, which was used in the present study. ARPE-19 cells are probably more sensitive to this chemical compound of Se than selenomethionine, which is also widely used. Complementary studies in ARPE-19 cells comparing different Se formulations in different concentrations could be conducted to clarify this.

Similar results on the pro-apoptotic effect of BPA have already been described in the literature, including that in immortalized mouse myoblast C2C12 cells by Liu et al. (2021). They described that the pro-apoptotic effect of BPA was attenuated by supplementation with coenzyme Q10 (CoQ10), a powerful antioxidant, which can improve the integrity of the lysosomal membrane, lysosomal degradation function, and promote autophagy. Despite the anti-apoptotic effect of GSC and its individual components observed in this study, MN assay confirmed the anti-apoptotic effect of GSC and its individual components and how the oxidative state and frequency of DNA damage are affected by GSC in this context. In fact, the genotoxic effect of BPA might be organ and cell type-dependent, similar to that for human blood cells (Ruberto et al., 2022; Santovito et al., 2018) and some cell lines, such as human hepatoma (HepG2) cells (Kober et al., 2016; Yu and Liu, 2023), where the frequency of MN and other types of genomic damage increased in the presence of BPA.

Analyzing the effect of each individual component of GSC revealed some surprising results. First, GSC did not show additive or synergistic effects with those of its individual components. Second, a cyto-genotoxic effect was triggered by exposure to Se, which was not observed with the GSC multi-supplement. However, before further highlighting the action of Se on ARPE-19 cells, the beneficial effects of Gua and LC must first be elucidated. Gua powder is consumed by pre-Columbian people living in the Amazon and is currently widely used as a raw material to produce energy drinks given its high caffeine content. The functional properties of Gua are well established (Algarve et al., 2019; Cadoná et al., 2017; Araujo et al., 2021; Felin et al., 2022; Hack et al., 2023; Kober et al., 2016).

In one of our previous studies, Gua decreased apoptosis and necrosis and differentially modulated the mRNA and protein expression levels of caspases 1, 3, and 8 in ARPE-19 cells (Bonadiman et al., 2017). Thus, the cytoprotective effects of Gua against BPA are biologically plausible.

LC is an endogenous molecule involved in fatty acid metabolism and is biosynthesized within the human body using the amino acids L-lysine and L-methionine as substrates. LC can also be obtained from many foods, with red meat being the best choice followed by fish, poultry, and milk (Knottnerus et al., 2018). The therapeutic potential of LC has been examined in terms of its neuroprotective effects in some disease conditions (Ribas et al., 2014). Some data suggest that LC can be used as a food supplement to treat age-related macular degeneration associated with mitochondrial dysfunction (Moos et al., 2022). A study performed by Li et al. (2021) in ARPE-19 cells suggested that LC could attenuate EMT, an important underlying mechanism of proliferative vitreoretinopathy induced by TGF-β1 by inhibiting the Erk1/2 and JNK pathways and upregulating PPARγ expression. Therefore, our results corroborate prior findings regarding the functional role of LC in Retinal Pigment Epithelium.

In this study, Se was found to have an unexpected effect on ARPE-19 cells. Although Se is a metabolically relevant element and its deficiency is potentially harmful, the relationship between Se and human health is complex. Se has hormetic effects in humans. Moreover, the toxicological effects of Se can be related to its form (speciation). Previous studies on Se nanocapsules (Özkaya et al., 2021) and Selenomethionine (Ananth et al., 2020) revealed the antioxidant effects of Se on oxidative stress markers triggered by BPA exposure. However, in the present study, ARPE-19 cells were supplemented with an inorganic form of Se (Se bis-glycinate, 0.5%). Thus, the chemical form of Se and its corresponding concentration can influence its effects on ARPE-19 cells.

5. Conclusion

Despite the methodological limitations inherent to *in vitro* studies, our results suggest that the multi-supplement GSC can attenuate the changes in the oxidative and

genotoxic markers related to the exposure of ARPE-19 cells to BPA. However, our results revealed that the antioxidant, anti-apoptotic, and genoprotective properties of GSC were not universally shared by its individual components; only Gua and LC proved to be beneficial. Se did not exhibit any positive impact probably because the range of Se concentration that is beneficial to organisms is narrow.

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