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Ochratoxin A-induced DNA damage in human fibroblast: protective effect of cyanidin 3-*O*-β-D-glucoside

Alessandra Russo[a], Luca La Fauci[b], Rosaria Acquaviva[a], Agata Campisi[a], Giuseppina Raciti[a], Christian Scifo[a], Marcella Renis[a], Giacomo Galvano[c], Angelo Vanella[a], Fabio Galvano[b,*]

[a]*Department of Biochemistry, Medical Chemistry and Molecular Biology, University of Catania, Catania 95100, Italy*
[b]*Department of Agro-forestry, Environmental Science and Technology, Mediterranean University of Reggio Calabria, 89061 Gallina di Reggio Calabria, Italy*
[c]*Department of Agronomy, Chemistry and Animal Production, University of Catania, Catania 95123, Italy*

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Abstract

Ochratoxin A (OTA), a mycotoxin produced by *Aspergillus ochraceus* and other moulds, has recently received growing attention because of its carcinogenic, teratogenic and nephrotoxic properties in both humans and farm animals. Nevertheless, with regard to the mechanism of toxicity, the data in the literature are inconclusive. The aim of our work was to verify in human fibroblasts treated with different OTA dosages the involvement of oxidative pathway in the damage mechanism of this mycotoxin and the possible protective effect exerted by cyanidin 3-*O*-β-D-glucoside (C3G), an anthocyanin present in pigmented oranges, red wines, fruits and vegetables.

The addition of OTA at 25 and 50 μM concentrations for 48 h determined only a slight but significant ($P<.05$) increase in radical oxygen species, whereas a substantial increase in their production was observed at longer exposure, in particular, when the fibroblasts were treated with 50 μM OTA for 72 h. Under the same experimental conditions, our data showed a significant ($P<.05$) increase in the rupture of cellular membrane and high damage to genomic DNA, evaluated by single-cell gel electrophoresis (comet assay), thus confirming the involvement of oxidative stress in the OTA genotoxicity in agreement with other studies. Diversely, mitochondrial functionality does not appear influenced by OTA treatment. C3G (0.125, 0.250 mM) added to the cells treated with 50 μM OTA significantly reduced free radical species production and prevented genomic DNA damage.

Keywords: Ochratoxin A; Reactive oxygen species; DNA damage; Cyanidin-3-*O*-β-D-glucoside

1. Introduction

Ochratoxin A (OTA) is a ubiquitous secondary fungal metabolite primarily produced by some strains of *Aspergillus ochraceus* and, more commonly, by *Penicillium viricatum*. It frequently occurs in foods (cereal grains, coffee, soy and cocoa), beverages (wine and beer), mixed feeds, animal tissues [1], human blood [2] and milk [1,2]. Ochratoxin A has received growing attention in recent years because of its carcinogenic, teratogenic and nephrotoxic properties in farm animals [2], especially pigs [3], with the kidney being the target organ in both humans and animals. A positive correlation among human nephropathies and dietary OTA exposure or plasma concentrations arises from several epidemiological studies [4,5]. Furthermore, OTA has been implicated in a fatal human disease referred to as Balkan endemic nephropathy [6,7], characterized by tubule interstitial nephritis and associated with high incidence of kidney, pelvis, ureter and urinary bladder tumors. Sufficient experimental evidence for carcinogenicity in animal studies has led to the classification of OTA as a possible human carcinogen by the International Agency for Research on Cancer [8]. According to Kuiper-Goodman [9], although incomplete toxicological, epidemiological and exposure data determine some uncertainties in assessing effective human exposure and health risk and in establishing causal relationships between incidence of OTA in foods and human diseases in the risk management of OTA, as well as of other mycotoxins, it is necessary to take action before all this information is available. For all these reasons, several chemical, biological and dietary approaches have been

* Corresponding author. Tel.: +39 95 351201; fax: +39 95 234345.
E-mail address: f.galvano@unict.it (F. Galvano).

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experienced in the attempt to reduce the toxicity of OTA [9,10]. Since OTA is known to produce oxidative damage, the protective properties of some substances able to act as reactive oxygen species (ROS) scavengers have been investigated through various biological assays, reporting an overall protective effect of vitamins A, C and E [10]. Anthocyanins are included in the list of natural compounds known to work as powerful antioxidants. The anthocyanin antioxidant properties are due to their particular chemical structure; their electron deficiency makes them very reactive towards ROS. In recent years, considerable attention has been given to the possible protection against ROS-mediated tissue injury offered by natural antioxidants present in dietary plants. Since cyanidin and its glycosides represent one of the major groups of naturally occurring anthocyanins, their antioxidant and biological properties have been thoroughly investigated. Recent findings indicating the possibility that anthocyanins are adsorbed as glycosides have stimulated studies on their bioavailability, absorption, metabolic fate and excretion [11]. Particularly, the antioxidant activity of cyanidin 3-*O*-β-D-glucoside (C3G), an anthocyanin present in red oranges, red wines, fruits and vegetables, has been widely demonstrated by numerous studies using different in vitro and vivo systems [11,12].

The aim of our work was to verify in human fibroblasts the involvement of oxidative pathway in the damage mechanism of OTA and the possible protective effect exerted by C3G.

2. Methods

2.1. Chemicals

Cyanidin-3-*O*-β-D-glycoside (purity 99%) was purchased from Polyphenols Laboratory, Bergen, Norway. β-Nicotinamide-adenine dinucleotide (NADH) was obtained from Boehringer Mannheim, Germany. Ochratoxin A was obtained from Sigma Chemical, St. Louis, MO. All other chemicals were purchased from GIBCO BRL, Life Technologies (Grand Island, NY).

2.1.1. Cell culture and treatments

Human immortalized cell lines of gingival fibroblasts (HF1, ATCC, Manassas, VA) were suspended in Dulbecco's modified essential medium (GIBCO BRL, Life Technologies) supplemented with 10% heat-inactivated foetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, streptomycin (50 μg/ml) and penicillin (50 U/ml), and were incubated at 6×10^5 cells per dish (35 mm). The cells were grown at 37°C in humidified 5% CO_2/95% air mixture. The medium was replaced twice weekly and the experiments were performed at subconfluence. The cells were treated for different times (48 and 72 h) with different concentrations of OTA (Sigma) (25 and 50 μM for 48 h; 6, 25 and 50 μM for 72 h). Times of treatment were chosen since no effect of OTA was observed before 48 h of treatment, at least for parameters examined by us. Ochratoxin A was dissolved at a concentration of 25 mM and the final concentration (25 and 50 μM for 48 h; 6, 25 and 50 μM for 72 h) was obtained by appropriate dilution in the culture medium. C3G (purity 99%, Polyphenols Laboratory) was added to the cultures exposed to 50 μM OTA for 72 h. C3G was dissolved at a concentration of 3 mM and the final concentration (0.125, 0.250 mM) was obtained by appropriate dilution in the culture medium.

At the end of the treatment the cells were scraped, washed with phosphate-buffered saline (PBS) and immediately analyzed.

The concentration of proteins for the different assays was measured in the cellular lysate according to the method of Bradford [13]. In this condition, no interference of OTA was observed compared with control untreated cells.

2.2. Lactic dehydrogenase release

Lactic dehydrogenase (LDH) activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate–lactate transformation, as previously reported [14,15]. Cells were lysed with 50 mM Tris–HCl+20 mM EDTA pH 7.4+0.5% sodium dodecyl sulfate, further disrupted by sonication and centrifuged at 13,000*g* for 15 min. The assay mixture (1 ml final volume) for the enzymatic analysis contained 33 μl of sample (5–10 μg of protein) in 48 mM PBS pH 7.5 plus 1 mM pyruvate and 0.2 mM NADH. The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

2.2.1. MTT bioassay

To monitor cell viability, the fibroblasts were set up, 2×10^5 cells per well, in a 96-well, flat-bottomed 200-ìl microplate, as previously reported [14,15]. Cells were incubated at 37°C in a humidified 5% CO_2/95% air mixture and treated with OTA and/or C3G as reported earlier. Four hours before the end of the treatment time, 20 μl of 0.5% MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in PBS was added to each microwell. After incubation with the reagent, the supernatant was removed and replaced with 100 μl of acidified isopropanol and 20 μl of 3% (wt/vol) dodecyl sulfate in water. The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories, Helsinki, Finland) at 570 nm. The results were expressed as percentage of the control value.

2.3. Reactive oxygen species assay

Reactive species determination was performed by using a fluorescent probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), as previously described [14,15].

Table 1
Cell viability in human fibroblasts after treatment with ochratoxin A for 48 h (25, 50 μM) and for 72 h (6, 25, 50 μM)

Treatment	MTT (% of control)	% LDH released
Control	100	10.0±2.9
48 h		
OTA 25 μM	94.6±2.6	10.7±2.7
OTA 50 μM	96.7±2.9	11.5±2.8
72 h		
OTA 6 μM	98.6±1.6	15.8±4.7
OTA 25 μM	99.8±2.6	16.1±2.8
OTA 50 μM	95.6±3.4	21.6±4.7*

Values are mean±S.D. of three experiments performed in triplicate.
* Significant vs. untreated control cells ($P<.05$).

DCFH-DA diffuses through the cell membrane; it is enzymatically hydrolyzed by intracellular esterases and oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCFH) in the presence of ROS. The intensity of fluorescence is proportional to the levels of intracellular oxidant species. One hundred microliters of 100 μM DCFH-DA, dissolved in 100% methanol, was added to the cellular medium where the acetate group is not hydrolyzed [16], and the cells were incubated at 37°C for 30 min. After incubation, fibroblasts were lysed and centrifuged at 10,000*g* for 10 min. The fluorescence (corresponding to the radical-species-oxidized DCF) was monitored spectrofluorometrically using a Hitachi F-2000 spectrofluorimeter (Hitachi): excitation 488 nm, emission 525 nm. The total protein content was evaluated for each sample, so the results are reported as fluorescence intensity per milligram protein and compared to relative control.

2.3.1. DNA analysis by comet assay

The presence of DNA fragmentation was examined by single-cell gel electrophoresis (comet assay), according to Singh et al. [17], with little modification as previously reported [14,15]. Briefly, 10 μl [$(0.8–1)\times10^5$ cells] was mixed with 75 μl of 0.5% low melting agarose (LMA) and spotted on microscope slides previously rinsed with 1% solution of standard melting point agarose. A third layer of 85 μl LMA was then added. The "minigels" were maintained in lysis solution (1% *N*-laurosil-sarcosine, 2.5 M NaCl, 100 mM Na_2EDTA, 1% Triton X-100, 10% dimethyl sulfoxide, pH 10), used as a lysis buffer, for 1 h at 4°C, then denatured in a high-pH buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 20 min, and finally electrophoresed in the same buffer at 18 V for 45 min. At the end of the run, the minigels were neutralized in 0.4 M Tris–HCl, pH 7.5, stained with 100 μl of ethidium bromide (2 μg/ml) for 10 min and scored using a Leika fluorescence microscope (Leika, Wetzlar, Germany) interfaced with a computer. Software (Leica-QWIN) allowed us to analyze and quantify DNA damage by measuring (a) tail length (TL), intensity (TI) and area (TA); (b) head length (HL), intensity (HI) and area (HA). These parameters are employed by the software to determine the level of DNA damage as (1) the percentage of the fragmented DNA (TDNA) and (2) tail moment (TMOM) expressed as the product of TD (distance between head and tail) and TDNA.

2.4. Statistical analysis

Each experiment was repeated at least three times in triplicate. Statistical analysis of results was performed by using one-way analysis of variance followed by Dunnett's post hoc test for multiple comparisons with control. All statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat, Evanston IL).

3. Results

Table 1 reports the results of LDH release. This assay was performed to evaluate the presence of cell toxicity as a result of cell disruption after membrane breakdown.

No statistically significant increase in LDH release in treated cells was observed. A significant ($P<.05$) increase in LDH was observed only in cell cultures treated with 50 μM OTA for 72 h. C3G alone did not increase LDH release (data not shown).

MTT assay was performed to monitor cell viability, measuring the conversion of tetrazolium salt to yield colored formazan, the amount of which is proportional to the number of living cells. Results (Table 1) did not show significant modification in succinate dehydrogenase activity, the major mitochondrial enzyme responsible for the MTT formazan conversion. In fact, the values observed for OTA-treated fibroblasts did not differ from those of untreated ones, regardless of toxin concentration and exposure times. C3G alone did not affect succinate dehydrogenase activity (data not shown).

Radical oxygen species (ROS) were determined using a fluorescent probe, DCFH-DA. The probe diffuses into the

Table 2
Intracellular oxidants in human fibroblasts after treatment with ochratoxin A for 48 h (25, 50 μM) and for 72 h (6, 25, 50 μM) and after the combination of C3G plus OTA 50 μM for 72 h

Treatment	Fluorescence intensity per milligram protein (arbitrary units)
Control	427.3±52
48 h	
OTA 25 μM	510.7±67*
OTA 50 μM	541.5±48*
72 h	
OTA 6 μM	1090±97**
OTA 25 μM	1489±88**
OTA 50 μM	2563±87**
C3G 0.125 mM	360±45
C3G 0.250 mM	405±68
C3G 0.125 mM+OTA 50 μM	1225±109***
C3G 0.250 mM+OTA 50 μM	997±105***

Values are mean±S.D. of three experiments performed in triplicate.
* Significant vs. untreated control cells ($P<.05$).
** Significant vs. untreated control cells ($P<.01$).
*** Significant vs. treated cells with ochratoxin A at 50 μM concentration for 72 h ($P<.01$).

Table 3
Comet assay of genomic DNA of human fibroblasts untreated and treated with ochratoxin A for 48 h (25, 50 μM) and for 72 h (6, 25, 50 μM) and after the combination of C3G plus OTA 50 μM for 72 h

Treatment	TDNA	TMOM
Control	17.2±3.0	83.6±11
48 h		
OTA 25 μM	100±9.5*	720±26*
OTA 50 μM	88.1±6.8*	970±49*
72 h		
OTA 6 μM	89.1±15*	730±38*
OTA 25 μM	123±33*	1090±29*
OTA 50 μM	146±23*	1359±55*
C3G 0.125 mM	19.3±3	106±25
C3G 0.250 mM	22.6±2.7	118±30
C3G 0.125 mM+OTA 50 μM	66.4±8.9**	530±68**
C3G 0.250 mM+OTA 50 μM	38.4±6.9**	397±99**

Values are mean±S.D. of three experiments performed in triplicate.
* Significant vs. control untreated cells ($P<.01$).
** Significant vs. treated cells with ochratoxin A at 50 μM concentration for 72 h ($P<.01$).

cells, intracellular esterases hydrolyze the acetate groups and the resulting DCFH then reacts with intracellular oxidants resulting in the observed fluorescence. The intensity of fluorescence is proportional to the levels of intracellular oxidant species. As reported in Table 2, the intensity of fluorescence was 427.33±52 in the control samples. The addition of OTA at 25 and 50 μM concentrations for 48 h determined only a slight, but significant ($P<.05$), increase of these radical species, whereas a substantial increase in their production was observed at longer exposure. In particular, when the fibroblasts were treated with 50 μM OTA for 72 h, the fluorescence intensity was approximately six times that of controls. C3G (0.125, 0.250 mM) added to the cells treated with 50 μM OTA significantly reduced free radical species production (Table 2).

The results of the comet assay are reported in Table 3 and Fig. 1. The results of TDNA and TMOM representing the percentage of the fragmented DNA and the product of TD (distance between head and tail) and TDNA, respectively, clearly evidenced dose-dependent DNA damage for cells exposed to OTA for 72 h (Table 3). A lesser, but significant increase of TDNA and TMOM (Table 3) was also observed after 48 h of exposure to OTA. When added to the cells exposed to this mycotoxin (50 μM OTA), C3G significantly reduced DNA damage, in particular at 0.250 mM concentration (Table 3 and Fig. 1).

C3G alone did not affect the oxidant species levels (Table 2) or damage DNA at any of the concentrations tested (Table 3).

4. Discussion

For a long time, OTA was considered not to be genotoxic because of some negative results in short-term tests for mutagenicity. Recent findings have left few doubts about the genotoxicity of OTA. Indeed, OTA has been demonstrated to be a transplacental genotoxic carcinogen [18], since in pregnant mice it crosses the placenta and accumulates in fetal organs where it induces DNA adducts. It has also been hypothesized that consumption of OTA-contaminated foods during pregnancy or childhood induces lesions in testicular DNA and that testicular growth at puberty promotes these lesions to testicular cancer [19]. Various studies have reported that OTA induces SOS DNA repair and gene mutations in bacterial systems and sister chromatid exchange in cultured human lymphocytes [20,21] and porcine bladder epithelial cells [22]. In addition, OTA has been reported to cause unscheduled DNA synthesis both in rat and mouse hepatocytes and in pig urinary bladder epithelial cells [23,24]. Other authors indicate DNA adducts are induced by OTA treatment [25–27]. In contrast, both in vivo and in vitro studies using ^{3}H-labeled OTA showed no covalent OTA-derived DNA adducts in rat kidney and liver [28].

The lack of covalent DNA binding of OTA was observed in metabolically active rat and human hepatocytes, along with a decrease in the GSH–GSSG ratio [29], which may favor a prooxidant state of the cells. In any case, adduct formation could be considered a secondary effect. A direct genotoxic effect of OTA after metabolic activation has been demonstrated in cell free systems only recently [30–32]. The generation of ROS may be mediated via the formation of a Fe^{3+}–OTA complex that is reduced by NADPH-cytochrome P450 reductase to a Fe^{2+} complex, which in the

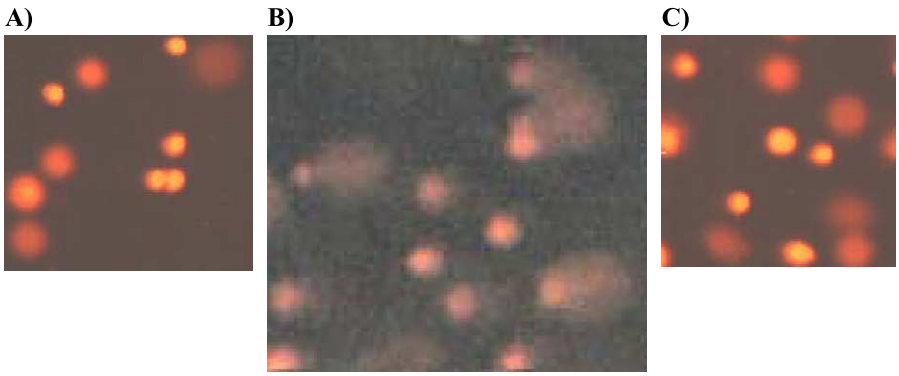

Fig. 1. Representative photomicrographs of microgel electrophoresed genomic DNA of human fibroblasts untreated (A), treated with ochratoxin A (50 μM) for 72 h (B), and after the combination of C3G (0.250 mM) plus OTA (C).

presence of oxygen provides the active species that initiates oxidative damage [33], notoriously related to carcinogenesis induction and progression. Radical species, particularly the hydroxyl OH., are able to bind to DNA, leading to strand breaks, deoxysugar fragmentation and base modification. Moreover, oxidation of lipids induced by $^{\cdot}OH$ and other reactive species can generate end products, such as malondialdehyde and unsaturated aldehydes that can bind to DNA to generate mutagenic adducts [34–36]. Lipid peroxides can also enter the nucleus where they react with Fe^{+2} to generate the alkoxyl radical, which attacks DNA [37]. Nevertheless, with regard to the mechanism of toxicity, as the data in the literature are inconclusive, it still remains an open question whether metabolic activation is necessary for the exertion of OTA toxicity and which enzyme system (cytochrome P450 enzymes or peroxidase activity of prostaglandin synthase and/or lipoxygenase) is responsible for the activation [32,38]. It is also not clear whether OTA acts as a genotoxic carcinogen or whether its carcinogenicity is due to an indirect mechanism such as induction of cytotoxicity, increased cellular proliferation and oxidative damage.

The involvement of oxidative pathways in the OTA toxicity mechanism is evidenced in many studies [32,39–42]. It been reported that some of the toxic effects of OTA in vitro and in vivo can be ameliorated or counteracted by vitamin E [43], acting as free radical scavenger and also decreasing the activities of several cytochrome P450 isoenzymes. Hoehler et al. [44] also reported that the production of hydroxyl radicals by OTA does not require the prior formation of an OTA–Fe complex, suggesting an indirect mechanism of ROS production for this mycotoxin.

The aim of our study was to verify, in human fibroblasts treated with different OTA dosages, the involvement of oxidative pathway in the damage mechanism of OTA and the possible protective effect exerted by C3G.

We demonstrated that the addition of OTA at 25 and 50 μM concentrations for 48 h determined only a slight, but significant ($P<.05$), increase of ROS, whereas a substantial increase in their production was observed at longer exposure. In particular, when the fibroblasts were treated with 50 μM OTA for 72 h, the fluorescence intensity was approximately six times that of controls. Under the same experimental conditions, our data showed a significant ($P<.05$) increase in LDH release (Table 1) and a high damage to genomic DNA (Table 3), thus confirming, after 72 h of exposure at the highest dose of OTA (50 μM), the involvement of oxidative stress in the OTA genotoxicity in agreement with other studies [32,39–42]. Diversely, the mitochondrial functionality measured by MTT test does not appear influenced by OTA treatment (Table 1).

Our data on DNA damage evaluated by comet assay, widely considered a versatile and highly effective tool in biomonitoring DNA integrity [45], are in agreement with the results previously reported by Lebrun and Follmann [32]. As evidenced by the TMOM and TDNA values, OTA treatment caused DNA breaks in a time- and dose-dependent manner.

In a study on rats, Gautier [28] observed the formation of early markers of oxidative stress, such as induction of the oxidative stress responsive gene heme oxygenase-1, decrease in plasma α-tocopherol levels and increase in oxidative metabolite α-tocopherylquinone, without the induction of either malonyl dialdehyde or 8-deoxyguanosine adduct production after OTA treatment, indicating that oxidative stress is probably evoked by a direct OTA action and not only by an unspecific mechanism related to OTA-induced cytotoxicity. Gross-Steinmeyer et al. [29] supported this notion, observing a lack of covalent DNA binding accompanied by a decrease in the reduced glutathione to oxidized glutathione ratio after incubation with OTA and a decline in the activity of glutathione reductase in cultured rat and human hepatocytes. In contrast, Schaaf et al. [40] observed that OTA contextually induces the elevation of ROS production, the formation of 8-oxyguanine adducts and the depletion of glutathione in rat proximal tubular and LLC-PK1 cells, thus providing, for the first time, evidence of DNA oxidative damage. Our data obtained for different treatment times and at various OTA concentrations indicate that DNA damage could probably be a direct effect of OTA treatment partially mediated by ROS production, at least at low dosages and treatment times. However, under these conditions, OTA treatment could stimulate the cell's protective response, modifying the expression of some stress genes as already reported by Gautier et al. [38]. The increased expression of these genes could counteract ROS production, at the same time activating endonuclease activities with consequent DNA fragmentation, as observed here. With increasing OTA concentration and time of treatment, cell defenses are unable to counteract ROS production, thus adding other DNA-damaging factors and inducing initial damage to the cell membrane, as evidenced by a significant ($P<.05$) increase in LDH release. This hypothesis seems to be supported by the observation of a clear DNA protection exerted by C3G when the experimental condition (50 μM OTA for 72 h) caused a marked increase of ROS production. As regards the protective mechanism exerted by C3G, diverse hypotheses can be advanced. C3G could protect the cell system and DNA from oxidative damage by acting as a free radical scavenger and for its property, although less marked, of chelating ions such as Cu(II) and Fe(II) [11], which can be involved in the oxidative activation of OTA [46]. Another remarkable mechanism of protection might be related to the ability of G3G to form a stable G3G–DNA copigmentation complex able to mutually protect G3G and DNA from oxidative damage, as reported by Sarma and Sharma [47]. In this sense, further confirmation arises from previous studies conducted in our laboratory showing the protective effect toward DNA damage exerted by red orange extract [12,48], notoriously rich in anthocyanins and C3G.

In conclusion, our results evidence the capability of C3G in counteracting, in human fibroblasts, ROS-mediated DNA damage induced by OTA treatment particularly when associated with ROS increase. These in vitro studies could provide a biochemical basis for use of nutritional supplements rich in this anthocyanin in preventing OTA genotoxicity.

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