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Chlorophyllin attenuates the effects of benzo[a]pyrene in human hepatoma HepG2/C3A cells

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ABSTRACT. Chlorophyllin, a semisynthetic compound derived from chlorophyll, has been a focus in cancer prevention because it exerts important biological activities, such as antigenotoxic, antioxidative and anticarcinogenic activities. The aim of this study was to evaluate the effect of chlorophyllin against benzo[a]pyrene toxicity in HepG2/C3A cells. Cells were co-treated (chlorophyllin plus benzo[a]pyrene) and the cell viability, cell growth kinetics, cell cycle, and apoptosis were evaluated. The mRNA levels of cell cycle and apoptotic genes were analyzed. Our results showed that chlorophyllin reduce the antiproliferative effects of benzo[a]pyrene in a multi-specific manner, restoring the normal distribution of the cell cycle and inhibiting the cell death induced by the xenobiotic. Gene expression analysis showed that benzo[a]pyrene decreased the mRNA levels of genes involved in cell cycle control (cyclins and cyclin-dependent kinases) and apoptosis. Cells co-treated with 200 µM of chlorophyllin and benzo[a]pyrene also showed a downregulation of mRNA levels of the genes, but the expression levels of the gene that encodes to tumor protein p53 were maintained near to control. Thus, chlorophyllin is a good candidate as a chemoprotective agent that mitigates the cytotoxic effects benzo[a]pyrene and, thus, might be a promising tool to prevent liver cancer.

Keywords: chlorophyllin; chemoprotection; apoptosis; cell cycle; cytotoxicity.

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Introduction

Various dietary components have been effectively used in cancer prevention and exhibit protective effects against a wide range of toxic compounds (Osman, Chittiboyina, & Khan, 2016). Chlorophyllin (Chl), a semisynthetic compound derived from chlorophyll, has been a focus in cancer prevention because it exerts important biological activities, such as antigenotoxic, antioxidative and anticarcinogenic activities (Das, Samadder, Mondal, Abraham, & Khuda-Bukhsh, 2016; Ozcan et al., 2019; Ozcan et al., 2021). In comparison to chlorophyll, Chl has greater stability and solubility because of the substitution of magnesium by a copper atom at the center of the ring and by the absence of the phytol group (Tumolo & Lanfer-Marquez, 2012).

Cancers are caused by both internal factors and by exposure to toxic substances present in the environment, such as tobacco, diet, radiation and others (Anand et al., 2008). Chl exhibits strong protective effects of cells against a wide range of mutagens and, notably, multiple mechanisms are liable for the protective activity of Chl (García-Rodríguez, López-Santiago, & Altamirano-Lozano, 2001; Hernaez, Xu, & Dashwood, 1997; Kumar, Shankar, & Sainis, 2004; Mata, Yu, Gray, Williams, & Rodriguez-Proteau, 2004). This compound exerts a high degree of antioxidant activity primarily by the capture of reactive oxygen species (ROS) (Kamat, Boloor, & Devasagayam, 2000). Moreover, the ability to form complexes with polycyclic aromatic compounds is another mechanism by which Chl neutralizes the effect of mutagens (Lagerqvist et al., 2011). These complexes are maintained by interactions between the flat aromatic molecules of the mutagen and the porphyrin rings of Chl. The formation of these complexes in aqueous solutions arrests the free molecules of the mutagenic compound, resulting in a decrease in the concentration of its active form (monomer) (Hernaez et al., 1997).

Benzo[a]pyrene (B[a]P) is a polycyclic aromatic hydrocarbon (PAH) compound, it is a widespread environmental toxic compound. Human exposure to B[a]P occurs through the burning of fossil fuels, cigarette smoke, intake of barbecue and smoked meat, and overheated oils (World Health Organization Regional Office for Europe, 2010). B[a]P is an agent well-known for inducing damage in tissues and cells and it is associated with an increase in the incidence of many types of cancers (Bukowska, Mokra, & Michałowicz, 2022). After metabolic activation, B[a]P is catalyzed into diol epoxides that are able to bind to DNA and form adducts, becoming an efficient mutagen and carcinogen (Kasala, Bodduluru, Barua, Sriram, & Gogoi, 2015).

Given that most sporadic cancers are related to environmental chemicals exposure and based on evidence that Chl has the ability to protect cells against carcinogenic effects of these compounds, this study aimed to evaluate the protective effect of Chl against benzo[a]pyrene toxicity in HepG2/C3A cells. The cellular ability to metabolize xenobiotics can change the response of cells to B[a]P exposure. HepG2/C3A cells performs hepatic function and are used as a model in toxicological studies due to their metabolizing ability (Mišík et al., 2019). To investigate the ability of Chl to mitigate the cytotoxic effects of B[a]P, the cells were co-treated and the cell viability, cell growth kinetics, cell cycle and apoptosis induction were evaluated. Besides, the mRNA levels of cell cycle components (cyclins and cyclin-dependent kinases - CDKs) and apoptotic genes were analyzed.

Material and methods

Chemical agents

Chlorophyllin (chlorophyllin sodium copper salt, CAS No. 11006-34-1 and degree of purity ⩾ 95.0%) and benzo[a]pyrene (B[a]P), CAS No. 50-32-8 and degree of purity ⩾ 96% were purchased from Sigma Aldrich.

Cell line

The hepatocellular carcinoma cell line (HepG2/C3A) was acquired from the Cell Bank of Rio de Janeiro and grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). The cells were maintained in a humidified incubator containing 5% CO_2 at 37°C. Under these conditions, the cell cycle was approximately 24 hours.

Resazurin assay

For the resazurin assay, Chl and B[a]P were individually evaluated for dose- and time-dependent cytotoxic effects. Next, we examined a combined treatment of Chl+B[a]P. Chl was used at concentrations of 25, 50, 100, 200, and 300 µM combined with B[a]P (20 µM). Cells were treated for 24 or 48 hours. Next, the culture medium was removed, and cells were incubated with 60 µM resazurin (7-hydroxy-10-oxide-phenoxazin-10-ium-3-one,) for 3 hours. Fluorescence was measured using the VICTOR 3 microplate reader (PerkinElmer) (530-560 nm excitation and 580–600 nm emission). The assay was performed in three repetitions and triplicate.

Real-time cell analysis (xCELLigence)

The cell proliferation analysis was performed using a real-time cell analyzer (RTCA, xCELLigence, Roche). RTCA system allows monitoring the effects of a compound in the cell index (CI). The CI reflects the change in the number of cells, viability, adhesion and cell morphology (Abassi et al., 2009). Cells were seeded into 96-well (*E-plates*) at a density of 2.5 × 104 cells well-1 and incubated at room temperature for 30 min., then the plate was placed in the RTCA station in the CO_2 incubator at 37°C. After 24 hours of stabilization, Chl (25, 50, 100, 200, and 300 µM); or Chl combined with B[a]P (20 µM) were added to the medium. The Cell Index (CI) was determined every 30 minutes for 96 hours. This experiment was performed in triplicate. The CI was calculated using RTCA software.

Cell cycle analysis using flow cytometry

Cells were treated with Chl (100 or 200 µM) with or without B[a]P (20 µM) for 24 hours. The Chl concentrations were chosen from the results of previous assays. The samples were treated with RNase (Life Technologies) (0.1 mg mL^{-1}) for 30 min. at 37°C. Next, the cells were resuspended in a hypotonic

fluorochrome solution: 50 mg mL^{-1} propidium iodide (CAS No. 25535-16-4 and degree of purity ⩾ 94.0%), 0.1% citrate sodium (CAS No. 6132-04-3 and degree of putity ⩾ 99.0%, and 0.1% Triton X-100 (CAS No. 9036-19-5) (Sigma Aldrich), and kept on ice for 30 min. while protected from light. The fluorescence of propidium iodide (PI) was estimated using flow cytometry (Accuri C6, BD Pharmingen™). The DNA content was analyzed, and the percentages of cells in the different phases of the cell cycle (Sub G1, G1, S, and G2/M) were estimated. A total of 10,000 events were acquired for each sample, and three independent experiments were performed.

Apoptosis analysis using Hoechst 33342 staining

Cells were treated with Chl (100 and 200 µM) with or without B[a]P (20 µM) for 24 hours. Next, the samples were incubated with Hoechst 33342 fluorochrome (CAS No. 23491-52-3, Sigma Aldrich) (5 µg mL^{-1}) for 20 min. in the dark. The nuclei were visualized with fluorescence microscopy (460× magnification, blue channel, 390/40 nm excitation, and 446/33 nm emission; FLoid® Cell Imaging Station, Life Technologies). Hoechst 33342 staining was performed to visualize morphologic characteristics of apoptosis, including nuclear condensation and the formation of apoptotic bodies. Three independent experiments were performed, and 500 cells were analyzed per sample.

Quantitative reverse transcription PCR (RT-qPCR) analysis

Cells were treated with Chl (200 µM) with or without B[a]P (20 µM) for 24 hours. Total RNA was isolated using RNeasy mini kit (Qiagen), according to manufacturer's recommendations. RNA integrity was confirmed using 1% agarose gel electrophoresis. The concentration and purity (A260/A280) were verified with a Nanodrop® 2000 spectrophotometer (Thermo Scientific). Three independent experiments were performed. Next, cDNA was synthesized from 500 ng of total RNA using dNTPs (Invitrogen, Life Technologies), Oligo-dT (Invitrogen, Life Technologies), and M-MLV reverse transcriptase enzyme (Invitrogen, Life Technologies).

The real-time PCR reactions were performed using a CFX96 TOUCH™ (Bio-Rad) PCR Detection System. The amplification of fragments was detected by the fluorescence emitted by the SYBR® Green Supermix (Bio-Rad) fluorophore. For data normalization, glyceraldehyde-3-phosphate (GAPDH) was used as the reference gene. We assessed the mRNA levels of cyclin A2, B1, D1, and E1 (CCNA2, CCNB1, CCND1, and CCNE1), cyclin-dependent kinases (CDK1 and CDK2), and of the genes involved in the apoptosis process, including BCL2-associated X protein (BAX), caspase 7 (CASP7), and tumor suppressor gene-tumor protein p53 (TP53). The primer sequences are shown in Table 1.

Table 1. Sequences of primers used in RT-qPCR.

Genes	Primer sequence (5'-3')	Reference
CCNA2	F: GAC CCT GCA TTT GGC TGT G R: ACA AAC TCT GCT ACT TCT GG	Hsieh (2006)
CCNB1	F: AGA GCA TCT AAG ATT GGA GAG R: CCA TGT CAT AGT CCA ACA TAG	Sigma*
CCND1	F: GCC TCT AAG ATG AAG GAG AC R: CCA TTT GCA GCA GCT C	Sigma*
CCNE1	F: GAC TTA CAT GAA GTG CTA CTG R: GAC GAG AAA TGA TAC AAG GC	Sigma*
CDK1	F: ATG AGG TAG TAA CAC TCT GG R: CCT ATA CTC CAA ATG TCA ACT G	Sigma*
CDK2	F: GAG ACC TTA AAC CTC AGA ATC R: TGG AAT AAT ATT TGC AGC CC	Sigma*
BAX	F: TTT CTG ACG GCA ACT TCA ACT GGG R: TGT CCA GCC CAT GAT GGT TCT GAT	Sigma*
CASP7	F: TCA CCA TGC GAT CCA TCA AGA CCA R: TTT GTC TGT TCC GTT TCG AAC GCC	Sigma*
TP53	F: TAC CAC CAT CCA CTA CAA CT R: GAC AGG CAC AAA CAC GCA C	Sigma*
GAPDH	F: GAA GGT GAA GGT CGG AGT C R: GGA AGA TGG TGA TGG GAT TT	Sugaya (2005)

*Primers obtained from Sigma-Aldrich (KiCqStart® SYBR® Green primers - predesigned primers for expression analysis gene).

Statistical analysis

The results were analyzed by analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California, USA). RT-qPCR data were normalized and analyzed according to Pfaffl, Horgan, and Dempfle (2002) using REST 2009 software (Pair Wise Fixed Reallocation Randomisation Test©).

Results

Chl protects against B[a]P-induced cytotoxicity

The cell viability (resazurin) and cell growth kinetics (RTCA) assays showed that Chl was cytotoxic at only the highest test concentration (300 µM). B[a]P (20 µM) reduced the viability and proliferation of HepG2/C3A cells in a time-dependent manner (Figure 1A). B[a]P (20 µM) exerted a high cytotoxic effect and the protective effect of Chl was clearly observed after 48 hours, since all Chl co-treatments effectively protected cells against the diminution in cell viability caused by B[a]P (Figure 1B).

Cell proliferation kinetics by RTCA revealed that the growth curves of cells treated with Chl at concentrations of 25, 50, 100, and 200 µM resembled those of the control (Figure 2A). B[a]P (20 µM) significantly reduced the cell index (CI) (Figure 2A) and Chl attenuated its effect as evidenced by the increased CI after co-treatment (Figure 2B). B[a]P reduced the CI after approximately 40 hours of exposure, and the protective effect of Chl (50, 100, and 200 µM) was observed following 72 hours of co-treatment. Based on these data, the concentrations of 100 and 200 µM of Chl were selected for subsequent assays.

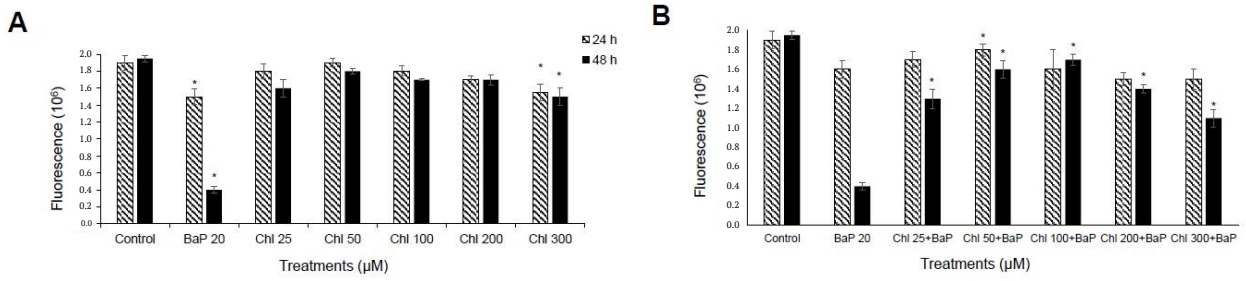

Figure 1. Cell viability of HepG2C3A after 24 and 48h of incubation measured using the resazurin assay. A) Cell viability after exposure to BaP 20 µM and Chl 25, 50, 100, 200, and 300 µM. B) Cell viability after exposure to BaP 20 µM alone and associated to Chl 25, 50, 100, 200, and 300 µM. Results are presented as the mean ± SD of three independent experiments. (A) * p > 0.05, ** p > 0.01, *** p > 0.001 relative to control; (B) * p > 0.05, ** p > 0.01, *** p > 0.001 relative to BaP 20 using ANOVA followed by Dunnett.

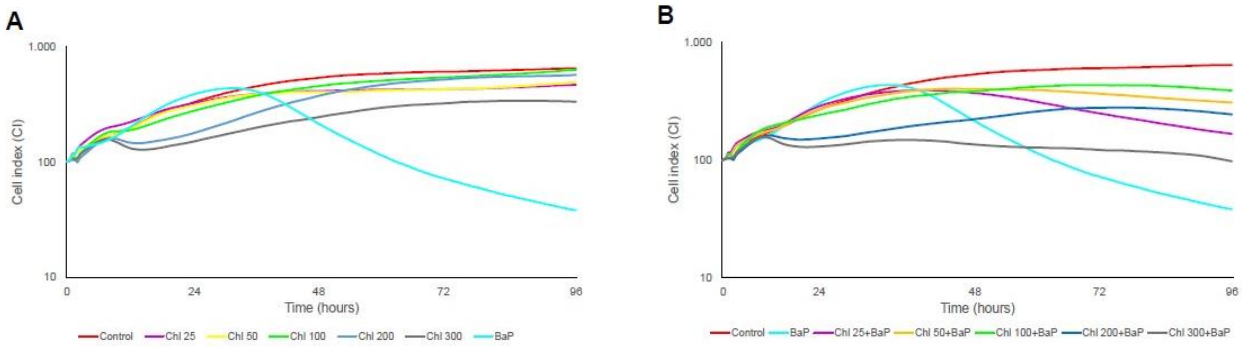

Figure 2. Real-time monitoring of HepG2/C3A cells obtained using the xCELLigence system demonstrating the kinetics of cell growth. A) Growth curves of HepG2/C3A cells exposed to Chl 25, 50, 100, 200, and 300 µM, and BaP 20 µM; B) Growth curves of HepG2/C3A cells exposed to BaP 20 µM alone and associated to Chl 25, 50, 100, 200, and 300 µM. The results are presented as the means of three replicates.

Chl protected against B[a]P-induced cell cycle alterations

Cell cycle analysis was performed to investigate the protective mechanisms of Chl against B[a]P antiproliferative effects (Figure 3). HepG2/C3A cells exposed to Chl (200 µM) showed a decrease of S phase (8%) in relation to control (16%). The other phases did not have significant alterations (G1: 57%, G2/M: 27.6 and Sub G1: 1.2%) compared with control (G1: 54%, S: 16%, G2/M: 26% and Sub G1: 1%).

B[a]P treatment increased the population of cells in the sub G1 phase (21%), highlighting the apoptotic effect of this compound. Furthermore, B[a]P increased the percentage of cells in the S phase (21%) and decreased the population in the G1 phase (32%). Cells exposed to B[a]P and co-treated with Chl at concentrations of 100 and 200 µM showed an expressive reduction in the number of cells in the sub G1 phase (4%). In addition, cells exposed to B[a]P and treated with the highest test concentration of Chl (200 µM) maintained the normal distribution of cells in the cell cycle (G1: 52%; S: 13%; G2/M: 28%).

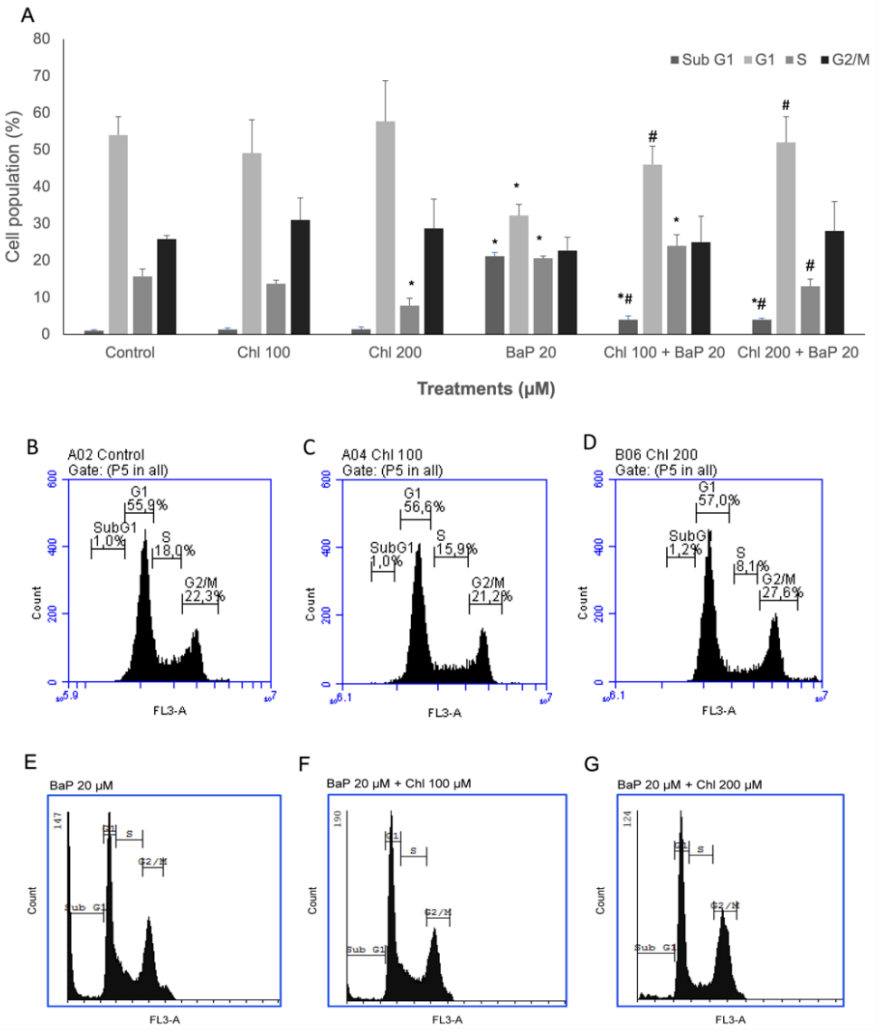

Figure 3. Cell cycle analysis (G1, S, G2/M and subG1) of HepG2/C3A by flow cytometry using propidium iodide labeling (PI). A) HepG2/C3A cell cycle distribution after 24 hours of treatment with Chl 100 µM and 200 µM, BaP 20 µM; Chl 100 µM + BaP 20 µM and Chl 200 µM + BaP 20 µM. Results are presented as the mean ± SD of three independent experiments. * $p < 0.05$ relative to control; # $p < 0.05$ relative to BaP 20 using ANOVA followed by Dunnett. Representative histograms indicate the number of cells (vertical axis) vs. DNA content (horizontal axis): B) Control; C) Chl 100 µM, and D) Chl 200 µM.

Chl protected against B[a]P-induced cell death

Morphological analysis using Hoechst 33342 was performed to investigate apoptosis (Figure 4). Untreated control cells showed typical oval nuclei and remained attached to the plate. After treatment with 100 and 200 µM Chl, the cells maintained control-like characteristics. Exposure to B[a]P (20 µM) increased cell detachment and the prevalence of condensed nuclei and/or apoptotic bodies (Figure 4B). There was an increase in the percentage of apoptotic cells after B[a]P (16%) exposure when compared to the control (1.1%). Chl effectively protected cells from the effects of B[a]P. Co-treatment with Chl 100 and 200 µM decreased the percentage of apoptotic cells to 2.6 and 1.6%, respectively (Figure 4A).

The expression of genes involved in cell cycle progression and apoptosis

There was a downregulation in *CCNA2*, *CDK1*, and *CDK2* mRNA levels in the cells exposed to Chl (200 µM) (Figure 5). The exposure of cells to B[a]P decreased mRNA levels of CCNA2, CCNB1, CCND1, and CCNE1

cyclins as well as CDK1 and CDK2 cyclin-dependent kinases. Cells co-treated with 200 µM Chl and B[a]P also showed a downregulation of mRNA levels of the genes.

B[a]P caused a downregulation of mRNA levels of BAX, CASP7, and TP53. The cells co-treated with B[a]P and 200 µM Chl showed a decrease in the levels of BAX and CASP7, and the TP53 mRNA level was restored (Figure 5). The cells exposed to 200 µM Chl showed no significant alterations in the mRNA levels of these genes.

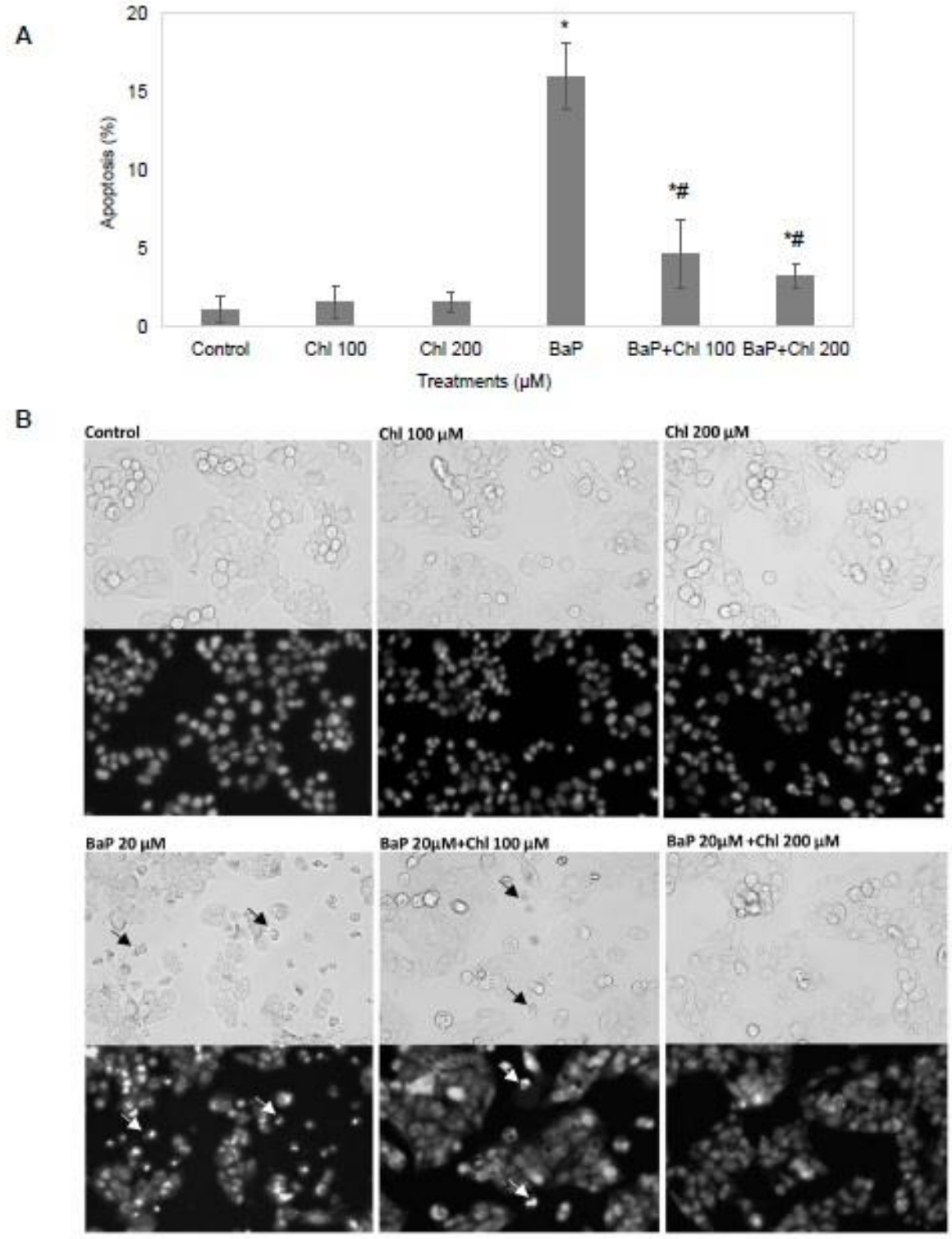

Figure 4. Apoptosis analysis by Hoechst 33342 staining. A) Percentage of HepG2/C3A apoptosis after 24 hours of treatment with Chl (100 µM and 200 µM), BaP (20 µM), BaP (20 µM) + Chl (100 µM) and BaP (20 µM) + Chl (200 µM). Results are mean ± SD of three independent experiments. * $p > 0.05$ compared to control, # $p > 0.05$ compared to BaP using ANOVA followed by Dunnett. B) Photomicrographs showing morphology and the staining with Hoechst 33342 of HepG2/C3A cells. Arrow indicates cells with condensed chromatin and/or apoptotic bodies (magnification= 460×).

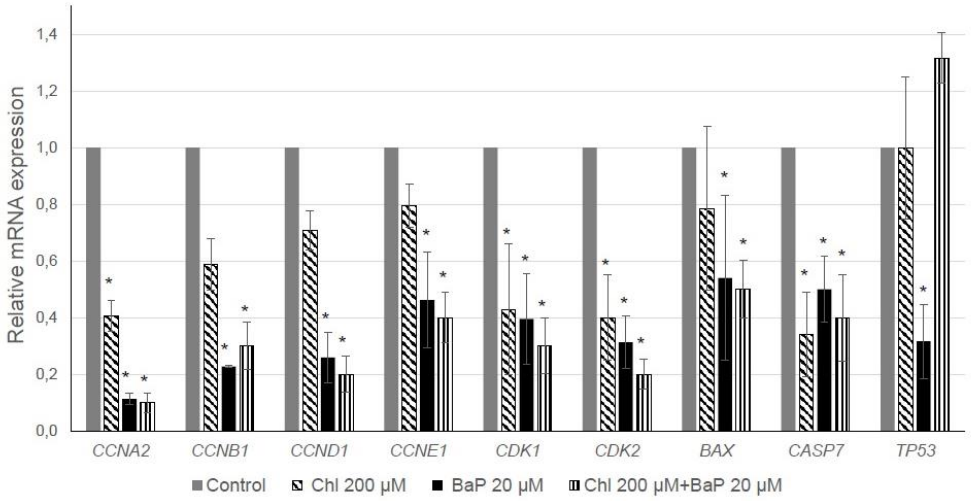

Figure 5. RT-qPCR analysis of genes involved in cell cycle and apoptosis regulation. Graphs shown the relative gene expression in HepG2/C3A cells after 24 hours of treatment (Cyclins, CDKs and apoptosis regulation genes). The relative expression of each target gene was normalized with the reference genes Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as relative expression to control group in three independent experiments (mean±SEM). Statistical differences compared to control are indicated by * (REST2009 software ®).

Discussion

In the present study, we investigated the cytoprotective mechanisms of Chl against the toxicity of environmental carcinogen B[a]P in human hepatoma HepG2/C3A cell line. Chl was able to reduce the cytotoxic and antiproliferative effects of B[a]P, restore the normal distribution of the cell cycle and inhibit the cell death induced by the xenobiotic.

The protective abilities and anticancer properties of Chl have been described *in vitro* and *in vivo* experiments, as shown by Das et al. (2016). The authors found that nano-encapsulated chlorophyllin (NCHL) inhibited the growth of A549 cells in a dose-dependent manner, it was able to increase reactive oxygen species and induce DNA damage, contributing further towards apoptosis. *In vivo*, NCHL reduced chromosome aberrations, micronuclei and DNA damage, it also showed efficacy in the recovery process of lung tissue damage and inhibition of tumor growth in sodium arsenite plus B[a]P treated mice.

In the present study RTCA growth curves obtained from the cells exposed to B[a]P showed an initial increase in CI followed by a decrease below control levels. This profile is compatible with compounds that interfere in DNA synthesis, transcription, and translation (Abassi et al., 2009). When the hepatocellular carcinoma cell line (HepG2/C3A) was co-treated with Chl, cell survival and proliferation were restored to levels similar to the control group. The morphology of cells co-treated with Chl and the environmental carcinogen was preserved and similar to control cells. Some authors have shown the potential of Chl to protect cells against the toxic effects of B[a]P (Ibrahim et al., 2007; John et al., 2010; Keshava et al., 2009; McCook, Dorogi, Vasily, & Cefalo, 2015). The hepatoprotective of Chl was described by (Patar, Sharma, Syiem, & Bhan, 2018) These authors observed the ability of Chl to restore morphological and cell alterations in hepatic tissue of the streptozotocin-induced diabetic mice. Said et al. (2022) also showed that Chl was able to reduce the hepatotoxicity induced by carbon tetrachloride in mice through anti-inflammatory and antioxidant activities.

Besides the viability and morphological results, in the present study, we could investigate how the treatments with Chl and B[a] act at the molecular level in hepatocellular carcinoma cells through the expression of mRNA levels of genes involved in cell cycle progression and apoptosis. This allowed us to better understand the Chl mechanisms of protection.

Chl acts in different mechanisms to produce its protector effect. One of these mechanisms is the ability of Chl to form complexes with diverse mutagenic agents (Pietrzak, Wieczorek, Stachelska, & Darzynkiewicz, 2003). These complexes are retained by the interaction between the mutagen molecules and Chl. The free molecules are captured to form these complexes in aqueous solution, thus reducing the concentration of the active form of the compound (Dashwood, 1997). Accordingly, Chl's ability in attenuates the toxic effects of B[a]P may be due to the capability of this molecule in capturing and neutralizing the tested molecules, and thus prevents uptake by cells. Furthermore, Chl acts by targeting numerous molecules and pathways involved in the metabolism of carcinogens, cell cycle progression, apoptosis and so forth (Nagini, Palitti, & Natarajan,

2015). In the current study, we investigated how Chl acts on the cell cycle and apoptosis pathway to play its protective effect on cells exposed to the toxic agent B[a]P.

Chl (200 µM) treatment caused downregulation of CCNA2, CDK1, and CDK2, and it may be related to the decrease of cells in the S-phase as cyclin A2/Cdk2 complex accumulation occurs early in the cell cycle and it is important for entry and completion of S phase. Cyclin A2/Cdk1 complex accumulation occurs in the late S or G2 phase (Merrick et al., 2008). Some authors have demonstrated the antiproliferative potential of Chl to inhibit cyclins (Chimploy et al., 2009; Chiu, Kong, & Ooi, 2003). Chimploy et al. (2009) found a downregulation of cyclins A, B, and E in HCT116 cells treated with Chl. Chiu et al. (2003) observed a reduction in the protein levels of cyclins D1 and E1 in MCF-7 cells exposed to Chl, and an increase in the number of cells in the G2/M phase. In our study, only the highest concentration of Chl was able to decrease cell proliferation of HepG2/C3a cells. Although we have observed downregulation of cyclins and CDKs mRNA levels, we did not observe cell cycle arrest after Chl treatment.

B[a]P, in turn, increased the percentage of cells in the S phase, decreased the cell population in the G1 phase and downregulated the mRNA levels of cell cycle regulators: CCNA2, CCNB1, CCND1, and CCNE1 cyclins as well as CDK1 and CDK2 cyclin-dependent kinases. The downregulation of cyclins and CDK genes can cause an inhibitory effect on the Cyclin/CDK complexes and alter the cell cycle regulation. Cell cycle arrest of HepG2 cells in the S-phase after B[aP treatment has been observed by other authors as a result of DNA damage (Stellas et al., 2014). The co-treatment with Chl (200 µM) restored the normal distribution of cells in the cell cycle, but the mRNA levels of cyclins and CDKs were kept downregulated.

After treatment with BaP, the rate of apoptotic levels was increased in HepG2/C3A cells, as observed by morphological assay and by the increase of the subG1 population. Our experiments showed that Chl effectively protected cells from the B[a]P-induced cell death. Several studies also showed that Chl has an anti-apoptotic effect against B[a]P and other cytotoxic drugs (Botelho et al., 2004; Ibrahim et al., 2007; Lagerqvist, Håkansson, Frank, Seidel, & Jenssen, 2011; Yu, Yang, & Kim, 2010; Zhang et al., 2008). Ibrahim et al. (2007) found that Chl reduced DNA fragmentation in liver samples from mice treated with cyclophosphamide and B[a]P. Botelho et al. (2004) observed a similar result that they attributed to the ability of Chl to bind to mutagenic agents and inhibit nuclear fragmentation. Zhang et al. (2008) reported that Chl protected against the cell death induced by hydrogen peroxide in human umbilical vein cells (HUVEC) via its antioxidant capacity. On the other hand, Yu et al. (2010) reported that Chl showed a small cytoprotective effect and low anti-apoptotic effect in human lung epithelial (A549) and leukemia (Jurkat T) cells treated with hydrogen peroxide. Lagerqvist et al. (2011) showed expressive protection against some polycyclic aromatic hydrocarbon diol epoxides derived from B[a]P; dibenzo[a,h]anthracene; dibenzo[a,l]pyrene and benzo[c]phenanthrene, increasing cell survival and reducing mutagenicity in Chinese hamster V79 cells.

Chl showed a cell survival response against apoptosis induced by B[a]P, however, the pro-apoptotic genes (CASP7 and BAX) were downregulated in both conditions, in the treatment with B[a]P and in the co-treatment with Chl. This downregulation could be related to other mechanisms that are involved in the toxicity response to B[a]P, such as the autophagy (Nikoletopoulou, Markaki, Palikaras, & Tavernarakis, 2013; Yuan, Liu, Deng, & Gao, 2017). Chl also causes downregulation of apoptotic genes, which could be a cytoprotective mechanism (Patar et al., 2018; Sharma, Kumar, & Sainis, 2007). Co-treatment did not alter these response patterns except for the normalization of TP53 levels. Chl associated with B[a]P could act to restore the levels of TP53 in response to genotoxic stress as the protein encoded by this gene can induce DNA repair, cell cycle arrest and apoptosis to protect the cells against carcinogenesis (Smit, Souza, Jennen, Kleinjans, & van den Beucken, 2017).

In summary, our results showed that Chl was able to reduce the cytotoxic and antiproliferative effects of B[a]P in a multi-specific manner, restoring the normal distribution of the cell cycle and inhibiting the cell death induced by the xenobiotic. Chl is a good candidate as a chemoprotective agent that mitigates the cytotoxic effects B[a]P and, thus, might be a promising tool to prevent liver cancer.

Conclusion

Chlorophyllin reduce the antiproliferative effects of benzo[a]pyrene in a multi-specific manner, restoring the normal distribution of the cell cycle and inhibiting the cell death induced by the xenobiotic. Gene expression analysis showed that benzo[a]pyrene decreased the mRNA levels of genes involved in cell cycle control (cyclins and cyclin-dependent kinases) and apoptosis. Cells co-treated with 200 µM of chlorophyllin

and benzo[a]pyrene also showed a downregulation of mRNA levels of the genes, but the expression levels of the gene that encodes to tumor protein p53 were maintained near to control. Thus, chlorophyllin is a good candidate as a chemoprotective agent that mitigates the cytotoxic effects benzo[a]pyrene and, thus, might be a promising tool to prevent liver cancer.

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