Renilton Aires Lima[1]
Eduardo Batista Cândido[2]
Flávia Perrim de Melo[3]
Josiane Barbosa Piedade[3]
Paula Vieira Teixeira Vidigal[4]
Luciana Maria Silva[3]
Agnaldo Lopes da Silva Filho[2]

Gene expression profile of ABC transporters and cytotoxic effect of ibuprofen and acetaminophen in an epithelial ovarian cancer cell line *in vitro*

Perfil da expressão gênica dos transportadores ABC e efeito citotóxico do ibuprofeno e acetaminofen em uma linhagem celular de câncer epitelial de ovário in vitro

Original Article

Keywords
Acetaminophen
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Cell proliferation
Chemoprevention
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Palavras-chave
Acetaminofen
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Transportadores de cassetes de ligação de ATP
Proliferação de células
Quimioprevenção
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Abstract

PURPOSES: To determine the basic expression of ABC transporters in an epithelial ovarian cancer cell line, and to investigate whether low concentrations of acetaminophen and ibuprofen inhibited the growth of this cell line *in vitro*. **METHODS:** TOV-21 G cells were exposed to different concentrations of acetaminophen (1.5 to 15 µg/mL) and ibuprofen (2.0 to 20 µg/mL) for 24 to 48 hours. The cellular growth was assessed using a cell viability assay. Cellular morphology was determined by fluorescence microscopy. The gene expression profile of ABC transporters was determined by assessing a panel including 42 genes of the ABC transporter superfamily. **RESULTS:** We observed a significant decrease in TOV-21 G cell growth after exposure to 15 µg/mL of acetaminophen for 24 (p=0.02) and 48 hours (p=0.01), or to 20 µg/mL of ibuprofen for 48 hours (p=0.04). Assessing the morphology of TOV-21 G cells did not reveal evidence of extensive apoptosis. TOV-21 G cells had a reduced expression of the genes ABCA1, ABCC3, ABCC4, ABCD3, ABCD4 and ABCE1 within the ABC transporter superfamily. **CONCLUSIONS:** This study provides *in vitro* evidence of inhibitory effects of growth in therapeutic concentrations of acetaminophen and ibuprofen on TOV-21 G cells. Additionally, TOV-21 G cells presented a reduced expression of the ABCA1, ABCC3, ABCC4, ABCD3, ABCD4 and ABCE1 transporters.

Resumo

OBJETIVOS: Determinar a expressão básica dos transportadores ABC em uma linhagem celular do câncer epitelial de ovário, e investigar se o acetaminofen e o ibuprofeno em baixas concentrações são capazes de inibir o crescimento desta linhagem celular *in vitro*. **MÉTODOS:** A linhagem celular TOV-21 G foi exposta a diferentes concentrações de acetaminofen (1,5 a 15 µg/mL) e ibuprofeno (2,0 a 20 µg/mL), de 24 a 48 horas. O crescimento celular foi avaliado utilizando-se um ensaio de viabilidade celular. A morfologia celular foi determinada por meio da microscopia de fluorescência. O perfil de expressão gênica foi estabelecido por um painel de 42 genes da superfamília de transportadores ABC. **RESULTADOS:** Observou-se um decréscimo significativo no crescimento das células TOV-21 G expostas a 15 µg/mL de acetaminofen durante 24 (p=0,02) e 48 horas (p=0,01), ou a 20 µg/mL de ibuprofeno por 48 horas (p=0,04). Ao avaliar a morfologia das células cultivadas, não foi observada evidência de apoptose extensiva. A linhagem de células estudada subexpressa os genes de ABCA1, ABCC3, ABCC4, ABCD3, ABCD4 e ABCE1 na superfamília de transportadores ABC. **CONCLUSÕES:** Este estudo fornece evidências *in vitro* referentes aos efeitos inibidores do crescimento de concentrações terapêuticas do acetaminofen e ibuprofeno na linhagem celular testada. Além disso, as células TOV-21 G apresentaram uma expressão reduzida de genes dos transportadores ABCA1, ABCC3, ABCC4, ABCD3, ABCD4 e ABCE1.

Correspondence
Agnaldo Lopes da Silva Filho
Department of Gynecology and Obstetrics of Medicine School of Universidade Federal de Minas Gerais
Avenida Professor Alfredo Balena, 190 – Santa Efigênia
Zip code: 30130-100
Belo Horizonte (MG), Brazil

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Department of Obstetrics and Gynecology, School of Medicine, Universidade Federal de Minas Gerais – UFMG – Belo Horizonte (MG), Brazil.
[1]Hospital das Clínicas de Minas Gerais – Belo Horizonte (MG), Brazil.
[2]Department of Obstetrics and Gynecology, Universidade Federal de Minas Gerais – UFMG – Belo Horizonte (MG), Brazil.
[3]Laboratory of Research and Development Management, Ezequiel Dias Foundation – Belo Horizonte (MG), Brazil.
[4]Department of Pathology, Universidade Federal de Minas Gerais – UFMG – Belo Horizonte (MG), Brazil.
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Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy[1,2]. Given the poor prognosis for women with EOC, it is imperative to continue exploring novel chemo-preventive and chemotherapeutic agents[3].

A range of findings, from epidemiological studies of patients to molecular studies of genetically modified mice, has led to a general acceptance that inflammation and cancer are linked[4,5]. Research focusing on colorectal and prostate cancers has provided strong evidence that nonsteroidal anti-inflammatory drugs (NSAID) are effective in both cancer prevention and treatment of established tumors[6-8]. Increasing evidence suggests that the inflammation significantly contributes to the etiology of EOC[5,9]. Collectively, hypotheses attributing the EOC to ovulation, gonadotropin release, and hormonal influences likely are not mutually exclusive as they all converge on the role of inflammation in promoting ovarian tumorigenesis[9,10]. These findings result in the initiation of a number of animal and clinical trials that examine the efficacy of cyclooxygenase (COX) inhibitors in primary and/or secondary prevention of cancer, both alone or as part of a combination therapy regimen for established tumors[11].

To date, 48 ATP-binding cassette (ABC) transporters that are divided into seven families (ABC A-G) have been identified in the human genome[12]. Several of these transporters can actively efflux a wide range of anticancer drugs and reduce intracellular drug concentrations. This phenomenon eventually confers cross-resistance to various chemotherapeutics drugs, resulting in multidrug resistance (MDR) acquisition[13,14].

The objective of this study was to determine the basic expression pattern of ABC transporters in an EOC cell line, and to investigate whether clinically relevant concentrations of acetaminophen and ibuprofen can inhibit the growth of EOC cells *in vitro*.

Methods

Cell culture

The human ovarian adenocarcinoma cell line TOV-21 G was obtained from the American Type Culture Collection (ATCC # CRL-11730), and spread according to their recommendations. These cells were maintained in a 1:1 mixture of culture medium 199 (Sigma # M2520) and MCDB 131 (Sigma # M8537), containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2.

Cytotoxicity

Cell viability was determined through the use of 3-[4,5-dimethylthiazol-2-yl]-2,55-diphenyltetrazolium bromide (MTT) colorimetric assay; in which MTT, a nontoxic pale yellow substrate, was converted to a dark blue formazan product by living cells. The formazan accumulation can be spectrophotometrically measured and it is directly proportional to the number of viable cells. All experiments were performed in quadruplicate. Cells were plated at $1x10^5$ cells/well in 96-well plates and incubated at 37°C, for 24 hours. The cells were washed in the following day with PBS and treated with acetaminophen at concentrations ranging from 1.5 to 15 µg/mL, or ibuprofen at 2.0 to 20 µg/mL. Control wells contained no drug. After incubation periods of 24 and 48 hours, 10 µL of MTT tetrazolium solution was added to each well and the cells were incubated at 37ºC for three hours. Plates were then centrifuged and the supernatant was removed. The formazan was solubilized with 50 µL of DMSO, and absorbance at 550 nm was measured. The results of the treated cultures are expressed as the percentage of viable cells compared with untreated controls.

Fluorescence staining

Cell morphology was established after exposure to concentrations of 1.5, 7.5 and 15 µg/mL of acetaminophen or 2, 10 and 20 µg/mL of ibuprofen for 48 hours through staining with DAPI, MitoTracker Orange and Live/Dead using a fluorescence microscope (Axiovert 200, Zeiss).

TOV-21G cells were treated with acetaminophen and ibuprofen, as previously described, fixed for one hour with 4% paraformaldehyde in PBS and then stained with DAPI solution for five minutes. DAPI is a fluorescent stain that binds strongly to DNA. When stained with DAPI, the DNA can be visualized with blue-white fluorescence. The cells were visually assessed for any morphological features of apoptosis, such as cell shrinkage, chromatin condensation, and formation of apoptotic bodies. To label mitochondria, cells were incubated with 50 nM MitoTracker Orange dye in pre-warmed medium, during 60 minutes at 37°C. This dye passively diffuses across the plasma membrane and accumulates in active mitochondria. The Live/Dead assay provides a two-color fluorescence cell viability assay, which is based on the simultaneous labeling of live and dead cells with two probes. Cells were washed with Hanks' balanced saline solution and stained with Live/Dead solution (1 µM Calcein AM and 2 µM Ethidium homodimer-1 in phosphate-buffered saline) for 10 minutes at room temperature in the dark. These labels allow the visualization of two recognized parameters of cell viability — intracellular esterase activity and plasma membrane integrity. The cell must be viable and functional so this fluorescence can happen. Live cells are distinguished by the presence of

ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is retained within live cells, producing an intense uniform green fluorescence in live cells. EthD-1 enters the cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells.

Real-time polymerase chain reaction

The quantitative polymerase chain reaction (PCR) was used to measure the expression level of human ABC transporter superfamily genes in TOV-21 G cells using a pre-made panel from Roche Applied Science. Each multi-well plate contains assays for 42 different ABC transporter genes in duplicate. Seven referential genes served as PCR controls and allowed for the quantification of the relative expression of target genes. The assays were carried out using a LightCycler® 480 instrument and 96 multi-well plates containing target-specific primers and a FAM-labeled Universal Probe Library hydrolysis probe, which has RNA. Total RNA was isolated from TOV-21 G cells using TRIzol® reagent. Two micrograms of total RNA were used to generate the first strand cDNA through reverse transcription.

Statistical analysis

In order to compare the mRNA expression of different ABC transporters, the results of gene expression were analyzed based on the cycle threshold (Ct) values normalized to beta-2-micro-globulin and 18 S probable dimethyladenosine transferase precursor genes. The Ct values of control genes were subtracted from those of the transporters to calculate a ΔCt value (e.g., Ct of transporter – CtB2M). The Student's *t*-test was performed to analyze the statistical significance of MTT values, and a one-way ANOVA was executed for pairwise comparisons. Statistical analyses were done using GraphPad Software, Prism 4.0 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant if $p<0.05$.

Results

Cell proliferation

Acetaminophen at concentrations of 1.5, 7.5 and 15 µg/L decreased TOV-21 G cell proliferation in 3.5 (p=0.10), 3.5 (p=0.08), and 7.2% (p=0.02), respectively, at 24 hours compared to untreated control cells considered to be of 100% proliferation (Figure 1A). After 48 hours, acetaminophen decreased cell proliferation in 7.6 (p=0.12), 5.2 (p=0.43), and 12.3% (p=0.01), as seen in Figure 1B.

When the cellular response was analyzed to 2, 10 and 20 µg/L of ibuprofen at 24 hours, decreases in cellular growth of 0.3 (p=0.96), 4.9 and 3.9% (p=0.05), respectively, were seen compared to controls (Figure 2A). These decreases were more apparent after 48 hours of ibuprofen exposure, at which time cellular growth was diminished to 7.8 (p=0.16), 9.52 (p=0.12), and 9.5% (p=0.04) compared to controls (Figure 2B).

Morphological changes

The morphological characteristics of cells were evaluated through fluorescent staining. Forty-eight hours after treatment, cells that were treated with acetaminophen and ibuprofen did not show any morphological signs of apoptosis than did the controls (Figures 3 and 4A to D). The Live/Dead assay revealed that 48 hours of treatment

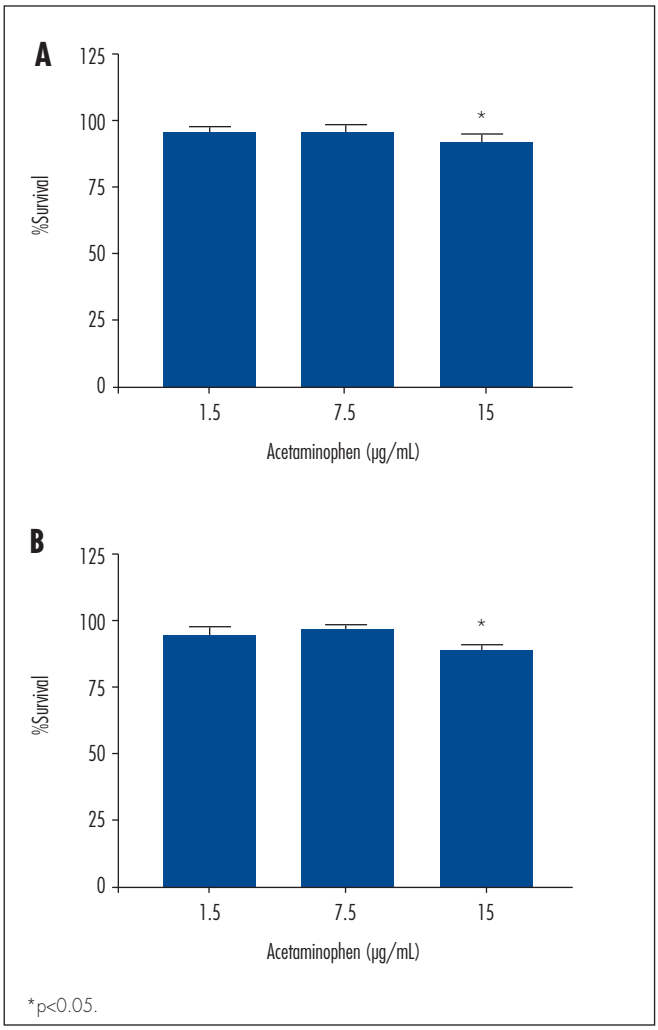

Figure 1. Cell viability in ovarian cancer cell lines exposed to acetaminophen in different time intervals. Bars represent the means of experiments performed in quadruplicate. Error bars indicate the standard deviation. Cell growth is expressed as a percentage of control. TOV-21 G cells showed a proliferation decrease at concentrations of 1.5, 7.5 and 15 µg/mL for drug exposure at (A) 24 and (B) 48 hours

with acetaminophen at 15 µg/mL or ibuprofen at 20 µg/mL did not increase the number of dead cells (Figures 3 and 4E to H).

MitoTracker Orange is an orange-fluorescent dye that stains mitochondria in live cells. Cells were considered MitoTracker Orange positive if a bright punctate orange fluorescence of the mitochondria was observed, and negative if cells exhibited a diffuse orange cytoplasmic staining. By 48 hours, cells treated with acetaminophen or ibuprofen showed no morphological changes in their mitochondria (Figures 3 and 4I to L).

Real-time polymerase chain reaction

TOV-21 G cells had reduced expression of the ABCA1, ABCC3, ABCC4, ABCD3, ABCD4, and ABCE1 transporter genes. The expression of ABCC4 (ΔCtB2M=-0.77 and ΔCt18S=-10.48) was not altered as much as that of ABCA1 (ΔCtB2M=-10.73 and ΔCt18S=-24,43), which was significantly under-expressed (Figure 5).

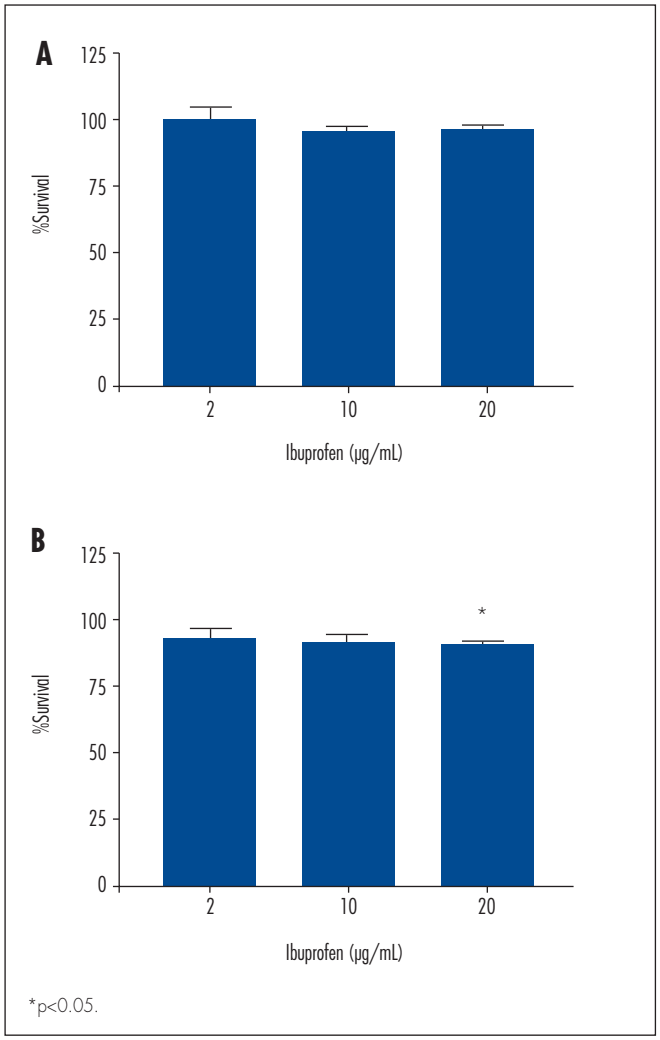

Figure 2. Cell viability in ovarian cancer cell lines exposed to ibuprofen in different time intervals. Bars represent the means of experiments performed in quadruplicate. Error bars indicate the standard deviation. Cell growth is expressed as a percentage of control. TOV-21 G cells showed a proliferation decrease at concentrations of 2, 10 and 20 µg/mL for drug exposure at (A) 24 and (B) 48 hours

Discussion

Testing different drugs on cell lines is a critical component of the antineoplastic drug development. MTT assays allow for testing different concentrations and durations of drug exposure. Limited data are available regarding the effects of NSAIDS on cell lines of EOC[15,16]. The published literature includes a wide range of drugs, doses and schedules, making it challenging to reconcile the differences and to understand how well a drug may work in the clinical practice[17-19].

From a clinically relevant perspective, ibuprofen is one of the most widely used analgesic, antipyretic and anti-inflammatory drugs nowadays[20]. It has relatively low risks for gastrointestinal, hepato-renal and other rare adverse drug reactions compared with other NSAIDs and coxibs[20]. Indeed, it has been described as "the mildest NSAID with the fewest side effects that has been in clinical use for a long time"[20]. Patients have been maintained on high doses of ibuprofen for years, without serious adverse effects[17]. Advanced age has a minimal influence on the pharmacokinetics of ibuprofen, and dosage apparently does not need to be adjusted for age[21]. The drug has, in general, predictable and reliable kinetic properties[20]. The maximum serum concentration (C_{max}) following the ingestion of 400, 600 and 800 mg doses is 15.4, 17.1 and 24.2 µg/mL, respectively[20].

Acetaminophen is one of the most popular and widely used drugs for the treatment of pain and fever, and does not produce gastrointestinal damage or untoward cardiorenal effects[22]. Despite much research, definitive proof that the analgesic and antipyretic effects of acetaminophen are dependent on COX inhibition is still lacking[22]. Indeed, inhibition of a third form of COX, COX-3, is one of the more recent proposals that have been put forward to explain the unusual effects of acetaminophen[22,23]. Plasma concentrations of 10 to 20 µg/mL have an assumed role to be therapeutic for acetaminophen[24].

To our knowledge, this is the first report describing the effect of ibuprofen and acetaminophen on the growth of an EOC cell line *in vitro*, using therapeutically relevant drug concentrations. Acetaminophen at a concentration of 15 µg/mL showed a significant inhibition of an EOC cell line after 24 and 48 hours of treatment. Ibuprofen inhibited growth at 20 µg/mL, but only 48 hours after exposure. The anti-proliferative effect exerted by ibuprofen on the EOC cell line studied here

is in accordance with previous observations[1]. The finding that therapeutic levels of acetaminophen and ibuprofen have effects on EOC cells, suggests the possibility of a chemo-preventive/chemotherapeutic strategy using these agents in dosages and schedules that will minimize the side effects, while yielding optimal ovarian cancer prevention/treatment.

This study demonstrated that acetaminophen and ibuprofen at therapeutics levels do not activate apoptosis in EOC cells. The mechanism(s) underlying the decrease in cell proliferation remains to be elucidated. It has been previously proposed that ibuprofen anti-proliferative actions are independent of COX and that p75NTR, a recently identified tumor suppressor, may in part be responsible for ibuprofen anticancer properties[1,25,26]. Previous studies suggest that acetaminophen is a tyrosine kinase substrate and intracellular glutathione depletion, reactive oxygen species formation and mitochondrial toxicity contributed to acetaminophen selective toxicity in melanoma cell lines[27,28].

Measuring ABC transporter gene expression may be useful in predicting anticancer drug response.

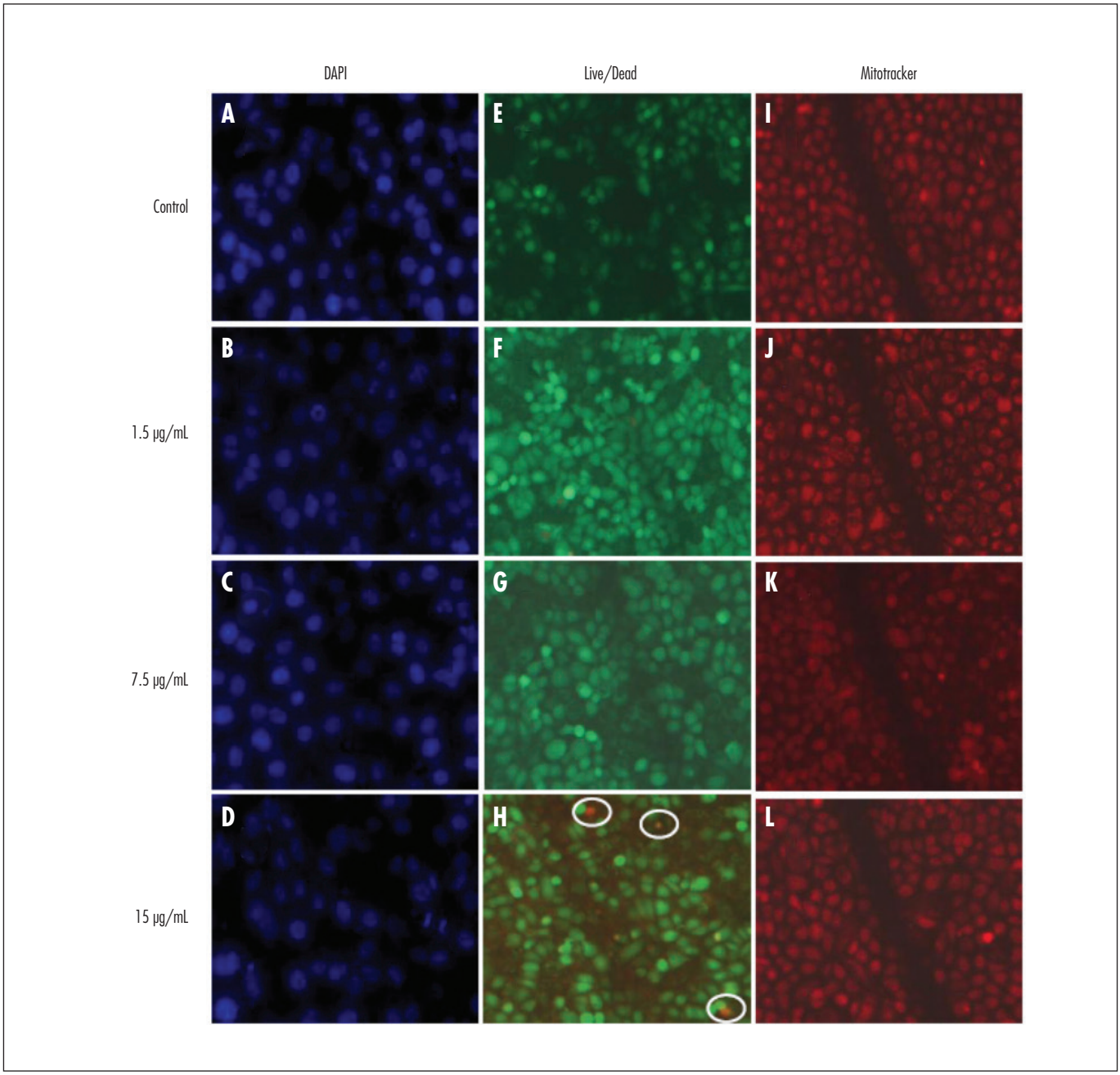

Figure 3. Representative images of DAPI (A to D), Live/Dead (E to H) and MitoTracker (I to L) staining of TOV-21 G cells treated with 1.5, 7.5 and 15 µg/mL of acetaminophen. The white circles indicate red dead cells

Real-time PCR is the most reliable and sensitive gene expression profiling technology for analyzing a panel of genes. Many studies have been carried out assessing the expression profile of ABC transporters. Thus, most of these investigations analyze only one or a small group of ABC transporters. In contrast, our method allows for the complete analysis of 42 ABC transporters. In this study, we focused on determining the basic expression profile of ABC transporters in TOV-21 G cells, an EOC cell line. Our data demonstrated that ABCA1, ABCC3, ABCC4, ABCD3, ABCD4 and ABCE1 were severely down-regulated. Yasui et al. found that ABCC4, ABCD3, ABCD4 and ABCE1 were amplified among 19 of the examined resistant cell lines[29]. ABCA1 is a major regulator of HDL metabolism, yet its mechanistic involvement in cancer cell proliferation and progression is unclear[30]. Data indicate that ABCC4, which was not altered largely, can release prostaglandins from cells. In addition to inhibiting prostaglandin synthesis, some NSAIDs might also act by inhibiting this release[31]. Therefore, an effect on ABCC4 may underlie the potentially beneficial effects of NSAIDs.

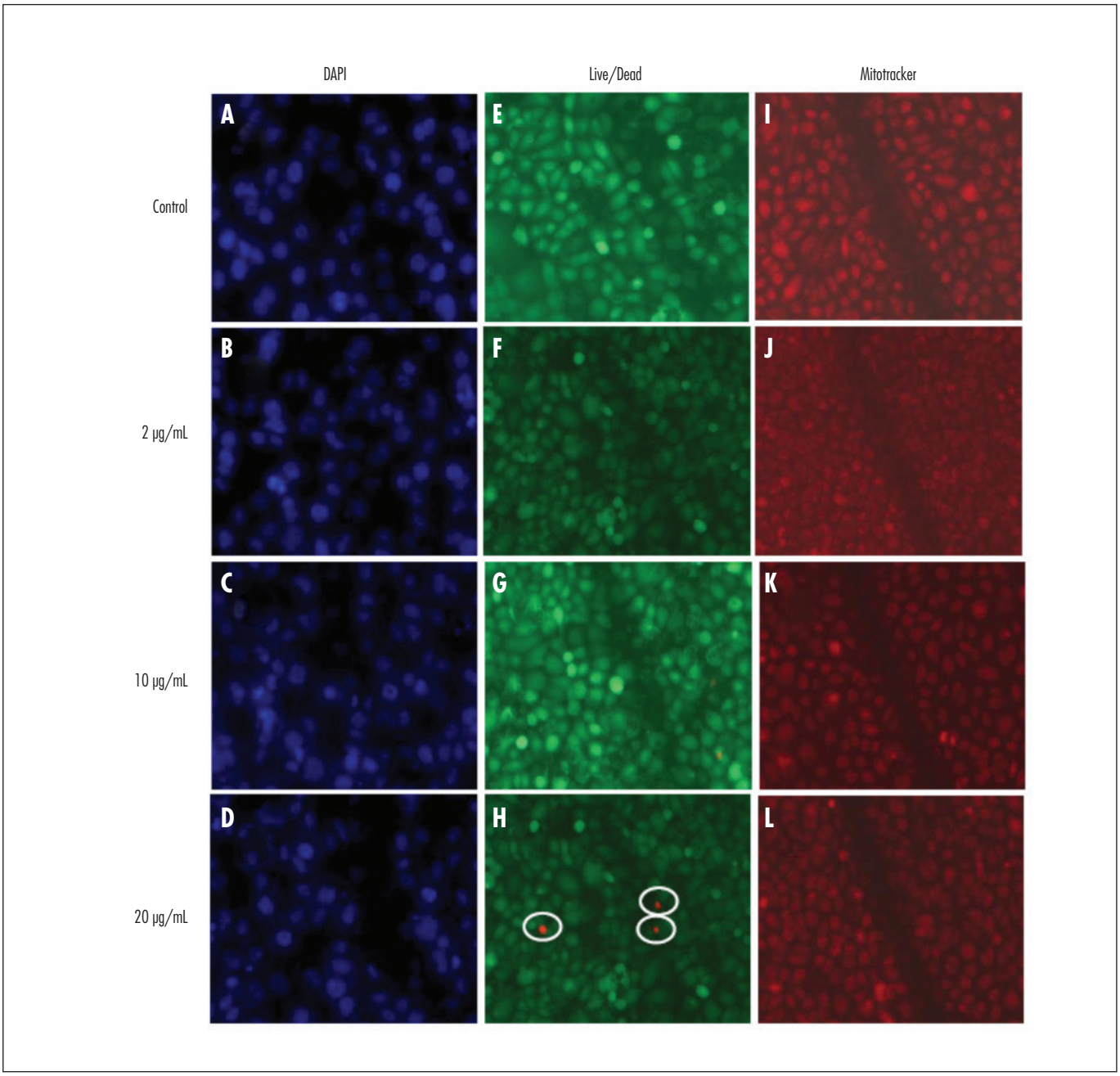

Figure 4. Representative images of DAPI (A to D), Live/Dead (E to H) and MitoTracker (I to L) staining of TOV-21 G cells treated with 2, 10 and 20 µg/mL of ibuprofen. The white circles indicate red dead cells

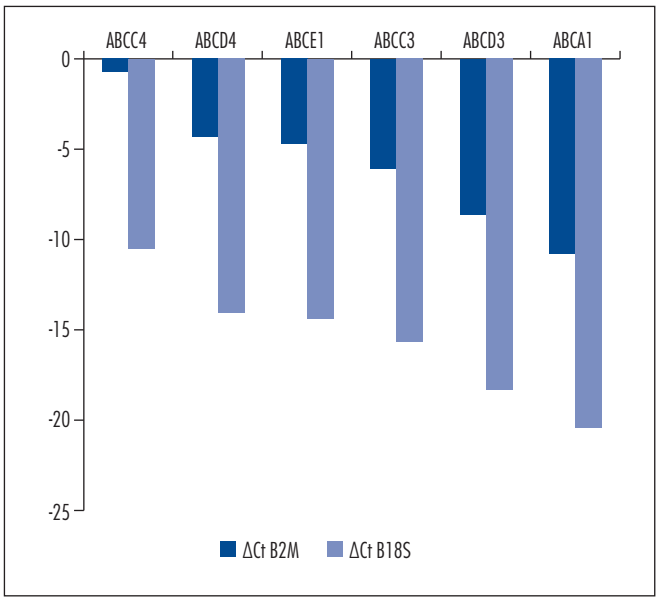

Figure 5. Gene expression profile of different ABC transporters normalized to a beta-2-micro-globulin precursor gene (ΔCt B2M=$Ct_{transporter}$–Ct_{B2M}) and an 18 S gene (ΔCt 18S=$Ct_{transporter}$–Ct_{18S})

An increased understanding of the mechanisms underlying drug resistance may lead to the development of more successful therapeutic protocols[29]. Hence, the expression profile of ABC transporter genes in cell lines used for drug screening is highly important for the antineoplastic drug discovery.

These findings ensure further studies on the physiologic role, clinical relevance, and potential use of ABCC4 as a therapeutic target. Due to the limitations of *in vitro* testing, these results should be confirmed in animal models for safety and efficacy. Using more than one cell type may help avoid making decisions based on tissue-specific responses.

Finally, the therapeutic concentrations of acetaminophen and ibuprofen directly inhibit EOC cell viability, which may significantly contribute to their antineoplastic effects. These results should encourage further research regarding the potential benefit of acetaminophen and NSAIDs in chemoprevention or as adjuvant therapies in EOC treatment.

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