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Differential Effects of Bisphenol A on Thyroid Cell Viability, Proliferation, and Migration Are Influenced by Genetic Background

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Abstract

Bisphenol A (BPA) is a common endocrine disruptor that poses a public health risk owing to its potential effects on thyroid function. However, its role in thyroid cancer development is unclear. This study investigated BPA's genotype-dependent effects of BPA on cellular processes in human thyroid cell lines. We exposed a non-tumorigenic cell line (Nthy-ori 3 − 1) and two papillary thyroid carcinoma cell lines, BCPAP (BRAF V600E) and TPC-1 (RET/PTC), to BPA concentrations (0.1–1.0 μg/mL) and assessed their viability, proliferation, and migration. The results indicate that BPA induces distinct, genotype-dependent responses. At higher concentrations (0.8-1.0 μg/mL), non-tumorigenic cells exhibited significant dose-dependent cytotoxicity but paradoxically showed enhanced migration compared to untreated cells. In cancer cell lines, TPC-1 cells exhibited relative resistance to BPA with no significant changes in migration, whereas BCPAP cells showed reduced proliferation and significant impairment of wound closure.

Our findings demonstrate that BPA exerts dual and opposing effects on thyroid cells, depending on the genetic background. Genotype-specific susceptibilities, cytotoxic and pro-migratory responses in normal cells, and a clear anti-migratory effect restricted to BRAF-mutant cells underscore the complex role of BPA in thyroid pathophysiology. These results suggest that genetic background is a critical determinant of cellular susceptibility to endocrine disruptors.

Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous compounds that interfere with the synthesis, secretion, transport, and action of natural hormones, thereby disrupting endocrine homeostasis (1, 2). These substances encompass diverse chemical classes, notably phenols and plasticizers, which are ubiquitous in modern environments. Dietary intake is a primary route of human exposure to EDCs, which have been increasingly linked to reproductive dysfunction, metabolic disorders, neuroendocrine disturbances, and various cancers (3–7).

Bisphenol A (BPA; 2,2-bis (4-hydroxyphenyl) propane) is a prominent member of the phenol class and is one of the most studied EDCs because of its extensive use in consumer products. It is a well-characterized xenoestrogen and a high-production-volume chemical, with global output projected to reach 9.3 million tons by 2030 (8–10). It is ubiquitous in products such as polycarbonate plastics, epoxy resin lining food containers, and medical devices (11). Human exposure to BPA is associated with the dysregulation of multiple hormonal pathways, including those of androgens, insulin, and thyroid hormones (12, 13). Epidemiological and experimental studies have further linked BPA to cardiovascular diseases, obesity, reproductive developmental disorders, and cancers of the breast and prostate, mediated through molecular and metabolic mechanisms (14–18).

Thyroid function is modulated by complex endocrine signaling, and BPA, as a structural analog of steroid hormones, may interfere with cellular homeostasis (19). While its structural mimicry suggests a potential interaction with estrogen receptors (ER) (20), BPA exposure in thyroid follicular cells has also been linked

to the disruption of thyroid hormone receptor (TR) signaling and related metabolic pathways. These multifaceted interactions underscore the broader endocrine-disruptive role of BPA that extends beyond estrogen-centric mechanisms.

Urinary BPA levels in patients with papillary thyroid carcinoma (PTC) were higher than those in healthy individuals, suggesting that BPA may be a risk factor for the development of PTC (21). In animal model studies, the synergistic effect between BPA and excess iodine was confirmed, which, together, may increase susceptibility to the development of thyroid tumors in rats (22). In Chinese women, elevated urinary BPA levels were associated with an increased risk of thyroid nodules (TNs), but only in those with positive thyroid autoantibody levels. This relationship was nearly linear, suggesting that BPA exposure is directly related to an increased risk of TNs (23).

BPA exposure upregulates the expression of critical genes involved in thyroid hormone synthesis, such as sodium-iodide symporter (NIS), thyroglobulin (Tg), and thyroid peroxidase (TPO), as well as key developmental transcription factors such as Pax8, Nkx2-1, and Foxe1 (24). While regulatory agencies have established tolerable daily intake (TDI) values for BPA, these vary significantly, from the European Food Safety Authority's (EFSA) value of 0.2 ng/kg/day to the U.S. EPA/FDA reference dose of 4 μg/kg/day (25, 26). This discrepancy, coupled with evidence of thyroid sensitivity to low EDC doses, underscores the necessity of assessing the effects of BPA over a range of physiologically relevant concentrations.

Existing data demonstrate that BPA can impair thyroid function, alter glandular morphology, and promote thyroid tumor cell proliferation (22, 27–29). However, molecular studies have reported contradictory findings, and a comprehensive comparison of BPA's functional impact across thyroid cell lines with distinct genetic backgrounds is lacking. Such investigations are critical for elucidating how genetic background influences cellular responses to this common environmental contaminant. The relationship between BPA and thyroid cancer remains inconclusive. This is particularly relevant given the high prevalence of thyroid nodules, which are detectable in up to half of the adult population and represent a substantial pool of follicular cells at risk of acquiring genetic alterations. This is crucial given the rising global incidence of thyroid cancer, largely attributed to papillary thyroid carcinoma (PTC). PTC is frequently driven by oncogenic mutations, such as BRAF V600E and RET/PTC rearrangements, which promote tumorigenesis through constitutive activation of the MAPK pathway (30, 31).

Although the effects of BPA are increasingly recognized as genotype-dependent, its functional impact on key cancer-relevant phenotypes, such as viability, proliferation, and migration, in thyroid cells with defined oncogenic drivers, remains poorly characterized. This gap is critical because the predominant oncogenic mutations in PTC, such as BRAF V600E mutation and RET/PTC rearrangements, constitutively activate divergent signaling cascades that may distinctly modulate cellular susceptibility to environmental stressors. Therefore, we hypothesized that the specific oncogenic background (defined by specific driver mutations), rather than genotype alone (referring to the broader genetic identity of the cell), is the primary determinant of the thyroid cellular response to BPA. To test this hypothesis, we characterized

these effects using a short-term in vitro exposure model designed to compare the relative cellular susceptibility across different genetic contexts using a normal human thyroid cell line and two PTC-derived lines harboring the canonical BRAF V600E mutation or RET/PTC rearrangement.

Results

BPA causes moderate reduction in cell viability in thyroid cells

In non-tumorigenic Nthy-ori 3 − 1 cells, viability assessed by Trypan Blue exclusion showed a downward trend even at the lowest BPA concentration (0.1 µg/mL), exhibiting a dose-dependent profile. However, statistically significant reductions were only detected at higher concentrations (0.8 and 1.0 µg/mL) after 24 h ($p \leq 0.05$). These effects were more pronounced at 48 h (0.8 µg/mL, $p \leq 0.05$; 1.0 µg/mL, $p \leq 0.001$), indicating a moderate but statistically significant cytotoxic effect of BPA on normal thyroid cells at these specific doses. In the PrestoBlue assay, Nthy-ori 3 − 1 cells displayed viability comparable to controls at low BPA concentrations (0.1, 0.2, and 0.4 µg/mL), with no statistically significant differences observed within this range ($p > 0.05$). Significant reductions were observed only at higher concentrations, specifically at 0.8 µg/mL ($p = 0.05$) and 1.0 µg/mL ($p \leq 0.001$). These findings were more pronounced after 48 h, which is consistent with the Trypan Blue results. Specifically, after 24 h of exposure to 1.0 µg/mL, cell viability decreased to 89.7% (a reduction of approximately 10.3%), and further declined to 82.2% after 48 h (a 17.8% reduction). At 0.8 µg/mL, viability remained relatively stable, showing a modest reduction of approximately 6.4% across both time points, with values around 93.6% (Fig. 1a). In the lower concentration range (0.1–0.4 µg/mL), the maximum reduction observed was less than 5%, reinforcing the lack of statistical significance at these doses.

TPC-1 cells exhibited a more gradual and modest reduction in viability in the Trypan Blue assay. At 24 h, slight increases in cell numbers were observed at 0.2 and 0.4 µg/mL, followed by a decrease at 0.8 µg/mL; however, these changes were not statistically significant ($p > 0.05$). After 48 h, reductions began at 0.1 µg/mL and reached significance at 0.8 and 1.0 µg/mL (≤ 0.05), indicating a smaller reduction in viability in TPC-1 cells than in Nthy-ori 3 − 1 cells. Cell viability, measured using PrestoBlue, remained stable at lower and intermediate concentrations (0.1–0.4 µg/mL), with no statistically significant differences observed and reductions remaining below 1.5% ($p \geq 0.05$). Specifically, at 0.8 µg/mL, viability decreased by approximately 1.7% to 2.2% ($p \leq 0.05$), whereas at 1.0 µg/mL, the reduction ranged from 2.5% to 3.0% ($p \leq 0.001$), consistent with the trends observed in the Trypan Blue assay (Fig. 1b).

BCPAP cells exhibited a variable response, with slight decreases in viability at lower BPA concentrations, particularly 24 h after treatment. A biphasic-like pattern was observed, characterized by a modest reduction at 0.1 and 0.2 µg/mL (approximately 0.5–1.0% reduction), followed by a return to near-control levels at 0.4 µg/mL (99.6% viability). At 48 h, minor reductions were observed at 0.2 µg/mL, while slight increases were noted at 0.4 and 0.8 µg/mL, potentially reflecting the intrinsic characteristics of BRAF-mutant cells. Metabolic activity after 24 h of exposure showed minor decreases at intermediate

concentrations, with 0.8 µg/mL and 1.0 µg/mL resulting in reductions of only 1.5% and 2.6%, ($p \leq 0.05$) and 2.6% ($p \leq 0.001$), respectively. However, these effects were time-dependent; at 48 h, the reductions became more pronounced, reaching approximately 10.4% at 0.8 µg/mL ($p \leq 0.05$) and 15.1% at 1.0 µg/mL ($p \leq 0.001$) (Fig. 1c).

BPA impairs thyroid cancer cell proliferation

The effects of BPA on thyroid cell proliferation were assessed using an EDU incorporation assay (Supplementary Fig. 1). Nthy-ori 3 − 1 cell proliferation was not significantly altered compared to of that the control after 48 h of treatment (0.8 µg/mL: 39.6%, $p = 0.485$; 1.0 µg/mL: 38.2%, $p = 0.732$), suggesting that BPA did not affect DNA synthesis in normal thyroid cells. In TPC-1 cells, proliferation remained near control levels at both 0.8 µg/mL (41.6%; 1.0% increase) and 1.0 µg/mL (41.6%; 1.0% decrease), with no significant differences ($p = 0.6730$ and $p > 0.9999$, respectively). These results suggest that exposure to BPA at concentrations of 0.8 µg/mL and 1.0 µg/mL does not significantly affect the proliferative capacity of this RET-rearranged cell line. In BCPAP cells, proliferation remained near control levels at 0.8 µg/mL (26.7%; 7.1% reduction), and no statistically significant difference was observed at this concentration ($p = 0.240$). However, a significant impairment of proliferative capacity was observed at the highest BPA concentration (1.0 µg/mL; 22.8%; 20.9% reduction; $p \leq 0.05$). Consequently, exposure to 0.8–1.0 µg/mL BPA reduced proliferation exclusively in BCPAP cells, whereas no such effect was observed in Nthy-ori 3 − 1 or TPC-1 cells.

BPA modulates thyroid cell migration

The closure of the cell-free area in Nthy-ori 3 − 1 cells progressed gradually in the control group, reaching near-complete closure (~ 0% open area) between 24 and 30 h of treatment. In the BPA-treated cells (0.8 and 1.0 µg/mL), area closure was accelerated, with consistently smaller open areas compared to the control (Supplementary Fig. 2). This stimulatory effect was most evident during the early migratory phase; specifically, the time required to reach 50% area closure was significantly shorter in the treated groups (3.5 h at 0.8 µg/mL and 4.5 h at 1.0 µg/mL) than in the control (6 h). At 0.8 µg/mL, significant differences in the open area were observed as early as 1.5–4.5 h ($p \leq 0.05$), with a peak effect between 5 and 9 h ($p \leq 0.01$), where mean differences reached approximately 21–22% relative to the control. After 10 h, although the differences remained significant ($p \leq 0.05$), the magnitude of the effect began to decrease as the control cells progressed toward total closure. These data, as detailed in Supplementary Fig. 2, indicate that BPA consistently enhances the early migratory capacity of non-tumorigenic thyroid cells.

In TPC-1 cells, which exhibited rapid baseline migration with full closure in the control group within 24 h, BPA exposure induced a subtle, non-significant acceleration of this process. This was reflected in the time to 50% area closure, which was reduced from 9 h in the control group to 8 h at 1.0 µg/mL and 8.5 h at 0.8 µg/mL. The progression toward full closure followed a similar trend, occurring at approximately 17

and 19 h for the 0.8 and 1.0 µg/mL doses, respectively, compared to 24 h in the control group. However, these differences did not reach statistical significance ($p \geq 0.05$), indicating that the impact of BPA on the migration of these tumorigenic cells was marginal under the tested conditions (Supplementary Fig. 3).

In BCPAP cells, the closure of the cell-free area occurred more slowly than that in other cell lines, requiring a total observation period of 72 h. BPA exposure significantly delayed this process, with full closure occurring at approximately 66 h (0.8 µg/mL) and 71 h (1.0 µg/mL) compared to 53 h in the controls. This inhibitory effect was further evidenced by the time to 50% area closure: while control cells reached this milestone at 18 h, the time increased to 19.5 h at 1.0 µg/mL concentration. Interestingly, at 0.8 µg/mL, the time to 50% closure was slightly reduced to 17.5 h, although the subsequent migration rate was significantly decreased. Statistical analysis confirmed that 0.8 µg/mL BPA significantly reduced the rate of area closure throughout the experiment, with mean differences reaching up to -6.3%. The most pronounced inhibitory effects were observed between 7.0 and 8.5 h ($p < 0.001$). These results indicate a dose-dependent and predominantly inhibitory effect of BPA on BCPAP cell migration (Supplementary Fig. 4).

Discussion

Our study demonstrates that BPA exerts cell type- and dose-dependent effects on human thyroid cell lines, influencing viability, proliferation, and migration in a manner that appears to be dictated by the genetic background of the cells.

The heightened sensitivity of non-tumorigenic Nthy-ori 3 − 1 cells was evident from the clear, dose-dependent decrease in viability at higher BPA concentrations (0.8–1.0 µg/mL), as confirmed by both Trypan Blue and PrestoBlue assays. This finding is consistent with previous reports that BPA disrupts thyroid function by interfering with signaling and metabolic homeostasis (32, 33). Intact endocrine regulatory pathways in normal cells may make them more susceptible to xenobiotic-induced stress and cytotoxicity. Despite the reduced viability, the proliferative capacity of Nthy-ori 3 − 1 cells remained constant. This suggests that BPA induces cytotoxic stress, leading to cell death in a subset of cells, while not arresting the cell cycle in others. A striking and paradoxical finding was the significant acceleration of migration in normal cells exposed to BPA A. This pro-migratory effect, particularly at 0.8 µg/mL, may represent an adaptive or compensatory response to a localized cytotoxic environment. BPA may activate pathways that promote cytoskeletal rearrangement and motility, such as estrogen receptor signaling or HIF-1α/VEGF pathways (34). However, further molecular studies are required to confirm whether this accelerated migration is a survival-driven adaptive mechanism or a direct consequence of disrupted intracellular signaling.

This apparent dichotomy, in which reduced viability coincides with increased migration, may represent an adaptive survival mechanism in normal cells that respond to toxic stressors. This compensatory behavior, commonly triggered by chemical stressors to preserve function (35), could be interpreted as an

attempt by the most resilient cells to escape or adapt to an unfavorable environment. Such stress-induced migration shares conceptual parallels with the invasive behavior of cancer cells during metastasis (36), highlighting the potential pro-tumorigenic effect of BPA, which may inadvertently prime a tissue environment conducive to disease progression.

A divergent effect was observed between the two cancer cell lines. TPC-1 cells harboring RET/PTC rearrangements did not show any inhibition of proliferation. In contrast, BCPAP cells carrying the BRAF V600E mutation displayed only modest but significant reductions in proliferation at the highest concentration of the drug. This finding is consistent with studies on prostate cancer models, where BPA induced antiproliferative effects via p53 regulation and upregulation of p21 and p27 expression. This relative resistance observed in BCPAP cells may reflect the constitutive activation of the MAPK/ERK pathway, which confers resilience to cytostatic effects (37). We hypothesized that the observed dichotomy in migratory capacity, where BCPAP cells showed impairment and normal cells showed enhancement, may result from BPA's interference with these specific oncogenic signaling nodes. Such responses could be mediated by the differential expression of estrogen receptors (ERα and ERβ) or GPER1, as observed in other cancer models (38). However, further studies quantifying the activation of downstream components such as pERK are necessary to confirm these specific mechanistic pathways. This suggests that BPA exerts opposing actions on cell motility depending on the internal oncogenic drivers and the balance of the signaling pathways.

The interpretation of the wound-healing assay requires consideration of inherent methodological constraints. Although proliferation was independently assessed by EdU incorporation, a proliferation inhibitor was not used during the migration assays. Consequently, wound closure should be interpreted as an integrated functional readout that reflects the combined contributions of cell migration, viability, cell cycle dynamics, and stress-associated signaling. This experimental design was intentional, as the aim was to capture the immediate and biologically relevant cellular response to BPA exposure, which affects multiple cellular processes.

Within this framework, the accelerated wound closure observed in Nthy-ori 3 – 1 cells occurred under conditions of concurrent cytotoxic stress and may represent a stress-associated adaptive response, rather than a purely migration-specific effect. Similarly, the reduced wound closure observed in BCPAP cells is interpreted as a predominantly functional antimigratory outcome, while acknowledging that it cannot be fully dissociated from concurrent alterations in the proliferation or stress signaling pathways.

The divergent responses observed among the three cell lines underscore the complex interplay between BPA and thyroid cell signaling pathways. A proposed model to explain these genotype-dependent effects involves the distinct estrogen receptor profiles of the cell lines. Owing to its structural similarity to estradiol, BPA can interact with estrogen receptors expressed in thyroid follicular cells, potentially modulating key processes such as proliferation and migration (50).

The BCPAP cell line exhibits high expression of estrogen receptor alpha (ERα) and comparatively low expression of estrogen receptor beta (ER_{β}) (39). In the literature, an increased ERα/ERβ ratio is

associated with enhanced proliferative, invasive, and migratory behaviors, supporting a pro-tumorigenic role for ERα and a counter-regulatory role for Erβ (40). Consistently, agents that downregulate ERα or upregulate ER_{β} have been shown to reduce BCPAP cell aggressiveness (41). The TPC-1 cell line expresses both ERα and ER_{β}, with levels varying according to the experimental context (42). In these cells, ERα is primarily linked to proliferative and pro-survival signaling, whereas ER_{β} is linked to differentiation and pro-apoptotic pathways (43). TPC-1 cells also express the membrane-associated G protein-coupled estrogen receptor (GPER1), which mediates rapid non-genomic responses to estrogenic stimuli (20).

We propose that the distinct receptor profiles of these cell lines provide a plausible mechanistic basis for the observed effects. Specifically, the balance of signaling between ERα, ER_{β}, and GPER1 may critically influence the cellular outcomes. This premise is supported by extensive research demonstrating the complex and context-dependent effects of BPA. For instance, BPA can exert both estrogenic and anti-estrogenic effects, such as promoting chemoresistance in breast cancer cells independent of ERα status (56) or exerting dual effects on processes such as wound healing, contingent on ER_{β} expression (57). Notably, BPA activation of ERα has been linked to increased proliferation and metastasis in ovarian cancer models (58), whereas its engagement with GPER1 can alter fundamental processes such as hepatic lipid metabolism (59).

Consequently, the interplay between ERα, ER_{β}, and GPER1 during BPA exposure may have significant implications for hormone-sensitive tissues, providing a logical framework for investigating the molecular mechanisms of this endocrine disruptor. The established literature underscores that BPA can modulate cancer-relevant phenotypes through genomic and non-genomic pathways. Its effects have been implicated in colorectal and breast cancers, where it influences nuclear receptors and pathways involved in tumor progression (60–65).

In the context of our results, the reported high ERα/ER_{β} ratio in BCPAP cells may increase their susceptibility to BPA-mediated proliferative or survival signals. Conversely, the differential signaling balance in TPC-1 or Nthy-ori 3 – 1 cells could underlie their distinct responses. However, this model requires direct experimental validation in future studies using receptor expression analysis, pharmacological inhibition and protein-level assays.

Furthermore, non-receptor-mediated mechanisms may have contributed to the observed effects. BPA-induced oxidative stress, DNA damage, and altered expression of thyroid-specific transcription factors (e.g., Pax8) can drive cytotoxicity and impair the function of susceptible cell types (33, 38).. In addition to classical hormonal signaling, BPA-induced oxidative stress has been increasingly linked to the overexpression of NADPH oxidase 4 (NOX4). NOX4 is the primary source of reactive oxygen species (ROS) in thyroid cells and is frequently upregulated in papillary thyroid carcinoma. It is possible that the heightened sensitivity and paradoxical migratory response observed in Nthy-ori 3 – 1 cells reflect a lower threshold for NOX4-mediated ROS production compared to tumor lines, which may have already developed robust antioxidant defenses. Thus, the conserved mechanism of NOX4 overexpression

potentially underpins the shift from physiological homeostasis to cytotoxic or pro-migratory states documented in our assays, providing a molecular link between BPA exposure and the specific cellular behaviors of each lineage (27).

This study has several limitations. Our in vitro model was designed for short-term exposure to compare cellular susceptibility across different genetic backgrounds rather than replicating chronic human exposure. The selected concentrations (0.1–1.0 µg/mL) align with high dietary intake scenarios and are consistent with the Specific Migration Limit (SML) established by ANVISA (0.6 mg/kg). Although these values fall within the range of older regulatory benchmarks, such as the previous US EPA/FDA TDI of 50 µg/kg/day, they are substantially higher than the more recent and restrictive TDI established by the EFSA (0.2 ng/kg/day). This distinction is critical: while our concentrations reflect the permissible migration levels in food packaging as set by Brazilian legislation, they do not correspond to the endogenous levels typically reported in human biomonitoring studies. Therefore, our findings should be interpreted cautiously.

Furthermore, the precise molecular mechanisms underlying these differential effects across cell types remain unresolved. The lack of experiments, such as immunofluorescence for receptor localization, detailed profiling of ERα, ERβ, and GPER1 expression, and analysis of downstream signaling pathways, precludes a deeper understanding of the underlying mechanisms. Similarly, when migration was analyzed, the absence of a proliferation inhibitor (e.g., mitomycin C) meant that the observed wound closure reflected an integrated functional outcome that may include contributions from altered proliferation and viability, particularly under cytotoxic stress.

Future studies incorporating receptor expression analysis, pharmacological inhibition, cell cycle profiling, and assays for specific cell death mechanisms are essential to validate the proposed hypotheses and clarify BPA's impact of BPA on thyroid cell function in genetically predisposed or neoplastic contexts.

Collectively, our data establish the genetic background as a critical determinant of cellular response to BPA, revealing mutation-specific susceptibilities relevant to thyroid cell biology. The limitations outlined above represent critical avenues for further investigation to achieve a deeper mechanistic understanding of the disease.

In conclusion, our data support the concept that genetic background is a critical determinant of cellular responses to BPA. Rather than acting as a uniform toxicant, BPA functions as a cellular modulator, the impact of which appears to be defined by the molecular identity of the cell. Within this limited panel of thyroid models, BPA induced cytotoxic stress and enhanced wound closure in non-tumorigenic cells, whereas RET/PTC-positive TPC-1 cells were relatively resistant. In contrast, significant antimigratory effects and impaired proliferation were restricted to BRAF-mutant BCPAP cells. These findings underscore the importance of considering genetic susceptibility in toxicological risk assessments. Furthermore, our results suggest that BPA exposure may have implications for thyroid cancer initiation and progression, depending on the underlying oncogenic background, a premise that warrants further investigation in vivo.

This study provides a useful comparative in vitro framework that highlights the nuanced, mutation-specific effects of endocrine disruptors. Future research incorporating a broader range of genotypes and exploring chronic low-dose exposure scenarios will be essential to model human risk and environmental health impacts more accurately.

Materials and Methods

Cell lines and culture conditions

Three human thyroid cell lines were employed in this study: Nthy-ori 3 − 1, an immortalized non-tumorigenic thyroid follicular epithelial line, served as a control, and BCPAP and TPC-1, derived from PTC, represented malignant models with distinct molecular backgrounds. BCPAP cells harbor the BRAF V600E mutation, whereas TPC-1 cells carry the RET/PTC rearrangement. The thyroid papillary carcinoma cell lines (BCPAP and TPC-1) were kindly provided by Professors Jacques Dumont and Marc Mareel, while the Nthy-ori 3 − 1 cell line was purchased from the European Collection of Authenticated Cell Cultures (ECACC). The identity of all cell lines was recently confirmed by Short Tandem Repeat (STR) profiling analysis, and all cultures were tested and found to be negative for mycoplasma contamination using a PCR-based detection kit.

Cells were maintained in RPMI-1640 medium (Capricorn Scientific) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic-antimycotic solution (Invitrogen™, USA) at 37°C in a humidified 5% CO_2 atmosphere. For routine maintenance in T25 flasks, Nthy-ori 3 − 1 cells were seeded at 3.0 x 104cells/mL, while BCPAP and TPC-1 were seeded at 1.5 × 104 cells/mL.

Specific seeding densities were optimized for each assay to ensure appropriate confluence at the start of BPA exposure: > * Cell Viability and Proliferation: For Trypan Blue exclusion assays, cells were seeded in 24-well plates at a density of 1.0 × 105 cells/well. For PrestoBlue and EdU assays, cells were seeded in 96-well plates at 1.0 × 104 cells/well, reaching approximately 60–70% confluence at the time of treatment. Wound Healing Migration: Cells were seeded into IBIDI® 2-well culture inserts in a 24-well plate (7. ×x 104 cells per insert chamber) to ensure a 90% confluent monolayer prior to insert removal and subsequent BPA exposure. In all experiments, the cells were allowed to adhere for 24 h before treatment.

BPA preparation

Bisphenol A (CAS No. 80-05-7, purity > 99%; Supelco, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) to prepare a 30,000 µg/mL stock solution. To ensure chemical stability and prevent degradation, aliquots were protected from light, and final working concentrations (0.1, 0.2, 0.4, 0.8, and 1.0 µg/mL) were freshly prepared by diluting the stock in culture medium immediately before each assay. Any unused working solutions were discarded. The final DMSO concentration was maintained at ≤ 0.1% across all groups, including the vehicle control.

BPA exposure protocol

BPA exposure protocol

After 24 h of seeding, the cultures were washed with pre-warmed PBS (1X) and treated with BPA-containing medium for 24 or 48 h. The selected exposure concentrations (0.1–1.0 µg/mL) were based on the Specific Migration Limit (SML) for Bisphenol A food-contact plastic materials, established at 0.6 mg/kg (approximately 0.6 µg/mL) by the Brazilian National Health Surveillance Agency (ANVISA) (RDC No. 17/2008) (36). These concentrations represent a physiologically relevant range for high dietary intake scenarios (~ 1 µg/mL). The cultures were maintained under standard conditions (37°C, 5% CO_2, and saturated humidity) throughout the treatment period.

Cell viability assays

1.Trypan Blue exclusion

For the Trypan Blue exclusion assay, the cells were detached, resuspended in PBS, and mixed 1:1 (v/v) with 0.4% Trypan Blue solution (Sigma-Aldrich, USA). Manual cell counting was performed by a single observer using a Neubauer chamber (hemocytometer) under an optical microscope. To ensure statistical representativeness, viable (unstained) and non-viable (stained blue) cells were counted in the four large corner squares of the chamber for each sample. Cell viability was calculated as the percentage of viable cells relative to the total number of cells used. Each treatment was performed in triplicate, and the average of the four squares was used to determine the final concentration for each sample.

2.PrestoBlue® assay

PrestoBlue® reagent (Invitrogen™, USA) was used to assess the metabolic activity of the cells. After BPA treatment, the medium was replaced with 100 µL of fresh medium containing 10% PrestoBlue®, and the plates were incubated for 1 h at 37°C, 5% CO_2, protected from light. Fluorescence was measured using a SpectraMax® microplate reader (excitation: 560 nm; emission: 590 nm). Relative viability was calculated in reference to the control condition (0.1% DMSO) after subtracting the blank fluorescence readings from both the control and treated samples. The resulting values were used to determine the percentage of viable cells under each condition.

Cell Proliferation (EdU Incorporation)

Cell proliferation was assessed using the Click-iT™ EdU Alexa Fluor™ 647 Imaging Kit (Invitrogen™, USA) according to the manufacturer's instructions. Briefly, after BPA exposure, the cells were incubated with 20 µM EdU for 2 h. The cells were then fixed in 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained using the Click-iT® reaction cocktail along with Hoechst nuclear counterstaining. Fluorescence imaging was performed using an Operetta® system, and proliferation was automatically quantified as the percentage of EdU-positive nuclei.

Cell migration (Wound Healing Assay)

Directional migration was assessed using a wound healing assay with Ibidi® 2-well culture inserts (Ibidi, Germany) placed in 24-well plates. To ensure a uniform and reproducible cell-free gap, cells were seeded into each insert chamber at a density of 7 × 104 cells (in 70 µL of medium). The cultures were incubated until a confluent monolayer (> 90%) was formed, typically within 24 h. The inserts were then carefully removed to create a standardized 500 µm cell-free gap, and the monolayer was washed with pre-warmed phosphate-buffered saline (PBS) to remove debris. The cells were then treated with a BPA-containing medium, and wound closure was monitored every 30 min for up to 72 h using the Opera Phenix® High-Content Screening System (PerkinElmer, USA), maintained at 37°C and 5% CO_2.

The wound area was quantified using an automated method. Images captured by the Opera Phenix® system were analyzed using the Harmony® High-Content Imaging and Analysis software (PerkinElmer, USA).

No proliferation inhibitors were used in the wound-healing assays. This experimental design was intentional, as the objective was to evaluate the integrated and immediate cellular response to BPA exposure, rather than isolating migration as a strictly proliferation-independent process. Given that BPA is known to simultaneously modulate cell viability, cell cycle dynamics, and stress-related signaling pathways, the wound-healing assay was interpreted as a composite functional readout, reflecting the combined contribution of these processes.

Migration rates were automatically quantified by measuring the reduction in the cell-free area over time. All experiments were performed in technical and biological triplicates to ensure reproducibility and reliability of the data.

Statistical analysis

Data were analyzed using GraphPad Prism 8.0.1 and are presented as mean ± standard error of the mean (SEM). For comparisons involving multiple groups, one-way ANOVA was performed, followed by Tukey's post-hoc test. Comparisons between the two groups were assessed using the Mann-Whitney U test or Student’s t-test, as appropriate. Statistical significance was set at $p < 0.05$.

Declarations

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Figures

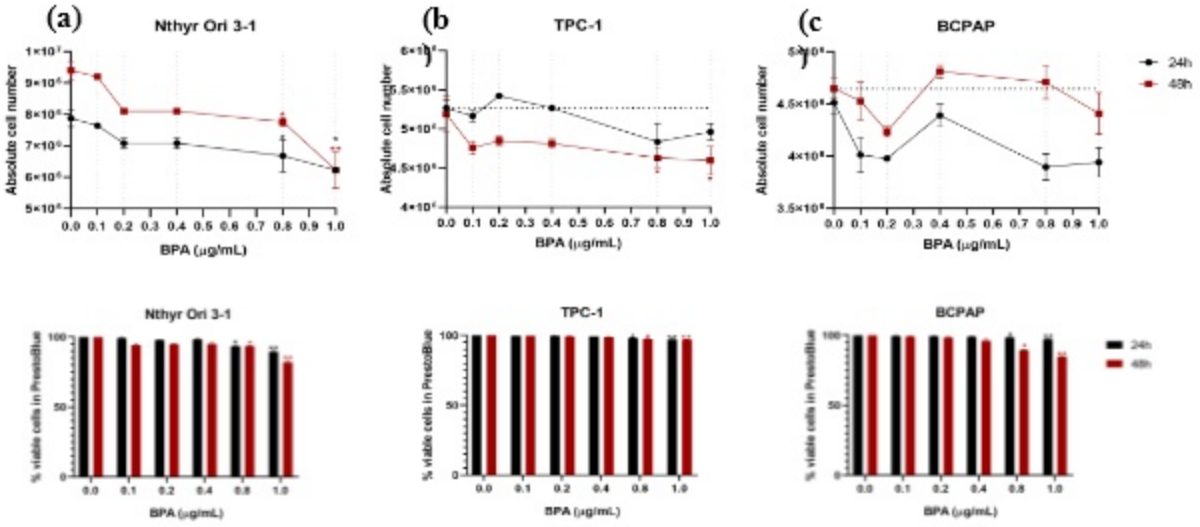


Figure 1

Effect of Bisphenol A on thyroid cell viability. Thyroid cell viability was measured after exposure to increasing BPA concentrations (0.1–1.0 µg/mL) for 24 and 48 h in Nthy-ori 3-1, TPC-1, and BCPAP cell lines using the Trypan Blue exclusion assay (upper panels) and PrestoBlue metabolic assay (lower panels). Data are presented as mean ± SEM from three independent experiments. *p ≤ 0.05, **p ≤ 0.001 vs. control.

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