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# Effects of bisphenol A on murine salivary glands and human tumor cell lines

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ABSTRACT

Bisphenol A (BPA) is an endocrine-disrupting chemical with a potential role in endocrine cancers. However, the effects of BPA on the salivary glands have been barely explored. We investigated the impact of *in vivo* sub-chronic exposure to BPA and its *in vitro* effects on human salivary gland mucoepidermoid carcinoma cell lines. Male and female mice were exposed to BPA (30 mg/kg/day). Sublingual and submandibular salivary glands from an estrogen-deficiency model were also analyzed. BPA concentration in salivary glands was evaluated by gas chromatography coupled to ion trap mass spectrometry. Immunohistochemical analysis using anti-p63 and anti-α-SMA antibodies was performed on mouse salivary gland tissues. Gene expression of estrogen receptors alpha and beta, P63 and α-SMA was quantified in mouse salivary gland and/or mucoepidermoid (UM-HMC-1 and UM-HMC-3A) cell lines. Cell viability, p63 and Ki-67 immunostaining were evaluated *in vitro*. BPA disrupted the tissue architecture of the submandibular and sublingual glands, particularly in female mice, and increased the expression of estrogen receptors and p63, effects that were accompanied by significant BPA accumulation in these tissues. Conversely, ovariectomy slightly impacted BPA-induced morphological changes. *In vitro*, BPA did not affect the proliferation of neoplastic cells, but augmented the expression of p63 and estrogen receptors. The present data highlight a potential harmful effect of BPA on salivary gland tissues, particularly in female mice, and salivary gland tumor cells. Our findings suggest that estrogen-dependent pathways may orchestrate the effects of BPA in salivary glands.

## 1. Introduction

Bisphenol A (BPA) is one of the highest-volume chemical compounds used worldwide in the production of numerous products such as plastics, thermal papers, and medical and dental materials (Frankowski et al., 2020; Geens et al., 2012; Marzouk et al., 2019). BPA is a ubiquitously distributed environmental pollutant (Ďurovcová et al., 2022) and has been consistently identified in plasma (Kaddar et al., 2009), placental fluids (Bonde et al., 2016), breast milk (Iribarne-Durán et al., 2022), semen (Castellini et al., 2022), urine (Castellini et al., 2022), and saliva (Gomes et al., 2020; Lopes-Rocha et al., 2022).

Multiple potential BPA-induced adverse effects have been

*Abbreviations:* Akt, serine threonine kinase; BPA, bisphenol A; CLARITY-BPA, Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide-$d_6$; ER, estrogen receptor; ERK, extracellular signal regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; NLRP3, NOD-like receptor protein 3; OVX, ovariectomized; p63, tumor protein 63; UM-HMC-1, University of Michigan-Human Mucoepidermoid Carcinoma-1; UM-HMC-3A, University of Michigan-Human Mucoepidermoid Carcinoma-3A; α-SMA, alpha-smooth muscle actin..

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documented in humans (Heindel et al., 2020; Patel et al., 2017; Prins et al., 2019; Vandenberg et al., 2020; Vom Saal and Vandenberg, 2021), such as a negative impact on male and female fertility (Bonde et al., 2016; Karwacka et al., 2019), the association with some endocrine disorders and increased occurrence of breast (Wan et al., 2022), endometrial (Caserta et al., 2022), and prostate cancer (Di Donato et al., 2017). In rodents, exposure to BPA can induce mammary carcinoma (Acevedo et al., 2013; Ayyanan et al., 2011; Durando et al., 2007; Wadia et al., 2013) and increase prostate sensitivity to adult hormone exposure and the risk of prostatic epithelial neoplasia (Almeida et al., 2021; Nelles et al., 2011; Nicholson et al., 2018; Taylor et al., 2020). Nonetheless, regulatory agencies such as the Food and Drug Administration continue to endorse that very low dose levels of BPA are safe and also that significant adverse effects of this chemical on humans are not biologically plausible (Vandenberg et al., 2020; Vom Saal and Vandenberg, 2021).

The oral cavity has been suggested to be the main route of contamination with BPA (Gayrard et al., 2013; Guignard et al., 2016; Teeguarden et al., 2015). Importantly, this chemical is able to interact with estrogen receptors (Acconcia et al., 2015; Matthews et al., 2001), which are expressed in oral epithelium and keratinocytes, particularly after BPA stimulation (Almeida et al., 2021; Emfietzoglou et al., 2019). Frequent exposure of oral tissues to BPA has raised questions about a possible link with oral and oropharyngeal carcinogenesis (Almeida et al., 2021; Emfietzoglou et al., 2019), as well as with cytopathological changes and apoptosis in the salivary glands (Çetin et al., 2021; Folia et al., 2013). Likewise, there is evidence that other estrogenic and anti-androgenic disrupting compounds, such as genistein and vinclozolin, disrupt salivary gland morphogenesis in rodents (Kouidhi et al., 2012; Kouidhi et al., 2018).

Recent literature has demonstrated that chronic exposure to BPA resulted in degeneration of acinar cells in the submandibular gland and duct system (Bashir et al., 2022). Along this line, it is suggested that the carcinogenic activity of BPA is anchored in the endoderm-derived glands (Cuomo et al., 2017), raising the hypothesis that the salivary glands are targets and potential sites of BPA accumulation. These findings may be associated with the effects of BPA as an endocrine-disrupting chemical or as an agent with pro-oxidative potential (Babu et al., 2013; Ďurovcová et al., 2022; Vandenberg et al., 2019; Vom Saal and Vandenberg, 2021). The ability of BPA to interfere with and/or prevent the binding of natural hormones to their receptors – even at low concentrations – may be linked to its deleterious effects on salivary gland homeostasis (Emfietzoglou et al., 2019; Folia et al., 2013; Heindel et al., 2020; Patel et al., 2017; Prins et al., 2019).

The expression of estrogen receptors in normal (Tsinti et al., 2009) and neoplastic human salivary glands (Aquino et al., 2018) may explain the interference of BPA with these tissues. Despite this, as far as we know, the literature is scarce with regard to the evaluation of the impact of BPA on the salivary glands (Bashir et al., 2022; Çetin et al., 2021; Folia et al., 2013; Sakabe et al., 2000; Selim et al., 2022), and no studies have examined the effects of BPA on human salivary gland tumor cell lines. The purpose of the present study was to examine BPA exposure in the major salivary glands of mice. The effects of BPA were also tested in an ovariectomy model that mimics conditions of low circulating estrogen (Baltgalvis et al., 2010). Our hypothesis was that, with the reduction of estrogen levels in ovariectomized animals, the greater availability of receptors would favor the BPA-estrogen receptor interaction, enhancing its actions on salivary gland tissues. The potential effects of this chemical on human salivary gland tumors were explored *in vitro*.

## 2. Material and methods

### 2.1. Animals and experimental protocols

Male and female C57BL/6 mice (5 weeks of age and weighing 15–25 g) were acquired from the Central Animal House of Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Animals were treated according to the regulations of the Institutional Ethics Committee (No. 387/2016), kept on a 12-h light/dark cycle under controlled temperature, receiving a standard diet and water *ad libitum*, and weighed weekly. The animals remained seven days in the institution's animal facilities for adaptation before the beginning of the experiments. The animals were kept in ventilated, autoclavable polypropylene cages with a maximum of five animals per cage, with males kept in separate cages from females.

The first set of experiments consisted of sub-chronic exposure to BPA. Male ($n = 20$) and female ($n = 20$) animals were randomly allocated to two groups: vehicle [0.05% dimethyl sulfoxide-$d_6$ (DMSO), Sigma-Aldrich, Burlington, MA, USA] and BPA [30 mg/kg/day; bisphenol A-(dimethyl-d6) - C15D6H10O2; Sigma-Aldrich] (Almeida et al., 2021). BPA was diluted in drinking water and only glass bottles were used throughout the assays. After six weeks, the animals were euthanized with an anesthetic overdose (300 mg/kg ketamine and 30 mg/kg xylazine) and the left sublingual and submandibular salivary glands were collected and fixed in 10% buffered formaldehyde solution. The right sublingual and submandibular salivary glands were immediately frozen in liquid nitrogen and stored at −80 °C.

The second set of experiments consisted of an ovariectomy-induced estrogen deficiency model. Female C57BL/6 mice ($n = 20$, 6 weeks of age and weighing 18–22 g) were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine) and randomly allocated to four groups: animals that ingested DMSO (sham/vehicle), animals exposed to BPA (sham/BPA), ovariectomized animals that ingested DMSO (OVX/vehicle), and ovariectomized animals exposed to BPA (OVX/BPA). Ovariectomy consisted of bilateral excision of the ovaries. Briefly, the ovaries were exposed and removed, and the skin was sutured immediately after the procedure (ovariectomized groups). In the surgical control group (sham), the ovaries were exposed but not removed and the skin was sutured later (Macari et al., 2015). The success of ovariectomy was confirmed by measuring uterine weight. One week after ovariectomy, the animals started ingesting BPA (30 mg/kg/day) or vehicle (0.05% DMSO) in drinking water and continued the treatment for six weeks. The animals were then euthanized with an anesthetic overdose and the major salivary glands were collected. Tissues were fixed in 10% buffered formaldehyde solution.

### 2.2. Histomorphometric assessment

Four-μm-thick sections of paraffin-embedded material obtained from each animal were stained with hematoxylin and eosin. A total of 10 fields on each slide were photographed under light microscopy at ×20 magnification (DM500, Leica Microsystems, Wetzlar, Germany) for morphometric evaluation using ImageJ software (ImageJ, US National Institutes of Health, Bethesda, MA, USA). Morphological analysis was conducted as previously defined (Folia et al., 2013). The number of mucous acini and excretory ducts per $mm^2$ was examined in the sublingual salivary gland, while the number of mucous acini and granulated convoluted tubules per $mm^2$ was evaluated in the submandibular salivary gland (Folia et al., 2013). All analyzes were performed in a blinded fashion by two calibrated authors (G.K.S. and T.F.A.A.). A third author (T.A.S.) resolved any discrepancies.

### 2.3. Immunohistochemistry

Serial sections approximately 3 μm thick were obtained with a microtome, mounted on polarized slides (StarFrost®, Waldemar Knittel Glasbearbeitungs GmbH, Germany) and subjected to immunohistochemistry. Immunohistochemical analyzes were performed using monoclonal antibodies against anti-p63 (clone 4A4; dilution 1:50; Medaysis, Livermore, CA, USA) and anti-α-SMA (clone 1A4; dilution 1:100; Zeta Corporation, Sierra Madre, CA, USA). For antigen retrieval, the sections were heated in citrate buffer, pH 6.0, in an electrical pressure cooker. The reaction was amplified with the streptavidin-biotin

complex and diaminobenzidine was used as a chromogen for color development. The sections were counterstained with Mayer's hematoxylin and cover slipped. Negative controls were obtained by the omission of primary antibodies, which were substituted by 1% PBS–BSA. All counts were performed in 10 alternate microscopic high-power fields (×40) using an integration graticule (4740680000000-Netzmikrometer 12.5, Carl Zeiss, Göttingen, Germany). Results were expressed as number of positive cells per $mm^2$.

### 2.4. BPA quantification in salivary glands

The right sublingual and submandibular salivary glands from female mice were used to determine BPA concentration by gas chromatography coupled to ion trap mass spectrometry (Fernandez et al., 2017; Gomes et al., 2020). Analyte extraction and concentration were carried out using the hollow fiber liquid phase microextraction method (Gomes et al., 2020).

### 2.5. In vitro assays

#### 2.5.1. Cells

The cell lines UM-HMC-1 (University of Michigan-Human Mucoepidermoid Carcinoma-1) and UM-HMC-3A (University of Michigan-Human Mucoepidermoid Carcinoma-3A) were used (kindly provided by Professor Fabio D. Nunes from the University of São Paulo, Brazil). Resulting cell populations were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Waltham, MA, USA) supplemented with 1% L-Glutamine (Invitrogen), 1% antibiotic/antimycotic solution (Sigma-Aldrich), 10% fetal bovine serum (Invitrogen), 20 ng/mL epidermal growth factor (Sigma-Aldrich), 400 ng/mL hydrocortisone (Sigma-Aldrich), 5 μg insulin (Sigma-Aldrich), 50 ng/mL nystatin (Sigma-Aldrich), and 1% amphotericin B (Sigma-Aldrich). The antimicrobial regimen was changed to 5% penicillin/streptomycin (Invitrogen). Cells were incubated at 37 °C in a 95% humidified atmosphere containing 5% $CO_2$ until reaching confluence.

#### 2.5.2. Resazurin cell viability test

Cells were plated in quadruplicate in two independent biological replicates at a cell density of $4 \times 10^3$ cells/well in 96-well microplates and incubated overnight at 37 °C in a 95% humidified atmosphere containing 5% $CO_2$. Different BPA concentrations (1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, and 10 μM) were tested and compared with the vehicle group (0.05% DMSO). Cells were assessed after 48 h by adding 0.01 mg/mL resazurin (AlamarBlue, Thermo-Fisher Scientific Inc., Abingdon, UK) in DMEM (Gibco, Thermo-Fisher Scientific Inc.). The plates were kept protected from light and incubated at 37 °C for 4 h in a 95% humidified atmosphere containing 5% $CO_2$. Absorbance was evaluated using a Synergy 2 Multi-mode Microplate Reader fluorometer (BioTek Instruments; Winooski, VT, USA) with 530/25 nm excitation and 590/35 nm emission. A well containing medium without resazurin was employed as a reaction blank control.

#### 2.5.3. Immunocytochemistry

UM-HMC-1 and UM-HMC-3A cells were plated at a cell density of $4 \times 10^5$ per well on a glass coverslip adapted to 12-well microplates and incubated at 37 °C in a 95% humidified atmosphere containing 5% $CO_2$ for 18 h. Cells were treated with 50 nM BPA and vehicle (0.05% DMSO) for 48 h. Immunocytochemical analysis was carried out in triplicate using the streptavidin-biotin-peroxidase complex technique with anti-p63 (clone MC0221; 1:50, Medaysis, Livermore, CA, USA) and anti-Ki-67 (clone MIB-1, 1:400, Dako Cytomation, Glostrup, Denmark) primary antibodies. Quantification of positive cells was undertaken by image acquirement with a light microscope (O600R, Opticam, São Paulo, SP, Brazil) at ×20 magnification using integrated software (Opticam Microscopy Technology OPTHD) and counted in 10 consecutive fields with ImageJ software.

### 2.6. mRNA extraction from mouse tissue and cell lines and quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of estrogen receptors alpha and beta, p63 and α-SMA was quantified by qRT-PCR in a pool composed of the right sublingual and submandibular salivary glands from female mice, and in UM-HMC-1 and UM-HMC-3A cell lines, expression of estrogen receptors alpha and beta and p63 were evaluated. Total mRNA was extracted with TRIzol reagent (Thermo-Fisher Scientific Inc.) followed by column purification (RNeasy Mini Kit, Qiagen Inc., Redwood City, CA, USA). The quality and integrity of mRNA samples were verified by analyzing 1 μL of total mRNA on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Complementary DNA was synthesized from 2.5 μg RNA using the SuperScript VILO Master Mix reverse transcriptase enzyme (Thermo-Fisher Scientific Inc.). The Rotor-Gene Q system (Qiagen Inc.) was employed for the analysis, and the SYBR® Green PCR Master Mix kit (Thermo-Fisher Scientific Inc.) was applied. The thermocycling protocol consisted of an initial pre-incubation stage at 95 °C for 10 min to increase detection sensitivity, 40 amplification cycles starting at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s, followed by a single cooling stage at 40 °C for 10 s. The results are described as the relative level of gene expression calculated in reference to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control using the Ct method and the $2^{-\Delta\Delta Ct}$ formula. Details of the target genes examined are provided in Table 1.

### 2.7. Statistical analysis

The GraphPad Prism software version 8.0 (GraphPad software, San Diego, CA, USA) was used for statistical analysis. Non-parametric and parametric data were submitted, respectively, to the Kruskal-Wallis multiple comparison test followed by Mann-Whitney test or One-Way analysis of variance (ANOVA) followed by the Dunnett post-hoc test, as well as Two-Way ANOVA with Tukey's multiple comparisons test. For all analyses, the level of significance was set at $<0.05$.

**Table 1**
Gene primer sequences designed based on nucleotide sequences in the Ensemble and National Center for Biotechnology Information databases.

| Target gene | Forward (F) and reverse (R) primer sequences | Bp |
|---|---|---|
| Cells of animal origin | | |
| *Esr1* (ERα) | F: TTGAACCAGCAGGGTGGC<br>R: AGGCTTTGGTGTGAAGGGTC | 128 |
| *Ers2* (ERβ) | F: CTGTGATGAACTACAGTGTTCCC<br>R: CACATTTGGGCTTGCAGTCTG | 80 |
| *p63α* | F: ACCCTTACATCCAGCGTTTCAT<br>R: AAGACCTCTGGGCTGAGGAA | 118 |
| *Smaα* | F: CCAGCCATCTTTCATTGGGAT<br>R: ACAGGACGTTGTTAGCATAGAGA | 113 |
| *Gapdh* | F: AGGTCGGTGTGAACGGATTTG<br>R: TGTAGACCATGTAGTTGAGGTCA | 159 |
| Cells of human origin | | |
| *ESR1* (ERα) | F: ATGAGAGCTGCCAACCTTTG<br>R: GGTTGGTCAGTAAGCCCATC | 190 |
| *ESR2* (ERβ) | F: TGGAGTCTGGTCGTGTGAAG<br>R: ACTTCACCATTCCCACTTCG | 167 |
| *P63α* | F: ACGGTGATGGTACGAAGCG<br>R: ACGGCCCCTCACTGGTAA | 116 |
| *GAPDH* | F: GAAGGTGAAGGTCGGAGTCAAC<br>R: CAGAGTTAAAAGCAGCCCTGGT | 71 |

**Note:** *Ers2*, *Mus musculus* estrogen receptor 2 beta (NM_207707.1); ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; *ESR1*, *Homo sapiens* estrogen receptor 1 (NM_001122742.1); *Esr1*, *Mus musculus* estrogen receptor 1 alpha (NM_00132531.1); *ESR2*, *Homo sapiens* estrogen receptor 2 (NM_001291723); *GAPDH*, *Homo sapiens* glyceraldehtde-3-phosphate dehydrogenase (NM_001289745.2); Gapdh, *Mus musculus* glyceraldehtde-3-phosphate dehydrogenase (NM_008084.3); *P63*, *Homo sapiens* transformation related protein 63 (NM_003722); *p63*, *Mus musculus* transformation related protein 63 (NM_001127259.1); *Smaα*, *Mus musculus* actin alpha 2 (NM_007392.3).

## 3. Results

### 3.1. Exposure to BPA resulted in its accumulation in the salivary glands and disruption of tissue morphology

Sub-chronic exposure to BPA (30 mg/kg/day) resulted in architectural changes in the female submandibular and sublingual glands. There was a significant reduction in the number of acini and granulated convoluted tubules in the submandibular glands, as well as a decrease in the number of acini and excretory ducts in the sublingual glands ($p < 0.05$) compared to vehicle ($p > 0.05$) (Fig. 1A and B). No morphological changes were observed in the sublingual or

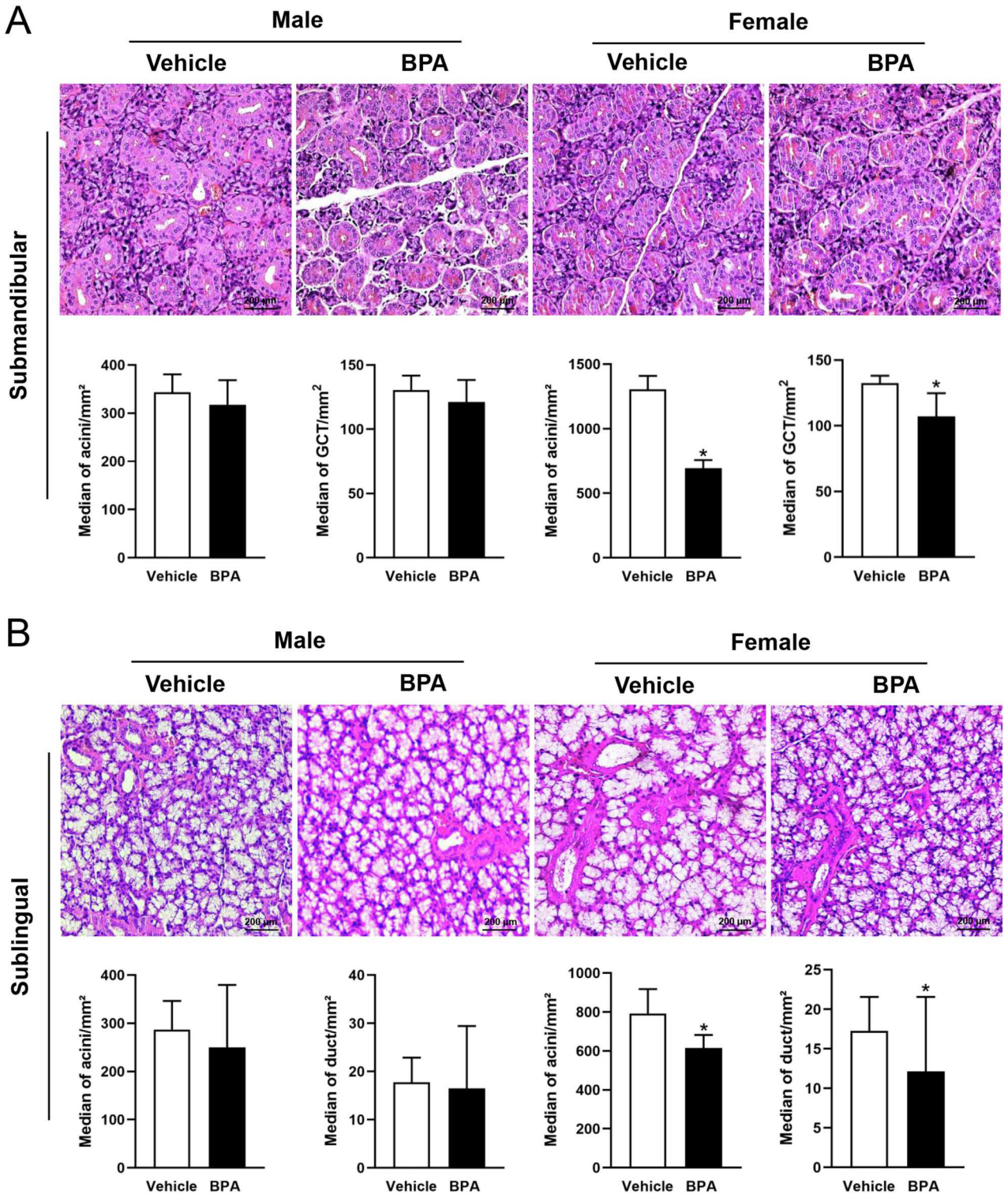


**Fig. 1.** Effects of exposure to bisphenol A (BPA) on the salivary glands of male and female mice. Representative hematoxylin and eosin-stained sections revealing the morphology and tissue architecture of **(A)** submandibular and **(B)** sublingual glands of male and female mice. Histomorphometry of acini, granulated convoluted tubes (GCT) and ducts of salivary glands of animals that received 0.05% dimethyl sulfoxide-$d_6$ (DMSO; vehicle) or BPA (30 mg/kg/day). Note: data ($n = 10$ per group) are shown as median with range. The Mann-Whitney test was used to determine statistical difference between groups and (*) indicates $p < 0.05$.

submandibular salivary glands of males exposed to BPA. Also, there were no changes in salivary gland weight between groups (data not shown).

Considering BPA-induced changes in salivary gland morphology, we then evaluated p63 and α-SMA expression to identify possible effects on the myoepithelial cell population. Although a significant increase in p63 positivity was observed in the salivary gland of male and female mice that received BPA ($p < 0.05$) (Fig. 2A), no change in α-SMA positivity was verified comparing the groups (Supplementary Fig. 1A). Consistently, an increase in p63 ($p < 0.001$) (Fig. 2B) and no change in α-SMA (Supplementary Fig. 1B) mRNA expression was found in female mice salivary glands.

Next, we examined BPA concentration in a pool of the sublingual and submandibular salivary glands of female mice. Further analyses involved only samples from female mice based on the fact that exposure to BPA revealed significant histopathological changes in females compared to males. Gas chromatography revealed a significant accumulation of BPA products in animals exposed to this chemical compared to animals that ingested only 0.05% DMSO (vehicle) ($p = 0.01$) (Fig. 3A).

### 3.2. Up-regulation of estrogen receptors in salivary glands after BPA exposure

Consistently with BPA-induced changes in salivary glands, enhanced levels of estrogen receptor mRNA for alpha ($p = 0.02$) and beta ($p = 0.01$) were observed in the salivary glands of female mice exposed to BPA compared to the vehicle group (Fig. 3B and C).

### 3.3. Estrogen deficiency modified the salivary gland response to BPA

Based on the estrogenic activity of BPA (Julien et al., 2018; Okuda et al., 2010), we hypothesized that reduction of estrogen availability by ovariectomy would boost the deleterious effects of BPA. Ovariectomized mice exposed to BPA showed similar number of acini of the submandibular glands compared to the respective vehicle ($p > 0.05$). In contrast, among the animals in the sham group, there was a decrease in the number of acini in the mice treated with BPA ($p = 0.03$). In addition, ovariectomy significantly contributed to decreasing the number of acini of the submandibular glands in the vehicle (sham vehicle *versus* OVX vehicle) ($p = 0.02$) but not in the BPA-treated group (sham BPA *versus* OVX BPA) ($p > 0.05$) (Fig. 4A). Regarding the values of granulated convoluted tubules in the group of ovariectomized animals, there was an increase in the animals exposed to BPA compared to the vehicle group ($p = 0.02$). In the sham group, the animals exposed to BPA had lower values of granulated convoluted tubules compared to the vehicle group ($p = 0.02$). When comparing the animals exposed to BPA, ovariectomized mice showed higher values granulated convoluted tubules values than the sham group ($p<0.001$) (Fig. 4B). Hence, ovariectomy induced a significant increase in the number of granulated convoluted tubules of submandibular glands in the BPA-treated group (sham BPA *versus* OVX BPA) ($p<0.001$) but not in vehicle group (sham vehicle *versus* OVX vehicle) ($p > 0.05$).

Females exhibited similar body weights after the 7-week period following ovariectomy and BPA or vehicle treatment (Fig. 4C). The uterine horns of ovariectomized mice showed marked atrophy in the DMSO and BPA groups compared to sham mice ($p < 0.05$). No difference was observed when comparing BPA *versus* vehicle in both sham or ovariectomized groups ($p > 0.05$) (Fig. 4D).

### 3.4. BPA did not affect the proliferation of neoplastic cells, but augmented the expression of p63 and estrogen receptors in vitro

In order to summarize the histopathology of malignant salivary gland tumors, we examined a pair of mucoepidermoid carcinoma cell lines, UM-HMC-1 and UM-HMC-3A (Warner et al., 2013). Primarily, we determined by the resazurin assay whether BPA at different concentrations (1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, and 10 μM) induced cytotoxicity. There was a significant reduction of cell viability only at the dose of 1 μM when compared to the vehicle in both cell lines, UM-HMC-3A ($p = 0.032$) and UM-HMC-1 ($p < 0.0001$). Although not significant, a decrease in cell viability was observed from 100 nM. Based on these results, the concentration of 50 nM was used for further assays (Fig. 5A and B).

We next evaluated the expression of Ki-67 and p63 by immunocytochemistry. No change in cell proliferation index measured by Ki-67

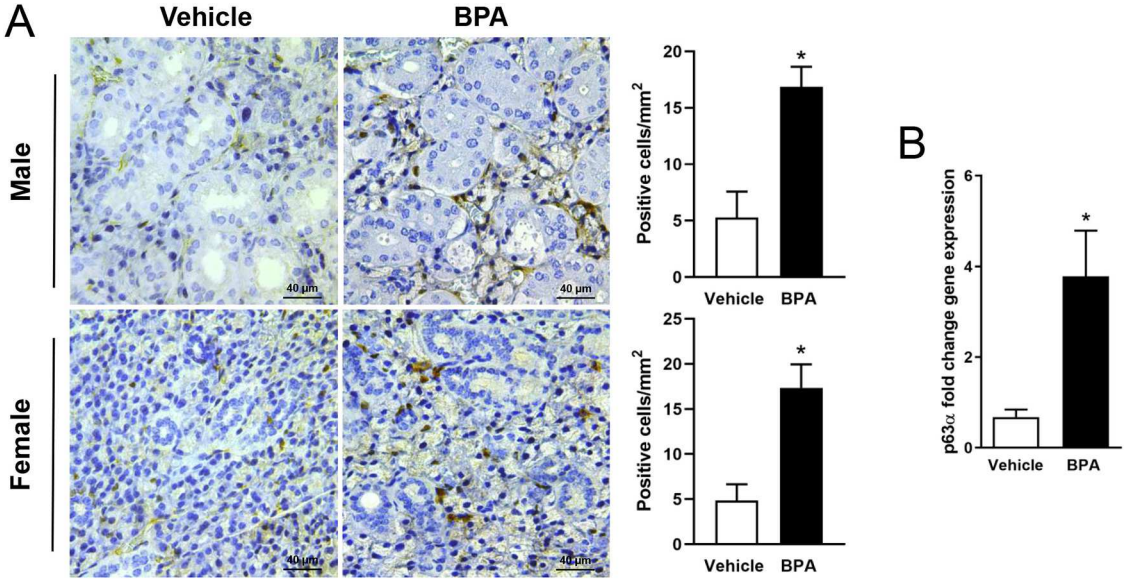


**Fig. 2.** Expression of p63 in the salivary gland of mice. **(A)** Representative immunohistochemical images of p63-stained sections of salivary glands of male and female mice that received 0.05% dimethyl sulfoxide-$d_6$ (DMSO; vehicle) or bisphenol A (BPA; 30 mg/kg/day). The number of positive cells per mm$^2$ is shown in bar graphs. **(B)** Up-regulation of p63 release by BPA in submandibular salivary glands of female mice. The p63 mRNA fold change in expression was determined in the submandibular salivary gland of mice exposed to BPA and 0.05% DMSO. Note: data ($n = 10$ and $n = 5$ per group, respectively) are shown as median with range. The Mann-Whitney test was used to determine statistical difference between groups and (*) indicates $p < 0.05$.

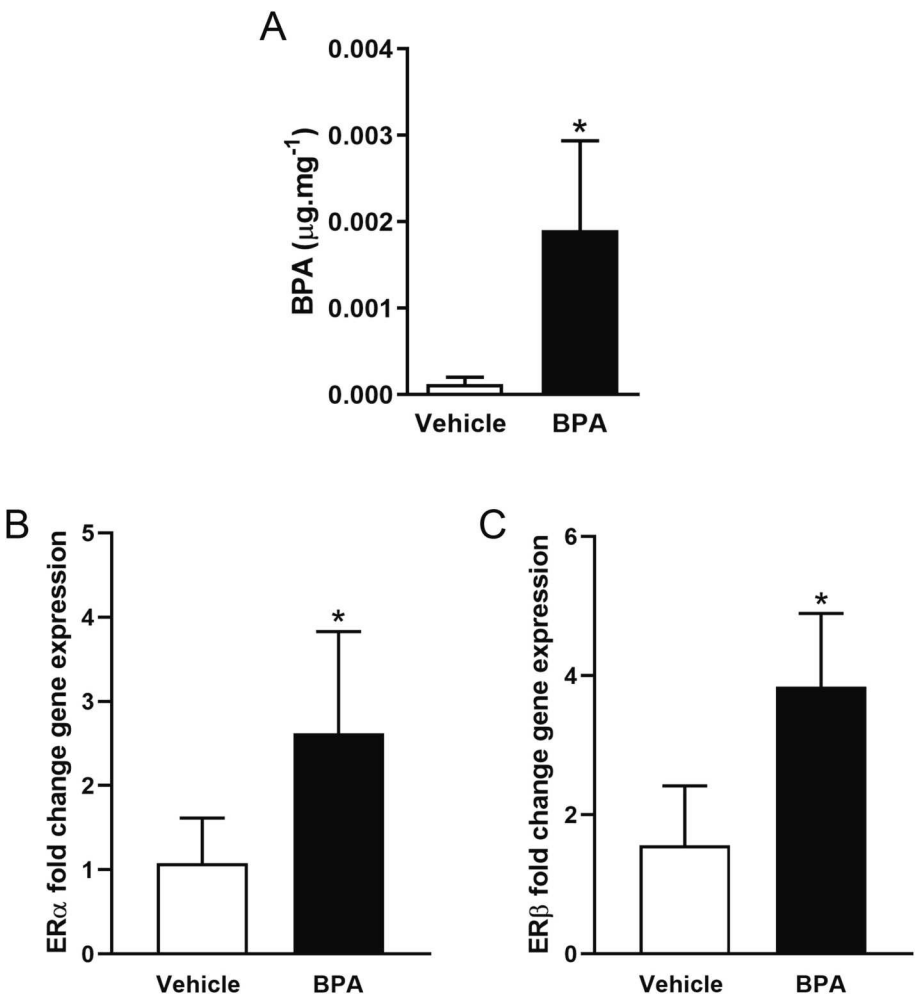


**Fig. 3. (A)** Bisphenol A (BPA) accumulation in submandibular and sublingual salivary gland tissues of female mice exposed to BPA in drinking water measured by gas chromatography coupled to ion trap mass spectrometry (GC/MS). Up-regulation of estrogen receptor (ER) alpha (α) and beta (β) release by BPA in submandibular salivary glands of female mice. The **(B)** ERα and **(C)** ERβ mRNA fold change in expression was determined in the submandibular salivary gland of mice exposed to BPA (30 mg/kg/day) and 0.05% dimethyl sulfoxide-$d_6$ (DMSO, vehicle). Note: data ($n = 5$ per group) are shown as median with range. The Mann-Whitney test was used to determine statistical difference between groups and (*) indicates $p < 0.05$.

percentage was observed for UM-HMC-1 and UM-HMC-3A cell lines compared to vehicle and BPA (data not shown). On the other hand, there was an increase in p63 expression in UM-HMC-1 and UM-HMC-3A cell lines treated with BPA compared to the vehicle ($p < 0.05$ and $p = 0.03$, respectively) (Fig. 5C and D). Analysis of p63 mRNA confirmed the immunocytochemistry findings, particularly by the significant expression in UM-HMC-1 cells treated with BPA ($p = 0.02$) (Fig. 5E). Additionally, we analyzed the expression of estrogen receptors alpha and beta in UM-HMC-1 and UM-HMC-3A cell lines treated with 50 nM BPA and 0.05% DMSO (vehicle) (Fig. 5F and G). An increased expression of estrogen receptor alpha was only observed in UM-HMC-3A treated with BPA compared to the respective vehicle group ($p < 0.001$) (Figs. 5F).

## 4. Discussion

Herein, we demonstrate that exposure to BPA resulted in its significant accumulation in the salivary glands, an effect accompanied by architectural changes in the submandibular and sublingual glands, particularly in female mice, and increased estrogen receptors expression. Nevertheless, the reduction in estrogen availability, *i.e.*, in the ovariectomy model, slightly impacted BPA-induced morphological changes, without additional modifications in the acini, but with an increase in the number of granulated convoluted tubules in the submandibular glands.

Rodents show remarkable molecular and functional sexual dimorphism of the salivary glands (Mukaibo et al., 2019), which reflects the effect of steroid hormones on the morphogenesis and secretory processes of these organs (Yamamoto et al., 2018). The findings of the present study revealed that females are more susceptible to salivary gland disruption induced by sub-chronic exposure to BPA than male mice. However, oral exposure to BPA (25 mg/kg, three times a week for 60 days) caused degeneration of acinar cells and duct systems in the submandibular glands of adult male rats (Bashir et al., 2022). In another study, 60-day-old male rats exposed to BPA (130 mg/kg/day for 28 days) exhibited cytopathological changes and cellular apoptosis in the salivary glands, in addition to a significant increase in tissue oxidative stress parameters (Çetin et al., 2021). Selim et al. (2022) reported that 8-week-old male rats exposed to BPA (10 mg/kg/day for 45 days) also exhibited a variable degree of degenerative changes in the submandibular salivary glands. This was accompanied by a significant increase in oxidative stress and inflammatory cytokines, along with activation of the Wnt/β-catenin pathway. According to a previous study conducted on adult male rats, BPA induced significant microscopic changes in the acinar structures of the submandibular glands in a dose-dependent manner, while few effects were observed on morphometry in the granulated convoluted tubules, even at high doses of BPA, *i.e.*, a daily dose of 12.5 mg/kg/day for 6 weeks (Folia et al., 2013). Despite the effects of BPA on salivary gland morphology, we expected changes in myoepithelial cell markers such as α-SMA, but no differences were observed between groups. In contrast, exposure to BPA has been shown to increase levels of several mesenchymal markers, including α-SMA in neoplastic breast cancer cells (Ansari et al., 2022).

Potential mechanisms that mediate the effects of BPA on the salivary glands comprise its pro-oxidative properties (Çetin et al., 2021; Selim et al., 2022), the activation of inflammatory pathways (Selim et al., 2022), and its role as an endocrine-disrupting chemical (*e.g.*, *via*

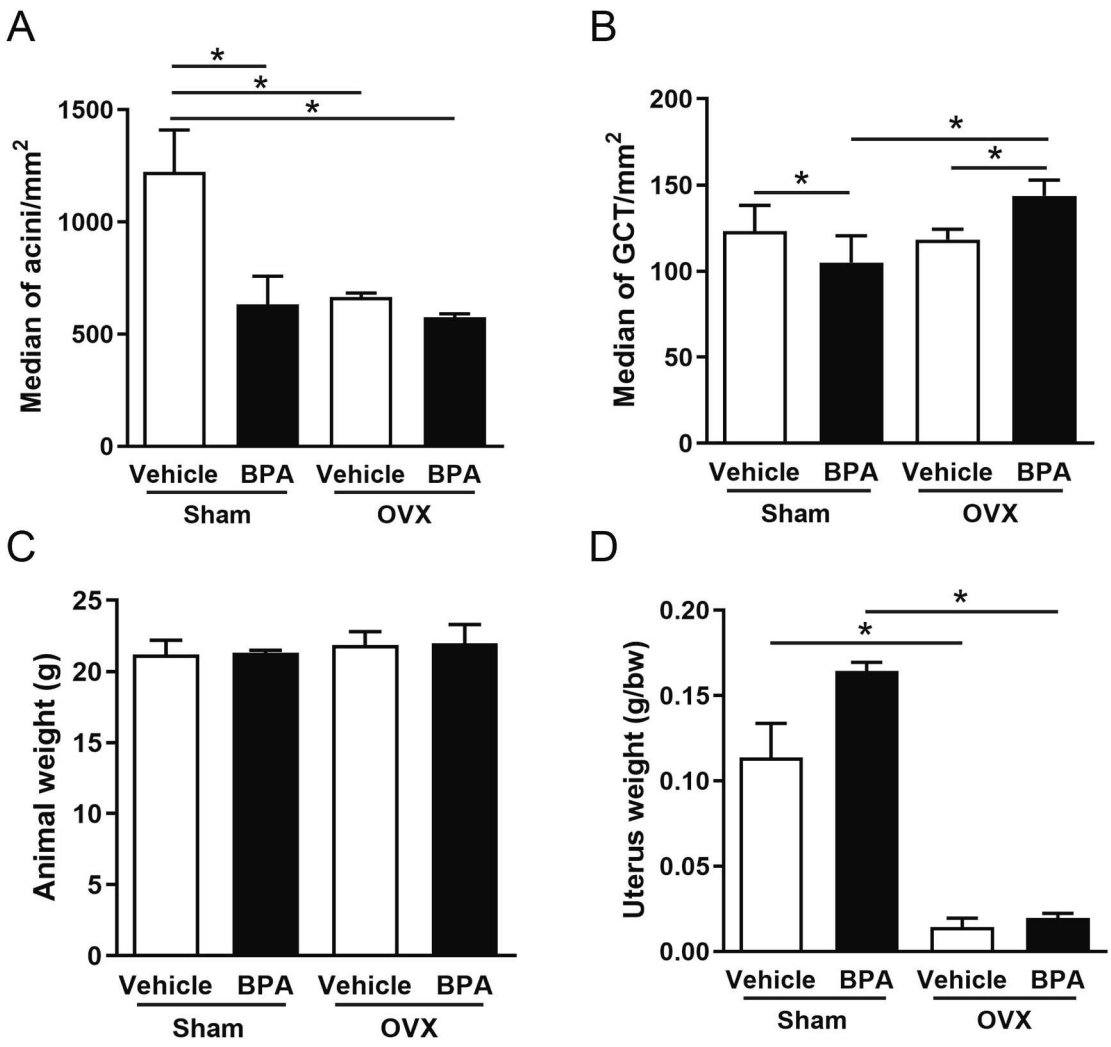


**Fig. 4.** Effects of ovariectomy-induced estrogen deficiency on the submandibular salivary glands of mice exposed to BPA (30 mg/kg/day) and 0.05% dimethyl sulfoxide-$d_6$ (DMSO, vehicle). Histomorphometry of **(A)** acini and **(B)** granulated convoluted tubes (GCT) of the glands. **(C)** Weight of animals and **(D)** weight of the uterus representing the efficacy of ovariectomy. Note: data ($n = 5$ per group) are shown as median with range. Two-Way ANOVA with Tukey's multiple comparisons test was used to determine statistical difference between groups and (*) indicates $p < 0.05$.

estrogen receptor signaling) (Vandenberg et al., 2019; Vom Saal and Vandenberg, 2021). Our data suggests that, at least in part, the BPA-induced changes in the salivary glands encompass estrogen receptors, which are expressed under steady-state conditions (Tsinti et al., 2009) and up-regulated by the exposure to BPA. Consistently, the ability of BPA to interfere with and/or prevent the binding of natural hormones to their receptors – even at low concentrations – may be linked to the deleterious effects of this chemical on salivary gland homeostasis (Emfietzoglou et al., 2019; Folia et al., 2013). In salivary gland tumors such as mucoepidermoid carcinoma and acinic cell carcinoma, estrogen receptor beta expression was detected at the nuclear and cytoplasmic levels, but there was no association with disease-free survival or overall survival (Aquino et al., 2018). On the other hand, in inflammatory conditions that affect the salivary glands such as Sjögren syndrome, the actions of BPA *via* estrogen receptors caused alterations in the morphology of monocyte-derived dendritic cells and in the immune-inflammatory events implicated in disease progression and deterioration (Wang et al., 2020). Furthermore, the female predominance of Sjögren syndrome and certain forms of salivary gland cancer may reflect sex-based differences since comparative analysis of salivary gland epithelial cells from patients with Sjögren syndrome demonstrated a significantly reduced responsiveness to 17β-estradiol (Manoussakis et al., 2012). Our findings, however, demonstrate that female salivary glands seem to be more susceptible to BPA. This suggests a role for sexual dimorphism in the salivary gland response to endocrine disruptors, a postulate that should be further explored in human and animal studies.

In the current study, we examined the actions of BPA on a pair of mucoepidermoid carcinoma cell lines and observed that, notably, this chemical triggered estrogen receptor alpha expression in UM-HMC-3A but not in UM-HMC-1. This corroborates the fact that multifocal mammary gland carcinoma of female rodents due to BPA exposure tends to be estrogen receptor alpha-positive (Ruiz et al., 2021). Earlier data have revealed that BPA does not induce apoptosis in human submandibular cell lines, but reduces the number of viable cells in a concentration-dependent manner (Terasaka et al., 2005). On the other hand, it has been suggested that BPA could act as a disruptor of inflammatory activity in a murine macrophage cell line, regulating the NF-κB/MAPK (mitogen-activated protein kinase) pathways and NLRP3 (NOD-like receptor protein 3) inflammasome activation (Lee et al., 2020). However, the significance of BPA-induced activation/inactivation of signaling pathways deserves further investigation, particularly concerning the differential effects of BPA in promoting estrogen receptor expression in the analyzed cell lines. Alternatively, oral squamous carcinoma cells stimulated with BPA showed increased phosphorylation of Akt, ERK, and NF-κB proliferation (Almeida et al., 2021).

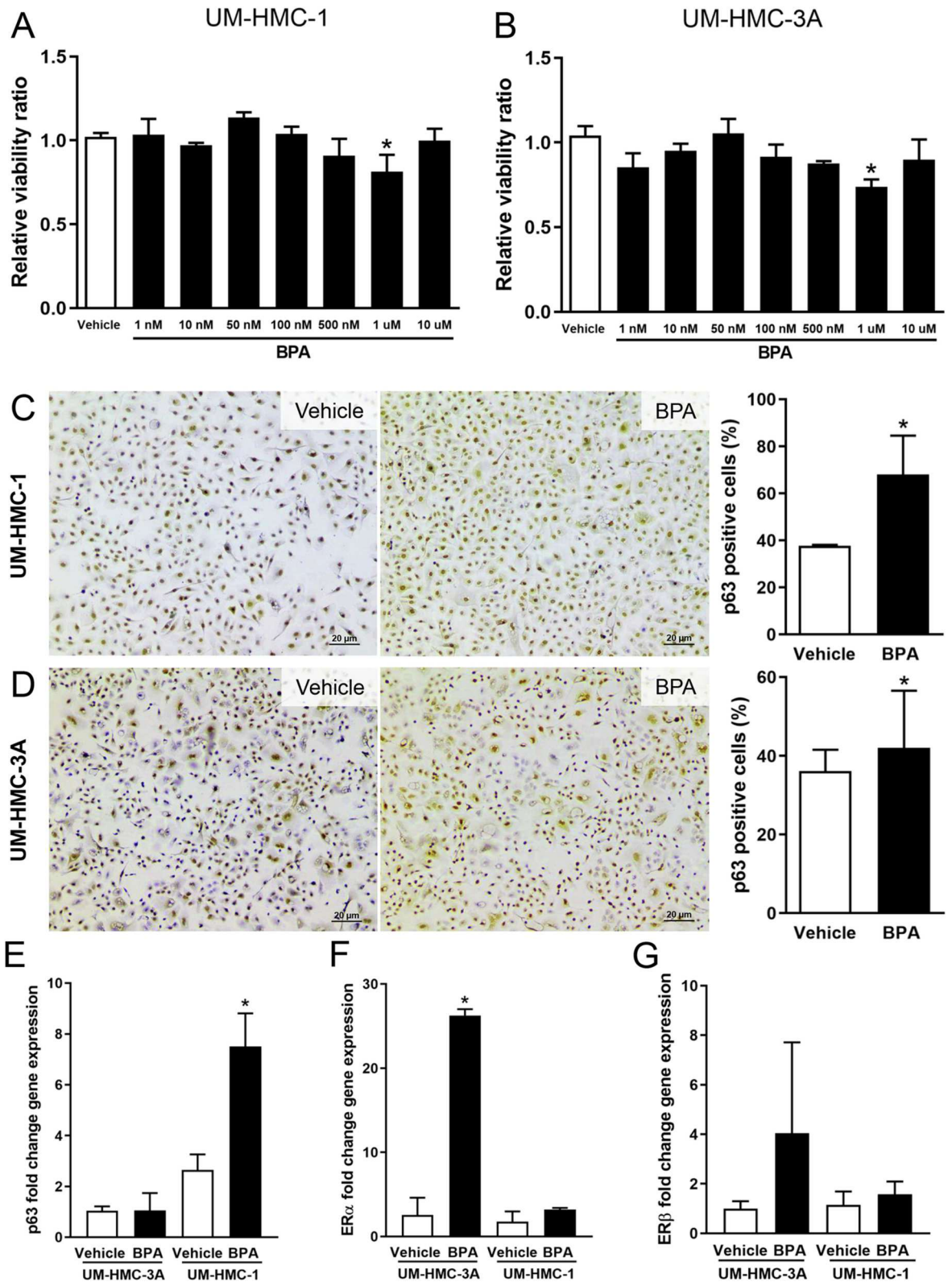


**Fig. 5.** Relative cell viability determined by the resazurin assay in the cell lines **(A)** UM-HMC-1 (University of Michigan-Human Mucoepidermoid Carcinoma-1) and **(B)** UM-HMC-3A (University of Michigan-Human Mucoepidermoid Carcinoma-3A). Representative immunocytochemical images of p63 staining in **(C)** UM-HMC-1 and **(D)** UM-HMC-3A in the respective groups, *i.e.*, 0.05% dimethyl sulfoxide-$d_6$ (DMSO, vehicle) and 50 nM bisphenol A (BPA). The relative ratio of p63 positive cells in vehicle and BPA in UM-HMC-1 and UM-HMC-3A is shown in bar graphs. Effects of **(E)** p63, estrogen receptor (ER) **(F)** alpha (α) and **(G)** beta (β) released by 50 nM BPA or 0.05% DMSO (vehicle) in UM-HMC-3A and UM-HMC-1 cell lines. Note: for relative cell viability, data ($n = 3$ in two independent experiments) are shown as mean ± standard deviation. One-Way analysis of variance (ANOVA) followed by the Dunnett post-hoc test was used to determine statistical significance among the groups and (*) indicates $p < 0.05$ in relation to vehicle. For immunocytochemical analysis and gene expression, data ($n = 2$ in two independent experiments) are shown as median with range. The Kruskal-Wallis test followed by Mann-Whitney test was used to determine statistical difference between groups and (*) indicates $p < 0.05$.

BPA also triggered p63 expression in salivary glands of mice, mainly female, that received BPA and in both neoplastic cell lines, UM-HMC-3A and UM-HMC-1, assessed in this study. The p63 gene is a member of the P53 family that plays an important role in stem cell identity and cellular differentiation, being a marker of myoepithelial differentiation in both benign and malignant salivary gland neoplasms (Sivakumar et al., 2022). p63 also inhibits cell apoptosis and functions as an oncogene in different oral tumors (Chen et al., 2018). For instance, its expression is significantly correlated with histological grade in mucoepidermoid carcinoma of salivary glands (Taghavi et al., 2020). In the mammary glands of female rodents, prenatal exposure to BPA increased the number of potentially malignant disorders, accompanied by hyperplastic ducts and p63-labeled myoepithelial cells (Durando et al., 2007). Likewise, developmental exposure to xenoestrogens disturbs the normal expression pattern of p63 and proper uterine cytodifferentiation, an effect related to glandular squamous metaplasia (Vigezzi et al., 2015).

The oral cavity is probably the main route of BPA contamination (Gayrard et al., 2013; Guignard et al., 2016; Teeguarden et al., 2015), which is consistent with a high concentration of this chemical detected in the oropharyngeal space, oral mucosa, and saliva (Almeida et al., 2021; Emfietzoglou et al., 2019; Gomes et al., 2020). Additionally, some dental restorative materials produce a significant release of BPA in saliva (Gomes et al., 2020; Lopes-Rocha et al., 2022; Tou et al., 2023). Recent literature has demonstrated that sub-chronic exposure to BPA disrupts oral keratinocyte homeostasis by altering proliferative activity and pro-survival and inflammatory pathways (Almeida et al., 2021). While for humans it is difficult to estimate exposure to BPA, divergent results and interpretations of data on this chemical in toxicology studies have been described in animal models, in which a wide range of doses is employed. For example, the Consortium Linking Academic and Regulatory Insights on Bisphenol A Toxicity (CLARITY-BPA) has tested dose levels from 2.5 to 25,000 μg/kg/B.W./day (Heindel et al., 2020; Leung et al., 2020), and others have tested even higher doses of this chemical (Camacho et al., 2015). It is worth mentioning that we adopted as a reference a former study that showed BPA-induced changes in the oral tissues of rats (Folia et al., 2013). The cited study tested daily doses from 5 μg/kg to 12,500 μg/kg of body weight and, at these doses, BPA had no effect on the oral epithelium (Folia et al., 2013). Hence, we carried out a pilot experiment (Almeida et al., 2021) with doses of 15 and 30 mg/kg and since no changes were observed at the dose of 15 mg/kg, the dose of 30 mg/kg was employed in the current study.

The present study has shortcomings that should be acknowledged. The results support short-term exposure to this chemical in both animal and *in vitro* models. It is not possible to define a parallel between the doses employed here and those that could be considered "safe" for humans (Vandenberg et al., 2019). Finally, due to the considerable heterogeneity in the parenchyma and stroma of normal salivary glands and derived neoplasms, it is not possible to attribute the BPA response to a particular cell component.

## 5. Conclusions

In summary, the present findings demonstrate that BPA accumulates in salivary glands of mice and causes morphological changes, particularly in females, and increase of p63 expression. Despite the increase in estrogen receptors seen in these tissues, the ovariectomy-induced reduction of estrogen levels slightly impacted the effects of BPA on salivary glands. In neoplastic cells from mucoepidermoid carcinoma, BPA increased estrogen receptors and p63 expression. These findings suggest an additional concern about the harmful effects of this chemical on salivary tissues, and potential long-term repercussions. Future studies on specific mechanisms of BPA-induced inflammation and signaling pathways should be performed with a focus on reducing and preventing damage caused by BPA.

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## CRediT authorship contribution statement

**Gabriela Kelly da Silva**: Formal analysis, Writing - original draft, Writing - review & editing. **José Alcides Almeida de Arruda**: Formal analysis, Writing - original draft, Writing - review & editing. **Tatiana Fernandes Araújo Almeida**: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing - review & editing. **Sicília Rezende Oliveira**: Formal analysis, Writing - original draft, Writing - review & editing. **Paula Alves da Silva Rocha**: Formal analysis, Software, Writing - review & editing. **Ricardo Alves Mesquita**: Formal analysis, Writing - original draft, Writing - review & editing. **Zenilda de Lourdes Cardeal**: Formal analysis, Software, Writing - review & editing. **Helvécio Costa Menezes**: Formal analysis, Software, Writing - review & editing. **Ivana Márcia Alves Diniz**: Formal analysis, Writing - original draft, Writing - review & editing. **Soraia Macari**: Formal analysis, Software, Writing - review & editing. **Andréia Machado Leopoldino**: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing - review & editing. **Tarcília Aparecida Silva**: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing - review & editing.

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## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

## Data availability

Data will be made available on request.

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