Upregulation of Clusterin in Prostate and DNA Damage in Spermatozoa from Bisphenol A–Treated Rats and Formation of DNA Adducts in Cultured Human Prostatic Cells

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Among endocrine disruptors, the xenoestrogen bisphenol A (BPA) deserves particular attention due to widespread human exposure. Besides hormonal effects, BPA has been suspected to be involved in breast and prostate carcinogenesis, which share similar estrogen-related mechanisms. We previously demonstrated that administration of BPA to female mice results in the formation of DNA adducts and proteome alterations in the mammary tissue. Here, we evaluated the ability of BPA, given with drinking water, to induce a variety of biomarker alterations in male Sprague-Dawley rats. In addition, we investigated the formation of DNA adducts in human prostate cell lines. In BPA-treated rats, no DNA damage occurred in surrogate cells including peripheral blood lymphocytes and bone marrow erythrocytes, where no increase of single-strand DNA breaks was detectable by comet assay and the frequency of micronucleated cells was unaffected by BPA. Liver cells were positive at transferase dUTP nick end labeling assay, which detects both single-strand and double-strand breaks and early stage apoptosis. BPA upregulated clusterin expression in atrophic prostate cells. On the whole, these experimental findings support mechanistically the hypothesis that BPA may play a role in prostate carcinogenesis and may, potentially, affect the quality of sperm.

Key Words: bisphenol A; DNA adducts; clusterin; Sprague-Dawley rats; prostate; spermatozoa.

The term endocrine disruptors (EDs) was coined 20 years ago to identify a large number of structurally diverse chemicals sharing the ability to disrupt the endocrine system of both humans and wild animals (Colborn et al., 1993; Wingspread Consensus Statement, 1992). Among them, estrogen-mimicking compounds or xenoestrogens are of particular concern because estrogens play important roles not only in the regulation of the physiological homeostasis but also because they are involved in the pathogenesis of various diseases including cancers of the genital system (Soto and Sonnenschein, 2010). Bisphenol A (BPA), 2,2-bis-4-hydroxyphenyl propane, is a prototype of xenoestrogenic ED. Due to its high production volume and widespread human exposure, BPA has received outstanding attention and has raised concern in the public opinion (Vandenberg et al., 2009). BPA is a monomer in the manufacture of epoxy resins, polycarbonate plastics, and flame retardants and is used as a dental sealant and for coating water pipe walls, food packaging, and plastic bottles (Staples et al., 1998). It is also a contaminant of food and water, which are the major sources of human exposure (Staples et al., 1998; Vandenberg et al., 2009).

BPA has been investigated for genotoxicity in a variety of test systems, both in vitro and in vivo, but the results are controversial (Izzotti et al., 2009; Vandenberg et al., 2009). It has been shown to bind DNA in acellular systems, in which this ED reacts with DNA after metabolic activation (Atkinson and Roy, 1995a; Edmonds et al., 2004; Izzotti et al., 2009); in cultured mammalian cells (Tsutsui et al., 1998); and in the liver of both rats (Atkinson and Roy, 1995b) and mice (Izzotti et al., 2009) treated in vivo. Moreover, we recently demonstrated that administration of BPA with drinking water results in the formation of DNA adducts and proteome alterations in the mammary tissue of mice (Izzotti et al., 2009, 2010). These results deserve attention also because BPA accumulates in the mammary cells of rodents after oral intake (Yoo et al., 2001). Interestingly, exposure to estrogens throughout life is a major risk factor for breast cancer (Pike et al., 1993), and the chemical structure of BPA resembles that of diethylstilbestrol (DES), a well known carcinogen in humans (International Agency for Research on Cancer, 1979). BPA can induce mammary carcinomas in prenatally exposed rats, and the early
exposure to this ED sensitizes the mammary gland to carcinogenic insults experienced later in life (Keri et al., 2007; Vandenberg et al., 2009).

Intriguingly, estrogens play a physiological role also during prostate development, growth, and differentiation (Carruba, 2007). Aromatization of androgens to estrogens may be involved in prostate carcinogenesis (Bosland, 2000; Carruba, 2007; Ellem et al., 2004), and depurinating estrogen-DNA adducts could serve as potential biomarkers to predict the risk of prostate cancer (Yang et al., 2009). Thus, estrogens might be involved in the etiology of prostate cancer and breast cancer with similar mechanisms (Yang et al., 2009). These types of cancers share many similarities, such as risk factors, biomolecular determinants, geographical distribution, and natural history (Carruba, 2007; Cavaliere and Rogan, 2006).

These premises prompted us to evaluate a variety of biomarkers in cells of male rats receiving BPA with drinking water. The investigated biomarkers included histological, immunohistochemical, and Western blot analyses of prostates for the detection of clusterin (CLU), whose gene has been cloned and identified as one of the most highly induced genes during the involution of the rat prostate gland following either surgical castration (Bettuzzi et al., 1989) or treatment with the anti-androgen finasteride (Astancolle et al., 2000). Besides many growth factors and hormones, CLU expression is under the control of estrogens (Filippi et al., 2002). In addition, we analyzed spermatozoa for the levels of reactive oxygen species (ROS) and of malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBARS) and for the assessment of DNA fragmentation by using the sperm chromatin dispersion (SCD) test (Fernández et al., 2003). Liver cells were analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Cyto genetic damage was evaluated in bone marrow erythrocytes, and single-strand DNA breaks (SSBs) were measured in peripheral blood lymphocytes. In addition, we evaluated in vitro the ability of BPA to form DNA adducts in two human prostatic cell lines. PNT1a cells are nontumorigenic epithelial cells, immortalized through stable transfection with the SV-40 large T antigen (Berthon et al., 1997), whereas PC3 cells are androgen-independent prostate cancer cells originated from bone metastasis of prostatic carcinoma (Kaighn et al., 1979).

MATERIALS AND METHODS

Rats. Sixteen male adult Sprague-Dawley rats, weighing on an average 250 g, were purchased from Harlan Italy (San Pietro al Natisone, Udine, Italy). The rats were housed in Makrolon cages on sawdust bedding and maintained on standard rodent chow (Teklad 2018, Harlan Italy) and tap water ad libitum. The animal room had a temperature of 23 ± 2 °C, a relative humidity of 55%, and a 12-h day/night cycle. Housing and treatment of rats were in accordance with our national and institutional guidelines.

Treatment of rats. After 10 days of acclimatization, eight rats were treated with BPA (Sigma Chemical Co., St Louis, MO), dissolved in absolute ethanol (25 mg/ml, wt/v) and then diluted in tap water to yield a calculated daily intake of 200 mg BPA/kg body weight. This dilution corresponded to a concentration of ethanol in drinking water of 4% (vol/vol). BPA was administered daily via drinking water for 10 consecutive days. Eight control rats received 4% ethanol in drinking water for the same period of time. At the end of the treatment, the body weights of control rats were 282.0 ± 16.0 g versus 274 ± 20.1 of BPA-treated rats.

Sacrific of rats and collection of biological samples. After overnight starving, all rats were anesthetized with diethyl ether and killed by cervical dislocation. Blood samples were collected from the lateral tail vein for isolating lymphocytes to be analyzed by comet assay. The livers were collected, and fragments were fixed in formalin and embedded in paraffin for TUNEL analyses. Immediately after surgical excision, prostates were weighed and divided into two parts. One was fixed in formalin for histological and immunohistochemical analyses, whereas the other was frozen at −80°C for protein extraction and Western blot analyses. The left femurs were removed, and bone marrow smears were prepared by means of a paintbrush for cytogenetical analyses. The testes were removed, and mature spermatozoa were collected from epididymal heads and used for evaluating DNA fragmentation and concentrations of ROS and TBARS.

Histological, immunohistochemical, and Western blot analyses of prostates. Five micrometer sections of paraffin-embedded prostates were stained with hematoxylin and eosin for evaluating prostate histology. For immunohistochemical analyses, formalin-fixed prostate fragments were transferred to 70% ethanol and then embedded in paraffin. Sections (5 μm) were cut and mounted onto slides, which were hydrated with xylene and graded alcohol and equilibrated in phosphate-buffered saline (PBS). Antigen retrieval was performed with 10 mM sodium citrate, pH 6.0, using a microwave for 3 × 5 min at 700 W. Endogenous peroxidase was quenched with 3% H2O2 in H2O. A specific binding was blocked with swine serum diluted 1:10 in 1% bovine serum albumin (BSA) in PBS. Immunostaining was performed using monoclonal antibodies anti-rat CLU (Millipore Corporate, Billerica, MA), diluted 1:50 in 1% BSA in PBS and incubated for 1 h at room temperature. From the secondary antibody to the chromogen reaction, the Universal LSAB+System-HRP kit (DakoCytomation, Milan, Italy) was used. Negative controls were prepared by excluding the primary monoclonal antibodies from the reaction. Counterstaining was performed with hematoxylin, and cover slips were mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

For protein extraction and Western blot analyses, lysates from frozen mouse prostates were homogenized in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% Na deoxycholate, 1% NP-40, 50 μg/ml DNase, and 50 μg/ml RNase), supplemented with Complete protease inhibitor according to manufacturer’s instruction (Roche Diagnostics Corporation, Milan, Italy), and heated at 100°C in SDS–polyacrylamide gel electrophoresis loading buffer. The equivalent of 50 μg of total protein was loaded on each lane and resolved by electrophoresis on 10% polyacrylamide gel and blotted onto a polyvinylidene fluoride membrane (Millipore Corporate, Billerica, MA). Membranes were incubated in Western Blocking Reagent (Roche Diagnostics, Mannheim, Germany) diluted 1:10 in Tris-buffered saline (TBS) for 1 h at room temperature, then either in anti-β-CLU primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:5000, kept overnight at + 4°C, or in anti-β-actin (Millipore Corporate, Billerica, MA), diluted 1:4000, for 1 h at room temperature. After 4 × 10 min washes in 0.1% Tween-20 in TBS, the membranes were incubated with secondary antibodies conjugated with peroxidase, anti-goat (PIERCE, Rockford, IL) diluted 1:25000 for β-CLU and anti-mouse (Sigma-Aldrich) diluted 1:5000 in blocking solution for 1 h at room temperature. Blots were washed 4 × 15 min in Tween-20 in TBS. Immunoreactive bands were detected with the Chemiluminescence Blotting Substrate (Roche Diagnostics Corporation).

Analysis of spermatozoa for ROS, TBARS, and SCD. Spermatozoa suspensions from each rat sample were assayed for ROS by using the membrane-permeable lipophilic fluorochrome 2′,7′-dichlorofluoresceindiacetate (DCF-DA). The cell suspensions were incubated with 1 μM DCF-DA for
postlabeling, as described previously (Izzotti et al. 1999). Cell suspensions were pooled in such a way to have approximately 10^6 cells per tube in triplicate. The cells were centrifuged at 1200 g for 5 min. After removal of the supernatant, the pellet was frozen at −80°C. In other experiments, PNT1a cells were treated at a final concentration of 1 nM, and after 2 months of treatment, the cells were processed as described above. Control cells were incubated with the medium containing 0.1% ethanol.

**DNA extraction and detection of DNA adducts.** DNA was extracted from control cells and BPA-treated cells by means of a commercially available kit using phenol-free reagents (Genuclea DNA MiniPrep kit, Sigma). The 260/280 absorbance ratio was > 1.70 and < 1.85 in all samples, as determined by fiber optic spectrophotometry (NanoDrop Technologies, Wilmington, DE), thus showing the lack of protein and RNA contamination. Bulky lipophilic DNA adducts were enriched by nuclease P1 digestion and detected by ^32^P postlabeling, as described previously (Izzotti et al., 1999). DNA adducts, detected by ^32^P-imaging (InstantImager, Packard, Meriden, CT), were quantified by calculating the ratio between cpm detected in DNA adducts and cpm in normal nucleotides. Their levels were expressed as adducts per 10^8 nucleotides.

**Statistical analysis.** All quantitative results were expressed as means ± SD either of the results obtained within the mice composing each experimental group for in vivo data or of replicates within each cell line and treatment for in vitro data. The overall statistical significance was evaluated by ANOVA, followed by Student’s t-test for paired data. The significance of the relative increase of DNA adduct levels within each line treated with BPA was evaluated by Student’s t-test comparing the difference between the means before and after treatment and the relative weighed SD.

## RESULTS

### Rat Prostate Biomarkers

The average weight of prostates was not significantly different in control rats (0.36 ± 0.03 g; prostate weight per animal weight ratio 0.76 ± 0.07 × 10^-3) and in rats receiving BPA with drinking water for 10 days (0.36 ± 0.05 g, prostate weight per animal weight ratio 0.79 ± 0.13 × 10^-3).

The histological analysis of prostate sections, examples of which are shown in Figure 1 (A and B), provided evidence for a normal appearance of the prostate from control rats (Fig. 1A), whereas the samples from BPA-treated rats were extensively atrophic. In addition, extravasation of lymphocytes was observed in some samples (Fig. 1B, arrows).

The immunohistochemical analysis of CLU protein in prostate sections showed that, in samples from control rats, the protein is well detectable in epithelial cells, with cytoplasmic and apical localization (Fig. 1C). In contrast, the signal was more intense in controls and BPA-treated rats, either untreated (A, C) or receiving BPA (200 mg/kg body weight) with drinking water for 10 consecutive days (B, D). The arrows in B show extravasation of lymphocytes. The asterisks show the cytoplasmic and apical localization of CLU in control rats (C) and positivity for CLU in the whole cytoplasm and sometimes in the nucleus of cells in atrophic areas of BPA-treated rats (D).
the atrophic areas of BPA-treated rats, staining the whole cytoplasm and sometimes the nucleus (Fig. 1D). However, when prostate homogenates were assayed by Western blot analysis for CLU and actin (Fig. 2), the CLU/actin densitometric ratio was not significantly different in control rats (1.02 ± 0.30) and BPA-treated rats (1.11 ± 0.25).

**Rat Spermatozoa Biomarkers**

The results relative to the analysis of ROS, TBARS, and SCD in mature spermatozoa are summarized in Table 1. ROS concentrations were higher, but not to a significant extent, in spermatozoa from BPA-treated rats, whereas BPA treatment resulted in a significant 3.4-fold increase of TBARS. Moreover, BPA caused a significant 2.1-fold decrease of the nuclear spreading factor, evaluated by SCD, which provides evidence for an increased BPA-related fragility of spermatozoa. Figure 3 shows the dispersion of SCD data relative to the 538 cells per rat analyzed from untreated rats and the 562 cells per rat from BPA-treated rats. These data show at a glance the homogeneity of results within each one of the two experimental groups and the sharp difference between controls and BPA-treated rats.

**TUNEL Assay in Liver Cells**

As shown in Table 1, the frequency of TUNEL-positive cells was increased 3.8-fold in the liver of BPA-treated rats as compared with control rats.

**Cytogenetical Analyses in Bone Marrow Erythrocytes**

The frequency of MN PCE was similar in the bone marrow of control rats and BPA-treated rats (Table 1).

**Table 1**

### Intermediate Biomarkers in Various Cells of Controls and BPA-Treated Rats

<table>
<thead>
<tr>
<th>End point (unit)</th>
<th>Controls</th>
<th>BPA</th>
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<tbody>
<tr>
<td><strong>Spermatozoa</strong></td>
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<tr>
<td>ROS (DCF-DA emission/μg protein)</td>
<td>1.4 ± 0.30</td>
<td>1.8 ± 0.41</td>
</tr>
<tr>
<td>TBARS (nmol MDA/μg protein)</td>
<td>1.7 ± 1.98</td>
<td>5.7 ± 3.73**</td>
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<tr>
<td>SCD (nuclear spreading factor)</td>
<td>1.4 ± 0.83</td>
<td>0.7 ± 0.18***</td>
</tr>
<tr>
<td><strong>Liver cells</strong></td>
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<tr>
<td>TUNEL-positive cells (%)</td>
<td>0.4 ± 0.52</td>
<td>1.5 ± 1.31*</td>
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<tr>
<td>Bone marrow erythrocytes</td>
<td></td>
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<tr>
<td>MN PCE (%)</td>
<td>0.5 ± 0.76</td>
<td>0.6 ± 0.74</td>
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<tr>
<td><strong>Peripheral blood lymphocytes</strong></td>
<td></td>
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<tr>
<td>SSB (tail moment at comet assay)</td>
<td>1.6 ± 0.74</td>
<td>1.2 ± 0.43</td>
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</table>

*Note.* The data are means ± SD within each experimental group.

* *p* < 0.05.

**p* < 0.01, significant increase as compared with controls.

***p* < 0.05, significant decrease as compared with controls.

**Comet Assay in Peripheral Blood Lymphocytes**

The comet assay did not show any significant difference in the intensity of SSBs from untreated rats and BPA-treated rats (Table 1).

**Formation of DNA Adducts in Cultured Human Prostatic Cells**

Table 2 summarizes the results relative to the measurement, in triplicate 32P postlabeling analyses, of bulky DNA adducts in two human prostatic cell lines, either untreated or treated with BPA at two different doses and exposure times. The background levels of DNA adducts were slightly higher in PC3 metastatic carcinoma cells than in PNT1a nontumorigenic epithelial cells, but not to a significant extent. Both types of cells responded to treatment for 24 h to a high dose of BPA.
The most remarkable alterations produced by BPA in vivo, among those investigated, affected possible target cells, such as prostate cells and spermatozoa. In the prostate, alterations of CLU protein were not so intense to be detected by Western blot analysis of whole organ homogenates. However, an accumulation of CLU was well evident in epithelial cells affected by BPA-related atrophy, in agreement with our previous conclusion that CLU overexpression is associated with atrophy of rat prostate epithelial cells (Marinelli et al., 1994). Increased levels of CLU are typical of cells doomed to die by apoptosis (Bettuzzi and Rizzi, 2009), to such an extent that plasmatic sCLU might be proposed as a marker of prostate cancer (Girard et al., 2004). The CLU gene has been found to express, through mechanisms that are not completely understood, a complex protein profile composed of extracellularly secreted CLU (sCLU) and intracellular nucleus-targeted CLU (nCLU). sCLU is probably cytoprotective, whereas nCLU is clearly proapoptotic (Caccamo et al., 2004, 2005). It is now believed that the balance between sCLU and nCLU may drive the fate of the cell (Bettuzzi and Rizzi, 2009), to such an extent that plasmatic sCLU might be proposed as a marker of prostate cancer (Girard et al., 2010). In the present study, the CLU signal was more intense in the atrophic prostate epithelium of BPA-treated rats and affected the whole cytoplasm and sometimes the nucleus. It is noteworthy that, after neonatal exposure to BPA, prostates from aged rats exhibited an increased incidence and score of prostate intraepithelial neoplasia as compared with controls (Prins et al., 2011).

### DISCUSSION

The results of the present study provide evidence that BPA is able to form DNA adducts in cultured human prostatic cells and to induce a variety of alterations in cells of rats receiving this ED with drinking water.

The observed formation of DNA adducts in PNT1a and PC3 cells after a short (24 h) treatment with high-dose BPA and, to a lesser extent, in PNT1a cells after a long-lasting (2 months) treatment with a much lower BPA dose indicates that these human prostatic cells possess the metabolic machinery needed to activate this ED. Recently, formation of DNA adducts has been reported to occur in human prostate carcinoma cells (LNCaP line) after treatment with the polycyclic aromatic hydrocarbon benzo(a)pyrene (Hrubá et al., 2010), a well known carcinogen.

Both in humans and in experimental animals, BPA is metabolized to its glucuronide and hydroxylated derivatives, mainly 3-hydroxy-BPA (3-OH-BPA or BPA catechol), which is then oxidized to its ortho-quinone, i.e., BPA-3,4-quinone (BPAQ) (Atkinson and Roy, 1995b; Edmonds et al., 2004). It is noteworthy that oxidation of catechols to semiquinones and quinones is a mechanism of tumor initiation for endogenous estrogens as well as for synthetic estrogens such as DES (Cavalieri and Rogan, 2006). The fact that the BPA-related increase of DNA adducts was greater in nontumorigenic prostate cells than in cancer cells, with a difference that was close to the statistical significance threshold, is consistent with the notion that, in general, metabolic activation of procarcinogens and formation of DNA adducts are less pronounced in cancer cells as compared with the corresponding healthy tissue (van Schooten et al., 1990).

In rats receiving BPA with drinking water for 10 consecutive days, we did not observe any DNA damage in surrogate cells. These included peripheral blood lymphocytes, where no increase of SSB levels was detected by SCGE, and bone marrow erythrocytes, where the frequency of MN PCE was not increased after BPA treatment. Conversely, a significant increase of TUNEL-positive cells occurred in liver cells from BPA-treated rats. The liver is the major organ of BPA metabolism, where this ED has been shown to form DNA adducts in both mice (Izzotti et al., 2009) and rats (Atkinson and Roy, 1995b) and to induce generation of ROS in male rats, even at low doses (Bindhumol et al., 2003). The TUNEL assay detects both SSBs and double-strand DNA breaks as well as early stage apoptosis. The specificity of this assay for the detection of apoptosis in liver tissue has been questioned (Stähelin et al., 1998). Clearly, evaluation of apoptosis in liver cells of BPA-treated rats would have required additional methodological approaches, but this end point was not the primary goal of the present study. Nevertheless, the results obtained are likely to be interpreted as the consequence of liver cell death in BPA-treated rats, in which hepatotoxicity has been ascribed to binding of BPAQ with DNA (Atkinson and Roy, 1995b).

<table>
<thead>
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<th>Table 2: Levels of Bulky DNA Adducts in Human Prostatic Cells</th>
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<td><strong>Treatment</strong></td>
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<td>----------------</td>
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<tr>
<td>Controls</td>
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<tr>
<td>BPA (200–250nM) for 24 h</td>
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<tr>
<td>BPA (1nM) for 2 months</td>
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</table>

**Note.** NT, not tested.

*P = 0.08.

**P < 0.05.

***P < 0.01, as compared with the corresponding controls.
Administration of BPA to rats resulted in evident alterations of spermatozoa. Although the increase in ROS levels was not statistically significant, concentrations of lipid peroxidation products, in the form of TBARS, were considerably elevated in spermatozoa from BPA-treated rats. Moreover, an enhanced fragility of spermatozoa was documented by a significant decrease of SCD. The male germ line is one of the most sensitive tissues to the damaging effects of toxicants (Bonde, 2010), and BPA has been shown to affect the reproduction of male rodents (Chitra et al., 2003). In previous studies, the oral treatment of male rats with BPA for 44–45 days resulted in disorganization, distortion, and degeneration of late spermatids (Takahashi and Oishi, 2001) and in a reduction of epididymal sperm motility and sperm count, with increased levels of H$_2$O$_2$ and lipid peroxidation products, accompanied by loss of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase (Chitra et al., 2003). Administration of BPA to rats with drinking water for 1 week increased the production of ROS in sperm cells, an effect that was inhibited by the antioxidant N-acetylcysteine (Minamiyama et al., 2010). In humans, one study in a workplace in China showed inverse relationships between urinary BPA levels and sperm concentration, count, vitality, and motility (Li et al., 2011). However, in another study in men attending an infertility clinic in the United States, the association between urinary BPA and sperm concentration, motility, and morphology was less evident (Meeker et al., 2010).

The results observed in experimental test systems with high doses of compounds cannot be automatically transferred to the human situation. Nevertheless, the BPA-related formation of DNA adducts in the mammary tissue of female mice (Izzotti et al., 2009, 2010) and the alterations detected in prostate cells and spermatozoa of male rats, together with the evidence that BPA forms DNA adducts in cultured human prostatic cells, as shown here, deserve attention. They suggest, as postulated with natural estrogens (Bosland, 2000; Cavalieri and Rogan, 2006; Carruba, 2007; Ellem et al., 2004; Yang et al., 2009), that the estrogen-mimicking compound BPA may be involved in mammmary carcinogenesis and prostate carcinogenesis with similar mechanisms. Moreover, the DNA damage observed in spermatozoa of rats treated with this ubiquitous ED may be a mechanism contributing to explain the epidemiological finding that the quality and quantity of human sperm has decreased during the last six decades (Maffini et al., 2006; Soto and Sonnenschein, 2010).

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