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The response of adult rat Sertoli cells, immortalized by a temperature-sensitive mutant of SV40, to 1,2-dinitrobenzene, 1,3-dinitrobenzene, 2,4-dinitrotoluene, 3,4-dinitrotoluene, and cadmium

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Abstract

In this study we test the hypothesis that immortalized adult rat Sertoli cells respond to known testicular toxins in a similar manner to Sertoli cells tested *in vivo* and in primary culture. This cell line was developed by immortalizing adult rat Sertoli cells with the temperature-sensitive mutant of SV40, ts255, such that the cells proliferate at the permissive temperature of 33°C but express differentiated characteristics at the nonpermissive temperature of 40°C. Confluent monolayers, grown at 33°C or 40°C, were exposed to a range of concentrations of dinitrobenzene (DNB) or dinitrotoluene (DNT) isomers or to cadmium chloride. Cellular response was assessed by neutral-red cell viability assay and ultrastructural changes. Cells grown at 40°C were sensitive to lower concentrations of each toxicant than were cells grown at 33°C. 1,2-DNB was more toxic than 1,3-DNB, and 3,4-DNT was more toxic than 2,4-DNT, as judged by the neutral-red cell viability assay. Ultrastructurally, cells treated with 1,2-DNB or 2,4-DNT showed increased numbers of autophagic vesicles compared to controls. Intercellular penetration of ruthenium red demonstrated breached tight junctions in 1,2-DNB and cadmium-treated cells. From these observations, we conclude that this cell line can serve as a model for studying toxic mechanisms in adult Sertoli cells.

Abbreviations: ASC-19D, adult Sertoli cells focus #19 and clonal line D; DMSO, dimethyl sulfoxide; 1,2-DNB, 1,2-dinitrobenzene; 1,3-DNB, 1,3-dinitrobenzene; 2,4-DNT, 2,4-dinitrotoluene; 3,4-DNT, 3,4-dinitrotoluene; ECM, extracellular matrix; F-12:DMEM, nutrient mixture F-12:Dulbecco's Modified Eagle Medium; NR, neutral red; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; TEM, transmission electron microscopy

Introduction

Sertoli cells provide support and sustenance to developing germ cells during spermatogenesis. Investigations *in vivo* and *in vitro* have identified these epithelial cells as the target of several testicular toxicants. In rats, gavage-administered 1,3-dinitrobenzene depressed sperm count, reduced testicular weight, and caused vacuolization of Sertoli cell cytoplasm (Linder et al., 1990). 1,2-Dinitrobenzene spared the testes at a similar dose (Blackburn et al., 1988). The lanthanum tracer method showed that 1,3-DNB breached the blood–testes

barrier in rats (Shinoda et al., 1989). In primary rat Sertoli cell culture (Williams and Foster, 1988) and rat Sertoli–germ cell coculture (Foster, 1989; Reader and Foster, 1990; Allenby et al., 1990), increased lactate production and cytopathology demonstrated the same pattern of toxicity for 1,3-DNB and 1,2-DNB as seen *in vivo*. Likewise, 3,4-dinitrotoluene was more potent than 2,4-dinitrotoluene when administered to rat Sertoli–germ cell cocultures (Reader and Foster, 1990). *In vivo*, 2,4-DNT produced various-sized vesicles, swollen mitochondria, and distended endoplasmic reticulum in rat Sertoli cells (Bloch et al., 1988). Cadmium altered the integrity of the blood–testes barrier *in vivo* (Setchell and Waites, 1970). The same results were observed in rat Sertoli cell monolayers grown in two-compartment chambers (Janecki et al., 1992).

Cell culture studies have the advantage that extratesticular factors can be eliminated and the use of live animals reduced. However, primary cultures of Sertoli cells do not proliferate, which limits their widespread and convenient application to the study of toxic mechanisms. To obviate this drawback, mature rat Sertoli cells were immortalized with a temperature-sensitive mutant of SV40 (Boekelheide et al., 1993; Roberts et al., 1995). These cells proliferate at the permissive temperature of 33°C and are reported to differentiate to a mature version at 40°C, as evidenced by their increased expression of transferrin and sulfated glycoprotein (SGP-2) mRNA at the higher temperature. Such a cell line might prove to be a useful model for investigating the mechanisms of toxicity of Sertoli cell toxicants.

The purpose of this study was to characterize the ultrastructural morphology of an immortalized Sertoli cell line obtained from sexually mature rats, ASC-19D (Roberts et al., 1995), at 33°C and 40°C and its response to 1,3-DNB, 1,2-DNB, 3,4-DNT, 2,4-DNT, and cadmium. Neutral-red assays and ultrastructural assessments were employed to determine cellular response to the toxicants. The effects were compared to previously reported manifestations generated by the same toxicants *in vivo* and in primary culture.

Materials and methods

Cell culture

Immortalized adult rat Sertoli cells, ASC-D19 (provided by Kenneth P. Roberts, University of Minnesota Medical School, Minneapolis, MN, USA), were seeded at a density of 5×10^6 cells per well into 24-well cell culture plates. The cultures were grown in a one-to-one mixture of F-12 and DMEM plus glutamine (Gibco BRL, Rockville, MD, USA) and supplemented with 10% newborn calf serum (Hyclone, Fort Collins, CO, USA) and 1% penicillin–streptomycin. Cells were incubated at 33°C in 5% CO_2 until confluency. At that time, cells destined for the 40°C studies were moved to that temperature for two days prior to experimentation.

In an effort to induce characteristic Sertoli cell columnar morphology, some cells were grown at either temperature on top of 100 µl of a one-to-one mixture of extracellular matrix (ECM) prepared from Engelbreth Holm-Swarm sarcoma in mice (Sigma, St. Louis, MO, USA) and F-12:DMEM (Anthony et al., 1989; Hadley et al., 1988; McGuiness et al., 1994).

Chemical exposures

Isomers of DNB and DNT (Sigma-Aldrich) were dissolved in DMSO (Sigma-Aldrich) and then mixed to the appropriate concentrations in serum-free medium. Volumes of DMSO were added such that all wells received a total of 2% DMSO. For the neutral-red assays, cells were exposed to a concentration range of 25–

200 μmol/L 2,4-DNT or 3,4-DNT or to 100 μmol/L 1,2-DNB or 1,3-DNB for 4, 8, or 12 h. Solvent control wells were exposed to 2% DMSO. Other control wells were left untreated. In a separate neutral-red study, cells were exposed to either 1,2-DNB or 1,3-DNB at a concentration range of 30–240 μmol/L for 12 h. During the exposures, cultures were incubated at either 33°C or 40°C. In each experiment, four culture wells of each treatment were assessed. Each experiment was repeated three times.

For electron microscopy, a damaging, yet sublethal, exposure level was selected based on prior neutral-red assays. Thus, the cells were exposed to 100 μmol/L concentrations of 1,2-DNB or 2,4-DNT for 6 h. Control wells were exposed to 2% DMSO or left untreated. Other wells were treated with 10 μmol/L cadmium chloride (Sigma-Aldrich) for 6 h.

Neutral-red assay

Cell viability was determined by neutral-red (NR) assay as described by Borenfreund and Puerner (1985). Stock neutral-red (Sigma-Aldrich) was dissolved in F-12:DMEM to give a final concentration of 50 μg/ml. This NR medium was incubated overnight at 37°C and then filtered (0.22 μm) to remove precipitate. After chemical exposures, culture medium was replaced by NR medium, and cells were μincubated at either 33°C or 40°C for 3 h to allow for incorporation of the dye into viable cells. After incubation, the cells were washed with PBS to remove unincorporated dye. Extraction buffer (1% glacial acetic acid, 50% ethanol, and 49% distilled water) was then added and the plates were rotated for 15 min to facilitate dissolution of incorporated NR. The NR incorporation was measured spectrophotometrically at an absorbance of 540 nm using a Bio-Tech ELx800UV Automated Microplate Reader.

Statistical analysis

Absorbance levels were expressed as fold differences in their means from the solvent controls plus or minus their percentage deviation from the mean, or as their mean plus or minus their standard deviations. Significant differences were determined by the *t*-test.

Transmission electron microscopy

Immediately after chemical exposure, one well from each condition was processed for TEM. Cell monolayers were fixed in 2.5% glutaraldehyde and 0.1% ruthenium red in 0.1 mol/L cacodylate buffer, pH 7.4. They were postfixed in 1% osmium tetroxide and 0.1% ruthenium red in buffer. The cells were *en bloc* stained with an aqueous, saturated solution of uranyl acetate, dehydrated in a graded series of ethanol, embedded in Epon, and polymerized at 60°C (Hyat, 1981; Bozzolla and Russell, 1992). Vertical ultrathin sections of the Sertoli cell monolayers were poststained with uranyl acetate and lead citrate and examined on a Philips EM208 or Philips CM100 electron microscope. Apical junctional areas between 80–120 contiguous cells from one well of each condition were classified as intact or breached based on the exclusion or penetration, respectively, of ruthenium red tracer dye. Autophagosomes and autophagic lysosomes were counted in the same cells as above.

Results

Morphology

Controls

Untreated and solvent control ASC-D19 Sertoli cells, grown at either 33°C or 40°C, were morphologically indistinguishable. They were all squamous in shape and had large nuclei

with finely granular chromatin and large, prominent nucleoli. They possessed normal mitochondria, distended rough endoplasmic reticulum, and extensive, budding Golgi (Figure 1). They retained characteristic Sertoli cell gap junctions, interdigitating cytoplasmic processes, and ectoplasmic specializations (Figures 2a,b,c). Occasional autophagosomes and autophagic lysosomes were seen (Figures 3a,b). Intercellular exclusion of ruthenium red tracer dye demonstrated intact tight junctions between almost all of the cells (Figure 4a).

1,2-DNB

1,2-DNB treated cells, grown at either temperature, possessed increased numbers of autophagosomes and autophagic lysosomes compared to control cultures. In addition, intercellular penetration of ruthenium red demonstrated breached tight junctions at twice the frequency of controls in 33°C cultures and five times the frequency of controls in 40°C cultures (Figure 4b). All other morphology resembled that of control cells.

2,4-DNT

Cells, grown at 33°C and exposed to 2,4-DNT, resembled control cultures in appearance. Cells grown at 40°C under the same exposure condition showed elevated numbers of autophagosomes and autophagic lysosomes compared to controls. Tight junctions remained intact at both temperatures. All other morphology resembled that of control cells.

Cadmium

Cadmium chloride-treated cells grown at 33°C exhibited the same frequency of autophagosomes and autophagic lysosomes as control cultures. However, they showed a 2-fold elevation in number of breached tight junctions

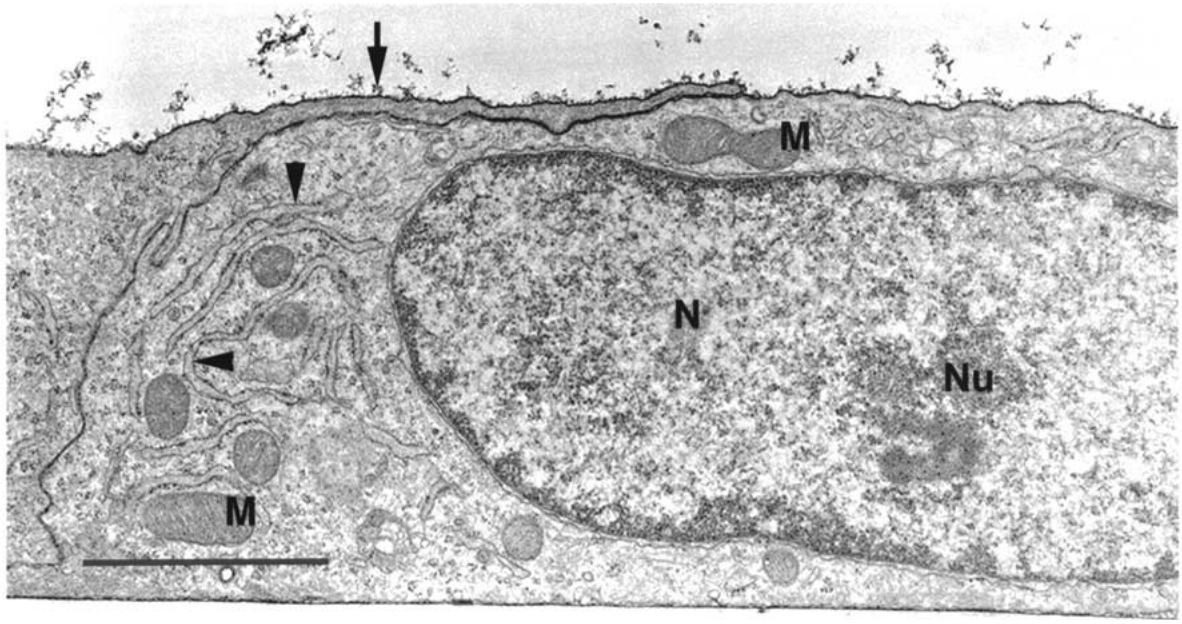
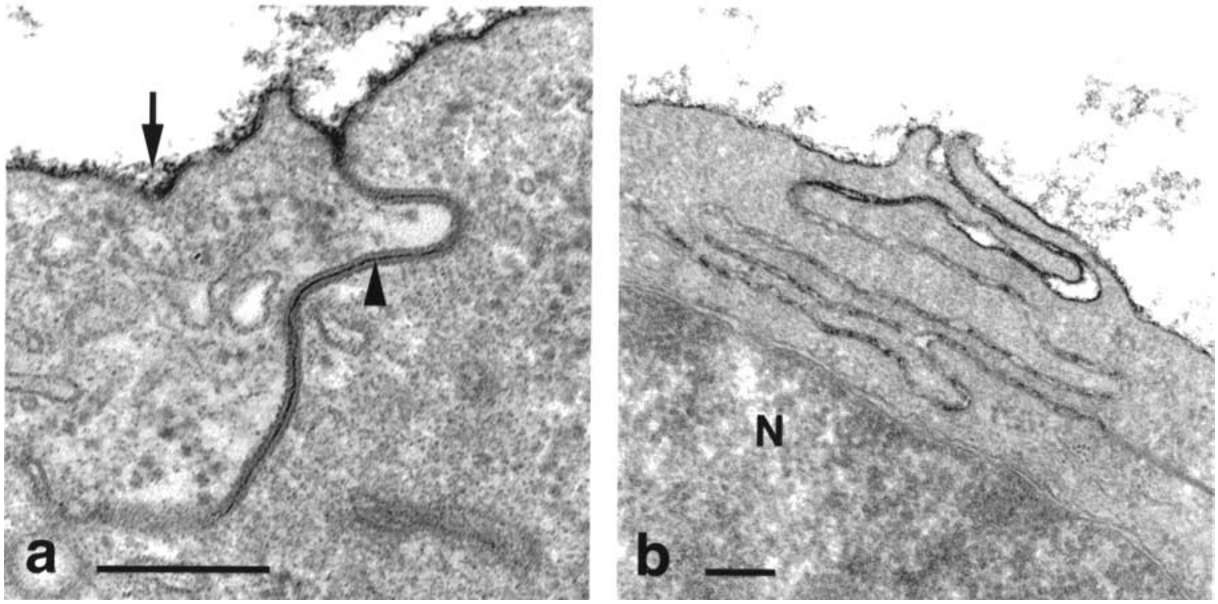


Figure 1. ASC-D19 control cell. Cells grown at 33°C or 40°C are squamous in shape. They have large nuclei (N) with finely granular chromatin and prominent nucleoli (Nu). Normal mitochrondria (M) and bulging rough endoplasmic reticulum (arrowheads) are present. Ruthenium red stains the apical glycocalyx (arrow). Scale bar = 2 μm.



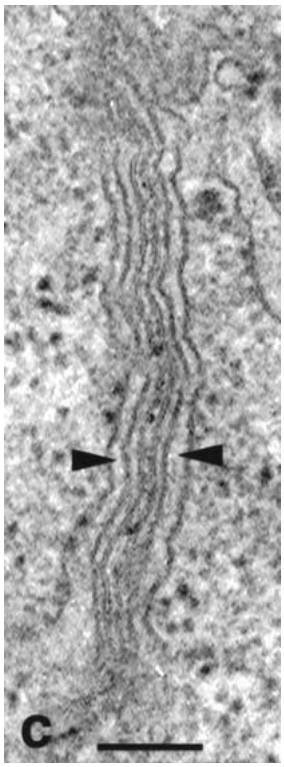


Figure 2. Characteristic Sertoli cell structures observed in ASC-D19 cells grown at either 33°C or 40°C and from all treatments. (a) A gap junction is demonstrated by a uniform 4 nm intercellular space that is stained with ruthenium red (arrowhead). Ruthenium red also stains the glycocalyx (arrow). Scale bar = 30 nm. (b) Interdigitating cytoplasmic processes are observed both apically (shown) and basally. (N) nucleus. Scale bar = 200 nm. (c) Ectoplasmic specializations are comprised of smooth endoplasmic reticulum (SER) located on opposing sides of a tight junctional area. Arrowheads indicate SER lumens. Scale bar = 200 nm.

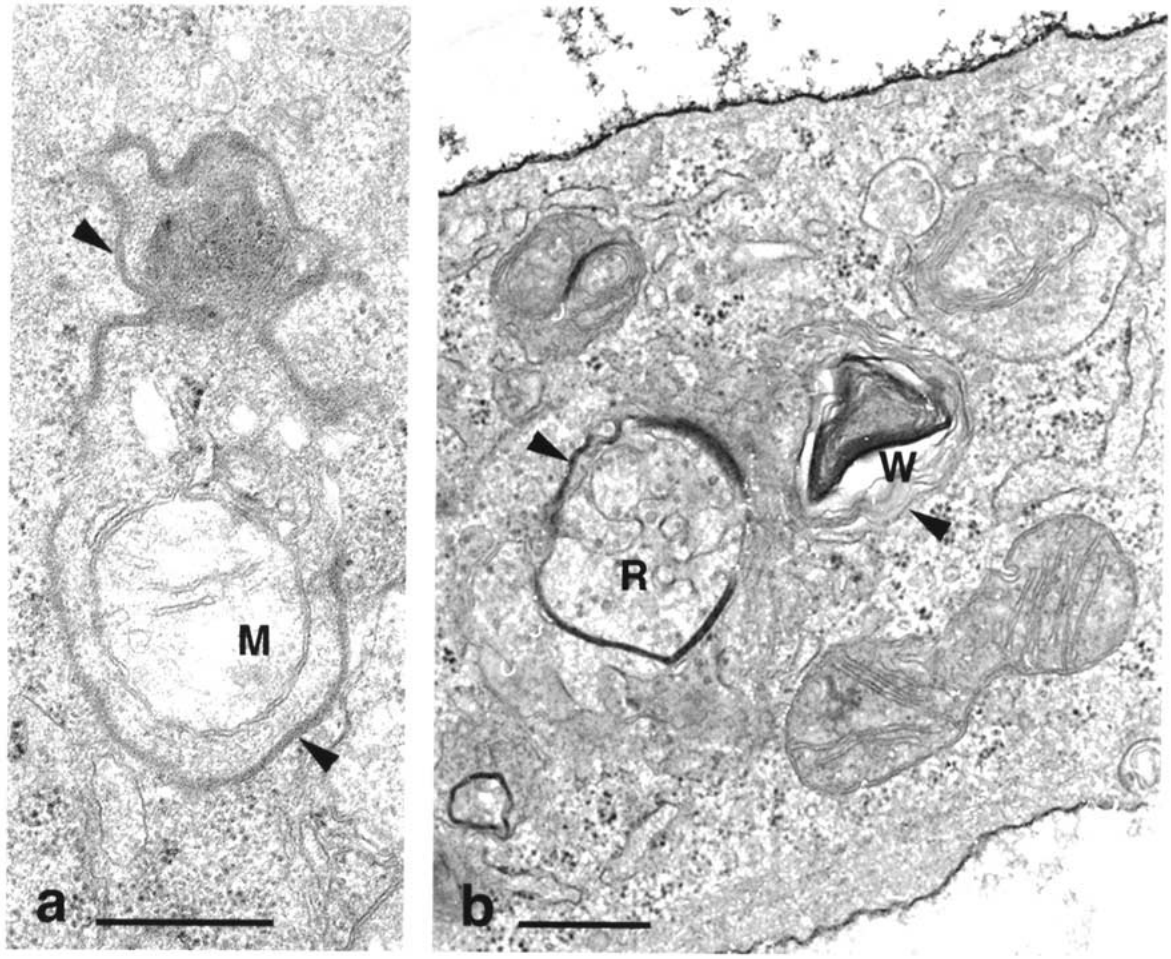


Figure 3. Autophagosome (a) and autophagic lysosomes (b) in ASC-D19 cells. These structures are seen in ASC-D19 control cultures, but they are seen in increased numbers in 100 μmol/L 1,2-DNB- and 2,4-DNT-treated cultures. (a) This autophagosome is comprised of a double-membrane-bound vesicle (arrowheads) that sequesters a mitochondrion (M) for subsequent degradation. Scale bar = 500 nm. (b) These autophagic lysosomes are identified by their single-membrane-bound vesicles (arrowheads). Inside the vesicles, cell organelles degenerate further to form whorls (W) or residual bodies (R). Scale bar = 500 nm.

compared to controls. Cadmium chloride-treated cells grown at 40°C lifted off of the substrate and were not available for examination by TEM.

In other exposure conditions and in controls, however, occasional apoptotic cells remained above the monolayers and were captured by TEM. They exhibited classic apoptotic morphology, such as cell rounding, plasma membrane blebbing, chromatin condensation, nuclear blebbing, and vacuolization (Figure 5).

Neutral-red assays

1,2-DNB

ASC-D19 grown at either 33°C or 40°C and treated with 1,2-DNB showed a dose- and time-dependent reduction in cell viability. Cells

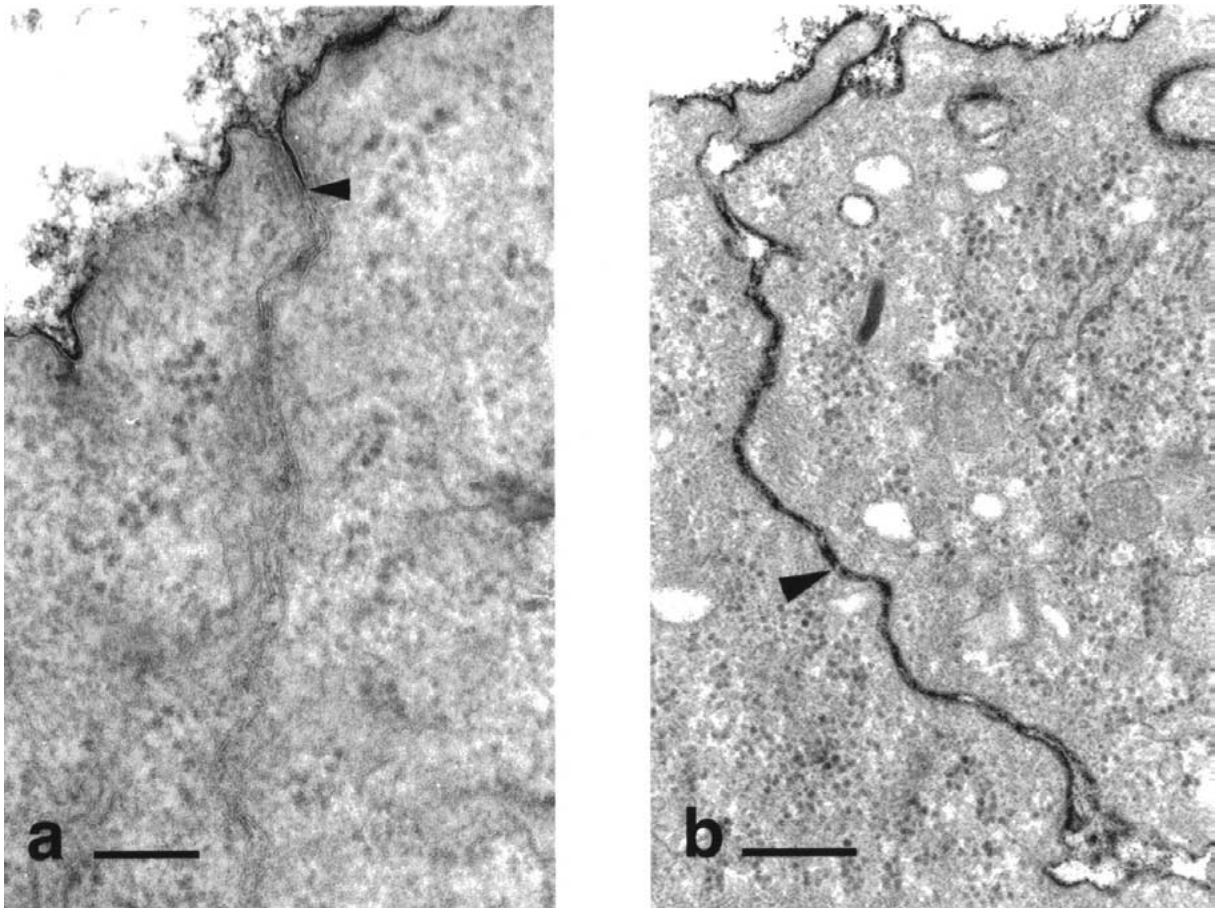


Figure 4. ASC-D19 cells showing intact (a) or breached (b) tight junctions. (a) An intact tight junction (arrowhead) prevents ruthenium red tracer dye penetration into the intercellular space. Control cells and cells treated with 100 μmol/L 2,4-DNT for 6 h retain intact tight junctions. Scale bar = 200 nm. (b) A breached tight junction permits intercellular penetration by ruthenium red (arrowhead). Cells grown at either 33°C or 40°C and exposed to 100 μmol/L 1,2-DNB for 6 h demonstrate breached tight junctions at two or five times the rate, respectively, of control cultures. Cells treated with 10 μmol/L cadmium chloride for 6 h show breached tight junctions at twice the rate of controls. Scale bar = 300 nm.

grown at 40°C showed a significant reduction in viability at a lower dose and shorter μexposure time compared with cells grown at 33°C (Figures 6 and 7). Cells grown at 33°C and exposed to 100 μmol/L 1,2-DNB showed a significant decrease in cell viability after an 8- or 12-h exposure. Cells grown at 40°C showed a significant decrease in cell viability after only 4 h (Figure 7). Twelve-hour exposure to increasing doses of 1,2-DNB significantly decreased cell viability in 33°C cells at 240 μmol/L concentration and in the 40°C cells at levels as low as 120 μmol/L (Figure 6).

1,3-DNB

Treatment with 1,3-DNB, at the full range of dose levels and exposure times, produced no significant effect on viability of cells grown at either temperature (Figures 6 and 7).

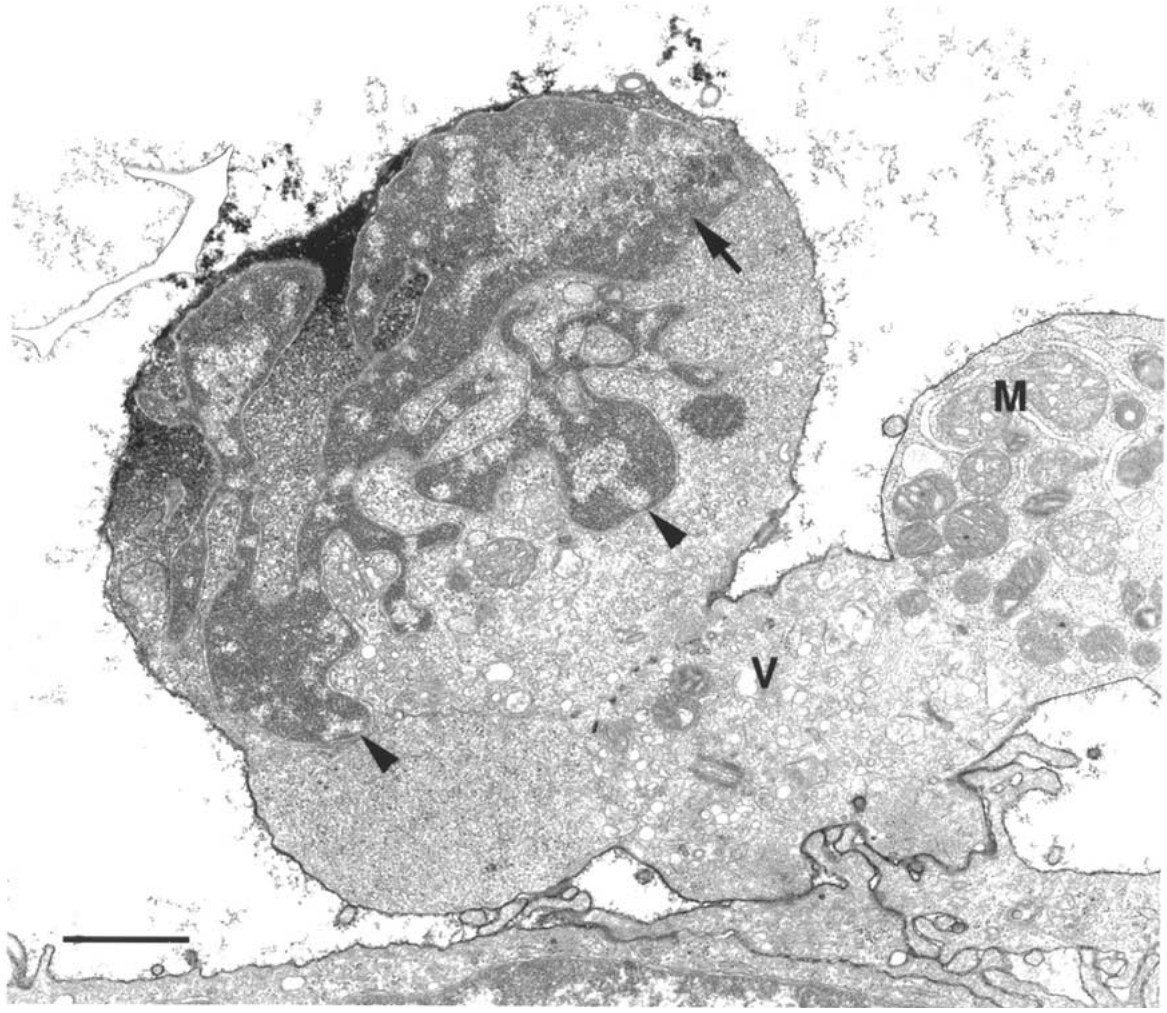


Figure 5. Apoptotic ASC-D19 cell. Most dead cells lifted off the substrate and were not available for electron-microscopic examination. However, occasional apoptotic cells were captured in all cultures, including controls. Classic apoptotic features are seen, such as cell rounding, plasma membrane blebbing, chromatin condensation (arrow), nuclear blebbing (arrowheads), and vacuolization (V). Mitochondria (M). Scale bar = 1 μm.

2,4-DNT

Treatment with 2,4-DNT produced no μsignificant decrease in cell viability at any concentration or exposure time in cells grown at either temperature (Figure 7).

3,4-DNT

Treatment of 33°C cells with 3,4-DNT produced no significant change in cell viability at any dose or exposure length. The 40°C cells showed significant decreases in viability after just 4 h at concentrations as low as 100 μmol/L (Figure 7).

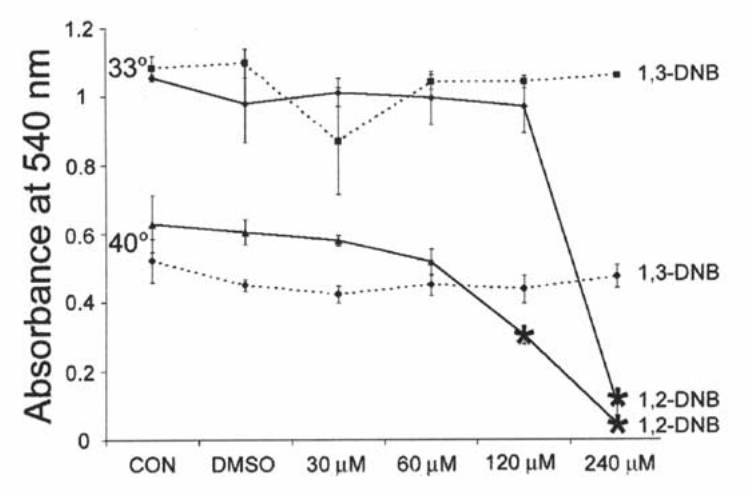


Figure 6. The effect of 1,2-DNB or 1,3-DNB on neutral-red assays of ASC-D19 cells grown at 33°C or 40°C. Cells were exposed for 12 h. Cells grown at 40°C showed a lower viability and greater sensitivity to 1,2-DNB than cells grown at 33°C. 1,2-DNB significantly reduced cell viability at the 240 µmol/L dose level in the 33°C cells and at the 120 µmol/L level in the 40°C cells. 1,3-DNB produced no significant change in cell viability at either temperature at concentrations up to 240 µmol/L. Values are expressed as mean absorbances at 540 nm plus or minus the standard error of the mean. Asterisks designate significant change compared to solvent controls.

Discussion

The adult rat Sertoli cell line ASC-D19 offers a promising and convenient model for investigating the effects of Sertoli cell toxicants. Grown at either 33°C, where they proliferate, or 40°C, where they differentiate, ASC-D19 cultures possess several morphological features of Sertoli cells *in vivo* (Gilula et al., 1976; Byers et al., 1991) and in primary culture (Onoda et al., 1990; Janecki et al., 1992). For example, they exhibit tight and gap junctions, interdigitation of cytoplasmic processes, and ectoplasmic specializations. At the same time, they are squamous rather than columnar in shape. Primary Sertoli cell cultures grown on plastic share this characteristic. Some authors have induced the columnar form in primary cultures by growing them on reconstituted extracellular matrix (Anthony and Skinner, 1989; Hadley et al., 1988; Onoda et al., 1990; Suarez-Quian et al., 1984). Efforts to recover the columnar form of ASC-D19 cells by growing them at either temperature on extracellular matrix were unsuccessful. Rather than assuming a columnar form, the proliferating cells overlaid one another and retained their squamous shape (data not shown). In fact, the squamous, rather than columnar, form might be inconsequential to its regulatory function, as demonstrated in a study by Anthony and Skinner (1989) in which no differences in cellular production of transferrin or androgen-binding protein were observed between primary cultures of Sertoli cells grown in columnar form on ECM or in flattened form on plastic.

Untreated and solvent control cultures grown at either temperature were morphologically indistinguishable. However, the 40°C cells were more susceptible than the 33°C cells to Sertoli cell toxicants. They exhibited a higher mortality rate at lower exposure levels and times, and a higher incidence of tight-junctional breaches and autophagosomes and

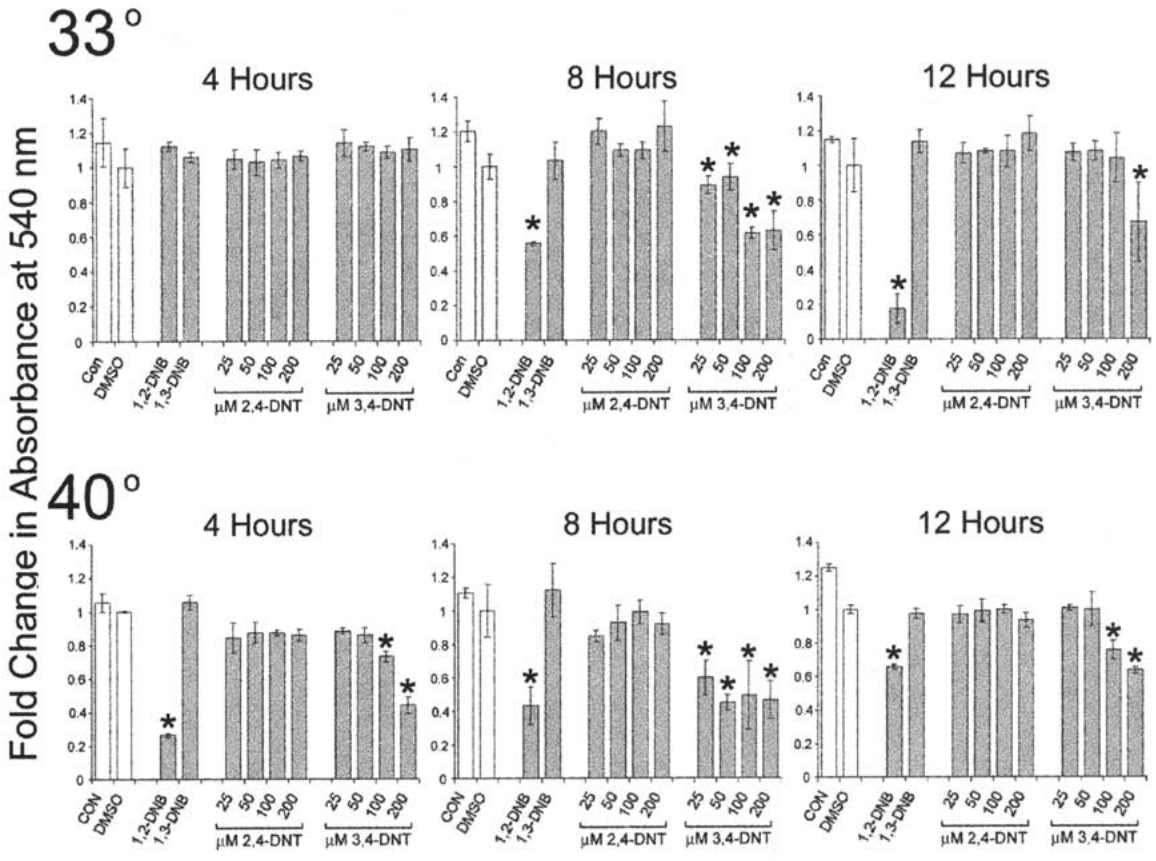


Figure 7. The effect of 4-, 8-, or 12-h exposure to isomers of DNB and DNT on cell viability of ASC-D19 cells grown at 33°C or 40°C. Cells grown at 40°C showed a greater sensitivity to toxicants than cells grown at 33°C. Exposure to 100 µmol/L 1,2-DNB lowered cell viability after 8 h in the 33°C cells and after only 4 h in the 40°C cells. Treatment with 100 µmol/L 1,3-DNB or 2,4-DNT at all dose levels and exposure times produced no significant effect on viability of cells grown at either temperature. Treatment with 3,4-DNT gave a significant reduction in cell viability after 8 h in cells grown at 33°C and after only 4 h in cells grown at 40°C. Values are expressed as fold differences in their means from the DMSO controls, plus or minus their percentage deviation from the mean compared to solvent controls. Asterisks designate significant changes in viability compared to solvent controls.

autophagic lysosomes than did the 33°C cells. This increased sensitivity might be attributed to a higher metabolic rate at the higher temperature. Another factor could be that the cells grown at 33°C remain in a proliferative state in which other metabolic functions are downregulated. Perhaps, at 40°C, the biotransformation of parent compounds into more potent metabolites (Cave, 1990; Ellis and Foster, 1992) is facilitated.

Neutral-red assay demonstrated that, in ASC-D19 cells, 1,2-DNB was more potent than 1,3-DNB. In both *in vivo* (Linder et al., 1990; Blackburn et al., 1988) and *in vitro* (Williams and Foster, 1988; Reader and Foster, 1990) studies, 1,3-DNB was the more potent of the two isomers. Exposure concentrations in the present study were in the same range as those used by previous authors. In rat primary hepatocyte cultures (Cossum and

Rickert, 1985), Sertoli–germ cell cocultures (Foster, 1989), and oral dosage of Fisher-344 strain rats (Nystrom and Rickert, 1987), 1,3-DNB was reduced to the end product, 3-nitroaniline. However, Foster (1989) reported that 3-nitroaniline was not toxic when returned to Sertoli–germ cell cocultures, suggesting that an intermediate metabolite is the toxic species in 1,3-DNB metabolism. In Sertoli–germ cell cocultures, depletion of glutathione increased the toxicity of 1,3-DNB. This phenomenon suggests that formation of a glutathione conjugate is a detoxification pathway for a reactive intermediate, possibly nitroso-nitrobenzene, generated during 1,3-DNB metabolism (Foster, 1989). However, glutathione–1,3-DNB conjugates were not detected in any of these studies. On the other hand, the predominant route of metabolism of 1,2-DNB is by its conjugation with glutathione (Cossum and Rickert, 1985; Nystrom and Rickert, 1987). Thus, the two isomers' different metabolic pathways produce different metabolites with different toxicities to Sertoli cells *in vivo* and in primary cultures. The studies of biotransformation pathways for the DNBs have not been completed in the ASC-D19 cell line. Whether the reversal of toxicity observed between the DNB isomers can be attributed to a difference in metabolism remains an open question.

The neutral-red assay showed that 3,4-DNT was more toxic than 2,4-DNT to ASC-D19 cells, as has been reported *in vivo* (Reader and Foster, 1990). In 2,4-DNT-treated cells, NR assay showed no increased cell mortality at either temperature. In addition, tight junctions remained intact. However, elevated numbers of autophagic bodies were seen in the 40°C, 2,4-DNT-treated cells. This finding demonstrates, again, the increased sensitivity of the 40°C cells compared to the 33°C cells. These observations also show that ASC-D19 cells at both temperatures are capable of tolerating this level of 2,4-DNT and resisting tight-junctional damage and cell death.

Control cells from either temperature showed occasional autophagosomes and autophagic lysosomes. These structures degrade the cells' own retired organelles and portions of cytosol. This normal physiological process occurs in virtually all eukaryotic cells (Dunn, 1994; Xue et al., 1999). ASC-D19 cells treated with 1,2-DNB at either temperature or with 2,4-DNT at 40°C showed elevated numbers of autophagosomes and autophagic lysosomes. Perhaps these increased numbers result from the removal of cellular components damaged by the toxicants. These structures observed by TEM probably correlate with previously reported vacuolar structures seen by light microscopy in DNB-exposed cells (Blackburn et al., 1988; Allenby et al., 1990; Linder et al., 1990) and DNT-exposed cells (Bloch et al., 1988; Reader and Foster, 1990).

Autophagy has been reported as an alternative method to apoptosis for cell death (Clark, 1990; Xue et al., 1999). Other authors have suggested that there is overlap between the two mechanisms (Zakeri et al., 1995) or that the presence of active autophagy is associated with a greater tendency to undergo apoptosis (Jia et al., 1997). In ASC-D19 cells exposed to 1,2-DNB, the neutral-red assay illustrated widespread cell death. Examination of these cells by TEM revealed structures potentially ascribed to either pathway.

Altered integrity of the blood–testes barrier upon exposure to cadmium has been reported in both *in vivo* (Setchell and Waites, 1970) and in primary Sertoli cell cultures (Janecki et al., 1992). Similarly, cadmium-treated ASC-D19 cells showed compromised tight junctions in the 33°C cells. The same dosage devastated the 40°C cells, again reflecting the greater sensitivity of the cells grown at the higher temperature. Low-level cadmium exposure has been shown to interfere with cadherins in MDCK cell cultures, resulting in the loss of ability to retain electrical resistance across the monolayer (Prozialeck et al., 1991; Prozialeck, 2000). A

similar mechanism might operate in ASC-D19 cells.

The immortalized adult rat Sertoli cell line ASC-D19 retains several characteristics of differentiated adult Sertoli cells. For example, the cells produce transferrin and sulfated glycoprotein (Roberts et al., 1995). They also possess tight and gap junctions, interdigitation of cytoplasmic processes, and ectoplasmic specializations. Neutral-red assay and ultrastructural pathology demonstrate that the immortalized adult rat Sertoli cell line ASC-D19 mimics Sertoli cells *in vivo* and in primary culture in response to cadmium and two isomers of DNT. However, the cells' susceptibility to 1,2-DNB compared to 1,3-DNB is contrary to that previously reported in Sertoli cells *in vivo* and in primary culture. This easily sustainable cell line's adherence to characteristic adult Sertoli cell morphology, protein production, and response to several known Sertoli cell toxicants makes it a useful tool for studying toxic mechanisms in adult Sertoli cells.

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