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# Use of a rat *ex-vivo* testis culture method to assess toxicity of select known male reproductive toxicants

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ABSTRACT

Due to the complex physiology of the testes, *in vitro* models have been largely unsuccessful at modeling testicular toxicity *in vivo*. We conducted a pilot study to evaluate the utility of the Durand *ex vivo* rat seminiferous tubule culture model [1–3] that supports spermatogenesis through meiosis II, including the formation of round spermatids. We used this system to evaluate the toxicity of four known testicular toxicants: 1,3-dinitrobenzene (DNB), 2-methoxyacetic acid (MAA), bisphenol A (BPA), and lindane over 21 days of culture. This organotypic culture system demonstrated the ability to successfully model *in vivo* testicular toxicity (Sertoli cell toxicity and disruption of meiosis) for all four compounds. These findings support the application of this system to study molecules and evaluate mechanisms of testicular toxicity.


## 1. Introduction

Evaluating testicular toxicity during nonclinical safety assessment is a significant challenge within the pharmaceutical industry. Testicular toxicity has delayed or halted compound development for most pharmaceutical companies within the last 5 years [4]. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use ICH S5(R2) guidelines recommend repeat dose studies over 2–4 weeks for assessing male reproductive toxicity [5]. These *in vivo* studies require a significant amount of compound and animals. Therefore, to support a reduction in animal use, more efficient identification of potential testicular toxicity, and an exploration of mechanism, it would be beneficial to employ an *in vitro* model.

Sertoli cell only culture systems do not enable the study of the complex interactions between Sertoli and germ cells that are found *in vivo*. We evaluated an improved *ex-vivo* organotypic culture system, developed by Durand's team [1–3], which utilizes isolated seminiferous tubules including Sertoli, peritubular and germ cells from prepubertal rats, co-cultured in bicameral chambers. This culture system has shown physiological cellular organization and supports germ cell maturation through meiosis. The physiological properties of this germ cell-Sertoli cell coculture system have been validated over the last twelve years and applied towards determining the mechanisms of action of cadmium and hexavalent chromium [1,6,7]. The improvements in testicular cell culture enable testing for integrity of the Sertoli–Sertoli junctional barrier (the primary component of the blood-testis barrier (BTB) *in vivo*), cell viability and proliferation, the time course of germ cell division, and expression of select genes over a four-week culture period [1,3,7].

In this paper, we present the results of a pilot study to characterize the utility of this co-culture system to identify the potential for testicular toxicity *in vivo*, as well as the underlying mode of action across diverse mechanisms of testicular toxicity. We selected four well-characterized testicular toxicants, 1,3-dinitrobenzene,

*Abbreviations:* DNB, 1,3-dinitrobenzene; MAA, 2-methoxyacetic acid; BPA, bisphenol A; BTB, blood–testis barrier; 2-ME, 2-methoxyethanol; TEER, *trans*-epithelial electrical resistance; Cx43, connexin-43; Cldn11, claudin-11; SpG, spermatogonia; SpC, spermatocyte; RS, round spermatocyte.

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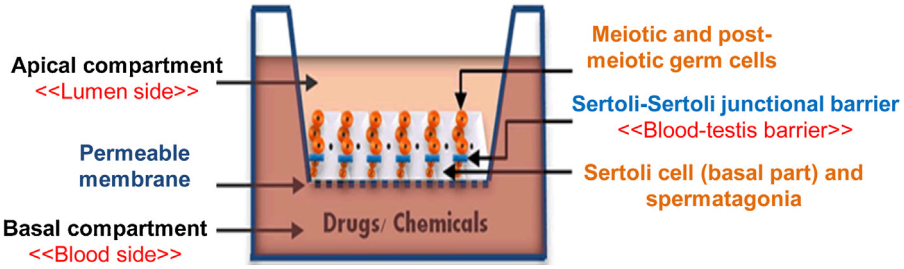


**Fig. 1.** Schematic of seminiferous tubule culture system.
Seminferous tubules from 20–22 day old SD rats were cultured on bicameral plates. Cells were treated with compound addition in the basal compartment.

2-methoxyacetic acid, bisphenol A, and lindane, to determine if exposure in the seminiferous tubule culture model could produce adverse effects similar to those published following *in vivo* exposure to these same compounds. We also evaluated how well the model worked in identifying the target cell population and providing insight into the mechanism of toxicity.

The toxicants selected encompass a variety of testicular toxicities. 1,3-dinitrobenzene (DNB) is a nitroaromatic compound commonly used in the manufacture of plastics, pesticides, and dyes. A single oral dose of DNB in adult rats induces Sertoli cell vacuolation and spermatocyte depletion within 24 h. With extended dosing, severe effects are produced including degeneration of pachytene spermatocytes, multinucleated and misshapen spermatids, and Sertoli cell vacuolation [8,9]. *In vitro*, DNB induced apoptosis and G2/M cell cycle arrest in TM4 Sertoli cells [10]. 2-methoxyacetic acid (MAA) is the toxic metabolite of 2-methoxyethanol (2-ME), a solvent used in printing inks, varnishes, and as a de-icing additive. 2-ME directly targets pre-meiotic and meiotic germ cells *in vivo* through histone hyperacetylation [11–13]. *In vitro*, MAA directly affects Sertoli cells, resulting in germ cell apoptosis [14,15]. Bisphenol A (BPA) is a plasticizer commonly found in many consumer and industrial products. BPA is an endocrine disruptor due to its structural similarity to estrogen. Although Leydig cells are a target of BPA [16], *in vitro* studies using primary Sertoli cells demonstrated direct targeting through disruption of cell–cell signaling [17,18]. Lindane is a pesticide used in both agriculture and parasiticidal treatment for lice. Lindane induces apoptosis in Sertoli cells as well as spermatogonia and spermatocytes [19].

By assessing the *in vitro* response of the different cell populations within the seminiferous tubule to these toxicants, we evaluated the ability of this *ex-vivo* system to recapitulate the testicular effects produced through a variety of mechanisms. In all cases, concentrations tested *in vitro* were selected to approximate concentrations reported to produce testicular pathology in rodent studies. We measured a variety of endpoints including germ cell count and viability, Sertoli cell tight junction integrity, as well as the progression of spermatogenesis through development of early spermatids (step 5 of spermiogenesis) [20].

Characterization of the *ex vivo* model will provide scientists with the ability to explore testicular toxicity mechanisms in an isolated system. The pilot study described demonstrates the use of this *ex-vivo* organotypic spermatogenic culture system to model *in vivo* testicular toxicity in response to compounds with diverse mechanisms and testicular pathologies and to decipher their mechanisms/modes of action. Although we present limited data on the validation of the system for screening molecules, based on the data collected to date, this assay has that potential. In order to use this as a screen, more studies are clearly needed to determine the accuracy and identify the limitations of the system to predict *in vivo* testicular toxicity.

## 2. Materials and methods

### 2.1. Animals

Cultures of seminiferous tubules were derived from naïve 20–22-day-old Sprague-Dawley rats (Charles River, provided by Janvier France). In order to account for inter-animal variations, testes from six to ten rats were pooled in each experiment, and were immediately used as described below. All procedures were approved by the Prefecture du Département du Rhône (approval number 692661226) and conducted in accordance with recommendations of the European Economic Community (EEC) (86/609/EEC) for the care and use of laboratory animals.

### 2.2. Preparation and culture of seminiferous tubules

This technique has been previously published [1–3] and is summarized in the schematic diagram in Fig. 1. Cultures were carried out in triplicate or sextuplicate at 33 °C in the culture medium, supplemented as described in references cited above, in a water-saturated atmosphere of 95% air and 5% $CO_2$. When required, toxicants were added in the basal compartment of the bicameral chamber of culture beginning from day 2 of the cultures. Stock solutions of BPA, DNB or lindane were prepared in absolute ethanol, and then diluted sequentially to obtain the working solutions in 0.1% ethanol. This concentration has previously been shown to be non-toxic in the model (unpublished data). A stock solution of MAA was prepared in culture medium and adjusted to pH 7.2–7.4 with NaOH.

Basal media (with or without the toxicant) were renewed every 2 days. At selected days of culture, cells were detached from the culture dishes and isolated with trypsin for flow cytometry. An aliquot of the cell suspension was used to determine the number of cells and to assess cell viability by trypan blue exclusion and counting with a hemocytometer. Each compound was tested at two concentrations based on previously published *in vitro* data—a high concentration that was anticipated to cause significant cellular effects in the absence of cytotoxicity, and a low concentration that was anticipated to induce minimal cellular effects. Table 1 summarizes the rationale for selecting the compound concentrations used in this study.

### 2.3. Trans-*epithelial electrical resistance (TEER)*

*Trans*-epithelial electrical resistance (TEER) measurement was performed with an EVOM2 (World Precision Instruments, Florida, USA). The culture was equilibrated at room temperature before assay. The TEER was calculated according to the following equation: $\text{TEER} = (R_{\text{total}} - R_{\text{control}}) \times A$ ($\Omega\,\text{cm}^2$), where $R_{\text{total}}$ is the resistance measured, $R_{\text{control}}$ is the resistance of the control insert (insert alone with the culture medium), and $A$ is the surface area of the insert. TEER was measured for 6 replicate wells. One-way ANOVA was