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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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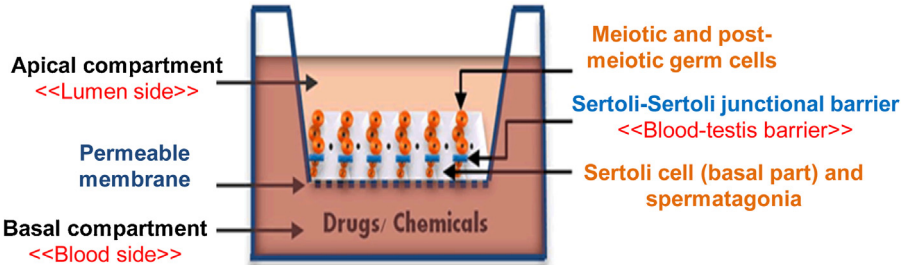

Fig. 1. Schematic of seminiferous tubule culture system.
Seminferous tubules from 20–22 day old SD rats were cultured on bicameral plates. Cells were treated with compound addition in the basal compartment.

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_{total} is the resistance measured, R_{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

Table 1
Justification of concentrations used.

Compound	Concentration justification
1,3-Dinitrobenzene (6 μM and 60 μM)	The top concentration was set at 60 μM based on induction of apoptosis in TM4 Sertoli cell culture at 60 μM [10] and stimulation of lactate and pyruvate secretion in primary Sertoli cells treated with 100 μM DNB [21]. The lower concentration was set at 10% of the high concentration based on a lack of literature at lower concentrations.
Methoxyacetic acid (0.5 mM and 2.5 mM)	The top concentration of 2.5 mM is based on 3 mM MAA causing a decrease in lactate in Sertoli cell cultures [15]. We used a low concentration of 0.5 mM based on concentrations of at least 1 mM inducing spermatocyte degeneration in tubule cultures [22].
Bisphenol A (5 μM and 50 μM)	A high concentration of 50 μM has been shown to increase lactate production in Sertoli cells [23]. Higher concentrations of 100 μM and 150 μM are cytotoxic to Sertoli cells cause detachment of cells in primary cell culture [24]. A low concentration of 5 μM was used based on 10 μM causing increased glutathione levels without cell death [25].
Lindane (5 μM and 30 μM)	The high concentration of 30 μM was selected based on a disruption of gap junctions in the 42GPA9 Sertoli cell line [26] and increased lactate production in Sertoli cells [23]. 5 μM was set as a low concentration that does not increase lactate production in Sertoli cells [23].

performed using GraphPad Prism version 6.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

2.4. Immunolabeling of cultured cells for flow cytometric analysis

To distinguish germ cells from somatic cells (mainly Sertoli cells, as rats possess only one layer of peritubular/myoid cells [27]), fixed cultured cells were immunolabeled with a monoclonal antibody against vimentin (clone V9; DAKO SA, Trappes, France) which was conjugated with a red fluorochrome, Alexa 647, by using a Zenon Mouse labeling kit (Life Technologies) [28]. Cells were incubated with the labeled anti-vimentin-Alexa 647 antibody at 1/42 dilution for 30 min at room temperature. Before analysis, Hoechst 33342 (Sigma–Aldrich, St Quentin Fallavier, France) was added to the labelled cell suspension at a final concentration of 0.12 mg/ml for 40 min on ice. After immunolabeling, cells were analyzed using a FACSVantage SE cell sorter (BD Biosciences, CA) equipped with an Enterprise II argon ion laser, which simultaneously emitted a 50 mW line tuned to UV (351 nm). A second line was tuned to 448 nm at 130 mW to excite Hoechst. Emission of Hoechst fluorescence 33342 (λ.reem = 424) was acquired after linear amplification through a 424/44 nm filter. Acquisition and analysis were performed using the CellQuest Pro™ 4.0.2 software as previously detailed [29]. The vimentin-positive somatic cells (mainly Sertoli cells, since there is only one layer of peritubular cells in the rat) and the vimentin-negative 4C, 2C and 1C germ cells were separated with the bivariate analysis of DNA content/vimentin. Using the bivariate linear forward light scatter and linear side angle light scatter analyses it was possible to identify spermatogonia, young spermatocytes, middle-to-late pachytene spermatocytes, secondary spermatocytes, and round spermatids [29]. Each acquisition was performed on 50,000–100,000 cells. Although treatment was performed using replicate wells per timepoint, individual wells did not have enough cells to ensure a representative number of cells from each subpopulation. Three to five wells were pooled for flow cytometry. Pooling of wells did not permit an estimate of measurement error in the flow cytometry data, thus, statistical analysis was not performed.

2.5. Immunofluorescence assay for connexin-43 (Cx43) and claudin-11 (Cldn11)

Expression of the junctional proteins, Cx43 and Cldn11 was measured on culture day 5. After several days of culture, there is a decrease in the Cx43 signal intensity [6], and therefore measurements were limited to day 5. Immunofluorescence analyses were performed as described previously [30]. Briefly, cultured tubules were fixed in methanol at −20 °C for 6 min, permeabilized and saturated with 0.1% of Triton × 100 in PBS with 0.1% BSA for 30 min. The tubules were then incubated for 2 h with the primary antibodies anti-claudin-11 (Life Technologies, 2.5 μg/ml), and anti-Cx43 (from BD Transduction Laboratories or from Sigma, 5 μg/ml). After washing, slides were subsequently incubated for 1 h with goat anti-rabbit/anti-mouse FITC or TRITC conjugated antibodies (1:100). To label nuclei, the seminiferous tubule fragments were mounted in Vectashield medium with DAPI (BioValley). Image collection was performed on a confocal microscopy (LSM 510 Zeiss, Germany, courtesy of C. Klein, INSERM IFR58) or with a wide field deconvolution microscope Nikon TE-2000E (SCM, University Paris Descartes, France). Representative images can be found in the Supplemental file, S1. Semi-quantitative evaluation of the immunoreactive signals was performed with a specific minicomputerized densitometric program developed for use with ImageJ analysis software as previously described [31].

3. Results

3.1. 1,3-Dinitrobenzene

Using TEER for evaluating the integrity of tight junctions between Sertoli cells, Fig. 2A shows that DNB disrupted the Sertoli–Sertoli cell junctions in a time and concentration dependent manner. This effect was seen at both concentrations, with disruption occurring after day 7 and progressing to complete disruption after 14 days of culture at the highest concentration. By 2–3 weeks of treatment, adherent cells at the highest concentration had completely dissociated from the culture plate. Dissociation from the plate occurred in the absence of overt cytolethality (Fig. 2C); however, the increased viability on day 21 may actually be a reflection of cell loss where the dead cells (mainly germ cells) have been phagocytosed by the Sertoli cells. Dinitrobenzene had no effect on the junctional proteins (Cx43 or Cldn11) when measured on day 5 (Fig. 2B), which was consistent with the absence of effects on TEER on day 5.

In order to evaluate whether DNB affected Sertoli cells and/or germ cells, the numbers of somatic cells (Sertoli plus peritubular cells) and germ cells were evaluated using flow cytometry. DNB induced an early increase in somatic cells (Fig. 2D) at day 7. From day 14 onward, there was no difference in the number of Sertoli cells at either concentration. Looking at the effects of DNB on spermatogenesis, exposure to either concentration resulted in a decrease in meiotic and post-meiotic germ cells (Fig. 2E).

3.2. Methoxyacetic acid

Methoxyacetic acid had a weak and transient effect on TEER early during the culture (Fig. 3A); returning to control levels after approximately 14 days of culture. On culture day 5, MAA exhibited a concentration-dependent decrease in the expression of Cx43 which corresponded with the early effects on TEER (Fig. 3B). Interestingly, levels of Cldn11 were not affected by MAA treatment, suggesting

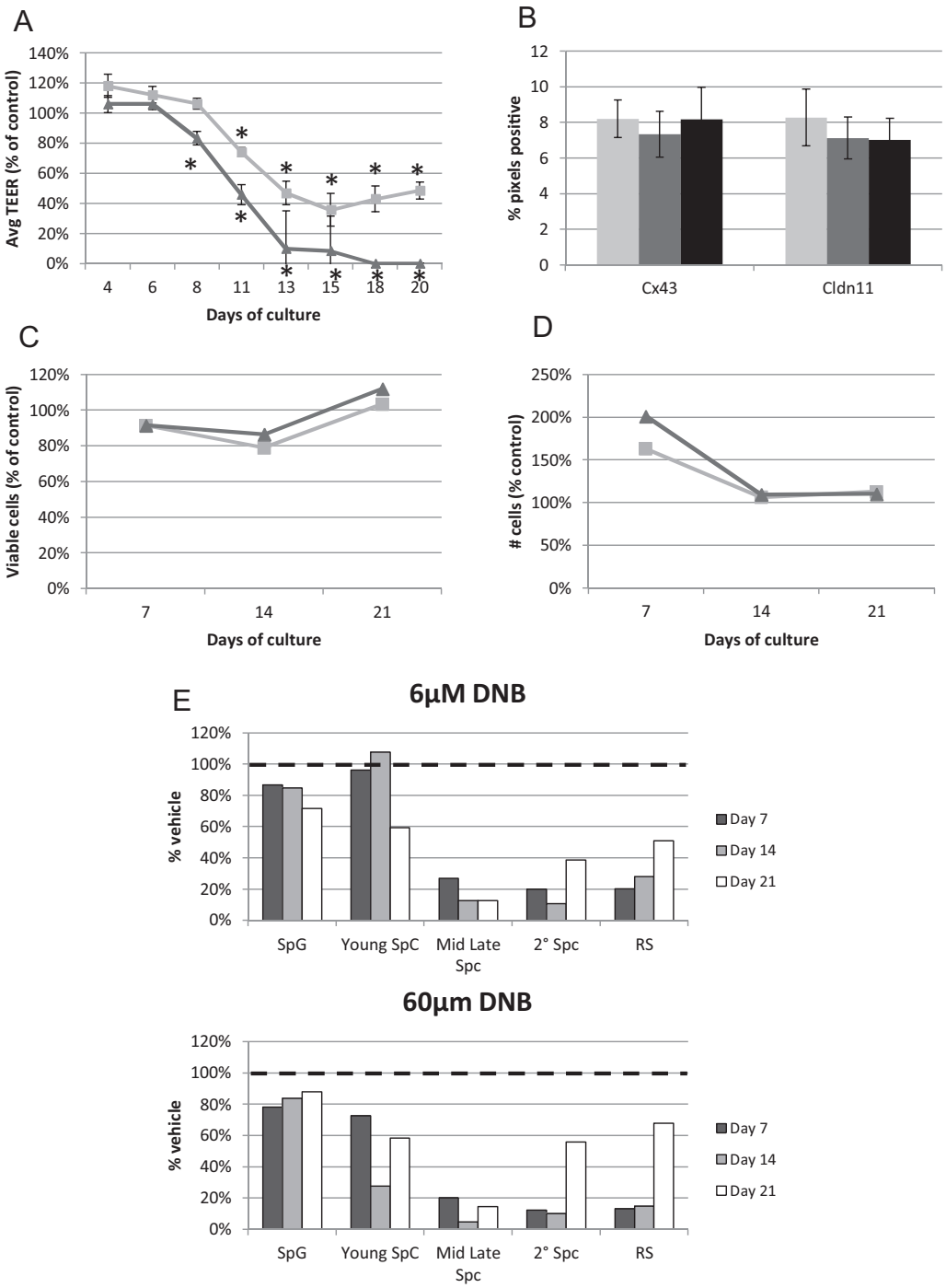

Fig. 2. Dinitrobenzene.
(A) TEER measurement as an *in vitro* surrogate for the blood-testis-barrier *in vivo*. Average TEER measurement in wells treated with either 6 μM (light grey) or 60 μM DNB (dark grey). Error bars reflect the standard error of the mean ($n = 6$). *$p < 0.05$ by one-way ANOVA. (B) Average Cx43 and Cldn11 positive pixels ($n = 25$ pixels; 2 experiments) in wells treated with either Vehicle (light grey), 6 μM (dark grey), or 60 μM DNB (black) on culture day 5. Error bars reflect the standard error of the mean. **$p < 0.05$ by ANCOVA (analysis of covariance). (C) Viability of all cells in the culture treated with either 6 μM (light grey) or 60 μM DNB (dark grey) relative to control wells. (D) Number of vimentin-positive somatic cells (primarily Sertoli cells) relative to control wells. 6 μM DNB (light grey) or 60 μM DNB (dark grey). (E) Relative number of cells in different stages of spermatogenesis over 21 days of culture. Dashed line highlights control values of 100%. Germ cell populations were separated on a FACS cell sorter based on ploidy and forward and side scatter as previously described [29]. Spermatagonia (SpG); young spermatocytes (Young SpC); mid-late spermatocytes (Mid Late SpC); secondary spermatocytes (2° Spc); round spermatids (RS).

some selectivity for MAA on the early disruption of the formation and maintenance of the Sertoli–Sertoli junctions.

The cells remained viable in culture over 3 weeks treatment with MAA (Fig. 3C). Somatic cell counts (predominantly Sertoli cells) were inversely correlated to the TEER (Fig. 3D). MAA caused an early increase in Sertoli cells. Although Sertoli cell counts dropped back to vehicle levels at the low concentration, they remained elevated at the high concentration for all 21 days. Treatment with 2.5 mM MAA showed an increase in young spermatocytes for the first 14 days of culture, followed by a dramatic decrease in

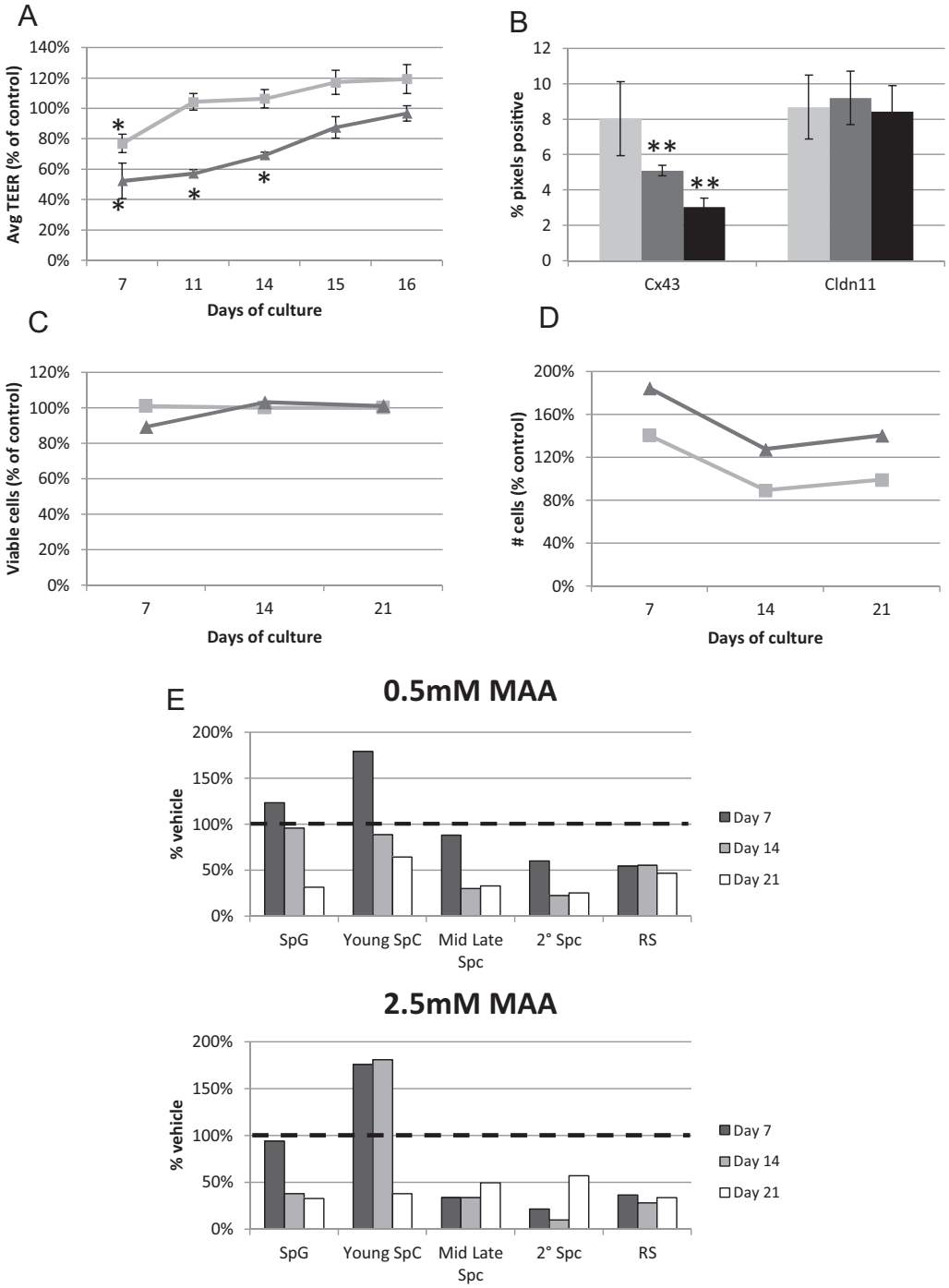

Fig. 3. Methoxyacetic acid.
(A) TEER measurement as an *in vitro* surrogate for the blood-testis-barrier *in vivo*. Average TEER measurement in wells treated with either 0.5 mM (light grey) or 2.5 mM MAA (dark grey). Error bars reflect the standard error of the mean ($n = 6$). $^{*}p < 0.05$ by one-way ANOVA. (B) Average Cx43 and Cldn11 positive pixels ($n = 25$ pixels; 2 experiments) in wells treated with either Vehicle (light grey), 0.5 mM (dark grey), or 2.5 mM MAA (black) on culture day 5. Error bars reflect the standard error of the mean. $^{**}p < 0.05$ by ANCOVA. (C) Viability of all cells in the culture treated with either 0.5 mM (light grey) or 2.5 mM MAA (dark grey) relative to control wells. (D) Number of vimentin-positive somatic cells (primarily Sertoli cells) relative to control wells. 0.5 mM MAA (light grey) or 2.5 mM MAA (dark grey). (E) Relative number of cells in different stages of spermatogenesis over 21 days of culture. Dashed line highlights control values of 100%. Germ cell populations were separated on a FACS cell sorter based on ploidy and forward and side scatter as previously described [29]. Spermatagonia (SpG); young spermatocytes (Young SpC); mid-late spermatocytes (Mid Late SpC); secondary spermatocytes (2° Spc); round spermatids (RS).

middle to late pachytene spermatocytes, secondary spermatocytes and round spermatids (Fig. 3E). After 3 weeks of treatment, toxic effects were seen in young spermatocytes as well. The response to MAA was concentration-responsive; exhibiting a similar, but less severe pattern at the low concentration of 0.5 mM.

3.3. Bisphenol A

Fig. 4A shows that BPA minimally affected the TEER at 5 μM, but dramatically reduced the TEER at the 50 μM concentration, suggesting a nearly complete disruption of the Sertoli–Sertoli barrier.

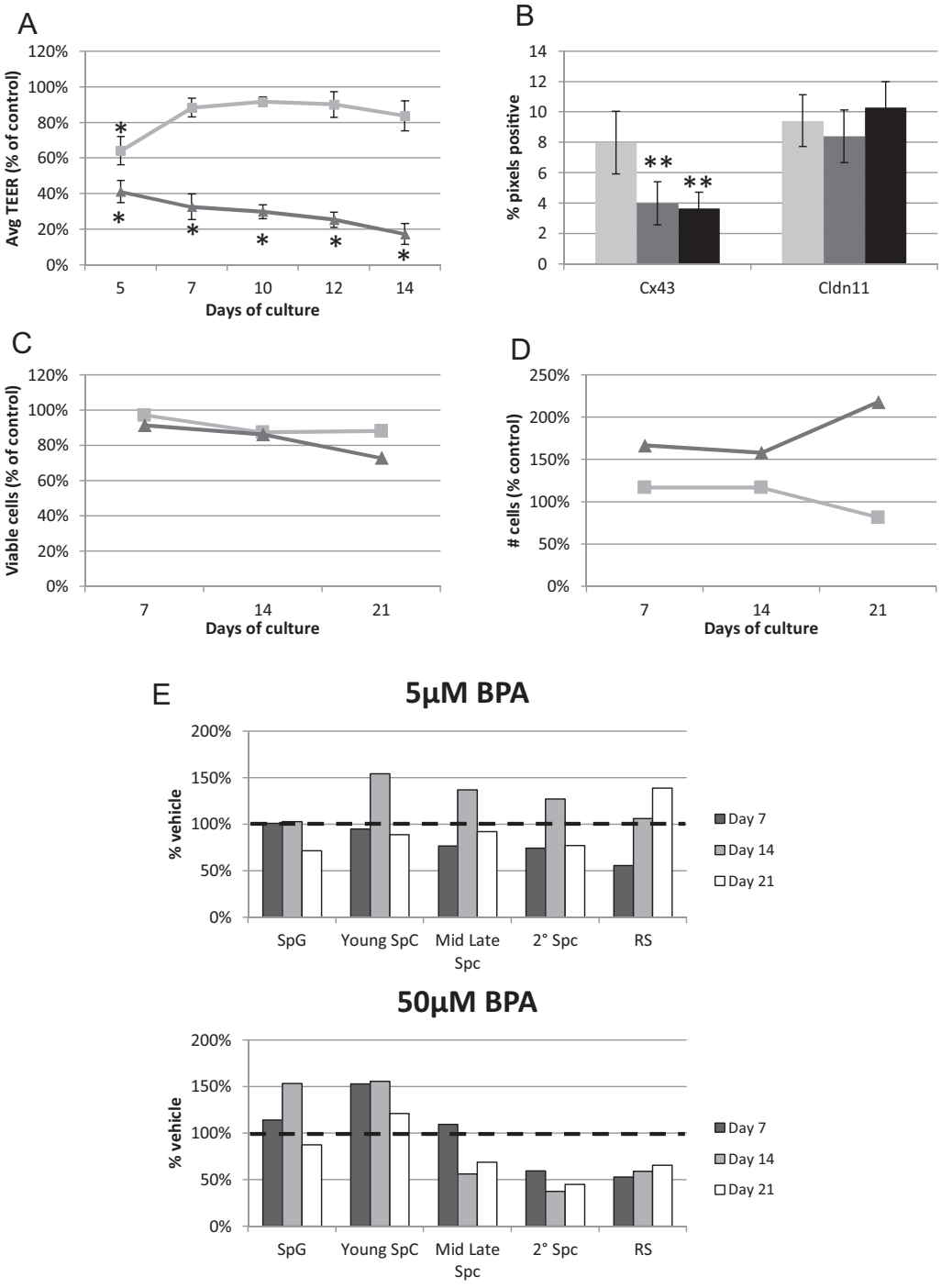

Fig. 4. Bisphenol A.
(A) TEER measurement as an *in vitro* surrogate for the blood-testis-barrier *in vivo*. Average TEER measurement in wells treated with either 5 μM (light grey) or 50 μM BPA (dark grey). Error bars reflect the standard error of the mean ($n = 6$). $^{*}p < 0.05$ by one-way ANOVA. (B) Average Cx43 and Cldn11 positive pixels ($n = 25$ pixels; 2 experiments) in wells treated with either Vehicle (light grey), 5 μM (dark grey), or 50 μM BPA (black) on culture day 5. Error bars reflect the standard error of the mean. $^{**}p < 0.05$ by ANCOVA. (C) Viability of all cells in the culture treated with either 5 μM (light grey) or 50 μM BPA (dark grey) relative to control wells. (D) Number of vimentin-positive somatic cells (primarily Sertoli cells) relative to control wells. 5 μM BPA (light grey) or 50 μM BPA (dark grey). (E) Relative number of cells in different stages of spermatogenesis over 21 days of culture. Dashed line highlights control values of 100%. Germ cell populations were separated on a FACS cell sorter based on ploidy and forward and side scatter as previously described [29]. Spermatagonia (SpG); young spermatocytes (Young SpC); mid-late spermatocytes (Mid Late SpC); secondary spermatocytes (2° Spc); round spermatids (RS).

As with MAA, both concentrations of BPA decreased expression of Cx43, but not Cldn11 (Fig. 4B). This is consistent with the observed TEER disruption for both compounds. After 14 days of treatment with 50 μM BPA, we began to see decreased viability (Fig. 4C). BPA had no effect on Sertoli cell counts at the low concentration, but induced a large proliferative response at the high concentration (Fig. 4D).

The response of developing germ cells to BPA at 50 μM, was similar to that seen with MAA, but was much less pronounced (Fig. 4E). After 14 days of culture, 50 μM BPA induced

approximately a 50% increase in spermatogonia and young spermatocytes and a decrease in middle to late pachytene spermatocytes, secondary spermatocytes and round spermatids. The loss of meiotic cells was still evident after 21 days of treatment. At 5 μM, the effects seen within the 21 days of treatment were variable, and there was no clear adverse effect on spermatogenesis.

3.4. Lindane

Lindane had a weak and transient effect on TEER (Fig. 5A). Until day 11, the TEER was similar (or slightly lower with 30 μM) in the presence or in the absence of lindane. After day 11, the TEER was higher than the control in the presence of 5 μM lindane ($p < 0.02$), and similar to the control at 30 μM lindane. The small Cx43 decrease in response to lindane treatment (Fig. 5B) in conjunction with the small TEER changes seen early after treatment suggest that lindane induces an early and reversible weak disruption of Sertoli cell function. The highest concentration of lindane resulted in a small decrease in viability over the 3 weeks of culture (Fig. 5C). As seen in Fig. 5D, lindane induced a concentration-responsive increase in Sertoli cell proliferation in the first week of treatment, which dropped as the culture progressed, but at 30 μM Sertoli cells remained elevated over control.

Within the first 7 days of culture, at both concentrations, lindane induced a clear decrease in total germ cells (data not shown). This decrease in total germ cells was due to a decrease of middle to late pachytene spermatocytes, secondary spermatocytes, and round spermatids (Fig. 5E). An apparent decrease in spermatogonia seen at day 14 was due to an unexplained increase of spermatogonia in the vehicle sample, rather than a true decrease in this cell population. This was likely an artifact of flow cytometry possibly due to low cell numbers and not a true reflection of the cells in culture.

4. Discussion

We conducted a pilot study to characterize the application of the Durand *ex vivo* rat seminiferous tubule culture model [3,7] for assessing chemically induced testicular toxicity. For this pilot study, we evaluated four well characterized compounds to determine how well an organotypic spermatogenic model correlates to known *in vivo* toxicity. The data presented here from a limited set of testicular toxicants demonstrated that this *ex vivo* culture system successfully identified all molecules as testicular toxicants and provided insight into the underlying mechanisms for testicular toxicity.

4.1. 1,3-Dinitrobenzene is a potent testicular toxicant targeting both Sertoli and germ cells

DNB is known to directly target Sertoli cells [8,32], and there is evidence that it has direct effects on developing germ cells [33]. Our data shows a direct toxicity on both Sertoli and germ cells in response to DNB. DNB was a potent disruptor of the TEER after a week of treatment at both concentrations. The lack of an effect on Cx43 and Cldn11 on day 5 is notable given the lack of an effect on TEER until day 8. The TEER measurement is a surrogate for the blood-testis-barrier and Sertoli cell function, supporting the known toxicity of DNB on Sertoli cells. *In vivo*, DNB causes a degeneration of pachytene spermatocytes, multinucleated and misshapen spermatids, and Sertoli cell vacuolation [8,9] which has been shown to be due to Sertoli cell induced apoptosis [33–35]. In this culture model, we saw a rapid depletion of germ cells at these same stages after 7 days of dosing-mid-late pachytene spermatocytes, secondary spermatocytes and round spermatids. The reduction in spermatids by DNB in our system is likely also due to injury to the preceding pachytene and secondary spermatocytes. DNB reduced the numbers of meiotic and post-meiotic cells at both concentrations within 7 days of culture- when the Sertoli–Sertoli junctional barrier was fully intact, as evidenced by TEER and Cx43. *In vivo*, a functional BTB is required to support spermatogenesis [36]. Given that the late stage meiotic germ cell toxicity precedes disruption of the TEER, we conclude that DNB may also directly target germ cell meiosis. Future experiments to more fully evaluate Sertoli cell function relative to meiotic germ cell viability will provide more definitive evidence as to whether or not germ cell degeneration is only a response to Sertoli cell injury. Although it may be present in our culture system, Sertoli cell vacuolation that is seen after treatment with DNB *in vivo*, was not visible in this system in the absence of vacuole-specific staining (*e.g.*, neutral red or eosine). Overall, the *ex vivo* system modeled the *in vivo* testicular toxicity of DNB-spermatocyte degeneration and Sertoli cell injury.

4.2. Methoxyacetic acid targets Sertoli cells and meiotic germ cells

MAA has been shown to disrupt histone acetylation during the prophase of meiosis [12] and may also induce germ cell apoptosis due to Sertoli cell injury [14,15]. In the current study, MAA disrupted the TEER within the first week of treatment, particularly at the high concentration. Interestingly, the Sertoli–Sertoli cell junctions were still able to recover as the culture progressed and actually formed a more stable barrier after 2 weeks of treatment with 0.5 mM MAA. At this time point, there were more Sertoli cells in the culture. Therefore, the reformation of tight junctions between these cells may be tighter (*i.e.*, increased TEER) than would be seen with fewer cells. Concurrent with the disruption of the Sertoli–Sertoli junctional barrier, we saw a concentration-responsive decrease in Cx43 expression on day 5. Cx43 is involved in the functional dynamics of the BTB and regulates other junction proteins within the seminiferous tubule [17,30,37]. *In vivo*, Cx43 knockouts still have a functional BTB, but disrupted spermatogenesis [38]. MAA may repress Cx43 enabling overexpression of downstream junction proteins such as occludin, resulting in an increased TEER. This would also explain the observed "recovery" of the Sertoli–Sertoli junctional barrier at the higher concentration of MAA. Along with TEER, we saw the total number of Sertoli cells return to near vehicle levels after 2 weeks of treatment. In spite of the apparent recovery of the Sertoli–Sertoli junctional barrier, the germ cell data confirms the toxicity of MAA. During the first week of treatment, MAA induced an increase in the number of spermatogonia and young spermatocytes with a corresponding decrease in middle to late stage pachytene spermatocytes, secondary spermatocytes and round spermatids relative to control. This suggests that MAA treatment is preventing young spermatocytes from completing meiosis, thus we see the accumulation of these cells. With longer time in culture, MAA causes a decrease in spermatogonia and young pachytene spermatocytes reflecting a potentially direct cytotoxic effect of MAA. All of the effects seen *in vitro* with MAA are aligned with previously published *in vivo* and *in vitro* data that MAA has a direct effect on both Sertoli cells and germ cell meiosis [12,14,15] and confirms that the toxic effect of MAA clearly targets pachytene spermatocytes [22].

4.3. BPA

BPA has been shown to have estrogenic properties and to have direct effects on both Sertoli cell tight junctions and spermatogenic meiosis [18,24,39–41]. In our *in vitro* study, BPA disrupted Cx43 and TEER at both concentrations on day 5. Similarly, exposure to BPA *in vivo* has been shown to disrupt the BTB through impaired expression of junction proteins, including Cx43, between Sertoli cells [42,43]. Similar to MAA, treatment with 5 μM BPA induced a transient disruption of TEER which returned to near normal

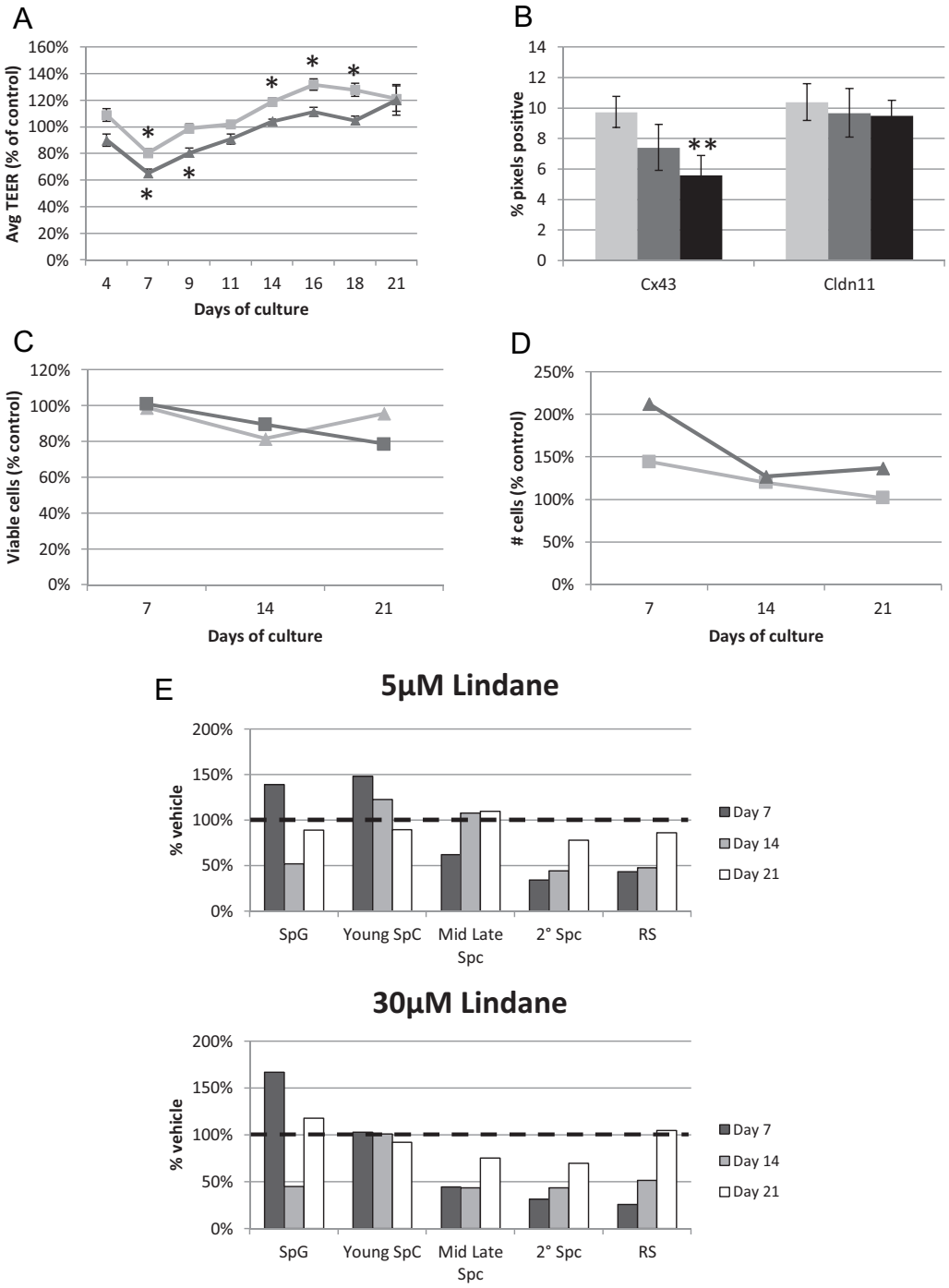

Fig. 5. Lindane.
(A) TEER measurement as an *in vitro* surrogate for the blood-testis-barrier *in vivo*. Average TEER measurement in wells treated with either 5 μM (light grey) or 30 μM lindane (dark grey). Error bars reflect the standard error of the mean ($n = 6$). *$p < 0.05$ by one-way ANOVA. (B) Average Cx43 and Cldn11 positive pixels ($n = 25$ pixels; 2 experiments) in wells treated with either Vehicle (light grey), 5 μM (dark grey), or 30 μM lindane (black) on culture day 5. Error bars reflect the standard error of the mean. **$p < 0.05$ by ANCOVA. (C) Viability of all cells in the culture treated with either 5 μM (light grey) or 30 μM lindane (dark grey) relative to control wells. (D) Number of vimentin-positive somatic cells (primarily Sertoli cells) relative to control wells. 5 μM lindane (light grey) or 30 μM lindane (dark grey). (E) Relative number of cells in different stages of spermatogenesis over 21 days of culture. Dashed line highlights control values of 100%. Germ cell populations were separated on a FACS cell sorter based on ploidy and forward and side scatter as previously described [29]. Spermatagonia (SpG); young spermatocytes (Young SpC); mid-late spermatocytes (Mid Late SpC); secondary spermatocytes (2° Spc); round spermatids (RS).

levels within 14 days, but without proliferation of Sertoli cells. These results suggest that low concentrations of BPA delay the reformation of the Sertoli–Sertoli junctions after the collagenase digestion used to prepare the culture. With 50 μM BPA, the TEER disruption was maintained throughout all 21 days of culture, along with apparent Sertoli cell proliferation. Mature Sertoli cells are generally thought to be terminally differentiated, however a number of publications have provided evidence that Sertoli cells can be induced to return to an immature state. Sertoli cell proliferation has been associated with disruption during cell culture, male infertility, and BTB disruption [44–47]. Sertoli cells have also been shown to proliferate in response to disruption of the gap junction

[48], as have retinal epithelial cells in response to knockdown of the junctional protein, occuldin [49]. We hypothesize that the Sertoli cell population is proliferating in response to the disruption of Sertoli–Sertoli cell communication.

BPA had a concentration-dependent impact on spermatogenesis. We saw minimal, if any, effect with 5 μM BPA, but a significant effect at 50 μM. 50 μM BPA disrupted spermatogenic progression from young spermatocytes to pachytene spermatocytes; there was an increased number of young spermatocytes, along with a decreased number of mid-late pachytene spermatocytes, secondary spermatocytes and round spermatids. Similarly, *in vivo*, BPA causes reduced sperm counts, and an increased number of stage VII tubules, and a decrease in stage VIII tubules [50,51]. A recent publication demonstrated that BPA directly disrupts completion of germ cell meiosis I [51] through estrogen receptor signaling and germ cell apoptosis.

4.4. Lindane

Lindane directly targets the Sertoli cell, leading to the inability to maintain spermatogenesis. Defamie et al. demonstrated that, *in vitro*, lindane disrupts Sertoli cell signaling through gap junctions at concentrations ≥50 μM [26]. In our study, we observed a concentration-responsive disruption of Cx43 within 5 days of treatment, accompanied by a mild reduction in TEER. As we saw with other treatments, Sertoli cells proliferated in response to the loss of Sertoli–Sertoli cell junctions.

In our study, lindane induced disruption of meiosis within 7 days at 30 μM, but had no effect on young spermatocytes even after 21 days of treatment. In addition to the direct effects on Sertoli cells, lindane also induces oxidative stress in the testes [52–55]. Lue et al. demonstrated that heat-induced oxidative stress causes germ cell apoptosis specifically at the pachytene spermatocytes stage, which is the specific stage where spermatogenesis was disrupted in our study [53]. Interestingly, we did not observe accumulation of spermatogonia and early spermatocytes which is consistent with the cells undergoing apoptosis during the pachytene stage, as opposed to inhibition of meiosis. In contrast, both MAA and BPA exhibited a characteristic increase in young spermatocytes as meiotic progression was impaired, causing early germ cells to become "stuck" at these stages of development, demonstrating impairment of the Sertoli cell's ability to regulate meiosis with MAA and BPA treatment.

4.5. The Durand model can be used to provide mechanistic insight and study molecules for testicular toxicity risk

In this study, we demonstrated that the Durand *ex vivo* rat seminiferous tubule culture model [3,7] was able to reliably reproduce testicular toxicity reported *in vivo* for four compounds. Table 2 compares the *in vitro* results from this study with the testicular toxicity reported *in vivo*. The table shows that the *in vitro* findings correlated well with previously published *in vivo* and *in vitro* findings. We have included *in vivo* exposure data for the compounds to provide context, however, these exposures would not necessarily be directly relevant to *in vitro* concentrations. The plasma exposure is not necessarily the same as the tissue exposure, nor were they collected from the published *in vivo* toxicity studies. Factors such as route of administration and vehicle can significantly affect exposure levels. All four compounds had diverse underlying mechanisms of toxicity that manifested in this study. The different responses seen *in vitro* demonstrate that mechanistic hypotheses can be developed from these data, guiding further studies for validation. From the pilot studies presented here, we were unable to clearly demonstrate whether the compounds had a direct effect on germ cells or if they impaired the ability of Sertoli cells to support spermatogenesis. This could be assessed using a modified co-culture system, where Sertoli cells are treated with compound in the absence of germ cells, followed by the addition of germ cells after compound wash-out [56].

Recent studies revealed that the gap junction protein Cx43, is crucial for spermatogenesis [48,57–59]. In addition, it has been reported that chemicals could alter Sertoli/germ cell interaction through disruption of Cx43 gap junctions, as well as other junction types leading to BTB destabilization [17,38,42,60]. All four compounds evaluated were shown to directly target Sertoli cells as seen by the disruption of TEER and Cx43. In the BTB *in vivo*, Cx43 is highly intermingled with proteins of tight (*e.g.*, occludin, claudin, ZO-1) and adherens junctions (*N*-cadherin) [57]. Occludin- and claudin-based tight junctions are the major components of the blood–testis barrier [61,62]. Among the claudin family, the claudin-11 isoform is the crucial protein for tight junction formation, barrier integrity in the testis and fertility [63]. The lack of an effect on claudin-11 in the present studies warrants further investigation.

All four compounds were shown to target developing germ cells. The observed differences between the response to each compound highlight the different mechanisms of toxicity, as seen with MAA and BPA compared to lindane. All four compounds blocked spermatogenesis at the meiotic transition to late spermatocytes, but only lindane had no effect on the pre-meiotic population. These findings showed organ-specific effects on developing germ cells. At the concentrations tested, all four compounds have been shown to be non-cytolethal in other cell lines [64–70]. Similarly, none of the compounds were cytolethal to somatic cells, and we did not observe overt cytolethality other than a 30% loss of viability with BPA.

The design of the studies presented here help to address whether or not a compound disrupts spermatogenesis. Previous work by Durand et al. [1,71–73] has demonstrated the reproducibility of the model. Given the objectives of the pilot study, we ran the experiment much like a standard *in vivo* toxicity study, including replicates within the study. Like live phase studies, repeating these experiments would require increased consumption of animals without significantly altering the interpretation of the data. The three week design of the study in conjunction with spermatogenesis progression provides insight into the mechanism of toxicity, as well as internal validation of the results, even in the absence of replicate studies. The progressive nature of the different cell types (*e.g.*, young spermatocytes maturing to round spermatids) provides another layer of confidence in these results. Compound-induced effects on specific cell populations at the lower concentration were also seen at the higher concentration.

Pilot experiments as represented here, provide a basis for secondary, more specific, experiments that expand upon these initial findings. For example, MAA caused a "backup" of spermatagonia and young spermatocytes without progression to the pachytene spermatocyte stage. Although this is concordant with inhibition of meiotic progression, focused studies would be required to specifically address the nature of this inhibition. A second *in vitro* system developed by Durand's team provides a platform to focus on specific stages of spermatogenesis (see for example, [20,30,72,74]). A caspase or TUNEL assay could be used to confirm that apoptosis was occurring in response to lindane, but not MAA and BPA. Other techniques such as siRNA or antibody studies can be used to identify on- *vs.* off-target toxicity; cells can be harvested for gene expression studies or western blots.

Other *in vitro* models have been developed for predicting testicular toxicity, most commonly Sertoli cell cultures of transformed mouse Sertoli cells, but additional models are still needed. In fact, a recent HESI workshop highlighted this need for new models, and went so far as to propose biomedical engineering as a solution [79]. Although several current models are useful in understanding the

Table 2
Summary of *in vitro* results compared with *in vivo* testicular toxicity.

Compound	Published *in vivo* effects and mechanism	*In vitro* findings in this study	*In vivo* exposure		
			Ref.	Dose (route)	Exposure
1,3-Dinitrobenzene (6 and 60 μM)	Sertoli cell vacuolation at 25 mg/kg [8,9]	Sertoli cell injury evident by TEER disruption at 6 μM and 60 μM Unable to assess vacuolation without specifically staining for vacuoles	[75]	25 mg/kg (IP)	AUC = 144 μM h C_{max} = 50 μM
	Severe spermatocyte degeneration at 25 mg/kg [8,9]	Loss of pachytene spermatocytes and round spermatids with 6 μM within 1st week At 60 μM, all meiotic cells degenerated within 1st week			
Methoxyacetic acid (0.5 and 2.5 mM)	Sertoli cell vacuolation and decreased lactate levels with ≥3 mM MAA *in vitro* [15] Degeneration of pachytene spermatocytes at 150 mg/kg [11] Targets meiosis *via* histone hyperacetylation [12]	Concentration-responsive TEER disruption in 1st week and decreased Cx43 expression indicates Sertoli cell injury "Backup" of spermatagonia and young spermatocytes without progression to pachytene spermatocyte stage is concordant with inhibition of meiotic progression	[76]	250 mg/kg 2-ME (oral)	C_{max} = 4.83 mM
Bisphenol A (5 and 50 μM)	Neonatal exposure to 400 μg/kg (subq) reduced Cx43 expression and fertility [43] Disrupts Sertoli cell-cell signaling at 50 mg/kg *in vivo* [42]	Concentration-responsive TEER disruption in 1st week and decreased Cx43 expression indicates Sertoli cell injury	[77]	100 mg/kg (oral)	AUC = 1.5 μM h C_{max} = 208 nM
	Disruption of meiotic progression at 200 μg/kg [51] Disruption of meiosis through ER signaling [43]	Disruption of spermatogenic progression from young spermatocytes to pachytene spermatocytes at 50 μM indicates disruption of meiosis			
Lindane (5 and 30 μM)	Disruption of Sertoli cell gap junctions *in vitro* [26]	Disruption of Sertoli cell gap junctions evident by concentration-responsive disruption of Cx43 within 5 days of culture, and mild reduction in TEER	[78]	3 mg (topical)	AUC = 49 nM h
	Induces apoptosis in Sertoli and germ cells at 40 mg/kg [54]	Loss of pachytene spermatocytes and spermatids within 7 days is consistent with apoptosis of pachytene spermatocytes		120 mg (topical)	AUC = 161 nM h
	Leydig cell toxicity at 40 mg/kg [54]	Culture system does not contain Leydig cells			

mechanism of a Sertoli cell toxicant, they are limited by the inability to assess the impact on spermatogenesis. The ability to evaluate effects on spermatogenesis using this organotypic *ex vivo* seminiferous tubule model may provide insight into more subtle effects that are not obvious with a single cell type assay. Attempts have also been made to integrate a battery of *in vitro* assays to predict *in vivo* reproductive toxicity. For example, the ReProTect project (www.reprotect.eu) integrates a battery of 14 assays to predict male and female fertility disruption, and developmental toxicity. The battery of assays was not able to reliably predict male fertility toxicity, including the inability to predict MAA and nitrobenzene toxicity, and partially predicted BPA toxicity [80].

As with all *in vitro* assays, one needs to understand the limitations of the model. The current system does not include Leydig cells, and is therefore, unable to detect compounds that primarily target this cell population in the testes. Although the correlation to adult testes has been previously demonstrated [1] cells are collected from prepubertal animals and may not fully model the adult testes. This is particularly evident when interpreting the TEER results, where the TEER may be reduced due to a true disruption of already formed Sertoli–Sertoli junctions, or an inhibition of the formation of these junctions. The assay effectively supports spermatogenesis, but this makes it a long term assay, reducing the throughput. We have demonstrated that the first 2–3 weeks are enough time to identify disruption of meiosis and Sertoli cell function. As with many cell culture systems, the cells are intolerant of high concentrations (>0.3%) of solvents, such as DMSO or ethanol. Compound metabolism in this culture system has not been thoroughly evaluated, but we do not anticipate extensive metabolism. Because of this, the model only evaluates potential testicular toxicity of the added compound. This may be beneficial since testing of parent compound and specific metabolites could help to guide the structural changes to limit testicular liability.

In conclusion, we demonstrated the ability of this organotypic *ex-vivo* rat seminiferous tubule culture model to simulate *in vivo* histological changes in response to four prototypical toxicants. We observed Sertoli cell toxicity caused by all four compounds. Disruption of spermatogenesis, as seen *in vivo*, was also determined *in vitro*, and we were able to hypothesize different mechanisms of spermatogenic toxicity that aligns with the known mechanism of toxicity. This data suggests that pursuit of additional studies to further validate the use of this model to predict compound-induced *in vivo* testicular toxicity are warranted. If successful, this model has merit for use identifying potential testicular toxicants and as a mechanistic mode of action tool.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2016.01.003.

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