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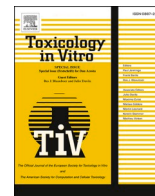
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A comparative study of the effects of 3 testicular toxicants in cultures of seminiferous tubules of rats or of domestic cats (veterinary waste): An alternative method for reprotoxicology

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ABSTRACT

In response to the EU cosmetics directive regulation and REACH legislation which encourage cell culture methods in order to reduce or replace the use of animals in toxicology studies, we settled the culture of prepubertal domestic cat seminiferous tubules in our validated BioAlter® model, usually used with prepubertal rat, called here BioAlter®-rat, by opposition to BioAlter®-cat settled here. We carried out a comparative study on the effects of 3 testicular toxicants, 1,3-dinitrobenzene at 60 µM, 2-methoxyacetic acid at 2.5 mM and carbendazim at 50 nM or 500 nM in both BioAlter®-cat and BioAlter®-rat over a 3-week culture period. Sertoli cell or each germ cell populations as well as the levels of Sertoli cell or germ cell specific mRNAs were studied. The harmful effects of the 3 toxicants on pre-meiotic, meiotic and post-meiotic cell numbers and on Sertoli or germ cell specific mRNAs were clearly observed in the two species, even if there might be some small differences in the intensity of the effects on some of the studied parameters. Hence, BioAlter®-cat might be a solution to the requirements of the EU cosmetics directive and REACH legislation for male reproductive toxicology studies.

1. Introduction

In vivo reproductive toxicology studies generally require rodents (mice or rats) and non-rodents (beagle dogs, rabbits, mini pigs or non-human primates) for reviews (Andersen, 2005; Son et al., 2020). In response to the requirements of the EU cosmetics directive regulation and REACH legislation which encourage cell culture methods in order to reduce or even replace the use of animals, we have settled the culture of prepubertal (6-month-old) domestic cat (*Felis catus*) seminiferous tubules in our validated BioAlter® model which is used with 20–22-day-old rat seminiferous tubules (Staub et al., 2000), called in this paper BioAlter®-rat by opposition to BioAlter®-cat we settled in this work. Domestic cats are usually neutered (veterinary waste) between 6 and 7 months because the spermatogenic function in male cats matures at 8 to 10 months of age (Tsutsui et al., 2004). Indeed, compared with entire male cats, neutered cats have a greater longevity of 1.8 years (O'Neill et al., 2015). Thus, by using BioAlter®-cat, we could answer to the

European directives.

Our Bio-Alter® technology (BioAlter®-rat) is a 3D cell culture process which allows achieving 80% of spermatogenesis *in vitro* in a serum free culture medium (Hue et al., 1998; Staub et al., 2000). This culture process ensures the maintenance (or reformation) of the blood-testis barrier and allows studying the mitotic and meiotic phases of spermatogenesis and the beginning of spermiogenesis (Durand et al., 2020; Staub et al., 2000). The Sertoli cells remain polarized which is crucial for the differentiation of germ cells. Thus, the introduction of a substance in the basal medium of the bicameral chamber of culture can mimic the access constraints to meiotic germ cells, as it occurs, *in vivo*, in the testis (Perrard et al., 2009b, 2010). Our model has been carefully validated on a physiological (Geoffroy-Siraudin et al., 2010; Perrard et al., 2003, 2010) and a toxicological points of view (Goldstein et al., 2016), and is relevant to investigate the mechanisms of action on spermatogenesis of heavy metals (Carette et al., 2013; Geoffroy-Siraudin et al., 2010, 2012) pesticides or pollutants either separately or in cocktail (Ali et al., 2014;

Abbreviations: DNB, 1,3-dinitrobenzene; MAA, 2-methoxyacetic acid; CBZ, Carbendazim; DMSO, dimethylsulfoxyde; TH2B, Testis specific histone 2B; STMN1, Stathmin 1; TP1, Transition Protein 1; TP2, Transition Protein 2; AR, Androgen receptor; TJP1, Tight junction protein 1; Erα, Estrogen receptors alpha; Erβ, Estrogen receptors beta.

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Blondet et al., 2022; Carette et al., 2015; Durand et al., 2017, 2020; Pisani et al., 2016); during a 3-week culture period. In addition, BioAlter®-rat decreases the number of sacrificed rats by 20 to 30 as compared to *in vivo* studies; in agreement with the European REACH directive.

In the present study, we settled first the culture conditions for cat seminiferous tubules (BioAlter®-cat). Then, we carried out a comparative study on the effects of three well-characterized testicular toxicants, 1,3-dinitrobenzene (DNB), 2-methoxyacetic acid (MAA) and carbendazim (CBZ) in both BioAlter®-cat and BioAlter®-rat. It is important to note that the three tested compounds had not previously been examined for effects on the cat reproductive system although they had undergone extensive study in the rat (see below). DNB is a nitroaromatic compound used in the production of polymers, pesticides and dyes. *In vivo* studies have shown that DNB induces severe effects including Sertoli cell vacuolation, spermatocyte depletion and multinucleated and misshapen spermatids (Elkin et al., 2010; Hess et al., 1988). MAA is the toxic metabolite of 2-methoxyethanol, a solvent used in printing inks, varnishes, and as a de-icing additive. The toxic effect of MAA on pachytene spermatocytes, has been described *in vivo* (Chapin et al., 1984; Wade et al., 2008). CBZ is a broad-spectrum benzimidazole, a widely used fungicide, known to induce *in vivo* testicular alterations such as the decrease of germ cell number (Hess et al., 1991; Yu et al., 2009). With BioAlter®-rat, we have also shown that the most striking effect of low doses (50 nM and 500 nM) of CBZ is a dose-dependent reduction of the round spermatid number, associated to an endocrine-disrupting effect by regulating the mRNAs of the androgen-dependent round spermatid-specific genes, TP1 and TP2 (Carette et al., 2015).

2. Material and methods

2.1. Animals

Experiments were performed with either 20–22-day-old male Sprague-Dawley rats (Charles River, Saint-Germain-Nuelles, France) having undergone no treatment, or testes (veterinary waste) from 6 month-old domestic (European shorthair) cats weighing 2.5 to 3 kg and living in an apartment or outside obtained from a veterinary clinic.

For rats, the experimental protocol was designed in compliance with recommendations of the European Economic Community (EEC) (86/609/EEC) for the care and use of laboratory animals. Testes from four 20–22-day-old rats were pooled in every culture experiment.

Cats were neutered by veterinarians of a veterinary clinic close to the laboratory. Cat owners and their cat were received the same day, early in the morning, by veterinarians so that seminiferous tubule preparation and culture can be performed quickly. Immediately after castrations, cat testes were put in culture medium (15 mM HEPES-buffered F12/DMEM supplemented with antibiotics) and stored at +4 °C during the 20 min of travel to the laboratory. Testes from 2 or 3 cats were pooled in every culture for each toxicant.

2.2. Histological study of cat testes

Before putting in culture, small pieces of cat testes were fixed in Bouin's fixative for 12–24 h, dehydrated and embedded in paraffin. Sections (4 µm) were deparaffinized and rehydrated. The nuclei of cells were stained with Harris hematoxylin (Merck, Darmstadt, Germany).

2.3. Preparation and culture of rat or cat seminiferous tubule segments

Rat seminiferous tubule segments were obtained and cultured as previously described, (Hue et al., 1998). Cat seminiferous tubule segments were prepared as previously described for the rat, seeded (Day 0) at about 300,000 cells/cm² in bicameral chambers (Sigma, Saint-Quentin-Fallavier, France) and cultured in the same medium as for the rat but supplemented with 0.2% fetal calf serum (Thermo Fisher

Scientific, Villebon sur Yvette, France).

When required, toxicants were added in the basal compartment of the bicameral chamber of culture from day 2 of the cultures. Stock solutions of DNB or CBZ were prepared in DMSO as described previously (Carette et al., 2015; Goldstein et al., 2016), and then diluted sequentially to obtain the working solutions in 0.1% or 0.003% DMSO for DNB 60 µM or CBZ (500 nM or 50 nM) respectively. The higher concentration of DMSO (0.1%) has previously been shown to be nontoxic 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 (Goldstein et al., 2016) then diluted in culture medium to obtain the working concentration: 2.5 mM.

The medium in the basal compartment was renewed every 2 days. At selected days of culture, cells were detached from the culture dishes and isolated with trypsin for determining cell number, and cell viability by trypan blue exclusion or cell flow cytometry analysis.

DNB and MAA were tested at concentrations based on previously published *in vitro* data. For DNB, the concentration was 60 µM based on induction of apoptosis in TM4 Sertoli cell culture at 10 µg/ml (60 µM) (Lee et al., 2009). For MAA, the concentration of 2.5 mM was based on 3 mM MAA causing a decrease in lactate in Sertoli cell cultures (Beattie et al., 1984). In regards to CBZ, 50 nM and 500 nM were the concentrations we tested previously in the rat (Carette et al., 2015).

2.4. Immunolabeling of cultured cells for flow cytometric analysis

Cultured cells from seminiferous tubules were fixed in 70% ethanol. To distinguish germ cells from somatic cells, the latter are mainly Sertoli cells as rat and cat possess only one layer of peri-tubular/myoid cells [(França and Godinho, 2003; Maekawa et al., 1996)] cells were immunolabeled with a monoclonal antibody against vimentin (clone V9; DAKO SA, Trappes, France) that was conjugated with a green fluorochrome, Alexa 488, by using a Zenon Mouse labeling kit (Life Technologies, Cailloux-sur-Fontaines, France) as previously described (Durand et al., 2017; Perrard et al., 2009a). Then, cells were analyzed using an Attune Acoustic Focusing Cytometer (Applied biosystems) as described (Carette et al., 2015; Durand et al., 2017). Acquisition and analysis were performed using the Attune Cytometric Software. The vimentin-positive somatic/Sertoli cells and the vimentin-negative tetraploid, diploid and haploid germ cells were separated with the bivariate analysis of DNA content/vimentin. Then, the bivariate linear forward light scatter and linear side angle light scatter analyses allowed the identification of the different populations of cells (Durand et al., 2017; Godet et al., 2000; Perrard et al., 2009a).

2.5. RT-qPCR

At selected days of culture, total RNA was extracted from cultured seminiferous tubule cells using TRI-REAGENT (Euromedex, Souffelweyersheim, France) according to the manufacturer's protocol. All samples were treated by DNase I before the reverse transcription; first strand cDNA was synthesized from 2 µg RNA with Super Script III in the presence of oligodT and Random Primer (all from Thermo Fisher Scientific, Villebon sur Yvette, France). Realtime PCR was performed in a Step One Plus instrument (Applied biosystems) by using SYBR-Green (Qiagen, Courtaboeuf, France). Rat or cat primers sequences are presented in Table 1 or Table 2 respectively. Mitochondrial ribosomal proteins S18 and S16 mRNAs were used as housekeeping gene controls.

2.6. Statistical analyses

Statistical analyses were performed using Graphpad and R softwares. Data are presented as the means ± S.E.M. Data were analyzed by paired Student's *t*-test to compare treated cells with their corresponding controls. When required, values were logarithmically transformed to eliminate heterogeneity of variance and achieve a reasonable assumption of

Table 1
Sequence of the primers used for rat qPCR.

	fwd-5'	rev-5'
TP1	CGACCAGCCGCAAATAAAG	ATCATCGCCCGTTTCCTAC
TP2	GGCCTCAAAGTCACACCAAT	CTTGCTCACTTTCCCTTCCA
STMN1	AAGGATCTTTCCTGGAGGA	TTCTCCTCTGCCATTTGCT
Clusterin	TTCAATCCCTCCAGTCCAAG	CACGAGAGGGGACTTCTGAG
Vimentin	AGATCGATGTGGACGTTTCC	CACCTGTCTCCGGTATTCGT
AR	GTGTCGTCTCCGAAATGTT	GGAATCAGGCTGGTTGTTGT
TJP1	TGCTCCAGCAGGTCCCTAAGT	TGGTAGCTGAGGGCAGAACT
Occludin	GAGGGTACACAGACCCAGA	CAGGATTGCGCTGACTATGA
Claudin-11	CTGGTGGACATCCATCATCCT	GCGCATACAGGAAACCAGAT
Claudin-4	GATGGTCATCAGCATCATCG	GAAGCCACCAGAGGGTTGTA
Cx 43	TCCTTGGTGTCTCTCGCTTT	GAGCAGCCATTGAAGTAGGC
16S	AGTCTTCGGACGCAAGAAAA	AGCCACCAGAGCTTTTGAGA
18S	ATCATCTTCCATGCCCATTA	AAGTTCAACCCGACAACAAC

normal distribution (Bland and Altman, 1996). $P < 0.05$ was considered significant.

3. Results

3.1. Settlement of the cat model: BioAlter®-cat

3.1.1. Histological study of the testes

In 6-month-day-old cat seminiferous tubules, a lumen was observed

and the seminiferous epithelium presented its characteristic stratification with the presence of meiotic cells and some rare round spermatids which were usually the most mature germ cells observed (Fig. 1) (França and Godinho, 2003). Thus, the most differentiated cat germ cells present at the beginning of the culture were pachytene spermatocytes and some rare round spermatids.

3.1.2. Culture of the cat seminiferous tubules

After 9 days of culture, the shape of the seminiferous tubules tended to disappear. Sertoli cells and germ cells have migrated from the seminiferous tubules and spread out on the membrane of the bicameral chamber (Fig. 2). The cell layer thus remained stable over the 3 weeks of culture.

3.1.3. Analysis of the cat cell populations

A representative flow cytometry analysis of the seminiferous tubule cell populations of a pool of two 6-month-old cats on day 0 (before the culture) is shown in Fig. 3. After elimination of the somatic cells on the basis of their positive reaction with an anti-vimentin antibody (see Material and Methods), the separation of germ cells according to their forward light scatter (FSC) and side angle light scatter (SSC) allowed the identification of 4 different cell populations: (i) spermatogonia; (ii) 4C cells including primary (leptotene, zygotene pachytene and diplotene) spermatocytes; (iii) secondary spermatocytes which are newly formed or at the beginning of prometaphase II and doublets of round spermatids

Table 2
Sequence of the primers used for cat RTqPCR.

	fwd-5'	rev-5'
TP1	GTCGACCGCCGCAAATTCAA	TCGGACTGCCCTTCCGGTATT
TP2	CCGAGGCACCAGAAACACAGCAT	GCCCTGAGTTCGCCCTCTTTGT
STMN1	TGCGGGAGAAGGACAAAGCACAT	GCTTCAGTTTCATCAGCAGGGTCTT
Clusterin	GGCTGTGTGTGACCTGTGAGAAT	AGCAGGGATTTGCGCTCTTCGT
Vimentin	TGGAAGGCGAGGAGAGCAGGAT	AGTGAGTGTCAACCAGAGGAAGTGA
AR	AGCCGCTGAAGGGAACAGAAGT	GCTTCCGGGTCCAGAGTCA
TJP1	AGGCGGATGGTGCTACAAGTGA	CCCGCTTCTGTATCTGTGTCTTCA
Occludin	ACGGAACCGGCTACGGCTAT	AGGCCACCATGGCCAGGAGAA
Claudin-11	TCGTGGGTGGCTGTGTATCATCGT	TGGAGCCCAAGAGTAGTAGAAACG
Claudin-4	ATCGTGGCGGGTGTGGTGT	GCGGGTGTAGAAGTCCCGGATGA
Cx 43	GCCTGTGCGAACCTACATCA	CTGTGGGACGAGAGGAAGCA
ER α	GCCAGTCGGGCTCGCCTAT	GGGCTCGGAGACACGCTGTTG
ER β	TTGGCAACGACTCAAGGTT	GGGTGCATACTGGAGTTGAG
16S	CGCCTTGCCCTGAGTGGCTGTA	CGGCCATCCCTAGGGCACTT
18S	CGATGGGCGGGAAGATAGC	TGAGCTCTCTGCCCTCTTGGT

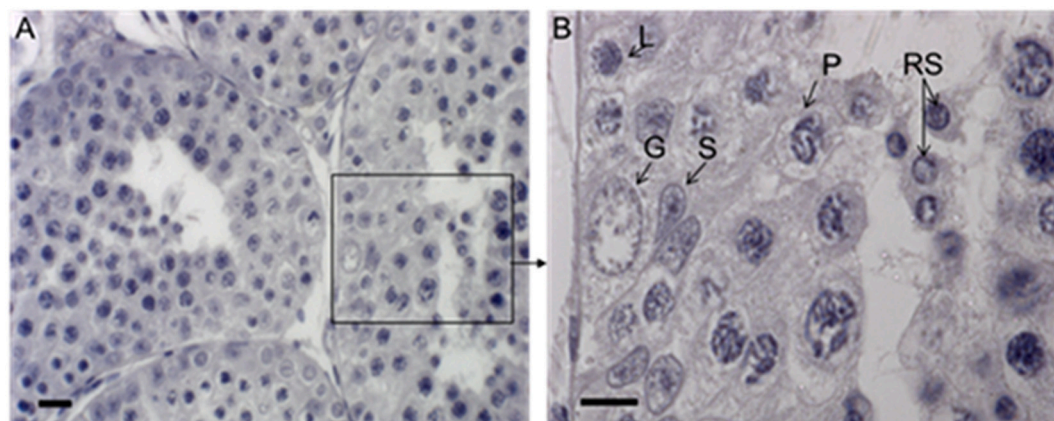


Fig. 1. Cross section from a 6-month-old cat testis.

1A: A lumen was observed in the seminiferous tubules.

1B: At a higher magnification of the inset of 1A, a stage V of the seminiferous epithelium was visualized. Sertoli cells (S), a spermatogonia A (G), meiotic cells (leptotene and pachytene spermatocytes (L and P respectively)) and some round spermatids (RS) were identified according to the size and aspect of their nuclei (França and Godinho, 2003).

Bars represent 10 μ m.

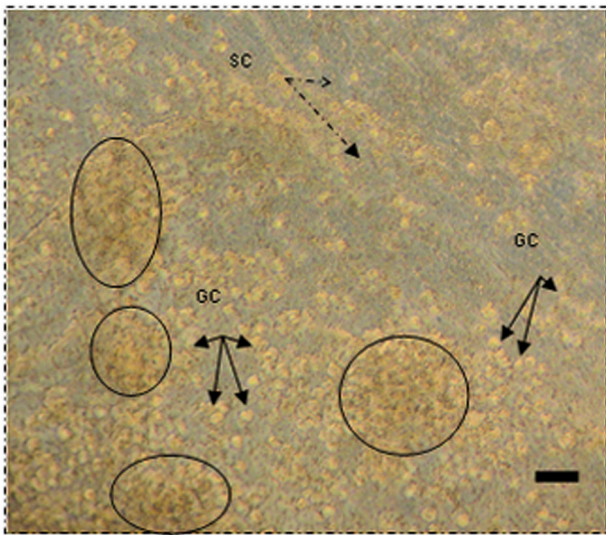


Fig. 2. Morphological aspect of cat seminiferous tubules cultured for 9 days. The oval lines showed remains of seminiferous tubules which have adhered to the membrane of the bicameral chamber. Somatic cells (SC) have migrated from the seeded seminiferous tubules to form the light gray carpet on the insert membrane (dotted arrows). Germ cells (GC) were carried by Sertoli cells outside the seminiferous tubules. GC were round and yellow (solid arrows). The bar represents 50 μm .

and (iv) round spermatids. These four germ cell populations were further characterized by microscopic examination (Fig. 3). Only one 4C cell population was observed in the cat while two 4C cell populations were present in the rat (Godet et al., 2000). Actually, the diameters of cat or rat leptotene, zygotene spermatocytes were 8 to 10 μm . The only one 4C

cell population in the cat was due to (i) the homogenous sizes of cat pachytene spermatocytes (10 μm to 12 μm), unlike heterogeneous sizes of rat pachytene spermatocytes (10 to 16 μm); (ii) the smaller size of cat diplotene spermatocytes (13–15 μm) as compared to that of rat diplotene spermatocytes (17–18 μm). In order to be able to compare the effect of toxicants on the cat or rat 4C cell populations, the two rat 4C cell populations were pooled (see below).

3.1.4. Evolution of the cat cell populations during the 3 weeks of culture

Fig. 4 shows the changes in the numbers of total cells, somatic cells, and of germ cells in 4 different control cultures. Each culture was performed by pooling the testes from 2 cats. As expected, the number of each type of cat tubular cells varied according to the number of total cells seeded and also the yield of each step of the spermatogenic differentiation, as it does in BioAlter®-rat (Blondet et al., 2022; Carette et al., 2015). Nevertheless, for every experiment, it was the same population of tubular cells which was exposed (treated cells) or not (control cells) to the toxicant (paired samples). Hence, in the following figures, the results are expressed as a percent of the values obtained with the corresponding control cells, as performed previously for the rat (Blondet et al., 2022; Carette et al., 2015).

3.2. Comparative study on the effect of DNB or MAA on cell viability and on the somatic or germ cell populations in BioAlter®-rat and BioAlter®-cat

3.2.1. Effect of DNB

Cell viability was not affected at any day of culture by DNB for either cat (between $83 \pm 5\%$ and $93 \pm 2\%$) or rat (between $78 \pm 3\%$ and $85 \pm 3\%$). Similarly, the numbers of total cells were not modified for either cat or rat (Fig. 5). The number of somatic cells was increased only on day 7 in the rat ($p \leq 0.05$) and on day 14 ($p \leq 0.05$) in the cat. By contrast, the number of total germ cells was decreased by DNB from day 7 onward ($p \leq 0.02$ to $p \leq 0.01$) in the rat, and from day 14 onward ($p \leq 0.02$ to

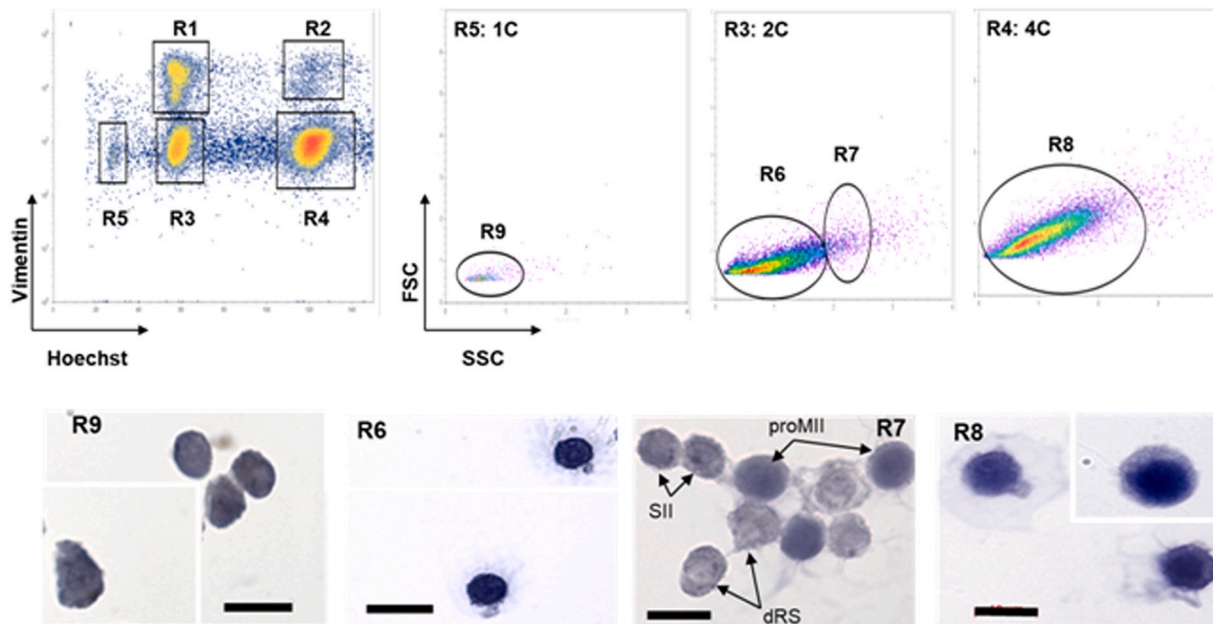


Fig. 3. Representative flow cytometry analysis of the cell populations of testis germ cells obtained from two pooled six-month-day-old-cats. Distribution of vimentin-positive diploid (R1) and tetraploid somatic cells (R2) which are in division or in doublets (Fouchécourt et al., 2006; Perrard et al., 2009a) or vimentin negative diploid (R3), tetraploid (R4), and haploid (R5) populations of germ cells according to their ploidy. The different germ cell populations of pubescent cats were subsequently characterized according to their forward light scatter (FSC) and side angle light scatter (SSC). R6: spermatogonia; R7: secondary spermatocytes and doublets of round spermatids; R8: primary spermatocytes (leptotene, zygotene, pachytene and diplotene); R9: round spermatids. Gated populations were sorted, stained with hematoxylin and identified as spermatogonia (R6), secondary spermatocytes newly formed (SII) or at the beginning of prometaphase II (proMII) and doublets of round spermatids (dRS) (R7), primary spermatocytes (R8) and round spermatids (R9). Bars represent 10 μm . Identical results were obtained in two separate experiments.

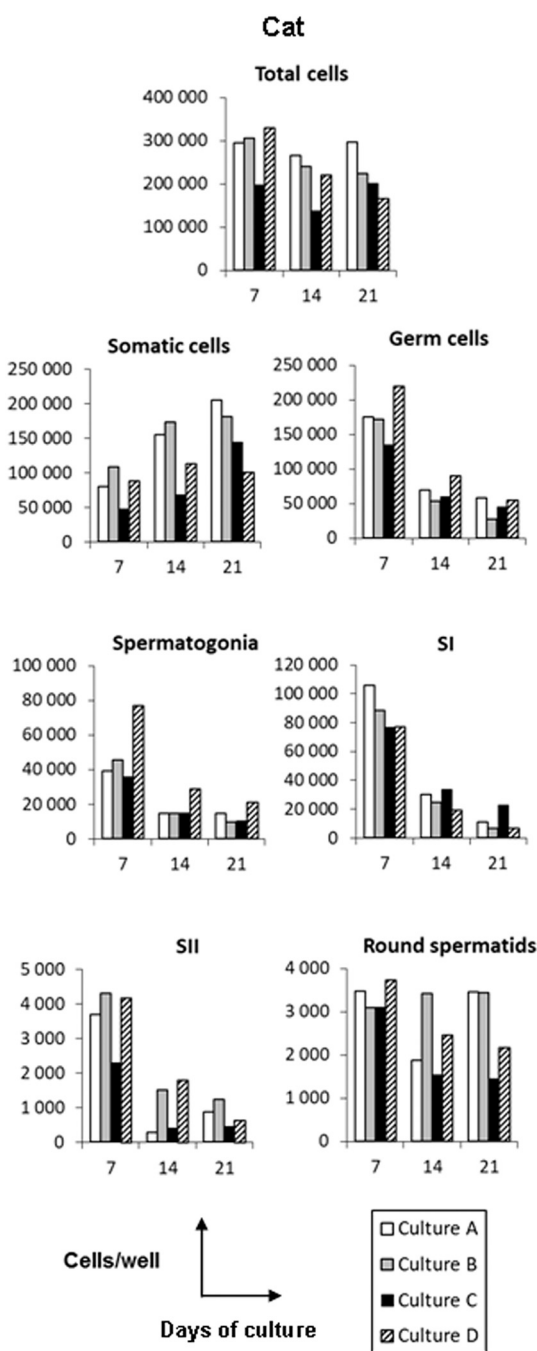


Fig. 4. Flow cytometry study of the changes in the number of total cells, somatic cells, and of germ cells in control cultures of 6-month-old cat seminiferous tubule segments. Individual values of four different cultures are presented. Each culture was performed by pooling the testes from 2 cats. Germ cells are spermatogonia, primary spermatocytes (SI), secondary spermatocytes (SII), and round spermatids.

< 0.01) in the cat. The number of spermatogonia was decreased in the two species ($p \leq 0.05$ on Day 7 and $p \leq 0.01$ on Day 14 in the rat) and ($p \leq 0.05$ on Day 21 in the cat). The number of primary spermatocytes was dramatically decreased in the rat from Day 7 onward ($p \leq 0.01$ to $p \leq 0.001$) and also decreased but less strongly in the cat on Day 14 and Day 21 (both $p \leq 0.02$). The number of secondary spermatocytes was decreased in the rat only on Day 7 ($p \leq 0.05$) and was not interpretable on Day 21, while the number of cat secondary spermatocytes was not modified at any day of culture. The number of round spermatids was

decreased in the rat on Day 7 ($p \leq 0.01$) and not interpretable on Day 21. In the cat, the number of round spermatids was decreased at every day of culture ($p \leq 0.05$ to $p \leq 0.02$). The presence of cellular debris in the presence of DNB may have distorted the flow cytometry analysis of both secondary spermatocytes and round spermatids at the end of the rat cultures.

3.2.2. Effect of MAA

Cell viability was not affected at any day of culture by MAA either for cat (between $94 \pm 1\%$ and $97 \pm 1\%$) or rat (between $91 \pm 2\%$ and $94 \pm 1\%$). The number of total cells was not modified by MAA during the first 2 weeks of culture both for rat and cat, but on Day 21 it was increased ($p \leq 0.05$ in the rat) or slightly decreased ($p \leq 0.02$ in the cat) (Fig. 6). The numbers of rat somatic cells were very increased by MAA throughout the culture ($p \leq 0.01$ to $p \leq 0.001$) while they were increased by MAA only on Day 14 ($p \leq 0.05$) in the cat. By contrast, the number of total germ cells was decreased by MAA in both species: from Day 7 onward ($p \leq 0.05$ to $p \leq 0.001$) in the rat and from Day 14 onward ($p \leq 0.001$) in the cat. The number of spermatogonia was decreased by MAA in the two species: on Day 7 ($p \leq 0.001$) and on Day 14 ($p \leq 0.01$) in the rat, and from Day 14 onward in the cat ($p \leq 0.01$). The number of primary spermatocytes was decreased in the rat from Day 7 onward ($p \leq 0.02$ to $p \leq 0.001$) and from Day 14 onward in the cat ($p \leq 0.01$). The number of secondary spermatocytes was decreased on Day 7 and on Day 14 (both $p \leq 0.01$) in the rat, but only on Day 7 ($p \leq 0.02$) in the cat. Nevertheless, the numbers of round spermatids were greatly decreased from Day 7 onward ($p \leq 0.01$ to $p \leq 0.001$) in the rat and from Day 14 onward in the cat ($p \leq 0.01$).

3.3. Effect of CBZ on cell viability, on the somatic and germ cells in BioAlter®-cat

Cell viability of cat seminiferous tubular cells was not affected at any day of culture by CBZ up to 500 nM (between $94 \pm 2\%$ and $97 \pm 1\%$). The numbers of total cells or of Sertoli cells were not modified by CBZ 50 nM at any day, while they were both decreased by CBZ 500 nM at the end of the culture ($p \leq 0.01$) (Fig. 7). The number of total germ cells was decreased by the two concentrations of CBZ from day 14 onward ($p \leq 0.05$ to $p \leq 0.01$). The number of spermatogonia was decreased by CBZ 50 nM on day 14 ($p \leq 0.01$) while it was decreased by CBZ 500 nM only at the end of the culture ($p \leq 0.01$). The number of primary spermatocytes was decreased by CBZ 50 nM only on Day 21 ($p \leq 0.05$) but was decreased on Day 14 by CBZ 500 nM ($p \leq 0.01$). The number of secondary spermatocytes was not modified but a decrease by CBZ 50 nM on Day 14 ($p \leq 0.05$). Nevertheless, the number of round spermatids was decreased by CBZ 50 nM from Day 14 onward ($p \leq 0.05$ to $p \leq 0.01$) or by CBZ 500 nM from day 7 onward ($p \leq 0.05$ to $p \leq 0.001$).

3.4. Comparative study on the effect of DNB or MAA on the levels of germ cell or Sertoli cell specific mRNAs in BioAlter®-rat and BioAlter®-cat

To provide additional information to the cytometric analyses, mRNA levels were measured in rat or cat seminiferous tubules cultured for 7 or 14 or 21 days in the presence or absence of DNB or MAA. For STMN1 mostly expressed by the primary spermatocytes and for TP1 and TP2 which are specifically expressed by round spermatids (Marret et al., 1998), the numbers of transcripts were corrected by the % of germ cells. For Clusterin, Vimentin, AR and the tight junction proteins (TJP1, Occludin, Claudin 11 and Claudin 4) specific of Sertoli cells, the numbers of transcripts were corrected by the % of somatic cells. Because Connexin 43, specific of gap junctions, is expressed by both Sertoli cells and germ cells, its mRNA transcripts were not corrected by any cell population. Indeed, the relative cell composition of the cultures changes according to the day of sampling, as it does *in vivo* during development (Almstrup et al., 2004). Hence, the measured levels of a specific mRNA depend on both the amount of mRNA produced by the specific cells and

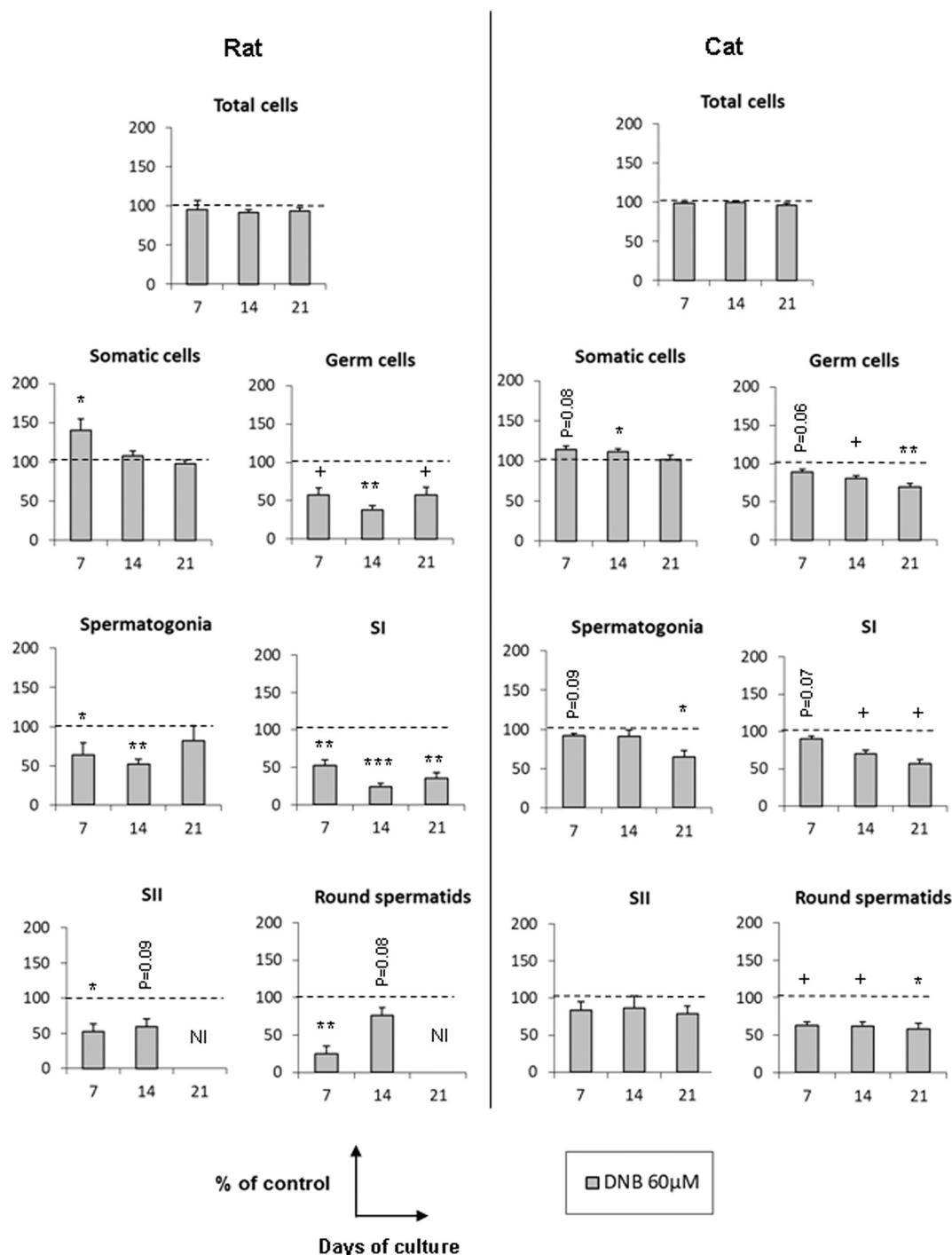


Fig. 5. Flow cytometry study of the effects of DNB 60 μM on the numbers of total cells, of somatic cells and of germ cells in BioAlter®-rat or BioAlter®-cat. Germ cells are: spermatogonia, primary spermatocytes (SI), secondary spermatocytes (SII) and round spermatids. As the number of seeded cells varied from one culture to another, results were expressed relatively to the numbers of each of the different cell types under control conditions which were given as an arbitrary value of 100. Each value is the mean ± SEM of 4 or 6 cultures for the cat or the rat respectively. * p ≤ 0.05; + p ≤ 0.02; ** p ≤ 0.01; *** p ≤ 0.001 vs control.

the proportion of these cells in the sample. Taking into account these two parameters provide more significant data (Plotton et al., 2005, 2006).

3.4.1. Effect of DNB

In BioAlter®-rat (Fig. 8), the numbers of transcripts of TP1 or TP2 were dramatically decreased from day 7 onward (p ≤ 0.01 to p ≤ 0.001

for TP1, and p ≤ 0.05 to p ≤ 0.01 for TP2). In BioAlter®-cat (Fig. 8) TP1 or TP2 mRNAs were (or tended to be) decreased by DNB from day 14 onward (p ≤ 0.05 on day 14 and p = 0.06 on day 21 for TP1, and p = 0.06 on day 14 and p = 0.08 for TP2). The numbers of transcripts of STMN1 were decreased in BioAlter®-rat on Day 7 (p ≤ 0.02) and on Day 21 (p ≤ 0.05), but never modified by DNB in BioAlter®-cat. In regards to the mRNAs specific of Sertoli cells: Clusterin mRNAs were increased on

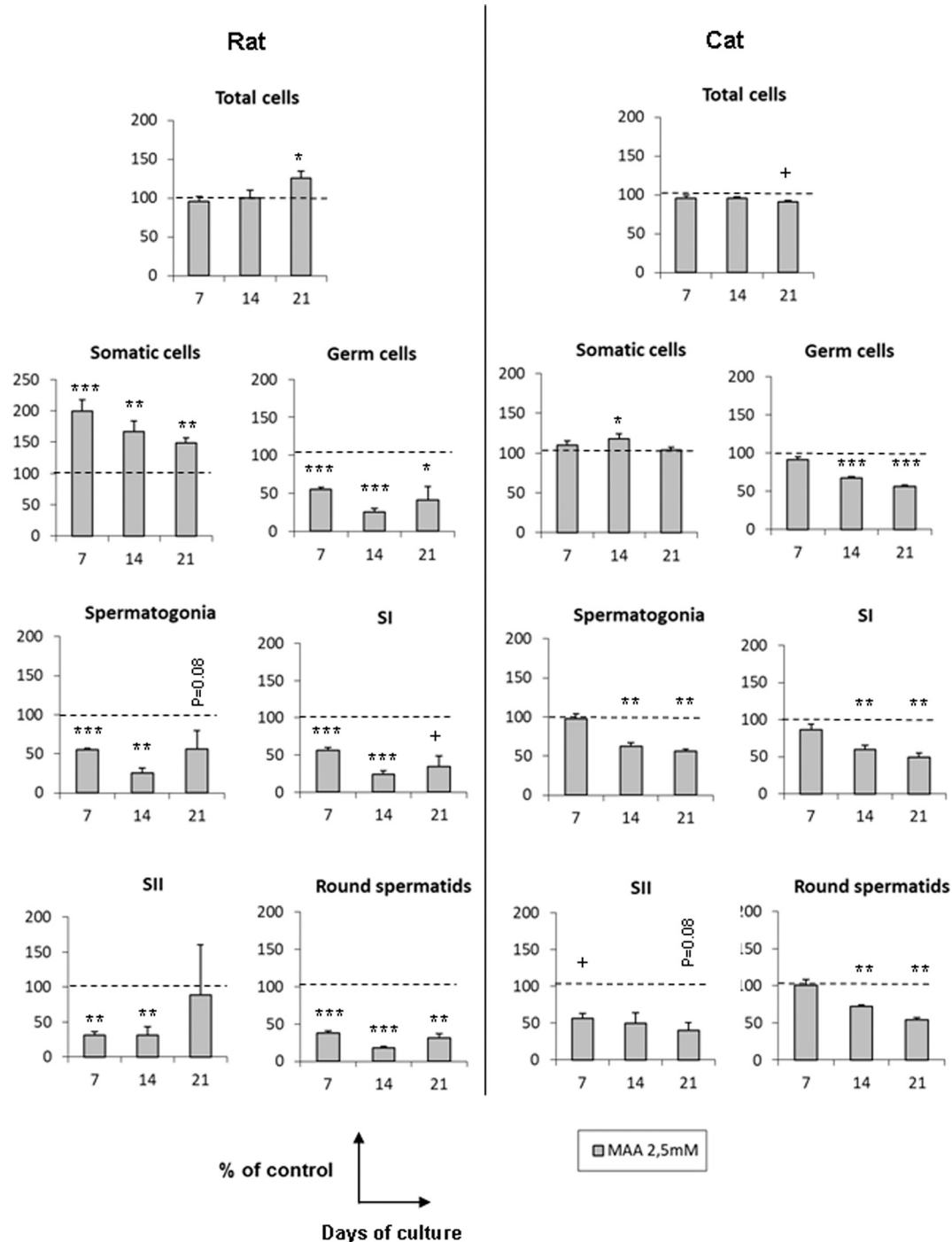


Fig. 6. Flow cytometry study of the effects of MAA 2.5 mM on the numbers of total cells, of somatic cells and of germ cells in BioAlter®-rat or BioAlter®-cat. For details see legend to Fig. 5.

Each value is the mean ± SEM of 4 cultures for the cat, and 4 to 6 cultures for the rat.

* p ≤ 0.05; + p ≤ 0.02; ** p ≤ 0.01; *** p ≤ 0.001 vs control.

Day 14 (p ≤ 0.05) in BioAlter®-rat but were decreased on day 7 (p ≤ 0.05) in BioAlter®-cat, while vimentin mRNAs were never modified by DNB in the two species. AR mRNAs were increased by DNB only on day 21 (p ≤ 0.05) in BioAlter®-rat and not modified in BioAlter®-cat. TJP1 mRNAs were never modified in both species. Occludin mRNAs were decreased by DNB only at the end of the culture (p ≤ 0.01) in BioAlter®-cat. Claudin 11 mRNAs were increased on Day 7 in the two species (p ≤ 0.05 in BioAlter®-rat and p ≤ 0.01 in BioAlter®-cat), while Claudin 4 mRNAs were increased only on day 7 in BioAlter®-rat (p ≤ 0.001).

Connexin 43 mRNAs were never modified in the two species.

3.4.2. Effect of MAA

In BioAlter®-rat (Fig. 9), the numbers of transcripts of TP2 were increased on Day 7 (p ≤ 0.05), while TP1 or TP2 mRNAs were not modified on Day 14 but were dramatically decreased at the end of the culture (p ≤ 0.05 for TP1 and p ≤ 0.02 for TP2). In BioAlter®-cat (Fig. 9), the numbers of transcripts of TP1 and TP2 were not modified on Day 7 and on Day 21, but TP1 mRNAs were decreased (p ≤ 0.01) and

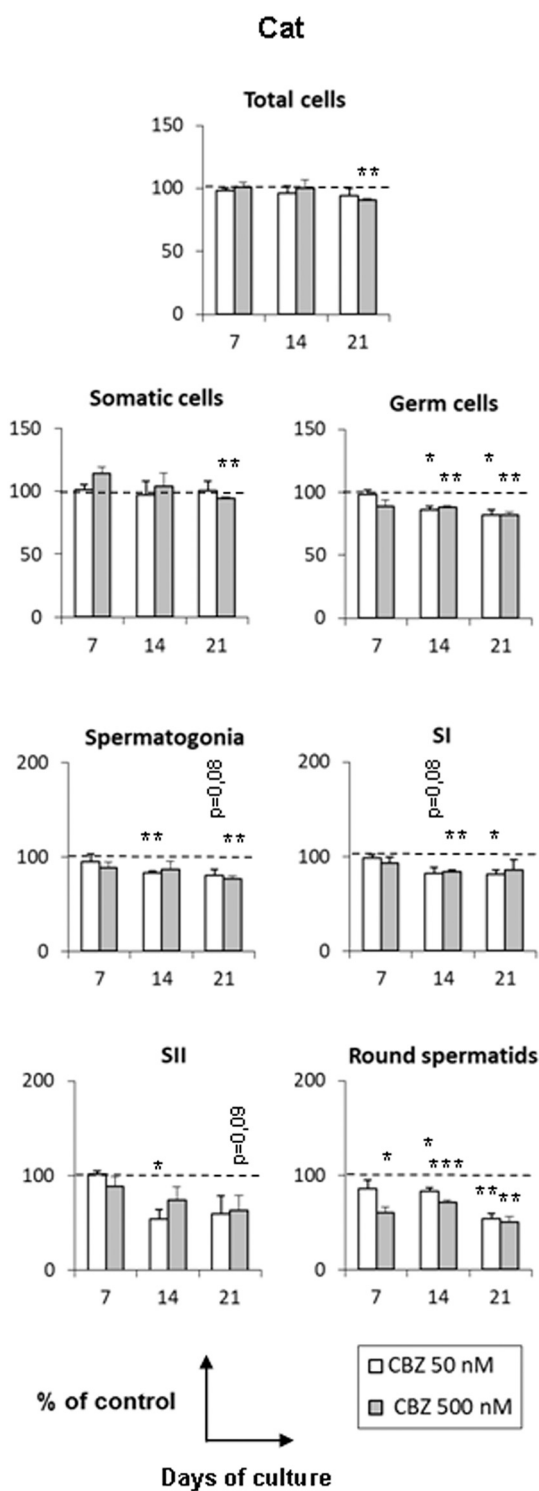


Fig. 7. Flow cytometry study of the effects of CBZ 50 nM or 500 nM on the numbers of total cells, of somatic cells and of germ cells in BioAlter®-cat. For details see legend to Fig. 5.

Each value is the mean \pm SEM of 4 cultures.

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ vs control.

TP2 mRNAs tended to be decreased ($p = 0.07$) on Day 14. The number of transcripts of STMN1 tended to be increased at the end of the culture ($p = 0.06$) in both species. Clusterin's mRNAs tended to be decreased on Day 14 ($p = 0.06$) and were decreased on day 21 ($p = 0.01$) in BioAlter®-rat, and were only decreased on Day 14 ($p \leq 0.05$) in BioAlter®-cat. Vimentin mRNAs were decreased on Day 7 and on Day 21 (both $p \leq$

0.05) in BioAlter®-rat, while they were decreased from Day 7 onward ($p \leq 0.02$ to $p \leq 0.01$) in BioAlter®-cat. The numbers of transcripts of AR were never modified at any day in BioAlter®-rat but only decreased on Day 14 ($p \leq 0.05$) in BioAlter®-cat. In regards to the mRNAs specific of tight junctions: TJP1 or Occludin mRNAs were never modified in BioAlter®-rat but TJP1 mRNAs were decreased from Day 14 onward ($p \leq 0.02$ to $p \leq 0.05$) and Occludin mRNAs tended to be increased on Day 7 ($p = 0.07$) and were increased from Day 14 ($p \leq 0.01$ to $p \leq 0.001$) onward in BioAlter®-cat. Both Claudin 11 and Claudin 4 mRNAs were decreased on Day 7 ($p \leq 0.05$) and Claudin 4 mRNAs tended to be decreased on day 14 ($p = 0.07$) in BioAlter®-rat. The numbers of transcripts of Claudin 11 were decreased ($p \leq 0.02$) and those of Claudin 4 tended to be decreased ($p = 0.07$), only on Day 14 in BioAlter®-cat. Connexin 43 mRNAs were never modified at any day in BioAlter®-rat while they were decreased only on day 7 ($p \leq 0.02$) in BioAlter®-cat.

3.5. Effect of 50 or 500 nM CBZ on the relative expression levels of TP1, TP2, STMN1, androgen receptor, and estrogen receptors α and β mRNAs in cat seminiferous cultures

In Fig. 10A the numbers of transcripts of TP1 or TP2 or STMN1 were expressed by % of germ cells. No effect of CBZ 50 nM was observed on these transcripts at any day of culture. By contrast, CBZ 500 nM increased TP1 mRNAs on Day 7 ($p \leq 0.05$) and increased or tended to increase TP2 mRNAs from Day 7 onward ($p \leq 0.02$ on Day 7, $p \leq 0.05$ on Day 14 and $p = 0.06$ on Day 21). STMN1 mRNAs were increased only on day 7 ($p \leq 0.02$) by CBZ 500 nM. To dwell further this latter results, the numbers of transcripts of TP1, TP2 were also expressed by % of round spermatids, and STMN1 mRNAs were also expressed by % of primary spermatocytes (Fig. 10B) as previously done in BioAlter®-rat (Carette et al., 2015), because round spermatids or primary spermatocytes were rather numerous at the end of these cultures. In this way, TP1 or TP2 mRNAs were increased (or tended to be increased) from day 7 onward by CBZ 500 nM: ($p \leq 0.01$ on Day 7 and on Day 21 and $p = 0.07$ on Day 14 for TP1 and $p \leq 0.05$ to $p \leq 0.01$ for TP2). The transcripts of STMN1 were only increased by CBZ 500 nM only on Day 7 ($p \leq 0.05$).

Because spermatogenesis is an androgen and estrogen-regulated process, the levels of AR, ER α , and ER β mRNAs were further determined (Fig. 10C). For AR the numbers of transcripts were expressed by % of Sertoli cells. Because ER α and ER β are expressed by both Sertoli cells and germ cells, their mRNA transcripts were not corrected by any cell population. The AR mRNA levels were never modified by CBZ treatment. By contrast, 500 nM CBZ induced an increase of both ER α and ER β mRNAs on day 7 ($p \leq 0.05$), while CBZ 50 nM did not induce any modification.

4. Discussion

The germ cells which are present in the 6-month-old cat testis are close to those found in the 20–22-day-old male Sprague-Dawley rats, the most mature germ cells of which are pachytene spermatocytes (stages XI–XII of the seminiferous epithelium) (Clermont and Perey, 1957). Hence, for a similar number of rat or cat cells seeded, the numbers of somatic cells and of all germ cell types identified by the flow cytometry analysis were similar in the 2 species (see Fig. 4, in the present paper, and Fig. 2 in (Blondet et al., 2022)).

Cat pachytene spermatocytes have similar sizes (see results), while three classes of rat pachytene spermatocytes were defined: early pachytene spermatocytes; middle pachytene spermatocytes and late pachytene with diplotene spermatocytes (Perrard et al., 2003; Vigier et al., 2004).

We previously published with Lilly Research Laboratories that BioAlter®-rat successfully mimicked the *in vivo* testicular toxicity of DNB and MAA (Goldstein et al., 2016), but only one experiment was performed for flow cytometric analysis, for each toxicant. In the present study, in order to compare the effects of DNB and MAA, in the rat and the

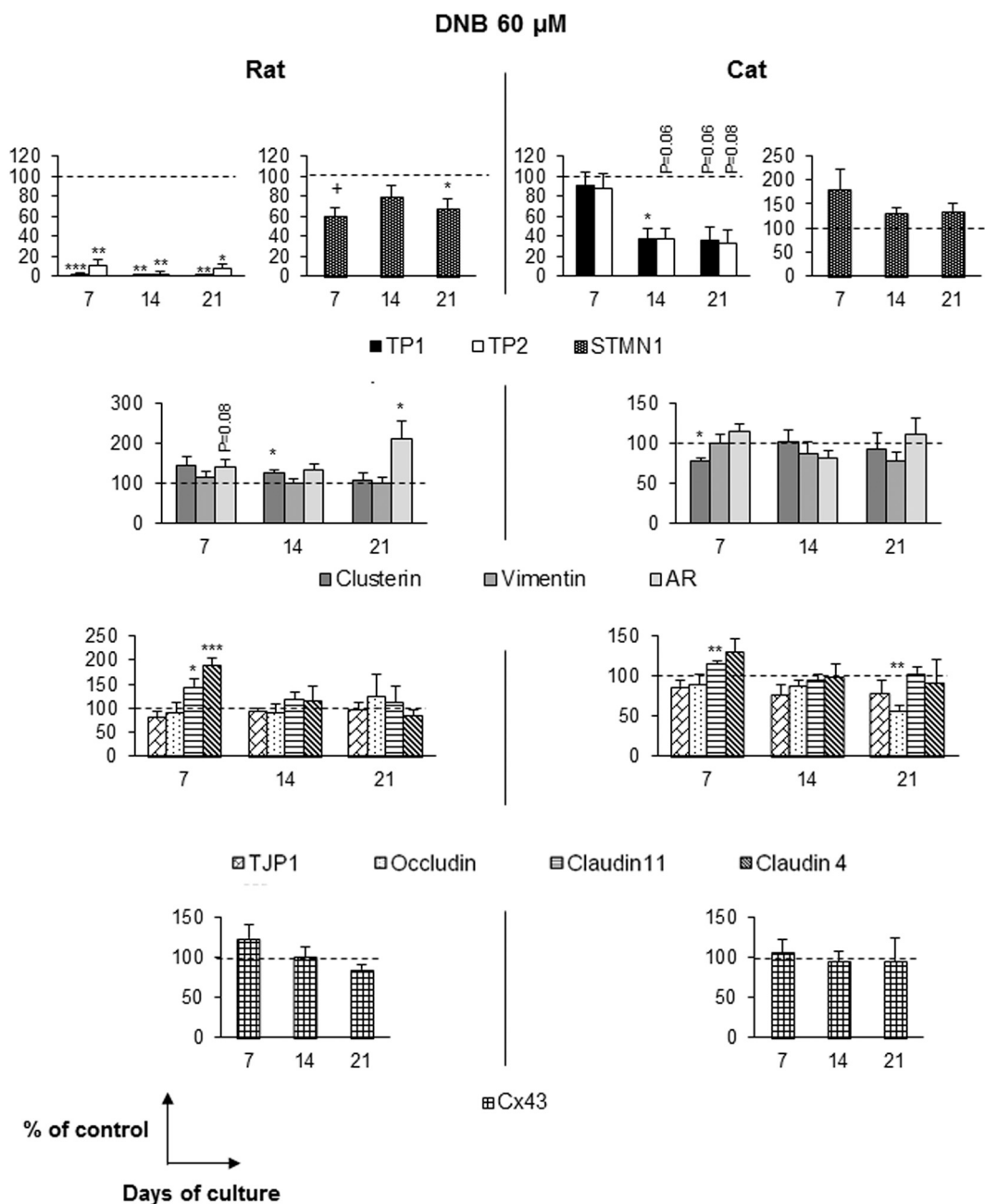


Fig. 8. Changes in the levels of mRNAs related qPCR products of TP1, TP2, STMN1, Clusterin, Vimentin, AR, TJP1, Occludin, Claudin11, Claudin 4 and Cx43 in rat or cat seminiferous tubule segments cultured for 7, 14 or 21 days in the absence (control) or presence of 60 μM DNB.

For TP1, TP2 and STMN1, the numbers of transcripts were expressed by % of germ cells and for Clusterin, Vimentin, AR, TJP1, Occludin, Claudin11, Claudin 4 by % of sertoli cells, in the cultures. Given that Cx43 is expressed by both Sertoli cells and germ cells, their mRNA transcripts were not corrected by any cell population. Results are expressed as a percentage of values obtained from cells cultured under control conditions.

Each value is the mean ± SEM of 3 to 5 cultures for BioAlter®-rat and 4 different cultures for BioAlter®-cat.

* p ≤ 0.05; + p ≤ 0.02 ** p ≤ 0.01 and *** p ≤ 0.001 vs. control.

cat, 4 to 6 cultures were conducted for each species. Moreover a comparative study on the effect of DNB or MAA on the levels of germ cell or Sertoli cell specific mRNAs was also performed in the two species. To the best of our knowledge, this is the first toxicological study with DNB, MAA or CBZ in the cat.

4.1. Effects of DNB

The decreases in the numbers of meiotic and post-meiotic germ cells in the cat and in the rat are in agreement with previous rat *in vivo* studies: Hess et al. showed that the major effects during the first hours

posttreatment were degeneration or exfoliation of pachytene spermatocytes and round spermatids and the retention of step 19 spermatids (Hess et al., 1988). Strandgaard and Miller showed that administration of DNB induced a severe apoptosis in rat seminiferous tubules and that spermatocytes were the primary cell population affected (Strandgaard and Miller, 1998). Our results are also in agreement with the *in vitro* study using BioAlter®-rat (Goldstein et al., 2016). We confirmed that exposure to DNB at 60 μM induced an early increase in somatic cells on Day 7 and a decrease in meiotic and post-meiotic germ cells.

It is important to note that the DNB-induced decrease in the numbers of total germ cells and of primary spermatocytes was greater in the rat

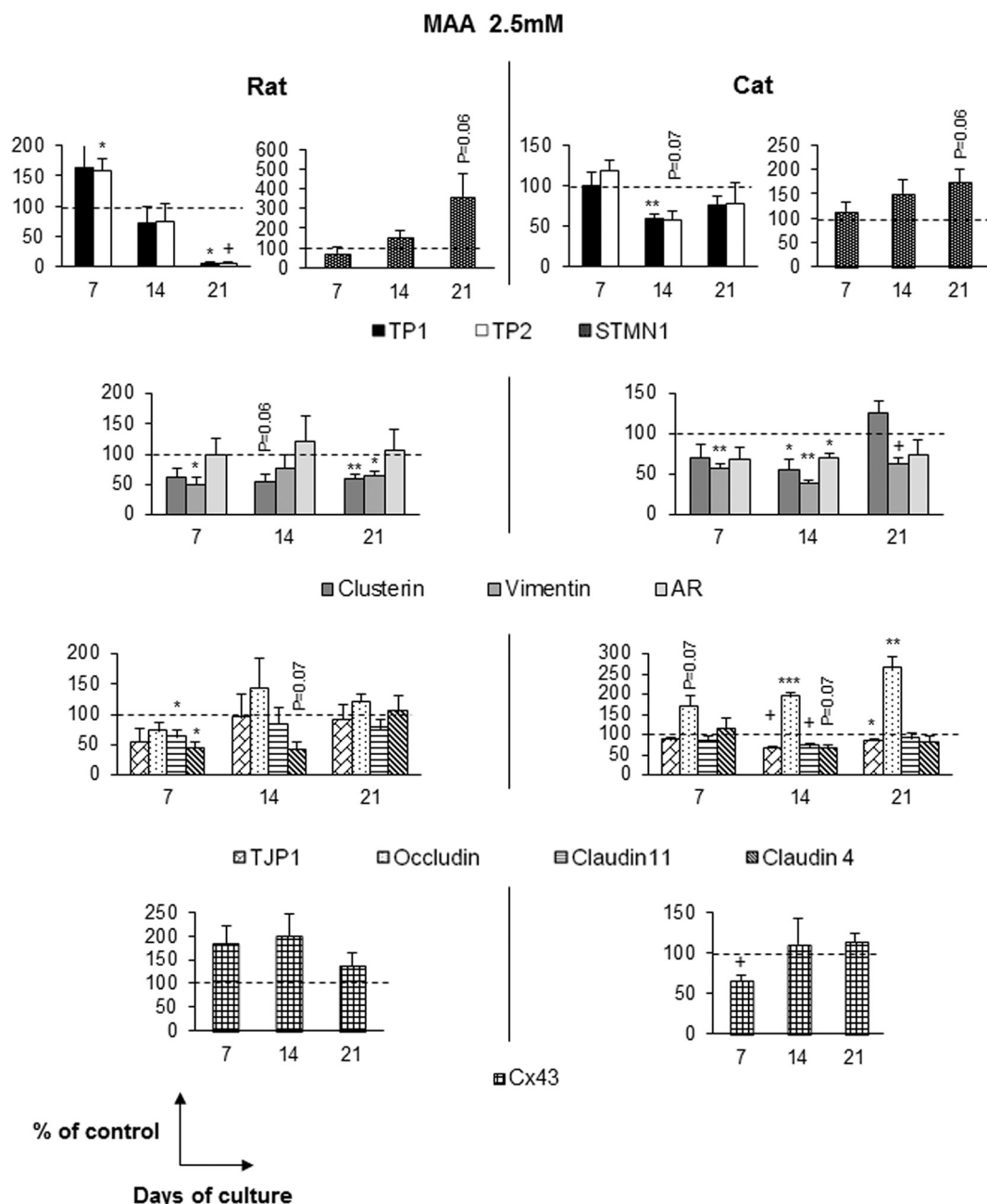


Fig. 9. Changes in the levels of mRNAs related qPCR products of TP1, TP2, STMN1, Clusterin, Vimentin, AR, TJP1, Occludin, Claudin11, Claudin 4 and Cx43 in rat or cat seminiferous tubule segments cultured for 7, 14 or 21 days in the absence (control) or presence of MAA 2.5 mM.

For details see legend to Fig. 8.

Each value is the mean ± SEM of 4 different cultures for both BioAlter®-rat and BioAlter®-cat.

* p ≤ 0.05; + p ≤ 0.02; ** p ≤ 0.01; *** p ≤ 0.001 vs control.

than in the cat while the effect on round spermatids was more visualized in the cat than in the rat. Indeed, at the end of the rat cultures the toxic effect of DNB resulted in a high quantity of debris which polluted the populations of secondary spermatocytes and of round spermatids. The toxic effect of DNB on round spermatids was confirmed by the decrease of TP1 or TP2 mRNAs, in both species. Since STMN1 is mostly expressed by spermatocytes and at very low level in spermatogonia (Guillaume et al., 2001; Marret et al., 1998), the decrease of the relative expression levels of STMN1 in the rat, but not in the cat, confirms the strong DNB-induced damage of rat spermatocytes or spermatogonia. It is important to underline that the mRNA levels of TH2B, specific of pachytene spermatocytes easily detectable in the rat (Marret et al., 1998), were

undetectable in control conditions in the cat, so we were unable to study its regulation by toxicants.

DNB induced some modifications of mRNA levels of some tight junction proteins in the two species. Our results are in agreement with previous *in vitro* results, in the rat, showing that DNB at 60 μM disrupted the trans-epithelial electrical resistance after one week of treatment (Goldstein et al., 2016). Indeed, as shown in Bio-AlteR®-rat, the trans-epithelial electrical resistance assesses the maintenance/formation of tight junctions and thus mimicks *in vitro* the development of the blood-testis-barrier (Carette et al., 2013, 2015). In addition, a microarray analysis *in vivo* in the rat has revealed that, during early testicular toxicity induced by DNB, several genes involved in tight junction

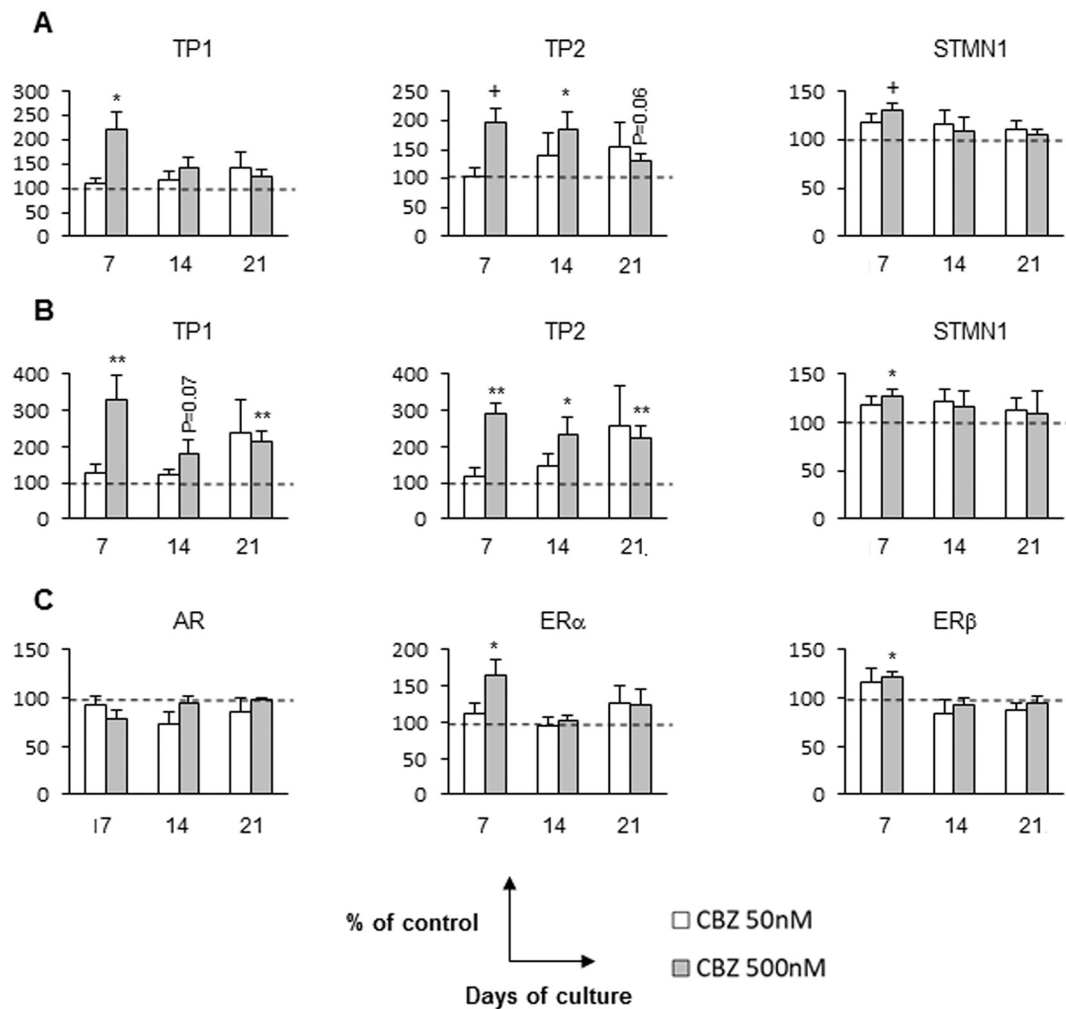


Fig. 10. Changes in the levels of mRNAs related qPCR products of TP1, TP2 and STMN1 (A-B) and of AR, ER α , and ER β (C) in cat seminiferous tubule segments cultured for 7, 14 or 21 days in the absence (control) or presence of 50 or 500 nM CBZ.

A: For TP1, TP2 and STMN1, the numbers of transcripts were expressed by % of germ cells in the cultures.

B: For TP1, TP2, and STMN1, the numbers of transcripts were expressed by % of round spermatids and by % of spermatocytes respectively in the cultures.

C: For AR the numbers of transcripts were expressed by % of Sertoli cells. Given that ER α and ER β are expressed by both Sertoli cells and germ cells, their mRNA transcripts were not corrected by any cell population.

Results are expressed as a percentage of values obtained from cells cultured under control conditions. Each value is the mean \pm SEM of 4 different cultures in BioAlter®-cat.

* $p \leq 0.05$; + $p \leq 0.02$; ** $p \leq 0.01$ vs. control.

signaling pathways are differentially expressed (Oh et al., 2014).

4.2. Effects of MAA

The decrease of the number of spermatocytes in both species, resulting in the decrease of the number of round spermatids, is in agreement with previous *in vivo* and *in vitro* studies in the rat which showed that MAA induced spermatocyte death/apoptosis (Chapin et al., 1984; Foster et al., 1984; Li et al., 1996; Wade et al., 2008). Moreover, we confirmed that MAA induced an increase in somatic cells during the whole culture, and had a direct effect on spermatocytes (Goldstein et al., 2016).

In both species, TP1 or TP2 mRNAs were not necessarily decreased when the numbers of the round spermatids were decreased. We published previously that the amount of TP1 mRNAs transcribed by round spermatids *in vitro* was increased by testosterone (Vigier et al., 2004). Our hypothesis is that MAA could interact with the regulation of TP1 or TP2 mRNAs by testosterone. Indeed, an *in vitro* study, in a Leydig cell model (Bagchi et al., 2011), highlighted the complex interactions

between testosterone and MAA, and provided insight into the effects of MAA exposure on androgen-dependent processes.

MAA affected directly the Sertoli cells by decreasing Clusterin and Vimentin mRNAs in both species and increasing their number especially in the rat. Moreover, an *in vitro* study showed that MAA induced an inhibition of the production of lactate by rat Sertoli cells which could account for the inhibitory action of MAA on spermatogenesis (Beattie et al., 1984).

4.3. Effects of CBZ

Our results showed that CBZ induced a reduction of the number of cat round spermatids as we observed previously in the rat (Carette et al., 2015). However, there were some differences between the 2 species in the effects of CBZ on the other cell populations. These differences between rat and cat on the somatic and germ cell populations might be due to the fact that the seeded cat germ cells were a little more differentiated than those of the 20–22-day-old rats (see the §1 of results).

We previously reported in the rat that CBZ possessed an endocrine-

disrupting effect by regulating the levels of *Erα* or *Erβ* mRNAs and enhancing the mRNAs of the androgen-dependent round spermatid-specific genes, TP1 and TP2 (Carette et al., 2015). In the cat, despite the decrease in the number of round spermatids by both concentrations of CBZ, TP1 and TP2 mRNA levels were both increased by CBZ 500 nM. These results are in agreement with our previous rat study. This might indicate an androgen like effect of CBZ at the level of the cat seminiferous epithelium, as we have shown in BioAlter®-rat (Carette et al., 2015).

The effect of testosterone occurs in a stage-specific manner (Henriksen et al., 1995) and involves AR on Sertoli cells (Wang et al., 2009). A fine balance between androgens and estrogens is required for the good running of spermatogenesis (Carreau and Hess, 2010). Our data in the cat suggest that this balance was modified by CBZ 500 nM which induced an increase in the expression of both estrogen receptors. However, our study did not show any modification of AR mRNAs by CBZ. These results are in agreement with those we published previously in the rat (Carette et al., 2015).

5. Conclusion

We have settled the culture of prepubertal domestic cat seminiferous tubules (veterinary waste) in our validated BioAlter® model with the aim of reducing animal experimentation in male reproductive toxicology. BioAlter®-cat demonstrated its ability to successfully detect the testicular toxicity of DNB, MAA and CBZ. However, there might be some small differences between the rat and the cat in the intensity of the effects on some of the studied parameters. In conclusion, BioAlter®-cat might be a solution to the requirements of the EU cosmetics directive and REACH legislation for male reproductive toxicology studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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