Development of an *in vitro* test system for assessment of male, reproductive toxicity

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Abstract

There is a need for improved reproductive toxicology assays that do not require large numbers of animals but are sensitive and informative. Therefore, Staput velocity-sedimentation separation followed by culture of specific mouse testicular cells was used as such a system. The specificity of separation was assessed using immunocytochemistry to identify spermatids, spermatocytes and spermatogonia. The efficacy of the system to detect toxicity was then evaluated by analysing the effects of hydrogen peroxide (H_2O_2) by the terminal uridine-deoxynucleotide end-labelling (TUNEL) assay to show the rate of apoptosis induced among the different types of germ cells. We found that 2 h of treatment at both 1μM and 10μM induced increases of over ~10-fold in the percentage of apoptotic cells ($p \leq 0.001$), confirming that testicular germ cells are prone to apoptosis at very low concentrations of H_2O_2. It was also demonstrated for the first time for this compound that spermatogonia are significantly more susceptible than spermatocytes, which are more affected than spermatids. This reflects the proportion of actively dividing cells in these cell types, suggesting a mechanism for the differential sensitivity. The approach should thus form the basis of a useful test system for reproductive and genetic toxicology in the future.

1 Introduction

Testing germline-genotoxicity in the male is generally undertaken *in vivo*, partly because of the difficulty of achieving full spermatogenesis *in vitro* and partly because mating studies are currently the only reliable way of testing heritable effects. The associated expense and ethical issues mean there is a constant need for the development of novel *in vitro* assays (cf the European REACH regulation [EU, 2007]). This will require an *in vitro* test system that allows examination of individual germ cell types. It should also have high sensitivity and be suitable for the rapid screening of large numbers of chemicals. We propose that the use of Staput to separate highly enriched populations of spermatogonia, spermatocytes and spermatids, and their subsequent culture in the presence of putative genotoxins or reproductive toxins, coupled with the measurement of appropriate end-points of damage, has the potential to meet this need. These three germ cell categories contain the three major events occurring in spermatogenesis: mitotic proliferation (spermatogonia); meiosis (spermatocytes); and spermiogenic differentiation (spermatids). Therefore, even though each type contains a number of different sub-types, they make suitable groupings for toxicity analysis as all the parts of each process are covered within each cell population used.

The ability to study specific germ cell types will also be useful in more fundamental studies of reproductive biology. During spermatogenesis the male germ cell undergoes complex morphological, biochemical, and physiological changes, resulting in the formation of a mature spermatozoon. This dynamic procedure depends upon Sertoli cells that supply nutrients, hormones and structural support to the germ cells through their development, and on Leydig cells that synthesise the steroid hormones necessary for germ cell differentiation (Cheng and Mruk, 2010, O'Shaughnessy et al., 2009). Even after decades of research in the field of male fertility, critical spermatogenic events, including Sertoli cell–germ cell interaction and mechanisms of androgen action, remain to be completely understood. A more in-depth understanding of these spermatogenic events will require, for example, the ability to study specific molecular signatures of individual testicular cells. That in turn will require the isolation of purified populations of spermatogenic cells as one of the crucial steps to address these important issues. Over the years, a range of approaches have been used to successfully isolate testicular cells, including elutriation. Velocity sedimentation separation using Staput chambers is another of the approaches used to isolate spermatogenic cells (Han et al., 2001) and has been more widely used, presumably because of the high purity of fractions that is possible and relatively low unit-cost of the experiments.

Germ cell apoptosis is very common during the various stages of mammalian testicular development up to a point midway through spermatid development, when nuclear condensation has advanced too far to permit the *de novo* gene expression on which post-meiotic DNA repair and presumably apoptosis depends (Leduc et al., 2008). However, understanding of the mechanisms underlying male germ cell apoptosis is still limited (Koji and Hishikawa, 2003) although its role in removing genetically damaged cells from the germline is well accepted. Testicular cells are prone to oxidation by H_2O_2 and other reactive oxygen species (ROS) (Peltola et al., 1994) which represent probably the commonest form of exposure to genotoxins that most cells encounter. Reactive oxygen species are chemically reactive molecules containing oxygen. They form as a natural by-product of the metabolism of oxygen and have a central role in sperm maturation as well as the acrosome reaction when expressed at low levels (Schulte et al., 2010). One of the main ROS forms during germ cells is Hydrogen Peroxide (H_2O_2) (Moustafa et al., 2004). H_2O_2 constitutes the main ROS form in sperm but its effective role as an endogenous inducer of germ cell apoptosis continues to be investigated (Aitken et al., 1998). H_2O_2 is also known to modulate a variety of cell functions. It is a potent ROS, but its lower biological activity compared with many other ROS, combined with its capacity to cross membranes and diffuse away from the site of generation, makes it an ideal molecule in signal transduction, and it is involved in inducing the acrosome reaction in sperm (Hampton and Orrenius, 1997). The plasma membrane of testicular cells is rich in polyunsaturated fatty acids, thus making it prone to oxidation by H_2O_2 and other ROS's; oxidative stress is known to cause DNA damage (Agarwal and Saleh, 2002). H_2O_2 is the main form of ROS in sperm cells and previous studies determined male germ cells displayed a much higher sensitivity to H_2O_2 in comparison to other cells (Maheshwari et al., 2009).

Enriched populations of germ cells in the mouse thus seem suitable for analysis of the effects of genotoxins using the TUNEL assay. Since similar mechanisms could operate in the generation of pathological states in the testis, the approach may also have utility in studies of infertility in the future.

Abbreviations: Tp1 = transition protein 1; Scp3 = synaptonemal complex protein 3; GDNFR = glial cell line derived neurotrophic factor receptor.

2 Materials and Methods

2.1 Animals

Sexually mature NMRI mice (National Medical Research Institute) weighing 25-30g (10-12 weeks old) were used in this study. Animals were sacrificed by cervical dislocation under CO_2 anaesthesia. Animals were obtained from the Institute of Cancer Therapeutics (ICT), University of Bradford, UK where they were maintained under standard conditions. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2 Staput Isolation of Germ Cell Fractions

Mixed testicular germ cells were separated using the velocity sedimentation technique (Staput) according to procedures developed for murine spermatogenic cells (Romrell et al., 1976). Briefly, the testes were removed and decapsulated from four male adult (10-12 week-old) NMRI mice. They were then placed into ice-cold Dulbecco's modified Eagle's medium (DMEM). The decapsulated testes were minced with a scalpel blade and suspended in Dulbecco's Minimum Eagle's Medium (DMEM) containing collagenase (5mg/ml) and DNAse (1μg/ml) (both from Sigma, Poole, UK), and the flask was incubated at 32°C for 20 min in a water bath. After two washes in DMEM, the dispersed cells were washed twice with medium and filtered through an 80μm nylon mesh (Tetco Inc., Briarcliff Manor, NY), successively. The different types of germ cells were separated by sedimentation velocity at unit gravity at 4°C, by use of a 2-4% BSA gradient in DMEM. The cells were bottom-loaded into the chamber in a volume of 10ml, and a BSA gradient using 250ml of 2% w/v and 4% w/v BSA was generated. The cells were allowed to sediment for a standard period of 2.5h, and then 31 fractions each of 12ml volume were collected at 60s intervals. The cells in each fraction were examined under a phase contrast microscope, and fractions containing cells of similar size and morphology spun down by low-speed centrifugation and then resuspended in DMEM.

2.3 Culture and Treatment

The isolated testicular germ cells were seeded onto coverslips in 6-well plastic culture plates with DMEM containing 10% fetal bovine serum (FBS), 100 Unit/ml penicillin, and 100 mg/ml streptomycin (5×10^6 cells/ml; 1ml per well) at 37ºC then the medium was changed and they were serum starved in DMEM (with antibiotics) for 16 h to allow the cells to attach to the coverslips. They were then incubated for 2h with or without H_2O_2. Incubations with H_2O_2 were made at final concentrations of 0, 1, and 10μM in triplicate. A temperature of 37ºC would not be suitable for long-term cultures of spermatogenic cells, which thrive best at

a temperature 1-2 degrees below core body temperature in humans. Attempts to recreate spermatogenesis *in vitro* typically use a culture temperature of 35°C, often maintained for several weeks (Reuter et al., 2013). Cells cultured for a single day at 37°C are healthy in appearance and only minimal numbers fail to survive, so these conditions were deemed suitable for the short-term experiments reported here. Treated and untreated cells were fixed with 4% formaldehyde for 10 min and washed twice, each for 5 min, in PBS containing 0.5% BSA and stored at in 70% (v/v) ethanol until further use. Approximately 80% cells were viable in the group exposed to 10µM H_2O_2. Some of the untreated cells were processed for immunohistochemistry to determine transition protein 1 (Tp1, spermatids), synaptonemal complex protein 3 (Scp3, spermatocytes) and Glial cell line derived neurotrophic factor receptor (GDNFR, spermatogonia).

2.4 TUNEL Assay

The TUNEL assay for apoptosis evaluation (Gavrieli et al., 1992; Lobascio et al., 2007) was performed on separate cell samples of the same cell populations as follows. Briefly, the slides were incubated with TUNEL reaction mixture (30mM Tris pH 7.4; 140mM sodium cacodylate; 1mM cobalt chloride; 5µM biotin-16-deoxyuridine triphosphate; 0.3U/µl terminal deoxynucleotidyl transferase [Tdt]; all from Sigma) for 60 min (humidity chamber, 37°C) and then washed twice in PBS. (H_2O_2–blocking of endogenous peroxidases was not performed as the testis is low in peroxidases so it is rarely necessary.) After multiple washing steps, the cells were treated with extravidin peroxidase solution for 30 min (humidity chamber, 37°C), rinsed with PBS, and visualized by adding 3,3′-diaminobenzine (DAB) for 10 min at room temperature. They were washed in phosphate buffer saline (PBS), counterstained using hematoxylin staining, and finally, mounted for light microscopic observation. For the negative controls, sections were incubated with the reaction mix without Tdt instead of the full TUNEL reaction mixture.

2.5 Immunohistochemistry

The fractions of cells used were grown on coverslips in 6-well plastic culture plates with DMEM containing 10% FBS, 100 Unit/ml penicillin, and 100mg/ml streptomycin. The cells were serum starved in DMEM (with the antibiotics) for 16h to allow the cells to attach to the coverslips, fixed with 4% formaldehyde for 10 min and washed twice, each for 5 min, in PBS containing 0.5% BSA. A 1 h block in PBS containing 0.1% BSA, 0.05% Triton X-100, and 1% goat serum was performed. Anti-SCP3 rabbit polyclonal antibody (1:400; Abcam,

Cambridge, UK), rabbit polyclonal anti-Tp1 antibody (1:50; Abcam, Cambridge, UK), rabbit polyclonal anti- GDNFR (1:100; Abcam, Cambridge, UK), were used as the primary antibodies. Briefly, the incubation was at 4°C overnight, followed by washing with PBS. The slides were incubated with secondary, biotinylated anti-rabbit-IgG antibody for 30 min at room temperature. Signals were developed with 3, 3'-diaminobenzine (DAB) for 10 min and counterstained with hematoxylin (Hsu *et al*. 1981; Khalfaoui *et al*. 2011) and mounted with Histomount (Fisher Scientific, Fair Lawn, NJ). Preparations of cells representing each fraction were scored for the presence of cells positive for each of the three markers and their total number per fraction calculated. Only fractions showing suitable purity of a specific cell type were used to set up the cultures (see below).

2.6 Cell Counting

The isolated testicular germ cells were assessed for morphology and staining, The following findings were considered to represent apoptosis: marked condensation of chromatin and cytoplasm clearly staining strongly brown or brown/black; The TUNEL- positive cells were scored in several fields on each coverslip to yield a total of at least 100 cells under a 40X objective of an Olympus CKX31 microscope. Values represent percentages from at least 100 cells from each culture. Using the one–way ANOVA test differences were considered as significant at $p<0.001$.

2.7 Statistical analysis

Data are expressed as mean ± S.D. of at least three independent experiments with three replicates *per* experimental group. Comparisons were made by one-way ANOVA; *P* values < 0.05 were considered significant.

3 Results

3.1 Purification of germ cells

Microscopic examination of each 12 ml fraction collected from the Staput chamber indicated that spermatids were concentrated in fractions 15–24, spermatocytes in the fractions 25–29, and spermatogonia in fractions 27–36, as shown in Figure 1. This confirmed that different types of germ cells could be separated from each other on the basis of their density and size using Staput.

Immunocytochemistry analysis was used successfully in this study to identify the principal classes of male germ cells following separation via Staput: examples of cells labelled with the different antibodies are shown in Figure 2.

It was critical to determine the purity of the fractions so cells from each of the fractions could be scored after binding to different antibodies. It was found that specific fractions contained high purities of the individual cell types: spermatids in fraction F19; spermatocytes in F27; spermatogonia in F30; (Figure 3) so only these fractions were used for cell culture.

3.2 TUNEL assay

The outcome of H_2O_2 treatment on mouse testis was evaluated by the TUNEL assay, and results, expressed as percentages of apoptotic cells, are shown in Figure 4. The TUNEL assay revealed that all cells types had undergone significant levels of apoptosis compared with the controls ($p \leq 0.001$). Representative apoptotic cells from treated and non-treated samples are illustrated in Figure 5.

The results of the induction of apoptosis by H_2O_2 treatment of different germ cell types are shown in Figure 4. After treatment with H_2O_2 (1 and 10μM) for 2 hours, a significant increase ($p \leq 0.001$) in spermatogonial apoptosis to 47% was observed when cells were treated with 1μM H_2O_2. Moreover, the apoptosis of spermatogonial cells treated with 10μM H_2O_2 showed a further significant increase to 62% when compared with control ($p \leq 0.001$). Following treatment with 1μM H_2O_2 spermatocytes cells showed an increase in cell apoptosis to 38%, which was statistically significant when compared with the corresponding controls ($p \leq 0.001$). A further increase to 51% in cell apoptosis was observed when cells were treated with 10μM H_2O_2. This increase was significant compared with controls ($p \leq 0.001$). Cell apoptosis was significantly increased to 29% when cells were treated with 1μM H_2O_2 ($p \leq 0.001$). The apoptosis of spermatids treated with 10μM H_2O_2 was significantly increased to 40% compared to the corresponding controls ($p \leq 0.001$).

Figure 4 additionally shows that while the levels of spontaneous apoptosis are similar for all three germ cell types in the control group, the response of spermatids to both 1 and 10μM H_2O_2 was markedly lower than that of spermatogonia, with spermatocytes intermediate between them in both cases. That difference was statistically significant ($p \leq 0.001$) between the cell types.

4 Discussion

Despite recent advances in the study of male germ-line cells in terms of genetics and development, our understanding of the effect of toxins on specific cell types has been largely limited to what can be achieved by, often cumbersome, *in vivo* studies to make such distinctions. The results presented here demonstrate that a suspension of mouse germ cells can be obtained from testicular tissue and fractionated into large homogeneous populations of spermatogonia, spermatocytes, spermatids and spermatozoa using Staput.

Validation of the system involved using immunohistochemistry to determine the purity of the cells populations isolated by Staput (Bellve et al., 1977), using antibodies against: Tp1 to detect spermatids; Scp3 to detect spermatocytes; and GDNFR to detect spermatogonia. Tp1 is an important nuclear protein in spermatids as histones are replaced by protamines during spermiogenesis. Its specificity to the haploid phase of spermatogenesis makes it a useful marker for spermatids (discussed in Meistrich and Hess, 2013). Spermatocytes can be located by the presence of Scp3. Synaptonemal complexes are structures formed between homologous chromosomes during meiotic prophase, thought to be involved in chromosome pairing and recombination. They comprise lateral and central elements and of the lateral elements, two components have been identified in rodents, one of which is the Scp3 (Dobson et al., 1994, Lammers et al., 1995). Spermatogonia can be labeled by GDNFR. Sertoli cells secrete a ligand to GDNFR called GFRα-1 (Viglietto et al., 2000) .The binding of this substrate-ligand complex activates the Ret receptor tyrosine kinase (Tadokoro et al., 2002). This mediates an intracellular response that is linked to the proliferation of an undifferentiated type A spermatogonia and is therefore considered a good marker for these types of spermatogonia (Meng et al., 2000).

Hydrogen peroxide (H_2O_2) has been found to induce apoptosis in a diversity of cells and although the sensitivity of germ line cells to H_2O_2 is not fully understood, DNA strand breakage by the production of free radicals is the trigger for the programmed cell death. The results of the present study show that H_2O_2, even at a low concentration of H_2O_2 of 1μM, has the ability to induce apoptosis in testicular germ cells *in vitro*. This is in line with what has been reported previously, demonstrating concordance between our approach to preparing testicular germ cells and previous methodologies (Maheshwari et al., 2009). In the present work, following 2 hours of treatment with 10μm H_2O_2, a tenfold increase in the proportion of apoptotic cells was found. There was a statistically significant induction of apoptosis in germline cells ($p<0.001$). The spermatogonia were significantly more affected by H_2O_2 than the spermatocytes, which were significantly more affected than spermatids. This correlates

with the proportion of dividing cells expected to be present in these populations. Thus, if dividing cells are more susceptible to genetic damage than non-dividing cells, this could account for the lowest amount of apoptosis occurring in the latter population. Indeed, there are a number of different types of cells within spermatogonia, spermatocytes and spermatids, each of which could have different susceptibilities to genetic damage. Therefore, one challenge for the future will be to examine chemicals that react primarily with each germ cell type, and to refine the Staput separation so as to isolate more specific and well defined populations. Furthermore, such a clear difference in sensitivity may be highly important in the toxicity assessment of other chemicals but would be very difficult to demonstrate with *in vivo* studies. Therefore, we believe that our approach holds significant advantages for the development of sensitive and specific, *in vitro* reproductive and genetic toxicology assays.

5 Conclusion

Staput separation of specific germ cell types, coupled with short term *in vitro* culture shows potential for the rapid assessment of toxins in multiple germ cell types with high sensitivity. Notably, it allows the examination of high numbers of cells such as spermatogonia, which are normally present in relatively low amounts *in vivo*, compared with spermatocytes and spermatids. Overall, the present work shows that this in vitro system has potential to be a sensitive, rapid screen for reproductive toxins.

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7 Conflict of Interest Statement

The authors have no conflicts of interest with regard to the funding of this research.

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9 Figure legends

Figure 1: Velocity sedimentation separation of germ cells of the mouse. Spermatids, characterised by TP1 antibody staining, sediment first, followed by spermatocytes (SCP3 antibody staining), then spermatogonia (GDNFR antibody staining). Microscopic examination of each fraction isolated by Staput showed that the maximum concentration of spermatids was in the fractions (15–24), followed by spermatocytes in the fractions (25–29), spermatogina in the fractions (37–36).

Figure 2: Immunohistochemistry staining was performed on Staput purified mouse testicular cells were stained with antibodies for specific proteins. Spermatids were detected with anti-Tp1 (Panel A); spermatocytes were detected anti-SCP3 (Panel B) and spermatogonia were detected with anti-GDNFR (panel C). Viewing magnification X400.

Figure 3: Assessment of the purity of the fractions. Cultured cells from all fractions were stained for each of the three antibodies and scored to determine their relative proportion in each fraction. The results for the three fractions showing the highest purity for each cell type are shown.The numbers are for total numbers of each cell type per fraction.

Figure 4: Effect of H_2O_2 treatment on germ cells evaluated in the TUNEL assay. Columns represent the mean percentages +/- SD of apoptotic cells for each of the three concentrations of hydrogen peroxide used (0, 1.0, 10µM). Data were obtained from three independent experiments. Each dose level within a cell type has been compared with the respective 0µM group. *** = $p < 0.001$.

Figure 5: Effect of H_2O_2 treatment on germ cells of the mouse evaluated by the TUNEL assay. The treated cells with H_2O_2 (A) were compared with untreated cells (A1). Arrows indicate representative TUNEL-positive (apoptotic) cells in each case. Viewing magnification X400.

10 Figures

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Figure 1(S)

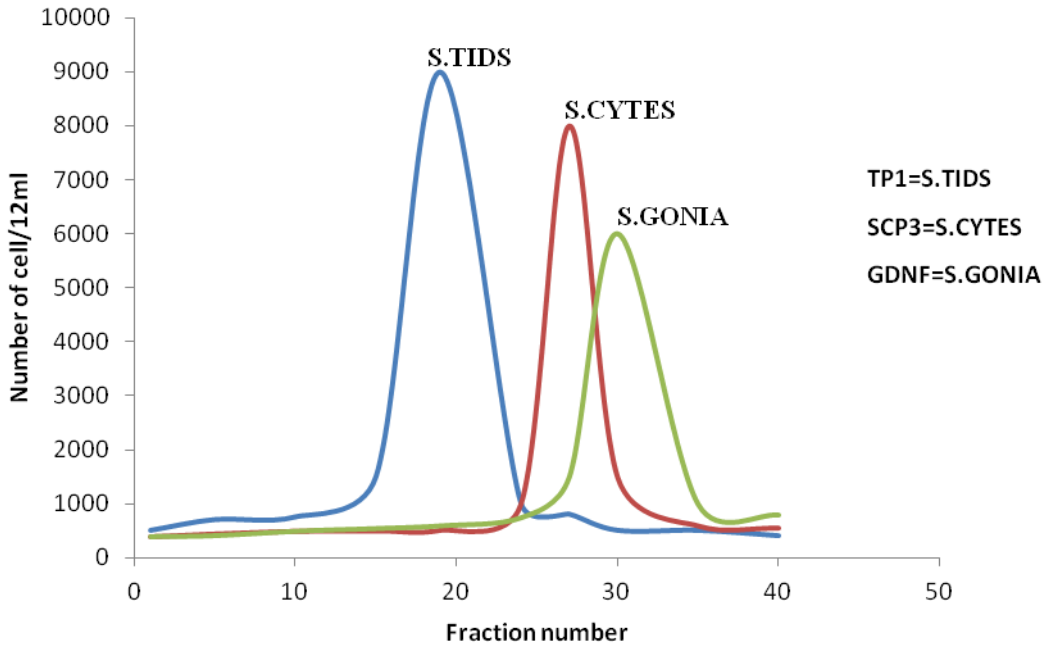


Figure 2

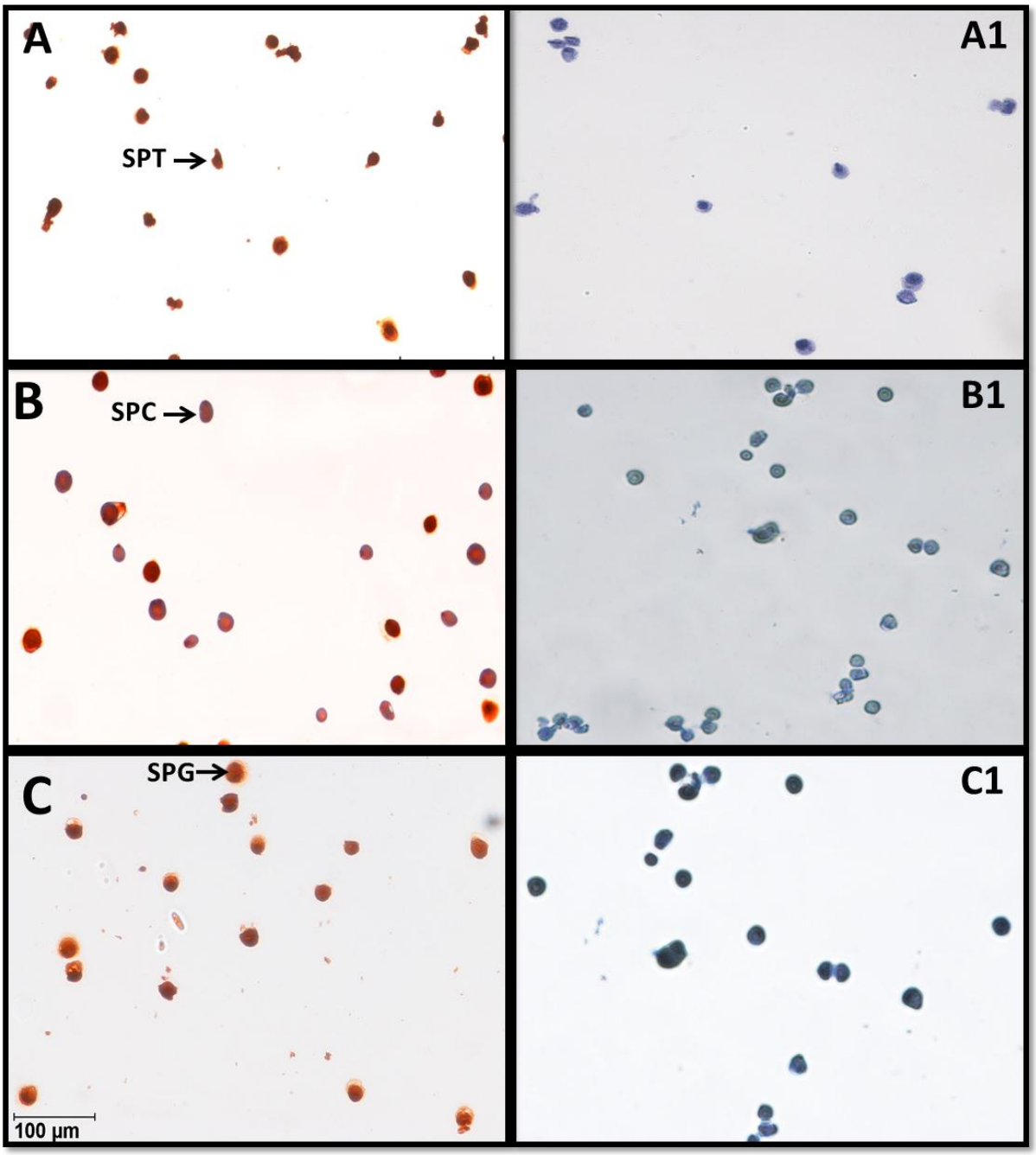
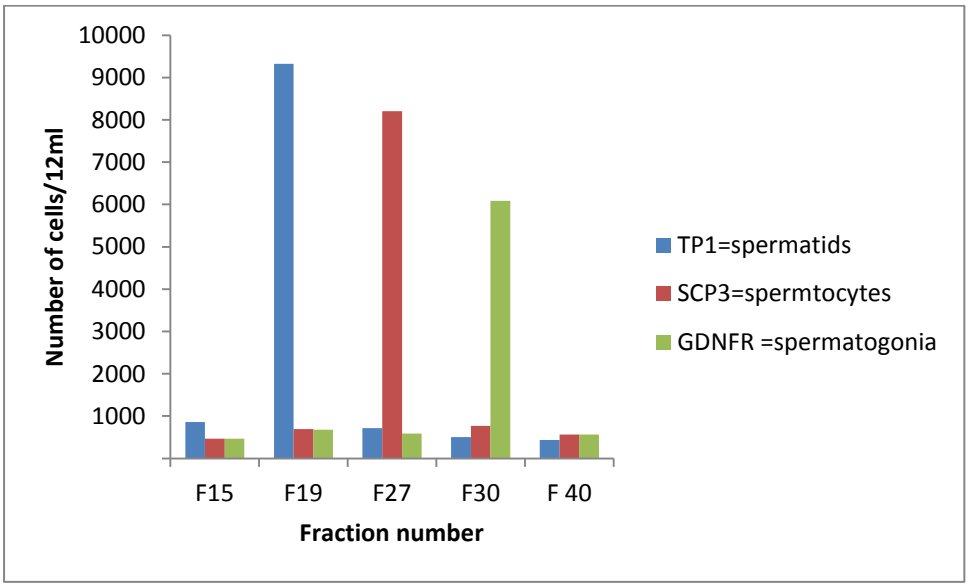


Figure 3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Number of cells/12ml
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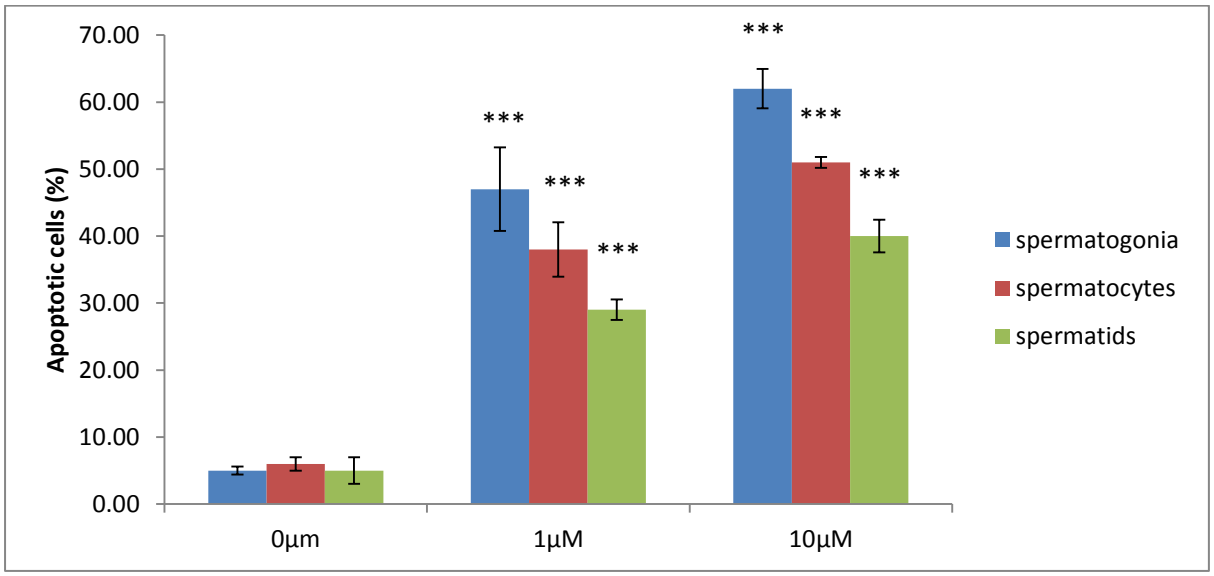
Fraction number
TP1=spermatids
SCP3=spermtocytes
GDNFR =spermatogonia

Figure 4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10µM
spermatogonia
spermatocytes
spermatids

Figure 5