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

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HIGHLIGHTS

- There is a need for new *in vitro* alternatives to current *in vivo* reproductive toxicity tests.
- Staput-separated male germ cell types were treated with 0, 1.0 or 10.0 μM H_2O_2 and analysed for apoptosis.
- H_2O_2 induced highly significant increases in apoptosis even down to 1.0 μM in all the germ cell types.
- Spermatogonia were the most susceptible, then spermatocytes, then spermatids, reflecting levels of cell division in each.
- The approach has great potential as a sensitive and useful method for the rapid 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 1 and 10 μM 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 (as demonstrated by the European REACH regulation [EU, 2007], for example). 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 provide supply nutrients, hormones and

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

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structural support to the germ cells and temporal regulation of 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; Meistrich & Hess, 2013). 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 ROSs; 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.

2. Materials and methods

2.1. Animals

Sexually mature NMRI mice (National Medical Research Institute) weighing 25–30 g (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 (5 mg/ml) and DNAse (1 μg/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 80 μm 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 10 ml, and a BSA gradient using 250 ml of 2% (w/v) and 4% (w/v) BSA was generated. The cells were allowed to sediment for a standard period of 2.5 h, and then 31 fractions each of 12 ml volume were collected at 60 s 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% foetal bovine serum (FBS), 100 Unit/ml penicillin, and 100 mg/ml streptomycin (5×10^6 cells/ml; 1 ml per well) at 37 °C 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 2 h with or without H_2O_2. Incubations with H_2O_2 were made at final concentrations of 0, 1, and 10 μM in triplicate. A temperature of 37 °C would not be suitable for long-term cultures of spermatogenic cells, which thrive best at a temperature 1–2° 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 10 μM 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 (30 mM Tris pH 7.4; 140 mM sodium cacodylate; 1 mM cobalt chloride; 5 μM biotin-16-deoxyuridine triphosphate; 0.3 U/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 visualised by adding 3,3′-diaminobenzine (DAB) for 10 min at room temperature. They were washed in phosphate buffer saline (PBS), counterstained using haematoxylin 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 100 mg/ml streptomycin. The cells were serum starved in DMEM (with the antibiotics) for 16 h 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 haematoxylin (Hsu et al., 1981; Khalfaoui et al., 2011) and a coverslip was applied using Histomount mounting medium (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).