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Assuring safety without animal testing: The case for the human testis in vitro

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ABSTRACT

From 15 to 17 June 2011, a dedicated workshop was held on the subject of in vitro models for mammalian spermatogenesis and their applications in toxicological hazard and risk assessment. The workshop was sponsored by the Dutch ASAT initiative (Assuring Safety without Animal Testing), which aims at promoting innovative approaches toward toxicological hazard and risk assessment on the basis of human and in vitro data, and replacement of animal studies. Participants addressed the state of the art regarding human and animal evidence for compound mediated testicular toxicity, reviewed existing alternative assay models, and brainstormed about future approaches, specifically considering tissue engineering. The workshop recognized the specific complexity of testicular function exemplified by dedicated cell types with distinct functionalities, as well as different cell compartments in terms of microenvironment and extracellular matrix components. This complexity hampers quick results in the realm of alternative models. Nevertheless, progress has been achieved in recent years, and innovative approaches in tissue engineering may open new avenues for mimicking testicular function in vitro. Although feasible, significant investment is deemed essential to be able to bring new ideas into practice in the laboratory. For the advancement of in vitro testicular toxicity testing, one of the most sensitive end points in regulatory reproductive toxicity testing, such an investment is highly desirable.

1. Introduction

We live in a time of rapid change, and it is not just that it *seems* faster than 40 years ago, it *is* faster than 40 years ago [1]. In the field of assuring the safety of chemicals used in all aspects of modern society, many independent threads appear to be converging to allow for a possible solution to a problem that has dogged the field of toxicology for years: how to identify compounds which are toxic to the male reproductive system without costly animal tests. This convergence suggests that now is the time for focused action.

It is the job of toxicologists to understand the likely human health impacts of exposure to any given chemical or group of compounds. Animal models are used to test for the toxicity of chemicals because it would not be ethical to conduct testing in humans and because in test species such as rats we can explore the full range of targets across the entire reproductive cycle. The use of rodent test species also allows a full exploration of dose–response, comparative organ toxicity, and limited comparisons with human response while at the same time considering interspecies differences in metabolism and physiology However, there has been a rising concern in respect of animal rights and a general desire to use fewer animals in safety testing [2]. But there has also been an evaluation of how well these animal tests have done at predicting human safety [3], and the answer is that animals predict correctly only 50–70% of the time. This suggests that some dangerous chemicals are allowed into commerce (either as industrial chemicals, pesticides, or pharmaceuticals), while other truly innocuous compounds are dropped during discovery and development because of an erroneous toxicity signal from animals.

At the same time, there have been advances made in the area of cell and tissue culture [4–6] which suggest the possibility of creating in vitro models of the tissues of concern using human cells, and thus avoiding the inter-species extrapolation problem. Use of cell-based test systems requires confirmation that the results can be extrapolated to the whole human (i.e. in vitro to in vivo extrapolation.)

The authors of this paper assembled from 15 to 17 June 2011 under the sponsorship of the Dutch Assuring Safety without Animal Testing initiative, to consider these issues with regards to spermatogenesis, and whether (a) this was an area of public safety concern, (b) whether animals can adequately predict human response now, and (c) whether existing animal alternatives would answer the need, and if not, (d) what new alternatives might be evaluated to meet that need and their likely chances of success.

2. Human evidence

There is considerable evidence from the public health and clinical literature that spermatogenesis and testicular structure/function are impacted by environmental exposures. For example, recent reviews and specific examples are cited as representatives of studies that have evaluated semen quality in adult men as an indicator of environmental or pharmacological effects [7–12]. There is growing evidence that altered testis development can result in reduced adult function. Testicular dysgenesis syndrome, which includes cryptorchidism, hypospadias, impaired spermatogenesis, and testicular cancer, reflects altered development, and can result from a altered androgen signaling or primary

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testicular failure in the fetus [13]. Many measures of testicular dysgenesis are increasing [14,15]. Studies across countries and migration studies support a strong environmental component for these effects [16–18]. Human epidemiology surveillance studies also support this [19,20].

3. Animal evidence

It is difficult to obtain a precise count of how many chemicals have produced testicular toxicity in animal toxicology studies. Ulbrich and Palmer [21] published a literature evaluation, which compared various methods of identifying male reproductive toxicants. Their database contained 117 substances or mixtures that are reported to have male reproductive effects in preclinical species or in humans.

The recent WHO report on endocrine disrupters and child health 'Possible developmental early effects of endocrine disrupters on child health' lists observed effects in the human reproductive system and in the animals [22]. Although the tables in the document are not comprehensive, they show both the abundance of readily available data and the profound lack of information about the effects of current exposures.

A relatively superficial scan of the National Toxicology Program's Database of Reproductive Assessment by Continuous Breeding (RACB) studies found that of the 85 studies in the accessible database, 42 of those showed evidence of male reproductive toxicity. Given the level of detail available in the abstracts (and the specifics of the protocol: many animals were not examined histologically), it is not possible to state with certainty how many of these had damage to the seminiferous epithelium, but by making reasonable inferences from the abstracts, it seems likely that at least 75% of these admittedly high-dose exposures produced damage to the seminiferous epithelium. We know that this does not represent a random selection of environmental chemicals [23]: chemicals were nominated based on some possible toxicity to reproduction (male or female) or to neonatal development, or because it was part of a structural series of related chemicals, or it showed up positive in a screening study [24].

In pharmaceuticals, a more recent effort by Sasaki et al. [25] found that many companies encounter 1–3 drug candidates per year that cause testicular toxicity sufficient to delay or halt the development of that compound. This suggests a recurring problem. Different companies will experience this to a greater or lesser degree, depending on serendipity or the therapeutic areas in which they are working (Jane Stewart, personal communication). If the margin is sufficiently large, that is, if there is a big enough multiple between the intended therapeutic human blood level and the levels which caused the toxicity in the preclinical species, then the risk can be managed by tightly controlling human exposure, or perhaps performing a human semen study early in development to determine whether human spermatogenesis is, in fact, affected by the compound, as reflected by a change in ejaculated sperm counts.

In our experience in pharmaceuticals, unmanageable testicular toxicity is rare, but those programs beset by this effect could benefit enormously from having an in vitro screening method which could identify a "clean back-up," a compound with the same intended pharmacologic activity but without the attendant toxicity. This is valuable in pharmaceuticals because each new compound must be synthesized, purified, and chemically characterized prior to being evaluated for safety. It is *much* less expensive to do this for the 100–200 mg of compound required for in vitro studies than it is to do it for 50–100 g necessary for an in vivo study. The same is true in new pesticide development.

Finally, we must face the issue of extrapolation between animals and humans. The most widely quoted paper here is by Olson et al. [3], who found that, depending on how one looked at the data, there was a correlation that ranged between 50% and 70% for the ability of animals to predict the effects seen in humans across multiple organ systems. In an attempt to get around this problem, and in an attempt to take advantage of recent advances in technology and understanding cell biology, a panel convened by the National Academies of Science recommended that future toxicity testing should bypass the phenomenological description of lesions, and distinguish toxic from non-toxic compounds by their effects on gene or biological pathways in cells in culture [26]. There is no conceptual reason why these cells could not be of human origin, thus neatly sidestepping the entire species-extrapolation issue (and replacing it with an in vitro–in vivo extrapolation issue). Applying this vision to the testis is being done by only a few leading investigators; we are at the very beginning of knowing the pathways of toxicity in the testis. For this reason, a new model that recapitulated in vitro much the same sort of selective cell death which is seen in the testis in vivo would be exceptionally useful.

Table 1
In vitro models of testicular function.

Species	Predominant cell types	References
Rat	Sertoli, spermatogonia, spermatocytes	[27,28,36,71–73]
Rat	Seminiferous tubule or tubular cells	[74,75]
Rat	Leydig cells	[76,77]
Rat	Seminiferous tubular explants	[29,38,41]
Rat	Sertoli and Leydig cells	[32]
Rat	Organ culture	[78]
Mouse	Organ culture	[49]
Mouse	Leydig cells	[79,80]
Mouse	Tubular reconstructions	[81]
Bull	Tubular cells	[82]
Human	Spermatids from stem cells	[83]
Human	Germ cells on Vero cells	[84]
Human	Sertoli and Leydig cells	[85]
Human	Spermatogonia with tubular cells	[86]
Human	Tubular cells	[87]

4. Existing alternatives

Cells from the testis have been cultured in vitro for many years. Some of these models can recapitulate some of the in vivo responses to chemical exposures: the cell death or release of germ cells from Sertoli cells [27,28], inhibited spermiation [29], or inhibited testosterone release from Leydig cells [30]. There are a wide variety of in vitro models that have appeared in the scientific literature, most for a limited purpose (Table 1). The shortcomings of these previous in vitro models are that they are not sustainable: they cannot sustain germ cell differentiation and maturation, nor do they maintain cell division that is a key feature of in vivo spermatogenesis. Indeed, all in vitro models are valuable only in limited duration: from the beginning of the culture, the Sertoli cells are de-differentiating and the germ cells are slowly but progressively dying. Thus, they are of very limited use. A co-culture model which contains mostly spermatogonia and spermatocytes could be useful for screening for spermatogonial proliferation and germ cell death in vitro (manifested as the release into the medium of the dead germ cells), but the absence of Leydig cells or round spermatids or mature spermatids from the culture means that this model would be useless in capturing adverse effects on steroidogenesis, or round spermatid sloughing, or the inhibited spermiation occasionally induced at the end of spermatogenesis, respectively. Specialized cultures containing those cell types would be necessary to capture those sorts of effects. These can be generated as primary cultures from animals, but they come with the unsustainable, "constant rate of decay" issue that plagues all primary testis cultures. Alternatively, cell lines from various testicular cell types can be used, but their