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Assessing Male Reproductive Toxicity during Drug Development

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Abstract

Pharmaceutical development is a crucial research field that attends to the population healthcare needs, comprising several preclinical and clinical steps before the approval of a new compound. Interestingly, the lack of efficacy and toxicity are known major failure causes during pharmaceutical drug development. Toxicity screening is therefore a mandatory procedure during pharmaceutical development. Renal and hepatic toxicity are the most common toxicity matters encountered during the development of new compounds. Nonetheless, the male reproductive system may also be a target for drug toxicity. However, developmental and reproductive toxicology testing is rarely performed both in pre-clinical and clinical trial phases. As a result, there are several risks for human fertility brought on by putative toxic chemical compounds. As there is a low incidence of reproductive and testicular toxicity (TT) found at the early stages of pharmaceutical development, several companies devote the majority of research investments to more frequent areas of occurrence. Such prioritization has resulted in scarce rigorous efforts aimed at improving detection methods to understand the causes of TT. Pharmaceutical companies should include comprehensive studies and more precise methods for TT evaluation when developing new pharmacological drugs, and should focus on the effects of the new chemical compound on male's reproductive functions.

Keywords: Testicular toxicity; Pharmaceutical development; Male infertility

Introduction

Male factor infertility represents practically 50% of all infertility cases worldwide [1]. Male aging, as well as genetic, environmental, behavioral, nutritional imbalance and metabolic disturbances are responsible for most of the reported infertility cases [2-5]. In fact, several drugs have been found to induce testicular toxicity (TT), due to histomorphological and/or hormonal alterations of the male reproductive system, leading to a decrease in semen quality and compromising the production of fully competent spermatozoa (Figure 1) [6].

Moreover, other extrinsic factors, like the use of therapeutic drugs, can be blamable for adversely affecting human fertility [6,7]. Through the various life stages of a man, his reproductive tract is under a regulated process of development. This, however, can unfortunately be disturbed following exposure to several toxic compounds. The exposure impacts on male reproductive potential may only be fully understand if it is known when the exposure occurred (during in utero development and/or puberty and adulthood). This different exposure may share a common "Testicular Dysgenesis Syndrome" etiology but may lead to reproductive congenital defects, such as cryptorchidism, hypospadias, and anomalies of the epididymis, vas deferens and reproductive accessory glands; or adult alterations, such as in spermatogenesis, and testicular tumors (Table 1) [8].

Pharmaceutical development is a crucial research field that attends to the population healthcare needs and comprises several mandatory steps before the approval of a new compound. The primary purpose of toxicity testing is to identify the dose range over which toxic effects are usually observed, as well as the nature of the drug toxicity. As therapy side-effects are a known safety issue, several researchers are focusing their attention on the negative effects of drugs within the defined safety therapeutic dose range [9]. Even so, the major failure causes during drug development are the lack of efficacy, and toxicity, with the latter accounting for approximately 30% of all failures [10]. Thus, toxicity screening during pharmaceutical development is essential to identify potentially toxic compounds and to minimize adverse side-effects. As a result, although TT is a sporadic issue during pharmaceutical development, it must also be an evaluated parameter when assessing overall drug toxicity [11]. Renal and hepatic toxicity are the most common toxicity matters encountered during drug-development. Nonetheless, the male reproductive system may also be a target for drug toxicity [12]. TT represents a challenging issue in the field of pharmaceutical investigation during preclinical stages, due to the lack of simple and robust screening methods [13,14]. During pharmaceutical development, histopathological, hormonal, and semen parameters evaluation are the most commonly employed methods to assess TT and general male genital tract toxicity [15,16]. While animal histopathological procedures are an accepted method to evaluate TT, these procedures are mainly descriptive, being unable to measure the toxicity degree and to discriminate between TT and nontoxic testicular changes (related to immaturity or to spontaneous conditions) [17,18]. Hence, it is important to identify supplementary quantitative approaches for TT evaluation during drug development. Over the last years some studies have been evaluating the efficiency of other precise methods such as the evaluation of the testicular cells proliferation index using immunohistochemistry assays [19], or via alterations to gene and protein expression [20-22] or epigenetic regulation [23,24]. In this review, we will review the effects of pharmaceutical growth on TT as

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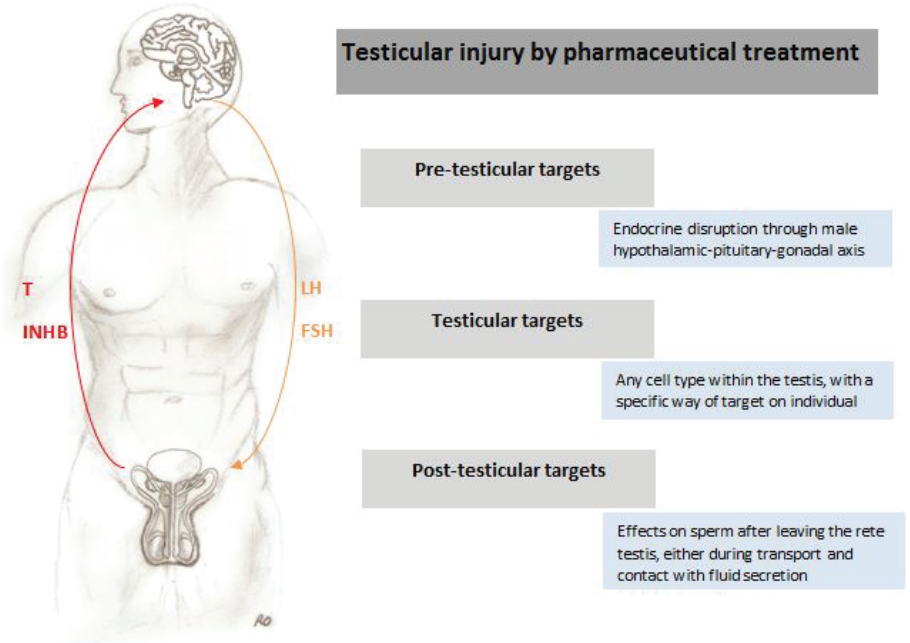

Figure 1: Potential effects of pharmaceutical drugs on male reproductive system. Testicular function, including androgen secretion and the production of fertile sperm, can be adversely affected by pre-testicular, testicular and post-testicular mechanisms. FSH, Follicle Stimulating Hormone; INHB, Inhibin B; LH, Luteinizing Hormone, T, Testosterone.

	Drugs	Species/Reference	Possible outcome
In utero development	-Chemical pesticides	Human [55,56]	- Disruption of endocrine system
	-Polychlorinated biphenyls	Fish [57,58]	- Increase of congenital anomalies (cryptorchidism and hypospadias)
	-Dioxin	Mice and rats [59-61]	
	-Receptor antagonists used in treatment of cancer		
	-Derivatives of estradiol		
Puberty and adulthood	-Environmental endocrine disrupting chemicals	Humans [62]	- Alteration of endocrine/paracrine status (influence on accessory male sex glands)
	-Industrial solvents	Mice [63]	- Alteration of testicular function (spermatogenesis) and histology (Leydig and Sertoli cells)
		Rats [64-68]	- Disruption of blood-testis barrier
			- Testicular cancer development

Table 1: Male reproductive tract toxicity during man's developmental stage.

well as the current methods involved in TT evaluation during the drug development process.

Method

Drug development a step-by-step process

Pharmaceutical therapy has been developed in response to population healthcare needs and comprises several mandatory procedures, to attest its efficacy and safety, in order to approve and legalize a new chemical entity [9]. The approval process of a pharmaceutical drug begins with a preclinical test. At this point, a new compound is tested *in vitro* and subsequently, *in vivo*, using experimental animal models [25]. Firstly, the preclinical assessment involves exploratory toxicology, and is of a dose-ranging nature, typically acute or short-term. The aim of this phase, that may exceed a 4 year period, is to identify the major target organs and physiological systems that are affected by the drug, screening for the drug's toxicity. This procedure also involves the evaluation of pharmacological effects of the new chemical. In addition, during the same period of time drug's genotoxicity is also investigated [9,26]. Nonetheless, a number of pharmaceutical compounds are unable to surpass the preclinical development stages due to the lack of evaluation tools that can accurately monitor toxicity in preclinical and clinical studies [27,28]. Thus, efforts have been made to improve the now available organ-specific toxicity detection tools through the identification and characterization of toxicity biomarkers [26,29]. At the end of the preclinical trial stage, if the chemical is reported as promising the investigator creates an Investigational New Drug Application with the Food and Drug Administration (FDA), showing the chemical's pharmacological profile and presenting the preclinical results of short-term toxicity in no less than two animal species [9,30]. If the application is approved, human clinical trials can be started after a 30 day period. Clinical trials are subdivided into 3 different phases including: Phase I-studies that are conducted in a small number of normal and healthy volunteers to determine the safe dosing range and

toxicity of a compound [31]; Phase II-if the drug is safe in the previous individuals, it will go on to Phase II testing, in which the drug is tested in a larger sample of volunteers who have the medical condition that the product is intended to treat [32]; Phase III-is achieved if the compound is still considered promising after phase II trials [33]. In this phase the drug will be tested in a larger sample of subjects with the disease of interest and several dose quantities or schedules than those used in the previous phase will be tested. The principal aim of this final phase of drug-development is to clinically demonstrate the efficacy and safety of the new chemical. Nonetheless, as phase III trials involve more individuals than phase II it is more likely to observe negative effects. At this stage, if the compound still remains promising the results of all clinical stages are presented to the FDA, and the producer will submit a New Drug Application to the FDA [9,26,34].

Male reproductive function concerns during the drug development phase

The human male reproductive system is very sensitive to toxicant-induced injury and the available procedures for detecting TT are fairly limited [35]. Toxicant-induced testicular injury is usually asymptomatic, which may pose a problem during pharmaceutical development [29]. Hence, it is important to establish clear strategies for toxicity evaluation during drug development. Currently, evaluation of human testicular function and sperm quality depends on hormonal profile and semen parameters assessment. Nevertheless, monitoring the exposure effects to a testicular toxicant using these traditional methods is problematic due to parameters variability and assays relatively insensitive [16]. Therefore to guarantee the reliability and value of the semen analyses it would be of great importance that a single centre prosecute and analyze all the samples during the trial. Moreover and according to World Health Organization guidelines [36] several controls, such as a group of men with normal semen analysis and proven fertility as well as control groups subjected to placebo and/or presenting subnormal semen parameters, should also be studied. As for hormonal profile evaluation, the Sertoli cell product, Inhibin B, has been proposed as a potential biomarker of TT in rodent toxicity studies [37]. Besides, the potential correlation between changes in plasma Inhibin B and Sertoli cell toxicity to allow a better linkage between animal study results and subsequent monitoring of testicular function in clinical trials during pharmaceutical development has also been reported [38]. Nonetheless, hormonal measurements are generally unreliable for the measurement of mild testicular injury, meaning that only severe and potentially irreversible injuries can be detected with this method [29]. Consequently, more sensitive tools are needed, to allow the translation of findings in preclinical species to humans, and to predict the testicular dysfunction at an earlier and reversible stage of injury. On the other hand, histopathology studies are a more sensitive method used for testicular injury measurement and can be performed in animal models. However, it is a very invasive procedure which makes it impractical to monitor exposures in humans [29]. In the early onset of injury, the majority of testicular toxicants show a cell-specific and a stage-specific pattern of damage. Accordingly, thorough morphological and molecular evaluations of the testis and the epididymis are essential for TT evaluation [39]. Efforts have been made to identify the potential value of measuring sperm molecular biomarkers when monitoring TT. The sperm membrane protein, SP22, a sperm protein that declines in abundance after exposure to both epididymal and testicular toxicants, is an example of the animal investigations that have been made for this purpose [40,41]. Nevertheless, there are some problems concerning this biomarker, such as the reduced amounts of sperm protein available for testing and the limitations that relying on a single biomarker instead of a group of indicators convey. In addition to SP22, several other molecular biomarkers have been studied. For instance, alterations in human sperm mRNA transcript content [22,42] and DNA methylation profile during human spermatogenesis [43,44] that are related with male fertility profile, have been investigated. Interestingly, there is strong evidence that a specific group of sperm mRNA transcripts can detect and predict low level exposures to Sertoli cell toxicants in the rat [45]. Evaluation of these sperm molecular biomarkers can provide comprehensive knowledge into the testicular response to toxic exposures and help translate findings to pharmaceutical drug development. In the last 5 years it was reported that cases of TT only occurred in just one to three of the drug development programs. However, some programs contained several drug candidates, and unknown TT was multifactorial and probably caused by environmental exposure to other toxic substances [15,16]. Usually, during pharmaceutical development there is a low incidence of TT and reproductive toxicity found at the early stages. Consequently, numerous companies devote the majority of research investments to more frequent areas of occurrence, such as hepatic or renal toxicity. Such prioritization has resulted in scarce rigorous efforts aimed at improving detection methods to understand the causes of TT [15]. Thus, more attention to TT should be applied during drug development not only on the recognition of nonclinical signs of TT but also to weigh the likely for such toxicity in humans. Additionally, the improvement of new biomarkers or a panel of biomarkers to assess earliest stages of TT, should be a major concern for pharmaceutical companies during drug development.

Male reproductive function evaluation during drug development

Drug development protocols include several different stages that aim to fully assess and comprehend a drug's efficiency and potential side-effects. As human reproduction is dependent on complex interactions between numerous cells and organs, in vitro testing falls short when evaluating drug toxicity, and in vivo research is necessary. Even though cooperative efforts have been made to establish strict guidelines for reproductive toxicity assessment during drug development, protocols are not yet standardize and discrepancies between research centres and between countries still exist [46]. Drug safety establishment must rely on a well-designed battery of studies that account for genetic, carcinogenic, and reproductive and development toxicity [47]. In order to identify the best approach for TT assessment during drug development, researchers have conducted numerous validation studies intended to calculate the current screening tools effectiveness. Some findings have already been made on the matter, and, for instance, it was reported that a 2-week drug treatment was sufficient to analyse drug toxicity-induced damages to rat male reproductive organs [46]. However, and since one cycle of spermatogenesis takes about 76 days the TT analysis should not be done only at the beginning and end of drug administration but 12-13 weeks after the beginning of drug delivery regardless of whether the treatment has already been or not finished. Drug development comprises several phases that are crucial steps in testing a drug's safety for male reproductive health. During its early stages, preclinical safety trials evaluate *in vitro* toxicity allowing for the development of safer and more effective animal testing trials. Nonetheless, the animal models used in testing should be relevant, data must be clearly interpreted and any confounding factors must be taken into account [47]. There are several characteristics that must be pursued when selecting a species

for toxicological studies in order to achieve relevant results. Firstly, its biology should be broadly understood and it should generally have similar toxic reactions to those of humans. Moreover, it should have a good reproductive capacity and present strong predictive values. Also, several aspects as the number of test subjects required and the cost associated, and the compound metabolisation should be taken into consideration when drawing TT animal trials [48,49]. During the preclinical animal trial phase, the translation to humans is the interest of the experimental design though it should be concerned with minimizing animal suffering and loss so long as doing so does not interfere with the validity and robustness of the results. All endpoints must carefully be considered and evaluated taking into account that results must be clinically relevant for latter human testing. The animals used in the preclinical trial phases must be sexually mature, and the presence of semen must be checked prior to drug exposure, as prepubertal animals may erroneously be misinterpreted as impaired spermatogenesis. Though as spermatogenesis relies on a pool of spermatogonia stem cells, prepubertal animals should also be studied. Other aspect to take into consideration is the number of animals used, that varies depending on the experimental design. Several other aspects can also influence the research results, such as the length in treatment and the dosages used, that must be sufficient to address possible effects on all germ cell stages. Lastly, all qualitative and quantitative measurements, such as hormonal levels, subject weight and overall health status must be carefully examined, and late onset events such as chronic toxicity must be taken into consideration in trial design [50]. As most requirements for animal subject selection and study design cannot be fully met, it comes as no surprise that evidences suggest that human based experiments are more likely to portray clear definite findings [51]. Several techniques, such as organ slice evaluation, would diminish the differences found between animal models and human experimentation models, as they would allow for a more clear cross-species comparison of the achieved results. Still, the use of human tissue provides important data that is clinically relevant in identifying drug toxic events and can also aid in the identification of cellular stress biomarkers, that can be latter used for drug toxicity monitoring [52]. Therefore, in vitro systems that are based on reproductive cells may be of greater value when testing drug properties related to TT. Even so, researchers must acknowledge that all in vitro findings are somewhat subjective as they do not represent relevant physiological conditions and are clearly limited by a lack of organ and tissue interactions [46]. Drug safety testing usually focus on common side-effects, such as hepatic, nephrotic and neuro toxicity. Although fertility is an important issue for most people, developmental TT testing is rarely performed both in preclinical and clinical trial phases [53]. Nonetheless, there are several risks for human fertility brought on by chemical compounds, like fertility loss and embryo development issues. In males, the reproductive tract can be affected by several chemical compounds, what can lead to transient or permanent infertility. Some of the most common drug-induced lesions found on males exposed to therapeutic drugs included, epididymis issues, sperm retention, seminiferous tubules morphological alteration, overall testicular atrophy, aspermia, germ cells alterations, Sertoli cell-only tubules and immature spermatozoa [15]. Even though these issues are a main concern for patients who might possibly be treated with these new drugs, few pharmaceutical companies study their new drugs effects on male fertility, as most developmental TT testing focus its attention on pregnancy outcome and embryo development aspects. Thus, pharmaceutical companies should include comprehensive studies of reproductive toxicity when developing new pharmacological drugs. These studies should focus not only on pregnancy and developmental risks associated with drug exposure, but also assess the effects of the new chemical compounds on male's reproductive functions (Table 2).

Nevertheless, for potential new medicines that are to be taken by those of reproductive age (and especially for those to be taken in repeat dose fashion), TT testing is mandated prior to beginning widespread exposure to patients. Though, many putative medicines are not tested

Pre-Clinical Phase			**Clinical Phase**
In vitro	***In vivo***		***In vivo***
Diagnostic tools			**Possible lesions associated with drug toxicity**
General physical andrological examination	Testicular size, volume and palpation		- Hiper-atrophy and atrophy - Testicular tumor - Orchitis
	Epididymus and Vas deferens palpation		- Atrophy, obstruction or inflammation
	Prostate retroperitoneal palpation		- Hiperplasia - Carcinoma
	Penis		- Size changes - Erectile dysfunction
	Secondary sexual characteristics		- Alterations of body proportion, fat distribution and musculature - Voice and hair mutations - Gynecomastia (indicative of endocrinologically active testicular tumor)
Molecular and Cytogenetics	Karyotyping Fluorescence in situ hybridization Molecular genetic diagnostics		- Structural chromosome abnormalities (such as Y chromosome microdeletions) - Genetic mutations
Endocrine Laboratory Diagnosis	Gonadotropins [Follicular Stimulating Hormone (FSH) and Luteinizing hormone (LH)]	↑ levels + ↓ T levels	- Primary hypogonadism
		↓ levels	- Secondary hypogonadism
	Testosterone (T)		- Alterations on reproductive performance - Alterations on social behavior - Alterations of the secondary sexual characteristics
	Estradiol (E2)		- Alterations on reproductive performance
	Prolactin		- Alterations on reproductive performance
	Inhibin B		- Sertoli cell dysfunction - Impaired spermatogenesis
	Anti-Mullerian hormone (AMH)		- Alterations of Sertoli cell number, function and maturation

Semen analysis	Macroscopic examination	Volume pH Appearance	- Infection - Obstruction of the efferent system - Semen secretions production dysfunction
	Count		- Azoospermia and/or Aspermia - Sertoli cell-only syndrome
	Concentration		
	Motility		- Asthenozoospermia
	Morphology		- Maturation arrest
	Immunological tests	Sperm agglutination	- Testicular inflammation (cytotoxicity) - Sperm motility disorders
	Biochemical analysis	Zinc, citric acid and prostatic acid phosphatse measurement	- Prostate dysfunction
		Prostaglandins and fructose measurement	- Seminal vesicles dysfunction
		Neutral α-glucosidase, L-carnitine and glycerophosphocholine measurement	- Epididymal dysfunction - Distal obstruction of the efferent system
	Sperm function	Vitality	- Metabolic Dysfunction
		DNA integrity	- Sperm DNA fragmentation and / or immature chromatin
		Reactive oxygen species	- Infection - Impaired motility
Hystophatology	Target cell type	Sertoli cell	- Tubular atrophy - Impaired spermatogenesis - Sertoli cell-only syndrome
		Leydig cell	- Impaired spermatogenesis - Hyperplasia - Testicular weight decrease
		Germ cell	- Hipospermatogenesis to maturation arrest - Disturbed differentiation of spermatids
	Target organ	Testis	- Morphological alterations - Tumors - Infection - Obstruction
		Epididymis	
		Seminal vesicles	
		Prostate	
Fertility assessment by fertilization and / or pregnancy rates		Animals	Feasible but there is the need to use pubertal animals
		Humans	Not ethically feasible but may be circumvented if during clinical trials evaluations the control group besides presenting normal semen parameters present also proven fertility
To evaluate the drug effect, the above studies should be conducted before and after (at least at the end of a spermatogenic cycle) exposure to determine the reversibility or permanent toxic effects.			

Table 2: Monitorization of the potential male reproductive toxicity of a drug in development.

because they have been winnowed from consideration by the finding of adverse results in earlier testing.

Discussion and Conclusion

During drug toxicity assessment it is important to evaluate the male's hormonal profile and reproductive tract function in order to identify any alterations that might result in spermatogenesis and spermatozoa defects. Nonetheless, the current methods for evaluating TT and semen quality are extremely variable or invasive, and in most cases, are only observed when the injury is severe and potentially irreversible.

The current lack of any reliable biomarkers for evaluation of TT, either in animal models or in the clinical trials phase, is a problem for the drug development industry. Nevertheless, the focus on this issue is increasing and several new researches now being done in this area may provide vital developments. In the future, toxicity evaluation during pharmaceutical development will be more effective with decreased time and resources needed to evaluate the safety of a new chemical compound. Many decades and continuing TT research as already identified and quantified the effects of male reproductive system's toxicants but will produce new insights on the mechanism of action for the toxicants in question.

The development of sperm-specific molecular biomarkers represents a very important research strategy. It may provide a reliable and sensitive monitoring method for therapeutic settings, and has the ability to facilitate the translation of preclinical animal study findings into the clinical trials phase of new pharmaceutical chemicals.

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