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Reproductive and Developmental Toxicity Prediction by *In-Vitro* Methods: A Review on Alternatives for Laboratory Animal Research

D.Sivaraman[1]*, R.Selvaraj[1], B.J.Dare[1], N.Mohana[1], R.Narasimha Raghavan[1], G.Vignesh[1].

[1]Centre for Laboratory Animal Technology and Research, Sathyabama University, Jeppiaar Nagar, Rajiv Gandhi road, Chennai– 600119, Tamil Nadu, India.

Abstract: The term reproductive toxicity (RT) in current scenario grabs more attention due to its increased prevalence and also improving awareness among the general public. Since some drugs and chemicals during administration in human or in animals which has a tendency to disturb the normal sexual behavior, production and maturation of spermatozoa, implantation and development of embryo including growth of the fetus. There are certain chemicals like Altretamine, Benzene, Aspirin, Carbon disulfide, Clobetasol propionate, which causes developmental reproductive toxicity as listed by the environmental protection agency (EPA). This review mainly focuses on foretelling, some of the important alternative methods available for predicting the reproductive toxicity nature of a chemical without using animals. Alternative assay like embryonic stem cell test (EST), Estrogen receptor binding assay, Computer-Assisted Sperm Analysis (CASA), Leydig cell culture, Androgen receptor chemicallyactivated luciferase expression reporter gene assays (AR CALUX) are available in recent time for evaluating the RT of a particular chemical. There are certain regulatory bodies which governs the accuracy and precision of the above mentioned test like, the organization for economic co-operation and development (OECD), EPA, European centre for the validation of alternative methods (ECVAM). According to the recent survey the accuracy of this assays ranging from 70-88% in animals and humans.
Key words: Reproductive toxicity, *In-vitro* toxicity test, EST, Estrogen receptor binding assay, Leydig cell culture, AR CALUX, CASA.

Introduction

According to OECD guideline for testing of chemicals for developmental and reproductive toxicity a test substance should prove its safety with respect to all the aspect of reproduction in both the sex of a species[1]. Further, most of the regulatory authorities strongly recommended the scale of safety at the no observed adverse effect level (NOAEL) that is the highest test dose of a drug at which there is no statistically significant level of toxicity or adverse events /biological changes in a test organism, this could also be helping for the scientist in predicting the dose response of a particular drug in a biological system[2].

According to the recent demography 2.2 million casualties and 1.06 lakh death incidence were reported annually[3] and most of the United states food and drug administration (FDA) approved molecules are called back due to unexpected adverse effects in humans. Hence safety data on reproductive toxicity testing is of utmost importance before proceeding to the efficacy study in any biological system. It is estimated, the global

annual usage of non-human vertebrates ranging from 115.3 to 126.9 million in various levels of preclinical testing[4].

The main problem concerning the usage of animals for research is i.Time consuming ii.Very expensive iii. Involved number of animal iv.Stringent regulation on usage of animals. Other factors, including maintenance and disposal of the waste materials collected each day and also over a period of time .The expense of maintenance covers the food and water consumption, animal bedding, excrement collected, food wastage, chemicals and other pathogenic waste disposal may contribute to air and water pollution[5]. Chances of occurrence of zoonotic infection like ringworm, Q fever, cat scratch disease, ectoparasites and simian foamy virus from lab animals to humans should also be considered at this point[6,7].

By considering the above mentioned facts and also some limitations in *in-vivo* experiments, it's a right time to consider the *in-vitro* testing in reproductive toxicological research since the researcher has good control on the evaluating the results and also can able to overcome the biological variation in the animals by adapting to *in-vitro* estimation[8]. Another main advantage in this method is cost effective, consumes less time and manpower when compare to *in-vivo* animal test.

According to the new regulation of the European union legislation the usage of animals for cosmetics research is completely replaced by the *in-vitro* test like EST designed by ECVAM[9]. ECVAM validates the reliability, reproducibility and accuracy of each *in-vitro* test and the main aim is to replace, reduce or refine the use of laboratory animals in the test process. Tracking system for alternative test methods review (TSAR), validates and approves the methodology of the test with respect to EU regulations on chemicals.

Embryonic stem cell test (EST)

EST becomes a global model for evaluating the embryo toxicity of a drug molecule. The EST first established in 1997[10]. Two permanent mice cell lines (ES cell line D3, 3T3 fibroblasts) were used in the EST. ES considerably the most suitable for evaluating the embryotoxic potential of a chemical/drug, since the method adopted for differentiating the ES cell *in-vitro* is similar to that of the embryo development in *in-vivo*. The results anticipated from the study categorized into three classes: strongly, weakly, or non-embryotoxic. As an outcome of this ES study on compounds like hydroquinone, eugenol, dibutyl phthalate and antimony used in cosmetics, these compounds may likely have embryotoxic potential[11].

The test compound is evaluated based on the concentration that inhibits 50% of the ES cell differentiation into cardiomyocyte (ID50) and also concentration that inhibits 50% of ES cell viability (IC50 ES) or concentration that inhibits 50% of 3T3 fibroblasts (IC50 3T3).

Estrogen receptor (ER) binding assay

Certain chemicals and toxicants commonly called as endocrine disruptors will alter the process of the endocrine system, including blockage of hormone action, modulating the production of endogenous hormones or by altering receptor population. These disruptions have a tendency to cause cancer, birth defects and other developmental disorders.Endocrine disruptors may be associated with the development of learning disabilities, severe attention deficit disorder, cognitive and brain development problems[12].

It is a specific test carried out in order to predict the estrogen receptor binding affinity of the test compound.The results anticipated from the study is that molar concentration of test chemical IC50 which inhibits 50% of binding affinity of the radioligand which has been calculated based on binding curve that fit to a four-parameter Hill equation[13,14]. Organic toxicants and heavy metals either from the environmental source or through drug may reach the system circulation and gained access to the endocrine system, thereby it disturbs the process of the reproduction either directly or indirectly[15,16].

Male infertility due drugs and chemical become a clinical issue globally these compounds may alter the following process of reproduction like production, the number and viability of sperm, volume of semen, ejaculatory dysfunction, competing with receptors of male reproductive hormones thereby disturbs the signals for the production of new sperm or causes dysfunction in prostate glands[17].

Drugs like finasteride, dutaseride, and propecia belongs to the category of 5-alpha-reductase inhibitors cause reduction in sperm numbers, similarly Silodosin, Tamsulosin, Alfuzosin, Hytrin, Cardura belongs to the category of alpha blockers has a tendency to decrease semen volume. Some anti-fungal agents like ketoconazole hurts testosterone production and decreases the sperm production. Anabolic steroids commonly used to develop the muscle mass may interfere with the hormone signals that are needed to produce sperm. Supplemental testosterone will also have a strong negative impact on sperm production[18]. By considering all these facts, it becomes highly mandatory that a compound should prove its level of safety pertains to male reproductive toxicity like CASA.

Computer Assisted Sperm Analysis (CASA)

CASA is one of the most valuable tools for analyzing sperm characters like motion, velocity, and morphology. The following parameters will be evaluated like percentage of motile spermatozoa, the percentage of progressive motility, DAP (distance average path, μm), DCL (distance curved line, μm), DSL (distance straight line, μm), VAP (velocity average path, μm/s), VCL (velocity curved line, μm/s), VSL (velocity straight line, μm/s), STR (straightness, VSL/VAP, %), LIN (linearity, VSL/VCL, %), WOB (wobble, VAP/VCL, %), ALH (amplitude of lateral head displacement,μm), and BCF (beat cross frequency, Hz)[19].

Leydig cell culture

Testosterone hormone plays a significant role both in animals and humans, as it requires for the development and maintenance of sexual organs, maturation of sperms and also for normal sexual behavior. Leydig cells are primarily responsible for synthesis and secretion of hormone called androgen. Hence isolation and culturing of leydig cells are highly beneficial in analyzing the toxic nature of a compound on the regulatory mechanism of male reproductive process[20]. A number of techniques are available for successful culturing of Leydig cells, these include monolayer cell culturing [21] Suspension cell culture[22-24] or on Cytodex beads[25].

The potential of toxicants can be made by evaluating the dose-responsiveness of Leydig cells quantitatively this will be useful in analyzing the Leydig cell function and viability. Leydig cell preparations may be used in mechanistically based response modeling following biologically relevant *in-vivo* exposures. This *in-vitro* test enables one to discriminate accurately between specific and nonspecific inhibitors of steroidogenesis while screening for potential testicular toxins[26].

AR CALUX reporter gene assay

In-vitro method of screening drugs for chemicallyactivated luciferase expression reporter gene assays for androgens (AR CALUX) is very ideal for the first line screening of molecules, to identify the correct positive hit for extensive analysis.The basic logic behind this assay is to identify the interaction of the test compound with the receptor of interest further the antagonist and agonist nature of drugs and chemical towards androgen receptor can be evaluated by AR CALUX cell lines.In these reporter gene assay, DNA sequences containing specific hormone-responsive elements are linked to the gene of an easily measurable protein[27-30]. The reporter gene from firefly luciferase, when introduced in a cell line will express the cognate receptor, or by double transfection with a receptor of interest[31,32].

The results of this assay expressed in terms of Log EC50 values of relative agonistic activity (RAA).This tool may also influence the researcher to concentrate more on the structural activity relationship aspect of the test compound since alteration in position of the functional group will have high impact on binding of the compound with the target hormonal receptor, so not only the toxicity nature, even efficacy of the test drug will also be analyzed and optimized for the therapeutic target for certain disorders.

Summary and Conclusion

Toxicity testing becomes highly essential in order to predict the possible adverse effect of the compound upon exposure and also to predict the dose response curve of the test compound. Toxicity tests are designed to minimize variance, bias and the potential for false-positive and false-negative results.

Certain i*n-vivo* model may produce false results, this is due to the biological variance among the test animals or due to interaction of the enzymes with the test drug. 3- hydroxy substitution of androgens due to 3beta-hydroxysteroid dehydrogenase leads to inactive androgens, while UDP-glucuronosyl transferase enzymes may inactivate estrogenic compounds. Thus the process of metabolism makes the test compound either become non-selective or inactive in such case prediction of toxicity in *in-vivo* becomes more complex.

There are some evidence based case were the results of *in-vivo* experiment gone wrong and as an outcome of this several drugs had withdrawn from the market like benoxaprofen[33,34] pemoline, phenylbutazone[35],troglitazone and amrinone[36]. Hence, in order to minimize these error researcher now rely more on *in-vitro* methods for more accuracy in evaluating the toxicity of the chemicals rather that *in-vivo*. Inherent complexity of the female reproductive system makes the evaluation procedure much difficult in *in-vivo* models. Similarly, there are some studies in male reproductive toxicity cannot be evaluated in rodent models like semen analysis due to difficulty in the collection of samples from rodents in such situation *in-vitro toxicity* studies are highly useful.

Usage of animals as sentinels for predicting the potential risk of the drugs in humans is one of the earliest method, but now a day due to increased advancement in the field of reproductive toxicology more number of *in-vitro* assays have been identified with high accuracy and precision. ECVAM validated the *in-vitro* methodology not only for evaluating pharmaceuticals, but also for medical device, chemical, cosmetic, personal care and household etc.

In-vitro reproductive toxicity test may also have some marginal limitation such as more chances of contamination, lack of biotransformation ability and immunological capability which is the actual scenario in whole animal experiments, which will affects the precision of the toxicity study[37,38]. Alternative assay like embryonic stem cell test (EST), Estrogen receptor binding assay, Computer-Assisted Sperm Analysis (CASA), Leydig cell culture, AR CALUX reporter gene assay will be useful for the toxicologist to exactly predict the target on which the compound likely to bind it may be a receptor, hormone or enzymes of interest. Since these are the key regulator which controls the process of reproduction, so any alteration in the physiology of the regulator will disturb various phases of the reproductive cycle.

Male reproductive toxicity evaluation can be achieved by the following *in-vitro* techniques like CASA, Leydig cell culture, AR CALUX reporter gene assay. In CASA structural characterization of the sperm will be achieved with respect to its number, movement, shape, viability and also its complete morphology. Male infertility may due to any one of the above mentioned reasons and that can be promptly identified with the help of CASA.

In Leydig cell culture test the direct effect of the toxicant on the production of androgen is well justified and also the dose required for the compound to halt this process may be quantified accurately. AR CALUX reporter gene assay substantiates the researcher in categorizing the toxicant whether it could be agonist or antagonist after binding on to the androgen receptor in the live cell culture. Hence, along with the identification of the toxic compound potential lead may also be invented through this *in-vitro* assay.

Female reproductive physiology is much more complex than the male; hence identifying the exact physiology of the toxicant is highly essential since it effects the fetus growth and development. EST will be useful in evaluating the concentration of the test substance which inhibits the embryonic cell viability and differentiation, so dose exposure level of the same toxicant on humans can be easily measured. Affinity of the compound towards the estrogen receptor can be analyzed using ER binding.

In conclusion *in-vitro* model cannot completely replace the whole animal experiments such as evaluation of mating behavior, but the results obtained from the *in-vitro* study will substantiate the researcher in enumerating the difference between toxic and non-toxic compounds. Toxicant like drugs and chemicals interact with the macromolecular protein, hormones and specialized reproductive cells like sperm will causes an alteration in the regular reproductive function ultimately leads to reproductive and developmental toxicity. Provision for analyzing the exact mechanism of the toxicant in *in-vivo* model is very limited, so through alternative methods toxicologist will able to predict the exact mechanism of action of the test compound and also stage of male and female reproduction process which is under threat. One can also predict the dose and time required for a toxicant that produces an undesirable effect in male and female reproductive cycle.Several

agencies justified the accuracy of the data's from the *in-vitro* study by comparing with the result of the *in-vivo* model. Hence, the *in-vitro* toxicological analysis is considered as a potential tool for a researcher in making a better decision on the toxicity nature of a compound in animals and humans.

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