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Can *Drosophila melanogaster* represent a model system for the detection of reproductive adverse drug reactions?

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Once a molecule is identified as a potential drug, the detection of adverse drug reactions is one of the key components of its development and the FDA approval process. We propose using *Drosophila melanogaster* to screen for reproductive adverse drug reactions in the early stages of drug development. Compared with other non-mammalian models, *D. melanogaster* has many similarities to the mammalian reproductive system, including putative sex hormones and conserved proteins involved in genitourinary development. Furthermore, the *D. melanogaster* model would present significant advantages in time efficiency and cost-effectiveness compared with mammalian models. We present data on methotrexate (MTX) reproductive adverse events in multiple animal models, including fruit flies, as proof-of-concept for the use of the *D. melanogaster* model.

Introduction

An important focus in pharmaceutical research is the detection of adverse drug reactions at early stages of drug discovery and development. Evaluating drug-induced developmental and reproductive toxicities represent a major step in the drug development process, since any reproductive system impairments may result in infertility, teratogenicity, mutagenocity and carcinogenicity [1]. Although *in vitro* cell culture systems may appear attractive for initial large-scale screening of toxicity, it is difficult to extrapolate *in vitro* results to mammals, owing to the lack of physiological context. Accordingly, animal models remain an important tool to researchers for use in the establishment of drug safety before human administration. There are a variety of animal models, ranging from worms to primates that can be used for the detection of adverse effects [2,3]. Although mammalian systems may represent a more accurate evaluation tool of short-term and long-term drug safety, they are frequently laborious and costly at early stages of drug discovery and development. The *D. melanogaster* model system, however, is time-efficient and cost-effective and is often overlooked in drug development.

In this paper, we propose increased use of the *Drosophila* model for the early detection of reproductive adverse effects. Using methotrexate (MTX) as a test compound, we present supporting data on drug-induced adverse reactions on the *Drosophila* reproductive system and demonstrate that these results are consistent with those obtained from human, rodents, and other *Drosophila* studies.

Non-mammalian model systems for reproductive adverse drug reactions screening

Currently, the most commonly used non-mammalian model systems during early stages of drug discovery and development are the *Caenorhabditis elegans*, *D. melanogaster*, and *Danio rerio*. Although worms and flies are increasingly used in disease-oriented screening of compound libraries, their use in the screening of drug-induced adverse effects is not explored.

C. elegans have been used as a model organism in biological research for 40 years. At approximately 1 mm long with a life span of about 3 days, they are easy to handle in the laboratory. With respect to pharmacology, *C. elegans* have been successfully used to identify new bioactive chemicals and candidate targets for such compounds [4]. The model provides sufficient neuronal and muscular physiological pathway complexity and these pathways are highly conserved. This level of complexity makes *C. elegans* an attractive candidate to screen for adverse drug reactions for these

☆ In this paper we present assays that can be used to detect adverse drug reactions in the early stages of drug development with the use of *Drosophila melanogaster*.

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1359-6446/06/$ - see front matter. Published by Elsevier Ltd. doi:10.1016/j.drudis.2009.05.010

pathways; however, the *C. elegans* reproductive system differs significantly from mammals. These worms are hermaphrodites, replicating asexually through self-fertilization, making *C. elegans* a less desirable organism to evaluate reproductive toxicity.

Zebrafish, *D. rerio*, are small, 3 cm vertebrate freshwater fish commonly used to study development and have recently been used to study drug-induced developmental toxicity. The embryos of this organism are transparent allowing for developmental changes to be observed without disturbing the embryo. Developmental toxicity studies in zebrafish are performed by adding compounds directly into the water in which the embryo is developing and then evaluating abnormal morphological changes in the embryo [5]. These studies provide valuable teratogenicity data, but they are not capable of determining the impact of maternal consumption of the compound on embryonic development, which is also important.

D. melanogaster has been used for the past 100 years as a powerful tool in genetic, behavioral and molecular biology research programs. Owing to the model's many conserved physiological, biological and pharmacological pathways, it has also been used in recent years in disease-oriented molecule screens [6,7]. Equivalent human and *Drosophila* disease genes have been identified and targeted for the treatment of cancer, neurological disorders, endocrine disorders, renal disorders, and cardiac diseases [8]. Recently, the value of *Drosophila* in pharmacology has been evaluated and validated across a wide spectrum of medical conditions, including seizures, aggression, sleep, pain, and psychoactive drug addiction [9–13].

The *D. melanogaster* model is not without its limitations, though. A major concern in using *Drosophila* for any pharmacological study is to evaluate drug uptake by the insect through food consumption. To verify adequate drug intake, a variety of methods can be employed: the addition of food dye to the food and drug mixture, administration of radiolabeled drugs, and measurement of drug concentration in fruit flies. Mass spectrometry and enzymatic assays are currently used to test for the presence of chemicals in fruit flies; however, these techniques are not widely used or validated for measuring drug concentrations quantitatively. Hence, currently there are no standardized methods to test drug concentrations in target tissues in adult fruit flies. An alternative to oral drug consumption is the injection of drug intra-abdominally into fruit flies via cell injectors and micromanipulator [14], but these methods are time consuming and, unless automated, not appropriate for high-throughput screening (HTS) of adverse drug reactions. In spite of the uncertainties of using food intake for drug delivery in fruit flies, this method is still considered the preferred mode of drug delivery. Although, we cannot quantify the amount of drug ingested by the fly, it is possible to observe dose-dependent increases in the severity of adverse drug reactions, suggesting that the fruit fly does indeed consume compound while consuming its food. Despite the challenges with drug uptake, the *Drosophila* model has several advantages that make it a valid approach to screening adverse drug reactions before the testing of vertebrate model systems.

Sexual differentiation in mammals and *D. melanogaster*

Although *Drosophila* has been used as a model system for HTS of disease-oriented screening of libraries of compounds [15], its use for the screening of drug-induced adverse effects is not yet commonplace. Owing to the similarities between *D. melanogaster* and mammalian reproductive systems, fruit flies appear to be a better model system over zebrafish and worms to test for reproductive and developmental toxicities. In order to validate this system as a potential model system, sexual organ development and differentiation comparisons between *Drosophila* and mammalian reproductive systems need to be determined. In mammals, sexual differentiation is a result of the interaction between genes and hormones, which direct the development of the bipotential gonad. The *SRY* gene, found on the Y sex chromosome, leads to the differentiation of the biopotential gonad into testis [16]. If *SRY* is not present, the bipotential gonad will form into an ovary. Differentiation of the male and female gonads is not, however, solely dependent on the presence or absence of *SRY*. In males, anti-Müllerian hormone and androgens play key roles in male differentiation. In females, the genes *DAX1* and *Wnt4a*, as well as the hormones estrogen and androgen, are needed for successful female differentiation [17,18]. Additionally, Steroidogenic Factor 1 (SF-1) levels in the testes or ovaries plays an important role in gonadal differentiation.

Drosophila sexual differentiation has traditionally been thought to be different than the corresponding mammalian processes, involving only the alternative splicing of sex gene mRNAs, without the influence of any hormones. Similar to mammals, however, *Drosophila* begin sexual differentiation with the formation of a bipotential gonad [19]. Development of the bipotential gonad into male-specific and female-specific organs begins with the expression of *sexlethal* (*sxl*). In females, the *sxl* mRNA stop codon is excised – processing that does not occur in the male – resulting in the production of sex-specific gene products. This differential expression of *sxl* also results in the alternative splicing of another sex gene, *transformer* (*tra*), leading to the production of two gene products in females and one gene product in males. The *tra* gene products influence the differential splicing of *doublesex* (*dsx*) giving rise to female and male-specific *dsx*, with the female form of *dsx* resulting in the initiation of transcription of female-specific genes [20].

D. melanogaster and mammalian sexual differentiation is more similar than previously believed, though. While it is clear that alternative splicing of mRNAs is a key component in *Drosophila* sexual differentiation, it is not clear if at any point in the differentiation process if endocrine differences between males and females arise. *Drosophila* do possess a rather complex endocrine system with several identified hormones including, estrone, estradiol, estratriol, testosterone, dihydroxytestosterone, androsterone, pregnenolone, progesterone and cortisol [21,22]. Despite the presence of these hormones, none has yet been explicitly implicated in *D. melanogaster* sexual differentiation and reproduction [23]. Others have challenged the conception that *Drosophila* do not posses sex hormones, proposing that ecdysone and 20-hydroxyecdysone are counterparts to the mammalian sex hormones testosterone and estrogen, respectively [24], and that the formation of mature oocytes requires the *Drosophila* hormones 20-hydroecdysone and juvenile hormone [25]. Further elucidation of the role of *Drosophila* sex hormones would reinforce the sexual development similarities between fruit flies and mammals.

There is also increasing evidence of conserved sexual development genes between *Drosophila* and mammals. In mammals, the interaction between *SRY* and SF-1 leads to the upregulation of SOX9 transcription factors [26]. SOX9, in turn, interacts with FGF9 growth factor, causing bipotential gonad cell differentiation into sertolli cells, thereby initiating the male-specific sex differentiation processes [27,28]. In the absence of SOX9, sertolli cells are not formed and the female-specific pathway is initiated [28]. The *Drosophila SOX9* ortholog *Sox100B* works in a manner similar to *SOX9* and is required for testis formation [29]. The authors of this work, Nanda *et al*. argue that these findings suggest 'that the molecular mechanisms regulating sexually dimorphic gonad development may be more conserved than previously suspected.'

Additionally, alternative splicing has also been shown to be involved in mammalian sex development [30]. It has been observed that pre-mRNA splicing in mammalian cells can be facilitated by SRY and SOX factors [31,32]. Wilms' Tumor 1 (WT1), a tumor suppressor gene that is implicated in genitourinary development, has also been shown to interact with splicing factors and splicesomes, with more than 16 different isoforms, nine of which are generated by alternative splicing [33,34]. Further, WT1 interacts with WT1-assiating protein, the mammalian homologue to *Drosophila* female-lethal(2)d, which is required for female-specific splicing of *sxl* and *tra* pre-mRNAs [35]. Thus, not only are there evolutionary-conserved sex determination genes, but there is evolutionary-conserved use of pre-mRNA splicing as a regulatory mechanism among distant phyla [30]. On the basis of the observed similarities between mammalian and *Drosophila* reproductive systems, the fruit fly model may be of use in the screening and evaluation of drug-associated adverse events.

In animals where fertilization occurs internally, including *Drosophila* and humans, ovulation and sperm storage are two vital reproductive steps. Ovulation is a cascade of events resulting in oocyte maturation and accessibility for fertilization. In mammals, female ovulation occurs cyclically, directed by levels of lutenizing hormone and follicle-stimulating hormone [36]. Sperm storage occurs in both male and female mammals with long-term storage occurring in the male epididymis and very brief storage in the female oviducts [37]. In *Drosophila*, ovulation is not cyclic, but is initiated within 90 minutes of mating [38]. Similar to humans, however, oocyte maturation is a result of the regulation of two hormones: 20-hydroxyecdysone and juvenile hormone [39]. Unlike mammals, both short-term and long-term sperm storage occur in the female reproductive tracts in three sperm storage vesicles; the seminal receptacle, the paired spermathecaea, and the spermathecal glands also known as the female accessory glands [39]. Recent studies by Allen and Sprading have shown that sperm vesicle development and sperm maturation are more conserved than previously believed [40]. They observed that the *Drosophila* nuclear hormone receptor *Hr39* has similar functions to the mammalian nuclear hormone receptor SF-1, both facilitating the normal development and function of sperm storage vesicles and the accessory glands. Additionally, sperm maturation is also conserved, with proteins secreted from the accessory glands functioning in sperm maturation. Although the location of sperm storage and maturation differ between *Drosophila* and humans, the molecular mechanism by which this occurs is very similar.

In *Drosophila*, the female reproductive system can be used as a surrogate for drug-induced adverse effects on physiological and morphological changes. Fruit fly reproductive organs include a pair of ovaries, oviducts, a uterus, making it structurally similar in many regards to mammalian reproductive organs [41]. Reproductive toxicity in *Drosophila* may be determined by observing the number of eggs laid, the number of eggs that hatch and the morphology of ovaries.

Chemically induced reproductive adverse effects in *D. melanogaster*

In spite of many similarities between fly and human female reproductive organs, the *D. melanogaster* model is not commonly used to screen for drug-induced reproductive toxicities. Several studies have, however, reported chemically induced adverse effects in reproductive organs of *D. melanogaster*. The ability to induce such reproductive changes in *Drosophila* makes this model system ideal for detecting adverse drug reactions. Environmental chemicals, such as pesticides, have been a growing concern for human and wildlife health [42]. For example, organophosphates, used as insecticides in agriculture, are commonly used because of their known biodegradability. *Drosophila* grown on food with dichlorvos and chloropyrifos, two organophosphate compounds, showed a decrease in female egg production and a delay in emergence of adult flies. Trypan blue staining exhibited tissue damage in ovaries of adult flies exposed to dichlorvos [43]. Additionally, genotoxicity has been reported in somatic and germ cells of *Drosophila* exposed to chloropyrifos [44]. Flies grown on food with another commonly used insecticide, the synthetic pyrethroid, cypermethrin, resulted in a decrease of mean number of daily eggs laid per female, delay in emergence of adult flies, and decrease in number of flies emerged. These results suggest that this insecticide affects development of the fly. In this study, Trypan blue also exhibited damage to ovaries of adult female flies in certain doses [45]. Insecticides are not the only potentially toxic environmental factor. Argemone oil is a less expensive and readily available oil added to mustard oil to increase the yield. Argemone oil and its main alkaloid fraction administered to *Drosophila* larvae, however, delayed both the emergence and number of emerging flies. This study also indicated a decrease in fecundity in females grown on food containing argemone oil [45].

It is evident that researchers are able to manipulate and induce changes in the reproductive organs of *Drosophila* by administering pharmaceutical agents. This capability further makes *D. melanogaster* a respectable model organism for the detection of reproductive adverse drug reactions in a non-target model system.

Effects of MTX on fertility and ovarian development of *D. melanogaster*

MTX is a folate analog and a folate antagonist that results in folic acid deficiency, a harmful condition in pregnancy. Since it reversibly inhibits dihydrofolate reductase and interferes with DNA synthesis, cell reproduction, and cell division in proliferating cells, the drug has traditionally been used as a chemotherapeutic agent [46]. This compound is also used in the treatment of a range of ailments associated with autoimmune diseases, although the precise molecular mechanism by which MTX diminishes inflammation is unknown. MTX reproductive adverse effects have been

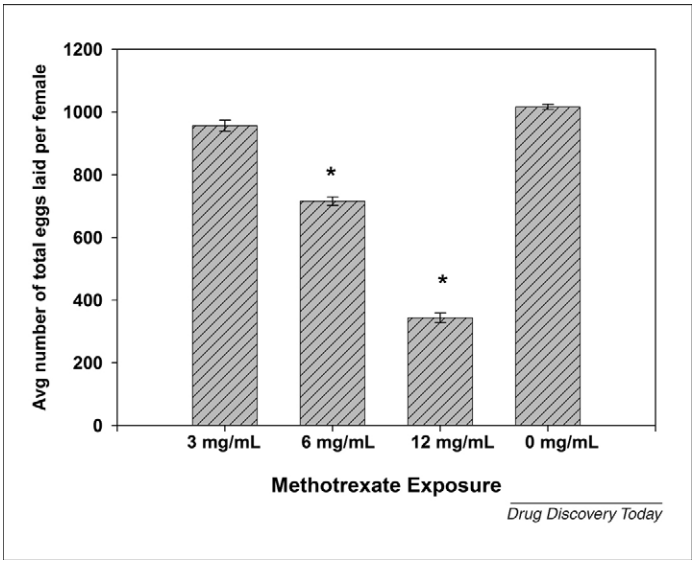

FIGURE 1

Two-day average reproductive output per each female fly with continuous treatment of 0, 3, 6, and 12 mg/mL of methotrexate. Doses of 6 and 12 mg/mL resulted in a 30% and 66% decrease ($P < 0.05$) in the total number of eggs laid. The impact of methotrexate on reproductive output was assessed statistically using a one-way ANOVA. A sample size of 10 females per dose was used, and error bars represent standard deviation.

observed across species, and in almost all organisms MTX has been shown to be a potent teratogen [47]. In mice, MTX displayed feticide properties leading to intrauterine deaths and congenital defects and the incidence of fetal death increased with an increase in dose [48]. Additionally, owing to MTX's ability to inhibit folic acid synthesis, administration leads to non-viable rat fetus development when administered before pregnancy. The number of fetal deaths was directly proportional to the duration of the MTX-induced folic acid deficiency before impregnation [49].

The effects of MTX on ovarian development and teratogenesis are seen in multiple studies, suggesting that these negative effects are conserved among species. In a study evaluating the impact of MTX on *Drosophila* development, female fecundity, and gene expression, MTX administration significantly decreased fecundity and resulted in ovaries that were flaccid and small with undeveloped follicles. Of the eggs that hatched and made it to adulthood, most had tumors as well as bristle, and eye and leg defects that were similar to mammalian birth defects, including small size, prominent eyes and abnormal limbs [50]. In another study, ovarian tissues from MTX-treated *Drosophila* had many revised and affected transcripts, with significant overlap between affected genes in both *Drosophila* and human cells treated with MTX [51]. Other studies suggest that once MTX administration is halted, the risk of developmental damage in *Drosophila* is decreased. This is similar to human mothers that were treated for four months with antifolate, similar to an anti-MTX drug [49,52]. Exposure to MTX also results in malformalities and defects in embryos of mice, rabbits, monkey and rats [49]. These results strengthen the argument that teratogenesis is conserved among species.

Case study: using a D. melanogaster *model to determine reproductive adverse effects*

In order to validate further the use of the *D. melanogaster* model as a non-mammalian system for the detection of reproductive system adverse events, we used MTX as a test compound. Through the use of various pharmacological assays to evaluate drug-induced phenotypic changes in *Drosophila*, we are now able to evaluate the impact of drugs on the fly nervous system, metabolic rate and reproductive system. To evaluate the effects of MTX on the fruit fly reproductive system, MTX was mixed in yeast in a 1:1 mixture and 75 μL of this mixture was pipetted into vials containing banana-molasses food, as previously detailed [53]. Drug uptake was verified by the presence of food coloring in the fly abdomen, which was added to the drug:yeast mixture. MTX administration resulted in a dose-dependent decrease in reproductive output across all doses (Figure 1). There was a significant decrease in the number of eggs laid per female, with a 66% reduction in eggs laid at the highest

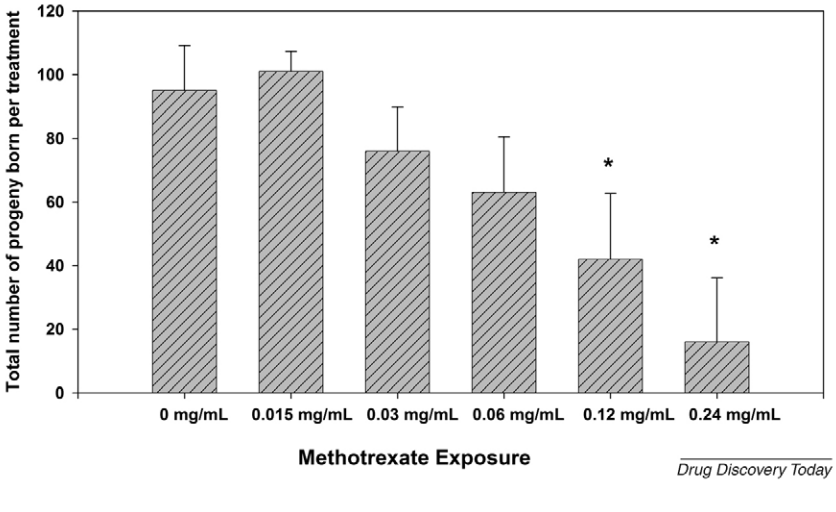

FIGURE 2

The total number of progeny born after the treatment of female flies with 0.015, 0.03, 0.06, 0.12, and 0.24 mg/mL of methotrexate. Sample size of 20 pairs of males and females used per dose. Treatment doses of 0.12 and 0.24 mg/mL result in a significant decrease in emerging progeny ($P < 0.0001$). Statistical analysis was completed using one-way ANOVA and Bonferroni's multiple comparison post-test vs 0.00 mg/mL.

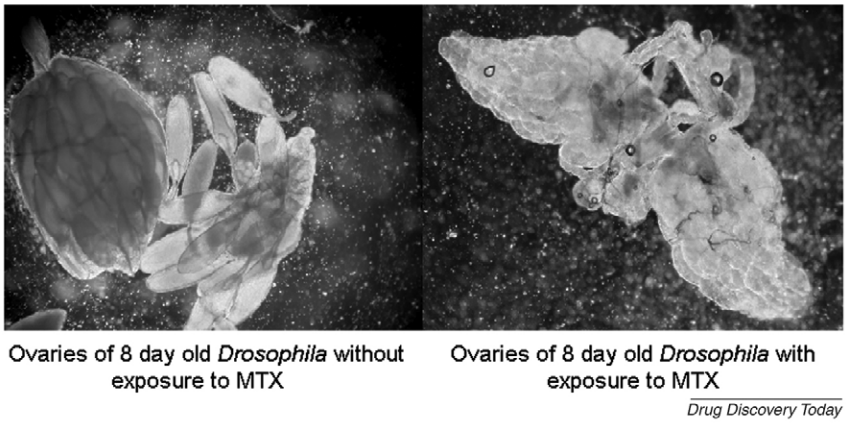

FIGURE 3

Ovaries from 8-day-old *Drosophila melanogaster* with and without 12 mg/mL methotrexate exposure. Ovaries from control flies are normal with long ovarian follicles that can be deposited as fertilized eggs. Ovaries that were exposed to 12 mg/mL methotrexate were withered and lacked mature oocytes.

level of MTX exposure (12 mg/mL) compared with untreated controls. These data suggest successful drug administration in the *Drosophila* model, as well as confirms the impact of MTX on the reproductive capacity in this model. Additionally, flies exposed to MTX exhibited a dose-dependent, drug-induced decrease in the number of progenies (Figure 2). At 0.12 mg/mL of MTX exposure, the number of viable progeny was 38% when compared with the observed progeny for untreated controls. We also examined the ovaries of female flies treated with 12 mg/mL MTX, which revealed major morphological changes. Ovaries exposed to MTX were withered and often lacked mature oocytes (Figure 3).

Our findings of ovarian impairment adverse events were consistent with the other studies exposing *Drosophila* to MTX [50–52]. Additionally, our findings parallel those observed for other animal models and humans. A review of MTX use among humans found some evidence of increased abortions and significant teratogenicity [49]. Similarly, we observed decreased viability among progeny, including dose-dependent decreases in reproductive output and number of progenies. This has also been observed in murinae models, where MTX-treated mice and rats had dose-dependent intrauterine death and congenital defects [48,49]. We also observed morphological changes in MTX-treated fruit fly ovaries, including deficiencies in oocyte development. By comparison, a recent study of women undergoing *in vitro* fertilization (IVF) while receiving MTX found that when an IVF cycle occurred within 180 days of MTX exposure, there was a significant decline in retrieved oocytes [54]. The shared results observed across these diverse animal models and human trials further confirm the *D. melanogaster* model as a useful non-mammalian system for the evaluation of reproductive adverse events in the drug development process.

Conclusion

In this paper, we propose using *Drosophila* as a model system to screen for adverse drug reactions, specifically reproductive events, during early stages of drug development. Pharmaceutical researchers are often challenged with discovering a compound with therapeutic benefits in rodents that cannot be studied in humans owing to its adverse effects. Although rodent studies are labor intensive and costly, they are widely used to evaluate drug induced adverse effects even during screening phases. We believe that evaluation of adverse effects in fruit fly assays is more time-efficient and cost-effective than in rodents. Unlike working with *Drosophila*, working with rodents is timely and requires a large housing facility, occasional veterinary care, expensive behavioral equipment, and approval of a number of animal protection protocols. With an average gestation period of 19–20 days, a mouse study that assays for the effects of a compound on fertility, conception and pup viability would be enormously time consuming and may take over three months to be completed [55]. By contrast, a *Drosophila* study that assays for the same effects would take about two weeks.

With the current funding climate in biomedical research, more than ever we should be concerned with developing cost-effective research programs. We hope that our work results in enthusiasm in pharmaceutical research community to further develop *D. melanogaster* as a model system to detect adverse drug reactions. We believe that this model system will result in tremendous cost and time savings in the drug development process.

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