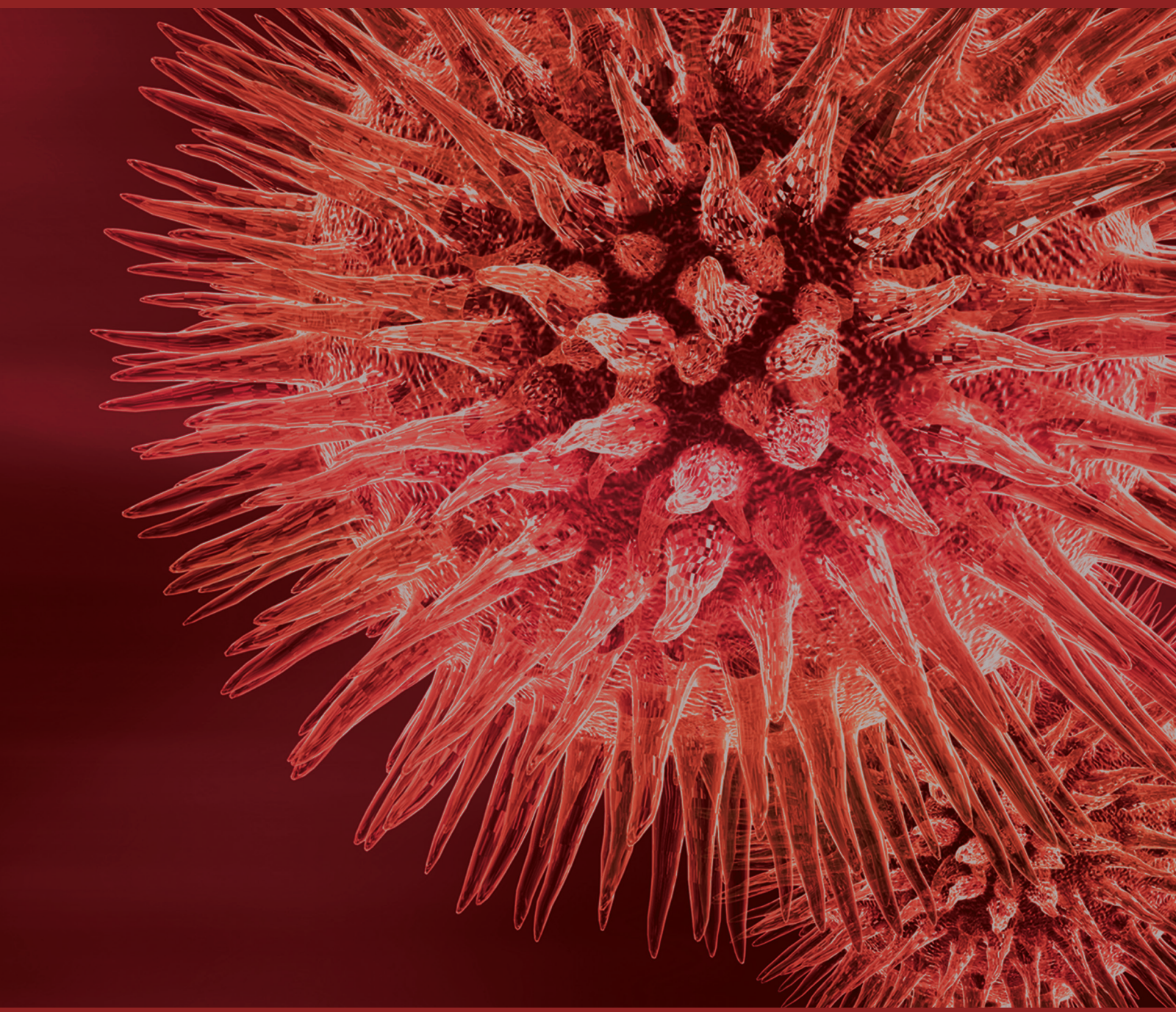
BioMed Research International

The Toxic Effects of Xenobiotics on the Health of Humans and Animals

Guest Editors: Yanzhu Zhu, Alex Boye, Mathilde Body-Malapel, and Jorge Herkovits



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Editorial

The Toxic Effects of Xenobiotics on the Health of Humans and Animals

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With the development of the society, the xenobiotics have brought high potential risk to human and animal. The study focused on the adverse health effect of xenobiotics which plays an important role in addressing public health challenge. The special issue has focused on the toxicity of xenobiotics. We call for manuscripts describing recent findings and future perspectives in the toxic field of xenobiotics on the human and animal health. Until the deadline of the special issue, 20 manuscripts have been received worldwide. After the review process, 7 manuscripts have been accepted by this special issue. The accepted research manuscripts have focused on the insecticides (Organochlorine Pesticides, Furia ®180 SC (Zeta-cypermethrin) and Bulldock 125®SC (β-Cyfluthrin) pyrethroid insecticides), sterilant (zinc gluconate-based chemical sterilant), toxic component of plant (usnic acid from *Cladonia substellata* Vainio and swainsonine from locoweed), and ethyl maltol and iron complexes. The accepted review manuscripts have described the enzymes and proteins which contributed to IDILI and the various assays and current trends in developing comprehensive models. They represent the important research finding and will contribute to the development of toxicology.

Insecticides bring high risk to human and animal through food, water, and air. The World Health Organization have estimated 3 million cases of severe acute poisoning (including suicides) and reported cases of intoxication responsible for approximately 2.2 million deaths. The number of incidents of exposure to pyrethroid insecticides has increased. Organochlorinated compounds (OCPs) are one of the important pesticides and have been used globally for many years. OCPs are not efficiently eliminated from the human body and may accrue in tissues because of enterohepatic reabsorption and affinity to adipose tissue. There has been limited work on interventions to facilitate elimination of OCPs. Evidence confirmed that enhanced mobilization and excretion via induced perspiration reduced the body burden of hexachlorobenzene (HCB) and polychlorinated biphenyl congeners. Removal of OCPs has also been facilitated by specific interventions which interrupts the enterohepatic circulation. To confirm that, sweat analysis may be useful in detecting some accrued OCPs, not found in regular serum testing. This method supplements the detection method of the OCPs. But perspiration is hard to collect. It maybe restricts its extensive use. Pyrethroid insecticides are another one of the important pesticides. Pyrethroid insecticides represent more than 30% of the worldwide insecticide market. Furia 180 SC is an isomer of cypermethrin insecticide. Bulldock 125 SC is an isomer of cyfluthrin. However, there is little information concerning the toxic effects of zeta-cypermethrin. But the research on the toxic effect of the *Furia 180 SC (Zeta-cypermethrin)* and Bulldock 125 SC (β-cyfluthrin) is still necessary in the future.

Sclerosing agents as zinc gluconate-based chemical sterilants (Infertile®) are used for chemical castration, but there is not enough evidence of its safety profiles for the receivers. Infertile at 60 mM presented risk for animal health. It suggests that the optimal dose must be less than 6 mM. The future studies must be continued in order to clarify the safety of Infertile on cells and tissues, in order to better understand the pathophysiological mechanisms of this drug.

The toxic component of plant often attracts the attention from food safety. Usnic acid is a compound of natural origin resulting from lichen secondary metabolism. It is considered one of the most important biologically active metabolites with important pharmacological properties: antitumor, antibiotic, antiviral, antioxidant, tuberculostatic, anti-inflammatory, and molluscicide. But limited studies showed toxicological potential of usnic acid. The teratogenic effect of usnic acid is in the period of organogenesis. It reveals the importance of evaluating the toxic effects of natural substances for elucidating the care in their indication as drug, particularly during pregnancy. Swainsonine is the primary toxin in locoweeds which is toxic plants. Its toxicity often occurs during pregnancy in livestock, and it may have different effects on embryonic development depending on the conceptus phase and maternal conditions during acute intoxication. Limited data showed the potential reproductive and developmental toxicity caused by swainsonine. The swainsonine caused reproductive and developmental toxicity in both parents and offspring mice. It suggests that severe reproductive toxicity and developmental toxicity are associated with swainsonine poisoning.

Ethyl maltol and iron complexes are products of ethyl maltol and the iron found in the cooking pots used to prepare the Chinese dish, hot-pot. Limited data showed their safety. The ethyl maltol and iron complexes were toxic, and the targeted endocrine organs were the liver and kidneys. It suggests that alternative strategies for preparing the hot-pot, including the use of non-Fe-based cookware, need to be developed and encouraged to avoid the formation of the potentially toxic complexes. This experiment focused on the toxic of ethyl maltol and iron complexes, which is related to the Chinese dish, hot-pot. It is significant and it will attract more attention on these complexes.

Idiosyncratic drug-induced liver injury (IDILI) is a significant source of drug recall and acute liver failure (ALF) in the United States. While current drug development processes emphasize general toxicity and drug metabolizing enzyme-(DME-) mediated toxicity, it has been challenging to develop comprehensive models for assessing complete idiosyncratic potential. The enzymes and proteins contributed to IDILI, including ones that affect phase I and phase II metabolism, antioxidant enzymes, drug transporters, inflammation, and human leukocyte antigen (HLA) which are important. It is imperative to develop various assays to detect individual reactions focusing on each of the mechanisms. The current trends in developing comprehensive models focused on examining the mechanisms. This review about IDILI is comprehensive and deeply showed the literature of potential mechanisms and predictive assays in idiosyncratic drug-induced liver injury. This review is significant and presents high value.

Acknowledgments

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Alex Boye
Mathilde Body-Malapel
Jorge Herkovits

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Research Article

Teratogenic Effect of Usnic Acid from *Cladonia substellata* Vainio during Organogenesis

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Studies about toxicological potential of usnic acid are limited. This way, the vast majority of data available in the literature are related only to biological activities. This is the first study that aimed to evaluate the oral toxicity of usnic acid during the period of organogenesis. Females rats were distributed in the control groups, treated I and II, at doses of 15 and 25 mg/kg, administered by gavage during the 6° to 15° days of pregnancy. After 20 days the fetuses were removed and analyzed. A reduction in weight gain during pregnancy, increased resorption, reduction in the number of viable fetuses, and their body weight were observed. Morphological changes in the litter were visualized as exposure of the eye and atrophy of the limbs at the dose of 25 mg/kg. Histological analysis of the liver of the fetus showed reduction in the number of megakaryocytes between experimental groups and increase in the number of hepatocytes in a dose of 25 mg/kg. The experimental model used in this study reveals teratogenic effect of usnic acid in the period of organogenesis. Since this achievement, the importance of evaluating the toxic effects of natural substances is imperative, in order to elucidate the care in their indication as drug.

1. Introduction

Usnic acid [2,6-diacetyl-7,9-dihydroxy-1,3-dimethyl-8-9b (2H, 9α/βH)-dibenzo-furandione; $C_{18}H_{16}O_7$] is a compound of natural origin resulting from lichen secondary metabolism. It is considered one of the most important biologically active metabolites with important pharmacological properties: antitumor, antibiotic, antiviral, antioxidant, tuberculostatic, anti-inflammatory, and molluscicide [1–10].

Despite the diverse pharmacological activities attributed to this compound, its use has shown damage to the main organ that acts in the detoxification of endo- and xenobiotics, the liver [11]. It was reported that usnic acid is one of the active ingredients of the supplement Lipokinetix®, causing hepatocellular damage [12]. Data claim that these people who used this product had acute liver failure [13]. Already *in vitro* studies, isolated rat hepatocytes treated with different concentrations of usnic acid showed almost 100% of necrosis [14–16].

The mechanism of action of usnic acid is not fully elucidated. Studies mention this ability of decoupling of the chain of electron transport, affecting mitochondrial function and cellular respiration, effect similar to what happens to carbon tetrachloride [12, 14, 17, 18].

To evaluate the toxicity of naturally occurring substances, such as usnic acid, preclinical trials in order to evaluate the potential of the substance to cause adverse effects on exposed organisms are included, in particular the effects of exposure during pregnancy which is one of the basic requirements for the use of bioactive molecules [19, 20].

Experimental studies using animal models, traditionally, provide the basis for screening teratogenic potential of

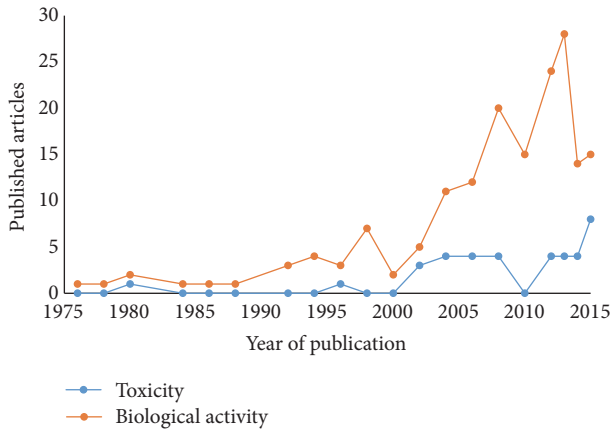


Figure 1: Scientific research on biological activity and toxicity of usnic acid. Source: Scopus.

substances to the verification of a particular agent to be considered toxic. These investigations have the key role in the elucidation of the principles and mechanisms involved in teratogenesis, which may be related to drug exposure conditions, stage of development of the embryos, and pathogenic mechanism of each agent [21].

In recent years there has been a breakthrough in the number of scientific researches to demonstrate the effectiveness of the various biological activities attributed to usnic acid (Figure 1).

However, studies about its toxicological potential are limited, so that the vast majority of data available in the literature are related only to the biological activities of compound [22–26].

Therefore, this is the first study that aimed to evaluate the oral toxicity of purified usnic acid from *Cladonia substellata* on the reproductive development of female rats during the period of organogenesis.

2. Material and Methods

2.1. Lichens Samples. Samples of *Cladonia substellata* Vainio were collected on sandy soils of "tabuleiro," savannah like vegetation, in Atlantic rainforest domain, along of Federal highway (BR 101), in the municipality of Mamanguape, Paraíba State, Northeast Brazil, coordinates $06^{\circ}42'42{,}4''$S and $35^{\circ}07'07{,}0''$WGr.

The usnic acid, the main substance of the species, was extracted using the eluotropic series of solvents, diethyl ether, chloroform, and acetone [27]. Sequentially, it was isolated and purified in silica gel column from ether extract and characterized as preestablished methodology in the Natural Products Laboratory of the Department of Biochemistry, through analyses of HPLC, ^{1}HNMR, and melting point [10]. Quantitation of usnic acid was carried out through HPLC, by injecting different amounts of purified usnic acid, and calculated by direct calibration. In addition, optical rotation of usnic acid was determined in a Jasco P2000 polarimeter in Analytical Centre of Fundamental Chemistry Department of Federal University of Pernambuco.

2.2. Experimental Animals. Wistar rats ($n = 18$) provided by the biotherium of the Department of Nutrition at the Federal University of Pernambuco, weighing about 300 g with 60 days of life, were used. The animals were kept in cages lined with air-conditioned environment in photoperiod (12 h light/12 h dark) at a controlled temperature of 25 ± 2°C with exhaust air and free food access ad libitum water. The experimental protocols were approved by the Ethics Committee of the Federal University of Pernambuco (process number 23076.029828/2013-94).

2.3. Experimental Procedure. Initially, females were submitted to the study of the estrous cycle in order to determine the fertile period. The analysis was carried out by vaginal swab technique for obtaining slides containing vaginal epithelial cells which were stained by hematoxylin-Shorr. Subsequently, using an Olympus microscope (BH-2, Japan) was identified the period of ovulation of the female, called estrus in which the females were matched with male and the mating was confirmed by the presence of "plug" (whitish mass spermatozoa in the vaginal opening) or the presence of sperm in the vaginal smear. The first 24 h after confirmation of mating were considered day zero (D0) of gestation. Females were randomly distributed in the control groups ($n = 6$) treated I ($n = 6$) and treated II ($n = 6$). The control group received 1 mL of physiologic solution, while the treated groups I and II received the purified usnic acid in doses of 15 mg/kg and 25 mg/kg body weight, respectively. Doses were based on 50% of LD_{50} (lethal dose that kills 50% of the animals) determined to rats [28], and 25 mg/kg, as recommended by Merck® index [29]. The doses of purified usnic acid were dissolved in 10 mL of PBS buffer Merck, and the product was administered orally (gavage), in daily doses, from 6th up to 15th day of pregnancy. During administration females were weighed to assess body mass gain at 0°, 6°, 10°, 14°, and 20 days of gestation. After this period, the mice were euthanized by overdose of the anesthesia with Urethane (1.25 mL/kg). Uteri were dissected, and after opening them, fetuses were removed and examined in the craniocaudal direction, to evaluate the presence of external malformations. Then, fetuses and their placentas were weighed.

The deployment sites were identified by iodine solution (Lugol 2%). In free wombs of their content the deployment sites were visualized by contrast to the uterine tissue. Deployments appeared as bright spots that were located and accounted for. The number of resorptions was the result of the number of implantations sites minus the number of fetuses.

2.4. Histomorphometric Analysis. For hepatotoxicity registration after euthanasia the livers of pregnant rats and fetuses were dissected and weighed and then subjected to histopathological analysis. The collected material was kept in buffered formalin solution (10%) for 24 h and then processed for routine histological technique. The blocks were cut into 4 mm thick, by Manson trichrome stained, and analyzed by light microscopy (Olympus BH-2, Japan).

For histomorphometric analysis slides were photographed under fixed focus and clarity of field through Motic®

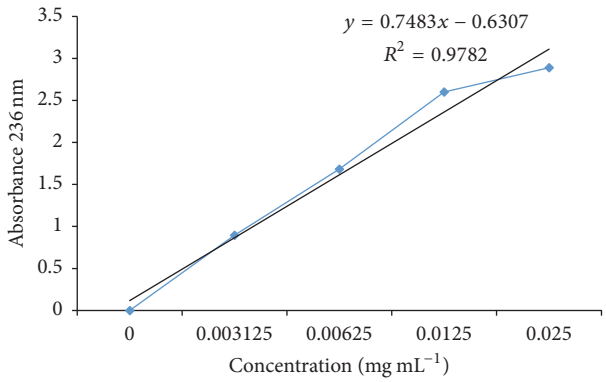


Figure 2: Calibration line of purified usnic acid from *Cladonia substellata*.

Images Plus 2.0 software with a digital camera attached to an optical microscope (Olympus BH-2, Japan) and connected to the computer. We obtained 30 photomicrographs per slide at full 400x magnification.

The photomicrographs were submitted to appropriate measurements using ImageJ software version 1:44 (Research Services Branch, U.S. National Institutes of Health, Bethesda, MD, USA). For the fetus, the following measurements were considered: number of hepatocytes (NH) and number of megakaryocytes (NM), and for pregnant rats: number of hepatocytes (NH) and number of Kupffer cells (NCK).

2.5. Statistical Analysis. Data were expressed as mean ± standard deviation (SD). Statistical significance was determined by one-way ANOVA followed by Tukey's test $P < 0.05$ significant analysis. All analyzes were performed using Prism software (GraphPad Software, Inc., San Diego, CA, version 5.01).

3. Results

3.1. Chemical Analyses of Usnic Acid. Data of extraction, purification, and spectroscopic analyses of usnic acid used in this study were previously described [10]. The optical rotation was α_{25}^{D}: +478.2200 (c 1.0 acetone). This way, the usnic acid used in our study is dextrorotary.

The thallus of *C. substellata* presented 95% of usnic acid, by direct calibration (Figure 2), and HPLC analyses showed 95% of purity.

3.2. Reproductive Variables Attributed to Pregnant Rats. During oral administration of purified usnic acid at 15 mg/kg and 25 mg/kg, no death was registered during treatment. The pregnant rats in the experimental groups showed a significant reduction in body weight gain at 10, 14, and 20 days of gestation (Table 1). With respect to the total number of fetal resorptions, an increase in the group exposed to the dose of 25 mg/kg, the presence small placentas, and further reduction in the number of fetuses were observed (Figure 3). The group exposed at 15 mg/kg remained close to the control group (Table 1).

Table 1: Reproductive variables attributed to pregnant rats.

	Groups		
	G1 (Control)	G2 (15 mg/kg)	G3 (25 mg/kg)
Mean body weight (g)			
Day 0	208 ± 4.3	209 ± 5.6	208 ± 5.2
Day 6	227 ± 1.4	227 ± 3.1	226 ± 5.8
Day 10	255 ± 3.4	236 ± 3.8*	224 ± 5.8***
Day 14	276 ± 2.3	250 ± 2.8***	229 ± 6.9***
Day 20	299 ± 2.5	279 ± 3.6***	247 ± 6.6***
Number of implantations	12 ± 0.5	10 ± 1.0*	8 ± 1.4***
Number of resorptions	1.1 ± 0.4	3.5 ± 0.5*	5.0 ± 1.0***

Data are expressed as mean ± standard deviation. ANOVA-Tukey. * $P < 0.05$, *** $P < 0.001$ compared to the control group.

Table 2: Parameters of prole.

	Groups		
	G1 (Control)	G2 (15 mg/kg)	G3 (25 mg/kg)
Mean body weight (g)	5.7 ± 0.3	4.6 ± 0.1*	3.5 ± 0.1***
Liver weight	0.5 ± 0.02	0.3 ± 0.02*	0.2 ± 0.05***

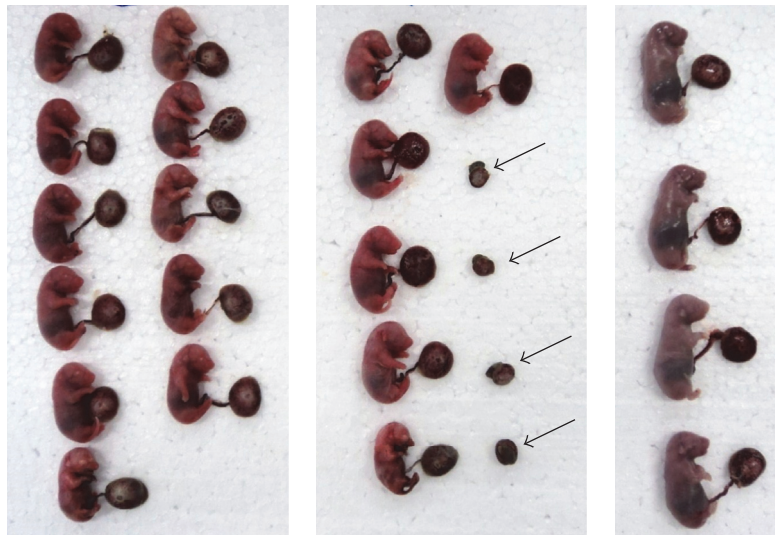
Data are expressed as mean ± standard deviation. ANOVA-Tukey. * $P < 0.05$, *** $P < 0.001$ compared to the control group.

3.3. Assigned Parameters to Prole. The treatment with usnic acid changed the parameters assigned to the offspring (average body weight, average weight of the livers, and external malformations). The average weight of fetuses and their placenta showed decrease by approximately 20% at 15 mg/kg, while at 25 mg/kg a reduction of about 39% was observed (Table 2). At 15 mg/kg a 32% reduction in the weight of fetal livers was observed. By the other hand, a relevant reduction of about 60% was observed in animals treated at 25 mg/kg (Table 2).

Concerning the external morphology, the fetuses treated at 25 mg/kg presented with abnormal patterns with disruption in their development (Figure 4), protrusion of the eyeball (Figure 5(a)), substantial mass proliferation in the region top of the face and neck (Figure 5(b)), and atrophy of the upper and lower limbs (Figure 5(c)). At 15 mg/kg none of those changes were displayed.

In the microanatomic examination of the liver of experimental groups, liver lobes consisting of cords of hepatic cells anastomosed to capillary sinusoids which were covered by endothelial cells and Kupffer cells were exhibited. A limit clear between the hepatic lobules with typical organization structures of port spaces was observed. It was not possible to visualize the space of Disse and Ito cells, indicating that there was no damage to the liver structure.

The liver of fetuses from experimental groups showed anastomosing cords of hepatic cells to capillary sinusoids.



(a) (b) (c)

Figure 3: Fetuses obtained from experimental and control groups and treated at 25 mg/kg: (a) Control group, (b) treated group at 25 mg/kg. Arrows showing embryos with interruption in the development and tiny placentas, (c) treated at 25 mg/kg presenting reduction in the number of viable fetuses.

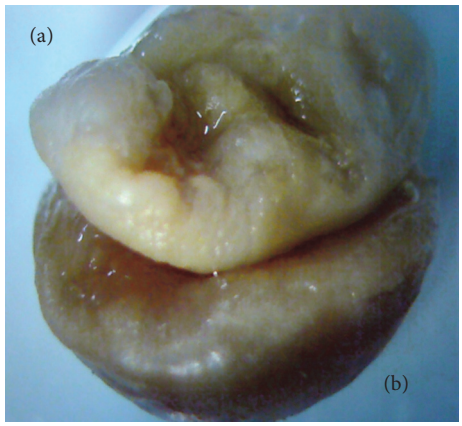


Figure 4: Fetus obtained from female treated with purified usnic acid at 25 mg/kg presenting interruption in embryonic development: (a) mass developing and (b) placenta.

However, it was not possible to identify a lobular organization, which is considered normal for this gestational age (20 days). The sinusoid capillaries were completely filled with blood, preventing the display of Ito cells and Kupffer cells. The structures of door spaces were not identified. Among the liver cells a large number of blood cell lineages were found at different stages of maturation. Among these, the megakaryocytes were easily identifiable.

3.4. Histomorphometric Analysis. The results from the histomorphometric analysis of the liver of pregnant rats exposed to usnic acid demonstrated significant changes in the average of the total number of hepatocytes per animal. The animals exposed at 15 mg/kg showed an increase of about 21%, while those exposed at 25 mg/kg had a 29% increase (Table 3).

Table 3: Histomorphometric analysis of the liver of pregnant rats treated with usnic acid.

	Groups		
	G1 (control)	G2 (15 mg/kg)	G3 (25 mg/kg)
NH	90.44 ± 15,61	109.38 ± 16,02*	116.96±12.67***
NCK	3.26 ± 2.26	4.03 ± 2.62	5 ± 2.83***

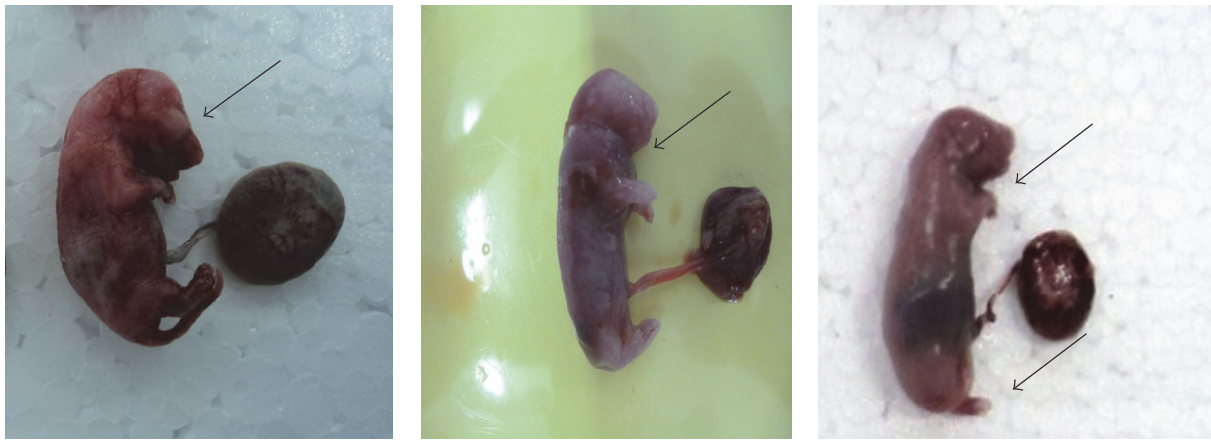
NH: number of hepatocytes; NCK: number of Kupffer cells. Data are expressed as mean ± standard deviation. ANOVA-Tukey. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the control group.

The count of Kupffer cells for animals exposed at 15 mg/kg did not show any change in the total number of such cells per animal. The animals treated at 25 mg/kg showed an increase of about 53%, when compared to the control group (Table 3).

The histomorphometry of fetus liver relative to the total number of hepatocytes did not show any change in the population of these cells at 15 mg/kg. In contrast, at 25 mg/kg a mean increase of approximately 24% was achieved. Regarding the counting of megakaryocyte, doses of 15 mg/kg and 25 mg/kg caused a decrease of about 30% and 53%, respectively (Table 4).

4. Discussion

The studies related to the toxicology of development using natural products are concentrated in the predeployment phase, *in vitro* culture of embryos in animal models, and in physical maturation and postnatal of the descendants. Little



(a) (b) (c)

Figure 5: Malformations in fetuses obtained from treated females with purified usnic acid at 25 mg/kg: (a) Arrow showing protrusion of the eyeball, (b) Arrow showing cell mass in the upper region of the fetus, (c) Arrows showing atrophy of the upper and lower limbs.

Table 4: Histomorphometric analysis of the liver of the fetus from rats treated with usnic acid during pregnancy.

	Groups		
	G1 (control)	G2 (15 mg/kg)	G3 (25 mg/kg)
NH	33.4 ± 8.6	33.2 ± 9.9	41.6 ± 6.0***
NM	0.17 ± 0.01	0.12 ± 0.01*	0.08 ± 0.01***

NH: number of hepatocytes; NM: number of megakaryocytes. Data are expressed as mean ± standard deviation. ANOVA-Tukey. *$P < 0.05$, ***$P < 0.001$ compared to the control group.

is elucidated with respect to the study of toxic action during the period of organogenesis, which is considered the most important of the intrauterine stage [30–34].

The assessment of the effects of a drug on the reproductive period includes research to analyze toxicity on the maternal organism and its offspring. Signs of maternal toxicity may be related to the reduction of body weight, followed or not by a decrease in food consumption; changes in weight and/or morphology of the bodies; and the occurrence of deaths during the treatment period. Our results showed that administration of usnic acid in both doses tested interfered in maternal weight gain during pregnancy, signaling an indication of maternal toxicity of the compound [35].

Similar results can be found in reproductive toxicity studies with β-lapachone, natural compound which has some pharmacological properties similar to usnic acid. Exposure to β-lapachone at doses of 40 mg/kg, 60 mg/kg, and 160 mg/kg for organogenesis is able to reduce body weight gain during pregnancy, induce the onset of skeletal defects in offspring, and decrease the number of fetuses per pregnancy [36].

Reduction in the number of viable fetuses per pregnancy, that is, those who continued the development after birth, is outside the normal range, because according to Krinke [37], Wistar rats of childbearing age have an average of 12 to 14 fetuses per litter.

Many of the changes observed in fetuses during intrauterine development can arise as a result of maternal exposure to teratogenic agents. This way, there is no necessarily a direct effect on progenitors. The effects of chemicals or compounds of natural origin during pregnancy can manifest as miscarriages, abnormalities, and delayed development. A very significant reduction in body weight of the conceptus is a fetotoxicity indication, inducing a delay of intrauterine development, since according to Sharp and Laregina [38], fetal body weight at birth is around 6 g.

Interruptions in intrauterine development, when usnic acid was administered at 25 mg/kg, were similar to reproductive toxicity studies of *Duguetia furfuracea* extracts. This product (1 mL), when administered orally during the preimplantation period to complete organogenesis, caused a direct effect on fetuses, delayed fetal development, presence of placenta atrophied without fetus, which clearly indicates that this was reabsorbed, and beyond external malformations, which are most obvious changes during the period of organogenesis [39].

The decrease in the weight liver of fetuses is an indication of hepatotoxic action of the usnic acid, in view of the natural and/or synthetic substances that can easily penetrate the fetal circulation through the liver before reaching the systemic circulation, and this exposure can trigger hepatotoxic effects on individuals exposed [40].

In microanatomic analyses of the liver of all animals treated with usnic acid no change was observed in hepatic tissue, such as necrotic areas. On the other hand, some studies have shown extensive lesions in the liver of animals subjected to subchronic exposure of usnic acid [4].

Histomorphometric analysis reveals a hepatotoxic effect induced by the administration of usnic acid at 25 mg/kg, triggering cell proliferation of hepatocytes for a possible regeneration of liver parenchyma, a regenerative mechanism involving the increase in the number of these cells. The abnormal proliferation of liver cells can occur in various pathological and/or experimental situations, influenced by

many factors, and in the liver an immense regenerative capacity when submitted to aggression. The regenerations of the hepatic tissue are dependent on 70% of proliferation of hepatocytes to restore normal liver architecture, in pathological conditions [41–43].

The increase in Kupffer cells in the hepatic tissue may be involved in the pathogenesis of some liver injury. According to some authors, these cells release biologically active substances, such as cytokines, which promote the pathogenic process in the tissue immediately after activation [44–46]. The morphometric study of Kupffer cells to pregnant rats exposed at 25 mg/kg is an indication of inflammation and increased liver damage due to exposure to the compound. The megakaryocytes are responsible for originating the blood platelets [43]; the reduction of multinucleated cells, megakaryocytes, in the offspring of the experimental groups confirms the toxicity of usnic acid, by changing the process of formation of these cells which can lead to a deficiency in the early stages that takes the route of blood coagulation in the body.

5. Conclusion

The experimental model used in this study reveals teratogenic effect of usnic acid in the period of organogenesis, and the dose of 25 mg/kg was considered the more toxic, being the dose of 15 mg/kg a candidate for new experimental research. It reveals the importance of the evaluation of the toxic effects of natural substances in order to elucidate the care in their indication as drug, particularly during pregnancy.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Pharmacokinetic and Toxicological Evaluation of a Zinc Gluconate-Based Chemical Sterilant Using In Vitro and In Silico Approaches

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Sclerosing agents as zinc gluconate-based chemical sterilants (Infertile®) are used for chemical castration. This solution is injected into the animal testis, but there are not enough evidences of its safety profiles for the receivers. The present work aimed to establish the pharmacokinetics and toxicological activity of Infertile, using in vitro and in silico approaches. The evaluation at the endpoint showed effects in a dose-dependent manner. Since necrosis is potentially carcinogenic, the possible cell death mechanism could be apoptosis. Our data suggested that Infertile at 60 mM presented risk for animal health. Even though Infertile is a licensed product by the Brazilian Ministry of Agriculture, Livestock and Supply, it presented a high mutagenic potential. We suggest that the optimal dose must be less than 6 mM, once, at this concentration, no mutagenicity or genotoxicity was observed.

1. Introduction

The absence of canine birth control for domestic or stray dogs in big cities represents a public health and animal welfare problem [1]. When abandoned, these animals are at risk of being attacked by humans or other animals or can even become reservoirs of zoonotic diseases [2, 3]. In order to control the canine population, numerous strategies have been described and used to prevent/stop the reproductive cycle, including surgery, hormonal modulation, and chemical and immunological methods. Surgical intervention is a guaranteed but expensive method, as it requires a hospital setting and involves risks associated with anesthesia and surgical wound infection [4].

Many dog owners argue that this method is invasive and incompatible with animal welfare [5]. As a tactical control method, hormonal steroids (such as estrogen-progesterone or progesterone only) have been orally administered to laboratory dogs to suppress ovulation [6, 7]. However, there are side effects, such as pyometra, an inflammatory reaction in the uterus, followed by bacterial infection, and cystic endometrial hyperplasia [8].

Chemical castration is performed by injecting a sclerosing agent into the animal's testis, epididymis, or *vas deferens*. This procedure is irreversible due to its action on germ cells, but no side effects have been reported [9]. Sclerosing agents act via systemic immune response, causing the rupture of the testis barrier and death of Sertoli cells. These agents can also induce local inflammation and the release of testis antigens [10]. Solutions are injected into the animal's testis, resulting in testicular germ cell atrophy, impairment of spermatogenesis, and fibrous occlusion of the *vas deferens* or the epididymis [11].

Zinc is an important mineral for spermatogenesis and semen constitution. Nevertheless, in very high concentrations, zinc acts as an inhibitor of germ cell division

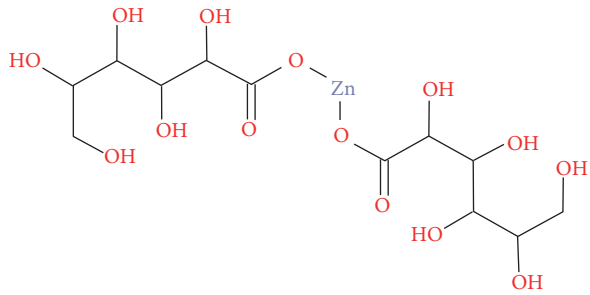

Figure 1: Zinc gluconate chemical structure.

and replication and leads to nucleus and cell membrane fragmentation [12], as reported in other studies that show zinc toxicity during animal development and breeding [13]. Chemical sterilants have been on the market since the year 2000, when Neutersol® was approved by the Food and Drug Administration (FDA) [14, 15]. Still, they have been tested in dogs since the 1970s [16–18] and other animals since the 1950s [19]. Recently, several chemical castration agents have been approved by the FDA and other health and sanitation agencies around the world, including zinc gluconate-based products (Figure 1), such as Testoblock® (BioRelease Tech., Birmingham, AL, USA) [6] and Infertile (RhobiPharma Ind. Farm., Hortolândia, SP, BR). A study demonstrated that Infertile is an effective sterilant for it induces changes in testis germ cells, producing fewer sperm cells and high rates of morphological defects [20].

All products used for human and animal health should be evaluated for their potential to induce DNA damage. The recommendation is to proceed with at least two in vitro genotoxicity tests before performing animal testing. In general, the first test to assess the toxicity of chemical compounds is the *Salmonella*/Microsome test, or Ames test, which shows patterns of mutation in DNA structure. Even if no positive results are observed for the Ames test, it is necessary to evaluate clastogenicity and chromosomal aberrations (damage to coiled nuclear DNA) in eukaryotic cells using micronucleus test [21].

The license from Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) (9427/2008) does not provide enough data on the mutagenicity and carcinogenicity of Infertile. As specified in the ordinance MAPA 74/1996, the agency determines that possible mutagenic, carcinogenic, and teratogenic effects must be declared by the manufacturers of veterinary pharmaceuticals. According to Brazilian animal health law, there is no need to evaluate these endpoints to obtain the license. Since 2006, though, Brazil is a cosignatory of OECD, so all products used for human and animal health have to be tested for their mutagenic, genotoxic, carcinogenic, and teratogenic potential. Chemical sterilization has been more economically and practically feasible. However, little is known about its mutagenic and genotoxic potential. The present work aims to investigate the pharmacokinetic and toxicological potential of zinc gluconate, using in silico and in vitro methods.

2. Materials and Methods

2.1. In Silico Approach. We used a modular toxicological predictive QSAR framework algorithm (LAZAR in silico toxicology, https://lazar.in-silico.ch/predict) [22] based on similarity of chemical alerts. To perform the toxicological prediction in LAZAR, we designed zinc gluconate chemical structure using ChemDraw and obtained the SMILE string. With the SMILE string, we predicted absorption, distribution, metabolism, excretion, and toxicological parameters (ADMET) based on QSAR similarity, using the pharmacokinetic algorithm pkCSM (http://bleoberis.bioc.cam.ac.uk/pkcsm/prediction) [23].

2.2. In Vitro Biological Approach

2.2.1. Test Compound. Toxicological tests were performed with ampoules of Infertile, lot 001/09, kindly provided by Dr. Helena de Godoy Bergallo, Laboratory of Small Mammal Ecology, Rio de Janeiro State University (UERJ). Dimethylsulfoxide (DMSO) at 10% was used to dilute the compound for the tests. The presence of many hydroxyl radicals in zinc gluconate structure may contribute to biological activity and solubility of the compound.

2.2.2. Salmonella/Microsome Mutagenicity Test. The features of *Salmonella enterica* serovar Typhimurium standard strains TA97, TA98, TA100, TA102, and TA104 from the authors' stock were used as described by Cardoso et al. [24] in the mutagenicity assay.

The test tube contained a mixture of 100 μL of one of the Infertile concentrations (6, 12, 30, and 60 mM) plus either 500 μL sodium-phosphate buffer (27.6 g/L $NaH_2PO_4 \cdot H_2O$ and 28.4 g/L Na_2HPO_4; 0.2 M, pH 7.4) or a metabolic fraction (S9 mix 4%; Molecular Toxicology Inc., MoltoxTM, USA) composed of a homogenate of Sprague-Dawley rat liver cells pretreated with polychlorinated biphenyl (Aroclor 1254), as well as 100 μL of the bacterial suspension (2×10^9 cells/mL). After 20 minutes of preincubation at 37°C, 2 mL of top agar (7 g/L agar; 5 g/L NaCl; 0.0105 g/L L-histidine; 0.0122 g/L biotin; pH 7.4, 45°C) were added to the test tube, and the final mixture was poured onto a Petri dish with minimal agar (15 g/L agar, Vogel-Bonner E medium 10x (10 g/L $MgSO_4 \cdot 7H_2O$; 100 g/L $C_6H_8O_7 \cdot H_2O$; 500 g/L K_2HPO_4; 175 g/L $Na(NH_4)HPO_4 \cdot 4H_2O$) containing 20 g/L glucose. This final mixture was incubated at 37°C for 72 h, and the His^+ revertant colonies were counted. The positive controls for assays in the absence of S9 mix were 4-Nitroquinoline-N-oxide (4-NQNO) (CAS: 56-57-5) at 1.0 μg/plate, for TA97 and TA98; sodium azide (SA) (CAS: 26628-22-8) at 0.5 μg/plate, for TA100; Mitomycin C (MM C) (CAS: 50-07-7) at 0.5 μg/plate, for TA102; Methylmethane sulfonate (MMS) at 50 μg/plate (CAS: 66-27-3) for TA104. In the presence of S9 mix, the positive controls were 2-Aminoanthracene (2-AA) (CAS: 613-13-8) at 1.0 μg/plate for TA97 and TA100; and Benzo[a]Pyrene (B[a]P) (CAS: 50-32-8) at 20 μg/plate for TA98, TA102, and TA104. All the chemicals were purchased from Sigma Co. (St. Louis,

USA). The substance or sample was considered positive for mutagenicity when the number of revertant colonies in the assay was at least twice the number of spontaneous revertants (mutagenicity index, MI ≥ 2) and when a significant response to the analysis of variance (ANOVA, $P \leq 0.05$) and reproducible positive dose-response curve ($P \leq 0.01$) were found. MI was calculated by dividing the number of His^+ induced in the sample by the number of His^+ in the negative control. All the experiments were done in triplicate and repeated at least twice [25, 26].

2.2.3. Survival Experiments. Quantitative evaluations were made to determine the cytotoxic effects for all the drug concentrations. In this step, 10 μL of the treated bacterial suspension was diluted in a saline solution (NaCl 9 g/L-0.9%). Then, 100 μL of the solution was put on a Petri dish with Luria Bertani (LB) agar and incubated at 37°C for 24 h. The total dilution was 10^{-7} fold. Colonies were counted and a survival percentage was calculated in relation to the negative control. The compound was considered cytotoxic when its survival rate was lower than 70% of bacterial survival, a significant response to one-way analysis of variance (ANOVA, $P \leq 0.05$) and reproducible dose-response curve ($P \leq 0.01$) [24].

2.2.4. Micronuclei in Cell Culture. The RAW264.7 macrophages were cultured in circular coverslips at 24-well plates with 950 μL essential Minimum Eagle Medium (MEM) Ca^{++}, 1.8 mM, pH 7.6 (Gibco), supplemented with 1.76 g/L $NaHCO_3$, 0.88 g/L pyruvate, 21.6 mg/L aspartic acid, and 16.8 mg/L L-serine with fetal bovine serum (FBS 10%), both at 37°C, and 50 μL cell suspension, for a final cell density of 2×10^5 cells/mL. This suspension was maintained in MEM Eagle 1.8 mMCa^{++} containing FBS (10%), streptomycin (100 mg/L), and penicillin (70 mg/L). Then, the plates were placed in an incubator with an atmosphere of 5% CO_2 at 37°C for 24 hours, for adhesion of macrophages. For cell treatment, the equivalent of 10% of the total volume (100 μL) of negative (DMSO final concentration = 1%) or positive controls or Infertile at 6, 12, 30, and 60 mM was added, and the plates were incubated (atmosphere of 5% CO_2 at 37°C) for 3 hours. After this period, the medium was removed and the plates were washed with 1 mL MEM Eagle 1.8 mM Ca^{++}. 1 mL Eagle MEM medium 1.8 mM Ca^{++} with FBS (10%) was added and the medium was incubated for 24 hours in an atmosphere of 5% CO_2. The negative control used in the assay was 100 μL DMSO, while the positive control was 100 μL N-methyl-N-nitro-N-nitrosoguanidine (MNNG) at a concentration of 0.5 mM. To determine the mitotic index and the number of micronuclei, the MEM Eagle 1.8 mM Ca^{++} solution was replaced with cold Carnoy's fixative (3 : 1 methanol to glacial acetic acid) for 15 minutes. The coverslips were washed with McIlvaine's buffer (MIB) (21.01 g/L citric acid and 35.60 g/L Na_2HPO_4, pH 7.5) for 2 minutes and left to dry at room temperature. The cells were then stained with 4′-6-diamidino-2-phenylindole (DAPI) (0.2 μg/mL) dissolved in MIB for 40 minutes, washed with MIB for 2 minutes, and briefly rinsed with distilled water. To determine the mitotic index, the number of cells with micronuclei and the percentages of necrosis and apoptosis, 1000 cells per concentration, were analyzed under a fluorescence microscope (Reichert Univar) with an excitation wavelength of 350 nm. Cells that glowed brightly and had homogenous nuclei were considered as having normal phenotypic morphology. Apoptotic nuclei were identified by the condensed chromatin at the periphery of the nuclear membrane or by fragmented nuclear body morphology. Necrotic cells presented chromatin forms with irregularly shaped aggregates, a pyknotic nucleus (shrunken and darkly stained), and cell membrane disruption, with cellular debris spilled into the extracellular milieu. The experiment was conducted in triplicate [24, 27, 28].

2.2.5. Statistical Analysis. The one-way ANOVA, followed by Tukey's posttest was performed using GraphPad Prism 5.0 for bacterial and eukaryotic cell models. For *Salmonella*/Microssome assay, we also performed Bernstein's correlative analysis using SALANAL software.

3. Results and Discussion

The pharmacokinetic properties of Infertile are presented in Table 1. According to pkCSM in silico prediction, zinc gluconate is poorly absorbed and consequently presents low distribution volume and is chemically inert to CYP isoenzymes.

The predictive results presented by pkCSM and LAZAR algorithms were compared and presented in Table 2. Both predictive strategies pointed Infertile as nonmutagenic in Ames toxicity test. LAZAR prediction suggested carcinogenic effect of zinc gluconate in rodents in general and to mice and rats separately. The maximum tolerated dose in humans and the toxicity to fathead minnows were predicted in the same range using both strategies.

These pharmacokinetic aspects of the compound favor its low hepatotoxic profile, once the prediction suggests no interaction between zinc gluconate and CYP enzymes, both used as substrate and inhibitor [29]. According to LAZAR's prediction, the carcinogenicity propensity of Infertile was determinant to the following investigation of the genetic toxicological profile of this compound.

Although the predictive results using the in silico approach indicated the absence of mutagenicity in Ames test, we performed the bacterial reversion assay (Ames test) using *Salmonella* strains and observed a mutagenic response for Infertile (Figure 2). In the absence of metabolic activation (−S9), TA98 strain indicated a positive mutagenic response (MI ≥ 2, Mutagenic Slope; 1,27 revertants/mM to Infertile at 30 mM and 60 mM; $P < 0.01$). A cytotoxic effect was observed for TA104 (60% survival) at the 60 mM concentration. With metabolic activation (+S9), no mutagenicity was detected for the strains used. However, cytotoxicity was detected for TA102 (65% survival at 30 mM and 45% survival at 60 mM) in the presence of the compound. Infertile showed negative mutagenic responses for strains TA97, TA100, TA102, and TA104 (−S9/+S9). Moreover, no cytotoxic effect was observed for TA97a, TA98, and TA100 strains in the presence

Table 1: Infertile's pharmacokinetic (ADME) properties prediction in pkCSM analysis of similarity.

Property	Model name	Predicted value	Unit
Absorption	Water solubility	2.99	Numeric (mmol/L)
Absorption	Caco2 permeability	−0,8980	Numeric (log Papp in 10^{-6} cm/s)
Absorption	Intestinal absorption (human)	0,0000	Numeric (% Absorbed)
Absorption	Skin permeability	−2.7350	Numeric (log Kp)
Absorption	P-glycoprotein substrate	Yes	Categorical (yes/no)
Absorption	P-glycoprotein I inhibitor	No	Categorical (yes/no)
Absorption	P-glycoprotein II inhibitor	No	Categorical (yes/no)
Distribution	VDss (human)	44.9	Numeric (mL/kg)
Distribution	Fraction unbound (human)	0.8810	Numeric (Fu)
Distribution	BBB permeability	−2.2160	Numeric (log BB)
Distribution	CNS permeability	−6.1620	Numeric (log PS)
Metabolism	CYP2D6 substrate	No	Categorical (yes/no)
Metabolism	CYP3A4 substrate	No	Categorical (yes/no)
Metabolism	CYP1A2 inhibitor	No	Categorical (yes/no)
Metabolism	CYP2C19 inhibitor	No	Categorical (yes/no)
Metabolism	CYP2C9 inhibitor	No	Categorical (yes/no)
Metabolism	CYP2D6 inhibitor	No	Categorical (yes/no)
Metabolism	CYP3A4 inhibitor	No	Categorical (yes/no)
Excretion	Total clearance	4.06	Numeric (ml/min/kg)
Excretion	Renal OCT2 substrate	No	Categorical (yes/no)

VDss: volume of distribution at steady state; BBB: brain blood barrier; CNS: central nervous center; CYP: cytochrome P; OCT: organic cation transporter.

Table 2: Comparison between pKCSM and LAZAR toxicity prediction of Infertile.

Model Name	pkCSM		LAZAR	
	Unit	Prediction	Unit	Prediction
AMES toxicity	Categorical (yes/no)	No	Categorical (yes/no)	No
Hepatotoxicity	Categorical (yes/no)	No	n.a.	—
Skin sensitisation	Categorical (yes/no)	No	n.a.	—
hERG I inhibitor	Categorical (yes/no)	No	n.a.	—
hERG II inhibitor	Categorical (yes/no)	No	n.a.	—
Oral rat acute toxicity (LD50)	Numeric (mol/kg)	0.29	n.a.	—
Oral rat chronic toxicity (LOAEL)	Numeric (mg/kg/day)	22.49	n.a.	—
Carcinogenicity (rat)	n.a.	—	Categorical (Yes/No)	Yes
Carcinogenicity (mouse)	n.a.	—	Categorical (Yes/No)	Yes
Carcinogenicity (rodents)	n.a.	—	Categorical (Yes/No)	Yes
Max. tolerated dose (human)	Numeric (mg/kg/day)	16.11	Numeric (mg/kg/day)	7.93
T. pyriformis toxicity	Numeric (log ug/L)	1.93	n.a.	—
Fathead minnow toxicity	Numeric (mol/L)	1,164	Numeric (mol/L)	1,567

hERG: human Ether-à-go-go-Related Gene; LD50: lethal dose of 50%; LOAEL: lowest observed adverse effect level; n.a.: not analyzed.

or absence of exogenous metabolism in all tested concentrations.

Infertile demonstrated mutagenic potential for the strain TA98 in the absence of exogenous metabolism system, which suggests frameshift mutation by G:C pair deletion. Infertile will probably not represent a risk for animal health because the Sertoli cells in animal testis exhibit a reasonable metabolic capacity to convert the compound into nontoxic metabolites [30]. Besides, the presence of the Zn^{2+} cation in the chemical structure of Infertile has a central role in the toxicity of the molecule, because heavy metals such as zinc, lead, cadmium, and mercury can increase the mutagenicity and cytotoxicity of various compounds in bacterial reverse mutation evaluations. It is likely that, in the +S9 experiments, no mutagenic concentration was observed because of the presence of metalloproteins in the liver homogenate, which bind to metallic cations and reduce their availability [31].

There is evidence that Infertile is a clastogenic or aneugenic compound, because, at 12 mM, almost 10% of the cells were found with micronucleus. At 30 and 60 mM, the increased rate of necrosis and apoptosis suggests high cytotoxicity. The increased rate of apoptosis at 30 mM suggests

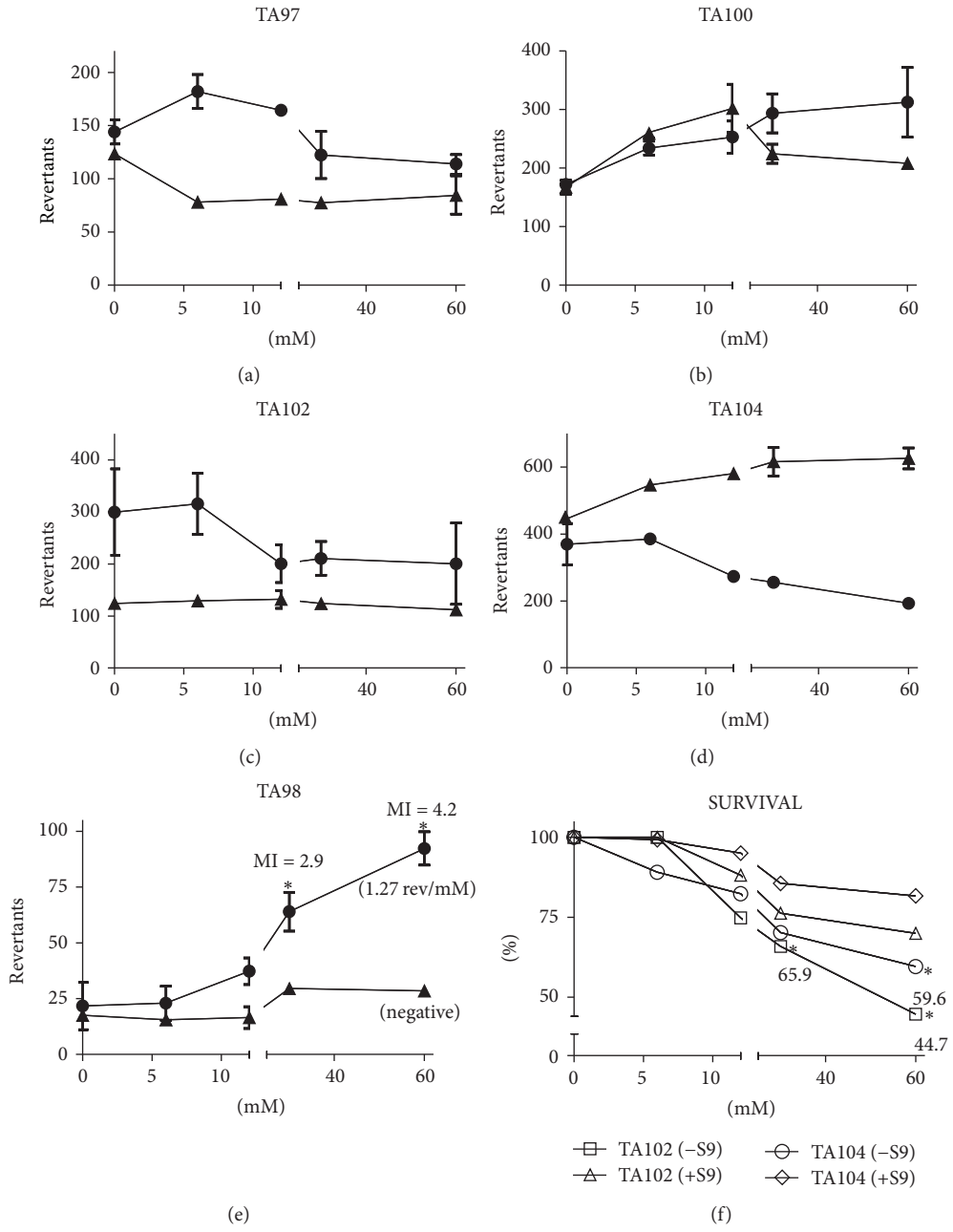

Figure 2: Mutagenicity and cytotoxicity evaluation of Infertile. The graphs ((a), (b), (c), and (d)) show that, in both absence (−S9, ●) and presence (+S9, ▲) of exogenous metabolism, there were no mutagenic concentrations to TA97, TA100, TA102, and TA104. On the other hand, in (e), mutagenic activity to TA98 (−S9) was detected. No cytotoxic (survival ≤ 70%) response was observed to TA97, TA98, and TA100 (−S9/+S9, data not shown). In (f), there was a decrease of survival to TA102 and TA104 at 60 mM (* < 0.01 versus negative control; n = 3 in triplicate; one-way ANOVA followed by Tukey's post hoc test).

that silent cell death occurs at this concentration, whereas, at 60 mM, there was an increase in necrosis, pointing out to abrupt cell death, which masks genotoxic response. This fact is crucial for the sterilant activity of Infertile, once the death of the epithelial and Sertoli cells in the seminiferous tubules is necessary for castration to occur [6]. The damage caused by the compound leads to a delay in cellular cycle, which could be the reason of the alteration in the mitotic index.

The results of the macrophage micronucleus assay are shown in Figure 3. At 12 mM, there was an increase in micronucleated cells rate (8%). In higher concentrations (30 and 60 mM), a prevalence of apoptotic and necrotic cells (cell death) was observed.

Infertile is registered by the Brazilian Ministry of Agriculture, Livestock and Supply and the usage dose ranges from 200 mM to 1000 mM [32], which indicates that it underwent

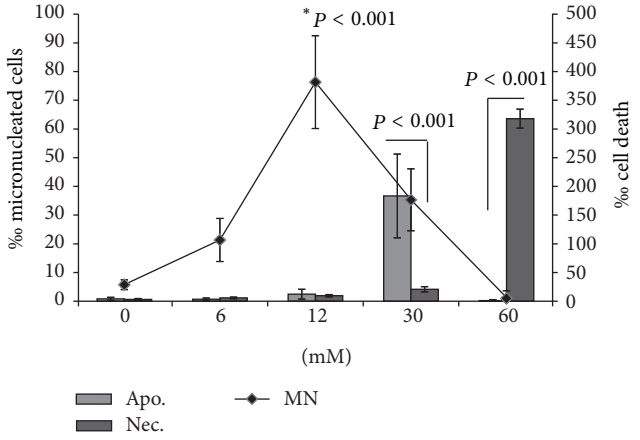

Figure 3: Infertile genotoxicity and cytotoxicity evaluation using RAW267.4 macrophage cell lineage. There are three curves showing the percentage of micronucleated cells (MN) in apoptosis (Apo) and necrosis (Nec). It can be observed that, at 12 mM, there is predominance of micronucleated cells (85/1000 cells), at 30 mM, cells in apoptosis predominate (143/1000 cells) and, at 60 mM of Infertile, there were 318 cells in necrosis/1000. It is important to note that when an event prevails, others tend to diminish. It is possible to observe that there is a turnover among the cell death events (* < 0.01 versus negative control; $n = 3$ in triplicate; one-way ANOVA followed by Tukey's post hoc test).

a strict quality control before it could be in the market. So far, chemical castration methods, as the intratesticular injection of Infertile, have not promoted any major harm to animals; therefore, they have the approval and support of specific organs, such as the Alliance for Contraception in Cats & Dogs and the Regional Council of Veterinary Medicine of the State of São Paulo [33]. However, either the product or the method still needs to be improved.

A study in Galapagos Islands showed that some animals treated with the zinc gluconate procedure presented tissue necrosis. These complications were attributed to improper injection techniques or inaccurate after-treatment management, besides the intrinsic characteristics of the local environment [34]. The evidence of tissue necrosis was linked to external events whereas it could have been related to an endogenous reaction based on inflammatory response. The presence of inflammation after the intratesticular injection of 200 mM zinc gluconate was reported, and anti-inflammatory drugs were prescribed to minimize this side effect [35].

The detection of cytotoxic concentrations of Infertile on eukaryotic cells in the present study corroborates previous findings for in vivo experiments [34, 35].

When cell death is silent, as in apoptosis, there is no inflammatory response, and dying cells contract into an almost invisible corpse that is soon consumed by neighboring cells. On the other hand, necrosis releases proinflammatory signals to the surrounding tissue microenvironment, unlike apoptosis [36]. As a consequence, necrotic cells induce an immune response to recruit inflammatory cells. Moreover, necrotic cells can release chemokines and bioactive factors that can stimulate viable cells to proliferate, with the potential, once again, to facilitate neoplastic progression [37].

Necrosis has a crucial role in inflammatory response. Once cellular disruption occurs, several damage associated molecular patterns (DAMPs) are released, such as mitochondrial DNA (mtDNA), which exerts immunogenic function and can recruit neutrophils to the area of necrosis [38]. The presence of DAMPs originated from cell debris at necrotic sites (necrotaxis signals) has been described as being more important than chemotactic stimuli for establishing leukocyte migration and inflammatory response [39]. A chronic necrotic-induced inflammatory environment causes the emergence of DNA damage induced by oxidative stress. This phenomenon can be mediated by ROS, such as superoxide radicals ($O_2^{\bullet -}$) and RNS, derived from nitric oxide radicals ($NO^{\bullet}$).

Oxidative damage can lead to single- or double-strand breaks, frameshift and point mutations, and chromosome abnormalities, and more than 30 different products of DNA and RNA nucleobases produced by oxidative damage have been identified [40]. Besides, TNF-α, an inflammatory cytokine, can trigger inflammation-mediated carcinogenesis. The molecular basis possibly involves induction of reactive oxygen. Reactive oxygen in the form of NO is often generated by inflammatory cytokine induction of NO synthase. NO can directly oxidize DNA, resulting in mutagenic changes, and it may cause damage to some DNA repair proteins. Inflammatory cytokines may also affect genome integrity via inhibition of cytochrome P450 or glutathione S-transferase isoenzymes [41].

Recently, it was demonstrated that nanoparticles of zinc oxide can delay apoptosis, reinforcing an oxidative cellular microenvironment and an increase in proinflammatory response, by enhancing the secretion of IL-1β and IL-8 in human cells [42]. The combination of these three factors suggests that zinc can favor a procarcinogenic environment in exposed tissues.

4. Conclusions

The cytotoxic effect of the zinc gluconate-based product, Infertile, is an important factor, considering its use as a canine sterilization agent. Cell death mechanism would have to be apoptosis, since necrotic processes are potentially carcinogenic. Although Infertile is licensed for use on animals, its genotoxic and cytotoxic effects, shown in the in vitro toxicological evaluation, demonstrate that the highest dose (60 mM) presents a risk for animal health by necrosis induction. Studies must be continued in order to clarify the activity on cells and tissues involved in the sterilant activity of Infertile and the cell damage induced, in order to better understand the pathophysiological mechanisms of this drug.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

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Research Article

Toxicity Studies of Ethyl Maltol and Iron Complexes in Mice

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Ethyl maltol and iron complexes are products of ethyl maltol and the iron found in the cooking pots used to prepare the Chinese dish, hot-pot. Because their safety is undocumented, the toxicity study of ethyl maltol and iron complexes was conducted in male and female Kunming (KM) mice. The animal study was designed based on the preliminary study conducted to determine the median lethal dose (LD_{50}). The doses used in the study were 0, 1/81, 1/27, 1/9, and 1/3 of the LD_{50} (mg kg body weight $(BW)^{-1}$ day^{-1}) dissolved in the water. The oral LD_{50} of the ethyl maltol and iron complexes was determined to be 743.88 mg kg BW^{-1} in mice. The ethyl maltol and iron complexes targeted the endocrine organs including the liver and kidneys following the 90 D oral exposure. Based on the haematological data, the lowest-observed-adverse-effect level (LOAEL) of the ethyl maltol and iron complexes was determined to be 1/81 LD_{50} (9.18 mg kg BW^{-1} day^{-1}) in both male and female mice. Therefore, we suggest that alternative strategies for preparing the hot-pot, including the use of non-Fe-based cookware, need to be developed and encouraged to avoid the formation of the potentially toxic complexes.

1. Introduction

Ethyl maltol ($C_7H_8O_3$, Figure 1) is an important food additive and the main component of a type of incense added to food. It is widely used to enhance the flavour of commercial food products such as candy, cookies, chocolate, and beverages [1–3] and extend the product shelf-life, inhibit the growth of melanoma, and enhance film uniformity in beverages, food, cigarettes, cosmetics, pharmaceuticals, and photosensitive materials [4]. In previous studies, ethyl maltol was found to be approximately six times more potent than its homologue maltol, in improving the aroma and flavour of food [3].

Hot-pot is an original traditional Chinese dish, and its most important characteristic is its fragrance. Ethyl maltol is the key food additive and flavour enhancer that imparts the characteristic hot-pot fragrance. In the process of preparing hot-pot, the businesses involved often randomly add a drop of ethyl maltol-containing incense to the bottom of the cooking pots, which when fabricated with Fe can react with the steam produced during the boiling process to produce iron oxide (Fe_3O_4). In previous studies, ethyl maltol was confirmed to combine with Fe^{3+} in different ratios, namely, 1 : 1, 1 : 2, and 1 : 3 [5]. In the process of making the well-loved Chinese hot-pot dish more flavourful, the ethyl maltol inevitably comes in contact with the Fe to form iron complexes that are kinetically labile. Although the ethyl maltol enhances the scent of foods, a high dietary intake may lead to headaches, nausea, and vomiting and could affect the liver and kidney functions [2, 3, 6].

Gralla et al. [7] evaluated the biological safety of ethyl maltol following acute, subacute, and chronic administration, and their results suggested that ethyl maltol is safe, but their information on the toxicity of ethyl maltol and iron complexes was limited. Therefore, this present study was designed to evaluate the acute and subchronic toxicity of ethyl maltol and iron complexes, to provide information on their safety for addressing the clinical implications of their consumption.

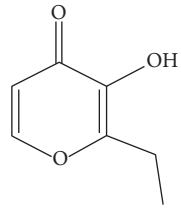

Figure 1: Ethyl maltol ($C_7H_8O_3$, EMA).

2. Materials and Methods

2.1. Test Article. The ethyl maltol was purchased from Beijing Newt Biotechnology Development Co., Ltd, with a purity of 99.5% that was detected using the ultraviolet (UV)-2501 UV-visible (VIS) spectrophotometer. The deionized water used in the tests was prepared using the HB-0.25-200T equipment (China) while the distilled water was sterilized at 0.15 kPa at 121°C for 15 min.

To simulate hot-pot production, the ethyl maltol was completely dissolved in hot distilled water and boiled in an iron pan for 1 h to ensure the formation of the red-purple complexes, and then the mixture was brought to 25°C. The stability of the ethyl maltol and iron complexes was maintained for up to 1 day at room temperature, and they were prepared daily at 7 a.m.

2.2. Animals. The 70 and 100 Kunming (KM) mice of both sexes (6- and 4-week-old, resp.) used in this study were obtained from Shanxi Medical University (China). Following their arrival, the health condition of all the animals was examined to confirm their suitability for use in the study and they were allowed to acclimate to the laboratory environment for 5 days. The animals were housed by sex in groups of five per cage in an environment-controlled animal room and were provided with standard commercial diet and drinking water ad libitum. Except for minor variations, all the animal rooms were monitored and maintained under a 12 h light-dark cycle with temperature ranging from 20 to 25°C and relative humidity of 40%–60%. All the animal procedures performed in this study were reviewed and approved by the Animal Experimental Welfare and Ethical Inspection Committee of the Chinese Center for Disease Control and Prevention (Approval number IACUC#15-026).

2.3. Experimental Design. In a preliminary dose-finding study, 70 mice were stratified by weight and assigned randomly to seven groups of five males and five females each. The mice in the control and groups 1, 2, 3, 4, 5, and 6 were given the ethyl maltol and iron complexes at doses of 0, 425.98, 532.48, 665.6, 832, 1040, and 1300 mg kg BW^{-1}, respectively, by gavage for 14 days at 9 a.m. daily. The mice were monitored to observe their general behaviour and signs of toxicity continuously for 3 h after the gavage. The mice were further observed once a day for up to 14 days for behavioural changes and signs of toxicity and death. The surviving mice were euthanized while those that died from the poisoning were necropsied. We calculated the median lethal dose (LD_{50}) of the ethyl maltol and iron complexes selected from the study doses based on the value obtained.

One hundred mice of both sexes were stratified by weight and randomly divided into five groups of 10 males and 10 females each, which were given a mixture of water and the ethyl maltol and iron complexes, at single doses of 1/81, 1/27, 1/9, and 1/3 LD_{50} mg kg BW^{-1} by gavage for 90 days. In addition, the control group was also given the same amount of distilled water (without ethyl maltol) boiled in the iron pan. The general cage-side observations for clinical signs were performed twice during the acclimation period, once daily after administration of the test article, and the detailed clinical observations were performed once daily. The animals were observed closely for any behavioural changes daily and were examined individually outside their home cage environments ensuring they were undisturbed. The body weights of the animals and food consumption were monitored for 10 days during the study period.

2.4. Measurement of Biochemical and Haematological Parameters. On day 45 (D45), the animals were fasted for 4 h, and blood samples were collected from their tail tips for haematological and biochemical analysis while 24 h urine samples were also collected. On D90, the animals were fasted for 4 h, and then their body weights were measured, and whole blood samples were collected while the 24 h urine samples were also collected. Then, the mice were euthanized and subsequently necropsied.

2.4.1. Haematological Parameters. The blood samples were analysed using an automatic blood analyser (MEK-6318K, Japan) for the red blood cell (RBC) and white blood cell (WBC) counts, haemoglobin (Hb), and percentage of leukocytes including lymphocytes (L), neutrophil (N), monocyte count (M), eosinophil (E), and basophil (B) counts.

2.4.2. Serum Biochemistry. The whole blood biochemistry of the mice including alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), lactate dehydrogenase (LDH), total protein (TP), albumin (ALB), globulin (GLO), albumin/globulin ratio (A/G), blood urea nitrogen (BUN), and creatinine (Cr) was analysed. The blood samples were collected from the orbital artery of mice anesthetized with pentobarbital sodium by intraperitoneal injection, centrifuged at 3000 rpm for 10 min at 4°C, and subsequently analysed using an automatic chemical analyser (HITACHI7180, China).

2.5. Organ Weight and Histopathology. After the blood collection, the animals were euthanized, and the organs including the hearts, livers, spleens, kidneys, and lungs were harvested for histological examination. We also determined the absolute and relative organ weights (based on terminal body

Table 1: Mortality rate in acute toxicity study in mice ($n = 10$).

Groups	Control group	1	2	3	4	5	6
Dose (mg kg^{-1})	0	425.98	532.48	665.6	832	1040	1300
Mortality rate	0	1	2	4	6	8	9
Mortality (%)	0	10	20	40	60	80	90
P^2	0	0.01	0.04	0.16	0.36	0.64	0.81

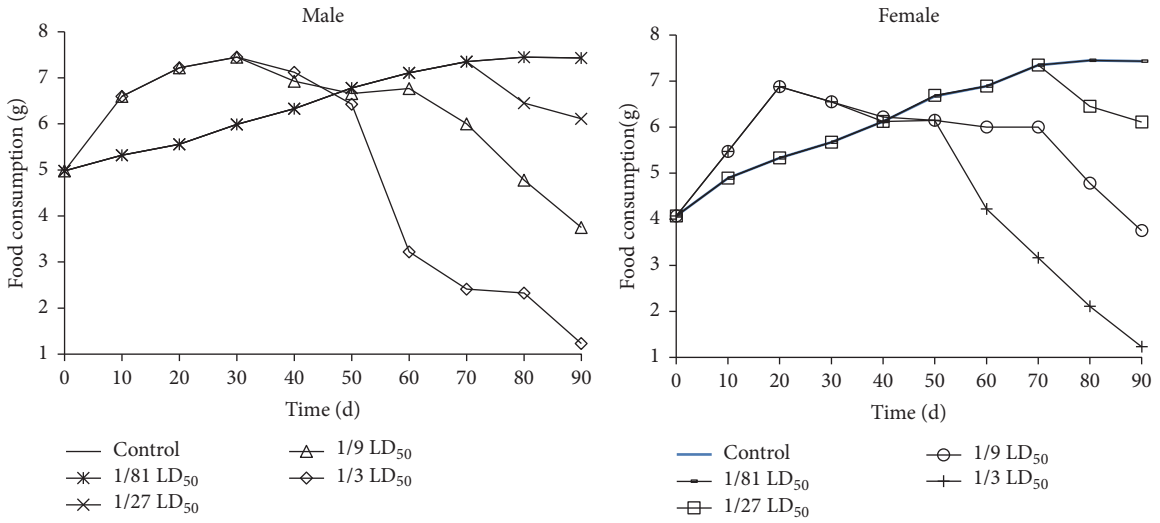

Figure 2: Food consumption changes for male and female mice for 90 days ($n = 10$). Data expressed as means ± standard deviation (SD).

weights) of the heart, liver, spleen, kidneys, and lungs, which were calculated as follows:

$$\text{Relative organ weight (\%)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight (g)}} \times 100. \quad (1)$$

The histopathological examinations were conducted on all the organs and the liver and kidneys tissues of all animals that showed obvious gross lesions were fixed in 10% buffered formalin for 24 h and then they were routinely processed for paraffin embedding [8].

2.6. Statistical Analysis. All the data are expressed as the means ± standard deviation (SD) and comparisons among the different groups were performed using an analysis of variance (ANOVA) and the IBM statistical package for the social sciences (SPSS) statistics 22 software was used for all the analysis. The significance level was set at 5 and 1% ($P < 0.05$ and $P < 0.01$, resp.). The LD_{50} value was determined according to the Bliss method [9].

3. Results

3.1. Preliminary LD_{50} Determination of Ethyl Maltol and Iron Complexes. Compared with the control group mice, the main behavioural signs of toxicity observed in the complex-treated mice were righting reflex disappearance, asthenia, and locomotor activity reduction (Table 1). The histological investigation showed different degrees of degeneration in the liver and kidneys cells, as well as oedema and haemorrhage in the liver and kidneys. No mortality was observed in 24 h, after which the mice in group 6 showed the first mortality followed by the other groups. After 1 week, no additional deaths were observed. LD_{50} of the ethyl maltol and iron complexes, which was evaluated using the Bliss method, was 743.88 mg/kg. According to the standard acute toxicity classification, the ethyl maltol and iron complexes belong to level 3 category, suggesting they possessed low toxicity.

3.2. Effect of Ethyl Maltol and Iron Complexes on Feed Intake and Growth of Mice. No mortality was observed at any of the tested dose levels during the 90-day treatment period. At the beginning of the study, the mice in the 1/3 LD_{50} dose group showed increased food intake (Figure 2), whereas that of the other treatment groups was similar to the control group. After 60 days, a reduction in food intake and activities consisting of lethargy and asthenia were observed in the 1/3 LD_{50} dose group. After 70 days, the toxicity symptoms including cowering together, a loss of appetite, lethargy, slow reaction time, and reduced food intake were observed in the 1/9 LD_{50} dose group. After 80 days, a loss of appetite, lethargy, and a slow reaction time were observed in the 1/27 LD_{50} dose group.

Figure 3 shows that during the first 50 days, the weights of the 1/3 and 1/9 LD_{50} treatment groups increased rapidly. In addition, after day 50, the weights of the 1/3 LD_{50} group increased slowly and by day 80, the weights in the 1/9 LD_{50}

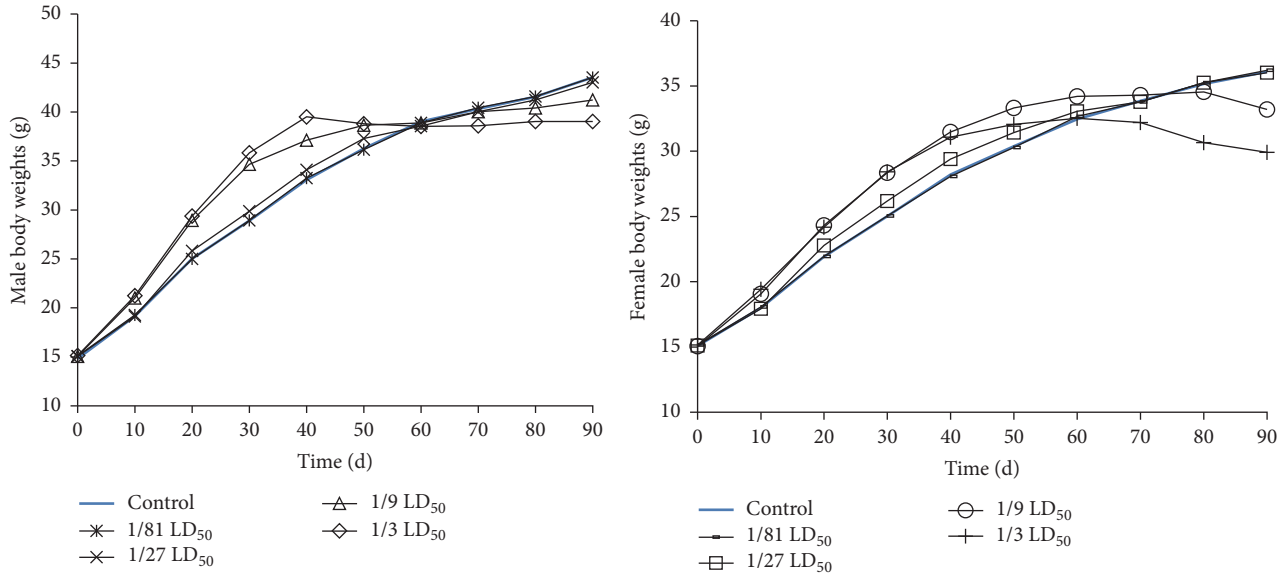

Figure 3: Body weights changes of male and female mice after 90-day treatment ($n = 10$). Data expressed as means ± standard deviation (SD).

Table 2: Effect of ethyl maltol complexes on male and female mouse organ coefficients ($n = 10$).

Organs	Control group	Test groups			
		1/81 LD_{50}	1/27 LD_{50}	1/9 LD_{50}	1/3 LD_{50}
Males					
Heart	0.55 ± 0.01	0.55 ± 0.01	0.55 ± 0.01	0.55 ± 0.01	0.55 ± 0.01
Liver	5.33 ± 0.04	5.38 ± 0.03	5.48 ± 0.03*	5.74 ± 0.04*	5.73 ± 0.03*
Spleen	0.46 ± 0.01	0.45 ± 0.01	0.46 ± 0.01	0.46 ± 0.01	0.46 ± 0.01
Lung	0.66 ± 0.01	0.67 ± 0.01	0.67 ± 0.01	0.67 ± 0.01	0.67 ± 0.01
Kidneys	1.46 ± 0.04	1.50 ± 0.02	1.55 ± 0.03	1.72 ± 0.04*	1.74 ± 0.03*
Females					
Heart	0.53 ± 0.01	0.53 ± 0.01	0.53 ± 0.01	0.52 ± 0.01	0.53 ± 0.01
Liver	5.26 ± 0.06	5.25 ± 0.06	5.61 ± 0.22*	5.65 ± 0.06*	5.75 ± 0.02*
Spleen	0.44 ± 0.01	0.44 ± 0.01	0.44 ± 0.01	0.44 ± 0.01	0.44 ± 0.01
Lung	0.62 ± 0.01	0.62 ± 0.01	0.62 ± 0.01	0.62 ± 0.01	0.62 ± 0.01
Kidneys	1.20 ± 0.02	1.22 ± 0.02	1.30 ± 0.01*	1.51 ± 0.02*	1.50 ± 0.02*

Results are expressed as means ± standard deviation (SD). *$P < 0.05$ versus the vehicle group.

treatment group increased slowly ($P < 0.05$). All the values were considered to be ethyl maltol complex-related due its toxicity.

3.3. Effect of Ethyl Maltol and Iron Complexes on Organ Index in Mice. The mean and relative organ weights for both male and female mice in this study were determined following the termination of the 90-day study and are presented in Table 2. Compared with the control group, the liver weights of the male and female mice in the 1/27 LD_{50} group had increased significantly by the end of the treatment period ($P < 0.05$). The kidney weights of the male mice in the 1/9 LD_{50} group increased significantly while those of females of the 1/27 LD_{50} group did not.

3.4. Effect of Ethyl Maltol and Iron Complexes on Haematological Parameters of Mice. Compared with the values of the control group mice, the Hb of the 1/27, 1/3, and 1/9 LD_{50} treatment group male mice differed significantly ($P < 0.05$) while that of the females in the 1/3 and 1/9 LD_{50} treatment groups did not. A significant increase in the RBC, WBC, N, and E counts was observed in the 1/9 LD_{50} treatment group male mice ($P < 0.05$). In addition, similar changes in these indices were observed in the 1/9 and 1/3 LD_{50} treatment group female mice.

The haematological parameters are shown in Tables 3 and 4. The Hb, RBC, and L of the mice of both sexes in the 1/27 LD_{50} treatment group were significantly decreased ($P < 0.05$), as were the WBC, N, and E of the 1/9 LD_{50} group

Table 3: Haematological parameters of mice after 45-day treatment with ethyl maltol and iron complexes ($n = 10$).

Haematological indices	Control group	Treatment			
		1/81 LD_{50}	1/27 LD_{50}	1/9 LD_{50}	1/3 LD_{50}
Males					
Hb ($g\,L^{-1}$)	146.09 ± 2.13	147.09 ± 1.99	152.25 ± 1.10*	160.09 ± 0.76*	156.97 ± 0.65*
RBC ($10^{12}\,L^{-1}$)	8.63 ± 0.22	8.53 ± 0.20	8.45 ± 0.22	9.22 ± 0.09*	8.94 ± 0.09
WBC ($10^{9}\,L^{-1}$)	7.58 ± 0.31	7.75 ± 0.34	7.49 ± 0.38	8.43 ± 0.17*	7.77 ± 0.17
N	28.66 ± 0.41	28.88 ± 0.20	28.84 ± 0.10	29.45 ± 0.18*	28.98 ± 0.13
E	2.91 ± 0.08	2.89 ± 0.08	2.95 ± 0.02	2.71 ± 0.07*	2.80 ± 0.08
DC (%) B	0.14 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	0.17 ± 0.01
M	2.38 ± 0.11	2.39 ± 0.09	2.44 ± 0.08	2.41 ± 0.11	2.61 ± 0.04
L	65.90 ± 0.51	65.66 ± 0.23	65.60 ± 0.11	65.26 ± 0.18	65.43 ± 0.11
Females					
Hb (g/L)	131.53 ± 2.2	131.27 ± 2.12	133.92 ± 1.74	139.31 ± 1.30*	137.27 ± 1.74*
RBC (10^{12}/L)	7.90 ± 0.08	7.85 ± 0.05	8.02 ± 0.05	8.58 ± 0.11*	8.18 ± 0.08*
WBC (10^{9}/L)	5.88 ± 0.071	5.87 ± 0.09	6.00 ± 0.06	6.94 ± 0.06*	6.22 ± 0.09*
N	27.83 ± 0.52	27.98 ± 0.48	29.71 ± 0.29*	33.16 ± 0.49*	31.11 ± 0.08*
E	3.13 ± 0.03	3.15 ± 0.03	2.99 ± 0.02*	2.46 ± 0.07*	2.76 ± 0.04*
DC (%) B	0.91 ± 0.05	0.93 ± 0.03	0.90 ± 0.04	0.84 ± 0.06	0.91 ± 0.04
M	2.04 ± 0.03	2.06 ± 0.03	2.10 ± 0.03	2.36 ± 0.03	2.15 ± 0.03
L	66.09 ± 0.53	65.89 ± 0.50	64.30 ± 0.31	61.20 ± 0.55	63.06 ± 0.08

The results are expressed as means ± standard deviation (SD). Hb: haemoglobin; RBC: red blood cell; WBC: white blood cell; N: neutrophil; E: eosinophil; B: basophil; M: monocyte; L: lymphocytes. *$P < 0.05$, versus the vehicle group.

Table 4: Haematological parameters of mice after 90-day treatment with ethyl maltol and iron complexes ($n = 10$).

Haematological indices	Control group	Treatment			
		1/81 LD_{50}	1/27 LD_{50}	1/9 LD_{50}	1/3 LD_{50}
Males					
Hb ($g\,L^{-1}$)	158.06 ± 1.03	158.16 ± 0.91	146.31 ± 0.74*	124.60 ± 1.93*	108.33 ± 3.13*
RBC ($10^{12}\,L^{-1}$)	10.33 ± 0.34	10.24 ± 0.25	8.37 ± 0.15*	7.61 ± 0.15*	6.35 ± 0.18*
WBC ($10^{9}\,L^{-1}$)	8.37 ± 0.23	8.12 ± 0.15	8.43 ± 0.16	10.18 ± 0.26*	12.36 ± 0.25*
N	28.68 ± 0.27	28.28 ± 0.23	29.20 ± 0.23	32.40 ± 0.37*	34.56 ± 0.28*
E	2.83 ± 0.05	2.86 ± 0.03	2.75 ± 0.03	2.52 ± 0.03*	2.44 ± 0.04*
DC (%) B	0.93 ± 0.03	0.93 ± 0.03	0.96 ± 0.03	0.91 ± 0.45	0.93 ± 0.02
M	2.19 ± 0.06	2.20 ± 0.04	2.18 ± 0.05	2.12 ± 0.03	2.15 ± 0.04
L	65.37 ± 0.23	65.73 ± 0.22	64.93 ± 0.23*	62.05 ± 0.34*	59.91 ± 0.26*
Females					
Hb ($g\,L^{-1}$)	140.87 ± 0.82	140.53 ± 0.78	134.98 ± 0.48*	126.35 ± 1.08*	115.56 ± 2.26*
RBC ($10^{12}\,L^{-1}$)	9.35 ± 0.17	9.71 ± 0.23	8.21 ± 0.18*	7.08 ± 0.09*	6.19 ± 0.11*
WBC ($10^{9}\,L^{-1}$)	7.93 ± 0.14	7.91 ± 0.11	8.28 ± 0.07	10.36 ± 0.28*	12.34 ± 0.30*
N	27.18 ± 0.38	27.50 ± 0.33	29.15 ± 0.32	32.33 ± 0.25*	34.14 ± 0.17*
E	2.88 ± 0.03	2.86 ± 0.03	2.75 ± 0.04	2.65 ± 0.04*	2.42 ± 0.02*
DC (%) B	0.97 ± 0.05	0.94 ± 0.02	0.95 ± 0.04	0.94 ± 0.03	0.95 ± 0.04
M	2.23 ± 0.05	2.18 ± 0.03	2.15 ± 0.05	2.11 ± 0.04	2.11 ± 0.03
L	66.73 ± 0.44	66.50 ± 0.31	65.19 ± 0.30*	62.06 ± 0.23*	60.55 ± 0.13*

The results are expressed as means ± standard deviation (SD). *$P < 0.05$ versus the vehicle group.

($P < 0.05$). All the significant differences observed were compared with those of the control group and are presented in Table 3.

3.5. Effect of Ethyl Maltol and Iron Complexes on Biochemical Parameters of Mice. Compared with the levels of the mice in the control group, the ALB, TBIL, ALT, ALP, AST, BUN, and creatinine levels of the 1/27 LD_{50} treatment group mice of both sexes were significantly different ($P < 0.05$). In particular, the TBIL, ALT, AST, BUN, and creatinine levels were increased in a dose-dependent manner relative to the control male and female mice, and the 1/9 LD_{50} and 1/3 LD_{50}

Table 5: Biochemical parameters of mice of both sexes after 90-day treatment with ethyl maltol and iron complexes ($n = 10$).

Biochemical parameters	Control group	Treatment			
		1/81 LD_{50}	1/27 LD_{50}	1/9 LD_{50}	1/3 LD_{50}
Males					
TBIL (μmol L^{-1})	1.93 ± 0.07	1.95 ± 0.06	2.17 ± 0.05*	2.75 ± 0.06*	3.04 ± 0.06*
ALT (U L^{-1})	46.48 ± 2.40	46.16 ± 2.64	63.44 ± 2.45*	86.19 ± 2.25*	95.34 ± 2.82*
ALP (U L^{-1})	111.54 ± 3.44	111.38 ± 2.93	96.12 ± 2.10*	84.46 ± 2.0*	74.42 ± 2.0*
AST (U L^{-1})	145.23 ± 3.17	146.94 ± 2.85	159.78 ± 2.79*	201.86 ± 3.44*	236.58 ± 6.13*
BUN (mmol L^{-1})	10.33 ± 0.31	10.49 ± 0.29	11.78 ± 0.34*	14.05 ± 0.47*	16.28 ± 0.41*
creatinine (μmol L^{-1})	13.60 ± 0.28	13.37 ± 0.45	14.88 ± 0.38*	18.14 ± 0.38*	19.45 ± 0.26*
LDH (U L^{-1})	889.96 ± 4.48	888.80 ± 4.26	905.53 ± 3.35*	970.73 ± 5.41*	970.73 ± 5.41*
Females					
TBIL (μmol L^{-1})	1.70 ± 0.02	1.71 ± 0.01	1.80 ± 0.01*	1.89 ± 0.02*	2.15 ± 0.03*
ALT (U L^{-1})	44.40 ± 4.21	46.09 ± 1.74	65.38 ± 3.18*	71.62 ± 1.69*	111.22 ± 4.44*
ALP (U L)	143.09 ± 4.01	138.33 ± 2.20	112.14 ± 3.37*	98.46 ± 3.62*	78.51 ± 3.60*
AST (U L^{-1})	142.71 ± 3.60	149.90 ± 1.09	178.61 ± 3.47*	192.30 ± 1.60*	215.21 ± 7.30*
BUN (mmol L^{-1})	8.42 ± 0.31	8.63 ± 0.30	8.93 ± 0.19*	10.64 ± 0.38*	12.66 ± 0.25*
creatinine (μmol L^{-1})	11.16 ± 0.55	11.50 ± 0.49	12.99 ± 0.45*	13.84 ± 0.28*	14.71 ± 0.31*
LDH (U L^{-1})	640.69 ± 6.49	637.72 ± 4.78	650.97 ± 4.87	670.83 ± 4.48*	660.86 ± 5.15*

Results are expressed as means ± standard deviation (SD). *$P < 0.05$ versus the vehicle group.

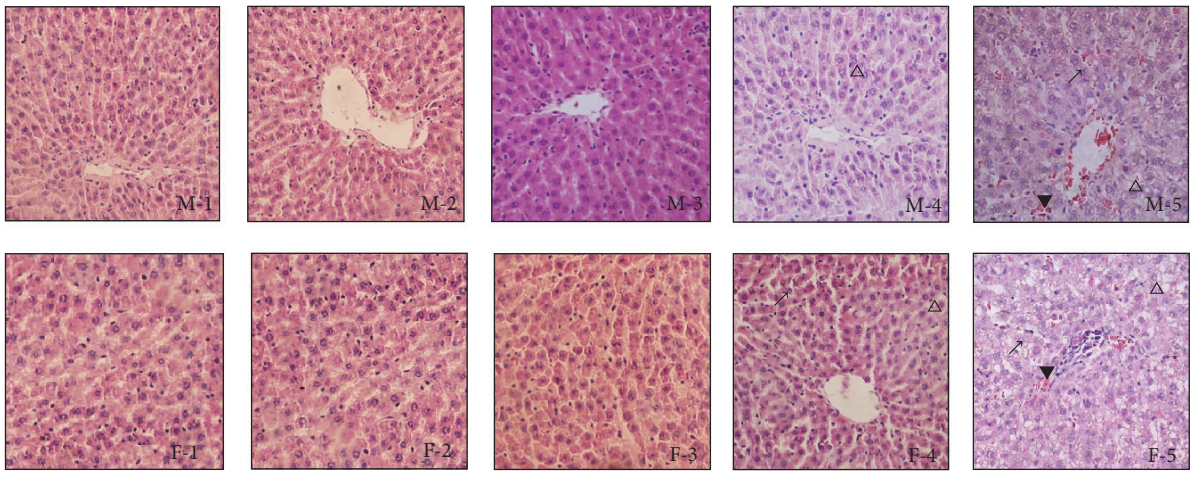

Figure 4: Histopathological examination of livers of male and female mice (haematoxylin and eosin, HE ×400) ($n = 10$). ↗ means granular denaturation, △ means vacuolar degeneration, and ▼ means haemorrhage. M-1, M-2, M-3, M-4, and M-5 were, respectively, from liver of male control, 1/81, 1/27, 1/9, and 1/3 LD_{50} treatment groups; F-1, F-2, F-3, F-4, and F-5 were, respectively, from liver of female control, 1/81, 1/27, 1/9, and 1/3 LD_{50} treatment groups.

treatment group values were statistically significant (Table 5). The LDH level of the male mice in the 1/27 LD_{50} treatment group was significantly increased ($P < 0.05$), while the level of the female 1/9 LD_{50} treatment group mice was significantly different ($P < 0.05$). All the changes reported as statistically significant at the study termination provide evidence of the effect of the ethyl maltol and iron complexes.

3.6. Effect of Ethyl Maltol and Iron Complexes on Histopathological Data in Mice. Following treatment with the ethyl maltol and iron complexes, the kidney tissues of the male and female mice were histopathologically examined at the end of the 90-day administration period (Figure 4). Lesions were observed in the livers of the 1/9 and 1/3 LD_{50} treatment groups. The hepatocytes were swollen, and the cap between adjacent hepatocytes was not obvious. There were numerous tiny empty bubbles and protein grain showing a honeycomb or reticular pattern with nuclei suspended among them. The nuclei and the surrounding boundary were not clearly demarcated, and the presence of nuclear enrichment, fusion, membrane disappearance, and granular denaturation, as well as vacuolar degeneration, were also observed in the 1/3 LD_{50} treatment group (Figure 4(M-5)). The hepatocytes were swollen, and there was a disordered sinusoidal pattern with similar lesions in the 1/9 LD_{50} treatment group (Figure 4(M-4)), but the lesions and the colour of the hepatocytes were

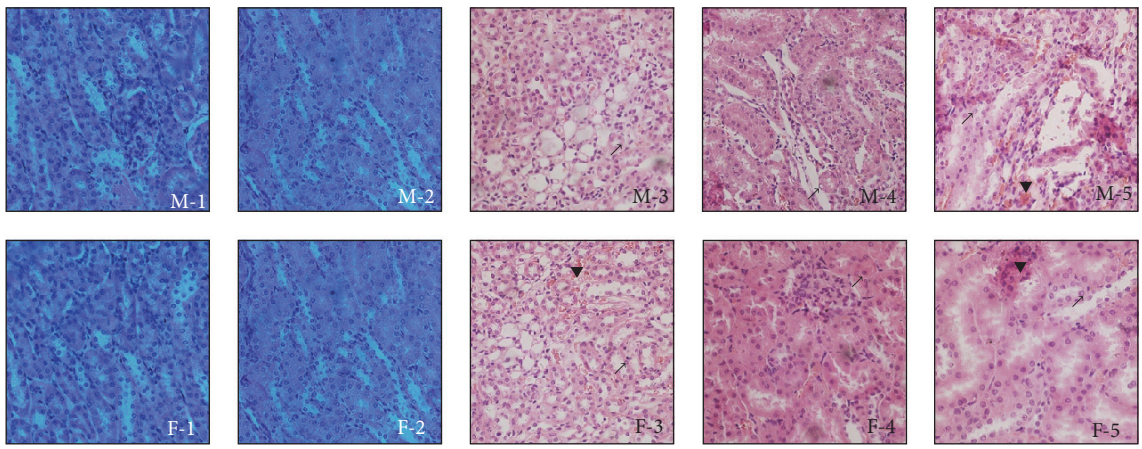

FIGURE 5: Histopathological examination of kidneys of male and female mice (haematoxylin and eosin, HE ×400) (n = 10). ↗ means granular denaturation; ▼ means haemorrhage; M-1, M-2, M-3, M-4, and M-5 were, respectively, from kidneys in male control, 1/81, 1/27, 1/9, and 1/3 LD_{50} treatment groups; F-1, F-2, F-3, F-4, and F-5 were, respectively, from kidneys in female control, 1/81, 1/27, 1/9, and 1/3 LD_{50} treatment groups.

milder. Furthermore, lesions were also observed in the female mice in the treatment groups. However, compared to the male mice, the lobular architecture had disappeared, while numerous hepatocytes showed necrosis and a similar nuclear fusion was also observed in the female mice in the 1/3 LD_{50} treatment group (Figure 4(F-5)). Compared to the treated male mice, the lobular architecture disappeared, and the phenomena of granular denaturation and vacuolar degeneration were more serious in the female mice of the 1/9 LD_{50} treatment group (Figure 4(F-4)).

The kidneys showed evidence of swelling and the tissue sections were muddy. There were numerous RBC in the kidney tubular lumen while the renal epithelial cells were swollen, and granular denaturation, nucleus enrichment, and interstitial hemorrhage in renal tubule were observed with nuclear swelling in the 1/3 LD_{50} treatment group (Figure 5(M-5)). The renal epithelial cells were swollen and showed evidence of necrosis while granular denaturation was observed with lesions that narrowed the lumen, which showed the presence of a protein-like substance in the 1/9 LD_{50} group (Figure 5(M-4)). We observed similar lesions in the female mice in the treatment groups, but compared to the males the lesions in the 1/3 LD_{50} treatment group (Figure 5(F-5)) were more serious than those of the male mice. The tubular structure was disordered, the lumen had narrowed, the renal epithelial cells were swollen, and granular denaturation was also observed in the 1/9 LD_{50} group (Figure 5(F-4)). Finally, the renal epithelial cells were swollen and showed evidence of necrosis while granular denaturation, haemorrhage, and protein-like substance were evident in lumen of the 1/27 LD_{50} group (Figure 5(F-3)).

4. Discussion

It is well known that the resulting 1 : 3 (metal-ligand) complexes can partition readily across cell membranes and, thereby, may facilitate Fe transport across the intestinal wall [10]. Ethyl maltol also can hold Fe(III) in solution and subsequently donate it to an endogenous uptake system. In light of these points, we considered that the rapid growth of the 1/3 and 1/9 LD_{50} treatment groups was caused by the ethyl maltol and iron complexes while the decreased body weight was due to the accumulation of toxic ethyl maltol and iron complexes. Furthermore, the increase in the organ weights indicates the presence of organ hyperaemia, oedema, hyperplasia, and hypertrophy [11, 12]. In our study, the liver and kidneys weights consistently increased, which was in agreement with our theory that the ethyl maltol and iron complexes are poisonous. Furthermore, the changes in the body and organ weights were a dose-dependent increase.

Ethyl maltol combines with Fe^{3+} in different ratios, namely, 1 : 1, 1 : 2, and 1 : 3 [5], and it can combine with Fe^{3+} in the blood. Therefore, the lack of Fe^{3+} induced the dose-dependent decrease in the RBC count and Hb level while a dose-dependent increase in the WBC count was observed in the 1/3 LD and 1/9 LD_{50} treatment groups on D90. We considered that the haematological parameters change observed on D45 might be attributable to the unstable dynamics of the ethyl maltol and iron complexes. When the dose of ethyl maltol and iron complexes was 1/27 LD_{50}, the complexes likely separated because of the unstable dynamic, and the haematological parameters increased as well. Collectively, these results provided strong evidence indicating the low risk of the ethyl maltol and iron complexes inducing toxicity following oral exposure.

ALT and AST, which exist mainly in the liver cells and rarely occur free in the blood, are important biological indicators of liver injury [13–15]. Ennulat et al. [16] and Adenuga et al. [17] also reported that ALT and TBIL were consistently identified as having the highest diagnostic accuracy for biliary injury with or without concurrent hepatocellular necrosis. However, ALT (Table 4) is considered a more specific and sensitive indicator of direct hepatocellular injury, and evidence shows that it is associated with negative diagnostic outcomes

in humans when it is elevated in the serum concurrently with elevated serum levels of TBIL [17, 18]. In our study, the ALP and AST but not ALT and TBIL levels (Table 4) were also significantly elevated in the mice of both sexes in the 1/27 LD_{50} treatment group. Therefore, we excluded the negative outcomes and considered that the liver function was damaged by the ethyl maltol and iron complexes. creatinine (Table 4), which is an important biological indicator of the kidney function, is produced by muscle metabolism and is completely excreted in the urine [14, 19]. The increase in creatinine and BUN was caused by the degeneration of the tubular epithelial cells and lesions of the glomerulus while the kidney function was damaged [20]. We detected a dose-dependent increase in BUN and creatinine in both male and female mice and, therefore, concluded that the kidney function was disrupted by the ethyl maltol and iron complexes, which can, therefore, be considered poisonous. Furthermore, all the alterations in the biochemical parameters were caused by the toxicity of the ethyl maltol and iron complexes.

The kidney and liver lesions were clear [21–23]. In the 1/3 and 1/9 LD_{50} treatment groups, swollen hepatocytes, disordered sinusoidal pattern, granular denaturation, vacuolar degeneration, and nucleus fusion were observed. The 1/3, 1/9, and 1/27 LD_{50} treatment groups exhibited swollen renal epithelial cells, which were necrotic while granular denaturation was also observed. The lesions were dose- and sex-dependent, since those of the female mice were more serious than the males in the 1/3 and 1/9 LD_{50} groups. The lesions of the liver and kidneys were caused by the toxicity of the ethyl maltol and iron complexes, which are poisonous.

5. Conclusion

In summary, oral LD_{50} of the ethyl maltol and iron complexes was determined to be 743.88 mg kg BW^{-1} in mice. The ethyl maltol and iron complexes targeted the endocrine organs including the liver and kidneys following the 90 D oral exposure. Based on the haematological data, the lowest-observed-adverse-effect level (LOAEL) of the ethyl maltol and iron complexes was determined to be 1/81 LD_{50} (9.18 mg kg BW^{-1} day^{-1}) in both male and female mice. Therefore, we suggest that alternative strategies for preparing the hot-pot, including the use of non-Fe-based cookware, need to be developed and encouraged to avoid the formation of the potentially toxic complexes.

Competing Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Review Article

Idiosyncratic Drug-Induced Liver Injury (IDILI): Potential Mechanisms and Predictive Assays

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Idiosyncratic drug-induced liver injury (IDILI) is a significant source of drug recall and acute liver failure (ALF) in the United States. While current drug development processes emphasize general toxicity and drug metabolizing enzyme- (DME-) mediated toxicity, it has been challenging to develop comprehensive models for assessing complete idiosyncratic potential. In this review, we describe the enzymes and proteins that contain polymorphisms believed to contribute to IDILI, including ones that affect phase I and phase II metabolism, antioxidant enzymes, drug transporters, inflammation, and human leukocyte antigen (HLA). We then describe the various assays that have been developed to detect individual reactions focusing on each of the mechanisms described in the background. Finally, we examine current trends in developing comprehensive models for examining these mechanisms. There is an urgent need to develop a panel of multiparametric assays for diagnosing individual toxicity potential.

1. Introduction

Adverse drug reactions (ADRs) are among the five leading causes of death in the United States, with hepatotoxic events being the most common site of ADRs, owing to the fact that the liver is the organ associated with clearance of toxic substances [1]. While the majority of ADRs that contribute to acute liver failure (ALF) are considered to be dose-dependent or "intrinsic," roughly 10–15% of ALF can be attributed to individual effects that are not dependent on dose [1]. These idiosyncratic adverse drug reactions (IADRs) are responsible for a significant amount of drug withdrawals during and after postclinical marketing trials [1]. Additionally, the cost for successful drug development can range from $160 million to $1.8 billion, and it takes ten to fifteen years from lead compound discovery to clinical evaluation [2]. This cost increases significantly when drug candidates fail at the late stage of clinical trials or drugs are withdrawn from the market due to unexpected ADRs. With roughly a postmarketing failure rate of one drug per year, there is a critical need to reduce the human and fiscal cost by decreasing incidence of IADRs.

While significant resources have been put into developing toxicity screens, there is very little in the way of predicting IADRs [3]. Many of the current technologies for detecting hepatotoxicity focus on cytotoxicity screens for in vitro hepatocyte cultures as a method to weed out drug candidates before clinical trials [3]. Subsequent follow-up with animal models is used to reduce the possible drug failure [4–6]. Unfortunately, these screens often have poor predictive value in assessing hepatotoxicity potential [7]. This problem becomes exacerbated when accounting for IADRs, as many of the preclinical trials focus on models that utilize healthy livers with fully functioning drug metabolizing enzymes (DMEs) [8–10].

It is believed that propensities for IADRs and IDILI can be increased by both genetic and nongenetic factors. Such nongenetic factors could include current disease states, pregnancy, other drugs being simultaneously taken with the drug causing the adverse reaction, and age [11]. Potential genetic factors focus on polymorphisms affecting the various DMEs, enzymes that reduce reactive oxygen species (ROS), drug transporters, the inflammation response in the liver, and the major histocompatibility complex (MHC) class of

proteins [5, 11]. Aside from several of the mechanisms that govern immune responses, all of these genetic responses can be localized to the liver, although a few other mechanisms can be found in other organs.

In this review, we address the basis for drug metabolism and disposition in the liver and the proteins and enzymes involved in these processes. We discuss any polymorphisms that have been correlated with (and potentially causative of) ADRs and assays that detect potential for ADRs due to these polymorphisms. The potential mechanisms addressed will include mutations that affect drug metabolism, drug disposition, antioxidant mediating enzymes, and the immune system. In addition, we will address what demographics are most likely to contain these mutations. Next, we discuss the various assays that can be implemented for measuring responses associated with dysfunction in critical proteins. Finally, we discuss the design of future platforms that can help integrate these assays to generate a complete profile for predicting ADRs.

2. Mechanisms for Idiosyncratic Hepatotoxicity

The natures of ADRs are complex. While an individual can have an adverse reaction to a drug, it is difficult to determine the exact cause of the reaction. Many ADRs are caused by the parent drug, the metabolized drug, or byproducts of drug metabolism. The drug metabolism process and the potential triggers for cellular toxicity are illustrated in Figure 1. Drug metabolism relies on an initial transport of drug into the hepatocyte via influx transporters [13]. The drug is metabolized by phase I DMEs, often creating more reactive metabolites [14]. This is followed up with modification via phase II DMEs with a bulkier side chain to deactivate them [14]. The parent drug, reactive metabolites, and heavier products will then all be transported into the bile by efflux transporters compared to those that transport the drug into the cell [13, 14]. Any forms of the drug may produce ROS, which need to be reduced to prevent damage to the cell [15]. Additionally, the drug and its metabolites may conjugate to proteins, forming haptens, which can be presented on the surface of the cell, making it recognized by the immune system as a damaged cell [16]. Any polymorphism that affects drug metabolism, drug transport, antioxidant defense, and immune responses is a potential mechanism associated with increased risk to IDILI.

2.1. Phase I Drug Metabolizing Enzymes. Phase I metabolism revolves around modification of the parent drug to create reactive metabolites via formation of alcohol and aldehyde groups (Table 1) [14]. While the parent drug itself has some reactivity, these alcohol and aldehyde functional groups are more reactive with proteins, forming adducts with the proteins that can lead to lymphocyte-signaled apoptosis [18]. Additionally, ROS produced by these functional groups can subsequently damage proteins and DNA via oxidative mechanisms and peroxidize lipids on the cell membrane [19]. These mechanisms can signal caspase-mediated apoptosis and necrosis, respectively [20].

Polymorphisms in phase I DMEs are one of the better characterized mechanisms that are responsible for idiosyncratic reactions. The majority of the proteins that are classified as phase I DMEs are part of the cytochrome P450 oxidase (CYP450) family. The mechanism of metabolism of CYP450 isoforms utilizes a heme to stabilize the intermediate state along with adjacent residues [21]. Each of the different isoforms of CYP450 present in hepatocytes is capable of drug metabolism, but with different specificities for the drugs. There are several isoforms of CYP450 that are clinically relevant in idiosyncratic reactions. Among these isoforms are CYP450 1A2 (CYP1A2), CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 [22, 23]. In addition to CYP450 enzymes, flavin monooxygenase (FMO), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH), monoamine oxidase (MAO), and several peroxidases are involved in phase I drug metabolism [14]. While all of these enzymes play roles in drug metabolism, the majority of incidences of ADRs are due to mutations in the phase I DME genes which result in different polymorphisms of the affected proteins that have altered activity of drug metabolism [22, 24].

For example, CYP1A2 metabolizes many drugs, including caffeine, clozapine, and fluvoxamine [22]. In addition, CYP1A2 assists in the metabolism of bilirubin and several hormones [25]. Two polymorphisms of CYP1A2 have been found to affect metabolism. The CYP1A2*1C polymorph is found to decrease caffeine demethylation, while the *1F polymorph is found to increase demethylation [22, 25].

The CYP2 family contains six enzymes with clinically relevant polymorphisms. CYP2B6 metabolizes methadone, cyclophosphamide, and nevirapine [26]. The most common variant of this DME is the *6 allele, which is present in 15–60% of the population (variation is based on ethnicity and race) [22, 27]. This allele and the *18 allele both show decreased activity compared to the wild type 2B6 allele [22, 27]. CYP2C8 mediates the modification of several anticancer, antidiabetic, and antimalarial drugs, including paclitaxel, troglitazone, and amodiaquine [28]. In CYP2C8, the *2 form and *3 form both impact paclitaxel clearance and turnover [29, 30]. CYP2C9 metabolizes an even broader range of drugs than CYP2C8, and several polymorphisms are known to either reduce or eliminate enzyme activity [22, 31]. CYP2C19 metabolizes proton pump inhibitors and several antidepressants [22, 24]. All of the mutant forms, excluding *17, completely eliminate the activity of the enzyme [32]. Ironically, the CYP2C19*17 form increases the metabolism of omeprazole, while eliminating the metabolism of the same drugs targeted by wild type CYP2C19 [22, 32]. CYP2D6 metabolizes about 25% of all known drugs, including tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and tamoxifen [22, 33]. There are over one hundred different variants of CYP2D6, and all of the clinically relevant variations show decreased or lack of metabolism by CYP2D6 [33, 34]. Finally, CYP2E1 metabolizes a variety of different drugs, including antitubercular compounds, alcohol, and anesthetics [35, 36]. However, there are mutant genotypes of CYP2E1 that have reduced metabolism of antitubercular drugs [22, 35, 37].

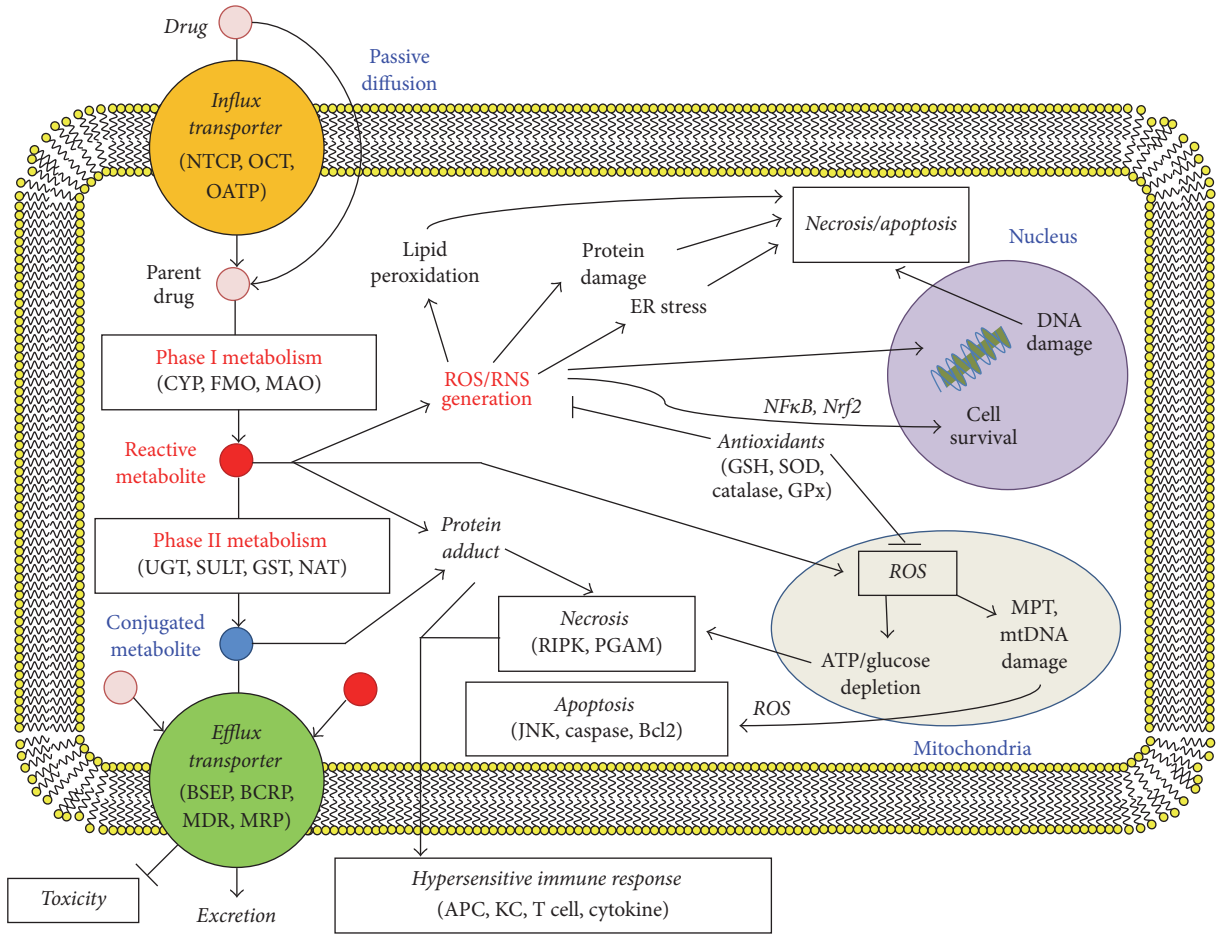

Figure 1: Simplified mechanisms of drug metabolism in liver cells with potential pathways towards toxicity. Abbreviations are used as follows: Na^+-taurocholate cotransporting polypeptide (NTCP), organic cation transporter (OCT), organic anion transporting polypeptide (OATP), bile salt export pump (BSEP), breast cancer resistance protein (BCRP), multidrug resistance protein (MDR), multidrug resistance-associated protein (MRP), cytochrome P450 (CYP), flavin-containing monooxygenase (FMO), monoamine oxidase (MAO), UDP-glucuronosyltransferases (UGT), sulfotransferase (SULT), glutathione S-transferase (GST), N-acetyl transferase (NAT), reactive oxygen species (ROS), reactive nitrogen species (RNS), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), nuclear factor erythroid 2-related factor 2 (Nrf2), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), mitochondrial pore transition (MPT), mitochondrial DNA (mtDNA), receptor-interacting serine/threonine protein kinase (RIPK), phosphoglycerate mutase (PGAM), c-Jun N-terminal kinase (JNK), B-cell lymphoma 2 (Bcl2), antigen-presenting cell (APC), and Kupffer cell (KC) [12].

The CYP3A family of CYP450s are the most abundant CYP450 isoforms, metabolizing close to sixty percent of known drugs [38]. Of these, CYP3A4 metabolizes the most drugs, though it has some overlapping activities with CYP3A5 and CYP3A7 [22, 38]. All of the polymorphisms that affect CYP3A4 and CYP3A5 activity either severely reduce or eliminate the activity, while the only known polymorphisms that affect CYP3A7 appear to increase the enzyme's activity [22, 38, 39]. Interestingly, most individuals do not carry a functional CYP3A5, but when it is present, it is responsible for one-third of the CYP3A activity [22, 38, 39].

The other major class of phase I DMEs that have polymorphisms that create susceptibility to ADRs is flavin monooxygenase (FMO). FMO catalyzes the oxygenation of many compounds, particularly at nitrogen sites [40]. FMO3 is both the most abundant of all the isoforms of FMO and the isoform with the most known clinically relevant polymorphisms [40]. Loss of function of FMO3 results in trimethylaminuria, a condition in which the body cannot break down trimethylamine, leading the individual to develop a naturally "fish-smelling" body odor and leaving the individual more susceptible to liver injury [41].

The susceptibility to IDILI due to dysfunction of phase I drug metabolism stems heavily from race. The *1C variant of CYP1A2 is more commonly found in Japanese populations, while the *1F variant appears more frequently in Caucasians [25]. 2B6 allelic expression is heavily tied to race, with the *6 variant and *18 variants most commonly expressed in New Guineans and Japanese, respectively [22, 42]. The *2 allele of CYP2C8 is more common in African populations, while the *3 allele is more common in Caucasian populations [43]. In CYP2C9, the *2 and *3 alleles are more common in Caucasian populations, while the majority of other mutations that reduce CYP2C9 activity are most commonly found in

Table 1: Examples of drug metabolizing enzymes (DMEs) [17].

Classification	Enzymes	Overall reactions
Oxidative DMEs (Phase I reactions)	Cytochrome P450 (CYP450)	*Carbon oxidation* $RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$
	Flavin-containing monooxygenase (FMO)	*N (or S) oxidation* $R\text{-}NH\text{-}R' + O_2 + NADPH + H^+ \rightarrow R\text{-}NOH\text{-}R' + H_2O + NADP^+$
	Monoamine oxidase (MAO)	*Oxidative deamination* $R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + H_2O_2 + NH_3$
	Alcohol dehydrogenase	*Alcohol oxidation* $R\text{-}CH_2OH + NAD^+ \rightarrow R\text{-}CHO + NADH + H^+$
	Aldehyde dehydrogenase	*Aldehyde oxidation* $R\text{-}CHO + NAD(P)^+ + H_2O \rightarrow R\text{-}COOH + NAD(P)H + H^+$
	Aldehyde oxidase	*Aldehyde oxidation* $R\text{-}CHO + O_2 + H_2O \rightarrow R\text{-}COOH + H_2O_2$
Conjugative DMEs (Phase II reactions)	UDP-glycosyltransferase (UGT)	*Glucuronidation* R + UDP-glucuronic acid → R-glucuronide + UDP
	Glutathione S-transferase (GST)	*Glutathione conjugation* $R + GSH \rightarrow GS\text{-}R$ $R\text{-}X + GSH \rightarrow GS\text{-}R + HX$
	Sulfotransferase (SULT)	*Sulfation* $R\text{-}XH + PAPS \rightarrow R\text{-}SO_4 + \text{phosphoadenosine} + H^+$
	N-Acetyltransferase (NAT)	*Methylation* $R\text{-}NH_2 + CoA\text{-}S\text{-}COCH_3 \rightarrow R\text{-}NCOCH_3 + CoA\text{-}SH$ $R\text{-}NHOH + CoA\text{-}S\text{-}COCH_3 \rightarrow R\text{-}NHOCOCH_3 + CoA\text{-}SH$

Xenobiotics (R), β-nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide (NAD), uridine 5′-diphosphate (UDP), glutathione (GSH), 3′-phosphoadenosine 5′-phosphosulfate (PAPS), and coenzyme A (CoA).

African populations [43, 44]. All of the clinically relevant polymorphisms of CYP2C19 have varying frequency that depends on race, with the *2 form being the most prevalent of the polymorphisms that is found in Caucasians [22]. In the CYP3A family, CYP3A4 does not have significant polymorphisms that are race dependent, but wild type CYP3A5 is more commonly found in African descent and less in Caucasian descent [22, 45]. Additionally, some European populations have a 20% incidence of a double null mutation in FMO3 [22].

2.2. Phase II Drug Metabolizing Enzymes. Phase II DMEs modify the drugs following phase I DME modification of the parent drug. The action of phase II DMEs is to replace the reactive aldehyde and alcohol functional groups with less reactive and larger functional groups to direct the drug towards clearance from the body. Phase II DMEs broadly consist of uridine 5′-diphospho-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), and N-acetyltransferases (NATs). All of the phase II DMEs utilize cofactors to conjugate to reactive metabolites to make ready for clearance. While the parent drug and drugs metabolized by phase I and phase II DMEs can all be cleared, clearance of phase II metabolized drugs provides the most stable form of the drug and has a general tendency to lead away from cholestasis and drug-induced liver injury (DILI) [21, 22].

UGTs represent the most diverse class of phase II DMEs. UGTs utilize uridine diphosphate glucuronic acid (UDP-GA) as a cofactor for glucuronidation [46]. Glucuronides are more stable than the reactive intermediates created by phase I DMEs but are significantly less stable than drugs metabolized by other phase II enzymes, particularly reactive acyl glucuronides [46]. There are twenty-four known isoforms of UGT: nine belong to the UGT1 family and fifteen belong to the UGT2 family [46]. Of all of the UGT isoforms, UGT1A1 has the most clinically relevant polymorphisms, owing to the fact that UGT1A1 metabolizes vast classes of chemotherapeutics and bilirubin [47]. The UGT1A1*6, *28, *33, and *34 mutations all result in decreased glucuronidation [48]. Having the UGT1A1*28 variant results in Gilbert's syndrome and UGT1A1*33 or UGT1A1*34 results in Crigler-Najjar syndrome, both of which are characterized by hyperbilirubinemia [22, 48]. Certain polymorphisms in UGT2B7 have potentially led to susceptibility to diclofenac induced liver injury, though no other UGTs polymorphisms have been found to lead to IDILI [49].

SULTs are found in the cytosol and perform a sulfation reaction using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as a cofactor [50]. There are twelve known human SULT enzymes, with SULT1 and SULT2 existing as the most common SULT families in humans [51]. SULT1A1 and SULT1A2 have the greatest number of clinically significant polymorphisms of any of the SULTs [51, 52]. SULT1A1 sulfonates 4-hydroxy-tamoxifen, which actually increases the efficacy of individuals carrying the wild type SULT1A1*1 form [52]. This is decreased with individuals carrying the SULT1A1*2 form [52].

GSTs have several distinct polymorphisms that carry the potential for idiosyncratic hepatotoxicity. GST conjugates

glutathione to drugs for easy clearance [53]. This family of phase II DMEs consists of seven subfamilies denoted by the drugs that they metabolize [54]. The GSTT1 and GSTM1 double null phenotype has been found to lead to troglitazone induced liver injury [55]. GSTP1 is a common metabolizing enzyme of chemotherapeutics, and several polymorphisms have been associated with increased risk of leukemia or susceptibility to chemotherapeutic injury [22].

Humans contain two isoforms of NAT: NAT1 and NAT2 [56]. Both isoforms of NAT function to acetylate parent drugs and reactive metabolites using acetyl coenzyme A (CoA) as a cofactor [56]. Substrates for NAT1 include p-aminobenzoic acid (PABA) and p-aminosalicylic acid (PAS), while substrates for NAT2 include isoniazid, hydralazine, and sulfonamides [22]. While there are approximately twenty-five known polymorphisms of NAT, mutations in NAT2 appear to have a more dramatic effect on acetylation reactions [22]. Slow acetylation has been observed in the NAT1*14 and *17 phenotypes, but isoniazid toxicity has been observed in people with null phenotypes of NAT2, and increased toxicity potential towards amonafide was observed in higher NAT2 activity [22].

Incidents of polymorphisms of phase II DMEs have been found to be race dependent. Slow acetylation from NAT2 is found to occur in about half of Caucasians, but only 10% of Japanese [57, 58]. Incidences of polymorphisms in SULT2A1 are significantly higher in people of African descent, but these polymorphisms have not been found to have clinical relevance [45]. Additionally, SULT1A1 polymorphisms that affect activity are found to be represented in different ethnic groups at different frequencies [52]. UGT1A1*6 is common in people of Asian descent, while 1/3 of Caucasians and a significant portion of people of African descent are carriers of the UGT1A1*28 gene [48, 59]. Additionally, the wild type variant of GSTP1 is present in 60–90% of the population; a statistic that is race dependent [53].

2.3. Antioxidant Enzymes. Antioxidant enzymes play a key role in the prevention of cellular injury by reactive ROS (Figure 2). ROS that exist inside the cell include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) among others [60, 61]. A cell's defense to oxidative injury includes the abundance of glutathione (GSH), as well as antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), peroxiredoxin (Prx), and thioredoxin (Trx) (Figure 2) [62, 63]. GSH, as a relatively small peptide, can rapidly react with ROS to protect the cell from oxidative injury. However, once approximately 90% of the GSH is depleted within a cell, its susceptibility to oxidative death increases significantly [64, 65]. Thus, the roles of these enzymes in the reduction of ROS are critical to cell survival of oxidative stress.

The antioxidant enzymes within the hepatocytes will generally exist in the mitochondria or the cytosol. However, many of the effects of ROS and the malfunctions of antioxidant enzymes can be observed in mitochondrial behavior. During a hepatotoxic event, mitochondria undergo an event known as mitochondria permeability transition (MPT) [15]. In MPT, stress causes mitochondria pores to swell, allowing passage of solutes of up to 1500 Da, including ROS, Ca^{2+} efflux, and the influx of potentially reactive metabolites [15]. Subsequent events of this include decreased levels oxidative phosphorylation, mitochondrial depolarization, swelling, and subsequent death of the cell by either apoptosis via cytochrome c release or necrosis via ATP depletion [66–68]. Both mechanisms of cell death have been observed to be caused by various hepatotoxicants. Though necrosis is generally found in more cases of DILI, apoptosis is heavily tied with the mechanisms related to immune mediated hepatotoxicity as the role of cytokines in inflammation can exacerbate much of the oxidative stress occurring within the cell [69, 70]. By mitigating ROS, antioxidant enzymes can decrease the frequency of this occurrence.

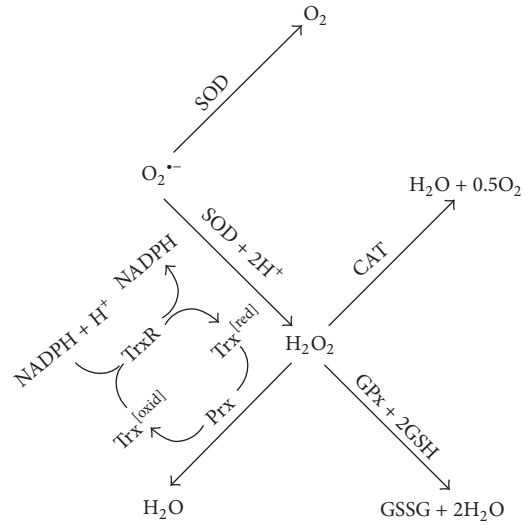

Figure 2: Mechanisms of mediating ROS within the liver. Abbreviations are used as follows: superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (Prx), thioredoxin (Trx), thioredoxin reductase (TrxR), glutathione peroxidase (GPx), glutathione (GSH), and glutathione disulfide (GSSG).

The mechanisms of these antioxidant enzymes serve to either convert more reactive ROS to H_2O_2 or convert all ROS to H_2O. The enzymatic antioxidants in the body all contain a metallic core to stabilize and catalyze the conversion of ROS to H_2O. In the case of SOD, the metals at the core can be copper and zinc or manganese for humans, where CuSOD and ZnSOD are in the cytosol or are extracellular, and MnSOD is in the mitochondria [71]. SOD generally converts superoxide anion and water to O_2 and hydrogen peroxide [71]. Humans contain three isoforms of SOD: SOD1, SOD2, and SOD3. SOD1 is a dimer located in the cytosol, SOD2 is a tetramer located in the mitochondria, and SOD3 is an extracellular tetramer [71]. SOD1 and SOD2 are the more clinically relevant isoforms in regard to idiosyncratic hepatotoxicity since SOD3 is extracellular and affects all organs. Specifically, organisms homozygous for a deletion in SOD2 die due to oxidative damage of the liver, while those heterozygous for functional SOD2 are still at risk for injury [62]. Additionally, people who are heterozygous for

either SOD1 or SOD2 deletions are at increased risk for hepatocellular carcinoma [62].

GPx is another enzyme that plays an important role in mitigating oxidative stress, but if deleted may also make an individual more susceptible to hepatotoxicity. GPx serves to catalyze conversion of hydrogen peroxide to water by creating a sulfide bond between two GSH molecules, creating glutathione disulfide (GSSG) [72]. This enzyme works in conjunction with glutathione reductase (GR), which reduces GSSG to GSH via oxidation of NADPH [72, 73]. GPx uses a selenium core to assist in the catalysis. There are eight known isoforms of GPx (denoted GPx1–8), but the two isoforms most involved in hepatoprotection are GPx1 and GPx4 [62, 73]. GPx1 exists in the cytoplasm and mitochondria, while GPx4 is uniformly distributed throughout the cell [62]. Besides converting peroxides to water, GPx also functions in removing peroxides from lipids. Most deletions in GPx have a greater impact on lipid peroxidation than in standard DILI, and fatty liver disease is present in individuals with less functional GPx. Mutations in GPx1 have been shown to potentially induce cholestasis and toxicity due to drug accumulation in the bile [62]. GPx4 mutations generally affect bone tissue more significantly, but there are impacts to liver tissue as well [62].

Among other antioxidant enzymes of note are CAT, Prx, and Trx. CAT is a heme-based tetramer protein that catalyzes the conversion of hydrogen peroxide to water within the peroxisome of the cell [74]. While CAT does play an important role in reducing ROS, deficiencies in CAT do not have correlation with increased risk of idiosyncratic hepatotoxicity, nor is it considered to put an individual at risk for other diseases [62].

Unlike the aforementioned enzymes, Prx, Trx, and thioredoxin reductase (TrxR) do not catalyze the reduction of ROS using metals. Instead, the mechanism relies on interactions with sulfide bonds in cysteine residues on Prx [75]. Prx and Trx work in conjunction with each other, where Prx converts hydrogen peroxide to water. Then, Trx reduces the oxidized form of Prx, and Trx is itself reduced by thioredoxin reductase (TrxR), coupling the oxidation of NADPH to NADP [75]. There are six isoforms of Prx that can be localized to the mitochondria, cytosol, peroxisome, or extracellular space. Deletions in Prx isoforms do not seem to affect hepatotoxicity, though circulating erythrocytes are affected by mutations in Prx. Deletions in any of the genes that code for Trx are lethal, and studies have shown that mice with elevated levels of Trx live longer. Additionally, a recent study has suggested that deletions in genes coding for TrxR1 show to put organisms at greater risk of DILI [76].

The most prevalent cause for susceptibility to ADRs due to problems with antioxidant enzymes is age. The suspected reason for this age related susceptibility is the general decreased ability of cells to deal with oxidative stress. The mitochondria itself is more likely to break down and functions with antioxidant enzymes in the mitochondria, including SOD2 and GPx1 [62]. Additionally, evidence suggests that higher levels of antioxidant enzymes can slow down the body from physiologically aging. This decreased level of antioxidant activity upon aging is a reason suspected for why older patients are at greater risk for experiencing ADRs. In addition to age, postmenopausal females are at a higher risk for DILI than premenopausal females and males that are older than fifty [62]. The hypothesis behind this is that higher levels of muscle tone (particularly lean muscle) promote antioxidant activity. While muscle does degrade with age, women are more susceptible to this injury due to lower initial muscle tone.

2.4. Hepatic Transporters. Drug transporter proteins are crucial for clearance of reactive metabolites in the liver, kidney, intestine, and brain (Figure 3) [77]. Hepatic transporters can be classified by mechanism of action and by location on the hepatocyte cell membrane. Transport of drugs into or out of the cell can be governed by the ATP-binding cassette (ABC) transporter family of proteins or the solute carrier (SLC) family of proteins [13]. Generally, SLC proteins are considered to be influx transporters [78], moving drugs from the plasma into the cell. ABC transporters are efflux transporters, moving metabolized drugs from within the cell into the bile [79–81].

The transporter families of greatest clinical relevance within the hepatocyte include organic anion transporting polypeptides (OATPs), organic cation transporters (OCTs), multidrug resistance proteins (MDRs), breast cancer resistance protein (BCRP), the bile salt export pump (BSEP), and multidrug resistance-associated proteins (MRPs) [82, 83]. The OCTs and the OATPs are part of the SLC class of transporter proteins, relying on coupled cation/anion transport, as a mechanism of facilitated diffusion down a concentration gradient [82]. Generally, these proteins are characterized as having twelve transmembrane hydrophobic domains that stabilize its structure within the cell membrane. Both classes of proteins function to carry drugs into the cell [14]. While OCT1 is an important liver influx transporter, it is also found within the intestine, so idiosyncratic reactions can occur in both organs [13, 24]. The OATP1B subfamily of proteins (specifically OATP1B1 and OATP1B3) is strictly found in the liver, making it an ideal protein to study for strict hepatotoxicity. Additionally, the Na^+-taurocholate transporting peptide (NTCP) is another SLC protein that plays a role in influx transport that is localized to the liver [77, 84]. Of all the influx transporters, OATP1B and NTCP are found to have the greatest effect on drug transport [77, 84].

Idiosyncratic reactions are not significantly associated with the SLC transporters. However, OCT1 and OATP1B1 are both found to have clinically relevant polymorphisms. People with defective OCT1 have trouble transporting cationic substrates of metformin [85], while mutations in OATP1B1 have been shown to cause increases in the accumulation of sulfoconjugated troglitazone [13, 24, 86]. Additionally, OATP1B1 mutations have been implicated with increased susceptibility to hyperbilirubinemia [87].

MDRs, BCRP, BSEP, and MRPs are all efflux transporters of the ABC family with clinical relevance in hepatotoxicity. MDR1 (also known as P-glycoprotein) transports a significant amount of xenobiotics and biological compounds into the bile [88–90]. While MDR1 can be found in many tissues, it also has many substrates, making it an important efflux transporter [13]. Additionally, MDR3 is strictly found in

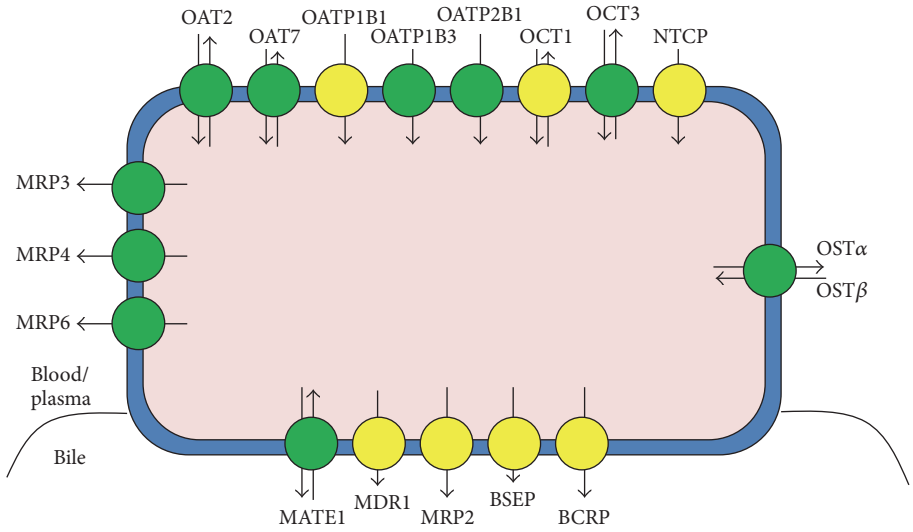

Figure 3: Transporter mechanisms on the cell membranes of hepatocytes. Proteins that are labeled yellow have known clinically relevant polymorphisms, while green labeled proteins do not have known clinically relevant polymorphisms. Abbreviations are used as follows: organic cation transporter (OCT), organic anion transporter (OAT), organic anion transporting polypeptide (OATP), Na^+-taurocholate cotransporting polypeptide (NTCP), breast cancer resistance protein (BCRP), bile salt export pump (BSEP), multidrug resistance protein (MDR), multidrug resistance-associated protein (MRP), organic solute transporter (OST), and multidrug and toxin extrusion protein (MATE) [13].

hepatocytes, working with ATP8A1 to regulate the transport of phospholipids across the cell membrane [91]. BCRP plays roles in both porphyrin transport and secretion of vitamins in breast milk [92]. While this protein is also found in many cell types, porphyrin release is the most common function found in hepatocytes [92, 93]. BSEP is a protein localized to hepatocytes that functions in the transport of bile salts into the bile canaliculi [94]. As a result, BSEP is an important regulatory in bile flow. Finally, MRP2, 3, 4, and 6 individually act as efflux transporters of hepatocytes, but only MRP2 conducts xenobiotics into the bile [77].

While mutations can occur in both families of transporters, the mutations affecting ABC transporters are considered to be more clinically relevant. Mutations in ABC transporters mean that metabolized drugs cannot leave the hepatocytes, leading to impaired canalicular bile flow. This generally manifests in the form of cholestasis and fatty liver disease [94, 95]. MDR1, BSEP, BCRP, and MRP2 all have polymorphisms that have been associated with idiosyncratic ADRs. Mutations in MDR1 have been implicated in efflux transport of verapamil [89]. Altered expression of BCRP has affected patients taking anticancer drugs or weight loss drugs, including gefitinib, irinotecan, topotecan, and diflomotecan [24, 96]. Mutations in BSEP have been implicated in early onset hepatocellular carcinoma, along with an increase in susceptibility to cholestatic injury from carbocyclic compounds with aromatic rings [97]. Generally, inhibition of BSEP has been correlated with the incidence of cholestatic liver disease [98, 99]. MRP2 mutations can also result in increased susceptibility to hyperbilirubinemia, as well as increased susceptibility to injury due to methotrexate [24, 100] and pravastatin [24, 101].

Age and race both play a significant role in the risk for ADRs from variations in the expression of transporter genes. Increasing age can reduce the expression of several efflux transporter proteins. In particular, mRNA levels of MDR1 are decreased in elderly patients. The expression of MDR1 is generally heterogeneous, and this expression becomes even more pronounced with age [102, 103]. Additionally, OATP1B1 has about fourteen known mutations that have been found to exist within individual populations of people from either European, African, or Asian descent [13]. Frequent polymorphisms of OATP1B1 found in Caucasians include OATP1B1*1b and OATP1B1*4, but mutations resulting in OATP1B1p.L193R show decreased transport compared to the other forms of OATP1B1 [13]. Individuals with the OATP1B1*15 haplotype, which is found in Japanese people, have general reduced transport activity of OATP1B1 [13, 92]. MRP2 mutations found in Korean people have also been found to increase the susceptibility to IDILI by herbal medicines [24].

2.5. Immunological Mechanisms. Immunological mechanisms towards drug reactions represent a diverse set of idiosyncratic reactions. Livers have a resident set of macrophages called Kupffer Cells (KCs), which initiate inflammation in the liver after drug exposure [61, 104]. Additionally, the liver is permeated with small populations of other lymphocytes, including other macrophages, T cells, B cells, and natural killer (NK) cells [3, 16, 70]. Owing to the liver's role in detoxification, these cells are necessary for developing foreign body responses.

During drug metabolism, damaged proteins may be expressed on the surface of hepatocytes. Lymphocytes can

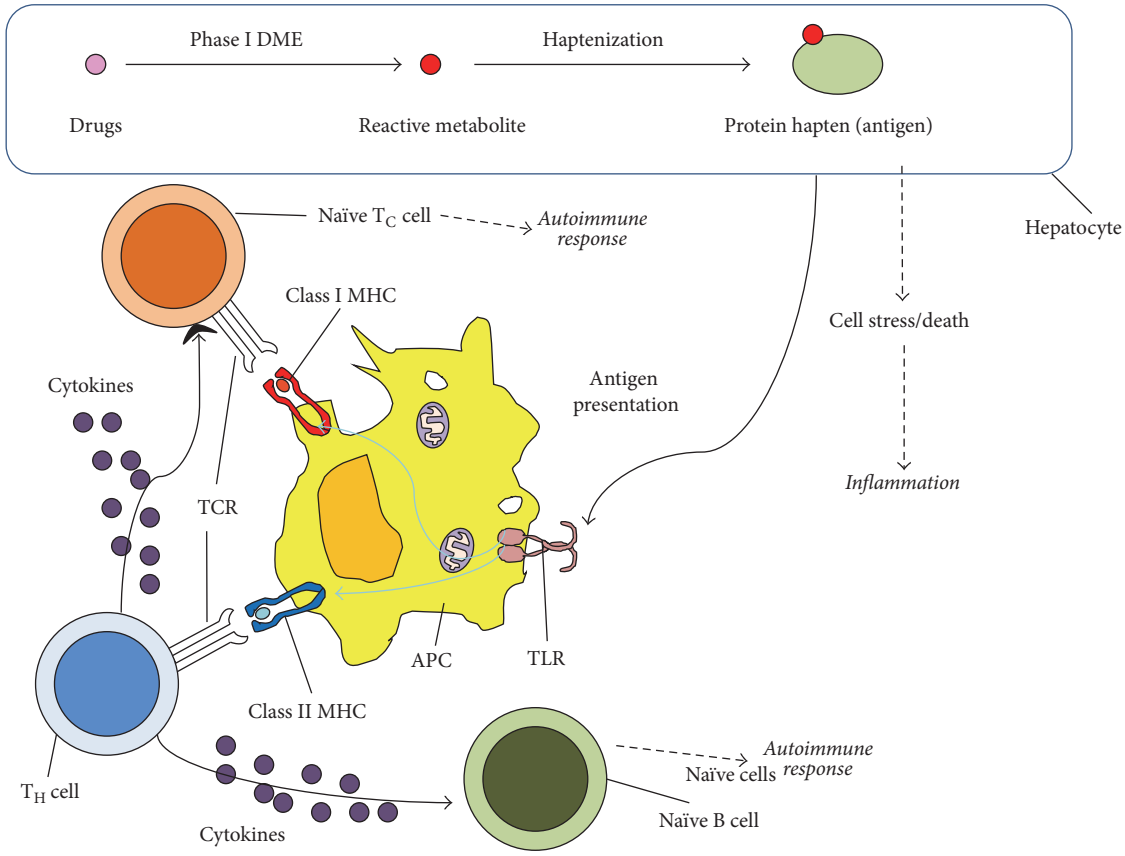

Figure 4: Simplified mechanisms of inflammation signaling caused by adverse drug reactions (ADRs) in the liver. Abbreviations are used as follows: toll-like receptor (TLR), major histocompatibility complex (MHC), T cell receptor (TCR), drug metabolizing enzymes (DMEs), antigen-presenting cell (APC), cytotoxic T lymphocyte (T_C cell), and helper T lymphocyte (T_H cell).

initiate and regulate inflammation by recognizing drugs and hepatocytes that have metabolized drugs as foreign bodies, either via recognizing the drug in solution or by recognizing damaged proteins on the surface of hepatocytes [61, 105] (Figure 4). During long-term drug exposure, a healthy liver may experience some inflammation that subsides with time. However, ADRs can manifest with significant inflammation in the liver, leading to drug-induced autoimmune hepatitis (DIAIH) or acute liver toxicity [70, 106]. Generally, this mechanism has been considered to be the "failure-to-adapt" model [10]. This model assumes that injury occurs after long-term exposure to drugs and is regulated by the levels of cytokines present during the adverse reaction.

IDILI associated with the failure-to-adapt model assumes the dysfunction in the expression of cytokines. In standard inflammation, a subset of macrophages called M1 macrophages (or, in the case of the liver, KCs) phagocytoses antigens derived from dead or dying cells called damaged-associate molecular patterns (DAMPs) before presentation on major histocompatibility complex (MHC) [107]. In addition to KCs, circulating macrophages, dendritic cells (DCs) neutrophils, and subsets of B and T cells with innate activity can respond to the presence of antigen [107]. Macrophages can present on MHC I or MHC II, which will either lead to the initiation of a cellular immune response by the CD8+ T cytotoxic (T_C) cells, or a humoral immune response by T helper (T_H) cells [107]. T_H1 cells, a variant of T helper cells, also help activate T_C cells in a delayed hypersensitivity response via secretion of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) [108]. Another subset, T_H17 cells, promotes inflammation by secreting interleukin 17 (IL-17) cytokines [109]. In a healthy individual, this inflammation subsides via activity of T regulatory (T_{reg}) cells, another T_H cell subset that secretes regulatory cytokines, including IL-10 and transforming growth factor beta (TGF-β) [108].

The deregulation of cytokines is a potential mechanism for IDILI. In inflammation-based liver injury, the excess of proinflammatory cytokines and the reduction of regulatory cytokines cause general cell death within the liver rather than targeting the liver cells that express damaged motifs [110, 111]. Inducing expression of inflammatory cytokines IL-1α and the use of anti-TNF-α antibodies increase the susceptibility to DILI [108]. IL-1α is proinflammatory, whereas TNF-α is regulatory, so the absence of antibodies against TNF-α actually increases the chance for inflammation. In addition, IFN-γ and IL-1α both appear to induce inflammation during acetaminophen-induced liver injury [104, 108]. It has also been found that mutations in anti-inflammatory cytokines IL-4 and IL-10 can increase the risk of diclofenac induced

liver injury in humans [112, 113]. While individual mutations can increase susceptibility to injury, double mutations provide an even greater risk to IDILI [11, 114]. Additionally, the source of cytokine deregulation is debated, as mutations can either affect master transcriptional regulators [115] or the actual cytokine [116]. This inflammation deregulation can be found in nonsteroidal anti-inflammatory drugs (NSAIDs) [116, 117] and antibiotics [69, 118].

While KCs are the predominant lymphocyte in the liver, there is a belief that deregulation often stems from defects in T_H17 cells, leading to autoimmune responses [109]. T_H17 deregulation has been implicated in other autoimmune diseases, including juvenile diabetes, Crohn's disease, multiple sclerosis, and rheumatoid arthritis [109]. It has been found that levels of proinflammatory cytokines IL-17 and IL-22 are elevated in the presence of penicillamine [114, 119]. In addition, the level of circulating T_H17 cells is also increased, corresponding with the increases in IL-17 and IL-22 [114]. T_H17 cell production is induced by IL-6 secretion of macrophages, which is pleiotropic [109]. Thus, there is significant interplay between the types of cells in the immune system and the potential for idiosyncratic drug reactions.

Another potential mechanism of IDILI is that human leukocyte antigen (HLA) haplotype will affect the recognition of the immune system of cells presenting damaged motifs. HLA codes for the MHC in humans. In adults, the HLA-A, HLA-B, and HLA-C genes govern the structure of MHC I, while HLA-DP, HLA-DQ, and HLA-DR govern the structure of MHC II [120]. While there is one gene for each of the MHC I coding regions, there are eight genes responsible for MHC II [120]. Individually, this leaves 1000–2000 different allotypes for each of the HLA-A, -B, and -C genes and 2–860 allotypes for each of the eight -D genes [120]. This yields a total of 1.7 billion haplotypes for MHC I and 10^{15} haplotypes for MHC II.

Generally, HLA polymorphisms associated with increased susceptibility to IDILI are found on the MHC II locus. This means that signaling from CD4+ T_H cells is directly affected, as these cells have direct interaction with MHC II. Additionally, because of the significant role of T_H cells in regulatory signaling, there are effects seen in the behavior of other lymphocytes, including T_C cells and KCs. The majority of known polymorphisms associated with HLA are found on HLA-DRB1, though there is a significant presence of polymorphisms affecting HLA-DQ as well [24, 121, 122]. While most of these polymorphisms are associated with increased disposition towards cholestatic liver injury, there are at least three known polymorphisms associated with increased toxicity towards amoxicillin [121, 122]. There are a set of mutations in HLA-DQ that predispose towards lumiracoxib-related liver injury [123]. Additionally, HLA-B has at least one known polymorphism to increase susceptibility to flucloxacillin [124, 125], and others that are more predisposed towards antiepileptic drugs [111]. Owing to the fact that HLA-DQ and HLA-DR polymorphisms code for polymorphisms in MHC II, it is possible that many of the deregulated cytokines are as a result of indirect effects from MHC II with altered functionality.

IADR predisposition via possible immune-based mechanisms can be found as a function of gender, age, race, and current immune state. Often, those that are immune compromised, such as people with autoimmune illnesses affecting inflammation will be more at risk for IDILI due to the dysfunction of cells that govern both mechanisms. It has been found that women with breast cancer with the HLA-DQA1*02:01 are more at risk to develop an IADR to certain breast cancer therapeutics than women with breast cancer with a different HLA haplotype [126]. Japanese are more at risk for HLA-mediated ticlopidine IDILI [127]. Additionally, age plays a significant role in cytokine-mediated ADRs, as DNA is more susceptible to mutation and autoimmune diseases inducing inflammation become overly prevalent [106, 109, 114].

3. Assays Detecting Susceptibility for Idiosyncratic Reactions

In clinical trials and in vivo tests, the assessment of ADR potential is developed by use of measuring biomarkers alanine transferase (ALT), aspartate transaminase (AST), and bilirubin [128, 129]. ALT and AST are general biomarkers for protein catabolism, while bilirubin is an indicator of cholestasis and bile flow. While other biomarkers have been developed to assess liver behavior, the combination of elevated ALT, AST, and bilirubin levels is the general indicator for potential of liver injury. The rule for assessing if DILI will occur was developed by Hy Zimmerman: it states that liver injury is likely to occur when an organism is exposed to drugs exhibits five times the upper limit of normal (ULN) for ALT activity, 3 × ULN for AST, and 2 × ULN for total bilirubin (TBL) found in serum [130]. While positively identifying that the markers for Hy's Law correlate with increased risk for DILI, the absence of these markers does not rule out liver injury [128]. Additionally, it is possible that the combination of factors may not even result in injury to the liver. Thus, reliance on other markers to predict and detect injury is necessary for proper drug development.

Developing assays for detecting the potential for IDILI relies on screening individual events. While conventional assays may characterize drug metabolite formation and the general potential for ADRs; very little emphasis has been put on detecting the potential for IADRs. Assays for detecting inflammation in vivo and CYP450 activity assays in vitro have been developed for clinical and industrial use, but very few assays have been developed to predict ADRs in individuals with polymorphisms in transporters, phase II DMEs, or antioxidant enzymes. Here, we will describe assays that can be used for detecting IADRs, with emphasis on in vitro systems, preferably ones that can be used for high-throughput screening (HCS) of potentially toxic compounds (see Table 2).

3.1. CYP450 Activity Assays. Measuring individual enzyme activity can provide information on potential mutation of DMEs indirectly. Coumarin-based substrates have been used to determine the activity of various CYP450 isoforms

Table 2: Assays used for determining IDILI potential.

Mechanisms	Proteins	Relevant assays
Phase I DMEs	CYP450	Coumarin metabolism Fluorescein (CYP2C9) Human liver microsomes (HLMs) High pressure liquid chromatography (HPLC)/Mass spectrometry (MS)
Phase II DMEs	UGT	Coumarin metabolism
	GST (GSTT1 and GSTM1)	CDNB, NBC, DCNB, EPNP, DCM, PBO GSTT1/GSTM1 single and double knockouts
	UGT/GST/NAT/SULT	Covalent binding level (CBL) HPLC/MS
Antioxidant enzymes	GPx1/SOD2	Monochlorobimane (mBCl) Oxygen consumption rate (OCR) Fluorescein Tetramethyl rhodamine (TMRM) Cytochrome c release $SOD2^{+/-}$ mice
Transporters	BSEP	Vesicular transport assay (VTA) ATPase assay
	MRP2	Fluorescein
	MDR1/MRP2	Flow cytometry
	OCT	OCT1 and OCT2 knockouts
	BSEP/BCRP/MDR/MRP/OATP/OCT/NTCP	HPLC/MS Drug uptake assays
Inflammation	TNFR1/IFN-γ/TLR9/IL-1/IL-4/IL-6/IL-10/IL-13	LPS-induced inflammation Lymphocyte transformation test (LTT) Cytokine production Cytokine knockouts
	CD69	Flow cytometry
HLA	MHC I/MHC II	Flow cytometry

Cytochrome P450 (CYP450), uridine 5′-diphosphate (UDP) glycosyltransferase (UGT), glutathione S-transferase (GST), N-acetyl transferase (NAT), sulfotransferase (SULT), glutathione peroxidase (GPx), superoxide dismutase (SOD), 1-chloro-2,4-dinitrobenzene (CDNB), p-nitrobenzyl chloride (NBC), 1,2-chloro-4-nitrobenzene (DCNB), 2-epoxy-3-(p-nitrophenoxy)propane (EPNP), dichloromethane (DCM), trans-4-phenyl-3-buten-2-one (PBO), lipopolysaccharide (LPS), tumor necrosis factor R1 (TNFR1), interferon gamma (IFN-γ), toll-like receptor (TLR), interleukin (IL), cluster of differentiation (CD), major histocompatibility complex class I (MHC I), and class II (MHC II).

(Figure 5). Each isoform can metabolize a different substrate, though there could be some substrate overlap [23, 131]. For example, 7-ethyloxymethyloxy-3-cyanocoumarin (EOMCC) can be metabolized via CYP1A2, CYP2D6, or CYP2E1, along with other CYP450 isoforms. The listed reactions occur via hydroxylation of the ethoxy or methoxy oxygen on the fluorescent compound. Additionally, all of the metabolites excite at 355 nm, but the HFC emits at a different wavelength than the metabolites formed by CYP1A2 and CYP3A4 metabolism, allowing for two CYP activities to be measured in one well. The drawback to these substrates is that if the cell line is capable of significant phase II metabolism, inhibitors need to be added to the solution to properly assess CYP450 activity.

While CYP2C activity might not be modeled by the coumarin fluorogenic substrates, activity can still be measured using conversion of dibenzylfluorescein (DBF) to fluorescein by CYP2C8 and CYP2C9 [23]. DBF requires low concentrations for drug metabolism and generally gives a strong fluorescent signal when CYP2C9 metabolism is present. Additionally, CYP3A4 activity may be quantified using Luciferin-based luminescent reagents [132].

In addition to standard fluorogenic substrate assays, drug metabolites can be pooled from media and quantified using high performance liquid chromatography (HPLC) and mass spectrometry (MS) [133–135]. The advantage of this is that endpoint activity can be quantified along with cell viability to determine if a therapeutic is potentially toxic to individuals or whole populations. However, this system is generally better suited for endpoint therapeutic studies, and there is great financial cost associated with performing HPLC and MS to quantify CYP450 activity.

In addition to recombinant individual DMEs used, human liver microsomes (HLMs), the centrifuged product of lysed liver tissues, are commonly used to measure a desired activity. Generally, HLMs contain a large concentration of CYP450s and some phase II DMEs such as UGTs, and HLMs can be used to measure the expression levels of CYP450s in

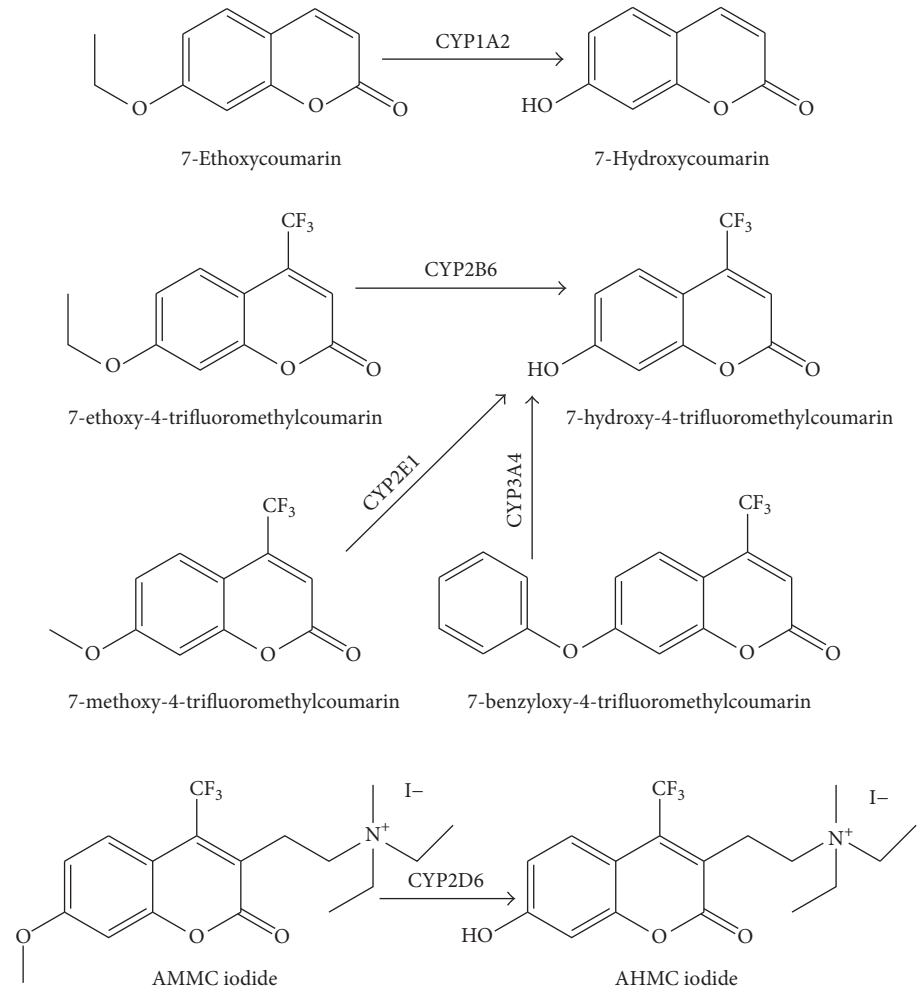

Figure 5: Metabolism of coumarin by CYP450 isoforms. Abbreviations are used as follows: 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin (AMMC), and 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin (AHMC).

the liver tissues from which they were isolated. Walsky and Obach used HLMs to monitor metabolic activity over a range of compounds that can be metabolized by CYP450s [131].

3.2. Phase II DME Assays. Specific protein assays that are relevant to IADRs are targeted towards measuring UGT and GST activity. One way to measure UGT activity is the fluorescent substrate 4-methylumbelliferone (4-MU), which is a coumarin derivative that undergoes glucuronidation [136, 137]. UGT's role in clearance of steroids makes steroidal compounds good substrates to assay for UGT specific activity as well [82]. In the case of GST, levels and activity can be quantified via the removal of chlorine from substrate 1-chloro-2,4-dinitrobenzene (CDNB) [138]. While CDNB can be used as a substrate for total GST activity, *p*-nitrobenzyl chloride (NBC), 1,2-chloro-4-nitrobenzene (DCNB), and trans-4-phenyl-3-buten-2-one (PBO) can be used to detect GSTM activity, and 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP) and dichloromethane (DCM) can be used as substrates for GSTTs [139, 140].

A functional assay can be used to determine idiosyncratic reactions in phase II DMEs in determining the covalent binding level (CBL) [55]. Because drugs that have been only metabolized by phase I DMEs are more reactive than the parent drug or drugs that have been successfully conjugated with bulkier functional groups, computation of CBL can be used to assess either reactive metabolite formation potential or the rate at which the metabolite is not being cleared from the hepatocyte.

Glucuronidation can be quantified using methods such as HPLC and MS [141]. Surendradoss et al. used HPLC and MS to quantify valproyl 1-O-acyl-glucuronide (VPA-G), the phase II metabolized drug of valproic acid [142, 143]. Additionally, diclofenac conjugated to GSH has been measured via HPLC to assay for GST activity [140]. The drawback to using these technologies is that metabolites can only be quantified in relative terms without giving exact values as to the forms of drug present in a solution. This leads metabolite analysis to be performed only for diagnosing an idiosyncratic event rather than determining its severity.

For reactive metabolite counts, GSH trapping can be used to quantify the level of oxidative metabolites before being run through HPLC and MS [144]. Additionally, adduct formation of proteins can also be measured using LC-MS, providing insight towards the mechanism of injury [117, 145].

In in vivo models, the GSTT1 and GSTM1 single-null and double null mutation has been thoroughly studied in regard to troglitazone induced ADRs in mice [35, 55]. These particular models are beneficial because there has been shown an additive effect towards generating IDILI with these responses.

3.3. Mitochondrial Activity Assays. One of the difficulties associated with determining idiosyncratic reactions with antioxidant enzymes is that very few of the assays can target specific protein functions, and they are designed for general ADRs as mitochondrial dysfunction is often a symptom (rather than the cause) of hepatotoxicity. Due to the fact that ADRs blamed on antioxidant toxicity are associated with proteins found in the mitochondrion, it is the primary indicator for liver functionality tests. Certain assays, such as the cupric ion reducing antioxidant capacity (CUPRAC) assay [146] or the MitoTracker dye [147], can rely on calculating general antioxidant capacity within a cell, or confocal microscopy to examine individual mitochondrial behavior. However, general assays are more geared towards calculating antioxidant potential associated with smaller molecules, including ascorbic acid and tocopherol. Additionally, 2′,7′-dichlorodihydrofluorescein (H_2DCF) can be used to measure general oxidative stress [148, 149]. However, this too is not specific for idiosyncratic mechanisms.

Mitochondrial dysfunction is a considerable target for drug toxicity screens. For general mitochondrial toxicity assays, measuring mitochondrial membrane potential (MMP) via fluorescent compound tetramethyl rhodamine (TMRM) can be used to quantify apoptosis as MMP depolarization is symptomatic of the issue [148]. Besides MMP, cytochrome c release and variation in the oxygen consumption rate (OCR), which is the turnover of O_2 during metabolism, may be symptomatic of mitotoxicity [150–153]. MitoTox has been used as a phosphorescent probe for the general measurement of OCR [151–153]. Additionally, Liang et al. managed to purify mitochondria and assay for GPx activity, though the method is not durable for high-throughput applications [138].

Owing to the role of mitochondria in reduction of ROS, antioxidant GSH is an adequate substrate for assessing mitotoxicity. One commonly used compound, monochlorobimane (mBCl), is fluorogenic in the presence of glutathione [154]. This substrate reacts with thiol based compounds, so fluorescence can correlate well with substrate depletion [154, 155]. While GSH acts as a general indicator for oxidative stress, this can be sufficient enough to indicate dysfunction with antioxidant enzymes, or even the general presence of an IADR. Other protocols are able to discriminate between GSH and GSSG based on the GS-GPx oxidation-reduction mechanism [101, 156]. The protocol however is reagent intensive and is not compatible with high-throughput drug screening.

One of the more common mutations studied is a heterozygous SOD2 mouse [117, 154]. A homozygous knockout for SOD2 is lethal to the organism due to SOD2 being the major enzyme responsible for removal of oxidant sources in the cell. Yet, a heterozygous expression of normal SOD2 results in a viable mouse with a decreased ability to reduce intracellular ROS. The SOD2 heterozygous mouse has been used to study cardiac function, aging, and drug metabolism. While significant studies have been done to show the potential for gene knockouts in SOD2, very few studies have been done for GPx1, which has also been shown to have polymorphisms with clinical relevance.

3.4. Transporter Assays. In the assessment of transporter activity, BSEP is commonly assayed because its dysfunction is correlated with cholestasis. Common BSEP assays include the vesicular transport assay (VTA) and the ATPase assay [94], both of which rely on the presence of ATP as a to drive the protein function and hence the assay. Because both assays rely on ATP, this makes VTA and the ATPase assay also suitable for ABC transporter proteins. In addition to VTP and ATPase, taurocholate may be used for measuring BSEP transport [157].

Drug uptake and release assays are also available for other compounds and transporter proteins [158]. Owing to the common regulation of MDR1 and CYP3A4 via the pregnane X receptor (PXR), there is significant overlap in compounds that can be used to assay transporter activity [77, 86, 88]. Examples of this are rifampicin and verapamil as inducers and inhibitors of MDR1 activity, respectively [77]. In the influx transporter system, OATP1B1 and OATP1B3 have broad substrate specificity that can be used to measure activity, including rifampicin [159, 160], taurocholate [161], and pitavastatin [82]. For other transporters, MRP2 is inhibited via a fluorescent compound, 5(6)-carboxy-2′,7′-dichlorofluorescein (CDCF) [82, 157, 162]. Additionally, OCT knockouts have been generated in mice, including double knockouts of OCT1 and OCT2 [77].

Flow cytometry can be used to diagnose potential for idiosyncratic reactions via transporter and immune mediated mechanisms. The principle of flow cytometry utilizes cell counting and cell sorting based on immunofluorescent labeling of cell surface markers. Transporter proteins and lymphocytes are ideal detection markers for flow cytometry because they exist on the cell surface and are stainable with fluorescent antibodies. Saab et al. used microvolume flow cytometry to assess the behavior of MDR1 and MRP2 as associated risks with IDILI [89]. By staining for efflux transporters, they were able to detect toxicity potential due to synergistic effects of several known idiosyncratic drugs with known effects to induce inflammation. Perez et al. used flow cytometry to quantify the behavior of several efflux transporters by examining ROS production due to mitochondrial deregulation [90].

3.5. Inflammation Assays and Immune System Dysfunction. Cytokine analysis is an effective tool for quantifying ADRs for long-term drug administration. In addition, cytokines can

be readily quantified in both serum and media. A common practice is to administer agonists that induce inflammation, such as lipopolysaccharide, (LPS) to activate toll-like receptor- (TLR-) mediated inflammation and apoptosis [163, 164]. Caspase 3/7 activity assays are a common assay used to measure inflammation-mediated apoptosis [19]. Because of the diversity of symptoms associated with IDILI, time dependence is a concern for most clinicians on the response. However, inflammation turnaround can be rather quick compared to other methods of IDILI. The significant drawback to inflammation assays is the complexity. Common methods for quantifying cytokine levels include ELISA and RT-PCR [163]. Due to toxicity occurrence coming from the immune system rather than the liver, multiple cell systems and pathways need to be monitored to determine the dysregulated cytokines.

Yano et al. attempted to look at the complexity of this issue by stimulating inflammation in mouse livers [163]. They added drugs at known concentrations and measured cytokine levels from mouse models to compare with the current elevations in standard liver biomarkers ALT and AST. Additionally, liver inflammation markers were compared in in vitro monocyte cultures with and without the presence of heat-inactivated HLMs by observing gene expression. Ultimately, the results showed a time dependent responsiveness towards cytokine development, with several of the elevated proinflammatory markers (IL-1β) clearly elevated within a 24-hour time window. Additionally, a transcription factor that promotes inflammation that is also found in plasma, high-mobility group protein B1 (HMGB1), was only found in high quantities of drugs that are considered hazardous to the liver. However, the biggest take away was the combined effects on the expression of MRP8, MRP9, IL-1β, and cryopyrin (NALP3), and receptor for advanced glycation end products (RAGE) would be elevated in the presence of drugs with increased susceptibility to liver injury. All of these markers are direct results of upregulation of the nuclear factor kappa B (Nf-κB) pathway.

There are several other assays besides direct quantification cytokine levels. Master transcriptional regulators may become a target for measuring specific lymphocyte activity [115]. By measuring the levels of master transcriptional regulators, scientists and clinicians have ideas of which cells are proliferating and which pathways are stimulated. Another test that can be used to assess drug toxicity is the lymphocyte transformation test (LTT) [121, 165]. Here, cells proliferate in response to the presence of particular antigens or drugs.

Additionally, flow cytometry can be used to quantify adverse behavior in lymphocytes. It has been used to quantify the presence of KCs and other cells that are involved in inflammation-based toxicity [166], cytokine synthesis during inflammation due to drug allergy [165], and the upregulation of cluster of differentiation 69 (CD69) [165], a biomarker for inflammation. Thus, injury can be assessed from in vivo and in vitro models using flow cytometry.

In addition to drug metabolite quantification, metabolism is a method for specifying immune cell activity. Broadly, lymphocytes can be categorized based on aerobic versus anaerobic glycolysis and whether or not oxidative phosphorylation is taking place. The presence of aerobic glycolysis without oxidative phosphorylation indicates the increased activity of cells often found at the site of inflammation, including neutrophils and KCs.

Cytokine knockouts have been used to monitor the potential for ADRs in mouse and rat models. Common knockouts that have been used to increase susceptibility to IDILI include TNFR1 [108, 167], IL-13 [108, 168], and double knockouts of IL-4 and IL-10 [108, 169]. In addition, IFN-γ and toll-like receptor 9 (TLR9) individual knockouts seem to decrease the susceptibility to IDILI [108]. In addition, while the double knockout of IL-4 and IL-10 increases susceptibility to ADRs, having a triple knockout of IL-4, IL-6, and IL-10 decreases susceptibility to IDILI [108, 169].

3.6. Genetic Regulation and Clustering. Creating genetic profile maps can give a diversified predictability of IADRs. Focus has been maintained on clusters of simultaneously regulated genes [170, 171] or genes that could potentially contribute towards IDILI [117]. Aside from genetic profiling, only flow cytometry may be able to predict IADRs due to HLA haplotypes [172], providing a necessary function for predicting some mechanisms of IDILI. Clustering in particular is important for assessment of commonly regulated genes and finding mutations within pathways. Targets for clustering analysis include xenobiotic metabolism, bioenergetics, and mechanisms for inflammation and injury [173]. Another target for genomic analysis is study of the peroxisome proliferator-activated receptors (PPARs), which are nuclear receptor proteins that control for transcription of various enzymes involved in drug metabolism [60, 174]. Additionally, the AmpliChip CYP450 Test has been developed to detect CYP450 polymorphisms [175, 176]. While gene regulation can give indication towards missing enzymes or downregulated pathways, clusters do not necessarily directly correlate with protein expression or activity at either the DNA or the RNA level as posttranscriptional modifications and RNA degradation may occur. Even so, models that focus on pathway regulation and gene analysis related to mechanisms of injury can be good predictors of idiosyncratic compounds [170, 177].

3.7. Predictive Models. While focus has been given towards in vitro and in vivo test, computational results can rule out a significant amount of drugs that have toxic potential. Of particular importance is the prediction of reactive metabolite formation [178] and how those metabolites may interact with intracellular molecules [31, 179], or looking at drugs that affect major regulatory molecules for drug metabolism [180, 181]. These models, while maintaining importance towards predicting general ADRs, may also be used if certain classes of compounds create toxicity for individuals with any clinically relevant polymorphisms.

4. Towards Integrative Platforms for Comprehensive IADR Predictability

While several of the current systems and assays emphasize detection of single mechanisms for hepatotoxicity, many of

the current in vitro systems fall short at generating complete individual profiles for predicting IADRs. While in vivo models can be used to generate comprehensive profiles for ADRs and simultaneous events, they generally have poor predictability and are more expensive than in vitro assays [150]. The rest of this review will focus on developing in vitro and ex vivo cellular models that can be used to assess cumulative idiosyncratic potential with applications in diagnosing idiosyncratic potential and drug development.

4.1. Controlled DME Expression Systems. Significant advantages of utilizing hepatomas or immortalized cell lines are the well-characterized expression of proteins from these cell lines and the affordability they present for clinical and industrial tests. While hepatocytes contain many of the significant proteins for drug metabolism, maintenance of these cultures is expensive and often the cells will lose their in vivo protein expression hours after being cultured. Thus, controlling the expression of DMEs, transporters, and antioxidant proteins via manipulation of the genome in immortalized cell lines provides a cost-effective and reproducible method for characterizing idiosyncratic potential. Additionally, using cells that either have expressions that can be easily altered or adequately reflect in vivo behavior of hepatocytes is the most important for any in vitro toxicity assay.

Among the most utilized forms of transformable liver cell lines are epithelial cells transformed with SV40 large T antigen (THLE cells). These cells have very low expression of phase I and phase II DMEs, making them ideally suited for transducing genes that express DMEs [182]. Viral transduction can be readily performed on these cells to express the desired DMEs. Kwon et al. successfully transduced recombinant adenoviruses expressing several clinically relevant CYP450 isoforms into THLE-2 cells on a miniaturized platform that is effective at generating dose-response curves for tested therapeutics [183]. Various combinations of DMEs expressed in THLE-2 cells on the chip can be used to potentially simulate systematic compound metabolism and toxicity, including idiosyncratic ADRs [183]. Thompson et al. also used THLE-2 cells transformed with CYP450 enzymes to successfully assess idiosyncratic risk of several drugs [157]. While this approach allows for tunability in enzymatic expression for several of the key DMEs, using compounds or short hairpin RNAs (shRNAs) to inhibit activity of specific DMEs within hepatic cells is also a reliable approach [184].

Besides THLE cells, HepaRG have been used to quantify drug metabolic activity. Unlike most other cell lines, HepaRG cells are derived from progenitor cells and retain much of the activity of in vivo human hepatocyte cultures, including CYP450 activities [136]. While not as easily transformable as the THLE cell expression system, HepaRG cells have some tunability with expression of clinically relevant sources of IDILI [185, 186]. Anthérieu et al. induced differentiation in HepaRG cells towards successfully mimicking hepatocyte behavior, including both phase I and phase II DME activity [187]. As illustrated by Mueller et al., HepaRG cells also have the potential for 3D growth in hanging droplet experiments, and activity of critical enzymes was increased compared to monolayer controls [188]. This is not to limit cells used for viral transduction to affect DME expression, as HepG2 have also been successfully transduced with adenoviral vectors containing DNA coding for the expression of CYP450s [189].

4.2. Hepatic Coculture Systems. Liver coculture systems provide the advantage of giving a more complete profile of liver behavior in the presence of drugs. Since the liver contains multiple cell types, the coculture system allows examination of the effects of drugs on different functions. While the liver is primarily composed of hepatocytes, there are other cells present, including several different kinds of lymphocytes, sinusoidal endothelial cells, and stellate cells [190]. In addition, the liver is organized based on proximity to blood vessels. Cells closer the portal vein are more involved in oxidative metabolism, β-oxidation of fatty acids, ureagenesis, and gluconeogenesis, whereas cells further from major blood vessels are involved in biotransformation of drugs, glutamine synthesis, lipid synthesis, and glycolysis [191]. As a result, development of models for liver structure in hopes for predicting idiosyncratic ADRs is a complex process.

Kostadinova et al. implemented a coculture system by using porous layered nylon scaffolds designed to fit 24-well plates, which allowed the cells to generate their own 3D matrix and growth factors by expansion between the pores (Figure 6(a)) [192]. Additionally, this scaffold had smaller pores to allow for nutrient regeneration and removal. Cells cultured on this scaffold included hepatocytes, stellate cells, KCs, and endothelial cells. Ultimately, this scaffold was successful at maintaining in vivo conditions for normal liver function for eleven weeks and was able to induce proinflammatory cytokine production via addition of LPS to scaffolds. Additionally, CYP450 assays and drug uptake were effectively measured in this system to account for several mechanisms that an affect idiosyncratic responses. While this system utilized a small scale, moderate throughput design, scaling down for 96-well and 384-well plate assays would be difficult due to the maneuverability of the scaffold.

Novik et al. present a coculture system that utilizes microfluidic cocultures of hepatocytes and nonparenchymal cells (NPCs) for clearance studies (Figure 6(b)) [193]. The advantage of this system is that there is tunability in O_2 parameters and flow rates, which is often missing in conventional high-throughput screens. Novik et al. were able to assess that phase I DME activity was elevated in the presence of flowed cocultures compared to static cultures and cultures with just hepatocytes. Additionally, the bile canaliculi formation was successfully imaged to indicate that transporter activity could be measured. However, there are limits to flow systems for high-throughput detection, making this particular system less advantageous for industrial use.

Several groups have implemented fibroblasts to facilitate the creation of a microenvironment that best mimics the liver (Figure 6(c)). Khetani et al. implemented micropatterned cocultures using fibroblasts [196]. Ultimately, while assays restored some liver functions, there were several false negatives with detecting compound toxicity, owing to the lack of diversity and monolayer culture used for these cells. Wang et al. micropatterned cocultures like Khetani et al., but utilize general stromal cells instead of fibroblasts, measuring their

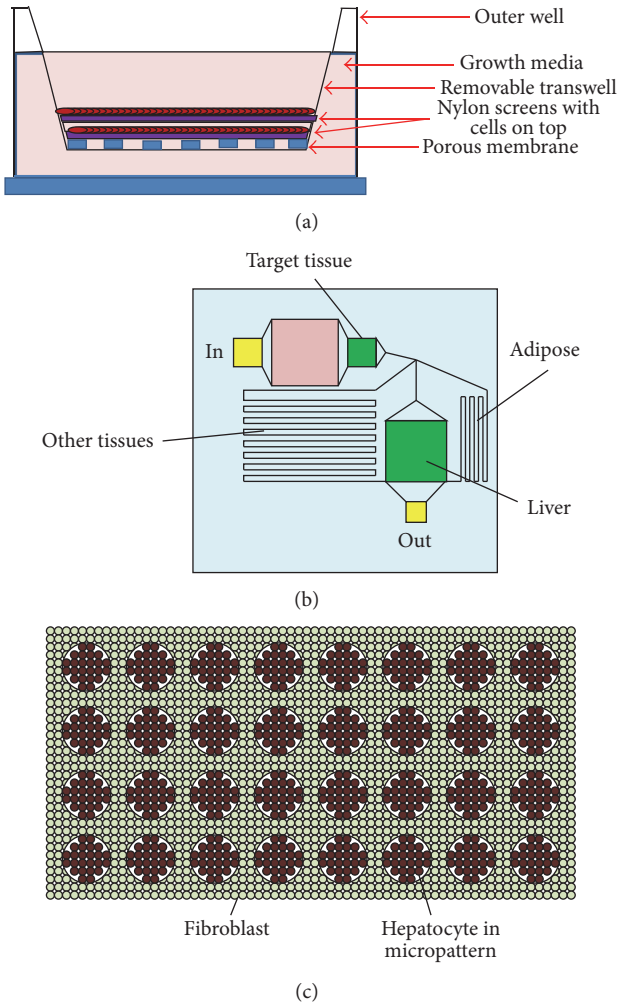

Figure 6: Hepatic cell coculture systems with applications in hepatotoxicity: (a) porous nylon membrane scaffold system [192], (b) the microfluidic system [193, 194], and (c) micropatterned hepatic cocultures [195].

results by quantifying circulating metabolites two and seven days after incubation [197]. While success rates were higher for detecting phase I and phase II metabolites compared to microsome analysis or suspension cultures, toxicity results were accurately predicted only about 70% of the time in two-day cultures and 80–85% of the time in week-long cultures. Thus, cocultures with fibroblasts in the absence of KCs, stellate cells, and endothelial cells may not be ideally suited for drug metabolism studies. More recently, Rose et al. have developed a system using plated hepatocytes cocultured with KCs to model the inflammation response within a 48-well collagen-coated plate [132]. The group was successful in measuring the concentrated immune response as well as CYP3A activity using the previously mentioned luciferin substrate [132].

Disadvantages of coculture platforms involve the complexity of the scaffolds to accurately mimic the in vitro liver environment, as well miniaturization being limited due to the number of cells needed to accurately determine toxic effects. The assay miniaturization is of particular importance with microfluidic platforms and the removable inserts, as this also impacts scalability and cost. Even integrating all cell types on a 96-well plate platform is challenging because decreasing the number of cells limits the interaction observed between different cell types. Thus, size of culture optimization should be considered when designing small scale liver tissues for toxicity testing.

4.3. Stem Cells for Modeling DILI. Besides coculture platforms, stem cells have become a larger part of the investigation into mechanisms that cause DILI and ADRs. In particular, the use of induced pluripotent stem cells (iPSCs) can be used to generate the necessary cell types to mimic the adult liver and model disease states. In iPSCs, adult somatic cells are reverted to behaving like embryonic stem cells (ESCs) via introduction of several key transcription factors [198]. While iPSCs require dedifferentiation with subsequent redifferentiation to derive appropriate cell lines, they provide an innovative way to both model liver disease and assess how certain polymorphisms may affect drug metabolism and disposition in vitro. Additionally, human iPSCs (hiPSCs) have a significant amount of commercial availability for applications in liver toxicity testing and have the potential to be used in coculture with hepatocytes and fibroblasts to give an accurate model for predicting potential liver toxicity [199–201].

It is becoming more important that the role of iPSCs is not only to derive hepatocyte-like cells, but also to recapitulate their function and potential polymorphisms to generate accurate models. Szkolnicka et al. have recently demonstrated the ability of redifferentiated iPSCs to express CYP1A and CYP3A DMEs and express similar responses to drugs as compared to cryopreserved hepatocytes cultured in vitro [202]. Individualized toxicity assessments have been completed by various groups as Choi et al. used patient-specific cell lines for individuals deficient in the alpha-1 antitrypsin (A1AT), a protease inhibitor that when absent has been correlated with liver cirrhosis and heptocellular carcinoma [203]. While maintaining levels of expression is an ongoing issue in the field of using stem cells and iPSCs in particular, there has been recent progress made towards having redifferentiated cells express appropriate levels of key DMEs to be used as models in drug metabolism [204].

4.4. Precision-Cut Liver Slices. As an alternative to standard in vitro models, precision-cut liver slices can be studied to determine the interactions of various cells in the liver in order to recapitulate conditions that can lead to IDILI. The main advantage of this ex vivo technique is that with all cells present, the goal of coculture is achieved without reconstructing the entire liver from the bottom up. Additionally, most DME and transporter expression are preserved during the toxicity testing [205]. Hadi et al. have demonstrated this use for detecting inflammation-induced liver injury while quantifying the levels of GSH and proinflammatory cytokines in mouse and human-derived liver slices [156, 206]. In addition to ADRs, liver disease states can be modeled, ranging from fibrosis to obesity [207, 208]. The disadvantage

of this technique is that because precision-cut liver slices need to be obtained from whole organisms, surgery or sacrifice of specimens is required, and achieving HTS for adequate drug modeling is difficult. While these are issues, precision-cut liver slices do provide an adequate way to model liver disease states.

5. Summary

With at least six general causes that have been potentially tied to IADRs, the need for predicting IADRs before in vivo experiments is critical. While individual drugs that might induce inflammation or have variant metabolism based on CYP450 expression are generally predicted before clinical trials, less focus has been give towards other potential theories regarding IADRs. There are currently assays available for high-throughput applications that can be used for predicting certain IADRs based on the individually described mechanisms. Simulating simultaneous idiosyncratic events requires platforms that can control the behavior of multiple events at once, or ones that can mimic the in vivo environment of the human liver. Future research should be focused on developing high-throughput platforms and utilizing assays that can predict ADRs in vitro.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Reproductive Toxicities Caused by Swainsonine from Locoweed in Mice

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Swainsonine is the primary toxin in locoweeds. It causes intention tremors, reproductive dysfunction, emaciation, and death. The objective of the present study was to evaluate the potential reproductive and developmental toxicities caused by swainsonine in mice. The treatment groups consisting of three generations of mice were given a range of concentrations of swainsonine by intraperitoneal injection (2.50 mg/kg body weight (BW), 1.20 mg/kg BW, 0.60 mg/kg BW, and 0 mg/kg BW). The 0 mg/kg BW group exhibited significantly fewer estrous cycles and an increased number of estrous ones compared to the 2.50 mg/kg BW, 1.20 mg/kg BW, and 0.60 mg/kg BW groups ($P < 0.05$). All three generations of mice treated with swainsonine had significantly higher spleen, liver, and kidney indices and significantly lower body weights compared to the 0 mg/kg BW group ($P < 0.05$). For the first and second generations of treatment group, the copulation indices and the numbers of live pups on postnatal days (PND) 0, 4, and 15 were significantly decreased compared to those of the 0 mg/kg BW group ($P < 0.05$). The fertility and gestation indices of the treatment group of the first generation were significantly increased compared to the 2.50 mg/kg BW, 1.20 mg/kg BW, and 0.60 mg/kg BW groups of the second generation ($P < 0.05$). Cumulatively, these results indicate that swainsonine may cause reproductive and developmental toxicities in mice in both parents and offspring.

1. Introduction

Locoweeds (*Astragalus* and *Oxytropis* spp.), a taxa of the Legume family, are toxic plants in the western United States that frequently poison livestock [1]. Consumption of locoweeds by grazing animals can result in locoism, which is characterized by emaciation, staggering gait, lack of muscular coordination, reproductive disturbances, immune system impairment, and death [2, 3]. The economic cost owing to locoism reaches several million dollars per year due to death, abortion, reproductive problems, and lack of weight gain [4–6]. Swainsonine, an indolizidine alkaloid, is the primary toxin in locoweeds [3]. Swainsonine is an effective inhibitor of both lysosomal α-mannosidase and Golgi α-mannosidase II [7]. Swainsonine toxicity caused by consumption of locoweed causes intention tremors, generalized depression, nervousness, proprioceptive deficits, aberrant behavior, reproductive dysfunction, emaciation, and death [8]. Additionally, locoweeds cause embryonic and fetal lethality, abortions, generalized reproductive dysfunction, and occasional birth defects. Swainsonine from locoweed causes acute intoxication in horses, goats, and sheep, in addition to chronic poisoning in rabbits, rats, and mice. Its toxicity often occurs during pregnancy in livestock and may have different effects on embryonic development depending on the conceptus phase and maternal conditions during acute intoxication. Therefore, we observed swainsonine effects on mouse reproductive performance during chronic poisoning. The objective of the present study was to evaluate the effect of three different doses of swainsonine in female mice during estrus, gestation, childbirth, and lactation. We also treated the first (F0), second (F1), and third (F2) generations of mice

with three different doses of swainsonine during growth and development. We report the results of the reproductive and developmental toxicities of swainsonine.

2. Material and Methods

2.1. Animals and Housing Conditions. Female Wistar mice (6 weeks old) were supplied by the Animal Center of the Fourth Military Medical University. During the experiment, the animals were housed individually in polypropylene cages with laboratory-grade pine shavings as bedding. The mice were maintained under controlled temperatures (±23°C). Lighting conditions consisted of a 12 : 12 hour light : dark cycle in a controlled environment with >8 air exchanges/hour and temperature and relative humidity in the ranges of 19–25°C and 40–70%, respectively. The experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the China College of Animal Experimentation and were approved by the College of Veterinary Medicine Northwest A&F University.

2.2. Extraction of Swainsonine from Locoweed

2.2.1. Plant Materials. The aerial portion of *Oxytropis kansuensis* was collected from a grassland in Tianzhu City of Gansu province in July 2011. The plants were then taxonomically identified by Zhao Bao-Yu, College of Veterinary Medicine, Northwest A&F University, China. The plants were subsequently dried in the shade, finely ground, and comminuted.

2.2.2. Isolation of Alkaloids from Oxytropis kansuensis. The plant sample (1.5 kg) was thermally refluxed in 10 L of distilled water into an ultrasonic cleaner for approximately 12 hours. The sample was subjected to heat treatment for approximately 1 hour at 50°C in an ultrasonic cleaner. A total of 18 L of the water extract was collected and reduced over heat to 2 L. The solution was centrifuged at 1500 r/min for 10 min and the solid impurities were removed. The sample was then concentrated to 1 L at 95°C, followed by recovery of the EtOH under reduced pressure to obtain a crude extract for isolation of the alkaloids. The extract was dissolved in 1 N HCl, and the filtrate was extracted with chloroform and alkalized. The aqueous fraction was successively extracted with chloroform, ethyl acetate, and n-butanol. The raw alkaloids were collected with n-butanol, dissolved in methyl alcohol, and filtered. The residuals were removed. The solvents were recovered using methanol and pressure, and the extractions with alkaline chloroform were repeated. We collected crude swainsonine with chloroform. We used silica gel column chromatography for purification. For the dry sample, we used a chloroform-methyl alcohol-water gradient elution and thin-layer chromatography (TLC) detection. For the collection of swainsonine, the extract was decompression-drained. Finally, a light yellow powder of swainsonine was collected, and pure swainsonine was obtained using a fractional distillation method [9].

2.2.3. Analysis of Swainsonine. TLC detection was performed on plates precoated with silica gel G using developing solvents (chloroform : methanol : ammonia : water [70 : 26 : 2 : 2, v/v], chloroform : methanol : ammonia : water [70 : 26 : 10 : 10, v/v], and methanol : ethyl acetate : ammonia [4 : 1 : 1, v/v]) and either a modified potassium heptaiodobismuthate reagent or H_2O_2/10% acetic anhydride in EtOH/Ehrlich's reagent as the chromogenic agent.

The extracts were dissolved in methanol and spotted onto the GF254 silica gel G precoated plates. The plates were developed with an ascendant run after saturation with the mobile phase in an s glass chamber for 5–10 min. The plates were dried when the mobile phase was 10 mm from the front edge of the plates. The plates were stained successively with a spray of H_2O_2 (heated for 10 min in an oven at 115°C), a spray of 10% acetic anhydride in dehydrated alcohol (heated at the same temperature until the smell of acetic anhydride disappeared), and finally a spray of Ehrlich's reagent (heated for 15 min at 120°C). The color of the spots in each plate was recorded, and the Retardation factor (R_f) was determined [9].

2.3. Study Design. To assess the reproductive and developmental toxicity of swainsonine, three generations of mice were used. The study design was as follows.

2.3.1. Experiment 1: Observation of the Estrous Cycle. Female mice ($N = 40$, 6 weeks old, 10 animals per group, consisting of 2.50 mg/kg BW, 1.20 mg/kg BW, 0.60 mg/kg BW, and 0 mg/kg BW mice) were treated 14 days before mating and throughout the mating period. After 14 days of treatment, 40 female mice were observed for the estrous cycle using the vaginal smear method. The vaginal lavage sample from each female was evaluated daily for the estrous cycle during the next 15 days of treatment. We collected the reproductive organs and recorded the estrous parameters after 15 days of treatment. Estrous cycles of 4-5 days were considered normal, and any other cycle lengths were considered irregular. In particular, cycles with more than 7 days of diestrus were considered continuous diestrus [10].

2.3.2. Experiment 2: The Observation of Reproductive Performance Test. Female mice ($N = 40$, 6 weeks old) were divided into four groups (10 animals per group: F0-I: 2.50 mg/kg BW; F0-II: 1.20 mg/kg BW; F0-III: 0.60 mg/kg BW; and F0-IV: 0 mg/kg BW). The F0-IV group was considered the control group, and the other three groups were the treatment groups.

Mice were given swainsonine by intraperitoneal injection 14 days before the mating period. After this premating period, 40 female mice (10 per group) were transferred to the home cage of male mice in the same group and cohabited on a 1 : 1 basis until a mating period of 2 weeks elapsed. During the mating period, vaginal plugs were examined daily for the presence of sperm, and a vaginal plug was considered evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. We recorded pregnancy rates of the female mice. A confirmed pregnant mouse continued to receive swainsonine throughout the parturition and

Table 1: Body and reproductive organ weights of female mice given swainsonine during the estrous cycle.

		Body weight (g)	Ovarian index	Uterus index	Estrous cycle (day)	Number of estrous mice
Day 7	2.50 mg/kg BW	26.15 ± 1.14^{a}	0.028 ± 0.002^{a}	0.198 ± 0.004^{a}	6.92 ± 0.23^{a}	3.53 ± 0.21^{a}
	1.20 mg/kg BW	28.04 ± 1.33^{b}	0.029 ± 0.002^{a}	0.208 ± 0.003^{a}	6.54 ± 0.15^{a}	4.01 ± 0.17^{a}
	0.60 mg/kg BW	29.58 ± 1.55^{c}	0.035 ± 0.002^{b}	0.210 ± 0.003^{a}	5.88 ± 0.14^{b}	5.81 ± 0.21^{b}
	0 mg/kg BW	31.41 ± 1.10^{d}	0.048 ± 0.003^{c}	0.300 ± 0.002^{b}	4.20 ± 0.16^{c}	7.54 ± 0.19^{c}
Day 15	2.50 mg/kg BW	27.05 ± 1.66^{a}	0.030 ± 0.002^{a}	0.209 ± 0.003^{a}	7.64 ± 0.25^{a}	2.99 ± 0.24^{a}
	1.20 mg/kg BW	28.89 ± 1.78^{b}	0.031 ± 0.002^{a}	0.216 ± 0.003^{a}	6.91 ± 0.14^{b}	3.98 ± 0.31^{b}
	0.60 mg/kg BW	30.5 ± 1.25^{c}	0.037 ± 0.003^{b}	0.311 ± 0.004^{b}	6.00 ± 0.15^{c}	4.55 ± 0.25^{c}
	0 mg/kg BW	32.19 ± 1.12^{d}	0.051 ± 0.002^{c}	0.364 ± 0.003^{c}	4.45 ± 0.11^{d}	7.66 ± 0.21^{d}

The values are the mean ± SD.
Ovary viscera index = ovary weight/body weight × 100 (mg/g).
Uterus viscera index = uterus weight/body weight × 100 (mg/g).
[a,b,c,d]Significantly different from each dosage group of same generation ($P < 0.05$).

lactation periods. When the newborn mice began weaning, the female mice (F0) were sacrificed, and the liver, kidney, heart, spleen, lungs, uterus, and ovaries were collected.

Newborn female mice (F1) were treated with swainsonine after delactation (40 F1 mice total, 10 per group: F1-I: 2.5 mg/kg BW; F1-II: 1.20 mg/kg BW; F1-III: 0.60 mg/kg BW; and F1-IV: 0 mg/kg BW). They were selected from the four groups of the F0 generation. When the F1 mice were 8 weeks old, they were mated with healthy male mice and cohabited on a 1 : 1 basis until a mating period of 2 weeks elapsed. All F1 mice were treated with swainsonine before mating and throughout the mating, gestation, and lactation periods. When newborn mice began weaning, the F1 female mice were sacrificed, and the liver, kidney, heart, spleen, lungs, uterus, and ovaries were collected.

Newborn female mice (F2) were selected from each of the four groups of the F1 generation. The F2 mice were not treated with swainsonine until delactation. They were then sacrificed, and the liver, kidney, heart, spleen, lungs, uterus, and ovaries were collected.

All experimental animals received intraperitoneal injections of swainsonine once every 3 days under aseptic condition. For each mouse that was sacrificed, the liver, kidney, heart, spleen, lungs, uterus, and ovaries were trimmed of extraneous fat and weighed immediately. The organ weight and terminal body weights were recorded.

2.4. Data Analysis. Statistical Product and Service Solutions (SPSS) software v11.3 was used to determine whether there were any statistically significant differences between the test groups and the control group. A one-way ANOVA was used to evaluate the data, and Dunnett's multiple comparison test was then applied. Values of $P < 0.05$ were considered significant. The data are presented as the group mean values ± standard deviation (SD).

3. Results

3.1. TLC Detection. All extracts were collected using column chromatography, which was placed on the thin-layer plate using the capillary sample. Figure 1 shows a developed TLC plate. The purple spots are swainsonine and the rose red spots are the swainsonine analogs as determined by comparison with the swainsonine standard (Retardation factor $[R_f] = 0.50$).

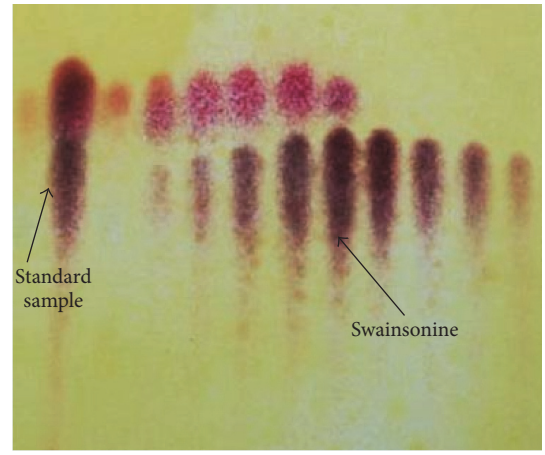

Figure 1: Thin-layer chromatography of swainsonine. The standard swainsonine sample (left arrow). Swainsonine is represented by the purple spots (right arrow).

3.2. Experiment 1: Body and Reproductive Organ Weights during the Observation of the Estrous Cycle. The body and reproductive organ weights increased for each group during the estrus cycle, as reported in Table 1. After 14 days of swainsonine treatment, we began to observe the estrus cycle for the four groups of mice over the course of 15 days. The mice were given swainsonine once every 3 days.

The body weights of the 2.50 mg/kg BW, 1.20 mg/kg BW, and 0.60 mg/kg BW groups were significantly lower than those of the 0 mg/kg BW group after 7 and 15 days of swainsonine treatment ($P < 0.05$). Furthermore, the body weights of the 2.50 mg/kg BW group were significantly lower than those for the 1.20 mg/kg BW and 0.60 mg/kg BW groups at 7 and 15 days of treatment ($P < 0.05$).

The ovarian index of the 2.50 mg/kg BW and 1.20 mg/kg BW groups of mice was significantly decreased compared to that of the 0.60 mg/kg BW and 0 mg/kg BW groups at 7 and

15 days of treatment. The ovarian index of the 0.60 mg/kg BW group was lower than that for the 0 mg/kg BW group ($P < 0.05$). The ovarian index decreased from 0 mg/kg BW group to 2.50 mg/kg BW group.

The uterine index of the 2.50 mg/kg BW, 1.20 mg/kg BW, and 0.60 mg/kg BW groups of mice was significantly decreased compared to that of the 0 mg/kg BW group at 7 days of treatment ($P < 0.05$). The uterine index of the 2.50 mg/kg BW, 1.20 mg/kg BW, and 0.60 mg/kg BW groups was not significantly different from each other at 7 days of treatment ($P > 0.05$). However, the 2.50 mg/kg BW and 1.20 mg/kg BW mice displayed a uterine index lower than that of the 0.60 mg/kg BW and 0 mg/kg BW groups at 15 days of treatment ($P < 0.05$). The uterine index for the 0.60 mg/kg BW mice was also significantly lower than that of the 0 mg/kg BW group at 15 days of treatment ($P < 0.05$).

With respect to the length of the estrous cycle (in days), the 2.50 mg/kg BW and 1.20 mg/kg BW groups displayed longer cycles than those of the 0.60 mg/kg BW and 0 mg/kg BW groups at 7 days of treatment ($P < 0.05$). The 0.60 mg/kg BW mice had significantly longer cycles compared to those of the 0 mg/kg BW group ($P < 0.05$). The estrous cycle length increased for the 2.50 mg/kg BW group compared to that of the 0 mg/kg BW group at 7 days of treatment. The 2.50 mg/kg BW group had significantly longer cycles compared to those of the 1.20 mg/kg BW, 0.60 mg/kg BW, and 0 mg/kg BW groups at 15 days of treatment ($P < 0.05$). The 1.20 mg/kg BW and 0.60 mg/kg BW groups also had longer cycles than those of the 0 mg/kg BW group, and there was a significant difference between the 1.20 mg/kg BW and 0.60 mg/kg BW groups at 15 days of treatment ($P < 0.05$).

The number of mice experiencing estrous cycle was lower in the 2.50 mg/kg BW and 1.20 mg/kg BW groups than in the 0.60 mg/kg BW and 0 mg/kg BW groups at 7 days of treatment ($P < 0.05$). The number of mice undergoing estrous cycle in 0.60 mg/kg BW group was significantly fewer than that in the 0 mg/kg BW group ($P < 0.05$). This number also decreased from the 2.50 mg/kg BW group to 0 mg/kg BW group at 7 days of treatment. The 2.50 mg/kg BW group had significantly fewer mice experiencing estrous cycle than 1.20 mg/kg BW, 0.60 mg/kg BW, and 0 mg/kg BW groups at 15 days of treatment ($P < 0.05$). Finally, the 1.20 mg/kg BW and 0.60 mg/kg BW groups had fewer mice in estrous cycle than the 0 mg/kg BW group, and there was a significant difference between the 1.20 mg/kg BW and 0.60 mg/kg BW groups at 15 days of treatment ($P < 0.05$).

3.3. Experiment 2: The Reproductive Performance Test

3.3.1. The Relative Organ Weights in Mice. The relative organ weights and body weights of the mice are shown in Table 2. The relative heart weight and relative lung weight of the four groups of mice from the F0, F1, and F2 generations were not significantly different among each group ($P > 0.05$).

For the relative liver weights of the F0, F1, and F2 generations, the experimental group had a significantly higher weight compared to that of the control group mice ($P < 0.05$). In the F0 and F1 generations, the liver weights of the mice in groups I, II, and III were significantly higher compared to those of the group IV mice ($P < 0.05$). There was no significant difference among mice of the groups I, II, and III ($P > 0.05$). For the F2 generation, the F2-III and F2-IV mice had significantly lower liver weights compared to those of groups F2-III and F2-IV ($P < 0.05$); however, there was no significant difference between the F2-I and F2-II groups and between the F2-III and F2-IV groups ($P > 0.05$). The relative liver weights of the F1 generation were higher than those of the F0 generation, except for the group IV mice.

For the spleen and relative kidney weights of the F0, F1, and F2 generations, the groups I, II, and III mice had significantly higher values compared to those of group IV ($P < 0.05$); however, there was no significant difference among groups I, II, and III ($P > 0.05$). The spleen and kidney indices of the F1 generation were higher than those of the F0 generation, except for the group IV mice.

For the relative ovary and uterus weights of the F0 and F1 generations, groups I, II, and III had significantly higher values compared to the group IV mice ($P < 0.05$). However, in the F2 generation, mice in groups I, II, and III had significantly lower values compared to those of the group IV mice ($P < 0.05$).

For body weights in the F0, F1, and F2 generations, groups I, II, and III were significantly lower compared to those of group IV ($P < 0.05$); however, there were no significant differences among groups I, II, and III ($P > 0.05$). The body weights of the F1 generation were lower than those of the F0 generation, except for the group IV mice.

3.3.2. Reproductive and Developmental Index. The developmental findings are shown in Table 3. For the F0 and F1 generations, the number of live pups for groups I, II, and III decreased from PND 0 to PND 15. The number of live pups obtained from the F0-IV and F1-IV groups was not significantly different from PND 0 to PND 15. For the F0 and F1 generations, the number of live pups on PND 0 for groups I, II, and III was significantly lower compared to that of group IV ($P < 0.05$). The number of live pups on PND 4 for group IV was significantly higher compared to that of groups I, II, and III ($P < 0.05$). The number of live pups on PND 4 for group III was significantly higher compared to that of group I ($P < 0.05$). The numbers of live pups on PND 15 for groups III and IV were significantly higher compared to those of groups I and II ($P < 0.05$). The number of live pups on PND 15 for group IV was higher than that of group III ($P < 0.05$).

The body weights of the live mice in the F0 generation were higher than those for the F1 generation during the same period. For the F0 and F1 generations, the body weights of the live mice of groups I, II, and III were significantly lower compared to those of group IV on PND 0, 4, and 15 ($P < 0.05$). There was no significant difference observed among groups I, II, and III ($P > 0.05$).

The reproductive findings are shown in Table 4. For the F0 and F1 generations, the copulation index for group IV was significantly increased compared to that of groups I, II, and III ($P < 0.05$). The copulation index of group III was significantly increased compared to that of groups I and II ($P < 0.05$). For the F0 and F1 generations, there were no significant

Table 2: Relative organ weights of mice given swainsonine by intraperitoneal injection.

	Heart index	Liver index	Spleen index	Lung index	Kidney index	Ovary index	Uterus index	Body weights (g)
F0-I	0.581 ± 0.05^{a}	5.411 ± 0.88^{a}	0.558 ± 0.06^{a}	0.581 ± 0.04^{a}	1.682 ± 0.21^{a}	0.079 ± 0.002^{a}	0.482 ± 0.02^{a}	30.8 ± 1.22^{a}
F0-II	0.588 ± 0.04^{a}	5.401 ± 0.95^{a}	0.535 ± 0.05^{a}	0.587 ± 0.05^{a}	1.674 ± 0.18^{a}	0.076 ± 0.003^{a}	0.479 ± 0.03^{a}	31.7 ± 1.25^{a}
F0-III	0.579 ± 0.06^{a}	5.390 ± 0.85^{a}	0.524 ± 0.05^{a}	0.586 ± 0.06^{a}	1.671 ± 0.23^{a}	0.071 ± 0.002^{a}	0.468 ± 0.02^{a}	32.8 ± 1.31^{a}
F0-IV	0.589 ± 0.05^{a}	5.013 ± 0.92^{b}	0.435 ± 0.06^{b}	0.588 ± 0.05^{a}	1.102 ± 0.19^{b}	0.061 ± 0.003^{b}	0.356 ± 0.03^{b}	35.1 ± 1.55^{b}
F1-I	0.598 ± 0.04^{a}	5.448 ± 0.96^{a}	0.561 ± 0.05^{a}	0.583 ± 0.06^{a}	1.692 ± 0.20^{a}	0.081 ± 0.002^{a}	0.498 ± 0.02^{a}	29.3 ± 1.33^{a}
F1-II	0.593 ± 0.03^{a}	5.428 ± 0.89^{a}	0.555 ± 0.06^{a}	0.591 ± 0.05^{a}	1.684 ± 0.22^{a}	0.079 ± 0.002^{a}	0.488 ± 0.03^{a}	31.5 ± 1.13^{a}
F1-III	0.596 ± 0.05^{a}	5.410 ± 0.91^{a}	0.532 ± 0.05^{a}	0.582 ± 0.06^{a}	1.668 ± 0.23^{a}	0.075 ± 0.003^{a}	0.479 ± 0.02^{a}	32.1 ± 1.25^{a}
F1-IV	0.591 ± 0.04^{a}	5.088 ± 0.89^{b}	0.441 ± 0.05^{b}	0.579 ± 0.05^{a}	1.152 ± 0.22^{b}	0.062 ± 0.002^{b}	0.361 ± 0.03^{b}	34.9 ± 1.45^{b}
F2-I	0.505 ± 0.04^{a}	5.231 ± 0.95^{a}	0.512 ± 0.06^{a}	0.533 ± 0.05^{a}	1.581 ± 0.18^{a}	0.039 ± 0.002^{a}	0.274 ± 0.02^{a}	14.2 ± 1.12^{a}
F2-II	0.511 ± 0.05^{a}	5.188 ± 0.91^{ac}	0.501 ± 0.06^{a}	0.551 ± 0.06^{a}	1.534 ± 0.21^{a}	0.041 ± 0.002^{a}	0.289 ± 0.03^{a}	15.7 ± 1.41^{a}
F2-III	0.513 ± 0.03^{a}	5.022 ± 0.89^{bc}	0.499 ± 0.04^{a}	0.561 ± 0.05^{a}	1.414 ± 0.19^{a}	0.042 ± 0.003^{a}	0.288 ± 0.03^{a}	16.1 ± 1.10^{a}
F2-IV	0.514 ± 0.03^{a}	4.858 ± 0.88^{b}	0.418 ± 0.05^{b}	0.563 ± 0.07^{a}	1.005 ± 0.24^{b}	0.053 ± 0.003^{b}	0.339 ± 0.03^{b}	18.8 ± 1.08^{b}

The values are the mean ± SD.

[a,b,c]Significantly different from each dose group for the same generation ($P < 0.05$).

The kidney weight is the weight of both kidneys.

The organ index = organ weight/body weight × 100 (mg/g).

Table 3: Developmental findings for mice given swainsonine by intraperitoneal injection.

	Number of live pups on PND 0 (%)	Number of live pups on PND 4 (%)	Number of live pups on PND 15 (%)	Body weights of live pups on PND 0 (g)	Body weights of live pups on PND 4 (g)	Body weights of live pups on PND 15 (g)
F0-I	80.5 ± 3.5^{a}	75.21 ± 5.6^{a}	73.70 ± 4.88^{a}	1.60 ± 0.32^{a}	2.51 ± 0.55^{a}	6.87 ± 0.89^{a}
F0-II	83.6 ± 4.1^{a}	80.45 ± 5.1^{ab}	77.34 ± 4.55^{a}	1.63 ± 0.35^{a}	2.67 ± 0.61^{a}	6.95 ± 0.93^{a}
F0-III	87.6 ± 3.6^{a}	84.67 ± 5.8^{b}	83.66 ± 4.18^{b}	1.64 ± 0.41^{a}	2.70 ± 0.71^{a}	7.04 ± 0.95^{a}
F0-IV	98.5 ± 5.1^{b}	95.70 ± 5.9^{c}	95.70 ± 5.90^{c}	1.74 ± 0.44^{b}	3.54 ± 0.74^{b}	8.55 ± 0.88^{b}
F1-I	75.5 ± 4.2^{a}	70.55 ± 4.89^{a}	65.66 ± 4.98^{a}	1.58 ± 0.36^{a}	2.31 ± 0.65^{a}	6.54 ± 0.97^{a}
F1-II	80.59 ± 4.07^{ab}	76.48 ± 4.23^{ab}	70.23 ± 4.18^{a}	1.60 ± 0.42^{a}	2.41 ± 0.53^{a}	6.74 ± 0.92^{a}
F1-III	88.33 ± 5.1^{b}	85.56 ± 4.68^{b}	83.56 ± 4.51^{b}	1.62 ± 0.41^{a}	2.58 ± 0.68^{a}	6.95 ± 0.97^{a}
F1-IV	98.1 ± 5.5^{c}	96.70 ± 5.10^{c}	96.70 ± 5.10^{c}	1.75 ± 0.39^{b}	3.61 ± 0.74^{b}	8.31 ± 0.89^{b}

The number of live pups on PND 0 (%) = number of live pups on PND 0/total number of pups born × 100 [postnatal day (PND)]. The number of live pups on PND 4 (%) = number of live pups on PND 4/total number of pups born × 100. The number of live pups on PND 15 (%) = number of live pups on PND 15/total number of pups born × 100. The sex ratio of live pups = number of live males/total number of live pups. The values are the mean ± SD. ^{a,b,c}Significantly different from each dose group for the same generation ($P < 0.05$).

Table 4: Reproductive findings in mice given swainsonine by intraperitoneal injection.

	Number of pairs	Number of pairs with successful copulation	Copulation index	Number of pregnant females	Fertility index	Gestation index	Gestation length (days)
F0-I	10	7.56^{a}	75.6^{a}	7.41	98.02^{A}	83^{A}	20.10 ± 1.23^{A}
F0-II	10	8.34^{ab}	83.4^{ab}	8.11	97.24^{A}	90^{A}	19.85 ± 1.21^{A}
F0-III	10	8.86^{b}	88.6^{b}	8.77	98.98^{A}	96^{A}	20.55 ± 1.25^{A}
F0-IV	10	9.87^{c}	98.7^{c}	9.87	100^{A}	100^{A}	20.41 ± 1.36^{A}
F1-I	10	7.20^{a}	72.0^{a}	6.32	87.78^{B}	76^{B}	19.87 ± 1.30^{A}
F1-II	10	7.98^{ab}	79.8^{ab}	6.91	86.59^{B}	85^{B}	20.21 ± 1.29^{A}
F1-III	10	8.23^{b}	82.3^{b}	7.32	88.94^{B}	93^{B}	19.98 ± 1.31^{A}
F1-IV	10	9.93^{c}	99.3^{c}	9.93	100^{A}	100^{A}	20.14 ± 1.34^{A}

The values are the mean ± SD. The copulation index (%) = number of copulated rats/number of pairs × 100. The fertility index (%) = number of pregnant females/number of pairs with successful copulation × 100. The gestation index (%) = number of dams with live pups/number of pregnant females × 100.
^{a,b,c}Significantly different from each dose group for the same generation ($P < 0.05$).
^{A,B}Significantly different from each dose group for different generations ($P < 0.05$).

differences among groups I, II, III, and IV with respect to gestation length ($P > 0.05$). The fertility and gestation indices of groups F0-I, F0-II, and F0-III were significantly higher compared to those of groups F1-I, F1-II, and F1-III ($P < 0.05$). There was no significant difference between F0-IV and F1-IV ($P > 0.05$).

4. Discussion

In this study, we successfully isolated swainsonine from *Oxytropis kansuensis*. The cationic structure of swainsonine is similar to that of mannose, and its high affinity to mannosidase allows swainsonine to inhibit lysosomal α-mannosidase [11]. In animal cells, vacuole degeneration occurs upon swainsonine poisoning. Cellular vacuolation occurs in most tissues because of the incomplete degradation of the carbohydrate portion of glycoproteins and the accumulation of undressed oligosaccharides [12, 13]. The objective of this study was to determine the effects of swainsonine toxicity on reproduction and development in mice. Doses of 2.50 mg/kg BW, 1.20 mg/kg BW, and 0.60 mg/kg BW were given to mice, which were sufficiently high to induce general toxic effects in the parents and offspring. Furthermore, these dosages of swainsonine produced measurably toxic effects on estrus in these mice.

Previous studies suggest that long-term ingestion of plants containing swainsonine induces significant toxic effects on reproduction in livestock [14]. However, these studies did not demonstrate any effects of swainsonine on reproductive performance or the estrous cycle for three consecutive generations of mice. In experiment 1, after 14 days of swainsonine treatment, we observed estrus regulation for four groups of mice over 15 days with continued administration of swainsonine. Changes in the estrous cycles were observed for groups receiving 2.50 mg/kg BW, 1.20 mg/kg BW, 0.60 mg/kg BW, and 0 mg/kg BW treatments of swainsonine. A prolonged estrous cycle and decreased ovarian and uterine indices were observed after 15 days of treatment. The number of estrous mice decreased and there was a reduction in body weight after 15 days of treatment. The ingestion of plants containing swainsonine can decrease serum progesterone concentrations and subsequently disrupt ovarian function, accompanied by a delayed estrus cycle, increased estrous cycle length, delayed conception, and abortion [15]. We found that the number of estrous mice decreased and the estrous cycles were lengthened by the increasing accumulation of swainsonine in the body. Cumulatively, swainsonine affects the estrous cycle and the number of estrous mice, leading to dysfunctional estrous and no estrus performance.

In experiment 2, changes in organ weights were observed for the kidney, liver, and particularly the spleen in the F0, F1, and F2 generations. The kidneys displayed mean swainsonine concentrations of 154 ng/g when histologic lesions were first observed. Liver lesions were observed when the liver had a mean swainsonine concentration of 319 ng/g [16]. The weights of the livers in groups I, II, and III were higher than those of group IV; however, the weights of livers and kidneys in mice of the F1-I, F1-II, and F1-III generations were higher than those of the F0-I, F0-II, and F0-III generations. The same results were observed for the F0 and F1 generations, and the mice of the F2 generation displayed similar results. Swainsonine poisoning causes hepatomegaly and kidney enlargement. However, the changes in the spleen were more pronounced than the changes in the liver and kidneys. The weights of the spleens in the mice of the F1-I, F1-II, and F1-III generations were higher than those of the F0-I, F0-II, and F0-III generation. The mice of the F2 generation also displayed similar results. Monocytes and macrophage-like cells appear to be very sensitive to locoweed-induced changes [17]. Because the spleen contains lymphoreticular tissue, the immune cells in the spleen may also accumulate swainsonine. The macrophages and reticular cells in the spleen and other lymphoid tissues are some of the first cells to develop the characteristic vacuolation associated with locoweed poisoning [18, 19]. This pathological change is initially observed in these tissues because they accumulate swainsonine more readily than other tissues. The ability of these tissues to

accumulate swainsonine primarily depends on differences in metabolism, and swainsonine exhibits varying affinities for different types of mannose [20–22]. Swainsonine is water-soluble and rapidly distributes into many parts of the body. In a previous study, swainsonine concentrations varied widely in different tissues and organs of sheep that ingested locoweed [23]. When livestock graze upon swainsonine-containing plants, the molecule accumulates at high concentrations in certain tissues and organs, which might impair their function and cause the typical symptoms associated with swainsonine poisoning.

Natural and experimental long-term ingestion of locoweed causes serious dysfunction in the reproduction of livestock (cattle, sheep, horses, and goats) in both males (mating behavior and libido) and females (failure to conceive, early embryo loss, or abortion) [24]. Ingestion of locoweed also decreases serum progesterone concentrations and subsequently disrupts ovarian function. This is accompanied by delayed estrus cycle, increased estrous cycle length, delayed conception, and abortion [25]. The weights of the ovaries and uterus for mice in groups I, II, and III were significantly higher compared to those of group IV for the F0 and F1 generations. However, the weights of the ovaries and uterus for mice in groups I, II, and III were significantly lower compared to those of group IV for the F2 generation. This result indicates that the ovaries and uterus of pregnant female mice are enlarged after swainsonine treatment, while those of nonpregnant female mice become smaller after swainsonine treatment. Therefore, swainsonine has a significant impact on the uterus and ovaries before and after pregnancy. We hypothesize that swainsonine causes shrinking of the ovaries and uterus, leading to disordered estrous cycle and decreased copulation and fertility rates before the pregnancy period. The higher concentrations of swainsonine significantly inhibited production of progesterone in a concentration- and time-dependent manner. The inhibition of progesterone production can be attributed to a reduction in luteal cell viability [26]. Flow cytometry and DNA fragmentation analyses indeed confirmed that higher concentrations of swainsonine inhibited luteal cell growth due to induction of cell cycle arrest and apoptosis [27]. Because swainsonine affects the estrus cycle, which leads to estrus dysfunction, the numbers of mating pairs displaying successful copulation for mice in groups I, II, and III were fewer compared to those of group IV for the F0 and F1 generations. The fertility index and gestation index for mice in groups I, II, and III of the F0 generation were significantly higher compared to those of the F1 generation. The number of pregnant female mice decreased with higher accumulations of swainsonine in the body. Furthermore, swainsonine caused several abnormalities, such as enlargement of the ovaries and uterus after the pregnancy period. We hypothesize that swainsonine will reduce the ability of female mice to conceive a second time, but there have been no reports of this thus far.

Feeding locoweed to pregnant ewes induces fetal cardiac dysfunction, delayed placentation, reduced placental and vascular development, hydrops amnii, abnormal cotyledonary development, interruption of fetal fluid balance, and abortion [28]. We showed that groups I, II, and III of the F0 and F1 generations of pregnant female mice birthed live pups, but the pups were weak or dead for groups I, II, and III of the F0 and F1 generations. The number of live pups on PND 15 for groups I, II, and III was significantly lower compared to those in group IV for the F0 and F1 generations. The body weights of the live pups on PND 15 of groups I, II, and III for F0 were higher than those of the F1 generation mice. Locoweed causes embryonic and fetal death, abortions, generalized reproductive dysfunction, and occasional birth defects [29]. In this study, the fetuses underwent autolysis, and the number of live pups on PND 0 for groups I, II, and III was lower than that of group IV for the F0 and F1 generations. Swainsonine exposure in utero also had severe effects on fetal goats, and the ultrasound observations were consistent with observed progesterone levels [30]. It is postulated that this cytoplasmic vacuolation interferes with the transport of nutrients across the placental barrier. Vacuolation itself may be associated with the death of the conceptus and subsequent abortion. A contributing factor may be that luteal cells of the ovary are also extremely vacuolated in ewes poisoned by locoweed and may not be producing sufficient progesterone to maintain pregnancy [31].

In conclusion, severe reproductive and developmental toxicities are associated with swainsonine poisoning. Specifically, swainsonine accumulation in the body prolonged the estrous cycle, decreased the number of live pups obtained on PND 0, 4, and 15, and reduced copulation and fertility indices in mice. Furthermore, the weights of the spleen significantly increased, and the body weights significantly decreased for mice in groups I, II, and III for the F0, F1, and F2 generations. Cumulatively, swainsonine toxicity significantly disrupts reproductive performance in the parent and offspring.

Competing Interests

The authors declare that there are no competing interests.

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Research Article

Human Elimination of Organochlorine Pesticides: Blood, Urine, and Sweat Study

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Background. Many individuals have been exposed to organochlorinated pesticides (OCPs) through food, water, air, dermal exposure, and/or vertical transmission. Due to enterohepatic reabsorption and affinity to adipose tissue, OCPs are not efficiently eliminated from the human body and may accrue in tissues. Many epidemiological studies demonstrate significant exposure-disease relationships suggesting OCPs can alter metabolic function and potentially lead to illness. There is limited study of interventions to facilitate OCP elimination from the human body. This study explored the efficacy of induced perspiration as a means to eliminate OCPs. *Methods*. Blood, urine, and sweat (BUS) were collected from 20 individuals. Analysis of 23 OCPs was performed using dual-column gas chromatography with electron-capture detectors. *Results*. Various OCPs and metabolites, including DDT, DDE, methoxychlor, endrin, and endosulfan sulfate, were excreted into perspiration. Generally, sweat samples showed more frequent OCP detection than serum or urine analysis. Many OCPs were not readily detected in blood testing while still being excreted and identified in sweat. No direct correlation was found among OCP concentrations in the blood, urine, or sweat compartments. *Conclusions*. Sweat analysis may be useful in detecting some accrued OCPs not found in regular serum testing. Induced perspiration may be a viable clinical tool for eliminating some OCPs.

1. Introduction

Organochlorinated compounds have been used globally for many years as solvents, fumigants, and insecticides. In the 1930s, Swiss chemist and Nobel Prize recipient Paul Muller first discovered that the most well-known organochlorine agent dichlorodiphenyltrichloroethane (DDT) had significant insecticide properties [1]. Since then, this pest control agent has been utilized around the world to prevent the transmission of many vector-born diseases including malaria and typhus [2]. Alongside DDT, several other organochlorinated pesticides (OCPs) were subsequently developed and were also utilized to control various pests. With unfolding toxicological research, however, evidence of potential human health risks associated with exposure to these agents began to appear. Like many OCPs, it was eventually confirmed that DDT elicited toxic effects that harmed nontarget species, bioaccumulated in the animal food chain, and had a very slow rate of environmental degradation. As a result, the use of DDT was eventually banned in the United States in 1972 [3].

Due to the ability of OCPs to accumulate in body tissues, their long half-life of elimination from the body, and emerging evidence of potential toxicity to human health, many countries throughout the world went on to ban many of the agents within the OCP family [4]. The sequelae of the OCP experiment, however, continue to linger as human contamination with these chemical compounds is still evident throughout the globe. Various factors are contributing to this reality, including the following: (i) the ongoing use of OCPs in some countries, (ii) the persistence of these agents within the environment and within the human body, (iii) the widespread use of international travel resulting in potential OCP exposure when visiting or relocating to jurisdictions

Table 1: Organochlorine pesticide groupings based on chemical structure.

Group	Constituents
(i) DDT and analogues	DDT DDE DDD Methoxychlor
(ii) Hexachlorobenzene	Hexachlorobenzene
(iii) Hexachlorocyclohexane	α-HCH, β-HCH, δ-HCH, and γ-HCH
(iv) Cyclodiene	Endosulfan I and endosulfan II Heptachlor Aldrin Dieldrin Endrin
(v) Chlordecone, Kelevan, and Mirex	Chlordecone, Kelevan, and Mirex
(vi) Toxaphene	Toxaphene

where OCPs are still used, (iv) the global exchange of potentially contaminated foodstuffs originating from jurisdictions where OCPs are still used, and (v) the potential for vertical transmission of OCPs from contaminated mothers to offspring during pregnancy and lactation [5]. As a result, OCPs continue to be found in individuals and population groups, including infant children. Individuals contaminated with OCP compounds remain at risk for adverse health consequences and some will potentially pass on these agents to subsequent generations through vertical transmission.

From a clinical perspective, the question remains about how to assess and manage people who have health issues related to xenobiotic contamination [6–8]. As many persistent toxicants including OCPs can sequester and accrue in tissues rather than remaining in the blood compartment [9], biomonitoring for such chemical agents through unprovoked blood and urine analysis does not necessarily reflect the actual body burden of these chemicals [10]. Furthermore, as persistent organochlorine compounds have been associated with myriad health risks, it might be possible to diminish the risk of adverse health sequelae and transgenerational spread of OCPs if means were identified to facilitate elimination of these agents from the human body altogether.

Existing medical literature suggests that elimination of organochlorine compounds and other persistent chemical agents from the human body can have significant clinical benefit in ameliorating health problems [10–12]. Accordingly, the value of establishing interventions to facilitate elimination of toxic chemical agents is thus apparent. In this research study, we provide an overview of literature linking some OCPs with potential human health effects, we endeavor to demonstrate that routine blood testing for OCPs may be inadequate for biomonitoring body burdens, and we provide evidence that transdermal depuration through perspiration facilitates elimination of various OCP compounds.

2. Background

OCPs are lipophilic contact insecticides; they have low vapour pressures and slow rates of environmental degradation. These properties make them highly penetrable, long lasting, and extremely effective pesticide agents [13]. These properties also contribute to bioaccumulation within the human organism by the ready absorption into the body and subsequent deposition into adipose tissue. Like medications, the toxicity of OCPs is related to pharmacokinetics, bioavailability, and the molecular topology which is, in turn, related to their molecular size, volatility, and lipophilicity. These factors determine the absorption, distribution, metabolism, excretion, and toxicity (ADMET) of each chemical agent. Additionally, toxicity also depends on the age, nutritional status, and innate detoxification capacity of the host as well as the frequency, intensity, and nature of the toxic exposures [14]. Based on their chemical structures, OCPs can be grouped into 6 categories (Table 1). Similar chemical structures within each group account for the similar chemical properties and comparable ADMET outcomes.

Uptake of OCPs into the human body may occur by ingestion, by inhalation, by vertical transmission, or by transdermal mechanisms. In addition, exposure to OCPs in combination with other pollutants, like inorganic toxic elements, may facilitate an additive or synergistic effect [15]. An interesting study of 702 adults over the age of 70 years, for example, reported significantly higher mortality levels in smokers in the 2nd or 3rd tertile of OCP blood levels but not among smokers in the lowest tertile of exposure [16].

In clinical practice, patients presenting with the sequelae of toxicant exposures are often challenging and misdiagnosed as "they will seldom present with a history of toxic exposure; their symptoms are multisystem and multifactorial; and the findings of the physical exam may provide little confirmation of the intake" [17]. Additionally, there is limited research on the full long-term impact of OCPs and other accrued toxicants within the human body. As a result of the unfolding health problems associated with bioaccumulation of adverse agents [18, 19] and the likelihood of vertical transmission [20] and potential transgenerational impact [21], however, there is emerging attention to the development of effective interventions to facilitate elimination of toxic compounds [6, 22].

To date, it has been realized that organochlorine insecticides exert pathobiological impact including carcinogenicity, neurotoxicity, hormone disruption, and other toxic effects [5, 23–28]. Some studies have linked OCP exposure to significantly higher rates of cancers of the breast, liver, testicles, and lung, as well as sarcoma and non-Hodgkin's lymphoma [26, 29]. OCP exposure has also been linked to higher rates of endometriosis in women [28] and increased risk for diabetes and obesity [30–32], as well as higher risk for neurological disorders like Parkinson's disease [33, 34]. Studies with children and adolescents have linked OCP exposure to neurological and psychiatric sequelae including abnormal reflexes, reduced cognitive development, depression, and behavioral problems [35]. The potential to prevent and/or treat such conditions by identifying individuals with a body

burden of these chemical agents, and then treating them to facilitate elimination of their accrued load of toxicants, is worthy of exploration and further research.

2.1. Biochemical and Pathophysiological Mechanisms of Harm. Although designed as acute neurotoxins, OCPs have been found to dysregulate human metabolic processes through several different pathways. These pathophysiological mechanisms include mitochondrial damage [24, 36], oxidative stress [37], cell death [38], endocrine disruption [39], and epigenetic modification [40, 41]. As discussed, there is also evidence that concomitant contamination with OCPs along with other toxic chemical agents may exhibit synergistic effects [15].

Additionally, OCPs also exhibit synaptic dysregulation by altering the cation channels (e.g., sodium) at the synapse in nerve cells [42]. Although this effect establishes their efficacy against insect pests, the similarity between organismal synaptic cation channels between species results in nonspecificity and inadvertent harm to nontarget organisms [43]. Another potential pathway of harm is the recently established mechanism entitled "toxicant induced loss of tolerance" (TILT) [44]. This pathophysiologic mechanism resulting in human multimorbidity is characterized by a toxicant burden (e.g., persistent OCPs) resulting in impaired tolerance and hypersensitivity [45]. When triggered by an antigenic incident from the environment (e.g., pollen, chemical agent, or food), an immune response ensues potentially producing antibodies and proinflammatory cytokines. A multisystem response resulting in a condition entitled "Sensitivity Related Illness" (SRI) is often the result [44]. Unfolding research continues to expound on various pathophysiological mechanisms of damage to the human organism.

2.2. Human Exposure. There is mounting evidence that individuals and population groups are currently being exposed to a multitude of different types of chemical toxicants, some of which may accrue within the body. The Centers for Disease Control, for example, recently released a study confirming accrual of multiple pollutants in most American adults and children [46]. In a similar nation-wide epidemiological study in Canada, Health Canada also reported on an assortment of stockpiled chemical toxicants within the general population [47].

An issue of particular concern is fetal and early life exposure to xenobiotics at critical times of development as a result of maternal exposure and bioaccumulation [48]. Although OCPs were not specifically measured, a recent cord-blood study revealed an accumulation of various toxicants in neonates at birth [49]. Studies on OCPs within lactating women have confirmed OCP levels in some women that exceed Health Canada's tolerable daily intake (TDI) guidelines [50]. The potential clinical sequelae of such accrual within reproductive-aged women can be seen, for example, in a large observational study examining specific OCP exposures (dicofol and endosulfan) [51]. This research suggests a correlation of increased risk of autistic spectrum disorder based on the distance between maternal residence and the location of pesticide application [51]. Such emerging work on gestational and lactational exposures led to the recent "Special Communication" by the International Federation of Obstetrics and Gynecology [20] (an organization which oversees much of the maternity care throughout the world) in an effort to bring global attention to the reality of widespread vertical transmission of toxicants and the myriad pediatric health problems resulting from maternal xenobiotic pollution.

Although restrictive policies have been implemented for OCP usage in many jurisdictions, these agents continue to be found within individuals and population groups. This is, as discussed, due to continued usage in some areas of the world, vertical transmission to offspring, and persistence within the human organism. Why do they persist? Following exposure, OCP compounds are dechlorinated and conjugated in the liver where biliary excretion is the main mechanism for elimination. However, organochlorine compounds are reabsorbed to some degree in the enterohepatic circulation and this recycling phenomenon accounts for the persistence within the human body [52].

As a result of such persistence and the lipophilic nature of OCPs, these chemical agents tend to store and bioaccumulate in adipose tissues. Although the complete range of toxicity relating to OCP exposure and bioaccumulation is not fully understood and remains somewhat controversial, many adverse sequelae have been linked to exposure to such agents as previously mentioned. Accordingly, interventions are required to assist in facilitating elimination of OCPs, in order to prevent or overcome adverse clinical sequelae resulting from the potential physiological disruption caused by the enduring presence of these toxicants.

Thus far, there has been limited work on interventions to facilitate elimination of OCPs [7, 52–55]. Schnare et al. examined the use of induced perspiration as a means to expedite elimination of PCBs, PBBs, and OCPs [56]. Their work confirmed that enhanced mobilization and excretion via induced perspiration reduced the body burden of hexachlorobenzene (HCB) and 10 polychlorinated biphenyl congeners [57]. Removal of organochlorine compounds has also been facilitated by specific interventions which interrupt the enterohepatic circulation [7, 52, 58]. The main purpose of this study is to determine whether induced perspiration can be used clinically to facilitate decorporation of the range of both parent OCPs as well as their metabolites.

3. Materials and Methods

3.1. Participant Recruitment. Nine males and 11 females with mean ages of 44.5 ± 14.4 years and 45.6 ± 10.3 years, respectively, were recruited to participate in this study after appropriate ethical approval was received from the Health Research Ethics Board of the University of Alberta. 10 participants were patients with various clinical conditions and 10 were otherwise healthy adults. Participants with health issues were recruited from the first author's clinical practice by invitation and both healthy and sick individuals were selected as samples of convenience by availability, desire to participate, and ease of contact. Each participant in the study provided informed consent and volunteered to give one 200 mL

Table 2: Participant demographics and general clinical characteristics.

Participant	Gender	Age	Clinical diagnosis	Technique used for sweat collection
1	M	61	Diabetes, obesity, hypertension	Exercise
2	F	40	Rheumatoid arthritis	Steam sauna
3	M	38	Addiction disorder	Steam sauna
4	F	25	Bipolar disorder	Steam sauna
5	F	47	Lymphoma	Steam sauna
6	F	43	Fibromyalgia	Steam sauna
7	F	48	Depression	Steam sauna
8	F	40	Chronic fatigue	Infrared sauna
9	F	68	Diabetes, fatigue, obesity	Steam sauna
10	M	49	Chronic pain, cognitive decline	Exercise
11	M	53	Healthy	Exercise
12	M	23	Healthy	Infrared sauna
13	M	21	Healthy	Infrared sauna
14	F	47	Healthy	Infrared sauna
15	M	53	Healthy	Infrared sauna
16	F	43	Healthy	Infrared sauna
17	F	51	Healthy	Infrared sauna
18	M	46	Healthy	Infrared sauna
19	M	57	Healthy	Infrared sauna
20	F	50	Healthy	Infrared sauna

random sample of blood, one sample of first morning urine, and one 100 mL sample of sweat. Demographic and clinical characteristics of all research participants are provided in Table 2.

3.2. Samples Collection. All blood samples were collected at one DynaLIFE laboratory site in Edmonton, Alberta, Canada, with vacutainer blood collection equipment (BD Vacutainer, Franklin Lakes, NJ 07417, USA) using 21-gauge stainless steel needles which were screwed into the "BD Vacutainer One-Use Holder" (REF 364815). The 10 mL glass vacutainer was directly inserted into the holder and into the back end of the needle. This process and the use of glass blood collection tubes were used to prevent contamination. Blood was collected directly into plain 10 mL glass vacutainer tubes, allowed to clot, and after 30 minutes were centrifuged for 10 minutes at 2,000 revolutions per minute (RPM). After serum was separated off, samples were picked up by ALS laboratories (about 3 kilometres from the blood collection site) for storage pending analysis. When received at ALS, serum samples were transferred to 4 mL glass vials and stored in a freezer at −20°C, pending transfer to the analytical laboratory.

For urine collection, participants were instructed to collect a first morning midstream urine sample directly into a provided 500 mL glass jar container with Teflon-lined lid on the same day that blood samples were collected. Urine samples were delivered by the participants directly to ALS laboratories, Edmonton. Samples were transferred to 4 mL glass vials and stored in a freezer at −20°C, pending transfer.

For sweat collection, participants were instructed to collect perspiration from any site on their body directly into the provided 500 mL glass jar container with Teflon-lined lid, by placing the jar against their prewashed skin (with toxicant-free soap, water, and nonplastic brush) when actively sweating or by using a stainless steel spatula against their skin to transfer perspiration directly into the glass jar. Stainless steel, made up primarily of iron, chromium, and nickel, was chosen as it is the same material as the needles used in standard blood collections and is reported not to off-gas or leach at room or body temperature. All but one of the participants (19/20) provided 100 mL of sweat. Each of the glass bottles used for sampling in this study was provided by ALS laboratories and had undergone extensive cleaning and rinsing. The containers were deemed appropriate for sweat collection with negligible risk of contamination: laboratory-grade phosphate-free detergent wash; acid rinse; multiple hot and cold deionized water rinses, oven dried, capped, and packed in quality-controlled conditions.

Sweat was collected within 1 week before or after collecting the blood and urine samples. No specifications were given as to how long sweating had commenced before collection. 10 participants collected sweat inside a dry infrared sauna; 7 collected sweat inside a steam sauna, and 3 collected sweat during and immediately after exercise; no specific instruction was given regarding the type or location of exercise. Participants were educated about the research and were asked to meticulously avoid exposure to any potential sources of toxicants around the time of collection. Sweat was delivered by the participants directly to ALS laboratories. Samples were transferred to 4 mL glass vials and stored in a freezer at −20°C, pending analysis. No preservatives were used in the jars provided for sweat and urine collection nor in the serum storage vials.

Table 3: Organochlorine pesticides analyzed.

Group	Parent	Metabolite
DDT and analogues	DDT	DDE, DDD
	Methoxychlor	NA
Hexachlorobenzene	Hexachlorobenzene	NA
Hexachlorocyclohexane	α-BHC, β-BHC, δ-BHC, and γ-BHC	NA
Cyclodiene	Endosulfan I, endosulfan II	Endosulfan sulfate
	Aldrin	Dieldrin
	Endrin	Endrin ketone
		Endrin aldehyde
	Heptachlor	Heptachlor epoxide
	cis-chlordane, *trans*-chlordane	*trans*-nonachlor
Chlordecone*, Kelevan*, and Mirex	Mirex	NA
Toxaphene*		

*Did not test for this specific compound in this study.

3.3. Laboratory Method Description. Of the 6 categories of OCPs, 5 were analyzed (Table 3, toxaphene was not included in this study). Organochlorine pesticide concentrations of parent and metabolite compounds were analyzed using dual-column gas chromatography with electron-capture detectors (GC-ECD). A pair of surrogate standards, tetrachlorometaxylene (TCMX) and decachlorobiphenyl (DCB), were used to monitor the performance of the method.

The methodology for determining the selected organochlorine pesticides was as follows. Serum samples were weighed into glass tubes (8 g) and 8 mL of methanol was added to the serum samples. Sweat and urine samples were weighed into glass tubes (5 g) and 5 mL of methanol was added to each of the samples. Serial extraction of the bioactive compounds was performed on serum, sweat, and urine samples 3 times by adding 12 mL of an ethyl ether : hexane solution (1 : 1, v/v) and removing the supernatant via centrifugation. The extract was then put through a sodium sulfate column to dry. The resulting extracts were combined and concentrated to 1 mL and put through a 12 g, 2% deactivated florisil column. Florisil was used to eliminate coeluting chlorophenols. External standard calibration was used for quantification. Method blanks were used to ensure quality control, water and calf serum samples. Instrument detection limits were determined to be 0.10 μg/kg. Pentachloronitrobenzene (PCNB) was added to the extracts as an internal standard and the samples were analyzed by dual-column gas chromatography with electron-capture detectors (DB-5 and DB-1701).

4. Results and Discussion

The results of the study can be found in Tables 4 and 5. The parent OCP compounds showed differential detection (Tables 4(a)–4(c)) and compounds detected in at least one participant's serum sample include aldrin, DDT, endosulfan I and endosulfan II, heptachlor, mirex, hexachlorobenzene, α-HCH, β-HCH, and γ-HCH. Endrin, δ-HCH, and methoxychlor were not detected in any of the participant's serum samples. The parent compounds detected in at least one participant's urine sample include aldrin, DDT, endosulfan I and endosulfan II, endrin, mirex, γ- HCH, and methoxychlor. Heptachlor, α-HCH, β-HCH, δ-HCH, and hexachlorobenzene were not detected in any participant's urine sample. All of the parent compounds except the isomers of hexachlorocyclohexane were detected in at least one participant's sweat sample.

The metabolite compounds also showed differential detection. The metabolite compounds detected in at least one participant's serum sample include DDE, endrin ketone, endrin aldehyde, and heptachlor epoxide. The metabolites, DDD, endosulfan sulfate, and dieldrin, were not detected in any serum samples. The metabolite compounds detected in at least one participant's sweat sample include DDE, DDD, endosulfan sulfate, dieldrin, and endrin ketone. Neither endrin aldehyde nor heptachlor epoxide was detected in any sweat samples. The metabolite compounds detected in at least one participant's urine sample include DDD, endosulfan sulfate, dieldrin, endrin aldehyde, and heptachlor epoxide. The metabolites DDE and endrin ketone were not detected in any urine samples.

Of the parent compounds, DDT, methoxychlor, and endrin as well as the metabolites DDE, DDD, and endosulfan sulfate appear to be readily excreted into sweat as they are found in over half of the participants examined (Table 5). Additionally, with the exception of DDE, these OCPs were not readily detected in blood testing while still being excreted and identified in sweat. This may indicate that these OCP compounds are stored and sequestered in tissues, not evident in blood testing, but are mobilized and excreted during perspiration. In contrast, endosulfan I was almost exclusively detected in urine samples of over half of the participants (Table 5). Collectively, the findings suggest that these participants have been commonly exposed to DDT, methoxychlor, endrin, and endosulfan I and are also carrying a body burden of the metabolites DDE, DDD, and endosulfan sulfate.

Table 4: (a) Distribution of parent compounds in serum (SE), sweat (SW), and urine (U) (μg/kg). (b) Distribution of parent compounds in serum (SE), sweat (SW), and urine (U) (μg/kg). (c) Distribution of parent compounds in serum (SE), sweat (SW), and urine (U) (μg/kg).

(a)

	SE Aldrin	SW Aldrin	U Aldrin	SE DDT	SW DDT	U DDT	SE Endosulfan I	SW Endosulfan I	U Endosulfan I
n^*	2	5	2	2	14	3	1	1	13
Mean**	—	0.15	—	—	0.49	—	—	—	0.14
Median**	—	0.14	—	—	0.42	—	—	—	0.14
Std. Dev.**	—	0.028	—	—	0.23	—	—	—	0.018
Range	0.17–0.66	0.10–0.17	0.10–0.15	0.22–0.23	0.27–1.1	0.19–0.39	0.11	0.12	0.12–0.18

*n represents the number of participants with a detectable amount of the OCP from the 20 total participants examined.
**For matrices having fewer than 5 individuals with detectable OCP levels, the mean, median, and standard deviation measurements were not provided.

(b)

	SE Endrin	SW Endrin	U Endrin	SE Heptachlor	SW Heptachlor	U Heptachlor	SE Mirex	SW Mirex	U Mirex
n^*	0	13	1	8	1	0	2	3	2
Mean**	—	0.18	—	0.37	—	—	—	—	—
Median**	—	0.15	—	0.33	—	—	—	—	—
Std. Dev.**	—	0.083	—	0.17	—	—	—	—	—
Range	—	0.11–0.18	0.12	0.15–0.67	0.11	—	0.12–0.18	0.10–0.17	0.11–0.16

*n represents the number of participants with a detectable amount of the OCP from the 20 total participants examined.
**For matrices having fewer than 5 individuals with detectable OCP levels, the mean, median, and standard deviation measurements were not provided.

(c)

	SE Methoxychlor	SW Methoxychlor	U Methoxychlor	SE Hexachlorobenzene	SW Hexachlorobenzene	U Hexachlorobenzene
n^*	0	16	3	6	1	0
Mean**	—	0.26	—	0.29	—	—
Median**	—	0.22	—	0.30	—	—
Std. Dev.**	—	0.13	—	0.11	—	—
Range	—	0.13–0.70	0.14–0.19	0.18–0.49	0.11	—

*n represents the number of participants with a detectable amount of the OCP from the 20 total participants examined.
**For matrices having fewer than 5 individuals with detectable OCP levels, the mean, median, and standard deviation measurements were not provided.

Most of the compounds examined were detected in less than half of the participants regardless of the matrix (Table 5). This likely reflects a reduced exposure level to these compounds. However, hexachlorobenzene, heptachlor, and endrin ketone were detected in at least 30% of participants and were all predominantly detected in serum samples (Table 5). Although some compounds were detected in participant urine, these levels typically occurred around the limit of detection and at much lower levels than either serum or sweat analysis. With the exception of endosulfan I, this suggests that urine analysis is not reliable as an analytic tool to measure the body burden of these compounds.

The partitioning of these compounds in the body seems to be related to their lipophilicity. With the exception of endosulfan I and its metabolite endosulfan sulfate, all of the compounds detected in over half of the participants have $\log K_{ow}$ values above 5 (Table 6). This indicates that they are highly lipophilic and fat soluble and are expected to sequester in fat tissue. In comparison to the compounds found predominantly in sweat, hexachlorobenzene and heptachlor have reportedly similar degrees of lipophilicity but were predominantly found in blood (Tables 4(a)–4(c)). Therefore, these findings may suggest that lipophilicity is not the only factor affecting the partitioning of these compounds.

There are limitations to the study and to interpretation of the results. There has been discussion in lay circles that differing means of thermal depuration by varying types of sauna (such as infrared sauna versus steam sauna), exercise, or other perspiration induction activities may result in differences in excretion rates through skin. This study did not have an adequate data set to control for potential differences in the excretion of the range of OCPs between usages of far-infrared sauna, regular sauna, or exercise. Several other issues may impact the reliability and reproducibility of perspiration analysis that were not considered including body site of sampling, timing of collection while perspiring, differences in skin characteristics between individuals, temperature and humidity of the surroundings, and factors such as diet,

Table 5: Percentage of individuals with detection of organochlorine pesticides within serum, sweat, and urine ($n = 20$).

	Serum	Sweat	Urine
4,4′-DDT	10	70	15
4,4′-DDE	95	70	0
4,4′-DDD	0	65	35
Methoxychlor	0	80	15
Endosulfan I	5	5	65
Endosulfan sulfate	0	85	10
Endrin	0	50	5
Aldrin	10	25	10
Dieldrin	0	15	10
trans-nonachlor	0	5	0
BHC (hexachlorobenzene)	30	5	0
α-BHC	10	0	0
β-BHC	15	0	0
δ-BHC	0	0	0
γ-BHC	1	0	1
Endosulfan II	10	10	5
Endrin ketone	35	5	0
Endrin aldehyde	5	0	5
Heptachlor epoxide	5	0	15
Heptachlor	40	5	0
cis-chlordane	5	5	0
trans-chlordane	10	15	10
Mirex	5	15	15

pharmaceutical intake, and supplement use. Accordingly, attempts to accurately quantify the relative amount of toxicants released into perspiration are limited.

Areas for future research in relation to induced perspiration might include an examination of (i) differences in rates of excretion into perspiration of assorted xenobiotics from diverse sites in the body, (ii) whether there is diurnal variation in the toxicant content of perspiration, (iii) whether hydration status affects the xenobiotic concentration of perspiration, (iv) dermal reabsorption potential of toxicants after perspiration, and (v) the release of xenobiotics in those in fasting states compared to those without caloric restriction.

5. Conclusion

As DDT, DDE, DDD, methoxychlor, endosulfan sulfate, and endrin appear to be readily excreted into sweat, induced perspiration appears to be a potential clinical tool to diminish the body burden of these agents. With the exception of DDE, however, these agents are not readily detected in blood testing. This suggests that common blood analysis may not truly represent the body burden of these compounds. As the routine use of unprovoked blood testing may thus be inadequate for biomonitoring body burdens of OCPs, there may be clinical advantages to the induction of perspiration through methods like sauna and/or exercise in order to collect samples for biomonitoring and diagnosis of many retained OCP compounds.

Table 6: Octanol/water partition coefficient of key OCPs [65–70].

Compound	$\log K_{ow}$
Aldrin	6.50
Endrin	5.2
Endosulfan I	3.83
Endosulfate	3.66
p,p′-DDT	6.91
p,p′-DDE	6.51
p,p′-DDD	6.02
Methoxychlor	5.08
Heptachlor	6.10
Hexachlorobenzene	5.73

In conclusion, the previous four papers in this "blood, urine, and sweat (BUS)" series have demonstrated that induced perspiration is effective at facilitating the removal of many toxic elements as well as various organic compounds, but not all [59–62]. This OCP study provides evidence that transdermal depuration through perspiration facilitates elimination of some parent and metabolite OCP compounds, but not all. While the absolute amount of each OCP compound released into sweat may be small according to this data, an average adult may sweat more than one liter per hour during exercise. Under thermal stress, maximal rates of sweating may be as high as two to four liters/hour [63]; and sweating rates for "acclimatized" people who regularly use saunas may be as high as two liters/hour [64]. Accordingly, regular sessions of induced perspiration should be considered cumulatively as a potential clinical modality to diminish body burdens of many xenobiotics, including OCP compounds.

Additional Points

Key Findings

(a) DDT and/or its metabolite(s) were found in nearly every participant regardless of age suggesting that exposure is very common.

(b) Nearly all organochlorine pesticide (OCP) parent compounds and several metabolites were detected in perspiration suggesting that sweating may be efficacious in diminishing the body burden of many of these toxicants.

(c) There were some parent OCPs, such as endosulfan I and hexachlorobenzene, and some metabolites, such as endrin ketone and heptachlor, that were not readily excreted into perspiration.

(d) Lipophilicity appeared to be a major factor influencing the efficacy of transdermal OCP elimination but not the exclusive determinant.

(e) Only endosulfan I appeared to be predominantly detected in urine suggesting that urine analysis has limited value in detection of retained OCPs.

Competing Interests

There is no conflict of interests regarding the publication of this paper.

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