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REVIEW ARTICLE

Progress and promise of alternative animal and non-animal methods in biomedical research

Irlan Almeida Freires[1] · David Fernando Colon Morelo[2] · Lélio Fernando Ferreira Soares[3] · Isabela Silva Costa[3] · Leonardo Pereira de Araújo[4] · Isadora Breseghello[3] · Henrique Ballassini Abdalla[5] · Josy Goldoni Lazarini[1] · Pedro Luiz Rosalen[1,6] · Suzane Cristina Pigossi[3] · Marcelo Franchin[3,7]

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Abstract

Cell culture and invertebrate animal models reflect a significant evolution in scientific research by providing reliable evidence on the physiopathology of diseases, screening for new drugs, and toxicological tests while reducing the need for mammals. In this review, we discuss the progress and promise of alternative animal and non-animal methods in biomedical research, with a special focus on drug toxicity.

Research Highlights

- Alternative methods can be effectively used to screen for toxic materials/drug dosages.
- The main advantages of the 3D cell culture include a high structural complexity, the simulation of cell-to-cell interactions, and the physiological behavior of cells in tissues.
- Invertebrate animals have been successfully used in scientific experimentation, with some outcomes similar to those observed in mammals.

Keywords 3D cell culture · *Galleria mellonella* larvae · Zebrafish · Brine Shrimp (*Artemia salina*) · Roundworms (*Caenorhabditis elegans*) · Fruit fly (*Drosophila melanogaster*)

✉ Irlan Almeida Freires
irlan.almeida@gmail.com

1 Department of Biosciences, Piracicaba Dental School, University of Campinas, Piracicaba, SP, Brazil

2 Institute of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, University of Bonn, Bonn, Germany

3 School of Dentistry, Federal University of Alfenas, Alfenas, Brazil

4 School of Pharmaceutical Sciences, Federal University of Alfenas, Alfenas, Brazil

5 Laboratory of Neuroimmune Interface of Pain Research, São Leopoldo Mandic Institute and Research Center, Campinas, SP, Brazil

6 Graduate Program in Biological Sciences, Federal University of Alfenas, Alfenas, Brazil

7 Bioactivity and Applications Lab, Department of Biological Sciences, Faculty of Science and Engineering, School of Natural Sciences, University of Limerick, Limerick, Ireland

Introduction

Historically, mammalian models have provided relevant evidence on the pathophysiology of numerous diseases (Andersen and Winter 2019). Animal experiments have been widely used for drug discovery and development by providing highly accurate pharmacological and toxicological evidence before clinical testing (Freires et al. 2017). Meanwhile, the use of mammalian models for scientific experimentation has been questioned for different reasons, including ethical issues, little representativeness of the results due to the low reproducibility of human biology, in addition to high costs (Meigs et al. 2018).

More recently, there have been great advances in the ethical use of laboratory animals with the establishment of the 3Rs rule (Refinement, Reduction, and Replacement). This approach has substantially changed the methodological outlines of scientific research worldwide (Russell and

Burch 1959; Robinson et al. 2019). The 3Rs contribute significantly to reducing the use of animals for testing while encouraging the implementation of alternative animal and non-animal models (Fontana et al. 2021) that are as much more sophisticated and closer to the in vivo condition (Nakamura et al. 2018; Rim 2020; Wang et al. 2021; Khabib et al. 2022).

In this review, we discuss the progress and promise of alternative animal and non-animal methods in biomedical research, with a special focus on drug toxicity. We shed light on the use of 3D cell culture (organoids) and invertebrate animal models, such as *Galleria mellonella* larvae, zebrafish, Brine Shrimp (*Artemia salina*), roundworms (*Caenorhabditis elegans*), and Fruit fly (*Drosophila melanogaster*). Collectively, the evidence gathered herein may support the development and/or implementation of innovative alternative methods that can substantially reduce the need for mammalian models in biomedical research.

3D cell-based assays

Generally, 2D and 3D cell culture models are used to screen for the pharmacological activity of a novel drug and its preliminary toxicological assessment, as well as for the elucidation of cellular pathology and physiology (Ravi et al. 2015; Belfiore et al. 2021). Importantly, cell-based assays have helped reduce the number of mammals in scientific experimentation. The 3D cell culture is more realistic as compared to the 2D model and provides evidence closer to that generated by in vivo models (Ravi et al. 2015). The main advantages of the 3D cell culture include a high structural complexity, the simulation of cell-to-cell interactions, and the physiological behavior of cells in tissues (Moysidou et al. 2021). 3D culture techniques can be sorted into scaffold-based and scaffold-free cultures (Souza et al. 2016). Scaffold-based 3D cultures are generated using natural or synthetic hydrogels that resemble the composition of the ECM (extracellular matrix), enabling cell growth, proliferation, cell-to-cell interaction, and transport of nutrients in a 3D environment. Therefore, 3D-based cultures provide a new plethora of possibilities for drug discovery and toxicological assessments (Bielecka et al. 2017; Kim 2005; Tomas-Bort et al. 2020; Zhang et al. 2020). Natural scaffolds are frequently fashioned with fibrinogen, collagen, gelatine, Matrigel, or hyaluronic acid, and their synthetic counterparts are typically assembled with polyethylene glycol (PEG), polylactic acid (PLA), or poly (vinyl acetate) (PVA) (Jensen and Teng 2020). The techniques for building scaffolds have evolved rapidly, from electrospinning, freeze-drying, and stereolithography to 3D printing and robotic micro-assembly (Lv et al. 2017). As for scaffold-free cultures, cells by themselves create their matrix by accumulating multicellular structures called spheroids (Knight and Przyborski 2015). This particular formation generates hypoxic areas in the center of the spheroids while the proliferative and oxygen-enriched areas on their surface are more similar to what is observed in solid tumors. For these reasons, scaffold-free culture systems have been extensively used in cancer research and drug resistance studies (Knight and Przyborski 2015).

Recent improvements in the field of 3D cell culture have led to the development of a technology called organoids (Fig. 1) (Sato et al. 2009). Organoids are stem cell-derived 3D cultures fashioned by seeding stem cells in a 3D environment in vitro (Bar-Ephraim et al. 2020). Organoids can be generated from different tissues and even species, and their culture condition recapitulates tissue complexity in terms of architecture and cellular composition (Dye et al. 2015; Huch et al. 2013; Lancaster et al. 2013; Mullenders et al. 2019; Tuveson and Clevers 2019). Organoid cultures can be generated from pluripotent stem cells (PSC) or adult

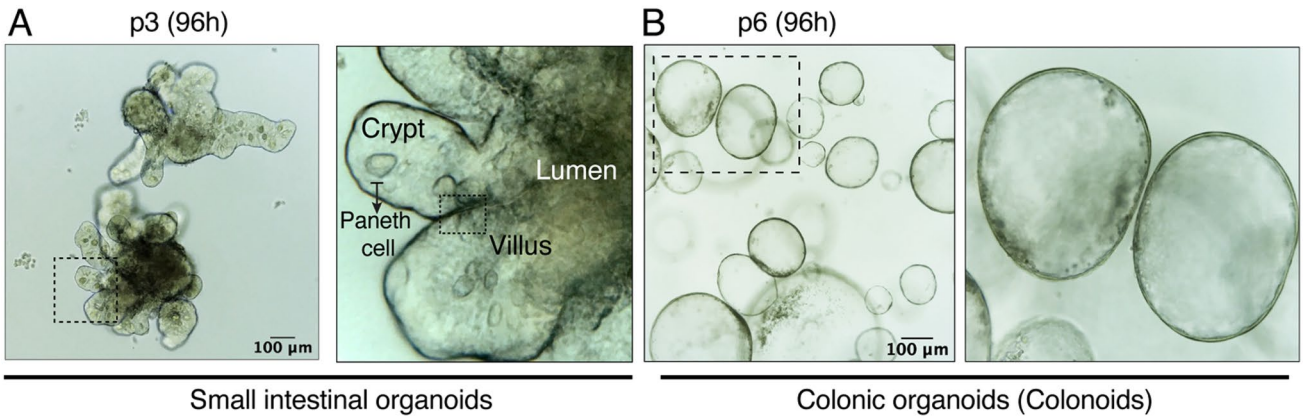

Fig. 1 Organoids derived from primary tissues. **A**, **B** Brightfield images of organoids prepared from the small intestine and colon. Images represent the organoids after 96 h in culture and 3–6 passages, respectively. These images were donated by Dr. David Colón, listed as an author

stem cells (ASCs) maintaining long-term near-native 3D epithelial organization while holding genetic stability and high heterogeneity (Tuveson and Clevers 2019). Furthermore, in contrast to the traditional 2D cell culture, which cannot mimic endogenous structural and physiological features (Jensen and Teng 2020), the versatile feature of organoid cultures, especially patient-derived tumor organoids (PDOs), has afforded a reliable platform for high-throughput drug-screening procedures and drug toxicity testing (Driehuis et al. 2021; Li et al. 2020; Tuveson and Clevers 2019). Indeed, compelling evidence validated the use of patient-derived tumor organoids as a predictive tool for a patient's treatment response whereas normal tissue-derived organoids enabled the screening for drug toxicity testing (Boretto et al. 2019; Driehuis et al. 2021; Fang and Eglen 2017; Huang et al. 2015; Richards et al. 2020; Saito et al. 2019; Vlachogiannis et al. 2018). Likewise, the in vitro response of patient-derived tumor organoids is predictive of patient response to therapy (Ooft et al. 2019; Vlachogiannis et al. 2018; Yao et al. 2020). Remarkably, the organoids technology has been used to generate a collection of patient-derived cultures, recently called "living biobanks" (Van de Wetering et al. 2015), which have opened avenues to predict patient response and embrace great promise for precision and personalized medicine (Grönholm et al. 2021). Lastly, a new dynamic 3D-based culture system, called organ-on-a-chip (OOCs), was developed. This system combines microfluidic-based systems, advanced 3D tissue-engineered constructs with cultured human cells that replicate a human organ of interest such as nephron, proximal tubules, or liver sinusoid (Bhise et al. 2014; Inamdar and Borenstein 2011).

Due to their features to mimic organs, 3D cell-based cultures have enabled cutting-edge advances in the field of toxicology. Lee et al. (2021) established uniformly sized hepatocyte-like cell spheroids (3D-uniHLC-Ss) using the microwell culture approach. Newly generated spheroids exhibited a high expression of hepatic gene markers (CYP2C9, CYP2C19, CYP3A4, and UGT2B7) and showed no significant signs of cell death. With the aid of imaging-based drug toxicity technology, the authors found that hPSC-3D-uniHLC-Ss exhibited enhanced sensitivity to various hepatotoxicants (tamoxifen, sunitinib malate, troglitazone, acetaminophen, cyclosporin A, mefenamic acid, nefazodone, sulindac, Rac-perhexiline maleate, and phenformin HCl) when compared to hepatocyte-like cells differentiated under 2D conditions. These findings indicate that 3D hPSC-derived liver spheroids could be used as an effective tool for high-throughput drug screening that more accurately reflects human-specific drug toxicity (Lee et al. 2021). Likewise, Bircsak et al. (2021) reported the development and validation of a high-throughput 3D microfluidic on-a-chip system (OrganoPlate LiverTox) for hepatotoxicity screening. They tested 159 compound libraries and found that the liver on-a-chip system was sufficiently robust to identify putative hepatotoxins in a high-throughput manner (Bircsak et al. 2021). Furthermore, Suter-Dick et al. (2018) recently fashioned an organotypic culture of human conditionally immortalized proximal tubule epithelial cells overexpressing the organic anion transporter 1 (ciPTEC-OAT1) in a three-channel OrganoPlate under microfluidic conditions. The authors exposed the cultures to four well-known nephrotoxicants (cisplatin, tenofovir, cyclosporine A, and tobramycin), and the NAG release (N-acetyl-beta- D-glucosaminidase). Moreover, a novel panel of four miRNAs (mir-21, mir-29a, mir-34a, and mir-192) was assessed as potential biomarkers of proximal tubule damage. Remarkably, the detection of kidney damage biomarkers and miRNA levels in the culture medium rendered this method very effective for in vitro nephrotoxicity assessments (Suter-Dick et al. 2018). Ishikawa and Ito (2017) demonstrated in a 3D co-culture model of human bronchial epithelium that repeated exposure to cigarette smoke (CS) decreased the number of ciliated cells and goblet cell differentiation. These findings were consistent with a significant increase in pro-inflammatory cytokines such as IL-8, IL-1β, and GM-CSF. Remarkably, the production of these inflammatory mediators was boosted with the repetition of cigarette smoke exposure (Ishikawa and Ito 2017), suggesting that this in vitro 3D model can be useful for toxicity assays.

Table 1 lists studies in which organoids derived from various human tissues were used in toxicological research. In detail, Sgodda et al. (2017) established scalable ESC-derived 3D hepatic organoids which were more sensitive to acetaminophen-induced toxicity than the conventional 2D-cultured ESC-derived hepatic cells. Moreover, Shinozawa et al. (2021) reported the development and validation of a high-fidelity drug-induced liver injury screening system using human pluripotent stem cell-derived organoids. Using this organoid system, they tested 238 marketed drugs at 4 different concentrations. Bile acid production, viability, cholestatic, and mitochondrial toxicity were assessed as readouts. The results revealed high predictivity, with 88.7% sensitivity and 88.9% specificity. Additionally, they demonstrated that liver organoid-based toxicity positively predicts genomic predisposition (CYP2C9 $*$ 2) for bosentan-induced cholestasis, indicating that susceptibilities based on the polymorphism or SNPs can be addressed using organoids (Shinozawa et al. 2021). Furthermore, Archer et al. (2018) created a 3D human cardiac microtissue using suspensions of human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CM), cardiac endothelial cells (hCMEC), and cardiac fibroblasts (hCF) to assess changes in cardiac pathology. The authors evaluated several FDA-approved structural cardiotoxins and non-structural cardiotoxins. They demonstrated that 3D human cardiac microtissues were able to detect changes in cardiac structure at clinically relevant

Table 1 3D cell culture-based toxicity evaluation

Organoid type	Organoid sources	Methods	Compounds tested	Toxicology readout	References
Liver organoid	Pluripotent stem cell (PSC)	PSCs differentiated toward a foregut endoderm progenitor population and then further maturated into hepatic cells, reflecting a midgestational, fetal phenotype by the WNT signaling pathway	Acetaminophen	Viability (WST-1)	Sgodda et al. (2017)
	Induced pluripotent stem cells (iPCS)	Human liver organoid (HLO) generated from storable foregut progenitors from pluripotent stem cell (PSC) lines with reproducible bile transport function	Two hundred and thirty-eight marketed drugs at four different concentrations	Bile acid production, viability (ATP assay), cholestatic and mitochondrial toxicity	Shinozawa et al. (2021)
Cardiac organoid	Human-induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs), cardiac endothelial cells (hCMEC), and cardiac fibroblasts (hCF) hiPSC-CMs, hFCs, HUVECs, hADSCs.	Cell suspensions of hiPS-CM, hCF, and hCMEC at a final cell ratio of 4:2:1, respectively, seeded onto 384 well ultra-low attachment spheroid microplates	Fifteen FDA-approved structural cardiotoxins and fourteen FDA-approved non-structural cardiotoxins	Mitochondrial membrane potential, endoplasmic reticulum integrity, and cellular viability	Archer et al. (2018)
		Organoid cell suspensions comprise 50% hiPSC-CMs and 50% non-myocyte (at a 4:2:1 ratio of FBs, HUVECs, and hADSCs, respectively), seeded in an agarose hydrogel scaffold	Doxorubicin	Contraction and apoptosis	Richards et al. (2020)
Intestinal organoid	Normal adult human ileal small intestinal tissue	Human primary ileal tissue was digested, and intestinal crypts were isolated by passing the chopped tissue sections through a filter mesh, and then suspended in Matrigel and cultured under specialized conditions	Thirty-one diarrheagenic drugs	Viability	Belair et al. (2020)
	Primary human small intestinal epithelial cells and fibroblasts	Primary human small intestinal epithelial cells grown in Transwell plates on a fibroblast layer, allowing apical and/or basolateral drug treatment and 96-well throughput	Thirty-nine commercial drugs	Viability (MTT)	Peters et al. (2019)
Kidney organoid	Human-pluripotent-stem-cell-derived kidney cells (hPSC-KCs)	PSCs were differentiated into segmented, nephron-like kidney organoids by GSK3b, CHIR, RB minus insulin (RBNI), IWP2, featured by proximal tubular, podocyte, and endothelia	Gentamicin and cisplatin	Nephrotoxicity by assessing the biomarker Kim-1	Freedman et al. (2015)

Table 1 (continued)

Organoid type	Organoid sources	Methods	Compounds tested	Toxicology readout	References
	Human umbilical vein endothelial cells (HUVEC), adult renal fibroblasts, and Primary human RPTEC	Cultured renal fibroblasts and HUVEC were combined at a 50:50 ratio, and then printed onto 0.4 µm Transwell clear polyester membrane inserts in a 24-well plate, afterward, primary RPTEC cells were added to the tissues in a suspension	Cisplatin	Viability (LDH) and expression of fibrosis gene markers	King et al. (2017)
	Human pluripotent stem cells (hPCS) and human embryonic stem cells (hESCs)	hPSCs were resuspended in a differentiation medium supplemented with CHIR and FGF9, and placed in 96-well, to further induce peritubular and renal vesicles	Gentamicin and cisplatin	Nephrotoxicity by assessing the biomarker Kim-1	Morizane et al. (2015)

concentrations, even with greater accuracy than 2D-cultured human iPSCs (Archer et al. 2018). Likewise, using a similar cardiac organoid system, Richards et al. (2020) demonstrated that produced organoids can mimic several pathological hallmarks of myocardial infarction (in particular, pathological metabolic shifts, fibrosis, and calcium handling) and can also recapitulate enhanced doxorubicin cardiotoxicity. Intestinal organoids are also used in toxicology studies. Belair et al. (2020) demonstrated that 3D spherical ileal organoids can recapitulate the different degrees of clinical incidence of diarrhea induced by 31 diarrheagenic marketed drugs, using cell viability as a toxicity readout. Interestingly, Peters et al. (2019) demonstrated in a 3D human gastrointestinal microtissue system, that 3D cultures not only are suitable for addressing classical cytotoxicity endpoints, such as ATP and LDH, which are often used in toxicity studies, but can also be used in gut barrier toxicity assays. This 3D system was successfully used as a predictor of drug-induced diarrhea. Furthermore, Freedman et al. (2015) and Morizane et al. (2015) examined the effectiveness of human kidney organoids for toxicity assessment under cisplatin and gentamicin treatment. They observed that kidney organoids were reactive to cisplatin and gentamicin. Nephrotoxic chemical injuries were confirmed by the upregulation of the kidney injury molecule-1 (Kim-1). Similarly, using a 3D proximal tubule tissue culture containing a combination of renal fibroblasts, endothelial cells, and human kidney proximal tubule epithelial cells (PTECs), King et al. (2017) recapitulated key hallmarks of nephrotoxicity after cisplatin treatment, suggesting that 3D kidney organoids provide valuable information for the prediction of drug-induced kidney toxicity in humans.

Alternative animals

Invertebrate animals have been successfully used in scientific experimentation, with some outcomes similar to those observed in mammals (Table 2). In the following sections, we describe some of the most relevant invertebrate models and their application in biomedical research.

Galleria mellonella larvae

Galleria mellonella, also known as honeycomb moth or wax moth, is part of the order Lepidoptera, family Pyralidae and subfamily Galleriinae (Kwadha et al. 2017). It is described worldwide where beekeeping is practiced and lives naturally in hives where it feeds on wax and pollen, causing bee galleriosis (Singkum et al. 2019). *G. mellonella* is used for toxicity studies of new biologically active agents as it is a low-cost, ethical, and reliable method, that provides preliminary evidence of the toxicological profile of new molecules or natural products before conducting pharmacological studies

Table 2 Alternative animal models and their toxicological applications in biomedical research

Model	Methods	Compounds tested	Toxicology readout	Ref
Galleria mellonella larvae	Assessment of acute toxicity for 72 h after administration of compounds and a subfraction	Gibberellin A4 and A7	Larval death (myelination or absence of movement upon touch)	Nani et al. (2022)
	Assessment of systemic toxicity of a purified subfraction (S8) of *Eugenia selloi* for 72 h	*Eugenia selloi* purified subfraction (S8)	Larval death (absence of movement upon touch)	Lazarini et al. (2022)
	Assessment of the relative toxicity of physiologically relevant doses of okadaic acid by direct injection into the hemocoel (body cavity) and/or gavage (force-feeding) for an experimental period of 96 h	Okadaic acid	Larval survival, total hemocyte counts and viability, phenoloxidase activity, REDOX-associated activity, superoxide dismutase activity, and lipid peroxidation	Coates et al. (2019)
	Experimentation to establish the conditions under which indomethacin induces gut damage (0.5–7.5 µg/larva) via intrahemocoelic injection and gavage (force-feeding)	Indomethacin	Larval survival, hemocyte counts and viability, superoxide dismutase activity, glutathione S-transferase (GST) activity, and gut permeability assessments	Emery et al. (2019)
	Assessment of the efficiency of *Galleria mellonella* as a model for examining nanoparticle toxicity using gavage	Silver, selenium, and functionalized gold nanoparticles	LD_{50} calculation, hemocyte proliferation, nanoparticles distribution, behavioral changes, and histological alterations	Moya-Andérico et al. (2021)
Zebrafish	Assessment of the effect of polystyrene microplastics at different concentrations (10 and 100 µg L^{-1})	Polystyrene microplastics	Oxidative stress indices (reactive oxygen species and lipid peroxidation), antioxidant enzymes (catalase activity, superoxide dismutase activity, glutathione peroxidase activity, glutathione S-transferase activity), acetylcholinesterase activity, gene expression, and histological analysis	Umamaheswari et al. (2021)
	Assessment of the effects of acrylamide (10, 30, 100, or 300 mg/L) on the neurotoxicity, and developmental and behavioral toxicity of Zebrafish embryos	Acrylamide	Lethality and developmental toxicity, behavior (locomotor activity), and neurotoxicity	Park et al. (2021)
	Assessment of the effects of ethanol (2–2.5%, v/v) on transgenic [hb9:GFP (green fluorescent protein)] Zebrafish embryos at 28 hpf (h post-fertilization)	Ethanol	Automated fluorescent image acquisition in vivo (axon lengths evaluation)	Kanungo et al. (2011)

Table 2 (continued)

Model	Methods	Compounds tested	Toxicology readout	Ref
Brine Shrimp (Artemia salina)	Assessment of venom and antivenom toxicity profiles after 72 h	Naja ashei venom and antivenoms	Lethality assay	Okumu et al. (2020)
	Assessment of the lethality of two new series of 17a-aza-D-homo -androster derivatives	Twenty-eight novel 17a-aza-D-homo-androster-17-one derivatives	Lethality assay	Hong et al. (2021)
	Assessment of the cytotoxic activity of fourteen novel steroidal C-17 pyrazolinyl derivatives 9a-g and 10a-g	Fourteen novel steroidal C-17 pyrazolinyl derivatives 9a-g and 10a-g	Lethality assay	Fan et al. (2013)
	Assessment of the effects of different concentrations of aluminum oxide nanoparticles after 24 and 96 h of exposure	Alpha (50 nm and 3.5 µm) and gamma (5 nm and 0.4 µm) aluminum oxide nanoparticles	Accumulation, toxicity, and depuration	Ates et al. (2015)
Roundworms (Caenorhabditis elegans)	Investigation of essential oils commonly used in acute, developmental, and reproductive toxicity assays as well as mucous membrane irritation assessments	Rosemary, citrus, and eucalyptus essential oil	Lethality, reproduction, development, reproductive toxicity assays, and gene expression analysis	Lanzerstorfer et al. (2021)
	Assessment of the toxicity effects of uranyl nitrate	Uranyl nitrate	Lethality, reproduction, locomotion, feeding behavior, and stress resistance assays, DAergic neurodegeneration measurement, basal slowing response, and gene expression analysis	Lu et al. (2020)
Fruit fly (Drosophila melanogaster)	Assessment of the interaction between aging, alcohol toxicity, and circadian function	Alcohol vapor	Behavior assays (loss-of-righting reflex, sedation, recovery, and mortality)	De Nobrega et al. (2017)
	Assessment of the toxicity and genotoxicity of different concentrations of native Concanavalin A (4.4, 17.5, and 70 µg/mL)	Mannose/glucose-binding lectin from *Canavalia ensiformis*	Survival assay and locomotor performance, oxidative stress markers, cell viability, lipid peroxidation, free Fe^{2+} content, total protein thiols (PSH) and non-protein thiols (NPSH), nitric oxide, and comet assay	Dos Santos et al. (2022)

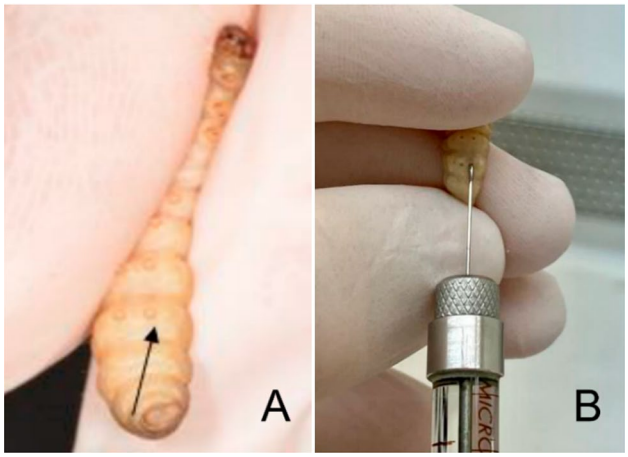

Fig. 2 Administration of a drug into the hemocoel of a *G. mellonella* larva in the last left-side proleg (**A**) using a microsyringe (**B**). These images were donated by Dr Masaharu Ikegaki (UNIFAL/MG)

in other animal models, such as rats or mice (Freires et al. 2017). Larval death is determined based on high melanization or the absence of movement upon touch. The administration of the test compounds is performed into the hemocoel of each larva via the last left leg using a micro syringe (Fig. 2) (Lazarini et al. 2020).

Among the examples of studies that used the *G. mellonella* model for toxicity assessment, Nani et al. (2022) found that two natural plant hormones (Gibberellin A4 and A7) did not show toxicity at the tested doses during the 72-h experimental period. The authors pointed out that the doses injected into *G. mellonella* larvae were extremely high and not consumable by humans, suggesting that consumption of the two natural plant hormones is probably non-toxic to mammals. In another study, Lazarini et al. (2022) evaluated the toxicity of a purified subfraction (S8) of *Eugenia selloi* in *G. mellonella* larvae and demonstrated that the natural product had no toxic effect at the tested doses. These results were essential to further study the anti-inflammatory effects of the subfraction (S8) of *Eugenia selloi* on mice at non-toxic doses. The *G. mellonella* can be also used for marine toxins investigation as demonstrated by Coates et al. (2019). The authors evaluated the relative toxicity of okadaic acid (using physiologically relevant doses) in *G. mellonella.* Okadaic acid is a polyether toxin that causes diarrhetic shellfish poisoning in humans. The results showed that okadaic acid at concentrations ≥ 75 ng/larva reduced larval survival and circulating hemocyte (blood cell) numbers within 24 h post-inoculation. Okadaic acid also reduced hemocyte viability and increased phenoloxidase and superoxide dismutase activity and malondialdehyde (i.e., lipid peroxidation) levels. Emery et al. (2019) also used the *G. mellonella* model in toxicology experimentation to establish the conditions under which indomethacin (0.5–7.5 µg/larva) induced gut damage. Higher levels of gut leakiness were detected by tracking fluorescent microspheres in the feces and hemolymph (blood equivalent) after 4 to 24 h. Moreover, tissue damage was observed in histological sections of the insect midgut, including epithelial sloughing and cell necrosis. Degeneration of the midgut and a significant increase in detoxification-associated activity (superoxide dismutase and glutathione-S-transferase) were also described. Lastly, *the G. mellonella* model was also used for nanoparticle (NP) toxicity assessment. Silver, selenium, and functionalized gold nanoparticles were synthesized and evaluated in *G. mellonella* larvae by Moya-Andérico et al. (2021). The study showed that the toxic effects produced by the NP were efficiently measured by larval indicators including LD50 calculation, hemocyte proliferation, NP distribution, behavioral changes, and histological alterations, confirming the efficiency of *G. mellonella* as a nanotoxicological model.

Zebrafish

Zebrafish (*Danio rerio*) is a small (3–5 cm long) tropical freshwater fish, native to northern India, northern Pakistan, and some regions of southern Asia, that was discovered in the rivers of the northern Himalayas (India). It has distinct linear pigmented stripes which resemble those of the zebra (Katoch and Patial 2021). Zebrafish has been proposed as a potential biological model because it exhibits complex behavioral interactions, ease of genetic manipulation, low maintenance cost, and high yield (Kalue 2017). The use of zebrafish offers several advantages for developmental neurotoxicity testing (DNT) studies (Nishimura et al. 2015), for instance, a range of simple and complex neurobehaviors, such as spontaneous swimming, startle responses, and learning (Farrell et al. 2011; Ingebretson and Masino 2013). Zebrafish can absorb a wide range of chemicals from the environment in which they swim (Diekmann and Hill 2013). Because it is prolific and small, DNT using the zebrafish model can be performed in 96-well plates, which makes it possible to evaluate detailed dose–response relationships over a large number of samples. This increases the statistical power and detects subtle yet significant changes in the fish exposed to low concentrations of neurotoxicants affecting their development (Nishimura et al. 2015). Several DNT assays using zebrafish have been developed, including the assessment of disturbances in gene expression, neurobehavioral profile, and neural morphogenesis through Abagyan.

The modulation of gene expression by exposure to chemicals at subtoxic concentrations can be detected in zebrafish without observable phenotypic changes (Sukardi et al. 2010). Therefore, defining a set of markers related to developmental neurotoxicity and quantifying the expression of these markers may be a rapid and sensitive means of performing DNTs (Nishimura et al. 2015). Umamaheswari

et al. (2021) evaluated the effect of polystyrene microplastics (PS-MPs) at different concentrations (10 and 100 µg L^{-1}) in zebrafish. The results showed that PS-MPs at different concentrations induced reactive oxygen species (ROS) generation and significantly inhibited neurotransmission in zebrafish. In addition, antioxidant genes (*cat, sod1, gpx1a,* and *gstp1*) were altered significantly, and *p53*, *gadd45ba,* and *casp3b* gene expression were upregulated, resulting in apoptosis. The PS-MPs also significantly upregulated *TNF* and *ptgs2a* expression which are essential gene markers in the inflammatory mechanism. Park et al. (2021) evaluated the effects of an industrial chemical called acrylamide (10, 30, 100, or 300 mg/L) on the neurotoxicity, developmental, and behavioral toxicity of zebrafish embryos. The study showed that acrylamide caused developmental toxicity characterized by scoliosis, curvature of the body, yolk retention, and swim bladder deficiency. The compound also reduced locomotor activity, measured as swimming speed and distance traveled. Moreover, 100 mg/L acrylamide shortened the brain and spinal cord width, demonstrating neuronal toxicity.

Another advantage is the use of zebrafish for the evaluation of developmental disturbances by neuroimaging due to the translucent nature of the eggs and embryos. This allows the investigation of changes during various stages of development (Hill et al. 2005). Zebrafish remain transparent from fertilization until 2 dpf (O'Malley et al. 2004), in addition to the existence of zebrafish mutants lacking pigment (Nishimura et al. 2013). These resources allow researchers to evaluate morphological changes in the central and peripheral nervous system induced by chemical exposure using fluorescence technology (Nishimura et al. 2015). Furthermore, transgenic strains expressing fluorescent proteins in specific neuronal subpopulations have been developed (Si 2008). Kanungo et al. (2011) developed a transgenic lineage of zebrafish that expresses green fluorescent protein (GFP) associated with the *hb9* transcription factor (selectively expressed in developing motor neurons in zebrafish). In this protocol, motor neurons were visualized in vivo by neuro-specific expression of GFP under the control of the regulatory sequence of the *hb9* gene. After exposure of the transgenic fish to ethanol during development, axon length reduction was observed in a dose-dependent manner. Similarly, early exposure to ethanol is also consistent with impaired motor coordination in humans (Driscoll et al. 1990; Kalberg et al. 2006).

Zebrafish have also been used in pesticide research (Goncalves et al. 2020). As reviewed by Goncalves et al. (2020), the development of accurate and rapid tests to assess the toxicity of thousands of commercial chemicals is urgent in the face of government decisions around the indiscriminate use of pesticides in countries that depend on the export of agricultural products. In this context, zebrafish have been used at various stages (embryo, larva, adult, cells, tissues, and organs) to evaluate pesticides of different classes (e.g., triazine, organophosphate, pyrethroid), use types (e.g., herbicide, insecticide), and environmental contaminants, either in their form or in combination (Goncalves et al. 2020). Furthermore, the adverse effects commonly evaluated are developmental toxicity, oxidative stress, neurotoxicity, endocrine disruption, behavioral changes, embryotoxicity, and organ toxicity. Secondarily, parameters such as energy metabolism, reproductive toxicity, immunotoxicity, genotoxicity, teratogenicity, and cytotoxicity are also described in the literature reviewed by Goncalves et al. (2020). Thus, this diversity of parameters evaluated consolidates the importance of zebrafish as a versatile model to assess the multitude of effects of pesticides and other toxic substances (Hill et al. 2005).

Brine shrimp (*Artemia salina* L.)

Brine Shrimp (*Artemia* Sp.) are invertebrate, branchiopod crustaceans found in a variety of saltwater ecosystems (Darbyshire et al. 2019). They play an important role in the energy flow of the food chain, being one of the most popular sources of nutrition for many fish and aquatic invertebrates (Sanchez-Fortun et al. 1995). In addition, *Artemia* Sp. has increasingly become a more popular and efficient model for toxicity testing, particularly in acute toxicity testing of toxic materials such as heavy metals and pesticides, as well as nanoparticles, bioactive molecules, and plant-derived natural extracts (Banti and Hadjikakou 2021; Chan et al. 2021; Logarto Parra et al. 2001).

In general, the genus *Artemia* contains six species (El-Magsodi et al. 2005), with *Artemia salina, Artemia franciscana, and Artemia urmiana* being the most commonly used species as a model for studying biological and cytotoxicity activities (Ntungwe et al. 2020). Furthermore, their short life cycle, ease of culture, high pupal production, commercial availability of their cysts, and low cost are additional advantages of using the present model (Adamski et al. 2019; Banti and Hadjikakou 2021; Doke and Dhawale 2015; Ntungwe et al. 2020).

Toxicity studies primarily explore the nauplii stage (planktonic larval stage) of *Artemia*. During this life stage of the crustacean, nauplii exhibit greater sensitivity to toxic agents compared to adult *Artemia* (Trompeta et al. 2019). Thus, determining toxicity by estimating the mean lethal concentration (LC50) can be useful as a rapid and simple test to predict the toxicity of plant extracts and guide their phytochemical fractioning (Lewan et al. 1992; Logarto Parra et al. 2001; Massele and Nshimo 1995). The method is commonly used as a safe approach when studying the antitumor and cytotoxicity activity of natural or synthetic compounds (Zhu et al. 2018). Moreover, among other animal model

assays, the *Artemia* lethality assay is the most user-friendly and efficient (Banti and Hadjikakou 2021; Meyer et al. 1982; Živković et al. 2016), making it a simple and suitable model system (Trompeta et al. 2019). Okumu et al. (2020) determined the enzymatic and toxic activity of Naja ashei venom and the capacity of antivenoms manufactured in different countries. The toxicity profiles of the venom and antivenoms were evaluated in a lethality assay with *Artemia*. Fivefold determinations and the surviving larvae were counted after 24, 48, and 72 h. Thus, the LC50 of the samples indicated that intermediate and high doses of the venom exhibited similar mortality rates and the test sera were generally non-toxic. Hong et al. (2021) evaluated the toxicity and antitumor activity of different compounds synthesized for cancer treatment. The authors indicate that like Fan et al. (2013), the chosen model has been important for different toxicity tests and LC50 estimation, which can be extrapolated to more complex animal models with greater safety. Similarly, Ates et al. (2015) used the test model in investigating the toxicity of aluminum oxide nanoparticles (Al_2O_3 NPs) on marine microorganisms. As *Artemia* are non-selective filtering crustaceans, they can easily ingest fine particles smaller than 50 µm (Hund-Rinke et al. 2006), highlighting *Artemia* also as a robust test model to study the ecological risks of nanomaterials in marine ecosystems. Thus, the main objective of using this aquatic invertebrate and other alternative models applies precisely in the preliminary evaluation of toxicity that can be translated to organisms of greater complexity, such as vertebrates (Freires et al. 2017). Further, studies indicate a correlation between dose–response and LC50 for mice and rats, which enables the future execution of applied toxicity tests with more safety and fewer animals involved (Logarto Parra et al. 2001; Naidu et al. 2014).

Roundworms (*Caenorhabditis elegans*)

Nemathelminths are non-segmented roundworms. As one of the most abundant groups of metazoans, they live in terrestrial or aquatic environments, sediments, and water columns (Bhadury et al. 2006). *Caenorhabditis elegans* is a free-living soil nematode. By becoming a model experimental organism over 20 years ago, *C. elegans* has contributed to the study of biological processes such as apoptosis, gene regulation by RNAi, and the function of microRNAs; being the first metazoan with a sequenced genome (Blaxter 2011; Hunt 2017; Kiontke and Fitch 2013). In addition to studies related to biological and genetic development, the model also shows promise in toxicity studies, with the determination of EC50 and LD50 (Cole et al. 2004; Lanzerstorfer et al. 2021; Leung et al. 2008). Lanzerstorfer et al. (2021) evaluated the toxicological properties of different essential oils used in the pharmaceutical and food industries. For this purpose, a robust investigation was conducted with a combination of in vitro (cell culture) and in vivo (*C. elegans*) analyses. Gene expression by RT-PCR, LC50, and LD50 determination, and other investigations were sufficient to prove the presence of toxic properties of the essential oils investigated. Furthermore, a recent study by Lu et al. (2020) reported the risks and concerns regarding an emerging heavy metal pollutant, depleted uranium. Using *C. elegans* as an alternative animal model for toxicity tests, the authors chronically exposed the worms to the pollutant and described inhibition of the expression of antioxidant genes, degeneration of dopaminergic neurons, and promotion of α-synuclein aggregation, besides dopaminergic neurotoxicity. According to the authors, the findings may raise public concern, since this pollutant could be considered an etiologic agent of Parkinson's disease, as shown by its potential neurotoxicity.

Fruit fly (*Drosophila melanogaster*)

Drosophila melanogaster is one of the most studied eukaryotic organisms in different areas of biological research. Drosophila has a short life span of about 10 days and produces a large number of offspring. They are small, have low experimental costs, and are easy to manipulate under standard laboratory conditions (Moraes and Montagne 2021; Tennessen et al. 2014). Although the body constitution of the insect is simpler than that of mammals, the anatomy of Drosophila includes organ systems with functions equivalent to the mammalian brain and peripheral nervous system—heart, intestine, gonads, lung (trachea system in the fly), kidney (Malpighi tubules in the fly), and liver (fat body in the fly) (Calap-Quintana et al. 2017; Ong et al. 2015; Pandey and Nichols 2011). Drosophila also constitutes a practical model for conducting toxicity studies, particularly the analysis of metal accumulation by the organism (Affleck and Walker 2019; Chifiriuc et al. 2016). This model can also be used to investigate cell cycle alterations, circadian rhythms, enzyme pathways, impairment in DNA repair, and genotoxicity (Demir 2020). De Nobrega et al. (2017) also demonstrated how Drosophila proved to be a valuable model for studies of aging, the circadian clock, and alcohol neurobiology. The study lays a foundation for future research on the cellular and physiological mechanisms by which the circadian clock and aging influence alcohol-induced toxicity in the body. Similarly, dos Santos et al. (2022) evaluated the mechanisms involved in the in vivo toxicity of the mannose/glucose-binding lectin from *Canavalia ensiformis* seeds, a medicinal plant explored in mitogenic and antitumoral activity. In this study, Drosophila was used to assess the toxicity and genotoxicity of different concentrations of the plant extract. The authors reported locomotor impairment and death, which occurred in parallel with oxidative stress and reduced cell viability. In this context, this animal model proved suitable and safe, pointing to the importance of toxicological

evaluations of substances extracted from plants with therapeutic potential before their pharmacological use. In general, the possibility of conducting studies with the present model also encompasses toxicogenomics, carcinogenesis, and radiation biology tests (Freires et al. 2017), including somatic mutation assessments and recombinant tests, comet assay, aberrant crypt foci assay, as well as LD50 determination (Augustyniak and Gladysz 2016).

Conclusion

Novel animal and non-animal models have been developed and validated as a result of technological advances and financial investments in the field. Alternative methods (*e.g.,* 3D cell-based culture and non-mammalian animals) should preferably be considered as a preliminary or substitute approach to in vivo models based on their effectiveness and simplicity of predicting toxicity in humans. In addition, these methods can also be effectively used to screen for toxic materials/drug dosages. The use of in vivo models to test products that are not commercially feasible should be avoided. Researchers should be familiar with the overall characteristics of each model, and their specificities, to choose the adequate alternative model according to the study purpose. Yet, experimentation in mammals continues to be essential in science due to the greater complexity of the organisms and biological responses. Further research is needed to standardize and validate new/optimized alternative models for scientific experimentation.

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