

A Review on Recent Developments in Acute and Subacute Toxicity Studies

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ABSTRACT

Acute and subacute toxicity studies are essential for evaluating the safety of pharmaceuticals, chemicals, and natural products. Acute toxicity studies assess the harmful effects of short-term exposure, whereas subacute studies investigate the effects of repeated exposure, typically over 28 days. Recent developments include in vitro assays, organ-on-chip technology, computational toxicology, physiologically based pharmacokinetic modelling, and omics-based approaches. These innovations aim to improve the accuracy and human relevance of toxicity assessment while reducing animal use in accordance with the 3Rs principle. However, challenges remain regarding method validation, reproducibility, and extrapolation of experimental results to humans. This review highlights recent advances, limitations, and future perspectives in developing more reliable, ethical, and effective toxicity testing methods.

Keywords: Acute toxicity, subacute toxicity, toxicological evaluation, OECD guidelines, alternative methods, organ-on-chip.

1. INTRODUCTION

Toxicity testing is a fundamental component of drug development, chemical safety evaluation, environmental health assessment, and the investigation of medicinal plants and other bioactive substances. Its primary objective is to identify potentially harmful effects, determine exposure–response relationships, characterise target-organ toxicity, and provide evidence for estimating safe exposure levels. Acute and subacute toxicity studies are particularly important because they provide early information about a substance's potential hazards before extensive development or human exposure.

Acute toxicity generally refers to adverse effects occurring after a single exposure or multiple exposures within a short period, commonly within 24 hours. Acute toxicity testing helps identify immediate toxic manifestations, clinical signs, mortality, and approximate dose ranges associated with adverse effects. Historically, median lethal dose (LD50) determination was widely used to characterise acute toxicity. However, conventional LD50 testing has increasingly been replaced or restricted in many applications because of ethical concerns, extensive animal use, and the limited relevance of lethality as the sole measure of toxicity. OECD Test Guidelines 420, 423, and 425 provide alternative approaches for acute oral toxicity assessment, while OECD Test Guideline 401, which described the conventional acute oral LD50 test, has been deleted (OECD, 2001a; OECD, 2001b; OECD, 2001c).

Subacute toxicity studies investigate the consequences of repeated exposure over a relatively short duration, commonly 28 days in standard experimental protocols. These studies can reveal cumulative toxicity, dose-dependent changes in physiological function, organ damage, and effects that may not become apparent after a single exposure. OECD Test Guideline 407 describes a repeated-dose 28-day oral toxicity study in rodents and provides a standardised framework for evaluating systemic toxicity, clinical observations, body weight, food consumption, haematology, clinical biochemistry, organ weights, and histopathological changes (OECD, 2008).

Recent developments in toxicology reflect a shift from traditional animal-centred testing towards integrated approaches that combine mechanistic biology, computational predictions, human-relevant cellular systems, and carefully designed animal studies when necessary. The National Research Council emphasised the long-term need to transform toxicity testing through predictive biological models and mechanistic understanding rather than relying exclusively on high-dose animal experiments (National Research Council, 2007). Subsequently, the development of new approach methodologies (NAMs), including organoids, organ-on-chip platforms, computational toxicology, and high-throughput screening, has created opportunities to improve the efficiency and human relevance of safety assessment (Ewart et al., 2022).

The present review aims to examine the scientific basis of acute and subacute toxicity studies, summarise significant developments in experimental and alternative testing methods, evaluate their strengths and limitations, and identify future research priorities for reliable, ethical, and human-relevant toxicological assessment.

2. REVIEW OF LITERATURE

2.1. ACUTE TOXICITY STUDIES: TRADITIONAL APPROACHES AND RECENT DEVELOPMENTS

Acute toxicity studies are designed to identify the immediate harmful effects of a substance following a single administration or a short period of exposure. Common endpoints include mortality, behavioural changes, respiratory abnormalities, neurological manifestations, body weight changes, and gross pathological findings. Depending on the intended use of the substance, acute toxicity may be evaluated through oral, dermal, inhalation, or other relevant exposure routes.

Historically, the LD₅₀ test was widely used to estimate the dose expected to cause death in 50% of a test population. Although this approach provided a quantitative measure of acute toxicity, it required relatively large numbers of animals and focused primarily on mortality. Modern guidelines have therefore promoted alternative procedures that improve animal welfare while retaining useful hazard-classification information.

The OECD introduced three major approaches for acute oral toxicity assessment: the Fixed Dose Procedure (TG 420), the Acute Toxic Class Method (TG 423), and the Up-and-Down Procedure (TG 425). These approaches use sequential or stepwise dosing strategies to reduce animal use and avoid unnecessary exposure to highly lethal doses (OECD, 2002a; OECD, 2002b; OECD, 2022).

Recent progress in acute toxicity testing includes improved clinical observation protocols, more systematic use of humane endpoints, computational prediction of acute hazard, and the integration of physicochemical properties with existing toxicological databases. In silico tools can assist in prioritising substances for testing, although their predictive performance depends on the availability of relevant training data, chemical similarity, and the biological mechanisms represented in the model.

An important development is the increased use of integrated approaches to testing and assessment (IATA), which combine existing information, computational predictions, in vitro evidence, and targeted experimental testing. Such approaches can improve the efficiency of hazard assessment, particularly when a chemical belongs to a well-characterised group or when reliable historical data are available (OECD, 2002; OECD, 2024).

2.2. SUBACUTE TOXICITY STUDIES AND REPEATED-DOSE ASSESSMENT

Subacute toxicity studies evaluate the adverse effects of repeated exposure over a limited period, commonly 28 days. Unlike acute toxicity studies, which primarily characterise immediate hazards, repeated-dose studies help identify cumulative effects, functional disturbances, and organ-specific injury that develops following continued exposure.

OECD Test Guideline 407 provides a standardised framework for repeated-dose 28-day oral toxicity testing in rodents. The study generally includes multiple dose groups and a control group, with systematic monitoring of clinical signs, body weight, food and water consumption, haematological parameters, clinical biochemistry, organ weights, gross pathology, and histopathology. The resulting information supports the identification of target organs and the characterisation of dose–response relationships (OECD, 2008; OECD, 2025).

One of the important outcomes of repeated-dose toxicity studies is the identification of the no-observed-adverse-effect level (NOAEL), when supported by the study design and available evidence. The NOAEL is the highest tested dose at which no biologically significant adverse effects are observed under the conditions of the study. Interpretation requires consideration of dose selection, study power, variability, biological relevance, and the distinction between adaptive and adverse responses. A NOAEL should not automatically be interpreted as a universally safe dose for humans.

The lowest-observed-adverse-effect level (LOAEL) may also be identified when adverse effects are observed at the lowest dose tested. When suitable dose–response data are available, benchmark-dose modelling can provide an alternative approach by estimating the dose associated with a predefined change in response. This method may make better use of the complete dose–response dataset than reliance on a single threshold dose (EFSA Scientific Committee, 2017).

Recent developments in repeated-dose toxicity studies include the incorporation of functional observations, more sensitive biomarkers, digital pathology, and molecular profiling. These approaches can identify early biological changes before conventional histopathological abnormalities become evident. Nevertheless, biomarkers should be validated and interpreted alongside conventional endpoints because a molecular alteration does not necessarily indicate an adverse health effect.

2.3. APPLICATION OF THE 3RS PRINCIPLE IN TOXICITY TESTING

The principles of replacement, reduction, and refinement (3Rs) have become central to the modernisation of toxicological research. Russell and Burch (1959) introduced this framework to promote humane scientific experimentation.

- **Replacement:** Using validated non-animal methods, including human cell cultures, computational models, organoids, and organ-on-chip systems, wherever scientifically appropriate.
- **Reduction:** Designing experiments to obtain reliable information using fewer animals, through sequential testing, improved statistical methods, and reuse of relevant existing data.
- **Refinement:** Improving animal care, monitoring, experimental procedures, and humane endpoints to minimise pain, distress, and unnecessary suffering.

The OECD acute oral toxicity guidelines illustrate the practical implementation of reduction and refinement by using sequential testing approaches rather than relying on conventional LD50 testing (OECD, 2002a; OECD, 2002b; OECD, 2022).

The 3Rs do not simply represent ethical principles; they also encourage better study design and more efficient use of scientific resources. Their implementation should be accompanied by appropriate validation, transparent reporting, and consideration of the regulatory requirements applicable to the substance under investigation.

2.4. IN VITRO TOXICITY TESTING AND HUMAN-RELEVANT CELLULAR MODELS

In vitro toxicity testing has developed substantially through advances in cell culture, stem-cell biology, tissue engineering, and high-throughput screening. Traditional two-dimensional cell cultures are relatively simple and inexpensive, but they may not adequately reproduce tissue architecture, cellular interactions, metabolic competence, or physiological gradients.

Three-dimensional cell cultures, spheroids, and organoids can better reproduce selected features of human tissues. Liver models are particularly important because hepatic metabolism can activate or detoxify chemicals, influencing their overall toxicity. Renal, cardiac, intestinal, and neural models can provide complementary information about organ-specific effects.

The National Research Council (2007) proposed a transformation of toxicity testing towards pathway-based biological assessment, emphasising mechanistic information and human-relevant systems. This vision has supported the development of high-throughput screening platforms and alternative methods that investigate molecular initiating events, cellular stress responses, and adverse biological pathways.

However, in vitro assays have limitations. These include restricted exposure duration, inadequate representation of systemic metabolism, variation among cell sources, and difficulties reproducing whole-body interactions. Consequently, in vitro results should be interpreted with respect to assay applicability, exposure relevance, and the biological processes represented by the model.

2.5. ORGAN-ON-CHIP TECHNOLOGY IN TOXICITY EVALUATION

Organ-on-chip technology combines microfluidic engineering with living cells to reproduce selected structural and functional features of human organs. Unlike conventional static cultures, these systems can incorporate fluid flow, mechanical forces, tissue interfaces, and controlled chemical exposure.

Nitsche et al. (2022) reviewed the application of organ-on-chip systems to next-generation risk assessment, highlighting their potential for studying skin, intestinal, and liver responses to chemicals. The review also identified important implementation challenges, including the need for robust devices, reproducible culture conditions, and standardised experimental procedures.

Liver-on-chip systems are particularly promising for repeated-exposure studies because they may support more physiologically relevant metabolic activity than some conventional cell models. Ewart et al. (2022) evaluated the performance of a human Liver-Chip for predictive toxicology, contributing evidence on its potential utility and practical considerations. Messelmani et al. (2022) also reviewed liver-on-chip technologies and their applications in drug and chemical toxicity testing.

Despite these advances, organ-on-chip systems are not yet universal replacements for animal studies. Important challenges include donor-to-donor variability, incomplete representation of immune and endocrine systems, limited long-term stability in some models, and differences in device materials and operating procedures. Their value is therefore greatest when they are used within a clearly defined testing strategy with appropriate reference compounds and performance criteria.

2.6. COMPUTATIONAL TOXICOLOGY AND PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELLING

Computational toxicology uses mathematical models, chemical descriptors, machine learning, and existing experimental datasets to predict the potential harmful effects of substances. Quantitative structure–activity relationship (QSAR) models estimate biological activity from chemical structure, while read-across approaches use information from sufficiently similar substances to address data gaps.

These approaches can support the prioritisation of chemicals, selection of dose ranges, and interpretation of experimental findings. However, predictions may be unreliable for chemicals that fall outside the model's applicability domain or possess mechanisms poorly represented in the training dataset.

Physiologically based pharmacokinetic (PBPK) models describe the absorption, distribution, metabolism, and excretion of a substance through interconnected physiological compartments. They can help relate administered doses to concentrations in blood and target tissues and support extrapolation between experimental systems and humans.

When integrated with in vitro data, PBPK models can assist in estimating the internal exposure associated with a toxic response. Nevertheless, their reliability depends on the quality of input parameters, the validity of model assumptions, and independent evaluation against suitable experimental data. Computational predictions should therefore be considered part of an integrated evidence framework rather than automatic substitutes for all experimental testing (National Research Council, 2007; OECD, 2024).

2.7. OMICS TECHNOLOGIES, BIOMARKERS, AND MECHANISTIC TOXICOLOGY

Omics technologies have expanded the scope of toxicity evaluation beyond conventional clinical and pathological endpoints. Transcriptomics measures changes in gene expression, proteomics investigates protein abundance and related modifications, and metabolomics evaluates changes in small-molecule metabolites.

These methods can identify biological pathways affected by chemical exposure and may reveal early indicators of cellular stress, mitochondrial dysfunction, oxidative damage, inflammation, or altered lipid metabolism. In acute studies, molecular profiling may help explain early toxic responses. In subacute studies, repeated measurements and tissue profiling may clarify whether molecular changes are transient, adaptive, progressive, or associated with organ injury.

The development of adverse outcome pathways provides a framework for connecting an initial molecular event with a sequence of biological responses that may ultimately lead to an adverse outcome. AOP-based analysis can help organise evidence from mechanistic assays, computational models, and conventional toxicity studies.

However, the interpretation of omics data requires careful control of false-positive findings, appropriate statistical analysis, biological replication, and independent validation. Molecular changes should not be classified as adverse merely because they differ statistically from controls. Their significance must be established through mechanistic evidence and their relationship to adverse outcomes.

2.8. COMPARATIVE OVERVIEW OF ESTABLISHED AND EMERGING METHODS

Method	Principal application	Major advantage	Key limitation
Acute toxicity testing (OECD TG 420, 423, 425)	Immediate hazard identification	Standardised hazard assessment with refined testing strategies	Limited information about repeated exposure
Repeated-dose testing (OECD TG 407)	Short-term systemic toxicity	Integrated clinical, biochemical, and pathological evaluation	Animal use and species-to-human extrapolation
Conventional in vitro assays	Cellular toxicity and mechanism screening	Relatively rapid and controllable	Limited systemic physiological representation
Organoids and organ-on-chip	Human-relevant organ responses	Improved tissue-level modelling	Standardisation and validation challenges
QSAR and read-across	Hazard prediction and data-gap filling	Efficient prioritization of chemicals	Applicability-domain and data-quality limitations
PBPK modelling	Internal exposure and dose extrapolation	Connects administered dose to tissue exposure	Depends on reliable physiological and kinetic parameters
Omics and AOP approaches	Mechanistic investigation	Sensitive pathway-level information	Complex data interpretation and validation requirements

3. CRITICAL REVIEW AND DISCUSSION

The literature indicates that acute and subacute toxicity assessment is transitioning from conventional animal-based methods towards integrated, mechanistic, and human-relevant approaches. Nevertheless, traditional toxicity studies remain important for evaluating complex biological responses that cannot yet be adequately represented by individual alternative methods.

3.1. IMPROVING THE PREDICTIVE ACCURACY OF TOXICITY STUDIES

A major limitation of conventional toxicity testing is the difficulty of extrapolating findings from experimental animals to humans. Differences in metabolism, enzyme activity, tissue sensitivity, and exposure conditions may result in different toxicological responses across species. The National Research Council (2007) therefore advocated the development of toxicity-pathway-based methods and computational approaches to improve the relevance of toxicological findings to human health.

Combining in vitro toxicity assays with PBPK modelling may improve interpretation by linking biological responses to realistic internal exposure concentrations. Similarly, organ-on-chip models may provide more physiologically relevant information about selected organs than conventional two-dimensional cultures.

However, the reliability of these approaches depends on experimental validation, reproducibility, and the biological processes captured by each model.

3.2. IMPROVEMENTS IN DOSE–RESPONSE ANALYSIS

Dose–response assessment is fundamental to determining the relationship between exposure and adverse effects. Traditionally, NOAEL and LOAEL values have been widely used to interpret repeated-dose toxicity studies. However, these values depend on the doses selected, sample size, variability, and statistical power of the experiment.

The European Food Safety Authority recommends the benchmark-dose approach as a scientifically advanced method for deriving reference points when suitable data are available. This approach models the dose–response relationship across the observed dataset and provides estimates of uncertainty through benchmark-dose confidence limits (EFSA Scientific Committee, 2017).

Integrating benchmark-dose modelling with molecular biomarkers and mechanistic information may improve the interpretation of early toxic responses. Nevertheless, molecular endpoints require careful validation before they can reliably support regulatory decisions.

3.3. BARRIERS TO THE ADOPTION OF ALTERNATIVE METHODS

Despite technological progress, several barriers continue to limit the widespread adoption of alternative toxicity methods:

- **Biological complexity:** Individual cellular models may not reproduce interactions among organs, immune responses, hormonal regulation, and whole-body metabolism.
- **Standardisation:** Variations in cell sources, culture media, exposure protocols, and analytical procedures can affect reproducibility.
- **Regulatory acceptance:** Alternative methods require evidence of reliability, relevance, and applicability to the intended regulatory purpose.
- **Data interpretation:** High-throughput screening and omics technologies produce complex datasets requiring robust statistical and biological interpretation.
- **Resource requirements:** Advanced cellular models, microfluidic systems, and computational infrastructure may require specialised expertise and investment.

These challenges indicate that a gradual, evidence-based transition is preferable to assuming that any single emerging technology can replace all conventional tests. OECD guidance on integrated approaches demonstrates the importance of combining multiple sources of evidence within a defined decision-making framework (OECD, 2024).

4. CONCLUSION

Acute and subacute toxicity studies remain essential for identifying toxic hazards, evaluating dose–response relationships, characterising target-organ effects, and supporting the safety assessment of pharmaceuticals, chemicals, pesticides, and natural products. Acute toxicity testing has evolved from conventional lethal-dose determination towards refined procedures that emphasise hazard classification, sequential dosing, and improved animal welfare. Subacute toxicity testing continues to provide important information about the effects of repeated exposure, including clinical, biochemical, haematological, and histopathological changes.

Recent advances in in vitro assays, organoids, organ-on-chip technology, computational toxicology, PBPK modelling, and omics-based analysis have expanded the possibilities for improving the predictive accuracy and

human relevance of toxicity assessment. These methods can provide mechanistic insights, reduce unnecessary animal use, and support more efficient testing strategies. However, their limitations—including incomplete physiological representation, variability, uncertain extrapolation, and the need for validation—must be addressed.

Overall, the most effective approach is an integrated toxicity assessment framework in which existing information, computational predictions, in vitro evidence, and appropriately designed in vivo studies are combined according to the scientific question and regulatory requirements. Continued investment in standardisation, validation, transparent reporting, and mechanistic research will be essential for establishing more predictive, ethical, and scientifically robust toxicity testing systems.

5. FUTURE DIRECTIONS

Future research should focus on the following priorities to strengthen acute and subacute toxicity assessment.

5.1. Development of integrated testing strategies

Future toxicity assessment should combine conventional toxicological endpoints with in vitro assays, computational predictions, and mechanistic evidence. Tiered testing strategies could prioritise substances with greater potential hazards and use more complex or resource-intensive studies only when justified by the available evidence.

5.2. Expansion of human-relevant organ-on-chip models

Further development of liver, kidney, cardiac, intestinal, and neural organ-on-chip systems may improve the detection of organ-specific toxicity. Multi-organ platforms could provide information about metabolism, tissue distribution, and interactions between organs. Greater standardisation and independent validation will be required before their broader use in regulatory safety assessment.

5.3. Integration of artificial intelligence and computational toxicology

Artificial intelligence and machine learning could improve chemical hazard prediction by integrating chemical structures, biological assay results, toxicological databases, and molecular information. Future models should incorporate transparent validation procedures, uncertainty estimates, and clearly defined applicability domains to reduce the risk of misleading predictions.

5.4. Improved dose–response modelling

Greater use of benchmark-dose modelling, uncertainty analysis, and internal exposure estimates may improve the interpretation of acute and repeated-dose toxicity findings. Combining these methods with PBPK models could help establish more biologically meaningful relationships between experimental doses and human exposure.

5.5. Standardisation and validation of new approach methodologies

International collaboration is needed to develop standardised protocols, reference chemicals, quality-control criteria, and performance benchmarks for emerging testing methods. Harmonisation across laboratories and regulatory agencies would improve reproducibility and facilitate acceptance of reliable alternative methods.

5.6. Greater emphasis on the 3Rs principle

Researchers should prioritise replacement, reduction, and refinement throughout the design and conduct of toxicity studies. Existing data should be evaluated before new experiments are initiated, sample sizes should be justified statistically, and humane endpoints should be incorporated into animal protocols.

5.7. Long-term and repeated-exposure prediction

Although acute and subacute studies provide valuable information, they may not adequately predict the consequences of chronic exposure, developmental effects, or delayed toxicity. Future research should investigate how short-term molecular and cellular responses relate to longer-term adverse outcomes and determine when additional testing is necessary.

5.8. Improved reporting and data sharing

Open access to well-annotated toxicological datasets, standardised reporting, and transparent analytical methods would support independent verification and computational model development. Researchers should report negative findings, uncertainty, methodological limitations, and the biological relevance of observed effects to strengthen the overall evidence base.

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