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REVIEW ARTICLE

Advances in acute toxicity testing: strengths, weaknesses and regulatory acceptance

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ABSTRACT
Safety assessment of chemicals, pharmaceuticals, food and food ingredients, cosmetics, industrial products is very crucial prior to their approval for human uses. Since the commencement of toxicity testing (about 500 years ago, since 1520), significant advances have been made with respect to the 3Rs (reduction, refinement and replacement) alternative approaches. This review is focused on the update in acute systemic toxicity testing of chemicals. Merits and demerits of these advances were also highlighted. Traditional LD_{50} test methods are being suspended while new methods are developed and endorsed by the regulatory body. Based on the refinement and reduction approaches, the regulatory body has approved fixed dose procedure (FDP), acute toxic class (ATC) method and up and down procedure (UDP) which involves few numbers of animals. In terms of replacement approach, the regulatory body approved 3T3 neutral red uptake (NRU), the normal human keratinocyte (NHK), and the 3T3 neutral red uptake (NRU) phototoxicity test for acute phototoxicity. However, other promising replacement alternatives such as organ on chip seeded with human cells for acute systemic toxicity and 3T3 neutral red uptake (NRU) cytotoxicity test for identifying substances not requiring classification, as well as the *in silico* approaches are yet to receive regulatory approval. With this backdrop, a collaborative effort is required from the academia, industries, regulatory agencies, government and scientific organizations to ensure speedily regulatory approval of the prospective alternatives highlighted.

KEY WORDS: acute toxicity, toxicity testing, *in silico*, *in vitro*, *in vivo*, 3Rs principles, regulatory approval

Introduction

Advancement in science and technology have brought significant development in the field of toxicity testing. Improvement of the conventional methods through application of up-to-date techniques is the issue of the present day.

In toxicity assessment of chemicals, there is no doubt that the best test species for humans are humans since accurate extrapolation of animal data directly to humans may not be guaranteed due to interspecies variation in anatomy, physiology and biochemistry (Gallagher, 2003). However, due to ethical reasons, such chemicals are to be tested using animal models before they are subjected to trials in humans (Parasuraman, 2011).

The conventional acute toxicity test which involves the use of large numbers of animals is being replaced by alternative methods. The methods require that fewer numbers of animals or other models that do not require the use of animals (such as *in silico* and *in vitro* approaches) are employed (Jen-Yin *et al.*, 2015; Doke & Dhawale, 2015).

In this review we described the various methods of acute toxicity testing, from history till the present. Merits and demerits of such methods were underscored while approaches that are yet to receive regulatory approval were also emphasized.

Acute toxicity (LD_{50}) test

Acute systemic toxicity evaluates the adverse effects that occur following exposure of organisms to a single or multiple doses of a test substance within 24 hours by a known route (oral, dermal or inhalation) (Saganuwa, 2016). After administration, the test substance is absorbed and distributed to various parts of the body before it elicits systemic adverse effect (EURL-ECVAM, 2017). The regulatory body requires the acute toxicity test report for labeling and classification of substances for human use (Gallagher 2003; Peers *et al.*, 2012; Arwa & Vladimir, 2016).

The LD_{50} (median lethal dose) test was introduced in 1927 by J. W. Trevan to estimate the dose of a test

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substance that produces 50% death in a given species of animals. It is usually the first test conducted for every chemical before further toxicity tests are carried out. It is used for estimating the potential hazards of chemicals on humans. Although its major endpoint is death, non-lethal acute effect may occur as signs of toxicity depending on the chemical being tested (Maheshwari & Shaikh, 2016).

Assessment of the acute toxic potential of substances is required to determine their adverse effects that might occur due to accidental or deliberate short-term exposure (Clemedson *et al.*, 2000). Results from acute toxicity test serve as a guide in dosage selection for long term toxicity studies as well as other studies that involve the use of animals (Maheshwari & Shaikh, 2016).

From the result of an acute toxicity test, a conclusion can be made on the toxicity status of the test substance. As depicted in table 1, substances with LD_{50} below 5 mg/kg are classified to be highly toxic while substances with LD_{50} above 15,000 mg/kg are termed relatively harmless (Loomis & Hayes, 1996).

History and timeline of acute toxicity testing

1920s: The conventional or Classical LD_{50} test

This is the first acute toxicity test that was developed in the 1920s. It was called "Classical LD_{50}" where large numbers of animals, up to 100 animals for five dose-groups is used. Animals are dosed with the test chemical to determine the dose that would result in 50 percent deaths (Maheshwari & Shaikh, 2016, Deora *et al.,* 2010).

1931: Karbal method

This method was introduced in 1931 and it involves the use of 30 animals which are divided into six groups of five animals each. The animals are dosed with the test substance and observed for the first four hours, 24 hours and daily for 14-days for signs of toxicity. At the end of 14 days the total number of death is recorded. The sum of the product of dose difference and mean death is divided by the number of animals in each group and the resulting quotient is subtracted from the non-lethal dose to obtain the LD_{50} value.

$LD_{50} = LD_{100} - \{\Sigma$ [Dose difference × Mean dead]$\}$/ Number of animals per group (Enegide *et al.,* 2013).

Table 1. Classification of LD_{50} based on dose range.

LD_{50}	Classification
<5 mg/kg	Extremely toxic
5–50 mg/kg	Highly toxic
50–500 mg/kg	Moderately toxic
500–5,000 mg/kg	Slightly toxic
5000-15,000 mg/kg	Practically non-toxic
>15,000 mg/kg	Relatively harmless

Loomis & Hayes, 1996

1938: Arithmetical Method of Reed and Muench

After the conventional LD_{50} test, the Arithmetical Method of Reed and Muench was introduced in 1938. In this test, animals are exposed to the chemical and the log dose, number of death and survival, cumulative death and survival are calculated. These are used for LD_{50} determination. However, in 2011, a modification which involved calculation of test animals that die and survive was made by Saganuwan. In this approach, forty test animals were divided into four groups of 10 animals each (Saganuwan, 2011).

1940: Approximate lethal dose

Due to the limitations characterized by high mortality rate in the conventional LD_{50} test, an alternative procedure for the determination of approximate lethal dose (ALD) was developed in the early 1940s. The animals are administered with the dose of the test substance that increases by 50 percent over the previous dose (Ekwall, 1999).

1944: Miller and Tainter method

The Miller and Tainter method was established in 1944. It involves the use of fifty animals which are divided into five groups of ten animals each. Signs of toxicity and death are observed and recorded after administration and the LD_{50} is calculated using probit analyses table. Probit values are plotted against log doses and the dose corresponding to probit five (5) becomes the LD_{50} value (Randhawa *et al.,* 2009; Saganuwa, 2016). Some of the traditional methods of LD_{50} estimation are depicted in Table 2.

Synopsis 1: Some of the older methods of LD_{50} determination (Arithmetical method of Reed and Muench, Karbal method, Lorke's method and Miller and Tainter methods) are depicted in Table 2. These methods lack regulatory acceptance and they are not in conformity with the 3RS (reduction, refinement and replacement) principle.

1960s – 1980s: Limitations posed by the conventional LD_{50} methods and development of alternative methods

The inspiration about alternative methods to toxicity testing in animals became overwhelming in the 1960s, 1970s, and 1980s when governments, academia and industry became more involved in the debate of improving toxicity testing guidelines. The alternative to animal testing kicked off in the 1980s when animal rights activists motivated the cosmetic industry to begin researching on unconventional methods to animal tests (Chapman *et al.,* 2013).

The term "alternative" came into limelight following the publication of a book titled "The Principles of Humane Experimental Technique" authored by William Russell and Rex Burch. They recommended that proper experimental design should reflect on methods that could reduce, refine and also replace (3Rs) the current techniques (Russel & Burch, 1959; Erkekoglu *et al.,* 2011; Jen-Yin *et al.,* 2015; Gertrude-Emilia, 2017).

Table 2. Comparison of various conventional methods used for LD_{50} determination.

	Conventional methods of LD_{50} estimation			
	Karbal method	**Reed and Muench**	**Lorke's Method**	**Miller and Tainter**
Year Introduced	1931	1938	1983	1944
Accuracy	Less	Less	Less	Less
Number of animals	Many (30)	Many (40)	Few (13)	Many (50)
Expenditure	High	High	Less	High
Simplicity	Complicated	Complicated	Simple	Complicated
Duration	Less	Less	Less	Less
Reproducibility	No	No	No	No
Endpoint (s)	Signs of toxicity and death	Signs of toxicity and death	Signs of toxicity and death	Signs of toxicity and death
Regulatory Approval	No	No	No	No

Enegide *et al.,* 2013; Saganuwa, 2016; Maheshwari & Shaikh, 2016

Table 3. Comparison of various alternative methods used for LD_{50} estimation:

	Alternative methods of LD_{50} estimation			
	FDP (OECD 420)	**ATC (OECD 423)**	**UDP (OECD 425)**	**Enegide *et al.***
Year Introduced	1992	1996	1998	2013
Accuracy	Higher	Higher	Higher	Higher
Number of animals	Few (10–40)	Few (3–12)	Few (2–15)	Few (6–12)
Expenditure	Less	Less	Less	Less
Simplicity	Complicated	Simple	Simple	Simple
Duration	Less	Less	Less	Less
Reproducibility	Yes	Yes	Yes	Yes
Endpoint (s)	Signs of toxicity	Signs of toxicity and death	Signs of toxicity and death	Signs of toxicity and death
Regulatory Approval	Yes	Yes	Yes	No

Enegide *et al.,* 2013; Saganuwa, 2016; Maheshwari & Shaikh, 2016

The 3Rs alternatives

Reduction

Reduction approach implies that the number of animals employed in a given test should be minimized while still maintaining consistency and accuracy with scientific practices that would yield convincing and valid results (Robinson, 2005).

Refinement

Refinement approach is geared towards providing better welfare to animals by minimizing pain (by using appropriate anesthetic and analgesics), distress and provision of a suitable environment for animals (Robinson, 2005; Brown & White, 2009; Erkekoglu *et al.,* 2011). Some reduction and refinement alternative methods are depicted in Table 3.

Replacement

Replacement approach involves methods other than the use of animals (Table 4). Such methods include, *in vitro* and *in silico* approaches (Broadhead & Combes, 2001; EURL-ECVAM, 2017).

Implementation of the 3Rs principles had significantly reduced the number of animals as well as reduction in drug failure rate in the discovery and development pipeline (Russell & Burch, 1959; Robinson, 2005; Chapman *et al.,* 2013).

1981: Incorporation of the LD_{50} tests into the OECD guideline

In 1981, the Organization for Economic Cooperation and Development (OECD) incorporated the LD_{50} test into its new test guidelines. It involves the use of 30 animals for 3 doses. In 1987 the OECD further modified this method by reducing the number from 30 to 20 animals where five animals per dose level are selected based on sighting studies or from historical data of the chemical to be tested.

An upper dose level limit of 5,000 mg/kg was also introduced essentially for substances whose LD_{50} values exceed 5,000 mg/kg. Similar guidelines were also published for acute dermal and inhalation toxicity (Ekwall, 1999). The limit test is usually employed whenever a test substance is

suspected to be non-toxic based on historical information about the test substance. In this regard, determination of the precise LD_{50} would not be necessary, but the limit test can be employed. This involves the exposure of few numbers of animals to large dose (5,000 mg/kg) of the test chemical. If animals survive, the LD_{50} is estimated to be above 5,000 mg/kg and no further acute toxicity testing is required (Maheshwari & Shaikh, 2016).

1983: Lorke's method

This method introduced in 1983 involves the use of thirteen animals in 2 phases. In the first phase, nine animals are divided into three groups of three animals each and are administered 10, 100 and 1,000 mg/kg body weight of the test substance in order to establish the dose range producing any toxic effect. The number of deaths in each group is recorded after 24-hours. In the second phase, four doses of the test substance are selected based on the result of phase 1 and are administered to four (4) groups of one animal each. After twentyfour hours, the number of deaths is recorded and the LD_{50} is calculated as the geometric mean of the highest non-lethal dose (a) and the least toxic dose (b). $LD_{50} = \sqrt{a \times b}$ (Lorke, 1983; Enegide *et al.,* 2013).

1992: Fixed dose procedure (FDP)

Fixed dose procedure (FDP) was introduced in 1992. The test substance is given at one of the four fixed-doses (5, 50, 500, and 2,000 mg/kg) to five male and five female animals of the same species. The objective of the FDP is to identify a dose that produces clear signs of toxicity but no mortality. Depending on the results of the first test, either no further testing is required or a higher or lower dose is tested. If mortality occurs, retesting at a lower dose level is necessary except if the original dose chosen is 5 mg/kg (OECD, 2001).

1996: Acute toxic class (ATC) method

This method (OECD 423) was introduced in 1996 and it is based on a sequential dosing in which one dose group 5, 50, 300 or 2,000 mg/kg body weight is used at a time. The sequential testing procedure uses three animals of one sex per step at any of the defined dose levels. Depending on the mortality rate, three but not exceeding six animals is used per dose level. The result of this approach is reproducible and the number of animals used is reduced by 40–70% compared to the traditional methods. In Germany, more than 85% of all tests conducted in 2003 employed the acute toxic class (ATC) method (OECD 2001). In this method, death is not used as the only end point, but signs of toxicity in its stepwise approach are also used for estimating the LD_{50} (OECD, 2001; Saganuwa, 2016).

Table 4. List of replacement (*in vitro* and *in silico*) alternative methods for LD_{50} determination.

Replacement	Regulatory Approval
3T3NRU cytotoxicity test	Yes
NHK neutral red uptake (NRU) cytotoxicity test	Yes
3T3 neutral red uptake (NRU) phototoxicity test	Yes
3T3 neutral red uptake (NRU) cytotoxicity test	No
In silico approach	No
Neuroblastoma SiMa cell line approach for botulinum neurotoxins acute toxicity	No

NRU: neutral red uptake, **NHK**: normal human keratinocyte (Maheshwari & Shaikh, 2016; PISC, 2017).

1998: Up and down procedure (UDP)

In this method that was introduced in 1998, the LD_{50} value of a test substance is estimated by testing individual animals sequentially, with the dose for each animal being regulated up or down based on the results of the preceding tests. Animals are dosed one at a time. The dose for the next animal is increased by a factor of 3.2 if the preceding animal survives, while the dose is decreased by a factor of 3.2 if the animal dies. It takes 1 or 2 days to observe each animal before dosing the next animal. Thereafter, animals that survive the test are monitored for delayed toxicity for 7 days (Enegide *et al.,* 2013). When a test substance is suspected to be relatively safe, either a 2,000 mg/kg or 5,000 mg/kg dose can be administered to the first animal and then observed for 48 hours for toxicity. If death is recorded, the initial dose is divided by a factor of 3.2 and a second and third animal can be dosed concomitantly and observed for 48 hours. If death is still observed with these two animals, further reduction can be done until no death is observed. However, if death is not recorded at 2,000 or 5,000 mg/kg starting doses, then the LD_{50} can be estimated to be above the limit range 2,000 or 5,000 mg/kg (Enegide *et al.,* 2013; Saganuwan, 2016).

2002: Deletion of the conventional LD_{50} test and introduction of the OECD TGs

After many years of controversy and debate over the LD_{50} test, other advanced methods were explored and implemented. This led to the suspension (deletion) of the conventional LD_{50} tests on December 17th, 2002. This was followed by the implementation of the currently used OECD TGs for acute toxicity tests where the number of test animals is reduced to a range of 2–15 animals (Sanganuwa, 2016). The test guidelines, already described above, include; Fixed Dose Procedure (OECD TG 420), Acute Toxic Class (OECD TG 423) and Up and down procedure (OECD TG 425). They had also given rise to significant improvements in animal welfare, in particular when evident signs of toxicity are used as the relevant endpoint instead of death. They also provide more information on target organs and possible mechanisms of toxicity (Saganuwa, 2016). They had also received regulatory approval by the regulatory body (PISC, 2017).

Synopsis 2: Deletion of the conventional LD_{50} test methods paved way for the implementation of the three OECD test guideline (fixed dose procedure, acute toxic class method and up and down procedure) which had gained regulatory acceptance. The reduction and refinement approaches were the drivers of these implementations (Table 3).

2013: Proposed acute toxicity test method of Enegide *et al.* (2013)

In 2013, Enegide and co-workers proposed a new method for the assessment of acute toxicity. The test method is divided into three stages. Outcome of the first stage determines the next step to take (i.e, whether to terminate the test or proceed to the next stage).

Stage 1 (the initial stage) requires four animals which are divided into four (4) groups of one animal each at 10, 100, 300 and 600 mg/kg or 50, 200, 400 and 800 mg/kg of the test substance. If mortality is not observed in this stage, the testing proceeds to stage 2.

Stage 2 (the second stage) involves three animals which are divided into three groups of one animal each receiving different doses higher than those used in the first stage. If no mortality occurs, testing proceeds to stage 3.

Stage 3 (the final stage) requires the use of three animals which are distributed into three groups of one animal each. Higher doses (not exceeding 5,000 mg/kg) of the test substance are administered to the different animals. When no signs of toxicity and mortality are recorded at this final stage of testing, the LD_{50} of the test substance is said to be greater than 5,000 mg/kg.

However, a confirmatory test is usually carried out whenever death of an animal is recorded at any stage by administering the lowest dose that cause mortality to two animals, followed by observation. Where at least a single animal from the two animals dies, the confirmatory test is validated. Also, if no mortality is still recorded at 5,000 mg/kg, a confirmatory test is also carried-out by administering 5,000 mg/kg to two animals.

This confirmatory test can also be carried-out to verify the substances with already established LD_{50} values in the literature. In the Enegide *et al.* method, the following formula is usually employed to estimate the LD_{50}.

LD_{50}=[M0+M1]/2,

where M0 = highest dose of test substance that produced no mortality, M1 = lowest dose of test substance that produced mortality.

Although this method requires the use of a lower number of animals (12), its sequence of testing is in 3 phases, unlike that of up and down procedure (UDP) and fixed dose procedure where testing can be started at any stage. Also, the Enegide *et al.* method is yet to undergo validation by the regulatory bodies for international acceptance (Enegide *et al.,* 2013; Maheshwari & Shaikh, 2016).

Acute toxicity for topical preparations

Ocular toxicity test

The ocular toxicity test identifies substances that are ocular corrosive or irritating to the eye (ICCVAM, 2006). Injury caused by irritation is reversible while that caused by corrosion is not reversible.

This test was developed in 1944 following a series of reports that women were suffering permanent eye injuries from cosmetic products. The Draize irritation test is performed by placing the test substance (solid, 0.5 g or liquid, 0.5 ml) on the eye of a rabbit, without local anesthetic. The other eye is used as a control. Clips are placed on the eyelids to hold the eyes open and to keep the animals from blinking the test substance away. The animals are placed in restraining stocks to prevent them from moving during the test period (ICCVAM, 1999).

Dermal toxicity test

For dermal toxicity test, animals are placed into at least three dose levels of five animals each and the test substance (solid, 0.5 g or liquid, 0.5 ml) is applied to the shaved skin (≥10% area of the body surface). Animals used in this test include rats, rabbits or guinea pigs. A 14-day observation is made and death of animals is used to estimate the LD_{50} (Maheshwari & Shaikh, 2016). A limit dose of 2,000 mg/kg can also be used for this test (EURL-ECVAM, 2017). Results of the dermal acute toxicity test aid in establishing dosage regimen for chronic dermal toxicity test and other studies.

Acute toxicity test for inhalation

This test is performed for aerosol-like preparations. Animals, usually rats, are exposed to the test substance for a minimum of four (4) hours and are then monitored for a period of 14-days. Animals that die during the study are autopsied. At the end of the study, animals are sacrificed and observed for pathological changes (Maheshwari & Shaikh, 2016). Inhalation toxicity, OECD TG 436 had received regulatory acceptance (PISC, 2017).

Limitations of the conventional LD_{50} testing

Results obtained from acute toxicity test may vary greatly from species to species and from laboratories to laboratories. LD_{50} is not tested on humans and relation to humans is only a guess because the human lethal dose may not be predicted exactly from animal studies (Maheshwari & Shaikh, 2016).

Signs recorded during acute toxicity studies

Some signs that should be observed and recorded during acute toxicity testing include: analgesia, tremors, increased motor activity, anesthesia, tonic extension, lacrimation, arching and rolling, salivation, clonic convulsions, straub reaction, muscle spasm, loss of righting reflex, writhing, hyperesthesia, ataxia, depression, sedation, stimulation, hypnosis and cyanosis (Botham, 2004; Saganuwan, 2016). Endpoints of ocular acute toxicity test include: redness, hemorrhage, ulcerations, discharge, blindness and swelling, while those of derma toxicity test include: erythema and edema. Various signs are associated with various chemicals (Maheshwari & Shaikh, 2016).

Alternative replacement approaches to LD_{50} tests

Chemical testing in laboratory animals had been performed for barely over a century (since 1920). However,

inter species variations between human and animals had limited this test due to failure of several drugs in clinical trials (Shanks *et al.*, 2009). Nine out of ten promising drug candidates that undergo phase 1 clinical trials do not achieve regulatory and marketing approval due to inconsistency in translating animal findings to human situation (Fisher, 2013).

Research also revealed that about 52 percent of all new drugs marketed during a 10-year period, revealed serious toxic or even fatal effects that were not predicted by animal tests (Jannuzzi *et al,* 2016). Animal studies are costly, time consuming and cruel (Russell & Burch, 1959; Jen-Yin *et al.,* 2015).

Recognition of these limitations in animal studies had resulted in the development of replacement alternative techniques which involve the use of non-animal methods. These replacement methods include *in vitro* and *in silico* techniques.

In vitro method

This alternative technique involves the use of cell and tissues which are cultured under controlled situation using 2 or 3 dimensional cell co-cultures. The use of human cell lines is preferred because they can easily predict possible effect in human (EURL-ECVAN, 2017). This test produces data that are more relevant to humans than the LD_{50} value obtained from animal studies (EURL-ECVAN, 2017).

The Multicenter Evaluation of *in vitro* Cytotoxicity (MEIC) has been working on *in vitro* alternatives to acute toxicity tests since 1989 and their evaluation revealed that *in vitro* human cell lines can predict acute toxicity in humans for most chemicals tested (Blais, 1993). Cellular models of toxicity are more rapid and can easily be adapted for high throughput screening. For instance, the acute *in vitro* effects of cisplatin, gentamycin, cephalosporins, cysteine conjugates, butyl hydroperoxide, mercuric chloride and cadmium chloride had been studied using primary cultures (Blais, 1993). The major advantage of this method is that it is specific on target organs.

Recently, an acute toxicity assay was developed by L'Oréal and CeeTox. This assay utilizes rat hepatoma cell line (H4IIE) in conjunction with concentration responses which measure cellular health and receptor binding. This assay is cheap, its specificity ranges from 84 to 90% and it could be a replacement alternative in the near future (Dayna *et al.,* 2017).

The use of "organ on chip" seeded with human cells is a replacement alternative to acute systemic toxicity testing (Dayna *et al.,* 2017). Organs on chip are microfluidic devices with ability to mimic human organ physiological system (Marx *et al.,* 2016). For instance, the development of multiple organs on chip (lung, liver, gut, kidney and heart) is presently ongoing at the Wyss Institute of Biologically Inspired Engineering at the Harvard University (Dayna *et al.,* 2017).

Due to the poor predictive power of animal studies which may lead to failure in the late stage of clinical trials, numerous pharmaceutical companies and government agencies are now developing interest in the organ on chip model of systemic acute toxicity prediction (Esch *et al.,* 2010; Dayna *et al.,* 2017). Examples of organs, tissues and system chips include: Heart, Brain, Intestine, Kidney, Eye, Liver, Skin, Placenta, Lung, Blood-Brain Barrier, Blood Vessels, Bones, Cervix, Fat, Marrow, Muscles and Nerve (PISC, 2017). Although the "organ on-chip" technique had undergone series of validation, it has not received regulatory approval as a replacement alternative method for acute systemic toxicity determination.

Also, the IC_{50} test, which determines the cytotoxicity of a chemical in terms of the chemical's ability to inhibit the growth of half of a population of cells had been introduced. The IC_{50} test is useful for comparing the toxicity of chemicals in human cells. It produces data that are more relevant to humans than the LD_{50} results obtained from animals (PISC, 2017).

Recently, the 3T3 neutral red uptake (NRU) cytotoxicity test, normal human keratinocyte (NHK), neutral red uptake (NRU) cytotoxicity test for establishing starting doses for oral acute systemic toxicity and 3T3 neutral red uptake (NRU) phototoxicity test for acute phototoxicity were approved by the regulatory body for acute toxicity prediction (table 4).

Although the 3T3 neutral red uptake (NRU) cytotoxicity test for identifying substances not requiring classification with LD_{50} above 2,000 mg/kg had received EURL ECVAM recommendation in 2013, it is yet to receive approval from the regulatory body as replacement alternative to acute systemic toxicity test (EURL-ECVAM, 2017; PISC, 2017).

A recent workshop comprised of academia, regulatory agencies, industry and no-governmental organizations was organized to explore new methods of evaluation of acute toxicity using non-animal methods which could aid in comprehending acute toxicity mechanism(s), thereby enhancing the generation of adverse outcome pathways. The attendees suggested the need to eliminate dermal toxicity studies on new pesticide formulations. They also emphasized education of personnel on interpreting results derived from *in vitro* and *in silico* methods (Hamm *et al.,* 2017).

Replacement alternative for the detection of Botulinum neurotoxin acute toxicity

Recently, a novel neuroblastoma SiMa cell line approach, which allows vesicle-associated membrane protein (VAMP) molecules for the detection of Botulinum neurotoxins (BoNTs), was developed as an alternative to the mouse LD_{50} bioassay. It involves the use of luminescent enzymatic reaction with sensitivity comparable to the mouse LD_{50} bioassay. The assay is useful for the detection of new botulinum drugs (tetanus vaccines) (Rust *et al.,* 2017). However, it is yet to receive a regulatory approval.

In silico approach

This involves the use of computational tools to predict toxicity of test chemicals. It complements *in vitro* and *in*

vivo toxicity screening, reduces the number of animals as well as the cost of toxicity testing. With this approach, toxicity of chemicals can be predicted before such chemicals are synthesized (Arwa & Vladimir, 2016).

Knowledge of the properties of a few representative substances can be deduced from the literature on existing compounds. Substances with similar chemical structures would often have similar biological and toxicological properties. The required calculations are performed using specially developed computer programs. This approach would help to narrow down the number of substances to be tested and the selected substance(s) can then be tested using the legally prescribed test methods (Valerio-Jr, 2009).

In this field, quantitative *in vitro* to *in vivo* (QIVIVE) extrapolation is required to predict systemic acute toxicity for chemicals and drugs. Computerized quantitative structure-property (QSPR) and computerized modeling based on quantitative structure–activity relationship models (QSAR models) are needed to create a biochemical model. With the availability of QIVIVE, *in vivo* human toxicity estimations can be made. In the near future, these *in silico* techniques may replace some of the animal tests (Daneshian, 2012).

Structure-activity relationships (SARs) and quantitative structure-activity relationships (QSARs), are also capable to predict acute toxicity. The OECD QSAR Toolbox, HazardExpert, Topkat, CASE Ultra, T.E.S.T, Derek Nexus and ACD/Percepta are some SAR software packages that contain models for the prediction of acute systemic toxicity (Cronin, 2002; Kleandrova *et al.,* 2015).

Advantages of replacement alternatives to acute toxicity testing

In vitro acute toxicity testing has several advantages. It involves small set-ups that allow little test substance, low costs, high-number of replicates as well as ease of interpretation of results obtained. *In vitro* methods are useful for elucidating the mechanisms of toxicity of a test substance. Cell models for practically almost all tissues or laboratory animal species are now available (PISC, 2017).

In silico model is very advantageous. It is cheaper, highly reproducible, can undergo constant optimization, and has also potentials to replace the use of animals in the near future (Valerio-Jr, 2009; Kleandrova *et al.,* 2015).

Disadvantages of the replacement alternatives to acute toxicity testing

The limitations associated with *in vitro* acute toxicity approach are such that most cell systems are representing only one cell type when compared to whole animal experiment, where hundreds of tissues interact with one another physiologically. Degeneration of cells due to continuous depletion of nutrients, accumulation of waste products, and insufficient oxygen supply resulting in anaerobic culture conditions are often common with some *in vitro* conditions (Shanks *et al.*, 2009). Sometimes cell lines placed in the banks may be contaminated due to poor storage conditions. Also ethical issues in relation to donation of human tissues could arise (Coecke *et al.*, 2006; Hartung & Daston, 2009).

Limitations associated with the *in silico* model include; lack of available toxicity data of some substances in the library of existing compounds, inappropriate (simplistic) modeling of some endpoints, poor domain applicability of models and non-approval by the regulatory body (Cronin, 2002; Ambuja *et al.,* 2013; Kleandrova *et al.,* 2015).

Synopsis 3: The three replacement approaches that have received regulatory acceptance include; 3T3NRU cytotoxicity test, NHK neutral red uptake (NRU) cytotoxicity test and 3T3 neutral red uptake (NRU) phototoxicity test. However, 3T3 neutral red uptake (NRU) cytotoxicity test as well as *in silico* approach are yet to receive regulatory acceptance (Table 4).

Conclusion

The present review evaluated the progress in acute toxicity testing. Traditional and alternative techniques were described. Limitations posed by the traditional methods prompted the implementation of the 3Rs techniques which involve the use of few or no animals for systemic acute toxicity test.

In light of the forgoing, researchers should be encouraged to utilize the 3Rs techniques. Also, collaborations from federal agencies, scientific organizations, academia and industries is required to effectively incorporate the alternative replacement methods (*in vitro, in silico*) into acute systemic toxicity assessment and linking the observed effect to *in vivo* situation.

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