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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.

REFERENCES

Ambuja SB, Elaina K, Thomas JF, John CL, Donna LM, Thomas H, Geoffrey WP. (2013). *Correlating in vitro data to in vivo findings for risk assessment. Altex* **31***:* 1/14. Symposium report.

Arwa BR, Vladimir BB. (2016). *In silico* toxicology: computational methods for the prediction of chemical toxicity. *Wiley Interdiscip Rev Comput Mol Sci.* **6**: 147–172.

Blais A, Morvan-Baleynaud J, Friedlander G, Le Grimellec C. (1993). Primary culture of rabbit proximal tubules as a cellular model to study nephrotoxicity of xenobiotics. *Kidney Int* **44**: 13–18.

Botham PA. (2004). Acute systemic toxicity—prospects for tiered testing strategies. *Toxicol in Vitro* **18**: 227–230.

Broadhead CL, Combes RD. (2001). The current status for food additives toxicity testing and the potential for application of the Three Rs. *ATLA* **29**: 471–485.

Brown MJ, White WJ. (2009). Reflections on improved health status of rodents bred for research: contributions to the reduction and refinement of animal use. *Altern Lab Anim* **37**: 187–189.

Chapman KL, Holzgrefe H, Black LE, Brown M, Chellman G, Copeman C, Couch J, Creton S, Gehen S, Hoberman A, Kinter LB, Madden S, Mattis C, Stemple HA Wilson S. (2013). Pharmaceutical toxicology: Designing studies to reduce animal use, while maximizing human translation. *Regul. Toxicol. Pharmacol* **66**: 88–103.

Clemedson C, Barile FA, Chesne C, Cottin M, Curren R, Eckwall B, Ferro M, Gomez-Lechon MJ, Imai K, Janus J, Kemp RB, Kerszman G, Kjellstrand P, Lavrijsen K, Logemann P, McFarlane-Abdulla E, Roguet R, Segner H, Thuvander A, Walum E, Ekwall B. (2000). MEIC evaluation of acute systemic toxicity. Part VII. Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further in vitro assays. *ATLA* **28**: 159–200.

Coecke S, Ahr H, Blaauboer BJ, Bremer S, Casati S, Castell J, Combes R, Corvi R, Crespi CL, Cunningham ML. (2006). Metabolism: A bottleneck in *in vitro* toxicological test development. *ATLA*. **34**: 49–84.

Cronin MTD. (2002). The current status and future applicability of quantitative structure–activity relationships (QSARs) in predicting toxicity. *ATLA* **30**: 81–84.

Daneshian M. (2012). A roadmap for the development of alternative (non-animal) methods for toxicokinetics testing. *ALTEX* **29**: 16–26.

Dayna KM, Alan MG, Albrecht P, Michael FW, Joseph C, James M, Kelly PC, Richard H, Ronald B, Harald FK, Anthony B, Thomas H. (2017). From *In Vivo* to *In Vitro*: The Medical Device Testing Paradigm Shift. t4 workshop report*. ALTEX Online first published, version 2 doi: 10.14573/altex.1608081.

Deora PS, Mishra CK, Mavani P, Asha R, Shrivastava B, Rajesh KN. (2010). Effective alternative methods of LD50 help to save number of experimental animals. *J Chem Pharm Res* **2**(6): 450–453.

Ekwall B. (1999). Overview of the Final MEIC Results: II. The *In Vitro–In Vivo* Evaluation, Including the Selection of a Practical Battery of Cell Tests for Prediction of Acute Lethal Blood Concentrations in Humans. *Toxicol in Vitro* **13**: 665–673.

Enegide C, David A, Fidelis SA. (2013). A New Method for Determining Acute Toxicity in Animal Models. *Toxicol Int* **20**(3): 224–226.

Erkekoglu P, Giray BK, Basaran N. (2011). 3R Principle and Alternative Toxicity Testing Methods. *FABAD J Pharm Sci* **36**: 101–117.

Escher SE, Tluczkiewicz I, Batke M. (2010). Evaluation of inhalation TTC values with the database RepDose. *Regul Toxicol Pharmacol* **58**: 259–274.

EURL-ECVAM (2017).European Union Reference Laboratory for Alternative to Animal Testing https: //eurl-ecvam.jrc.ec.europa.eu/glossary/glossary/in-vitro-methods. Accessed on 17th November, 2017**.**

Fisher G. (2013). Import Ban on Animal-Tested Products Goes Into Effect. The Times of Israel, 1 January 2013. https: //www.timesofisrael.com/import-ban-on-animal-tested-products-goes-into-effect/. Accessed on November, 2th, 2017.

Gallagher ME. (2003). Toxicity testing requirements, methods and proposed alternatives. *Environs* **26**(2): 257–273.

Gertrude-Emilia C. (2017). Advances in science: next generation of lab tools, models and testing platforms used in predictive toxicology. *Molecular Life* **1**(1): 22–28.

Hamm J, Kristie S, Amy JC, Judy S, Shannon B, Barun B, Bas B, Warren C, David D, Anna F, Natàlia GR, Sean G, Rabea G, Jon H, Anna L, Joanna M, Elissa R, Louis S, David A. (2017). Alternative approaches for identifying acute systemic toxicity: Moving from research to regulatory testing. *Toxicol in Vitro* **41**: 245–259.

Hartung T, McBride M. (2011). Food for thought. on mapping the human toxome. *ALTEX* **28**: 83–93.

Hartung T, Daston G. (2009). FORUM Are *In Vitro* Tests Suitable for Regulatory Use? *Toxicol Sci* **111**(2): 233–237.

ICCVAM (2006). Test Method Evaluation Report – *In Vitro* Ocular Toxicity Test Methods for Identifying Ocular Severe Irritants and Corrosives. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). NIH Publication No.: 07-4517. Available: [http: //iccvam.niehs.nih.gov/methods/ocutox/ivocutox/ocu_tmer.htm].

ICCVAM (1999). Corrositex®. An *In Vitro* Test Method for Assessing Dermal Corrosivity Potential of Chemicals. The Results of an Independent Peer Review Evaluation Coordinated by ICCVAM, NTP and NICEATM. NIEHS, NIH Publication (No. 99-4495.)

Jannuzzi AT, Ozcagli1 E, Kovatsi L, Goumenou M and Tsatsakis AM. (2016). Using Stem Cells in Toxicological Assessments. *J Med Toxicol Clin Forens Med* **2**(1): 1–2.

Jen-Yin G, Richard JW, Libby D, Nicola JP, Ruth AR (2015). Development and use of *in vitro* alternatives to animal testing by the pharmaceutical industry 1980–2013. *Toxicol Res* **4**: 1297–1307.

Kleandrova VV, Luan F, Speck-Planche A, Cordeiro MN (2015). *In silico* assessment of 26 the acute toxicity of chemicals: recent advances and new model for multitasking 27 prediction of toxic effect. *Mini Rev Med Chem* **15**: 677–86.

Loomis TA, Hayes AW. (1996). Loomis's essentials of toxicology. 4th ed., California, Academic press: 208–245.

Lorke D. (1983). A new approach to practical acute toxicity testing. *Arch Toxicol* 54: 275–87.

Maheshwari DG, Shaikh NK. (2016). An overview on toxicity testing method. *Int J Pharm Technol* **8**(2): 3834–3849.

Marx U, Andersson TB, Bahinski A. (2016). Biology-inspired microphysiological system approaches to solve the prediction dilemma of substance testing using animals. *ALTEX* **33**: 272–321.

OECD (2001). Acute Oral Toxicity – Acute Toxic Class Method. Test No. 423. Adopted 17th December, 2001.

Parasuraman S. (2011). Toxicological screening. *J Pharmacol Pharmacother* **2**(2): 74–79.

Peers IS, Ceuppens PR, Harbron C. (2012). In search of preclinical robustness. *Nat Rev Drug Discov* **11**(10): 733–734.

PISC (2017). Peta International Science Consortium Ltd. Alternatives to animal testing. www.piscltd.org.uk.

Randhawa MA. (2009). Calculation of LD_{50} value from the method of Miller and Tainter, 1944. *J Ayub Med Coll Abbottabad* **21**(3): 1–3.

Robinson V. (2005). Finding alternatives: an overview of the 3Rs and the use of animals in research. *School Sci Rev* **87**: 1–4.

Russell WMS, Burch RL. (1959). The Principles of Humane Experimental Technique. Methuen, London. Stephens ML, Goldberg AM, Rowan AN, 2001. The First Forty Years of Alternatives Approach: Refining, Reducing, and Replacing the Use of Laboratory Animals. In: Salem DJ, Rowan AN (Eds.), The State of Animals. Humane Society Press, Washington, DC. 121–135.

Rust A, Doran C, Hart R, Binz T, Stickings P, Sesardic D, Peden AA Davletov B. (2017). A Cell Line for Detection of Botulinum Neurotoxin Type B. *Front Pharmacol* **8**: 796: 1–8.

Saganuwan AS. (2011). A modified arithmetical method of Reed and Muench for determination of a relatively ideal median lethal dose (LD_{50}). *Afr J Pharm* **5**(12): 1543–1546.

Saganuwan SA. (2016). Toxicity study of drugs and chemicals in animals: An overview. *BJVM*. 2016 online first. ISSN 1311-1477.

Shanks N, Greek R, Greek J. (2009). Are animal models predictive for humans? Philos. *Ethics Humanit Med* **4**(2): 1–20. Available from: http://www.pehmed.com/content/4/1/2.

Sonali K, Doke S, Dhawale C. (2015). Alternatives to animal testing: A review. *Saudi Pharm J* **23**: 223–229.

Valerio-Jr, LG. (2009). *In silico* toxicology for the pharmaceutical sciences. *Toxicol Appl Pharmacol* **241**: 356–370.

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ORIGINAL ARTICLE

Pixantrone, a new anticancer drug with the same old cardiac problems? An *in vitro* study with differentiated and non-differentiated H9c2 cells

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ABSTRACT
Pixantrone (PIX) is an anticancer drug approved for the treatment of multiple relapsed or refractory aggressive B-cell non-Hodgkin's lymphoma. It is an aza-anthracenedione synthesized to have the same anticancer activity as its predecessors, anthracyclines (*e.g.* doxorubicin) and anthracenediones (*e.g.* mitoxantrone), with lower cardiotoxicity. However, published data regarding its possible cardiotoxicity are scarce. Therefore, this work aimed to assess the potential cytotoxicity of PIX, at clinically relevant concentrations (0.1; 1; and 10 µM) in both non-differentiated and 7-day differentiated H9c2 cells. Cells were exposed to PIX for 48 h and cytotoxicity was evaluated through phase contrast microscopy, Hoescht staining and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction and neutral red (NR) uptake assays. Cytotoxicity was observed in differentiated and non-differentiated H9c2 cells, with detached cells and round cells evidenced by phase contrast microscopy, mainly at the highest concentration tested (10 µM). In the Hoechst staining, PIX 10 µM showed a marked decrease in the number of cells when compared to control but with no signs of nuclear condensation. Furthermore, significant concentration-dependent mitochondrial dysfunction was observed through the MTT reduction assay. The NR assay showed similar results to those obtained in the MTT reduction assay in both differentiated and non-differentiated H9c2 cells. The differentiation state of the cells was not crucial to PIX effects, although PIX toxicity was slightly higher in differentiated H9c2 cells. To the best of our knowledge, this was the first *in vitro* study performed with PIX in H9c2 cells and it discloses worrying cytotoxicity at clinically relevant concentrations.

KEY WORDS: Pixantrone; H9c2 cells; cardiotoxicity

ABBREVIATIONS:
DMEM: Dulbecco´s Modified Eagle medium; **DOX:** Doxorubicin; **EDTA:** Ethylenediamine tetraacetic acid; **EMA:** European Medicines Agency; **FBS:** Fetal bovine serum; **FDA:** Food and Drug Administration; **HBSS:** Hanks' balanced salt solution; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **MTX:** Mitoxantrone; **NHL:** Non-Hodgkin's lymphoma; **NR:** Neutral Red; **PBS:** Phosphate buffered saline; **PIX:** Pixantrone; **RA:** Retinoic acid; **ROS:** Reactive oxygen species

Introduction

Chemotherapy is still one of the most frequent strategies used against cancer. Anticancer drugs, however, lack total selectivity, which results in serious side effects (Hrynchak *et al.,* 2017; Reis-Mendes *et al.,* 2015). Cardiotoxicity is one of the most worrying side effect found in patients treated with anticancer drugs of several pharmacological groups, like anthracyclines [*e.g.* doxorubicin (DOX)] and anthracenediones [*e.g.* mitoxantrone (MTX)] (Hrynchak *et al.,* 2017; Reis-Mendes *et al.,* 2015). DOX has been used for almost 50 years, but cardiotoxicity has been a critical and limiting side effect in its clinical use. MTX was synthesized with the purpose to maintain an effective anticancer potential yet without the cardiotoxicity, which characterized DOX (Conner, 1984). However, early data of

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MTX demonstrated that cardiac toxicity was still a matter of concern (Stuart-Harris *et al.,* 1984). It was thus necessary to study the cardiotoxicity mechanisms of these compounds in order to find new ones that would be, in fact, less cardiotoxic. The mechanisms of cardiotoxicity of MTX are still poorly known, however several hypotheses have been suggested. The 5,8-dihydroxyphenyl ring of MTX was implicated in its cardiotoxicity through the formation of free radicals (Corbett *et al.,* 1981; El-Helw & Hancock, 2007). Additionally, MTX can form complexes with Fe^{3+} and lead to oxidative stress, although the Fe^{3+}-MTX complex is eight times less potent than Fe^{3+}-DOX in the production of the hydroxyl radical (Herman *et al.,* 1997; Malisza & Hasinoff, 1995). Actually, the oxidative stress hypothesis was the first considered as the culprit for MTX-induced cardiotoxicity, possibly because it has been linked to DOX-induced cardiotoxicity (Costa *et al.,* 2013). Therefore, the 5,8-dihydroxyphenyl ring of MTX was replaced by a pyridine ring to create an aza derivative. Moreover, the (ethylamino)-diethylamino side chains were substituted for (hydroxyethylamino)-ethylamino side chains, forming a powerful cytotoxic agent against *in vitro* and murine models of cancer, BBR 2778. This compound was later named pixantrone (PIX) (6,9-bis [(2-aminoethyl) amino] benzo[g]isoquinoline-5, 10-dione) (Krapcho *et al.,* 1994) (Figure 1).

PIX (Pixuvri®) was approved by the European Medicines Agency (EMA) in monotherapy in adult patients with relapsed or refractory aggressive B-cell non-Hodgkin's lymphoma (NHL) (EMA 2017; Péan *et al.,* 2013). PIX seems to have similar anticancer mechanisms when compared with MTX and anthracyclines, namely DOX (Volpetti *et al.,* 2014). Anthracyclines or anthracenediones kill tumor cells by intercalating in the DNA, inhibiting topoisomerase IIα, and forming double-strand breaks (Beggiolin *et al.,* 2001; Hasinoff *et al.,* 2016). These mechanisms are essentially due to the presence of the planar ring system that is composed by the four rings in anthracyclines and an amino-sugar, daunosamine, and the three rings in anthracenediones without any amino-sugar (Beggiolin *et al.,* 2001; Hasinoff *et al.,* 2016). Moreover, the nitrogen atom in PIX was added in the anthracenedione chromophore to create an additional hydrogen bond, and in turn, modulate the DNA affinity and the interaction with topoisomerase II (El-Helw & Hancock, 2007). In fact, PIX works as a topoisomerase II inhibitor, as PIX induces formation of linear DNA in a cleavage assay with topoisomerase IIα and topoisomerase IIβ. In K562 cells, PIX also showed to cause DNA double-strand breaks (Hasinoff *et al.,* 2016).

While some efficacy has been demonstrated in relapsed aggressive NHL (Borchmann *et al.,* 2003; Pettengell *et al.,* 2012), the Food and Drug Administration USA (FDA) has not approved PIX use and it has only been conditionally approved in the EU for aggressive NHL (EMA, 2017). In fact, data regarding its cardiotoxicity are scarce, and lack of FDA approval is, in part, due to the inconsistencies shown by some of the studies carried out so far (FDA, 2010). In a Phase I clinical trial performed by Faivre *et al.* six patients with advanced solid tumors received a cumulative dose of 500 mg/m^2 (range, 560–1 460 mg/m^2) of PIX without any evidence of cardiotoxicity (Faivre *et al.,* 2001). Although not so widely used as its analogues, from the cardiac point of view, most authors consider PIX a safer drug than DOX or MTX. However, in a study by Dawson and collaborators, a patient treated with total dose of 1 120 mg/m^2, four cycles, showed signs of decreased left ventricular ejection (Dawson *et al.,* 2000) and a Phase III clinical trial using lower cumulative doses showed decreased ejection fraction in 19% of the patients treated with PIX (Pettengell *et al.,* 2012). As reported by the FDA, Phase II and Phase III clinical studies showed several inconsistences regarding PIX cardiotoxicity (FDA, 2010). There are also few *in vitro* studies aimed to evaluate the effects of PIX on cardiac cells (Hasinoff *et al.,* 2016), thus a better clarification concerning its possible cardiotoxicity is essential. Therefore, the main purpose of this work was to study PIX cardiotoxicity *in vitro* in non-differentiated and differentiated H9c2 cells as to elucidate its possible cardiotoxicity.

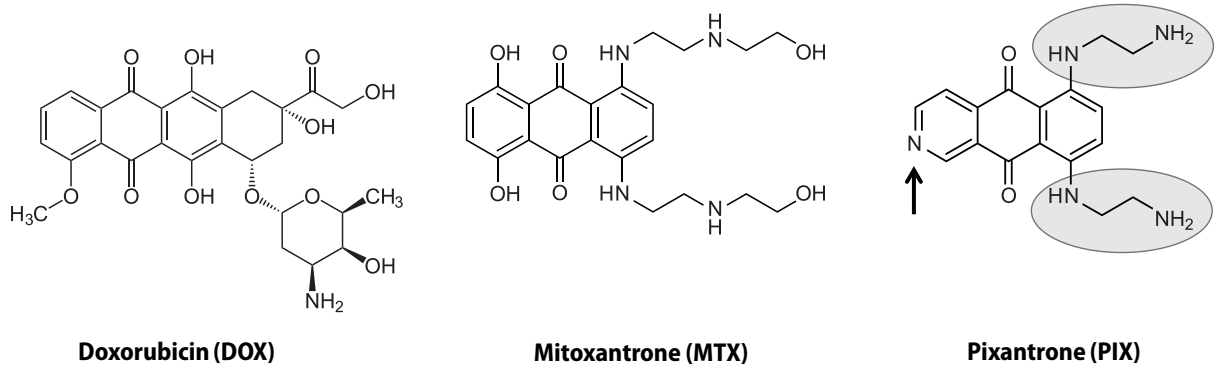

Figure 1. Chemical structure of DOX, MTX and PIX. DOX is composed by a tetracyclic quinone-hydroquinone chromophore, a carbonyl-containing side chain, and an aminosugar (daunosamine). MTX is a three-ring quinone-hydroquinone anthracenedione; its side chains lack carbonyl groups and MTX also lacks daunosamine. PIX differs from MTX in its lack of the hydroquinone, insertion of a nitrogen heteroatom in the same ring (black arrow), and substitution of (ethylamino)-diethylamino for (hydroxyethylamino)-ethylamino side chains (grey circles).

Materials and methods

Materials

Trypsin-EDTA solution, trypan blue solution 0.4% (w/v) and Dulbecco´s Modified Eagle Medium (DMEM) high glucose, sodium dodecyl sulphate, hydrochloric acid, sodium bicarbonate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), neutral red (NR) solution, Hoescht 33258 solution, dimethyl sulfoxide, retinoic acid (RA) and paraformaldehyde were obtained from Sigma-Aldrich (Germany). PIX was acquired from Abcam (United Kingdom). All sterile plastic material was obtained from Corning Costar (USA). Penicillin/streptomycin (10 000 units/mL/10 000 µg/mL) and the phosphate buffer solution (PBS) without calcium and magnesium were obtained from Biochrom (Germany). Fetal bovine serum (FBS), PBS with calcium and magnesium and Hank's saline solution (HBSS or "Hanks' balanced salt solution") were obtained from Gibco (United Kingdom).

Cell culture

Experiments were performed using H9c2 cells, a subclone of the original cell line derived from embryonic BD1X rat heart tissue (Kimes & Brandt, 1976). The rat cardiomyocyte derived H9c2 cell line was obtained from the European Collection of Cell Cultures [H9c2 cell line from rat (BDIX heart myoblast), Sigma-Aldrich (Germany)]. H9c2 cells have morphological similarities with immature embryonic cardiomyocytes, while preserving characteristics of adult cardiac cells (Hescheler *et al.,* 1991) and their metabolic characteristics are similar to those of the rat heart (Zordoky & El-Kadi, 2007). The cells are not fully differentiated in cardiomyocytes and remain in the proliferative state when 10% FBS is present in the culture medium (Rossato *et al.,* 2013). They are one of the most widely used *in vitro* model to study cardiotoxicity (Rossato *et al.,* 2013; Sardão *et al.,* 2009; Zordoky & El-Kadi, 2007). To obtain differentiated H9c2 cells, the cells are maintained in a low FBS (1%) medium enriched with 10 nM RA for 7 days (medium change every other day). This procedure enhances the cells' cardiac adult phenotype and decreases myogenic differentiation and cellular division (Menard *et al.,* 1999). Since PIX acts on cellular division, this later model can be seen as a good model to avoid the bias of high proliferation rates.

Experimental procedures in cell culture

Cell maintenance

While proliferating, cells were placed at 37 °C with 5% CO_2 in DMEM high glucose enriched by 10% of FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. To prevent loss of myoblast cells, cell cultures were subcultured through trypsinization before reaching 70–80% confluence and were used before passage 29 (Reis-Mendes *et al.,* 2017; Ruiz *et al.,* 2012).

H9c2 were used in two differentiation states: non-differentiated and differentiated. The trypsinization step was performed prior to each seeding and to maintain cells in culture. Non-differentiated H9c2 cells were seeded at a density of 8 750 cells/cm^2 (Sardão *et al.,* 2009). Twenty-four hours after seeding, the cells were incubated with different PIX concentrations (0.1, 1 and 10 µM) for 48 h. The concentrations were chosen based on the plasma values of PIX found in a Phase I clinical trial (Faivre *et al.,* 2001). Maximum concentration (Cmax) of PIX 1 h after intravenous doses (37.5 to 150 mg/m^2) ranged between 1–7 µM (Faivre *et al.,* 2001).

H9c2 cells were also differentiated with DMEM supplemented with 1% FBS, RA 10 nM and antibiotics, with medium change every other day for 7 days, after being seeded in a density of 6 000 cells/cm^2 (Reis-Mendes *et al.,* 2017). After the 7-day differentiation, cells were incubated with PIX (0.1, 1 or 10 µM) for 48 h.

Stock solutions of PIX were prepared in sterile PBS and PBS was used as control. PIX solutions were made monthly and frozen at –20 °C to guarantee stability.

Phase contrast microscopy

Phase-contrast microscopy morphological evaluation was performed to determine the effects of PIX after a 48-h incubation in both non-differentiated and differentiated cells. A Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera (Japan) was used.

Hoescht staining

To evaluate the effects of PIX on the nuclear morphology of both non-differentiated and differentiated H9c2 cells, Hoechst staining was performed, as previously described (Reis-Mendes *et al.,* 2017). Cells were examined in a Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera (Japan) using a fluorescent filter (λexcitation = 346 nm and λemission = 460 nm).

Cytotoxicity evaluation

For the evaluation of PIX putative cytotoxicity, two assays were used: the NR lysosomal uptake and the MTT reduction assays. Both methods were performed 48 h after the cells' exposure to PIX and the assays are described as follows.

MTT reduction assay

The MTT colorimetric assay is based on the reduction of the tetrazolium salt with formazan formation and it was performed as previously described (Reis-Mendes *et al.,* 2017; Rossato *et al.,* 2013). The spectrophotometric measurement of the formazans was done at 550 nm in a multi-well plate reader (PowerWaveXS, BioTek Instruments Inc., USA).

Neutral red lysosomal uptake assay

The NR dye enters the viable cells and concentrates in lysosomes. The NR uptake assay was performed as previously described (Reis-Mendes *et al.,* 2017). The absorbance was read at 540 and 690 nm (reference) on a multi-well plate reader (Biotech Synergy HT, USA).

Statistical analysis

The results are expressed as mean ± standard deviation. Outliers were evaluated by the ROUT test. The D'Agostino & Pearson normality test was used to evaluate data distribution. A parametric analysis of variance (ANOVA) was performed when data distribution was normal, followed by the Tukey's *post-hoc* test. When data did not follow a normal distribution, statistical analysis was performed using the Kruskal-Wallis test, followed by the Dunn's *post-hoc* test when a significant *p*-value was reached. Statistical significance was reached when $p<0.05$. All statistical analyses were performed in GraphPad Prism 7 software (USA). All details of the statistical analyses can be found in the figure legends.

Results

Morphological evaluation of pixantrone-exposed non-differentiated H9c2 cells showed cytotoxicity at the highest concentration tested with no signs of condensed nuclei

Cells were morphologically assessed through phase-contrast microscopy after a 48-h exposure to PIX. H9c2 cells were incubated with PIX (0.1, 1 and 10 µM), as described in the Methods section. Control cells had mostly cells with polygonal form attached to the bottom of the wells with very few detached cells (Figure 2A). At 48 h, PIX 0.1 and 1 µM had no signs of cellular damage (Figures 2B and 2C), while PIX 10 µM caused a clear cytoplasmic injury in non-differentiated H9c2 cells and most cells were round and detached (Figure 2D). Hoescht staining was used to evaluate the nuclear morphology after the 48-h incubation. A slight decrease in the number of cells in PIX 0.1 µM (Figure 2F) and PIX 1 µM (Figure 2G) exposed cells was observed when compared to the control wells (Figure 2E), with some nuclear fragmentation in non-differentiated H9c2 cells incubated with PIX 0.1 µM (Figure 2F). PIX 10 µM caused a marked decrease in the number of cells, with very few fluorescent nuclei in the field (Figure 2H).

Pixantrone caused significant cytotoxicity in non-differentiated H9c2 cells according to the neutral red and MTT assays

The NR uptake and the MTT reduction assays were done after the 48-h incubation in non-differentiated H9c2 cells (Figure 3). In the NR assay, PIX caused a concentration dependent decrease in the uptake of the vital dye: 0.1 µM presented values 87.07±6.07%, 1 µM of 76.25±8.21% and 10 µM of 23.08±13.53% (Figure 3A). In the MTT assay, PIX produced a significant mitochondrial dysfunction at 0.1 µM (89.32±7.49%), 1 µM (84.06±8.82%) and 10 µM (47.00±7.39%) concentrations when compared to control cells (100.0±3.51%) (Figure 3B).

Pixantrone caused higher cellular damage in differentiated than in non-differentiated H9c2 cells

The differentiation with 10 nM RA and 1% FBS for 7 days led to morphological changes, making H9c2 cells more resembled to the cardiac phenotype. The differentiation protocol dramatically reduced cell division, and the cell bodies became smaller and fusiform. Overall, differentiated control cells showed a more organized network, bearing a cardiac-like morphology, as shown by phase-contrast morphology (Figure 4A). In differentiated H9c2 cells at 48 h, PIX incubation caused cell injury with cytoplasmic injury observed at PIX 1 µM and PIX 10 µM. In the latter concentration, the cellular damage was substantial (Figure 4D). In the Hoescht staining, a slight decrease in the number of cells for PIX 0.1 µM (Figure 4F) and PIX 1 µM (Figure 4G) was observed when compared to the control wells (Figure 4E). PIX 10 µM was able to cause a marked decrease in the number of cells, with very few fluorescent nuclei observed in the field (Figure 4H).

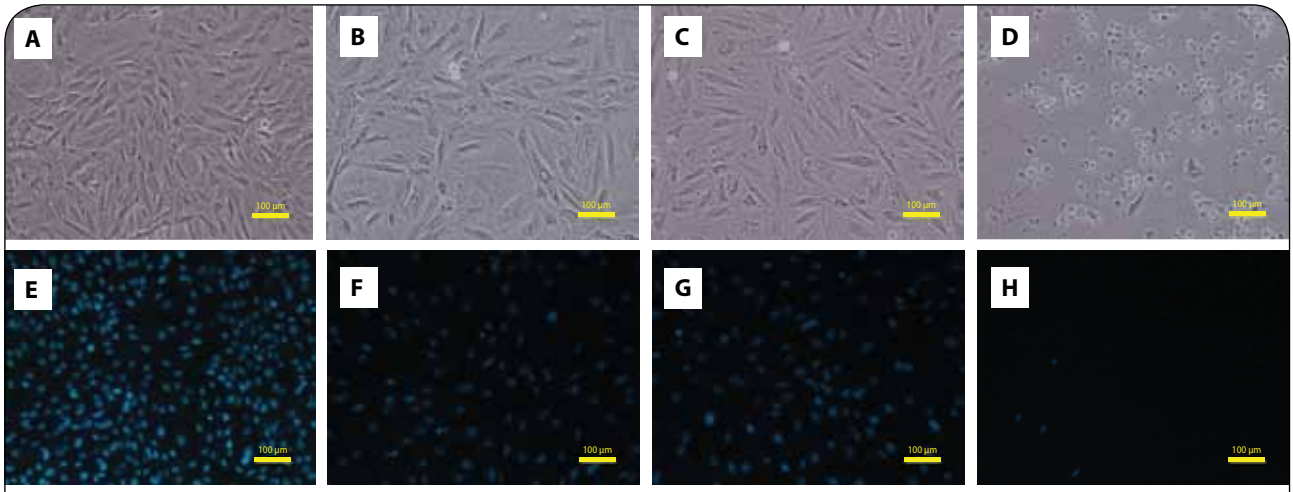

Figure 2. PIX causes cytotoxicity in non-differentiated H9c2 without signs of condensed nuclei. Phase contrast microscopy (A, B, C, D) and fluorescence microscopy (Hoechst 33258 staining) (E, F, G, H) images of non-differentiated H9c2 cells after a 48-h exposure to PBS (A and E), 0.1 µM PIX (B and F), 1 µM PIX (C and G) or 10 µM PIX (D and H). Images are representative of two independent experiments (scale bar 100 µm).

Pixantrone caused a concentration-dependent mitochondrial and lysosome dysfunction in differentiated H9c2 cells

In the NR assay performed in differentiated H9c2 cells, the highest concentration of PIX (10 µM) caused a substantial impairment of lysosomal uptake of the vital dye (7.44±3.83%) when compared to control cells (100.0±2.03%). NR uptake in PIX 0.1 µM was 93.51±3.22% and in PIX 1 µM, it was 86.15±3.06% (Figure 5A). In the MTT reduction assay, PIX 10 µM (39.54±2.40%) caused a significant mitochondrial dysfunction when compared to control cells (100.0±2.90%) (Figure 5B). The highest concentration of PIX (10 µM) caused a significant higher cytotoxicity when compared to PIX 1 µM or PIX 0.1 µM (89.42±6.29% and 91.59±5.27%, respectively).

Discussion

The present study is the first report on the toxicity of pharmacologically relevant concentrations of PIX (0.1; 1 and 10 µM) in H9c2 cells. PIX was cytotoxic at all concentrations tested, as evaluated by phase contrast microscopy, MTT reduction and NR uptake assays, in both differentiated and non-differentiated H9c2 cells.

A Phase III, multicentre, open-label, randomized trial was used to test the efficacy and safety of PIX in heavily pre-treated patients with relapsed or refractory aggressive NHL. The frequency of cardiac adverse events was higher in the PIX group than in the comparator group, where patients were treated with vinorelbine, oxaliplatin,

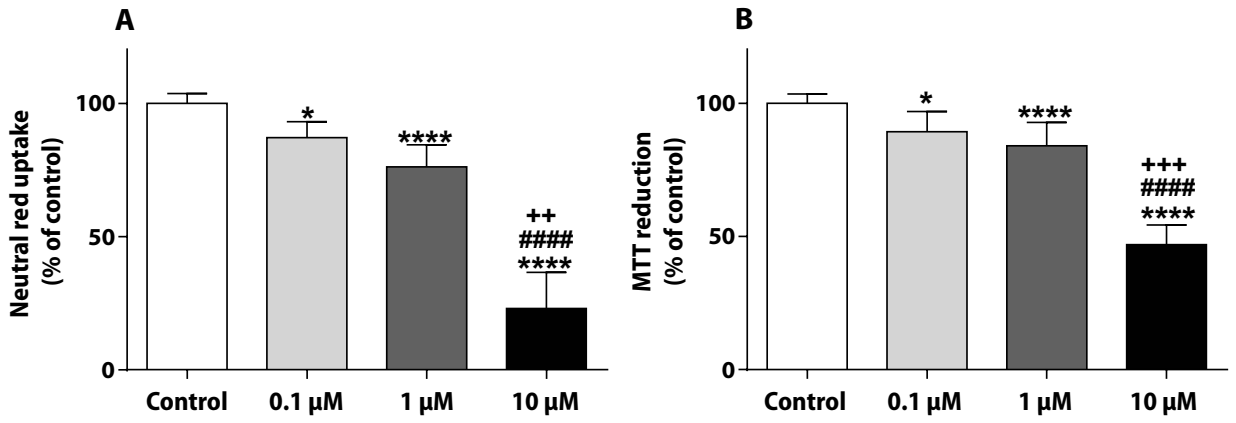

Figure 3. PIX leads to lysosomal and mitochondrial damage in non-differentiated H9c2. NR uptake (A) and MTT reduction (B) assays in non-differentiated H9c2 cells exposed to 0.1, 1 or 10 µM PIX for 48 h. Values are expressed as percentage of control and are shown as mean ± standard deviation. The results were obtained from 4–5 independent experiments using 20–24 wells. The statistical analyses were made using the Kruskal-Wallis test, followed by the Dunn's *post-hoc* test ($^{*}p<0.05$ and $^{****}p<0.0001$ versus control; $^{\#\#\#\#}p<0.0001$ versus PIX 0.1 µM; $^{++}p<0.01$, $^{+++}p<0.001$ versus PIX 1 µM).

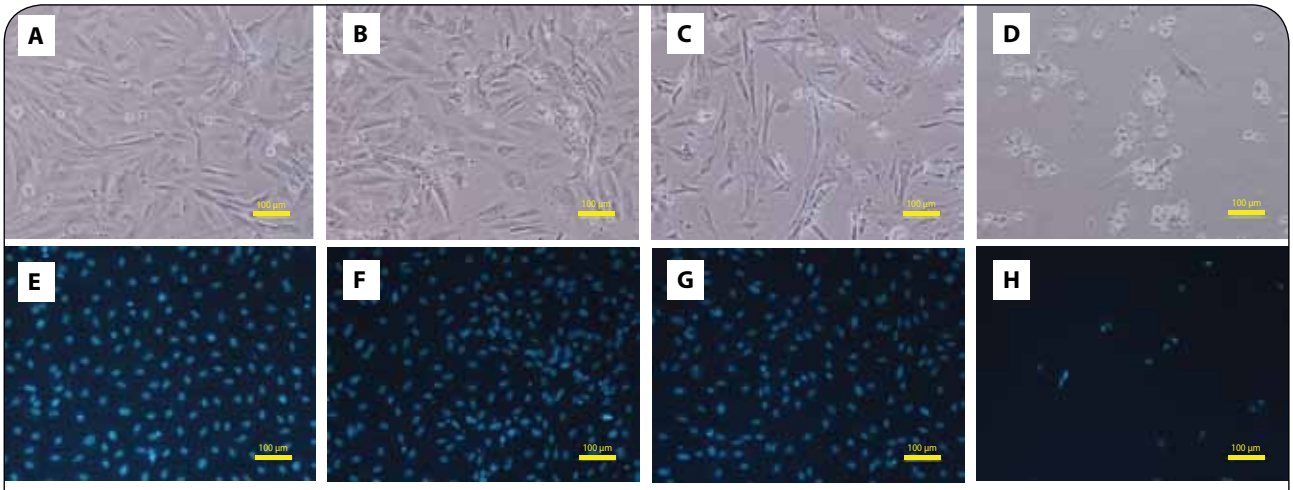

Figure 4. PIX evokes cellular damage in a concentration dependent manner in differentiated H9c2 cells. Phase contrast microscopy (A, B, C, D) and fluorescence microscopy (Hoechst 33258 staining) (E, F, G, H) images of 7-day differentiated H9c2 cells after a 48-h exposure to PBS (A and E), 0.1 µM PIX (B and F), 1 µM PIX (C and G) or 10 µM PIX (D and H). Images are representative of two independent experiments (scale bar 100 µm).

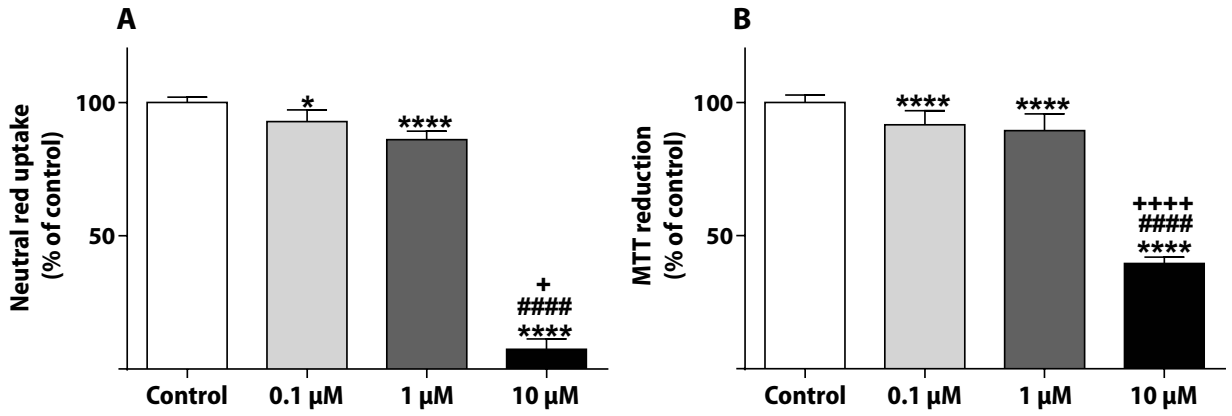

Figure 5. PIX induces lysosomal and mitochondrial disruption in differentiated H9c2 cells. NR uptake (A) and MTT reduction (B) assays in differentiated H9c2 cells exposed to 0.1, 1 or 10 µM PIX for 48 h. Values are expressed as percentage of control and are shown as mean ± standard deviation. The results were obtained from 4 independent experiments using 20 wells. The statistical analyses were made using the Kruskal-Wallis test, followed by Dunn's *post-hoc* test (A) and the ANOVA test, followed by the Tukey *post hoc* test (B) ($^{*}p<0.05$ and $^{****}p<0.0001$ versus control; $^{\#\#\#\#}p<0.0001$ versus PIX 0.1 µM; $^{+}p<0.05$, $^{++++}p<0.0001$ versus PIX 1 µM).

ifosfamide, etoposide, MTX, or gemcitabine in a single agent therapy. The authors justified the higher frequency of cardiac adverse events in the PIX group by the presence of five patients within this group (compared with none in the comparator group) with previous histories of congestive heart failure or cardiomyopathy at the time of study enrolment (Pettengell *et al.,* 2012). However, two major issues, in our point of view, must be discussed that make PIX eligible as culprit of those cardiac events. More cardiac adverse events occurred in the PIX treatment group [24 of 68 (35.3%)] than in the comparator [14 of 67 (20.9%)], even when the comparator group had a higher median previous DOX dose equivalent of 315.5 mg/m^2 (range 15–681 mg/m^2), when compared to 292.9 mg/m^2 (range 51–472 mg/m^2) in the PIX group (Pettengell *et al.,* 2012). Moreover, patients in the comparator arm were treated with acknowledged cardiotoxic drugs (Pettengell *et al.,* 2012) but presented lower incidence of cardiotoxicity. In fact, FDA raised several issues regarding PIX cardiotoxicity, suggesting that PIX is indeed cardiotoxic, but gave no conclusions regarding the comparison of its toxicity to anthracyclines/anthracenediones (FDA, 2010). Our results support the idea that PIX is, in fact, cardiotoxic. In the morphological evaluation done through phase contrast microscopy, cellular damage was not evident following a 48-h exposure to 0.1 or 1 µM PIX, in non-differentiated H9c2 cells. Only at 10 µM PIX a significant damage was observed. Despite the clear reduction in the number of nuclei, especially seen in the highest PIX concentration tested (10 µM), in both differentiated and non-differentiated cells, no significant changes on nuclear morphology were evident when compared to control cells. These data indicate that PIX cytotoxicity may not elicit apoptosis in H9c2 cells in the time-point analyzed. Moreover, in the present work, two methods were used to evaluate the cytotoxicity of PIX, the MTT reduction and the NR uptake assays, which are extremely sensitive in assessing cellular homeostasis impairment. The MTT colorimetric assay is based on mitochondrial activities of NADH dehydrogenase (complex I) and/or succinate dehydrogenase (complex II) on the tetrazolium salt with formazan formation (Costa *et al.,* 2009), whereas the NR uptake assay is based on the ability of viable cells to uptake the supravital dye that penetrates cell membranes and concentrates in lysosomes (Repetto *et al.,* 2008). Significant cytotoxicity was reported in both assays at all concentrations tested, being more pronounced for 10 µM PIX at 48 h, both in differentiated and non-differentiated H9c2 cells. The concentrations used here are clinically relevant, since 1 h after intravenous administration of doses ranging between 37.5 to 150 mg/m^2, the Cmax of PIX on the plasma of patients in a Phase I clinical trial was between 1–7 µM (Faivre *et al.,* 2001). The recommended dose of PIX is 50 mg/m^2 administered on days 1, 8, and 15 of each 28-day cycle for up to 6 cycles for the treatment of adult patients with multiple relapsed or refractory aggressive NHL (Péan *et al.,* 2013).

To the best of our knowledge, there is only one study with PIX in cardiac cellular models (Hasinoff *et al.,* 2016). For that reason, our results will be compared with the published data of other acknowledged cardiotoxic anticancer drugs, DOX and MTX, in *in vitro* models. Non-differentiated H9c2 cells exposed to 1 µM DOX at several time points (between 0 and 48 h) evidenced an increase in cell death with increasing time of exposure, evaluated through phase contrast microscopy (Zhang *et al.,* 2015). Regarding MTX, our group assessed the cytotoxicity of 5 µM and 2 µM MTX in differentiated H9c2 cells. After a 48-h exposure, an increase in cell death with increasing MTX concentrations was found (Reis-Mendes *et al.,* 2017). Based on these morphological data, PIX seems to cause lower damage when compared to DOX or MTX in the same cellular model, since only at the highest concentration tested (10 µM), a significant

damage was seen. Moreover, in our study with MTX, no signs of nuclear morphological alterations were observed after exposure to 2 or 5 μM MTX for 48 h in differentiated H9c2 cells (Reis-Mendes *et al.,* 2017), as evidenced here with PIX. However, in non-differentiated H9c2 cells, a 24-h exposure to 1.60 μM MTX or 0.9 μM DOX caused morphological signals of apoptosis (Kluza *et al.,* 2004) and caspase 3 activation occurred in non-differentiated H9c2 cells exposed for 24 h to 100 nM and 1 μM MTX (Rossato *et al.,* 2013).

In non-differentiated H9c2 cells, DOX elicited mitochondrial dysfunction as seen through the MTT reduction assay, following a 24-h exposure to 0.2, 1 and 5 μM (Zhang *et al.,* 2015). Regarding MTX, also in non-differentiated H9c2 cells, it led to a time- and concentration-dependent cytotoxicity (Rossato *et al.,* 2013). At 48 h, 10 μM MTX had values of around 20% when compared to control (100.0%) in the MTT assay, whereas 1 and 0.1 μM MTX showed values of approximately 80 and 90%, respectively, when compared to control cells (Rossato *et al.,* 2013). In the study carried out by our group using differentiated H9c2 cells exposed to various concentrations of MTX (0.01, 0.1, 1, 2 and 5 μM) for 48 h, 5 μM MTX showed values of around 75% in the MTT reduction assay, whereas 1 and 0.1 μM of 80% and 90%, respectively, when compared to control cells (Reis-Mendes *et al.,* 2017). The cytotoxicity caused by MTX is, in fact, very similar to what was observed in the present work with similar concentrations of PIX in non-differentiated and differentiated cells. Moreover, PIX, as did DOX, decreased the mitochondrial membrane potential of cardiac rat neonatal cardiomyocytes, thus demonstrating the ability of PIX to alter mitochondrial homeostasis (Hasinoff *et al.,* 2016) and its cardiotoxic potential.

Moreover, in the present work, 10 μM PIX caused a higher cytotoxicity in the NR assay in differentiated H9c2 cells when compared to non-differentiated cells. This assay also seems more sensitive to detect PIX cytotoxicity. On the other hand, using the same assay, differentiated H9c2 cells exposed for 48 h to 1 μM MTX incorporated 60% of NR when compared to control cells (set to 100%) (Reis-Mendes *et al.,* 2017), whereas with PIX, at the same incubation time and using the same concentration, the NR uptake was 76.25±8.21% in non-differentiated cells and 86.15±3.06% in differentiated cells. Additionally, 0.1 μM MTX did not cause any significant toxicity in the NR assay at 48 h in differentiated H9c2 cells (Reis-Mendes *et al.,* 2017), while 0.1 μM PIX decreased the dye's cellular incorporation to 87.07±6.09% in non-differentiated cells and to 93.51±3.22% in differentiated cells at the same incubation time point, further demonstrating the cardiotoxic potential of PIX. Most authors agree that using several cytotoxicity tests with different inherent mechanisms can help elucidate the underlying cytotoxicity of the drugs tested (Lopes *et al.,* 2008; Reis-Mendes *et al.,* 2017; Soares *et al.,* 2013).

The mechanisms involved in PIX putative cardiotoxicity are still undisclosed. In human leukemia K562 cells and contrasting with DOX, PIX produced no detectable semi-quinones or reactive oxygen species (ROS), possibly due to a lower PIX accumulation in those cells. The intracellular accumulation of PIX (1.5 nmol) was shown to be low when compared to MTX (8.1 nmol) or DOX (6.5 nmol) under the same experimental conditions (1 h incubation at 37 °C in a final concentration of 10 nmol) (Hasinoff *et al.,* 2016). Also, the spectrophotometric titrations of PIX suggested that, unlike what happens with DOX or MTX, PIX does not bind to Fe^{3+}, probably due to its lack of hydroquinone functionality (Herman *et al.,* 1997; Malisza and Hasinoff 1995). Fe^{3+} causes oxidative stress via the Fenton reaction and through formation of the highly toxic hydroxyl radical (Costa *et al.,* 2011). It is widely known that, in the heart, DOX forms a semiquinone free radical and Fe^{3+}-DOX complexes, which are responsible for a futile redox cycle that creates oxidative stress (Costa *et al.,* 2013). MTX does not form ROS easily, but it affects the heart mitochondria (Reis-Mendes *et al.,* 2017; Rossato *et al.,* 2014; Rossato *et al.,* 2013), with the heart being largely susceptible to energetic dysfunction. According to Rossato *et al.*, the endpoint of toxicity induced by MTX is the electron transport chain and its dysfunction can create a mild oxidative stress that results in fatal cardiotoxicity (Rossato *et al.,* 2014; Rossato *et al.,* 2013). As stated before, PIX is able to decrease the mitochondrial membrane potential of cardiac rat neonatal cardiomyocytes (Hasinoff *et al.,* 2016) and mitochondria can be targets for PIX.

Additionally, compared to MTX and anthracyclines, PIX shows better selectivity for topoisomerase IIα (the form present in cancer cells) instead of IIβ (the form present in cardiomyocytes) at the 1–10 μM concentration range (Hasinoff *et al.,* 2016). The higher selectivity of PIX towards topoisomerase IIα led to the belief that PIX elicits low cardiotoxicity, since topoisomerase IIβ is the isoform present in cardiomyocytes and its inhibition can be responsible, at least partially, for the cardiotoxicity of DOX and MTX (Capranico *et al.,* 1992; Hasinoff *et al.,* 2016; Zhang *et al.,* 2012). However, the cardiac concentration of PIX after therapy is not known, and it may be sufficient to inhibit the physiological cardiac form of topoisomerase and thus cause cardiac dysfunction. In fact, cardiac dysfunction has been observed after PIX therapy, even if its use is not largely disseminated (Pettengell *et al.,* 2012; Volpetti *et al.,* 2014).

Overall, the two cytotoxicity assays performed in the present work show to be more sensitive to detect PIX-induced toxicity than Hoescht staining or phase contrast microscopy. In addition, differentiated H9c2 cells are slightly more prone to PIX induced cytotoxicity than non-differentiated H9c2 cells. Thus PIX toxicity is not dependent on its effects on cellular division, as the former model has a reduced proliferative state (Menard *et al.,* 1999; Reis-Mendes *et al.,* 2017). Despite the structural changes performed in the synthesis of PIX and its lower potential to form ROS and toxic drug metal complexes in human myocardial strip (Salvatorelli *et al.,* 2013), our work clearly shows its potential cardiac toxicity. This contradicts some of the data obtained in the scarce

clinical and preclinical studies done with PIX, where PIX is claimed as a cardiosafe drug when compared with its analogues (Beggiolin *et al.,* 2001; Borchmann *et al.,* 2003; Cavalletti *et al.,* 2007; Hasinoff *et al.,* 2016; Pettengell *et al.,* 2012). Actually, compared to the reports made for its predecessors, mainly with the works done in our group with MTX in the same cellular models (Reis-Mendes *et al.,* 2017; Rossato *et al.,* 2013), the data obtained here show that PIX has similar cardiac toxicity as MTX and should be regarded as a potential cardiotoxic drug.

Acknowledgments

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REFERENCES

Beggiolin G, Crippa L, Menta E, Manzotti C, Cavalletti E, Pezzoni G, Torriani D, Randisi E, Cavagnoli R, Sala F, Giuliani FC, Spinelli S. (2001). Bbr 2778, an aza-anthracenedione endowed with preclinical anticancer activity and lack of delayed cardiotoxicity. *Tumori* **87**: 407–416.

Borchmann P, Morschhauser F, Parry A, Schnell R, Harousseau JL, Gisselbrecht C, Rudolph C, Wilhelm M, Gunther H, Pfreundschuh DM, Camboni G, Engert A. (2003). Phase-II study of the new aza-anthracenedione, BBR 2778, in patients with relapsed aggressive non-Hodgkin's lymphomas. *Haematologica* **88**: 888–894.

Capranico G, Tinelli S, Austin CA, Fisher ML, Zunino F. (1992). Different patterns of gene expression of topoisomerase II isoforms in differentiated tissues during murine development. *Biochim Biophys Acta* **1132**: 43–48.

Cavalletti E, Crippa L, Mainardi P, Oggioni N, Cavagnoli R, Bellini O, Sala F. (2007). Pixantrone (BBR 2778) has reduced cardiotoxic potential in mice pretreated with doxorubicin: comparative studies against doxorubicin and mitoxantrone. *Invest New Drugs* **25**: 187–195.

Conner CS. (1984). Mitoxantrone: a replacement for doxorubicin? *Drug Intell Clin Pharm* **18**: 479–480.

Corbett TH, Griswold DP Jr., Roberts BJ, Schabel FM Jr. (1981). Absence of delayed lethality in mice treated with aclacinomycin A. *Cancer Chemother Pharmacol* **6**: 161–168.

Costa VM, Carvalho F, Bastos ML, Carvalho RA, Carvalho M, Remião F. (2011). Contribution of catecholamine reactive intermediates and oxidative stress to the pathologic features of heart diseases. *Curr Med Chem* **18**: 2272–2314.

Costa VM, Carvalho F, Duarte JA, Bastos ML, Remiao F. (2013). The heart as a target for xenobiotic toxicity: the cardiac susceptibility to oxidative stress. *Chem Res Toxicol* **26**: 1285–1311.

Costa VM, Silva R, Tavares LC, Vitorino R, Amado F, Carvalho F, Bastos ML, Carvalho M, Carvalho RA, Remiao F. (2009). Adrenaline and reactive oxygen species elicit proteome and energetic metabolism modifications in freshly isolated rat cardiomyocytes. *Toxicology* **260**: 84–96.

Dawson LK, Jodrell DI, Bowman A, Rye R, Byrne B, Bernareggi A, Camboni G, Smyth JF. (2000). A clinical phase I and pharmacokinetic study of BBR 2778, a novel anthracenedione analogue, administered intravenously, 3 weekly. *Eur J Cancer* **36**: 2353–2359.

El-Helw LM, Hancock BW. (2007). Pixantrone: a novel aza-anthracenedione in the treatment of non-Hodgkin's lymphomas. *Expert Opin Investig Drugs* **16**: 1683–1691.

EMA. (2017). *Pixuvri (pixantrone): EU summary of product characteristics*. European Medicines Agency, London, UK.

Faivre S, Raymond E, Boige V, Gatineau M, Buthaut X, Rixe O, Bernareggi A, Camboni G, Armand JP. (2001). A phase I and pharmacokinetic study of the novel aza-anthracenedione compound BBR 2778 in patients with advanced solid malignancies. *Clin Cancer Res* **7**: 43–50.

FDA. (2010). *FDA ODAC Briefing Document: Pixuvri (pixantrone).* In: U.S.D.o.H.a.H. Services (Ed), Maryland, USA.

Hasinoff BB, Wu X, Patel D, Kanagasabai R, Karmahapatra S, Yalowich JC. (2016). Mechanisms of action and reduced cardiotoxicity of pixantrone; a topoisomerase II targeting agent with cellular selectivity for the topoisomerase II alpha isoform. *J Pharmacol Exp Ther* **356**: 397–409.

Herman EH, Zhang J, Hasinoff BB, Clark JR Jr., Ferrans VJ. (1997). Comparison of the structural changes induced by doxorubicin and mitoxantrone in the heart, kidney and intestine and characterization of the Fe(III)-mitoxantrone complex. *J Mol Cell Cardiol* **29**: 2415–2430.

Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. (1991). Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res* **69**: 1476–1486.

Hrynchak I, Sousa E, Pinto M, Costa VM. (2017). The importance of drug metabolites synthesis: the case-study of cardiotoxic anticancer drugs. *Drug Metab Rev* **49**: 158–196.

Kimes BW, Brandt BL. (1976). Properties of a clonal muscle cell line from rat heart. *Exp Cell Res* **98**: 367–381.

Kluza J, Marchetti P, Gallego MA, Lancel S, Fournier C, Loyens A, Beauvillain JC, Bailly C. (2004). Mitochondrial proliferation during apoptosis induced by anticancer agents: effects of doxorubicin and mitoxantrone on cancer and cardiac cells. *Oncogene* **23**: 7018–7030.

Krapcho AP, Petry ME, Getahun Z, Landi JJJr, Stallman J, Polsenberg JF, Gallagher CE, Maresch MJ, Hacker MP, Giuliani FC, Beggiolin G, Pezzoni G, Menta E, Manzotti C, Oliva A, Spinelli S, Tognella S. (1994). 6,9-Bis[(aminoalkyl) amino]benzo[g]isoquinoline-5,10-diones. A novel class of chromophore-modified antitumor anthracene-9,10-diones: synthesis and antitumor evaluations. *J Med Chem* **37**: 828–837.

Lopes MA, Meisel A, Dirnagl U, Carvalho FD, Bastos ML. (2008). Doxorubicin induces biphasic neurotoxicity to rat cortical neurons. *Neurotoxicology* **29**: 286–293.

Malisza KL, Hasinoff BB. (1995). Production of hydroxyl radical by iron(III)-anthraquinone complexes through self-reduction and through reductive activation by the xanthine oxidase/hypoxanthine system. *Arch Biochem Biophys* **321**: 51–60.

Menard C, Pupier S, Mornet D, Kitzmann M, Nargeot J, Lory P. (1999). Modulation of L-type calcium channel expression during retinoic acid-induced differentiation of H9c2 cardiac cells. *J Biol Chem* **274**: 29063–29070.

Péan E, Flores B, Hudson I, Sjöberg J, Dunder K, Salmonson T, Gisselbrecht C, Laane E, Pignatti F. (2013). The European Medicines Agency review of pixantrone for the treatment of adult patients with multiply relapsed or refractory aggressive non-hodgkin's b-cell lymphomas: Summary of the scientific assessment of the committee for medicinal products for human use. *Oncologist* **18**: 625–633.

Pettengell R, Coiffier B, Narayanan G, de Mendoza FH, Digumarti R, Gomez H, Zinzani PL, Schiller G, Rizzieri D, Boland G, Cernohous P, Wang L, Kuepfer C, Gorbatchevsky I, Singer JW. (2012). Pixantrone dimaleate versus other chemotherapeutic agents as a single-agent salvage treatment in patients with relapsed or refractory aggressive non-Hodgkin lymphoma: a phase 3, multicentre, open-label, randomised trial. *Lancet Oncol* **13**: 696–706.

Reis-Mendes A, Gomes AS, Carvalho RA, Carvalho F, Remiao F, Pinto M, Bastos ML, Sousa E, Costa VM. (2017). Naphthoquinoxaline metabolite of mitoxantrone is less cardiotoxic than the parent compound and it can be a more cardiosafe drug in anticancer therapy. *Arch Toxicol* **91**: 1871–1890.

Reis-Mendes AF, Sousa E, de Lourdes Bastos M, Costa VM. (2015). The role of the metabolism of anticancer drugs in their induced-cardiotoxicity. *Curr Drug Metab* **17**: 75–90.

Repetto G, del Peso A, Zurita JL. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc* **3**: 1125–1131.

Rossato LG, Costa VM, Dallegrave E, Arbo M, Silva R, Ferreira R, Amado F, Dinis-Oliveira RJ, Duarte JA, de Lourdes Bastos M, Palmeira C, Remiao F. (2014). Mitochondrial cumulative damage induced by mitoxantrone: late onset cardiac energetic impairment. *Cardiovasc Toxicol* **14**: 30–40.

Rossato LG, Costa VM, Vilas-Boas V, de Lourdes Bastos M, Rolo A, Palmeira C, Remiao F. (2013). Therapeutic concentrations of mitoxantrone elicit energetic imbalance in H9c2 cells as an earlier event. *Cardiovasc Toxicol* **13**: 413–425.

Ruiz M, Courilleau D, Jullian JC, Fortin D, Ventura-Clapier R, Blondeau JP, Garnier A. (2012). A cardiac-specific robotized cellular assay identified families of human ligands as inducers of PGC-1alpha expression and mitochondrial biogenesis. *PLoS One* **7**: e46753.

Salvatorelli E, Menna P, Paz OG, Chello M, Covino E, Singer JW, Minotti G. (2013). The novel anthracenedione, pixantrone, lacks redox activity and inhibits doxorubicinol formation in human myocardium: insight to explain the cardiac safety of pixantrone in doxorubicin-treated patients. *J Pharmacol Exp Ther* **344**: 467–478.

Sardão VA, Oliveira PJ, Holy J, Oliveira CR, Wallace KB. (2009). Doxorubicin-induced mitochondrial dysfunction is secondary to nuclear p53 activation in H9c2 cardiomyoblasts. *Cancer Chemother Pharmacol* **64**: 811–827.

Soares AS, Costa VM, Diniz C, Fresco P. (2013). Potentiation of cytotoxicity of paclitaxel in combination with Cl-IB-MECA in human C32 metastatic melanoma cells: A new possible therapeutic strategy for melanoma. *Biomed Pharmacother* **67**: 777–789.

Stuart-Harris RC, Bozek T, Pavlidis NA, Smith IE. (1984). Mitoxantrone: an active new agent in the treatment of advanced breast cancer. *Cancer Chemother Pharmacol* **12**: 1–4.

Volpetti S, Zaja F, Fanin R. (2014). Pixantrone for the treatment of adult patients with relapsed or refractory aggressive non-Hodgkin B-cell lymphomas. *Onco Targets Ther* **7**: 865–872.

Zhang S, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, Yeh ET. (2012). Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* **18**: 1639–1642.

Zhang YY, Meng C, Zhang XM, Yuan CH, Wen MD, Chen Z, Dong DC, Gao YH, Liu C, Zhang Z. (2015). Ophiopogonin D attenuates doxorubicin-induced autophagic cell death by relieving mitochondrial damage in vitro and in vivo. *J Pharmacol Exp Ther* **352**: 166–174.

Zordoky BN, El-Kadi AO. (2007). H9c2 cell line is a valuable in vitro model to study the drug metabolizing enzymes in the heart. *J Pharmacol Toxicol Methods* **56**: 317–322.

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ORIGINAL ARTICLE

Toluene abuse markers in marginalized populations

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ABSTRACT

Toluene abuse is one of the most common addictions among marginalized Roma. The aim of the study was the comparison of urinary toluene metabolite levels in marginalized population of Eastern Slovakia as compared to the majority population, and to verify the validity of the answers, given in the questionnaires, regarding toluene abuse. The study was carried out as part of the HEPA-META project aiming to map the prevalence of health problems in marginalized Roma. The majority of people living outside the area of the segregated Roma communities comprised the control group. The total number of study participants was 770. Statistically significant differences in the levels of hippuric acid and *o*-cresol were found between Roma and the majority population. Variations in urinary hippuric acid levels in addition to toluene abuse can be caused also by dietary factors, medical treatment as well as alcohol consumption, which is frequent (not only) in marginalized communities.

KEY WORDS: toluene abuse; Roma; *o*-cresol; hippuric acid

Introduction

Toluene (methylbenzene) is a colorless, volatile liquid with a characteristic sweet odor. It occurs naturally in crude oil, and is produced industrially either from gasoline or coal. Toluene is mainly used as a precursor to benzene (over 50%), benzoic acid, *p*-xylene and is also used in the production of toluene di-isocyanate, precursor for polyurethane foams. Furthermore, toluene is widely used as a solvent for various polymers, resins, and certain pharmaceuticals. Its use as a solvent in adhesives, paints and inks is preferred over benzene due to its much lower toxicity.

The current Occupational Safety and Health Administration standard for toluene is 200 ppm for an 8-hour workday. Toluene levels of 500 ppm are considered dangerous to life and health (Public Health Statement for Toluene, 2015). Due to its lipophilic nature, toluene is absorbed relatively rapidly through the skin, the respiratory and gastrointestinal tract resulting in its rapid distribution throughout the body. In the brain, toluene selectively affects some ion channels. It increases the release of dopamine and the activity of dopaminergic neurons.

Acute intoxication is recognizable thanks to the symptoms of CNS disorders, including euphoria, hallucinations, delusions, tinnitus, dizziness, confusion, headache, vertigo, seizures, ataxia, stupor, and coma (Camara-Lemarroy *et al.*, 2015). Repeated chronic exposure can lead to white matter damage.

Toluene is detectable in the arterial blood after few seconds of inhalation. Approximately 80% of a dose is metabolized in the liver to hippuric acid and is excreted in urine. Ring hydroxylation to cresols is a minor metabolic pathway (Figure 1). On the other hand, hippuric acid is also a degradation product of different aromatic compounds in the digestive tract, while cresols are exclusive degradation products of toluene.

The highest prevalence of volatile solvent misuse worldwide is in marginalized populations [Beckley and Woodward, 2013). Its background is associated with different conditions. Low educational level, indifferent attitude toward drug use, poor social status, poor health, and efforts to escape from the given problems. Toluene abuse has become widespread, especially among marginalized young Roma in our region due to its availability and relatively low cost.

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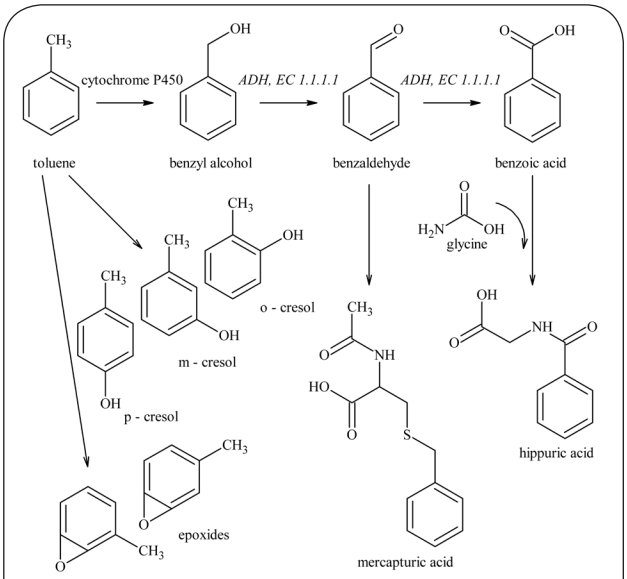

Figure 1. Metabolism of Toluene. Adapted by Yacob & Zinalibdin (2010).

Materials and methods

Participants and data collection

The participants were Roma people living in the segregated settlements in Eastern Slovakia in the Košice County as well as representatives of the majority population from the same region aged 18–55 years, with an average age of 34±8 years (Madarasová Gecková *et al.*, 2014b). The participants recruited via local Roma community workers and general practitioners were divided into 3 categories based on the place of residence:

- Category 1: people from segregated Roma communities, identified as Roma, N=422 (155 men and 267 women)
- Category 2: representatives of the majority population living in the vicinity of the segregated Roma communities, N=134 (62 men and 72 women);
- Category 3: representatives of the majority population living outside the area of segregated Roma communities, N = 214 (107 men and 107 women).

All participants were asked to fill in a questionnaire which contained the following statements regarding toluene abuse: "never tried", "tried once or two times", "toluene user, frequency: less than once a month", "toluene user, frequency: several times a month", "toluene user, frequency: once a week", "toluene user, frequency: several times a week", "toluene user, frequency: daily".

On the day of urine analysis, the participants (after at least 8 hours fasting) obtained detailed instruction about first-morning urine sample collection. Compliance with this request was confirmed upon arrival.

Analysis

The analysis of urinary hippuric acid, *o*-cresol and creatinine was performed in first morning urine samples by reverse phase HPLC (Shimadzu, Japan) on a modified silicagel column with nonpolar octadecyl functional group (Nucleosil standard Expert Column; 100-3 C18, ID 4 mm, length 250 mm, Macherey – Nagel). Urine samples were injected manually in the volume of 40 μL at a flow rate of the mobile phase 0.8 mL/min at 30 °C. Analytes were detected by UV/Vis detectors at 280 nm and at 220 nm as well as by fluorescent detector (excitation wavelength 280 nm, emission detected at 350 nm). The mobile phase composition during the isocratic mode of the elution was as follows: acetonitrile (ACN)/water 15/85 (v/v) with 0.05% addition of formic acid. The method performance was evaluated in terms of linearity, limit of detection and limit of quantification for every analyte. Urinary hippuric acid levels were quantified using UV/Vis detector at 220 nm, retention time 8.0 min. The quantification of *o*-cresol was performed based on the fluorescent detector signal at retention time 30 min. Correction according to the hydration was made by calculation of analyte concentration on urine creatinine concentration.

Data were analyzed using IBM SPSS Statistics 22 data analysis software. Statistical analysis was performed via the following statistical tests: Kolmogorov-Smirnov test of normality; Shapiro-Wilk test of normality, non-parametric Wilcoxon test of statistical significance to compare the urine parameters in Roma and in the control groups.

Participation in the study was voluntary and anonymous. Detailed information about our study and its procedures was given to all respondents, and informed consent was obtained prior to medical examination.

Ethic committee approval

The Ethics Committee of the P. J. Šafárik University in Košice, Faculty of Medicine, approved the study (No. 104/2011).

Results

Levels of hippuric acid showed a log normal distribution of values. The median values were in the majority population lower in men compared to women in all investigated populations. The 75th percentile of the values of urinary hippuric acid in Roma men as well as in Roma women was above 570 mg/g creatinine and 690 mg/g creatinine, respectively; while in the majority population the value of the 75th percentile was lower. Non-parametric Wilcoxon test indicated a statistically significant difference in urinary hippuric acid levels in Roma men (Category 1) when compared to men at group 3 (p=0.003). Statistically significant difference was observed in Roma women and majority women in Category 2 (p=0.0001) as well as in Category 3 (p=0.019, Table 1).

Similarly, to the hippuric acid, levels of *o*-cresol showed a log normal distribution of values (both Kolmogorov-Smirnov test of normality as well as the Shapiro-Wilk test of normality had a p-value <0.01). The median of the values in the categories did not differ and has a value close to zero μg/g of creatinine. We detected differences in the inter-quartile ranges (75th percentile – 25th percentile)

Table 1. Urinary hippuric acid level of the study respondents.

Category		N	Median [mg/g creatinine]	percentile [mg/g creatinine] 25th	75th	Test of Normality K – S[a] / S – W[b]	Non-parametric Wilcoxon test
Men	1	155	338	227.0	573.3	1.09×10^{-19}/ 4.60×10^{-14}	
	2	62	302	180.9	449.5	3.24×10^{-4}/ 5.25×10^{-7}	p=0.227
	3	107	250	159.8	416.6	1.17×10^{-2}/ 2.27×10^{-5}	p=0.0295**
Women	1	267	506	345.7	698.5	0.200/ 0.702	
	2	72	331	225.1	512.4	4.83×10^{-4}/ 6.22×10^{-9}	p=0.000140***
	3	107	436	275.1	596.2	4.46×10^{-5}/ 9.96×10^{-5}	p=0.0186*

a) K – S: Kolmogorov-Smirnov test of normality; b) S – W: Shapiro-Wilk test of normality; p-values are rounded to three significant digits

Table 2. Urinary o-cresol level of the study respondents.

Category		N	percentile [µg/g creatinine] 25th	75th	Test of normality K – S[a] / S – W[b]	Non-parametric Wilcoxon test
Men	1	155	0.000	0.331	6.25×10^{-23}/ 5.58×10^{-14}	
	2	62	0.000	0.315	4.16×10^{-27}/ 6.38×10^{-16}	p=0.471ns
	3	107	0.000	0.000	8.05×10^{-35}/ 4.07×10^{-15}	p=0.0138**
Women	1	267	0.000	0.316	1.28×10^{-20}/ 2.20×10^{-12}	
	2	72	0.000	0.000	2.76×10^{-38}/ 2.98×10^{-14}	p=0.0636ns
	3	107	0.000	0.000	8.09×10^{-28}/ 2.68×10^{-14}	p=0.813ns

a) K – S: Kolmogorov-Smirnov test of normality; b) S – W: Shapiro-Wilk test of normality; p-values are rounded to three significant digits

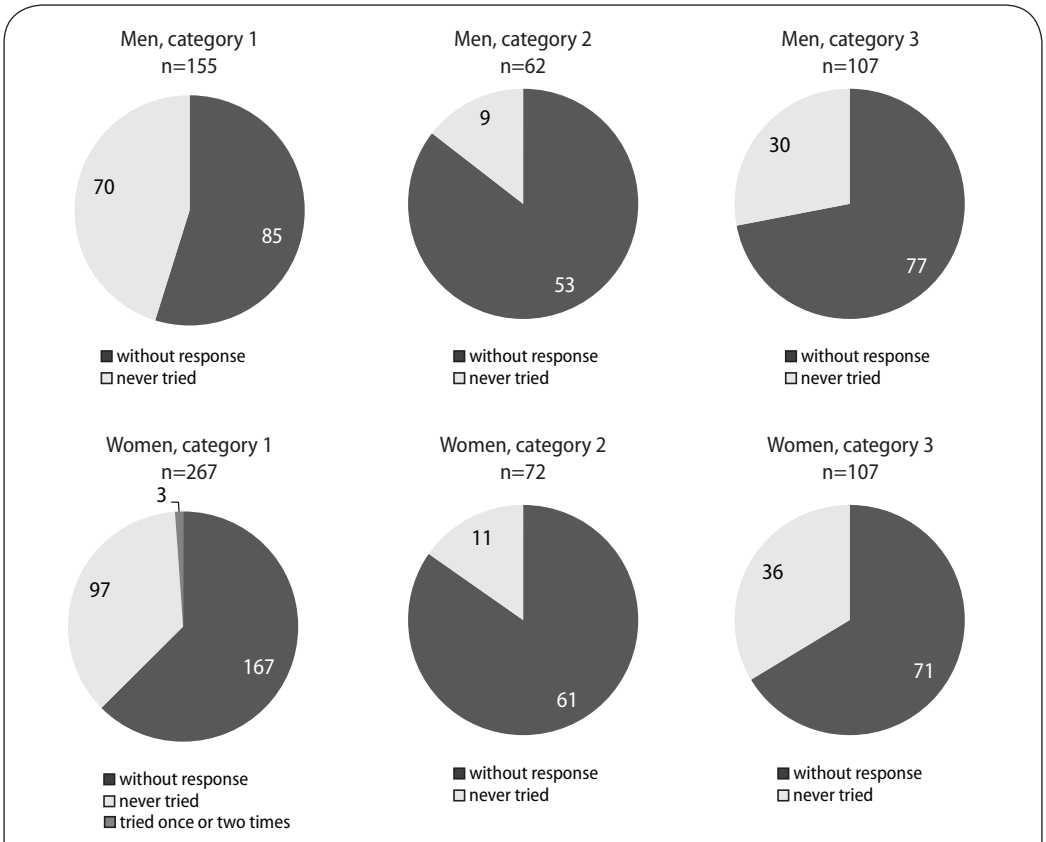

Figure 2. Evaluation of the answers given in the questionnaire regarding toluene use.

of the values among the categories. Non-parametric Wilcoxon test indicated a statistically significant differences in *o*-cresol in Roma men (Category 1) compared to men in Category 3 (p=0.014; Tabdle 2).

Results of the questionnaire monitoring toluene use among study participants is shown in Figure 2. The majority of participants did not respond to this question (66.7% of total participants, 66.3% of men and 67.0% of women) at all, or answered "never" (32.9% of total participants, 33.6% of men and of women 32.3%). Answer " tried once or two times " was responded only by three Roma women of Category 1.

From this perspective, it was interesting to compare the urinary toluene metabolite levels with the denied or negative answers given in the questionnaire. The statistical comparison of respondents who admitted toluene use with the other participants was hampered with extremely low number of admitters. The three values of urinary hippuric acid in subjects who admitted toluene use were 191.44, 346.1, and 1049.1 mg/g creatinine. The same was true for the urinary levels of *o*-cresol. The values of urinary *o*-cresol levels of probands who reported experiences with toluene were 0.426, 0.112, and 2.343 μg/g creatinine, respectively.

Discussion

Survey conducted by Važan *et al.* (2011) in seven Roma settlements in Eastern Slovakia showed a 2% incidence of chronic toluene users. In our study, during the evaluation of the questionnaire we detected only three women of the Roma ethnic minority admitting toluene use (0.39% of total study participants and 1.12% of Roma women). Elevated urinary toluene metabolites were observed in 21 cases (2.73% of total study participants), of which 14 were Roma women and 1 Roma man. This comprises 5.24% and 0.65% of Roma women and men, altogether 3.55% of the Roma participants. The reason for the controversy between the results of questionnaire and the metabolite analysis could be as follows:

- Weak response rate – only one participant from three responded to this question at all, although the overall response rate to other questions regarding the health status was much higher (Madarasová Gecková *et al.*, 2014a).
- Feeling of shame.
- Fear of possible consequences after admitting to toluene abuse.
- Participants were aware of the fact that their urine will be analyzed and stopped toluene use some days before the investigation.
- Differences in toluene metabolism between men and women and other factors affecting toluene degradation.
- Gouva *et al.* (2015) devoted a study to shame and anxiety feelings of Roma living in Greece. According to their findings, Roma women are subject to shame more likely than Roma men are. In our study, we find the opposite, although the numbers are low. If anxiety and shame is associated with increased outrightness when responding a question on drug abuse or not, is an open question

The study of Huang *et al.* (1994) is devoted to metabolization of toluene in 233 toluene-exposed workers. They found that at the same level of toluene exposure, the concentration of urinary toluene metabolites is higher in women compared to men. They also described a lower urinary excretion of hippuric and methyl-hippuric acid in smokers and alcohol consumers after toluene exposition as compared to nonsmokers and abstainers.

Unfortunately, there are no data documenting the relationship between smoking, alcohol consumption and conversion of toluene to cresol. So far, no study was addressed to gender based cresol formation. Our results indicate that the *o*-cresol formation following toluene exposure is similar to the formation of hippuric acid, and is increased in women compared to men.

The poor socio-economic status has undoubtedly considerable influence on the quality of life and is the most common cause of marginalization. From a medical point of view, the marginalized populations represent the most threatened group of the society. The isolated Roma communities constitute the largest group of marginalized people in Eastern Slovakia. Loss of employment, disability, volatile compound – or alcohol addiction, or lack of adaptability to the customs of the majority are the causes but also the consequences of a vcious circle of the lifestyle of marginalized Roma. Their worse health status in comparison with the majority is also confirmed (Hubková *et al.*, 2014). They more likely to suffer from chronic hepatitis B and metabolic syndrome (Janicko *et al.*, 2014), have a higher risk of cardiovascular diseases (Babinska *et al.*, 2013), Roma women have a higher incidence of nephropathy compared to non-Roma females (Rosenberger *et al.*, 2014).

There is a considerable effort to improve the social and health status of marginalized Roma. Some of the projects emerging from the surveys conducted in Roma settlements, such as integration of Roma children in the education process, are already under implementation. However, most of them require long time to achieve perceptible result. Some former ineffective attempt to tackle inhalant abuse ought to be replaced by specifically targeted educational projects focusing on parental and early childhood education.

In contrast to the answers given in the questionnaires about toluene abuse, our work confirmed the suitability of HPLC detection of urinary *o*-cresol as a marker of toluene abuse. Our results indicate that the formation of *o*-cresol after exposure to toluene is more pronounced among women than among men. Furthermore, the results clearly point to the need of using objective laboratory tests and the establishment of cut-off values for the metabolites of toluene as markers of exposition and abuse. From point of view of the public health, the objective HPLC method is a valuable tool for the evaluation of the health status of marginalized population groups in order to address their health problems arising from toluene abuse effectively.

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REFERENCES

Agency for Toxic Substances and Disease Registry Division of Toxicology and Human Health Sciences. [webpage on the Internet]. Public Health Statement for Toluene. Published on September 2015. [updated 2015 January 21; cited 2017 June 29]. Available from: http:// www.atsdr.cdc.gov/phs/phs.asp?id=159&tid=29

Babinska I, Veselska ZD, Bobakova D, Pella D, Panico S, Reijneveld SA, Jarcuska P, Jarcuska P, Zezula I, Geckova AM, HEPA-META team. (2013). Is the cardiovascular risk profile of people living in Roma Settlemets Worse in Comparison with the Majority Population in Slovakia? *Int J Public Health* **58**: 417–425.

Beckley JT, Woodward JJ. (2013). Volatile Solvents as Drugs of Abuse: Focus on the Cortico-Mesolimbic Circuity. *Neuropsychopharmacol* **38**: 2555–2567.

Camara-Lemarroy CR, Rodríguez-Gutiérrez R, Monreal-Robles R, González-González JG. (2015). Acute toluene intoxication – clinical presentation, management and prognosis: a prospective observational study. *BMC Emerg Med* **15**: 19.

Gouva M, Mentis M, Kotrotsiou S, ParalikasT, Kotrotsiou E. (2015). Shame and Anxiety Feelings of a Roma Population in Greece. *J Immigr Minor Health* **17**: 1765–1770.

Huang MY, Jin C, Liu YT, Li BH, Qu QS, Uchida Y, Inoue O, Nakatsuka H, Watanabe T, Ikeda M. (1994). Exposure of workers to a mixture of toluene and xylenes. I. Metabolism. *Occup Environ Med* **51**: 42–46.

Hubková B, Maslanková J, Stupak M, Guzy J, Kovácová A, Pella D, Jarcuska P, Mareková M, HepaMeta Team. (2014). Assessment of Clinical Biochemical Parameters in Roma Minority Residing in Eastern Slovakia Compared with the Majority Population. *Cent Eur J Public Health* ***S*2014**: S12–S17.

Janicko M, Senajová G, Drazilová S, Veselíny E, Fedacko J, Siegfried L, Kristian P, Virág L, Pella D, Mareková M, Gecková AM, Kalanin P, Jarcuska P, Halánová M, HepaMeta Team. (2014). Association between metabolic syndrome and hepatitis B Virus Infection in the Roma Population in Eastern Slovakia: A population-based Study. *Cent Eur J Public Health* **S2014**: S37–S42.

Madarasová Gecková A, Babinská D, Veselská ZD, Bosáková L, Kolarcik P, Jarcuska P, Pella D, Halánová M, HepaMeta Team. (2014a). Socioeconomic Characteristics of the Population Living in Roma Settlements and Their Association with Health and Health-related Behaviour. *Cent Eur J Public Health* **S2014**: S57–S64.

Madarasová Gecková A, Jarcuska P, Mareková M, Pella D, Siegfried L, Jarcuska P, Halánová M, HepaMeta Team. (2014b). HepaMeta – Prevalence of Hepatitis B/C and Metabolic Syndrome in Population Living in Separated and Segregated Roma Settlements: a Methodology for a Cross-sectional Population-Based Study Using Community-Based Approach. *Cent Eur J Public Health* **S2014**: S6–S11.

Rosenberger J, Majrníková M, Jarcuska P, Pella D, Mareková M, Gecková AM, Halánová M, HepaMeta Team. (2014). Higher Prevalence of Nephropathy in Young Roma Females Compared with Non-Roma Females. *Cent Eur J Public Health*. **S2014**: S28–S31.

Važan P, Khan MR, Poduška O, Stastná L, Miovský M. (2011). Chronic toluene misuse among Roma youth in Eastern Slovakia. *Subst Use Misuse* **46**: 57–61.

Yacob AR, Zinalibdin MR. (2010). High Performance Liquid Chromatography Determination of Urinary Hippuric Acid and Benzoic Acid as Indices for Glue Sniffer urine. *World Academy of Science Engineering and Technology International Journal of Chemical, Molecular, Nuclear, Materials and Metallurgical Engineering* **4**: 237–242.

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ORIGINAL ARTICLE

Exploration of teratogenic and genotoxic effects of fruit ripening retardant Alar (Daminozide) on model organism *Drosophila melanogaster*

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ABSTRACT
Alar (Daminozide) is a plant growth regulator which is widely used as a fruit preservative for apple and mango to prevent pre-harvest fruit drop, promote color development and to delay excessive ripening. The aim of the present work was to demonstrate the effect of Alar on several life history traits, adult morphology, Hsp70 protein expression and *in vivo* DNA damage in the brain of the model organism *Drosophila melanogaster*. We assessed the life history and morphological traits including fecundity, developmental time, pupation height, egg-to-adult viability and mean wing length, body length, arista length and sternopleural bristle number of the emerging flies. The results showed a significant delay in the developmental milestones, increase in body length, wing length, arista length, a decrease in fecundity, pupal height and variation in sternopleural bristle number in the treated flies in comparison to the controls. Overexpression of Hsp70 protein suggests alar induced subcellular molecular stress and comet assay validates genotoxicity in the form of DNA damage in the treated larvae. Mutation screening experiment revealed induction of X lined lethal mutation.

KEY WORDS: Alar; drosophila melanogaster; life history traits; Hsp 70; comet assay; DNA damage

Introduction

Alar (Daminozide, Figure 1) is used as plant growth regulator and considered a "stop-drop" wonder chemical as its application prevents fruits from prematurely falling. Alar is manufactured by mixing succinic anhydride with 1,1-dimethylhydrazine (UDMH), a toxic component of rocket fuel. The chemical is widely used in processing major varieties of red apples (like Mclntosh, Cortand, Jonathan and Red Delicious) and mangos (like Langra and Dashehary, etc). Though it is not a pesticide, it earns popularity among the farmers as it provides economic benefits. Moreover, the Alar treated fruits appear lucrative to consumers owing to its cosmetically enhanced color and crunchiness. Alar was first used in the USA and was continued until 1989 when the US Environmental Protection Agency suggested a ban of the chemical owing to increasing reports on cancer incidence among consumers of Alar treated apples. Alar penetrates the apples' pulp and cannot be washed or peeled off. Thus the chemical enters the living system following its consumption with fruits. Moreover, upon heating for making apple sauce or sterilizing apple juice or following digestion, Alar is degraded into UDMH, which is 1000 times more carcinogenic than Alar itself (Gordon, 2011).

As far as published literature is concerned, studies on adversities of Alar in biological systems are tantalizingly lacking. The present work has been conducted to fill such a void with experimental outcome and consequent scientific understanding. As the mutagenicity of various compounds used in agriculture have been tested through assays performed on the model organism *Drosophila melanogaster*, we have taken this opportunity considering the ease of experimentation with this insect model and to cope with ethical issues. The use of *Drosophila* in research has been recommended by the European Centre for the Validation of Alternative Methods (Festing *et al.*, 1998). Our present work represents the very first attempt to prove that the fruit preservative Alar is not safe to consume and it inducts various teratogenic and genotoxic effects at molecular level in higher eukaryotes.

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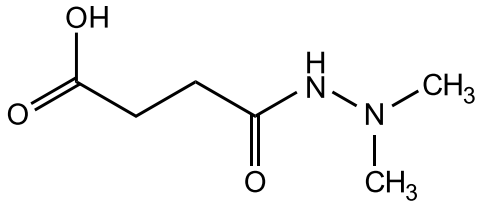

Figure 1. Chemical Structure of Alar [4-(2,2-dimethylhydrazinyl)-4-oxobutanoic acid].

Materials and Methods

Fly Strain

The wild type Oregon R strain and a transgenic strain Bg^9 of *Drosophila melanogaster* were used for this study. In the Bg^9 transgenic strain, the wild type hsp70 sequence is tagged with reporter gene construct through P-element induced insertional mutagenesis. Here the reporter gene is bacterial lacZ which expresses β-galactosidase along with induced expression of Hsp70 protein following exposure to stress. These stocks were maintained in drum vials measuring 30 mm in diameter and 105 mm in height and on standard yeast-agar-cornmeal culture medium at 24±1 °C and 65–70% relative humidity (RH)in BOD incubator (Ashburner, 1989).

Chemicals

Analytical grade Alar or Daminozide, IUPAC Name 4-(2,2-dimethylhydrazinyl)-4-oxobutanoic acid (purity 99%) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Sisco Research Laboratoty (Mumbai, India).Agarose, Low Melting Point Agarose, Ethidium Bromide and Collagenase were from Sigma Chemical Co. (St. Louis, MO USA). Phosphate Buffered Saline (PBS) (Ca^{2+}, Mg^{2+}free) was procured from Hi-Media Pvt. Ltd. (Mumbai, India). All other chemicals were of molecular biology reagent grade.

Preparation of Alar for treatment

Alar or 4-(2,2-dimethylhydrazinyl)-4-oxobutanoic acid was dissolved in distilled water to prepare a high concentration stock solution and mixed with standard *Drosophila* culture medium at different concentrations (200, 400, 10000, 20000 ppm) by making necessary dilutions from the stock solution.

Determination of acute LC_{50}

To investigate the acute toxicity of Alar in *Drosophila melanogaster* in terms of LC_{50}, twenty late 2^{nd} instar larvae were introduced in each food vial containing 5 ml of standard *Drosophila* food mixed with different concentrations of Alar (2000, 4000, 8000, 12000 and 13000 ppm). Similarly, control sets were set up with the food vials without Alar. Triplicate sets of each treated and control group were maintained at 24 °C and 65–70% RH till eclosion of the adult. Death of larvae and pupae were recorded by counting the pupae following final molt and by recording hatched imagoes, respectively.

Morphological and life history parameters

Flies were reared in culture medium containing Alar at a concentration of 400 ppm under standard laboratory conditions for successive five discrete generations (fresh vials for each generation) and freshly eclosed flies of generation 6 were then subjected to study. Simultaneously control sets were maintained in a culture medium prepared with distilled water instead of Alar solution. Uninterrupted rearing of the flies for five successive generations in Alar containing medium intuitively ensured homogeneous integration of Alar molecules in subcellular organization of treated flies. We collected 10 pairs of virgin males and females from the freshly eclosed 6^{th} generation and transferred each pair to 10 respective vials containing Alar free medium. They were allowed to mate and oviposit for the next 24 hours. On the very next day the male fly from each vial was removed and the female fly was transferred to fresh vials every day (for 24 hours) for the next 20 days and the number of eggs laid daily by those 10 females was recorded for each day. We estimated total fecundity (in terms of number of eggs laid) and mean egg production by a female through the range of 20 days (Mukhopadhyay *et al.*, 2003)to take the accounts of respective life history traits for the entire period of reproductive age of the *Drosophila* females.

The pupal height (the level of puparium on the wall of glass vial) of each larva was measured as the distance from the surface of the food to the midpoint between the spiracles on the puparium (Sokolowski, 1985). Emergence of the adults from the puparium was counted to estimate egg-to-adult viability and sexing of adult was also done. All flies were observed carefully under stereo zoom microscope to find any morphological distortions such as changes in eye color, wing shape, body pigmentation, *etc.* and photographs were taken with Olympus stereo zoom microscope model SZ-11. A single wing was removed from each adult fly and the distance between the alula notch and the tip of the wing was measured using a dissecting microscope fitted with a graduated eyepiece. Besides, the mean wing length and body length of the emerging adults were measured following the method by Santos *et al.* (1994). The time needed to complete the pupation and imago emergence (the day of pupation and adult emergence) was recorded as parameter of developmental time. As the eggs are hatched at any time within 24 hours following oviposition, we synchronized our record on adult emergence time and day of pupation initiating the time count from the time of hatching of the first instar larvae. This experimental regimen was followed for all the replicates in treated and control categories.

We observed distorted morphological features among some treated flies and subsequently evaluated whether those distorted features were hereditary by crossing two identical dysmorphic flies and by performing test cross

between F1 progeny and either of the parental flies. For all the experimental design the generation count was started following inception of the experiments.

Effects of temperature on adversity of Alar

We reared the 10 pairs of male and female flies in 200 ppm Alar-mixed culture medium at two different temperatures (23 °C and 28 °C) to test whether imperilments inducted by Alar get exacerbated in interaction with increasing temperatures. Other experimental conditions remained the same as mentioned in the life history and morphological study above.

In situ molecular stress assessment by Hsp70 expression level

As the Hsp 70, the molecular chaperon of eukaryotic system, exhibits overexpression under any kind of cellular stress, we intended to study the expression pattern of this protein under the exposure of Alar. Late 2nd instar larvae of Bg^9 stock of *Drosophila melanogaster* were reared in Alar-mixed culture medium (200, 400 and 10000 ppm) till their 3rd instar larval stage was reached. Wandering 3rd instar larvae feeding on different concentrations of Alar were collected and washed thoroughly in Poel's salt solution (PSS). These larvae were dissected, fixed in 2.5% glutaraldehyde followed by washing in 50 mM sodium phosphate buffer (pH 8.0), stained in X-gal staining solution (Sarkar *et al*., 2015) in grooved slides, kept overnight at 37 °C in a humidified chamber and observed under stereo-zoom microscope for differential Hsp70 protein expression.

Genotoxicity and neurotoxicity assessment by alkaline comet assay

In vivo genotoxicity, DNA damage in particular and neurotoxicity of Alar in *Drosophila melanogaster* was assessed using single cell gel electrophoresis or comet assay following the method of Dhawan *et al.* (2009) with minor modifications. Comet slides were prepared by using 10 late 2nd instar larvae fed with sub-lethal (400 and 10 000 ppm) and lethal (20 000 ppm) concentration of Alar as well as control larvae for 60 hrs in culture vial at standard laboratory condition (24±1 °C temperature, 65–70% relative humidity).After 60 hrs, wandering 3rd instar larvae from both treated and control groups were removed from the culture medium and washed with Dulbecco's Phosphate Buffered Saline (PBS pH 7.4). Cerebral ganglia from 10 larvae each from control and treated groups were explanted in PSS (pH 6.8) and treated with 300 µl of collagenase (0.5 mg/ml in PBS, pH 7.4) for 15 min at 24±1 °C. The cells were then passed through nylon mesh (60 µm), washed with PBS with gentle shaking and finally suspended in 80 µl of PBS. For positive control, cells were exposed to 25 µM H_2O_2 for 5 min at this stage.

The cell suspension was then mixed with equal volume of 1.5% low melting point agarose (LMPA) and layered on top of the end-frosted slides that were precoated with 1% normal melting point agarose (NMA). The slides were cooled at 4 °C for 5 min. After lysis for 2 h at 4 °C in lysing solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, pH 10 and 1% Triton X-100, pH 10), the slides were transferred to a horizontal electrophoresis apparatus filled with freshly chilled electrophoresis buffer (1 mM of Na_2 EDTA and 300 mM of NaOH, pH>13) and left for 10 min to facilitate unwinding of DNA. Electrophoresis was performed for 15 min at 300 mA, 25 V at 4 °C. After electrophoresis, the slides were immediately neutralized with 0.4 M of Tris buffer (pH 7.5) and stained with ethidium bromide (20 µg/ml) for 10 min in dark. After washing in chilled distilled water the slides were examined under a fluorescent microscope (Leica, Germany) at 40X magnification and the images were analyzed using CASP version 1.2.3beta2 (Comet Assay Software Project, http://casplab.com/). Each experiment was performed in triplicate with 10 larvae and the slides were prepared in duplicate. Three different parameters were used as indicators of DNA damage – tail moment (TM; arbitrary units), tail DNA (%) and tail length (µm).

Assessment of the potential of Alar for inducing X-linked lethal mutation

We employed attached X method (Figure 9) to screen any X-lined lethal mutation induced by Alar exposure. Freshly eclosed 50 male flies of attached X stock (BDSC stock no. 43329) were isolated. Males were kept in separate vial having culture media mixed with Alar of concentration 1 000 ppm for 48 hours. Individual males were then crossed with two virgin females of attached X stock and reared at 24±1 °C and 65–70% RH for 48 hours and then the flies were removed from the vials. The vials were kept till the emergence of F1 flies. The F1 flies were examined thoroughly for the presence of males. We scored lethality following the deviation of female:male from 1:1 value.

Statistical analysis

Probit analysis was performed for the determination of LC_{50} of Alar in late 2nd instar larvae of *Drosophila melanogaster*. The treated and control sets were compared using 2×2 ANOVA for the morphological parameters having sex specific difference. We performed 'two-way ANOVA' to find if there was an interaction between culture temperature and Alar exposure on the morphological and life history trait of *Drosophila melanogaster*. One-way ANOVA was performed for life history parameters, where sex is not a significant variable. We recorded mean and median fecundity and compared the values for treated and control sets using t-test. Daywise fecundity is also recorded and compared using one-way ANOVA. Variations in the number of sternopleural bristles on both left and right body sides of control and treated flies (males and females separately) were compared and analyzed by t-test. For testing male lethality we compared the female:male ratio value by Chi square method.

The values of three selected parameters for DNA damage (Tail Length, % Tail DNA and Tail Moment) through comet assay were compared using Student's t-test. Statistical analyses were performed in SPSS (for Probit analysis), VassarStats (for ANOVA) and GraphPad (for t-test) statistical software.

Results

Determination of acute LC_{50}

The Probit parametric estimates are presented in Table 1. Figure 2 displays the Probit line graph of acute toxicity of Alar in *Drosophila melanogaster* larvae and pupae. The estimated mortality for control set was nearly zero. The calculated lower and upper limits for the LC_{50} values were 6705.600 and 45181.211 ppm, respectively for larvae and 4280.964 and 27921.125 ppm, respectively for pupae at 95% confidence interval. This suggests that *Drosophilia melanogaster* showed 50% pupal death at the lower concentration (8011.601 ppm) of Alar treatment in comparison to larvae, which exhibited 50% death at higher concentration (10842.477 ppm). There is a positive relation between toxicity and dose of Alar (Figure 2).

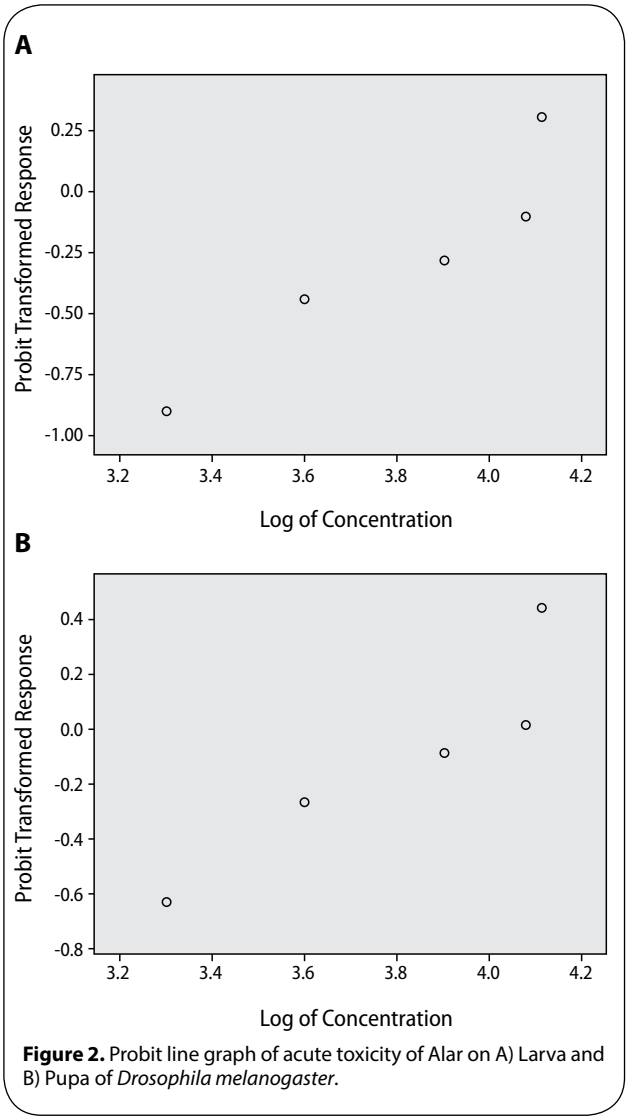

Figure 2. Probit line graph of acute toxicity of Alar on A) Larva and B) Pupa of *Drosophila melanogaster*.

Morphological and life history parameters

Summary of the results is presented in Table 2. Alar treated flies exhibit a significant delay in pupation time and adult emergence time (p=0.003 and <0.0001 respectively in One-way ANOVA) in all experimental conditions than do the controls. We scored pupation time for control group as 4.9±0.2 days in contrast to treated group which attained pupal phase at 6.0±0.2 days reared in 400 ppm Alar containing medium. Similarly, the treated group needed 9.9±0.3 days to emerge as imago in contrast to 8.93±0.2 days for the control. We observed a significant reduction in pupation height in Alar treated flies (0.4±0.2 cm.) in contrast to the control (4.0±0.04 cm) in 400 ppm Alar medium. Similar results were obtained in other experimental conditions too (Table 2). We recorded a significant reduction (p=0.0009 in One-way ANOVA) in mean daily egg production in Alar treated groups with a span of over 20 days (Tables–3; Figure 3). Egg to adult survival rate was also decreased significantly in treated groups when compared to control (Tables 2–3; Figure 3).

We assessed various morphometric traits including wing length, body length and arista length in sex specific manner. We scored female arista length for control as 274±0.5 µm and for treated 294±2.3 µm. For male sex, the estimates are 251±1.6 µm for control in contrast to 260±0.8 µm in treated reared in 400 ppm Alar containing medium. Similarly, body and wing also exhibited increase in length (Tables 2 and 4).

Beside morphometric variations between control and treated groups, we recorded various distortions and teratogenic effects on wing structure (Figure 4). These traits were found to be non hereditary in nature upon crossing between flies of similar trait up to F2. Abnormality in the formation of pupa was also found in F1 generation (Figure 5). Variation in the number and arrangement of sternopleural bristles (both unilateral and bilateral asymmetry) was found to be significant (p<0.0001 in t-test) between control and Alar treated F1 flies.

Table 1. Parameter estimates for the Probit analysis.

						95% Confidence Interval	
PROBIT[a]	Parameter	Estimate	Std. Error	Z	Sig.	Lower Bound	Upper Bound
For Larval LC_{50}	Concentration	1.203	0.139	8.627	0.000	0.930	1.476
	Intercept	−4.854	0.536	−9.062	0.000	−5.390	−4.318
For Pupal LC_{50}	Concentration	1.043	0.134	7.773	0.000	.780	1.306
	Intercept	−4.070	0.513	−7.933	0.000	−4.583	−3.557

[a] PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10 000 logarithm)

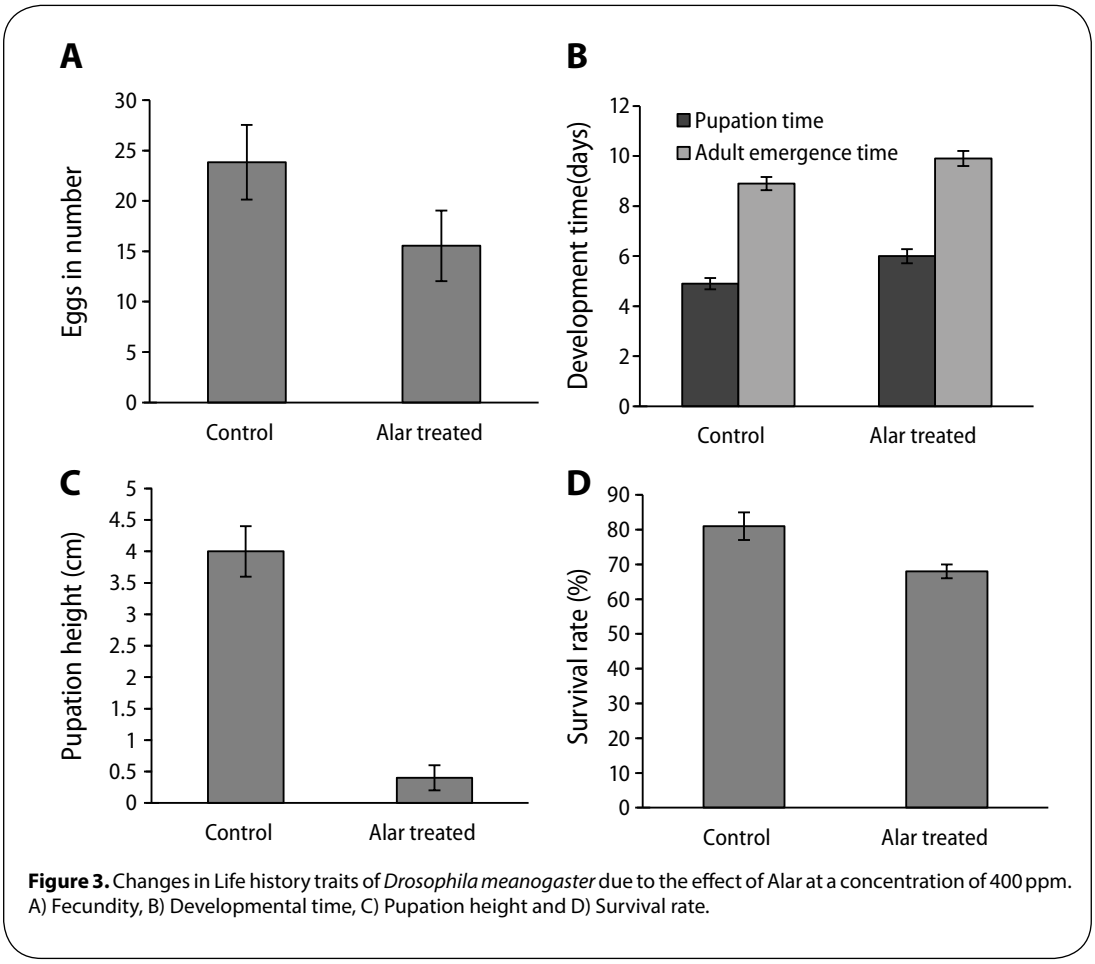

Figure 3. Changes in Life history traits of *Drosophila meanogaster* due to the effect of Alar at a concentration of 400 ppm. A) Fecundity, B) Developmental time, C) Pupation height and D) Survival rate.

Table 2. Effect of Alar on morphological and life history traits of *Drosophila melanogaster* at different culture temperatures.

Morphological traits	Sex	Control at 23 °C	Treated at 23 °C 400 ppm	Treated at 23 °C 200 ppm	Control at 28 °C	Treated at 28 °C 200 ppm
Body length (mm)	Female	2.51±0.02	2.58±0.07	2.58±0.07	2.46±0.05	2.53±0.09
	Male	2.17±0.03	2.2±0.14	2.21±0.12	2.06±0.06	2.10±0.15
Wing length (mm)	Female	2.4±0.004	2.44±0.01	2.44±0.01	2.33±0.04	2.39±0.12
	Male	2±0.001	2.08±0.01	2.08±0.01	1.9±0.03	1.98±0.07
Arista length (µm)	Female	274±0.5	294±2.3	294±2.3	265±2.6	285±4.9
	Male	251±1.6	260±0.8	260±0.8	244±1.4	253±6.2
Life history traits						
Fecundity (in no.)		23.83±3.7	15.54±3.5	18.75±1.8	20.72±1.1	15.54±3.5
Pupation time (day)		4.94±0.2	6±0.2	6.9±0.04	3.89±0.18	5.93±0.02
Adult emergence time (day)		8.93±0.2	9.9±0.3	10.94±0.01	7.84±0.08	9.85±0.02
Pupation height (cm)		4±0.04	0.4±0.2	2.04±0.3	3.01±0.3	0.45±0.1
Survival rate (%)		81±3.2	68±2.7	72±3.1	78±3.1	68±2.9

Values represent mean ± SD.

Effects of temperature on adversity of Alar

Result of the experiment to study temperature dependent Alar effect (200 ppm) exhibited interesting observations. For all morphological traits studied an increase in size occurred at a temperare lower than 23 °C and higher than 28 °C (Table 2). Similarly, for life history traits much delay was recorded in pupation time and adult emergence time in temperatures lower than 23 °C compared to temperature higher than 28 °C. On the contrary, fecundity and survival rate scored lower value at higher temperature.

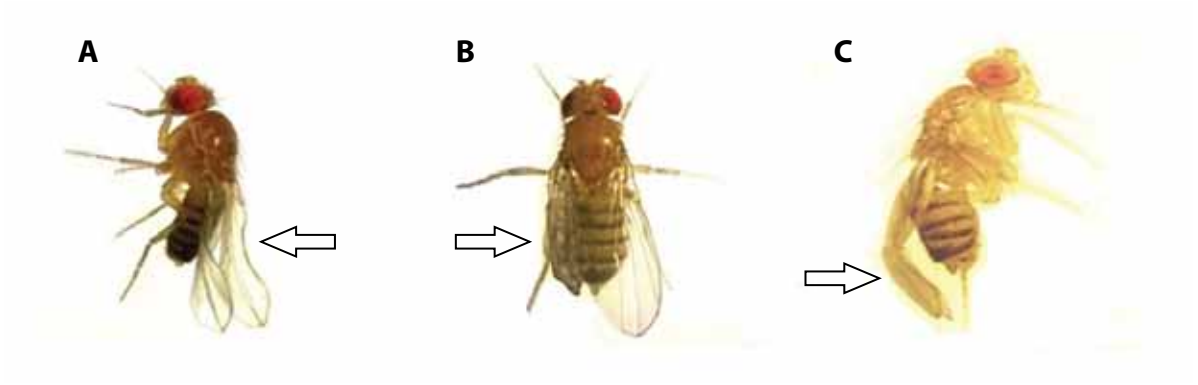

Figure 4. Wing abnormalities in Alar treated (400 ppm) flies (A, B, C).

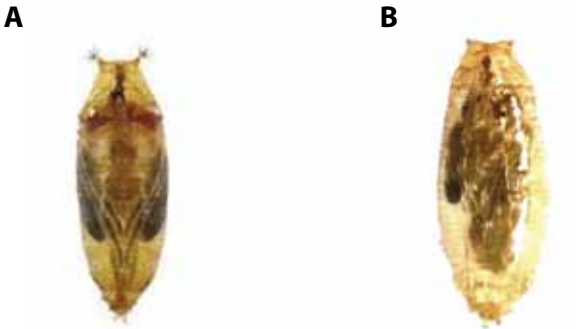

Figure 5. Abnormally formed Pupa A) Control, B) Alar treated (400 ppm).

Table 3. Effect of Alar (400 ppm) on selected life history traits of *Drosophila melanogaster* analysed by one-way ANOVA (Sex ignored).

Life history traits	Sum of squares (SS)	df	Mean square (MS)	F	*p*-value
Fecundity (in no.)	687.24	1	687.24	12.77	0.0009
Pupation time (day)	22.71	1	22.71	860.82	<0.0001
Adult emergence time (day)	18.52	1	18.52	488.78	<0.0001
Pupation height (cm)	301.35	1	301.35	663.37	<0.0001
Survival rate (%)	3366	1	3366	12.02	0.0008

Table 4. Effect of Alar (400 ppm) on selected morphological traits of *Drosophila melanogaster* analysed by two-way ANOVA.

Traits	Source of variation	Sum of squares (SS)	df	Mean square (MS)	F	*p*-value
Wing length (mm)	Alar	0.16	1	0.16	848.64	<0.0001
	Sex	5.86	1	5.86	30451	<0.0001
	Interaction	0.02	1	0.02	84.24	<0.0001
	Error	0.03	156	0		
Body length (mm)	Alar	0.09	1	0.09	12.75	0.0005
	Sex	5.27	1	5.27	741.27	<0.0001
	Interaction	0.01	1	0.01	1.7	0.1942
	Error	1.11	156	0.01		
Arista length (μm)	Alar	9030	1	9030	3929	<0.0001
	Sex	33235	1	33235	14462	<0.0001
	Interaction	1199	1	1199	521.75	<0.0001
	Error	358.5	156	2.3		

The effects of temperature and exposure to Alar were found to be significant for all the selected life history traits. However, no two-way interaction between temperature condition and exposure to Alar was found (Table 5; Figure 6). As there are sex specific differences in morphometric traits, we performed two-way ANOVA separately for males and females. The individual effects of temperature condition and exposure to Alar were found to be significant for both sexes. Their interaction, however was not significant (Table 5).

In situ molecular stress assessment by Hsp70 expression level

Figure 7 (A–D) shows β-galactosidase activity following X-gal staining as a signature of Hsp70 expression level in third instar larvae of Bg^9 stock of *Drosophila melanogaster* exposed to different concentrations of Alar (200, 400, 10000 ppm Alar). The intensity of blue coloration is proportional to the level of Hsp70 expression and molecular stress and apparent in different parts of the digestive tract, especially in the midgut (MG) and hindgut (HG) (Figure 7: B–D) of the Alar exposed larvae. The untreated

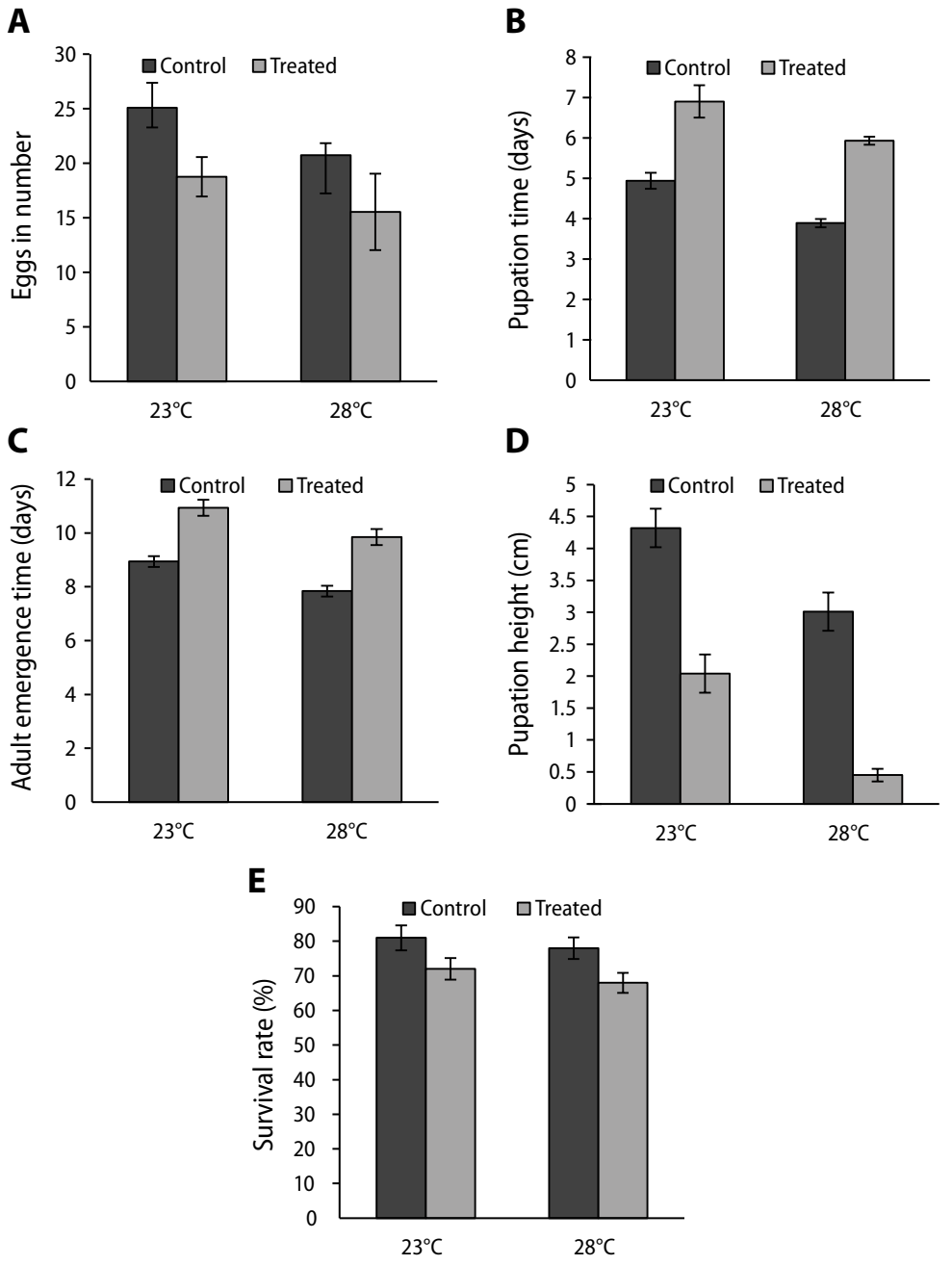

Figure 6. Effect of Temperature on adversity of Alar at a concentration of 200 ppm. A) Fecundity, B) Pupation time C) Adult emergence time, D) Pupation height and E) Survival rate.

larvae (control), on the other hand, expressed much less or no blue coloration in their digestive tract (Figure 7A).

Genotoxicity and neurotoxicity assessment by alkaline comet assay

Larvae exposed to H_2O_2 (positive control) and to different concentrations of Alar (400, 10 000, 20 000 ppm) showed a significant increase in DNA damage in the cells of cerebral ganglia (Table 6). Figure 8A represents images of brain cells of third instar larvae after 60 hours of treatment with different concentrations of Alar. Figure 8B is the graphical representation of the data of three selected DNA damage parameters (Tail Length, % Tail DNA, and Tail Moment) obtained from comet assay.

Assessment of the potential of Alar for inducing X-linked mutation

We isolated 1734 F1 living flies from the 50 experimental vials and sorted them carefully under microscope for sexing. We found 1087 female flies in contrast to 647 male flies and tested by chi-square method for the deviation from 1:1 female:male ratio. A significant deviation

Table 5. Effect of Temperature on adversity of Alar (200 ppm) on selected morphological and life history traits of *Drosophila melanogaster* analysed by two-way ANOVA.

Traits	Sex	Source of variation	Sum of squares (SS)	df	Mean square (MS)	F	*p*-value
Body length (mm)	Female	Temperature	0.1	1	0.1	22.34	<0.0001
		Alar	0.18	1	0.18	37.94	<0.0001
		Interaction	0.00	1	0.00	0.02	0.8877
		Error	0.73	156	0.00		
	Male	Temperature	0.46	1	0.46	39.07	<0.0001
		Alar	0.07	1	0.07	6.34	0.0128
		Interaction	0.00	1	0.00	0.01	0.9205
		Error	1.82	156	0.01		
Wing length (mm)	Female	Temperature	0.16	1	0.16	33.85	<0.0001
		Alar	0.1	1	0.1	21.67	<0.0001
		Interaction	0.00	1	0.00	0.34	0.5607
		Error	0.72	156	0.00		
	Male	Temperature	0.39	1	0.39	232.26	<0.0001
		Alar	0.24	1	0.24	146.48	<0.0001
		Interaction	0.00	1	0.00	0.83	0.3637
		Error	0.26	156	0.00		
Arista length (µm)	Female	Temperature	3506.26	1	3506.26	374.22	<0.0001
		Alar	16060.06	1	16060.06	1714.09	<0.0001
		Interaction	8.56	1	8.56	0.91	0.3416
		Error	1461.63	156	9.37		
	Male	Temperature	1890.63	1	1890.63	167.48	<0.0001
		Alar	3724.9	1	3724.9	329.96	<0.0001
		Interaction	0.4	1	0.4	0.04	0.8417
		Error	1761.05	156	11.29		
Fecundity (in no.)		Temperature	286.9	1	286.9	50.61	<0.0001
		Alar	661.83	1	661.83	116.76	<0.0001
		Interaction	6.56	1	6.56	1.16	0.2849
		Error	430.8	76	5.67		
Pupation time (day)		Temperature	40.8	1	40.8	1839.72	<0.0001
		Alar	160.4	1	160.4	7231.92	<0.0001
		Interaction	0.06	1	0.06	2.89	0.0911
		Error	3.46	156	0.02		
Adult emergence time (day)		Temperature	48.11	1	48.11	2493.64	<0.0001
		Alar	161.6	1	161.6	8375.49	<0.0001
		Interaction	0.00	1	0.00	0.02	0.8877
		Error	3.01	156	0.02		
Pupation height (cm)		Temperature	84.33	1	84.33	195.51	<0.0001
		Alar	234.45	1	234.45	543.53	<0.0001
		Interaction	0.8	1	0.8	1.84	0.1769
		Error	67.29	156	0.43		
Survival rate (%)		Temperature	576.6	1	576.6	6.14	0.0143
		Alar	3765.91	1	3765.91	40.11	<0.0001
		Interaction	0.03	1	0.03	0.00	1
		Error	14645.52	156	93.88		

(χ^2=56.7; p<0.05) was recorded which suggests mortality of male resulting from probable induction of X lined lethal mutation in treated male by Alar.

Discussion

Indiscriminate use of food additives and preservatives poses severe health problems in human and model organisms. Unfortunately, characterization of adversity of many such food additives and fruit preservatives remains outside the scientific research interest. Alar, *i.e.* Daminozide, is such a chemical which is almost ubiquitous for its use in apple preservation and finds its route of entry into the human body and that of other species who are natural consumers of fruits through apple slices and juice. We determined the LC_{50} value of Alar for both the larval and pupal stages of *Drosophila* and the result showed that LC_{50} value for pupae was lower than the LC_{50} value for larvae. The reason may be that the pupal stage

Table 6. Effect of Alar on comet parameters in cells of brain ganglia of *D. melanogaster*. Values are median ± SE of three experiments.

Group	Tail DNA (%)	Tail Length (µm)	Tail Moment (arbitrary units)
Negative Control	11.107±0.65	11±0.54	1.221±0.09
400 ppm Alar	16.112±0.58	23±1.11	3.705±0.19
10 000 ppm Alar	20.48±1.55	35±3	7.167±0.94
20 000 ppm Alar	34.07±0.53	96±0.54	31.038±0.54
Positive Control	67.34±2.9	189±9.35	125.23±8.7

p<0.0001 in comparison with control.

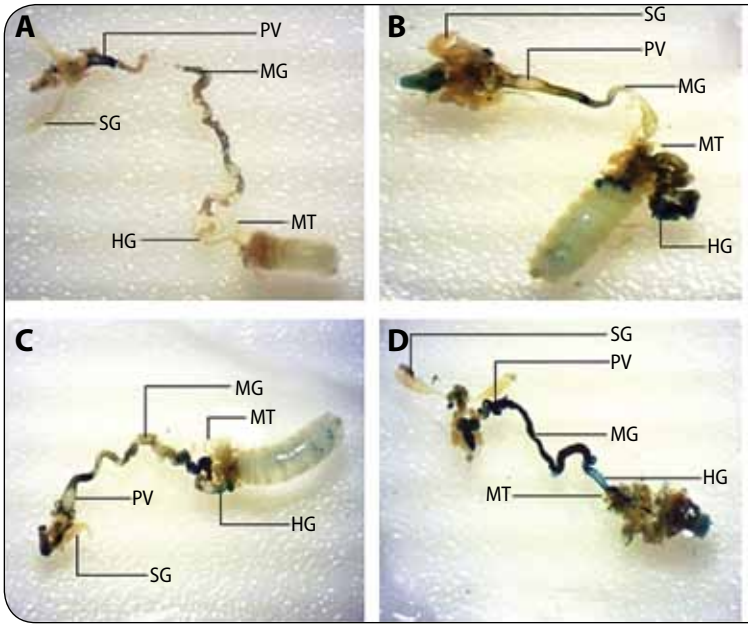

Figure 7. X-gal staining of third instar larvae of transgenic *Drosophila melanogaster* (Hsp70-LacZ) Bg^9 exposed to different sub-lethal concentrations of Alar. A) Control, B–D) 200, 400 and 10000 ppm Alar. (SG–Salivary Gland, PV–Proventriculus, MG–Midgut, MT–Malpighian tubules, HG– Hindgut).

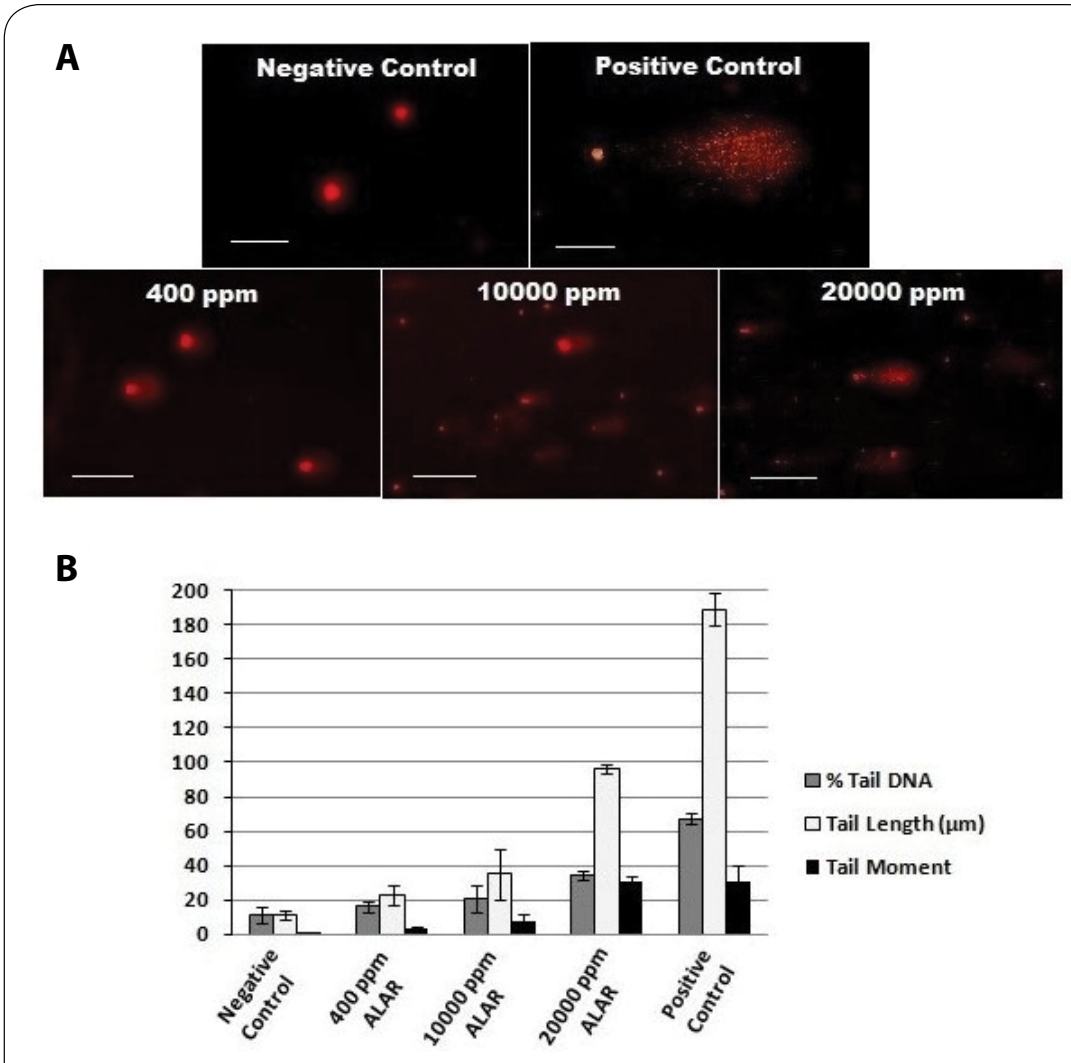

Figure 8. Comet assay in the cells of brain ganglia of third instar larvae of *D. melanogaster*. A) Images of brain cells after comet assay (Scale bar = 100 µm, 40X magnification) and B) Graph showing parameters of comet assay performed with different concentrations of Alar (Error bars indicate the standard errors from the means).

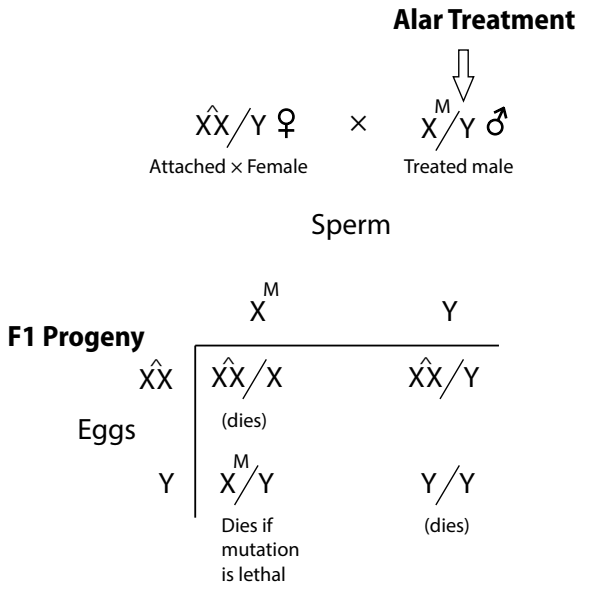

Figure 9. Scheme of cross involving attached X stock for screening X lined recessive lethal mutation induced by Alar.

is the metabolically active developmental stage devoid of feeding where maximum tissue rearrangements are taking place. Thus, food already ingested in the larval life happens to manifest its effect on pupae (Dad *et al.*, 2011). Moreover, the pupal stage is non-excretory and hence chance of removal of Alar from the body is nil and thus comparatively less concentration of Alar exerts lethality in the pupal stage.

In the present work, Alar was found to be a toxic agent for life history and morphological traits and probably acts as teratogenic agent in *Drosophila melanogaster*. The results showed delayed developmental regimen and significant increase in body, wing and arista length. This modulation of life history and morphological traits may be local adaptation of the insect against Alar. It may be possible that the larger body size is adaptive and a fly with such features somehow manages to survive in the stressful Alar containing medium. Similarly, extension of developmental phases beyond the normal schedule may be another survival strategy in unfavorable cultural conditions. Alternately, Alar may have toxic effects on the genes that regulate ontogenetic development. Intuitively, genes of TOR pathway or activating lifespan extending genes such as sir2 (Roy *et al.*, 2017) may be the target, though other genes may also get affected. We recorded a drop in fecundity which suggests strong effect of Alar on germinal tissue and shortening of pupation height which is the indicator of pre-adult fitness (Casares *et al.*, 1997). Alternatively, low mean puparium height among the treated larvae is indicative of neurotoxicity. It may be possible that neurotoxicity induced by Alar affected the mobility of larvae due to which they cannot move up to the upper part of the culture vial, the preferred site of control larvae for pupation. It is possible that Alar might have created lethal conditions for some larvae. Surviving larvae might have adjusted or adapted to this toxicant by altering their morphology and life history traits.

The present study also revealed an effect of temperature on the adversity of Alar on morphological and life history traits of *Drosophila melanogaster*. The results are consistent with previous studies on the effect of the pesticide (Das *et al.*, 2010; Podder & Roy, 2015) and temperature condition (Schnebel & Grossfield,1992; Rezaei, 2012; Chen *et al.*, 2013; Chang *et al.*, 2014) on life history traits. We observed developmental delay among the flies treated at the lower temperature of 23 °C than the flies reared at the higher temperature at 28 °C. Additionally, a greater increase in body length, wing length and arista length was recorded at lower temperature than in flies reared at higher temperature. At this point it is very difficult to explain the cause behind the temperature effects on adversity of Alar. More incisive analyses are needed to unravel the exact etiology.

Alar was found to be highly toxic at higher concentrations, as revealed by Hsp70 expression in transgenic *Drosophila melanogaster* (hsp70-lacZ) strain Bg^9. Usually, Hsp70 expression increases in response to subcellular molecular stress and means to maintain the cellular and molecular homeostasis (Hightower, 1991). Considerable numbers of studies have used Hsp70 as reporter gene in evaluating the toxic potential of some toxicants (Nazir *et al.*, 2001; Mukhopadhyay *et al.*, 2002; Siddique *et al.*, 2013). In the present study, we observed overexpression of Hsp70 in larval proventriculus, midgut and hindgut. Exposure of the larvae to Alar occurs through their feeding and hence the gut tissues express Hsp70 immediately to mitigate the imperilments induced by Alar following its consumption.

We recorded DNA damage through comet assay in the cerebral ganglionic cells of mature third instar larvae exposed to different concentrations of Alar. This observation unambiguously revealed that Alar is a neurotoxicant causing neurodegeneration in the cells of cerebral ganglia of treated larvae. Though we did not perform this experiment on the other tissues from different parts of larval body, but predictively we can say this kind of genotoxicity in form of DNA damage may have induced by Alar in other body tissues as well. We found X-lined recessive lethal mutations as we recorded selective excess mortality of male sex (Figure 9) in our genetic screening experiment involving cross using attached X stock. That suggests alar has mutagenic potential too.

In recent years, *Drosophila melanogaster* has been emerged as a popular model eukaryote for in vivo genotoxicity studies (Mukhopadhyay *et al.*, 2004; Siddique *et al.*, 2005). The fly has worldwide distributions in orchards and markets and therefore, it becomes an innocent victim of pesticides or preservatives used indiscriminately in those orchards and fruit processing units. These chemicals enter into human body through direct or indirect routes and also affect other non-target living animals like *Drosophila*. These facts justify the use of *Drosophila*

melanogaster in present study. In summary, the present work is the first ever systematic study on the adversity of fruit preservative Alar on living system and the results help realize that the effects of this chemical cannot be ignored and further research attempts are warrant to characterise its toxicity at molecular level.

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REFERENCES

Ashburner M. (1989). *Drosophila*: a laboratory manual. CSHLPress,New York, USA. Casares P, Carracedo MC, Garcia-Florez L. (1997). Analysis of larval behaviours underlying the pupation height phenotype in *Drosophila simulans* and *D melanogaster. Genet Sel Evol* **29**(6): 589–600.

Chang CJ, Gill MK, Liu S, Mang SL, Yung E. (2014). The effect of temperature on development time and body size in male and female wild-type Oregon-R and mutant *ort-1 Drosophila melanogaster. The Expedition* **4**.

Chen SY, Leask KP, MacKinnon SW, Ramanaden YJ, Yoon JH. (2013). The effects of temperature on the time to maturation of *Drosophila melanogaster. The Expedition* **3**.

Dad I, Yousuf MJ, Anjum SI. (2011). Determination of LC50 of Chlorpyrifos and Neem extract on third instar larvae of house flies and their effect on fecundity. *J Basic ApplSci* **7**(2): 169–174.

Das SK, Podder S, Roy S. (2010). Effect of fungicide, Thiovit®Jet on several life history trait of *Drosophila melanogaster* (Diptera: Drosophilidae). JABS **4**: 31–36.

Dhawan A, Bajpayee M, Parmar D. (2009). Comet assay: are liable tool for the assessment of DNA damage in different models. *Cell Biol Toxicol* **25**: 5–32.

Festing MFH, Baumans V, Combes DR, Halder M, Hendricsen FM, Howard BR. (1998). Reducing the use of laboratory animals in biomedical research: Problems and Possible solutions. *Alt Lab Anim* **26**: 283–301.

Gordon W. (2011). The True Alar Story: Part I. http://www.onearth.org/blog/the-true-alar-story

Hightower LE. (1991). Heat shock, stress proteins, chaperones and proteotoxicity. *Cell* **66**:191–194.

Mukhopadhyay I, Saxena DK, Chowdhuri DK. (2002). Toxicity of cypermethrin: *hsp70* as a biomarker of response in transgenic *Drosophila*. *Biomarkers* **7**: 501–510.

Mukhopadhyay I, Saxena DK, Chowdhuri DK. (2003). Hazardous effects of effluent from the chrome plating industry: 70kDa heat shock protein expression as a marker of cellular damage in transgenic *Drosophila melanogaster* (hsp70-lac Z). *Environ Health Perspect* **3**: 1926–1932.

Mukhopadhyay I, KarChowdhuri D, Bajpayee M, Dhawan A. (2004). Evaluation of in vivo genotoxicity of cypermethrin in *Drosophila melanogaster* using the alkaline Comet assay. *Mutagenesis* **19(2)**: 85–90.

Nazir A, Mukhopadhyay I, Saxena DK, KarChowdhuri D. (2001). Chlorpyrifos induced hsp70 expression and effect on reproductive performance in transgenic *Drosophila melanogaster* (*hsp70-lacZ*) *Bg9*. *Arch Environ ContamToxicol* **41**: 443–449.

Podder S, Roy S. (2015). Study of the changes in life cycle parameters of *Drosophila melanogaster* exposed to fluorinated insecticide, cryolite. *Toxicol Ind Health* **31**(12): 1341–1347.

Rezaei A. (2012). The effect of different temperature on the viability and fertility of *Drosophila melanogaster. Egypt Acad J Biolog Sci* **5**(1): 81–87.

Roy A, Mandi M, Roy S. **(**2017).Rotenone induced alterations in lifecycle parameters and compound eye morphology of *Drosophila melanogaster. Toxicol Int* **24**(1): 46–53.

Santos M, Fowler K and Partridge L. (1994). Gene–environment interaction for body size and larval density in *Drosophila melanogaster*: an investigation of effects on development time, thorax length and adult sex ratio. *Heredity* **72**: 515–521.

Sarkar S, Dutta M, Roy S. (2015). Potential toxicity of flubendiamide in *Drosophila melanogaster* and associated structural alterations of its compound eye. *Toxicol Environ Chem* **96**: 1075–1087.

Schnebel EM, Grossfield J (1992) Temperature Effects on Pupation-Height Response In Four *Drosophila* Species Group Triads. *J Insect Physiol* **38**(10): 121–132.

Siddique YH, Fatima A, Jyoti S, Naz F, Rahul, Khan W, Singh BR, Naqvi AH. (2013). Evaluation of the Toxic Potential of Graphene Copper Nanocomposite (GCNC) in the Third Instar Larvae of Transgenic *Drosophila melanogaster* (hsp70-lacZ)Bg9. *PLoS ONE* **8**(12): e80944.

Siddique HR, KarChowdhuri D, Saxena DK, Dhawan A. (2005). Validation of *Drosophila melanogaster* as an in vivo model for genotoxicity assessment using modified alkaline Comet assay. *Mutagenesis* **20**(4): 285–290.

Sokolowski MB. (1985). Genetics and ecology of *Drosophila melanogaster* larval foraging and pupation behavior. *J Insect Physiol* **31**: 857–864.

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ORIGINAL ARTICLE

New insights into morphological, stereological and functional studies of the adrenal gland under exposure to the potent goitrogen thiourea

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ABSTRACT
Thiourea (thiophen-3-yl-acetic acid) is a well established antithyroid drug used for treating hyperactivity of the thyroid gland as it blocks the conversion of thyroxine (T4) to triiodothyronine (T3) in peripheral tissues. Human exposures to thiourea include contaminated drinking water and vegetables for its extensive use in fertilizers. Chronic thiourea exposure can cause thyroid dysfunction leading to redox imbalance. However, such effects on morphological, quantitative, functional and hypothalamo-pituitary-adrenocortical axis (HPA) analysis of the adrenal gland are yet to be explored. The aim was to explore the effect of thiourea on structural and functional status of the adrenocortical region with special reference to the HPA axis. Control rats were fed a normal laboratory standardized diet whereas to experimental rats, thiourea at a dose of 0.3 mg/day/Kg body weight was administered orally, once every day for consecutive 28 days. Histology and histometry, including morphometry of the adrenal, adrenal Δ5 3β HSD and 17β HSD activity, LPO level and serum corticosterone profile were assessed. Statistical significance was studied by 'Mann-Whitney U' test at $p<0.05$. Hypertrophy and hyperplasia of the adrenocortical cells was found especially in the layer zona fasciculata ($p=0.0027$) and enhanced adrenal Δ5 3β HSD activity ($p=0.0067$) in comparison to that of the control. Increased lipid peroxidation ($p=0.0054$) and up-regulated corticosterone release ($p=0.0064$) through adrenocortical stress signalling pathway were also noted. Stereological analysis of the left adrenal gland showed significant increase in volume ($p=0.0025$) and mass of cells ($p=0.0031$) in adrenocortical region in comparison to that of control animals. This study concludes that thiourea, in addition to its antithyroidal activity, develops stress in the adrenal as evident by enhanced lipid peroxidation in the gland that in turn through the HPA axis causes hypertrophy and hyperplasia of adrenocortical cells to enhance synthesis and release of corticosterone secretion to counteract the stress developed under the influence of this potent chemical agent.

KEY WORDS: adrenal gland; oxidative stress; thiourea; thyroid hormones; corticosterone

Introduction

Thiourea is a potent antithyroidal drug and in pharmacological doses it is used in management of hyperthyroidism and/or Grave's Disease. Altered thyroidal function is reported to be closely associated with both hypothalamo-hypophyseal-gonadal and hypothalamo-hypophyseal-adrenal axis which may lead to the generation of oxidative stress in the long run (Weng *et al.,* 2007; Poncin *et al.,* 2010). The important neuroendocrine mechanism in a stress reaction is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in a rapid increase in circulating corticotrophin (ACTH) and subsequent rise in glucocorticoids, which are critical for successful adaptation (Miller *et al.,* 2007). Thus, plasma levels of ACTH and glucocorticoids are a good indicator of stress response intensity, particularly in its acute phase (Otis *et al.,* 2007). Incidentally, thiourea is added to fertilizers to inhibit the nitrification process (Wang *et al.,* 2017) and under conditions not favoring biotic or abiotic removal; thiourea may be present in surface waters and sediments over longer periods (Mutic *et al.,* 2017). Therefore thiourea contaminated drinking water and food are a potent route of exposure to humans in relation to the harmful side effects of this chemical.

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Oxidative stress generation is a resultant of increased production of reactive oxygen species (ROS) and there are reports which suggest that in various organs, including the adrenal gland, lipid peroxidation is increased in conditions of oxidative stress (Chakraborty *et al.,* 2014). ROS are chemically reactive molecules containing oxygen which form as a natural by-product of the normal metabolism of oxygen and have important roles in cell signalling and homeostasis. Examples include oxygen ions and peroxides. Cumulative effects of ROS may result in significant damage to cell structures and have been implicated as an underlying agent in various pathological conditions (Chakraborty *et al.,* 2016).

Thiourea, due to its antithyroidal properties, might alter adreno-cortical physiology in the long run as adrenal and thyroid are functionally interrelated. However the effect of sub-chronic exposure to thiourea in adrenal gland function is yet to be clearly elucidated experimentally. No conclusive experimental or clinical data are available about its effects on adrenergic stress signalling pathway on regular ingestion. Taking all this into consideration, as well as the fact that there are very few reports on morphological, quantitative and functional analysis of adrenal glands after thiourea exposure, the present study has been undertaken to determine its effects on adrenal gland morphology and functions in adult rats.

Material and methods

Animal maintenance and grouping for the study

For the investigation, twelve adult female virgin albino rats of 110±10 g were used. They were housed in two cages with six rats each in an air-conditioned room and 12 hrs light/12 hrs dark cycles were maintained. The rats were acclimatized to housing conditions for at least one week prior to experiment. All animal experiments were performed in accordance to the ethical guidelines approved by the Institutional Animal Ethics Committee, Department of Physiology, University of Calcutta [Approval no. IAEC/Project Proposal (PG)/ENDO-(SP)/2013–2014]. The rats were allowed free access to drinking water and basal diet made of locally available wheat flour, Bengal gram powder, milk powder, vegetable oil and recommended amount of vitamins. Approximately 10 g of food was fed per 100 g of body weight (Vento *et al.,* 2008). The rats were divided into two groups of six animals each. The experimental group was orally administered thiourea for 28 days and was paired with a control group.

Control rats were fed normal laboratory standardized diet, whereas the experimental rats received thiourea at a dose of 0.3 mg/day/Kg body weight fed to treated rats orally, according to body weight once every day for 28 days (Hamden *et al.,* 2008). Simultaneously, animals of the control group were provided equal quantity of sterile water by the same route and for the same duration. The animals were housed in hygienic polypropylene cages and maintained in a controlled environment at a temperature of 22±2 °C and relative humidity (40–60%) in an animal house with a constant 12 hrs light/12 hrs dark schedule. The animals were fed a standardized diet, which consisted of 70% wheat, 20% Bengal gram, 5% fish meal powder, 4% dry yeast powder, 0.75% refined sesame oil, 0.25% shark liver oil, and water ad libitum (Chakraborty *et al.,* 2014).

Sacrifice of the animals

The animals were sacrificed at the end of the experimental period of 28 days, following institutional ethical procedure. Anesthesia was given using ether prior to sacrificing the animals. Blood was collected separately from each animal of both groups from the hepatic portal vein and the serum was separated by centrifugation. Then the adrenal gland was collected from each animal and was stored separately. Before sacrifice the body weight of each rat were recorded.

Histological study

On the day of the sacrifice of the animals, the adrenal gland of both the groups of animals were removed and weighed and fixed in Bouin's solution. For dehydration, the tissues were kept in various ascending gradations of alcohols followed by absolute alcohol for an hour each. The tissues were then transferred to xylol for 15 mins (two times) and then to paraffin (56–58 °C), which contained 50% melted paraffin and 50% xylol. Finally the tissue was kept in melted paraffin and blocks were prepared. The tissue sections were cut using microtome and the sections were taken on a slide. These sections were stained in hematoxylin and eosin and each slide was examined under a light microscope (Model – CH20i Olympus; serial no. 8A06177) at ×400 magnification for histopathological examination. The photomicrographs of the sections were taken using the Nikon Cool Pix P1500 digital camera (Chakraborty *et al.,* 2014).

Morphometric analysis of adrenal gland

A single paraffin section containing both the cortical and medullary area was chosen for adrenal gland morphometry. In this section 50 test areas of the outer zona fasciculata (ZF) were counted (Sarwar *et al.*, 2008). Since the adrenocortical cells of rats are mononucleated, the numerical density of the nuclei corresponds to the number of cell per mm^3 (Bozzo *et al.*, 2006). The test area was measured with a 10X objective using the Image-Pro Plus software (Rockville, USA). To measure these areas, a circular area was selected with the aid of the software, in which the nuclei were counted. After this procedure, the area of the ZF adrenocortical cells of the adrenal gland was calculated by dividing the number of nuclei counted by the circular area measured.

Assay of Δ^5 3β hydroxysteroid dehydrogenase (HSD) and 17β HSD activity

For measurement of adrenal steroidogenic enzymes Δ^5 3β-HSD and 17β-HSD, the adrenals were collected and homogenized in homogenizing fluid (20% spectroscopic grade glycerol, 5 mM potassium phosphate and 1 mM ethylenediaminetetraacetic acid (EDTA) at a tissue

concentration of 100 mg/mL homogenizing mixture). The homogenized sample was then centrifuged at 10,000 rpm at a constant temperature of 4 °C. Then the supernatant was used for the assay. The activity of adrenal steroidogenic enzymes Δ^5 3β HSD was determined by optical measurement of the rate of reduction of nicotinamide adenine dinucleotide (NAD). The final volume of reaction system was 3.0 mL which contained 1 mL of 100 M of sodium pyrophosphate (pH 8.9), 20 L of 30 g of substrate (17-estradiol) of 3 each in 0.02 mL of purified doxin and a suitable quantity of enzyme (100–500 mL). The reaction was initiated by adding 1 mL of 0.5 mM of NAD (Chandra *et al.,* 2011). The activity of adrenal 17 HSD was determined by using the same supernatant fluid as described earlier. The reaction system contained 1.5 mL of 440 M of sodium pyrophosphate (pH 8.9), 0.5 mL of 5% BSA (bovine serum albumin), 40 L of 0.3 M of substrate (17-estradiol) of 17 each in 0.02 mL of purified doxin and a suitable quantity of enzyme (100–500 L) to initiate the reaction finally by adding 1 mL of 1.35 M of NAD (Mondal *et al.,* 2013). Both the reactions were carried out in the silica cuvettes having 1.0 cm light path, in a spectrophotometer at 340 nm absorbance, as mentioned in the preceding paragraph. The activities were measured at 15 s intervals against a blank (containing all components except the tissue homogenate).

Measurement of lipid peroxidase (LPO) activity

Two (2) mL of thiobarbituric acid-trichloroacetic acid-hydrochloric acid (TBA-TCA-HCl) reagents was added to 2 mL of tissue extract and mixed thoroughly. The solution was heated for 15 min in boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 10,000 rpm for 10 min. Malonaldehyde (MDA) forms adducts with thiobarbituric acid (TBA), which was measured spectrophotometrically in a spectrophotometer (UV-1240 Shimadzu, Japan) at 532 nm against a blank containing 50 mM phosphate buffer (pH 7.4) instead of biological sample. MDA, a product of LPO, was measured as a standard. An extinction coefficient of 156,000 $M^{-1}cm^{-1}$ was applied for calculation (Chandra *et al.,* 2010).

Assay of serum corticosterone

Serum corticosterone was assayed using ELISA kit developed by National Institute for Health and Family Welfare (NIHFW), Govt. of India, New Delhi, India. Briefly, the solid phase enzyme immunoassay for corticosterone is a competitive type immunoassay wherein horseradish peroxidase labelled corticosterone (HRP-corticosterone) competes with corticosterone present in the sample for a fixed and limited number of antibody sites immobilized on the wells and washed. The HRP-corticosterone fraction bound to the antibody in the solid phase is measured by adding a chromogen/substrate solution which is converted to a blue compound. After 15 min incubation, the enzymatic reaction is stopped with 1 M sulphuric acid which changes the solution to yellow color. The absorbance of the solution photometrically measured at 450 nm is inversely related to the concentration of corticosterone present in the sample.

Table 1. Thiourea induced alteration in body weight in female rats.

Groups	Initial body weight (g)	Final body weight (g)	Gain in body weight percent
Control	95.52±1.79	136.11±2.31	42.49
Thiourea-treated	97.19±2.09	126.16±2.49	29.80*
p-value	0.872	0.003	

(Values are Mean ± SEM; n=6); The comparison between control and thiourea-treated groups was done following two tail Mann-Whitney U test and significant difference was found between the two groups ($p<0.05$).

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). Differences between-group were established using Mann-Whitney U test. A value of $p<0.05$ was interpreted as statistically significant. Statistical analyses were performed using Origin 8.1 (Northampton, MA, USA) and MS-Office Excel 2007 software packages.

Results

Body weight

The body weight of the control animals increased progressively throughout the period of investigation, with a net body weight gain of +42.49% (Table 1). However, the net body weight gain of the animals of thiourea-treated group was only +29.80% at the end of the total experimental period.

Adrenal gland weight and morphometry

There was a significant increase in relative adrenal weight in thiourea-treated group of rats followed by morphometric alterations in all the three cortical layers as well as in the medulla compared to control group of rats ($p=0.0028$) (Table 2).

Adrenal Δ^5 3β hydroxysteroid dehydrogenase (HSD) and 17β HSD activity

The activity of the adrenal Δ^5 3β HSD enzyme was significantly increased ($p=0.0067$) and the activity of the adrenal 17β HSD enzyme was also increased, however not significantly in the thiourea-treated group in contrast to control ($p=0.8745$) (Table 3).

Serum corticosterone and lipid peroxidation (LPO) assay

Serum corticosterone level was increased in thiourea-treated group as compared with control ($p=0.0064$). Similarly in the thiourea treated groups there was an elevation of LPO in comparison to the control ($p=0.0054$), indicating generation of oxidative stress on its exposure (Table 4).

Histological changes of adrenal gland

In all examined sections, the adrenal gland consisted of three cortical zones clearly identifiable by arrangement and stainability of cells: zona glomerulosa (ZG), zona

fasciculata (ZF) and zona reticularis (ZR) (Figure 1). The thickness of the overall cortex and the respective zones remained fairly hypertrophied in adrenals after thiourea administration in comparison to normal. Just under the ZG, oval or cuboidal epithelial cell lining was also noticed. ZF, covering almost 2/3rd volume of the cortical cross-sections, possessed long cords, mainly with cuboidal cells of light frontier cytoplasm and round nucleus. ZR was formed by epithelial cells lying long ways of thinly wall blood vessels (Figure 2).

Discussion

In the study, the effect of a potent synthetic antithyroidal drug, thiourea, was evaluated on the morphological and functional status of the adrenal gland in rats. The investigated parameters were detailed morphology and morphometry of the gland along with evaluation of adrenocortical steroidogenic enzyme activities and with lipid peroxidation level, as well as serum corticosterone profile in both control and thiourea exposed groups of animals.

Exposure to thiourea markedly reduced the net body weight gain of the experimental animals as compared to the control animals. Similar observations have been reported earlier on the potentiality of thiourea as an inhibitor of morphometric growth and body weight in other species; which may be due to its positive association with hypothyroid condition, in which both syntheses of proteins and growth rate is retarded resulting in weight loss despite a normal appetite (Gupta *et al.*, 2013). In another study, the decrease in body weight has been suggested to be positively associated with plasma glucose-lowering activity in vivo by thiourea derived compounds (Zhang *et al.*, 2009), which may be also related with the decreased in body weight of experimental animals, corroborating the observations as found in this study.

The thiourea treated group of animals showed an increase in relative adrenal gland weight compared to the control group of animals. Detailed morphometry of the adrenal revealed that the volume of all three respective areas, i.e. zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) were increased in the treated group of rats in comparison to their control counterparts. Cells of the zona glomerulosa have little cytoplasm containing only a small amount of lipid. The ZF is the broadest of all the three zones of the adrenal, which makes up about 75% of the total cortex. Its cells are polyhedral and have many intracytoplasmic lipid droplets (Ulrich-Lai *et al.*, 2006). These lipids, mostly cholesterol and cholesterol esters, are used as substrates for steroidogenesis (Miller and Bose, 2011). These lipid droplets are mobilized from the stores to mitochondria for the synthesis of steroids. In this study, marked histological involvement was found in each of the zona layers of the adrenal cortex; particularly the zona fasciculata was disorganized under the effect of thiourea administration. This adrenocortical layer produces corticosterone (in rodents) under the influence of adreno-corticotrophic hormone (ACTH). The cells of this

Table 2. Detailed morphometric alterations of the cortex and medulla of left adrenal gland of control and thiourea-treated rats.

Parameters	Control	Thiourea treated	*p*-value
Adrenal gland weight(mg)	25.69 ±0.58	29.84±0.38*	0.0028
Volume (mm^3) the entire gland	159.7±0.64	181.9±0.96*	0.0025
Zona glomerulosa	12.22±0.42	14.7±0.30*	0.0041
Zona fasciculata	90.51±0.56	101.23±0.29*	0.0027
Zona reticularis	25.13±0.68	46.43±0.19*	0.0026
Medulla	22.41±0.38	18.64±0.14*	0.0037
Relative volume of the gland (%)			
Zona glomerulosa	7.65±0.57	8.08±0.60	0.7489
Zona fasciculata	62.31±0.56	55.65±1.04*	0.0051
Zona reticularis	15.73±1.07	25.52±0.53*	0.0052
Medulla	14.03±0.70	10.24±0.39*	0.0064
Average cell volume (mm^3)			
Zona glomerulosa	23±0.57	29±1.78*	0.0052
Zona fasciculata	75±1.46	89±1.02*	0.0025
Zona reticularis	58±0.89	71±1.06*	0.0037
Distribution of adrenocortical cells (%)			
Zona glomerulosa	14.4±0.42	15.95±0.69	0.0031
Zona fasciculata	46.98±0.88	59.9±0.65*	0.0074
Zona reticularis	36.31±0.77	39.03±0.62*	0.0054
Number of adrenocortical cells (1×10^3)			
Zona glomerulosa	277.7±1.31	299.2±0.55*	0.0098
Zona fasciculata	788.43±1.24	858.21±0.92*	0.0045
Zona reticularis	277.12±0.83	301.76±0.74*	0.0069
Total	1343.25±54.87	1459.17±87.06	

Results are expressed as means ± SEM

Table 3. Thiourea induced alteration in adrenal Δ^5 3β and 17β HSD activity in female rats.

Groups	$\Delta^5$3β HSD activity (Δ OD/min/mg protein)	17β HSD activity (Δ OD/min/mg protein)
Control	0.13 ±0.008	0.18 ±0.004
Thiourea-treated	0.17 ±0.020*	0.21 ±0.007
p-value	0.0067	0.8745

(Values are Mean ± SEM; n=6); The comparison between control and thiourea-treated group was done following two tail Mann-Whitney U test and significant difference was found between the two groups ($p<0.05$).

Table 4. Thiourea induced alteration in serum corticosterone level and adrenal lipid peroxidation level.

Groups	Serum corticosterone level (µg/dl)	Lipid peroxidation level (nmole TBARS/mg protein)
Control	23.1±1.71	4.16±0.93
Thiourea-treated	45.4±2.38*	9.51±0.49*
p-value	0.0064	0.0054

(Values are Mean ± SEM; n=6); The comparison between control and thiourea-treated groups was done following two-tail Mann-Whitney U test and significant difference was found between the two groups ($p<0.05$).

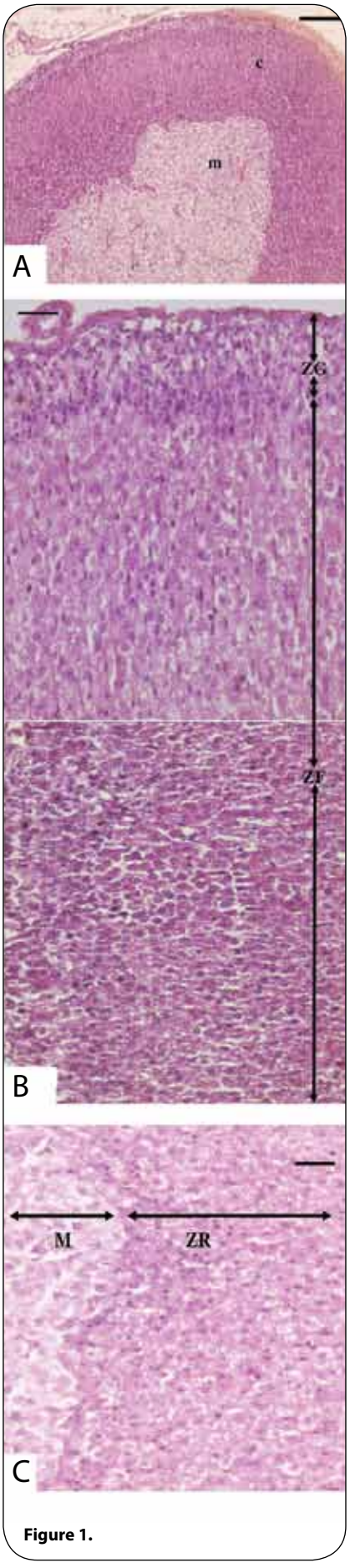

Figure 1.

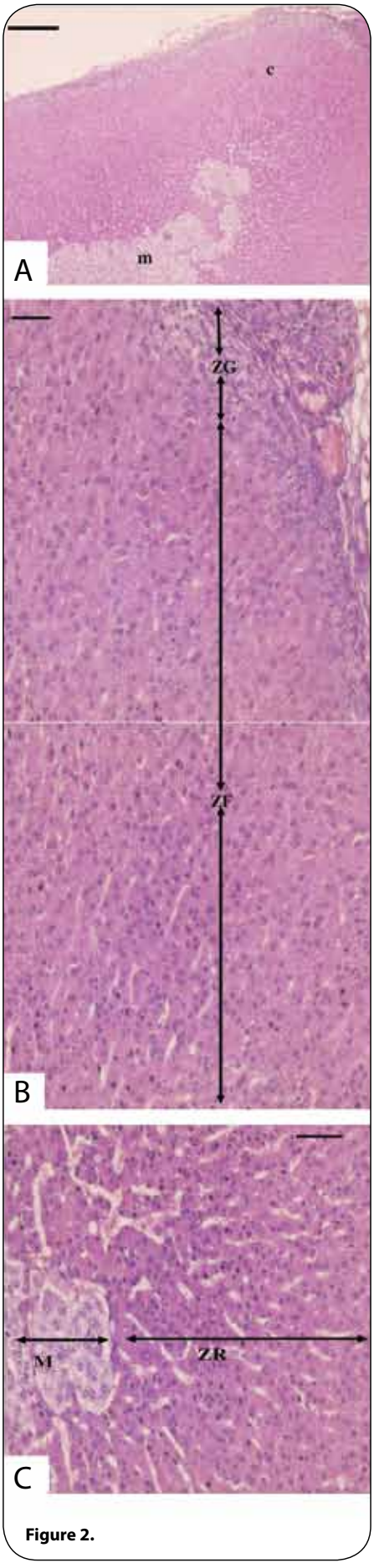

Figure 2.

Figure 1. Photomicrographs of adrenal gland sections are shown at 40× after H&E staining (**A**) Control Scale bar=18.13 µm (approx). Distinctive features of ZG and ZF (**B**) and ZR (**C**) of control adrenal gland after H/E stain at 400×. c=cortex, m&M=medulla; ZG=zona glomerulosa, ZF=zona fasciculata and ZR=zona reticularis.

Figure 2. Photomicrographs of adrenal gland sections are shown at 40X after H / E staining **(A)** Thiourea treated. Scale bar=18.13µm (approx). Distinctive features of ZG and ZF (**B**) and ZR (**C**) of thiourea treated adrenal gland stained with H/ E stain at 400×. ZG in thiourea rats, showing hypertrophic and hyperplastic appearance of adrenocortical cells. In ZF cells are cuboidal with round nucleus; in ZR cells are distorted having irregular arrangement. Scale bar 15 µm. c=cortex, m&M=medulla; ZG=zona glomerulosa, ZF=zona fasciculata and ZR=zona reticularis.

layer remain arranged in cords in control, but are anarchically arranged after thiourea exposure. All these indicates that prolonged exposure to thiourea caused structural and functional (Kapoor *et al.*, 2006) modifications in the adrenal gland. A similar finding has been reported in rats exposed to different stressful external stimuli. The average size along with the distribution and number of the cells especially in ZF and ZR were significantly increased in the adrenal of thiourea treated groups, indicating their hypertrophy and hyperplasia. Thus thiourea administration over a period of time has significant impact on adrenal gland morphology and morphometry, with possible occurrence of endocrine dysfunction in the long run.

There is a direct functional relationship between adrenal gland activity and increased gland weight (Hui *et al.*, 2009). Hypertrophy and macroscopic hyperemia (increase of blood flow) have been previously described in response to administration of ACTH or following prolonged exposure to stress (Hui *et al.,* 2009). In this investigation the adrenal gland weight was increased following thiourea administration. It was reported previously that such a condition can arise as a result of the stress response, yet it may also occur due to deficient glucocorticoid feedback regulation of ACTH due to toxicity to the adrenal cortex (Chakraborty *et al.*, 2016). Thus excess thiourea as used in this study might be responsible for producing oxidative stress in the adrenal resulting in the cellular and subcellular alterations. There was a significant elevation of adrenal lipid peroxidation (LPO) level when compared to the control group of rats. As widely known, LPO refers to oxidative degradation of lipids. It is a gradual process in which free radicals steal electrons from the lipid in the cell membrane. Malonaldehyde (MDA) levels thus formed indicate the intoxication and generation of oxidative stress in this organ, considered an endpoint of oxidative stress formation (Chakraborty *et al.*, 2016). This clearly suggests that thiourea exposure caused elevation of LPO level or in other words generation of oxidative stress resulting in adrenal gland weight and subsequent changes in the activity of the gland by feedback mechanism to ACTH.

In parallel to adrenal weight elevation there was also subsequent increase in adrenal Δ^5 3β hydroxydehydrogenase (HSD). It is one of the important regulatory enzymes of the adrenal steroidogenic pathway. Thiourea at the dose used in this study caused significant increase in adrenal weight followed by hyperactivity of adrenal Δ^5 3β-HSD activity with concomitant rise in corticosterone level, possibly an ameliorative effect to the developed stress under the influence of thiourea. Recent reports suggest an important endocrinological concept from the above, most relevant to the pathological manifestations of toxicity or stress in the adrenal cortex, the feedback regulation of the hypothalamic–pituitary axis by glucocorticoids. This reflects the possible involvement of hypothalamo-pituitary-adrenal axis following thiourea administration in experimental animals. Similarly, adrenal 17β HSD was concomitantly elevated, though not significantly, following thiourea exposure, also indicating its hyperactivity when compared to control animals. There are reports of an increase in adrenal 17β HSD positively correlating with adrenal adenoma, an adrenal tumor in humans (Gangkak *et al.*, 2015) suggesting additional evidence for hyperplasia and weight gain in the gland after thiourea administration.

A major neuroendocrine mechanism in a stress reaction is the activation of the hypothalamic-pituitary-adrenal axis (HPA), resulting in a rapid increase in circulating corticotropin (ACTH) and subsequent rise in glucocorticoids, which are critical for successful adaptation (Miller *et al.*, 2007). Thus, plasma levels of ACTH and glucocorticoids are good indicators of stress response intensity, particularly in its acute phase (Otis *et al.*, 2007). Acute as well as chronic stress excites the HPA axis to stimulate the release of corticotrophin releasing hormone (CRH) from the hypothalamus that triggers the release of ACTH from the pituitary, resulting in enhanced corticosteroid synthesis and release from the adrenal cortex (Rodríguez-Gutiérrez *et al.,* 2014). Increased activity of adrenal Δ^5 3β-HSD was found in parallel with the rise in the level of ACTH in serum (Markov *et al.*, 2009). This study demonstrates an increase in adrenal 3β-HSD parallel with the rise in serum corticosterone, confirming that thiourea in the doses as used in this study elevates the adrenal stress signaling pathway leading to oxidative stress in the gland, which was further confirmed by increase in adrenal LPO level.

The overall results revealed that the chronic thiourea exposed group of animals develop morphological and functional changes of the adrenal gland (especially in the area of zona fasciculata) with increased level of lipid peroxidation, enhanced adrenal Δ^5 3β HSD activity followed by elevated serum corticosterone level. Thus thiourea in pharmacological doses as in this study, does not only suppress thyroid gland function but also modulates the activity of the adrenal cortex, which is a major concern in health and disease.

It would have been better if the investigation had been carried out in patients using thiourea as a drug, followed by evaluation of the activity status of the adrenal. Further for the non-availability of rat ELISA kits, one of the most important parameters, ACTH, was not assayed.

However these studies are likely to be helpful to understand the functional status of the adrenal gland in hypothyroid conditions, as in the patients who are consuming anti-thyroid drugs.

Conclusion

Based on the observation of the study it may be concluded that though thiourea has antithyroidal activity yet simultaneously develops stress in the adrenal, as evident by enhanced lipid peroxidation in the gland, that in turn through the hypothalamo-pituitary-adrenal axis it causes hypertrophy and hyperplasia of adrenocortical cells to stimulate the synthesis and release of corticosterone to counteract the generated stress under the influence of this chemical agent. This study provides novel insights

and raises new concerns about the hyperactivation of the adreno-cortical functional status linked with oxidative stress in populations exposed to excess thiourea for considerable long periods.

Several studies found it necessary to understand the structural and functional status of the adrenal under the influence of other antithyroidal drugs like propyl thiouracil, marcapto imidazole, etc.

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REFERENCES

Bozzo A, Soñez CA, Mugnaini MT, Pastorino IC, Rolando AN, Romanini MC, Gauna HF. (2006). Chronic stress effects on the apoptotic index of the adrenal cortex of pregnant rats. *Bio cell* **30**: 439–45.

Chakraborty A, Mandal J, Mondal C, Sinha S, Chandra AK. (2016). Effect of excess iodine on oxidative stress markers, steroidogenic – enzyme activities, testicular morphology and functions in adult male rats. *Biol Trace Elem Res* **172**(2): 380–94.

Chakraborty A**,** Mondal C, Sinha S, Mandal J, Chandra AK. (2014). Amiodarone induced oxidative stress in stress – vulnerable organs of adult male rats. *Asian J Clin Pharm Res* **7**(4): 177–83.

Chandra AK, Choudhury SR, De N, Sarkar M. (2011). Effect of green tea (Camellia sinensis L.) extract on morphological and functional changes in adult male gonads of albino rats. *Indian J Exp Biol* **49**: 689–97.

Chandra AK, Sinha S, Choudhury SR. (2010). Thyroxine induced stress and its possible prevention by catechin. *Indian J Exp Biol* **48**: 559–65.

Gangkak G, Yadav S, Verma S, Agarwal N, Yadav SS, Tomar V. (2015). Pure androgen secreting adrenal adenoma: a case report and review of literature. *Clin Nephrol Urol Sci* **2**: 1–5.

Gupta KK, Gattani A, Moolchandani A, Sareen M. (2013). The influence of experimental hypothyroidism on hepatic and renal function in rams in an arid tropical environment. *Vet Arhiv* **83**: 161–70.

Hamden K, Masmoudi H, Ellouz F, ElFeki A, Carreau S. (2008). Protective effects of Peganum harmala extracts on thiourea-induced diseases in adult male rat. *J Environ Biol* **9**(1): 73–7.

Hui XG, Akahira J, Suzuki T, Nio M, Nakamura Y, Suzuki H, Rainey WE, Sasano H.(2009). Development of the human adrenal zona reticularis: morphometric and immunohistochemical studies from birth to adolescence. *J Endocrinol* **203**(2): 241–52.

Kapoor A, Dunn E, Kostaki A, Andrews MH, Matthews SG. (2006). Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. *J Physiol* **572**: 31–44.

Markov GV, Tavares R, Dauphin-Villemant C, Demeneix BA, Baker ME, Laudet V. (2009). Independent elaboration of steroid hormone signaling pathways in metazoans. *Proc Natl Acad Sci U S A* **106**: 11913–8.

Miller GE, Chen E, Zhou ES. (2007). If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. *Psychol Bull* **133**: 25–45.

Miller WL, Bose HS. (2011). Early steps in steroidogenesis: intracellular cholesterol trafficking. *J Lipid Res* **52**(12): 2111–35.

Mondal S, Mukherjee S, Chaudhuri K, Kabir SN, Kumar Mukhopadhyay P. (2013). Prevention of arsenic-mediated reproductive toxicity in adult female rats by high protein diet. *Pharm Biol* **51**(11): 1363–71.

Mutic AD, Baker BJ, McCauley LA. (2017). Deleterious Effects from Occupational Exposure to Ethylene Thiourea in Pregnant Women. *Workplace Health Saf* **1**: 2165079916687312.

Otis M, Campbell S, Payet MD, Gallo-Payet N. (2007). Expression of extracellular matrix proteins and integrins in rat adrenal gland: importance for ACTH-associated functions. *J Endocrinol* **193**: 331–47.

Poncin S, Van Eeckoudt S, Humblet K, Colin IM, Gérard AC. (2010). Oxidative stress: a required condition for thyroid cell proliferation. *Am J Pathol* **176**(3): 1355–63.

Rodríguez-Gutiérrez R, González-Velázquez C, González-Saldívar G, Villarreal-Pérez JZ, González-González JG. (2014). Glucocorticoid functional reserve in full-spectrum intensity of primary hypothyroidism. *Int J Endocrinol* 1–8.

Sarwar G, Parveen S, Aslam siddiqui M. (2008). Comparison of the effects of experimentally induced hypothyroidism in adrenal glands morphology in pre and postnatally treated albino rats. *Pak Jour Pharmacol* **25**(1): 13–8.

Ulrich-Lai YM, Figueiredo HF, Ostrander MM, Choi DC, Engeland WC, Herman JP. (2006). Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *Am J Physiol Endocrinol Metab* **291**: E965–73.

Vento PJ, Swartz ME, Martin LB, Daniels D. (2008). Food intake in laboratory rats provided standard and fenbendazole-supplemented diets. *J Am Assoc Lab Anim Sci* **47**: 46–50.

Wang Y, Jin X, He L, Zhang W. (2017). Inhibitory effect of thiourea on biological nitrification process and its eliminating method. *Water Sci Technol* **75**(12): 2900–2907.

Weng Q, Saita E, Watanabe G, Takahashi S, Sedqyar M, Suzuki AK, *et al.* (2007). Effect of methimazole-induced hypothyroidism on adrenal and gonadal functions in male Japanese quail (Coturnix japonica). *J Reprod Dev* **53**: 1335–41.

Zhang Z, Schreiner PR. (2009). (Thio)urea organocatalysis--what can be learnt from anion recognition? *Chem Soc Rev* **38**(4): 1187–98.

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ABSTRACT BOOK

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L-01

DEVELOPMENT AND VALIDATION OF THE EPIDERM *IN VITRO* SKIN IRRITATION PROTOCOL FOR THE ASSESSMENT OF MEDICAL DEVICES EXTRACTS

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Assessment of dermal irritation is an essential component of the safety evaluation of medical devices. Reconstructed human epidermis (RhE) models have replaced rabbit skin irritation testing for neat chemicals (OECD TG 439). However, medical device (MD) extracts are dilute solutions with low irritation potential, therefore the validated RhE-methods needed to be modified to reflect needs of ISO 10993.

A protocol employing RhE EpiDerm was optimized in 2013 using known irritants and spiked polymers (Casas et al., TIV, 2013). In 2014 a second laboratory assessed the transferability of the assay. After the successful transfer and standardization of the protocol, 17 laboratories worldwide were trained in the use of the protocol in the preparation for the validation. All laboratories produced data with almost 100% agreement of predictions for the selected references.

In 2016, an international round robin validation study was conducted to confirm the ability of the RhE models to correctly predict the intra-cutaneous irritation of extracts from MDs. Four irritant polymers and three non-irritant controls were tested. Blinded polymer samples were extracted with sesame oil and saline per ISO 10993-12. Positive and negative solvent controls were included.

The EpiDerm tissues were able to correctly identify virtually all of the irritant polymer samples either in the saline or in the sesame oil or in both solvent extracts. Our results indicate that RhE tissue models can detect the presence of skin irritants at low concentrations in dilute medical device polymer extracts. The use of the reconstructed tissue models, as replacements for the rabbit intra-cutaneus test if being implemented into the ISO 10993 standards used to evaluate medical device biocompatibility. The work will be published in a special issue of Toxicology *in Vitro* in 2018.

L-02

RECONSTRUCTED 3D HUMAN SMALL INTESTINE MODEL FOR PREDICTION OF GASTROINTESTINAL TOXICITY AND DRUG ABSORPTION

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Small intestine is an important gateway through which many nutrients, drugs and other substances enter blood flow. In fact, about 90% of orally administered drug absorption occurs in the small intestine. Thereby there is a need of good and reliable *in vitro* model capable to predict drug toxicity and absorption/metabolism patterns. However, currently available *in vitro* intestine models are neither organ nor species-specific, relying predominantly on the use of cell lines generated from the colon or kidney. In addition they lack a proper 3D architecture and functionality, which in turn affects their ability to properly predict drug absorption and toxic effects.

Here we present the reconstructed 3D human small intestine model – EpiIntestinal, which mimics morphology and cell-type composition of normal human small intestine. As opposed to organoid models, EpiIntestinal is polarized and allows studying bidirectional drug penetration through intestinal wall. It expresses proteins involved in active drug transport and metabolism at physiological levels, which makes it ideal for modelling of complex drug absorption profiles, including the permeation, metabolism, drug-drug interaction and adverse effects of drugs on epithelium.

Comparative studies revealed that the absorption of drug in EpiIntestinal mimics the in vivo profile much closer than the currently used Caco-2 model. In another study aimed at adverse effects of drugs, EpiIntestinal was able to predict toxicity with much higher specificity and sensitivity than animal model. All in all, this model represents a promising tool to model complex processes occurring in small intestine.

L-03

SUCESFULL EVALUATION OF TWO EPIOCULAR EYE IRRITATION TEST PROTOCOLS IN THE INTERNATIONAL PROJECT CON4EI - CONSORTIUM FOR *IN VITRO* EYE IRRITATION TESTING STRATEGY

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Assessment of the acute eye irritation potential is part of the international regulatory requirements for testing of chemicals. The objective of the CON4EI (CONsortium for *in vitro* Eye Irritation testing strategy) project is to develop tiered testing strategies for eye irritation assessment for all drivers of classification. For this, a set of 80 reference chemicals (38 liquids and 42 solids) was tested with eight different alternative methods. Here, the results obtained with reconstructed human cornea-like epithelium (RHCE) EpiOcular and the EpiOcular Eye Irritation Test (EIT) -adopted as OECD TG 492 - are shown.

The primary aim of this study was an evaluation of the performance of the test method to discriminate chemicals not requiring classification for serious eye damage/eye irritancy (No Category) from chemicals requiring classification and labelling (Category 1 and 2). In addition, the predictive capacity in terms of *in vivo* driver of classification was investigated. In a second step, it was investigated whether the EpiOcular EIT can be used as part of a tiered-testing strategy for eye

irritation assessment. The chemicals were tested in two independent runs by MatTek *In Vitro* Life Science Laboratories.

For the EpiOcular EIT (OECD TG 492), a sensitivity of 96.9% and specificity of 86.7% with an accuracy of 95% was obtained overall and for both runs separately (100% concordance). For the EpiOcular ET-50 method, the overall accuracy of 74.5%, an FNR of 3.1% (Classified versus Not classified) and FPR of 3.4% (Classified versus Not classified) were achieved. Furthermore, about 79% of the Cat 1 liquids and 69% of the Cat 1 solids and 68% of the Cat 2 liquids and about 61% of the Cat 2 solids were identified correctly. The results of these studies seem promising with regard to the evaluation of inclusion of these test methods in an integrated testing strategy (ITS) for eye irritation assessment.

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L-04

SELECTED BISPHENOLS AND PHTHALATES SCREENED FOR ESTROGEN AND ANDROGEN DISRUPTION BY *IN SILICO* AND *IN VITRO* METHODS

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Endocrine disruptors are substances capable to bind to specific receptors similarly to endogenous hormones, thus potentially contributing to endocrine system disturbances and consequent system disorders. Sources of exposure may come from industry or agriculture, including consumer products, e.g. food packaging materials, thermopaper, plastics, household products or cosmetics. Interaction of ligands with receptors is a molecular initiation event that leads to complex effects. The physiological receptor mechanism may be affected either by direct receptor binding of the exogenous ligand to the receptor, resulting in activation (agonistic activity) or inhibition (antagonistic activity), or consequent modulation of associated signaling pathways regulation. Human receptors may share ligands with variable affinity and efficacy. Certain substances may be persistent, resulting in bioaccumulation in the food chain as well as in the organism. Others may be quickly metabolized and act for a limited time. Therefore, exposure to endocrine disruptors cannot be simply investigated in humans since it is influenced also by individual environmental, medical and social factors. These facts complicate the detection of negative effects *in vivo*. Development and use of *in silico* screening tools and *in vitro* methods is therefore effective for first-level screening and should be used more intensively. In our pilot study, selected bisphenols and phthalates were tested using OECD QSAR Toolbox, Stably Transfected Transactivation *In Vitro* Assay to Detect Estrogen Receptor Agonists (OECD TG 455) and yeast-based microplate assay (in compliance with Draft ISO/DIS 19040) in order to determine the interactions of the tested chemicals with human estrogen and androgen receptors. *In vitro* results correlated well with *in silico* prediction for phthalates predicted as non binders, while predictions for bisphenols differed slightly. Both *in vitro* biological methods exhibited good concordance of results regarding the estrogenic activity. Minor discrepancies were detected for certain bisphenols due to cytotoxicity elicited in higher concentrations. Substances showing strong estrogenic activity exhibited parallel activity on the androgen receptor. The research article also summarizes recent developments in legislation with reference to *in vitro* methods suitable for screening of endocrine disruption.

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L-05

FORMULATION OF APOFERRITIN NANOCARRIER WITH ENCAPSULATED ELLIPTICINE AND STUDY OF ITS PROPERTIES

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One of the approaches to decrease the adverse effects of drugs is their encapsulation inside a suitable nanocarrier, allowing for a targeted delivery to tumour tissue whereas avoiding healthy cells. Apoferritin is the iron-free form of ferritin, a naturally occurring iron-storage protein. Using apoferritin as a nanocarrier has the potential to move undetected through the body without inducing any resistance from the immune system of the patient. Furthermore apoferritin can be modified with recognition ligands to achieve tumor-specific targeting. The simple-to-use encapsulation protocol (creating ApoElli) was developed and the prepared nanocarrier was characterized. The nanocarrier exhibits narrow size distribution, which suggests being suitable for entrapping of the hydrophobic molecule of ellipticine. The release of ellipticine at acidic (6.5) and neutral (7.4) pH was studied. Ellipticine is gradually released from its ApoElli form into the water environment under acidic pH; more than 80% ellipticine was released after 48 hrs incubation at pH 6.5. In contrast at pH 7.4 less than 20% was released. ApoElli is also stable after its storage at physiological pH (7.4) up to 1 month at 4 °C. The presence of membrane particles accelerates release

of ellipticine from Apo-Elli and makes it possible to be transferred into microsomes even at pH 7.4. Microsomal cytochromes P450 are capable of oxidizing free ellipticine and/or its ApoElli form to its metabolites and generating covalent ellipticine-derived DNA adducts, both under pH 7.4 and 6.5. The form of ellipticine plays essentially no role in these processes. The ApoElli is toxic to UKF-NB-4 neuroblastoma cancer cells but exhibits significantly lower toxicity for non-malignant cells (non-malignant fibroblasts, HDFn cells).

The work was supported by GACR (17-12816S) and Charles University (204025/2012).

L-06

APOFERRITIN NANOCAGE FOR ELLIPTICINE DELIVERY TO NEUROBLASTOMA CELLS

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Recently, nanoparticles have been widely investigated for delivery of anticancer drugs. Apoferritin is a natural nontoxic iron carrier and has a natural hollow structure that can be used to store small molecules such as cytostatics. The aim of our study was to compare the cytotoxic effects of anticancer drug ellipticine loaded in apoferritin (APOELLI) and free ellipticine (ELLI) on neuroblastoma cells UKF-NB-4, chemoresistant sublines derived from this cell line, and normal human fibroblasts (HDFn), as a model of non-malignant cells. We show here that the cytotoxicity of APOELLI is lower than cytotoxicity of free cytostatic, but APOELLI induces more double strand breaks than free ELLI in neuroblastoma cells. Moreover, cytotoxicity of drug loaded apoferritin is significantly lower for HDFns. Further, using fluorescence microscopy, we have shown that apoferritin can deliver drugs inside cells and the drug exerts their effect thereof. Fluorescence intensity of ELLI/APOELLI in nuclei of neuroblastoma cells is significantly higher than in those of HDFn, because ELLI/APOELLI is more sequestrated in lysosomes in fibroblasts. The extent of APOELLI enter into the UKF-NB-4 cells correlated with formation of covalent ELLI-derived DNA adducts in these cells; the levels of ELLI-DNA adducts generated by APOELLI were 67% of those formed by free ELLI. The results found in this study seem to be promising, because encapsulation does not affect toxicity of cytostatic and improves drug stability. We suppose that apoferritin with encapsulated ELLI is targeted to the several cancer cells including neuroblastoma through receptors TfR 1 and/or SCARA 5 which are expressed in many cancers.

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L-07

DNA ADDUCTS FORMATION BY PLANT ARISTOLOCHIC ACID IS UNIQUE BIOMARKER OF EXPOSURE AND EXPLAIN THE INITIATION PHASE OF UPPER UROTHELIAL CANCER

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Aristolochic acid (AA) is an alkaloid causing aristolochic acid nephropathy (AAN) and Balkan endemic nephropathy (BEN) that are renal diseases often associated with upper urothelial cancer (UUC). The formation of covalent DNA adducts by carcinogens is considered to be one of the earliest steps in the initiation phase of cancer development. Moreover, the covalent binding of carcinogens to DNA, which is causally related to tumorigenesis, is now considered as a central dogma of chemical carcinogenesis. This belief is supported by various observations, such as the facts that: (i) the carcinogenic properties of many carcinogens is dependent upon their activation to reactive electrophilic derivatives, which react with nucleophilic sites within DNA; (ii) the extent of DNA adduct formation can frequently be correlated with the magnitude of carcinogenic responses; and (iii) mutations in certain tumour suppressor genes and the activation of several proto-oncogenes can be mediated by the interaction of carcinogens with DNA. However, since humans are exposed not only to one but to a complex mixture of carcinogens, direct proofs of an association of exposure to the development of a specific cancer type are rare. The plant carcinogen AA is one of the rare examples where a distinct environmental exposure is linked to tumour development in humans. This study demonstrates the significance of AA-derived DNA adducts in the aetiology of UUC leading to specific A:T to T:A transversion mutations (mutational signature) in AAN/BEN-associated tumours, which are otherwise rare in individuals with UCC not exposed to AA. Therefore, such DNA damage produced by AA-DNA adducts is one rare example of the direct association of exposure and cancer development (UUC) in humans, confirming that the covalent binding of carcinogens to DNA is causally related to tumourigenesis. Even though aristolochic acid I (AAI), the major component of the natural plant extract AA, might directly cause interstitial nephropathy, enzymatic activation of AAI to reactive intermediates capable of binding to DNA is a necessary step leading to the formation of AA-DNA adducts and subsequently AA-induced malignant transformation. Therefore, AA-DNA adducts can not only be utilized as biomarkers for the assessment of AA exposure and markers of AA-induced UUC, but also be used for the mechanistic evaluation of its enzymatic activation and detoxification. Differences in AA metabolism might be

one of the reasons for an individual's susceptibility in the multi-step process of AA-mediated carcinogenesis and studying associations between activities and/or polymorphisms of the enzymes metabolising AA is an important determinant to identify individuals having a high risk of developing AA-mediated UUC.

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L-08

CHANGES IN EXOSOME PRODUCTION AFTER CHEMOTHERAPY TREATMENT

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Intercellular communication is one of the most important processes commonly found among all organisms. It is secured by extracellular vesicles among which we include exosomes. Exosomes are nanosized vesicles of intracellular origin with size in range of 30-150 nm. They mediate communication between cells by transferring their cargo.

The aim of our work was to test isolated vesicles from ovarian and breast cancer cell lines for exosome markers and to measure the size and concentration of isolated vesicles. As additional objectives, we chose to test the effect of drugs on the production of exosomes form various cell lines and their effect on viability of cells. We verified the expression of CD63, CD9 or TSG101 as markers enriched on exosomes, while we registered significant changes in their expression as well as change in the amount of produced exosomes while being affected by several specific drugs - cisplatin (CP) and inhibitor of survivin (YM155). The treatment of cells with specific drugs also changed the amounts of exosomes produced into the extracellular milieu, which size and concentration were tested on NanoSight NS500 device. The potential of exosomes to mediate intercellular communication was tested as the ability of a tumor or non-tumor cell line to internalize heterologous. This effect was observed by confocal microscopy. Alterations in exosome secretion reflect different cell and tissue states in the whole organism and adapt to the individual's lifestyle. In the future, tailored validation of patients' exosome profiles will be necessary.

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L-09

ALTERED EXPRESSION OF BIOTRANSFORMATION ENZYMES IN HEPATOCELLULAR CARCINOMA

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Hepatocellular carcinoma (HCC) is killing 250,000 to 1 million patients per year and originates in the key cell population for biotransformation, the hepatocytes. Despite that, studies on biotransformation enzymes in HCC are scarce. It is known that malign transformation leads to a significant deregulation of gene expression and alteration of metabolic functions in hepatocytes. Advanced stages of the disease are frequently associated with liver failure and threaten the patients with a severe impairment of (drug) metabolism. The impact on efficacy and toxicity of drug in such patients is unknown. This study aimed at analysis of the most important enzymes of both phases of drug metabolism, in hepatocellular carcinoma samples as compared to their para-tumour non-cancerous tissue. Whole transcriptome profiles were obtained for protein coding genes and long non-coding RNAs using gene expression microarrays Agilent SurePrint G3 8x60k and for a panel of 754 miRNAs using qPCR TaqMan Low Density Arrays v3.0. Selected genes were validated at mRNA level using qPCR and at protein level by western blotting. Results show significant alteration of several drug metabolism-associated pathways as well as a significant drop of gene expression across the whole P450 superfamily (up to several orders of magnitude) in the HCC tissue compared to surrounding non-cancerous tissue in approximately half of the patients, correlating with expression of expression levels of regulative nuclear factors and the histological grade of the tumours.

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L-10

INSIGHTS ON THE RELATIONSHIP BETWEEN STRUCTURE OF PHENYLETHANOID GLYCOPYRANOSIDES AND THEIR ACTIVITIES USING CELL-FREE ASSAYS AND HUMAN CELLS CULTURED *IN VITRO*

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Prevention of cancer remains the most promising strategy for reducing both its incidence and the mortality. The study of protective potential of selected natural compounds and their synthesized analogues, which could be used in the prevention and health protection, might be therefore of great importance. Plants are a rich source of phytochemicals possessing such properties. Salidroside as the main natural phenylethanoid glycopyranoside

(PEGP) present in plants of the genus *Rhodiola* is characterized by many beneficial pharmacological effects. The structure of the compound, a wide range of its biological activities and limited availability of the most productive species has inspired organic chemists to synthesize salidroside (SALI - tyrosol β-D-glucopyranoside) and PEGPs: tyrosol β-D-galactopyranoside (TYBGAL), tyrosol α-D-galactopyranoside (TYAGAL), tyrosol α-D-mannopyranoside (TYAMAN), hydroxytyrosol α-D-mannopyranoside (HOTAMA), homosyringyl β-D-glucopyranoside (HSYGLU), hydroxytyrosol β-D-xylopyranoside (HOTXYL) and hydroxysalidroside (HOSALI).

The objectives of our study were (i) to prepare PEGPs by chemical or less conventional enzymatic procedures; (ii) to determine their reducing power, radical scavenging and chelating capacities using cell-free approaches and (iii) in experimental system utilizing human hepatoma HepG2 cells to evaluate their cytotoxicity (MTT test) and protective potential against lesions induced by hydrogen peroxide (H_2O_2; comet assay).

Glycosylated hydroxytyrosols (HOSALI, HOTAMA, HOTXYL) and HSYGLU manifested the highest reducing power and DPPH radical scavenging capacity and they were most active in protection of HepG2 cells against free-radicals generating agent H_2O_2, particularly at the lower concentrations used. On the other hand, pre-treatment of HepG2 cells with SALI had protective effects even though SALI displayed neither reducing power nor DPPH radicals scavenging activity. We suppose that protection induced by SALI is achieved by the effects on other cellular processes distinct from antioxidant action.

Differences in the effectiveness of the phenylethanoid glycopyranosides found in this study revealed that structures of their molecules in terms of aglycone combined with sugar moiety can affect and contribute to their activities.

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L-11

METABOLISM OF POLYCYCLIC AROMATIC HYDROCARBONS PLAYS A MAJOR ROLE BOTH IN THEIR GENOTOXICITY AND IN THEIR NON-GENOTOXIC EFFECTS

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Polycyclic aromatic hydrocarbons (PAHs) are an important group of abundant environmental pollutants formed by a variety of combustion processes. Both individual PAHs, such as benzo[a]pyrene (BaP), and their complex mixtures, have been associated with adverse human health effects, including cancer. Their carcinogenicity is primarily attributed to their genotoxic effects, linked with formation of covalent DNA adducts, for which PAHs require activation to their electrophilic metabolites, dihydrodiol epoxides, in order to exert mutagenic or carcinogenic effects. However, other metabolites of PAHs have received considerably less attention, although they may induce a number of toxic effects linked with generation of oxidative stress, alter the activity of important intracellular signaling pathways (including e.g. activation of receptor tyrosine kinases or mitogen-activated protein kinases) or activate specific intracellular receptors. This presentation aims to provide an overview of known non-genotoxic effects of PAHs, which could be linked with their metabolism. In the second part, we will illustrate the important role of metabolism in estrogen-like effects of PAHs and/or their mixtures. We studied both metabolism and the estrogen receptor (ER)-mediated effects of model PAHs, such as BaP (and benz[a]anthracene; BaA, in human breast cancer cell models in the presence or in the absence of enzymatic activity required for their metabolization. In cells without active PAH metabolism, BaP formed significantly lower amounts of most of its metabolites, including hydroxylated-BaPs; this was linked with suppression of estrogen-like effects, such as ER-dependent modulation of cell cycle progression or induction of the ER-dependent luciferase reporter gene. These results suggest that more attention should be paid to the role of metabolism of PAHs in their toxic modes of action, as it may alter also their non-genotoxic effects.

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L-12

ANTIGENOTOXIC ACTIVITY OF *POLYSCIAS FILICIFOLIA* EXTRACTS

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Searching for antigenotoxic compounds, which are able to decrease or even remove the mutagenic effects, represents a rapidly expanding field of the cancer research. Traditional medicinal plants are an important source of active compounds with a potential antigenotoxic activity. Biotechnological methods in plant cell and organ cultures offer the possibilities to improve productivity of useful compounds, one of them is the elicitation. We evaluated the antigenotoxic potential of extracts of south Asian traditional herb, *Polyscias filicifolia* Bailey, growing *in vitro* with an addition of elicitors. The extracts contain wide range of biological active compounds like phenolic acids: chlorogenic acid (CGA), caffeic acid (CA) and ferulic acid (FA) derivatives

or triterpenoid saponins, which aglycone is oleanolic acid (OA). Additionally, we evaluated the antigenotoxic activity of phenolic acids and OA. The evaluation was made using short-term bacterial *umu*-test, toward three different mutagens: 4-nitroquinoline-N-oxide, mitomycin C and 2-aminoanthracene (2AA). The tested extracts exhibited high antigenotoxic potential if the assay was performed with 2AA and metabolic activation. However, the extracts after a hydrolysis of saponins were slightly active. Phenolic acids: FA and CA slightly decreased 2AA-genotoxicity, while CGA increased the effect. Therefore, phenolic acids probably are not responsible for the extracts activity and more attention should be put on triterpenoid saponins. We evaluated antigenotoxic activity of free aglycone (OA) and no effect was observed. Based on the obtained results it can be conclude that *Polyscias filicifolia* extracts are a potential source of antimutagenic compounds, but further studies are necessary to demonstrate the activity of triterpenoid saponin fractions.

L-13

CYANOBACTERIA – BASED DIETARY SUPPLEMENTS QUALITY MONITORING

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The dietary supplement Spirulina, used worldwide as a natural product for overall health improvement, represents a dried biomass of cyanobacteria. It has no therapeutic value, but does have value as a source of nutrients, e.g. vitamins, minerals and other substances with a nutritional or physiological effect. *Cyanobacteria* are prokaryotic gram-negative bacteria whose rapid and excessive growth associated with water eutrophication can lead to the formation of aggregations, referred to as a "water blooms". Some of these bloom-forming cyanobacteria genera produce cyanotoxins which may cause various health problems. Spirulina is the common name for cyanobacteria–based dietary supplements that contain, in particular, dried biomass from two species, *Arthrospira platensis* and *Arthrospira maxima.* These non-toxic cyanobacteria grow under controlled conditions, so are generally considered to be safe. They must meet the requirements of European and Slovak food legislation, however, which sets limits for levels of contaminants. Based on the confirmed contamination of other cyanobacteria-based products, concerns have also been raised about the safety of Spirulina dietary supplements. To ensure the protection of human health, the Public Health Authority of the Slovak Republic in Bratislava assessed the levels of selected contaminants (heavy metals and polycyclic aromatic hydrocarbons). In addition to the legislation, the declared cyanobacteria *Arthrospira* was microscopically examined in eight randomly selected samples, where its presence was confirmed, but with observed differences in level within individual samples. The samples were also analysed for the presence/absence of the specific cyanotoxins, microcystins (LR, YR, RR). Ecotoxicity monitoring optimised extract preparation conditions and ecotoxicity testing, and evaluations were made. The results of ecotoxicological tests performed on the test organisms *Vibrio fischeri, Thamnocephalus platyurus* and *Sinapis alba* revealed the possible presence of contaminants in some of the cyanobacteria-based dietary supplement samples.

L-14

PROLONGED EXPOSURE TO NON-LETHAL DOSES OF DDT AND DDE SHOWED DIFFERENT EFFECTS ON INSULIN PRODUCTION AND PROTEIN EXPRESSION IN PANCREATIC BETA CELLS

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Diabetes is one of the most prevalent diseases of civilization nowadays. An influence of pollutants on development of diabetes worldwide is suspected but an exact level and mechanism of their effect is yet to be determined. Among compounds with suspected pro-diabetic effects belong also pesticide DDT and its metabolite DDE. The aim of the present work was to investigate effects of prolonged exposure to non-lethal doses of DDT and DDE on insulin production and protein expression in pancreatic beta cells. To achieve that, we exposed rat pancreatic beta cells INS1E to non-lethal doses (10 μM) of DDT or DDE for 1 month. Changes in glucose-induced insulin secretion were determined by ELISA kit and changes in protein expression were examined using 2D-electrophoresis and western blot. Cells exposed to DDT for 1 month completely failed to increase insulin secretion after exposure to higher glucose concentrations. WB showed significant decrease in expression of both proinsulin and hexameric insulin in cells exposed to DDT. Cells exposed to DDE for 1 month secreted insulin at the level of control cells. WB showed significant decrease in expression of proinsulin but not of hexameric insulin in cells exposed to DDE. 2D-electrophoresis showed one protein with upregulated expression in both cells exposed to DDT and cells exposed to DDE: vitamin D-binding protein (VDBP). To conclude, DDT and DDE showed different effect on insulin secretion. A possible modulator of DDT and DDE effect on expression of proinsulin in pancreatic beta cells can be vitamin D-binding protein. Further work needs to be done to elucidate mechanisms of described phenomena.

L-15

TOXICITY OF HOSPITAL WASTEWATER ASSESSED BY DIFFERENT BIOASSAYS

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The purpose of this study was to determine toxicity of wastewater from hospitals in the Czech Republic using traditional and alternative toxicological methods.

The pilot study comprised weekly dynamics of sewage ecotoxicity of treated wastewater from one large hospital in two different seasons (May vs. November). A detailed investigation of wastewater ecotoxicity, genotoxicity and reprotoxicity followed in five different hospitals. The toxicity classification system based on the calculation of TU (toxic unit) was applied to evaluate ecotoxicity.

The seven following bioassays were used in this study: algal growth inhibition test with *Desmodesmus subspicatus* (EN ISO 8692), *Vibrio fischeri* luminescent test (EN ISO 11348-2), immobilization test with *Daphnia magna* (EN ISO 6341), *Allium cepa* assay, Bacterial Reverse Mutation Test – Ames Agar Plate Test (OECD TG 471), Comet assay (single-cell gel electrophoresis) and YES/YAS – Yeast Based Reporter Gene assay.

In the pilot study, the wastewater ecotoxicity during one week showed no significant differences in separate working days, however, higher toxicity values were recorded in May compared to November. In the following study, samples from two of the five hospitals were classified as toxic (III.toxicity class), the others as non toxic (I.toxicity class). Genotoxicity has not been confirmed neither by Ames test, nor Comet assay in any sample. In several cases, wastewater samples exhibited agonist activity to the estrogen and androgen receptors.

The study demonstrated different levels of toxicity of treated hospital wastewater. Variable sensitivity of individual bioassays for tested wastewater samples was recognized. It can be assumed that the results of Ames test, Comet and YES/YAS assays may be influenced by sample sterilization (by filtration) which might have caused a loss of genotoxic and reprotoxic activity as certain chemicals may be captured on the filters. The study will continue with optimization of sample preparation. A more extensive study including proposal for improvement of hospital wastewater treatment within the Czech Republic can be recommended with the aim to decrease the discharge of toxic chemicals into the local sewage system and environment.

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L-16

FORMATION AND ELIMINATION OF *N,N*-DIMETHYLFORMAMIDE ADDUCTS WITH BLOOD PROTEINS IN HUMANS

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N,N-Dimethylformamide (DMF), an industrial solvent with hepatotoxic properties, undergoes metabolism to a reactive intermediate, *N*-methylisocyanate, that binds covalently to blood protein globin to produce stable adducts, *N*-methylcarbamoylvaline (MVU) at the globin *N*-terminus and N_{ε}-methylcarbamoyllysine (MLU). Earlier we have shown in rats that physiological removal of the erythrocytes is followed by a hydrolytic cleavage of globin resulting ultimately in the excretion of free MVU and N_{α}-acetyl-MLU (MLU-Ac) in the urine. In the current study with human volunteers, DMF was used as a testing adduct-forming compound to verify a theoretical model based on our previous experiment on rats, which describes the fate of the protein adducts in blood including their degradation and excretion in the urine. Following ingestion of 500 mg DMF in aqueous solution, the subjects (n=7) provided multiple blood and urine samples during next five months. In addition to MVU and MLU in globin, protein-bound MLU was also measured in total plasma. Both globin and plasma were worked-up by acidic hydrolysis followed by HPLC-ESI-MS^2 or GC/MS analysis. Maximum levels of MVU and MLU in globin were attained 7 days post-exposure (17.5±3.7 nmol/g and 15.7±3.5 nmol/g, respectively) and then followed by an almost linear decline to the pre-exposure levels ca. 130 days later, reflecting the life span of human erythrocytes. MLU levels in plasma peaked on day 3 (3.8±0.7 nmol/ml) and then declined with a half-life of ca. 25 days, close to turnover rate of human serum albumin. Analyses of urine samples for MLU-Ac and MVU, based on SPE clean-up and HPLC-ESI-MS^2 or GC/MS, are currently in progress. In addition to their role in deriving the above toxicokinetic model in humans, urinary MVU and MLU-Ac are considered as promising non-invasively accessible biomarkers of cumulative exposure to DMF in occupational biomonitoring.

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L-17

NANOPARTICLES – POTENTIAL ECOTOXICOLOGICAL THREAT FOR THE ENVIRONMENT

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Nanotechnologies and different kinds of nanomaterials belong nowadays to intensively investigated materials due to their unique properties and several potential applications as electronics, medicine or imaging methods. However, with the research of their possitive properties there is a growing demand for investigation and evaluation of their impact on the environment and possible harmful properties for organisms. This report summarizes possible toxic effects of developed nanomaterials on environment and living organisms. It deals with their possibility of bioaccumulation in plants or animals, ecotoxicity and mechanisms of toxicity. Main goal of this report is to highliht the potential environmental complications in case of nanomaterials use and the necessity of research about their toxic effects.

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L-18

MICROPLASTICS AS CONTAMINANTS - SCOPE, FATE, AND ENVIRONMENTAL IMPACTS

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Microplastics represent emerging pollutants of global importance. They are small enough to be ingested by a wide range of organisms and at nano-scale, they may cross some biological barriers. As well as microplastics, chemical additives added to plastics during manufacture which may leach out upon ingestion, can enter food chains and potentially cause humans serious health problems. In this article, we discuss the sources of plastic microparticles, summarize known informations about their behaviour at wastewater treatment plants and potential impacts of microplastics in the environment.

Supported by the grant scheme for supporting excellent teams of young researchers under the conditions of STU in Bratislava: „Mikroplasty a ich účinné odstránenie pomocou progresívnych postupov."

L-19

A COMPARISON OF THE REACTIVATING, THERAPEUTIC AND NEUROPROTECTIVE EFFICACY OF TWO NOVEL BISPYRIDINIUM OXIMES (K305, K307) WITH TRIMEDOXIME AND THE OXIME K203 IN RATS AND MICE POISONED WITH TABUN

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The ability of three original bispyridinium oximes (K305, K307, K203) and one currently available oxime (trimedoxime) to reactivate tabun-inhibited acetylcholinesterase and reduce acute toxicity of tabun including neurotoxic signs and symptoms was evaluated in tabun-poisoned rats and mice. The oxime-induced reactivation of tabun-inhibited acetylcholinesterase was measured in diaphragm and brain of tabun-poisoned rats. The results showed that the reactivating efficacy of two recently developed oximes (K305, K307) does not achieve the level of the reactivation of tabun-inhibited acetylcholinesterase induced by the oxime K203 and trimedoxime. Generally, the reactivating efficacy of all oximes studied is higher in diaphragm compared to the brain. The therapeutic efficacy of all oximes studied roughly corresponds to their reactivating efficacy. While both recently developed oximes were able to reduce acute toxicity of tabun less than 1.55 fold, another original oxime K203 and commonly used trimedoxime reduced the acute toxicity of tabun more than 1.6 fold. Thus, the differences between therapeutic efficacy of all oximes studied are not so high as in the case of reactivating efficacy. Only one newly developed oxime (K307) combined with atropine was able to markedly decrease tabun-induced neurotoxicity in the case of sublethal poisoning although it did not eliminate all tabun-induced acute neurotoxic signs and symptoms. Its ability to decrease tabun-induced acute neurotoxicity was only slightly lower compared to the oxime K203 and trimedoxime. On the other hand, the neuroprotective efficacy of the oxime K305 was negligible. In conclusion, the reactivating, therapeutic and neuroprotective efficacy of both newly developed oximes does not prevail the effectiveness of the oxime K203 and trimedoxime and, therefore, they are not suitable for their replacement of commonly used oximes for the antidotal treatment of acute tabun poisoning.

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L-20

THE MONOQUARTERNARY REACTIVATORS FOR THE TREATMENT OF ORGANOPHOSHOROUS INTOXICATION

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Mono- and bis-pyridinium aldoximes are the only causal antidotes that are designated for the treatment of organophosphate (OP) poisoning. Intoxication by OPs is caused either by pesticides or by the nerve agents, the latter belong to group of chemical warfare agents. These compounds irreversibly inhibit enzyme acetylcholinesterase (AChE) that is no more able to fulfill its physiological function. Mono- and bis-pyridinium aldoximes are able to restore catalytic function of AChE. The

reactivating ability of aldoximes is limited by several drawbacks like low blood-brain barrier permeation, low reactivation potency against specific nerve agents etc. In order to obtain efficient treatment of OP, the introduction of novel AChE reactivators raised as an important issue. For over 60 years of intensive research, none of the reactivators reached sufficient activity. Herein, we present novel mono quaternary reactivators that possess excellent *in vitro* activity to restore AChE activity after intoxication with different nerve agents as well as pesticides. The molecular docking simulations, total synthesis and biological evaluation will be discussed.

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L-21

OXIME ADMINISTRATION AT 100% MTD OR 5% LD_{50} DOSES- THEIR THERAPEUTIC EFFICACY IN SARIN INTOXICATION TREATMENT

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The poisoning with nerve agent (e.g. sarin, tabun, VX) represents a life threating danger. Compounds with oxime moiety attached to a quaternary nitrogen pyridinium ring are able to reactivate acetylcholinesterase (AChE; 3.1.1.7) inhibited by organophosphorus agents. However, there are some evidence, other mechanisms not related with reactivation may lead to survival.

The main aim of this work was a comparison of efficacy two different doses (100% maximal tolerated dose-MTD and 5% lethal dose-LD_{50}) of obidoxime and HI-6 after sarin intoxication. 100% MTD is higher than standardly used 5% LD_{50}. Thus, the higher concentration of oxime in the central nervous system and their direct effect on cholinergic receptors were assumed. Accordingly, behavior mice Balb/C and their symptoms of sarin intoxication were observed. *In vivo* determination of reactivation in peripheral (blood) and central (brain) compartment showed that administration of 100% MTD does not provide significant benefit.

This work was supported by the Long-term organization development plan Medical Aspects of Weapons of Mass Destruction of the Faculty of Military Health Sciences, University of Defence.

L-22

DEVELOPMENT OF NOVEL DISINFECTANTS AGAINST PATHOGENS BASED ON QUATERNARY AMMONIUM SALTS

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In this project, we tried to develop new compounds or mixture of compounds based on quaternary ammonium salts (QAS) with a strong disinfectant potential against bacterial, fungal and viral pathogens which ma occur in the hospital environment. We have prepared, approximately 60 analogues of quaternary ammonium salts (QAS) containing quaternary nitrogen and long carbon chain. It is assumed that their effect is based on ability to interfere with the stability and functionality of microbial cell membranes of a wide range of infectious agents, which is ideal for topical use as disinfectants. According to the literature and our preliminary data, QAS were found to be effective against both bacteria and fungi. We observed that the most promising anti-G+ and G-bacteria agents were the series 31, 32, 27, 20 and 18. Against anaerobic bacteria the highest efficacy showed series 33 and 32. Although efficacy on viruses in quaternary ammonium salts is relatively rare, one of 32 series reduces the virus titer by 5 orders of magnitude after 5-minute virus exposure. A little less effective was series 28 and 33. Highest efficacy against yeasts was observed for series 32, 18, 16 and 32, against fibrous fungi then for series 18 and 16. Supplemental efficacy against green algae was very high for series 32, 27, 18 16 and 27. On the basis of the identified efficacy, combinations of substances were proposed to cover the entire spectrum of pathogens. These mixtures have been formulated and their efficacy against bacteria, viruses and fungi was confirmed. Furthermore, less skin irritability has been observed for novel mixtures in comparison to standard Ajatin at 0.1%.

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L-23

DECONTAMINATION OF WARFARE AGENTS

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Decontamination of chemical, biological and radiological agents is one of the basic measures to prevent poisoning or infection of affected persons. CBRN agents can be misused during war conflict (e. g. Gulf War, Syrian Civil War) or for terrorist reasons. The need for new combined decontamination and disinfecting agent results both from the obsolescence of existing solutions and from the requirements for improved protection of soldiers and civilians. The armed forces of advanced countries are usually equipped with primary sorption agents such as IPB-80. The sorbent is commonly inorganic material or modified activated carbon (charcoal). Relatively rare are the sorbents combined with reactive agents. In addition, various types of decontamination solutions are used - from purely aqueous solutions through water/organic solvent mixtures to completely non-aqueous solutions. Active decontamination agents are most often based on nucleophilic reagents such as alcoholates, phenolates, organic or inorganic peroxides, oximes and complex metal compounds. Liquid agents introduced into military equipment in the last decades

are characterized by the use of highly viscous polyethylene glycol-type solvents (Canadian RSDL or Russian IPP-11). At present, the Czech Army is equipped for personal decontamination by the IPB-80 anti-chemical package, which contains activated montmorillonite and is based on the sorption principle. Although this package device has a high exploitation value and low production cost, its weaknesses are known. It has limited use in the interior of combat vehicles, sporadic efficiency in decontamination of worn equipment and open wounds. Decontamination system, based on solutions of chemically reactive (active) agents can circumvent these handicaps. Furthermore, they combine antimicrobial efficacy with hydrolytic activity and by that can be used as polyvalent agents against both biological and chemical warfare agents.

We are focused on the development of novel quaternary ammonium compounds as a part of decontamination means. Quaternary cationic surfactants are compounds that are widely used in many industries. This large group of structurally different chemicals provides a number of interesting features. The most commonly used group are benzalkonium salts (*N*-alkyl, *N*-benzyl and *N*,*N*-dimethylamines, where the alkyl is most often in between C_{10}-C_{18}). We have prepared many of novel compounds that will be tested soon.

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L-24

HYDROCORTISONE REDUCES VESICATION BY MECHLORETHAMINE IN VIVO

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Extravasation reactions (EVRs) occur in approximately 6% of cancer patients receiving intravenous chemotherapy, with dermatotoxic effects ranging from tissue swelling and localized inflammation, to blistering (vesication) and ulcer formation. Current treatments aimed at reducing undesired EVRs remain grossly inadequate. Our long-term goal is to decipher the molecular mechanisms that regulate vesication and cutaneous inflammatory responses to cytotoxic chemotherapy drugs such as mechlorethamine (HN2), and to identify novel medical interventions to reduce EVRs. The purpose of the present study was to determine the vesicant countermeasure potential of hydrocortisone (HC). To this end, the mouse ear vesicant model (MEVM) was used, with male Swiss Webster mice serving as the test strain. Compared to control ears, mouse ears exposed to a single dose of HN2 (0.500 µmol/ear) showed an increase in wet weights, ear thickness, edema, hyperplasia, vesication and inflammatory cell infiltration after 24 h. Tissue expression of inducible nitric oxide synthase (iNOS) and matrix metalloproteinase-9 (MMP-9) were upregulated in response to HN2. Fluorescence microscopy of TUNEL stained sections showed that the occurrence of apoptosis extended from the epidermis of the HN2 treated side all the way to the contralateral epidermis. In contrast, HN2 exposed ears treated topically with HC at a test dose of 0.031 mg/ear showed a significant decrease in wet weight (12.6% less than HN2 alone), morphometric thickness (16.5% less than HN2 alone) and vesication (60.0% for HN2 reduced to 33.3% after HC). Taken together, our studies suggest that lose-dose HC may serve as an effective countermeasure to chemotherapy EVRs.

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L-25

SEARCHING FOR NEW OPPORTUNITIES IN THE TREATMENT OF DEPRESSION AND ANXIETY: PRECLINICAL EVALUATION OF COMPOUNDS WITH PYRIDOINDOLE STRUCTURE

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DAnxiety and depression represent a serious mental health problem with globally increasing prevalence. Although the etiology of depression and anxiety is not yet completely understood, it is well established that serotonin (5-HT), norepinephrine (NE), and dopamine (DA) systems play a key role in their treatment.
Using *in silico* analysis of molecular structures, the pyridoindole derivatives (PDs) with code number 144, 143 and 104 were designed to test their potential antidepressant (PD144) and anxiolytic (PD143, PD104) properties. All PDs properties were tested in Wistar rats, subjected to behavioral assessment after acute administration (open field – OF, elevated plus maze – EPM, light/dark test – L/D, stress-induced hyperthermia – SIH and forced swim test – FST). In addition, in selected brain regions HPLC-ED analysis of monoamines levels, Fos-immunostaining and *in vivo* electrophysiology were evaluated.

PD144 caused significant changes of SIH and distance travelled in OF as well as decreased immobility time in FST. 5-HT and NA levels were increased in the hippocampus, whereas HVA/DA turnover was increased in the prefrontal cortex and the striatum.
Anxiolytic properties of PD143 were observed in all tests designed to identify the anxiety-like behavior, whereas PD104 showed significant changes only in the OF. Both, PD143 and PD104, decreased DOPAC/DA as

well as 5HIAA/5HT turnover in the hypothalamus and the amygdala. In addition, PD104 induced an increase in 5-HT level in the hippocampus and the hypothalamus. All tested substances following an acute administration increased the number of immunoreactive cells displaying c-Fos expression in the central nucleus of the amygdala and the hypothalamic paraventricular nucleus. Both of these structures are involved in the pathogenesis of depression and anxiety.

Electrophysiological investigations showed that PD144 dose-dependently suppressed the excitability of 5-HT neurons of the dorsal raphe nucleus, NE neurons of the locus coeruleus, and DA neurons of the ventral tegmental area. These results associate with the mechanism of action of the triple reuptake inhibitors.
In conclusion, our results represent a unique approach for preclinical evaluation of the tested compounds. We were able to identify anxiolytic and antidepressant properties, whereas the best results were achieved by PD144.

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L-26

MODULATING THE EFFECT OF PRENYLATED PHENOLIC COMPOUNDS ON PANCREATIC INS-1E CELLS

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Phenolic compounds are of considerable biomedical interest due to their antioxidant properties and potential in prevention and possibly treatment of many chronic diseases. The fruits, leaves and root bark of Morus nigra (Moraceae), the black mulberry tree, have a long history of use for various therapeutic purposes in traditional medicine worldwide. The roots of the plant are known to be a rich source of phenolic compounds with a particularly high chemical diversity [1]. Seven prenylated phenolic agents isolated from Morus nigra were tested for SERCA activity in non-cellular system, and in pancreatic ß cells for viability and apoptosis. Albanol A and B, kuwanon E and U and morusin induced reduction of SERCA activity in non-cellular system which correlated with decrease of cell viability and initiation of apoptosis in pancreatic β cells. These properties may be useful in cancer treatment. On the other hand, moracin P and R only slightly decreased SERCA activity, increased cell viability and did not induce apoptosis, suggesting that they might have a potential use against cardiovascular diseases and/or diabetes.

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[1] Zoofishan, Z., Hohmann, J. & Hunyadi, A. Phytochem Rev (2018). https://doi.org/10.1007/s11101-018-9565-1

L-27

IMPACT OF PRE-GESTATIONAL STRESS ON OFFSPRING NEUROBEHAVIORAL DEVELOPMENT AND HIPPOCAMPAL FUNCTIONING

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Depression during pregnancy or even prior to gestation can negatively affect offspring´s neurobehavioral development. Several studies have shown, that offspring who had experienced excessive stress during gestation had higher rates of cognitive, social and mood disorders later during adolescence or even in adulthood. Hippocampal neurons play a crucial role in the regulation of behavior, mainly anxiety-related behaviors and spatial learning and memory. Excessive stress could interfere with sensitive developmental processes in the brain and may affect hippocampal functioning with severe neurobehavioral consequences in later life. The aim of this work was to investigate the effects of pre-gestational stress on emotional and cognitive behaviors of the offspring. We also investigated primary neuronal cultures prepared from hippocampi of the newborn offsprings of the stressed dams to investigate hippocampal excitability. Moreover, we studied neurogenesis in the dentate gyrus of hippocampus in adolescent and adult offspring. Our results have shown, that pregestational chronic unpredictable stress affected selected reproductive and neurobehavioral variables of the rat offspring. Offspring exposed to maternal stress prior to conception showed altered behavior in a new and anxiogenic environment, spatial memory deficit and reduced neurogenesis in the dentate gyrus of hippocampus. Pre-gestational stress also affected both spontaneous and depolarization-activated action potential firing of hippocampal neurons. This work suggests, that maternal stress even prior to gestation can interfere with functional brain development of the offspring and can cause long-term behavioral changes at the level of neurobehavioral adaptations.

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L-28

ANALYSIS OF ECG RECORDED FROM RATS PRENATALLY EXPOSED TO STRESS AND VENLAFAXINE

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The prevalence of gestational depression currently oscillates between 10–18% among pregnant women all over the world. The most frequently prescribed antidepressants are selective serotonin reuptake inhibitors (SSRI) and selective serotonin and noradrenaline reuptake inhibitors (SNRI), including venlafaxine. Venlafaxine is not considered as a human teratogen. However, it has been documented, that prenatal exposure to venlafaxine, could possibly lead to congenital malformations, especially heart defects. On the other hand, untreated depression also represents a great risk for both mother and her child. The aim of this study was to focus on the effect of the maternal stress as a model of depression and treatment with/without venlafaxine (10 mg/kg/day, p. o.) on the electrical activity of the heart of both male and female rat offspring. The ECG was recorded from anesthetized rats. In males, prenatal exposure to stress led to significant decrease in P wave amplitude, but on the other hand, P wave and PQ interval prolongation, compared to control. Prenatal exposure to venlafaxine caused significant increase in duration of P wave and QRS complex. Venlafaxine showed tendency to potentiate the effect of stress, which resulted in significant prolongation of P wave, PQ interval. Compared to the control group, we observed no significant changes in QT or QTc interval duration in any of male groups, however QRS complex duration was prolonged. Female offspring prenatally exposed either to stress or venlafaxine did not display significant changes of selected ECG parameters. Prenatal administration of venlafaxine combined with prenatal exposure to stress led to significant increase in P wave amplitude, but decrease in duration of P wave and PQ interval. Moreover, QRS complex shortening and QT interval prolongation was present in this group, compared to control. In conclusion, we observed differences between males and females in reactions to prenatal exposure to stress or venlafaxine, but it is important to remark that both factors, when present during pregnancy, may affect the electrical activity of the heart of offspring.

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L-29

PROPOSED *IN SILICO* APPROACHES FOR MIXTURES

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Various safety assessment approaches are used for botanicals varying from reliance on post market surveillance of adverse events to tiered strategies. As the first tier in a newly proposed decision tree approach for the risk analysis for botanicals, the literature is searched for the available safety data on the botanical and its primary constituents. Data gaps exist for most botanicals, in particular, individual chemical constituents and repeat exposure information and vulnerable subpopulations (children, pregnant women, elderly). This lack of data drives one to a second tier in which the plant's compounds are assessed individually, including extraction methods. These chemicals need to be identified and evaluated for toxicity. Tools are constantly being developed that identify what often are called 'compounds of concern' or compounds with known biological activity. Additionally, harvesting methods and seasons, plant parts, and extraction methods all lead to different extracts which in turn may lead to different chemicals of concern. This approach together with better chemical characterization allows for more precise identification of these compounds. In the third step, these identified compounds undergo individual and specific risk assessment. Key elements for evaluating the safety of botanicals include better information on history of use, systematic assessment of weight of evidence, use of *in silico* approaches, inclusion of threshold of toxicological concern considerations, and adoption of *in vitro* and *in vivo* physiological based pharmacokinetic modeling.

L-30

ZEBRAFISH AS A MODEL FOR IDENTIFICATION OF TOXIC MECHANISMS WITH SINGLE CHEMICALS AND MIXTURES

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The zebrafish, *Danio rerio*, is an increasingly used model organism in biomedical research, high-throughput pharmacological screenings and toxicology due to several reasons: genetic tools are available, its transparent larval stages are amenable for imaging techniques, high fecundity and fast development allow investigating a large number of animals without long waiting periods and their small body size allows for the continuous measurement of behavior with full control of the environment. Moreover, major brain structures and physiological processes are conserved among phyla, allowing basic findings to be translated to other vertebrates including humans. Toxicity tests with zebrafish embryos and early larval stages have been gaining more attention through the standardization of testing methods in an OECD guideline (zFET OECD 236). The rapid extrauterine development of this vertebrate model enables the convenient assessment of numerous sub-lethal and lethal endpoints. Moreover, since larval stages up to 120 hours post fertilization, i.e. upon feeding independently, are not protected by law, they are considered alternatives to animal testing. Next to dissecting detailed toxic mechanisms with single chemicals and mixtures using the toolbox available, larval stages are amenable to high-throughput automatic devices which allow exposing the animals to a wide range of defined chemical mixtures. While zebrafish is an excellent model organism for the mechanistic dissection of complex processes, there are certain limitations with respect to exposure routes and application of chemicals through the medium. However, because of its numerous advantages the zebrafish can be used as a model to study both human and environmental health.

L-31

ADVANCED *IN VITRO* APPROACHES FOR COMPARATIVE ASSESSMENT OF HEATED TOBACCO PRODUCTS

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This presentation highlights the successful integration of omics approaches with cellular-based assays for the comparative assessment of a candidate modified-tobacco product, the Tobacco Heating System 2.2 (THS2.2) *in vitro*. Advanced human air-liquid interface cellular models of the aerodigestive tract (buccal, bronchial, and nasal organotypic cultures) were exposed to THS2.2 aerosol or cigarette smoke (CS) at similar nicotine concentrations. Less cytotoxicity and lower concentrations of secreted inflammatory mediators were observed in all cultures following exposure to THS2.2 aerosol compared with those following exposure to CS at comparable doses of nicotine. Global gene expression profiling and computational network enrichment analysis demonstrated mechanistic changes following exposure to CS including the impact on xenobiotic metabolism response, oxidative stress, and inflammatory response; at comparable nicotine concentrations, THS2.2 aerosol exposure elicited reduced and more transient effects on these processes. The identification of mechanisms by which potential modified risk tobacco products can reduce the impact on biological systems compared to cigarette smoke exposure is of great importance in understanding the molecular basis of the harm reduction paradigm. The presented systems toxicology approach is a robust methodology enabling detection of not only subtle exposure effects, but also identification of relevant biological mechanisms across tissues and exposure conditions.

L-32

EXTRAPOLATING NEW APPROACHES INTO A TIERED APPROACH FOR MIXTURE RISK ASSESSMENT

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Current risk assessments of chemicals do not generally consider exposure to multiple substances but rely instead on the assessment of individual substances in individual foods and other commodities. This is consistent with approaches to the assessment of chemical mixtures in most environmental media, such as soil, water and air. In reality, humans are routinely exposed simultaneously to numerous chemicals during normal daily activities. These mixtures can be variable and constantly changing and defining them presents a challenge. Several approaches to this problem have been proposed, including the use of high through-put assays or Tox21 approaches. In addition, structure activity relationships, read-across procedures and the Threshold for Toxicological Concern (TTC) concept can often shed light on the toxicity of otherwise obscure contaminants. *In vivo* models such as the nematode, *C. elegans,* or the zebrafish, *Danio rerio,* assays are rapid and inexpensive, and might be used to facilitate mixture assessment, either independently or in an integrated manner to assess health impacts. Exposure to chemical mixtures is the rule, not the exception. Although guidelines exist to sort our way though this sticky wicket, improvements are always welcome and, indeed, needed. Fortunately, new methods offer a significant improvement in the hazard assessment of chemical mixtures, and with the appropriate exposure determination, should continue to promote the credible protection of public health and the environment.

EFFECT OF HOP-DERIVED PRENYLFLAVONOIDS ON EFFICIENCY OF SELECTED CYTOSTATICS *IN VITRO*

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Prenylflavonoids, a unique class of naturally occurring flavonoids, are secondary metabolites of plants. They are formed by attaching of prenyl group to the flavonoid backbone. Various biological activities of prenylflavonoids have been described, including anti-cancer, antibacterial, anti-inflammatory, osteogenic, estrogen-like and antioxidant activity. Prenylflavonoids possess also inhibitory effect on a number of enzymes. Their biological activity and presence in food make interaction, both positive and negative, with concurrently used therapy possible. In the present study, we explored whether prenylflavonoids xanthohumol, isoxanthohumol, 6-prenylnaringenin and 8-prenylnaringenin could enhance the antitumor effect of classical cytostatics 5-fluorouracil, irinotecan and oxaliplatin in two colorectal carcinoma cell lines *in vitro*. Effect of the prenylflavonoids was compared to that of naringenin, their structural analogue lacking prenyl group. Two isogenic human cell lines, one established from a colorectal adenocarcinoma (SW480) and the other from its lymph node metastasis (SW620), were used. Effect of studied compounds on the cancer cell viability, activity of caspases and reactive oxygen species (ROS) formation was evaluated. Cell viability was monitored by neutral red uptake test, activity of caspases was determined by Caspase glo assay kit, and ROS formation was assessed using dichlorofluorescein assay. Combinations of prenylflavonoids and naringenin with individual cytostatics were set according to the recommendation of Chou and the type of interaction was evaluated by CalcuSyn software. IC_{50} of prenylflavonoids were determined below 40 μM. Most of the prenylflavonoids exerted antagonistic effect with classical cytostatics, except for the 8-prenylnaringenin, which showed synergistic effects with 5-fluorouracil and irinotecan. The 8-prenylnaringenin was also the only prenylflavonoid, which was able to significantly increase activity of caspases 3, 7, 8, and 9. Described *in vitro* interactions indicate possibility of side effects during chemotherapy. This should be verified by *in vivo* experiments.

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LEVELS OF SELECTED CONTAMINANTS IN FISH MUSCLE FROM UPPER NITRA RIVER

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The purpose of this study was to determine the levels of Zn, Cu, Ni, Cr, Pb, Cd and Hg in the muscle of three common fish species – brown trout (Salmo trutta m. fario), Alpine bullhead (Cottus poecilopus) and grayling (Thymallus thymallus), which inhabiting the upper course of the Nitra River. The concentrations of analysed metals ($mg.kg^{-1}$ wet weight) ranged as follows: brown trout – Zn 5.86–12.97, Cu 0.51–0.76, Ni 0.00–0.37, Cr 0.18–0.41, Pb 0.00–0.34, Cd 0.03–0.13, Hg 0.04–0.07; Alpine bullhead – Zn 7.02–13.68, Cu 0.34–0.62, Ni 0.00–1.13, Cr 0.19–0.24, Pb 0.00–0.37, Cd 0.03–0.09, Hg 0.06–0.18; grayling – Zn 3.38–6.36, Cu – 0.46–0.62, Ni 0.04–0.22, Cr 0.13–0.22, Pb 0.00–0.25, Cd 0.02–0.09, Hg 0.05–0.12, respectively. Statistically significant differences among individual fish species have been recorded. For individual fish species the statistically significant correlations between analysed metals and standard length, weight and age were detected. Permissible limits for safe consumption in the case of Pb, Cd and Hg were exceeded in 10%, 63% and 0%, respectively.

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THE EFFECT OF ANTHOCYANIN RICH WHEAT ON RAT CYTOCHROMES P450

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Wheat is one of the most important agricultural crops worldwide. The nutritional quality of wheat grain may be improved by the use of colored varieties with the presence of polyphenols, particularly anthocyanins. Anthocyanins, a subclass of flavonoids, are beneficial for human and animal health being e.g. mild antioxidants. Anthocyanins exhibit anti-inflammatory and anti-carcinogenic effect, prevent cardiovascular disease, control obesity and mitigate diabetes properties. Because of the possibility of metabolic interaction of anthocyanins with other foreign compounds, we decided to determine whether the consumption of wheat enriched with anthocyanins does not affect the activity of phase I biotransformation enzymes, namely cytochromes P450 (CYP) in the liver.

Rats were fed by Novosibirskaya wheat (control), ANK-28A, ANK-28B (red grain colour) and Aoi-Yu (blue grain colour) and had an access to tap water *ad libitum*. After 75 days, rats were anesthetized and exsanguinated. Livers were taken for further analyses. Microsomal fractions were prepared (pooled from each cage) and used for the study of CYP activity. Activities of rat CYPs (CYP1A, CYP2E1, CYP3A, CYP2D, CYP2C, CYP2B) were measured using their specific substrates [1].

Experiments have shown that eating foods enriched with anthocyanins does not result in clinically significant changes of CYP activity. On the contrary, a slight inhibition of some enzymes, e.g. CYP2E1 by anthocyanins, may have protective effect on the organism, as inhibition of CYP2E1 may be reflected in decrease of ROS formation.

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[1] Philips IR, Shephard EA. Cytochrome P450 Protocols (second edition), Methods in Molecular Biology 2006, **320**.

SEX AND AGE SPECIFIC EFFECTS OF MATERNAL DEPRESSION AND VENLAFAXINE TREATMENT ON ANXIETY-LIKE BEHAVIOR AND HIPPOCAMPAL NEUROGENESIS OF RAT OFFSPRING

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The prevalence of mental stress-related disorders, such as depressive and anxiety disorders, is rising, particularly in developed countries. Women are twice as vulnerable as men to experience a major depressive disorder during their lifetime. The character of early development in the uterus, and perhaps earlier, creates conditions for effects of risk factors for the physical and mental health of offspring. Risk factors include maternal depression as well as antidepressants that pass through the placenta, haematoencephalic barrier and also into the breast milk. Thus, gynecologists and obstetricians have a dilemma whether to treat or not to treat depression during pregnancy. At present, more findings suggest that risk of antidepressant medication during pregnancy and lactation is lower compared to risks of untreated depression. However, the effects of prenatal and early postnatal treatment with antidepressants on late postnatal development are poorly understood. Venlafaxine, the serotonin and noradrenaline reuptake inhibitor, is a new line of antidepressant therapy in pregnancy. However, the effects of venlafaxine treatment in gestation and lactation on the fetal and neonatal development are not fully known.

The aim of the present work was to study effects of maternal depression (chronic unpredictable stress) and venlafaxine (10 mg/kg/day from day 15 of gestation to day 21 *post partum*) during gestation and lactation on behavioral variables with focus on anxiety-like behavior and hippocampal neurogenesis of rat offspring in various postnatal developmental stages.

The results of our studies showed that maternal depression had anxiolytic effect on behavior in adolescent but anxiogenic effect in adulthood, exclusively in males. Maternal depression reduced neurogenesis in the dentate gyrus of hippocampus in males. Venlafaxine treatment affected only anxiety-like behavior in adolescent females.

The results of our studies suggest that the risks of untreated maternal depression may outweigh the risks of antidepressant treatment. The negative effects of untreated maternal depression can not occur immediately after birth, but under challenging conditions in adulthood.

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NITRIC OXIDE-DEPENDENT AND INDEPENDENT MECHANISMS OF EPICATECHIN-INDUCED VASORELAXATION

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(–)-Epicatechin (Epi) is a major bioactive cocoa flavanol, which is absorbed well in the gastrointestinal tracts of both humans and rodents. This study elucidated the mechanisms underlying vasodilatory effects induced by chronic and acute Epi treatment in the femoral arteries (FA) of adult spontaneously hypertensive rats (SHR).

We determined mechanisms associated with depressor effect of Epi in SHR after 10-day Epi treatment (250 mg/kg/day) and underlying mechanism after the acute Epi administration (up to 10 mmol/l) in the isolated FA using the wire myograph. Chronic Epi-treatment led to significant blood pressure reduction, elevated nitric oxide (NO) production and increase of NO-dependent component of acetylcholine-induced vasorelaxation without the alterations in the vascular smooth muscle cell function. Acutely administered Epi fully relaxed the FA pre-contracted with high-potassium salt solution (KPSS) or serotonin. However, NO synthase and soluble guanylate cyclase inhibitors (L-NMMA, ODQ) or estrogen receptor antagonists (fulvestrant, G15) failed to attenuate the relaxant effect of Epi. In contrast, Epi fully relaxed KPSS pre-contracted FA similarly as did nifedipine (specific L-VDCC inhibitor). Moreover, pre-treatment with Epi or nifedipine substantially inhibited the KPSS-induced contractions.

In conclusion, the results showed different vascular mechanisms of Epi when administered chronically *per os* or acutely on isolated arteries. Chronic Epi-treatment was associated with improved vascular NO bioavailability and NO-dependent mechanisms of vasorelaxation. In contrast, *in vitro* studies failed to confirm direct NO-dependent mechanism of Epi action, which was associated with a blockade of calcium entry through L-VDCC located on the vascular smooth muscle cells.

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INFLUENCE OF HUMULENE, B-CARYOPHYLLENE AND CARYOPHYLLENE OXIDE ON THE MRNA AND PROTEIN EXPRESSION OF PHASE I XENOBIOTIC-METABOLIZING ENZYMES IN PRECISION-CUT HUMAN LIVER SLICES

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Sesquiterpenes α-humulene (HUM), β-caryophyllene (CAR) and caryophyllene oxide (CAO) are important constituents of *Humulus lupulus* (hops) and *Syzygium aromaticum* (clove) essential oils. They are often present in various folk medicines and dietary supplements. Numerous biological activities, including anticancer, anti-inflammatory and pro-apoptotic, of these sesquiterpenes have been reported. Moreover, their inhibitory effect on the activity of several phase I xenobiotic-metabolizing enzymes in human and rat subcellular fractions have been described. Therefore, the aim of this study was to evaluate their influence of selected phase I drug-metabolizing enzymes in precision-cut human liver slices. In our experiments, five human liver samples received from surgery were used to obtain precision-cut liver slices, which were cultivated for 24 hours in presence of HUM, CAO and CAR in the 10 μM concentration. The mRNA expression of selected isoforms of cytochrome P450 (CYP 3A4, 2B6, 2C), carbonyl reductase 1 (CBR1) and aldo-keto reductase 1C (AKR1C) were detected using real-time quantitative PCR and protein levels of these enzymes was detected using immunoblotting. Studied compounds slightly altered the level of mRNA as well as protein of CYP3A4, CYP2B6 and CYP2C. HUM caused significant inhibition in CBR1 and AKR1C mRNAs in one liver sample. In addition, protein expression of CBR1 was remarkably inhibited by HUM in all liver samples, while that of AKR1C3 was markedly increased in three samples. The observed effects of HUM, CAR and CAO were inconsistent, and they reflected rather inter-individual variability among liver donors.

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BIRT TRAUMA – STILL A CURRENT PROBLEM IN NEONATOLOGY

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As the birth trauma is refer foetal and neonatal injury during labour caused by mechanical forces. We may notice it during the first investigation of the newborn, or it may also be a hidden injury that will occurs during hours or days. The worst complication of the birth injury is asphyxia. Risk factors are divided into maternal, foetal and joined with delivery.

Retrospective analysis of the causes of birth trauma in newborns admitted to Neonatal Department of Intensive Medicine (NDIM). 123 newborns were analysed retrospectively during January 1st, 2015 – December 31st, 2016. The analysis was focused on the presence of risk factors (gestational age, birth length and weight; maternal risk factors – primipara, hypertension, infection, preeclampsia, diabetes mellitus, cefalopelvic disproportion, drug abuse, not cooperated mother during delivery; foetal risk factors – macrosomia; form of delivery – instrumental delivery, the necessity of using external expression, prolonged delivery, pelvic position of the newborn) and form of the birth trauma.
From 123 newborns (36.58% premature newborns; 63.42% term newborns) 68.30% were born spontaneously and 31.70% by caesarean section (in two cases in pelvic position and in 6 cases delivery was complicated by dystocia of arms). Primipara was the most frequent risk factor (63.41%). Macrosomia was confirmed in 12.20% (n=15) of cases of prenatally hypertrophic newborns. In less then 5% – dislocation of the nasal cartilaginous portion of the septum, bleeding into the adrenal glands, suffusion, subarachnoid bleeding, subdural bleeding, incision, fracture of femur, fracture of humerus, torticollis, torsion of testes, lesion of brachial plexus, fracture of clavicle; ≥5–10% – haemorrhage into retina, oedema, haemorrhage; ≥10–20% – caput succedaneum, cefalhaematoma, haematoma of soft tissue and ≥20% – haematoma and incisions different forms of birth trauma were presented.
Despite the increasing level of perinatal care, we are still experiencing traumatic neonatal injury. Birth injuries of the newborn may affect the newborn's quality of life both in the short and the long follow up. Some forms of postpartum trauma leave permanent consequences. Through monitoring of the foetus during pregnancy and delivery and early recognition of possible complications contribute to a reduction in the incidence of postnatal trauma as well as of severe asphyxia.

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EFFECT OF BISPHENOLS ON SELECTED NUCLEAR RECEPTOR EXPRESSION IN HUMAN GRANULOSA CELL LINE COV434

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Bisphenols are chemical plasticizers and monomeric constituents of polycarbonate plastics and epoxy resins. Bisphenol A (BPA) is a well-known endocrine disrupting compound, capable of affecting the normal function and development of the reproductive system, breast, adipose tissue, etc. Recently, other bisphenols have been introduced as replacements for BPA usage in many applications but the data show that they might also exert various adverse biological effects. In spite of the diverse identified bisphenol effects, little is known about the

molecular mechanisms of their action but experimental data show that BPA binds with a significant number of different receptors. Members of the nuclear receptor superfamily play roles in numerous physiological and patophysiological processes, including reproduction, metabolism, and in the genesis and progression of cancer.

In the present study, we used the human ovarian granulosa cell line COV434 to investigate whether bisphenols BPA and BPS are able to influence the viability of the cells and the expression of the selected nuclear receptors related to reproductive processes (*LRH-1, NURR1, RORA, TRA*). The agents were tested in several concentrations (1 nM, 100 nM, 10 μM) and the cells were treated for 48 h. The results indicate that bisphenols might afflict cellular processes via their influence on nuclear receptor ecosystem, and further detailed studies are needed for elucidating the consequent effects.

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THE EFFECT OF SILVER NANOPARTICLES AND IONS ON ZEBRAFISH EMBRYOS (*DANIO RERIO*)

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In present days, significant advancement in the field of nanotechnologies is noted. Silver nanoparticles are used in many different ways, for example in medicine, agriculture, construction and production of variety of consumer goods, such as electronics and textiles. It is due to their characteristics, such as antibacterial, antivirotic and antimycotic activity. With growing production and usage, a rise of concentrations of silver nanoparticles in the environment is expected. There is a concern, that silver nanoparticles in the ecosystem might have a negative impact on non-target species, such as fish. Therefore, we conducted fish embryo toxicity tests according to the modified OECD 236 guideline, using two different species of silver nanoparticles and silver nitrate, as a source of silver ions. 96hLC_{50} values were recorded, showing great differences in toxicological effects of tested substances. 96hLC_{50} for silver nitrate was 58.44 μg/l. 96hLC50 calculated for silver nanoparticles stabilized with maltose and gelatine was nearly 100 times higher – 4.31 mg/l. 96hLC50 for silver nanoparticles stabilized with polyvinylpyrrolidone exceeded 100 mg/l. Other sublethal effects, such as presence of oedemas and deformations, bradycardia, lags in development and delayed hatching, were observed as well.

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UNPREDICTABLE CHRONIC MILD STRESS AS AN ANIMAL MODEL OF DEPRESSION

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Depression is characterized by affective, cognitive and behavioral impairments and it affects approximately 20% of pregnant and lactating women. Untreated depression during gestation represents a higher risk for maternal morbidity, including arterial hypertension leading to preeclampsia or eclampsia, suicide attempts and *post-partum* depression. Most of the experimental models resemble the dysfunctions observed in human depression, such as significant weight loss, increased anxiety, as well as anhedonia, although, hallmarks of the disorder such as depressed mood, low self-esteem or suicidality are hardly accessible in non-humans. Therefore, the need for well-controlled animal studies dealing with consequences of pre- and perinatal effects of stress is crucial. For experimental purposes there is a need for reliable and reproducible tests to measure behavioral phenotypes in animals, such as depression and anxiety–like behavior, anhedonia and behavioral despair. The objective of the study was to induce depression status in pregnant and lactating female rats.

Female Wistar rats were subjected to two-week chronic unpredictable stress induced by random stressors. Moreover, they were treated with SNRI antidepressant venlafaxine orally at a dose of 5 mg/kg twice a day from day 15 of gestation to day 21 *post-partum*. Mothers were tested in the open field eight weeks after chronic unpredictable stress procedure in a single 15-min session as well as in sucrose preference test to evaluate anxiety status. Subsequently, hippocampal neurogenesis was investigated by immunohistochemistry essay using DCX staining. Results of the present study showed that two-week chronic unpredictable stress altered behavior of the dams demonstrating elevated anxiety-like behavior and lead to a lower litter numbers. Stressed dams had lower hippocampal neurogenesis, while venlafaxine treatment reversed this lowering.

Our results suggest that stress and antidepressant therapy can have significant impact on behavior and hippocampal neurogenesis in rat dams and that unpredicted mild chronic stress represents reliable animal model of depression.

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ASSESSING THE TIME DEPENDENT DERMATOXICITY OF MECHLORETHAMINE USING THE MOUSE EAR VESICANT MODEL

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Mechlorethamine (HN2) is an alkylating agent and sulfur mustard mimetic which is also used in anticancer therapy. Dermal exposure of HN2 is associated with extravasation and tissue blistering reactions that can lead to secondary infections in treated patients, thereby limiting the clinical use of this chemotherapeutic agent. The purpose of the present study was to investigate the time dependent dermatotoxicity of HN2 using the mouse ear vesicant model (MEVM). To this end, our operational definition of dermatotoxicity included tissue responses to HN2 consistent with an increase in the wet weights of mouse ear punch biopsies, an increase in the morphometric thickness of H&E stained ear sections and an elevation in histopathological scoring values for tissue edema, hyperplasia, inflammatory cell infiltration and vesication. The ears of male Swiss Webster mice were exposed to a single dose of HN2 (0.500 µmol/ear) or DMSO and the mice were then euthanized at 15 min or 1, 2, 4, 8, 12 and 24 hr following HN2 exposure. Mouse ears exposed to HN2 at all time points showed an increase in wet weight, morphometric thickness, edema, inflammatory cell infiltration and signs of vesication. The incidence in tissue vesication sharply increased between 4 and 8 hr after exposure, revealing that tissue vesication is well established by 8 hr and remains elevated at 12 and 24 hr after exposure. It is worthy to note that ears treated with DMSO vehicle also exhibited an increase in wet weight and morphometric thickness at 15 min, 1, 2 and 4 hr following treatment; however, these vehicle effects eventually subsided by 8 hr. The majority of published studies using the MEVM have examined tissue responses at 24 or 48 hr after vesicant exposure. The results of the present study provide a more holistic understanding of the kinetics of vesication and indicate that time points earlier than 24 hr may be useful to assess the effects of medical countermeasures to mustards, as well as investigate the toxic mechanisms involved in the process of vesication.

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CHARACTERIZATION OF CADMIUM INDUCED APOPTOSIS

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Cadmium (Cd) is a heavy metal which is highly toxic. Cadmium has nephrotoxic and hepatotoxic effects linked with induction of oxidative stress. Acute cadmium exposure causes apoptotic cell death. Cd in cells activates pro-apoptotic factors and leads to the activation of caspases and DNA damage, hi our study, we focused on comparison of methods for detection of apoptosis in cells treated with Cd. We used Human Kidney-2 cells (HK-2) treated with Cd at concentrations 25–100 pM $CdCl_2$ for time periods (6–24 h). In our work, we used various methods for detection of apoptosis such as: detection of mitochondrial membrane potential (JC-1 probe). DNA condensation (Hoechst 33258), detection of DNA fragmentation (TUNEL assay) and detection of caspase 3/7 activity (Caspase 3/7 kit). We observed the decrease of mitochondrial membrane potential in cells treated with 100 pM Cd after 24 h. We observed condensation of nucleus in both concentrations of Cd (25 and 100 pM) at both time duration (6 and 24 h). DNA fragmentation was observed in cells treated with 100 pM Cd after both time periods. Finally, caspase activity was increased at both tested Cd concentrations after 6 h. We conclude, all four methods detected apoptosis in Cd treated cells. After comparison of the methods, we found a strong correlation – the most sensitive apoptosis assay in Cd toxicity characterization was caspase 3/7 activity assay.

THE EFFECT OF THE EXPOSURE OF RATS TO THE ANTICANCER AGENTS VANDETANIB, LENVATINIB AND ELLIPTICINE ON THE EXPRESSION OF CYTOCHROMES P450

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Inhibitors of tyrosine kinases (TK) have become the novel tool in cancer therapy because of their ability to disrupt cell signaling pathways that regulate the processes of tumor growth and metastasis in cancer tissues. The TK inhibitors vandetanib and lenvatinib are oral anticancer drugs used for the treatment of certain tumors as so-called targeted therapy. Very little is known about the impact of these two compounds on the expression of biotransformation enzymes crucial for metabolism of drugs. Because combination of drugs is often used in the anticancer treatment and drug-drug interactions may play an important role in therapeutic effect of the administrated drugs, the Wistar rats were exposed to vandetanib or lenvatinib either alone or in combination with DNA-damaging cytostatic chemotherapeutic drug ellipticine to investigate the effect of these drugs on gene and protein expression of major biotransformation enzymes *in vivo*.

First, we focused on the expression of cytochromes P450 (CYP) 1A and 3A, the enzymes dominantly responsible for metabolizing drugs, including all three studied compounds, and other xenobiotics, in rat livers. Our results confirmed that ellipticine is a potent inducer of CYP1A1 and CYP1A2 that was reflected in the significantly increased gene and protein expression as well as activity of these enzymes. Co-treatment of vandetanib or lenvatinib with ellipticine had no effect on ellipticine induction potential. Interestingly, vandetanib and lenvatinib alone enhanced the mRNA and protein levels of CYP1A1 but at least 10-times less efficiently than ellipticine. All treatments led to slightly increased gene expression of CYP3A1 and marker activity of CYP3A, 6β-hydroxylation of testosterone. Furthermore, we studied the effect of the drugs on the gene expression

of CYP family 2 that also plays an important role in metabolism of drugs. Among rat isoforms CYP2A2, 2B1, 2C11, CYP2D1 and CYP2E1, only the gene expression of *CYP2C11* was 2-times decreased by ellipticine administrated to rats either alone or in combination with TK inhibitors.

It should be taken into account that by altering the expression of the enzymes involved in the metabolism of the administrated drugs, their pharmacological efficacy might be modulated.

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ACCUMULATION OF SELECTED METALS POLLUTION IN AQUATIC ECOSYSTEMS IN THE BASIN SMĚDÁ, CZECH REPUBLIC

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In 2016 was carried monitoring the environmental pollution of the river Smědá. The study present the concentrations of Hg, Pd, Co and Cd in the water, sediment and muscle tissue of brown trout– *Salmo trutta* morpha *fario* at 10 localities. The potential ecological risk of heavy metal concentrations in the sediments indicated that five sites in the middle and lower reaches posed small bis moderate ecological risk, but do not exceed the legal limits valid in the Czech Republic. Only two sites (2,3) represents a potential higher ecological risk (Hg content in sediment 0.057 mg/kg and Pb in sediment mg/kg) caused by the historical occurrence of the glassworks factory.

The contents of the analysed metals in fish muscles were low for all sites and did not exceed the values of limits admissible in the Europien comision regulation 1881/2006/ES and 629/2008/ES.

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IMPACT OF ANTHELMINTICS ON ACTIVITY OF ANTIOXIDANT ENZYMES IN SOYA (*GLYCINE MAX*)

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Veterinary anthelmintics, widely used drugs against parasitic worms, represent risk to environment as they may affect non-target organisms including plants. Plants are exposed to veterinary pharmaceuticals in pastures with treated animals or in fields fertilized with dung from treated animals. Anthelmintics enter plant body and can induce stress and consequent response. The production of reactive oxygen species (ROS), alterations in plant cell redox state and antioxidant mechanisms are usually the first responses to environmental stress. The antioxidant enzymes, including superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione peroxidase , glutathione reductase, and glutathione S-transferase, which are responsible for ROS elimination, may be affected as well. The aim of this project was to find out the impact of anthelmintics unwantedly occurring in environment (fenbendazole, FBZ; ivermectin, IVM) on soya (*Glycine max*) antioxidant enzymes. The changes in activities of antioxidant enzymes were measured in different parts of soya plant, in roots, leaves, seeds, and pods. The soya plants were harvested after the cultivation with 10 μM FBZ or 10 μM IVM for 3 months in greenhouse, and 20,000 g supernatant was prepared. The results showed the significant decrease of peroxidase and ascorbate peroxidase activity after FBZ and IVM treatment of *Glycine max* in roots, leaves, seeds, and pods. The activity of glutathione S-transferases was increased in soya treated with both anthelmintics. In addition, FBZ and IVM also markedly increased glutathione reductase activity in soya seeds. Some changes in the activity of other antioxidant enzymes were observed as well. In conclusion, veterinary anthelmintics can induce stress in plants and might affect their antioxidant systems.

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BIOSYNTHESIS OF MAGNETIC NANOPARTICLES USING PLANT EXTRACTS AND THEIR POTENTIAL ANTIBACTERIAL ACTIVITY

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Plant contains abundant natural compounds such as alkaloids, flavonoids, saponins, steroids, tannins and other nutritional compounds. These naturally occurring biomolecules have been identified as a potential reducing and capping agent for wet synthesis of nanomaterials in non-hazardous ways. In addition, green fabrication of magnetic nanoparticles is single step, rapid and cost-effective as compared to other physico-chemical technologies, which make it a promising technology. In this study, eco-friendly method was introduced to synthesize magnetite iron oxide nanoparticles using the aqueous extracts of geen tea leaves and *Cannabis sativa* seeds in alkaline conditions. The synthesized nanoparticles were characterized by UV–VIS spectroscopy and XRD analysis. These biogenic nanoparticles exhibited potential antibacterial activity against pathogenic bacteria presented in real waste water, therefore could be beneficial for potential applications in various fields

such as drug delivery, antibacterial drug, biomedical fields or wastewater treatment.

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HOMO- AND HETERODIMERS OF ACRIDINE DERIVATIVES AS INHIBITORS OF ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE IN ALZHEIMER´S DISEASE

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In the last decade much attention has been paid to the development of homo- and heterodimers containing two identical or different structural units linked by chain for simultaneous interaction with the active and the peripheral binding sites of acetylcholinesterase (AChE). A series of novel monotacrine, tacrine-tacrine, tacrine-acridine, tacrine-coumarin, acridine-coumarin and tacrine-quinoline ligands were designed, synthesized, and biologically evaluated as inhibitors of both AChE and butyrylcholinesterase (BChE) [1–3]. Among of monotacrine ligands, compound in which tacrine is connected to an (benzylpiperazinyl)etyl unit exhibited excellent inhibitory activity against hBChE (IC_{50}=0.4 nM) [2]. From tacrine-tacrine homodimers, inhibitors with buthylene-thiourea and hexylene-thiourea linker showed a strong acetylcholinesterase activity, with an IC_{50} value of 2 and 8 nM, resp [2]. The most effective inhibitors of hAChE within tacrine-acridine dimers were the derivatives joined with alkylenepiperazine linker with an IC_{50} value of 3 and 6 nM [2]. The structure-activity relationship studies showed clear correlation between the structure of homo/heterodimers and their inhibition potential against hAChE/BChE.

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[1] S. Hamulakova, et al. Eur. J. Med. Chem. **55** (2012) 23–31.
[2] S. Hamulakova, et al. Int. J. Biol. Macromol. **70** (2014**)** 435–439.
[3] S. Hamulakova, et al. J. Med. Chem. **57** (2014) 7073–7084.

ESTIMATION OF REDOX STATUS IN KIDNEY CELLS TREATED WITH CADMIUM

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Cadmium is a highly toxic heavy metal. The toxic effect of cadmium is most commonly detected in kidney. Acute exposure of the cells to cadmium can lead to apoptosis. Cadmium-induced cell death is linked with oxidative stress. Glutathione is the main intracellular nonprotein thiol with antioxidant properties in cells playing an essential role in protection against oxidative stress. Therefore, glutathione levels may be reduced in presence of cadmium-induced oxidative stress. Our aim was to characterize the redox changes in cells after cadmium treatment. A human proximal tubular cell line was used for studying nephrotoxicity *in vitro.* Proximal tubular cells were treated with $CdCl_2$ at concentrations 0–200 μM for 2, 6 and 24 h. To evaluate the induction of oxidative stress after $CdCl_2$ treatment, we measured intracellular reactive oxygen species (ROS) production and glutathione levels using intracellular probes and bimanes, respectively. ROS production increase was detected in cells treated with 100 and 200 μM $CdCl_2$ after 2 and 6 h. After 24 h, no increase in ROS production was detected in 100 μM and 200 μM $CdCl_2$ treated cells. After 24 h of treatment with 25 μM $CdCl_2$, we found substantial enhancement of ROS production. Significant depletion of intracellular glutathione appeared in all incubation periods following the increase in ROS production. However, the finding of ROS production increase was not linked with glutathione depletion after 24 h of treatment with 25 μM $CdCl_2$. We conclude that $CdCl_2$ is a potent inducer of oxidative stress in tubular cells associated with ROS production and reduction of glutathione levels. The level of oxidative stress is depends on the dose and time of $CdCl_2$ treatment.

THE HIGH-FRUCTOSE DIET DIFFERENTLY AFFECTS MICRORNAS EXPRESSION IN LEAN AND OBESE MICE

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Fructose is a highly lipogenic sugar naturally occurring mainly in fruits, vegetables or honey. The major problem is steady increase in fructose intake in soft drinks, sweets or prepackaged food. Fructose intake, compared to glucose, does not increase the levels of insulin and leptin, hormones involved in long-term regulation of energy homeostasis and body adiposity. In addition, dietary fructose is transported to the liver, where it bypasses the major control point of glycolysis, enzyme phosphofructokinase, and this way is becoming an unregulated source of glycerol-3-phosphate and acetyl-CoA. Fructose accelerates de novo lipogenesis and due to the molecular instability of furanose ring, it promotes the formation of reactive oxygen species (ROS). Taking together, the excessive consumption of fructose might contribute to the development of many serious metabolic disorders and diseases. The molecular mechanisms of these effects have not been fully elucidated yet. In our study, we focused on changes in miRNAs expression caused by high-fructose intake. MiRNAs have emerged as a key regulators of metabolic homeostasis over the past decade and the abberant

expression may be associated with many disorders and diseases, including obesity and related pathologies. These small, endogenous, single stranded, non-protein coding RNAsgene products, present in genomes of all eukaryotic organisms regulate RNA silencing and post-transcriptional gene expression through binding mainly to 3´UTR region of mRNA. For our experiments, we used lean mice and mice with obesity induced by high-fat diet, both with or without fructose administration in drinks. The panel of tested miRNAs was selected based on their involvement in obesity, metabolic syndrome, NAFLD and other related pathologies. The changes in miRNAs expression we observed in plasma, liver tissue, and white, brown and subcutaneous adipose tissues.

IMPACT OF PLATINUM NANOPARTICLES ON THE AQUATIC ORGANISMS

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In recent years several nanoparticles types have been studied intensively, but only very few studies have focused on the toxic effect of platinum nanoparticles (PtNPs) to aquatic organisms. PtNPs have many industrial and biomedical applications. Due to this extensive use, emission of PtNPs into aquatic environment must be expected. This may lead to undesirable environmental effects. Therefore, investigating the potential aquatic toxicity of nanomaterials has become an important issue. In this study we investigated the toxicity of three PtNPs of nominal size (3.1–24.4 nm) on organisms representing all trophic levels of the aquatic ecosystem: Duckweed *Lemna minor*, water crustacean *Daphnia magna* and marine bacteria *Vibrio fischeri*. The experiments were carried out on the basis of OECD 221; OECD 202 guideline and ISO 11348–2. Concentrations for each organism were chosen on the basis of the range finding test. The marine bacteria responded most sensitively to the presence of platinum nanoparticles. The effective concentration of Pt1 (3.1–10 nm) that caused 50% inhibition in bioluminescence of *Vibrio fischeri* was 135.47 $\mu g.L^{-1}$ and the effective concentration of Pt3 (8.7–24.4 nm) was 254.64 $\mu g.L^{-1}$. The concentration of Pt1 that caused 50% growth rate inhibition after 168 hours ($168hEC_{50}$) to *Lemna minor* was 10.67 $\mu g.L^{-1}$ and the concentration of Pt3 that caused 50% growth rate inhibition was <130 $\mu g.L^{-1}$. The acute toxicity ($48hEC_{50}$) of Pt1 for *Daphnia magna* caused concentration <410 $\mu g.L^{-1}$, Pt2 (4.2–21 nm) <415 $\mu g.L^{-1}$ and for Pt3 775.32 $\mu g.L^{-1}$. The ecotoxicity of platinum nanoparticles varies considerably according to the test organisms and particle size. The lowest toxicity of all tested samples was observed in *Daphnia* while the highest toxicity was in bacteria.

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HUMAN GUT MICROBIOME – XENOBIOTICS METABOLISING SYSTEM

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There is accumulating evidence indicating functional interactions between the human gut microbiome and ingested xenobiotics. These interactions may result in changes of effects of therapeutics and increase of toxicity and carcinogenicity of environmental compounds. Thus, it is getting accepted that human gut microbiome acts as an „additional organ" involved in metabolism of xenobiotics. Myricetin (MYR) is one of the key flavonoids present in various human foods and beverages including vegetables, teas and fruits. Its reductive analogue, dihydromyricetin (DHM), is highly effective in counteracting acute EtOH intoxication and in reducing excessive EtOH consumption. As some 2,3-unsaturated flavonoids undergo reductive metabolismus by gut microbiota, e.g. reduction of quercetin to taxifolin, we tested whether MYR is converted to reductive metabolite DHM, too. Our experiments with human fecal microbiota indicate that MYR was metabolized very fast, even when applied into the reaction mixture as a solid. Only negligible amount of the parent compound was left after 6 hrs. It seems that under anaerobic conditions MYR may serve as a convenient source of energy for microbiota. Alas, no DHM was detectable in the reaction mixture either. Thus MYR does not undergo reductive metabolism and cannot be used as a DHM precursor. In addition, the microbiota composition has changed in response to the MYR presence. It is not clear whether the shift in bacteria composition is originating from MYR antimicrobial effect on some bacterial genera and/or whether MYR is promoting the growth of other genera.

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THE IMPACT OF ANTIDEPRESSANTS ON FISH

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Even so the usage of antidepressants in world is on the rise, the information about impact on water organisms is limited. Psychopharmaceuticals are commonly found in surface waters in amount between ng/l to µg/l, thus they were chosen for this study for assessment of the potential impact on non-target organism *Danio rerio*. Embryos in different stages of development were

used and two different concentrations of venlafaxine were chosen; the 300 ng/l representing the environmental concentration (low) and the 30 µg/l as 100x higher (high) concentration for evaluation of dose depending effect. For the gene expression evaluation, genes ABCB4, CYP1A, CYP3A65, GST, ABCC1 and PXR were chosen, each of them representing one of the phase zero to III of xenobiotic biotransformation. The results of assay showed that the impact of venlafaxine on the zebrafish embryos is the most evident in the time of hatching (96 hours post fertilization); this period is one of the most threating period for the embryo development due to the first contact with the exogenous environment. In this time, the results of gene expression showed increase in mRNA amount of ABCB4 and GST in both concentrations of venlafaxine. The CYP1A, CYP3A65, ABCC1 and PXR gene revealed increase in mRNA amount in high concentration of venlafaxine; however, in contrast the low concentration for these genes revealed the decrease in mRNA amount. The second increase in gene expression was observed in 144 hours post fertilization for both concentrations of venlafaxine and all genes; this period is the time of transition to exogenous nutrition of embryo and thus the first oral exposure to xenobiotics in environment. To sum it, the study showed that venlafaxine can affect the gene expression of biotransformation enzymes of *Danio rerio* embryos already in environmentally relevant concentration.

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EXPRESSION AND PURIFICATION OF HUMAN ACETYLCHOLINESTERASE

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Human acetylcholinesterase (*h*AChE, EC 3.1.1.7) is the target of numerous natural and synthetic inhibitors, including therapeutics and a wide range of toxic esters as organophosphorus pesticides and nerve agents.

Current study is focused on new Expi293 expression system which is used for production of recombinant protein. This expression model promised to achieve expression levels near to 1 g/L of protein. The Expi293F™ cells were transfected with a pcDNA™3.4 TOPO® vector encoding the *h*AChE and incubated at 37°C8% CO2, 80% humidity and 120 rpm stirring speed. The cells produced and secreted complete *h*AChE within seven days. Progressive level of protein was supervised by Ellman's method. Protein was purified by Sepharose/Procainamide affinity chromatography. Subsequently, protein was analyzed by western blot using an anti-His tag antibody. The purity enzyme was determined via SDS-PAGE. The kinetic parameters of a recombinant enzyme were validated by value of IC_{50} of standard inhibitor e.g. donepezil, which was compared with published results.

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OPTIMIZATION OF PREPARATION OF VANDETANIB ENCAPSULATED IN AN APOFERRITIN

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Vandetanib is a once-daily oral agent using for the treatment of tumours of the thyroid gland. It acts as a tyrosine kinase inhibitor affecting signalling of epidermal growth factor receptor, vascular endothelial growth factor receptor or rearranged during transfection. Adverse effects connected with this drug are vomiting, nausea, insomnia, etc. The most dangerous adverse effect is connected with long QT interval. One way how to minimize side effects is targeting of drugs to tumour tissues. An apoferritin, the apo-form of naturally occurring protein ferritin, represents a suitable transporter for such targeting. It is a protein composed of 24 polypeptide subunits, structurally arranged to create an internal cavity with size of 8 mm in diameter. The structure is remarkably stable and is able to withstand biologically extreme temperatures (up to 70°C) and a wide pH range (pH 2–10). Furthermore, apoferritin can move undetected through the body without any immune response. It is also possible to modify its surface by ligands specific for targeting tissues. Unfortunately, such modification can stimulate immune responses.

Herein, the ability of the apoferritin to encapsulate vandetanib (creating ApoVan) was studied. At a constant concentration of drug and an increasing concentration of apoferritin more vandetanib is incorporated into apoferritin internal cavity. With increasing concentration of drug, the concentration of ApoVan reaches the maximum and starts to decline. These results indicate that the efficiency of encapsulation is dependent on ratio of vandetanib to apoferritin. The prepared ApoVan samples were characterized by transmission electron microscopy and quasielastic dynamic light scattering. The nanocarrier exhibits narrow size distribution and spherical shape. The surface zeta potential (ζ-potential) was also determined. All the characteristic indicate that apoferritin is a suitable nanotransporter for vandetanib.

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METABOLISM OF A TYROSINE KINASE INHIBITOR VANDETANIB BY HUMAN CYTOCHROMES P450 AND FLAVIN MONOOXYGENASES *IN VITRO* AND ITS EFFECT ON FORMATION OF DNA ADDUCTS GENERATED BY AN ANTICANCER DRUG ELLIPTICINE

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Vandetanib is a tyrosine kinase inhibitor (TKI) indicated for the treatment of symptomatic or progressive medullary thyroid cancer in patients with unresectable locally advanced or metastatic disease. It inhibits signalling of epidermal growth, vascular endothelial growth factor or rearranged during transfection. In this study, oxidation of vandetanib by human hepatic microsomes and recombinant cytochromes P450 (CYPs) and flavin-containing monooxygenases (FMOs) expressed in Supersomes™ was studied. The vandetanib oxidation products were separated by HPLC and identified by mass spectroscopy. Human hepatic microsomes oxidize vandetanib to *N*-desmethylvandetanib, but not to vandetanib-*N*-oxide. Of all tested human CYP enzymes, the CYP1A1, 2C8, 2D6, 3A4 and 3A5 enzymes, mainly in the presence of cytochrome b_5, oxidize vandetanib to *N*-desmethylvandetanib. No vandetanib-*N*-oxide was generated by tested human CYPs. However, FMO enzymes were able to generate this metabolite. Of three human FMOs tested (FMO1, FMO3 and FMO5), FMO1 and FMO3 oxidize vandetanib to vandetanib-*N*-oxide. FMO1 was more effective than FMO3 in this reaction. The results found in this study approved the knowledge showed by the preliminary studies, suggesting that vandetanib is oxidized to *N*-desmethylvandetanib and vandetanib-*N*-oxide, and specified the efficiencies of individual CYPs and FMOs in the reactions. Moreover, they indicated an essential role of cytochrome b_5 in oxidation of vandetanib to *N*-desmethylvandetanib by CYP3A4. Because this CYP is the most important enzyme activating also another anticancer agent that is effective against certain tumours of the thyroid gland, DNA-damaging drug ellipticine, the effect of vandetanib on metabolic activation of this drug was investigated. An inhibition effect of vandetanib on the most efficient anticancer effects of ellipticine, formation of covalent ellipticine-derived DNA adducts, was found. Cytochrome b_5 plays an important role also of this CYP3A4-mediated activity.

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TYROSINE KINASE INHIBITORS VANDETANIB, LENVATINIB AND CABOZANTINIB MODULATE METABOLISM OF AN ANTICANCER AGENT ELLIPTICINE BY CYTOCHROMES P450

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Vandetanib, lenvatinib and cabozantinib are tyrosine kinase inhibitors (TKIs) targeting VEGFR subtypes 1 and 2, EGFR and the RET-tyrosine kinase, thus considered as multiple TKIs. These TKIs have already been approved for treating patients suffering from thyroid cancer and renal cell carcinoma, and further clinical trials are ongoing for prostate cancer and glioblastoma multiforme. Ellipticine and its derivatives are other anticancer agents that are effective against certain tumors of the thyroid gland (anaplastic thyroid carcinoma), ovarian carcinoma, breast cancer and osteolytic breast cancer metastasis. Ellipticine anticancer efficiencies are dependent on its metabolism leading both to the activation metabolites causing DNA damage (covalent DNA adducts) and their detoxification to products that are excreted. Ellipticine is oxidized by microsomal cytochrome P450 (CYP) enzymes and peroxidases. Oxidative activation by CYP3A4 leads to formation of 12-hydroxy- and 13-hydroxyellipticine, reactive metabolites that dissociate to ellipticine-12-ylium and ellipticine-13-ylium, binding to DNA, while formation 9-hydroxyellipticine by CYP1A1 and the ellipticine dimer by peroxidases are considered to be detoxification products. A number of studies testing the effectiveness of individual anticancer drugs alone or in a combination with other cytostatics demonstrated that such combination can have additive and/or even synergistic effects on treatment regimen. The aim of this study was to study the effect of TKIs vandetanib, lenvatinib and cabozantinib on oxidative metabolism of ellipticine. All tested TKIs inhibit oxidation of ellipticine catalyzed by hepatic microsomes and individual CYPs, but not by peroxidases (horseradish peroxidase, lactoperoxidase and myeloperoxidase). The mechanism of these effects is studied in details. The study might provide a rationale for the clinical evaluation of the combination of TKIs and DNA-damaging anticancer drugs.

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CYTOTOXICITY OF ENDOCRINE DISRUPTORS IN TM3 LEYDIG CELL LINE

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Environmental contaminants altering the function of the endocrine system and exhibiting adverse health effects on the organism are defined as endocrine disruptors (EDs). Alkylphenol ethoxylates, a class of non-ionic surfactants, are microbially degraded into alkylphenol diethoxylates and alkylphenol monoethoxylates. These are subsequently degraded into alkylphenols (4-octylphenol; 4-nonylphenol) and along with other sub-products, are known to persist in the environment for a long time. Exposure to other EDs such as bisphenols has been also shown to cause adverse effects on the male reproductive system in humans and numerous animal species. A decrease in semen quality was the first reported alteration and from this moment on an informative expansion was launched on the potential consequences of bisphenol exposure. Both may disrupt not only spermatogenesis, by interfering with germ cells and sperm-supporting cells, but may also affect steroidogenesis occurring in Leydig cells. The aim of the present study was to investigate the potential impact of selected EDs on the male reproductive system in mice. Our study was initiated to evaluate the role of alkylphenols and bisphenols on Leydig cell function *in vitro* at lower experimental concentrations (0.04–5.0 μg/mL). Subsequently, we determined the metabolic activity, membrane integrity and lysosomal activity in TM3 cell line after 24 h cultivation. The cell viability was assessed using the metabolic activity (AlamarBlue^{TM}) assay, while the membrane integrity of exposed cells was evaluated by CFDA-AM assay. Determination of lysosomal activity was monitored by neutral red assay. A slight decrease in cell viability and membrane integrity of TM3 Leydig´s line was recorded after alkylphenols (1.0–2.5 μg/mL) treatment. Significantly decreased lysosomal activity was observed following exposure to the whole applied range of alkylphenols *(P<0.05; P<0.001).* Bisphenol´s exposure to TM3 Leydig cell line has shown a similar tendency. All the monitored parameters representing cellular health were negatively affected by experimental doses (0.04–5.0 μg/mL) of bisphenol A and bisphenol B.

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SURFACE WATER QUALITY AND THE EFFECT OF MUNICIPAL WASTEWATER TREATMENT IN THE SVRATKA RIVER (CZECH REPUBLIC)

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The aim of this work was to determine the selected physical-chemical indicators of surface water quality. Sampling was carried out during four seasons of the year at three different localities of the Svratka River – in the town of Tišnov, in the town of Veverská Bítýška, and close to the selected wastewater treatment plant (WWTP) in order to assess the impact of the selected town or location on the water quality in the river. At the same time, selected indicators were determined directly in the waste water from the WWTP near the town of Tišnov.

Among the parameters of interest were chosen basic elements set in the legislation such as chemical oxygen demand (COD), total nitrogen and total phosphorus, and some other substances such as nitrates, sulphates, chlorine, etc. These indicators were in surface water and wastewater determined by the method of spectrophotometry, using certain reagent and cuvette sets from Merck.

The results were compared with the permissible limit values specified in the present legislation of the Czech Republic, especially the Decree no. 401/2015 Coll., and with the values according to CSN 75 7221 – Classification of surface water quality. According to these regulations, we have classified the Svratka River to the class III of surface water quality (impure water). Based on the obtained results, it was also possible to assess the effectiveness and efficiency of selected WWTP to remove contaminants from waste water, which could negatively affect water quality in the river and thus the whole water ecosystem.

CYTOTOXIC EFFECTS OF NOVEL NITRO-HYDROXYNAPHTHANILIDES ON CANCER CELL LINES IN THE CONTEXT OF THE DIFFERENT SUBSTITUTION PATTERN

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The structure of three new series of nitro-substituted hydroxynaphthanilides was designed based on ring analogy with salicylanilides, derivatives possesing many pharmacological effects including promising anticancer activity, although their mechanism of action is still not fully understood. Our study focused on the evaluation of their potential effects on viability and proliferation of human cancer cell lines in the context of differences in the substitution pattern on naphthalene or anilide part of the structure.

Our results revealed that the position of the β-ring of naphthalene towards the carboxanilide or phenolic scaffolds and the position of nitro substituent both affected the intensity of cytotoxic activity in cancer cell lines. In all three series of compounds, we found their increased potency towards impaired cell viability and antiproliferative effect with the positioning of the substituent

as follows: *ortho* < *meta* < *para*. Their antiproliferative activity was also quantitatively comparable to that activity of another nitro-substituted salicylanilide, niclosamide. In addition, compounds with such effects induced accumulation of cells in G1 cell cycle phase in a dose-dependent manner in both THP-1 and MCF-7 cell lines. The higher concentration of 10 μmol/l of the most potent compound did not affect metabolic activity of nontumor cell line 3T3-L1 but at the same time it induced the phosphatidylserine externalization and activation of caspase 3 in cancer cell line THP-1. On the other hand, activation of caspase 8 upon that treatment was not observed. Further analysis showed the ability of that compound to induce cytochrome C release and cleavage of pro-caspase 9. That effects were also accompanied by a loss of mitochondrial membrane potential.

Thus our results indicated the pro-apoptotic effects of tested compounds in cancer cell lines, their significant advantage also lies in the fact that such an activity seems to be effectively modulated by appropriate structure modification.

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IN VITRO PAMPA AND MDCK MODELS FOR THE PREDICTION OF BLOOD BRAIN BARRIER PENETRATION

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The study of drugs acting on the targets in the brain can be precisely determined only by the *in vivo* study. Unfortunately, this way is very expensive and ethical aspect must be considered because of the high consumption of laboratory animals. The development of new potential drugs targeting the brain requires screening to assess permeability through the blood brain barrier (BBB). Two screening methods used for this purpose, PAMPA (Parallel Artificial Membrane Permeability Assay) and MDCK (Madin-Darby Canine Kidney) cells permeability assay, have been used in this experiment for evaluation of standard drugs set. Both PAMPA and MDCK permeability assays are used to evaluate the ability of compounds to diffuse from a donor compartment through the appropriate membrane into an acceptor compartment. PAMPA´s membrane is a brain polar lipid layer while MDCK assay uses MDCK cell monolayer. Then, the concentration in acceptor compartment has been determined by UV-VIS spectrophotometry. The transport through PAMPA is based exclusively on physico-chemical properties mostly represented by the logP but completely ignores the active mechanisms present in the BBB of living cells. These mechanisms involve active transport, efflux transporters and enzymes. This fact limits PAMPA´s use because a lot of compounds interact with them. MDCK BBB model improves evaluation using living cells which form the tight junction similarly to the BBB endothelial cells. Here we compare the two models for the BBB penetration assessment.

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EFFECT OF NEURAMINIDASE TREATMENT OF LUNG EPITHELIUM ON BINDING OF PSEUDOMONAS AERUGINOSA

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Cystic fibrosis (CF) is one of the most common human autosomal recessive diseases. This genetic disorder is associated with increased susceptibility of lungs to bacterial infections, which frequently develop to life-threatening conditions for CF patients. In this regard Pseudomonas aeruginosa (PA) is the most dangerous bacterial pathogen. Among PA virulence factors lectins seem to play crucial role in the PA adherence on CF airway epithelium. PA lectins, PAIL and PAIIL, showing high affinity for D-galactose and/or L-fucose, are assumed to be namely involved in the bacteria binding. Likely, the low-sialylation of glycoconjugates on CF epithelial cells provides exposed saccharide residues suitable for the lectins interaction. To study the PAIL and PAIIL involvement in the adherence of PA on airway epithelium the mouse model mimicking CF lung conditions is being developed. The status of low-sialylation was induced by the intratracheal instillation of neuraminidase, the enzyme cleaving terminal sialic acid. For our experiments lectins PAIL and PAIIL were recombinantly expressed and coupled with high-performance fluorescent label, DyLight 488. The saccharide binding ability of the DyLight 488 labeled lectins was checked by using a red blood cell agglutination assay. The PAIL and PAIIL labeling did not alter the lectin saccharide binding affinity. Next, tissue slices of lungs from neuraminidase-treated and untreated mice were mounted on microscope slides and incubated with DyLight 488 labeled lectins. Finally, tissue specimens were examined on an inverted fluorescence microscope (Nikon Eclipse TE2000-U). In the neuraminidase-treated group the cuboidal epithelium lining the surface of respiratory bronchioles showed an apparent fluorescence DyLight 488 labeled PAIIL when compared with untreated mice. On the other hand, when DyLight 488 labeled PAIL lectin was used (for neuraminidase treated mouse), fluorescence was observed both on the epithelium of bronchioles surface and on the pulmonary alveoli surface.These results support the assumption that PAIL and PAIIL mediate the PA binding on CF airway epithelium. Thus, lectins PAIL and PAIIL are a candidate target in preventing PA lung infections of CF patients.

IMPLEMENTATION OF *IN VITRO* METHODS FOR REGULATORY TOXICOLOGY TESTING AND RESEARCH AT HAMELN RDS A.S.

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Hameln has set up a research programme to implement toxicology methods that follow the 3Rs principles (Refine, Reduce and Replace animal testing) as requested by the EU regulation Directive 2010/63/EU. In this programme, hameln (as member of EU-NETVAL) implemented particular *in vitro* methods described by the regulatory standards.

Amongst the tests, the OECD TG 431 (*in vitro* skin corrosion test), TG 439 (*in vitro* skin irritation test), TG 492 (*in vitro* eye irritation test), TG 471 (bacterial reverse mutation test – Ames test), TG 473 (*in vitro* mammalian chromosomal aberration test), TG 476 (*in vitro* mammalian cell gene mutation test), TG 487 (*in vitro* mammalian cell micronucleus test) are those most frequently used *in vitro* assays at hameln. Implementation of these methods requires evaluation of benchmark materials and controls as defined by the OECD Test Guidelines. Hameln also actively collaborates with external parties to obtain trainings in the *in vitro* methods in order to accelerate their implementation.

This poster will present data and experiences obtained during this implementation process and will discuss the benefits and pitfalls of the implementation of the 3R methods into the GLP certified laboratory for both regulatory and scientific purposes.

EFFECT OF CEMTIRESTAT ON Ca-ATPASE (SERCA1) ISOLATED FROM ZUCKER DIABETIC FATTY RATS

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Cemtirestat, 3-mercapto-5H-1,2,4-triazino[5,6-b] indole-5-acetic acid, has been identified as one of the efficient inhibitors of aldose reductase, the enzyme involved in the polyol pathway, representing a promising therapeutic target in prevention of diabetic complications linked with glucose toxicity [1]. Impairment of calcium ATPase by high glucose or its derivatives was observed in human diabetes as well as in experimental animal models. Moreover, acute increases in glucose level strongly correlate with oxidative stress and may thus influence conformational changes in proteins [2]. We investigated sarcoplasmic reticulum (SR) calcium ATPase (SERCA1) which transports calcium ions from the cytoplasm into the SR, and thus plays a key role in calcium homeostasis and cell signaling. *In vivo* effects of cemtirestat on calcium pump SERCA1 isolated from skeletal muscles of Zucker diabetic fatty rats, a type 2 diabetes model, were examined. Results indicate a significant decrease of SERCA1 activity and attenuation of the enzyme expression accompanied by its post-translational modifications in diabetic rats compared to control animals. Yet treatment with cemtirestat failed to affect either activity decrease or declined expression of SERCA1 in diabetic rats.

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[1] Štefek M., et al. (2016). *International Journal of Advances in Science Engineering and Technology* **4**(3): 41–44.
[2] Horáková L., et al. (2013). *Free Radical Research* **47**(supp1): 81–92.

EFFECT OF BILBERRY EXTRACT (*VACCINIUM MYRTILLUS L.*) ON CONJUGATING ENZYMES IN RATS

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Vaccinium myrtillus L. (bilberry) fruit is a blue-colored berry with high content of anthocyanins, bioactive secondary metabolites composed of a sugar moiety and anthocyanidins (flavonoid structure). Bilberries are widely studied due to their health-promoting properties, especially for their positive effect on night vision, vascular permeability and capillary fragility. However, very little is known about bilberry effect on biotransformation enzymes. The aim of this study was to evaluate the effect of bilberry extract on the mRNA expression and activity of main phase II biotransformation enzymes: glutathione S-transferase (GST), sulfotransferase (SULT), UDP-glucuronosyl transferase (UGT) and catechol-O-methyltransferase (COMT) and predict possible impact on the action of together administered drugs. The *in vivo* study, where rats were exposed to bilberry extract (two concentrations, 0.15 or 1.5 mg/mL) in drinking water for 29 and 58 days, was performed to fulfil the objective. Mild decrease of COMT mRNA level was observed in samples with lower concentration of bilberry extract after 58 days. All other assayed mRNA levels remain unchanged. GST, COMT and UGT activities did not show response to bilberry extract. Slightly modified activity was observed in SULT, where activity was increased after 58 days by both concentrations (118±0.6% of control – in samples with lower concentration; 115±0.7% of control – in samples with higher concentration). The results suggest that bilberries as food supplement possess very low, if any, potential for food–drug interactions with respect to conjugating enzymes. This conclusion is positive particularly for bilberries regular consumers.

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ANTIEPILEPTIC DRUGS DURING PREGNANCY. CZTIS EXPERIENCE

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Antiepileptic drugs are suspect teratogens with various risk of malformations. They are used for epilepsy treatment but also for treatment of psychiatric diseases and migraine. Aim of our work was to evaluate spectra and changes over the time in indications for individual drugs. Majority were calls before planned pregnancy or during first trimester. Proportion of calls for valproate in epileptic women is stabile reaching approximately 20%. It is probably result of disease severity or maternal fears from malformation. Spectra of drugs has been changed to safer and newer drugs, i.e. lamotrigine and levetiracetam. Spectra of antiepileptic drugs for psychiatric indications are very similar with relatively high occurrence of valproic acid and lamotrigine. Topiramate and clonazepam were more frequently used in psychiatric indication then for epilepsy. Antiepileptic drugs in both indications are combined, more often in psychiatric indication. Psychiatric drugs are less studied, information on reproductive toxicity are not available, evaluation of the risk therefore cannot be exact. We add three cases of psychiatric patients (depression, bipolar disorder and obsessive-compulsive disorder) treated by antiepileptic drugs with follow up. Case 1: 33 years old woman suffering from depression was treated by alprazolam, venlafaxine, clonazepam, bupropion, zolpidem and trazodone. She gave birth girl smaller for gestational age with small hemangioma. Case 2: Woman 34 years old suffering from bipolar disease and sclerosis multiplex was treated by valproic acid, sertraline, and methylprednisone. She used sporadically paracetamol with caffeine. She gave birth healthy girl in term. Case 3: 28 years old woman suffering from obsessive- compulsive disorder was treated by sertraline, lamotrigine, flupentixole and sulpiride. She gave birth premature girl (36th week) without malformation.

Conclusions: We found that polytherapy, drugs without known effect on fetus, and anticonvulsant with risk of teratogenicity are used for treatment psychiatric indications. Improvement of knowledge about optimal pharmacotherapy during pregnancy in psychiatrists is therefore essential.

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THE CHANGES IN MICRO-RNA EXPRESSION IN PLASMA AND HEART OF MICE TREATED WITH CARDIOTOXIC ANTICANCER DRUGS

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Cardiovascular toxicity remains a major cause of drug failure during preclinical and clinical development and it also contributes to the post-approval withdrawal of medicines. Moreover, many drugs widely used in clinical practice have potentially toxic effects on the heart in some patients. MicroRNAs (miRNAs), small endogenous non-coding RNA, seem to be promising tool regarding drug-induced cardiotoxicity. Cardio-specific miRNAs can serve as markers in the identification of potentially toxic compounds through *in vitro* preclinical screening and the miRNAs circulating in plasma can be useful in the identification of patients with subclinical cardiotoxicity. In our project, we focused on two anticancer drugs, doxorubicin (DOX) with proven cardiotoxicity and imatinib (IMT) with potential cardiotoxicity. Mice were treated with DOX (3mg/kg, every other day, i.v.) or IMT (100mg/kg, every day, p.o.) for 9 days. The control groups were treated only with the solvents. The last day of experiment, mice were sacrificed, blood was sucked out and hearts were removed. Plasma samples and heart homogenates were used for miRNAs isolation and quantification using miRNA-microarray and qPCR. In plasma, concentration of troponin T was also assayed. The results showed significant increase of troponin T in mice treated with DOX. DOX treatment led to up-regulation of miR-208b and miR-367 in heart. In plasma of DOX-treated mice, levels of several miRNAs (namely miR-34a, miR-133a,b, miR-1a, miR-7058) were increased, while others (e.g. miR-6240, miR-339, miR-6236) were decreased in comparison to controls. IMT treatment caused also similar increase in troponin-T plasmatic level and miRNAs expression, but the changes were not statistically significant due to marked inter-individual differences.

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THE EFFECT OF PRENATAL AND PERINATAL EXPOSURE TO PHTHALATE MIXTURE ON SOCIABILITY, ANOGENITAL DISTANCE AND TESTOSTERONE PRODUCTION IN ADULT RATS

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Phthalates (Pht) are chemicals belonging to the group of endocrine disruptors that are capable of interfering with the endocrine system of animals. Testosterone (T) is the hormone important for brain development and its levels are negatively affected by exposure to Pht. For this reason, there is a growing concern of Pht having detrimental impact on brain development, and behaviour of individuals. Our goal was to investigate the effect of prenatal and perinatal exposure to Pht mixture on sociability, anogenital distance (AGD), and plasma T levels in adult rats. Pregnant Wistar rats were divided into two groups: control (Ctrl) and exposed to the mixture of phthalates (Ft) – Di(2-ethylhexyl) phthalate

(DEHP), Diisononyl phthalate (DiNP), and Di-n-butyl phthalate (DBP) in dose of 4.5 mg/kg/day each. Mixture was diluted in peanut oil as vehicle, and delivered to an animal orally from gestational day 15 to postnatal day 4. Social interaction test was performed in adult rats (Ctrl: ♂ n=12, ♀ n=10; Ft ♂ n=10, ♀ n=12) to assess sociability. AGD was measured between the groups (Ctrl: ♂ n=19, ♀ n=25; Ft ♂ n=21, ♀ n=22) to assess changes in prenatal T levels. Postnatal plasma T levels were measured in adulthood (Ctrl: ♂ n=15, ♀ n=26; Ft ♂ n=18, ♀ n=22) using radioimmunoassay. Our results show that offspring from Ft group spent a less time in social interaction (p<0.001), had a decreased frequency of social contacts (p<0.001), shorter AGD (p<0.05), and decreased levels of plasma T when compared to Ctrl (p<0.05). These results suggest a possible role of phthalates in decreasing T levels, which could lead to abnormalities in brain development, leading to changes in sociability, aspect of behaviour which was shown to be impaired in several neurodevelopmental diseases.

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IN VITRO CHARACTERIZATION OF ACETYLCHOLINESTERASE REACTIVATORS

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Acetylcholinesterase (AChE; 3.1.1.7) reactivators play a key role in the treatment of organophosphate poisoning. The reactivators disrupt the covalent bond between organophosphorus compounds and AChE and restore the physiological function of this enzyme. On the other hand, the reactivators possess their own toxicities, whose mechanisms are not fully understood.

The objective of our study was to compare the cytotoxicity of asoxime, methoxime, obidoxime, pralidoxime, trimedoxime, K027, K074, K075, and K203 using hepatocellular carcinoma cell line HepG2. MTT assay and real-time cell viability assay were used to measure cytotoxicity of selected compounds, which was expressed as toxicological index IC_{50}. Fluorogenic 2,7-dichlorofluorescein diacetate dye and dihydroethidium were utilized to measure generation of reactive oxygen species (ROS). The microcapillary flow cytometry was used for determination of change in apoptotic activity.

The tested reactivators showed different cytotoxicity with HI-6 being the most and K027 being the least toxic. Changes in intracellular ROS were also detected; however, they did not correlate with substances´cytotoxicity.

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THE TOXICITY OF SELECTED ANTIDEPRESSANTS ON THE *DANIO RERIO* LARVAE

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In recent years observed increased interest in the presence of active substances in surface waters. The presence of drugs in surface waters is associated with a significant and ever-increasing use of medicines worldwide, which in turn is mainly due to the increasing prevalence of civilization diseases and an aging population. The main source of pharmacologically active substances in surface waters are treated municipal wastewater, which are introduced into watercourses from surface waters treatment plants. Pharmacologically active compounds, when they enter surface waters, may affect aquatic organisms. It is worth noting that these organisms are often vulnerable not to one, but to several pharmacologically active compounds occurring in concentrations of μg/L and ng/L throughout their lives. A group of drugs that is particularly interesting are antidepressants. According to the global health estimates by WHO 2015 the proportion of the global population with depression in 2015 is estimated to be 4.4%. Antidepressants can even affect aquatic organisms even at low concentrations of ng/L.

The aim of this study was to determine the influence of selected antidepressants and their mixtures on larvae Zebrafish as well as to determine the effect of low concentrations of sertraline, paroxetine, fluoxetine and mianserine and their mixtures on physiological or histological responses of Danio rerio larvae.

The fish embryo toxicity test (FET) was carried out in 6-well plates according to OECD 236 guideline. The plates were incubated at 27–0.5°C.for 96 h. The proliferative activity of hepatocytes was analyzed in fish embryo tissues by immunohistochemical staining of the nuclear proliferation cell antigen (PCNA).

Exposition to the analyzed pharmaceuticals did not influence the survival of embryos and larvae during 96 h of the test at the concentrations tested (5–25 μg/L). In some cases it has been reported pathologies such as scoliosis or pericardial edema. PCNA test revealed that significantly lower proliferation (3-times) of hepatocytes occurred in larvae exposed to paroxetine, mianserin, sertraline and the mixture of the pharmaceuticals at the highest concentrations (25 μg/L of each compound).

The results suggests that selected antidepressants sertraline, paroxetine and mianserin may adversely affect the organogenesis of fish.

INFLUENCE OF NEWLY DEVELOPED HEMOSTATIC AGENTS ON THE INFLAMMATION RESPONSE IN TRAUMATICALLY INJURED KIDNEY

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Various surgical procedures require application of an effective hemostatic material. Appropriate agent should prove high hemostatic activity and besides also biocompatibility and potential bioresorbability. The aim of this study was evaluation of the local tissue reaction in rat's kidney after surgical intervention and application of hemostatic textiles. We used a common model of partial nephrectomy. We assessed new cellulose nonwoven textiles based on oxidized cellulose and sodium salt of carboxymethylcellulose in comparison with a fibrilar form of regenerated oxidized cellulose. Materials were attached to the wound to effectively stop the bleeding and left in the same location while the peritoneum was sutured. Main purpose was to investigate the influence on the renal parenchyma after 3 and 30 days from the surgery. We leaned on the results from renal histology and immunohistochemistry considering histological changes and kidney cytokines TNF-α, TGF-β as inflammation markers. Obtained results revealed better hemostatic activity of the new carboxymethylcellulose material together with beneficial effect on the healing tissue.

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INTERFERENCE OF EXOGENOUS BROMIDE WITH THE METABOLISM OF IODINE AND THYROID HORMONES IN THE RAT

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With the aid of several radioanalytical methods, using ^{82}Br and ^{131}I or ^{125}I radiotracers, we studied the effects of an enhanced bromide intake on various aspects of iodine metabolism and, consequently, on the metabolism of thyroid hormones in the rat. In particular, we followed the influence of both an extremely high bromide intake (> 160 mg bromide per animal per day) and also of lower intakes, under conditions of sufficient iodine supply (standard diet B) and of iodine deficiency (Remington type diet R). In addition to adult male rats, we used also lactating rat dams and their pups.

Here, we summarize the effects of excessive bromide intake on the thyroid function and on the whole-body metabolism of iodine. Discussed is especially the influence of a high bromide intake in lactating rat dams on the production rate of mother's milk and on iodine and bromide transfer through mother's milk to the suckling young. Moreover, the impact of high levels of bromide in the organism of lactating dams on their performance in the course of the lactation period and in particular on the prosperity of their pups is also described. We have proved that bromide, similarly to iodide, readily penetrated into rat milk and via mother's milk was transferred in a large extent into the body of suckling young. We have also confirmed the earlier observation that bromide toxicity is dependent on the state of iodine supply into the organism.

In the adult rats we followed the effects of exogenous bromide on the uptake of ^{131}I-iodide by the thyroid glands and by various others organs and tissues (for more comprehensive results, *see* [1]). In addition, we have also found a complex, biphasic relationship between the extent of bromide intake in the rats and the specific peroxidase activity in their thyroid glands (*see* the accompanying poster).

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[1] Pavelka S. in: COMPREHENSIVE HANDBOOK OF IODINE: NUTRITIONAL, BIOCHEMICAL, PATHOLOGICAL AND THERAPEUTIC ASPECTS (Preedy VR, Burrow GN and Watson RR, eds.). Oxford: Academic Press, 2009, pp. 199–206 (Chapter No. 20) and 587–595 (Chapter No. 61)

BIPHASIC INFLUENCE OF EXOGENOUS BROMIDE ON THE THYROID PEROXIDASE ACTIVITY

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Thyroid peroxidase (TPO) is the key enzyme in the biosynthesis of thyroid hormones in thyrocytes. Recently, we have proved potent goitrogenic effects of exogenous bromide and perchlorate ions in the rat [1] (*see* also the accompanying poster). Here, we followed in detail, with the aid of an improved radiometric enzyme assay for TPO, the influence of different bromide and/or perchlorate intake in the animals, maintained on diets with diverse iodine content, on their TPO activity.

Unexpectedly, we found that the influence of exogenous bromide on the TPO activity in the rat thyroids was complex, biphasic with regard to the extent of bromide intake. An increase (up to 3-fold) in TPO activity was measured in rats with a low or moderate bromide intake (below ca. 60 mg per animal per day), while in animals with very high bromide intake (over ca. 160 mg) its thyrotoxic effects prevailed and TPO activity was reduced. The inhibitory effect of bromide was markedly increased in animals maintained on iodine-deficient diet. Interestingly, elevated TPO activity was found in all rats administered with perchlorate alone, regardless of the type of diet (the content of iodine in the diet, respectively). At the whole-body level, in rats administered with bromide and perchlorate we measured a consistent increase in relative weight of their thyroids with increasing time and concentration

of applied bromide, and a sharp reduction of the 24-h uptake of [^{131}I]-iodide by their thyroids. In these animals, we also determined a steady decline in serum total thyroxine concentration.

Supported by the Academy of Sciences of the Czech Rep. (Research project No. AV0Z50110509)

[1] Pavelka S. in: COMPREHENSIVE HANDBOOK OF IODINE: NUTRITIONAL, BIOCHEMICAL, PATHOLOGICAL AND THERAPEUTIC ASPECTS (Preedy VR, Burrow GN and Watson RR, eds.). Oxford: Academic Press, 2009, pp. 199–206 (Chapter No. 20) and 587–595 (Chapter No. 61)

INTERACTION OF ANTIDEPRESSANT DRUG FLUOXETINE WITH THE METABOLISM OF TRI-IODOTHYRONINE

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Thyroid hormones (TH) are supposed to control the activity of some neurotransmitters (e.g., serotonin), which are hypothetically involved in the pathogenesis of depressive illness. Namely, one of the pathogenic factors of depression might be inadequate activities of brain enzymes iodothyronine deiodinases (IDs) that could lead to local insufficient concentration of 3,3',5-triiodo-L-thyronine (T_3). This hypothesis led to the development of a new group of non-tricyclic antidepressant drugs known as selective serotonine re-uptake inhibitors. The most frequently used representative of this group of pharmaceuticals is fluoxetine (Fluox). We studied the interaction of Fluox with the metabolism of TH in the rat with the aid of our newly developed radiometric assays for deiodinating enzymes, IDs of types 1, 2 and 3 (D1, D2 and D3), as well as of adapted radiometric enzyme assays for conjugating enzymes iodothyronine sulfotransferases (ST) and uridine 5'-diphospho-glucuronyltransferase (UDPGT). Effects of subchronic administration of Fluox by itself, T_3 alone or in combination with Fluox, on T_3 production and degradation in the CNS and in different peripheral rat tissues were followed both, at the level of whole organism and at the molecular level.

In samples of liver microsomes of rats treated with Fluox, we found about two-fold higher UDPGT activities in comparison with control rats. Even more profound changes in enzyme activities were found in case of IDs, especially in the pituitary and cerebellum. The treatment of rats with Fluox alone caused a moderate increase in D2 and, in turn, a slight decrease in D3 activities in cerebellum. On the other hand, the administration of T_3 by itself caused, in accordance with our expectation, a substantial decrease in pituitary D2 activity and a simultaneous increase in D1 and D3 activities practically in all tissues studied. In conclusion, the elaborated radiometric assays for IDs, UDPGT and ST were found very useful for the assessment of enzymatic changes at the molecular level, caused by the administration to the rats of Fluox, T_3 or the combination of these pharmaceuticals.

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GESTATIONAL HYPOXIA ALTERED POSTNATAL DEVELOPMENT AND INDUCED LONG-LASTING BEHAVIORAL CHANGES IN RATS

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Lowered blood oxygen level during pregnancy may have long-lasting or even permanent effect on health of the offspring. The aim of our study was to assess effect of gestational hypoxia on postantal development and behavioral changes (activity, anhedony, anxiety) of the rat offspring. Hypoxia (10.5% O_2) was induced on GD19 and 20 for 8 h per day. The weight of hypoxic group was significantly elevated compared to control group and this increase in weight persisted up to adulthood. Sensorimotor development of hypoxic pups was delayed, seen as significantly lower percentage of appropriate reaction at air-righting and startle reflex test. The activity of hypoxic pups in the open field at weaning was significantly lower than control, especially in female rats. However, this activity did not differ from control group on postnatal day 85. Although hypoactivity was normalized at adulthood, other hypoxia-induced behavioral changes were apparent. Hypoxic group showed anxiety-like behavior with significantly more entries to dark zone in light-dark test and anhedony in sucrose preference. In conclusion our model showed crucial role of oxygen supply within last days of gestation for proper behavioral development and coping strategies.

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CYTOTOXICITY AND ANTI-INFLAMMATORY EFFECT OF CHLOROQUINE AND HYDROXYCHLOROQUINE IN BV-2 MICROGLIAL CELLS: ROLE OF REDOX HOMEOSTASIS CHANGES

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Chloroquine (CQ), a commonly used anti-malaria drug, was also shown to have immunomodulatory properties. In addition, oxidative stress-promoting effects were reported for CQ cytotoxicity along with protective effects of antioxidant co-treatment. In this study, CQ and its derivative hydroxychloroquine (HCQ) were evaluated for their cytotoxic and anti-inflammatory properties in murine BV-2 microglial cells, an accepted model of the brain-resident macrophages playing a key role in neuroinflammation.

The 24 h incubation with CQ caused some more profound viability decline of BV-2 cells than did HCQ, as confirmed by MTT assay. However, this was not associated with upregulation of intracellular reactive oxygen species (ROS) levels, as confirmed by dichlorodihydro-fluorescein diacetate (DCFH-DA) assay and flow cytometry. The co-treatment with antioxidant SMe1EC2 suppressed the basal ROS levels and enhanced MTT conversion in CQ-treated BV-2 cells that might be ascribed to proliferation-promoting effect of SMe1EC2.

The non-toxic concentrations of CQ and HCQ showed a mild suppression of lipopolysaccharide (LPS)-stimulated NO release by BV-2 microglia, as documented by measurement of nitrite levels in media by Griess method. This effect was associated with promotion of intracellular ROS production with a more profound effect confirmed for CQ. The preliminary data from Western blot analysis point to negligible influence of either CQ or HCQ on expression of proteins iNOS, phospho-Erk1/2/Erk1/2 and HO-1. However, LC3-II/LC3-I ratio was increased in CQ- and HCQ-treated activated cells in comparison to LPS-stimulated control, pointing to inhibition of autophagy flux.

In conclusion, the results of our study point to a role of intracellular oxidative stress in modulation of inflammatory profile of microglial cells by CQ and HCQ with some higher relevancy for CQ.

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BIOTRANSFORMATION OF IVERMECTINE IN SOYA *(GLYCINE MAX)*

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Veterinary pharmaceuticals are used in large amounts in modern husbandry for treatment and prevention of diseases in animals. These drugs represent important source of environmental pollution as they can reach environment through the treatment processes, inappropriate disposal of used containers, unused medicine or livestock feed, and manufacturing processes. Pharmaceutical as well as other xenobiotics enter plant body and can induce stress and consequent response. The production of reactive oxygen species (ROS), which are associated with the direct damage of various biomolecules, and subsequent alterations in plant cell redox state and antioxidant mechanisms are usually the first responses to environmental. The ivermectine was chosen according to their importance in anthelmintic therapy, occurrence in waste water and documented negative effects on non-target organisms.

Soybean (Glycine) intake has received interest due to its health benefits, such as lowering the risk of chronic diseases – heart diseases and cancers, especially breast and prostate cancers, osteoporosis, and diabetes. Soy antioxidant activities and the role of soya antioxidants such as isoflavones have received increasing interest since it has been recognized that soybeans may have therapeutic activities in addition to health promotion.

The objectives of this study were to identify the IVM metabolites and IVM biotransformation pathways in soya (*Glycine max*). Plants were incubated with IVM (10 μM) for 2 and 4 weeks. Before analysis, homogenized samples were subjected to liquid–liquid extraction. The samples were analysed using UHPLC/MS (QqQ) in positive-ion mode. The results showed that IVM entered plant and enzymatic systems of plant were able to transform IVM via several reactions.

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ESTROGEN AND ANDROGEN RECEPTOR BINDING AFFINITY OF BISPHENOLS ESTIMATED BY QSAR

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Chemicals might interact with proteins such as the estrogen receptor (ER) or androgen receptor (AR) resulting in initiation of a cascade of biological effects and perturbation of the endogenous hormone system. Despite the complexity of the endpoints for reproductive impairment, it has been long appreciated that chemical binding to the ER or AR is one of the important mechanisms of interference with the reproductive process. Since reprotoxicity test methods are very expensive and time-consuming, alternative procedures are being developed.

It is also known that the ER or AR are much less of a lock-and-key interaction than highly specific receptors. The ER and AR are nonspecific enough to permit binding with a diverse array of chemical structures. There are three primary ER binding subpockets, each with different requirements for hydrogen bonding. The steroidal compounds usually interact at two points within the ER using two hydrogen-bonding groups. However, there are also chemicals with one hydrogen-bonding group that bind ER and cause subsequent gene activation. For AR the most important parameter seems to be distance between nucleophilic sites in the molecules, the oxygen atom connected to a cyclic carbon atom (associated with the 3-position in the steroidal skeleton) and the oxygen atom in the hydroxyl group (associated with the 17-position in the steroidal skeleton). Also partition coefficient log Kow (n-octanol-water) of binding chemical plays significant role.

Defining the boundaries of these chemicals is the challenge for (Q)SAR and computational chemistry. The aim of the presented work was to characterize the groups of bisphenols with potential to bind to ER or AR and cause adverse effects in the human organism. All the evaluated bisphenols showed high and even very high affinity to the ER binding subpockets.

These compounds may act as a gene activators and cause adverse effect in the human, especially during the pregnancy, breast-feeding and developing.

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NEW (PYRIDINE-2-SULFONATE) SILVER COMPLEX: DESIGN, DNA BINDING AND CYTOTOXIC ACTIVITY

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In recent years, more attentions focused on the coordination compounds as metallo-pharmaceuticals with antitumor activity. It culminated with the discovering of cisplatin and its derivatives as the antitumor drug and carried on with the finding of novel other non-platinum based chemotherapeutic agents. These pharmaceuticals emerging from the interface of pharmacology, toxicology, biochemistry and inorganic chemistry, have achieved access over traditional organic drugs.

Toward this goal, we have prepared new silver pyridine-2-sulfonate complex, which was characterized by X-ray and variety of analytical techniques. We have investigated the antiproliferative effect of new complex, free ligand and silver sulfadiazine on mouse leukaemia L1210 by MTT assay. The highest cytotoxic effect of Ag complex was observed after 48 h cultivation against S cells (IC_{50} = 1.4 μM) and R cells (IC_{50} = 1.2 μM). On the other side, no cytotoxic effect of free ligand was observed against all tested cells (IC_{50} >5 μM after 24, 48 and 72 h cultivation). Moreover, the DNA-binding properties of this new metal complex were investigated by electronic absorption, fluorescence, and CD spectra. To establish the mechanism of anticancer action of complex, also topoisomerase (Topo I) inhibition assay were conducted. Topo I inhibition study have shown that Ag complex inhibits its activity at concentration of 30 μM. Our results provide useful information about complex-DNA interactions, which are valuable for the rational drug designing having enhanced activity and greater clinical efficacy.

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DOSE-RESPONSE RELATIONSHIP OF SELECTED ANTIDEPRESSANTS ON PARAMETERS OF ISOLATED PERFUSED RAT HEARTS

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Depression is a very common and disabling disease with important social and economic implications affecting 16–17% of population – prognosis for 2020 is 40% (1). From economical point of view depression belongs among five most expensive diseases over the world (2). Mortality of mentally diseased people is higher in comparison to corresponding aged-matched individuals (3) and is probably related to high incidence of cardiovascular disorders (4). Antidepressants (AD) are drugs used for the treatment of major depressive disorder. The earliest and probably most widely accepted scientific theory of antidepressant action is the monoamine hypothesis which states that depression is due to an imbalance (most often a deficiency) of the monoamine neurotransmitters (namely serotonin, norepinephrine and dopamine). In our study we focused on selected AD citalopram (serotonin reuptake inhibitor), venlafaxine (serotonin–norepinephrine reuptake inhibitor), melatonin (affecting dual melatonergic-serotonergic pathway) and amitriptyline (tricyclic antidepressant) used as a standard and their dose-response relationship on functional parameters of isolated spontaneously beating perfused rat heart. Doses of applied substances were in concentration range between 10^{-9} – 10^{-4} mol.l^{-1} and each concentration was applied for 10 minutes. Increasing concentration of tested AD slowed heart rate, decreased the incidence of all forms of ventricular premature beats, except citalopram, in which opposite slope of dependence and even episodes of tachycardia were observed. There was a tendency to decrease the left ventricular developed pressure and contractility index, as well as heart product, with increasing concentration of AD. The coronary flow was mildly suppressed by tested drugs. According to the obtained data we determined 10^{-7} mol.l^{-1} as the effective concentration (EC50). The character of changes in selected parameters depended on the type of representative AD.

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[1] Kořinkova V., et al. (2007). *Metodický list racionálnej farmakoterapie* **42**: 11(3–4), 1–8; [2] Janů L., et al. (2007). *Medicína pro praxi* **1**: 24–27; [3] Walker ER, et al. (2015) *JAMA Psychiatry* **72**(4): 334–341; [4] Laursen TM, et al. (2009). Arch Gen Psychiatry. 66(7): 713–720.

EFFECTS OF SELECTED METAL NANOPARTICLES ON MOUSE TESTIS LEYDIG CELLS *IN VITRO*

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Despite a great potential benefit of silver (Ag) and gold (Au) nanoparticles (NPs) in the areas of biomedical, pharmaceutical and industrial applications, there has been an increased interest in studying their possible deleterious effects in biological systems. Current data have suggested that NPs may pose adverse effects on male reproductive functions, mainly due to modification of the testicular structure, impairment of spermatogenesis and alteration in the biosynthetic and catabolic pathways of testosterone.

The present study aimed to investigate a potential toxicity of two different sizes (20 nm and 100 nm) of Ag and Au NPs on mouse somatic Leydig TM3 cells, the testosterone-producing cells of the testis. TM3 cells were cultured with the different concentrations of Ag NPs (0.1–10 μg/ml) or Au NPs (0.16–16x10^{10} or 0.19–19x10^{8} particles/ml) in the absence or presence of luteinizing hormone (LH, 100 ng/ml) for 24, 48, and 72 h. Cell viability was assessed by MTT and CytoTox-ONE Homogenous Membrane Integrity (LDH) assays. The Apo-ONE Homogeneous Caspase-3/7 Assay was used to measure the activities of caspase-3 and -7 for assessment of cell apoptosis. Testosterone levels in culture media were measured by radioimmunoassay commercial kits. Treatment of TM3 cells with Ag and Au NPs induced a significant concentration- and time-dependent inhibition of cell viability and increase in cell apoptosis. The smaller NPs showed more deleterious effects. Alterations in testosterone secretion by TM3 cells were observed by the action of Ag and Au NPs. The mechanisms involved in Ag and Au NPs-induced toxicity should be further investigated.

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MICRORNAS IN DIAGNOSIS AND THE PREVENTION OF DRUG-INDUCED CARDIOTOXICITY

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Drug-induced cardiotoxicity is a serious problem associated with the administration of many drugs. Prediction of the onset of heart damage is still mainly based on the detection of a circulating cardiac troponin. MicroRNAs (miRNAs) have been reported to be affected by drugs and other xenobiotics, and the potential of miRNAs as biomarkers and diagnostic tools has been considered. In recent years, an association of certain miRNAs with the cardiotoxicity of some drugs, namely anthracyclines, bevacizumab, cyclosporine A and isoprenaline, has already been found.

We have reviewed available information about the changes in miRNAs levels induced by cardiotoxic drugs. We have focused on three aspects: the altered expression of miRNAs in the heart upon treatment with cardiotoxic drugs, circulating miRNAs as promising early biomarkers of cardiotoxicity, and the potential of miRNAs in the prevention and/or attenuation of drug-induced cardiotoxicity. The targeted changes in the level of certain miRNAs by antagomiRs and miRNA-mimics are also described and evaluated. In addition, the cardioprotective mechanism of various natural compounds via their effect on miRNA levels are examined.

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OXIDATION OF AN ANTICANCER DRUG ELLIPTICINE BY HUMAN AND RAT CYTOCHROMES P450 LEADING TO ITS DETOXIFICATION AND ACTIVATION: A COMPARATIVE STUDY

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Ellipticine is a drug exhibiting significant antitumor and anti-HIV activities. The prevalent mechanisms of antitumor, mutagenic and cytotoxic activities of ellipticine were suggested to be intercalation into DNA, inhibition of DNA topoisomerase II activity and formation of covalent adducts with DNA after being activated with CYPs. Here, we compare the efficiency of human and rat recombinant CYP enzymes expressed in Supersomes^{TM} (microsomes from Baculovirus transfected insect cells containing recombinantly expressed human CYPs and NADPH:CYP reductase with or without cytochrome b_5) to oxidize ellipticine to individual metabolites and to form DNA adducts. Moreover, we evaluated whether oxidation of ellipticine to metabolites generating DNA adducts (13-hydroxy-, 12-hydroxyellipticine and ellipticine N^2-oxide) correlated with formation of these adducts. All tested recombinant CYPs oxidized ellipticine to up to five metabolites: 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide. 13-Hydroxyellipticine, the metabolite forming the major ellipticine-deoxyguanosine adduct in DNA, was generated predominantly by a CYP3A subfamily of both humans and rats, followed by CYP1A1, 1A2, rat 2D1 and human CYP2D6*1. Differences were found in efficiencies of CYPs of a 2A subfamily in both species. While rat CYP2A1 and 2A2 oxidize ellipticine to 13-hydroxyellipticine with efficiency similar to CYP2D1, human CYP2A6 was much less active. 12-Hydroxyellipticine was produced by rat CYP2A1 with the highest efficiency, followed by human CYP2C19 and rat CYP1A1. Among the CYP enzymes tested, human recombinant CYP2D6*1 was the most efficient enzyme generating ellipticine N^2-oxide followed by human CYP3A4 forming about four times lower amount of this ellipticine metabolite. CYP3A2 generated the highest amounts of this metabolite among the rat CYP forms. Here, we also showed that all CYPs activate ellipticine to form DNA adducts. Two major DNA adducts found to be formed on deoxyguanosine from 13-hydroxy- and 12-hydroxyellipticine, were generated. Formation of 13-hydroxyellipticine correlated with generation of the DNA adduct 1. In the case of adduct 2, situation is more complicated as there are two ellipticine metabolites responsible for its production. Therefore, the correlation coefficients for the formation of adduct 2 and 12-hydroellipticine (or ellipticine N^2-oxide), were less significant.

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ANTHELMINTICS IN PLANTS – THE EFFECT ON TRANSCRIPTOME

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Anthelmintics, the drugs against parasitic worms, are widely used in human and veterinary medicine, nowadays. The usefulness of anthelmintic drugs is indisputable, but at the same time they pose a risk to ecosystems. With excrements of treated animals, anthelmintics can get into the environment and there affect non-target organisms – free-living invertebrates and wild plants. In our project, the most frequently used anthelmintics (albendazole, fenbendazole, flubendazole, ivermectin, monepantel) were used, and different plant species were tested, also the model plant *Arabidopsis thaliana (*wild type, *Brassicaceae).*

The presented work is the part of this project. The aim of the study is to get information about the effects of anthelmintics on hydroponics cultures of *Arabidopsis thaliana* and changes in plant transcriptome. The broad-spectrum benzimidazole anthelmintic fenbendazole was first used. Hydroponics cultures was stressed by 5 μM fenbendazole. The effect was studied after 24 and 72 hours of stress. The microarray analysis was performed. For general expression at the transcription level were used Agilent-based microarrays.

Exposure to fenbendazole in 5 μM concentration resulted in up-regulation of 104 and down-regulation of 64 transcripts in roots after 24 hours, up-regulation of 10 and down-regulation of 20 transcripts in roots after 72 hours. Significantly stronger response was recorded n rosettes where transcription of 193 genes was increased and 272 genes was decreased after 24 hours, 393 genes was increased and 403 genes was decreased after 72 hours.

Now we are working on the same experiment with the anthelmintic drug ivermectin. It is a macrocyclic lactone. It is used in human and veterinary medicine against parasitic nematodes and some ectoparasites. The results will be presented at the poster section. It is almost the first study of the effect of ivermectin on plants.

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PHARMACEUTICALS AND PLANTS: TOXIC EFFECT OF IVERMECTIN ON PLANT PROTEOME

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In environment, ivermectin (IVM), a broad spectrum anthelmintic used especially in veterinary medicine, may impact non-target organisms. The plants uptake, metabolise and accumulate IVM as another organic xenobiotics. Nevertheless, the informations about IVM effects in plants are limited. Therefore, we investigated the effect of IVM and its metabolites on the protein expression in model plant *Arabidopsis thaliana.*

Hydroponics cultures were stressed by 5 μM fenbendazole. The effect was studied after 24 and 72 hours of stress. Comparative proteomic analysis (nano LC-MS) was provided to identify the proteins expressed under this stress. More than 5600 proteins were identified there. The proteins with more than 2-fold change in expression in comparison to control, and proteins, which were expressed only in treated samples (at least 3 from 4) or only in control were selected. Exposure to IVM in 5 μM concentration resulted in up-regulation of 51 and 100 and down-regulation of 42 and 51 proteins in roots after 24 and 72 hours, respectively. In rosettes expression of 42 and 34 proteins was increased and expression of 79 and 80 proteins was decreased after 24 and 72 hours, respectively. In the roots were predominantly up-regulated proteins from plastids (loucoplats) involved in various biological processes as electron transport and energy generating pathways. On the other hand down-regulated were the proteins with structural molecule activity. In the leaves, enzymes localized in the cell wall were up-regulated, especially after 24 hours and down-regulated the proteins of endoplasmatic reticulum. Increased expression was found in only few biotransformation enzymes (e.g. superoxide dismutase). There was little difference between 24 and 72 hours.

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EFFECT OF *CIS*-NEROLIDOL, *TRANS*-NEROLIDOL AND FARNESOL ON THE MRNA AND PROTEIN EXPRESSION OF PHASE I XENOBIOTIC-METABOLIZING ENZYMES IN PRECISION-CUT HUMAN LIVER SLICES

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Sesquiterpenes *cis*-nerolidol (cNER), *trans*-nerolidol (tNER) and farnesol (FAR) comprise a group of plant secondary metabolites with numerous biological and pharmacological activities, among which an inhibitory effect on several phase I drug-metabolizing enzymes in humans have been observed. Being a part of plant essential oils, they are regularly consumed in food or dietary supplements. Moreover, they are officially approved food flavors and are widely used in cosmetic industry. They may, therefore, enter the human body in concentrations that may eventually lead to serious

drug interactions in treated patients. Accordingly, we investigated the effect of cNER, tNER and FAR on mRNA and protein expression of several important phase I drug-metabolizing enzymes in human liver. For the investigation, precision-cut human liver slices were cultivated in a medium supplemented by cNER, tNER or FAR in 10 μM concentration for 24 hours. Known inducers of cytochrome P450 enzymes (CYP) rifampicine and β-naphthoflavone were used as positive controls. The mRNA expression of three CYP isoforms, namely CYP 3A4, CYP 2B6 and CYP 2C, as well as the expression of carbonyl reductase 1 (CBR1) and aldo-keto reductase 1C (AKR1C) was detected using real-time quantitative PCR. Protein levels of these enzymes were detected using western blot technique. A statistically significant inhibitory effect of tNER and FAR on the mRNA expression in one human liver sample was observed. In both cases, the studied compounds inhibited the mRNA expression of CYP 3A4, CYP 2C, CBR1 and AKR1C. However, their effect on CYP 2B6 was rather contradictory. While tNER acted as an inhibitor and caused a significant decrease in the CYP 2B6 mRNA expression, FAR, on the other hand, caused a considerable increase in the mRNA expression. Moreover, the mRNA expression inhibition of CYP 2C and AKR1C by FAR in two different human liver samples was detected. The results, however, differ among individual human liver samples presumably due to possible inter-individual variability.

This study was supported by the Czech Science Foundation, Grant No. 18-09946S and P303/12/G163.

INTERACTION OF NEW POTENTIAL ANTICANCER DRUGS WITH HUMAN LIVER MICROSOMAL CYTOCHROMES P450

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Cytokinins are group of phytohormones that are involved in many processes in plants. These processes including e.g. growthing, differentiation and leaf senescence. However, they also have various activities in animals and humans. Three cytokinin derivates (BPA-302, BP-21 and BP-117) were tested for their potential to inhibit activities of human liver microsomal cytochromes P450 (CYP) *in vitro*. All activities (CYP1A2, CYP2A6 and CYP2C9) were determined according to established protocols. The results have shown no prominent inhibition of individual CYP activities with either compounds except in the case of CYP2C9 and BP-117. CYP2C9 plays a large part in drug biotranformation and its inhibition by another drug could lead to drug-drug interactions. However, this should be verified by further experiments and *in vivo*.

Supported by the grant IGA LF_2018_011 and LO1304 (NPU I)

WATER FLOWERS EXTRACTS OF *CORNUS MAS* AND *CORNUS KOUSA* INHIBIT ALDOSE REDUCTASE, WITHOUT ANY EFFECTS ON LIPOTOXICITY

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The effective inhibition of aldose reductase of leaves extracts from *Cornus mas* and *Cornus kousa* as well as some biological activities including antidiabetic activity of fruits from *Cornus* species were evaluated in the recent studies. In contrast, the biological effects of flowers from *Cornus* sp. have not been studied yet. The inhibitors of aldose reductase, the first enzyme in polyol pathway, are considered to be potential therapeutical agents in the development of chronic diabetic complications. Diabetes mellitus could be accompanied by elevated blood level of free fatty acids, which could cause lipotoxicity. Our study is focused on evaluation of potential inhibitory efficacy of water flower extracts from *Cornus mas* and *Cornus kousa* on isolated rat aldose reductase *in vitro*. The extracts were studied in the cell model of lipotoxicity as well, which presents a risk during diabetes. The cytotoxicity of the extracts on mouse fibroblasts cell line was evaluated. Both extracts showed effective inhibition of rat lens aldose reductase in non-toxic low concentrations. In contrary, the non-toxic concentrations of both extracts caused almost no effects in the lipotoxicity cell model induced by palmitic acid.

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COPPER-CHELATORS ARE ALSO RELATIVELY POTENT ZINC CHELATORS

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Zinc (Zn) is an essential metal that is involved in numerous physiological processes. It is required for the catalytic activity of approximately 100 enzymes and it plays a role in protein and DNA synthesis, cell division, wound healing and immunity. Zinc deficiency is characterized by growth retardation, loss of appetite, suppressed immune function, diabetes, etc. One of the causes of zinc deficiency can be the long-life use of metal chelators which are mostly non-selective. Such example are copper-chelators used in rare inherited disorder resulting in body copper excess – Wilson's disease.

The aim of this work was to study possible ability of D-penicillamine, trientine and ammonium tetrathiomolybdate (ATM) to bind zinc by a spectrophotometric method based on the competition between the tested compound and dithizone as an indicator. Various physiologically relevant pH levels ranging from 4.5 to 7.5 were tested. compound and dithizone as an indicator. Various

physiologically relevant pH levels ranging from 4.5 to 7.5 were tested.

All the compounds shown the non-selectivity for copper and the capacity to bind zinc. Experiments showed that the most potent Zn chelator was trientine. It can bind approximately 65% of Zn when is mixed with zinc ions in the molar ratio of 1:1 at pH 7.5. D-penicillamine and ATM showed lower chelating capacity. Surprisingly all of the tested compound showed higher capacity to form a complex with Zn ion in comparison with ability of D-penicillamine to chelate Cu ions.

Clinically used copper-chelators as well ATM are also relatively potent zinc chelators and hence they long term use can possibly result in toxicity associated with zinc deficiency.

INTERMITENT HYPOXIA *IN UTERO* AND FREQUENCY OF SKELETAL AND VISCERAL ANOMALIES IN THE RAT FOETUSES

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Chronic or intermitent hypoxia is a common pregnancy complication associated with intrauterine foetal growth restriction that may influence respiratory outcome at birth. An experimental model of intermitent intrauterine hypoxia is proposed. The objective of this study was to determine the effect of early (eGIH) and late gestational intermitent hypoxia (lGIH) on foetal growth, and incidence of skeletal and visceral anomalies. Pregnant Wistar/DV rats were exposed to the lower oxygen containing (10%O_2 for 8 hr/day) in a special hypoxic chamber during late gestational period (days 15–16 or 19–20 of gestation) or to the lowered oxygen containing environment for 12 hr on day 16 or 20 of gestation. On day 21 of gestation were assessed : the weight of live foetuses and frequency of skeletal and visceral anomalies. No gross malformations observed after repeated 8 –hour GIH or single 12- hour GIH. Pups exposed to intermitent hypoxia *in utero* weiged significantly less than the control pups only in the late GIH. Skeletal anomalies consists of anomalies of sternebrae (split, unossified, aditional, reduced ossification), phalanges (reduce ossification of metacarpals and metatarsals) and ribs (wavy, 13th or 14th pair of accessory thoracolumbar rudimentary ribs). Inspection of visceral anomalies revealed nasal anomalies (unseparate nasal conchae) brain and skull anomalies (internal hydrocephalus of moderate degree and not complet dilatation or undilatation of lateral brain ventricles). The frequency of skeletal and visceral anomalies is similar to the control group in early and late intermitent hypoxia without statistical significance. These data demonstrate that brief, intermitent periods of intrauterine hypoxia have no significant effect on incidence of skeletal and visceral anomalies in rat fetus.

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ANTIDEPRESSANT SERTRALINE AS A POTENTIAL POLLUTANT IN WATER ENVIRONMENT

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The aim of this article is to describe potential risk of sertraline as a pollutant in water environment. Sertraline is type of antidepressant from a group of selective serotonin reuptake inhibitors, which primarily affect the nervous system, mainly brain. Its usually taken by people as pharmaceuticum for the treatment of depression, panic disorders, anxiety disorders, anorexia, social phobia and panic stress. In recent years, due to to people‘s hurried lifestyle the rate of use sertraline and other antidepressants in the human population has been steadily increasing. Sertraline presents a certain ecology danger for aquatic environment, where it can continually get throw wastewater treatment plants and persists in range between ng/l to μg/l. So far many side effects of sertraline to non-target water organisms were described. Studies have shown that sertraline causes changes in swimming activity, behavioral modification, enzyme activity (SOD, CAT, GST, AchE), feeding rate and food consumption in fish. The main affected organs of fish were brain, kidneys and liver. For this reason it is very important to constantly monitor the environmental concentrations of sertraline as well as to study its other negative risks.

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STUDY OF PROTECTIVE EFFECTS OF ANTIOXIDANTS TARGETING AND NOT TARGETING MITOCHONDRIA

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3T3 cells and tumor cells MCF-7 were exposed to the action of various concentrations of ascorbic acid (0–6 mM) and cupric ions (0–6 μM) individually to show their effect. Viabilities of the cells were determined by using the MTT test. The results showed dose-dependent decrease in cell viability when examining ascorbic acid. Both cell strains were almost equally sensible to ascorbic acid. Similar results were observed when examining cupric ions alone. The percentages of the viability of 3T3 and MCF-7 cells damaged by ascorbic acid in the presence of cupric ions were ca. 50% and 30%, respectively.

The addition of *N*-acetylcysteine did not result either in higher viability of 3T3 cells or in higher damage of MCF-7 cells.

Further, 3T3 and VH10 cell lines were subjected to the oxidative damage by Cu(II) ions and ascorbate, which form hydroxyl radicals. MitoQ as a mitochondrially targeted antioxidant was examined to prevent cell death and was compared with trolox as a reference standard serving as an antioxidant. MitoQ at lower concentrations (up to 500 nM) increased viability of cells. In contrast, MitoQ at higher concentrations (over 500 nM) decreased the viability of both cell strains. The former observation indicates that the application of MitoQ should be evaluated also from the point of its cell-destructive potential.

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COMPARISON OF INHIBITORY EFFECT OF RESVERATROL AND ITS DERIVATIVES ON ENZYME ACTIVITIES IN HUMAN LIVER MICROSOMAL CYTOCHROME P450 3A4 USING TWO INDEPENDENT SUBSTRATES

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Resveratrol and its derivatives belong to stilbenoids which are polyphenols naturally occurring in grapes, wine, berries or peanuts. Stilbenoids are common parts of human diet and are known to be health benefit agents for human. Due to their anti-inflammatory, anti-asthmatic, anti-diabetic, hypolipidemic and antioxidant properties are these polyphenols in forefront of interest.

We investigated the comparison of mechanism of inhibitory effect of eight stilbenoids (dihydroresveratrol, oxyresveratrol, cis-resveratrol, trans-resveratrol, pinostilbene, pterostilbene, cis-piceatannol and trans-piceatannol) on human hepatic cytochrome P450 CYP3A4/5 present in human microsomes using two independent substrates (midazolam and testosterone). Final concentration of compounds examined was chosen in the range 0 – 100 micromolar in the reaction mixture.

All compounds studied influenced CYP3A4/5 activity with substrate testosterone. Oxyresveratrol has been shown to be a mixed inhibitor and pterostilben a competitive inhibitor of CYP3A4/5, other compounds tested have been found to be noncompetitive inhibitors of this activity of CYP3A4/5 with substrate testosterone. With substrates midazolam, only oxyresveratrol, trans-resveratrol and pinostilben influenced activity of CYP3A4/5 and these compounds have been shown to be the noncompetitive inhibitors of this activity of CYP3A4/5.

The differences in mechanism of inhibitory effect are related to the promiscuity of CYP3A4/5 active site. CYP3A4/5 has a relatively large and flexible active site cavity that can accommodate multiple substrate molecules to achieve optimal activity. Hydroxylation site of testosterone molecule is oriented differently to this site in midazolam molecule. This could be the reason why the mechanisms of inhibitory effects with substrates testosterone and midazolam are not the same.

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THE EFFECTS OF NATURAL PSEUROTINS ON SELECTED IMMUNE CELL FUNCTIONS

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Pseurotin A is a secondary metabolite produced by many species of fungi, mainly by Aspergillus sp. and Penicillium sp. During the pseurotin A biosynthesis, a large number of closely related bioactive compounds, such as pseurotin D or synerazol is also formed. Natural pseurotins have antimicrobial and antiparasitic activity. Interestingly, a few studies suggested effects of pseurotins in eukaryotes, such as antiangiogenic activity. In this study, we focused on effects of natural pseurotins on physiological functions of immune cells. Our results employing endotoxin-activated myeloid RAW264.7 cells (murine peritoneal macrophages) show that pseurotins (Pseurotin A, Pseurotin D and some structure analogs) were able to significantly reduce NO production in a concentration-dependent manner, both at the level of nitric oxide (NO) production and at the level of inducible NO synthase expression. These pseurotins also inhibited expression of early response cytokine interleukin (IL)-6 but not tumor necrosis factor α. Moreover, pseurotins were able to inhibit proliferation of RAW264.7. Other tested immune cells were mouse B-lymphocytes. They were isolated by sorter Aria II based on CD19 positivity. Interestingly, we show that pseurotins inhibited immunoglobulin E production of B-lymphocytes activated by a combination of E. coli endotoxin and IL-4. These effects were also related to changes in proliferation of B-lymphocytes via inhibition of JAK/STAT signaling pathway. We did not see any cytotoxic effects of pseurotins on these cells. It can be concluded that natural pseurotins are able to reduce oxidative stress, inhibit production of cytokines, NO and are able to modulate B-lymphocyte immune response.

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METABOLISM OF A TYROSINE KINASE INHIBITOR LENVATINIB BY HUMAN CYTOCHROMES P450 *IN VITRO*

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Lenvatinib is an oral, multitargeted tyrosine kinase inhibitor (TKI) of vascular endothelial growth factor receptors (VEGFR1-VEGFR3), fibroblast growth factor receptors (FGFR1-FGFR4), platelet-derived growth factor receptor (PDGFR)α, rearranged during transfection (RET), and v-kit (KIT) signaling networks implicated in tumor angiogenesis. It is used for treatment of certain types of tumors of the thyroid gland and metastatic renal cell carcinoma. Based on preliminary studies using human hepatic microsomes, lenvatinib was suggested to be oxidized by cytochromes P450 (CYPs), mainly by CYP3A4, to its *O*-demethylated metabolite, a desmethylated form of lenvatinib. However, no direct prove of this suggestion was demonstrated. Therefore, the aim of this study was to investigate the metabolism of lenvatinib by human microsomal enzymes *in vitro* in detail. The metabolism of lenvatinib by human hepatic microsomes and recombinant CYPs expressed in Supersomes^{TM} was investigated. The lenvatinib metabolites were separated by HPLC and identified by mass spectroscopy. Utilizing human hepatic microsomes *O*-desmethyllenvatinib, *N*-depropylated lenvatinib and one additional metabolite were produced. Of all tested human CYP enzymes, the CYP1A1, 1A2, 2C19 and 3A4 enzymes oxidize lenvatinib to its metabolites. *O*-desmethylated lenvatinib was generated by CYP1A1, 1A2 and 3A4, while CYP2C19 forms another metabolite; its structure has not yet been identified. CYP1A1 and 3A4 are also responsible for oxidation of lenvatinib to *N*-depropylated metabolite. Cytochrome b_5 plays an essential role in the CYP2C19 and 3A4 activities to oxidize lenvatinib. Besides CYPs, aldehyde oxidase (AO) oxidizes lenvatinib forming one metabolite; its structure has not yet been identified. Further characterization of structures of all lenvatinib metabolites formed by the tested enzymatic systems is under way in our laboratory.

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EFFECT OF HEXAHELICENE ON THE EXPRESSION OF CYP1A1

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The helicenes are polycyclic aromatic hydrocarbons (PAHs) with non-planar screw-shaped structures composed of ortho-fused benzene rings. Since many planar PAHs activate the aryl hydrocarbon receptor, this study examined whether [6]helicene and its derivative, 1-butyl-3-(2-methyl[6]helicenyl)-imidazolium bromide (compound **1**), affect the expression of cytochrome P450 1A1 (CYP1A1) in human hepatoma HepG2 cells. The MTT reduction assay showed that both [6]helicene and compound **1** significantly decreased the viability of HepG2 cells after 24 h of exposure. Compound **1** was less cytotoxic than [6]helicene with the IC50 values reaching 8.4 and 0.9 μM, respectively. After 24 h of HepG2 cell treatment with 5 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a prototypical CYP1A1 inducer, we found a 386-fold increase in the level of CYP1A1 mRNA and a 40-fold increase in CYP1A1 activity. In contrast, 24 h of exposure to [6]helicene (0.001–1 μM) and its derivative **1** (1–5 μM) resulted in mild or negligible changes in the mRNA levels and activity of CYP1A1. At the highest concentrations tested, CYP1A1 mRNA levels induced by [6]helicene and compound **1** increased 2.9-fold and 2.7-fold, respectively, and the activity of CYP1A1 reached 1.0-fold and 1.4-fold of the control values. We conclude that [6]helicene and its derivative **1** have a weak effect on the CYP1A1 pathway in HepG2 cells.

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CLIMATE CHANGE IMPACT ON THE TOXICITY OF PHENOXY HERBICIDES

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Climate change is a major concern for sustainable agriculture and food security. Crop productivity strongly depends on crop protection measures such as use of herbicides. Climate change could influence the fate and ecotoxicity of herbicides by altering their environmental partitioning and degradation, distribution and abundance of weeds and growth and development of weeds and crops. Differential responses of crops and weeds to elevated temperature and CO_2 may also cause shifts in their response to herbicidal application and their competitive interactions. The aim of the study was to examine the influence of elevated temperature and CO_2 on the effects of phenoxy herbicide (Chloro-2-methylphenoxyacetic acid (MCPA)) to spring barley (*Hordeum vulgare* L.) and common lambsquarter (*Chenopodium album* L.). Two climate scenarios were investigated: current climate (21 °C, 400 ppm CO_2) and future climate (25 °C, 800 ppm CO_2). *Ch. album* and *H. vulgare*, growing together in the microcosms, were sprayed with herbicide sprays solutions equivalent to 0.5–2 of field application rate. The growth and response of antioxidative defence system were evaluated after 2 weeks. Antioxidant enzymes superoxide dismutase

(SOD), catalase (CAT) and glutathione reductase (GR) were measured. Oxidative stress parameters, such as the concentrations of malondialdehyde were determined. Phenoxy herbicide severely inhibited the growth of *Ch. album*, altered activity of antioxidative enzymes and induced oxidative stress. Less pronounced effects of herbicides were also found in non-target *H. vulgare*, though the majority of effects were statistically insignificant. Moreover, reduced interspecific competition due to dramatic decrease in *Ch. album* growth at high herbicide dose led to an increase in *H. vulgare* biomass of roots and shoots. The results of our study show that the ongoing increases in temperature and atmospheric CO_2 concentration may have important consequences for crop-weed competition.

SELECTION OF SUITABLE REFERENCE GENES FOR GENE EXPRESSION STUDIES IN HUMAN LIVER SLICES

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Precision-cut liver slices are an interesting model due to its multicellular composition, preserved tissue architecture and intercellular communication. Its applicability to human tissues allows us to avoid interspecies differences and directly apply human tissues into multiple experimental designs. Despite a short time when liver slices are able to keep its viability, functionality and physiological processes (up to 24 hours), it is still a very good model for gene expression studies. However, there exists no study validating this model for selection of a suitable reference gene (or their combination). Therefore, we decided to perform a validation study, since selection of inappropriate reference gene can influence the trend and deviation of results. In our experiments, three human liver samples received from surgery were used to obtain precision-cut liver slices (8 mm diameter, 150 μm thickness), which were cultivated for 24 hours in 85% O_2/ 5% CO_2 atmosphere and samples were collected after 0, 4, 8, 12, 18 and 24 hours. Known cytochrome P450 (CYP) inducers β-naphthoflavone and rifampicine were used as positive controls at all time points. To verify the viability of liver slices, ATP content and lactate dehydrogenase leakage were measured. For reference gene validation, GAPDH, SDHA, ACTB, B2N, HPRT and YHWAZ were chosen. These genes were compared using RefFinder, a free web tool that uses several other softwares, such as geNorm, Normfinder, BestKeeper and the comparative Ct method, and gives those genes a comprehensive gene ranking. The gene stability was compared for the whole 24-hour interval and also for each time point separately. The gene expression of CYP3A4, 2B6, 1A2 and 2C was also determined.

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