Interdiscip Toxicol. 2019; **Vol. 12**(3): 111–119.
doi: 10.2478/intox-2019-0013

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Cichorium intybus attenuates streptozotocin induced diabetic cardiomyopathy via inhibition of oxidative stress and inflammatory response in rats

Manju SHARMA [1,4], Aiman AFAQUE [1], Shridhar DWIVEDI [2], Zeeba S. JAIRAJPURI [3], Yasmeen SHAMSI [5], Mohd Faiyaz KHAN [6], Mohd Ibrahim KHAN [7], Danish AHMED [7]

[1] *Department of Pharmacology, Faculty of Pharmacy, Hamdard University, New Delhi, India*
[2] *Department of Medicine and Preventive Cardiology, Hamdard Institute of Medical Sciences &Research, Hamdard University, New Delhi, India*
[3] *Department of Pathology, Hamdard Institute of Medical Sciences &Research, Hamdard University, New Delhi, India*
[4] *Department of Pharmacology, Hamdard Institute of Medical Sciences &Research, Hamdard University, New Delhi, India*
[5] *Department of Mahiyatul Amraz, Faculty of Medicine (Unani), Hamdard University, New Delhi , India*
[6] *Department of Clinical Pharmacy, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj, Kingdom of Saudi Arabia*
[7] *Department of Pharmaceutical Sciences, Sam Higginbottom University of Agriculture, Technology & Sciences, (SHUATS), Allahabad, India*

ITX120319A01 • Received: 04 August 2017 • Accepted: 04 April 2019

ABSTRACT

The aim of the present study was to investigate the effects of *Cichorium intybus* on lipid peroxidation activities of both enzymatic and non-enzymatic antioxidants, inflammatory mediators, myocardial enzymes and histopathology of cardiac tissues in experimental diabetic cardiomyopathy (DCM). DCM was induced by single intraperitoneal injection of streptozotocin (STZ) (40 mg/kg) combined with high energy intake in rats. Seed extract of *Cichorium intybus* (CIE) (250 mg/kg & 500 mg/kg) was administered orally once a day for 3 weeks. Phytochemical investigations of seed extract revealed presence of some active ingredients such as alkaloids, tannins, saponin, phenols, glycosides, steroids, terpenoids and flavonoids. Seed extract of *Cichorium intybus* confirmed a significant potency towards restoring the blood glucose, an elevation of the levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), blood glutathione (GSH), TNF-α and IL-6 and a reduction in the levels of catalase (CAT) was observed following the STZ treatment. Oxidative stress was accompanied by myocardial degeneration as evidenced by histopathological examination of cardiac tissues. Administration of CIE reduced the lipid peroxides level in heart. Serum levels of AST, GSH, LDH and SOD were brought down to physiological levels by CIE in STZ induced DCM rats. CIE also markedly down-regulated serum TNF-α and IL-6 levels. Catalase that was reduced in serum was brought back to near normal level. The extensive necrotic changes of cardiac tissue by STZ was minimized to normal morphology upon CIE administration. The study demonstrates the cardioprotective effect of CIE via inhibition of oxidative stress and pro-inflammatory cytokines.

KEY WORDS: *Cichorium intybus*; diabetic cardiomyopathy, streptozotocin

Introduction

India is pertinently considered as the diabetic capital of the world with the 60 million of diabetics when contrasted with the quantity of diabetic patients around the globe which is increasing quickly and is expected to achieve 439 million by 2030 (Shaw *et al.,* 2010). The long term complications of diabetes are of great concern. However, there is an increasing recognition that diabetes patients suffer from an additional complication termed diabetic cardiomyopathy (DCM). Diabetic cardiomyopathy, as an independent diabetic cardiovascular complication, is characterized by hypertension or valvular heart disease, the myocardial dysfunction in the absence of coronary artery disease. Past investigations reported that the pathophysiology of DCM results in increment of lipid accumulation, hyperglycemia, excessive generation of reactive oxygen species, cardiac inflammation and accumulation of cardiac fibrosis (Westermann *et al.,*

Correspondence address:
Danish Ahmed
Department of Pharmaceutical Sciences, Faculty of Health Science
Sam Higginbottom University of Agriculture Technology and Sciences, SHUATS
Post. Agriculture Institute, Naini, Allahabad - 211007, (U.P.), India.
TEL.: +91-9580001578 • E-MAIL: danish.ahmed@shiats.edu.in

2009; Falcão-Pires & Leite-Moreira, 2012). The existence of oxidative stress has been postulated in patients with diabetes. Lipid peroxidation (LPO) has been implicated in the pathogenesis of naturally occurring or induced diabetes (Baynes & Thorpe, 1996). Oxidative stress may be increased in diabetes owing to hyper-production of reactive oxygen species (ROS), such as $O_2^{\bullet}$, $OH^{\bullet}$ & H_2O_2 and/or a deficiency in the antioxidant defense system (Ahmed *et al.,* 2015). The cytotoxic action of STZ is allied with the generation of reactive oxygen species causing oxidative damage (Szkudelski, 2001). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH) provides support in scavenging the free radicals by breaking down the ROS (Kim JK *et al.,* 2009). Past investigation have reported a flaw in the scavenging machinery in diabetic patients as compared to the healthy control (Anwer *et al.,* 2012; Anwer *et al.,* 2007).

It has been revealed after extensive investigations within the last two decades that inflammation itself mediated most chronic illnesses such as cancer, neurological, autoimmune, diabetes and cardiovascular diseases. Series of genes expressed gets stimulated by activated macrophages during inflammation in host defense, which conclude the release of different inflammatory mediators, cyclooxygenase-2, nitric oxide, pro inflammatory cytokines etc. (Yoon *et al.,* 2009). Therefore, to prevent delay or treat the above ailment, inflammation should be suppressed. (Begum *et al.,* 2015).

In India, plants, either medicinal or non-medicinal, have been a vital source for treatment of various ailment along with diabetes. *Cichorium intybus*, usually called as Kasni, is used in Unani and Ayurvedic traditional system of medicine to treat hyperglycemia as well as other diseases in India. The genus *Cichorium (Asteraceae)* is made up of six species and it is a wild plant with major geographical native in Europe and is common in Australia, North America and China. Chicory, a common name to *Cichorium intybus,* is well known as a coffee substitute and also widely used medicinally to treat various diseases ranging from diabetes to common wounds. In Eurasian countries and some parts of Africa, *Cichorium intybus* is most commonly used medicinal plant. In spite of its traditional use, chicory is not described in the European Pharmacopoeia or in any official Pharmacopoeia of a European Union member state (European Medicines Agency, 2013). However, due to its ubiquitous distribution several parts of the plant have been used globally in traditional medicines (Süntar *et al.,* 2012).

With its wide therapeutic index different preparations of this plant are employed to treat various diseases. The juice extract is said to be a traditional remedy for tumors and for uterus cancer (Judžentienė & Būdienė, 2008). In South Africa, chicory syrup is used as a purifying medicine and tonic for infants (Van Wyk *et al.,* 1997) and stems, leaves, weed, and roots are made into a tea to treat jaundice. In Turkey, an ointment is made for wound healing from the leaves of chicory (Sezik *et al.,* 2001). The flowers of the chicory plant are used as an herbal treatment of everyday complaint such as appetite defects, sinus problems, cuts and bruises, and treatment of gallstones and gastroenteritis (Judžentienė & Būdienė, 2008). According to the European monograph, utilization of chicory roots includes the relief of symptoms related to mild digestive disorders like flatulence, slow digestion, abdominal fullness etc. and temporary loss of appetite (European Medicines Agency, 2010). The anti-malarial compounds, light-sensitive sesquiterpene lactones lactucin and lactucopicrin, were isolated and identified from chicory roots (Bischoff *et al.,* 2004, Pieroni, 2000). In India, various categories of folk medicine from chicory plant are used. Folkloric use of *c. intybus* has been well documented acting as a hepato-protectant. In a commercial Unani product of India, chicory seeds are one of the main ingredients of Jigrine, used for the treatment of various diseases of the liver (Ahmed *et al.,* 2003). Liv-52, a traditional Indian tonic used widely for hepato-protection (Huseini*et al.,* 2005) is also one of the herbal components of Ayurvedic system of Indian medicine.

Cichiroum intybus seed consist of Chicoric acid which is a major compound in methanolic extracts of chicory. Aliphatic compounds and their derivatives represent the major fractions while minor constituents of the plant comprised of terpenoids. The flowers of chicory comprise flavonoids, saccharides, essential oils, methoxy-coumarin cichorine etc. (Judžentienė & Būdienė, 2008). Numerous studies on *Cichiroum intybus* plant indicated that it possesses antimalarial, antiinflammatory, antimicrobial, antihelmintic, analgesic activity, antiallergic activity, antioxidant, tumor-inhibiting activity, gastro and hepatoprotective effects besides its positive influence in diabetes (Ahmed *et al.,* 2003; Huseini*et al.,* 2005; Nandagopal & Kumari, 2007; Miller *et al.,* 2011; Gürbüz *et al.,* 2002; Cavin *et al.,* 2005; Wesołowska *et al.,* 2006; Heimler *et al.,* 2009; Conforti *et al.,* 2008; Kim *et al.,* 1999; Pushparaj *et al.,* 2007). The antidiabetic effect of the aqueous seed extract of *Cichiroum intybus* has also been investigated in early-stage diabetic rats, chicory treatment led to the increase in insulin levels pointing toward the insulin-sensitizing action of chicory (Ghamarian *et al.,* 2012). However, there is a lack of experimental evidence on the favorable role of *Cichorium intybus* in STZ induced diabetic cardiomyopathy. In view of all the above reports we chose to evaluate chicory for possible beneficial actions in STZ induced diabetic cardiomyopathy.

Materials and methods

Experimental animals

Healthy Albino Wistar rats (8–10 weeks old), weighing about 150–180 g were procured from the Central Animal House Facility, Hamdard University, New Delhi, India. The animals had free access to standard laboratory food and water *ad libitum*, and they were housed in a natural light-dark cycle (12 h each). The experimental protocol was approved by the Institutional Animal Ethics Committee and the care of laboratory animal was taken as per the

guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Drugs and chemicals

The seeds of *Cichorium intybus* were procured from Hamdard Wakf Laboratories, India and was authenticated by chief scientist, Raw Material Herbarium and Museum, CSIR- NISCAIR (Ref. no. NISCAIR/RHMD/Consult/2014/2538/117). streptozotocin (STZ) was purchased from Sigma Aldrich, St. Louis, USA. The AST, LDH, SOD and CAT diagnostic kits were purchased from Span diagnostics, Surat, India. Rat TNF-α and IL-1β cytometric assay kits were purchased from BD Biosciences, India. All the other biochemicals and chemicals used in the present study were of analytical grade.

Induction of experimental diabetes

Experimental diabetic rats were induced by feeding with high fat diet (HFD) during the whole experimental period. HFD was prepared by adding 20% sucrose (w/w) and 20% lard (w/w) into normal pellet diet. After 8 weeks of dietary manipulations, rats were intraperitoneally injected with STZ (40 mg/kg) dissolved in 100 mM citrate buffer pH 4.5 bolus of STZ. Control rats were administered an equivalent volume of citrate buffer (Wang *et al.,* 2007). Blood glucose levels were measured 72 h after STZ injection and rats which had blood glucose values ≥11.6 mmol/l were used in the groups III–V.

Preparation of extract and preliminary phytochemical studies

Chicory seeds were cleaned and powdered using an electric mill. Every 200 g was soaked in 1 l of distilled water and refluxed for 20 minutes in a boiling water bath to make a 20% solution. The solution was allowed to cool at room temperature before being vacuum filtered through Whatman No. 1 filter paper. The filtrate was lyophilized and stored at –20 °C; every 100 g of powdered seed yielded about 8.2 g of lyophilized substance. The extract was suspended in distilled water and was administered orally to the animals using feeding tubes in doses of 250 mg/kg and 500 mg/kg. Different secondary metabolites are present in plant materials which exhibit various pharmacological activities. Crude extracts were subjected to phytochemical analysis for identification of alkaloids, cardiac glycosides, steroids, tannins, and saponins following standard protocol (Trease & Evans, 2002).

Experimental design

The rats were divided into five groups comprising of six animals in each group as follows:

- Group I: Healthy control, received citrate buffer (1 ml/kg, i.p.)
- Group II: *per se,* received only CIE (500 mg/kg, p.o.)
- Group III: Diabetic cardiomyopathy (DCM), STZ single dose (40 mg/kg, i.p.) + HFD
- Group IV: STZ + HFD + CIE (250 mg/kg, p.o.)
- Group V: STZ + HFD + CIE (500 mg/kg, p.o.)

Diabetic animals were treated with chicory seed extract (250 mg/kg and 500 mg/kg, p.o.) for 3 weeks. On the last day of experiment, blood samples were collected by retro-orbital puncture for biochemical estimations and animals were sacrificed by cervical decapitation under light chloroform anesthesia and organs were collected for histopathological examination.

Measurements of glycemic level in serum

By using digital glucometer (Gluco One) blood glycemic level was determined using serum.

Measurements of myocardial enzymes and inflammatory cytokines in serum

Aspartate Aminotransferase (AST) and Lacate Dehydrogense (LDH) were determined by colorimetric analysis using a spectrophotometer with the associated detection kits (Reitman & Frankel, 1957; McLauchlan DW *et al.,* 1988). TNF-α and IL-6 in serum were determined by cytometric method following the manufactures instructions (Brouckaert *et al.,* 1993).

Estimation of Superoxide Dismutase (SOD) and Malondialdehyde (MDA) in heart tissue

SOD activity was measured according to the method of Marlund and Marklund (Marklund & Marklund, 1974). The enzyme activity was expressed in unit/mg of protein and 1 unit of enzyme is defined as the enzyme activity that inhibits autoxidation of pyrogallol by 50%. The concentration of MDA was measured in heart using the method of Ohkawa *et al.* 1979 (Ohkawab *et al.,* 1979). Briefly, the heart tissues collected soon after sacrificing the rats, were suspended in 150 mM KCl and homogenized in Teflon homogenizer. The concentration of thiobarbituric acid reactive substances (TBARS) was expressed as nmol of MDA/mg of protein using 1,1,3,3,-tetraethoxypropane as standard. The protein were estimated by the method of Lowry *et al.* 1951 (Lowry *et al.,* 1951).

Determination of reduced and total glutathione (GSH)

Freshly collected heart tissues were weighed and 10% (w/v) homogenates were made in 1.15 M KCl using a motor driven Teflon-pestle. GSH was estimated using the method of Sedlak and Lindsay 1968 (Sedlak & Lindsay, 1968) that uses 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB). The total glutathione in blood was estimated by the method of Beutler *et al.*1963 (Beutler *et al.,* 1963). The absorbance was read at 412 nm.

Determination of CAT

CAT activity was estimated following the method of Claiborne (1985) based on a decrease in absorbance at 240 nm due to consumption of hydrogen peroxide (H_2O_2).

Determination of SGOT

Recombinogen kit was used for SGOT estimation. 2 ml of blood samples was collected in centrifuge tube and left to stand for 1 hour at 37 °C and further samples was cooled in refrigerator for 3 hours. The clot formed was then removed and serum samples were decanted out. These serum samples were then centrifuged at 3000 rpm

Table 1. Isolated and identified compounds from the extraction of *Cichorium intybus* seed.

S.No.	Compound
1.	11,13-Dihydrolactucopicrin
2.	11β,13-Dihydrolactucin
3.	Caffeic acid
4.	Chicoric acid
5.	Chlorogenic acid
6.	Cichorioside
7.	Cichorioside B
8.	Cyanidin
9.	Kaempferol-3-O-glucoside
10.	Kaempferol-3-O-glucosyl-7-O-(6″-O-malonyl)-glucoside
11.	Kaempferol-7-O-glucoside
12.	Lactucin
13.	Lactucopicrin
14.	Malic acid
15.	Pelargonidin-3-O-monoglucuronide
16.	Quercetin-3-O-glucuronide-7-O-(6″-O-malonyl)-glucoside
17.	Quercetin-7-O-(6″-O-acetyl)-glucoside
18.	Quercetin-7-O-galactoside
19.	Tricin-3-O-glucoside
20.	β-Sitosterol

Table 2. Effect of *Cichorium intybus* seed extract on blood glucose of rats.

S.No.	Treatment	Glucose (mg/dl)
1.	Control (1 ml/kg. i.p.)	100.67±6.19
2.	Per se (500 mg/kg, p.o.)	116.33±2.4
3.	STZ Single dose (40 mg/kg, i.p.)+HFD	306.33±10.18*
4.	STZ+HFD+CIE (250 mg/kg, p.o.)	121.33±4.01**
5.	STZ+HFD+CIE (500 mg/kg, p.o.)	106.67±3.26**

Data shown as means±SEM from 6 animals. All comparisons of group IV and V with group III by student's t test (*p<0.001). All comparisons of group I, II, IV and V with group III by ANOVA (**p<0.001).

Table 3. Effect of *Cichorium intybus* seed extract on glutathione in blood and heart muscle of rats.

Groups	Treatment	Whole Blood glutathione (mg %)	Tissue GSH (µmole/g. wt of tissue)
I	Control (1 ml/kg. i.p.)	1.09±0.053	1.89±0.082
II	Per se (500 mg/kg, p.o.)	1.14±0.107**	1.97±0.077**
III	STZ Single dose (40 mg/kg, i.p.)+HFD	2.10±0.071**	3.77±0.047**
IV	STZ+HFD+CIE (250 mg/kg, p.o.)	1.61±0.064**, *	3.28±0.098**,*
V	STZ+HFD+CIE (500 mg/kg, p.o.)	1.56±0.033***	2.87±0.08**, *

Data shown as means ± SEM from 6 animals. All comparisons of group IV and V with group III by student's t test (*p<0.001). All comparisons of group I, II, IV and V with group III by ANOVA (**p<0.001).

for 15 minutes. The supernatant after centrifugation was the serum sample used for analysis.

Histopathology

10% neutral buffered formalin fixed hearts were dehydrated in ascending grades of isopropyl alcohol embedded in paraffin wax and 5 µm sections were stained by haematoxylin and eosin stain.

Statistical analysis

Data were expressed as the mean ±SEM. For a statistical analysis of the data, group means were compared by one way ANOVA with *post hoc* analysis. The Tukey-Karmer *post hoc* test was applied to identify significance among groups; p<0.05 was considered to be statistically significant.

Results

Preliminary phytochemical screening

The results of the preliminary phyto-chemical screening of *cichorium intybus* seeds extract (CIE) showed the presence of alkaloids, phenolics, flavonoids, tannins, steroids, anthraquinones and saponin glycosides.

Chemical constituents

Various compounds from the extraction of *Cichorium intybus* have been isolated and identified and a summary of isolated and identified chemicals are listed below in (Table 1). (Street *et al.* 2013)

Effect of CIE on blood glucose in STZ diabetic rats

In group III (Diabetic group) the blood glucose was at highest level and after the administration of chicory decrease of level of blood glucose was observed. (Table 2).

Effect of CIE on myocardial enzymes in STZ diabetic rats

Myocardial enzymes SGOT and LDH are biochemical indicators of myocardial injury. A significant increase (p<0.01) (Figures 1–2) in the levels of these enzymes was observed in animals treated with STZ. Administration of CIE at doses 250 mg/kg and 500 mg/kg significantly reduced (p<0.01) the level of these enzymes when compared to STZ treatment alone.

Effect of CIE on STZ induced LPO

Freshly prepared heart homogenates were studied for the concentration of malondialdehyde (MDA). Rats treated with STZ alone had a significant increase (p<0.001) in the level of MDA. Simultaneous treatment of CIE at two concentrations reduced the level of MDA in heart (p<0.01) as shown in (Figure 3).

Effect of CIE on STZ induced changes in GSH contents

The glutathione levels in blood and reduced glutathione in heart homogenates are shown in (Table 3). The concentration of glutathione in animals treated with STZ was significantly increased both in blood and in

homogenates of heart (p<0.001) as compared to control rats. Co-administration of CIE extract in two doses (250 mg/kg and 500 mg/kg) decreased the concentration of total and reduced glutathione in blood and heart (p<0.001). However, the values remained at higher than physiological levels at the time of estimation.

Effect of CIE on STZ-induced changes in activity of antioxidant enzymes

Figure 4 and 5 demonstrate the activities of SOD and CAT in cardiac tissue of healthy control, CIE only treated rats, diabetic control, and CIE treated diabetic rats. Only CIE treatment did not register any significant change in the activities of antioxidant enzymes when compared with healthy control rats. A significant (p<0.001) decrease in the activity of CAT and a significant increase in the activity of SOD activity was a notable manifestation of STZ toxicity. Administration of CIE significantly (p<0.001) improved the activities of these enzymes in diabetic rats when compared with diabetic control rats.

Effect of CIE on inflammatory biomarkers in STZ diabetic rats.

To further elucidate the mechanism of action of CIE in myocardial inflammation, pro-inflammatory cytokines such as TNF-α and IL-6 were determined using Cytometric Bead Array technique. The serum level of TNF-α and IL-6 in STZ induced diabetic group was significantly (p<0.001) increased compared with the healthy control group as shown in (Figure 6–7). However, the levels were significantly lowered in animals treated concomitantly with two doses of CIE (p<0.001). No significant difference in IL-6 level was found at the lower dose of CIE.

Effect of CIE on STZ induced histological changes on heart.

Intraperitoneal injection of STZ along with HFD, induced myocardiopathy, characterized by extensive myocardial necrosis associated with massive leucocytic infiltrations predominantly lymphocytes with occasional polymorphs (Figure 8B). The changes were predominantly observed in the ventricular myocardium and subendocardial myocardium. CIE at the dose of 500 mg/kg had remarkable cardioprotective effect as evidenced by minimal myocardial

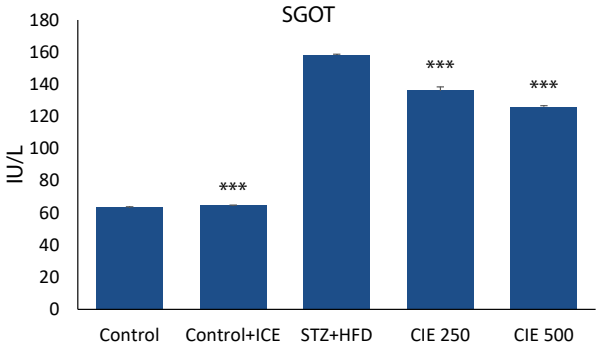

Figure 1. Effect of *Cichorium intybus* treatment on SGOT level in rats. Values are expressed as mean ± SEM (n=6). Significant differences are indicated by ***p<0.001; **p<0.01; *p<0.05 vs. group III.

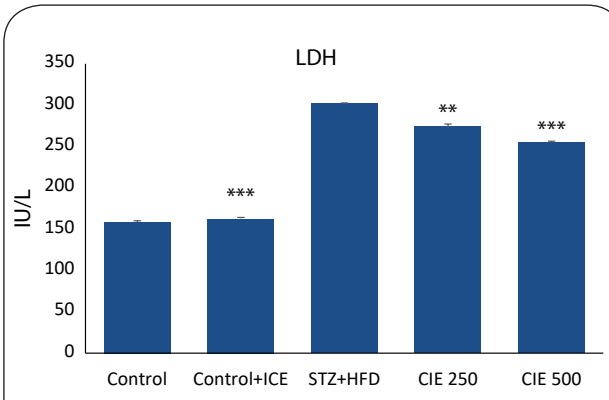

Figure 2. Effect of *Cichorium intybus* treatment on LDH level in rats. Values are expressed as mean ± SEM (n=6). Significant differences are indicated by ***p<0.001; **p<0.01; *p<0.05 vs. group III.

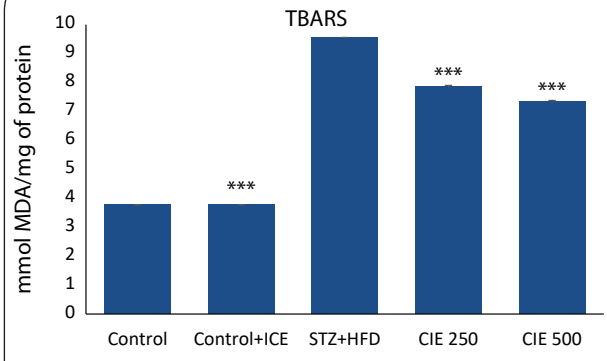

Figure 3. Effect of *Cichorium intybus* treatment on TBARS in the heart of rats. Values are expressed as mean ± SEM (n=6). Significant differences are indicated by ***p<0.001; **p<0.01; *p<0.05 vs. group III.

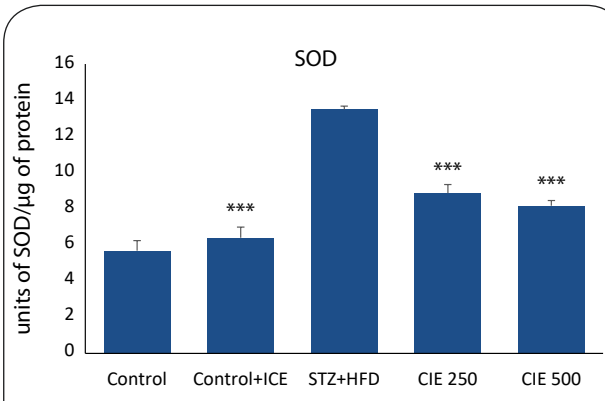

Figure 4. Effect of *Cichorium intybus* treatment on SOD in the heart of rats. Values are expressed as mean ± SEM (n=6). Significant differences are indicated by ***p<0.001; **p<0.01; *p<0.05 vs. group III.

necrosis and infiltrative changes (Figure 8D). CIE at dose 250 mg/kg was less effective in providing the protective effect on heart from STZ induced necrosis.

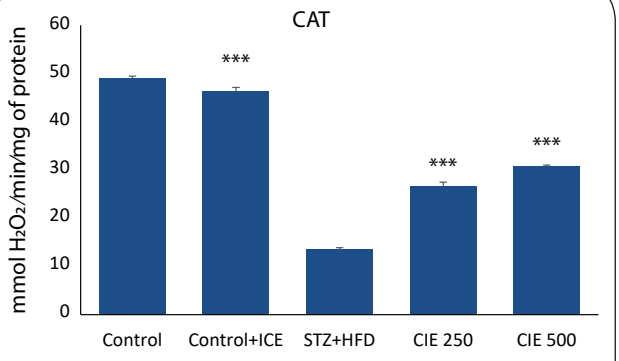

Figure 5. Effect of *Cichorium intybus* treatment on CAT in the heart of rats.Values are expressed as mean ± SEM (n=6). Significant differences are indicated by ***p<0.001; **p<0.01; *p<0.05 vs. group III.

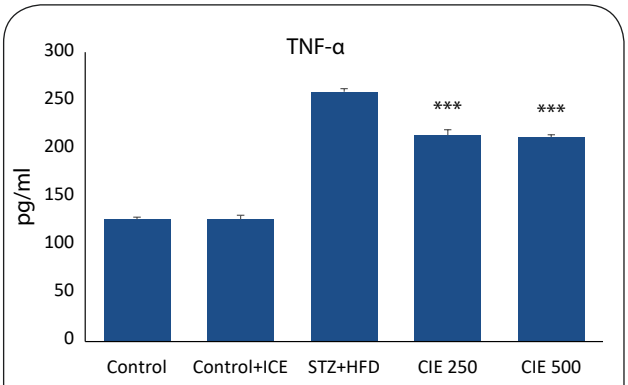

Figure 6. Effect of Cichorium intybus treatment on TNF-α levels in the heart of rats. Values are expressed as mean ± SEM (n= 6). Significant differences are indicated by ***p<0.001; **p<0.01; *p<0.05 vs. group III.

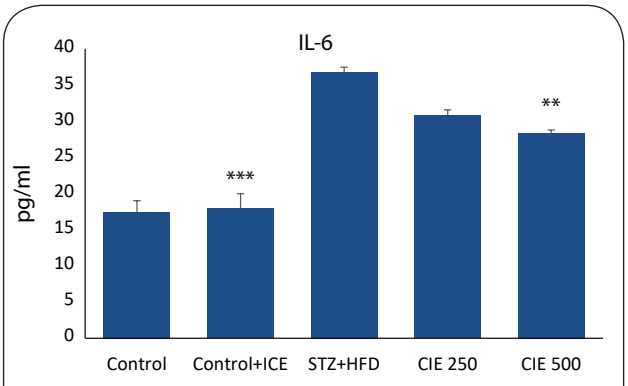

Figure 7. Effect of *Cichorium intybus* treatment on IL-6 level in the heart of rats. Values are expressed as mean ± SEM (n=6). Significant differences are indicated by ***p<0.001; **p<0.01; *p<0.05 vs. group III.

Discussion

The present study was undertaken to evaluate the protective effect of *cichorium intybus* on STZ induced diabetic cardiomyopathy in rats. Experimental diabetes produced by low dose of STZ combined with high energy intake is regarded as a general strategy to obtain type-2 diabetes animal model, since it stimulates the real course of human type-2 DM (Wang *et al.,* 2007; Ti *et al.,* 2011). The high energy intake induces insulin resistance at first and subsequently an injection of low dose STZ makes partial dysfunction of beta cells to suppress insulin secretion which works as a compensation to insulin resistance.

The cytotoxic action of STZ is associated with generation of ROS causing oxidative damage (Szkudelski, 2001). Disturbances of antioxidant defense systems in diabetes have been demonstrated, including alterations in the activities of antioxidant enzymes such as SOD, CAT and impaired glutathione metabolism (Maritim & Sanders, 2003). Chemicals with antioxidant properties and free radical scavengers may help in the regeneration of beta cells and protect pancreatic islets against cytotoxic effects of STZ (Alvarez *et al.,* 2004). Decreased LPO and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications (Armstrong *et al.,* 1996). Myocardial necrosis produced by STZ is also the consequence of increase LPO due to generation of free radicals. *Cichorium* seed extract protects the myocardium from peroxidative damage.

Chicory has reported antidiabetic activity (Pushparaj *et al.,* 2007; Ghamarian *et al.,* 2012) and as evidenced in the present study chicory seed extract possesses a strong antioxidant property. Decreased LPO and improved antioxidant status may be one mechanism by which dietary treatment contributes to the prevention of diabetic complications. The glucose lowering capacity of chicory has been attributed to its chemical composition including antioxidant compounds (Hassan & Yousef, 2010). Chicoric acid, a compound isolated from chicory, is also a new potential antidiabetic agent exhibiting both insulin-sensitizing and insulin-secreting properties (Tousch *et al.,* 2008).

The LDH and AST levels, which were significantly increased following myocardial injury by STZ, were reduced to physiological level in serum upon administration of CIE in two doses. However, the 500 mg/kg dose of chicory had better protective effect than the 250 mg/kg dose group. An elevation of reduced GSH in heart muscle and total glutathione in whole blood was observed in STZ diabetic rats. The demonstration of increased myocardial levels of total and reduced glutathione is consistent with an activation of the glutathione-peroxidase redox pathway secondary to the generation of and subsequent detoxification of STZ induced free radicals (Jackson *et al.,* 1984). Increase in cardiac muscle GSH suggest that the myocardial detoxification system is not overwhelmed as has been suggested. The presence of myocyte damage, as demonstrated histologically, suggests that injury has

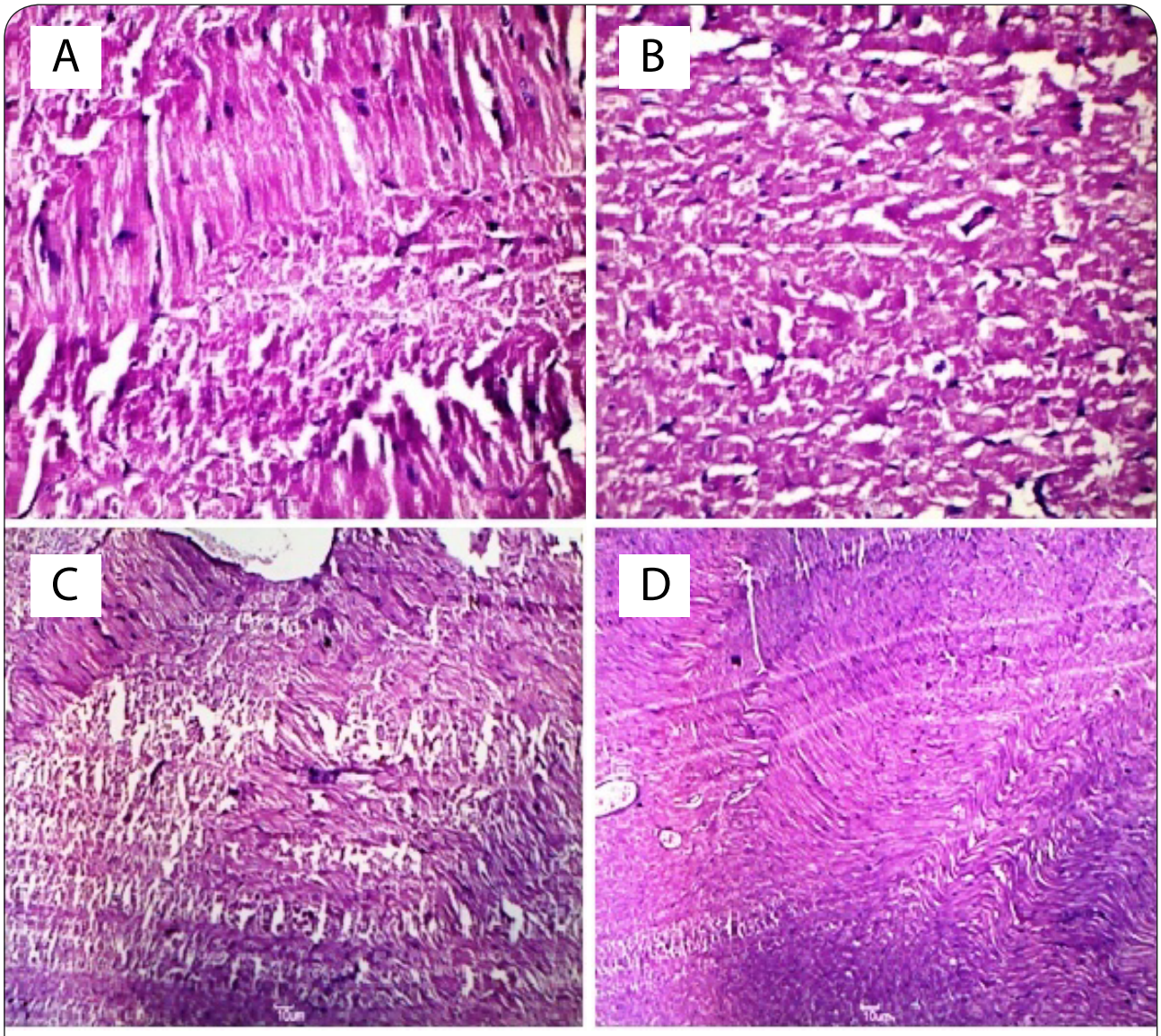

Figure 8. Histopathological changes in heart of rat and in animal treated with *Cichorium intybus*. Figure A- Normal rat heart shows normal morphology of cardiomyocyte. Figure B- Degenerative changes in many myocardial fibres and massive leucocytic infilterations are seen in heart of STZ diabetic rats. Figure C- CIE-250mg/kg treated heart shows mild peripheral necrosis and edema with occasional polymorphs. Figure D- A few myocardial fibres show degenerative changes and necrosis in CIE-500mg/kg treated rats and a minimal infilterative changes. Myocardial tissue sections stained with hematoxylin and eosin (magnification=400X).

occurred despite the elevation of GSH level. If the rise in GSH is in response to free radicals, it would appear that the system is able to cope adequately with their production indicating contributory effect of other mechanisms in cardiac damage by STZ. The lowering of the level of GSH by chicory suggests scavenging effect of chicory on free radicals responsible for the elevation of GSH in heart muscle. The role of chicory in this context requires further study.

Furthermore, cardiac inflammation, characterized by increased levels of pro-inflammatory cytokines, was suppressed by chicory as well. Pro-inflammatory cytokines such as TNF-α and IL-6 are chief mediators of inflammatory reaction and critically participate in the manifestation of DCM. Chicory has been used as an anti-inflammation agent for generations (Cha *et al.,* 2011). TNFα plays several important roles in inflammation based on its appearance at the inflammatory site and ability to induce certain mechanisms including activation and chemotaxis of leukocytes, expression of adhesion molecules on neutrophils and endothelial cells, and regulation of the secretion of other pro-inflammatory cytokines (Collins & Grounds, 2001). The TNF-α stimulates hyperlipidemia and hepaticlipogenesis simultaneously reducing the sensitivity to insulin in muscle tissues and finally the necrosis of target organs (Khanra *et al.,* 2015).

Biochemical observations were in keeping with the morphological changes in the heart. Supplementation with CIE prevented the disorganization of cell plates and restored the cardiac cytoarchitecture nearly similar to

that of normal rats. The extensive necrotic and abundant infiltrative changes in heart, which were consistently observed in STZ diabetic animals, were reduced to minimum with chicory co-treatment and further substantiated the protective effect of chicory in heart.

In summary, the present study shows that the administration of chicory extract shows remarkable hyperglycemic effect as well as reduces the tissue specific marker enzymes, lipid peroxides, GSH and pro-inflammatory cytokines which are elevated in response to acute administration of STZ and thereby demonstrates its cardioprotective effect. Analytical studies determining the active component of chicory responsible for cardioprotective effect are yet to be isolated and confirmed. Detailed mechanistic action of chicory, particularly the flavonoids present in CIE, on different free radicals requires further study.

Acknowledgements

Authors wish to acknowledge Jamia Hamdard for providing necessary facilities and other documentary works.

REFERENCES

Ahmed B, Al-Howiriny TA, Siddiqui AB. (2003). Antihepatotoxic activity of seeds of Cichoriumintybus. *J Ethnopharmacol* **87**: 237–240.

Ahmed D, Sharma M, Kumar V, Bajaj HK, Verma A. (2015). 2β-hydroxybetulinic acid 3β-caprylate: an active principle from Euryale FeroxSalisb. seeds with antidiabetic, antioxidant, pancreas & hepatoprotective potential in streptozotocin induced diabetic rats. *J Food Sci Tech.* **52**(9): 5427–5441.

Alvarez JF, Barbera A, Nandal B, Barcello-Batllori S, Piquer S, Claret M, Guninovart JJ, Gomis R. (2004). Stable and functional regeneration of pancreatic beta cell population in n-STZ rats treated with tungstate. *Diabetologia* **47**: 470–477.

Anwer T, Sharma M, Pillai K, Haque S, Alam M, Zaman M. (2007). Protective effect of bezafibrate on streptozotocin-induced oxidative stress and toxicity in rats. *Toxicology* **229**: 165–172.

Anwer T, Sharma M, Pillai KK, Khan G. (2012). Protective effect of Withaniasomnifera against oxidative stress and pancreatic beta-cell damage in type 2 diabetic rats. *Acta Pol Pharm* **69**: 1095–1101.

Armstrong AM, Chestnutt JE, Gormley MJ, Young IS. (1996). The effect of dietary treatment on lipid peroxidation and antioxidant status in newly diagnosed noninsulin dependent diabetes. *Free Rad Biol Med* **21**: 719–726.

Baynes JW, Thorpe SR. (1996). The role of oxidative stress in diabetic complications. Current Opinion in Endocrinology. *Diabetes and Obesity* **3**: 277–284.

Begum R, Sharma M, Pillai K, Aeri V, Sheliya MA. (2015). Inhibitory effect of Careyaarborea on inflammatory biomarkers in carrageenan-induced inflammation. *Pharmaceutical Biol* **53**: 437–445.

Beutler E, Duron O, Kelly BM. (1963). Improved method for the determination of blood glutathione. *J Lab Clinic Med* **61**: 882–888.

Bischoff TA, Kelley CJ, Karchesy Y, Laurantos M, Nguyen-Dinh P, Arefi AG. (2004). Antimalarial activity of Lactucin and Lactucopicrin. sesquiterpene lactones isolated from Cichoriumintybus L. *J Ethnopharmacol* **95**: 455–457.

Brouckaert P, Libert C, Everaerdt B, Takahashi N, Cauwels A, Fiers W. (1993). Tumor necrosis factor, its receptors and the connection with interleukin 1 and interleukin 6. *Immunobiol* **187**: 317–329.

Cavin C, Delannoy M, Malnoe A, Debefve E, ToucheA, Courtois D, Schilter B. (2005). Inhibition of the expression and activity of cyclooxygenase-2 by chicory extract. *Biochem and Biophy Res Communic* **327**: 742–749.

Cha DS, Eun JS, Jeon H. (2011). Anti-inflammatory and antinociceptive properties of the leaves of Eriobotrya japonica. *J Ethnopharmacol* **134**: 305–12.

Claiborne A. Catalase activity. In; *Greenwald* RA (Ed.) CRC Handbook for methods for Oxygen Radical Research (1985). CRC Press, Boca Raton F L. pp. 283–284.

Collins RA, Grounds MD. The role of tumour necrosis factor –alpha (TNF-α) in skeletal muscle regeneration. (2001). Studies in TNF-α (–/–) and TNF-α (–/–)/ LT-α (–/–) mice. *J Histochem Cytochem* **49**: 989–1001.

Conforti F, Ioele G, Statti G, Marrelli M, Ragno G, Menichini F. (2008). Antiproliferative activity against human tumor cell lines and toxicity test on Mediterranean dietary plants. *Food ChemToxicol* **46**: 3325–3332.

European Medicines Agency, "Assessment report on Cichoriumintybus L., radix," EMA/HMPC/113041/2010, 2013.

European Medicines Agency, "Community herbal monograph on Cichoriumintybus L., radix," EMA/HMPC/121816/2010, 2012.

Evans WC. *Trease and Evans Pharmacognosy.* 15 ed. Saunders Publishers London. (2002); 60: pp 357–359

Falcão-Pires I, Leite-Moreira AF. (2012). Diabetic cardiomyopathy: understanding the molecular and cellular basis to progress in diagnosis and treatment. *Heart Failure Reviews* **17**: 325–344.

Ghamarian A, Abdollahi M, Su X, Amiri A, Ahadi A, Nowrouzi A. (2012). Effect of chicory seed extract on glucose tolerance test (GTT) and metabolic profile in early and late stage diabetic rats *DARU J Pharmaceutical Sci* **20**: 1–9.

Gürbüz Il, Üstün O, Yeşilada E, Sezik E, Akyürek N. (2002). In vivo gastroprotective effects of five Turkish folk remedies against ethanol-induced lesions. *J Ethnopharmcol* **83**: 241–244.

Hassan HA, Yousef MI. (2010). Ameliorating effect of chicory (Cichoriumintybus L)- supplemented diet against nitrosamine precursors- induced liver injury and oxidative stress in rats. *Food Chem Toxicol* **48**: 2163–2169.

Heimler D, Isolani L, Vignolini P, Romani A. (2009). Polyphenol content and antiradical activity of Cichoriumintybus L. from biodynamic and conventional farming. *Food Chemistry* **114**: 765–770.

Huseini HF, Alavian S, Heshmat R, Heydari M, Abolmaali K. (2005). The efficacy of Liv-52 on liver cirrhotic patients: a randomized, double-blind, placebo-controlled first approach. *Phytomed* **12**: 619–624.

Jackson JA, Reeves JP, Muntz KH, Kruk D, Prough RA, Willerson JT, L. M. Buja. (1984). Evaluation of free radical effects and catecholamine alterations in adriamycin cardiotoxicity. *American J of Pathol* **117**: 140–153.

Judžentienė A, Būdienė J. (2008). Volatile constituents from aerial parts and roots of Cichoriumintybus L. (chicory) grown in Lithuania. *Chemija.* **19**: 25–28.

Khanra R, Dewanjee S, Dua TK, Sahu R, Gangopadhyay M, De Feo V *et al.* (2015). Abromaaugusta L.(Malvaceae) leaf extract attenuates diabetes induced nephropathy and cardiomyopathy via inhibition of oxidative stress and inflammatory response. *J Translational Med* **13**: 1–14.

Kim HM, Kim HW, Lyu YS, Won JH, Kim DK, Lee YM, Morii E, Jippo T, Kitamura Y, An NH. (1999). Inhibitory effect of mast cell-mediated immediate-type allergic reactions by *Cichorium intybus*. *Pharmacological Res* **40**: 61–65.

Kim KW, Suh SJ, Kim JD, Kim SS, Lee IS, Kim JK, Chang GT, Kim DS, Kim CH. (2009). Effects on lipid peroxidation and antioxidative enzymes of Euonymus alatus in cultured rat hepatocytes. *Basic & Clinic Pharmacol & Toxicol* **104**: 60–70.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Maritim AC, Sanders RA. (2003). Effects of alpha lipoic acid on biomarkers of oxidative stress in streptozotocin induced diabetic rats. *J Nutr Biochem* **14**: 288–294.

Marklund S, Marklund G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochemn* **47**: 469–474.

McLauchlan DW. Enzymes In: Gowenlock AH, McMurry JR, McLauchlan DM, edVarley's . *Practical Clinical Biochemistry.* 6th ed. London Heinemann. (1988): pp 477–549.

Miller M, Duckett S, Andrae J. (2011). The effect of forage species on performance and gastrointestinal nematode infection in lambs. *Small Ruminant Res* **95**: 188–192.

Nandagopal S, Kumari BR. (2007). Phytochemical and antibacterial studies of Chicory (Cichorium intybus L.)-A multipurpose medicinal plant. *Adv Biol Res* **1**: 17–21.

Ohkawa H, Ohishi N, Yagi K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95**: 351–358.

Pieroni A. (2000). Medicinal plants and food medicines in the folk traditions of the upper Lucca Province, Italy. *J Ethnopharmacol* **70**: 235–273.

Pushparaj P, Low H, Manikandan J, Tan B, Tan C. (2007). Anti-diabetic effects of Cichoriumintybus in streptozotocin-induced diabetic rats. *J Ethnopharmacol* **111**: 430–434.

Reitman S, Frankel S. (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transminases. *American J Clin Pathol* **28**: 53–56.

Sedlak J, Lindsay RH. (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Bio chem.* **25**: 192–205.

Sezik E, Yeşilada E, Honda G, Takaishi Y, Takeda Y, Tanaka T. (2001). Traditional medicine in Turkey X. Folk medicine in central Anatolia. *J Ethnopharmacol* **75**: 95–115.

Shaw JE, Sicree RA, Zimmet PZ. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Practical* **87**: 4–14.

Street RA, Sidana J, Prinsloo G. (2013). Cichorium intybus: Traditional uses, Phytochemistry, Pharmacology and Toxicology. *Evidence-Based Complimentary and Alternative Medicine* **579319**: 1–13.

Süntar I, Akkol EK, Keles H, Yesilada E, Sarker SD, Baykal T. (2012). Comparative evaluation of traditional prescriptions from Cichoriumintybus L. for wound healing: stepwise isolation of an active component by in vivo bioassay and its mode of activity. *J Ethnopharmacol* **143**: 299–309.

Szkudelski T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiological Research* **50**: 537–546.

Ti Y, Xie GL, Wang ZH, Bi XL, Ding WY, Wang J, Jiang GH, Bu PL, Zhang Y, Zhong M, Zhang W. (2011). TRB3 gene silencing alleviates diabetic cardiomyopathy in a type 2 diabetic rat model. *Diabetes* **60**: 2963–2974.

Tousch D, Lajoix AD, Hosy E, Azay-Milhau J, Ferrare K, Jahannault C, Cros G, Petit P. (2008). Chicoric acid, a new compound able to enhance insulin release and glucose uptake. *Bio chemic and Bio phy Res Communic* **377**: 131–135.

Van Wyk B-E, OudtshoornBv, Gericke N. Medicinal Plants of South Africa: Briza; Briza Publications, Pretoria, South Africa, 1997.

Wang HJ, Jin YX, Shen W, Neng J, Wu T, Li YJ, Fu ZW. (2007). Low dose streptozotocin (STZ) combined with high energy intake can effectively induce type 2 diabetes through altering the related gene expression. *Asia Pac J Clin Nutr* **16**: 412–417.

Wesołowska A, Nikiforuk A, Michalska K, Kisiel W, Chojnacka-Wójcik E. (2006). Analgesic and sedative activities of lactucin and some lactucin-like guaianolides in mice. *J Ethnopharmacol* **107**: 254–258.

Westermann D, Walther T, Savvatis K, Escher F, Sobirey M, Riad A, Bader M, Schultheiss HP, Tschöpe C. (2009). Gene deletion of the kinin receptor B1 attenuates cardiac inflammation and fibrosis during the development of experimental diabetic cardiomyopathy. *Diabetes* **58**: 1373–1381.

Yoon W-J, Ham YM, Kim S-S, Yoo B-S, Moon J-Y, Baik JS, Lee NH, Hyun C-G. (2009). Suppression of pro-inflammatory cytokines, iNOS, and COX-2 expression by brown algae Sargassum micracanthum in RAW 264.7 macrophages. *Eur Asia J Bio Sci* **3**: 130–143.

Interdiscip Toxicol. 2019; **Vol. 12**(3): 120–128.
doi: 10.2478/intox-2019-0014

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

General toxicity assessment of the novel aldose reductase inhibitor cemtirestat

Marta ŠOLTÉSOVÁ PRNOVÁ [1], Lucia RAČKOVÁ [1], Lucia KOVÁČIKOVÁ [1], Jana BALLEKOVÁ [1], Jana VISKUPIČOVÁ [1], Silvia MICHÁLIKOVÁ [1], Betul TAŞKOPARAN [2], Zübeyir ELMAZOĞLU [2], Tea LANIŠNIK RIŽNER [3], Cimen KARASU [4], Sreeparna BANERJEE [2], Milan ŠTEFEK [1]

[1] Department of Biochemical Pharmacology, Institute of Experimental Pharmacology and Toxicology, CEM, Slovak Academy of Sciences, Bratislava, Slovakia
[2] Department of Biological Sciences, Middle East Technical University, Ankara, Turkey
[3] Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Slovenia
[4] Department of Medical Pharmacology, Faculty of Medicine, Gazi University, Ankara, Turkey

ITX120319A02 • Received: 22 July 2019 • Accepted: 15 September 2019

ABSTRACT

Cemtirestat, 3-mercapto-5*H*-[1,2,4]-triazino[5,6-*b*]indole-5-acetic acid was recently designed and patented as a highly selective and efficient aldose reductase inhibitor endowed with antioxidant activity. The aim of the present study was to assess the general toxicity of cemtirestat using *in silico* predictions, *in vitro* and *in vivo* assays. ProTox-II toxicity prediction software gave 17 "Inactive" outputs, a mild hepatotoxicity score (0.52 probability) along with a predicted LD50 of 1000 mg/kg. Five different cell lines were used including the immortalized mouse microglia BV-2, the primary human fibroblasts VH10, the insulinoma pancreatic β-cells INS-1E, the human colon cancer cells HCT116 and the human immortalized epithelial endometrial cell lines HIEEC. In contrast to the clinically used epalrestat, cemtirestat showed remarkably low cytotoxicity in several different cell culture viability tests such as MTT proliferation assay, neutral red uptake, BrdU incorporation, WST-1 proliferation assay and propidium iodide staining followed by flow cytometry. In a yeast spotting assay, the presence of cemtirestat in incubation of Saccaromyces cerevisiae at concentrations as high as 1000 μM did not affect cell growth rate significantly. In the 120-day repeated oral toxicity study in male Wistar rats with daily cemtirestat dose of 6.4 mg/kg, no significant behavioral alterations or toxicological manifestations were observed in clinical and pathological examinations or in hematological parameters. In summary, these results suggest that cemtirestat is a safe drug that can proceed beyond preclinical studies.

KEY WORDS: cemtirestat; aldose reductase inhibitor; toxicity assessment

ABBREVIATIONS:

AKR1B1: Aldo-keto reductase family 1, member B1 (human aldose reductase); **HEPES:** (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); **BrdU:** Bromodeoxyuridine; **DMEM:** Dulbecco's modified eagle medium; **FBS:** Fetal bovine serum; **LC–MS:** Liquid chromatography–mass spectrometry; **MCF:** Median corpuscular fragility; **MDA:** Malondialdehyde; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NR:** Neutral red; **P/S:** Penicillin-streptomycin solution; **PBS:** Phosphate-buffered saline; **RPMI-1640:** Growth medium used in cell culture; **RBC:** Red blood cells; **WST:** Water soluble tetrazolium salt; **ZDF:** Zucker diabetic fatty; **YPD:** Yeast extract peptone dextrose

Correspondence address:
Marta Soltesova Prnova, PhD.
Department of Biochemical Pharmacology
Institute of Experimental Pharmacology and Toxicology
Centre of Experimental Medicine of the Slovak Academy of Sciences
Dubravska cesta 9, 84104 Bratislava, Slovakia
TEL.: +421232295712 • E-MAIL: marta.prnova@savba.sk

Introduction

Cemtirestat, 3-mercapto-5*H*-1,2,4-triazino[5,6-*b*] indole-5-acetic acid (Figure 1), was recently designed and patented (Stefek *et al.,* 2017) as a highly selective and efficient aldose reductase inhibitor. (Stefek *et al.,* 2015; Stefek *et al.,* 2016; Soltesova Prnova *et al.,* 2015; Zhan *et al.,* 2018). Aldose reductase (AKR1B1), the first enzyme of the polyol pathway, is a key mediator of glucose toxicity under hyperglycemic conditions (Yabe-Nishimura, 1998). Aldose reductase thus represents a promising therapeutic target and efficient aldose reductase inhibitors are sought as potential drugs to treat diabetic complications. In our recent study in ZDF rats, an animal model of type 2 diabetes, cemtirestat normalized symptoms of peripheral neuropathy with high significance (Soltesova Prnova *et al.* 2019). Inhibition of sorbitol accumulation in red blood cells and in the sciatic nerve along with markedly decreased plasma levels of thiobarbituric acid reactive substances pointed to the bifunctional effect of cemtirestat comprising both inhibition of flux of glucose through the polyol pathway and antioxidant action.

Cemtirestat is presently a subject of preclinical studies as a promising agent with a therapeutic potential in relation to diabetic peripheral neuropathy. The aim of the present study was to assess a general toxicity of cemtirestat using *in silico* predictions, cell culture *in vitro* assays and *in vivo* animal investigations.

Material and methods

Substance

Cemtirestat (3-mercapto-5*H*-1,2,4-triazino[5,6-*b*]indole-5-acetic acid) was custom synthesized by Apollo Scientific Ltd, Bredbury, UK. A purity of >95% has been established for cemtirestat substance by the LC–MS technique as described in Stefek *et al.* (2015). The reference aldose reductase inhibitor epalrestat was obtained from Sigma-Aldrich (St. Louis, MO, USA).

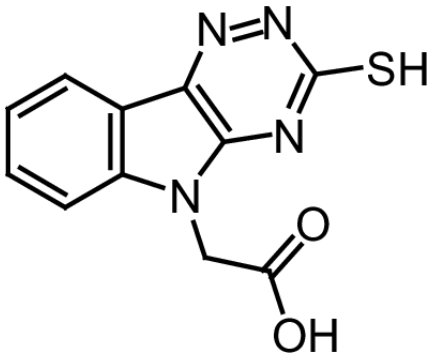

Figure 1. 3-Mercapto-5*H*-[1,2,4]-triazino[5,6-*b*]indole-5-acetic acid (cemtirestat).

In silico predictions

ProTox-II (http://tox.charite.de/protoxII), a webserver for the prediction of toxicity of chemicals was used.

Cell culture tests

Cell lines

The immortalized mouse microglial cell line BV-2 was kindly provided by Dr. Blasi at the University of Perugia (Blasi*et al.,* 1990) and was cultured under standard conditions in Dulbecco's modified eagle medium (DMEM, Sigma Aldrich), supplemented with 10% fetal bovine serum (FBS, PAA, Biotech, s. r. o., Bratislava, Slovakia), and 1% P/S (100 U/ml penicillin, 100 mg/ml streptomycin, K-Trade, s.r.o., Bratislava, Slovakia) and maintained in 5% CO_2 at 37 °C. Cells were used for 10 passages at maximum (Mrvova *et al.,* 2015).

Nonmalignant diploid human fibroblasts VH10 were obtained from Prof. A. Kolman (Laboratory of Radiology, University of Stockholm, Sweden) and were cultured under standard condition in DMEM, supplemented with 10% FBS, and 1% P/S (100 U/ml penicillin, 100 mg/ml streptomycin) and maintained in 5% CO_2 at 37 °C as described elsewhere (Slamenova *et al.* 2009).

Rat INS-1E insulinoma pancreatic β-cells were kindly provided by Prof. Claes Wollheim, University of Geneva) and were cultured in RPMI 1640 (11 mM glucose, Sigma Aldrich) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM Na pyruvate, 55 µM 2-mercaptoethanol, 10 mM HEPES, 1% non-essential amino acids, and 10% fetal bovine serum, pH 7.0–7.4. The cells were grown in a humidified incubator containing 5% CO_2 at 37 °C as described previously (Viskupicova *et al.,* 2017).

Human colon cancer cells (HCT-116) were kindly provided by Prof. Sreeparna Banerjee, Department of Biological Sciences, Middle East Technical University, Ankara, Turkey and were cultured as described previously in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific, Waltham MA, USA), 2 mM L-glutamine and 1% penicillin/streptomycin (Enayat *et al.,* 2016).

Human immortalized epithelial endometrial cell line (HIEEC) was kindly provided by Prof. Michael A. Fortier (Laval University, Qébec, Canada) and it was originally generated from a primary culture prepared from endometrial biopsy taken from a 37-year-old woman with confirmed absence of neoplasia and endometriosis, on day 12 of her menstrual cycle and were cultured in RPMI-1640 Medium (R5886; Sigma-Aldrich) as described elsewhere (Hevir-Kene and Rižner, 2015).

The *Saccharomyces cerevisiae* strain RDKY3615 (MAT a, ura3-52, his3Δ200, leu2Δ1, trp1Δ63, lys2ΔBgI, hom3-10, ade2Δ1, ade8, hxt13::URA3, Chen and Kolodner, 1999) was obtained from Dr. Hernan Flores Rozas from the College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Florida.

MTT viability test

To assess cell metabolic activity, the MTT assay was used which is based on the ability of cellular NAD(P)H-dependent oxidoreductases to reduce the tetrazolium dye MTT to insoluble formazan, which has a purple color (Stockert *et al.* 2018).The cells were grown in 96-well microplates in complete cell culture medium. At the end of the incubation with or without the compounds tested, the cells were incubated with MTT (0.5 mg/ml) for 120 min at 37 °C. Subsequently, 100 µl of 10% sodium dodecyl sulfate in HCl (0.01 M) was added and the cells were incubated at 37 °C overnight for complete dissolution of generated formazan. The absorbance was spectrophotometrically recorded at 570 nm using the reference 690 nm (Mrvova *et al.,* 2015; Viskupicova *et al.,* 2017).

Neutral red (NR) uptake assay

To determine the number of viable cells in culture, the NR uptake assay was used which is based on the ability of viable cells to incorporate neutral red dye within the lysosomes (Repetto *et al.,* 2008). One hundred microliters of NR solution (0.003%) in complete cell culture medium

was added to the cells and the dye accumulation was left to proceed for 4 hours. Next, the incubation medium was removed, the cells were washed twice with phosphate buffered saline (PBS) and the accumulated dye was solubilized with 2% acetic acid/ethanol mixture (1:1, v/v). The absorbance of the samples was read spectrophotometrically at 540 nm using a reference wavelength at 690 nm (Milackova *et al.* 2015).

BrdU incorporation assay

BrdU incorporation into the newly synthesized DNA (Vega-Avila and Pugsley, 2011) was used to detect proliferating HCT-116 cells according to the manufacturer's instructions as described elsewhere (Enayat *et al.,* 2016). Chemiluminescent BrdU cell proliferation kit was purchased from Roche (Mannheim, Germany).

WST-1 proliferation test

WST-1 proliferation test (Ishiyama, 1995) was used to determine the effect of cemtirestat on the proliferation of human immortalized epithelial endometrial cells (HIEEC). The reagent WST-1 (Roche Diagnostics, Germany) was employed following the manufacturer's instructions as described elsewhere (Kljun *et al.* 2016).

Cell cycle analysis

VH10 fibroblasts cultured under standard condition (Slamenova *et al.,* 2009) were fixed in 70% ethanol overnight at –20 °C; the cells were pelleted and resuspended gently in ice-cold phosphate-buffered saline (PBS) . After 2 washes, cells were suspended in propidium iodide staining solution (10 µg/ml propidium iodide, 100 µg/ml RNase, 0.1% (v/v) Triton-X in PBS for 15 min at 37 °C in the dark. The cell cycle was analyzed using a flow cytometer (Beckman Coulter FC500). Data were analyzed using MultiCycle AV software with a minimum of 5000 cells per sample.

Yeast spotting test

The yeast spotting test (Kwolek-Mirek and Zadrag-Tecza, 2014) was used to determine the effect of cemtirestat on yeast viability. An aliquot of the glycerol stock of the yeast strain was taken from –80 °C, streaked on a YPD plate and incubated at 30 °C for 16 h. A single colony was picked and inoculated into 3 ml of liquid yeast extract peptone dextrose (YPD) medium and grown overnight with shaking at 200 rpm at 30 °C. This overnight starter culture was then transferred to 10 ml of fresh liquid YPD and grown at 30 °C with shaking at 200 rpm until the OD 600 nm reached the value of 0.6. The culture was then divided into five separate tubes in equal numbers and either left untreated (Control) or treated with vehicle or 10, 100 and 1000 µM of cemtirestat for 24h in YPD. During treatment the cells were incubated at 30 °C with shaking at 100 rpm. The cells in each tube were washed extensively followed by 10-fold serial dilution in 100 µl final volume (Enayat *et al.,* 2016). 10 µl from each dilution was plated on an YPD-agar plate; the plate was air dried in a sterile manner and incubated at 30 °C for 48 h and photographed.

***In vivo* tests**

Animals

Male Wistar rats were supplied by our own breeding facility at the Department of Toxicology and Laboratory Animal Breeding, Centre of Experimental Medicine, Slovak Academy of Sciences Dobra Voda. The animals were fed standard chow (protein, 19.2%; carbohydrate, 65.1%; fat, 4.0%; fiber, 4.0% and ash, 7.7% of weight). The animals were randomly assigned to two groups: (C) control rats (n=6); (T) rats (n=6) treated with cemtirestat (100 mg/l in drinking water). The drug treatment proceeded for 120 days. During the experiment the animals were housed in groups of two in cages of type T4 Velaz (Prague, Czech Republic) with bedding made of wood parings (changed every other day). Tap water and pelleted standard chow were supplied *ad libitum*. The animal room was air-conditioned with 10 air changes per hour; the environment was maintained, with continuous monitoring, at 23±1 °C temperature and 40–70% relative humidity. After the completion of behavioral testing for each group at the end of the experiment, the rats were anesthetized with chloral hydrate (20 mg/100 g i.p.), blood was collected into heparinized tubes by heart puncture and organ samples were collected for biochemical assays. All tissues were rapidly frozen in liquid nitrogen and stored at –80 °C.

The study was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences and the State Veterinary and Food Administration of the Slovak Republic, and it was performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83-25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003).

Plasma assays

Frozen (–80 °C) samples of plasma were used for biochemical assays. Plasma glucose was analyzed by using an enzymatic colorimetric assay for glucose Glucose GOD 1500 (PLIVA-Lachema Diagnostika, Brno, CZ). Plasma cholesterol, triacylglycerides, urea, creatinine, Ca, P and Mg were assayed by Laboratoria s.r.o. Piešťany, Slovakia. Plasma levels of thiobarbituric acid reactive substances (TBARS), used as a marker of oxidative stress, were determined by modification of the method of Buege and Aust (Buege and Aust, 1978) as described previously (Soltesova Prnova *et al.,* 2019).

Sorbitol assay

Erythrocyte sorbitol assay was performed by modified enzymatic assay as reported previously (Soltesova Prnova *et al.,* 2019).

Behavioral tests

The rats were transferred to the experimental room and allowed to acclimatize for one hour. Hot water immersion tail-flick test, hot plate test and paw tactile responses test by using von Frey flexible filaments were performed as described previously (Soltesova Prnova *et al.,* 2019).

Osmotic fragility

The osmotic fragility was determined by the degree of hemolysis induced by the changes of osmotic pressure using a step-down protocol with decreasing concentrations of NaCl, as reported previously (Prnova *et al.* 2015). The median corpuscular fragility (MCF), used as quantitative marker of osmotic fragility, was calculated as a concentration (mM) of NaCl at which 50% hemolysis occurred.

Statistical analysis

Statistical comparisons were carried out by one way ANOVA followed by Tukey's post hoc test. $p \leq 0.05$ was considered statistically significant.

Table 1. ProTox-II toxicity prediction of cemtirestat.

No	Target	Prediction	Probability
1	Hepatotoxicity	Active	0.52
2	Carcinogenicity	Inactive	0.52
3	Immunotoxicity	Inactive	0.99
4	Mutagenicity	Inactive	0.61
5	Cytotoxicity	Inactive	0.66
6	Aryl hydrocarbon Receptor (AhR)	Inactive	0.62
7	Androgen Receptor (AR)	Inactive	0.99
8	Androgen Receptor Ligand Binding Domain (AR-LBD)	Inactive	0.97
9	Aromatase	Inactive	0.82
10	Estrogen Receptor Alpha (ER)	Inactive	0.86
11	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Inactive	0.98
12	Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)	Inactive	0.84
13	Nuclear factor (erythroid-derived 2)-like 2/antioxidant responsive element (nrf2/ARE)	Inactive	0.91
14	Heat shock factor response element (HSE)	Inactive	0.91
15	Mitochondrial Membrane Potential (MMP)	Inactive	0.81
16	Phosphoprotein (Tumor Supressor) p53	Inactive	0.83
17	ATPase family AAA domain-containing protein 5 (ATAD5)	Inactive	0.94

Table 2. Initial and final body weights and plasma glucose concentrations in male Wistar rats with or without cemtirestat treatment*.

	Body weight (g)		Blood glucose (mmol/l)	
	Initial	Final	Initial	Final
C (6)	262.5±15.1	450.8±38.1	8.1±1.9	10.6±1.2
T(6)	275.8± 8.0	465.8±38.3	7.5±1.5	8.6±2.6

*Group C, untreated control animals; Group T, rats treated by cemtirestat 6.4 mg/kg/day; Data are mean values±SD. Number of animals in each group is shown in parentheses. One way ANOVA followed by Tukey's *post hoc* test gave no significant differences between C and T groups.

Results and discussion

Toxicity predictions *in silico*

The aim of computational approaches in toxicology is to complement *in vitro* and *in vivo* toxicity assays to minimize the need for animal testing and to reduce the cost and time of toxicity tests (Raies and Bajic, 2016). As shown in Table 1, ProTox-II toxicity prediction software (Banerjee *et al.,* 2018) gave 16 "Inactive" outputs, a mild hepatotoxicity score (0.52 probability) along with a predicted LD_{50} of 1000 mg/kg. These outputs classify cemtirestat as a compound of acute toxicity class 4.

Cell culture studies

Remarkably low acute cytotoxicity of cemtirestat was observed in cell culture viability tests. To avoid method-related bias in viability tests (Pamies and Hartung, 2017) five principally different viability assays were used including MTT assay, NR uptake assay, BrdU incorporation assay, WST-1 proliferation test and propidium iodide

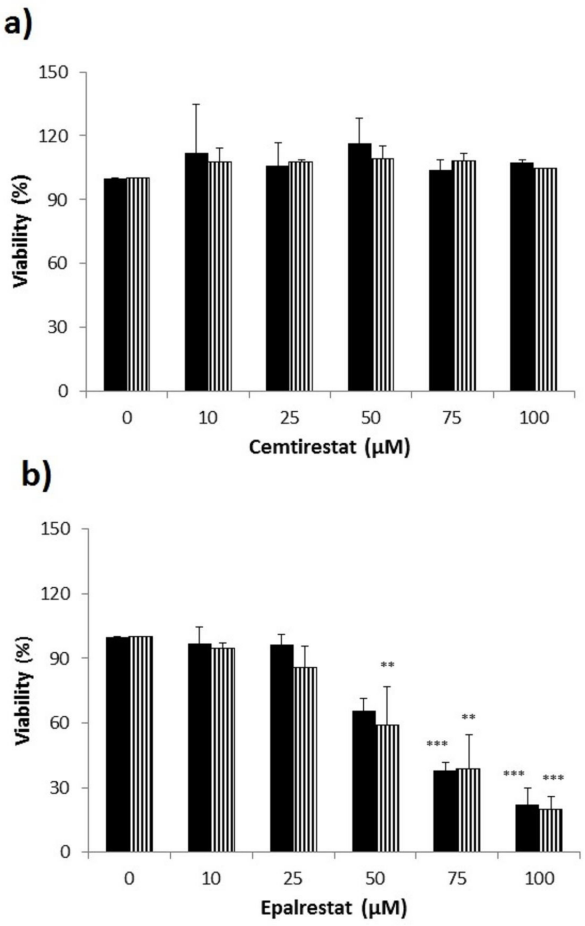

Figure 2. Viability parameters of BV-2 microglia subsequent to 24 h exposure to cemtirestat (a) in comparison with standard epalrestat (b). MTT test (black columns), NR uptake test (striped columns). Results are mean ± SD of at least three independent experiments run in three replicates.***p≤0.001, **p≤0.01 *vs.* control. One way ANOVA followed by Tukey's post hoc test.

DNA staining followed by flow cytometry. No significant effect of cemtirestat on cell viability was recorded with up to 100 µM of cemtirestat in the immortalized mouse microglia BV-2 (Figure 2a) and the human immortalized epithelial endometrial cell lines HIEEC (Figure 3). In contrast to cemtirestat, significant cell toxicity of the standard aldose reductase inhibitor epalrestat was recorded in the microglia at concentrations as low as 50 µM (Figure 2a). At this concentration the loss of viability estimated by MTT assay and NR uptake was 35% and 41%, respectively. A mild decrease in viability of Schwann cells in culture treated with 100 and 200 µM epalrestat was reported by Sato *et al.* (2014). Concentrations of cemtirestat up to 200 µM, were found to be without any effect on cell viability of the insulinoma pancreatic β-cells INS-1E (Figure 4) and on the proliferative capacity of the human colon cancer cells HCT-116 (Figure 5). Moreover, no significant cytotoxicity up to 1000 µM cemtirestat was recorded in the primary human fibroblasts VH10 (Figure 6). Chronic toxicity profile of cemtirestat applied every 12 hours over five consecutive days (9x 150 µM in total) in primary VH10 fibroblasts, shown in Figure 7, revealed

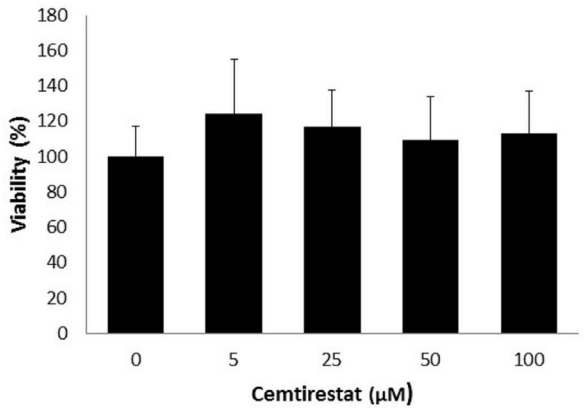

Figure 3. Viability parameters of HIEEC cells subsequent to 72 h exposure to cemtirestat. WST-1 proliferation test. Results are mean ± SD of at least three independent experiments run in three replicates. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences between the groups.

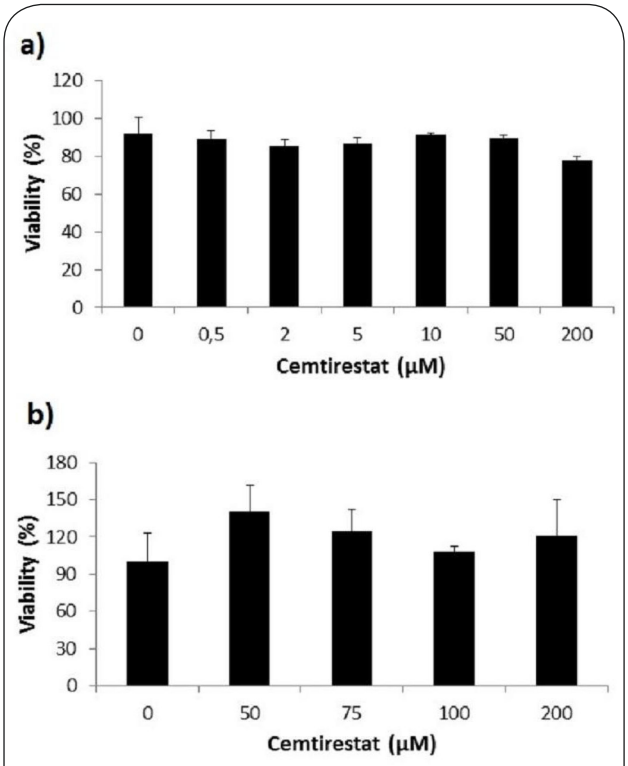

Figure 5. Viability parameters of colon cancer HCT116 cells subsequent to a) 24 h (MTT test), b) 48 h (BrdU incorporation assay) exposure to cemtirestat. Results are mean ± SD of at least three independent experiments run in eight replicates. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences between the groups.

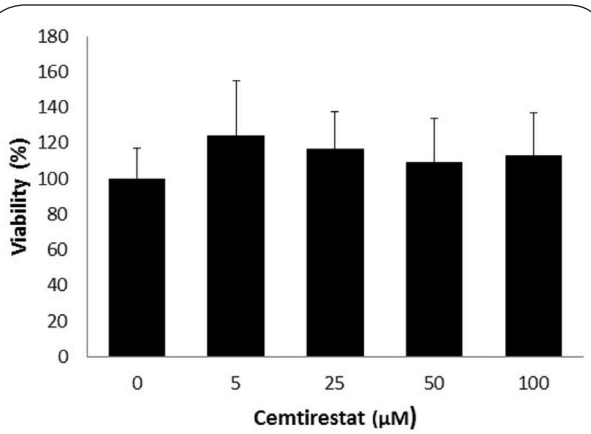

Figure 4. Viability parameters of pancreatic INS-1E cells subsequent to 24 h exposure to cemtirestat. MTT test. Results are mean ± SD of at least three independent experiments run in three replicates. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences the groups.

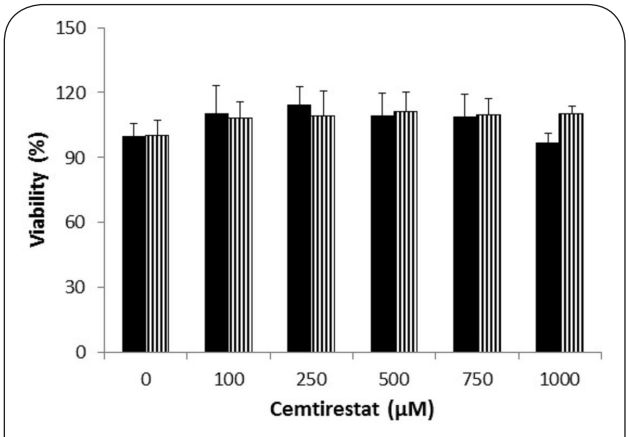

Figure 6. Viability parameters of VH-10 cells subsequent to 24 h exposure to cemtirestat. MTT test (black columns), NR uptake test (striped columns). Results are mean ± SD of at least three independent experiments run in three replicates. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences between the groups.

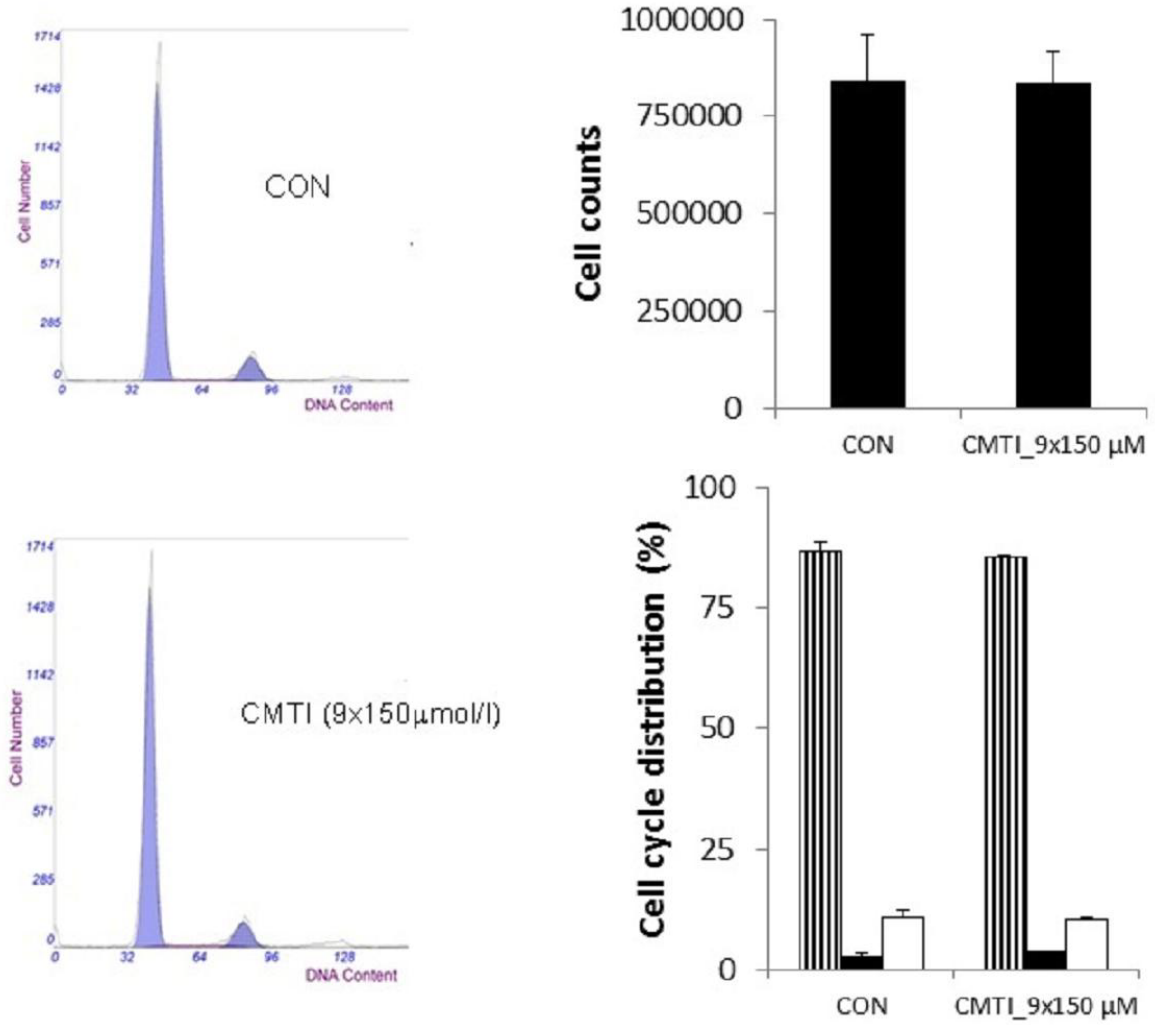

Figure 7. Effect of 5-day repetitive treatment with cemtirestat added into medium twice a day (150 µmol/l) on cell growth and cell cycle distribution in VH10 fibroblasts assessed by propidium iodide DNA staining and analysis of DNA content by flow cytometry. Data are mean ± SD of at least three independent experiments run in 3-4 replicates. One way ANOVA followed by Tukey's post hoc test gave no significant differences between the groups. Representative cell cycle histograms; G1 phase (stripped columns), S phase (black columns), G2/M phase (empty columns).

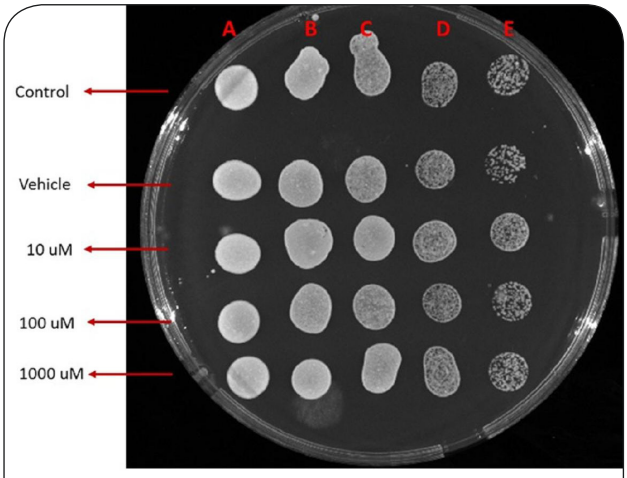

Figure 8. Yeast spotting test with cemtirestat (1 replicate). Concentration dependence. A: undiluted, B:1/10 diluted, C:1/100 diluted, D:1/1000 diluted, E:1/10000 diluted.

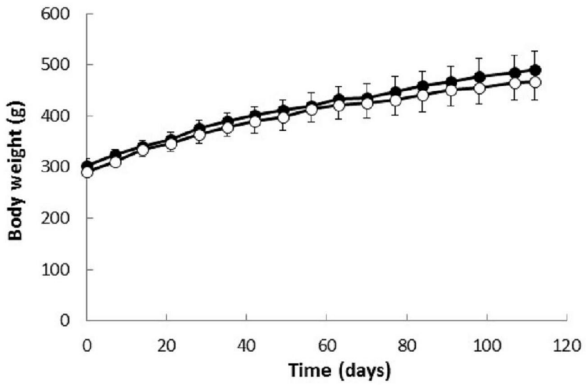

Figure 9. Body weights of male Wistar rats treated with cemtirestat. Group C, untreated rats(●); group T, rats treated with cemtirestat (○, 6.4 mg/kg/day). Results are means ± SEM from 6 (C, T) animals. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences between C and T groups.

Table 3. Relative organ weights (g/100 g b.w.) male Wistar rats at the end of the experiment*.

	C (6)	T(6)
Liver	2.734±0.093	2.623±0.139
Spleen	0.189±0.055	0.180±0.010
Adrenals	0.0091±0.0012	0.0087±0.0013
Kidney	0.516±0.031	0.518±0.042
Testes	0.923±0.060	1.001±0.069
Lens	0.023±0.003	0.026±0.003
Heart	0.224±0.011	0.230±0.008

*Group C, untreated control animals; Group T, rats treated by cemtirestat 6.4 mg/kg/day; Data are mean values±SD. Number of animals in each group is shown in parentheses. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences between C and T groups.

Table 4. Average chow/water consumption, plasma biochemical markers and erythrocyte sorbitol and MCF values of male Wistar rats recorded at the end of the experiment. Effect of cemtirestat treatment*.

	C (6)	T(6)
Average daily food consumption (g/100 g body weight)	7.59±1.19	8.14±1.99
Average daily water intake (g/100 g body weight)	6.32±0.66	6.39±1.11
Cholesterol (mM)	1.63±0.25	1.74±0.29
Triglycerides (mM)	1.45±0.25	1.06±0.34
Urea (mM)	7.21±0.50	7.93±0.58
Creatinine (μM)	30.28±4.31	34.27±4.47
TBARs in plasma (μM)	5.60±0.54	5.76±0.31
Ca	2.30±0.16	2.36±0.12
P	1.48±0.27	1.49±0.41
Mg	0.72±0.05	0.82±0.07
Sorbitol in red blood cells (nmol/ml packed RBC)	1.91±0.98	2.28±0.74
Erythrocyte MCF	58.63±2.83	57.10±2.69

*Measurements of chow and water consumption were made at 2-week intervals throughout the 4-month experiment. Group C, untreated control animals; Group T, rats treated by cemtirestat 6.4 mg/kg/day; MCF: The median corpuscular fragility was calculated as a concentration (mM) of NaCl at 50% hemolysis. Data are mean values±SD. Number of animals in each group is shown in parentheses. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences between C and T groups.

no significant cell cycle-dependent cytotoxic effect. These experimental data are also in accordance with previously reported absence of any effect of cemtirestat on osmotic fragility of isolated erythrocytes up to 250 μM concentration (Prnova *et al.*, 2015). As shown in Figure 8, cemtirestat up to 1000 μM concentration did not affect the viability of *Saccharomyces cerevisiae* in a yeast spotting test.

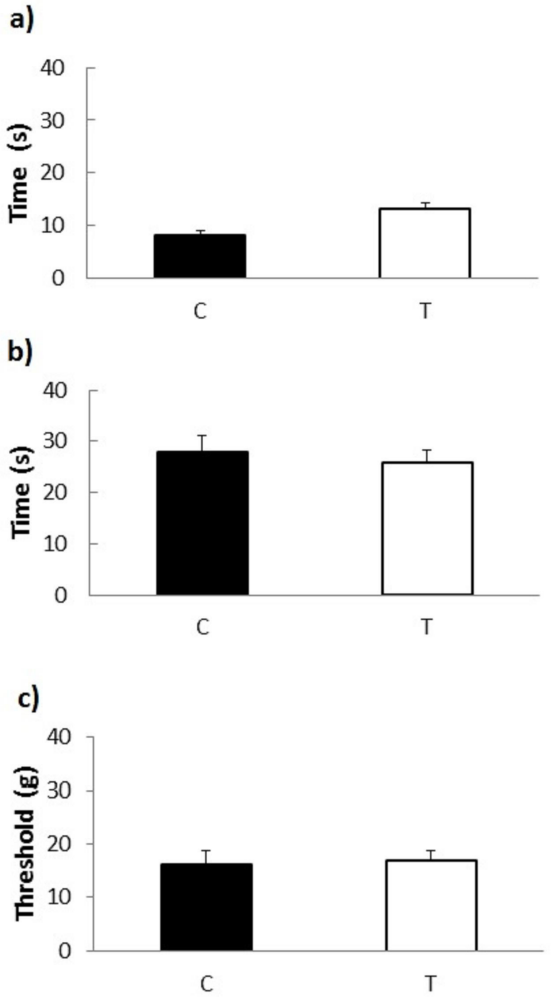

Figure 10. Peripheral nerve sensitivity tests in male Wistar rats treated with cemtirestat. Group C, untreated male Wistar rats; Group T, Wistar rats treated by cemtirestat (6.4 mg/kg/day); (a) Tail-flick test response latencies (50 °C); (b) Hot plate test response latencies (55 °C); (c) Tactile response thresholds as a result of stimulation with flexible von Frey filaments. Results are presented as means ± SEM from 6 (C, T) animals. One-way ANOVA followed by the *post-hoc* Tukey test gave no significant differences between C and T groups.

Animal studies

In our previous study in ZDF rats (Soltesova Prnova *et al.*, 2019), there were two groups of animals treated with cemtirestat. The drug was administered as a water solution by oral gavage in daily doses of 2.5 and 7.5 mg/kg. The dose of 2.5 mg/kg corresponds to the recommended daily dose of clinically used aldose reductase inhibitor epalrestat (Sharma and Sharma, 2008). In the present study, cemtirestat was administered as a solution in drinking water and the required daily dose was 7.5 mg/kg. Based on preliminary measurements of daily water consumption, the required concentration of cemtirestat in drinking water was estimated to be 100 mg/l. Yet the final average dose calculated on the basis of actual water consumption (Table 4) during the 4-month treatment was slightly lower, approx. 6.4 mg/kg/day.

Experimental data summarized in Table 2 and Figure 9 show that cemtirestat treatment had no significant effect on body weight gains of the experimental animals during the 120-day experiment. In addition, as shown in Table 3, cemtirestat treatment had no significant effect on relative organ weights of the animals. In agreement with these findings, cemtirestat also did not affect daily food and water consumption (Table 4). No signs or symptoms of cemtirestat toxicity developed during the 120-day treatment period. These observations are in accordance with our previously reported findings that revealed no acute toxicological manifestation of cemtirestat administered intragastrically (50 mg/kg/day) to male Wistar rats for five consecutive days (Soltesova Prnova *et al.* 2015a).

Extensive plasma assays revealed no significant effect of cemtirestat on the levels of plasma glucose (Table 2), plasma cholesterol, triglycerides, urea, creatinine, TBARs, Ca, Mg and P levels (Table 4). Experimental value of the median corpuscular fragility (MCF, quantitative marker of osmotic fragility of the erythrocytes) was not affected by cemtirestat. This result is in agreement with previously published results showing absence of any effect of 250 μM cemtirestat on osmotic fragility of isolated red blood cells (Prnova *et al.,* 2015).

Sensitivity of the peripheral nerves to painful stimuli (tail flick and hot plate tests) and to non-painful stimulation with flexible von Frey filaments was not significantly affected by 120-day treatment of the experimental rats with cemtirestat (Figure 10).

Conclusions

Based on ProTox-II toxicity prediction software, cemtirestat was classified as a compound of acute toxicity class 4 with mild hepatotoxicity. Cell culture viability tests performed on six different cell lines proved remarkably low cytotoxicity of cemtirestat. To avoid method-related bias in viability tests, five principally different viability assays were used. In the 120-day repeated oral toxicity study in male Wistar rats treated with the daily dose of 6.4 mg/kg of cemtirestat, no significant behavioral alterations or toxicological manifestations were observed in clinical and pathological examinations or in hematological parameters. In summary, these results suggest that cemtirestat is a safe drug that can proceed beyond preclinical studies.

Acknowledgements

Supported by VEGA 2/0005/2018, Slovak Research and Development Agency under the contract NO. APVV-15-0455, SAS-Tubitak JRP 2015/7 „GLUCOLIPOTOX", TUBITAK Project No: 215S19, Science Academy of Turkey Young Investigators Award (BAGEP) and N1-0066 from the Slovenian Research Agency.

Declaration of interest: *The authors report no conflict of interest.*

REFERENCES

Banerjee P, Eckert AO, Schrey AK, Preissner R. (2018). ProTox-II: a webserver for the prediction of toxicity of chemicals. *Nucleic Acids Res* **46** (W1): W257–W263.

Chen C, Kolodner R.D. (1999). Gross chromosomal rearrangements in Saccharomyces cerevisiae replication and recombination defective mutants. *Nat. Genet.* **23**: 81–85. .

Enayat S, Şeyma Ceyhan M, Taşkoparan B, Stefek M, Banerjee S. (2016). CHNQ, a novel 2-Chloro-1,4-naphthoquinone derivative of quercetin, induces oxidative stress and autophagy both in vitro and in vivo. *Arch Biochem Biophys***596**: 84–98.

Hevir-Kene N, Rižner TL. (2015). The endometrial cancer cell lines Ishikawa and HEC-1A, and the control cell line HIEEC, differ in expression of estrogen biosynthetic and metabolic genes, and in androstenedione and estrone-sulfate metabolism. *Chem Biol Interact* **234**: 309–19.

Ishiyama M. (1995). Advantages of WST-1compared to MTT agents. *In vitro Toxicology* **8**: 187–189.

Kljun J, Anko M, Traven K, Sinreih M, Pavlič R, Peršič Š, Ude Ž, Codina EE, Stojan J, Lanišnik Rižner T, Turel I. (2016). Pyrithione-based ruthenium complexes as inhibitors of aldo-keto reductase 1C enzymes and anticancer agents. *Dalton Trans* **45**(29): 11791–800.

Kwolek-Mirek M, Zadrag-Tecza R. (2014). Comparison of methods used for assessing the viability and vitality of yeast cells. *FEMS Yeast Res* **14**(7): 1068–1079.

Mrvová N, Škandík M, Kuniaková M, Račková L. (2015). Modulation of BV-2 microglia functions by novel quercetin pivaloyl ester. *Neurochem Int.* **90**: 246–54.

Milackova I, Rackova L, Majekova M, Mrvova N, Stefek M. (2015). Protection or cytotoxicity mediated by a novel quinonoid-polyphenol compound? *Gen Physiol Biophys* **34**(1): 51–64.

Pamies D, Hartung T. (2017). 1st Century Cell Culture for 21st Century Toxicology. *Chem Res Toxicol* **30**(1): 43–52.

Prnova MS, Ballekova J, Majekova M, Stefek M. (2015). Antioxidant action of 3-mercapto-5H-1,2,4-triazino[5,6-b]indole-5-acetic acid, an efficient aldose reductase inhibitor, in a 1,1'-diphenyl-2-picrylhydrazyl assay and in the cellular system of isolated erythrocytes exposed to tert-butyl hydroperoxide. *Redox Rep* **20**(6): 282–288.

Raies AB, Bajic VB. (2016). In silico toxicology: computational methods for the prediction of chemical toxicity. *WIREs Comput Mol Sci* **6**: 147–172.

Repetto G, del Peso A, Zurita JL. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat Protoc* **3**(7): 1125–31.

Sato K, Yama K, Murao Y, Tatsunami R, Tampo Y. (2013). Epalrestat increases intracellular glutathione levels in Schwann cells through transcription regulation. *Redox Biol* **2**: 15–21.

Sharma SR, Sharma N. (2008). Epalrestat, an aldose reductase inhibitor, in diabetic neuropathy: an Indian perspective. *Ann Indian Acad Neurol* **1**(4): 231–235.

Slamenová D, Horváthová E, Wsólová L, Sramková M, Navarová J. (2009). Investigation of anti-oxidative, cytotoxic, DNA-damaging and DNA-protective effects of plant volatiles eugenol and borneol in human-derived HepG2, Caco-2 and VH10 cell lines. *Mutat Res* **677**(1–2): 46–52.

Soltesova-Prnova M, Ballekova J, Gajdosikova A, Gajdosik A, Stefek M. (2015a). A novel carboxymethylated mercaptotriazinoindole inhibitor of aldose reductase interferes with the polyol pathway in streptozotocin-induced diabetic rats. *Physiol Res* **64**(4): 587–591.

Soltesova-Prnova M, Majekova M, Milackova I, Díez-Dacal B, Pérez-Sala D, Ceyhan MS, Banerjee S, Stefek M. (2015b). [5-(Benzyloxy)-1H-indol-1-yl]acetic acid, an aldose reductase inhibitor and PPARγ ligand. *Acta Biochim. Pol.* **62**(3): 523–528.

Soltesova-Prnova M, Svik K, Bezek S, Kovacikova L, Karasu C, Stefek M. (2019). 3-Mercapto-5H-1,2,4-Triazino[5,6-b]Indole-5-Acetic Acid (Cemtirestat) Alleviates Symptoms of Peripheral Diabetic Neuropathy in Zucker Diabetic Fatty (ZDF) Rats: A Role of Aldose Reductase. *Neurochem Res* **44**(5): 1056–1064.

Stefek M, Milackova I, Díez-Dacal B, Pérez-Sala D, Soltesova-Prnova M. (2017). Use of 5-carboxymethyl-3-mercapto-1,2,4-triazino-[5,6-b]indoles and their pharmaceutical composition. *Slovak Patent* No. 288508.

Stefek M, Soltesova-Prnova M, Ballekova J, Majekova M. (2016). Cemtirestat, a novel aldose reductase inhibitor and antioxidant, in multitarget pharmacology of diabetic complications. *International Journal of Advances in Science, Engineering and Technology*. IRAJ **4**(3): 41–44.

Stefek M, Soltesova-Prnova M, Majekova M, Rechlin C, Heine A, Klebe G. (2015). Identification of novel aldose reductase inhibitors based on carboxymethylated mercaptotriazinoindole scaffold. *J Med Chem* **58**(6): 2649–2657.

Stockert JC, Horobin RW, Colombo LL, and Blázquez-Castro A. (2018). Tetrazolium salts and formazan products in cell biology: Viability assessment, fluorescence imaging, and labeling perspectives. *Acta Histochemica* **120**: 159–167.

Vega-Avila E, Pugsley MK. (2011). An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. *Proc West Pharmacol Soc* **54**: 10–14.

Viskupicova J, Zizkova P, Rackova L, Horakova L. (2017). Pycnogenol Cytotoxicity in Pancreatic INS-1E β cells Induced by Calcium Dysregulation. *Phytother Res* **31**(11): 1702–1707.

Yabe-Nishimura, C. (1998). Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications. *Pharmacol Rev* **50**: 21–33.

Interdiscip Toxicol. 2019; **Vol. 12**(3): 129–135.
doi: 10.2478/intox-2019-0015

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Plasma paraoxonase1 activity in rats treated with monocrotophos: a study of the effect of duration of exposure

Raju NAGARAJU [1,2], Apurva Kumar R. JOSHI [1,3], Sowmya Giriyapura VAMADEVA [1], Rajini Padmanabhan SHARDA [1]

[1] Food Protectants and Infestation Control Department, CSIR- Central Food Technological Research Institute, Mysore, India
[2] Occupational Biochemistry, Regional Occupational Health Centre, Bangalore, Karnataka, India
[3] Department of Biochemistry, School of Sciences, Jain (deemed-to-be-university), Jayanagar 3rd Block, Bangalore, India

ITX120319A03 • Received: 13 November 2018 • Accepted: 22 July 2019

ABSTRACT

We have earlier demonstrated the potential of monocrotophos (MCP), a highly toxic organophosphorus insecticide (OPI), to elicit insulin resistance in rats after chronic exposure. Given the understanding of role of paraoxonase1 (PON1) in OPI toxicity and diabetes pathology, this study was envisaged to understand the effect of duration of exposure to MCP on plasma PON1 activity in rats. Rats were administered MCP *per os* at 1/20 and 1/10th LD_{50} as daily doses for 180 days. Interim blood samples were collected at 15, 30, 45, 90 and 180 d for analysis of plasma parameters. Exposure to MCP for 45 resulted in persistent trend of hyperinsulinemia, while significant increase in fasting glucose levels was observed after 180 days. MCP caused suppression of plasma cholinesterase activity though the study period, albeit extent of inhibition was more severe during the early phase of the study. Exposure to MCP for 180 d resulted in hypertriglyceridemia and marginal decrease in HDL-C levels. MCP failed to modulate PON1 activity in plasma during the early phase of the study (up to 45 d). However, prolonged exposure resulted in significant increase in the plasma PON1 activity. This suggests that manifestation of insulin resistance in rats subjected to chronic exposure to MCP is associated with increase in PON1 activity. Our work provides rationale for studying whether the increase in PON1 activity observed in the present study serves to counter the deleterious effect of long term exposure to organophosphorus insecticides on metabolic homeostasis.

KEY WORDS: paroxonase1 activity; monocrotophos; organophosphorus insecticide; insulin resistance; chronic exposure; metabolic homeostasis

Introduction

Organophosphorus insecticides (OPI) represent a major class of pesticide employed worldwide either for mitigating pests or preventing vector born disorders in agriculture and public health respectively. Although OPIs are less persistant than organochlorine insecticides, presence of OPI residues in various components of biosphere (Varo *et al.,* 2002; Sanghi *et al.,* 2003; Battu *et al.,* 2004; Kumari *et al.,* 2008; Joko *et al.,* 2018) clearly suggest that OPI are realistic toxicological threat. OPI act by inhibiting the enzyme acetylcholinesterase (AChE) and thereby causing cholinergic stress-mediated neurotoxicity (Fukuto, 1990; Sultatos, 1994; Sogorb & Vilanova, 2002). It is now well recognized that OPI exhibit potential to cause endocrine disruption (McKinlay *et al.,* 2008; Mnif *et al.,* 2011; Cecchi *et al.,* 2012). Activation of hypothalamus-pituitary-adrenal axis as evidenced by increase in circulating glucocorticoid hormones appears to be one of the most widely observed effects of OPI and other AChE inhibiting chemicals (Spassova *et al.,* 2000; Joshi & Rajini, 2009; Joshi & Rajini, 2012). In addition both clinical (revived by Joshi & Sukumaran, 2019) and experimental studies (Rahimi *et al.,* 2007; Joshi *et al.,* 2009) establish that OPI possess hyperglycemic potential. Cholinergic stress is one of the major factors responsible for hyperglycemic nature of OPI (MCP) (Joshi & Rajini, 2012) and can be completely attenuated by antagonists of cholinergic and adrenergic receptors (Joshi & Rajini, 2012; Joshi *et al.,* 2012). Epidemiological studies on cohorts of farmers and pesticide formulators reveal that chronic exposure to pesticides including OPI is associated with the development of symptoms of diabetes in exposed individuals and the duration of exposure determine the degree of symptoms in exposed individuals (Montgomery *et al.,* 2008; Raafat

Correspondence address:
Raju Nagaraju, PhD.
Occupational Biochemistry, Regional Occupational Health Centre
Bangalore, 562110 Karnataka, India
TEL.: 91-80-22172500 • FAX 91-080- 22172502
E-MAIL: raju86mys@yahoo.com

et al., 2012). Further, it is now established that OPIs elicit insulin resistance in experimental animals (Mostafalou *et al.,* 2012, Nagaraju *et al.,* 2015).

Human paraoxonase1 is a calcium-dependent hydrolytic enzyme and it is speculated that plays an important role in diseases such as diabetes and atherosclerosis. Low PON1 activity is associated with higher risk of cardiovascular diseases (Shunmoogam *et al.,* 2018). PON1 is implicated in hydrolytic detoxification of many OPIs and is known for hydrolyzing paraoxon, phenyl acetate and 4–nitrophenyl acetate as well as lactones (Costa *et al.,* 2005; Ceron *et al.,* 2014). PON is synthesized in the liver and secreted into the plasma where it associates with HDL particles. A 43kDa protein PON1 is known for polymorphisms arising from single nucleotide polymorphisms. While as many as 8 SNPs are known to occur in PON1, important ones are at positions 55 (Leu-Met) and 192 (Gln-Arg). These polymorphisms could either affect substrate specificity or biological activity of PON1 (Humbert *et al.,* 1993; Yamuda *et al.,* 2001; Shunmoogam *et al.,* 2018). PON1 is known for protecting LDL particles from oxidation, a property of PON1 that is majorly responsible for its cardioprotective effects (Mackness *et al.,* 1993; Mackness *et al.,* 2006). Studies suggest PON1 activity may be reduced in diabetic condition and owing to its ability of hydrolyzing OPIs, PON1 serves as a detoxification mechanism against OPI toxicity (see discussion). In our previous study, we observed the course of development of insulin resistance in rats subjected to chronic exposure. Exposure of rats to MCP for 45 days is required to elicit insulin resistance (Nagaraju *et al.,* 2015). Further, we observed that 180 days exposure to MCP is associated with hyperinsulinemic insulin resistance and elevated PON1 activity in rats (Nagaraju & Rajini, 2016). Therefore in this study, we analyzed the effect of duration of exposure to MCP on circulating PON1 activity (as measured by paraoxon hydrolysis) in an effort to understand the status of metabolic homeostasis in relation to circulating PON1 activity.

Materials and methods

Chemicals

Acetylthiocholine iodide (ATCI), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), and paraoxon (O, O-dimethyl-O-p-nitrophenylphosphate) were procured from Sigma Chemicals Co., (St. Louis, MO, USA). Orlistat was procured from Centurion Laboratories, Vadodara, India. Blood glucose, triglyceride, cholesterol and HDL-c kits were procured from Span Diagnostics (India). Insulin kit was procured from Crystalchem.,(USA). Monocrotophos (Technical grade, 75%) was a gift from Hyderabad Chemicals (Hyderabad, India). All other reagents and chemicals used were of analytical grade procured from SRL Pvt. Ltd (Mumbai, India).

Animals and experimental protocol

This study was approved by Institute Animal Ethical Committee, regulated by the CPCSEA, India. Adult male rats (CFT-Wistar strain, ~150±5 g) drawn from the Institute Animal House Facility at CSIR-CFTRI (Mysore) were divided into three groups. Rats of the first group served as control and received distilled water as vehicle, while second and third group were administered MCP at 0.9 and 1.8 mg/kg b.w/d respectively for 180 d. The blood samples were collected after 15, 30, 45, 90 and 180 exposures through orbital sinus method and used for measuring blood glucose, lipid profile, AChE and PON1 activity. The samples were collected 2 h after the last dose (Nagaraju *et al.,* 2015). Separate batches of rats from each group were fasted overnight and blood was collected via tail nick method for the estimation of glucose (Accu-Check) and insulin for analyzing insulin resistance via HOMA method.

Plasma parameters

Glucose, total cholesterol, and HDL-C were quantified by employing colorimetric kits based on action respective oxidases to generate hydrogen peroxide, which is quantified by use of peroxidase and a chromogen to obtain a red colored quinoneimine dye. The intensity of the dye was measured at 505 nm and results were expressed as mg/dl. For quantifying HDL-cholesterol, the LDL/VLDL fraction in serum was precipitated by using polyethylene glycol and supernatant was used to measure cholesterol using cholesterol kit. Plasma insulin was quantified using sandwich ELISA kit. Plasma triglyceride levels were measured with correction for free glycerol (Nagaraju *et al.,* 2013) using orlistat as lipoprotein lipase inhibitor and cholinesterase activity was quantified by measuring rate of hydrolysis acetylthiocholine iodide using DTNB (Galgani F, 1991). Paraoxonase activity in plasma was monitored by assessing the hydrolysis of paroxon in the presence of calcium chloride (Charlton-Menys *et al.,* 2006).

Homeostatic model of insulin resistance (HOMA-IR)

Fasting blood glucose and insulin values were used to calculate insulin resistance and beta cell function by employing HOMA model (Matthews *et al.,* 1985).

Statistical analysis

Mean and standard error (SE) were determined for all parameters and results were expressed as mean ± SE. The data were analyzed employing ANOVA followed by Tukey's *post-hoc* test for comparison of means to determine the significance of differences between the groups. A p-value below 0.05 was considered as significantly different.

Results

Impact of repeated oral doses of MCP on fasting blood glucose, insulin and insulin sensitivity

The impact of repeated oral doses of MCP on fasting blood glucose, insulin, and status of insulin resistance at different time intervals is tabulated in Table 1. The fasting blood glucose levels were considerably enhanced in treated

Table 1. Impact of repeated oral doses of monocrotophos on fasting blood glucose, insulin and beta cell function in treated rats.

Variable	MCP exposure (mg/kg b.w)	15	30	45	90	180
Fasting blood glucose (mg/dl)	0	77.00±1.1^a	84.01±2.0^a	74.67±1.2^a	74.67±2.91^a	71.00±2.08^a
	0.9	65.50±2.0^b	78.33±1.9^a	77.33±2.3^a	70.67±2.73^a	85.33±2.73^b
	1.8	61.88±5.0^b	73.67±1.8^a	77.67±4.2^a	71.33±3.14^a	94.33±2.85^b
Fasting plasma insulin (mu/L)	0	8.53±0.8^a	8.29±0.6^a	7.60±0.6^a	8.33±0.3^a	7.72±0.24^a
	0.9	4.10±0.5^b	8.35±0.8^a	10.90±0.2^b	13.08±0.3^b	16.44±0.54^b
	1.8	3.62±0.4^b	8.31±1.2^a	12.73±0.9^b	15.85±0.9^b	26.33±1.92^c
Insulin resistance (HOMA)	0	1.06±0.1^a	1.03±0.1^a	0.94±0.1^a	1.02±0.1^a	0.94±0.03^a
	0.9	0.47±0.1^b	1.04±0.1^a	1.35±0.1^b	1.57±0.1^b	2.03±0.05^b
	1.8	0.43±0.1^b	1.01±0.1^a	1.47±0.1^b	1.90±0.1^c	3.35±0.26^c
Beta cell function (HOMA)	0	138.8±5.6^a	114.6±1.9^a	138.1±11.0^a	147.3±12.0^a	124.13±8.9^a
	0.9	119.3±8.1^a	133.2±10.7^a	155.7±12.1^a	223.1±18.9^b	208.3±14.7^b
	1.8	123.1±10.6^a	149.6±14.1^a	180.5±16.0^b	247.5±19.5^b	200.6±1.9^b

Values are expressed as mean ± S.E (n=3); The columns with different alphabets are statistically different (p<0.05).

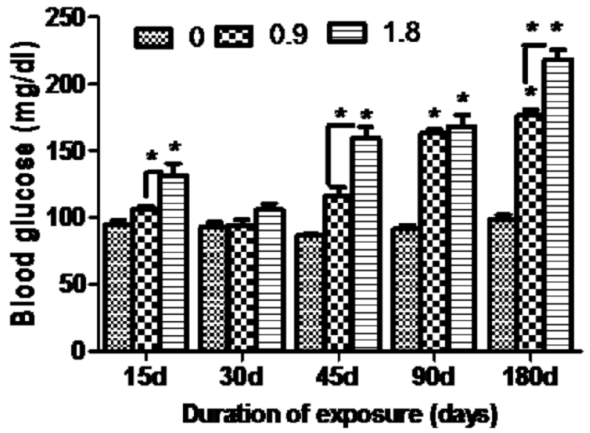

Figure 1. Effect of 0.9 and 1.8 mg/kg MCP on Blood glucose: The values are mean ± SE (n=6). Bars with (*) are significantly different (p<0.05)

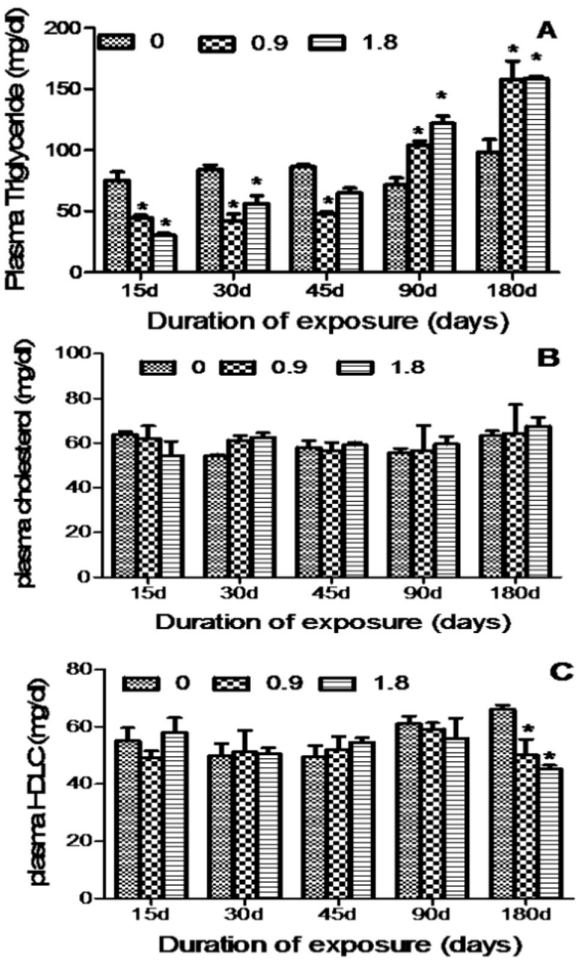

Figure 2. Impact of 0.9 and 1.8 mg/kg MCP on Lipid profile; Triglyceride (a), total cholesterol (b), High density lipoprotein cholesterol (c) Values are mean ± SE (n=6). Bars with (*) are significantly different (p<0.05)

rats after 180th dose (20–33% above control, p<0.05), while plasma insulin levels were significantly enhanced during the 45th dose of MCP administration (50–70% above control, p<0.001) in treated rats. The plasma insulin level was highly intensified as the duration of exposure continued and attained 2.7–3.5 fold above controls after 180 days exposure. Since enhanced fasting insulin is the surrogate marker of IR, we calculated IR using the HOMA model. MCP treated rats exhibit insulin resistance after 45 d of MCP exposure and insulin resistance worsened as the experimental regime continued and at the end of regime insulin resistance was found to be 3–4 fold above controls in treated rats. Similarly, aggravation of insulin resistance due to chronic exposure is associated with an increase in beta cell function (60–80%).

Effect of chronic exposure to MCP on plasma parameters.

Effect of duration of exposure to MCP on blood glucose, lipid profiles AChE, and PON1 activity in rats are depicted in Figures 1–4 respectively. The data represented in these figures are from samples collected 2 h after the respective last dose. Impact of MCP on blood glucose levels was persistent after 45 days of exposure and after 180 days

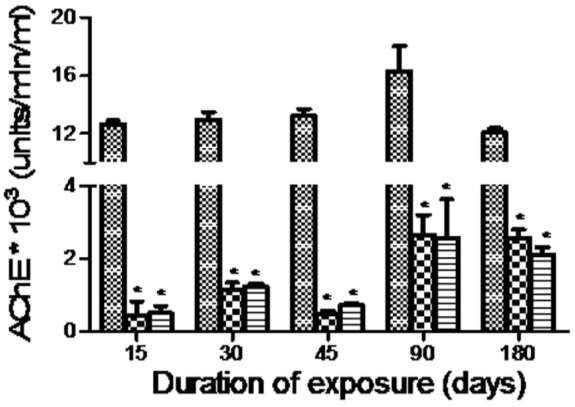

Figure 3. Effect of 0.9 and 1.8 mg/kg MCP on AChE activity: The Values are mean ± SE (n=6). Bars with (*) are significantly different (p<0.05)

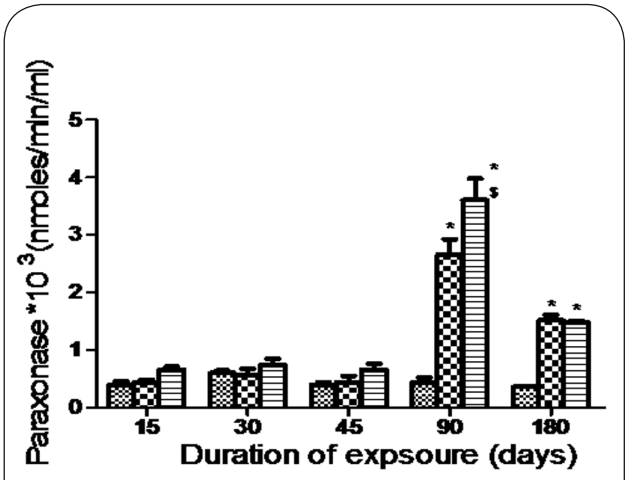

Figure 4. Paraxonase activity in control and 0.9 and 1.8 mg/kg MCP treated rats: The Values are mean ± SE (n=6). Bars with different symbols are significantly different (p<0.05)

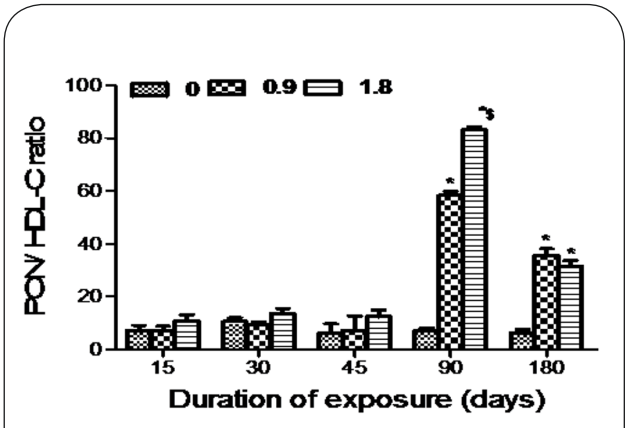

Figure 5. PON activity/HDL cholesterol ratio in control and 0.9 and 1.8 mg/kg MCP treated rats: The Values are mean ± SE (n=6). Bars with different symbols are significantly different (p<0.05)

of exposure, glucose levels were 90–110% above control (Figure 1). Figure 2 depicts data on triglycerides, total and HDL-cholesterol in plasma of rats from different groups. The plasma triglyceride levels were significantly declined in earlier phase (35–45% and 50–60% of control at 15 and 30 d respectively), while enhanced plasma triglycerides were noticed after 180 d which was found to be 50–60% above control. The plasma cholesterol levels remain unaltered, while a marginal decrease in HDL-c levels was noticed (60–75% of control) after 180 d and which might demonstrate the possibility of symptoms of dyslipidemia induced by chronic exposure to MCP in treated rats.

The AChE activity was suppressed throughout the study period in MCP-treated rats, although extent of inhibition was more severe up to 45 days (Figure 3). Interestingly, plasma PON1 activity remained comparable in rats of all groups up to 45 days of the study period. However, there was a phenomenal increase in circulating PON1 activity in plasma of rats exposed to MCP for 90 days. In rats exposed to MCP for 180 days, the plasma PON1 activity remained elevated, although extent of elevation was less compared to that of 90 days exposure group (Figure 4). Consequently, PON1 activity/HDL-c ratio was significantly increased in rats exposed to MCP for 90 and 180 days, although extent of elevation was more pronounced in rats exposed for 90 days.

Discussion

hyperglycemia and dyslipidemia are the common metabolic impairments associated with OPI toxicity in humans and experimental animals (Rahimi *et al.,* 2007; Karami-Mohajeri *et al.,* 2011; Joshi & Sukumaran, 2019). Many OPIs including MCP are known to induce hyperglycemia in experimental animals after exposure to a single dose (Seifert, 2001; Lasram *et al.,* 2008; Joshi & Rajini, 2009, Joshi & Rajini 2012; Joshi *et al.,* 2012; Acker *et al.,* 2012). An important feature of hyperglycemia induced by OPI appears to be its transient nature (Seifert, 2001; Lasram *et al.,* 2008; Joshi & Rajini, 2009, Joshi & Rajini 2012). In this study, we observed that the chronic exposure was associated with increased glycemic responses in the earlier phase which gradually enhanced and aggravated as the duration of exposure continued. These results are in agreement with our previous observations (Nagaraju *et al.,* 2015) of hyperglycemia caused by chronic exposure which was found to be associated with up regulation of substrate driven glucose synthesis and enhanced glycogenolysis in MCP treated rats. Further, in this study, MCP treated rats exhibited dyslipidemia, characterized by hypertriglyceridemia with marginal lower levels of HDL cholesterol. These results are in agreement with observations made by others (Slotkin *et al.,* 2005; Rezg *et al.,* 2010) on the effect of OPIs on lipid profile.

Plasma cholinesterase activity, a marker of OPI toxicity, was significantly decreased in MCP treated rats. Interestingly, the extent of inhibition is more severe in the early phase of the present study than chronic exposure

which might be due to enhanced PON1 activity in these rats. Mice lacking serum paraoxonase demonstrated more sensitivity to OPI toxicity via inhibition of acetylcholine esterase activity at the dose which is ineffective in wild animals (Shih *et al.,* 1998). Therefore, the enhanced PON1 activity might have an impact on AChE activity after chronic exposure. We observed that fasting blood glucose and insulin levels were significantly increased in treated rats after chronic exposure. Scientific studies have shown that chronic exposure to organophosphorus insecticides in farmers and pesticide formulators is likely to be associated with insulin resistance and type 2 diabetes which correlates with duration and frequency of exposures to such chemicals in their lifetime (Montgomery *et al.,* 2008; Raafat *et al.,* 2012). Further, OPI's may increase the risk of gestational diabetes (Saldana *et al.,* 2007). Gifford *et al.,* (2019) reported that OPI toxicity is associated with acute dysregulations in glucose homeostasis linked to changes in insulin action and secretion.

PON1 is a serum enzyme closely associated with high-density lipoproteins and is known for its antioxidant properties and metabolism of toxic lipid molecules associated with LDL and HDL molecules (Mackness *et al.,* 2006). Further, it also hydrolyzes various organophosphorus insecticides and lactone containing pharmaceutical compounds. PON1 polymorphism and activity are known to be a determinant or biomarker for the sensitivity to organophosphorus insecticides in human subjects. General observation from these studies is that lower activity or phenotypes with lower activity are associated with high risk for OPI toxicity (Lee *et al.,* 2003; Sirivarasai *et al.,* 2007). Further, mice lacking PON1 are reported to exhibit higher sensitivity of OPI toxicity and fail to prevent LDL oxidation when fed high levels of fat and cholesterol in diet (Shih *et al.,* 1998). In addition to modulating OPI toxicity, the role of PON1 as a critical determinant of metabolic health is now being understood. Studies have reported that diabetes is associated decreased PON1 activity (Mackness *et al.,* 1991; Abbott *et al.,* 1995; Inoue etal., 2000; Flekač *et al.,* 2008; Gupta *et al.,* 2011; Shakeri *et al.,* 2017). Animal studies shed light on association of PON1 activity with metabolic dyshomeostasis. Streptozotocin (STZ), a phamocological diabetogen used for generating experimental models of insulin-dependent diabetes, is known to cause hyperglycemia and decreased PON1 activity (Patel *et al.,* 1990). Administration of recombinant PON1 has been reported to reduce incidence of diabetes, lower glucose and increase circulating insulin levels in STZ-treated rats (Koren-Gluzer *et al.,* 2011). PON1 deficiency (knockout) has been reported to cause increase in fasting glucose and insulin levels in both normal diet and high fat diet fed mice (Koren-Gluzer *et al.,* 2013). Further, PON1 deficiency has been reported to aggravate STZ-induced diabetes in mice, while PON1 over expression offers protection against diabetes incidence and mortality in mice (Rozenberg *et al.,* 2008). Thus, PON1 activity may be perceived to play a major role in regulation of metabolic homeostasis. In our previous study, we reported that chronic exposure to MCP in rats is associated with the onset of insulin resistance after 45 days of exposure to daily doses and insulin resistance worsened as the duration of exposure continued (Nagaraju *et al.,* 2015). We demonstrated that insulin resistance after 180 days exposure to MCP is associated with augmented pancreatic beta cell response presumably to counter the metabolic effects of MCP (Nagaraju *et al.,* 2015; Nagaraju & Rajini, 2016). Further studies are needed to understand whether increase in PON1 activity reported by us serves as a counter against chronic MCP-induced metabolic dysregulations.

Conclusion

Many scientific studies have established a strong correlation between PON1 activity and the prevalence of metabolic syndrome in humans and animal studies. PON1 is also a determinant of OPI toxicity. In this study, plasma PON1 activity was significantly increased after chronic exposure to MCP which coincides with decrease in the extent of AChE inhibition along with an increase in fasting plasma insulin levels. Further studies are needed to understand the impact of increase in PON1 activity on metabolic status in rats subjected to chronic MCP exposure.

Acknowledgments

The authors wish to thank the Director, CSIR-CFTRI, Mysore, for his support to the study. The first author (RN) acknowledges financial support from ICMR, India in the form of research fellowship. M/s Hyderabad Chemicals Supplies is greatly thanked for the gift of technical grade sample of monocrotophos.
RN, AKRJ and PSR conceived and designed the study. Experiments and data acquisition were performed by RN and SV. RN, AKRJ and PSR wrote the manuscript. All authors have read and approved the manuscript.

REFERENCES

Abbott CA, Mackness MI, Kumar S, Boulton AJ, Durrington PN. (1995). Serum paraoxonase activity, concentration, and phenotype distribution in diabetes mellitus and its relationship to serum lipids and lipoproteins. *Arteriosclerosis, thrombosis, and vascular biology* **15**(11): 1812–1818.

Acker CI, Nogueira CW. (2012). Chlorpyrifos acute exposure induces hyperglycemia and hyperlipidemia in rats. *Chemosphere*. **89**(5): 602–608

Battu RS, Singh B, Kang BK. (2004). Contamination of liquid milk and butter with pesticide residues in the Ludhiana district of Punjab state, India. *Ecotoxicology and environmental safety* **59**(3): 324–331.

Charlton-Menys V, Liu Y, Durrington PN. (2006). Semiautomated method for determination of serum paraoxonase activity using paraoxon as substrate. *Clinical chemistry* **52**(3): 453–7.

Cecchi A, Rovedatti MG, Sabino G, Magnarelli GG. (2012). Environmental exposure to organophosphate pesticides: assessment of endocrine disruption and hepatotoxicity in pregnant women. *Ecotoxicology and environmental safety* **80**: 280–287.

Ceron JJ, Tecles F, Tvarijonaviciute A. (2014). Serum paraoxonase 1 (PON1) measurement: an update. *BMC veterinary research* **10**(1): 74.

Flekač M, Škrha J, Zidkova K, Lacinova Z, Hilgertova J. (2008). Paraoxonase 1 gene polymorphisms and enzyme activities in diabetes mellitus. *Physiological Research* **57**(5): 717–726.

Fukuto TR. (1990). Mechanism of action of organophosphorus and carbamate insecticides. *Environmental Health Perspective* **87**: 245–254.

Galgani F, Bocquene G. (1991). Semi-automated colorimetric and enzymatic assays for aquatic organisms using microplate readers. *Water Research* **25**(2): 147–150.

Gifford RM, Chathuranga U, Lamb T, Verma V, Sattar MA *et al.* (2019). Short-term glucose dysregulation following acute poisoning with organophosphorus insecticides but not herbicides, carbamate or pyrethroid insecticides in South Asia. *Clinical Toxicology* **57**(4): 254–264.

Gupta N, Binukumar BK, Singh S, Sunkaria A, Kandimalla R, Bhansali A, Gill KD. (2011). Serum paraoxonase-1 (PON1) activities (PONase/AREase) and polymorphisms in patients with type 2 diabetes mellitus in a North-West Indian population. *Gene* **487**(1): 88–95.

Humbert. (1993).The molecular basis of the human serum paraxonase activity polymorphism. *Nature genetics* **3**: 73–76.

Inoue M, Suehiro T, Nakamura T, Ikeda Y, Kumon Y, Hashimoto K. (2000). Serum arylesterase/diazoxonase activity and genetic polymorphisms in patients with type 2 diabetes. *Metabolism-Clinical and Experimental* **49**(11): 1400–1405.

Joko T, Anggoro S, Sunoko HR, Rachmawati S. (2018). Identification of Soil Properties and Organophosphate Residues From Agricultural Land in Wanasari Sub-District, Brebes, Indonesia. In *E3S Web of Conferences 2018* (Vol. 31, p. 06010). EDP Sciences.

Joshi AK, Rajini PS. (2009). Reversible hyperglycemia in rats following acute exposure to acephate, an organophosphorus insecticide: role of gluconeogenesis. *Toxicology* **257**: 40–45.

Joshi AK, Nagaraju R, Rajini PS. (2012). Insights into the mechanisms mediating hyperglycemic and stressogenic outcomes in rats treated with monocrotophos, an organophosphorus insecticide. *Toxicology* **294**(1): 9–16.

Joshi AK, Rajini PS. (2012). Hyperglycemic and stressogenic effects of monocrotophos in rats: evidence for the involvement of acetylcholinesterase inhibition. *Experimental and toxicologic pathology* **64**(1–2): 115–20.

Joshi AK, Sukumaran BO. (2019). Metabolic dyshomeostasis by organophosphate insecticides: insights from experimental and human studies. *EXCLI journal* **18**: 479–84.

Karami-Mohajeri S, Abdollahi M. (2011). Toxic influence of organophosphate, carbamate, and organochlorine pesticides on cellular metabolism of lipids, proteins, and carbohydrates: a systematic review. *Human & experimental toxicology* **30**(9): 1119–1140.

Koren-Gluzer M, Aviram M, Meilin E, Hayek T. (2011). The antioxidant HDL-associated paraoxonase-1 (PON1) attenuates diabetes development and stimulates β-cell insulin release. *Atherosclerosis* **219**: 510–518.

Koren-Gluzer M, Aviram M, Hayek T. (2013). Paraoxonase1 (PON1) reduces insulin resistance in mice fed a high-fat diet, and promotes GLUT4 overexpression in myocytes, via the IRS-1/Akt pathway. *Atherosclerosis* **229**(1): 71–78.

Kumari B, Madan VK, Kathpal TS. (2008). Status of insecticide contamination of soil and water in Haryana, India. *Environmental monitoring and assessment* **136**(1–3): 239–244.

Lasram MM, Annabi AB, Rezg R, Elj N, Slimen S, Kamoun A, El-Fazaa S, Gharbi N. (2008). Effect of short-time malathion administration on glucose homeostasis in Wistar rat. *Pesticide biochemistry and physiology* **92**(3): 114–119.

Lee BW, London L, Paulauskis J, Myers J, Christiani DC. (2003). Association between human paraoxonase gene polymorphism and chronic symptoms in pesticide-exposed workers. *Journal of Occupational and Environmental Medicine* **45**(2): 118–22.

Longnecker MP, Daniels JL. (2001). Environmental contaminants as etiologic factors for diabetes. *Environmental health perspectives* **109**(suppl 6): 871–876.

Mackness M, Harty D, Bhatnagar D, Winocour PH, Arrol S, Ishola M, Durrington PN. (1991). Serum paraoxonase activity in familial hypercholesterolaemia and insulin dependent diabetes mellitus. *Atherosclerosis* **86**: 193–199.

Mackness MI, Arrol S, Abbott C, Durrington PN. (1993). Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis* **104**(1–2): 129–135.

Mackness B, Quarck R, Verreth W, Mackness M, Holvoet P. (2006). Human paraoxonase-1 overexpression inhibits atherosclerosis in a mouse model of metabolic syndrome. Arteriosclerosis, *Thrombosis, and Vascular Biology*. **26**(7): 1545–1550.

Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**(7): 412–419.

McKinlay R, Plant JA, Bell JN, Voulvoulis N. (2008). Endocrine disrupting pesticides: implications for risk assessment. *Environment international* **34**(2): 168–183.

Mnif W, Hassine AI, Bouaziz A, Bartegi A, Thomas O, Roig B. (2011). Effect of endocrine disruptor pesticides: a review. *International journal of environmental research and public health* **8**(6): 2265–2303.

Montgomery MP, Kamel F, Saldana TM, Alavanja MC, Sandler DP. (2008). Incident diabetes and pesticide exposure among licensed pesticide applicators: Agricultural Health Study, 1993–2003. *American journal of epidemiology* **167**(10): 1235–1246.

Mostafalou S, Eghbal MA, Nili-Ahmadabadi A, Baeeri M, Abdollahi M. (2012). Biochemical evidence on the potential role of organophosphates in hepatic glucose metabolism toward insulin resistance through inflammatory signaling and free radical pathways. *Toxicology and industrial health* **28**(9): 840–851.

Nagaraju R, Joshi AK, Rajini PS. (2013). Estimation of plasma triglycerides with correction for free glycerol by orlistat inhibition of lipoprotein lipase activity. *Analytical biochemistry* **439**(1): 44–46.

Nagaraju R, Joshi AK, Rajini PS. (2015). Organophosphorus insecticide, monocrotophos, possesses the propensity to induce insulin resistance in rats on chronic exposure. *Journal of diabetes* **7**(1): 47–59.

Nagaraju R, Rajini PS. (2016). Adaptive response of rat pancreatic β-cells to insulin resistance induced by monocrotophos: Biochemical evidence. *Pesticide biochemistry and physiology* **134**: 39–48.

Patel BN, Mackness MI, Harty DW, Arrol S, Boot-Hanford RP, Durrington PN. (1990). Serum esterase activities and hyperlipidaemia in the streptozotocin-diabetic rat. *Biochimica et Biophysica Acta (BBA)-General Subjects* **1035**(1): 113–116.

Raafat N, Abass MA, Salem HM. (2012). Malathion exposure and insulin resistance among a group of farmers in Al-Sharkia governorate. *Clinical biochemistry* **45**(18): 1591–1595.

Rahimi, R, Abdollahi, MA. (2007). review on the mechanisms involved in hyperglycemia induced by organophosphorus pesticides. *Pesticide biochemistry and physiology* **88**: 115–121.

Rezg R, Mornagui B, Benahmed M, Chouchane SG, Belhajhmida N, Abdeladhim M, Kamoun A, El-Fazaa S, Gharbi N. (2010). Malathion exposure modulates hypothalamic gene expression and induces dyslipedemia in Wistar rats. *Food and chemical toxicology* **48**(6): 1473–1477.

Rozenberg O, Shiner M, Aviram M, Hayek T. (2008). Paraoxonase 1 (PON1) attenuates diabetes development in mice through its antioxidative properties. *Free Radical and Biological Medicine* **44**: 1951–1959.

Saldana TM, Basso O, Hoppin JA, Baird DD, Knott C, Blair A, Alavanja MC, Sandler DP. **(**2007**).** Pesticide exposure and self-reported gestational diabetes mellitus in the Agricultural Health Study. *Diabetes Care* **30**: 529–534.

Sanghi R, Pillai MK, Jayalekshmi TR, Nair A. (2003). Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. *Human & experimental toxicology* **22**(2): 73–76.

Seifert J. (2001). Toxicologic significance of the hyperglycemia caused by organophosphorous insecticides. *Bulletin of environmental contamination and toxicology* **67**(4): 463–469.

Spassova D, White T, Singh AK. (2000). Acute effects of acephate and methamidophos on acetylcholinesterase activity, endocrine system and amino acid concentrations in rats. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **126**(1): 79–89.

Shakeri R, Khajeniazi S, Marjani A. (2017). Association between promoter polymorphism (–108C> T) of paraoxonase1 gene and it's paraoxonase activity in patients with Type2 diabetes in northern Iran. *Clinica Chimica Acta* **474**: 34–37.

Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. (1998). Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* **394**: 284–287.

Shunmoogam N, Naidoo P, Chilton R. (2018). Paraoxonase (PON)-1: a brief overview on genetics, structure, polymorphisms and clinical relevance. *Vascular Health Risk Managment* **14**: 137–143.

Sirivarasai J, Kaojarern S, Yoovathaworn K, Sura T. (2007). Paraoxonase (PON1) polymorphism and activity as the determinants of sensitivity to organophosphates in human subjects. *Chemico Biological Interaction* **168**: 184–192.

Slotkin TA, Brown KK, Seidler FJ. **(**2005**).** Developmental exposure of rats to chlorpyrifos elicits sex-selective hyperlipidemia and hyperinsulinemia in adulthood. *Environmental Health Perspective* **113**: 1291–1294.

Sogorb MA, Vilanova E. (2002). Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicological Letters* **128**: 215–228.

Varo I, Serrano R, Pitarch E, Amat F, Lopez FJ, Navarro JC. (2002). Bioaccumulation of chlorpyrifos through an experimental food chain: study of protein HSP70 as biomarker of sublethal stress in fish. *Archives of environmental contamination and toxicology* **42**(2): 229–235.

Yamada A, Shoji T, Tahara H, Emoto M, Nishizawa Y. (2001). Effect of insulin resistance on serum paraoxonase activity in a nondiabetic population. *Metabolism-Clinical and Experimental* **50**(7): 805–811.

Interdiscip Toxicol. 2019; **Vol. 12**(3): 136–142.
doi: 10.2478/intox-2019-0016

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Positive and inverse correlation of blood lead level with erythrocyte acetylcholinesterase and intelligence quotient in children: implications for neurotoxicity

Nnenna Linda NWOBI [1], Solomon Kayode ADEDAPO [2], Olugbemi OLUKOLADE [3], Opebiyi Alexander OYINLADE [4], Ikeoluwa Abiola LAGUNJU [4], Nnodimele Onuigbo ATULOMAH [5], Ikechukwu Ambrose NWAZUOKE [6], John Ibhagbemien ANETOR [2]

[1] Department of Chemical Pathology, BenCarson School of Medicine, Babcock University, Nigeria
[2] Department of Chemical Pathology, College of Medicine, University of Ibadan, Ibadan, Nigeria
[3] Department of Family Medicine and Psychiatry, University College Hospital, Ibadan, Nigeria
[4] Department of Paediatrics, College of Medicine, University of Ibadan/University College Hospital, Ibadan, Nigeria
[5] Department of Public Health, School of Public and Allied Health, Babcock University, Nigeria
[6] Department of Special Education, Faculty of Education, University of Ibadan, Nigeria

ITX120319A04 • Received: 07 February 2019 • Accepted: 12 September 2019

ABSTRACT

Blood lead level (BLL) is insufficiently sensitive for early detection of Lead-induced neurotoxicity (LIN). This study determined the possible role of the combination of BLL, intelligent quotient (IQ) and erythrocyte acetylcholinesterase (AChE) activity in the early detection of LIN in Children. Apparently healthy children (n=309) from eight public primary schools in Ibadan, Nigeria were recruited and classified into: children with Elevated BLL (EBLL) and children with Acceptable BLL (control) based on CDC cut-off for childhood lead exposure. Neurological indices (speech, memory, cranial nerves and cerebellar functions), IQ, BLL and erythrocyte AChE activity were assessed using standard methods, Standard Progressive Matrices, AAS and HPLC respectively. Statistical analysis involved Student's t-test, Pearson's correlation and multivariate regression. $p<0.05$ was considered significant. There were 169 (54.7%) children with EBLL while there were 140 (45.3%) control children. Both groups exhibited normal speech, memory, cranial nerves and cerebellar functions. However, IQ was lower in EBLL children (85.9±11.6) compared with control (91.5±14.0) while BLL and AChE activity were higher in EBLL children (0.4±0.1 µmol/l; 117.5±25.5 µkat/l) compared with control (0.2±0.0 µmol/l; 59.4±10.2 µkat/l). BLL showed inverse correlation with IQ (r=−0.134, p=0.019) but positive correlation with AChE (r=0.978, $p\leq0.001$). 16.2% of the observed variation in BLL could be accounted for by AChE using the equation; [BLL=−0.007+0.003 AChE] $p<0.05$. Elevated blood lead level is prevalent among the school children and appears to have adverse effect on their IQ. Erythrocyte AChE could be a promising marker for early recognition of significant environmental lead exposure and lead-induced neurotoxicity in children.

KEY WORDS: erythrocyte acetylcholinesterase; intelligence quotient; lead-induced neurotoxicity

ABBREVIATIONS:
ACh: Acetylcholine; **AChE:** Acetylcholinesterase; **AAS:** Atomic absorption spectroscopy; **BLL:** Blood lead level; **CDC:** Centers for Disease Control and Prevention; **CNS:** Central nervous system; **EBLL:** Elevated BLL; **HPLC:** High-Pressure Liquid Chromatography; **Ha_2O_2:** Hydrogen peroxide; **IQ:** Intelligent quotient; **Pb:** Lead; **LIN:** Lead-induced neurotoxicity; **UI/UCH:** University of Ibadan/University College Hospital

Correspondence address:
Dr. Nnenna Linda Nwobi
Department of Chemical Pathology,
Ben Carson School of Medicine, Babcock University, Nigeria
TEL.: 234(0)8135388049 • E-MAIL: lindanwobi@yahoo.ca

Introduction

Lead (Pb) is a naturally occurring ubiquitous toxic metal whose uncontrolled use in multiple industrial, domestic, agricultural, medical and technological applications has brought about its wide environmental distribution, widespread human exposure and subsequent serious health and intellectual concerns particularly in children in developing countries and Nigeria is not an exception (Ogunseitan & Smith 2007; WHO, 2013; Anetor *et al.*, 2016; Nwobi *et al.*, 2019).

Children are exposed to Pb through contaminated food, water, air and dust which, after being absorbed into the blood, is distributed to virtually every organ and system where it causes broad range of toxicity (WHO,

2015). One of the systems of importance and greatest susceptibility is the central nervous system (CNS) where it has tendency to cause neurotoxicity particularly in the brain of children because of the high absorption rate of Pb coupled with increased penetration of Pb through the blood–brain barrier that is the most sensitive to damage (Bellinger, 2008; Mason *et al.,* 2014).

One of the mechanisms of lead-induced neurotoxicity (LIN) has been reported to be interference in the neurotransmission function of the CNS and of particular interest is its interference with the cholinergic system which may result in impairments in CNS function including neurocognition (Lidsky & Schneider, 2003; Flora *et al*., 2012; Sharma *et al*., 2014). Acetylcholinesterase (AChE) is a key enzyme involved in cholinergic neurotransmission which hydrolyses the neurotransmitter acetylcholine (ACh) in order to terminate normal synaptic transmission of impulse and avoid unnecessary stimulation of the nervous system (Saldanha, 2017). This enzyme is a type-B carboxylesterase found mainly in the neuromuscular junction and cholinergic brain synapses but also in human erythrocytes (Hajjawi 2012; Gupta *et al*., 2015). Several reports have shown that the activity of erythrocyte AChE does not only correlate positively with the activity of the brain AChE but also reflects neurochemical targets in brain (Ademuyiwa *et al*., 2007; Lionetto *et al*., 2013; Gupta *et al*., 2015).

One of the numerous brain functions include neurocognition (Hasselmo, 2006; Haense *et al*., 2012). Essential factors in neuro-cognition such as concentration, focus, memory and highly-ordered thought processes have been reported to be facilitated by acetylcholine (Hasselmo, 2006). Several reports have linked Intelligence quotient (IQ), a well recognised and widely used index of neurocognition, to blood lead level (BLL) (Slikker *et al.,* 2000; Haense *et al*., 2012; Liu *et al.,* 2013).

Blood lead level is the most widely used biomarker of lead exposure and achieving BLL limit of 0 µg/dl in children is rarely feasible (Sommar *et al*., 2014; WHO, 2015). Consequently, the US Centers for Disease Control and Prevention (CDC) since 2012 has identified BLL ≤5 µg/dl as acceptable and BLL >5 µg/dl as elevated blood lead level (EBLL) that should prompt further medical investigation in children (CDC, 2012). However, the use and interpretation of BLL for the early detection of LIN is not generally accepted owing to its short half-life of about 30 days and its ability to reflect primarily recent exposures without possibility of accurately assessing long term risk which has more insidious impact (Sommar *et al*., 2014; Nwobi *et al.,* 2019).

Lead-induced neurotoxicity is insidious and has the tendency to go unrecognized especially in the early years (WHO, 2009; WHO, 2013; WHO, 2015). This makes it, and its early detection, an area of exceptional importance and substantial current challenge, and therefore calls for the need for continuous search to identify accessible and simple biomarkers for early identification of LIN in children. However, it is worthy to note that the possibility that Pb-induced perturbation of erythrocyte AChE activity may precede alterations in IQ has been poorly explored in the search of biomarkers for LIN particularly in an environment with high chemical burden such as Nigeria (Anetor *et al*., 2008; Orisakwe, 2014). This study was therefore designed to determine the possible role of the combination of blood lead level, intelligent quotient and erythrocyte acetylcholinesterase activity in the early detection of lead-induced neurotoxicity in Children.

Materials and methods

Study area, study design and study population

This cross-sectional study involved 309 apparently healthy children (aged 8–10 years) who had been resident in Ibadan, Oyo State, South-West, Nigeria for ≥5 years. The children were selected from 8 public primary schools based on multistage random sampling technique while the recruitment of the children from the schools was based on parental consent, assent and physical presence on the day of sampling.

The participating children were grouped into two; children with elevated blood lead level (EBLL) (n=169; 83 boys and 86 girls) and children with Acceptable Blood Lead Level who served as control (n=140; 71 boys and 69 girls). Elevated blood lead level was defined as BLL >5 µg/dl (>0.2415 µmol/l) while acceptable blood lead level (control) was defined as ≤5 µg/dl (≤0.2415 µmol/l) based on the cut-off for childhood blood lead level recommended by the US Centre for Disease Control and Prevention (CDC, 2012).

Exclusion criteria for the children included history of lead exposure requiring chelation therapy, neurological or neurodevelopmental disorder including mental sub-normalties, such as autism, epilepsy, cerebral palsy. evidence of anaemia, malnutrition, liver dysfunction, renal dysfunction or any obvious pathology, intake of mineral supplements or medications such as anticonvulsants, previous failure or repeat in a class, inappropriate age for class and inability to perform the cognitive test for whatever reasons.

This study was approved by the University of Ibadan/University College Hospital (UI/UCH) Joint Research Ethics Committee, Nigeria with approval number: UI/EC/12/0064 as well as the Ministry of Education, Oyo State, Nigeria. The parents/guardian of all the participating children received oral and written information about the study protocol in both English language and their local dialect and signed written informed consent was obtained from those that agreed that their children should participate in the study. Assent from each participating child was also got.

Assessments and blood sampling

All assessments and blood sampling of the participants were carried out on site during regular school days before the beginning of classes between 8 and 10 a.m.

Assessments of anthropometry and neurological indices

Anthropometric, blood pressure and neurological indices such as speech, memory, cranial nerves and cerebellar functions were assessed by a Paediatric Neurologist using standard procedures before blood sampling.

Assessment of intelligence quotient

First, intelligence capacity was assessed by a Clinical Psychologist (before blood sampling) using Raven's Standard Progressive matrices (Raven *et al.,* 2000). This test is a widely used non-verbal test of intelligence capacity that relies on non-verbal problems that require abstract reasoning. It involves visuo-spatial reasoning, abstract thinking, deductive reasoning and general intelligence and covers widest range of mental ability of individuals (Raven *et al.,* 2000). The Raven's Standard Progressive matrices has been reported as one of the best instruments for the assessment of IQ in sub-Sahara African and has also been validated for use for Nigerian children (Daramola *et al.*, 2010; Iloh *et al.*, 2017).

Instrument: The Raven's Standard Progressive Matrices consisted of 60 problems divided into five sets (Set A, B, C, D and E), each made up of 12 problems. Each set consisted of matrices of increasing difficulty. While the earlier series required accuracy of visual discrimination, the later ones involved two-dimensional analogies which demanded permutation, alteration of pattern and perception of other logical relations for successful solution. Intelligence capacity was finally converted to Intelligence quotient (IQ) by a Psychometrist using the validated formula; IQi = (100+Zi) × 15 as described by Wicherts *et al.* (2010).

Blood sampling

Non-fasting blood samples (5 ml) of the participants were collected by a trained Paediatric Phlebotomist into heparinised tubes between 8 and 10 a.m. on site during regular school days, before the beginning of classes. Percentage hematocrit was determined immediately on site using Hawksley micro-HCT reader using Hawksley micro-HCT reader, Hemocue (Ängelholm, Sweden). Aliquots of blood samples (2 ml) were separated for lead analysis and the remaining blood samples (3 ml) were for erythrocyte AChE analysis. The obtained blood samples were stored in cooler boxes and transferred from the point of collection to the laboratory either for immediate analysis and/or storage at –20 °C.

Biochemical analyses

Lead

Lead analysis was based on the method of Miller *et al.*, 1987, using a graphite furnace Atomic Absorption Spectrometer Perkin-Elmer AAnalyst 800 with Zeeman-effect background correction (Norwalk, U.S.A). Sample preparation involved simple dilution (1+9) with a matrix modifier which contained 0.5% V/V Triton X-100, 0.2% V/V 16 M nitric acid and 0.2% m/V dibasic ammonium phosphate (Miller *et al.*, 1987).

Erythrocyte Acetycholinesterase Activity

The blood sample was centrifuged at 2500 RPM for 10 minutes to separate plasma and erythrocyte. Erythrocyte were washed twice with phosphate buffered saline before being used for AChE assay. Erythrocyte AChE activity was determined by the method of Miller & Blank. 1991, using Waters 616/626, High Pressure Liquid Chromatography (HPLC) analyser with electrochemical detector system (Young Lin, Seoul, South Korea). The reaction mixture was prepared by mixing 500 µl of 0.1 M phosphate buffer (pH 7.2), 50 µl of Ethylhomocholine bromide (internal standard), 25 µl of 500 mM acetylcholine and 25 µl of the hemolysate (prepared by diluting the washed cells 1:4 with deionized water). Acetylcholine as a substrate, was acted upon by cholinesterase to produce choline and acetate. The product choline was eluted into a post column reactor containing immobilized choline oxidase, where it was oxidized to produce electrochemically active H_2O_2. The H_2O_2 produced was directly proportional to the activity of AChE activity (Miller & Blank. 1991).

All reagents and standards used were of analytical grade. Internal controls, certified reference standards as well as samples spiked with known concentrations of the reference material were included in each batch of 20 samples. Calibration curves were obtained using 6 points with the certified standard. After each analytical run, calibration curves were obtained again in check for linearity and replication. A mean recovery rate of >95% was obtained for each element after two determinations. The samples were all analysed in one day and results were only acceptable when data obtained fell within expected quality control samples (X±2SD).

List of chemicals used

Reagents. Acetylcholine chloride, choline chloride, acetylcholinesterase, choline oxidase , diisopropyl phosphorofluoridate, and sesame oil were all purchased from Sigma Chemical Co., Ltd. (St. Louis, MO); potassium phosphate monobasic was purchased from Fisher Scientific Co. (Fair Lawn, NJ); tetramethylammonium chloride, sodium azide, tris(hydroxymethyl) aminomethane (Tris), and ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA) were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Bromoethane and 3-dimethylamino-l-propanol (Aldrich), were employed to synthesize the internal standard, ethylhomocholine bromide (N,N-dimethyl-N-ethyl-3-amino-1-propanol bromide), Octyl sodium sulphate was purchased from Eastman Kodak Co. (Rochester, NY). All other chemicals used were of analytical grade and of the highest available purity and used without purification.

Statistical analysis

The SPSS statistical software program version 21.0 (IBM Inc, Chicago, IL) was used for the statistical analysis. Values were assessed for normality by checking for skewness. Results were expressed as mean ± SD. Independent sample t-test was used to determine differences between children with EBLL and control. Pearson's product moment correlation analysis was used to evaluate the relationship among BLL, erythrocyte AChE activity and IQ. Step-wise Multiple Regression Analysis was used to model cause-effect relationship between BLL (independent variable) and the erythrocyte AChE activity and IQ (dependent variables). All tests were 2-tailed and p-value <0.05 was considered as statistically significant.

Table 1. Age, haematocrit levels, anthropometric and blood pressure indices and in children with elevated blood lead level and control.

Indices	Participants with EBLL (n=169)	Control (n=140)	t-value	*p*-value
Age (years)	8.6±1.6	8.9±1.5	–1.711	0.088
MUAC (cm)	17.2±1.6	17.1±2.6	0.376	0.707
OFC (cm)	51.3±2.4	51.6±1.6	–0.865	0.388
Weight (kg)	23.8±4.8	24.5±4.8	–1.015	0.311
Height (cm)	125.0±9.2	126.7±10.1	–1.285	0.200
BMI (kg/m^2)	15.1±1.5	15.3±1.8	–0.539	0.590
Heart rate/minute	92.2±13.2	91.9±10.8	0.134	0.894
SBP(mm/Hg)	96.5±13.4	96.8±11.1	–0.159	0.874
DBP (mm/Hg)	55.9±9.0	54.2±7.3	1.381	0.168
Haematocrit (%)	36.6±3.3	36.7±2.5	0.867	0.387

Results are presented as mean ± standard deviation, EBLL = elevated blood lead level, MUAC = mid upper arm circumference, OFC = occipital frontal circumference, BMI = body mass index, SBP = systolic blood pressure, DBP = Diastolic blood pressure.

Table 2. Blood lead level, erythrocyte acetylcholinesterase activity and intelligence quotient in children with elevated blood lead level and control.

Indices	Participants with EBLL (n=169)	Control (n=140)	t-value	*p*-value
BLL (µmol/l)	0.4±0.1	0.2±0.0	17.133	<0.001*
AChE (µkat/l)	117.5±25.5	59.4±10.2	17.545	<0.001*
IQ	85.9±11.6	91.5±14.0	2.884	0.004*

Results are presented as mean±standard deviation, EBLL = elevated blood lead level, * = Significant at $p< 0.05$ (2- tailed), BLL = blood lead level, AChE = erythrocyte acetylcholinesterase activity, IQ = intelligence quotient.

Table 3. Correlation of lead with erythrocyte acetylcholinesterase activity and intelligence quotient in the study participants.

Variables	Correlating pair BLL (µmol/l)	
	r-value	*p*-value
IQ score	–0.134	0.019*
AChE (µkat/L)	0.978	<0.001*

* = Significant at $p< 0.05$, BLL= blood lead level, AChE= erythrocyte acetylcholinesterase activity, IQ= intelligence quotient.

Table 4. Relationship between blood lead, cholinesterase activity and intelligence quotient in the study participant.

	β	t-value	p-value
Constant	–0.007	–1.519	0.130
AChE (µkat/L)	0.003	82.888	<0.001*
Excluded variable			
IQ	–0.002	–0.133	0.894

Where, BLL= independent variable, AChE and IQ=dependent variables. R=slope, R^2=coefficient of determination, B_0=intercept, β=slope of each variable. BLL=–0.007+(0.003) AChE; BLL in µmol/l, erythrocyte AChE in µkat/l; R=0.162, R^2= 0.026, p=0.005*. * = Significant at p<0.05, BLL= Blood lead level, AChE= erythrocyte acetylcholinesterase activity, IQ=intelligence quotient.

Results

The study population was stable with no attrition and all participants had normal neurological indices such as speech, memory, cerebellar and cranial nerves functions. Children with elevated blood lead level (EBLL) and control were properly matched for gender, age, haematocrit level, anthropometric and blood pressure indices that could be confounders. Thus, suggesting that both groups were comparable because they had relative homogeneity (p>0.05) (Table 1). Out of the 309 children that participated in this study, 169 (54.7%) exhibited elevated blood lead level, while 140 (45.3%) exhibited acceptable blood lead level which also served as the control (Table 1).

Blood lead level (BLL) and erythrocyte AChE activity were significantly increased in children with EBLL compared with control ($p< 0.05$) (Table 2, Figure 1, Figure 2). Remarkably, IQ was significantly decreased in children with EBLL compared with the control ($p< 0.05$) (Table 2 andFigure2). Blood lead level showed strong significant positive correlation with AChE activity ($p< 0.05$) but significant negative correlation with IQ score ($p< 0.05$) (Table 3).

Multiple regression analysis between BLL (dependent variable) and erythrocyte AChE activity and IQ

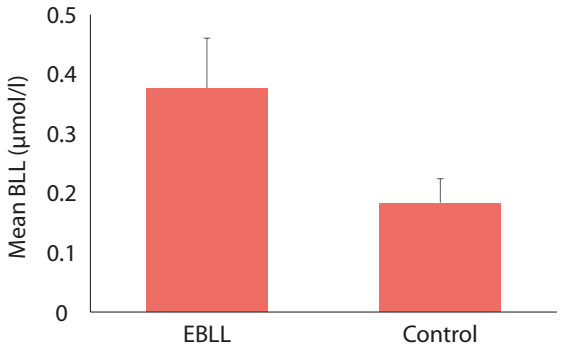

Figure 1. Mean blood lead level (BLL) in children with elevated blood lead level (EBLL) and control in Ibadan, Nigeria.

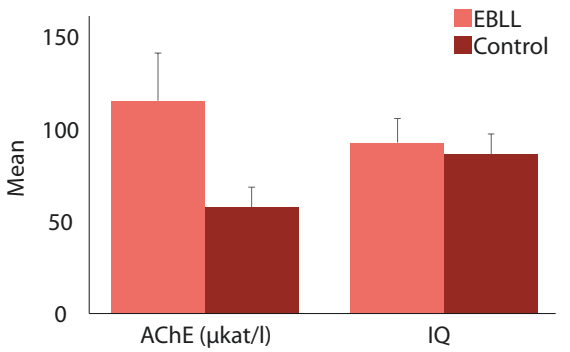

Figure 2. Mean erythrocyte acetylcholinesterase (AChE) activity and intellingence quotient (IQ) in children with elevated blood lead level (EBLL) and control in Ibadan, Nigeria

(independent variables) is shown (R=0.162, R^2=0.026, p=0.005) (Table 4). Blood lead level showed significant positive relationship with AChE (Model Coefficient: B=−0.007; β=0.003; p<0.001) (Table 4) which implies that 16.2% of the observed variation in BLL could be accounted for by AChE activity using the linear equation between BLL and AChE; BLL (µmol/l) = −0.007+0.003 AChE (µkat/l) (Table 4 and Figure 3). However, the model did not show significant relationship with IQ (Model Coefficient B=−0. 007; β=−0.002; p>0.05) (Table 4).

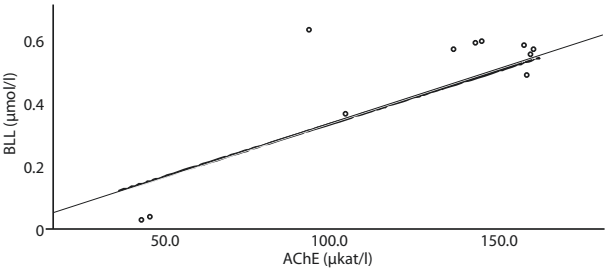

Figure 3. Scatter plot showing linear relationship between blood lead level (BLL) and erythrocyte acetylcholinesterase (AChE) activity in children in Ibadan, Nigeria.

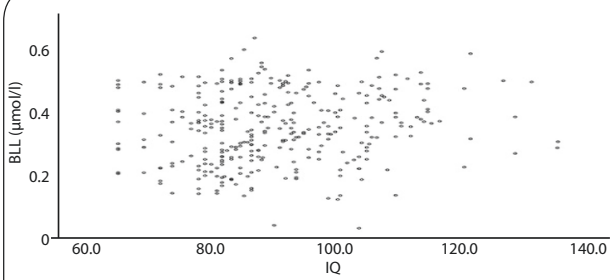

Figure 4. Scatter plot between blood lead level (BLL) and intellingence quotient (IQ) in children in Ibadan, Nigeria

Discussion

Lead-induced neurotoxicity (LIN), a condition whose insidious effect could be unrecognised particularly in the early years, remains a topic of substantial concern and interest particularly in developing countries where about 99% of 600,000 new cases of children with intellectual disabilities as a result of lead exposure reside (WHO, 2013; Sharma *et al.*, 2015). Thus, this study attempted to determine the possible role of the combination of blood lead level, intelligent quotient and erythrocyte acetylcholinesterase activity in the early detection of lead-induced neurotoxicity in Children.

The study showed that 54.7% of the total participants had blood lead levels greater than the current CDC cut-off for acceptable limit (5 µg/dl) (0.2415 µmol/l) (CDC, 2012), which implies a high prevalence of increased lead exposure in the children. The prevalence of childhood lead exposure in Nigeria was reported as 25% in 2008 (Nriagu *et. al.,* 2008). However, this prevalence was determined when BLL of 10 µg/dl (0.4830 µmol/l) was the accepted as the lowest level of medical concern for children (CDC, 991). The difference between observed prevalence in this study and the documented prevalence could at least in part, be explained by the recent reduction in the cut-off value for elevated blood lead level from 10 µg/dl to 5 µg/dl (CDC, 2012). Consequently, applying this recent CDC recommended cut-off in a chemical laden environment could make the burden of lead toxicity much greater especially given the fact that the already estimated health and educational cost of every 1 µg increase in blood lead level in Nigerian children is as high as US $0.38–$1.15 billion (Ogunseitan & Smith, 2007).

Lead toxicity may be explained by its interference with the activity of the most enzymes. Lead could bind to the thiol-groups of these proteins or displace some essential metal ions that are necessary for their normal functioning (Sharma *et al.*, 2015). However, the mechanism by which lead alters AChE activity is still incompletely understood as this enzyme does not contain free thiol groups in its structure to which Pb could bind (Rosenberry & Soggin, 1984; Ademuyiwa *et al.,* 2007). However, the mechanism may be dependent on calcium, a nutritionally essential element involved in the regulation of many neurological processes, with which Pb competes at protein binding sites (Florea *et al.*, 2013; Brini *et al.*, 2014; Nwobi *et al.*, 2019).

Calcium norrmally activates Protein Kinase C (PKC), a phospholipid-dependent enzyme that is found in high concentrations in neuronal tissues where it participates in several signal transduction cascades by regulating neurotransmitter release and neuronal ion channels such as calcium channels involved in cholinergic neurotrassmission (Hwang *et al.,* 2002; Brini *et al.,* 2014). However, reports have shown that Pb, even at picomolar concentrations, can substitute calcium in the activation of this enzyme possibly resulting to abnormally increased PKC activity (Long *et al.,* 1994; Hwang *et al.*, 2002; Brini *et al.*, 2014). The observed increased erythrocyte AChE activity in the children with EBLL coupled with its positive relationship with blood Pb may be accounted for, at least in part, by the breakdown in normal homeostatic function of calcium resulting to abnormally sustained lead-induced PKC activation. This possibly impacted the presynaptic calcium ion channel and increased the level of acetylcholine released into the synaptic cleft which subsequently resulted to the concomitant increase in AChE activity observed in this study (Florea *et al.*, 2013; Brini *et al.,* 2014).

Acetylcholine, a key factor in neuro-cognition (Soreq & Seidman, 2001; Hasselmo 2006), is a cholinergic neurotransmitter which binds briefly to the postsynaptic acetylcholine receptors for the chemically-gated ion channels in the postsynaptic membrane to open for impulse transmission. The observed reduced IQ in children with elevated blood lead levels and the inverse relationship between blood lead levels and IQ may, at least in part, be

accounted for by the lead-induced acetylcholine receptor desensitisation resulting to decreased responsiveness of the receptors to acetylcholine, diminished stimulatory effect of acetylcholine at the post-synaptic membrane and reduced efficiency of cholinergic neurotransmission which possibly manifested as the observed reduced IQ. Several reports have shown that lead not only desensitises but also reduces the operations and the aggregation of acetylcholine receptors (Morlry *et al*., 2003; Chen *et al*., 2005; Badawoud & Hassan, 2013). This inhibits the action of acetylcholine at the postsynaptic membrane and reduce ion flow (Quick & Lester, 2002) which regulates impulse transmission and inadvertently negatively impact intelligent quotient. This observation is in line with other reports that emphasised the existence of an inverse relationship between blood lead level and different measures of neuro-cognition (Canfield et al*.,* 2005; Tellez-Rojo *et al.*, 2006 and Surkan *et al*., 2007).

The reduced IQ may have some adverse societal consequences such as learning difficulties and increase in the number of children who are school drop-outs because they cannot cope well with intellectual abilities in school. The consequences may be worrisome to the affected families because of extra counselling and financial burden as these children may require several attempts, special education and remedial programs before they can succeed in their study. These children may also not contribute fully to the development of the society when they become adults resulting to societal poor economic development and leadership. Thus, there is need for sensitive, reliable early indicator of lead-induce neurotoxicity in the paediatric population particularly in our environment.

The significant multivariate regression analysis involving blood lead level, erythrocyte acetylcholinesterase and intelligent quotient showed that blood lead level could be predicted by erythrocyte acetylcholinesterase activity based on the equation [BLL (μmol/l) =−0.007+0.003 AChE (μkat/l) (R^2=0.026, p=0.005]. This relationship appears to suggest that erythrocyte AChE activity could account for BLL and may imply that erythrocyte AChE activity, if measured regularly in children exposed to similar duration and level of lead, might act as a surrogate index for LIN.

Taken together, elevated blood lead level is prevalent among the school children in Ibadan, South-West, Nigeria and appears to have adverse effect on their intelligence quotient. However, the multivariate regression analysis involving blood lead level, erythrocyte acetylcholinesterase and intelligent quotient showed that blood lead level could be predicted by erythrocyte acetylcholinesterase activity based on the equation [BLL (μmol/l) = −0.007+0.003 AChE (μkat/l) (R^2= 0.026, p= 0.005]. Thus, the activity of this enzyme appears to be a promising marker for early recognition of significant environmental lead exposure and lead-induced neurotoxicity in children. This study provides a valid scientific basis for the possible role of the combination of blood lead level, erythrocyte acetylcholineesterase activity and intelligence quotient in the early detection of lead-induced neurotoxicity in the paediatric population.

Acknowledgements

We acknowledge the contribution of the Psychometrist, Prof. Jelte M. Wicherts of the Department of Methodology and Statistics, Tilburg University, Tilburg, Netherlands for his kind assistance in generating the estimated IQ and Dr. Michael O. Akinosun of the Department of Chemical Pathology, University of Ibadan, Nigeria for his help during the laboratory analysis. We are very grateful to the children and their parents for voluntarily giving their assent and consent respectively. We also appreciate the efforts of the management of the selected schools in helping to organize the children and others who were of assistance during the field work. The authors have no conflict of interest to declare.

REFERENCES

Ademuyiwa O, Ugbaja RN, Rotimi SO, Abam E, Okediran BS, Dosumu OA, Onunkwor BO. (2007). Erythrocyte acetylcholinesterase activity as a surrogate indicator of lead-induced neurotoxicity in occupational lead exposure in Abeokuta. Nigeria. *Environ Toxicol Pharmacol* **24**(2): 183–188.

Anetor J I, Anetor GO, Iyanda AA, Adeniyi FAA. (2008). Environmental Chemicals and Human Neurotoxicity: Magnitude, Prognosis and Markers. *Afri. J. Biomed. Res*. **11**: 1–12.

Anetor JI, Igharo OG, Anetor GO, Nwobi NL, Iyaanda AA. (2016). The Zamfara lead poisoning episode in Nigeria: an indication for childrens environmental toxicology and micronutrient center. *Toxicol Digest* **1**: 23–33.

Badawoud MH, Hassan SM. (2013). Effects of exposure to lead on the peripheral motor system of the rat; An ultrastructural study. *Neurosci* **18** (1): 52–57.

Bellinger DC. (2008). Very low lead exposures and children's neurodevelopment. *Curr Opin Pediatr* **20**: 172–177.

Brini M, Calì T, Ottolini D, Carafoli E. (2014). Neuronal calcium signaling: function and dysfunction. *Cell Mol Life Sci* **71**(15): 2787–814.

Canfield RL, Jusko TA, Kordas K. (2005). Environmental lead exposure and children's cognitive function. *Riv Ital Pediatr* **31**(6): 293–300.

Centres For Disease Control and Prevention 1991. *Preventing Lead Poisoning In Young Children*, Retrieved 11/08/17 From https://wonder.cdc.gov/wonder/prevguid/p0000029/p0000029.asp

Centres For Disease Control and Prevention 2012. Response To Advisory Committee On Childhood Lead Poisoning Prevention Recommendations In "Low Level Lead Exposure Harms Children: A Renewed Call Of Primary Prevention". Retrieved 20/12/2016 From https://www.cdc.gov/nceh/lead/prevention/blood-lead-levels.htm

Chen SS, Lin CH, Chen TJ. (2005). Lead-induced attenuation in the aggregation of acetylcholine receptors during the neuromuscular junction formation. *Toxicol Lett* **159**: 89–99.

Daramola OO, Ayoola OO, Ogunbiyi OA. (2010). The comparison of intelligence quotients of atopic and nonatopic children in Ibadan, Nigeria. *Indian J Dermatol*. **55**(3): 221–224.

Flora G, Gupta D, Tiwari A. (2012). Toxicity of lead: A review with recent updates. *Interdiscip Toxicol* **5**2): 47–58.

Florea A, Taban J, Varghese E, Alost BT, Moreno S, Büsselberg D (2013). Lead (Pb2+) neurotoxicity: Ion-mimicry with calcium (Ca2+) impairs synaptic transmission. A review with animated illustrations of the pre- and post-synaptic effects of lead, Journal of Local and Global Health Science, 2013:4 http://dx.doi.org/10.5339/jlghs.2013.4

Gupta VK, Pal R, Siddiqi NJ, Sharma B. (2015). Acetylcholinesterase from Human Erythrocytes as a surrogate biomarker of lead-Induced neurotoxicity. *Enzyme Res*. 2015. Article ID 370705.

Haense C, Kalbe E., Herholz K, Hohmann C, Neumaier, B, Krais, R., Heiss WD. (2012). Cholinergic system function and cognition in mild cognitive impairment. *Neurobiol Aging*. **33** (5): 867–877.

Hajjawi OS. (2012). Acetylcholinesterase in Human Red Blood Cells. *Eur J Sci Res* **75**(4): 510–522.

Hasselmo ME. (2006).The Role of Acetylcholine in Learning and Memory. *Curr Opin Neurobiol* **16**(6): 710–715.

Hwang K, Lee B, Bressler JP, Bolla KI, Stewart WF, Schwartz BS. (2002). Protein Kinase C Activity and the Relations between Blood Lead and Neurobehavioral Function in Lead Workers. *Environ Health Perspect* **110** (2): 133–138.

Iloh KK, Emodi IJ, Ibeziako NS, Ikefuna AN, Ubesie, AC, Iloh, ON, Ilechukwu GC, Adaeze CA. (2017). Neurocognitive Function of School-aged HIV-infected Children in Enugu, Nigeria. *J Trop Pediatr* ***63***(6): 425–430.

Lidsky TI, Schneider JS. (2003). Lead neurotoxicity in children: basic mechanisms and clinical correlates. *Brain.* **126**(1): 5–19.

Lioneto MG, Caricao R, Calisi A, Giordano ME, Schettino T (2013). Acetylcholinesterase as a Biomarker in Environmental and Occupational Medicine: New Insights and Future Perspectives. *Biomed Res Int* **2013**.Article ID 321213.

Liu J, Li L, Wang Y, Yan C, Liu X (2013). Impact of Low Blood Lead Concentrations on IQ and School Performance in Chinese Children. *PLoS One* **8**(5): e65230.

Long GJ, Rosen JF, Schanne, FA. (1994). Lead activation of protein kinase C from rat brain. *J Biol Chem* **269**: 834–837.

Mason LH, Herp JP, Han DY. (2014). Pb Neurotoxicity: Neuropsychological Effects of Lead Toxicity. *Biomed Res Int* Article ID 840547.

Miller D, Paschal D, Gunter E, Stroud P, D'Angelo J. (1987). Determination of blood lead with electrothermal atomic absorption using a L'vov platform and matrix modifier. *Analyst* **112**: 1701–1704.

Miller RB, Blank CL.(1991). Determination of serum cholinesterase activity by liquid chromatography with electrochemical detection. *Anal Biochem* **196** (2): 377–384.

Morley EJ, Hirsch HV, Hollocher K, Lnenicka GA. (2003). Effects of chronic lead exposure on the neuromuscular junction in Drosophila larvae. *Neurotoxicol* **24**: 35–41.

Nriagu J, Afeiche M, Linder A, Arowolo T, Anna G, Stridhar MK. (2008). Lead poisoning associated with malaria in children of urban areas of Nigeria. *Int J Hyg Envir Heal* **211**(5–6): 591–605.

Nwobi NL, Adedapo KS, Oyinlade OA, Olukolade O, Lagunju IA, Atulomah NO, Nwazuoke IA, Anetor JI (2019). Urinary calcium: a promising predictive biomarker for early recognition of environmental lead exposure in children. *Int J Res Med Sci* **7**(6): 2265–2272.

Ogunseitan OA, Smith TR. (2007). The Cost of Environmental Lead Poisoning in Nigeria. *Afr J Environ Sci Tech* **1**(2): 27–36.

Orisakwe OE. (2014). Lead and cadmium in public health in Nigeria: physicians neglect and pitfall in patient management. *N Am J Med Sci* **6**(2): 61–70.

Quick MW, Lester RA. (2002). Desensitization of neuronal nicotinic receptors. *J Neurobiol* **53**(4): 457–478.

Raven J, Raven JC and Court JH. 2000. Raven Manual: Section 3. *Standard Progressive Matrices*. Oxford Psychologist Press, Oxford.

Rosenberry TL, Soggin DM (1984). Structure of human erythrocyte acetylcholinesterase. Characterization of inter-subunit disulfide bonding and detergent interaction. *J Biol Chem.* **259**: 5643–5652.

Saldanha C (2017). Human Erythrocyte Acetylcholinesterase in Health and Disease. *Mol* **22**(9): 1499. doi: 10.3390/molecules22091499

Sharma B, Singh S, Siddiqi NJ. (2014). Biomedical implications of heavy metals induced imbalances in redox systems. *Biomed Res Int* **2014**: Article ID 640754.

Sharma P, Chambial S, Shukla KK (2015). Lead and Neurotoxicity. *Indian J Clin Biochem*. **30**(1): 1–2.

Slikker W, Beck BD, Cory-Slechta, Paule MG, Anger WK, Bellinger D. (2000). Cognitive Tests: Interpretation for Neurotoxicity? (Workshop Summary). *Toxicol Sci* **58** (2): 222–234.

Sommar JN, Hedmer M, Lundh T, Nilsson L, Skerfving S, Bergdahl IA. (2014). Investigation of lead concentrations in whole blood, plasma and urine as biomarkers for biological monitoring of lead exposure. *J Expo Sci Environ Epidemiol*. **24**(1): 51–57.

Soreq H, Seidman S. (2001). Acetylcholinesterase – new roles for an old actor. *Nat Rev Neurosci* **2**: 294–302.

Surkan PJ, Zhang A, Trachtenberg F, Daniel DB, Mckinlay S, Bellinger DC. (2007). Neuropsychological function in children with blood lead levels <10µg/dL. *Neurotoxicol.* **28**(6): 1170–1177.

Téllez-Rojo MM, Bellinger DC, Arroyo-Quiroz C, Lamadrid-Figueroa H, Mercado-Garcia A, Schnaas-Arrieta L, Wright RO., Hérnandez-Avila, M, Hu, H. (2006). Longitudinal associations between blood lead concentrations lower than 10 mg/dL and neurobehavioral development in environmentally exposed children in Mexico City. *Pediatrics,* **118**(2): 323–330.

Wicherts JM, Dolana CV, van der Maas, HL. (2010). A systematic literature review of the average IQ of Sub-Saharan Africans. *Intelligence.* **38**(1): 1–20.

World Health Organization. (2009). *Global Health Risks: Mortality And Burden Of Disease Attributable To Selected Major Risks,* Retrieved January 12, 2017 From https://apps.who.int/iris/handle/10665/44203

World Health Organisation (2013). *International Lead Poisoning Prevention Week, Geneva.* Retrieved January 12, 2017 From https://www.who.int/mediacentre/news/notes/2013/lead-20131018/en/

World Health Organisation (2015). Lead Exposure in African Children. Retrieved January 12, 2017 From https://apps.who.int/iris/bitstream/handle/10665/200168/9780869707876.pdf?sequence=1

Interdiscip Toxicol. 2019; **Vol. 12**(3): 143–148.
doi: 10.2478/intox-2019-0017

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Evaluation of acute toxicity of vinasse by means of *Daphnia magna* and *Aliivibrio fischeri*: a comparative study

Möritz VELÁSQUEZ-RIAÑO, Juan Sebastían MENESES-SÁNCHEZ, Carel Elizabeth CARVAJAL ARIAS

Facultad de Ingeniería, Universidad El Bosque, Bogotá, Colombia

ITX120319A05 • Received: 03 September 2018 • Accepted: 24 August 2019

ABSTRACT

In the bioethanol industry, per liter of the produced alcohol 9 to 14 liters of vinasse are obtained as a byproduct. If the vinasse is directly shed into bodies of water without an adequate treatment, it may have negative effects on the existing biota and human health due to its high turbidity and color, low pH and high content of organic material. The purpose of this study was to assess the acute toxicity of vinasse by means of a rapid test with *Aliivibrio fischeri* and compare it with a standard immobilization assay with *Daphnia magna*. The standard assay of *D. magna* by means of its EC_{50} of 4.7% showed that organism was more sensitive to the contaminant, in comparison with the 69.6% obtained with the A. fischeri which suggests that it should be continuesly used as one of the organisms of first choice for the evaluation of the acute toxicity of this effluent.

KEY WORDS: wastewater distillery; bioindicators; water fleas; luminescent bacteria

Introduction

Vinasse is the industrial residue of the process of distilling alcohol produced by the fermentation of molasses (from sugar cane, beetroot, timbers) by yeasts like *Saccharomyces cerevisiae* (Parnaudeau *et al*., 2008; Velásquez-Riaño *et al*., 2013). Depending on their origin their characteristics vary but they are generally brown in color, have a high turbidity (due to suspended solids) and toxicity, a low pH (which ranges from 3.5–5.0) and a high content of dissolved and suspended organic material with values for biochemical oxygen demand (BOD) between 7,000 and 20,000 mg/l and values for chemical oxygen demand (COD) between 50,000 and 150,000 mg/l. They also contain a noticeable amount of inorganic salts composed of calcium sulfates and phosphates, potassium, sodium and magnesium (España-Gamboa *et al*., 2011).

In the bioethanol industry, for each liter of alcohol a residue of 9 to 14 liters of vinasse is obtained which is produced depending on whether a process of recirculating the vinasse is used or not (Jimenez *et al*., 2003). If the vinasse is directly shed into bodies of water without an adequate treatment, due to the above mentioned characteristics, it may have negative effects on the existing biota and human health (if that resource is used for consumption), (Figaro *et al*., 2006).

Over the years, several alternatives, either physico-chemical or biological, have been proposed for the use and degradation of vinasses. These strategies include the production of energy (methane) as in the case of anaerobic biodigestors (Marques *et al*., 2013; Choeisai *et al*., 2014; Formagini *et al*., 2014) and the production of microbial biomass or some metabolite of interest (Marques *et al*., 2013; Nitayavardhana *et al*., 2013; Sydney *et al*., 2014). However, there are few studies which have included parameters for the analysis of the toxicity of this byproduct after a physico-chemical, biological or coupled treatment was made. Evaluating these toxicological parameters is fundamental for a correct analysis of the treatment effectiveness for removing contaminating substances, since the traditional parameters on their own cannot show whether new substances, maybe even more

Correspondence address:
Möritz Velásquez-Riaño, DSc.
Facultad de Ingeniería, Universidad El Bosque,
Av. Cra. 9 No. 131A-02, Bogotá, 110121 Colombia
E-MAIL: mvelasquezri@unbosque.edu.co; moritzvr@yahoo.com

toxic than the original one, are or are not being produced (due to the treatment) (Rodrigues & Umbuzeiro, 2011; Romanholo-Ferreira *et al.*, 2011).

Among the studies which have evaluated the parameters of vinasse toxicity there is one from Romanholo-Ferreira *et al.* (2011) that analyzed the treatment of vinasses with *Pleurotus pulmonarius,* employing *Pseudokirchneriella subcapitata*, *Hydra attenuata*, *Daphnia magna* and *Daphnia similis* as toxicological indicators. This study concluded that the proposed treatment serves to reduce the color and degrade the complex compounds and, in turn, diminish the toxicity of vinasses. Grossi-Bothelo *et al.* (2012) analyzed the toxicity of vinasse in cladocerans and fish before and after an adjustment of the pH employing an acute toxicity assay. The average lethal concentration (LC_{50} 48h) of vinasse, before the adjustment of the pH, was 0.7% for *Ceriodaphnia dubia* and 0.8% for *D. magna,* and the average lethal concentration (LC_{50} 96h) for *Danio rerio* was 2.6%. After adjusting the pH, the values increased for all of the organisms in the study showing a decrease of the toxicity. Barba-Ho & García (2012) compared a photo-Fenton system (H_2O_2/Fe^{2+}) with an anaerobic biological system coupled with the photo-Fenton one with the aim to evaluate the effectiveness of such treatment in removing the organic material and toxic compounds (using *Daphnia pulex* as a toxicity assay). At the end of the experiment, they obtained high percentages of the removal of COD (83–97%), BOD_5 (96%), TOC (88%), phenols (99%), hardness (87%) and chlorides (91%). The evaluation of toxicity with *D. pulex* showed an increase in the toxicity of vinasse after the photo-Fenton treatment with a value of LC_{50} 48 h of 6.9% (v/v) for the crude vinasse at 5.5% (v/v) and a final value of 16.7% (v/v) for the one treated with the coupled system. They concluded that the coupled treatment with anaerobic microorganisms was the most suitable for removing the organic material and reducing the toxicity of this compound.

As showed above, most studies have evaluated the toxicity of vinasse before and after its treatment by using standard indicators belonging to the *Daphnia* genus. Although that test is relatively simple and economical, it requires the cultures to be maintained in suitable conditions including a constant cleaning of the culture vessels, the growing of their food (*Chlorella* sp.), their periodical feeding with *Chlorella* sp., and the synchronization of the cultures to maintain a sufficient amount of neonates for the implementation of the assays. Caring for the cultures thus takes a great deal of a time.

For that reason, the aim of this study is to broaden the battery of toxicity assays for vinasse, by means of a rapid assay with an analysis of the inhibition of the luminescence of *A. fischeri* and a comparison between its average effective concentration (EC_{50}) and the one obtained from a standard assay with *D. magna*.

Table 1. Main physical and chemical characteristics of the vinasse used in this study

Parameters	Value
Turbidity (NTU)	241
pH	4.8
Conductivity (NaCl) (ms/m)	0.3
COD (mg/l)	97,000
BOD_5 (mg/l)	33,236
Total phosphates (as PO_4) (mg/l)	40.7
Total Kjeldahl Nitrogen (mg/l)	1,059.6
Total solids (mg/l)	58,329.9
Total volatile solids (mg/l)	39,581.8
P (P_2O_5) (g/l)	0.5
K (K_2O) (g/l)	11.0
C (CaO) (g/l)	1.6
Mg (MgO) (g/l)	2.7
Cu (mg/l)	1.1
Mn (mg/l)	3.4
Fe (mg/l)	27.0
Cr (VI) (mg/l)	0.3

Materials and methods

The vinasse

The vinasse for this study was kindly supplied by the Fábrica de Licores de Antioquia (Antioquia Liquor Distillery, Medellín, Colombia), which was stored at 4 °C until it was used in the different assays (it was stored for 15 days). Table 1 shows the main physical and chemical characteristics of the vinasse used in this study.

The acute inmobilization test of *Daphnia magna*

The strain of *D. magna* used in this study was supplied by professor Maria Teresa Reguero Reza of the Universidad Nacional de Colombia (National University of Colombia, Bogotá). *D. magna* was fed with *Chlorella vulgaris* at a proportion of 0.1–0.2 mg of culture/day/*Daphnia* and it was kept at a photoperiod of 16:8 h of light-darkness at 22±1 °C. The assay followed the next TG202 protocol of the OECD (OECD, 2004) in which 20 neonates younger than 24 h of age in groups of 5 individuals/10 ml (4 replicas in plastic cells) were exposed to each of the vinasse concentrations evaluated: 100, 50, 25, 12.5, 6.3, 3.1 (% v/v); a negative control was reconstituted water and a positive control was 1.2 mg/l of $K_2Cr_2O_7$, while the temperature and initial photoperiod were maintained. The number of immobilized animals was recorded after 48 h. The daphnids which were unable to move after 15 s of a gentle shaking were regarded as immobile.

Inhibition assay using *Aliivibrio fischeri*

Acute toxicity was measured *in vitro* with the BioTox™ kit (Aboatox Oy, Finland). This kit employs bacteria which emits a natural luminescence (*A. fischeri* NRRL B-11177). For this assay, we followed the manufacturer´s

instructions; briefly, the flask which contains the lyophilisate of *A. fischeri* was reconstituted by the addition of a whole flask of the reagent at a dilution of +4 °C, balanced for a minimum of 30 min at +4 °C, and stabilized at +15 °C for a minimum of 30 min. The pH of the initial sample of vinasse was measured and adjusted to 7.0±0.2, the salinity was adjusted to 2% with a 20% of NaCl, and the sample was oxygenated to reach an initial concentration of dissolved oxygen to more than 3.0 mg/l. Afterwards, the sample of pure vinasse was diluted with a 2% of NaCl to obtain a series of dilutions of 3.1, 6.3, 12.5, 25, 50, 100 (% v/v). A sample of the 2% NaCl was used as a control. Later, 500 µl of the dilutions and the control were placed in the test tubes (in duplicate). The flask which contains the reconstituted bacteria was placed in a self-injector connected to a luminometer (Triathler, Hidex Oy) with a maximum count rate of 30,000,000 counts per second (CPS) previously calibrated. Following that, it proceeded to individually place each of the previously prepared tubes (starting with those which contain the control sample), into which the device injected 500 µl of bacteria, and after counting five seconds, the reading of the initial bioluminescence in CPS was made. Finally, each of the samples was subjected to a contact time of 30 min at +15 °C, and after that the bioluminescence was measured again.

Statistical analysis

Only nominal concentrations were used in this study because it is very difficult to quantify measured concentrations once the vinasse has been dilute because this effluent is a mixture of many compounds. In the tests of acute toxicity in *D. magna*, the EC_{50} value and its 95% confidence limits were calculated by the Probit analysis (Finney, 1971). For the acute inhibition assay with *A. fischeri*, the inhibition percentage (INH%) of each sample dilution was calculated in accordance with the equations shown below (1 and 2) and plotted on log- log scale. The EC_{50} value was determined by using a standard linear regression analysis of the linear comparison between the logarithm of the toxic concentration and the logarithm of the intensity of the lost/remaining light and with a Probit analysis (Finney, 1971).

1. $KF=IC_{30}/IC_0$
2. $INH\%=100-IT_{30}/(KF\times IT_0)\times 100$

Where:

KF = Correction factor.
IC_{30} = Intensity of luminescence of the control after the time of contact (30 min) in the CPS.
IC_0 = Maximum CPS value of the control during the 5-second kinetic measurement.
IT_{30} = Intensity of luminescence of test sample after time of contact (30 min) in the CPS.
IT_0 = Maximum CPS value of the sample during the 5-second kinetic measurement.

Results and discussion

The evaluation of the eco-toxicity of a substance is an essential stage in the analysis of its possible environmental impacts and serves as a tool for taking decisions about its final disposal. Unfortunately, an evaluation of the toxicity of these byproducts is not obligatory and even today it is still incorrectly thought that the traditional parameters for the degradation of a substance like the BOD, COD, color, turbidity, pH, etc. are sufficient to establish if those substances may or may not be harmful for the ecosystems into which they are shed (Rodrigues & Umbuzeiro 2011).

In the assay of acute immobilization with *D. magna*, the EC_{50} 48 h was only calculated with the Probit analysis method, obtaining a value of 4.7% (Figure 1). Figure 2 shows a curve which represents the changes in the emission of light from several concentrations of vinasse measured by the BioTox™ kit (and expressed as INH%). One can see that the emission of light declines as the concentration of the sample increases (inversely proportional) and at the same time the percentage of inhibition increases as the concentration of vinasse increases (directly proportional). The EC_{50} values given by el BioTox™ kit were calculated by means of a standard linear regression analysis of the

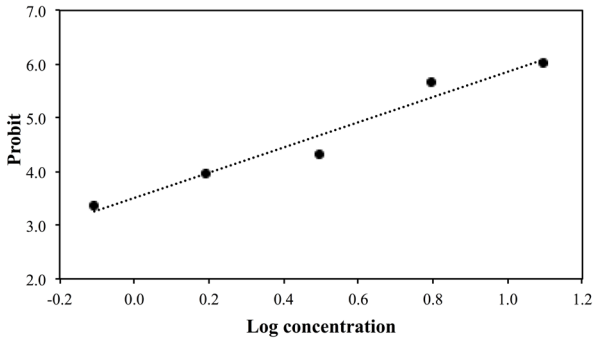

Figure. 1 Probits of lethality percentage values of *D. magna* plotted against Log10 concentrations of vinasse.

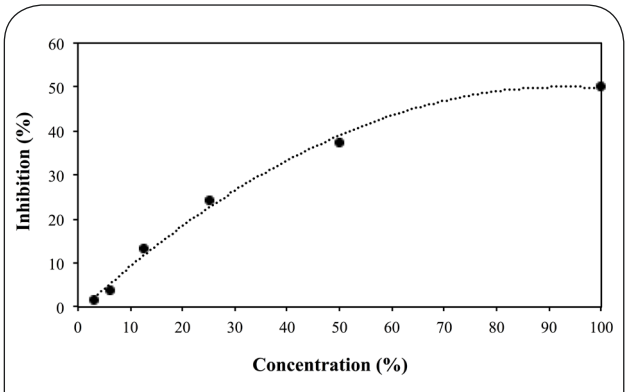

Figure. 2 Changes in *A. fischeri* light emission (INH%) as a function of toxicant concentration (%) for a contact time of 30 minutes.

linear comparison between the logarithm of the toxicant concentration and the logarithm of the intensity of the lost /remaining light, which yielded a linear plot. The EC_{50} value for the vinasse at an exposure of 30 min. was 69.6% (Figure 3). The EC_{50} value was also calculated by means of the Probit analysis. However, the EC_{50} value that method yielded differs from that obtained by the linear regression method, reaching a value of 85.6% (Figure 4).

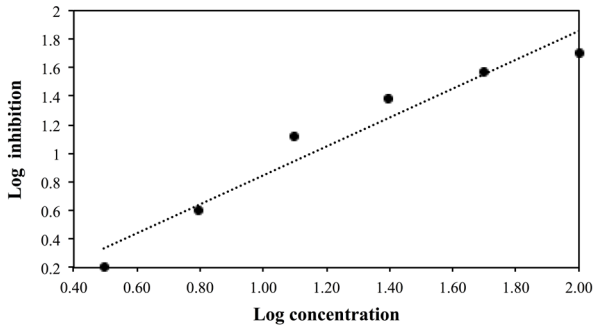

Figure. 3 Standard linear regression analysis of the linear comparison between the logarithm of the toxicant concentration and the logarithm of the intensity of the lost /remaining light in *A. fischeri*.

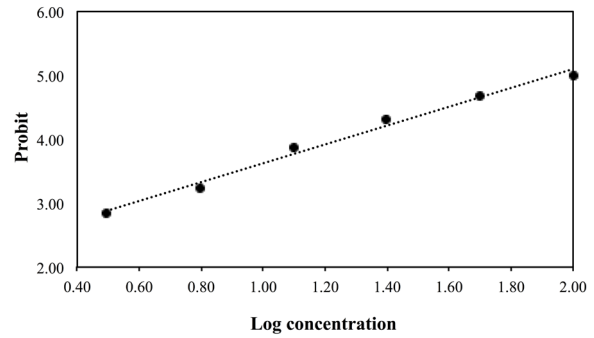

Figure. 4 Probits of inhibition percentage values of *A. fischeri* plotted against Log10 concentrations of vinasse..

Table 2. Toxicity bioassays conducted with vinasse at different trophic levels.

Bioassay	EC_{50} (%)	Source
D. magna	3.6	Romanholo-Ferreira *et al.* (2011)
D. similis	2.2	
H. attenuata	2.3	
P. subcapitata	1.6	
D. pulex	5.5	Barba-Ho and García (2012)
D. magna	0.8	Grossi-Botelho *et al.* (2012
C. dubia	0.7	
D. rerio	2.6	
A. cepa	11.2	Christofoletti *et al.* (2013)
D. pulex	3.9 (LC_{50})	Paz-Pino *et al.* (2014)
A. fischeri	28.5	Guerreiro *et al.* (2016)
D. magna	4.7	This study

As can be seen in the EC_{50} calculations, the organism which is the most sensitive to the vinasse by far was *D. magna*, with an EC_{50} 48 h value of 4.7%, which is 14.4 times less than that obtained from *A. fischeri* (69.6%). To undertake the evaluation of the eco-toxicity of a substance it is recommend the use of different organisms in the same study which correspond to different levels of the trophic chain (Choi & Meier 2001). Thus, the evaluation of the toxicity of vinasse has been done with different bacteria (*A. fischeri*) (Guerreiro *et al.*, 2016), algae (*Pseudokirchneriella subcapitata*) (Romanholo-Ferreira *et al.*, 2011), onion seeds (*Allium cepa*) (da Silva-Souza *et al.*, 2010; Christofoletti *et al.*, 2013), sugar cane (*Saccharum officinarum*) (Srivastava & Jain, 2010), cladocerans (*Ceriodaphnia dubia*, *Daphnia magna, Daphnia similis, Daphnia pulex*) (Romanholo-Ferreira *et al.*, 2011; Grossi-Botelho *et al.*, 2012; Barba-Ho & García, 2012; Paz-Pino *et al.*, 2014), cnidarians (*Hydra attenuata*) (Romanholo-Ferreira *et al.*, 2011), the eggs of nematodes (*Meloidogyne javanica* and *Meloidogyne incognita*) (Pedrosa *et al.*, 2005) and fish (*Channa punctatus*, *Danio rerio*) (Kumar & Gopal, 2001; Grossi-Botelho *et al.*, 2012) (Table 2).

The choice of the organism to be evaluated is going to depend on the environment which may be affected by the shedding of the substance: daphnids are mainly used in studies of the toxicity of water. Due to their sensitivity, these cladocerans are widely recommended as the standard organisms for evaluating acute toxicity by a variety of international organizations and agencies like EPA (2002), OECD (2004) and ISO (2012) and, as has been mentioned above, they have also been used in the evaluation of acute toxicity in vinasse. Among the assays, which use bacteria regarded as the main trophic level in many aquatic ecosystems in terms of energy flows and the cycling of nutrients, one of the most widely used on a world level and which is also standardized by the ISO (2007) is the assay of inhibition with *A. fischeri.* In view of the above, we decided to work with those two organisms in this study.

The characteristics of the vinasses in terms of conventional parameters like BOD, COD, pH, turbidity, color, etc., may differ depending on the process of fermentation and distillation used in each factory and the same applies to the different batches in the same factory (Kumar & Gopal 2001; Naik *et al.*, 2008) which may have a direct influence on their toxicity. The EC_{50} 48 h value obtained with *D. magna* in this study was 4.7% at a pH of 4.8. This value is 5.9 times greater than that reported by Grossi-Botelho *et al.* (2012), who, working with sugar cane vinasse with a pH of 4.0, obtained a LC_{50} 48 h value of 0.8%. These authors showed that the acute toxicity of vinasse changes as a function of the adjustment of the pH and that it is more toxic at a low pH (4.0) than a neutral pH which may explain the difference in the results. However, the EC_{50} 48 h value obtained in the present study was very similar to that obtained in previous ones which employed

D. pulex, with a value of 6.9% (v/v) (Barba-Ho & García, 2012) and 3.9% (v/v) (Paz-Pino *et al*., 2014).

At the start of the present study, no reports that would deal with the use of *A. fischeri* to evaluate acute toxicity in vinasse were found in the literature and that is the reason why we decided to assess the potential of this rapid assay for evaluating acute toxicity in this byproduct of the ethanol industry. However, a short while ago the first study which included the use of this bacteria (Guerreiro *et al*., 2016) was published and while the authors show that the toxicity to *A. fischeri* was eliminated after the proposed treatment was done, they did not take into account its toxicity for several organisms, since, as the present study has shown, *A. fischeri* is not an organism which is suitable for making tests of acute toxicity with vinasse. Because it has such a low sensitivity to this byproduct, it should not be used as a criterion in making the decision of evaluating its toxicity reduction and much less making decisions of how it should be disposal.

One of the probable reasons for its low sensitivity to this byproduct is that vinasse is a rather complex solution. This byproduct is made up of various elements which are needed for the growth of this bacteria, and which are also found in seawater, (the origin of this microorganism or a common medium for its culture in the laboratory) like Na, K, Mg and Ca, among others. It is also a good source of nitrogen which favors the growth of this bacteria (Romanholo-Ferreira *et al*., 2011). High sensitivity to this effluent by *D. magna*, which was previously demonstrated, must be due, in the first place, to the complexity of its mixture since it is made up of compounds like glycerol, lactic acid, sorbitol, citric acid, quinic acid, β-fructofuranose, α-glucopyranose, trehalose, saccharose, among others (such a mixture is a challenge for the metabolism of any organism) (Morales *et al*., 2000). In the second place, it must be due to its low pH which, as also been demonstrated, is a factor that negatively affects this cladoceran. And finally, it must be due its large amount of organic material and high turbidity which are directly related to the rapid exhaustion of the dissolved oxygen in the medium (Christofoletti *et al*., 2013) and in turn makes it very difficult for this organism to survive.

Although many authors describe the acute immobilization test for *D. magna* as "simple" and rapid, it needs a lot of time and effort to standardize the test in the laboratory. The daphnids must grow in optimum conditions of alkalinity, pH, temperature and photoperiod, they must be fed, usually with algae (which likewise should be grown in optimum conditions), and finally, at least 120 neonates less than 24 h old are needed for the test which means that this assay is really complex. Despite the above, due to its strong sensitivity to the vinasse, *D. magna* should be one of the top choices for an organism used for testing the toxicity of this byproduct in water, however, scientists should also continue to evaluate others organisms and rapid tests to see if they would be more sensitive, less complicated and cheaper to use in such assays than this cladoceran.

Conclusion

The standard assay of *Daphnia magna* by means of its EC_{50} of 4.7% showed that the organism was more sensitive to the contaminant in comparison with the 69.6% obtained with the *A. fischeri* which suggests that it should continue to be used as one of the organisms of first choice for the evaluation of the acute toxicity of this effluent.

REFERENCES

Barba-Ho LE, García LA. (2012). Evaluación de la factibilidad de acople de un sistema fotocatalítico biológico para el tratamiento de vinazas mediante estudios de toxicidad (Evaluation of the coupling feasibility of a biological photocatalytic system for the treatment of vinasse through toxicity studies). *EIDENAR* **11**: 63–71.

Choeisai P, Jitkam N, Silapanoraset K, Yubolsai C, Yoochatchava, W, Yamaguchi T, Onodera T, Syutsubo K. (2014). Sugarcane molasses-based bio-ethanol wastewater treatment by two-phase multi-staged up-flow anaerobic sludge blanket (UASB) combination with up-flow UASB and down-flow hanging sponge. *Water Sci Technol* **69**: 1174–1180.

Choi K, Meier PG. (2001). Toxicity evaluation of metal plating wastewater employing the Microtox® Assay: A comparison with cladocerans and fish. *Environ Toxicol* **16**: 136–141.

Christofoletti CA, Pedro-Escher J, Fontanetti CS. (2013). Assessment of the genotoxicity of two agricultural residues after processing by diplopods using the *Allium cepa* assay. *Water Air Soil Pollut* **224**: 1–14.

Da Silva-Souza TS, Hencklein FA, De Angelis DF, Fontanetti CS. (2013). Clastogenicity of landfarming soil treated with sugar cane vinasse. Environ Monit Assess 185: 1627–1636.

EPA. (2002). *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. Fifht edition, Washington, DC.

España-Gamboa E, Mijangos-Cortes J, Barahona-Perez L, Dominguez-Maldonado J, Hernández-Zarate G, Alzate-Gaviri L. (2011). Vinasse: characterization and treatments. *Waste Manag Res* **29**: 1235–1250.

Figaro S, Louisy-Louis S, Lambert J, Ehrhardt JJ, Ouensanga A, Gaspard S. (2006). Adsorption studies of recalcitrans compounds of molasses spentwash on activated carbons. *Water Res* **40**: 3459–3466.

Finney DJ. (1971). *Probit analysis*. Cambridge University Press, Cambridge.

Formagini EL, Marques FR, Serejo ML, Paulo PL, Boncz MA. (2014). The use of microalgae and their culture medium for biogas production in an integrated cycle. *Water Sci Technol* **69**: 941–946.

Grossi-Botelho R, Tornisielo VL, Alves de Olinda R, Maranho L A, Machado-Neto L. (2012). Acute toxicity of sugarcane vinasse to aquatic organisms before and after pH adjustment. *Toxicol Environ Chem* **94**: 2035–2045.

Guerreiro LF, Rodrigues CS, Duda RM, de Oliveira RA, Boaventura RA, Madeira LM. (2016). Treatment of sugarcane vinasse by combination of coagulation/flocculation and Fenton's oxidation. *J Environ Manage* **181**: 237–248.

ISO 11348. (2007). Water quality - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test). Part 3: Method using freeze-dried bacteria.

ISO 6341. (2012). Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) -Acute toxicity test.

Jimenez AM, Borja R, Martín A. (2003). Aerobic-anaerobic biodegradation o beet molasses alcoholic fermentation wastewater. *Process Biochem* **38**: 1275–1284.

Kumar S, Gopal K. (2001). Impact of distillery effluent on physiological consequences in the freshwater teleost *Channa punctatus*. *Bull Environ Contam Toxicol* **66**: 617–622.

Marques SS, Nascimento IA, Almeida PF, Chinalia, FA. (2013). Growth of *Chlorella vulgaris* on Sugarcane Vinasse: The Effect of Anaerobic Digestion Pretreatment. *Appl Biochem Biotechnol* **171**: 1933–1943.

Morales A, Larrahondo J, Victoria H, Jaramillo A. (2000). Compuestos orgánicos en vinaza (Organic compounds in vinasse). *Carta trimestral* **3**: 5–6.

Naik NM, Jagadeesh KS, Alagawadi AR. (2008). Microbial decolorization of spentwash: a review. *Indian J Microbiol* **48**: 41–48.

Nitayavardhana S, Issarapayup K, Pavasan, P, Khanal SK. (2013). Production of protein-rich fungal biomass in an airlift bioreactor using vinasse as substrate. *Bioresour Technol* **133**: 301–306.

OECD. (2004). Test No. 202: *Daphnia* sp. Acute Immobilization Test, OECD Publishing, Paris.

Parnaudeau V, Condom N, Oliver R, Cazevieille P, Recous S. (2008). Vinasse organic matter quality and mineralization potential, as influenced by raw material, fermentation and concentration processes. *Bioresour Technol* **99**: 1553–1562.

Paz-Pino OL, Barba-Ho LE, Marriaga-Cabrales N. (2014). Tratamiento de vinazas acoplando electrodisolución, heterocoagulación y digestión anaerobia (vinasse treatment by coupling of electrodissolution, heterocoagulation and anaerobic digestion). *DYNA* **81**: 102–107.

Pedrosa EMR, Rolim MM, Albuquerque PHS, Cunha AC. (2005). Supressividade de nematóides em cana-de-açucar por adição de vinhaça ao solo (Suppressivity of nematodes in sugarcane by adding vinasse to soil). *Revista Brasileira de Engenharia Agrícola e Ambiental* **9**: 197–201.

Romanholo-Ferreira LF, Aguiar MM, Messias TG, Pompeu GB, Lopez AM, Silva DP, Monteiro RT. (2011). Evaluation of sugar-cane vinasse treated with *Pleurotus sajor-caju* utilizing aquatic organisms as toxicological indicators. *Ecotoxicol Environ Saf* **74**: 132–137.

Rodrigues ES, Umbuzeiro GA. (2011). Integrating toxicity testing in the wastewater management of chemical storage terminals - a proposal based on a ten-year study. *J Hazard Mater* **186**: 1909–1915.

Srivastava S, Jain R. (2010). Effect of distillery spent wash on cytomorphological behaviour of sugarcane settlings. *J Environ Biol* **31**: 809–812.

Sydney EB, Larroche C, Novak AC, Nouaille R, Sarma SJ, Brar SK, Letti LA Jr, Soccol VT, Soccol CR. (2014). Economic process to produce biohydrogen and volatile fatty acids by a mixed culture using vinasse from sugarcane ethanol industry as nutrient source. *Bioresour Technol* **159**: 380–386.

Vélasquez-Riaño M, Lombana-Sánchez N, Villa-Restrepo AF, Fernández-Calle EP. (2013). Cellulose production by *Gluconacetobacter kakiaceti* GM5 in two batch process using vinasse as culture media. *Water Sci Technol* **68**: 1079–1084.

Interdiscip Toxicol. 2019; **Vol. 13**(3): 149–156.
doi: 10.2478/intox-2019-0018

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

Influence of estrous stages on electrocardiography, clinical pathology and ovarian weight of experimental beagle dogs: a retrospective analysis

Chitrang SHAH, Laxit BHATT, Ravichandra B.V., Viren KOTHULE, Shekhar KADAM, Nataraju G.J., Jitendra PATEL, Ramachandra RANVIR, Upendra BHATNAGAR, S. Rajesh SUNDAR, Mukul JAIN

Pharmacology & Toxicology, Zydus Research Centre, Ahmedabad, India

ITX120319A06 • Received: 18 May 2017 • Accepted: 09 January 2019

ABSTRACT

Estrous cycle is a repetitive phenomenon occurring during the reproductive life of a female dog. The duration of the canine estrous cycle is considerably longer than one in the most of the other animals and is broadly grouped into follicular phase (proestrus and estrus), luteal phase (diestrus) and non-seasonal anestrus. Dogs in the same stage of cycle can be inadvertently assigned to same group during routine safety and metabolic studies leading to possible erroneous interpretation of test-item related effects. This retrospective analysis was conducted by analyzing data of 86 female beagle dogs from control/placebo treated groups to correlate any possible effect of estrous stages with electrocardiography, clinical pathology and ovarian weight. Different estrous cycle stages of beagles were confirmed histologically by evaluating ovary, uterus, vagina and mammary glands. The incidence of beagles in diestrus was the highest, followed by anestrus, proestrus and estrus. No significant effect was noticed on heart rate, P–A, P–D, RR, QRS and QT intervals across different stages of estrous cycle. However, significantly higher PQ (PR) interval in dogs in proestrus stage was observed compared to dogs in anestrus and estrus. Marginally higher WBCs, neutrophils, lymphocytes, RBCs, hemoglobin, AST and lower hematocrit, lipid profile (total cholesterol, HDL, LDL, triglycerides), ALP level was evident in estrous period. Relative ovary weight was significantly higher in dogs in diestrus stage. Considering these results, one may need to exercise caution while interpreting experimental data from female beagle dogs.

KEY WORDS: estrous cycle; electrocardiography; clinical pathology; ovarian weight; beagle dogs

Introduction

Estrous cycle is a repetitive phenomenon occurring during the reproductive life of a female that involves a patterned sequence of structural, functional and hormonal changes in the reproductive system (Butinar *et al.*, 2004). Compared with the other laboratory animals, there are numerous reproductive features which are inimitable in female dogs. The duration of the canine estrous cycle is considerably longer than one in the mostof the other animals (Butinar *et al.*, 2004). The canine estrous cycle consists of 4 phases: proestrus, estrus, diestrus and anestrus. Broadly these different stages of estrous cycle can be grouped into follicular phase (proestrus and estrus), luteal phase (diestrus) and non-seasonal anestrus (Chandra & Adler, 2008). Anestrus length and ovarian cycle intervals, variable within and among bitches, are likely affected by neuroendocrine components of an endogenous circannual cycle. This is linked and controlled by cyclical fluctuations in the levels of FSH, LH, estrogen and progesterone (Butinar *et al.*, 2004). Hardly any organ in the body remains unaffected by these large hormonal fluctuations. Repeated cyclical changes and variations in the estrogen and progesterone level in the blood during different phases could affect the blood/plasma volume, cardiac activity and may also affect electrocardiographic pattern and reproductive organ weight. In routine safety studies and certain metabolic studies, due to small group size (3 to 6 dogs per dose group), dogs in the same stage of the cycle can be inadvertently

Correspondence address:
Laxit K. Bhatt
Department of Pharmacology & Toxicology,
Zydus Research Centre, Ahmedabad, INDIA
E-MAIL: laxitk.bhatt@zyduscadila.com

assigned to same group potentially leading to erroneous interpretation of drug-induced effects on the electrocardiography, clinical pathology and reproductive organ weight when the test chemical being tested has either direct or indirect effect on the female reproductive system/hormones. Recording of ECG in non-rodents has a particular importance during preclinical assessment of drugs: to extrapolate the potential cardiovascular risk in humans (Hammond *et al.*, 2001; Finley *et al.*, 2003). Multiple factors have been described to affect the ECG parameters (De Ponti *et al.*, 2002; Luo *et al.*, 2004). There is a paucity of literature which describes effect of estrous on ECG parameters in animals.

Keeping in view of all the above factors, the present retrospective analysis in beagle dogs from control/placebo groups of routine preclinical toxicity studies was undertaken to correlate and summarize any possible effect of estrous stage with electrocardiography, clinical pathology and reproductive organ weight.

Materials and methods

This retrospective analysis was conducted using data from 86 female beagle dogs which were used as control (vehicle/placebo treated) animals in toxicity studies

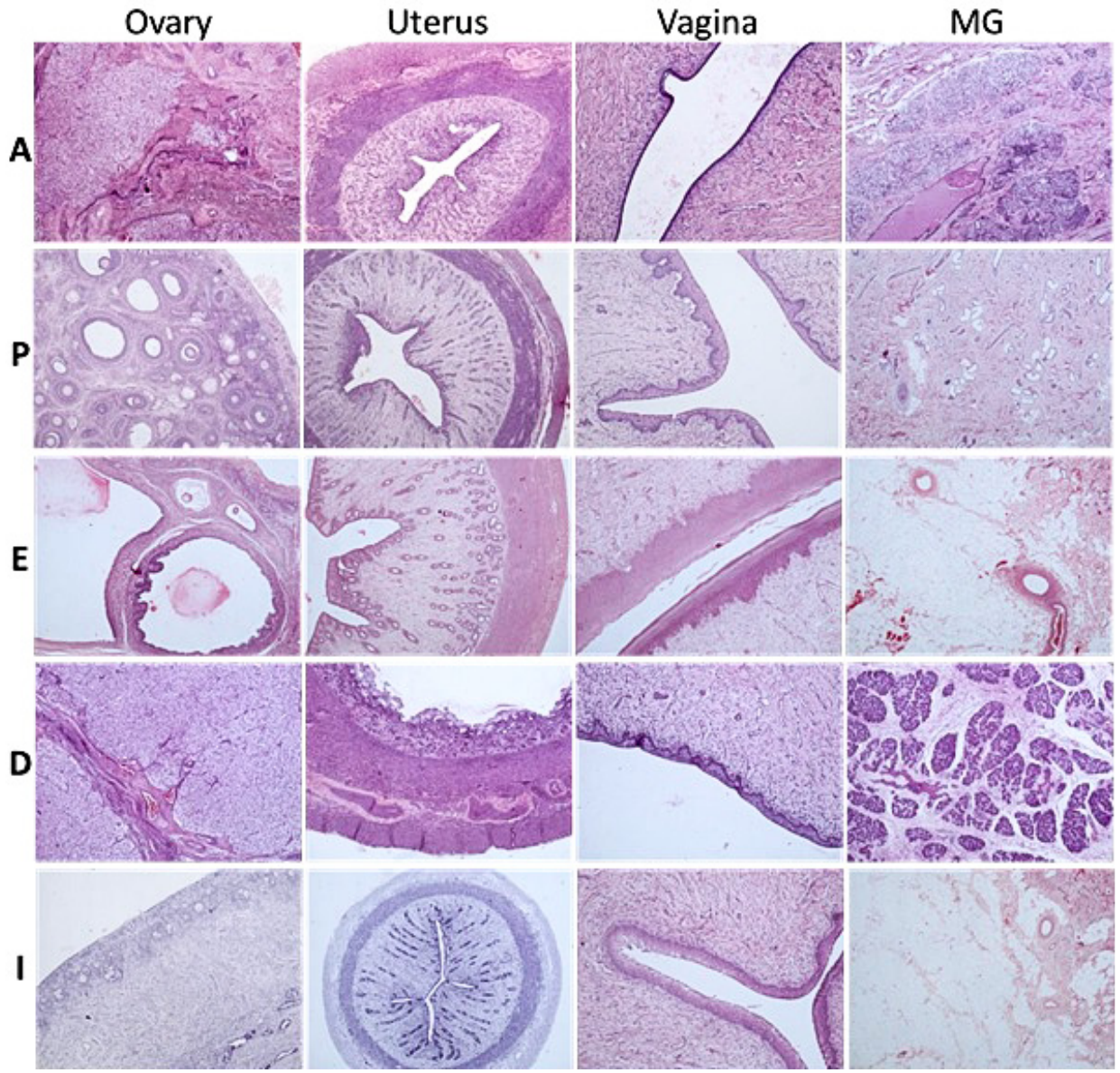

Figure 1. Typical histological feature (H&E Stain) of the ovary (40×), uterus (40×), vaginal mucosa (100×) and mammary gland (MG) (40×) in anestrus (A), proestrus (P), estrus (E), diestrus (D) and immature (I) beagle dogs.

ISSN: 1337-6853 *(print version)* | 1337-9569 *(electronic version)*

conducted between year 2005 and 2014 at Zydus Research Centre, Ahmedabad, India. Age at termination was in the range of 10–21 months. All dogs were supplied by Animal Research Facility of Zydus Research Centre and were housed individually in kennels, under identical housing and husbandry conditions at 22±3 °C temperature and 30% to 70% relative humidity with 12/12 hours light/dark cycle. Comingling was permitted daily for few hours. Standard dog feed (Pedigree, Mars International India Pvt. Ltd.) and purified water was provided to dogs. Periodical quality checking of feed and water were performed to ensure proper nutrient content and acceptable limits of total dissolved solute in water and microbial contamination as per Standard Operating Procedures of Zydus Research Centre.

Electrocardiographic (ECG) examination was performed on all dogs during the terminal stage of each study using CARDIOVIT AT-1(VET) Electrocardiograph Machine, Schiller AG, Switzerland. The ECG parameters viz. heart rate, P–A, P–D, RR, QRS, QT, QTc and PR intervals were recorded from Lead II. Blood was collected from cephalic/saphenous vein for hematology (Cell-DYN® 3700, USA) and clinical chemistry (Randox Daytona analyzer, Randox Laboratories Ltd., USA and EasyLyte electrolyte analyzer, Medica Corporation, USA). Animals were euthanized by intravenous injection of overdose of Thiopentone Sodium. At termination, animals were subjected to gross pathological examinations and tissues (ovary, uterus, vagina and mammary gland) were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm thickness and stained with hematoxylin and eosin. Stage of estrous was confirmed by histological examination of ovary, uterus, vagina and mammary glands and further data was grouped into proestrus, estrus, diestrus, anestrus and immature stages (Figure 1 and Table 1) (Harleman & Foley, 2001; Rehm *et al.*, 2007; Chandra & Adler, 2008).

The studies were conducted in AAALAC (Association for Accreditation and Assessment of Laboratory Animal Care) accredited facility, in compliance with Indian regulations (Committee for the Purpose of Control and Supervision of Experiments on Animals, 2005) governing the housing and use of animals and all the procedures used in these studies were reviewed and approved by the Institutional Animal Ethics Committee.

Results

Incidences of estrous stages

The percentage incidence of diestrus, anestrus, proestrus and estrus were 33.73, 26.51, 26.51 and 4.82 respectively. In addition, few dogs (8.43%) were found to be immature during histological evaluation (Table 2).

Electrocardiography

The electrocardiographic parameters such as P–A, P–D, RR, QRS and QT intervals did not reveal any significant changes at different stages of estrous cycle. In addition, no distinct pattern of variation in heart rate was evident at different phases of estrous cycle. However, higher PQ (PR) interval was observed in dogs of proestrus stage compared to dogs in anestrus and estrus stages (Table 3).

Table 1. Overview of estrous cycle with duration and hormonal dominance.

Stage	Duration (in days)	Hormonal dominance
Proestrus	9	Estrogen, FSH
Estrus	9	Estrogen, LH, FSH
Diestrus	60	Progesterone, Prolactin
Anestrus	100–150	FSH, Prolactin

FSH-Follicle Stimulating Hormone, LH- Luteinizing hormone

Clinical Pathology

There were no statistically significant changes in hematology and clinical biochemistry parameters. However, certain non-significant fluctuations such as marginally higher WBCs, neutrophils, lymphocytes, RBC counts, hemoglobin, AST and lower hematocrit, lipid profile (total cholesterol, HDL, LDL, triglycerides) and ALP level were evident in estrus stage. Lower triglycerides, HDL cholesterol, total protein and globulin and higher serum inorganic phosphorous were observed in immature animals (Tables 4 and 5).

Organ weight

The absolute and relative ovary weight was higher in diestrus stage and lower in immature animals.

Discussion

The beagle is essentially monoestric (Anderson & Simpson, 1973; Chastain & Ganjam, 1986; Concannon, 1993) with considerable variation in intervals between cycles and in hormonal profile during different stages of estrous cycle (Table 6). Non-rodent safety and metabolic studies are frequently performed in purpose-bred beagle dogs with a limited (three to six per group) number of dogs assigned randomly to individual treatment groups based on body weight.

The assigned stage of cycle for each bitch in the present analysis was based completely on the histomorphological findings in the reproductive organs viz. ovary, uterus, vagina and mammary glands (Harleman & Foley, 2001; Rehm 2007; Chandra & Adler, 2008). The present retrospective analysis indicated that diestrus stage was the most frequent in bitches at the time of termination in toxicity studies followed by anestrus, proestrus and estrus. The higher chances of occurrence of bitches in anestrus-diestrus are expected due to their longer duration in the cycle (Table 1) (Chandra & Adler, 2008; Van Cruchten *et al.*, 2003).

Table 2. Key histological features during different stages of estrous cycle in beagle dogs.

Organs	Proestrus	Estrus	Diestrus	Anestrus	Immature
Ovary	Moderately large ovarian antral follicles. Liquor folliculi lined by multiple layers of granulosa cells. Small, shrunken (atretic) corporal lutea (CL) with vacuolated cells.	Larger and cystic follicles. Two or three large tertiary follicles lined by stratified layers of elongated granulosa cells. Follicles lined by thick layers of rounded luteinizing cells amid loose stroma, and the cystic space containing eosinophilic material.	Large with large CL. Up to two large CLs with closely packed luteal cells containing amphophilic to eosinophilic cytoplasm. Late phase luteal cells rarefy to vacuolated cytoplasm.	In the early phase, small CLs with irregular outline, luteal cells containing cytoplasmic vacuoles and prominent vasculature (arterioles) and stroma. Late phase: shrunken (atretic) CLs containing lipofuscin pigment. Early follicular development with many primary and secondary follicles.	Absence of CL or its remnants.
Uterus	Clear and edematous endometrial stroma. Proliferation of superficial and deep endometrial glands. Thicker myometrium with hypertrophied eosinophilic smooth muscle cells.	Thick and eosinophilic endometrial stroma. Glandular and myometrial features similar to those in proestrus.	Thick myometrium composed of hypertrophied smooth muscle cells and thick endometrium. Initially, the superficial epithelial cells are columnar and eosinophilic, followed by a fine vacuolated appearance in the later part of this phase.	Atrophic Small cross-shaped lumen. Basophilic endometrial stroma. Compact myometrium.	–
Vagina	Five to seven layers of squamous epithelium.	Five to seven layers of squamous epithelium covered by four to six layers of keratin (hyperkeratosis and parakeratosis).	Variable histological features. Mucosa lined by three to four layers of cuboidal epithelium with neutrophils between the cell layers.	Thinner than in diestrus. Generally two cell layers thick. Conspicuous absence of leukocytes.	–
Mammary gland	Quiescent and inactive ducts. Hemosiderin pigment and occasional apoptosis. No mitosis. Compact stroma and inactive glands.	Inactive (quiescent) glandular tissue to very slight stromal/periductal edema.	Phase I: Stromal and ductal proliferation. Phase II: Early lobular development with branching ducts and alveolar proliferation. Phase III: Abundance of glandular tissue with large lobules containing secretory material. Phase IV: Early regression, increased interlobular connective tissue, and eosinophilic secretions in distended ducts and acini.	In the early part, ducts distended with secretions and acinar regression not complete. Apoptosis of acinar epithelium. In late anestrus, lobular architecture of the glandular tissue barely evident. Abundant mature connective tissue, and collapsed ducts.	Glandular tissue barely discernible in dermis.

Table 3. Electrocardiographic changes in different stages of estrous cycle.

Parameters	Follicular Phase		Luteal Phase	Sexually inactive	
	Estrus (e)	Proestrus (p)	Diestrus (d)	Anestrus (a)	Immature (i)
RR (msec)	480.7±96.6	473.7±92.8	547.3±138.7	457.3±76.4	496.6±70.4
HR (bpm)	127.4±25.6	131.5±27.7	118.2±30.2	135.1±26.5	122.8±16.0
P–A (mV)	0.3±0.0	0.2±0.1	0.2±0.1	0.3±0.1	0.2±0.1
P–D (msec)	40.0±2.0	42.6±5.7	43.3±12.0	41.8±4.4	37.1±4.9
QT (msec)	185.0±7.1	181.2±18.3	192.3±16.5	174.4±14.7	180.0±15.3
QTc(B) (msec)	268.4±16.8	265.5±26.7	266.1±24.3	259.2±14.5	256.1±15.7
QTc(F) (msec)	237.0±6.9	233.5±20.8	238.4±15.4	226.9±11.8	227.6±14.1
QTc(V) (msec)	230.2±1.3	227.0±15.7	228.2±19.6	213.1±18.8	221.8±16.5
QRS (msec)	50.0±2.2	51.8±4.4	49.8±8.8	48.4*^{i}±2.3	60.0*^{a}±1.0
PQ/PR (msec)	80.0*^{p}±14.1	107.8*^{a,e}±8.3	94.0±13.4	91.4*^{p}±12.2	94.3±15.1

All values in mean±SD, *: Statistically significant at $p<0.05$ as compared to respective phase superscripted

Table 4. Hematological changes in different stages of estrous cycle.

Parameters	Follicular Phase		Luteal Phase	Sexually inactive	
	Estrus (e)	Proestrus (p)	Diestrus (d)	Anestrus (a)	Immature (i)
WBC (10^3 cells/µl)	10.9±1.2	9.1*[i]±1.3	8.2±2.5	8.9*[i]±2.2	8.5*[p,a]±2.7
RBC (10^6 cell/µl)	7.0±1.4	6.5±0.8	6.2±0.9	6.4±0.8	6.2±0.3
Hemoglobin (g/dl)	16.2±2.1	14.8±1.2	14.7±1.6	15.4±1.6	14.5±1.0
Hematocrit (%)	40.0±2.0	43.6±4.1	42.7±5.4	45.1±5.2	41.6±3.0
MCV (fl)	68.4±3.46	67.1±2.7	68.7±3.3	70.8±3.6	67.3±2.0
MCH (pg)	23.4±1.8	22.8±1.1	23.8±1.3	24.2±1.2	23.4±0.6
MCHC (g/dl)	34.3±0.9	33.9±0.8	34.6±1.0	34.1±0.8	34.8±0.7
Platelets (10^3 cells/µl)	346.5±29.0	384.5±108.2	333.0±79.1	307.6±126.7	255.7±29.7
Neutrophils (10^3 cells/µl)	7.2±0.6	6.2±0.9	5.6±1.7	6.7±2.2	4.8±1.6
Lymphocytes (10^3 cells/µl)	3.2±0.1	2.4±0.5	2.1±0.9	1.8±0.8	2.8±1.1
Monocytes (10^3 cells/µl)	0.4±0.6	0.4±0.4	0.4±0.3	0.4±0.2	0.7±0.3
Eosinophils (10^3 cells/µl)	0.003±0.003	0.007±0.005	0.006±0.009	0.008±0.008	0.036±0.081
Basophils (10^3 cells/µl)	0.06±0.09	0.02*[i]±0.02	0.03±0.03	0.02*[i]±0.02	0.08*[p,a]±0.06
PT (sec)	8.2±1.1	8.1±0.9	9.1±3.3	7.94±0.5	8.1±0.7
APTT (sec)	10.6±0.7	11.7±1.4	11.2±1.2	12.5±1.9	11.3±0.9
Reticulocyte (%)	0.5±0.2	0.7±0.2	0.5±0.3	0.5±0.3	0.8±0.5
Reticulocytes (10^3 cells/µl)	26.0±11.2	43.3±17.7	34.2±23.5	43.0±23.5	46.6±30.8

All values in mean±SD, *: Statistically significant at $p<0.05$ as compared to respective phase superscripted.

Recording of ECG has a particular importance on cardiac repolarization during preclinical assessment of drugs. Dog shares certain similarities with the human electrical conduction system and hence used to evaluate the potential risk of arrhythmia in humans (Hammond *et al.* 2001; Finley *et al.* 2003), which cannot be assessed by other methods and have no morphological correlates visible in histopathological examination (Detweiler, 1981). Owing to association with Torsades de Pointes, drug-induced QT interval prolongation has been and remains a significant hurdle to the development of safe and effective drug. Drug regulatory agencies have showed increasing interest in the QT interval because certain drugs can prolong the QT interval to a level that produces ventricular arrhythmias. QT interval is a dynamic physiological variable that can be affected by the velocities of both the ventricular conduction and repolarization (Moss, 1999; Sheridan, 2000; De Ponti *et al.*, 2002). Multiple factors have been described to affect the QT interval such as cardiac cycle length, autonomic nervous system activity, age, gender, circadian rhythm, plasma electrolyte concentrations and variations in ion channels involved in cardiac repolarization (De Ponti *et al.*, 2002; Luo *et al.*, 2004). However, effect of estrous on QT and other ECG parameters have not been reported in experimental beagle dogs. In the present analysis, it was found that there was no statistically significant influence of estrous on QT interval in bitches.

The PQ (sometimes referred to as the PR interval as a Q wave is not always present) interval indicates how fast the action potential is transmitted through the atrioventricular node (AVN) from the atria to the ventricles. A prolonged PQ interval is a sign of a degradation of the conduction system or increased vagal tone (Bezold-Jarisch reflex), or it can be pharmacologically induced, characterized as 1st , 2nd or 3rd degree AV block depending on the severity (Hanton & Rabemampianina, 2006). In present analysis, PQ interval was found to be higher in proestrus dogs as compared to dogs in anestrus and estrus; nonetheless all the values observed were within historical data range. Reports suggest that in addition to the differences in PQ interval between genetic strains, there shall be high inter- and intra-animal (beat to beat) variability in PQ interval and values up to 169 ms may occur in healthy animals (Hanton & Rabemampianina, 2006). Review of various literatures suggests that PQ interval may get affected by heart rate in dogs which could be due to drug treatment or experimental conditions, in particular stress and excitation (Ettinger & Suter, 1970; Ganz & Knappen, 1976). In our study, no such findings correlate to fact that the prolonged PQ interval is associated with heart rate, as no changes were evident in heart rate among different

Table 5. Serum biochemical changes in different stages of estrous cycle.

Parameters	Follicular Phase		Luteal Phase	Sexually inactive	
	Estrus (e)	Proestrus (p)	Diestrus (d)	Anestrus (a)	Immature (i)
Glucose (mg/dl)	96.6±1.1	88.7±8.7	91.5±8.9	93.0±12.6	100.5±10.0
Triglycerides (mg/dl)	60.8±49.4	96.7±49.0	92.4±44.2	155.0*i±106.8	48.3*a±6.0
Total Cholesterol (mg/dl)	239.9±76.4	289.8±79.0	292.3±82.4	288.5±76.8	216.0±31.2
HDL Cholesterol (mg/dl)	187.0±68.5	184.3±21.8	191.0±35.0	196.4*i±35.5	148.7*a±26.9
LDL Cholesterol (mg/dl)	9.4±7.2	21.6±17.3	21.6±14.6	23.2±17.6	9.3±5.9
ALT (U/l)	50.0±2.2	28.5±5.2	26.9±7.1	31.6±6.6	35.6±11.2
AST (U/l)	46.2±1.2	39.2±21.3	37.5±29.6	33.3±12.9	32.3±11.2
ALP (U/l)	80.1±16.1	177.6±119.0	108.7±69.3	173.6±82.9	86.0±16.0
GGT (U/l)	1.6±0.5	2.7±1.1	3.3±1.7	2.8±1.3	2.2±1.5
Creatine Kinase (U/l)	120.2±34.6	205.4±124.9	188.7±116.9	198.8±69.6	241.2±73.7
Total Bilirubin (mg/dl)	0.3±0.3	0.1±0.1	0.2±0.1	0.2±0.1	0.3±0.2
Total Protein (g/dl)	6.0±0.6	5.8±0.6	6.0±0.5	6.2*i±0.3	5.3*a±0.3
Albumin (g/dl)	3.3±0.3	3.1±0.5	3.2±0.3	3.2±0.2	3.5±0.5
Globulin (g/dl)	2.7±0.4	2.8*i±0.4	2.8*i±0.4	2.9*i±0.3	1.9*p,d,a±0.6
A/G ratio	1.3±0.1	1.1*i±0.2	1.2*i±0.2	1.1*i±0.2	2.1*p,d,a±1.0
Urea (mg/dl)	23.1±3.0	22.7±4.3	24.4±8.4	26.8±17.0	30.6±4.9
Creatinine (mg/dl)	0.9±0.1	0.8±0.1	0.8±0.2	0.8±0.3	0.9±0.2
Phosphorus (mg/dl)	4.9±0.5	4.2±0.7	4.0*i±0.8	4.8±1.1	5.3*d±0.8
Calcium (mg/dl)	10.1±0.6	10.1±0.8	10.3±0.8	10.5±0.5	10.6±1.0
Sodium (mmol/l)	147.3±2.7	146.7±1.9	147.3±2.1	147.3±2.5	146.4±4.0
Potassium (mmol/l)	4.6±2.7	4.5±1.9	4.5±2.1	4.5±2.5	4.4±4.0
Chloride (mmol/l)	111.9±1.1	111.5±2.0	112.7±1.9	113.3±4.3	114.2±1.0

All values in mean±SD, *: Statistically significant at $p<0.05$ as compared to respective phase superscripted.

Table 6. Ovarian weight in different stages of estrous cycle.

Parameters	Follicular Phase		Luteal Phase	Sexually inactive	
	Estrus ^{e}	Proestrus ^{p}	Diestrus ^{d}	Anestrus ^{a}	Immature ^{i}
Absolute Ovary weight (grams)	1.22±0.11	1.52±0.65	1.94*i±0.63	1.32±0.55	0.85*d±0.12
Ovary weight (relative to body weight)	0.007±0.002	0.009*d±0.004	0.015*a,i,p±0.006	0.010*d±0.003	0.008*d±0.001
Ovary weight (relative to brain weight)	1.36±0.15	1.81±0.79	2.52*i±0.84	1.74±0.66	1.09*d±0.10

All values in mean±SD, *: Statistically significant at $p<0.05$ as compared to respective phase superscripted.

stages of estrous and all dogs used in the present analysis were from control group.

The cyclic changes that occur in the female reproductive tract are stimulated and regulated by ovarian steroid hormones, estrogen and progesterone, that in turn are controlled by an integrated hypothalamic-pituitary-ovarian (HPO) axis through release of FSH and LH. The ovary plays a pivotal role in the estrous cycle (Evans, 2009). Numerous studies have been undertaken to examine the fluctuation in clinical pathological parameters during the estrous cycle in dogs and other laboratory animal species. The present study reveals that hematological determinants show hardly any variation during the different stages of estrous cycle. However, total WBC, neutrophils, lymphocytes, RBC counts and hemoglobin values showed non-significant higher values at estrus

stage. Contrastingly, Willson *et al.,* 2012 reported lower hematocrit, RBC count and hemoglobin values in diestrus dogs. Interestingly, our analysis did not reveal any changes in the circulating eosinophil numbers in relation to the estrous stages. However, Willson *et al.,* 2012 reported that there was a 45.8% higher circulating eosinophils in diestrus dogs. Corroborative to our findings, few authors (Castrodale *et al.*, 1941; Crafts, 1948; Gaunt & Pierce, 1986) also reported increase in neutrophil production in the bone marrow, resulting in an increase in white blood cells in the peripheral blood after administration of exogenous estrogen.

All the minor changes in clinical pathological parameters during the estrous cycle may be associated with the presumptive changes in blood estrogen, progesterone, gonadotrophic hormones and/or body temperature. Similar to our findings, Günzel-Apel *et al.*, 1997 also reported that PT, APTT and hematocrit were unaffected during the estrous cycle in bitches, however, he concluded that the luteal phase of the nonpregnant and pregnant bitches exhibited significantly increased fibrinogen, the large number of platelets and the decreased antithrombin III activity which were attributed to direct or indirect effects of the high peripheral progesterone concentrations. Landshman & Bleiberg, 1979 reported that estrogen enhanced megakaryocytopoiesis and erythropoiesis in the bone marrow and spleen in mice. Rüberg *et al.,* 1990 studied the fluctuations in blood coagulation parameters during estrous cycle in experimental dog models and indicated that higher blood estradiol and progesterone were accompanied by impaired and enhanced coagulation respectively. They also observed increased platelet count during proestrus and estrus but they concluded it impossible to relate changes in coagulation to a given stage of estrous or ovulation due to individual variation.

With some limitations, the stage of the cycle can be determined through a combination of vaginal smears and serum hormone levels prior to initiating safety/toxicity studies (Fowler *et al.*, 1971; Vermeirsch *et al.*, 2001)

Conclusion

The long duration of the estrous cycle, monoestric behavior, and variable duration of individual estrous stages can be confounding factors in interpreting xenobiotic-induced effects on the female reproductive system in beagle dog. In conclusion, one should consider the overall endocrine system and unique reproductive features to correlate electrocardiographical, clinical pathological and ovarian weight data when interpreting data from safety/toxicity studies.

Acknowledgements

The authors wish to thank Mr. Rajesh J Patel, biostatistician at Zydus Research Centre, for his immense contribution in statistical analysis of the data. They would also like to thank Ms. Sweta Patel, Mr. Tushar Patel and Ms. Harshida Trivedi for their technical assistance in clinical pathology and Mr. Sandeep Patel, Mr. Kanu Bhavsar, Mr. Dinesh Patel, Mr. Satyam Patel, Mr. Hiren Acharya and Ms. Mittal for their histotechnology support.

REFERENCES

Anderson AC, Simpson ME. (1973). *The ovary and reproductive cycle of the dog (Beagle)*, xvii to 290, Geron-X, Inc., California.

Butinar, J., Mujagić, E. and Galac, S. (2004). The oestrus cycle in the bitch: a review article. *Slov Vet Res* **41**: 5–11.

Castrodale D, Bierbaum O, Halwig EB, MacBryde CM. (1941). Comparative studies of the effects of estradiol and stilbestrol upon the blood, liver and bone marrow. *Endocrinol* **29**: 363–372.

Chandra SA, Adler RR. (2008). Frequency of Different Estrous Stages in Purpose-bred Beagles: A Retrospective Study. *Toxicol Pathol* **36**: 944–949.

Chastain CB, Ganjam VK. (1986). *Clinical Endocrinology of Companion Animals*. Lea and Febiger, Philadelphia.

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for Laboratory Animal Facility. (2005). The Gazette of India. Government of India, New Delhi.

Concannon PW. (1993). Biology of gonadotrophin secretion in adult and prepubertal female dogs. *J. Reprod. Fertil Suppl* **47**: 3–27.

Crafts RC. (1948). The effects of estrogens on the bone marrow of adult female dogs. *Blood*. **3**: 276–285.

De Ponti F, Poluzzi E, Cavalli A, Recanatini M, Montanaro N. (2002). Safety of non-antiarrhythmic drugs that prolong the QT interval or induce torsade de pointes. *Drug Safety* **25**: 263–286.

Detweiler DK. (1981). The use of electrocardiography in toxicological studies with beagle dogs, in *Cardiac Toxicology,* (Balazs T ed) pp. 33–82, CRC Press, Florida.

Ettinger SJ, Suter PF. (1970). *Canine Cardiology*, WB Saunders Company, Philadelphia.

Evans GO. (2009). *Animal clinical chemistry: A practical handbook for toxicologists and biomedical researchers*. Taylor & Francis Group LLC, USA.

Finley MR, Lillich JD, Glimour RF, Freeman LC. (2003). Structural and functional basis for the long QT syndrome: relevance to veterinary patients. J *Vet Int Med* **17**: 473–488.

Fowler, EH, Feldman MK, Loeb WF. (1971). Comparison of histologic features of ovarian and uterine tissues with vaginal smears of the bitch. *Am J Vet Res* **32**: 327–334.

Ganz H, Knappen F. (1976). A contribution to the subject of the parameters PQ, QRS and QT being functions of heart rate in the ECG of the beagle dog (author's transl.). *Arzneimittelforschung* **26**: 2061–2065.

Gaunt SD, Pierce KR. (1986). Effect of estradiol on hematopoietic and marrow adherent cells of dogs. *Am J Vet Res* **47**: 906–909.

Gunzel-Apel AR, Hayer M, Mischke R, Wirth W, Hoppen HO. (1997). Dynamics of haemostasis during the oestrus cycle and pregnancy in bitches. *J Reprod Fertil Suppl* **51**: 185–193.

Hammond TG, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Redfern WS, Sullivan AT, Camm AJ. (2001). Methods of collecting and evaluating non-clinical cardiac electrophysiology data in the pharmaceutical industry: results of an international survey. *Cardiovas Res* **49**: 741–750.

Hanton G, Rabemampianina Y. (2006). The electrocardiogram of the Beagle dog: reference values and effect of sex, genetic strain, body position and heart rate. *Lab Ani* **40**: 123–136.

Harleman JH, Foley GL. (2001). Reproductive system, in *Pathobiology of the aging dog*, (Mohr U, Carlton WW, Dungworth DL, Benjamin SA, Capen CC, Hahn FF eds.) pp. 293–309, Iowa State University Press, IA.

Landshman N, Bleiberg I. (1979). Effect of estradiol on erythropoiesis and megakaryocytopoiesis in mice. *Isr J Med Sci* **15**: 140–146.

Luo S, Michler K, Johnston P, Macfarlaine PW. (2004). A comparison of commonly used QT correction formulae: The effect of heart rate on the QTc of normal ECGs. *J Electrocardiol* **37**: 81–90.

Moss AJ. (1999). The QT interval and Torsade de Pointes. *Drug Saf* **21**: 5–10.

Rehm S, Dinesh JS, Williams AM. (2007). Estrous cycle-dependent histology and review of sex steroid receptor expression in dog reproductive tissues and mammary gland and associated hormone levels. *Birth Defects Res B Dev Reprod Toxicol* **80**: 233–245.

Rüberg B, Günzel-Apel AR, Wirth W, Hoppen HO. (1990). Fluctuations in blood coagulation in bitches during the oestrous cycle. *Waltham Rep* **31**: 39–49.

Sheridan DJ. (2000). Drug-induced proarrhythmic effects: assessment of changes in QT interval. *Brit J Clin Pharmacol* **50**: 297–302.

Van Cruchten S, Van den BroeckW, Duchateau L, Simoens P. (2003). Apoptosis in the canine endometrium during the estrous cycle. *Theriogenol* **60**: 1595–1608.

Vermeirsch H, Simoens P, Coryn M, Van den Broek W. (2001). Immunolocalization of progesterone receptors in the canine ovary and their relation to sex steroid hormone concentrations. *Reprod* **122**: 73–83.

Willson CJ, Chandra SA, Kimbrough CL, Jordan HL. (2012). Effect of estrous cycle phase on clinical pathology values in beagle dogs. *Vet Clin Pathol* **41**: 71–76.

ISSN: 1337-6853 *(print version)* | 1337-9569 *(electronic version)*