RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences

(P-ISSN 1595-093X: E-ISSN 2315-6201)

http://dx.doi.org/10.4314/sokjvs.v16i4.8

Ahur *et al*./*Sokoto Journal of Veterinary Sciences*, **16**(4): 64 - 72.

Age-sex dimorphisms in the estimation of median lethal dose (LD50) of lead diacetate in rabbits using up-and-down procedure (Arithmetic method)

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Publication History:
Received: 08-05-2018
Accepted: 22-09-2018

Abstract

Lead had been known to be toxic since pre-antiquity. It causes neurodevelopmental, cardiovascular and renal pathologies. Other toxicological studies of lead (Pb) salts are studied in laboratory animals such as rat, mice, hamsters, rabbits and monkeys; however, there is paucity of information on the median lethal dose (LD_{50}) of Pb salts in rabbits especially when used as a model for molecular studies of Pb toxicity. In view of this, oral LD_{50} of lead (Pb) diacetate [$Pb(CH_3COO)_2$] was determined in both young (6 weeks old, weighing 0.40 ± 0.03 kg) and adult (34 weeks old, weighing 1.63 ± 0.10 kg), male and female New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*) using the revised arithmetic method of Up-and-Down Procedure (UDP). The estimated LD_{50} of Pb diacetate in young male and female NZW rabbits was 1214.20 ± 275.80 and 1214.20 ± 275.80 mg/kg body weight respectively. Also, the LD_{50} of the adult male and female rabbits was 1503.30 ± 342.90 and 1792.50 ± 354.40 mg/kg body weight respectively. Similarities in the estimated LD_{50} of the young rabbits could be attributed to poorly developed xenobiotic metabolic processes. Sex-dimorphism in the toxicity of Pb diacetate was observed in the adult animals, where the male animals were found to be more sensitive to the toxicant than the female. Hence, Pb diacetate is moderately toxic in NZW Rabbits according to "Gosselin, Smith and Hodge scale" of toxicity rating.

Keywords: Arithmetic mean, Dimorphism, Dose, Lead, Rabbits, Sex, Toxicity

Introduction

Toxicity studies of toxicants or pharmacological agents are conducted mostly in laboratory animals with the aim of determining acute, sub-chronic and chronic effects of an agent intended to be used either in humans or animals (NICEATM, 2000; Saganuwan, 2012). Acute toxicity test often refers to as lethal dose or *dosis letalis* (LD_{50}), is a fundamental component in defining the toxicity of a test material for hazard classification and labelling (Svarc-gajiae, 2009). Trevan (1927) first defined LD_{50} as the dose of an agent which can kill 50 % of test subjects. The LD_{50} has been used as a benchmark for comparing the toxicity of chemicals in laboratory animals and relating the toxicity to human health (NICEATM, 2000). Acute toxicity in laboratory animals can serve as an indicator of toxicity potential of a chemical compound in humans (Mugford & Kedderis, 1998). Several methods are adopted in the estimation of

LD_{50} ranging from "moving average" to "up-and-down procedure" (Karber, 1931; Reed & Muench, 1938; Miller & Tainter, 1944; Litchfield & Wilcoxon, 1949; Lorke, 1983; Weil, 1983; Bruce, 1985; ASTM, 1987; Dixon, 1991; Schlede *et al*., 1994, Saganuwan, 2014). The up-and-down procedure (UDP) is the most currently adopted method of estimating LD_{50} for chemicals and agents given as a single oral dose (OECD, 2001; NIH, 2001) to meet the principles of "3Rs"; Replacement, Reduction and Refinement in animal experiments (OECD, 2001; Burden *et al*., 2015). The UDP was first described by Bruce (1985) and adopted by OECD (OECD, 2000a; OECD, 2000b; OECD, 2001; NIH, 2001; Botham, 2004). It requires less number of animals (about 5-9), is faster and its findings are comparable to that of other acute toxicity methods (OECD, 2001).

Lead (Pb) is a heavy metal that has an old history of toxicity (Wedeen, 1984) and remains a significant health problem of both developed and developing nations (Struzynska, 2000; Rosner, 2016). Lead poisoning (plumbism) is re-emerging; in 2010, about 400 deaths were reported in Zamfara State (UNEP/OCHA, 2010) and in May of 2015, 28 deaths were reported in Rafi Local Government Area of Niger State (Faul, 2015) all in Nigeria, because of acute exposures to Pb from artisanal mining of gold in lead-rich ores. In May 2016, Pb poisoning was reported in Flint, Michigan, USA (Rosner, 2016). Acute signs of Pb toxicity range from severe abdominal pain, constipation, nausea, vomiting and anorexia especially among children, whilst, chronic manifestations are encephalopathy, seizures, attention deficit hyperactivity disorders (ADHD), decreased peripheral nerve impulse conduction, memory and other motor function losses (US ASTDR, 2010). Due to differences in species, age and existence of sexual dimorphism in xenobiotic metabolism (Mugford & Kedderis, 1998), there is need to investigate acute toxicity of Pb and its compound in laboratory animals, especially to guide researchers in biomedical experiments involving lead. Rabbits are laboratory animals used for biomedical research (Morton *et al*., 2003) and contribute a lot to livestock production sector (Mailafia *et al*., 2010), providing animal protein (about 20.8% crude protein levels) in most developing countries like Nigeria, where protein demand is crucial and paramount (Ajala & Balogun, 2004). Most biomedical researches on Pb toxicity are reported in mice, rats and non-human primates (Rice, 1990; Altmann *et al*., 1993; Mameli *et al*., 2001; Eneh & Akah, 2012), but, there is dearth of knowledge on the acute oral toxicity (LD_{50}) of Pb salts in NZW rabbits (Bersenyi, 2003; Raafat *et al*., 2009; Elgohary *et al*., 2009). Therefore, the objective of the study was to estimate the LD_{50} of Pb diacetate in young and adult, male and female NZW rabbits using the revised Arithmetic method of Up-and-Down Procedure (Saganuwan, 2014).

Materials and Methods

Laboratory animals

Twenty-four (24) New Zealand White (NZW) rabbits comprising 12 weaned, 6 weeks old and 12 adults, 34 weeks old respectively, procured from the Animal House of the National Veterinary Research Institute (NVRI), Vom Nigeria were used for the experiment. Ethical approval was given by Ethical Committee of College of Veterinary Medicine, Federal University of Agriculture, Makurdi, Nigeria with permit number (No. CVM/VPP/12/2014). Age selection was done based on the report of Masoud *et al*. (1986).

The weaned rabbits weighing 0.40 ± 0.03 kg (mean ± SD) were divided into 2 groups of 6 animals each, group 1 comprised 6 females and group 2, 6 males respectively. The adult rabbits weighting 1.63 ± 0.10 kg (mean ± SD) were also divided into 2 groups of 6 animals each. Group 3 comprised 6 adult female rabbits and Group 4 comprised 6 adult male rabbits respectively. The animals were kept in stainless steel cages and acclimatized for 2 weeks at ambient temperature 28 ± 5 °C, cyclical diurnal changes and relative humidity (70 ± 10 %). Standard commercial rat pellets (Vital Feed®) prepared by Grand Cereal and Oil Company Limited (GCOL), Jos, Nigeria and water were provided *ad-libitum*.

Treatment

The toxicity test was conducted using the arithmetic method of UDP revised by Saganuwan (2014). A default dose of 1000 mg/kg body weight of Pb diacetate was adopted (Lorgue *et al*., 1996) and administered to the 1st animal and the outcome of either death (X) or survival (O) was observed in 48 hours. Subsequent animals were dosed using a dose progression factor of 3.2 until 2 or 3 reversals were achieved (Table 1). Moribund animals were euthanized using pentobarbital sodium and considered dead (The Humane Society of United States, 2013). All the surviving animals were further observed for 12 days for signs of toxicity.

Statistical analysis

Data generated were expressed as arithmetic mean using SPSS version 17 statistic package (IBM®, NY

US). Student's t-test was used to differentiate between the means at 5 % level of significance (Kestenbaum, 2009).

Results

Treatments and treatment outcomes are presented in Tables 1 and 2. The estimated LD_{50} of Pb diacetate in young male and female NZW rabbits was 1214.2 mg/kg bwt, with standard deviation (SD) and standard error of mean (SEM) of 675.6 and 275.8, respectively. But default dose (Dd) progression factor and confidence interval (Ci) of 2.8 mg/kg bwt and 22.7 % respectively were deduced as described by Saganuwan (2014) (Table 1). The estimated LD_{50} of Pb diacetate of adult female NZW rabbits was 1792.5 mg/kg bwt with SD of 868.1 mg/kg bwt and SEM of 354.4 translating to a deduced Dd and Ci of 2.9 and 19.8 % respectively (Table 2). Similarly, the estimated LD_{50} of Pb diacetate of adult male NZW rabbits was found to be 1503.3 mg/kg bwt and 342.9 mg/kg bwt with a deduced Dd and Ci of 2.9 and 22.8 % respectively (Table 2). The LD_{50} values for adult female (1503.3 ± 342.9 mg/kg bwt) and adult male (1792.5 ± 354.4 mg/kg bwt) rabbits were higher, though not statistically ($p>0.05$) as compared to the values reported for the young rabbits (Table 1). The

Table 1: Treatment outcome and estimated LD_{50} of $Pb(CH_3COO)_2$ of weaned NZW rabbits using the arithmetic method of UDP

	Weaned females (Group 1)			Weaned males (Group 2)		
No.	Weight (kg)	Dose (mg/kg)	Survival status	Weight (kg)	Dose (mg/kg)	Survival status
1st	0.42	1000	X*	0.38	1000	O
2nd	0.38	850	O	0.41	2585	X*
3rd	0.43	1000	O	0.35	1000	X*
4th	0.40	2585	X*	0.45	850	O
5th	0.38	1000	X*	0.42	1000	X*
6th	0.41	850	O	0.39	850	O
Am (50% LD_{50})		1214.2			1214.2	
SD (Dd)		675.6 (2.8)			675.6 (2.8)	
SEM (Ci)		275.8 (22.7 %)			275.8 (22.7 %)	
LD_{50} (Am ± SEM)		1214.2 ± 275.8			1214.2 ± 275.8	

Values with different alphabet superscript are significant at $p<0.05$, X = Death, O = Survival, AM=Arithmetic mean, GM = Geometric mean, SD = Standard deviation, SEM = Standard error of mean, Dd = Default dose, Ci = Confidence interval, * = signs of toxicity observed (epistaxis, arched back, tremor, huddling at corners, paresis, coma and death)

Table 2: Treatment outcome and estimated LD_{50} of $Pb(CH_3COO)_2$ of adult NZW rabbits using the arithmetic method of UDP

	Adult females (Group 3)			Adult males (Group 4)		
No.	Weight (kg)	Dose (mg/kg)	Survival status	Weight (kg)	Dose (mg/kg)	Survival status
1st	1.52	1000	O	1.78	1000	O
2nd	1.48	2585	X*	1.59	2585	X*
3rd	1.70	1000	O	1.61	1000	X*
4th	1.67	2585	X*	1.72	850	O
5th	1.50	1000	O	1.59	1000	O
6th	1.66	2585	X*	1.75	2585	X*
Am (50% LD_{50})		1792.5			1503.3	
SD (Dd)		868.1 (2.9)			839.9 (2.9)	
SEM (Ci)		354.4 (19.8 %)			342.9 (22.8 %)	
LD_{50} (Am ± SEM)		1792.5±354.4			1503.3±342.9	

Values with different alphabet superscript are significant at $p<0.05$, X = Death, O = Survival, AM=Arithmetic mean, GM=Geometric mean, SD=Standard deviation, SEM=Standard error of mean, Dd=Default dose, Ci=Confidence interval, * = signs of toxicity observed (epistaxis, arched back, tremor, huddling at corners, paresis, coma and death)

estimated LD_{50} of the adult female rabbits (1792.5 ± 354.4 mg/kg bwt.) was higher ($p>0.05$) compared with the adult male (1503.30 ± 342.9 mg/kg bwt.) respectively; however, the means were not statistically different (Table 2). The estimated LD_{50} ranges (mean ± SEM) obtained for the young, adult male and female rabbits were 938.4 – 1490.0, 1160.4 – 1846.2 and 1438.1 – 2146.9 mg/kg between, respectively.

Discussion

Lead is a complex toxicant affecting several cells and organs, disrupting functional and structural mechanism of the biological systems like the nervous, cardiovascular, hematopoietic and renal systems (Khalil-Manseh *et al*., 1993; Zawia *et al*., 2000; Cory-Slechta, 2003; Vaziri, 2008). Mechanisms of toxicity are associated with disruption of Ca^{2+} fluxes and Ca^{2+}-regulated metabolisms (Sidhu and Nehru, 2003), induction of "ionic mimicry" and distortion of sulfhydryl (thiol) groups of important antioxidant enzymes and protein ligands (Lanphear *et al*., 2005, Xu *et al*., 2009).

Age-sex dimorphisms are reported to affect morphological, physiological, immunological and behavioural parameters, and hence, influence the outcome of experiments (Diedrich *et al*., 2007; Florez-Vargas *et al*., 2016). In the present study, the LD_{50} value of the young rabbits showed no sex-based dimorphism in response to acute Pb diacetate poisoning as their LD_{50} values were same ($p>0.05$). This may be attributed, but not limited to, lack of differentiation of the xenobiotic metabolizing enzymes (i.e. phase I and II enzymes) and other organic ligands in membrane proteins of these young rabbits (Mugford & Kedderis, 1998; Grace *et al*., 2008). Our finding agrees with the report of Lindahl *et al*. (1999) that young animals are vulnerable to the effect of Pb toxicity due to immaturity of blood brain barrier and poorly differentiated astroglia (a Pb sink), irrespective of sex differences. The magnitudes of Pb toxicity are known to be strongly dependent on the differentiation and developmental periods *in vivo* in which the biological system is exposed (IARC, 2006). Our findings support the above assertion as the LD_{50} values of adult male and female rabbits were found to be higher than the young rabbits suggesting an increased resistance to high dose of Pb diacetate administered acutely in the adult animals. The difference observed in adult rabbits may be attributed to a more complex metabolic process of the toxicant in adult rabbits (Trimbell, 1991; Mugford & Kedderis, 1998), a more matured blood brain barrier and differentiated cellular structures (Pueschel *et al*., 1996; Lindahl *et al*., 1999; Grandjean & Landrigan, 2006) and a decreased intestinal absorption capability for Pb ions (Skerfving & Bergdahl, 2007; Pokras & Kneeland, 2009). The difference may also be due to greater capacity to store Pb in an inactive form in the bones, while in the young animals, the active bone absorption/resorption mechanism contributes to their relatively high vulnerability to Pb toxicity (Hu *et al*., 1998; Hu *et al*., 2007). Age is one of the factors known to interplay in the clinical manifestations of Pb poisoning (Braide & Anika, 2007). Garg (2007) also reported that age dimorphism accounts for the differences in the response to toxic doses of Pb salts; which is usually due to differences in biotransformation.

Our findings also revealed that a higher LD_{50} value of the adult female rabbits is indicative of their low-susceptibility to the toxic effects of the compound as compared to the adult male rabbits. Differences in our treatment outcomes were generally not significant ($p>0.05$) probably due to a single animal per dose protocol as a UDP guideline in obeying the principles of Replacement, Reduction and Refinement (3Rs) in the use of animals for research (OECD, 2001; Burden *et al*., 2015). Quarterman (1987) reported that male animals are more prone to toxicity of Pb salts than female animals. In contrast, Mugford & Kedderis (1998) reported that female animals are more prone to the toxic effects of most xenobiotics. Male animals are reported to have higher rate of xenobiotic metabolism than the females and hence metabolize and excrete most toxic agents faster and so are less susceptible (Sipes & Gandolfo, 1991). The most important xenobiotic metabolic enzymes in mammals are the microsomal cytochrome P450 (e.g. CYP2A & 2E) enzymes, which catalyze the oxidation and reduction of exogenous compounds. However, there exist sex-dependent differences in the expression of these microsomal CYP450 enzymes, and their different manifestations are developmentally regulated and thus, manifest more in adult animals (Waxman *et al*., 1985). This could also be a reason why there were no differences between the LD_{50s} of the weaned rabbits. Furthermore, female animals are reported to have 10-30 % less total CYP450 enzymes compared to males (Mugford & Kedderis, 1998) which explains why females metabolize chemical compounds more

slowly and thereby more prone to toxic agents. However, the result of the present study showed male rabbits to be more prone to Pb toxicity than the females.

The results obtained using Saganuwan's revised method (Saganuwan, 2014) are comparable with the results obtained from other species using other methods of LD_{50} determination. The ranges of estimated LD_{50s} in the present work for the young, adult male and female rabbits are comparable to the ones estimated and reported by Lorgue *et al*. (1996) and JECFA (2000). Lorgue *et al*. (1996) reported LD_{50} ranges of 600 – 800, 400 – 600, 800 – 1000, 800 – 1000 and 200 – 600 mg/kg bwt for cattle, horses, pigs, dogs and poultry respectively, using methods like; traditional acute oral toxicity (TAOT or TG 401), fixed dose procedure (FDP or TG 420), acute toxic class method (ATCM or TG 423) and the conventional up-and-down procedure (UDP or TG 425) (Lipnick *et al*., 1995; Stitzel *et al*., 2002). JECFA (2000) also reported lethal dose range of 300 – 4000 mg/kg bwt of Pb salts in animals after multiple short term oral exposures. Although, Pb is known to cause chronic poisoning (EFSA, 2010), acute poisonings are still reported especially from contamination by Pb batteries, Pb-laden paints and Pb production industries (Frape & Pringle, 1984; O'Hara *et al*., 1995; UNEP, 2008). The outcome of the present studies revealed Pb diacetate to be moderately toxic in NZW rabbits according to the "Gosselin, Smith and Hodge scale" of toxicity rating (Svarc-gajiae, 2009).

In conclusion, the LD_{50} values reported in the adult rabbits were higher as compared to the young rabbits. The adult male rabbits were reported to be more prone to the toxic effects of Pb diacetate when compared to the adult females. This may be due to sexual dimorphism in the xenobiotic metabolic capability of the adult rabbits. Lead diacetate may be classified as moderately toxic in rabbits on the "Gosselin, Smith and Hodge scale" of toxicity rating (Svarc-gajiae, 2009).

Acknowledgement

Authors are grateful to Dr SA Saganuwan of the Department of Veterinary Physiology, Pharmacology and Biochemistry, College of Veterinary Medicine, Federal University of Agriculture, Makurdi, for his valuable contributions and revision of up-and-down procedure (UDP).

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