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REVIEW ARTICLE

The pathway of lead through the mother's body to the child

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ABSTRACT

Placenta, the organ on which great attention is concentrated during pregnancy, represents an ineffective barrier to the transfer of hazardous heavy metals, mainly lead, into the foetus. The presence of lead in the placenta is an environmental hazard for a person's future. Due to hormonal changes, lead is released during pregnancy into the bloodstream of the mother from deposits in the bones and in the teeth, where it has accumulated for years as a result of a contaminated environment. Since lead is a neurotoxic metal, exposure to lead during prenatal and postnatal development can cause serious neurocognitive damage and hence the development of an Attention Deficit Hyperactivity Disorder (ADHD) in a developing human. Our work provides an overall picture of the "toxic pathway" of lead through the mother's body, the risks arising from its transplacental transfer and its accumulation in the developing foetus as well as effective prevention to protect all newborns.

KEY WORDS: human placenta; lead; calcium; neurotoxicity; ADHD

INTRODUCTION

Sources of lead

Lead is the oldest cumulative toxic metal that seriously contaminates the environment. Because of its malleability, resistance to corrosion, and low melting point humans have used lead since prehistoric times to fabricate statues, jewelry, water pipes, and drinking vessels. The Romans used lead to sweeten wine (Eisinger, 1982). Lead solder was used as a welding material in the automotive industry and lead was also a part of some pesticides. At present, most of the world's lead production is used to produce lead accumulators in the automotive industry. Gasoline with lead additive has been used since 1923. 50–70% of lead from gasoline was received into the atmosphere as lead chloride and lead bromide by exhaust gases and lead was geo-accumulated in the soil. It is estimated that up to 4 million tons of lead was released into the air and soil by the combustion of leaded petrol which represents 90% of the lead in the atmosphere. Lead emissions from the combustion of leaded fuels and metals smelting have contributed significantly to the accumulation of atmospheric and soil Pb^{2}+ (lead ion). From 1923 to 1986, the combustion of leaded gasoline in the United States is estimated to have dispersed around 4 million metric tons of Pb^{2}+ into the atmosphere and eventually to the soil (Mielke, 1999). It is estimated by the Agency for Toxic Substances and Disease Registry (ATSDR) that the combustion of leaded gasoline has accounted for 90% of the Pb^{2}+ deposited in the atmosphere (Mielke, 1999). Pb^{2}+ contamination of soil is so extensive that it has been estimated that current soil Pb^{2}+ levels are 1000 times higher in contaminated areas than in certain pristine forest areas (Renberg *et al.,* 2000). Food is also one of the main sources of lead exposure (Hanning *et al.,* 2003). Drinking water can contain lead and thus be a source of poisoning. It may be contaminated from the environment or from pipes containing lead particles. Lead reaches the body mainly through inhalation, orally and exceptionally through resorption via broken skin. After absorption from the gastrointestinal tract or lungs, lead is distributed by blood to the tissues and organs of the human body. In blood, 99% of lead is transported on the surface of erythrocytes and 1% via plasma. Lead in the liver, where it causes hepatotoxicity, penetrates the bloodstream and transports into the kidneys, spleen, lungs, brain and mineralized tissues: bones and teeth. It has been shown that nutritional status is a significant biological factor of susceptibility for elevated Pb^{2}+ in body. Iron, zinc and calcium deficiencies increase the retention

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of ingested Pb^{2}+ and may also increase the gastrointestinal absorption of Pb^{2}+ (Goyer, 1996; Ruff *et al.,* 1996). Age at exposure is also considered a significant risk factor for Pb^{2}+ intoxication and its effects. Children absorb significantly more Pb^{2}+ in the intestine than adults. Pre- and perinatal exposure results in higher brain Pb^{2}+ levels than postnatal exposure due to an under-developed blood-brain barrier in early life (Goyer, 1996; Ruff *et al.,* 1996). Social environment has also been shown to alter susceptibility to the cognitive effects of Pb^{2}+ exposure. Poverty, residence in the inner city and minority status have all been demonstrated to result in the creation of a high-risk situation for Pb^{2}+ intoxication (Koller *et al.,* 2004; Ruff & Bijur, 1989; Ruff *et al.,* 1996). For example, the number of non-Hispanic black children who have increased blood Pb^{2}+ levels in the United States is from 3 to 13.5 times higher than in non-Hispanic white children with increased blood Pb^{2}+ levels (Bernard & McGeehin, 2003).

Accumulation of lead in tissues

Lead as well as other heavy metals can be accumulated in the body. The preservation of lead in human tissue creates the long-term exposure indictor of this element, as well as image of lead pollution sources. In chronic exposure, bone serves as the primary storage organ from which lead can be released during pregnancy, lactation, osteoporosis and developing conditions (Silbergeld, 1991). The half-life of the deposited lead in the bones is 32 years, the time in the trabecular bones being shorter. It is released from soft tissues in 20–30 days. Dentin, as we know, also accumulates lead from blood during early childhood (Gulson & Wilson, 1994).

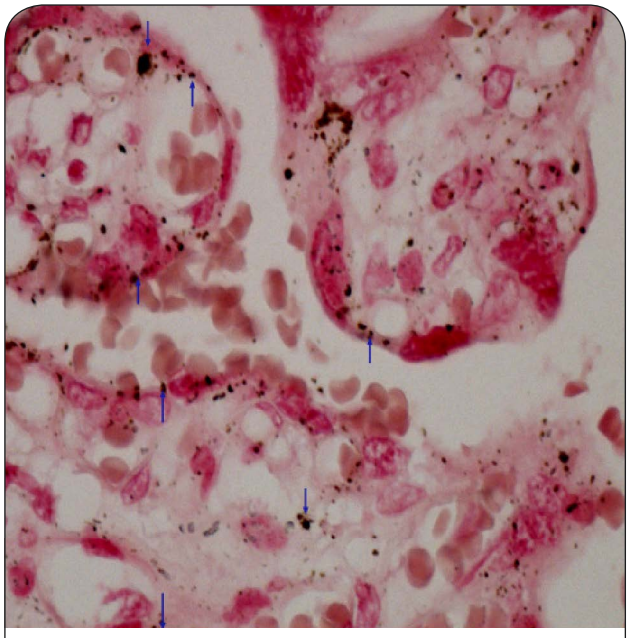

Figure 1. Placenta – proof of calcium. Method after Koss was used. Cumulation of calcium deposits in the chorium villus (↓) and in the syncytiotrophoblast (↑). Magnified 400×.

Mobilization of lead from the mother's skeleton

The skeleton is a potential endogenous source of lead during pregnancy and lactation. There are number of papers that describe the mobilization of lead in women during physiological stress such as pregnancy and lactation (Thomson *et al.,* 1985; Ernhart & Greene, 1992; Manton, 1992). The mobilization of lead from the skeleton in the postnatal period is more extensive than in pregnancy. Such increased release of skeletal lead into blood during the postnatal period is attributable to increased lead mobilization from maternal skeletal reserves during lactation, associated with increased bone resorption and may be associated with inadequate calcium intake in the diet. The maternal gestational age affects the metabolism of minerals in mother and child. The foetus produces 1,25-dihydroxyvitamin D, which controls the transport of calcium through the placenta. This activity is the highest in the last trimester of pregnancy which is a critical period due to the development of neuronal structures, synaptogenesis, bursitis and brain growth. Inhibition of synaptic structures and the blocking of neurotransmitter connections by lead may occur during this period (Silbergeld, 1991).

Calcium – an important carrier of lead when transported by the placenta

The calcium content in the foetus is potentially increasing during pregnancy. The transfer of calcium from the mother to the foetus occurs via an active mechanism, the calcium concentration being higher in foetal blood than in maternal. Heavy metals are known to act on calcium homeostasis through the perturbation of calcium channels and pumps as well as interference with the protein kinase C and calcium binding protein. Our histochemical finding points out that calcium plays a role of lead carrier (Figure1).

Lead transport is thought to be closely related to the movement of calcium ions through the syncytiotrophoblast. The presence of lead in maternal and umbilical blood modifies calcium transport into the syncytiotrophroblast (Lafond *et al.,* 2004). All cells contain similar systems for the permanent and temporary regulation of calcium ions and for calcium receptors. Poisons that affect mobilization and homeostasis of calcium will become a place for regulation of these processes. Lead, cadmium, zinc and other metals are transported across cell membranes via calcium transporters (Pounds, 1984; Cooper *et al.,* 1984; Atchison *et al.,* 1986; Koop, 1986; Simons, 1986; Hinkle *et al.,* 1987). Placental calcium transport is dependent on a series of transplacental proteins located in the syncytiotrophoblast, forming a barrier between the mother and the foetus. These proteins include 4 plasma membrane ATPase isoforms (PMCA 1–4), which are a step towards placental calcium transport. Placental calcium transport occurs in syncytiotrophroblasts (Belkacemi *et al.,* 2005). It continues through a well-controlled sequence of events consisting of an apical entry through the calcium channel, cytosolic calcium diffusion bound to calcium transport proteins,

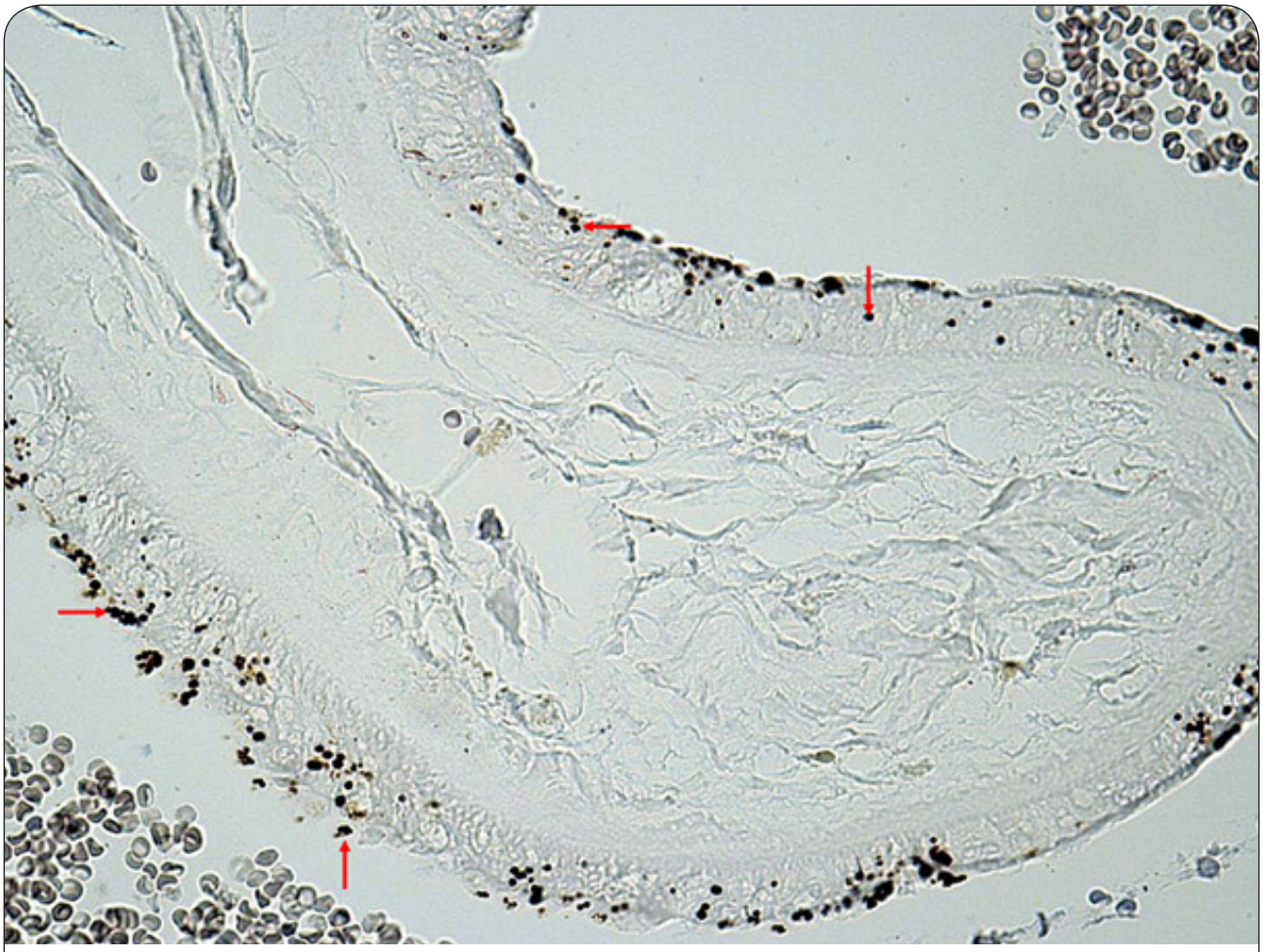

Figure 2. Placenta - Mallory and Parker method for proof of lead in the light microscope. Capturing of lead by resorption plasmodium of syncytiotrophoblast (←,→,↑). Available at 20 lenses using the Canon D60.

and basolateral calcium ion extravasation via the plasma membrane calcium-dependent ATPase (Stauffer *et al.,* 1993; Zylinska *et al.,* 2002).

Transport of lead through the placenta

The lead transport mechanism is not entirely clear. Several data suggest that lead transport may be a matter of a simple diffusion from maternal circulation into the foetal circulation.

The largest amount of lead levels measured in foetal tissues at various gestation periods were described in Barltrop's study (Barltrop, 1969). An important question is how early the lead transport takes place. In Barltrop's studies, lead transport starts around the 13th week of gestation and is prolonged to childbirth. His study has shown that lead contained in the brain and other tissues increases with an increasing organ size (Barltrop, 1969). Barltrop (1969) noted that the concentration of lead in the femur rapidly increased in the first trimester, which resulted in an initial loss of ossification and the depositing of calcium. Calcium deficiency increases the absorption of lead and its pathological effects (Mahaffey *et al.,* 1973). The primary mechanism for the transplacental transmission of lead is probably a simple diffusion. The concentration of lead in the developing baby's tissue, including brain tissue, is directly dependent on lead concentration in the umbilical cord blood. The lead content increases proportionately with the growing size of the foetus organs. The blood-brain barrier is immature which facilitates the entry of lead into the brain and therefore does not prevent a direct attack on the brain structures of the developing foetus.

Human placental Syncytiotrophoblast – an important place for lead transport

Syncytiotrophoblast is the epithelial layer separating maternal blood that flows around the villi. It is not composed of individual cells but is presented as a continuous multi-core surface layer without the separation of cellular boundaries. Syncytiotrophroblast is produced by fusing cells from the stem cells of the villous cytotrophoblast and then formed into true syncytia. The surface of the syncytia rises to irregular microvilli and the adjacent cytoplasm contains pockets formed by a smooth membrane. These pockets indicate intense pinocytosis which serves to transport substances from the mother's

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circulation to the foetus (Castellucci & Kaufmann, 1995). The microvilli covering the syncytiotrophroblast surface represent the maternal-foetal contact zone (Teasdale and Jean-Jacques, 1986). The syncytiotrophoblast is the most frequent place for the accumulation of lead and nickel deposits (Figure 2).

Interactions between lead and the cells of the human body

There is a considerable number of publications describing the adverse health effects of lead already at low exposure levels, with special attention paid to pregnant women and children (Carpenter, 2001; Factor-Litvak *et al.,* 1999; Goyer, 1996; Rosen, 1995). Lead represents a significant environmental risk for pregnant women and their offspring. Exposure to high environmental levels of lead may be associated with the preterm ruptures of foetal membranes, spontaneous abortions and preterm births (Angell *et al.,* 1982). Lead can make an impact by its toxicity even without the presence of clinical symptoms (Needleman *et al.,* 1979) and its toxicity may be manifested several years after exposure. Animal data show that a diet highly lacking in proteins and minerals increases lead absorption (Levander, 1979). It is proven that the deficiency of iron and zinc may increase the toxic effects of lead (Levander, 1979). Animal studies have shown that lead also interferes with collagen synthesis (Vistica *et al.,* 1977), energy metabolism and membrane structures (White & Selh, 1975) which may have an adverse effect on the integrity of the chorioamnion membranes, and may also be a predisposition for premature membrane rupture (Angell *et al.,* 1982). Lead is a well-known human reproductive toxin. Lead exposure negatively impacts the pregnancy outcomes of mothers and their newborns (Castellino *et al.,* 1995). Prenatal exposure to lead is manifested by toxic effects on the human foetus, including risk factors: premature birth, low birth weight and the impairment of mental development. Dawson *et al.* (1999), Tabacova & Balabaeva (1993) claim that exposure to lead during pregnancy may interfere with the etiology of preeclampsia. Baranowska (1995) found that high levels of lead in the placenta correlated with low Apgar scores. The effect of lead on cognitive and behavioral development is particularly critical in newborns (Goyer, 1996; Rosen, 1995). Foetal lead exposure may affect multi-organ systems in embryo development, including the retardation of cognitive development in early childhood (Banks *et al.,* 1997). Lead causes a significant increase in the frequency of chromatin changes in maternal bone marrow cells and the reduction of nucleolar organization, causing several specific chromosomal aberrations, changes in the maternal bone marrow and foetal cells (Nayak *et al.,* 1989). Prenatal exposure to high levels of lead causes foetal malformations, abortions, growth and mental retardation, and encephalopathy (Davis & Svendsgaard, 1987; Fahim *et al.,* 1976). Lead carcinogenic mechanism involves direct deoxyribonucleic acid (DNA) damage, DNA inhibition and may also generate reactive oxygen forms and thus cause oxidative damage to the DNA (Silbergeld *et al.,* 1999). Silbergeld *et al.* (2000) highlights the particular importance of transplacental lead exposure and the increase of cancer risk in later life.

Postnatal effects of lead on humans

The mechanisms by which lead damages the brain functions and the effects of increased sensitivity of the immature nervous system of the foetus are not yet fully understood. Lead is predominantly accumulated in the brain's endothelial cells (Toews *et al.,* 1978). High levels of lead exposure result in the loss of the normal blood-brain barrier function and plasma movement into interstitial brain spaces (Goldstein, 1984). The blood-brain barrier is a vulnerable place for neurotoxic lead activity. Edema, increased intracranial pressure and reduced brain perfusion may be the basis for irreversible brain damage. Exposure to already low levels of lead can change the microenvironment of the brain. Goldstein studies (1988) have confirmed that important signals in the blood-brain barrier structure arise from interactions between the endothelial cells and astrocytes. Astrocytes outweigh the amount of neurons in the brain, their protrusions encircling endothelial cells (Raine, 1988). The toxic effects of lead increase the interactions between the endothelial cells and astrocytes (Gebhart & Goldstein, 1988). It suggests that the foetal brain offers low resistance to lead toxicity because it lacks the lead-protein complexes in astrocytes that remove the lead from the mitochondria. These astrocytes are especially at high risk of lead toxicity during in utero since the immature endothelial cells that form the capillaries of the brain offer a decreased resistance to lead, and thereby easily allow fluids and ions, such as $Pb^{2}+$, to enter the brain. Lead toxicity during this development period has often been associated with cognitive impairment and learning malfunctions, as lead can accumulate in their nervous system as they develop (Gularte & McGlothan, 1998). Lead has already been shown to be a behavioral teratogen at low exposure levels. The high levels of lead exposure involve psychological harm, including mental retardation in children (Bornschein *et al.,* 1980; Rutter, 1980). The basal ganglia are sensitive to hypoxia, a change in metabolism, which plays an important role in the pathophysiology of a hyperkinetic disorder. Lead transported through the transplacental barrier into the foetus also attacks dopamine pathways in the middle brain (e.g. in the striatum). Dopamine neurons in the middle brain regulate motor control and emotions thereby engage in cognitive processes and different forms of memory. In the striatum region, dopamine is responsible for the proper coordination of limb movements. The degeneration of dopamine pathways and subsequent dopamine depletion, which may be caused by the neurotoxic poisoning by lead, result in hypokinesis. Dopamine is important in influencing psychomotor and attention which are dysfunctional in ADHD. Persons with ADHD have the hypoactivity of the cortical dopamine system and the hyperactivity of the striatal system (Drtílková & Šerý, 2007). Exposure to excessive amounts of inorganic lead during the toddler years may produce permanent adverse effects on the brain

functions. Maximum lead intake occurs at the age when most changes affect the density of the brain's synaptic connections. The development of synapse reorganization is partly provided by protein kinase. This enzyme is very susceptible to lead stimulation, so lead intoxication can interrupt the development of the neural network without producing obvious change factors. Protein kinase regulates the development of brain capillaries and the blood-brain barrier. The stimulation of this enzyme by lead can interrupt the development sequence and disrupt the precise regulation of the neuronal environment which is necessary for normal brain function. These claims have shown that the sensitivity of protein kinase to lead may partially enlighten the brain dysfunctions seen in children who have been intoxicated by this poison. Defects in cognitive and behavioral functions arise in children with higher blood levels of lead (McMichael *et al.,* 1988).

Attention deficit hyperactivity disorder (ADHD)

Recently, research has shown that lead, as a neurotoxic metal, can cause ADHD in children. The cause of this syndrome is the accumulation of lead in the striatum of the brain which contains myelin in the neurons. It is a small and diffused organic brain damage caused by various external influences in the prenatal period, during childbirth and early childhood but ADHD occurrence may also be genetically conditioned (Hudziak *et al.,* 2005; Kuntsi *et al.,* 2005; Sherman *et al.,* 1997). The external factors affecting ADHD can include smoking and alcohol drinking during pregnancy, a complicated and premature birth, head trauma, and also various environmental impacts: the increased incidence of heavy metals in the air and food. The presence of metallic protozoans in blood serum disrupts the metabolism of enzymes and this can cause hyperactivity in some children. Awareness of the etiology of this disease will help in the ADHD treatment which has an increasing trend of occurrence in the world. An early, rapid, correct diagnosis is the basis for therapy and the reality of how the ADHD incidence curve in the world will progress depends on it. The open question remains the establishment of centers for the detection of this disease the consequences of which are felt by all society.

Discussion

Polluted environment can leave changes on the microscopic structure of the placenta. It has not been a long time since lead petrol has ceased being used and so the accumulation of lead and its consequences are manifesting themselves in the generation of mothers who are 25 to 30 years old. These are women who lived during the full-scale use of leaded petrol. It is characteristic for heavy metals that they are very slowly released from the body. Lead and other neurotoxic metals are believed to be transported by simple diffusion from the maternal blood through the syncytiotrophoblast to foetal blood. During a woman's life, a pregnancy is when hormone disharmony occurs that triggers the release of lead from the bones and teeth, since the foetus needs the calcium. It is deriven from bone and teeth deposits for the development of its skeleton and the lead penetrates the bloodstream of the foetus with the calcium. Recently, attention has been paid to the importance of calcium transport in relation to lead transport. The direct exposure of the mother to lead may not always occur. The probability of lead intoxication is higher in mothers who stayed in places with an increased lead level during their lives, even during pregnancy. Lead is found in the environment almost everywhere. It can be detected in the air and dust, its levels are strictly monitored in drinking water. Therefore, as a precaution against this disease, we suggest taking an umbilical cord blood with a lead test for each newborn immediately after birth, setting up centers tracking the statistical occurrence of lead results and promptly addressing the cause behind the increase of ADHD.

Conflict of interest

The authors declare no conflicts of interest. The authors received no financial support for the research, authorship, and/or publication of this article.

Ethical approval

The Ethical Committee of the University Hospital Bratislava approved the study. All patients signed informed consent documents before participating in the study.

REFERENCES

Atchison WD, Joshi U, Thornburg JE. (1986). Irreversible suppression of calcium entry into nerve terminals by methylmercury. *J Pharmacol Exp Ther* **238**: 618–624.

Banks EC, Ferretti LE, Shucard DW. (1997). Effects of low level lead exposure on cognitive function in children: a review of behavioral, neuropsychological and biological evidence. *Neurotoxicology* **18**: 237–281.

Baranowska I. (1995). Lead and cadmium in human placentas and maternal and neonatal blood (in a heavily polluted area) measured by graphite furnace atomic absorption spectrometry. *Occup Environ Med* **52**: 229–232.

Barltrop D. (1969). Transfer of lead to the human foetus, in *Mineral metabolism in Paediatrics* (D.Barltrop, W.Burland, Eds.) pp:135–151, Blackwell Scientific, Oxford, England.

Belkacemi L, Bedard I, Simoneau L, Lafond J. (2005). Calcium channels, transporters and exchangers in placenta: a review. *Cell Calcium* **37**(1): 1–8.

Bernard SM, McGeehin MA. (2003). Prevalence of blood lead levels >or = 5 micro g/ dL among US children 1 to 5 years of age and socioeconomic and demographic factors associated with blood of lead levels 5 to 10 micro g/ dL. Third National Health and Nutrition Examination Survey, 1988–1994. P*ediatrics* **112**: 1308–1313.

Bornschein R, Pearson D, Reiter L. (1980). Behavioral effects of moderate lead exposure in children and animal models. *CRC Rev Toxicology* **8**: 43–99.

Carpenter DO. (2001). Effects of metals on the nervous system of humans and animals. *Int J Occup Med Environ Health* **14**(3): 209–218.

Castellino N, Castellino P, Sannolo N. (1995). *Inorganic lead exposure: metabolism and intoxication*. Boca Raton, FL: Lewis Publishers.

Castellucci M, Kaufmann P. (1995). Basic structure of the villous trees, in P*athology of the Human Placenta*, third edn. Springer Science + Business, New York: 57.

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Cooper GP, Suszkiw JB, Manalis RS. (1984). Presynaptic effects of heavy metals, in *Cellular and Molecular Neurotoxicology* (T.Narahashi, Ed.) pp: 1–21, Raven Press, New York.

Davis JM, Svendsgaard DJ. (1987). Lead and child development. *Nature* **329**: 297–300.

Dawson EB, Evans DR, Nosovitch J. (1999). Third trimester amniotic fluid metal levels associated with preeclampsia. *Arch Environ Health* **54**(6): 412–415.

Drtílková I, Šerý O. (2007). *Hyperkinetická porucha ADHD.* Praha, Galén, pp: 268.

Eisinger J. (1982). Lead and wine: Eberhard Gockel and the Colica Pictonum. *Medical History* **26**: 279–302.

Ernhart CB, Greene T. (1992). Postpartum changes in maternal blood lead concentrations. *Br J Ind Med* **9**: 11–13.

Factor-Litvak P, Wasserman G, Kline JK, Graziano J. (1999). The Yugoslavia prospective study of environmental lead exposure. *Environ Health Perspect* **107**(1): 9–15.

Fahim MS, Fahim Z, Hall OG. (1976). Effects of subtoxic lead levels on pregnant women in the state of Missouri. *Res Commun Chem Pathol Pharmacol* **13**: 309–331.

Gebhart AM, Goldstein GW. (1988). Use of an vitro system to study the effects of lead on astrocyte-endothelial cell interactions: a model for studying toxic injury to the blood-brain barrier.*Toxicol Appl Pharmacol* **94**: 191–206.

Goldstein GW. (1984). Brain capillaries: a target for inorganic lead poisoning. *Neurotoxicology* **5**: 167–176.

Goldstein GW. (1988). Endothelial cell-astrocyte interactions: a cellular model of the blood-brain barrier. *An N Y Acad of Sci* **529**: 31–39.

Goyer RA. (1996). Results of lead research, prenatal exposure and neurological consequences. *Environ Health Perspect* **104**(10): 1050–1054.

Gularte T, McGlothan J. (1998). Hippocampal NMDA receptor mRNA undergoes subunit specific changes during developmental lead exposure. *Brain Research* **790**: 98–107.

Gulson BL, Wilson D. (1994). History of lead exposure in children revealed from isotopic analyses of teeth. *Arch Environ Health* **49**: 279–283.

Hinkle PM, Kinsella PA, Osterhoudt KC. (1987). Cadmium uptake and toxicity via voltage-sensitive calcium channels. *J Biol Chem* **262**: 16333–16337.

Hudziak JJ, Derks EM, Althoffi RR, Rettew DC, Boomsma DI. (2005). The genetic and environmental contributions to attention deficit hyperactivity disorder as measured by the conners´ rating scales-revised. *American J Psychiatry* **162**: 1614–1620.

Koller K, Brown T, Spurgeon A, Levy L. (2004). Recent developments in low-level lead exposure and intellectual impairment in children. *Environ Health Perspect* **112**: 987–994.

Kopp SJ. (1986). Cadmium and the cardiovascular system, in H*andbook of Experimental Pharmacology.* Vol.80 (E.C.Foulkes, Ed.) pp: 195–280, Springer-Verlag.

Kuntsi J, Rijsdijk F, Roland A, Asherson P, Plomin R. (2005). Genetic influences on the stability of attention-deficit/hyperactivity disorder symptoms from early to middle childhood. *Biol Psychiatry* **57**: 647–654.

Lafond J, Hamel A, Takser L, Vaillancourt C, Mergler D. (2004). Low environmental contamination by lead in pregnant women: effect on calcium transfer in human placental syncytiotrophoblasts. *J Toxicol Environ Health* **67**: 1069–1079.

Levander OA. (1979). Lead toxicity and nutritional deficiencies. *Environ Health Perspect* **29**: 115.

Madison DV, Malenka RC, Nicoll RA. (1991). Mechanisms underlying long-term potentiation of synaptic transmission. *Annu Rev Neurosci* **14**: 379–397.

Mahaffey KR, Haseman JD, Goyer RA. (1973). Dose-response to lead ingestion in rats on low dietary calcium. *J Lab Clin Med* **83**: 92–100.

Manton WI, (1992). Postpartum changes in maternal blood lead concentrations. Correspondence. Br J Ind Med **49**: 671–672.

McMichael AJ, Baghurst PA, Wigg NR, Vimpani GV, Robertson EE, Roberts RJ. (1988). Port Pirie Cohort Study: environmental exposure to lead and children´s abilities at the age of four years. *N Engl J Med* **319**: 468–475.

Mielke H. (1999). Lead in Inner cities. *Am Sci* **87**: 62-73.

Nayak BN, Ray M, Persaud TVN, Nigli M. (1989). Relationship of embryotoxicity to genotoxicity of lead nitrate in mice. *Exp Pathol* **36**: 65–73.

Needleman HL, Gunnoe C, Leviton A, Reed R, Peresie H, Maher C, Barrett P. (1979). Deficits in psychologic and classroom performance of children with elevated dentine lead levels. *N Engl J Med* **300**: 689.

Pounds JG. (1984). Effect of lead intoxication on calcium homeostasis and calcium-mediated cell function: a review. *Neurotoxicology* **5**: 295–332.

Raine CS (1988). *Neurocellular anatomy*, 4th ed. (G. Siegel, B. Agranoff, R.W. Albers, P. Molinoff, Eds.) pp: 3–33, Raven Press, New York.

Renberg I, Brannvall M, Bindler R, Emeteryd, O. (2000). Atmospheric lead pollution history during four millennia (2000BC to 2000 AD). *Ambio* **29**: 152–158.

Rosen JF. (1995). Adverse health effects of lead at low exposure levels, trends in the management of childhood lead poisoning. *Toxicology* **97**(1–3): 11–17.

Ruff HA, Bijur PE. (1989). The effects of low to moderate lead levels on neurobehavioral functioning in children: toward and conceptual model. *J Dev Behav Pediatr* **10**: 103–109.

Ruff HA, Markowitz ME, Bijur PE, Rosen JF. (1996). Relationships among blood lead levels, iron deficiency, and cognitive development in two-year-old children. *Environ Health Perspect* **104**: 180–185.

Rutter M. (1980). Raised lead level and impaired cognitive behavioral functioning: A review of the evidence. *Devel Med Child Neurol* **22**: 1–25.

Sherman DK, McGue MK, Iacono WG. (1997). Twin concordace for attention deficit hyperactivity disorder: a comparison of teachers´ and mothers´ reports. *Am J Psychiatry* **154**: 532–535.

Silbergeld EK. (1991). Implications for toxicology during pregnancy and lactation. *Environ Health Pespect* **91**: 63–70.

Silbergeld EK, Waalkes M, Rice JM. (1999). Lead as an carcinogen: Experimental evidence and mechanisms of action. *Am J Ind Med* **38**: 316–323.

Silbergeld EK, Waalkes M, Rice JM. (2000). Lead as a carcinogen: Experimental evidence and mechanisms of action. *Am J Ind Med* **38**: 316–323.

Simons TJB. (1986). Cellular interactions between lead and calcium. *Br Med Bull* **42**: 431–434.

Stauffer TP, Hilfiker H, Carafolie E, Strehler EE. (1993). Quantitative analysis of alternative speicing options of human plasma membrane calcium pump genes. *J Biol Chem* **268**(34): 25993–26003.

Tabacova S, Balabaeva L. (1993). Environmental pollutants in relation to complications of pregnancy. *Environ Health Perspect* **101**: 27–31.

Teasdale F, Jean-Jacque G. (1986). Morphometry of the microvillous membrane of the human placenta in maternal diabetes mellitus. *Placenta* **7**: 81–88.

Thompson GN, Robertson EF, Fitzgerald S. (1985). Lead mobilization during pregnancy. *Med J Australia* **43**: 131.

Toews AD, Kolber A, Hayward J, Krigman MR, Morrell P. (1978). Experimental lead encephalopathy in the suckling rat: concentrations of lead in cellular fractions enriched in brain capillaries. *Brain Res* **147**: 131–138.

Vistica DT, Ahrens FA, Ellison WR. (1977). The effects of lead upon collagen synthesis and proline hydroxylation in the Swiss mouse 3T6 fibroblast. *Arch Biochem Biophys* **177**: 15.

White JM, Selh HS. (1975). Lead and the red cell. *Br J Haemato*l **30**: 133.

Zylinska L, Kawecka I, Lachowicz L, Szemraj J. (2002). The isoform-and location-dependence of the functioning of the plasma membrane calcium pump. *Cell Mol Biol Lett* **7**(4): 1037–1045.

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ORIGINAL ARTICLE

Non-clinical safety evaluation of a novel pharmaceutical salt, rosuvastatin ethanolamine, in Wistar rats

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ABSTRACT

Rosuvastatin, a second generation 3-Hydroxy-3-Methyl Glutaryl Coenzyme-A reductase inhibitor, is widely used for the management of hypercholesterolemia. Rosuvastatin ethanolamine, developed by Cadila Healthcare Ltd., is a novel, chemically stable, and pharmaceutically acceptable salt, having better physiochemical properties than commercially available Rosuvastatin salt. The objective of the present study is to evaluate safety, tolerability, and toxicokinetic profile of novel salt. Therefore, four weeks repeated dose oral (gavage) toxicity and toxicokinetic study of Rosuvastatin ethanolamine was carried out. The drugs were administered once daily at salt corrected dose of 15, 40, and 100 mg/kg for four weeks. No signs of toxicity were observed during repeated (four weeks) oral administrations of Rosuvastatin ethanolamine in rats up to 40 mg/kg. Single male mortality was observed at 100 mg/kg dose. Microscopy finding in liver was minimal to mild bile ductular proliferation, single cell necrosis, and hepatocellular vacuolation of cytoplasm with associated statistically significant serum elevation of transaminase enzymes; AST, ALT, ALP, and/or liver functional marker; total bilirubin with at ≥40 mg/kg. The systemic exposures (AUC_{0-24} and C_{max}) were not markedly different between males and females, or between the administration periods (except high dose, where exposure on day 28 was approximately 2 to 3 fold higher than that of day 1. In conclusion, Rosuvastatin ethanolamine exhibited toxicities to liver as the target organ at ≥40 mg/kg in this study. These adverse effects with associated exposures should be taken into consideration for the future assessing of potential Rosuvastatin toxicities.

KEY WORDS: Rosuvastatin ethanolamine, toxicity study, toxicokinetic study, Wistar rat

INTRODUCTION

Rosuvastatin (Crestor; licensed to AstraZeneca) markedly reduces low-density lipoprotein (LDL) cholesterol levels, increases high-density lipoprotein (HDL) cholesterol levels, and improves other parameters of the atherogenic lipid profile (Olsson *et al.,* 2002). The mechanism of action of statin class drugs is to competitively inhibit 3-Hydroxy-3-Methyl Glutaryl Coenzyme-A (HMG-CoA) reductase which catalyzes the rate limiting step in cholesterol synthesis, HMG-CoA to mevalonate (Buse, 2003). This *de-novo* decreases in hepatic cholesterol synthesis leading to an up-regulation of hepatic LDL receptors with subsequent increases in LDL uptake and resulting to reduced plasma LDL levels. In addition to reducing LDL levels, statins can also decrease triglyceride (TG), perhaps, by reducing the rate of very low-density lipoprotein (VLDL) synthesis and increasing its clearance (Buse, 2003). Intensive lipid-lowering therapy with rosuvastatin 40 mg per day provides greater LDL lowering efficacy than atorvastatin 80 mg per day, enabling more patients to achieve goal LDL level. Therefore, Rosuvastatin may improve achievement of goal LDL level in high-risk patients with hypercholesterolemia (Leiter *et al.,* 2007).

Rosuvastatin ethanolamine, developed by Cadila Healthcare Ltd., is a novel, chemically stable, and pharmaceutically acceptable salt of rosuvastatin. Rosuvastatin ethanolamine reveals better purity and physiochemical properties like melting point, solubility, and improved

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stability under various stress conditions than currently marketed rosuvastatin salt (Patent number – WO 2012/073256 A1, Cadila Healthcare Ltd., 2012). As it is quite imperative to evaluate safety of novel salt, repeated dose toxicity and toxicokinetics study of rosuvastatin ethanolamine (test article) was evaluated in Wistar rats at salt corrected equivalent dose of 15, 40, and 100 mg/kg. Findings from this study provide insights into the design and interpretation of data derived for toxicology studies with rosuvastatin.

Materials and methods

Drugs

Rosuvastatin ethanolamine was supplied by Cadila Healthcare Ltd., India. Dose formulations were prepared at salt corrected equivalent rosuvastatin concentrations of 1.5, 4.0, and 10.0 mg/ml in the vehicle composition of 5.0% polyethylene glycol 400 (PEG 400)/ 5.0% polyoxyethylene sorbitanmonooleate (Tween™ 80)/ 90.0% of 0.5 % (w/v) methylcellulose in reverse osmosis-treated water, which was dispensed into amber glass bottles. Dose formulations were prepared freshly on each day prior to the dosing.

Experimental animals and housing conditions

The study was designed to use minimum number of animals to meet scientific objectives, goals, and considerations of applicable regulatory requirements. Healthy young adult Wistar rats (age ~7 weeks; body weight range ~170–230 g for male and 120–165 g for female on day of receipt) were obtained from Animal Research Facility of Zydus Research Centre and were acclimatized for a minimum period of seven days. Animals were housed in individually ventilated cages in environmentally controlled rooms (temperature of 18–26 °C; relative humidity of 30–70%; 12 h light/dark cycle) with feed and water provided *ad libitum*. The experimental animals were provided with UV treated Teklad global diet supplied by Harlan Laboratory, USA and filtered drinking water (Reverse osmosis water filter system followed by UV treatment). Proximate analysis of nutrient content and microbial contaminant of feed was analyzed batch wise. Quality of water was periodically checked to ensure acceptable limits of total dissolved solute and microbial contamination. On the first day of dosing, rats were approximately 8 weeks of age; males weighed ~200–250 g and females weighed ~140–180 g. Animal used was in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals and protocol was approved by the Institutional Animal Ethics Committee. The study was conducted in AAALAC (Association for Accreditation and Assessment of Laboratory Animal Care) accredited facility.

Experimental design

The animals were randomized into different study groups (10/group/sex) to maintain the parity in different groups as per body weight. The difference between and within the groups was not more than 20% of mean body weight. The animals from the respective groups were administered for four weeks by oral gavage using a dose volume of 10 ml/kg with rosuvastatin ethanolamine at 15, 40, 100 mg/kg (test article). The animals from the control group (vehicle control) were administered with vehicle alone. An additional 6 animals/sex/group were added to each dose group for toxicokinetic evaluation.

In-life observations

Detailed clinical and mortality observations were performed daily on all animals throughout the treatment phase. Body weights were recorded during acclimatization, on first day of dosing as well as every week thereafter during the treatment phase. Food consumption was measured weekly during the treatment phase to coincide with body weight measuring.

Toxicokinetics

Toxicokinetic evaluation was carried out on day 1 and day 28 after treatment for evaluating plasma drug concentration. A serial blood collection was carried out at different time intervals such as pre-dose, 15 min, 30 min, 1, 2, 4, 8, 12, 18, and 24 h post dosing. The vehicle control group's animals were bled only for 2 time points (pre-dose and 1 h post dose). The blood samples were collected in saline diluted sodium heparin and placed on wet ice bath, followed by centrifugation (3 000 rpm, 15 min) in cold condition to obtain plasma. The plasma samples were stored frozen at –75±10 °C until analysis.

Bioanalysis

The estimation of rosuvastatin in the plasma samples were employed using a high performance liquid chromatography coupled with tandem mass spectrometry with turbo ion source (LC-MS/MS). The analytical method was validated in accordance to in-house procedure and guidance for industry on bioanalytical method validation (Available from: https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf). The alprazolam was used as an internal standard (0.1 µg/ml). The chromatographic separation of rosuvastatin and internal standard from endogenous matrix was carried on analytical column ACE 100, C18 50×4.6 mm, 5 µ (ACE, ML9 2QS, Scotland) using gradient elution with flow rate of 1.0 ml/min. The mobile phase was solvent mixture of; (A) 100 mg ammonium acetate in 1 l purified water and 100 µl TFA, (B) acetonitrile 100% v/v. The purification of plasma samples for analyte and IS was achieved using the protein precipitation extraction with methanol, followed by 5 µl injection of clear supernatant for analysis. The quantitative measurement was performed in multiple reaction monitoring (MRM) in positive ion mode with mass transition pair of m/z 482.1–258.1 for rosuvastatin and m/z 309.1–281.0 for internal standard. The calibration standard curve was linear over 1 to 1000 ng/ml with limit of quantitation (LOQ) 1.0 ng/ml. During the analysis of plasma samples, a quality control (QC) samples at low, medium, and high

levels were analyzed and distributed across the samples. The inter-run back calculated results of the QC samples indicated accuracy 100.45%, 99.52%, and 101.00% and precisions (%CV) 10.31%, 5.24%, and 5.50% at low, medium, and high QC levels, respectively. The unknown sample concentrations below the LOQ were set to 'zero' for evaluation of toxicokinetic parameters.

Ophthalmic examination

Ophthalmic examination was performed by a veterinarian prior to the initiation of dosing and during fourth week of the treatment phase by using an indirect ophthalmoscope and a slit lamp. Prior to examination, a mydriatic agent (Tropicamide ophthalmic solution 1%, Sunways Ltd.) was instilled into each eye.

Clinical pathology

Detailed clinical pathology investigations were carried out for all the animals immediately before scheduled necropsy, except animals which died during the treatment phase. All animals were fasted overnight (water allowed) before the blood collection. Blood samples were drawn from the retro-orbital plexus under a mild anaesthetic condition (Isoflurane). Following blood collection, samples were immediately placed on wet ice and centrifuged. Samples for hematologic analysis were collected into tubes containing plasma EDTA and the following parameters were measured: red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), platelet count (PLT), absolute reticulocyte count (RET), absolute differential leukocyte count, total leukocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Samples for coagulation were collected into sodium citrate tubes, centrifuged and the resultant plasma used to measure prothrombin time (PT) and activated partial thromboplastin time (APTT). Samples for serum clinical chemistry were collected in tubes containing no anticoagulant, centrifuged and the resultant serum was used to measure the following parameters: glucose (GLU), triglyceride (TG), total cholesterol (TCHOL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), urea nitrogen, creatinine (CRE), total bilirubin (TBIL), total protein (TP), albumin (ALB), calcium (Ca^{2+}), inorganic phosphorus (IP), sodium (Na^{+}), potassium (K^{+}), chloride (Cl^{-}), and creatinine kinase (CPK). Urine samples were collected overnight at the end of the treatment phase by placing animal individually in metabolic cages to evaluate the following parameters: volume/quantity, appearance/color, pH, glucose, blood, protein, bilirubin, urobilinogen, nitrite, ketones, specific gravity, and sediment examination.

Terminal procedures

Complete necropsies and gross pathology examinations were performed on animals that died during the treatment phase and all surviving animals at the conclusion of the study. At necropsy, major organs were evaluated for grossly visible lesions and various organs were weighed. Tissues from all major organs were fixed and preserved in 10% neutral buffered formalin, processed, trimmed, embedded in paraffin, and stained with hematoxylin and eosin for microscopic examination. The histopathological evaluation was performed by board certified toxicopathologist (Diplomat Indian Association of Toxicologic Pathologist affiliated to Society of Toxicologic Pathologist). All the tissues/organs collected from control and high dose groups were subjected initially to histopathological evaluation. The grading of the histological lesions was performed by following criteria. Severity of lesions was graded depending on the approximate percentage of tissue involved i.e. less than 20% as minimal, 21% to 50% as mild, 51% to 75% as moderate and 76% to 100% as severe. The treatment related changes observed at high doses were evaluated from next lower dose for the all respective tissue. All treatment related histopathological changes were peer reviewed by Board Certified Veterinary Pathologist (Diplomat Indian College of Veterinary Pathologist). Organs weighed included adrenals, heart, kidneys, liver, spleen, thymus, testes/ovaries with oviduct, epididymides/uterus with cervix, brain, prostate, and seminal vesicles with coagulating glands.

Statistical methods

Statistical analysis was performed using GraphPad Prism Software version 5.04 (GraphPad Software, Inc., CA, USA). Data were analyzed for dose wise comparison. Analysis of Variance (ANOVA) was used for the comparison of different dosage groups with the control group for different parameters. Comparison of dosage groups with the control group was done on the basis of individual group data. Bartlett's test for equal variances was performed for each parameter. *Post-hoc* test to analyze data after ANOVA was done using Dunnett's test. Feed consumption was analyzed using two way ANOVA procedure. Comparison was done on the basis of individual group data. All data are presented as the mean ± SD (n=10 for toxicity study group, n=6 for toxicokinetics group). TK parameters such as T_{max}, C_{max}, AUC_{0-24}, and $T_{½}$ were calculated using non-compartmental analysis model (NCA) of Win-Nonlin Software 5.3 (Pharsight, Mountain View, CA, USA).

Results

Mortality and in-life observation

Single case of male mortality was observed during the study at high dose of 100 mg/kg dose on day 14 which showed adverse signs of toxicity such as lean body condition, chromodacryorrhea, decreased motor activities, hunched back, and lethargy for few days prior to death.

There were no other adverse clinical signs noticed in the study. The weekly body weight recording revealed statistically significant and marginally lower group mean body weight (<10%) in females at 100 mg/kg dose from the

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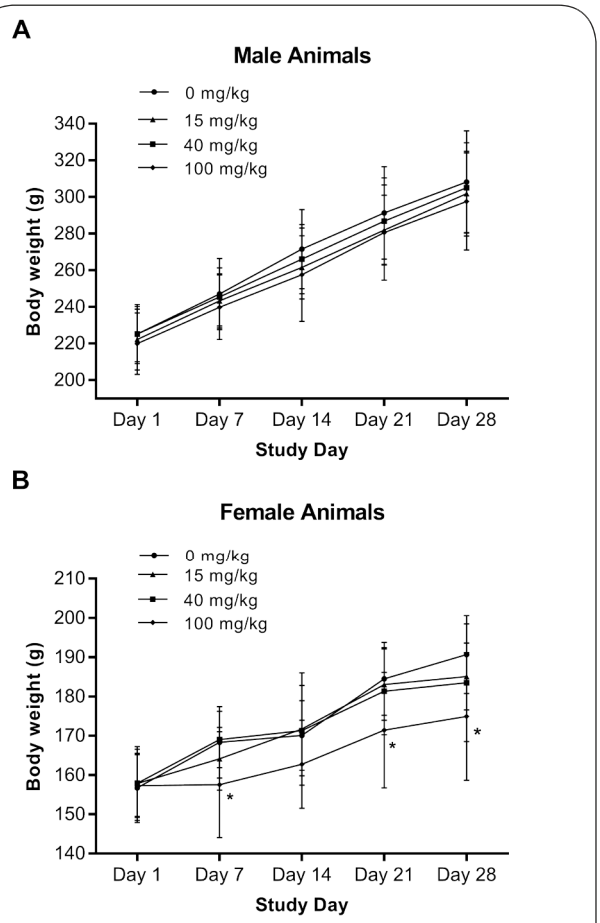

Figure 1. Body weight. Each value represent mean ± SD (n= 10), $p<0.05$ vs. vehicle control.

day 7 of treatment when compared with the concurrent control group (Figure 1B). Group mean feed consumption of each treatment groups was found to be comparable with the concurrent control group in both sexes. No drug related ophthalmic lesions were observed in all rats in any of the dose groups during the study.

Clinical pathology

Hematology

There were no drug treatment related significant changes observed in hematology and coagulation parameters in any dose group treated with rosuvastatin ethanolamine in the both sexes. The non-dose and/or sex dependent minor variations were noticed and are presented in the Table 1 & 2.

Clinical chemistry

Rosuvastatin ethanolamine treatment at 15 mg/kg dose resulted in minimal elevation of ALT (~39 %) in males, and minimal elevation of total bilirubin (~25–40%) in females. The treatment at 40 mg/kg in males resulted in a mild elevation of AST and ALT and decline in total protein albumin. The females treated at 40 mg/kg with rosuvastatin ethanolamine exhibited mild elevation of total bilirubin and albumin when compared with concurrent control group. In high dose group (100 mg/kg) of ethanolamine salt treatment showed moderate enzymatic elevation of AST, ALT, ALP, total bilirubin, and decline in total protein in males. In females, rosuvastatin ethanolamine treatment raised levels of AST, ALP, and total bilirubin as compared to control. All other statistically significant observations are presented in Tables 3 & 4.

Table 1. Group Mean Hematological Analytes (Sex: Male)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
WBC (10^3/µl)	8.4±1.7	8.6±2.7	8.8±1.0	9.9±1.8	3.7–12.3
RBC (10^6/µl)	8.3±0.2	8.3±0.4	8.2±0.6	8.5±0.4	6.6–9.1
HGB (g/dl)	14.9±0.3	14.7±0.7	14.9±1.2	15.1±0.6	12.7–16.1
HCT (%)	49.0±0.9	47.8±2.1	48.6±3.7	49.2±2.1	40.0–49.9
MCV (fL)	58.9±1.3	57.9±2.0	59.5±1.7	57.8±2.6	51.1– 63.7
MCH (pg)	17.9±0.5	17.8±0.6	18.3±0.6	17.8±0.6	16.3–20.3
MCHC (g/dl)	30.3±0.6	30.8±0.2*	30.7±0.4	30.8±0.4	30.6–33.3
PLT (10^3/µl)	582.1±58.1	613.6±45.8	563.4±29.9	602.8±72.2	508–1045
NEU (10^3/µl)	1.23±0.29	1.62±0.91	1.30±0.32	1.35±0.37	0.43–2.15
LYM (10^3/µl)	6.88±1.74	6.38±1.98	7.09±1.07	8.05±1.48	2.56–10.30
MONO (10^3/µl)	0.17±0.14	0.34±0.24	0.25±0.15	0.25±0.15	0.013–0.545
EOS (10^3/µl)	0.055±0.02	0.082±0.03	0.079±0.04	0.090±0.03*	0.029–0.232
BASO (10^3/µl)	0.095±0.04	0.133±0.07	0.108±0.05	0.092±0.05	0.029–0.265
RET (10^3/µl)	296.4±64.8	289.0±66.7	237.8±38.4	459.1±98.5*	121–622
PT (sec)	12.59±0.6	11.98±0.7	13.60±0.2**	13.04±0.9	9.5–15.4
APTT (sec)	18.0±2.2	18.1±2.6	18.1±3.9	17.2±2.5	10.9–30.0

* Significant at 5% level ($p<0.05$), ** Significant at 1% level ($p<0.01$)

Urinalysis

Treatment related changes were not observed in the parameters of urine analysis of animals treated with rosuvastatin ethanolamine. (Data not shown)

Pathology

Gross pathology examination at the completion of study did not reveal any gross lesions across all doses. The single male animal found dead on day 14 treated by high dose

Table 2. Group Mean Hematological Analytes (Sex: Female)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
WBC (10^3/µl)	5.9±1.7	4.8±1.6	5.2±1.6	5.1±1.3	2.37–9.36
RBC (10^6/µl)	7.6±0.7	7.6±0.4	7.6±0.3	8.0±0.6	6.42–8.42
HGB (g/dl)	13.9±1.3	13.9±0.4	13.9±0.6	14.4±1.0	12.8–15.2
HCT (%)	15.0±4.0	45.1±1.3	44.8±2.2	46.5±3.3	39.7–47.5
MCV (fL)	59.4±1.3	59.2±1.8	58.7±1.4	58.3±1.7	53.7–63.8
MCH (pg)	18.3±0.5	18.2±0.6	18.2±0.5	18.0±0.5	17.4–20.5
MCHC (g/dl)	30.8±0.4	30.8±0.3	31.0±0.5	30.9±0.2	30.5–33.6
PLT (10^3/µl)	602.6±75.5	610.7±63.1	606.7±75.7	602.4±80.6	545–1057
NEU (103/µl)	0.53±0.10	0.60±0.24	0.65±0.27	0.63±0.28	0.353–1.499
LYM (10^3/µl)	4.98±1.67	3.84±1.36	4.20±1.34	4.06±1.15	1.350–8.260
MONO (10^3/µl)	0.20±0.09	0.17±0.09	0.20±0.1	0.24±0.13	0.014–0.389
EOS (10^3/µl)	0.055±0.02	0.071±0.02	0.054±0.02	0.073±0.04	0.026–0.169
BASO (10^3/µl)	0.102±0.04	0.075±0.04	0.071±0.04	0.072±0.03	0.016–0.179
RET (10^3/µl)	400.0±152.1	458.2±176.3	434.9±119.5	529.2±154.8	139–936
PT (sec)	12.7±0.5	11.3±0.4	10.6±0.3	12.9±0.6	9.3–13.1
APTT (sec)	19.4±2.9	19.0±2.7	21.2±4.5	18.5±4.0	10.8–24.6

Table 3. Group Mean Clinical Chemistry Analytes (Sex: Male)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
GLU (mg/dl)	86.2±11.9	78.2±12.6	89.0±13.1	84.1±7.1	42.3–150.7
TG (mg/dl)	104.2±57.8	85.4±27.5	74.5±28.4	73.6±15.7	33.0–152.0
TCHOL (mg/dl)	52.6±6.5	62.4±14.8	62.3±6.0	64.4±10.2*	37.2–87.5
HDl (mg/dl)	21.0±2.3	21.8±4.5	22.0±2.3	23.4±2.9	12.7–33.8
LDl (mg/dl)	3.8±1.0	3.3±1.0	4.8±1.8	6.2±1.9**	1.4–7.1
AST (U/l)	94.3±5.6	118.5±17.0	127.5±34.3**	148.7±23.5**	74.6–197.0
ALT (U/l)	31.4±3.6	43.5±3.7**	46.4±14.8**	51.0±13.7**	20.6–48.8
ALP (U/l)	110.0±35.7	129.3±28.3	136.7±31.7	176.8±41.8**	53.5–246.8
TBIL (mg/dl)	0.16 ±0.03	0.16±0.02	0.19±0.02	0.23±0.04**	0.02–0.27
TP (g/dl)	6.35±0.27	6.12±0.25	6.06±0.23*	5.82±0.14**	5.3–6.9
ALB (g/dl)	3.69±0.14	3.50±0.11**	3.49±0.09**	3.59±0.08	3.2–4.0
UREA (mg/dl)	34.6±4.1	35.9±4.4	32.7±2.6	32.1±4.2	23.2–49.6
CRE (mg/dl)	0.71±0.04	0.65±0.05	0.69±0.06	0.70±0.04	0.42–0.82
CPK (U/l)	818.0±198.1	980.1±278.4	991.3±561.3	739.9±148.7	286.1–2098.8
Ca^{2+} (mg/dl)	10.3±0.3	10.1±0.3	9.8±0.3	10.2±0.2	9.0–11.0
IP (mg/dl)	6.5±0.4	5.7±0.4	6.0±0.5	6.2±0.4	4.2–8.3
Na^+ (mmol/l)	144.8±1.0	145.8±1.4	144.7±1.2	144.1±1.6	137.4–146.8
K^+ (mmol/l)	3.6±0.1	3.7±0.1	3.6±0.3	3.5±0.2	3.4–5.0
Cl^- (mmol/l)	102.7±0.9	104.6±1.1	102.7±1.0	103.1±1.5	99.7–107.6

* Significant at 5% level ($p<0.05$), ** Significant at 1% level ($p<0.01$)

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Table 4. Group Mean Clinical Chemistry Analytes (Sex: Female)

Analytes	Vehicle	Rosuvastatin Ethanolamine			Reference Values
	0 mg/kg	15 mg/kg	40 mg/kg	100 mg/kg	Lower Limit-Upper Limit
GLU (mg/dl)	66.4±17.2	89.4±10.4**	79.2±7.6	45.0±12.8**	53.3–135.2
TG (mg/dl)	33.5±10.1	42.0±13.1	32.8±7.3	25.0±7.5	22.0–81.3
TCHOL (mg/dl)	40.5±6.7	56.2±11.6**	45.9±10.6	39.7±13.5	26.0–71.9
HDl (mg/dl)	16.6±2.77	21.8±4.1*	17.9±3.4	18.1±6.1	10.1–21.7
LDl (mg/dl)	1.2±0.4	1.8±0.7	2.4±0.7**	2.0±1.3	0.50–4.40
AST (U/L)	131.0±41.1	106.5±30.1	105.4±28.5	218.2±101.2**	72.0–200.3
ALT (U/L)	27.9±4.3	28.1±4.6	27.0±5.5	35.1±11.9	16.6–38.8
ALP (U/L)	50.6±18.4	68.5±13.3	66.6±18.6	128.9±62.3**	19.1–162.7
TBIL (mg/dl)	0.16±0.02	0.20±0.03*	0.23±0.02**	0.29±0.06**	0.08–0.31
TP (g/dl)	6.4±0.4	6.5±0.5	6.6±0.2	6.2±0.3	5.4–7.3
ALB (g/dl)	3.73±0.20	3.84±0.25	3.95±0.12*	3.69±0.14	3.4–4.4
UREA (mg/dl)	38.7±4.3	37.7±3.9	36.3±2.7	37.0±4.5	31.5–61.4
CRE (mg/dl)	0.70±0.04	0.73±0.05	0.71±0.07	0.67±0.07	0.51–0.89
CPK (U/l)	1609.1±838.8	1065.4±918.7	808.9±517.9*	1272.6±188.9	520.6–1976.8
Ca^{2+} (mg/dl)	10.2±0.3	10.2±0.4	10.4±0.3	10.1±0.3	8.84–11.20
IP (mg/dl)	4.3±0.8	4.5±0.6	5.0±0.5*	5.2±0.7**	3.10–7.20
Na^{+} (mmol/l)	145.5±1.6	145.5±1.1	145.3±0.9	143.4±1.5**	137.4–146.4
K^{+} (mmol/l)	3.4±0.2	3.6±0.4	3.6±0.2	3.5±0.2	3.03–4.64
Cl^{-} (mmol/l)	104.6±1.3	104.8±1.1	104.8±1.0	103.6±0.9	101.1–108.2

* Significant at 5% level ($p<0.05$), ** Significant at 1% level ($p<0.01$)

Table 5. Microscopic Examination

Dose (mg/kg)		Vehicle		Rosuvastatin Ethanolamine					
		0		15		40		100	
Sex		M	F	M	F	M	F	M	F
Unscheduled Deaths (Number of Animals)		0	0	0	0	0	0	1	0
Liver									
Single cell necrosis	Minimal	0	0	0	0	3	5	2	5
	Mild	0	0	0	0	1	0	5	2
Bile ductular proliferation	Minimal	0	0	0	0	0	0	7	7
	Mild	0	0	0	0	0	0	0	0
Vacuolated cytoplasm	Minimal	0	0	2	2	4	6	4	3
	Mild	0	0	0	0	1	0	3	4
Thymus									
Atrophy and lymphoid depletion	Mild	0	0	0	0	0	0	1	0
	Moderate	0	0	0	0	0	0	0	0

showed, histopathologically, mild single cell necrosis, minimal bile ductular proliferation and cytoplasmic vacuolation of liver, mild atrophy and lymphoid depletion in thymus, mild single cell necrosis and vacuolation in pancreas, and minimal inflammatory cells and hyperkeratosis in the fore stomach.

Absolute and relative organ weight estimation revealed no treatment related adverse changes in animals treated with test and reference substance in both sexes (data not presented). The minor variations of organ weights such as higher weight of spleen, seminal vesicles with coagulating glands at 40 mg/kg of rosuvastatin ethanolamine, lower adrenals weight at 100 mg/kg of rosuvastatin ethanolamine in females were neither dose dependent nor of any pathological significance. Histopathological examination did not reveal any adverse changes in any of the major organs examined in this study except for treatment related adverse effects in liver at ≥40 mg/kg in the both sexes of rosuvastatin ethanolamine treatment groups (Table 5). Treatment and dose-related adverse effects noticed in liver were: minimal to mild bile ductular proliferation, single cell necrosis, and hepatocellular vacuolation of cytoplasm (Figures 2, C & D). The dose related relationship was clearly observed during the histopathology examination of liver, especially in terms of increased observation of sever findings. Incidence of stress related changes was noticed, such as minimal atrophy and lymphoid depletion of thymus in single male at 100 mg/kg dose.

Toxicokinetics

Mean plasma toxicokinetic parameters following oral administration of rosuvastatin ethanolamine in male and female rats are presented in Table 6. In the both males and females, the mean systemic exposure (C_{max} and AUC_{0-24})

Table 6. Toxicokinetic Parameters

Sex	Dose (mg/kg)	Day	T_{max} (h)	C_{max} (µg/ml)	$T_{1/2}$ (h)	AUC_{0-24} (h.µg/ml)	Fold increased exposure of low dose
Male	15	1 28	2.46±4.68 0.71±0.70	0.15±0.12 0.11±0.02	8.37±4.39 8.44±2.27	0.39±0.09 0.66±0.18	–
	40 (2.67 fold of low dose)	1 28	0.50±0.00 2.42±4.70	0.97±1.13 0.77±0.61	5.41±3.00 5.17±2.36	1.47±0.95 2.01±0.59	3.77 3.05
	100 (6.67 fold of low dose)	1 28	0.67±0.38 0.38±0.14	11.63±13.69 21.62±13.93	4.98±1.53 5.12±1.89	17.80±13.69 30.12±18.93	45.64 45.63
Female	15	1 28	0.50±0.27 0.29±0.10	0.12±0.04 0.21±0.10	7.56±3.51 3.90±0.48	0.37±0.13 0.73±0.09	–
	40 (2.67 fold of low dose)	1 28	0.33±0.13 0.46± 0.10	0.85±0.40 2.18±1.27	7.67±5.46 4.65±1.71	2.07±1.14 2.95±1.49	5.59 4.04
	100 (6.67 fold of low dose)	1 28	0.30±0.11 0.25±0.00	9.08±4.65 49.86±28.20	5.12±1.86 4.17±1.83	9.81±4.53 32.13±10.24	26.51 44.01

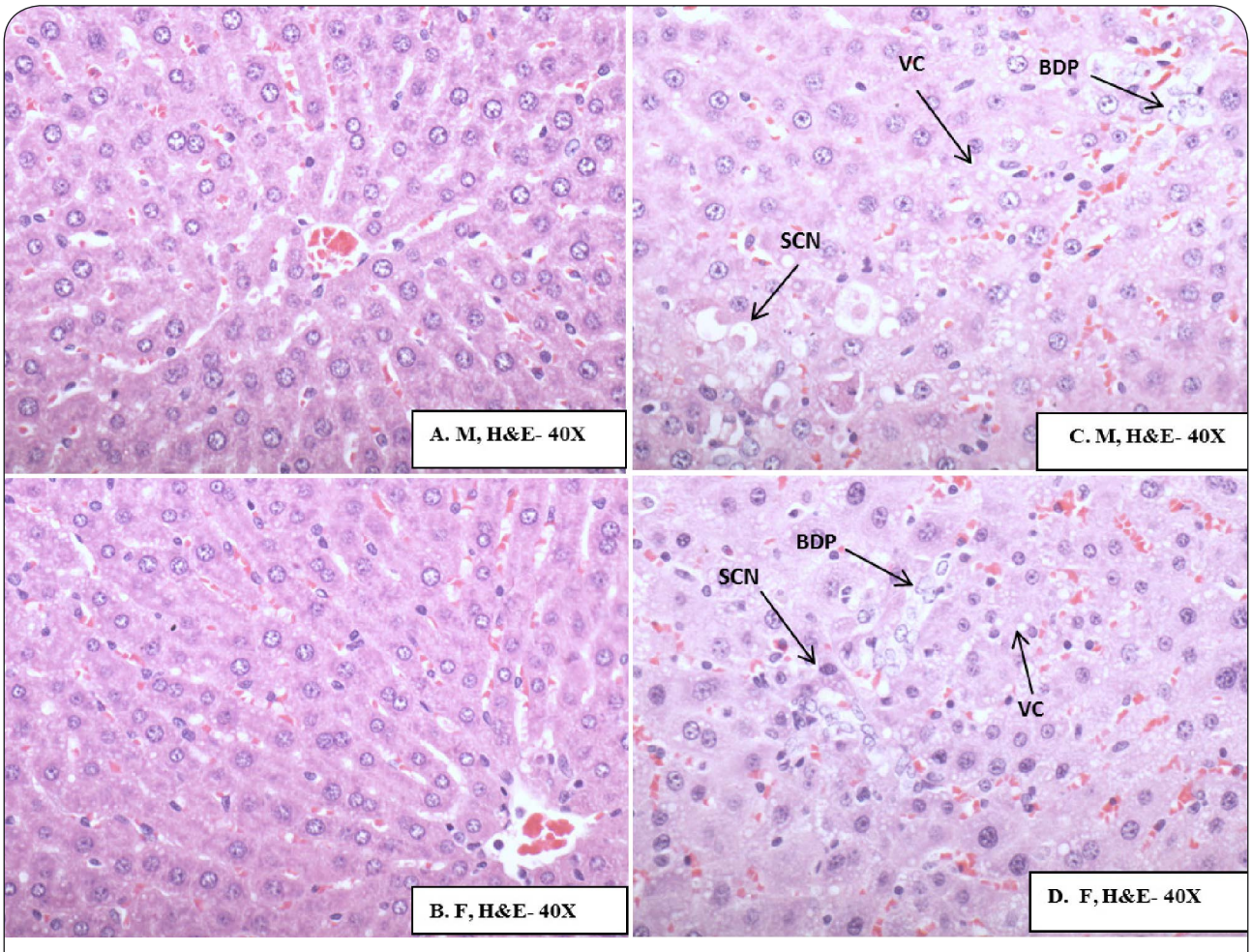

Figure 2. Adverse effects of Rosurvastatin ethanolamie in liver. A & B: control: normal histological appearance of liver; C & D: Rosrvastatin ethanolamine, 100 mg/kg. SCN – single cell necrosis; VC – vacuolated cytoplasm; BDP – bile duct proliferation; M – male; F – female.

to rosuvastatin following oral administration of ethanolamine salt was found to be more than a multiple of dose in both sexes on day 1 and 28 of treatment period. Significant gender specific differences were not noticed in this study. On day 28, the plasma exposure was higher by 2 and 3 folds approximately from that of day 1 in male and female rats respectively at 100 mg/kg. The median T_{max} ranged from 0.25 to 2.46 h following oral gavage administration of rosuvastatin ethanolamine with no apparent changes due to sex, day or dose level (Table 6). A second absorption peak was observed at about 8–12 h post dosing, which evident enterohepatic re-circulation of drug.

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Discussion

We conducted toxicity and toxicokinetic studies in Wistar rats to evaluate safety, tolerability, and toxicokinetic of novel compound, rosuvastatin ethanolamine. The toxicity test was conducted over four weeks using oral route of administration, which is the intended route of administration in humans. The toxicokinetic test was done separately from the toxicity test. In this four week dose repeated oral toxicity study, the study results were comparable between male and female rats.

The single male animal at high dose of 100 mg/kg showed overt signs of toxicity such as lean body condition, chromodacryorrhea, decreased motor activities, hunched back, and lethargy, which eventually resulted into the death. The animals found dead exhibited single cell necrosis, bile ductular proliferation & cytoplasmic vacuolation of liver, atrophy and lymphoid depletion in thymus, single cell necrosis and vacuolation in pancreas, and inflammatory cells and hyperkeratosis in the fore stomach. The similar types of lesions were reported with the previously conducted study (Pharmacology review(s) of rosuvastatin calcium, available from: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2003/21-366_Crestor.cfm). Hence, the cause of death of this animal could be attributed to the rosuvastatin treatment.

Rosuvastatin intends to reduce LDL-cholesterol and increase HDL-cholesterol levels by competitively inhibiting (HMG-CoA) reductase which catalyzes the rate limiting step in cholesterol synthesis, HMG-CoA to mevalonate, in hyperlipidemic conditions (Buse, 2003). But this action could not reflect in the clinical chemistry results because this study was carried out in the healthy test system having the normal levels of cholesterols.

Post-treatment clinical chemistry estimation revealed dose dependent enzymatic elevation of AST, ALT, ALP, and/or liver functional marker (total bilirubin) in the both sexes treated with rosuvastatin ethanolamine at doses from 40 mg/kg. Dose dependent findings of microscopic liver changes of the males and females, which included minimal to mild bile ductular proliferation, single cell necrosis, and hepatocellular vacuolation of cytoplasm correlated with the changes of clinical chemistry analytes. Elevated serum levels of transaminase enzyme and liver function marker (total bilirubin) along with liver histopathology are the standard biomarkers for the assessment of liver toxicity (Marrer & Dieterle 2010). Therefore based on the observed histopathological changes in liver and related clinical chemistry changes, liver was considered as target organ of toxicity for the rosuvastatin. This observed toxicity in liver is also a well-known safety concern with the statin therapy clinically (Famularo *et al.* 2007). Non-clinical toxicity for liver in rats treated with statin class of drugs was clearly categorized as exaggerated biochemical effect of HMG-CoA reductase inhibitors (Macdonald & Halleck 2004). The most serious adverse effects of statins are related to muscle toxicity (Pasternak *et al.* 2002 and Antons *et al.* 2006), contrastingly, in this study we did not observe any sign of muscle toxicity markers during either microscopic examination or serum creatinine kinase estimation. We concluded that this might be either due to short duration of treatment period or because the studied highest dose level was not sufficient to exhibit the muscle toxicity in rats under this experimental condition.

In conclusion, we investigated the standard parameters for characterizing the general toxicity and toxicokinetic profile of rosuvastatin ethanolamine. The novel salt, rosuvastatin ethanolamine, targeted liver as the main organ of its toxicity at ≥40 mg/kg in Wistar rats which was considered to be exaggerated biochemical effect of statin class of drugs.

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REFERENCES

Antons KA, Williams CD, Baker SK, Phillips PS. (2006). Clinical perspectives of statin-induced rhabdomyolysis. *Am J Med* **119**(5): 400–409.

Buse J. (2003). Statin Treatment in Diabetes Mellitus. *Clin Diabetes* **21**: 168–72.

Cadila Healthcare Ltd. (2012). Salts of Rosuvastatin. International patent application WO 2012/073256 A1. 2012.06.07.

Famularo G, Miele L, Minisola G, Grieco A. (2007). Liver toxicity of rosuvastatin therapy. *World J Gastroenterol* **13**(8): 1286–1288.

Guidance for Industry; Bioanalytical Method Validation, May 2001. U.S. Food and Drug Administration. Available from: https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf [Access 13 November 2017]

Leiter LA, Rosenson RS, Stein E, Reckless JPD, Schulte KL, Schleman M, Miller P, Palmer M, Sosef F. (2007). Efficacy and safety of rosuvastatin 40mg versus atorvastatin 80 mg in high-risk patients with hypercholesterolemia: Results of the POLARIS study. *Atherosclerosis* **194**(2): e154–e164.

Macdonald JS, Halleck MM. (2004). The Toxicology of HMG–CoA Reductase Inhibitors: Prediction of Human Risk. *Toxicol Pathol* **32**(S2): 26–41.

Marrer E, Dieterle F. (2010). Impact of biomarker development on drug safety assessment. *Toxicol Appl Pharmacol* **243**: 167–179.

Olsson AG, McTaggart F, Raza A. (2002). Rosuvastatin: a highly effective new HMG-CoA reductase inhibitor. *Cardiovasc Drug Rev* **20**(4): 303–328.

Pasternak RC, Smith SC, Bairey-Merz, CN, Grundy SM, Cleeman JI, Lenfant C. (2002). ACC/AHA/NHLBI clinical advisory on the use and safety of statins. *Stroke* **33**: 2337–2341.

Pharmacology review(s) of rosuvastatin calcium. U.S. Food and Drug Administration.available from: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2003/21-366_Crestor.cfm [Accesses 21 June 2017].

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ORIGINAL ARTICLE

Evaluating the perception of farmers towards pesticides and the health effect of pesticides: A cross-sectional study in the oil palm plantations of Papar, Malaysia

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ABSTRACT

Agriculture is an important occupation in Malaysia that generates a major portion of the national revenue. Similar to the rest of the world, pesticides are used to boost agricultural production in Malaysian farms. However, chemical pesticides are associated with human health hazard and are not environment-friendly as they persist in nature for long periods of time. Therefore, pesticide use should be reduced and farmers should be trained on correct and/or alternative ways of pesticide use. In this cross-sectional study, we surveyed 19 palm oil plantations in the Sabah district of Malaysia and evaluated the perception of the workers towards pesticide use and awareness regarding the health effects post-pesticide exposure. Analysis of the survey shows that most of the workers among the 270 respondents were 30-year-old males with average education, and belonged to the low income group. Majority opined that they were aware of the health hazards of pesticide use and suffered from symptoms (with mean duration of three days) such as vomiting, diarrhea, skin irritation, and dizziness. Surprisingly, the opinion was almost equally divided on whether they perceived pesticides to be the cause of their health problems, and a major percentage did not avail medical help. Most of the workers responded that they did not receive any training in pesticide handling and used partial personal protective equipment (glasses, hats, shirt, and gloves) during working hours. Interestingly, a large percentage responded that they would not read the safety material even if it was provided. These observations clearly highlight the urgency of improving the awareness, education, and attitude of these plantation workers towards the short- and long-term effects of pesticide use. They should also be educated about alternative and eco-friendly ways of farming. Finally, the plantation management should intervene and proactively advocate the use of safe farming practices.

KEY WORDS: chemical pesticides; human health hazard; neurobehavioral alterations; plantation management; Malaysia

Introduction

Agriculture is one of the largest sources of livelihood worldwide. Owing to population explosion and the demand for high quality and cosmetically superior food products, recent years have seen an increase in the use of pesticides in the agriculture sector. Pesticides are chemicals used for controlling pests and weeds, and include herbicides, insecticide, fungicide, antimicrobials, rodent repellants, *etc.* (US Environmental, 2007; Randall *et al.*, 2013; Hillocks, 2012). Most pesticides are used as plant protection agents that protect plants/crops from weeds, fungi, and insects. However, pesticides adversely affect human health, and cause acute and delayed symptoms in exposed people (Hillocks, 2012). Although people are aware of the short-term effect of these pesticides, knowledge regarding its long-term effects is limited. Praneet Vatakul *et al.* (2013) showed that regular exposure to pesticides can cause serious health issues such as asthma, sperm count reduction, decline in sperm quality, psoriasis, and dermatitis. A systematic review showed that non-Hodgkin lymphoma and leukemia are positively associated with pesticide exposure and suggested that cosmetic use of pesticides should be decreased (Bassil *et al.*, 2007). A growing body of evidence indicates associations between organophosphate insecticide exposure

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and neurobehavioral alterations (Jurewicz & Hanke, 2008; Weselak *et al.*, 2007; Wigle *et al.*, 2008; Mink *et al.*, 2011). Limited evidence also exist for other negative outcomes from pesticide exposure including neurological defects, birth defects and fetal death (Sanborn *et al.*, 2007). The Food and Agriculture Organization of the United Nations (FAO, 2016) has attempted to control the use of pesticides with its code of conduct by promoting pesticides that require little personal protective equipment. Moreover, the World Health Organization (WHO) has formulated a Recommended Classification of Pesticides by Hazard by categorizing pesticides according to their health hazard, ranging from "extremely hazardous" to "unlikely to present acute hazard". This is a very useful tool, especially for developing countries, for eliminating extremely toxic pesticides (WHO, 2016).

Pesticides have seen tremulous times in Malaysia. Since organic produce is being promoted globally, pesticide usage is gradually being reduced. The Malaysian government banned the use of Paraquatin 2002, which affected the entire agricultural industry, including Syngenta Malaysia Limited, the largest producer of Paraquat. However, the ban was lifted by the government in 2006 to facilitate an in-depth study on the multiple uses of Paraquat (Watts, 2011). In a detailed survey, Srinivasan (2003) observed that Malaysian farmers are against the ban as they believed that these chemicals are essential for quality farming. The study showed that the farmers were aware of the health and environmental hazards caused by pesticide, in particular, poisonous pesticides like Paraquat and agreed that safe practices should be adopted for pesticide use. However, they believed that an alternative to pesticides should be developed before final banning of such products (Srinivasan, 2003).

The Department of Occupational Safety and Health (DOSH) under the Ministry of Human Resources of Malaysia had recorded 2,648 cases of occupational poisoning and disease in the year 2014, whereas the number stood at 2,588 in 2013, which indicates a rising trend of occupational diseases due to poor working conditions and lack of proper control measures in Malaysia. However, most of the workers are unaware or negligent about the adverse health effects of prolonged pesticide exposure.

Thus, the aim of the study is to understand the perception of farmers towards pesticide use and evaluate the effect of pesticides on worker health. The study will also discuss the clinical issues associated with incorrect pesticide use and investigate whether farmers are aware about these effects. We focused on the palm oil plantations of Papar in the Sabah district of Malaysia, as palm oil is one of the most important agricultural products that have contributed to the economic development of the country. Use of pesticides such as Paraquat in palm oil plantations has eliminated the practice of manual weeding and enabled farmers to focus more on other aspects of farming and marketing. Thus, the response and attitude of palm oil plantation workers and owners is critical for understanding the trend in pesticide use and awareness in Malaysia.

Methods

Study design

This is a cross-sectional study covering 19 palm oil plantations in Papar, Sabah district of Malaysia, aimed at understanding the pesticide awareness of the plantation workers and its relationship with various demographic, educational, and socio-economic factors.

Study population

The workers in the 19 palm oil plantations were the target of this survey. In total, 950 workers (50 in each plantation) were targeted for the survey.

Inclusion and exclusion criteria

Workers who directly worked with pesticides for minimum six months irrespective of gender and age in any plantation in Papar were included in the study. Workers who were not exposed to pesticide or exposed for less than six months and administrative workers were excluded from the survey.

Sampling method

The random sampling method was adopted for collecting data from the respondents.

Sample size collection

The following formula was used to calculate the sample size:

$$n \geq (Z21-\alpha/2xp(1-p))/d2$$

where $\alpha = 0.05$ considering 95% confidence level, p is the estimated proportion, and d is the estimated error (0.05 in this case). The prevalence rate is considered as 0.80.

Considering that the total number of workers in 19 plantations is 950 (average 50 workers per plantation), the number of respondents for the sample size was 246. After adding the 10% rate for non-response to the survey questionnaire, the final sample size was 270.

Research data collection methods

We used a survey questionnaire for collecting data from the workers, whereas direct recorded interviews were used for obtaining the views of the plantation managers. The questionnaire was individually administered to the respondents, and was read out in cases where the respondent was not educated enough to complete the survey without assistance.

The survey questionnaire had five parts, namely, demographic profile of the workers, methods of applying pesticides, use of safety measures while applying pesticides, health profile, and perception about the environmental effects of pesticide usage. The questionnaire was translated into Bahasa Melayu by an expert translator and reversely translated to English after completion of the survey. Afterwards, the questionnaires were pre-tested with 40 respondents, where they were asked whether they understood the appropriateness of questions and their relevance to the community; changes to answers, if any,

were incorporated in cases of misunderstanding. The corrected and validated questionnaire was used in the study.

Data collection

Four teams of five members each collected the data. All the teams adhered to a standard method of data collection, and they were also briefed on ethical and soft skill methods to smoothly expedite the process.

Data analysis

Data was analyzed using Microsoft Excel and the SPSS software.

Ethical issues

The respondents were informed about the aim of the research. The information was kept confidential and the respondents were never pressurized into participating in the survey or expressing desired views. No monetary or non-monetary incentives were provided to the respondents. All respondents provided informed consent for their participation. All the plantation managers/owners were personally met and verbal consent was obtained before starting the data collection.

Results

Socio-demographic factors

Age and gender: The respondents belonged to different age groups (15–55 years), with 21–35 year-old workers being mainly represented in the survey. The mean age of the respondents was 30 years (Figure 1A). Gender wise, male respondents were in majority (80%) (Figure 1B).

Economic status: The income group statistics presented a mix of all income groups. Some workers owned the farm and hence had higher income, although 82.6% workers belonged to the low-income group, with the income below 3000 Rm (Figure 2A).

Education: Approximately 23% respondents were illiterate, 50.4% could read and write (basic primary school education), and only 26.6% had high school education (Figure 2B).

Pesticide-associated factors

Exposure time: The respondents were exposed to pesticides for a minimum of seven months and maximum of 40 months, with 18.9 months of mean duration of pesticide exposure (Table 1).

Mixing of pesticide brands: All the respondents agreed to mixing different brands of pesticide (Table 1). Paraquat was majorly used by most of the farmers.

Frequency of pesticide use: Majority (97.8%) of the respondents agreed that the usage of pesticide has increase over time (Figure 3A). The main reasons were: (a) they were told to do so by the manager or pesticide supplier (29.6%) and (b) everybody else had increased (25.6%) pesticide usage (colleague or another plantation) (Figure 3B).

Risk awareness of pesticide exposure: Surprisingly, 94.1% respondents agreed that they were aware of the risks and toxic side-effects of pesticide usage (Table 1).

Alternatives to pesticides: Awareness about the following alternatives to pesticide use was probed: decreased dosage of existing pesticides, decreased application of pesticides and change to less toxic pesticides, manual clearing, use of light traps and crop rotation, variation in sowing and harvesting time, cultivation of "enemy" plants, and use of biological methods of pest control. Among these, the workers preferred the use of manual clearing (74.8%), crop rotation (62.6%), and light traps (69.6%), whereas the percentage of workers who preferred using the other alternatives was low (Figure 4).

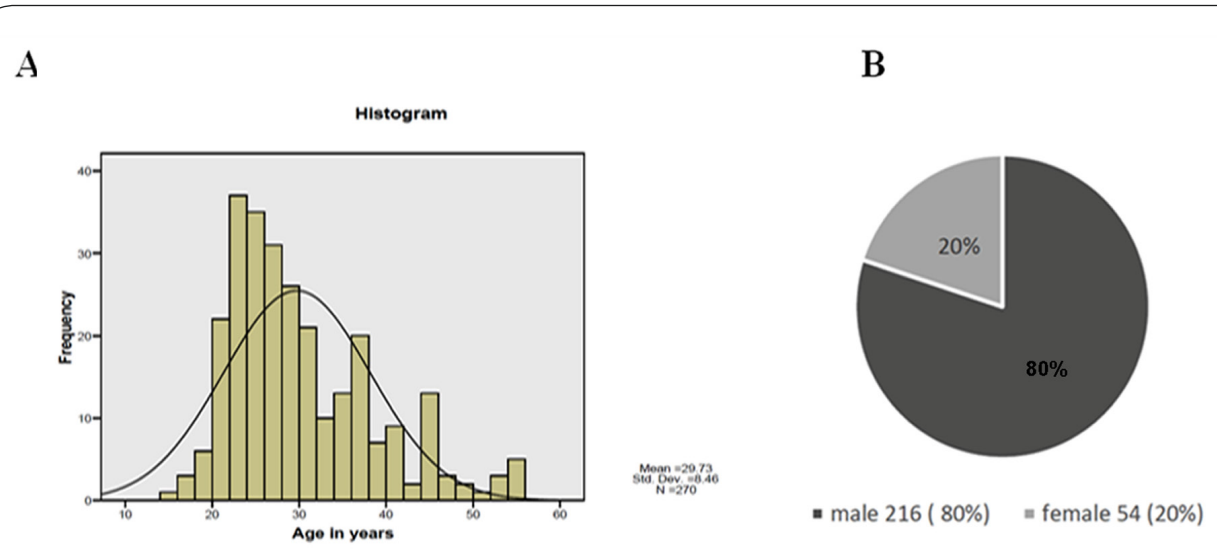

Figure 1. (A) Age distribution and **(B)** gender distribution of palm oil workers in Papar, Sabah, Malaysia.

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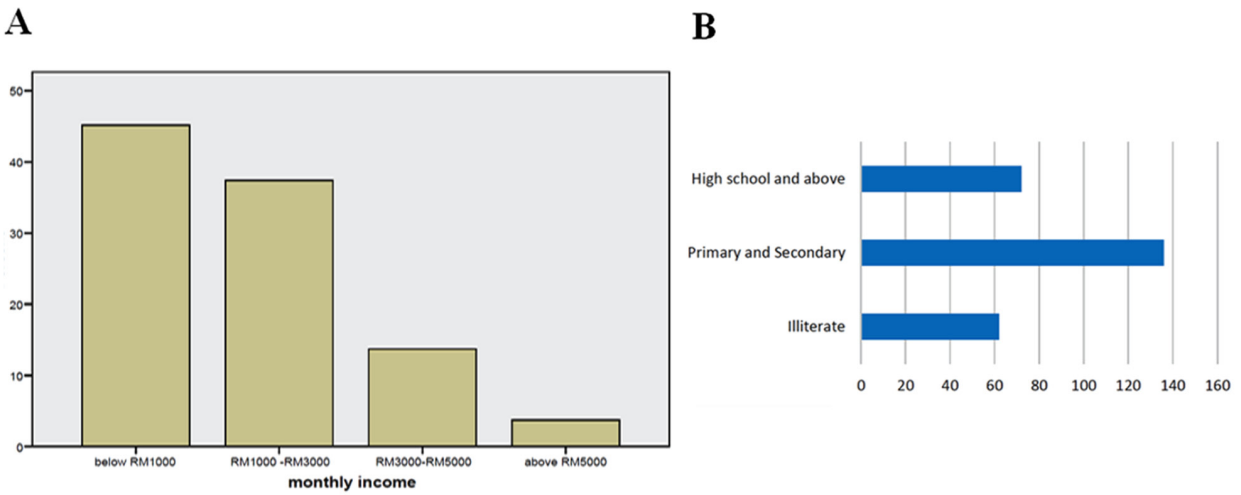

Figure 2. (A) Income distribution and **(B)** education level of palm oil workers in Papar, Sabah, Malaysia.

Table 1. Questionnaire and socio-demographic data.

S No.	Title	Question	Response	
01	Pesticide application duration.	Duration of applying pesticide?	Minimum 07 months	
			Maximum 40 months	
			Mean	18.9 months
			Standard Deviation	9.7 months
02	Assessment of mixed pesticide usage from the survey questionnaire.	Mix different brands of pesticide?	Yes	270
			No	0
03	Perception of pesticide exposure risk awareness from survey questionnaire.	How much risk do you think you are exposed to while using the pesticides?	No risk at all	16
			There is risk	254
			Total	270
04	Duration of medical symptoms after pesticide exposure.	After how many days of pesticide exposure you have developed the symptoms?	Mean	3days
			Standard Deviation	±0.9
05	Perception on effects of pesticide use.	Can this usage of pesticide cause any kind of long term or short term health effect?	No effect	38 (14.1%)
			I don't know	13 (4.8%)
			There is effect	219 (81.1%)
06	Turn-around time for entering field.	How long duration after applying pesticide do you re-enter the field?	Range	12–16 hours
			Mean	14.8
			Standard Deviation	±1.272
07	Assessment of skin contact with pesticide during pesticide application.	During mixing the pesticide does any part of your body come in contact with the liquid?	Yes	188
			No	70
08	Perception on effect of pesticides on the environment	Did you observed any dead animals, frogs, birds, and insects in or around field?	Yes	178 (64.8%)
			No	92 (35.2%)

Health effects of pesticides

All the workers mentioned suffering from at least one type of ailment during the course of pesticide usage. Skin irritation (74.4%), vomiting (90%), diarrhea (97.4%), head ache (66.7%), and dizziness (80.7%) were the most common symptoms, which mostly lasted for three days (± 0.9) (Figure 5A, Table 1). However, opinion regarding the involvement of pesticides in the emergence of these symptoms was divided; 55% of the respondents opined that they were sure that the pesticides were responsible for their ailments, whereas 45% were not sure (Figure 5B). Among these, only 48.5% respondents said that they visited the doctor when ill, which indicated that either they did not have access to basic health care or were averse to availing medical treatment for their condition (Figure 5C).

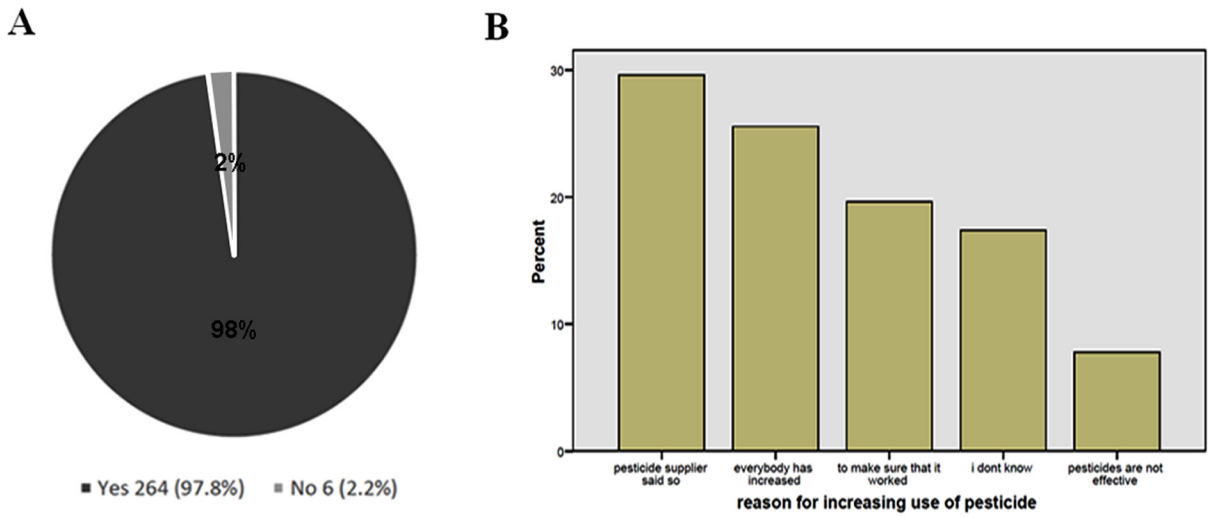

Figure 3. Frequency of pesticide use.

Perception on effects of pesticide use

When asked whether pesticide use can cause long- or short-term effects, most workers (81.1%) agreed that pesticides exerted harmful effects, whereas 14.1% answered by negative and 4.8% were unsure about the health effects of pesticides (Table 1).

Pesticide handling

Training and attitude: When asked whether they were provided with pesticide handling training, 76.7% respondents said that they did not receive any training prior to pesticide application (Figure 6A) and 68.1% claimed that they were not provided with CSDS or equivalent safety documentation (Figure 6B). The questionnaire further inquired whether the respondents would read the CSDS sheet if it was provided (Figure 6C). Surprisingly 73% claimed that they would not, which shows that attitude also plays a role in safe handling of pesticides.

Use of personal protective equipment (PPE): The respondents were asked whether they used safety boots, hats, gloves, shirts, and eyeglasses while working with pesticides. Unfortunately, most respondents wore partial PPE as some PPE items were deemed unnecessary or as sources of uneasiness. Eyeglasses were the least favorite PPE (73.7% did not wear protective glasses), followed by gloves (44.8%) and hat (34.1%) (Figure 7A). A large majority (69.6%) also claimed that the pesticides came in contact with their skin while mixing (Table 1).

Hygienic practices after pesticide use: We observed that 51.5% did not bathe (Figure 7B) and 41.4% did not change clothes after using pesticides (Figure 7C), highlighting that awareness about persistence of pesticide residues on the skin was moderate.

Turn-around time for re-entering the field: The turn-around time for re-entering the field ranged between 12–16 h, with a mean of 14.8 h (Table 1).

Compliance with standard application procedure: When asked if the workers mixed pesticides with a stick

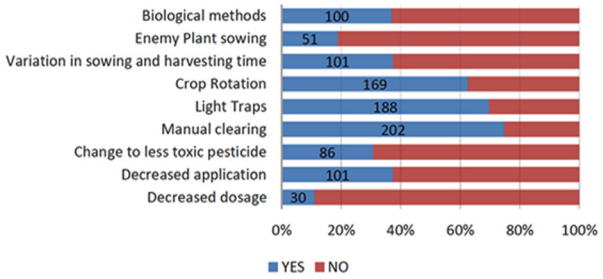

Figure 4. Alternative pest control methods.

while using full PPE (hand gloves and eye shield) 62.2% responded that they do not comply with this method. Next, they were asked whether they did not place their mouths on the spray nozzle while cleaning, and surprisingly, 62.2% claim they do not comply with this rule. Similarly, 66.7% opined that they did not comply with the "no smoking at work" rule, and 41.5% did not obey the "do not spray against the wind" rule (Figure 8).

Perception on effect of pesticides on the environment

The last part of the questionnaire probed awareness regarding the effect of pesticides on the environment. Respondents were asked whether they had seen any dead animals, birds, and insects at their spraying location, to which 64.8% replied in affirmative (Table 1).

Classification according to severity of symptoms

The respondents were divided into two groups based on the severity of physical ailment symptoms, namely the mild symptom (174 cases) and moderate-severe symptom groups (96 cases). Next, we ascertained the association of various above-mentioned factors with the severity of symptoms using statistical tests. The severity of the symptoms was not significantly associated with gender

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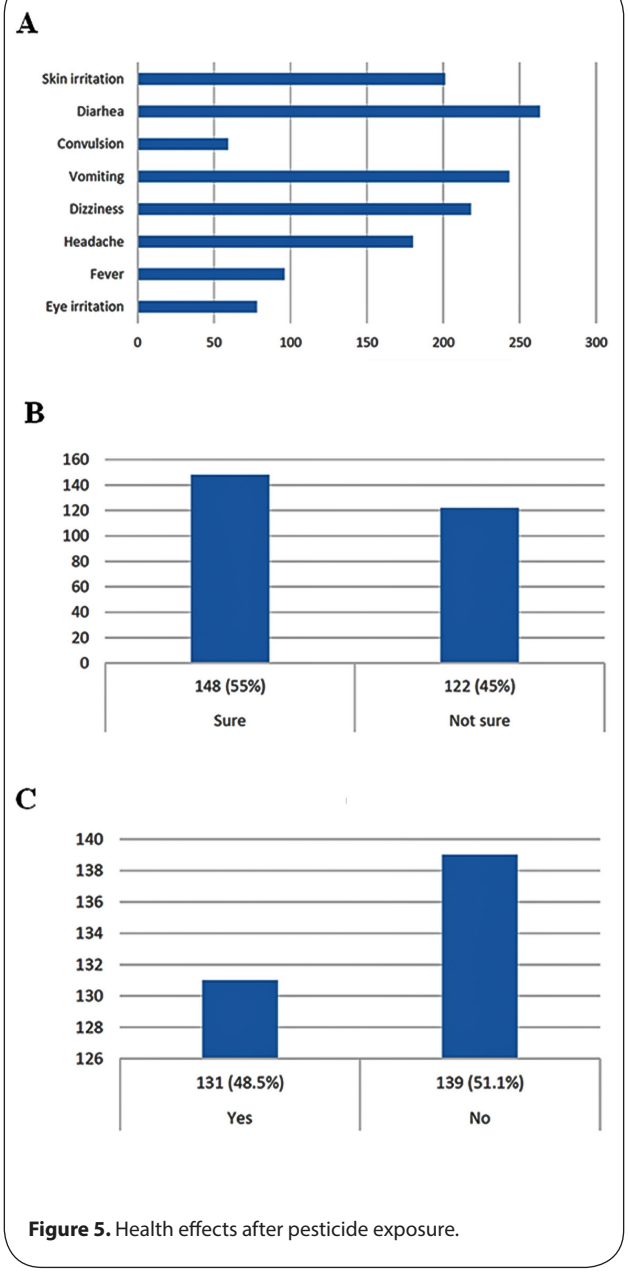

Figure 5. Health effects after pesticide exposure.

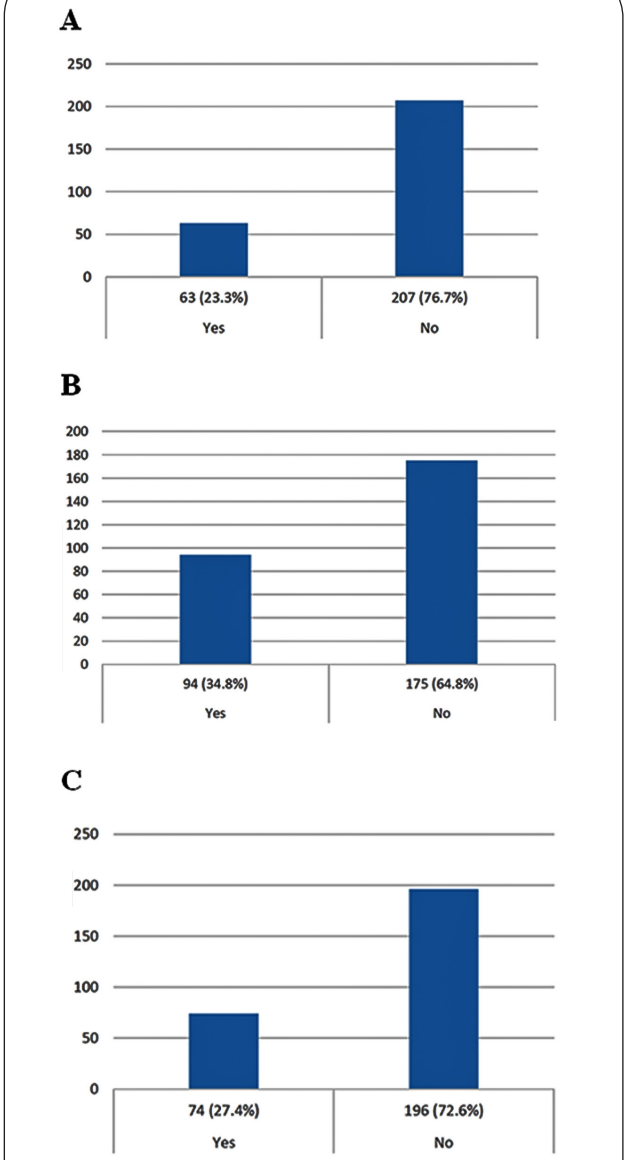

Figure 6. Assessment of pesticide handling. **(A)** training, **(B)** CSDS availability, **(C)** willingness to read CSDS.

(data not shown). The chi-square test showed a significant association between the income of the workers and the elicited symptoms, with lesser income being associated with severe symptoms (p=0.000) (Figure 9A). Similarly, there was a significant association between the literacy level of the workers and the severity of symptoms, with higher education being associated with mild symptoms (p=0.000) (Figure 9B). An independent t-test showed that longer duration of pesticide usage was associated with mild symptoms (p=0.000) (Figure 9C), whereas skin contact was directly associated with severe symptoms (Figure 9G). An inverse relationship was observed between training and symptom severity, with trained workers showing lesser symptoms (mild plus severe) than untrained ones (p=0.000) (Figure 9D). Use of PPE (glasses, shirts, and gloves) also correlated with occurrence of milder symptoms (p=0.000) (Figures 9E and F).

Co-relationship between use of PPE and symptoms

Significant association between the usage of gloves by workers and symptoms were observed post statistical analysis. From the survey it was found that 149 farmers put on the gloves and 121 do not use gloves while handling pesticides. Out of 149 farmers 138 used to develop mild while 11 developed moderate symptoms. In the same way out of 121 gloves non-users, 36 farmers developed mild and 85 developed moderate symptoms. Chi square and probability value were 115.170 and 0.000 respectively. Hence usage of gloves can significantly reduce the occurrence of symptoms in workers. Likewise notable association between the usage of protective shirt and symptoms were found. Usage of protective shirt can reduce the symptoms (Chi-Square test value at 1 degree of freedom was found to be 26.692, and p-value at 5% level of significance was 0.000). Similar result was observed between protective

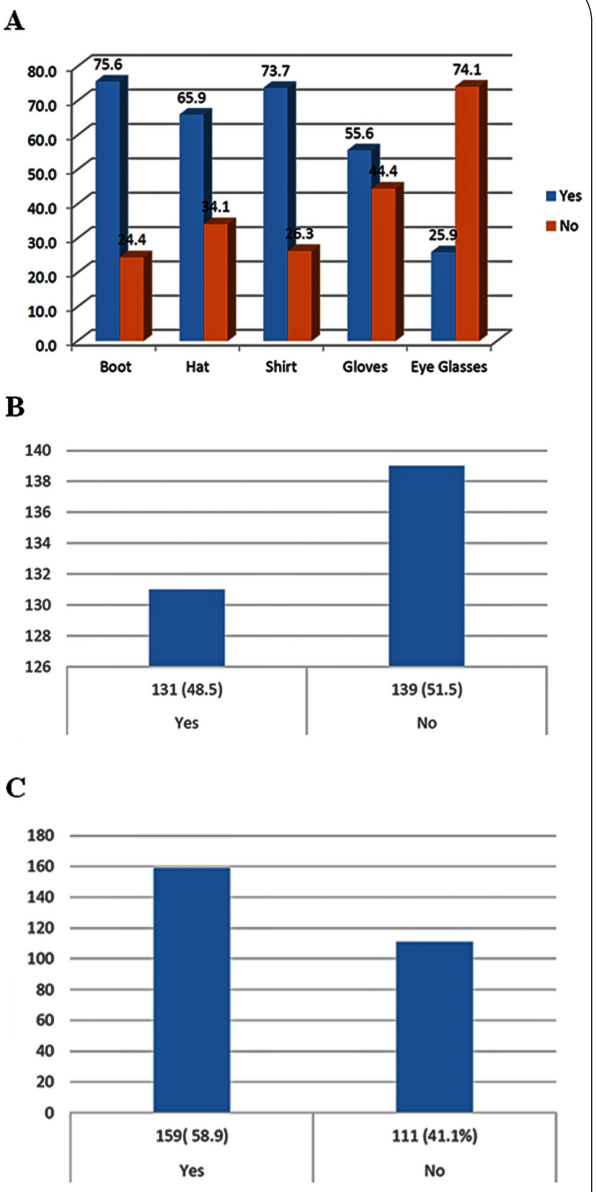

Figure 7. Use of safety and hygiene measures during pesticide handling. **(A)** personal protective equipment, **(B)** bathing, and **(C)** changing clothes.

eyeglass and symptom analysis. Chi-Square test value and p value were found to be 53.149 and 0.000 respectively. Hence usage of PEE during pesticide application can reduce the risk of developing symptoms of serious health hazards in farmers.

Discussion

In this cross-sectional study, we surveyed 19 oil palm plantations in the Sabah district of Malaysia and evaluated the perception of the workers towards pesticide use and awareness regarding the health effects post-pesticide exposure. We observed that most of the workers among the 270 respondents were approximately 30-year-old males with average education and belonged to the low income group. The majority opined that they were aware of the health hazards of pesticide use and suffered from symptoms such as vomiting, diarrhea, skin irritation, and dizziness. Surprisingly, the opinion was almost equally divided on whether they perceived pesticides to be the cause of their health problems, and a major percentage did not avail of medical help. Most of the workers responded that they did not receive any training in pesticide handling and used partial personal protective equipment (glasses, hats, shirt, and gloves) during working hours. Interestingly, a large percentage responded that they would not read the safety material even if it was provided. These observations clearly highlight the urgency of improving the awareness, education, and attitude of these plantation workers towards the short- and long-term effects of pesticide use.

Occupational pesticide poisoning is a major health issue among field and agricultural workers, (Hossain, 2010). Previous field studies from Indonesia, India, Vietnam, China, and South Korea have reported that occupational pesticide has a prevalence rate of 8.8% to 31% based on self-reporting, although the study period and definition of poisoning varies between studies (Bertolote *et al.*, 2006). Approximately 200,000–300,000 people die worldwide from pesticide poisoning every year with the majority of deaths occurring in developing countries (Gunnell *et al.*, 2007). Studies in Asian countries have highlighted the use of unauthorized pesticides and a lack of advice on alternatives to pesticide use (Konradsen, 2007), which contributes to pesticide poisoning. Gangemi S *et al.*, 2016, had critically reviewed the effect of pesticides on human health leading to immunotoxicity and the impact of cytokine levels on health, resulting in the development of numerous chronic ailments. In another study Fenga C, *et al.*, 2014, studied the effect of pesticide on the levels of IL-17 and IL-22 in serum of greenhouse workers. They found a significant augment in IL-22 concentration in the exposed subjects compared to controls. This fact confirmed that exposure to pesticide perhaps reduce host defense against cancer and infections. A study was conducted (Costa C *et al.*, 2015) to estimate the common genetic polymorphisms of the paraoxonase 1 (PON1) gene in a group of 55 farmers who were exposed to pesticides. Polymorphism of PON1 gene leads to atherosclerosis. In another experiment by Costa C *et al.*, 2013, immunotoxicity of the synthetic pyrethroid α-cypermethrin (αCYP) was evaluated in 30 green house workers who are occupationally exposed to pyrethroid. It was concluded from the study that pyrethroid exposure in green house may lead to decreased immune system in farmers. Experiment on hair sample by Knipe DW *et al.*, 2016, revealed the presence of diethyl phosphates (organophosphates) in more than 80% of the subjects from rural area of Sri Lanka. This was due to the expose of pesticide in fields. Koureas M *et al.*, 2016, estimated the levels of organochlorine pesticides (OCs) in general population residing in Larissa, Greece. They used optimized headspace solid-phase microextraction GC-MS, to detect and quantify OC levels in serum samples of 103 volunteers. The result revealed the presence

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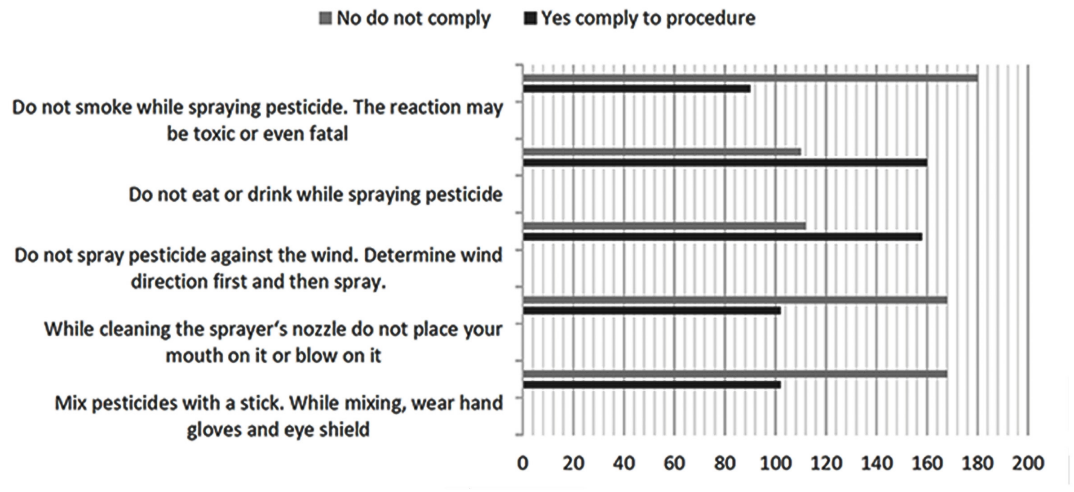

Figure 8. Compliance with standard safety measures while applying pesticides.

of p,p′-DDE (frequency 99%, median: 1.25 ng/ml) and hexachlorobenzene (frequency 69%, median: 0.13 ng/ml) in the serum samples.

Therefore, the use of pesticides in developing countries should be further investigated and clarified for providing guidance to the respective governments and international organizations during policy making. Many workers in Sabah are small-scale field workers with a property value of less than a few acres per household, who have formed organizations for promoting worker benefits and exchanging knowledge on plantation practices (Rajasuriar *et al.*, 2007).

In most cases, the increase in the percentage of pesticide intoxication is mainly because of the lack of knowledge regarding pesticide exposure, pesticide handling techniques, and misuse (or no use) of protective equipment. (Eddleston *et al.*, 2008). However, Yassin *et al.* (2002) observed that even though the farmers in the Gaza strip were knowledgeable about the health impact of pesticides, they did not practice safe handling measures. Therefore, change in attitude via intense awareness campaigns should also be considered. Other factors that may be responsible for pesticide poisoning are poor income and educational status, and poor quality of life of the workers.

The majority of oil plantation workers had not received any training and briefing about the harmful effects of pesticides and the preventive measures required to protect themselves and the environment from these effects, which has led to hazardous pesticide management practices. These results corroborate those of Austin *et al.* (2001), who observed that a large percentage of the laborer class work without any awareness about the hazards and threats associated with their profession. Therefore, training programs for farmers and pesticide applicators should be formulated, and a wide range of media, including radio, newspapers, posters, communication with extension officers, *etc*. should be used to communicate the relevant information to pesticide users. In addition, educating women, children, and health workers on good stewardship practices may influence pesticide applicators for safe and effective handling of pesticides.

We observed that even though 71% of the workers had received some form of formal education, only 23.3% workers had received training on pesticide management and handling, which corroborates the results of Dasgupta & Meisner (2005) in Bangladesh. In addition, the workers who did not receive training and show moderate-severe ailment symptoms is approximately twice (40%) of those who received training and show moderate-severe symptoms (21%). Therefore, we concluded that training positively affects workers' health. In addition, education plays a key role in augmenting the understanding of the health risks associated with pesticides. Byrness & Byrness indicated in their 1978 study that education enhances one's ability to receive, decode and understand information. Therefore, an educated worker would have the intellect to understand the usage and dosage of pesticides and its different components. In contrast, a poorly educated farmer may perform some critical and specialized tasks (*e.g.* calibration of sprayers, measurement and mixing of pesticides) with difficulty. Since, majority of the oil plantation workers had some form of formal education, there is a likelihood that they will better understand the principles of correct pesticide usage. Anang *et al.* (2013) and Boateng *et al.* (2014), reported an increase in the literacy rate and education level among cocoa farmers in Ghana, which might be because of education policy reforms and should also be introduced in the Malaysian system. Our results show that the education level of a worker is significantly associated with the severity of ailment symptoms. Higher education level correlated with awareness regarding the consequences of pesticide application, which decreased the severity of the symptoms.

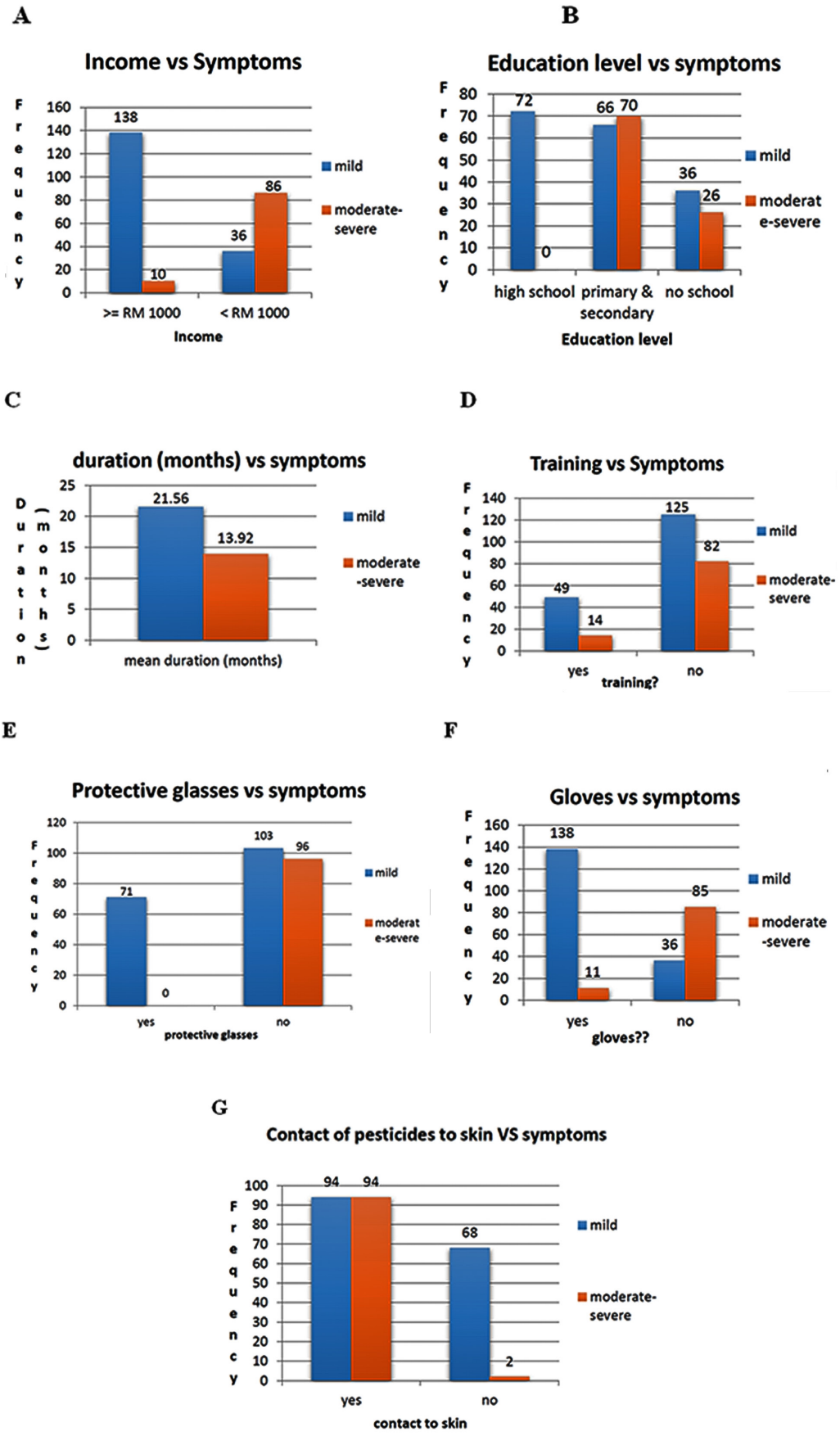

Figure 9. The associations of various factors with the severity of symptoms.

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Correct pesticide handling and post-handling hygiene play important roles in containing pesticide pollution. For example, the majority of the workers do not consider the direction of wind while spraying pesticides, which exposes the farmers to the health risk of pesticide intoxication, as the wind may blow the chemical towards the body, including the face of the farmer. This may also pollute the environment (soil and nearby water bodies) due to spray drift. Ntow *et al.* (2006) observed that poor spraying practices increased the exposure of farmers to chemicals via both skin contact and inhalation. Disposal of chemical containers, left-over spray solutions, and waste water from sprayer equipment also play important role in dermal pesticide persistence. Farmers commonly dispose empty pesticide containers, unwanted pesticides or left over spray solutions, and the water used for washing spraying equipment in unsafe ways, including disposal near water bodies. This represents a pollution problem for those who drink directly from these water sources as well as aquatic systems which are sources of livelihood for some communities (Antwi-Agyakwa *et al.*, 2013; Lekei *et al.,* 2014; Afari-Sefa *et al.*, 2015).

Operational habits during and after pesticide application, such as scooping or stirring pesticides with bare hands, chewing gum or stick, singing, receiving visitors, talking, removing/ blowing/sucking blockages in sprayer nozzles with mouth, eating, drinking water or alcohol, whistling, and smoking cigarette/tobacco pipes, also determines the extent of pesticide contact with skin/body. These practices readily expose the palm plantation workers to contamination through oral and dermal routes. Similar operational habits during pesticide application have been reported in other studies in developing countries (Lawal *et al.*, 2005; Tijani 2006).

Pesticides enter the human body by inhalation or dermal contact. Therefore, use of PPE during pesticide application has been recommended by the International Labor Organization (ILO) and the World Health Organization (WHO). We observed that majority of the palm oil plantation workers used partial PPE, with use of eyeglasses being the least. Therefore, rigorous training and stringent implementation of safety measures should be considered to improve PPE awareness among the workers, especially because PPE use was inversely related to severity of health symptoms.

In this study, we observed that the pesticide sprayers followed hygiene measures such as changing clothes, washing hands, and showering after spraying pesticides. This contrasts the findings of other studies (Jørs *et al.*, 2008) which documented a low percentage of farmers following appropriate hygiene measures. This difference might be related to availability of water or it might reflect the hygiene behavior of the general population.

We also observed that income plays an important role in deciding the effect of pesticides on worker's health and the workers' awareness about such effects. Workers in high income group can either buy better and less toxic pesticides or can purchase bigger land, resulting in an increase in pesticide usage. Furthermore, workers with higher income can purchase safety equipment, avail of higher education, or afford to pay doctor's fees when ill. Approximately 55% of the plantation workers in this study earn below RM1000 and there is a significant association between household income and the severity of symptoms, indicating that workers with better income have lower chances of developing severe symptoms.

Conclusions

In conclusion, we found that a sizable population of the working staff of the palm oil plantations was severely affected by pesticide exposure, and the health hazards ranged from mild skin irritations to lung cancer. The following measures should be implemented to stem pesticide poisoning-mediated health problems. First, the industry management should provide the workers with adequate safety measures such as at least two sets of PPE, one of which can be worn when the other one is being sterilized. Proper sterilization procedures must be made available in the campus to facilitate effective cleanup of the pesticide residue. The staff must be put on rotational shifts, in order to prevent over exposure to pesticides. They must be also given adequate leaves and breaks upon sensing any symptoms of pesticide toxicity. Regular health checkups must be made mandatory to identify any symptoms of pesticide poisoning in the early stages, thereby ensuring good health of the workers. Second, the workers should be encouraged to regularly practice hygienic measures after each shift in the field and use protective clothing and equipment irrespective of any inconvenience. Upon any physical discomfort, immediate medical help must be obtained and a break must be sought from work, if required. Overall, extensive educational and training programs have to be initiated to improve the worker's perception on pesticides and attitude towards use of safe and hygienic measures during and after pesticide application.

REFERENCES

Afari-Sefa V, Asare-Bediako E, Kenyon L, Micah JA. (2015). Pesticide use practices and perceptions of vegetable farmers in the cocoa belts of the Ashanti and Western Regions of Ghana. *Adv Crop Sci Tech* **3**: 174.

Anang BT, Mensah F, Asamoah A. (2013). Farmers' assessment of the government spraying program in Ghana. *J Econ Sustain Dev* **4**: 92–99.

Antwi-Agyakwa AK. (2013). Susceptibility of field populations of cocoa mirids,sahlbergella singularis haglund and distantiella theobroma (distant) to bifenthrin. (Unpublished Master's thesis). Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

Austin C, Arcury T, Quandt S, Preisser JS, Cabrera L. (2001). Training farmworkers about pesticide safety: issues of control. *J Health Care Poor Underserved* **12**: 236–249.

Antwi-Agyakwa, AK, Osekre, EA, Ninsin, KD, Adu-Acheampong, R. (2016). Insecticide handling in cocoa production in four regions in Ghana. *Journal of Science Education and Technology (Ghana)* **36**: 1–9.

Bassil KL, Vakil C, Sanborn M, Cole DC, Kaur JS, Kerr KJ. (2007). Cancer health effects of pesticides: Systematic review. *Can Fam Physician* **53**: 1704–1711.

Bertolote JM, Fleischmann A, Eddleston M, Gunnell D. (2006). Deaths from pesticide poisoning: a global response. *Br J Psychiatry* **189**: 201–203.

Boateng DO, Nana F, Codjoe Y, Ofori J. (2014). Impact of illegal small scale mining (Galamsey) on cocoa production in Atiwa district of Ghana. *Int J Adv Agric Res* **2**: 89–99.

Byrness FC, Byrness KJ. (1978). Agricultural extension and education in developing countries. *Rural Dev a Chang World* pp. 54–67

Costa, C., Gangemi, S., Giambò, F., Rapisarda, V., Caccamo, D., Fenga, C. (2015). Oxidative stress biomarkers and paraoxonase 1 polymorphism frequency in farmers occupationally exposed to pesticides. *Molecular Medicine Reports* **12**: 6353–6357.

Costa C, Rapisarda V, Catania S, Di Nola C, Ledda C, Fenga C. (2013). Cytokine patterns in greenhouse workers occupationally exposed to α-cypermethrin: an observational study. *Environ Toxicol Pharmacol* **36**(3): 796–800.

Dasgupta S, Meisner C. (2005). *Pesticide traders' perception of health risks: evidence from Bangladesh*. World Bank Publications, Washington, DC.

Eddleston M, Buckley NA, Eyer P, Dawson AH. (2008). Management of acute organophosphorus pesticide poisoning. *Lancet* **371**: 597–607.

Food and Agricultural Organization [webpage on the Internet]. Food and Agricultural Organization of the United Nations [cited 2016]. Retrieved 08 09, 2016. Available from: http://www.fao.org/home/en/

Fenga C, Gangemi S, Catania S, De Luca A, Costa C. (2014). IL-17 and IL-22 serum levels in greenhouse workers exposed to pesticides. *Inflamm. Res*. **63**: 895.

Gangemi S, Gofita E, Costa C, Teodoro M, Briguglio G, Nikitovic D, Fenga C. (2016). Occupational and environmental exposure to pesticides and cytokine pathways in chronic diseases (Review). *International Journal of Molecular Medicine* **38**(4): 1012–1020.

Gunnell D, Eddleston M, Phillips MR, Konradsen F. (2007). The global distribution of fatal pesticide self-poisoning: systematic review. *BMC Public Health* **7**: 357.

Hillocks R. (2012). Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. Crop Prot **31**: 85–93.

Hossain F. (2010). Effects of pesticide use on semen quality among farmers in rural areas of Sabah, Malaysia. *J Occup Health Psychol* **52**: 353–360.

Jørs E, Morant RC, Aguilar GC, Huici O, Lander F, Baelum J, Konradsen F. (2006). Occupational pesticide intoxications among farmers in Bolivia: a cross-sectional study. Environ Health. **2006** (21): 5–10.

Jurewicz J, Hanke W. (2008). Prenatal and childhood exposure to pesticides and neurobehavioral development: review of epidemiological studies. *Int J Occup Med Environ Health* **21**:121–132.

Knipe, D. W., Jayasumana, C., Siribaddana, S., Priyadarshana, C., Pearson, M., Gunnell, D., Tsatsakis, A. M. (2016). Feasibility of hair sampling to assess levels of organophosphate metabolites in rural areas of Sri Lanka. *Environmental Research* **147**: 207–211.

Konradsen F. (2007). Acute pesticide poisoning–a global public health problem. *Dan Med Bull* **54**: 58–59.

Lawal BO, Torimiro DO, Banjo AD, Joda AO. (2005). Operational habits and health hazards associated with pesticide usage by cocoa farmers in Nigeria: lessons for extension work. *J Hum Ecol* **17**:191–195.

Lekei EE, Ngowi AV, London L. (2014). Farmers' knowledge, practices and injuries associated with pesticide exposure in rural farming villages in Tanzania. *BMC Public Health* **14**:389.

Mink PJ, Mandel JS, Lundin JI, Sceurman BK. (2011). Epidemiologic studies of glyphosate and non-cancer health outcomes: a review. *Regul Toxicol Pharmacol* **61**: 172–184.

Ntow WJ, Gijzen HJ, Kelderman P, Drechsel P. (2006). Farmer perceptions and pesticide use practices in vegetable production in Ghana. *Pest Manag Sci* **62**: 356–365.

Praneetvatakul S, Schreinemachers P, Pananurak P, Tipraqsa P. (2013). Pesticides, external costs and policy options for Thai agriculture. *Environ Sci Policy* **27**: 103–113.

Rajasuriar R, Awang R, Hashim SB, Rahmat, HR. (2007). Profile of poisoning admissions in Malaysia. *Hum Exp Toxicol* **26**:73–81.

Randall C, Hock W, Crow E, Hudak-Wise C, Kasai J. (2013). *National Pesticide Applicator Certification Core Manual*. National Association of State Departments of Agriculture Research Foundation, Washington, DC.

Sanborn M, Kerr KJ, Sanin LH, Cole DC, Bassil KL, Vakil C. (2007). Non-cancer health effects of pesticides: Systematic review and implications for family doctors. *Can Fam Physician* **53**: 1712–1720.

Srinivasan P [webpage on the Internet]. (2003). Paraquat: A Unique Contributor to Agriculture and Sustainable Development [cited 2016 May 25]. Available from: http://paraquat.com/sites/default/files/Paraquat%20a%20unique%20contributor_0.pdf

Tijani AA. (2006). Pesticide use practices and safety issues: the case of cocoa. Farmers in Ondo State, Nigeria. *J Hum Ecol* **19**(3):183–190 .

US Environmental Protection Agency. (2007). Pesticides: Health and Safety. National Assessment of the Worker Protection Workshop #3.

US Environmental. (2007). What is a pesticide? epa.gov [cited 2007 September 15].

Watts M. (2011). Paraquat [cited 2016 May 28]. Available from: http://www.panna.org/sites/default/files/Paraquat%20monograph%20final%202011-1.pdf.

Weselak M, Arbuckle TE, Foster W. (2007). Pesticide exposures and developmental outcomes: the epidemiological evidence. *J Toxicol Environ Health B Crit Rev* **10**: 41–80.

WHO. (2016). World Health Organization [cited 2016 September 08]. Available from: http://www.who.int/en/

Wigle DT, Arbuckle TE, Turner MC, Bérubé A, Yang Q, Liu S, Krewski D. (2008). Epidemiologic evidence of relationships between reproductive and child health outcomes and environmental chemical contaminants. *J Toxicol Environ Health B Crit Rev* **11**: 373–517.

Yassin MM, Abu Mourad TA, Safi JM. (2007). Knowledge, attitude, practice, and toxicity symptoms associated with pesticide use among farm workers in the Gaza Strip. *Occup Environ Med* **59**: 387–393

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ORIGINAL ARTICLE

Acute and subacute toxicity of *Ammi visnaga* on rats

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ABSTRACT

Ammi visnaga (Av) is a source of khellin where a tea made from the fruit of this plant was used as herbal medicine for kidney stones in Egypt. In the present research, the acute and subacute toxicity studies with oral intake of 150, 300 and 600 mg/kg of Av seed ethanolic extract in rats were done. In acute toxicity test, 4 groups of rats (n = 6/group: 3 males and 3 females) were chosen and the first control group received tap water, while the other three groups received Av seed ethanolic extract dissolved in tap water at doses of 150, 300, and 600 mg/kg, and general behavior, adverse effects, and mortality were recorded for up to 14 days. In subacute toxicity study, 72 rats (36 males and 36 females) were divided into 4 major groups; group I received tap water (control group), while animals in groups II, III, and IV (test groups) received oral intake of Av seed ethanolic extract dissolved in tap water at doses of 150, 300 and 600 mg/ kg bwt, respectively. Each of this major group was subdivided consequently into 3 subgroups (n = 6/group: 3 males and 3 females) where brain tissue, blood sample, body and organs weights were recorded at the beginning and then after two and four weeks of the experiment for the determination of hematological, biochemical and histopathological changes in tissues (liver, kidney, brain, spleen, heart, testis and ovary). With regard to acute toxicity, Av seed ethanolic extract did not induce any toxic effects or death or any organ toxicity. In subacute toxicity study; oral intake with Av seed ethanolic extract did not reveal any change in body and organs weights, hematological parameters, serum glucose and cholesterol, brain neurotransmitters, liver and kidney functions, male and female hormones. In conclusion, Av seed ethanolic extract is nontoxic to liver, kidney, brain, spleen, heart, testis and ovary.

KEY WORDS: *Ammi visnaga*; Apiaceae; acute toxicity; subacute toxicity; rats

Introduction

In the last decade, the medicinal plants represent an important and rich source of new synthetic drugs. There is huge number of the world's populations depending upon medicinal plants as an alternative and complimentary drugs therapy for many known diseases. The most common plants uses involve application of plant extracts, which contain numerous of molecules with well-known biological effects (Yakob *et al.,* 2012). The medicinal plants are commonly used world-wide without informing about their possible unhealthy or toxic effects, the World Health Organization has recommended that traditional plants used for the treatment of diseases need further scientific investigation on their toxic side effects (WHO, 2008). The medicinal plants contain bioactive ingredients which act as defense mechanisms against many diseases but the plant itself may be toxic in nature (Roch *et al.,* 2001). So, the medicinal uses of herbs have been restricted by a lack of defined chemical classification, dose treatment, and well-known toxicity data to evaluate plant safety (Denga *et al.,* 2013). Consequently; it is very urgent and important to evaluate the toxicity of the medicinal plants used in medicinal and pharmacological applications.

Ammi visnaga (Av) belongs to family Apiaceae. Av is medically applied for prevention and treatment of urinary lithiasis and consequently Av represents an alternative and complementary medical application. Av is used in medical usage against the crystallization of calcium oxalate in kidney (Kachkoul *et al.,* 2018). It is a flowering plant that is traditionally known by numerous names depending on the different areas of cultivation and out of these names bisnaga and khella were used. It is cultivated in North Africa, Europe and Asia, but it can be cultivated throughout the world. Av plant is annual herb up to 80 centimeters in height. The plant leaves are

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20 centimeters long and are often oval or sometimes triangular in shape. The inflorescence, like other Apiaceae family, forms multiple umbel of white flowers. The fruit forms a compacted oval-shape (less than 3 millimeters long). Khellin is a main constituent obtained from Av and other Ammi species while khellin has a diuretic effect. Another important effect of khellin is relaxation of the smooth muscle but this function is limited due to its side effects (Waltenberger *et al.* 2016). Herbicidal activity was observed and recorded for both khellin and visnagin, constituents of Av. This herbicidal activity of khellin and visnagin was not a light-dependent effect. Both khellin and visnagin induced photosynthetic efficiency decrease, membrane destabilization, decline in cell division and death (Travaini *et al.,* 2016). Khellin showed very fast mortality effect for the larvae of Culex quinquefasciatus in various development stages so 18 minutes of khellin action induced a 54.3% mortality rate of the larvae within 24 hours (Pavela *et al.,* 2016). The famous and medically applicable two synthetic derivatives of khellin are amiodarone and cromoglycate. These two synthetic derivatives of khellin have fewer side effects so they are used in modern medicine. Av also contains visnagin as a chemical constituent (Vanachayangkul *et al.,* 2010). In Egypt, a tea from Av fruit is famously used as a popular herbal remedy for kidney stones.

This research was designated to study the acute toxicity (determination of LD_{50}) and subacute toxicity (clinical changes, hematological, biochemical and histopathological changes) in rats after daily oral administration with *Ammi visnaga* though the period of 28 days.

Materials and methods

Materials

All kits reagents used in this research were purchased from Bio-diagnostic Kits reagents through local Egyptian supplier with the exception of testosterone, dehydroepiandrosterone sulfate, 3β-hydroxysteroiddehydrogenase, 17-β-estradiol and estradiol-17-β-stearate kits that were purchased from Biosource Inc, Belgium. Further, the kits reagents used in this research include dehydroepiandrosterone sulfate and 3β-hydroxysteroiddehydrogenase kits that were achieved from EURO/DPC Ltd., United Kingdom while kits reagents used in the current research such as 17-β-estradiol and estradiol-17-β-stearate kits were purchased from Steraloids Inc., USA. All kits reagents used in this study were manufactured in 2018.

Plant material

In this research, *Ammi visnaga* (Av) seeds were delivered from Horticulture Department, Ministry of Agriculture, Dokki, Cairo, Egypt. The seeds were crushed, pulverized, and weighed in sequence to prepare extraction.

Preparation of seed ethanolic extract

Av seeds were air-dried in an oven at 40°C for 4 days and then the dry seeds were cut and pulverized. The dried seeds (500 g) were placed in 1000 ml of distilled boiling water and kept at room temperature for 15 minutes to yield dried powder. Then the dried powder was macerated for 7 days using 70% ethanol as a solvent. The solvent was then eliminated by a rotary vacuum evaporator under reduced pressure to obtain total alcoholic extract. The alcoholic extract obtained is lyophilized and represents a yield of 15% of the dry seeds extracted. Evaporation process of the extract was done to dryness to give dried seed total alcoholic extract (150 g) according to the method of Chopra *et al.* (1986).

Animals

Albino rats of *Sprague Dawley* strains (130±10 g, 10-weeks old) of both sexes were obtained from the animal house of the National Research Centre, Dokki, Cairo, Egypt and were kept in special plastic cages. The animals were maintained on a commercial balanced diet and tap water. The laboratory animal conditions were followed (Guide for the Care and Use of Laboratory Animals, 2011) such as 12 hours light/12 hours dark cycle, room temperature = 26–30 °C, humidity = 40–70%, Number of rats=3 rats /one cage and each group comprised 2 cages (3 males/cage and 3 females/cage). The animal room ventilation maintained the temperature for rats within 68 to 79 °F and minimized temperature fluctuations. All experimental procedures were done following the acceptance of the ethics committee of National Research Centre and in accordance with recommendations for the proper care and use of laboratory animals (NIH publication no 85:23 revised 1985).

Experimental design

Determination of LD_{50} of Av

There were 36 rats of both sexes (18 males and 18 females) used. The LD_{50} of Av seed ethanolic extract was determined in mg/kg body weight (bwt) for adult rats (Behrens & Karber, 1953), where $LD_{50} = DM - \Sigma Z.D/m$, DM is the highest dose used, Z is the number of dead rats from two successive doses divided by two, D is the difference between two successive doses, m is the number of rats in each group. The doses chosen for such study were 5%, 10% and 20% of the LD_{50} of Av seed ethanolic extract (Koriem *et al.,* 2010).

Acute toxicity study

Animals were divided into 4 groups (n=6 rats/group: 3 males and 3 females) were followed OECD 423 guideline (OECD, 2001). Control group rats received tap water while the other three groups received Av dissolved in tap water at doses of 150, 300, and 600 mg/kg. The above doses was selected on the basis of LD_{50} of Av. Animals were observed closely for first 6 hours, for any toxicity manifestation, like increased motor activity, salivation, convulsion, coma, and death. Animals were under more investigation for the period of 14 days and the number of rats that died within the study period was noted (Gautam *et al.,* 2012).

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Subacute toxicity study

The repeated doses (28 days) for oral toxicity studies were carried out in rats according to the OECD test guideline 407 (OECD, 2008). Seventy-two rats (36 males and 36 females) were used and divided into 4 major groups; group I received tap water (control group), whereas rats in groups II, III, and IV (test groups) received Av dissolved in tap water at doses of 150, 300 and 600 mg/kg bw/d, respectively. Each of this major group was subdivided consequently into 3 subgroups (n=6 rats / group: 3 males and 3 females) where brain tissue, blood sample, food consumption, water intake, body and organs weights were measured and recorded at the beginning and then after two and four weeks of the experiment. The Av seed extract dose was taken daily by oral gavage in the volume of 5 ml of tap water/kg bw/d, once daily for 28 consecutive days. Any death or abnormal clinical signs among animals during the experimental period were observed daily.

Biochemical analysis

Hemoglobin (Hb) concentration was determined by the method described by Van Kampen & Zijlstra (1961). Hematocrit (Hct) value, Red blood cells (RBCs) and white blood cells (WBCs) were carried out according to the method of Rodak (1995). Serum glucose was measured according to the enzymatic colorimetric method described by Trinder (1969). Allain *et al.* (1974) method was applied to determine serum total cholesterol while Reitman & Frankel (1957) method was used to calculate serum aspartate and alanine aminotransaminases (AST & ALT) activities. The colorimetric method of Kind & King (1954) was used to determine serum alkaline phosphatase (ALP) while Walter and Gerard (1970) method was applied to calculate serum total bilirubin. The method of Gornall *et al.* (1949) was used to calculate serum total protein while Drupt (1974) method was used to estimate serum albumin. The enzymatic method of Patton & Crouch (1977) was applied to estimate serum urea while kinetic method of Houot (1985) was used to calculate serum creatinine. The method of Kabasakalian *et al.* (1973) was applied to determine serum uric acid while Ciarlone & Smudski (1977) method was used to estimate serotonin (5-hydroxytryptamine; 5-HT), norepinephrine (NE), and dopamine (DA) in brain cerebral cortex. Maruyama *et al.* (1987) method was applied to estimate serum testosterone (Ts) and Dehydroepiandro-sterone sulfate (DHEA-SO_4) concentrations. Talalay (1962) method was applied to determine serum 3β-hydroxysteroiddehydrogenase (3βHSD) level while Vihma *et al.* (2001) method was used to estimate serum 17-β-estradiol (17β-E) and estradiol-17-β-stearate (E-17β-s) levels.

Histopathological investigation

Tissue samples of brain, liver, kidney, spleen, heart and ovary tissues were fixed at 10% neutral formalin solution while testes tissues were fixed in Bouin´s fluid and then processed for routine embedding in paraffin. Blocks were sectioned at a thickness of 5 µm and stained with hematoxylin and eosin for histopathological examination under microscope for any cellular damage or change in morphology of that particular tissue.

Statistical analysis

The results obtained in this research were tabulated as mean ± standard error mean (SEM). The variances between different groups were assessed by ANOVA using the SPSS 13 software package for Windows followed by *post-hoc* testing. The inter-group comparisons were performed using the least significant difference (Tukey) test; significance at $p \leq 0.05$.

Results

The LD_{50} of Av

The LD_{50} of Av was equal to 3000 mg/kg body weight, so 5%, 10% and 20% of the LD_{50} of Av seed extract equal to 150, 300 and 600 mg/kg, respectively.

Acute toxicity study

Av seed extract administrated at doses 150, 300 and 600 mg/kg bw/d respectively by oral gavage into normal male and female rats did not induce any death or toxic symptoms in treated rats. All animals were normal throughout the study and survived until the end of the 14-day experiment period.

Subacute toxicity study

General observations

No mortality and clinical changes were observed in both males and females during the test. Both male and female animals did not reveal any disturbance in animal behavior or other physiological activities.

Food consumption and water intake

No difference in food consumption and water intake were found between control and treated rats throughout the period of 28 day.

Body and organs weights

Slight changes were observed in animal body and organs weights in all the Av-treated animals in comparison with control rats after 28 successive days of Av oral intake.

Hematological parameters, serum glucose and cholesterol

It was obvious that Av seed extract did not induce any change in Hb, Hct, RBCs, WBCs, serum glucose and total cholesterol in all the treated animals compared to control group after 28 days of study period (see Table 1).

Liver function

It was clear that Av seed extract did not induce any change in serum AST, ALT, ALP, total bilirubin, total protein and albumin in all the treated animals compared to control group after 28 days of study period (see Table 2).

Kidney function and brain cerebral cortex neurotransmitters

It was observable that Av seed extract did not induce any change in serum urea, creatinine and uric acid, as well as, tissue serotonin, norepinephrine and dopamine in all the treated animals compared to control group after 28 days of study period (see Table 3).

Male and female hormones

It is clear that Av seed extract did not induce any change in serum Ts, DHEA-SO_4, 3βHSD, 17β-E and E-17β-s activities on treated male and female animals compared to control group after 28 days of study period (see Table 4).

Histopathological results

Histological structure of the liver in all treated rats was comparable with normal pattern of the organ in controls (Figure 1A). No changes of hepatic lobular architecture and hepatocytes were observed (Figures 1B, C&D).

Histological structure of the kidney in all treated rats was comparable with normal pattern of the organ in controls (Figure 2A). No changes of glomeruli and the renal tubules architecture were detected (Figures 2B, C&D).

Histological structure of the brain in all treated rats was comparable with normal pattern of the organ in controls (Figure 3A). No changes of the normal brain structure architecture were identified (Figures 3B, C&D).

Histological structure of the spleen in all treated rats was comparable with normal pattern of the organ in controls (Figure 4A). No changes within macrophages in the red pulp with normal structure architecture were found (Figures 4B, C&D).

Histological structure of the heart in all treated rats was comparable with normal pattern of the organ in controls (Figure 5A). No changes in cardiac muscle fibers, acidophilic cytoplasm and centrally located nuclei were observed (Figures 5B, C&D).

Histological structure of the testis in all treated rats was comparable with normal pattern of the organ in controls (Figure 6A). No changes in seminiferous tubules and germ cells architecture were identified (Figures 6B, C&D).

Histological structure of the ovary in all treated rats was comparable with normal pattern of the organ in controls (Figure 7A). No changes in small follicles and large follicles architecture were reported (Figures 7B, C&D).

Discussion

Orally taken medicinal plants without any ordinary dose in correlation with lack of sufficient scientific research on their medical safety attract more attention regarding their toxicity (Saad *et al.,* 2006). There are no published studies on Av toxicological data after subacute exposure of its seed extract.

In acute toxicity study, the oral administration of Av seed ethanolic extract at 150, 300 and 600 mg/kg bw/d respectively did not show any observable toxic effects in rats in terms of any deaths or abnormal symptoms which points to it being nontoxic and safe in rats. In subacute

Table 1. The effect of Av on blood parameters, serum glucose and cholesterol

Parameters		Day 0	Day 14	Day 28
Hb (gm/dl)	Control	13.79±0.45	14.12±0.27	15.24±0.61
	Av 150 mg/kg	13.62±0.37	13.84±0.61	14.28±0.45
	Av 300 mg/kg	14.18±0.60	14.68±0.37	15.16±0.29
	Av 600 mg/kg	14.25±0.83	14.90±0.56	15.38±0.70
Hct (%)	Control	43.81±1.50	45.16±1.82	46.41±2.13
	Av 150 mg/kg	40.56±1.73	42.18±1.73	43.26±1.90
	Av 300 mg/kg	41.38±1.92	44.50±2.04	45.28±1.74
	Av 600 mg/kg	45.12±2.07	46.34±1.85	47.02±1.64
RBCs (10^6 cells/mm^3)	Control	5.90±0.46	5.75±0.62	5.95±0.38
	Av 150 mg/kg	5.45±0.35	6.20±0.93	5.80±0.56
	Av 300 mg/kg	5.60±0.69	5.95±0.81	6.15±0.42
	Av 600 mg/kg	5.95±0.58	6.10±0.84	6.25±0.95
WBCs (10^3 cells/mm^3)	Control	9.45±0.75	9.60±0.91	9.85±0.53
	Av 150 mg/kg	9.25±0.48	9.85±0.67	10.15±0.82
	Av 300 mg/kg	9.50±0.69	9.95±0.90	10.20±0.74
	Av 600 mg/kg	9.65±0.75	9.70±0.86	9.85±0.63
Serum glucose (mg/dl)	Control	108.12±15.25	104.92±17.34	109.40±14.18
	Av 150 mg/kg	106.65±13.21	103.76±15.80	107.24±16.35
	Av 300 mg/kg	110.00±10.93	107.00±12.91	109.24±13.95
	Av 600 mg/kg	107.85±14.18	110.20±16.15	108.52±17.45
Total cholesterol (mg/dl)	Control	122.8±3.6	124.1±4.0	119.8±2.5
	Av 150 mg/kg	119.2±4.1	123.5±5.2	120.7±4.6
	Av 300 mg/kg	124.0±2.9	118.9±3.7	123.2±5.8
	Av 600 mg/kg	117.9±5.3	119.2±4.1	121.8±3.9

Values are expressed as mean ± SEM of 6 rats in each group

Table 2. The effect of Av on liver function

Parameters		Day 0	Day 14	Day 28
Serum AST (U/l)	Control	19.81±3.95	20.23±4.21	19.56±3.57
	Av 150 mg/kg	18.93±4.26	19.40±3.64	20.13±4.10
	Av 300 mg/kg	20.15±2.74	19.35±4.04	18.75±2.91
	Av 600 mg/kg	19..28±3.47	18..86±3.79	19.73±4.15
Serum ALT (U/l)	Control	9.27±3.23	8.69±4.06	9.42±3.61
	Av 150 mg/kg	8.69±2.82	9.14±4.02	8.90±3.57
	Av 300 mg/kg	9.17±3.51	8.76±2.80	9.35±3.94
	Av 600 mg/kg	9.60±2.94	9.39±4.17	9.43±2.75
Serum ALP (U/l)	Control	210.5±6.81	209.0±4.93	211.2±5.24
	Av 150 mg/kg	208.7±5.43	211.5±6.26	208.4±4.90
	Av 300 mg/kg	209.2±6.14	207.5±5.81	210.0±6.34
	Av 600 mg/kg	212.0±5.96	208.5±4.82	209.1±5.67
Total bilirubin (mg/dl)	Control	0.56±0.08	0.55±0.04	0.57±0.09
	Av 150 mg/kg	0.54±0.06	0.57±0.08	0.59±0.07
	Av 300 mg/kg	0.58±0.09	0.56±0.05	0.60±0.08
	Av 600 mg/kg	0.57±0.07	0.54±0.09	0.58±0.06
Total protein (g/dl)	Control	8.72±0.54	8.90±0.37	9.14±0.72
	Av 150 mg/kg	9.14±0.72	8.85±0.52	9.23±0.69
	Av 300 mg/kg	8.56±0.49	8.74±0.60	8.95±0.56
	Av 600 mg/kg	9.24±0.63	8.82±0.46	9.15±0.80
Serum albumin (g/dl)	Control	5.08±0.49	5.14±0.37	5.26±0.67
	Av 150 mg/kg	5.26±0.62	4.93±0.59	5.34±0.90
	Av 300 mg/kg	4.89±0.37	4.95±0.81	5.19±0.57
	Av 600 mg/kg	5.31±0.59	5.13±0.47	5.20±0.64

Values are expressed as mean ± SEM of 6 rats in each group

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Table 3. The effect of Av on kidney function and brain neurotransmitters.

Parameters		Day 0	Day 14	Day 28
Urea (mg/dl)	Control	26.5±2.83	28.1±2.48	27.5±3.16
	Av 150 mg/kg	28.2±3.27	26.9±2.74	29.0±3.84
	Av 300 mg/kg	27.0±2.54	29.1±2.96	25.8±2.69
	Av 600 mg/kg	25.9±1.96	28.90±3.52	27.3±2.50
Creatinine (mg/dl)	Control	0.75±0.08	0.70±0.07	0.68±0.09
	Av 150 mg/kg	0.69±0.06	0.74±0.08	0.71±0.05
	Av 300 mg/kg	0.72±0.09	0.68±0.06	0.74±0.07
	Av 600 mg/kg	0.78±0.05	0.72±0.09	0.76±0.06
Uric acid (mg/dl)	Control	8.21±0.43	7.95±0.68	8.15±0.36
	Av 150 mg/kg	7.87±0.62	8.27±0.59	7.96±0.52
	Av 300 mg/kg	7.92±0.50	8.17±0.42	8.25±0.71
	Av 600 mg/kg	8.34±0.71	7.98±0.69	8.13±0.45
Serotonin (g/g tissue)	Control	0.56±0.05	0.55±0.03	0.57±0.06
	Av 150 mg/kg	0.54±0.04	0.56±0.05	0.55±0.07
	Av 300 mg/kg	0.57±0.06	0.55±0.03	0.56±0.05
	Av 600 mg/kg	0.58±0.08	0.57±0.07	0.59±0.04
Norepinephrine (g/g tissue)	Control	2.49±0.19	2.46±0.16	2.48±0.15
	Av 150 mg/kg	2.46±0.17	2.48±0.14	2.45±0.19
	Av 300 mg/kg	2.50±0.14	2.45±0.17	2.47±0.16
	Av 600 mg/kg	2.48±0.23	2.47±0.20	2.50±0.24
Dopamine (g/g tissue)	Control	0.27±0.01	0.26±0.04	0.28±0.03
	Av 150 mg/kg	0.25±0.03	0.24±0.02	0.26±0.04
	Av 300 mg/kg	0.28±0.04	0.27±0.03	0.25±0.02
	Av 600 mg/kg	0.26±0.02	0.28±0.05	0.27±0.03

Values are expressed as mean ± SEM of 6 rats in each group

Table 4. The effect of Av on males and females hormones.

Parameters		Day 0	Day 14	Day 28
Ts (ng/ml)	Control	5.98±0.42	6.14±0.53	5.86±0.69
	Av 150 mg/kg	5.75±0.59	5.84±0.68	6.14±0.47
	Av 300 mg/kg	6.12±0.70	5.95±0.43	6.08±0.56
	Av 600 mg/kg	6.09±0.42	5.87±0.67	6.95±0.51
3βHSD (U/l)	Control	74.36±3.67	70.86±4.12	75.16±3.49
	Av 150 mg/kg	76.12±4.54	73.40±3.75	78.01±4.86
	Av 300 mg/kg	71.95±3.80	75.16±4.09	72.56±3.98
	Av 600 mg/kg	73.46±3.69	71.50±2.86	76.30±4.21
DHEA'S (µg/dl)	Control	197.5±23.7	200.1±21.8	198.2±24.9
	Av 150 mg/kg	201.2±26.9	196.8±24.6	199.7±19.8
	Av 300 mg/kg	195.7±28.5	199.0±25.9	201.4±27.1
	Av 600 mg/kg	202.0±25.3	198.5±27.0	200.0±24.9
17β-E (pmol/l)	Control	276.4±32.7	281.7±28.9	278.2±27.6
	Av 150 mg/kg	275.0±28.1	278.4±31.2	276.7±31.2
	Av 300 mg/kg	278.4±27.9	279.0±29.4	280.3±28.5
	Av 600 mg/kg	280.3±30.5	282.0±33.6	281.2±31.4
E-17β-s (pmol/l)	Control	122.8±20.5	124.0±24.1	125.0±21.9
	Av 150 mg/kg	120.6±19.7	121.7±23.4	123.5±25.7
	Av 300 mg/kg	123.1±23.2	125.0±26.0	127.0±19.2
	Av 600 mg/kg	124.0±19.6	123.9±25.1	126.2±27.4

Values are expressed as mean ± SEM of 6 rats in each group

toxicity research, animals that were orally administrated with 150, 300 and 600 mg/kg bw/d respectively of Av for 28 days did not show any mortality, body and organ weight, biochemical or histopathological changes in animal organs such as liver, kidney, spleen, heart and testis that support the safety protocol of Av seed extract. Subacute research did not reveal any biochemical change in liver, kidney, brain, heart, spleen, testis and ovary in both male and female rats after oral intake of 600 mg/kg bw/d Av seed ethanolic extract for 28 successive days. The hematopoietic system/bone marrow is one of the most sensitive targets for any toxic compounds or drugs and it is an important guide of any physiological and pathological changes in both man and animal (Mukinda & Syce, 2007). In subacute research, the hematological parameters are associated with toxic results since any variation in hematological system is a sensitive index for any human toxicity if the data obtained from animal studies are translated (Olson *et al.*, 2000). Subacute study of both male and female animals with daily oral intake of Av seed extract for 28 successive days showed small or minor variations in some biochemical and hematological parameters.

The main organ for metabolism, including drugs, is liver. The hepatic tissue is a place of cholesterol removal or degradation and, in the same time, liver is a main place of cholesterol synthesis. In this explanation, liver controls glucose synthesis and increase free glucose discharge from liver glycogen stores (Anderson & Borlak, 2008). Minor variations were recorded in both glucose and cholesterol levels in this research. Consequently, Av seed ethanolic extract had minor effect on the lipid and carbohydrate metabolism in both male and female animals. Further, drugs showing any toxicity affect the transaminases aspartate aminotransferase (AST) and alanine amino transferase (ALT) in liver which are well known enzymes used as good indicators of liver function (El Hilaly *et al.*, 2004) and biomarkers predicting possible toxicity (Rahman, 2001). The elevation or increase in serum AST and ALT enzyme activities indicate the destruction occurring in parenchymal hepatic tissue/cells (Anderson & Borlak, 2008). In this research, serum AST and ALT levels did not reveal any variation related to increase even at the higher dose (600 mg/kg bw/d) of Av seed extract compared to control group. The serum AST is originally from cytoplasm and mitochondria and consequently any variance in AST level give an indication of hepatic destruction that lead to discharge of AST from liver into serum (Mukinda & Eagles, 2010). Thus, no observed increases in both AST and ALT hepatic enzymes support the evidences that Av seed ethanolic extract subacute administration did not induce any change in hepatic cells or metabolism in both male and female animals.

The Av seed ethanolic extract did not show any histopathological alterations in hepatic, nephrotic and splenic tissues which indicate no observable effect on reticuloendothelial system. Av seed ethanolic extract neither showed any observable variances in the weight of the organs or color of organs nor affected the histopathological changes in organs like heart, brain, testis and ovary indicating minimum increasing toxic effects on reproductive, cardiac and brain tissues.

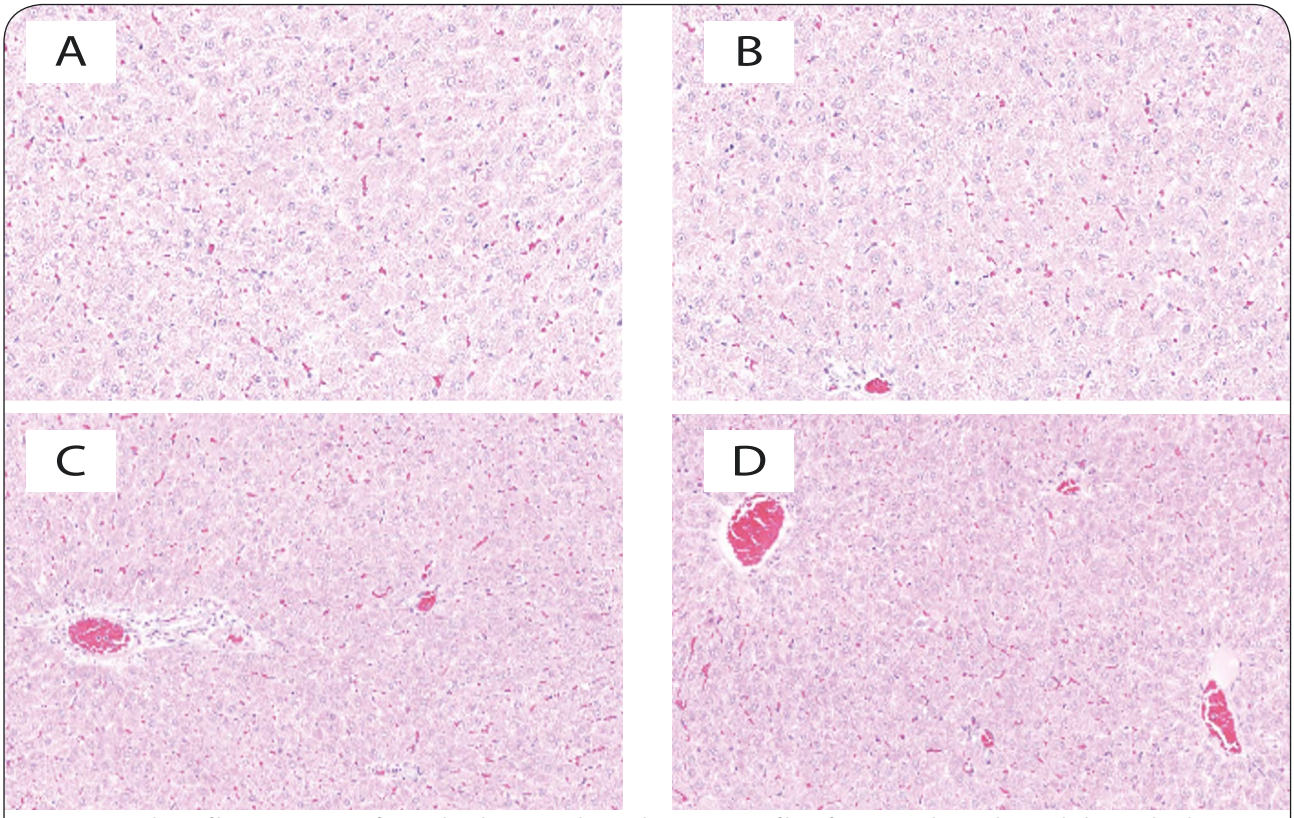

Figure 1. Histology of liver (H&E, 100x) of control and Av-treated animals. (A) Section of liver from control animals revealed normal architecture and hepatic cells with granulated cytoplasm; [(B), (C), and (D)] liver from Av (150, 300, and 600 mg/kg)-treated animals exhibited normal architecture of hepatocytes and hepatic cells with granulated cytoplasm.

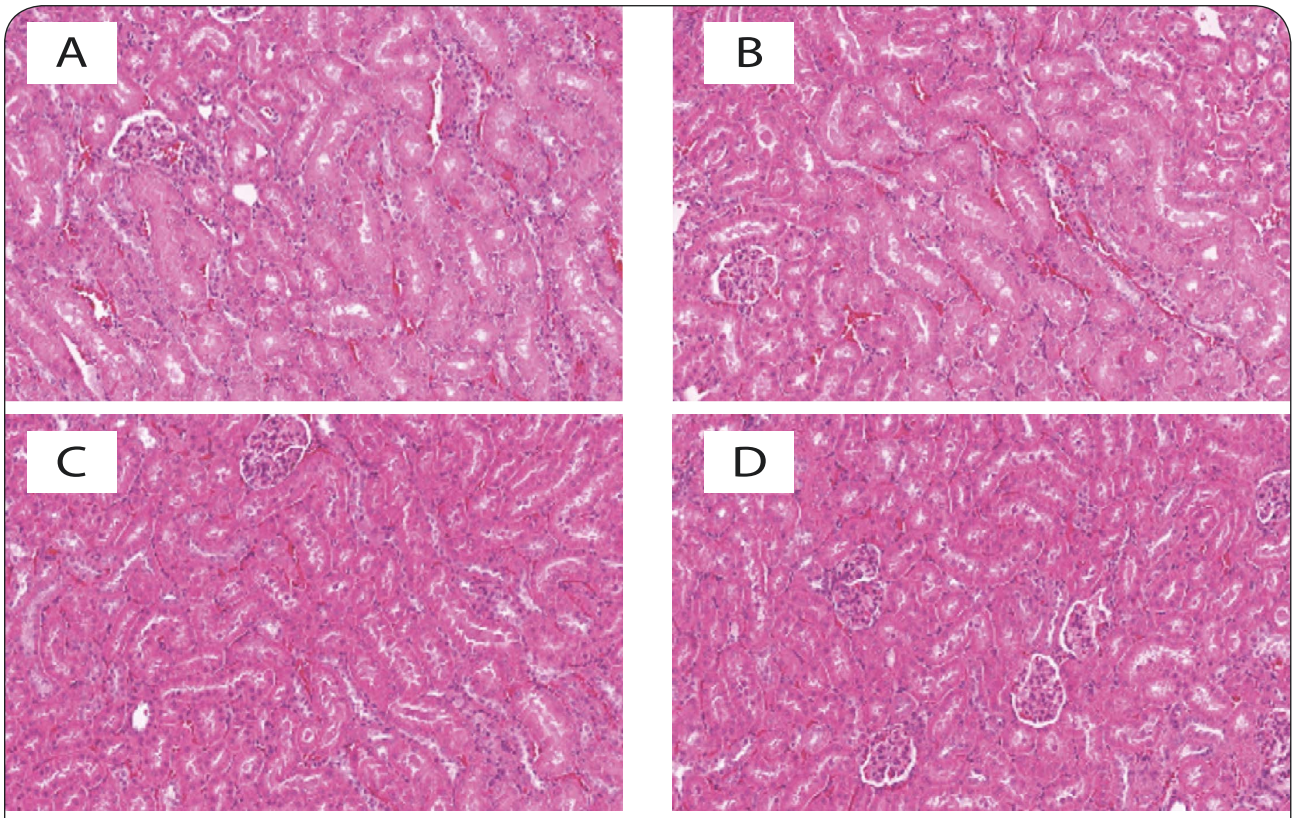

Figure 2. Histology of kidney (H&E, 100x) of control and Av-treated animals. (A) Section of kidney from control animals showed normal size of glomeruli with normal tubules; [(B), (C), and (D)] kidney from Av (150, 300, and 600 mg/kg)-treated animals exhibit normal size of glomeruli with normal tubules.

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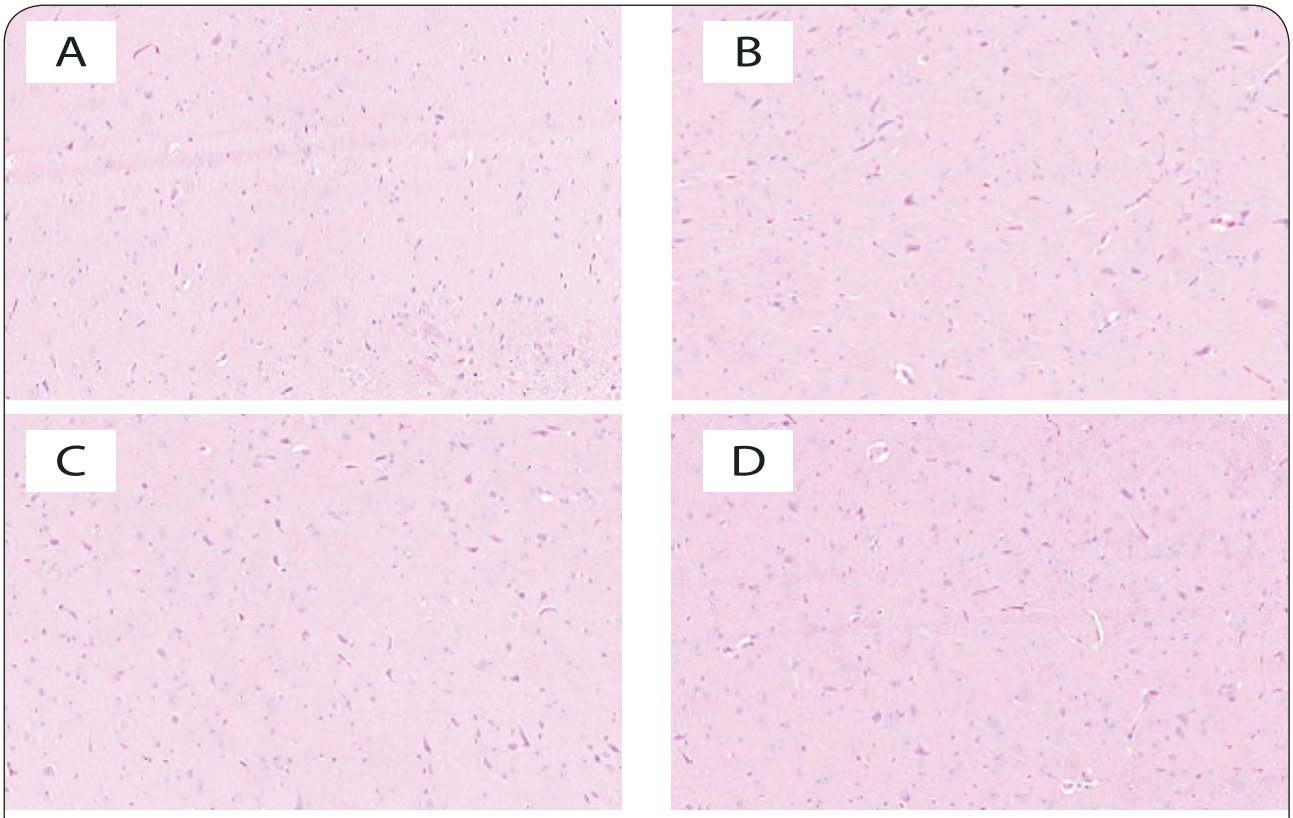

Figure 3. Brain sections of control rats showing the highly active nerve cells that have huge nuclei that relatively pale-stained (A). [(B), (C), and (D)] brain from Av (150, 300, and 600 mg/kg)-treated animals exhibit normal brain structure looks like control.

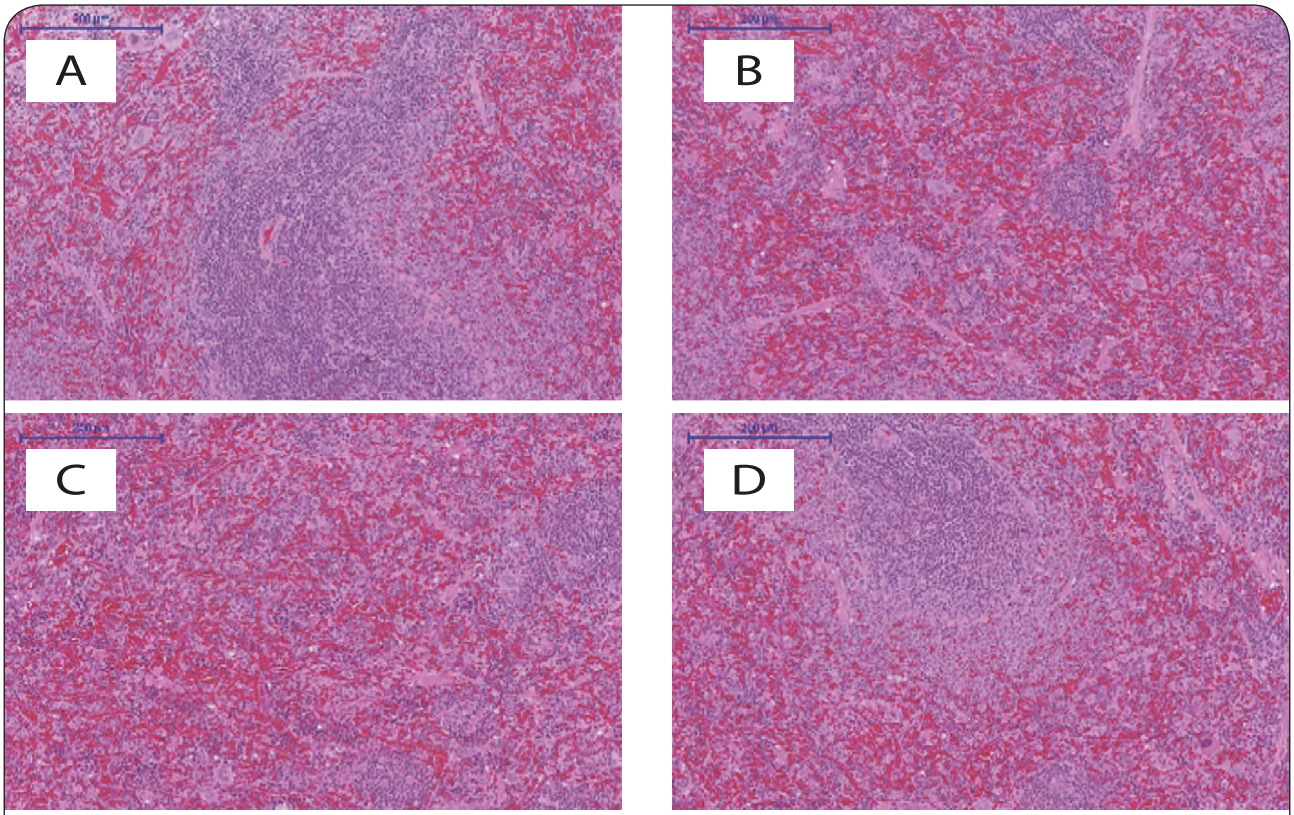

Figure 4. Histology of spleen (H&E, 100x) of control and Av-treated animals. (A) Section of spleen from control animals showed normal granular hemosiderin pigment predominantly within macrophages in the red pulp; [(B), (C), and (D)] spleen from Av (150, 300, and 600 mg/kg)-treated animals exhibit normal hemosiderin pigment predominantly within macrophages in the red pulp with normal structure.

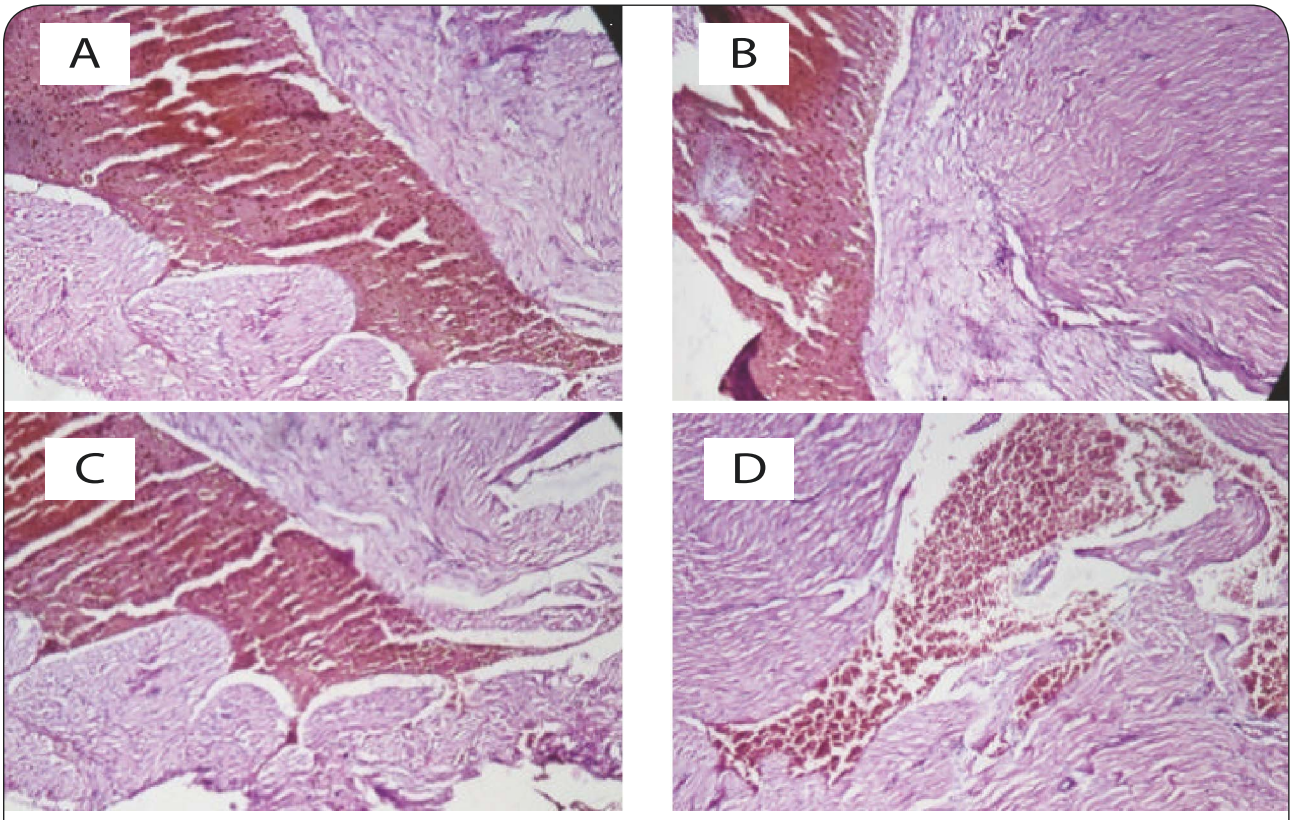

Figure 5. Histology of heart (H&E, 100x) of control and Av-treated animals. (A) Section of heart from control animals showed normalmuscle fibers with acidophilic cytoplasm and centrally located nuclei; [(B), (C), and (D)] heart from Av (150, 300, and 600 mg/kg)-treated animals exhibit normal muscle fibers with acidophilic cytoplasm and centrally located nuclei.

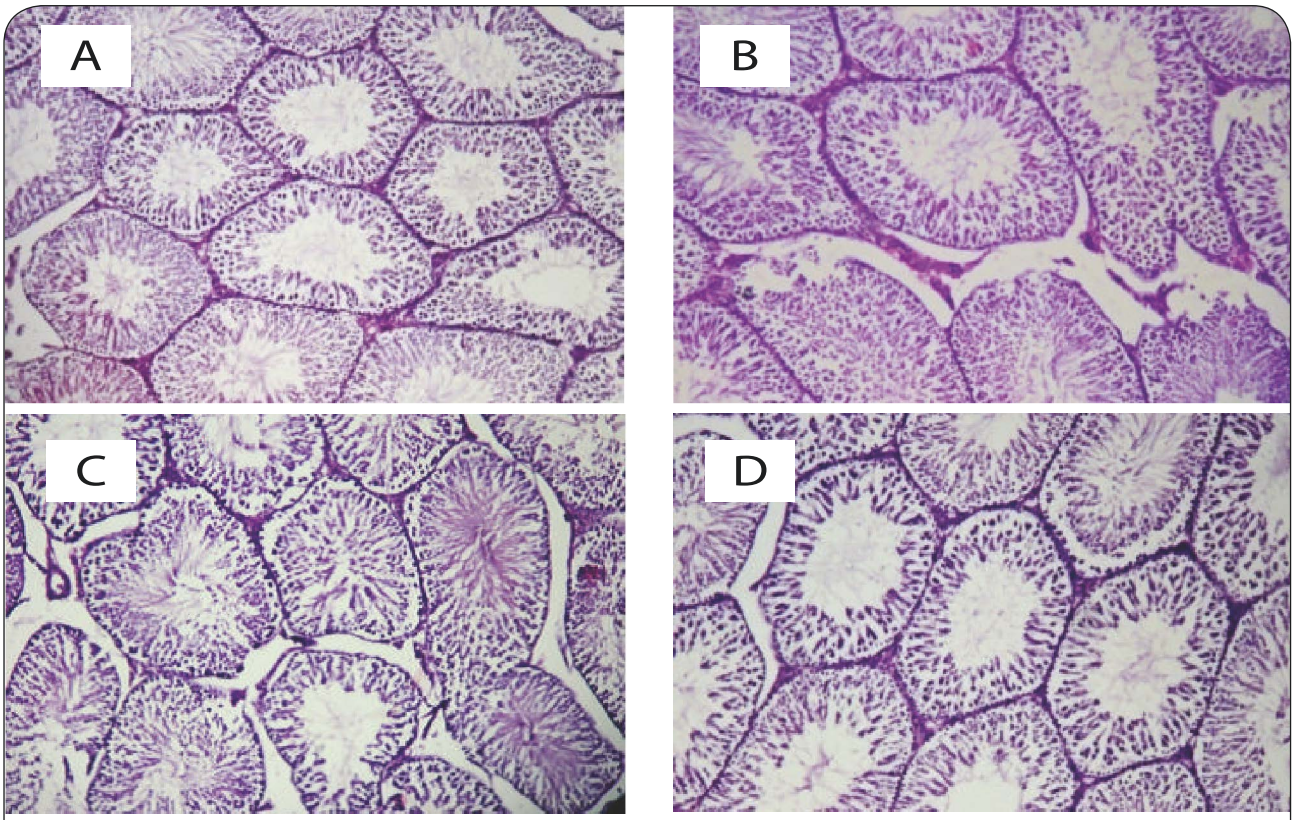

Figure 6. Histology of testis (H&E, 100x) of control and Av-treated animals. (A) Section of testis from control animals showed well-layered seminiferous tubules with germcell; [(B), (C), and (D)] testis from Av (150, 300, and 600 mg/kg)-treated animals exhibit normal seminiferous tubules with germ cell.

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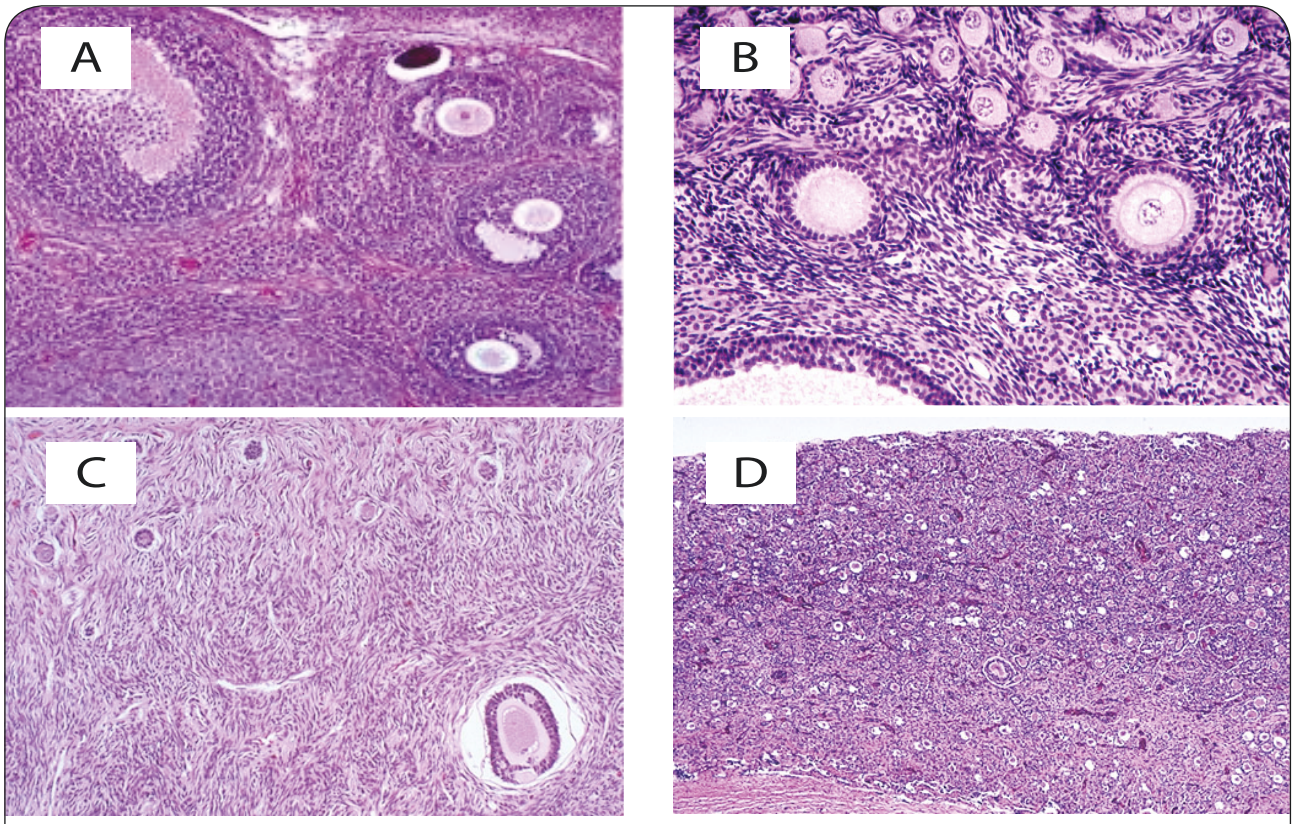

Figure 7. Histological of ovary (H&E, 100x) of control and experimental group of animals. (a) Section of ovary from control animal showed normal small follicles and large follicles; ((B), (C) and (D)) ovary from Av (150, 300, and 600 mg/kg)-treated exhibit normal small follicles and large follicles in the histology.

Finally, no observable alteration occurred in kidney function parameters represented by no variance in serum urea, creatinine and uric acid levels as well as histological changes in Av seed ethanolic extract-treated nephrotic tissues.

Conclusion

The study provides valuable data on the acute and subacute effects of *Ammi visnaga* in male and female rats. The doses of 5, 10 and 20% corresponding to 150, 300 and 600 mg/kg bw/d of Av did not cause any mortality or toxic effects in the acute toxicity study on rats. No significant clinical, hematological, biochemical, histopathological changes or levels of some hormones were observed in treated animals versus controls in 28-day oral toxicity study.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

REFERENCES

Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. (1974). Enzymatic determination of total serum cholesterol. *J Clin Chem* **20**: 470–475.

Anderson N, Borlak J. (2008). Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis. *Pharmacol Rev* **60**(3): 311–357.

Behrens H, Karber S. (1953). Determination of LD50. *Arch. Fur Exp Path Pharm* **3**: 177–372.

Benke D, Barberis A, Kopp S, Altmann KH, Schubiger M, Vogt KE, Rudolph U, Mohler H. (2009). GABAA receptors as in vivo substrate for the anxiolytic action of valerenic acid, a major constituent of valerian root extracts. *Neuropharmacol* **56**: 174–181.

Bent S, Padula A, Moore D, Patterson M, Mehling W. (2006). Valerian for sleep: a systematic review and meta-analysis. *Am J Med* **119**(12): 1005–12.

Boullata JI, Nace AM. (2000). Safety Issues with Herbal Medicine: Common Herbal Medicines. *Pharmacotherapy* **20**(3): 64–71.

Chopra RN, Nayar SL, Chopra IC. (1986). *Glossary of Indian Medicinal Plants (Including the Supplement)*. New Delhi, India: Council of Scientific and Industrial Research. pp. 402–405.

Chow NK, Fretz M, Hamburger M, Butterweck V. (2011). Telemetry as a tool to measure sedative effects of a valerian root extract and its single constituents in mice. *Planta Med* **77**: 795–803.

Ciarlone AE, Smudski JW. (1977). Lidocaine's influence on the accumulation and depletion rates of mouse brain amines. *J Dental Res* **56** (11): 1391–1394.

Denga Y, Caob M, Shia D. (2013). Toxicological evaluation of neem (Azadirachta indica) oil: acute and subacute toxicity. *Environ Toxicol Pharmacol* **35**: 240–246.

Drupt F. (1974). Determination of serum albumin by bromocresol green. *Pharma Biol* **8**: 77–99.

El Hilaly J, Israili ZH, Lyoussi B. (2004). Acute and chronic toxicological studies of Ajugaiva in experimental animals. *J Ethnopharmacol* **91**(1): 43–50.

ESCOP (European Scientific Cooperative on Phytotherapy). (2003). Valeriana officinalis. In: *ESCOP Monographs*, 2nd edition. Stuttgart: Thieme. pp. 539–546.

Gautam MK, Singh A, Rao CV, Goel RK. (2012). Toxicological evaluation of Murraya paniculata (L.) leaves extract on rodents. *Am J Pharmacol Toxicol* **7**(2): 62–67.

Gornall AC, Bardawill CJ, David MM. (1949). Determination of serum proteins by means of the Biuret-reaction. *J Biol Chem* **177**: 751–766.

Guide for the Care and Use of Laboratory Animals. Environment, Housing, and Management. (2011). National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. 8th edition. Washington (DC): National Academies Press (US). ; pp. 62–147.

Hadley S, Petry JJ. (2003). Valerian. *Am Fam Physician* **67**(8): 1755–8.

Hendriks H, Bos R, Woerdenbag HJ, Koster AS. (1985). Central nervous depressant activity of valerenic acid in the mouse. *Planta Med* **51**: 28–31.

Houot O. (1985). *Interpretation of clinical laboratory tests*. Edited by Siest G, Henny J, Schiele F, Young DS. Biomed Pub.; pp. 220–234.

Kabasakalian P, Kalliney S, Westcott A. (1973). Determination ofuric acid in serum, with use of uricase and atribromophenol-aminoantipyrine chromogen. *Clin Chem* **19**(5): 522–524.

Kachkoul R, Sqalli Houssaini T, Miyah Y, Mohim M, El Habbani R, Lahrichi A. (2018). The study of the inhibitory effect of calcium oxalate monohydrate's crystallization by two medicinal and aromatic plants: Ammi visnaga and Punica granatum. *Prog Urol* **28**(3): 156–165

Khom S, Baburin I, Timin E, Hohaus A, Trauner G, Kopp B, Hering S. (2007). Valerenic acid potentiates and inhibits GABAA receptors: molecular mechanism and subunit specificity. *Neuropharmacol* **53**: 178–187.

Khom S, Strommer B, Ramharter J, Schwarz T, Schwarzer C, Erker T, Ecker GF,Mulzer J, Hering S. (2010). Valerenic acid derivatives as novel subunit-selective GABAA receptor ligands – in vitro and in vivo characterization. *Br J Pharmacol* **161**: 65–78.

Kind PRN, King EG. (1954). Estimation of plasma phosphatase by determination of hydrolyzed phenol with amino antipyrine. *J Clin Pathol* **7**: 322–326.

Koriem KM, Arbid MS, El-Gendy NF. (2010). The protective role of Tropaeolum majus on blood and liver toxicity induced by diethyl maleate in rats. *Toxicol Mech Methods* **20**: 579–586.

Marder M, Viola H, Wasowski C, Fernández S, Medina JH, Paladini AC. (2003). 6-methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS. *Pharmacol Biochem Behav* **75**(3): 537–45.

Maruyama Y, Aoki N, Suzuki Y, Ohno,Y, Imamura M, Saika T, Sinohara H, Yamamoto T. (1987). Sex-steroid-binding plasma protein SBP, testosterone, oestradiol and dehydroepiandrosterone DHEA in prepuberty and puberty. *Acta Endocrinol. Copenhagen* **1141**: 60–67.

Mukinda JT, Eagles PFK. (2010). Acute and sub-chronic oral toxicity profiles of the aqueous extract of Polygala fruticosa in female mice and rats J *Ethnopharmacol* **128**(1): 236–240.

Mukinda JT, Syce JA. (2007). Acute and chronic toxicity of the aqueous extract of Artemisia afra in rodents. *J Ethnopharmacol* **112**(1): 138–144.

Murphy K, Kubin ZJ, Shepherd JN, Ettinger RH. (2010). Valeriana officinalis root extracts have potent anxiolytic effects in laboratory rats. *Phytomedicine* **17**: 674–678.

Neuhaus W, Trauner G, Gruber D, Oelzant S, Klepal W, Kopp B, Noe CR. (2008). Transport of a GABAA receptor modulator and its derivatives from Valeriana officinalis L. s. l. across an in vitro cell culture model of the blood-brain barrier. *Planta Med* **74**: 1338–1344

OECD. (2001). Acute Oral Toxicity: Acute Toxic Class Method. Test Guideline. pp. 423–435.

OECD. (2008). Repeated Dose 28-day oral toxicity study in rodents In "OECD Guidelines For the Testing of Chemicals, Section 4: Health Effects" OECD Publishing.

Olson H, Betton G, Robinson D. (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regulat Toxicol Pharmacol* **32**(1): 56–67.

Patočka, J, Jakl J. (2010). Biomedically relevant chemical constituents of Valeriana officinalis. *J Appl Biomed* **8**(1): 11–18.

Patton CJ, Crouch SR. (1977). Spectrophotometric and kinetic investigation of the Berthelot reaction for the determination of ammonia. *Anal Chem* **49**: 464–469.

Pavela R, Vrchotová N, Tříska J. (2016). Larvicidal activity of extracts from Ammi visnaga Linn. (Apiaceae) seeds against Culex quinquefasciatus Say. (Diptera: Culicidae). *Exp Parasitol*. **165**: 51–7.

Rahman MF. (2001). Effects of Vepacide (Azadirachta indica) on aspartate and alanine aminotransferase profiles in a subchronic study with rats. *Human Exper Toxicol* **20**(5): 243–249.

Reitman S, Frankel S. (1957). A colorimetric method for the determination of serum glutamic oxaloacetic acid and glutamic pyruvic transaminases. A*m J Clin Pathol* **28**: 56–63.

Roch ABD, Lopes RM, Schwartsmann G. (2001). Natural products in anticancer therapy. *Curr Opin Pharmacol* **1**: 364–369.

Rodak LC. (1995). Routing testing in haematology in: *Dignostic haematology* W.B. Saunders Comp. Philadelphia London, Toronto; pp.128–144.

Saad B, Azaizeh H, Abu-Hijleh G, Said O. (2006). Safety of traditional Arab herbal medicine. *Evidence-Based Complemen Altern Med* **3**(4): 433–439.

Talalay P. (1962). Hydroxysteroid dehydrogenase. In: Cloowick, S.P., Kaplan (Eds.), *Methods in Enzymology*, vol. 11. Academic Press, NY, pp. 512–516.

Travaini ML, Sosa GM, Ceccarelli EA, Walter H, Cantrell CL, Carrillo NJ, Dayan FE, Meepagala KM, Duke SO. (2016). Khellin and Visnagin, Furanochromones from Ammi visnaga (L.) Lam., as Potential Bioherbicides. *J Agric Food Chem.* **64**(50): 9475–9487.

Trinder P. (1969). Determination of glucose in blood using glucoseoxidase with an alternative oxygen acceptor. *Ann Clin Biochem* **6**: 24–27.

Vanachayangkul P, Byer K, Khan S, Butterweck V. (2010). An aqueous extract of Ammi visnaga fruits and its constituents khellin and visnagin prevent cell damage caused by oxalate in renal epithelial cells. *Phytomedicine* **17**(0): 653–658.

Van Kampen E, Zijlstra WG. (1961). Standardization of hemoglobinometry. II. The hemoglobin cyanide method. *Clin Chim Acta* **6**: 538–544.

Vihma V, Adlercreutz H, Tiitinen A, Kiuru P, Wähälä K, Tikkanen MJ. (2001). Quantitative determination of estradiol fatty acid esters in human pregnancy serum and ovarian follicular fluid. *Clin Chem* **47**(7): 1256 –1262.

Waltenberger B, Mocan A, Šmejkal K, Heiss EH, Atanasov AG. (2016). Natural Products to Counteract the Epidemic of Cardiovascular and Metabolic Disorders. *Molecules* **21**(6): 33 pages.

Walter M, Gerarde H. (1970). Ultra micro method for the determination of conjugated and total bilirubin in serum or plasma. *Microchem J* **15**: 231–238.

Weeks, Benjamin S. (2009). Formulations of dietary supplements and herbal extracts for relaxation and anxiolytic action: Relarian™. *Med Sci Monit* **15** (11): RA256–262.

WHO (World Health Organization). (2008). WHO Guidelines on Safety Monitoring of Herbal Medicines in *Pharmacovigilance Systems*, WHO (World Health Organization), Geneva, Switzerland, pp. 105–114.

Wills RBH, Shohet D. (2009). Changes in valerenic acids content of valerian root (Valeriana officinalis L. s.l.) during long-term storage. *Food Chem* **115** (1): 250–253.

Yakob HK, Uyub AM, Sulaiman SF. (2012). Toxicological evaluation of 80% methanol extract of Ludwigia octovalvis (Jacq.) P.H. Raven leaves (Onagraceae) in BALB/c mice. *J Ethnopharmacol* **142**: 663–668.

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ORIGINAL ARTICLE

Experimental study of imiprotrin allergic potency in case of inhalation

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ABSTRACT

Probable changes were studied in rats' immune status under experimental conditions with inhalation route of type I pyrethroid–imiprotrin administration, which is the main component of a number of household insecticidal agents. The drug at a concentration of 45.0 mg/m^3 interrupts immunological homeostasis in experimental animals. Nonspecific cellular component parameters of immune system have changed significantly. Imiprotrin is capable of inducing delayed hypersensitivity. Imiprotrin induces sensibilization under experimental conditions in more than half of the experimental animals, but the magnitude of the reactions to the intradermal administration of the drug has no probable differences, which allows imiprotin to be attributed to substances with moderate sensibilization potential

KEY WORDS: imiprotrin; immunological homeostasis; laboratory rats

Introduction

A steadily increasing tendency is observed within the last decade in the active use of household insecticides including use inside the houses (Sarwar, 2016; Titiek *et al.,* 2011). Pyrethroids are the active ingredients in a large list of these agents. It was reported that the route for these compounds in confined spaces through respiratory tract is a significant negative factor in human living environment (Bradberry *et al.,* 2005), and their long-term effect leads to the development of humoral and cellular reactions, skin hypersensitivity, and respiratory disturbances (Macan *et al.,* 2006). The studies in volunteers showed that after pyrethroids use under the recommended conditions and doses, the modulation of immune components within the physiological range towards the low values during the first days was observed. Although these changes were interpreted as manifestations of immunoregulation compensatory mechanisms (Hadnagy *et al.,* 2003), possible immune system changes caused by pyrethroids should not be neglected. The experiment in rats showed immunotoxic effects induced by aerosol which contained pyrethroids mixture of imiprotrin and deltametrin (Emara & Draz, 2007).

The purpose of these studies was to detect, under experimental conditions, the probable changes in the immune status of the experimental animals after inhalation of type I pyrethroid-imiprotrin, which is the main component in a number of household insecticides.

Methods

Experimental animals and their maintenance

Experimental study was carried out in 20 white, outbred male rats aged 3–3.5 months with a body weight of 180–200 g. Experimental animals received standard granular feed with unlimited access to drinking water. The animal studies were conducted in compliance with the bioethics principles, legislation, and requirements in accordance with the provisions of the "European Convention for the Protection of Vertebrate Animals used for Research and Scientific Purposes" (European Communities, 1986).

The animals were randomly divided into two groups of 10 animals in each: experiment and control.

Chemicals and treatment

Imiprotrin is a synthetic insecticide of the pyrethroid group, a derivative of cyclopropanecarboxylic acid. CAS Number 72963-72-5. Molecular weight – 318.37 mg/mol.

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Sensibilization in white rats was carried out by single intranasal administration of imiprotrin at a dose corresponding to a concentration of 45.0 mg/m^3. Concentration was chosen based on the previous experimental results, where this concentration was established as a threshold value (Hrushka & Turkina, 2017). The conversion of the administered dose into concentration was carried out by the formula:

D = C × V, where C – drug concentration, mg/m^3

D – drug dosage (mg) administered to animals

V – air volume inhaled by rats within 4 hours

Control animals were injected intranasally with a solvent (tween oil).

Clinical and immunological tests

Changes in peripheral blood parameters (WBC absolute count and WBC differential) were determined. Based on WBC differential the ratio of individual WBC populations was calculated. Hematological parameters were calculated: lymphocyte to monocyte ratio (LMR), neutrophil to monocyte ratio (NMR), neutrophil to eosinophile ratio (NER).

Humoral immunity parameters

Immunoglobulin (Ig) E in blood serum was determined by ELISA test according to the instructions provided with the enzyme-immunoassay analyzer "STAT FAX PLUS-303".

Circulating immune complexes (CIC) were determined by precipitation of large globular blood immune complexes with macromolecular polyethylene glycol, spectrofluorometric method.

Allergic tests *in vivo*

Ear swelling challenge test (ESCT) was performed one week after sensibilization to characterize the delayed-type allergic reactions.

ESCT setting: thickness of the middle external ear was pre-measured in mm using micrometer, then 25 ml of the experimental substance was applied on both surfaces of the middle third of the fixed ear in concentration that does not cause non-allergic contact dermatitis. Ear thickness was re-measured in 24 hours with the calculation of EST (swelling challenge test) value based on the thickness difference before and after application. The severity of compound's allergic effect was determined in comparison with the control by swelling intensity. EST is considered positive with a value of 0.03 mm or above. In order to exclude the influence of nonspecific factors on delayed hypersensitivity assessment by ESCT absolute terms in control and experimental animals, as well as to determine the integral evaluation criteria for delayed hypersensitivity (by incidence and severity of skin reactions) ESCT score was used: ESCT absolute value in mm with a gradation of 0.03–0.07; 0.08–0.12; 0.13–0.17; 0.18–0.22; 0.23 and corresponds to 1; 2; 3; 4; and 5 points, respectively

Immunological tests *in vitro*

In order to perform quantitative assessment, a detection method was used ten days after animal sensibilization of blood cells reaction to allergen *in vitro* – specific leukocyte agglomeration reaction (SLAR), specific leukocyte lysis reaction (SLLR) and neutrophil damage index (NDI). These tests allow detecting the delayed allergic reaction caused by cell sensibilization.

SLAR setting: two test tubes were added with 0.04 ml 5% citrate each. Citrate was added to test tube, which contained imiprotrin, the second tube was a control one. Then, 0.2 ml of blood was added to all test tubes and incubated at 37 °C for 2 hours. Subsequently, smears were prepared on degreased slides, dried at room temperature within 24 hours and dyed with 0.1% aqueous methylene blue solution for 10 minutes, then rinsed gently with water. 500 leukocytes were counted in each smear, estimating the number of cells forming aggregates of three or more cells. Then the percentage of agglomerated leukocytes was calculated.

SLLR setting: test tubes were added with 0.05 ml of saline solution, where the experimental test tube was added with saline solution and imiprotrin. Then, 0.1 ml of experimental animals' blood was added to the test tubes and incubated at 37 °C for 2 hours. The resulting solution was then transferred in 0.02 ml into plate wells containing 0.4 ml of Turk solution. The absolute number was counted and calculations made by the formula:

SLLR = (Lc–Le) / Lc*100, where L – absolute number of leukocytes in control (Lc) and experiment (Le).

The reaction was considered positive only with an indicator more than or equal to 10%.

For NDI setting as an anti-coagulant 5% aqueous solution of sodium citrate in saline solution was used. The working concentration of the experimental substance is prepared based on the same anticoagulant, which is added to the blood. First silicone centrifuge tube (experimental sample) was added with 0.1–0.2 ml of the experimental substance solution, second (control) was added with the same amount of the experimental animal blood, after which the test tubes are stirred and kept at room temperature for 1 hour. At the end of incubation smears are prepared on slides, which are fixed and stained using the method for leukocyte count. Count 100 neutrophils in immersion, taking into account the amount of chromatinolysis, picnose, fragmentation of nuclei, hyperchromatosis or carriolysis. The reaction rate is calculated by dividing difference in the number of damaged neutrophils by 100 in experimental and control samples.

Statistical analysis

Statistical processing of results was carried out using Microsoft Excel. The verification of obtained data compliance with the normal law of distribution was carried using Shapiro-Wilk's W test. Accuracy of changes received for comparative values was estimated using Student's t-test. Changes were considered accurate, if significance level exceeded 95% (p<0.05) (Glantz & Slinker, 1990).

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Results and discussion

Changes in leukocyte count and leukocytal indices

Imiprotrin inhalation route does not affect the total leukocyte count in experimental animals as compared to the control group. Leukocyte count analysis showed the destabilizing effect of the drug on blood functional state, which was manifested by an increase in the absolute and relative number of eosinophils and neutrophils, severe lymphopenia, with no changes in relative and absolute quantitative parameters of other leukocyte subpopulations (Table 1).

Allergic disorders caused eosinophilia by binding IgE antigen complexes to the surface of mast cells. It should be noted that eosinophilic granulocyte plays a significant role in the pathophysiological phase of hypersensitivity. Eosinophil cationic protein (ECP) induces the secretion of histamine from mast cells and basophils, actively involved in inflammatory reactions; inhibits the proliferation of T-lymphocytes, provides regulation of cell-mediated immune responses. In modern literature, it is considered an active actor in developing allergic diseases and an important element in maintaining immunological homeostasis (Hogan *et al.,* 2003).

In addition, substantial effect of imiprotrin on the immune status of experimental animals in our experiment is evidenced by an increase in the number and content of neutrophils. Neutrophils play one of the leading roles in developing and maintaining inflammatory reactions that can rapidly increase metabolic rate and can be used as an objective criterion for assessing the effector component of immune system. Due to antigen-presenting and effector functions, neutrophils can be the actors and regulators of immune response. The implementation significance of granulocytes functional potential in developing and overcoming allergic diseases is justified by the experimental data from several authors (Nathan, 2006). Engagement of eosinophils and neutrophils in allergen-specific reactions is confirmed by the presence of Fce receptors to IgE on their surface (Gounni *et al.,* 2001).

The main role in immune responses is played by lymphocytes that recognize antigens. Our study found relative lymphopenia in experimental animals against the background of neutrophilia. This indicates the defect in cellular immunity, namely the failure of the lymphocyte component. In clinical practice, this pattern is characteristic of acute allergenic pneumonia (Kurup *et al.,* 2006).

Hematological indices analysis shows an increase in NMR indicating an imbalance in the components of microfagal-macrophage system and LMR decrease indicating the suppression of the effector component of the immune response. In general, experimental animals show abnormal immunologic reactivity.

The pattern of leukocytes count found in experimental animals is characteristic of immune-dependent inflammatory processes in the body and suggests a shift in immunological homeostasis under imiprotrin effect. The analysis of identified changes allows classifying them as allergenic ones. The experiment results obtained are

Table 1. Changes in the immunological status of rats exposed to imiprotrin test

Parameters	Control	Experiment	t_{exp}
Leucocytes, G/L*	7.97±0.7	8.63±1.26	1.45
Leukocyte count:			
basophils,%	0.2±0.42	0.3±0.48	0.5
basophils, G/L*	0.0166±0.035	0.027±0.044	1.0
eosinophils,%	2.4±0.7	4.4±1.35	4.2
eosinophils, G/L*	0.19±0.06	0.39±0.17	4.0
neutrophils,%	17.1±2.47	23.4±3.4	4.7
neutrophils, G/L*	1.37±0.28	2.04±0.5	3.7
monocytes,%	2.2±0.42	2.4±0.52	1.0
Monocytes, G/L*	0.176±0.04	0.207±0.054	1.5
lymphocytes,%	78.1±3.14	69.6±3.44	5.8
lymphocytes, G/L*	6.22±0.48	5.98±0.7	0.9
Haematological parameters:			
NER	7.72±2.7	5.81±2.06	1.8
NMR	8.0±1.8	10.2±2.73	**2.1**
LMR	36.47±5.81	30.1±5.88	**2.4**
Humoral immunity:			
CIC, cu	91.10±39.53	124.10±30.63	0.8
IgE, IU d/ml	5.74±2.6	7.82±3.8	1.4

The values are expressed as mean ± SDev. (n=10); $p<0.05$ compared with respective control rats; Student's t-test – t_{th} (theoretical) = 2.1; t_{exp} – experimental t-value; *G/L – Giga cells per liter

Table 2. Results of allergic tests in vivo and in vitro in rats exposed to imiprotrin test.

Parameters	Control	Experimental	t_{exp}
specific leukocyte lysis reaction (SLLR)			
H	1/10	8/10	
Absolute count	7.74±0.44	9.80±0.80	1.59
%	6.29±1.06	12.11±1.20	1.46
Specific leukocyte agglomeration reaction (SLAR)			
H	7/10	10/10	
Absolute count	7.9±4.01	9.9±3.41	1.2
%	0.83±0.57	1.41±0.14	3.2
Neutrophil damage index (NDI)			
H	0/10	2/10	
Absolute count	5.8±0.9	7.9±0.56	1.96
%	0.013±0.002	0.04±0.006	3.66
Ear swelling challenge test (ESCT)			
H	4/10	9/10	
10-2 mm	0.18±0.06	0.24±0.06	0.4
score	1.6±2.27	3.6±0.5	1.1

Note: The values are expressed as mean ± SDev. (n=10); $p<0.05$ compared with respective control rats; Student's t-test - t_{th} (theoretical) = 2.1; t_{exp} - experimental t value; H – number of positive animals in numerator, denominator – total in experiment.

consistent with changes in cellular component of immune system in the experimental animals under imiprotrin effect, which we have discovered earlier (Hrushka & Turkina, 2017).

Humoral component of immune system

Humoral component of immune system showed no probable differences in the circulating immune complexes count in sensitized and control animals (Table 1). This shows the absence of immunocomplex pathology in experimental animals.

IgE is unchanged in relation to control, presumably because the pathological processes pass through IgE-independent pathway. This mechanism of respiratory hypersensitivity is discussed in modern scientific literature for non-atopic allergies (Cochrane *et al.,* 2015). It is important that in clinical practice with a confirmed diagnosis of allergenic pneumonia serum IgE in patients remain within the limits of normal (Kurup *et al.,* 2006).

Results of allergic tests *in vivo* and *in vitro*

The sensitized animals were found with an increase in blood cells response to imiprotrin effect, in particular, a significant increase in percentage of SLAR – 1.7-fold, NDI – 2.5-fold vs control (Table 2).

Agglomeration is the first phase of cells allergenic reaction. SLAR results indicate the development of delayed-type allergenic response after inhaled imiprotrin effect.

NDI changes from 0.02 to 0.07 were observed in the experimental group (critical value increase of 0.05 was observed in two animals from experimental group), while this index in all control animals did not exceed 0.02. NDI increase observed in experiment with imiprotrin action is due to the allergen effect on growth of neutrophil granulocytes, which have amoeboid activity, and indicates a specific sensitivity the body.

SLLR results are not unambiguous. No significant differences were found in leukolysis parameters of the experimental and control groups. At the same time, there was a steady tendency to this parameter increase in animals of the experimental group. 10% increase in its critical value was observed in 80% of experimental animals. It ranged from 0 to 8% in control, and only one animal exceeded the critical value of 10%.

ESCT increase in the experimental animals in the integrated assessment has no statistically significant differences compared to the control group. In absolute terms, after an intranasal administration of the drug, a positive ear swelling challenge test (ESCT) was detected in 9 out of 10 experimental animals. The average absolute value 1.5-fold exceeded the control values.

Thus, *in vivo* and *in vitro* tests confirmed the assumptions on allergic nature of changes in peripheral blood parameters in experimental animals. In general, based on the results obtained, the immune system reaction to imiprotrin inhalation effect can be attributed to a neutrophil-dependent type (Gounni *et al.,* 2001).

Consequently, the sensitizing effect analysis indicates that delayed hypersensitivity is the most probable outcome of body sensitivity with this imiprotrin route of administration at a dose of 45 mg/m^3. Taking into account the important role of immune system in preserving body balance and the risk of developing pathological conditions in case of its abnormality, it is important to develop regulations for imiprotrin in environmental setting (Cochrane *et al.,* 2015).

Conclusions

The inhalation effect of imiprotrin at a concentration of 45.0 mg/m^3 causes an impairment of immunological homeostasis in experimental animals. Nonspecific cellular component parameters of immune system have changed significantly due to imiprotrin effect, which reflects systemic processes occurring in the body as a whole.

The ability of imiprotrin to induce delayed hypersensitivity has been identified. Imiprotrin, under conditions of experimental reproduction and detection of delayed hypersensitivity, causes sensibilization in more than half of experimental animals, but the magnitude of reactions to intradermal administration of the drug has no probable differences, which allows imiprotrin to be classified as a substances with moderate sensitizing potential.

Thus, the data obtained indicate the need for further studies of imiproproline's lower concentrations in order to establish safe levels of its effect. It should be noted that at low concentrations, there appears a problem of controlling the factors that may lead to a lowered dose, namely mechanical loss in the esophagus, mechanical loss to other regions in the nose, and mechanical loss anteriorly from nose. Therefore, there is a need to control the concentration of the substance in the blood plasma. An emphasis of the future research should be put on sensitive tests for detection of the allergic alterations in the body at the interleukin or chemokine levels.

REFERENCES

Bradberry SM, Cage SA, Proudfoot AT, Vale JA. (2005). Poisoning due to pyrethroids. *Toxicol Rev* **24**(2): 93–106.

Cochrane SA, Arts JH, Ehnes C, Hindle S, Hollnagel HM, Poole A, Suto H, Kimber I. (2015). Thresholds in chemical respiratory sensitisation. *Toxicology* **333:** 179–194.

Emara AM , Draz EI. (2007). Immunotoxicological study of one of the most common over-the-counter pyrethroid insecticide products in Egypt. *Inhal Toxicol* **19**(12): 997–1009.

European Communities (EC) [webpage on the internet] (1986). – European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. Strasbourg, 18.III.1986. European Treaty Series No. 123. [accessed on 11 April 2005] Available from: www.conventions.coe.int/treaty/en/treaties/html/123.htm

Glantz SA, Slinker BK. (1990). *Primer of Applied Regression and Analysis of Variance*. New York: McGraw-Hill, Health Professions Division.

Gounni AS, Lamkhioued B, Koussih L, Ra C, Renzi PM, Hamid Q. (2001). Human neutrophils express the high-affinity receptor for immunoglobulin E (FcRI): role in asthma. *FASEB J* **15**(6):940–949.

Hadnagy W, Leng G, Sugiri D, Ranft U, Idel H. (2003). Pyrethroids used indoors – Immune status of humans exposed to pyrethroids following a pest control operation – a one year follow-up study. *Int J Hyg Environ Health* **206**(2): 93–102.

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Hogan SP, Rosenberg HF, Moqbel R, Phipps S, Foster PS, Lacy P, Kay AB, Rothenberg ME. (2008). Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy* **38**(5): 709–750.

Hrushka OI, Turkina VA. (2017). Досліження впливу іміпротрину на клітинний імунітет лабораторних тварин. *International research and practice conference "Innovative technology in medicine: experience of Poland and Ukraine"* 165–168.

Kurup VP, Zacharisen MC, Fink JN. (2006). Hypersensitivity pneumonitis. *Indian J Chest Dis Allied Sci* **48**(2): 115–128.

Macan J, Varnai MV, Turk, R. (2006). Zdravstveni učinci piretrina i piretroida. *Arh Hig Rada Toksikol* **57**: 237–243

Nathan C. (2006). Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**(3): 173–182.

Sarwar M. (2016). Indoor risks of pesticide uses are significantly linked to hazards of the family members. *Cogent Medicin* **3**(1): 1155373

Titiek SY, Hermanu T, Kooswardhono M, Nurmala K. P, dan Sjafrida M. (2011). Pesticide Use of Behavior: A Case Study of Household Pest Control in DKI Jakarta Urban Settlement. *Forum Pasca Sarjana* **34**(3): 195–212.

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ORIGINAL ARTICLE

Short communication: Chlorpromazine causes a time-dependent decrease of lipids in *Saccharomyces cerevisiae*

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ABSTRACT

Chlorpromazine (CPZ) is still a commonly prescribed antipsychotic which causes poorly understood idiosyncratic toxicity such as cholestasis, phospholipidosis and steatosis. CPZ has diverse cellular targets and exerts various toxicity mechanisms whose exploration is necessary to understand CPZ side effects. We report here that CPZ causes a decrease of total lipid content in *Saccharomyces cerevisiae* at the same dose range as that used on mammalian cells. The observed lipid decrease was obvious after 4 and 9 hours of treatment, and disappeared after 24 hours due to cells adaptation to the chemical stress. The inhibitory effect of CPZ was antagonized by the antioxidant N-acetyl L-cysteine and is likely caused by the parent compound. The obtained results demonstrate that yeast model is valid to investigate the involved CPZ toxicity mechanisms, particularly in terms of lipids alteration. This would contribute to understand CPZ side effects in simple model and reduce experimentation on animals.

KEY WORDS: phenothiazine; chlorpromazine toxicity; *Saccharomyces cerevisiae*; lipids; oxidative stress

Introduction

Chlorpromazine (CPZ) is a first generation antipsychotic phenothiazine which is still commonly prescribed (Dudley *et al.,* 2017). CPZ mediates its antipsychotic effects by blocking the dopamine D2 receptors (Suzuki *et al.,* 2013). The therapeutic use of CPZ is associated with poorly understood idiosyncratic adverse reactions, mainly hepatotoxicity, due to its numerous molecular targets. CPZ toxicity mechanisms are diverse with an emphasis on oxidative stress and alteration of the expression of genes involved in transport across the cell membrane and in lipid metabolism (Antherieu *et al.,* 2013; Dejanović *et al.,* 2017; Hu & Kulkarni, 2000). CPZ causes also membrane structure alteration (Kamada *et al.,* 1995; Morgan *et al.,* 2019).

In different experimental models, CPZ induces phospholipidosis and steatosis (Bachour-El Azzi *et al.,* 2014). Phospholipidosis is characterized by intra-cellular accumulation of phospholipids as lamellar bodies (Anderson & Chan, 2016). Moreover, phenothiazines, including CPZ, cause alteration of the lipid profile by affecting the activity of several enzymes involved in lipid metabolism (Hoshi & Fujino, 1992; Ide & Nakazawa, 1980; Martin *et al.,* 1986). In humans, treatment with phenothiazines elevates serum triglyceride and total cholesterol levels (Saari *et al.,* 2004). These changes in lipid metabolism could also be mediated by the effect of CPZ on microtubules and possibly the traffic of vesicles inside the cell (Thyberg *et al.,* 1977), in addition to the alteration of lipid metabolism-related enzymes.

Investigating the mechanisms of CPZ toxicity, such as cholestasis and lipids alteration, in human is difficult for ethical reasons. Although diverse *in vitro* models are in use (Morgan *et al.,* 2019), CPZ toxic effects remain ambiguous. CPZ toxicity may be due to the parent molecule as well as to its metabolites (Abernathy *et al.,* 1977; Parmentier *et al.,* 2013; Tavoloni & Boyer, 1980; Yeung *et al.,* 1993) such as (mono-N-demethylated CPZ, di-N-demethylated CPZ, CPZ sulfoxide). These metabolites are not detected in *Saccharomyces cerevisiae* (our unpublished data) probably due to the lack of cytochromes P450 homologous to CYP1A2 and CYP3A4 which catalyze CPZ

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biotransformation (De Filippi *et al.,* 2007; Wójcikowski *et al.,* 2010).

In this study on *Saccharomyces cerevisiae*, we investigated CPZ toxic effects on yeast lipid content and we indirectly assessed the involvement of oxidative stress. CPZ acted in a similar manner on *Saccharomyces cerevisiae* relative to mammalian cells in terms of oxidative stress involvement. Total lipid amount was lower in CPZ-treated cells. Our data validate the use of the yeast model to investigate CPZ toxicity mechanisms, especially regarding lipid alteration.

Materials and methods

Saccharomyces cerevisiae BY4741 wild type strain was supplied by Euroscarf-Germany and used in our experiments. Yeast cells were grown at 30°C in Yeast Peptone Dextrose (YPD, Sigma-Germany) rich medium (1% yeast extract, 2% peptone and 2% dextrose), pH=7.4. Yeast cells from the mid exponential growth phase were treated with CPZ (Abcam Biochemicals, USA) and growth was assessed by spectrophotometry (630 nm) in a dose- and time-dependent manner. N-acetyl L-cysteine (NAC) was from Armesco USA.

Yeast cells were lysed mechanically and total proteins were measured using the Bradford assay. Total lipid extraction was performed using ice-cold chloroform/methanol (2:1) as previously described (Knittelfelder & Kohlwein, 2017). Total lipid measurement was performed by gravimetry using a precision balance (Boeco, Germany, d=0.1 mg) as well as by spectrophotometry at 580 nm using Sudan black as previously described (Thakur *et al.,* 1989).

Table 1. Effect of CPZ ± NAC on lipid content in *S. cerevisiae*.

Lipid (relative to control)	CPZ 20 µM	CPZ 50 µM	NAC 5 mM	NAC+CPZ 50 µM
4 hours	0.30±0.23*	0.37±0.15*	nd	nd
9 hours	nd	0.53±0.08*	0.89±0.07	1.07±0.15#
24 hours	nd	0.85±.14	1.11±0.04	1.62±0.5

Yeast cells from the mid exponential growth phase were treated with the indicated doses and total lipids extracted and assessed by spectrophotometry and gravimetry at the indicated time points. The results were normalized to OD values of the culture at each time point, and then expressed relative to the value in the control untreated cells. The values correspond to the average±SEM of 4 to 9 independent experiments. Statistically significant difference compared to the control (*) and to CPZ 50 µM (#), $p<0.05$. nd, not determined.

Table 2. Effect of CPZ ± NAC on total protein content in *S. cerevisiae*.

	Control	CPZ 50 µM	NAC 5mM	NAC+CPZ 50µM
Proteins (mg/ml)	5.73±0.85	2.69±0.65*	5.68±0.37	4.42±1.72#

Yeast cells from the mid exponential phase were treated with the indicated dose for 24 hours and then collected and lysed. Total proteins were measured by spectrophotometry. The results are normalized to OD values of the culture. The values correspond to the average±SEM of 4 to 9 independent experiments. Statistically significant difference compared to the control (*) and to CPZ 50 µM (#), $p<0.05$.

Data were expressed as means ± SEM from four to nine independent experiments. They were statistically analyzed using GraphPad Prism software through analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test, or F-test. The criterion of significance for statistical tests was $p<0.05$. The half maximal inhibitory concentration IC50 of CPZ was calculated from nonlinear regression of dose-response data (doses used up to 200 µM) based on the four parameter logistic function, using GraphPad Prism software (GraphPad Software, La Jolla, CA).

Results and discussion

CPZ inhibited yeast growth in a dose and time-dependent manner. CPZ IC_{50} was 28.08±1.03 µM and 43.83±1.02 µM after 6 and 24 hours of treatment, respectively. This result is not graphically illustrated, but in agreement with our previous work (Sayyed *et al.,* 2019). CPZ toxic effect was antagonized by the antioxidant NAC, thereby demonstrating oxidative stress as a main toxicity mechanism. Yeast cells are less sensitive to CPZ after 24 hours due to their ability to develop a chemical stress resistance response, which is in agreement with previous reports (Al-Attrache *et al.,* 2018; De Filippi *et al.,* 2007; Simpson & Ashe, 2012). Since CPZ has many molecular targets *in vivo* and *in vitro* (De Filippi *et al.,* 2007; Ros *et al.,* 1979; Thyberg *et al.,* 1977), its toxic effects deserve investigation in order to help understanding its toxicity *in vivo*. Validity of *Saccharomyces cerevisiae* as a model to investigate CPZ toxicity is valuable since it helps reducing experimentation on animals.

Our results show that CPZ at 20 and 50 µM decreases total lipids up to 70% after 4 hours of treatment. Lower, but significant, decrease in percentage was obtained after 9 hours (46%) while the decrease was not significant after 24 hours (Table 1). In the presence of NAC, the effect of CPZ on lipid content disappeared suggesting the involvement of oxidative stress in the CPZ-caused lipid decrease. The lack of CPZ effect after 24 hours is probably attributed, at least partially, to an adaptation mechanism of yeast to the chemical stress. The effects of CPZ on lipid content of *S. cerevisiae* could be due to alteration of the activity of enzymes involved in lipid metabolism. In fact, in animal cells, it was reported that CPZ alters the expression of many lipid metabolism-related genes, including ADRP (Adipose Differentiation-Related Protein) and Perilipin-4 genes which are involved in the formation of lamellar vesicles and Acyl-CoA (Bachour-El Azzi *et al.,* 2014; Hoshi & Fujino, 1992; Jassim *et al.,* 2012; Martin *et al.,* 1986). In addition, CPZ inhibits triacylglycerol synthesis by inhibiting the enzyme phosphatidate phosphohydrolase and thus the inhibition of diacylglycerol formation in rats (Bowley *et al.,* 1977; Ide & Nakazawa, 1980, 1981). CPZ effects on yeast are likely due to the parent drug since this yeast does not have enzymes equivalent to those involved in chlorpromazine metabolism in human (e.g. CYP1A2, CYP3A4,…) (De Filippi *et al.,* 2007; Wójcikowski *et al.,* 2010). None of the CPZ metabolites

found in human (mono-N-demethylated CPZ, di-N-demethylated CPZ, CPZ sulfoxide, 7-OH CPZ) were detected in *Saccharomyces cerevisiae* (our unpublished data). Therefore, this yeast is a simple and valid model to investigate effects of CPZ itself on lipid alteration. In mammalian cell models, metabolites of CPZ contribute to its toxicity (Abernathy *et al.,* 1977; Parmentier *et al.,* 2013; Yeung *et al.,* 1993).

Our results also show that 50 µM of CPZ significantly decreases total protein content of *S. cerevisiae* by 52%, and that the co-treatment with NAC reverses this inhibition after 24 hours (Table 2). These results demonstrate that protein synthesis and/or turn over are targets of CPZ and that oxidative stress is involved in CPZ toxicity in *S. cerevisiae.* This result is in agreement with previous studies on *S. cerevisiae* and NIH-3T3 cells, suggesting involvement of similar mechanisms of CPZ toxicity in yeast and mammalian cells (De Filippi *et al.,* 2007; Deloche *et al.,* 2004). Protein synthesis is therefore a CPZ target since 30 µM CPZ significantly inhibits translation and higher concentrations (200 µM) completely blocks protein synthesis (De Filippi *et al.,* 2007). It is important to emphasize the fact that CPZ effect on lipids disappears after 24 hours while its effect on proteins is still present which suggests that distinct mechanisms are involved.

In conclusion, CPZ effect on yeast protein and lipid content are in agreement with those reported in animal cell models regarding the involvement of oxidative stress. A CPZ toxic dose causes a decrease of lipid content in yeast which resists the chemical stress after 24 hours. Total proteins are also decreased by CPZ and the effect persists at 24 hours. Mechanisms and actors that mediate the observed CPZ effects, as well as the decreased lipid types remain to be investigated. *Saccharomyces cerevisiae* is a simple not expensive model that is useful to better understand CPZ toxicity and to reduce animal use in experimentation.

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REFERENCES

Abernathy, CO, Lukacs, L, and Zimmerman, HJ. (1977). Adverse effects of chlorpromazine metabolites on isolated hepatocytes. *Proc Soc Exp Biol Med* **155**: 474–478.

Al-Attrache H, Chamieh H, Hamzé M, Morel I, Taha S, and Abdel-Razzak Z. (2018). N-acetylcysteine potentiates diclofenac toxicity in *Saccharomyces cerevisiae*: stronger potentiation in ABC transporter mutant strains. *Drug Chem Toxicol* **41**: 89–94.

Anderson GD, Chan L-N. (2016). Pharmacokinetic Drug Interactions with Tobacco, Cannabinoids and Smoking Cessation Products. *Clin Pharmacokinet* **55**: 1353–1368.

Anthérieu S, Bachour-El Azzi P, Dumont J, Abdel-Razzak Z, Guguen-Guillouzo C, Fromenty B, Robin M-A, and Guillouzo A. (2013). Oxidative stress plays a major role in chlorpromazine-induced cholestasis in human HepaRG cells. *Hepatol* **57**: 1518–1529.

Bachour-El Azzi P, Sharanek A, Abdel-Razzak Z, Anthérieu S, Al-Attrache H, Savary CC, Lepage S, Morel I, Labbe G, Guguen-Guillouzo C, Guillouzo A. (2014). Impact of inflammation on chlorpromazine-induced cytotoxicity and cholestatic features in HepaRG cells. *Drug Metab Dispos Biol Fate Chem* **42**: 1556–1566.

Bowley M, Cooling J, Burditt SL, Brindley DN. (1977). The effects of amphiphilic cationic drugs and inorganic cations on the activity of phosphatidate phosphohydrolase. *Biochem J* **165**: 447–454.

De Filippi L, Fournier M, Cameroni E, Linder P, De Virgilio C, Foti M, Deloche O. (2007). Membrane stress is coupled to a rapid translational control of gene expression in chlorpromazine-treated cells. *Curr Genet* **52**: 171–185.

Dejanović B, Vuković-Dejanović V, Stevanović I, Stojanović I, Mandić Gajić G, Dilber S. (2017). Oxidative stress induced by chlorpromazine in patients treated and acutely poisoned with the drug. *Vojnosanit Pregl* **73**: 312–317.

Deloche O, de la Cruz J, Kressler D, Doère M, Linder P. (2004). A membrane transport defect leads to a rapid attenuation of translation initiation in *Saccharomyces cerevisiae*. *Mol Cell* **13**: 357–366.

Dudley K, Liu X, De Haan S. (2017). Chlorpromazine dose for people with schizophrenia. *Cochrane Database Syst Rev 4*, CD007778.

Hoshi K, Fujino S. (1992). Difference between effects of chlorpromazine and perphenazine on microsomal phospholipids and enzyme activities in rat liver. *J Toxicol Sci* **17**: 69–79.

Hu J, Kulkarni AP. (2000). Metabolic fate of chemical mixtures. I. "Shuttle Oxidant" effect of lipoxygenase-generated radical of chlorpromazine and related phenothiazines on the oxidation of benzidine and other xenobiotics. *Teratog Carcinog Mutagen* **20**: 195–208.

Ide H, Nakazawa Y. (1980). Effect of chlorpromazine on the cytoplasmic phosphatidate phosphohydrolase in rat liver. *Biochem Pharmacol* **29**: 789–793.

Ide H, Nakazawa Y. (1981). Effect of chlorpromazine on intracellular transport of phospholipids. *Chem Biol Interact* **34**: 69–73.

Jassim G, Skrede S, Vázquez MJ, Wergedal H, Vik-Mo AO, Lunder N, Diéguez, C, Vidal-Puig A, Berge RK, López M, Steen VM, Fernø J. (2012). Acute effects of orexigenic antipsychotic drugs on lipid and carbohydrate metabolism in rat. *Psychopharmacology (Berl.)* **219**: 783–794.

Kamada Y, Jung US, Piotrowski J, Levin DE. (1995). The protein kinase C-activated MAP kinase pathway of *Saccharomyces cerevisiae* mediates a novel aspect of the heat shock response. *Genes Dev* **9**: 1559–1571.

Knittelfelder OL, Kohlwein SD. (2017). Lipid Extraction from Yeast Cells. *Cold Spring Harb Protoc, 2017.*

Martin A, Hopewell R, Martín-Sanz P, Morgan JE, Brindley DN. (1986). Relationship between the displacement of phosphatidate phosphohydrolase from the membrane-associated compartment by chlorpromazine and the inhibition of the synthesis of triacylglycerol and phosphatidylcholine in rat hepatocytes. *Biochim Biophys Acta* **876**: 581–591.

Morgan K, Martucci N, Kozlowska A, Gamal W, Brzeszczyński F, Treskes P, Samuel K, Hayes P, Nelson L, Bagnaninchi P, Brzeszczynska J, Plevris J. (2019). Chlorpromazine toxicity is associated with disruption of cell membrane integrity and initiation of a pro-inflammatory response in the HepaRG hepatic cell line. *Biomed Pharmacother Biomedecine Pharmacother* **111**: 1408–1416.

Parmentier C, Truisi GL, Moenks K, Stanzel S, Lukas A, Kopp-Schneider A, Alexandre E, Hewitt PG, Mueller SO, Richert L. (2013). Transcriptomic hepatotoxicity signature of chlorpromazine after short- and long-term exposure in primary human sandwich cultures. *Drug Metab Dispos Biol Fate Chem* **41**: 1835–1842.

Ros E, Small DM, and Carey MC. (1979). Effects of chlorpromazine hydrochloride on bile salt synthesis, bile formation and biliary lipid secretion in the rhesus monkey: a model for chlorpromazine-induced cholestasis. *Eur J Clin Invest* **9**: 29–41.

Saari K, Koponen H, Laitinen J, Jokelainen J, Lauren L, Isohanni M, Lindeman S. (2004). Hyperlipidemia in persons using antipsychotic medication: a general population-based birth cohort study. *J Clin Psychiatry* **65**: 547–550.

Sayyed K, Aljebeai A-K, Al-Nachar M, Chamieh H, Taha S, Abdel-Razzak Z. (2019). Interaction of cigarette smoke condensate and some of its components with chlorpromazine toxicity on *Saccharomyces cerevisiae*. *Drug Chem Toxicol* 1–11.

Simpson CE, Ashe MP. (2012). Adaptation to stress in yeast: to translate or not? *Biochem Soc Trans* **40**: 794–799.

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Suzuki H, Gen K, Inoue Y. (2013). Comparison of the anti-dopamine D_2 and anti-serotonin 5-HT(2A) activities of chlorpromazine, bromperidol, haloperidol and second-generation antipsychotics parent compounds and metabolites thereof. *J Psychopharmacol* **27**: 396–400.

Tavoloni N, Boyer JL. (1980). Relationship between hepatic metabolism of chlorpromazine and cholestatic effects in the isolated perfused rat liver. *J Pharmacol Exp Ther* **214**: 269–274.

Thakur MS, Prapulla SG, Karanth NG. (1989). Estimation of intracellular lipids by the measurement of absorbance of yeast cells stained with Sudan Black B. *Enzyme Microb Technol* **11**: 252–254.

Thyberg J, Axelsson JE, Hinek A. (1977). In vitro effects of chlorpromazine on microtubules and the Golgi complex in embryonic chick spinal ganglion cells: an electron microscopic study. *Brain Res* **137**: 323–332.

Wójcikowski J, Boksa J, Daniel WA. (2010). Main contribution of the cytochrome P450 isoenzyme 1A2 (CYP1A2) to N-demethylation and 5-sulfoxidation of the phenothiazine neuroleptic chlorpromazine in human liver--A comparison with other phenothiazines. *Biochem Pharmacol* **80**: 1252–1259.

Yeung PK, Hubbard JW, Korchinski ED, Midha KK. (1993). Pharmacokinetics of chlorpromazine and key metabolites. *Eur J Clin Pharmacol* **45**: 563–569.

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