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REVIEW ARTICLE

Aluminium toxicosis: a review of toxic actions and effects

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ABSTRACT

Aluminium (Al) is frequently accessible to animal and human populations to the extent that intoxications may occur. Intake of Al is by inhalation of aerosols or particles, ingestion of food, water and medicaments, skin contact, vaccination, dialysis and infusions. Toxic actions of Al induce oxidative stress, immunologic alterations, genotoxicity, pro-inflammatory effect, peptide denaturation or transformation, enzymatic dysfunction, metabolic derangement, amyloidogenesis, membrane perturbation, iron dyshomeostasis, apoptosis, necrosis and dysplasia. The pathological conditions associated with Al toxicosis are desquamative interstitial pneumonia, pulmonary alveolar proteinosis, granulomas, granulomatosis and fibrosis, toxic myocarditis, thrombosis and ischemic stroke, granulomatous enteritis, Crohn's disease, inflammatory bowel diseases, anemia, Alzheimer's disease, dementia, sclerosis, autism, macrophagic myofasciitis, osteomalacia, oligospermia and infertility, hepatorenal disease, breast cancer and cyst, pancreatitis, pancreatic necrosis and diabetes mellitus. The review provides a broad overview of Al toxicosis as a background for sustained investigations of the toxicology of Al compounds of public health importance.

KEY WORDS: aluminium; intoxication; pathology; toxicity; toxicosis

Introduction

Aluminium (Al) is the most widely distributed metal in the environment (Delhaize and Ryan, 1995; Ranjbar *et al.*, 2008; Exley and House, 2011) occurring naturally in the trivalent state (Al^{+3}) as silicates, oxides and hydroxides, but may combine with other elements such as chlorine, sulphur, fluorine, as well as form complexes with organic matter (Jones and Bennet, 1986; Ganrot, 1986; Martin, 1992). Environmental media may be contaminated by Al from anthropogenic sources and through the weathering of rocks and minerals. Weathering processes on rocks release more Al to the environment than human-related activities (Lantzy and MacKenzie, 1979). Exposures to Al occur in occupations associated with mining and processing of ore, scrap metal recycling, deployment and use of Al-containing compounds and products, and during engagement in Al metal cutting, sawing, filing and welding. Animals and humans living in environments contaminated by industrial wastes may also be exposed to high levels of Al (Sorgdrager *et al.*, 1998; Vandenplas *et al.*, 1998; Boran *et al.*, 2013).

Several chemical compounds with Al are in extensive use in various products and processes associated with human activities. These compounds are Al chloride, Al hydroxide (alumina trihyrate), Al nitrate, Al phosphate, Al sulfate (alum), Al potassium (potash alum), Al ammonium sulfate (ammonium alum) and Al silicate (Anon, 1982; Lewis, 2001). The compounds are used in crude oil refining and cracking of petroleum; manufacturing of cooking utensils and foils, parchment paper, printing ink, glass, ceramics, pottery, incandescent filaments, fireworks, explosives, photographic flashlight, electric insulators, cement, paints and varnishes, fumigants and pesticides, lubricants, detergents, cosmetics, pharmaceuticals (drugs), vaccines, as well as in water treatment and purification, treating sewage and fur, tanning leather, waterproofing clothes and concretes, industrial filtration, hemodialysis, measuring radiation exposure, in products as flame retardant and fireproofing, anticorrosion agent, food additives to prevent caking as well as components of baking powders and colorants (Anon, 1982, 2008a; Malakoff, 2000; Lewis, 2001; Soni *et al.*, 2001; Saiyed and Yokel, 2005).

The Al ion has no physiological role in metabolic processes (Exley and House, 2011) but it can be a metallic

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toxicant to humans and animals (Becaria *et al.*, 2002) when there is high body burden of the metal after natural or unnatural exposure (Exley, 2013). Al was considered unsafe to humans after the discovery of increased levels of Al in brain tissues of patients with encephalopathy, having been exposed to Al accumulation through dialysis (Alfrey and Solomons, 1976). Toxicosis due to Al accumulation in mammalian tissues was associated with various pathologic effects (Wills and Savory, 1983; Kaiser *et al.*, 1984; Boyce *et al.*, 1986; Drüeke *et al.*, 1986; Hewitt *et al.*, 1990; Bushinsky *et al.*, 1995; Reinke *et al.*, 2003; Abubakar *et al.*, 2004; Bogdanović *et al.*, 2008; Yousef and Salama, 2009; Khattab *et al.*, 2010; Blaylock, 2012; Buraimoh and Ojo, 2013; Sumathi *et al.*, 2013). Recent reviews on toxic effects of Al covered reproductive toxicity (Mouro *et al.*, 2017), pulmonary lesions (Kongerud and Søyseth, 2014; Taiwo, 2014), impact on the breast (Darbre, 2016), bone abnormalities (Chappard *et al.*, 2016; Klein, 2019), immunotoxity (Zhu *et al.*, 2014a) and neurologic disorders (Colomina and Peris-Sampedro, 2017; Morris *et al.*, 2017). This review is an abridged and global overview of toxic effects of Al and its compounds, covering some relevant aspects of exposure and updated systemic toxicosis in humans and animals, relevant as background for prospective toxicopathologic studies.

Literature search justification and methods

The initial goal in our study group was to explore the role of the ubiquitous Al ion in erythrocyte membrane dysfunction (Igbokwe, 2016) and metabolic dysregulation (Igwenagu, 2017). With the preliminary literature search starting in 2013 and looking backwards in time, research publications revealed a myriad of toxic actions of Al causing pathological conditions. Several narrative literature reviews, referred to in the introductory section, were discovered to have focused on Al toxicity of one system of the body in each review, covering the scope of nervous, reproductive, respiratory, mammary, skeletal and immune tissue toxicities. One review addressed the toxicities in bone, hematopoietic tissue and kidney (Jeffrey *et al.*, 1996) and another summarized the physiological alterations in the musculoskeletal, respiratory, cardiovascular, hepatobiliary, endocrine, urinary and reproductive systems(Nayak, 2000). Thus the question raised was whether Al toxicosis, as a disease entity, existed in the literature with current research information. The literature search for Al toxicosis as a narrative review (Green *et al.*, 2006) with a broad thematic approach was unproductive and this observation justified the need for the current review.

Toxicosis associated with Al exposure is the pathological condition or disease caused by the toxic actions of Al and its compounds. The literature search was intended to collate, synthesize and integrate the published reports on the subject matter without meta-analysis and critical evaluation of published data. For this review, the major themes for the literature search under the title included exposure modalities, toxic actions and effects in cells, tissues and systems of the body (Figure 1). These themes provided the key words and phrases for the internet search. The initial search platform was usually GOOGLE.COM with the linked GOOGLE SCHOLAR helping to search related articles. Subsequently, the search was extended to MEDLINE, PUBMED, PMC Europe, RESEARCHGATE, SCOPUS, SCIENCEDIRECT, SAGE, TANDFONLINE and SPRINGERLINK. On each platform, an article that was found could also have links to related articles and these links were followed to enrich the search outcomes. Every article was read as an abstract or full article after downloading to the literature bank. At this stage, "backward search" based on references of read articles could be done and "forward search" was required when new themes emerged that needed further exploration. The

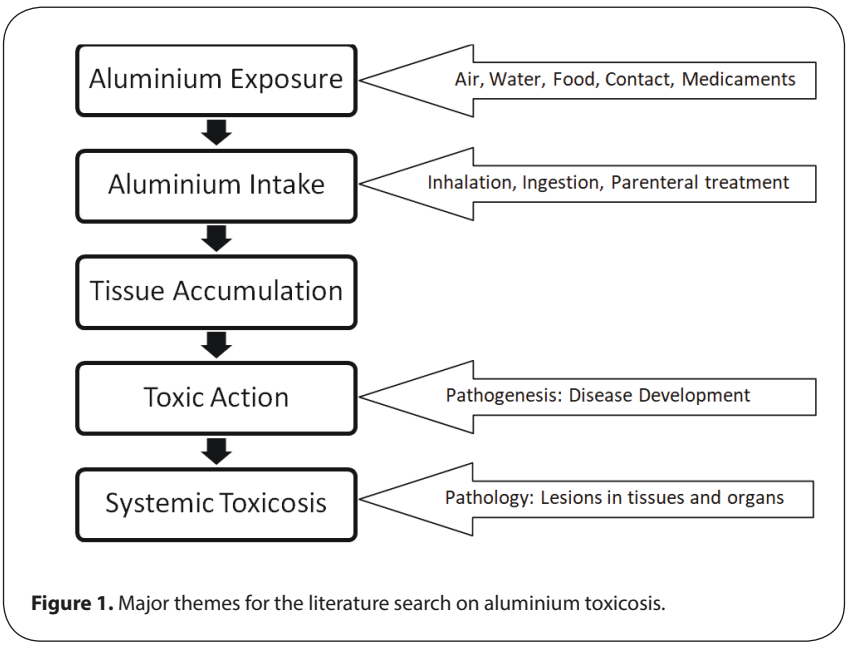

Figure 1. Major themes for the literature search on aluminium toxicosis.

integration of the literature search processes are illustrated in Figure 2. Occasionally, article request options were used to obtain restricted publications and the articles were received from authors. The literature collated from the search was read for content comprehension. The body of knowledge was summarized and narrated after cognitive reflection and integration to represent the current knowledge of the literature on Al toxicosis. The articles with thematic contents were included for the review when they were published in journals with reputable standing. The contents of the excluded articles were peripheral to the themes under review.

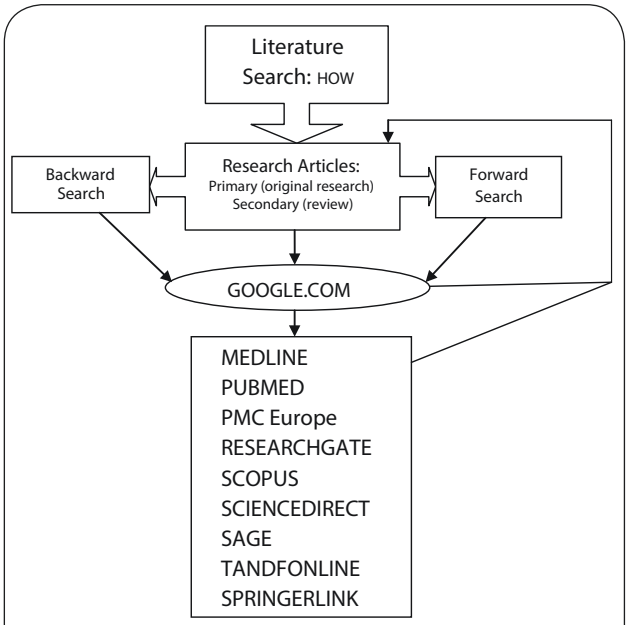

Figure 2. Integrated literature search process.

Exposure to aluminium

Aluminium intake

Aluminium in the air

The largest source of airborne Al-containing particles is the dust from soil and rocks (Lee and Von Lehmden, 1973; Sorenson *et al.*, 1974). Human activities, such as mining and agriculture, contribute to the dust in winds (Eisenreich, 1980; Filipek *et al.*, 1987). About 13% of atmospheric Al is attributed to anthropogenic emissions (Lantzy and MacKenzie, 1979). The major anthropogenic sources of Al-containing particulate matter include coal combustion, Al production, iron and steel foundries, brass and bronze refineries, motor vehicle emissions and other industrial activities such as smelting, filing, sawing, welding of Al metals (Lee and Von Lehmden, 1973; Ondov *et al.*, 1982; Que Hee *et al.*, 1982). Cigarette smoke may contribute to the concentration of Al in the air (Exley *et al.*, 2006; Kazi *et al.*, 2009; Pappas, 2011; Afridi *et al.*, 2015). The air containing Al particles or droplets becomes the source of Al in inhaled aerosols.

Aluminium in drinking water

Al occurs ubiquitously in natural waters due to weathering of Al-containing rocks and minerals and mobilization from terrestrial to aquatic environment (Campbell *et al.*, 1992). This mobilization of Al is often seasonal in nature and is associated with pH depressions (acidification) occurring during the spring snow melt or associated with erosion from specific storm events (Rosseland *et al.*, 1990; Nelson and Campbell, 1991; Campbell *et al.*, 1992). Al concentrations in surface waters can be increased directly or indirectly by human activities through industrial and municipal discharges, surface run-off, tributary inflow, groundwater seepage, and wet and dry atmospheric deposition (Eisenreich, 1980). Industrial release of Al in waste materials into surface waters from processing and manufacturing facilities could be toxic to aquatic life (Filipek *et al.*, 1987; Trieff *et al.*, 1995; His *et al.*, 1996; Gensemer and Playle, 1999). Acidic drainage from mines or acid rain may cause an increase in the dissolved Al content of the surrounding water bodies (Cronan and Schofield, 1979; Filipek *et al.*, 1987). The use of Al compounds as coagulating agents in the treatment of water for drinking could increase its Al content (Qureshi and Malmberg, 1985; Henshaw *et al.*, 1993; Cech and Montera, 2000). In pure water, Al has a minimum solubility in the pH range of 5.5–6.0 and concentrations of dissolved Al increase at higher or lower pH values (Browne *et al.*, 1990). The source of water for human and animal consumption and the purification process involved may influence the Al content of drinking water as source of exposure.

Aluminium in food

Al is present in foods naturally or from the use of Al-containing food additives (Sepe *et al.*, 2001; Flaten, 2002; Hayacibara *et al.*, 2004; Yokel *et al.*, 2008). The concentrations in foods and beverages vary widely, depending upon the food product, the type of processing used, and the geographical areas in which the food crops are grown (Sorenson *et al.*, 1974; Pennington and Schoen, 1995). The foods highest in Al are those that contain Al additives (Pennington, 1988; Greger, 1992; Saiyed and Yokel, 2005; Yokel and Florence, 2006; Yokel, 2012). The use of Al cookware, utensils and wrappings can increase the amount of Al in food (Liukkonen-Lilja and Piepponen 1992; Pennington and Schoen, 1995). The migration of Al from cookware into food increases with the acidity of the food and the duration of exposure (Valkonen and Aitio, 1997; Lin *et al.* 1997). Al was also reported to migrate into fish grilled on Al foil and the migration of Al into foods appeared to be dependent on factors such as temperature, duration of cooking, the composition and pH of the food, and the presence of other substances like organic acids and salts (Ranau *et al.*, 2001). Foods found to be naturally high in Al include potatoes, spinach and tea (Pennington and Schoen, 1995; Stahl *et al.*, 2011). Processed dairy products and flour may be high in Al if they contain Al-based food additives (Pennington and Schoen, 1995).

Daily intakes of Al in humans from food range from 3.4 to 9 mg/day (Pennington and Schoen, 1995; Biego *et al.*, 1998; Yang *et al.*, 2014). It is unlikely that Al-containing food additives are intentionally added to the diets of livestock and pets yet, Al contamination of some additives used in livestock and pet food is possible (Burgoin, 1992). Thus Al contents of harvested food products, processed foods, and cooked, baked or grilled foods may be sources of Al exposure.

Aluminium in pharmaceuticals and agrochemicals

The route of intoxications with pharmaceuticals and agrochemical sources may be through inhalation of aerosols, ingestion of medications or by parenteral administration. Humans and animals are exposed to Al-containing medications such as phosphate binders, antacids, buffered analgesics, antidiarrheal and antiulcer drugs (Lione, 1983, 1985; Yokel and McNamara, 2001; Krewski *et al.*, 2007). Various intravenously administered pharmaceutical products were reported to contain 684–5977 μg/g of Al (Sedman *et al.*, 1985). Many antacids contain 104–208 mg of Al per tablet, capsule or 5 ml of suspension (Zhou and Yokel, 2005). The use of other consumer items such as dentifrices, disinfectants, fumigants, pesticides, anti-perspirants and some cosmetics are sources of Al exposure (Lewis, 2001; Pineau *et al.*, 2014). Al hydroxide, Al phosphate, Al potassium sulfate (alum), and Al silicate (zeolite) are used in the preparation of a number of vaccines to adsorb antigenic components and to serve as adjuvant that enhance immune response (Lione, 1985; Tomljenovic and Shaw, 2011; Issa *et al.*, 2014). Adjuvant as a source of Al during vaccinations has been receiving attention in research (Malakoff, 2000; Keith *et al.*, 2002; Mitkus *et al.*, 2011; Glanz *et al.*, 2015) and it is presumed that there could be mistakes in adjusting Al content of vaccines to body weights of neonates who stand the risk of Al toxicity from vaccines (Lyons-Weiler and Ricketson, 2018). More Al was absorbed into blood by rabbits after intramuscular injection with adjuvant containing Al phosphate compared to Al hydroxide (Hem, 2002). It is unlikely that parenteral Al administrations are a major source of Al exposure to livestock or pets (Issa *et al.*, 2014).

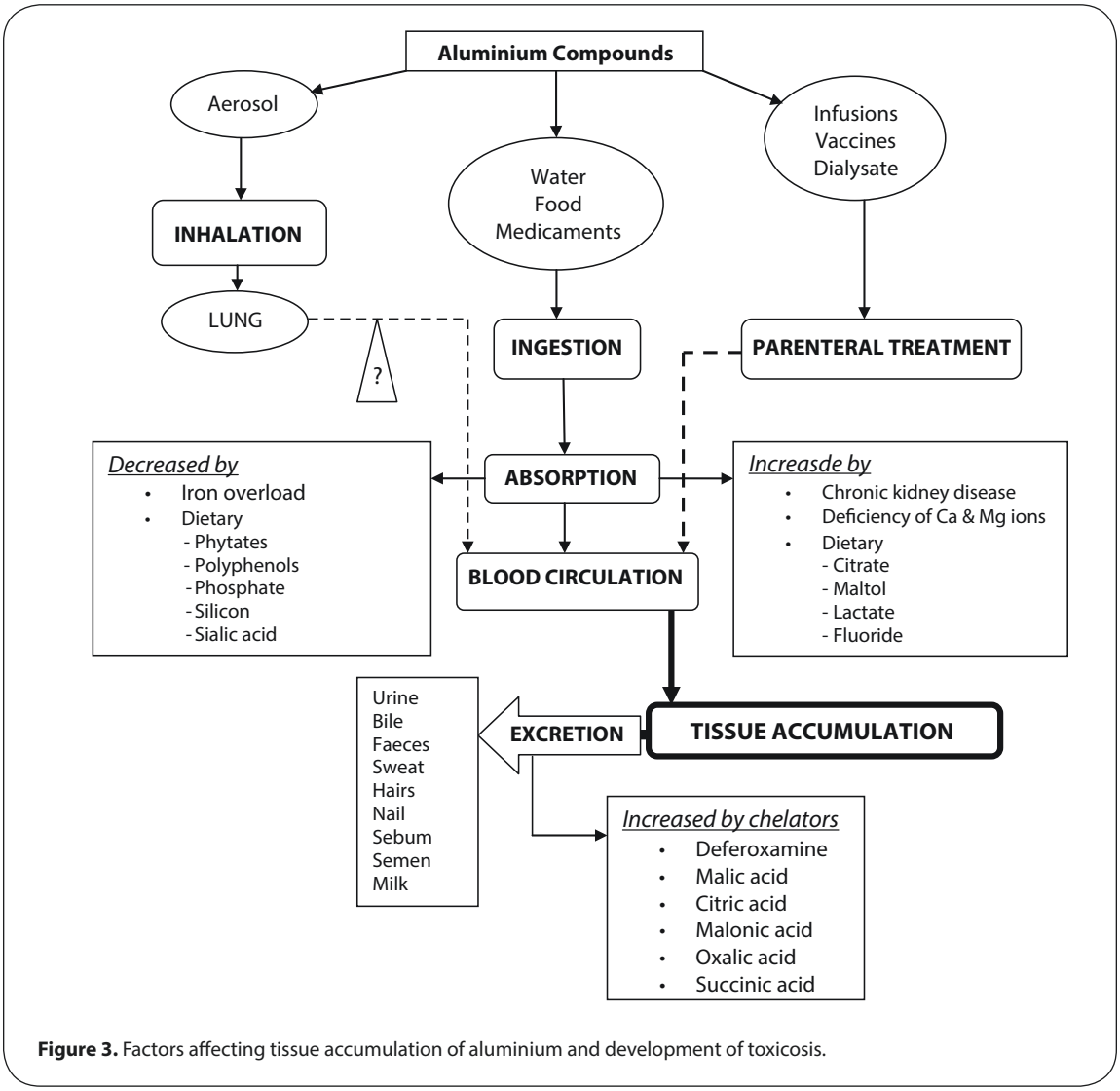

Figure 3. Factors affecting tissue accumulation of aluminium and development of toxicosis.

Food or water for livestock could be contaminated with Al when Al sulfate and zeolite are applied to litter and waste lagoons to reduce phosphorus loss from lands fertilized with the wastes and to reduce ammonia fumes in facilities (Moore *et al.*, 1999; Moore *et al.*, 2000; Codling *et al.*, 2002). Alum has also been added to dairy slurry to reduce ammonia emissions (Lefcourt and Mesinger, 2001). Thus, this section indicates that Al exposure can arise when certain pharmaceutical products are administered orally or parenterally to individuals or when agrochemicals contaminate food/feed and water taken by individuals or those in close proximity inhale aerosols from agrochemical fumigants and sprays.

Absorption, distribution and elimination of aluminium

The dynamic chain of Al intake, absorption and elimination determines the level of tissue accumulation and development of toxicosis (Figure 3). Inhalation and ingestion (via food and water) are the two main routes through which Al gets into the body (Alfrey, 1980; Teraoka, 1981; Jouhanneau *et al.*, 1997). Following inhalation, Al compounds are deposited in the lungs (Christie *et al.*, 1963; Stone *et al.*, 1979; Thomson *et al.*, 1986). The lungs continually receive Al mostly as particles of Al silicates and other poorly soluble compounds (Thomson *et al.*, 1986). The concentration of Al in the lungs tends to increase with age and may result in respiratory anomalies where the Al is localized (Alfrey, 1980; Teraoka, 1981; Taiwo, 2014). There is no available evidence in literature that particulate or soluble Al gets into the blood circulation from the lungs to be subsequently distributed to other organs of the body.

Gastrointestinal absorption, after ingestion, is the main route through which Al is systemically accumulated in animals and humans, and absorption occurs largely in the duodenum (Feinroth *et al.*, 1984; Steinhausen *et al.*, 2004). The absorption of Al is usually low and varied when compared with the amount ingested (Kawahara *et al.*, 2007). The uptake of Al through gastrointestinal pathway is complex and is influenced by various factors including individual differences, age, pH, stomach contents and type of Al compound (Priest *et al.*, 1996). Al absorption from water intake (about 0.3%) is greater than from food (about 0.1%) (Martyn *et al.*, 1989; Steinhausen *et al.*, 2004; Anon, 2008b; Zhou *et al.*, 2008).This was attributed to organic ligands in foods such as phytates and polyphenols that were suggested to form complexes with Al ion and inhibit its absorption (Reto *et al.*, 2007). Absorption of Al via the gastrointestinal tract can be enhanced in the presence of citrate, maltol, lactate and fluoride in water or food, and during chronic renal diseases, while the absorption is reduced in individuals with iron overload, or when ingested with phosphate, silicon, polyphenols and sialic acid (Brown *et al.*, 1987; Edwardson *et al.*, 1993; Anon, 2008c; Zhou *et al.*, 2008). However, there is complete Al uptake from parenteral fluids and vaccines with subsequent distribution to various parts of the body (Tomljenovic and Shaw, 2011).

About 90% of the Al circulating in the blood is transported bound to transferrin (iron-transporter protein), while the rest of Al binds to albumin and citrate in the blood (Day *et al.*, 1991; Harris and Messori, 2002; Hemadi *et al.*, 2003; Chen *et al.*, 2010). Cellular uptake of Al in tissues is relatively slow and is presumed to be mediated by endocytosis and intracellular transfer of the Al bound to transferrin (Hemadi *et al.*, 2003). However, Al-transferrin complex may not bind to the transferrin-receptor (Hemadi *et al.*, 2003; Sakajiri *et al.*, 2010), indicating the existence of an alternative mechanism of cellular uptake of Al (DeVoto and Yokel, 1994; Anon, 2011). The total body burden of Al in healthy humans has been reported to be approximately 30–50 mg/kg body weight and normal levels of Al in serum are approximately 1–3μg/L (Krewski *et al.*, 2007). The mean serum Al level in 44 non-exposed persons who did not use antacids was reported to be 1.6 μg/L (Valkonen and Aitio, 1997) and Chen *et al.* (2010) reported that values in hemodialysis patients were ten-fold higher than the values in unexposed individuals. About one-half of the total body Al is in the skeleton, and the levels in human bone tissue range from 5 to 10 mg/kg (Anon, 2008c). Al has also been found in human skin, lower gastrointestinal tract, lymph nodes, adrenals, parathyroid glands, and in most soft tissue organs (Anon, 2008b). In rats, accumulation of Al after oral exposure was higher in the spleen, liver, bone, and kidneys than in the brain, muscle, heart, or lungs (Anon, 2008b). It has also been reported that Al can reach the placenta and fetus and to some extent distribute to the milk of lactating mothers (Anon, 2008b). Al levels increase with age in tissues and organs (bone, muscle, lung, liver, and kidney) of experimental animals (Krewski *et al.*, 2007). Moreover, Al has been shown to rapidly enter the brain, extracellular fluid and the cerebrospinal fluid, with smaller concentrations in these organs than in the blood (Martin, 1992; Krewski *et al.*, 2007). The iron status is negatively correlated with Al accumulation in tissues and animal experiments have shown that calcium and magnesium deficiency may contribute to accumulation of Al in the brain and bone (Anon, 2011).

The Al ion in blood circulation is eliminated primarily by the kidneys (about 95%) in the urine, presumably as Al citrate (Shirley and Lote, 2005; Krewski *et al.*, 2007; Anon, 2008c). Tissue accumulation of Al is reduced by citrates and fluorides through renal excretion when the transferrin-Al binding capacity of the blood is exceeded (Anon, 2008b). Al is also excreted in the milk, bile, feces, sweat, hairs, nails, sebum and semen (Gorsky *et al.*, 1979; Greger and Sutherland, 1997; Exley, 2013). Urinary excretion of Al is enhanced by chemical chelators such as deferoxamine and malic, malonic, citric, oxalic and succinic acids, as reviewed in the later section on treatment of Al in this document. On the whole, it is noted that Al accumulation, which is responsible for Al toxicosis, is enhanced by exposure to Al and its continuous intake, as well as increased intestinal absorption and decreased excretion of the metal (Figure 3).

Toxic actions of aluminium

The toxic actions of Al responsible for the toxic effects of the toxicities are diverse and capable of causing a multifaceted systemic toxicosis. These toxic actions are summarized in Table 1. The molecular targets of action generate outcomes in the cell and disrupt cellular homeostasis with consequences that lead to lesions in the cell (Figure 4), which are responsible for systemic toxicosis associated with structural and functional abnormalities of organs.

Toxic effects of Al arise mainly from its pro-oxidant activity which results in oxidative stress, free radical attack and oxidation of cellular proteins and lipids (Exley, 2013). Protein polypeptides are transformed to secondary structures when Al ions interact with them through oxygen-containing amino acids, side chains and protein backbone leading to ultimate denaturation (Mujika *et al.*, 2018) or conformational or structural alteration (Exley *et al.*, 1993; Zatta *et al.*, 2005; Exley, 2006) as in β-amyloid. The aggregation and precipitation of β-amyloid is triggered and potentiated by Al exposure which is associated with Alzheimer's disease (Bondy and Truong, 1999; Zatta *et al.*, 2005; Exley, 2006) and this phenomenon may be responsible for neuritic plaque deposition, neuronal death and dysneurogenesis. Fibrillation and aggregation of human islet amyloid polypeptide hormone (amylin) was stimulated by Al exposure leading to formation of β-pleated sheet structure (Mirhashemi and Aarabi, 2011; Mirhashemi and Shahabaddin, 2011; Xu *et al.*, 2016), which may predispose pancreatic β-cell to damage. The proteolytic degradation of the amyloid peptides is also prevented by Al, thereby enhancing the accumulation of the amyloid (Sakamoto *et al.*, 2006). Extracellular surfaces and intracellular ligands may likely associate with Al to induce inhibitory or stimulatory effects (Exley and Birchall, 1992). Interaction of Al with metabolic and other enzymes causes inhibition or activation of the enzymes (Hofstetter *et al.*, 1987; Xu *et al.*, 1990; Exley *et al.*, 1994; Zatta *et al.*, 1999, 2000; Yang *et al.*, 2003; Mailloux *et al.*,

Table 1. Toxic actions associated with aluminium exposure.

Toxic action or effect	Selected references
Oxidative stress, lipid peroxidation	Kattab *et al.*, 2010; Exley, 2013; Abd-Elhady *et al.*, 2013; Zhang *et al.*, 2016; Yang *et al.*, 2018; Yu *et al.*, 2019
Pro-inflammatory: organ inflammation in lung, intestine, heart, and testis	Fogarty *et al.*, 1998; Verma *et al.*, 2007; Lerner, 2007; Exley, 2013; Taiwo, 2014; de Chambrun *et al.*, 2014; Gherardi *et al.*, 2016; Martinez *et al.*, 2017; Hangouche *et al.*, 2017
Immunosupression: induces lymphocyte apoptosis and dysfunction, inhibits lymphocyte proliferation, causes macrophage dysfunction	Nordal and Dahl, 1988; Kammalov *et al.*, 2011; She *et al.*, 2012; Zhu *et al.*, 2014; Zhuang *et al.*, 2016; Xu *et al.*, 2018; Yu *et al.*, 2019
Protein denaturation and transformation	Exley *et al.*, 2006; Mujika *et al.*, 2018
Enzymatic stimulation or inhibition	Ohsaka and Nomura, 2016
Metabolic impairment: impairs glycolysis and Kreb's cycle; promotes lipid and protein oxidation	Xu et al 1990; Mailloux *et al.*, 2006
Genotoxicity: reduced cell proliferation and differentiation, dysneurogenesis	Nam *et al.*, 2014
Amyloidogenic and anti-amyloidolytic	Sakamoto *et al.*, 2006; Xu *et al.*, 2016
Acts as metalloestrogen, promotes proliferation and migration of breast cancer cells	Bakir and Darbre, 2015; Darbre, 2016
Induces teratogenesis causing foetal and neonatal defects	Malekshah *et al.*, 2005; Wang *et al.*, 2012; El Mazoudy and Bekhet, 2016
Disrupts mineral metabolism of Fe, P, Ca, Zn, Cu by altering intestinal absorption and cellular uptake	Jeffery *et al.*, 1996; Contini, 2007; Kell, 2009; Fu *et al.*, 2014; Zhu *et al.*, 2014
Induces apoptosis, eryptosis, tissue necrosis	Niemoeller *et al.*, 2006; Xu *et al.*, 2018; Yang *et al.*, 2018; Yu *et al.*, 2019
Disrupts cell membrane permeability and receptor function, increases osmotic fragility, inhibits membrane ATPases	Fu *et al.*, 2014; Zhang *et al.*, 2016; Sun *et al.*, 2018; Gomes *et al.*, 2019
Endocrine disruption: parathyroid hormone, testosterone, luteinizing hormone, follicle stimulating hormone, estradiol, norepinephrine, cortisol, thyroid hormone, insulin	Díaz-Corte *et al.*, 2001; Chinoy and Patel, 2001; Gonzelez-Suerez *et al.*, 2005; Shahraki *et al.*, 2008; Orihuela, 2011; Sun *et al.*, 2011; Muselin *et al.*, 2016; Zhuang *et al.*, 2016; Mouro *et al.*, 2018; Wei *et al.*, 2018 Gomes *et al.*, 2019
Inhibits cartilage formation	Zhang *et al.*, 2017
Inhibits bone formation and mineralization by increasing osteoclastic activity and reducing osteoblastic activity	Cox and Dunn, 2001; Li *et al.*, 2012; Cao *et al.*, 2016; Song *et al.*, 2016; Sun *et al.*, 2016; Yang *et al.*, 2016; Huang *et al.*, 2017; Yang *et al.*, 2018; Xu *et al.*, 2018
Induces hypertension (systolic and arterial)	Zhang *et al.*, 2016
Causes ischaemic stroke and thrombosis	Abedini *et al.*, 2014
Induces contact allergy	Netterlid *et al.*, 2013
Inhibits the biological function of vitamin D in the intestine linked to calcium absorption	Dunn *et al.*, 1995

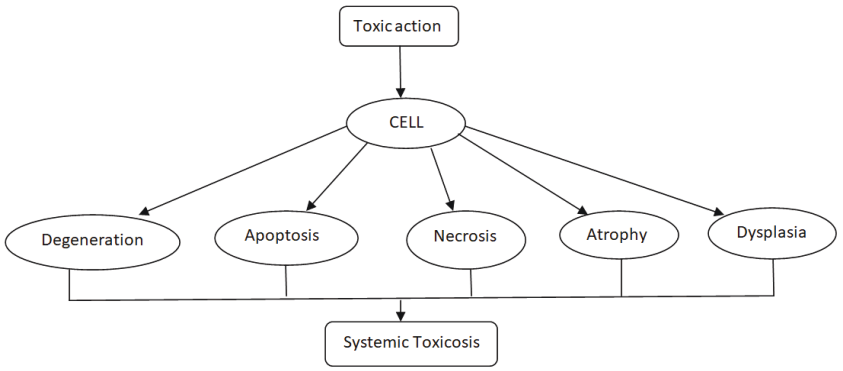

Figure 4. Cellular pathology in the systemic toxicosis of aluminium.

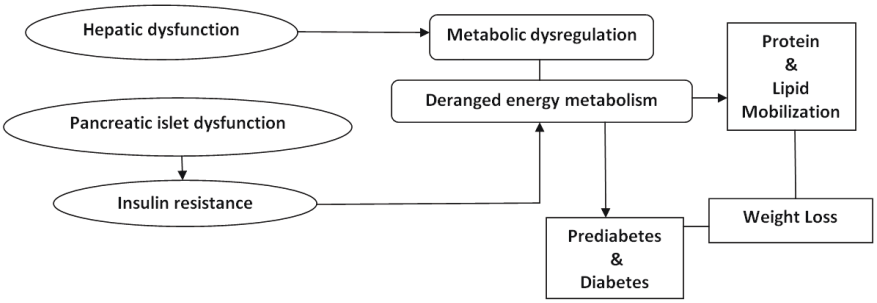

Figure 5. Metabolic abnormalities in aluminium toxicosis.

2006; Sushma *et al.*, 2007; Ohsaka and Nomura, 2016). Al binds to the phosphate groups of nucleotide such as adenosine triphosphate (ATP) and affects energy metabolism (Kawahara *et al.*, 2007). Exposure of hepatocytes to Al impedes ATP production, inhibits glycolysis, impairs the function of tricarboxylic acid (Kreb's) cycle and promotes lipid and protein oxidation (Xu *et al.*, 1990; Mailloux *et al.*, 2006) with a metabolic shift to lipogenesis in tissues (Han *et al.*, 2013). These metabolic perturbations (Figure 5) may be responsible for the reports of body weight loss and decreased production performance (like egg production) in animals exposed to Al (Wisser *et al.*, 1990; Capdevielle and Scanes, 1995; Li *et al.*, 2015).

Al exposure can cause the disruption of iron homeostasis leading to iron overload (Ward *et al.*, 2001; Contini *et al.*, 2007). Oxidative stress and injury, mediated by iron, seems to be facilitated by Al (Xiea *et al.*, 1996). Elevated concentrations of cellular iron can enhance oxidative damage to the cell and are linked to the pathogenesis of neurodegenerative disorders (Jang and Surh, 2002; Toyokuni, 2002; Adzersen *et al.*, 2003; Deugnier, 2003; Ng, 2004). Iron overload due to Al exposure has been shown to result in increased lipid peroxidation, DNA lesions, and apoptosis induced by reactive oxygen species (Bacon *et al.*, 1983; Oteiza, 1994; Kell, 2009). Apoptosis of erythrocytes (eryptosis), lymphocytes and osteoblasts is also stimulated by Al ions (Niemoeller *et al.*, 2006; Li *et al.*, 2012; Xu *et al.*, 2018; Yang *et al.*, 2018; Yu *et al.*, 2019). The oxidative injury was reported to activate the JNK apoptotic pathway in ostoblasts (Yang *et al.*, 2018). In culture, Al induced apoptosis of osteoblasts by inhibiting apoptotic Bcl-2 protein expression and increasing the expression of pro-apoptotic Bax, Bak and Bim proteins (Xu *et al.*, 2018). Al may decrease ferritin synthesis and increase the expression of transferrin receptors, thereby disrupting the normal synthesis of transferrin receptors with ferritin creating increased free iron levels in the cell, resulting in an increase of oxidative damage via the fenton reaction (Yamanaka *et al.*, 1999). The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) are affected by Al exposure because of oxidative stress (Oteiza *et al.*, 1993a; Julka and Gill, 1996; Campbell *et al.*, 1999). Abnormal increases in levels of malondealdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) were reported along with decreased levels of antioxidants such as GSH, GPx, SOD, and CAT in tissue homogenates of rats exposed to Al (Anane and Creppy, 2001; Gonzalez *et al.*, 2007; Newairy *et al.*, 2009; Khattab *et al.*, 2010; Bai *et al.*, 2012; Exley, 2013; Abd-Elhady *et al.*, 2013; Zhang *et al.*, 2016; Yu *et al.*, 2019).

Mutagenesis and alteration of gene function may arise from the toxic action of Al with changes in transcriptional expressions (Exley, 2013). Somatic and germinal

genetoxicity in mice exposed to Al was associated with chromosomal aberrations and depression of mitosis (D'Souza *et al.*, 2014). Neuronal gene expression is influenced by the binding of Al to DNA (Lukiw *et al.*, 1998) predisposing cells to or causing genotoxicity (Pogue and Lukiw, 2016) and thus Al exposure may lead to reduction of cell proliferation and differentiation (Nam *et al.*, 2014, 2016; Sun *et al.*, 2015; Cao *et al.*, 2016; Li *et al.*, 2016; Yang *et al.*, 2016; Sun *et al.*, 2016, 2017; Huang *et al.*, 2017). Neurogenesis was impaired by Al toxicity (Nam *et al.*, 2014, 2016). Osteoblastic proliferation and differentiation were inhibited by Al when there was downregulation and inhibition of Wnt/β-catenin signaling pathway (Sun *et al.*, 2015; Cao *et al.*, 2016; Huang *et al.*, 2017; Sun *et al.*, 2017). Osteoblast differentiation was also inhibited by Al through the inhibition of BMP-2 signaling pathway (Yang *et al.*, 2016). In addition, osteoblast mineralization in vitro was inhibited by Al-induced decline in transforming growth factor (TGF)-β1 expression and action, and upregulation of Smad7 expression (Sun *et al.*, 2016) along with decreased protein expressions of osteopontin, osteocalcin and osteosialoprotein (Song *et al.*, 2017). The mineralization of bone is impaired by decreased calcium absorption (Orihuela, 2007), because Al inhibits the synthesis of calbindin, a calcium-binding protein involved in transcellular transport of calcium in enterocytes and inhibits the stimulation of synthesis of osteocalcin (the bone matrix protein) in osteoblasts by vitamin D via cellular unresponsiveness (Fanti *et al.*, 1992; Jeffery *et al.*, 1996; Cox and Dunn, 2001). The expression of cartilage stimulating growth factors, TGF-β1 and BMP-2, were inhibited by Al, thereby suppressing cartilage growth and disrupting cartilage structure (Zhang *et al.*, 2017). The effects of Al on growth manifested in developmental abnormalities of fetuses due to teratogenesis in pregnant individuals (Malekshah *et al.*, 2005; Wang *et al.*, 2012; El Mazoudy and Bekhet, 2016; Yassa *et al.*, 2017).

The proliferative and migratory characteristics of the human breast cancer cell may be affected by Al when it acts as a metalloestrogen or increases the intracellular secretion of matrix metalloproteinase (MMP9) and levels of activated MMP14 that are involved in migratory and invasive properties of cancerous cells, thereby influencing the metastatic process (Darbre *et al.*, 2013a, b; Bakir and Darbre, 2015; Darbre, 2016). It is unclear whether Al has the capacity to initiate and promote any other carcinogenic process apart from the indirect evidence provided above with regard to breast cancer.

Pro-inflammatory actions of Al have been reported in various tissues (Fogarty *et al.*, 1998; Verma *et al.*, 2007; Lerner, 2007; Exley, 2013; Taiwo, 2014; de Chambrun *et al.*, 2014; Gherardi *et al.*, 2016; Martinez *et al.*, 2017; Hangouche *et al.*, 2017). It is triggered by Al-induced oxidative stress and free radical production (Milnerowicz *et al.*, 2015). Exposure to Al increased pro-inflammatory cytokine (interleukin-1β and tumor necrosis factor alpha) levels (Jangra *et al.*, 2015) and elevated gene expression of tumor necrosis factor alpha (TNFalpha) and macrophage inflammatory protein-1alpha (MIP-1alpha) in concentration-dependent manner (Johnson and Sharma, 2003). Genes that encode pro-inflammatory signaling elements were significantly up-regulated by Al (Lukiw *et al.*, 2005). These cytokines that are released due to Al exposure can recruit leukocytes, which secrete more pro-inflammatory cytokines and other chemokines, to exacerbate the inflammation (Milnerowicz *et al.*, 2015). The inflammation can be a chronic granulomatous type (Chen *et al.*, 1978; de Vuyst *et al.*, 1987; Forgarty *et al.*, 1998; Gherardi and Authier, 2012) and Al has been reported to cause granuloma formation in vitro (de Chambrun *et al.*, 2014). Chronic exposure of mice (5 months) to Al sulfate in drinking water elicited time-dependent systemic inflammation characterized by increased serum interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), C-reactive protein (CRP) and a triad of pro-inflammatory microRNAs (miRNA-9, miRNA-125b and miRNA-146a) and the biomarkers of inflammation indicated progressive chronic inflammation in the exposed animals (Pogue *et al.*, 2017). The inflammatory conditions associated with Al exposure are summarized in Figure 6.

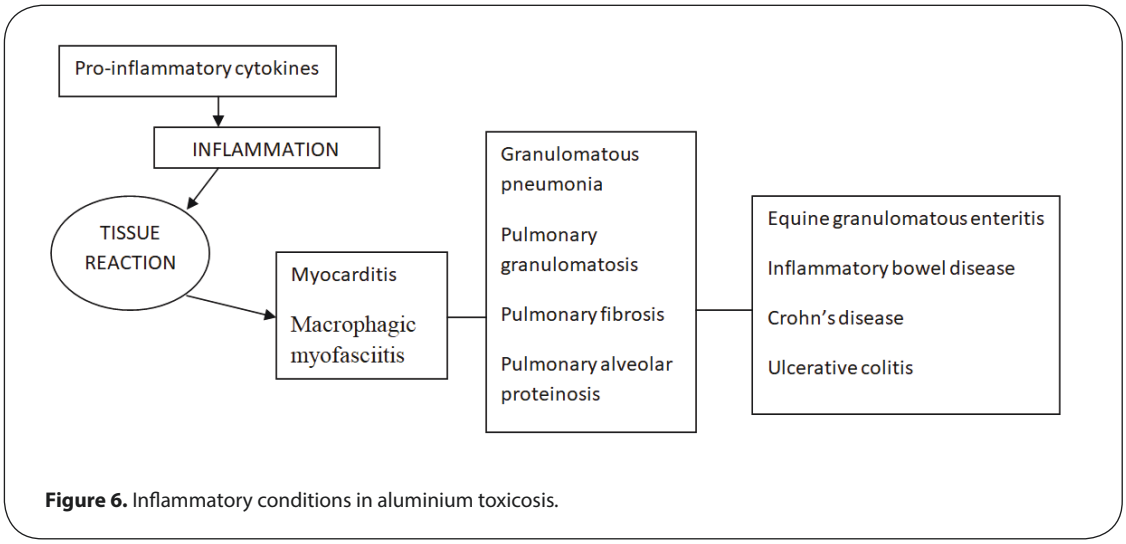

Figure 6. Inflammatory conditions in aluminium toxicosis.

Table 2. Effects of aluminium exposure on endocrine secretions in animals.

Hormone	Animal/human	Increase	Decrease	Normal	Reference
Plasma growth hormone	Duckling			+	Capdevielle *et al.*, 1995
Plasma insulin-like growth factor 1	Duckling			+	Capdevielle *et al.*, 1995
Plasma cortisol	Rat	+			Vasanthan and Joshi, 2018
Blood norepinephrine	Rat	+			Zhuang *et al.*, 2016
Serum estradiol	Mice		+		Chinoy and Patel, 2001
Serum testosterone	Gerbil (*Meriones unguiculatus*)	+			Reza and Palan, 2006; Gomes *et al.*, 2019
	Rat		+		Shahraki *et al.*, 2008; Sun *et al.*, 2011; Muselin *et al.*, 2016; Mouro *et al.*, 2018
Serum luteinizing hormone	Rat		+		Shahraki *et al.*, 2008; Sun *et al.*, 2011; Muselin *et al.*, 2016
	Rat	+			Reza and Palan, 2006
Serum follicule stimulating hormone	Rat			+	Reza and Palan, 2006; Sun *et al.*, 2011
	Rat		+		Shahraki *et al.*, 2008
Serum/plasma parathyroid hormone	Rat		+		Cannata *et al.*, 1983; Díaz-Corte *et al.*, 2001; Gonzelez-Suerez *et al.*, 2005
	Human patient with chronic renal failure, on haemodialysis	+			Sherrard *et al.*, 1985; Cournot-Witmer and Plachott, 1990
Serum thyroid hormone, T4	Rat		+		Orihuela, 2011
Serum thyroid hormone, T3	Rat		+		Orihuela, 2011
Serum thyroid hormone, free T4	Rat			+	Orihuela, 2011
Serum thyrotropin (TSH)	Rat			+	Orihuela, 2011
Insulin	Rat	+ (Acute)	+ (Chronic)		Wei *et al.*, 2018

As an adjuvant, Al in vaccines induces local inflammation that involves the NLRP3 inflammasome and NLRP3-independent pathways where macrophages, B- and T-lymphocytes play important roles in enhancing antigen-specific immune responses and increasing inflammatory cytokine production (Exley *et al.*, 2010; Hogenesch, 2013; Zlatkovic *et al.*, 2013; He *et al.*, 2015). Toxic exposure to Al causes immunotoxicity leading to inhibition of lymphocyte and macrophage functions (Nordal *et al.*, 1988; Zhu *et al.*, 2014a). Immunosuppression arises from oxidative stress which is associated with apoptosis of lymphocytes (Yu *et al.*, 2019) and damage to thymocytes and lymphocytes (Kamalov *et al.*, 2011). Immune functions of splenic B- and T-lymphocytes were inhibited in vitro by reduction of lymphocyte proliferation, cytokine secretion and proportions of CD-3(+) and CD-4(+) lymphocytes (She *et al.*, 2012). LPS-induced NLRP3 inflammasome activation, IL-1β, IL-6 and TNF-α expression and release in peritoneal macrophages were also suppressed by Al exposure (Xu *et al.*, 2018). Norepinerphrine release and activation of β-adenoceotors/cAMP pathway were promoted by Al in vivo, and this endocrine factor suppressed macrophage expressions of MIF and TNF-α (Zhuang *et al.*, 2016). Contact allergy to Al has been reported as an aberrant immune response in those having atopic dermatitis (Netterlid *et al.*, 2013).

The endocrine disruptions or hormonal changes associated with Al exposure are summarized in Table 2. Al accumulates in endocrine glands and causes damage to the glands through oxidative stress, thereby decreasing the level of the hormones secreted (Morrissey *et al.*, 1983) into the bloodstream for action at the target organs, causing organ hypofunction. For instance, there are reports of testicular and ovarian failures (Mohammed *et al.*, 2008; Fu *et al.*, 2014; Miska-Scramm *et al.*, 2017) from inadequate androgenic hormone levels (Chinoy and Patel, 2001; Shahraki *et al.*, 2008; Sun *et al.*, 2011; Muselin *et al.*, 2016; Mouro *et al.*, 2018) and decreased androgen receptor functions (Fu *et al.*, 2014; Sun *et al.*, 2018; Gomes *et al.*, 2019), bone pathology due to dysfunction of parathyroid gland (Cannata *et al.*, 1983; Sherrard *et al.*, 1985; Cournot-Witmer and Plachott, 1990; Díaz-Corte *et al.*, 2001; Gonzelez-Suerez *et al.*, 2005), prediabetes and diabetes due to pancreatic islet damage (Wei *et al.*, 2018). Parathyroid function can be impaired by the Al ion acting on calcium-sensing receptors when calcium level is low, with a higher efficiency than calcium and decreasing the expression of the receptors in the gland (Gonzelez-Suerez *et al.*, 2005). The metabolic effect of some levels of thyroxine (T3 and T4) decline without change in free T4 level is yet to be ascertained (Orihuela, 2011). The secretion of the hormone, sometimes increases when Al ion is stimulatory to the gland or because the target organs are refractory or unresponsive to the hormone when there is receptor depletion or decreased expression of the receptor on the cell membrane. The Al ions act as chemical stressor by promoting the release of norepinerphrine (Zhuang *et al.*, 2016) and cortisol (Vasanthan and Joshi,

2018); and the elevated levels of these hormones can cause increase in blood pressure (Zhang *et al.*, 2016). Elevated blood insulin level is associated with insulin resistance due to depletion of glucose transporter-4 protein expression in skeletal muscles during Al exposure (Wei *et al.*, 2018). Pseudohyperparathyroidism is associated with osteitis fibrosa in human patients with renal failure and hypercalcemia when exposed to Al intoxication (Sherrard *et al.*, 1985).

The cellular membrane is vital for the viability of the cell and Al exposure disrupts membrane activity via oxidative stress in various ways. In Alzheimer's disease associated with Al exposure, membrane fluidity increased in platelets and decreased in erythrocytes; and this observation was corroborated by a study where in vitro exposure of membrane suspensions to Al increased fluidity of platelet membranes and decreased the fluidity of erythrocyte membranes (van Rensburg *et al.*, 1992) with consequent effect on the viability of platelets (Neiva *et al.*, 1997) and erythrocytes (Vittori *et al.*, 2002). Erythrocyte membrane permeability and osmotic fragility are affected by *in vivo* and *in vitro* Al exposures (Igbokwe, 2018). Erythrocyte osmotic fragility decreased (Bazzoni *et al.*, 2005) or increased (Zatta *et al.*, 1989; Hernández *et al.*, 2008; Al-Qayim *et al.*, 2014; Oztürk and Ozdemir, 2015, Zhang *et al.*, 2016; Cheng *et al.*, 2018) depending on the Al speciation and type of erythrocyte injury. Eryptotic (apoptotic) injury reduces the erythrocyte aggregate size (Bazzoni *et al.*, 2005) because of the shrinking effect, thereby increasing osmotic resistance (Igbokwe, 2016). On the other hand, eryptotic injury which progresses to oncotic injury may cause swelling of the erythrocyte and increase osmotic fragility. It is therefore presumed that Al may cause either shrinking or swelling effect when the erythrocyte membrane is destabilized by Al exposure because of altered membrane permeability to intracellular and extracellular ions (Igbokwe, 2016). Cell membrane functions which regulate transmembrane transport of ions are expected to be disrupted when ATPase in cell membrane loses some level of activity during Al exposure. There are reports showing that Al inhibited activities of Na^+K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase in erythrocytes (Zhang *et al.*, 2016), vascular endothelial cells (Vorbrodt *et al.*, 1994), testes (Sun *et al.*, 2018) and ovaries (Fu *et al.*, 2014) of rats. The Al-induced change in erythrocyte size may also be accompanied by change in erythrocyte shape resulting in the formation of echinocytes (Suwalsky *et al.*, 2004), acanthocytes and stomatocytes (Vittori *et al.*, 2002) in vitro, due to altered membrane morphology (Lukyanenko *et al.*, 2013). After long-term oral intake of Al, schistocytes and target cells were observed in stained peripheral blood of rats (Vittori *et al.*, 1999). The lipid bilayer of the plasma and mitochondrial membranes was morphologically altered in lymphocytes (Skarabahatava *et al.*, 2015). The protein components of membranes are degraded or inadequately expressed during Al exposure, as observed in the loss of band 3 protein of erythrocyte membrane (Vittori *et al.*, 2002; Vota *et al.*, 2012; Cheng *et al.*, 2018), inaction of membrane-bound enzymes, inhibition of calbindin protein level in enterocytic membrane (Cox and Dunn, 2001) and downregulation of GLUT4 protein expression in the membrane of skeletal muscle (Wei *et al.*, 2018). The surface of cell membranes could be affected by Al exposure through externalization of phosphatidylserine after apoptosis (Vota *et al.*, 2012), inhibition of membrane receptor protein expression in gonads (Fu *et al.*, 2014; Sun *et al.*, 2018; Gomes *et al.*, 2019), dysregulation of erythropoietin receptor functions on erythroid progenitors (Vittori *et al.*, 2005) and loss of membrane surface sialic acid residues on vascular endothelial tissue with impaired intercellular junctions (Vorbrodt *et al.*, 1994).

Systemic toxicosis

Pulmonary effect

Pulmonary lesions in humans linked to Al exposure during production of Al products include granulomatous pneumonia, pulmonary granulomatosis, pulmonary fibrosis, pulmonary alveolar proteinosis and desquamative interstitial pneumonia (Chen *et al.*, 1978; Herbert *et al.*, 1982; Miller *et al.*, 1984; De Vuyst *et al.*, 1987; Jederlinic *et al.*, 1990; Taiwo, 2014; Iijima *et al.*, 2017). Asthma may be caused by Al exposure (Burge *et al.*, 2000), though the asthma among Al workers may be due to other chemical factors like gases and smoke (Taiwo *et al.*, 2006). Reactive airways dysfunction syndrome was rarely reported among Al smelter workers (Wesdock and Arnold, 2014). Acute-duration oral exposure to Al phosphide has been reported to cause pulmonary edema in persons following accidental or volitional ingestion (Chopra *et al.*, 1986; Khosla *et al.*, 1988). The toxicity was probably due to the formation of highly toxic phosphine gas rather than to Al exposure (Alter *et al.*, 2001; Kamanyire and Murray, 2003; Moghadamnia, 2012). Intermediate- and chronic-duration studies found no organ weight or histological changes in the lungs of rats exposed to 70 mg Al/kg/day as Al chloride in drinking water for 30, 60 or 90 days (Dixon *et al.*, 1979), rats exposed to 133 mg Al/kg/day as Al nitrate in drinking water for 30 days (Gomez *et al.*, 1986), rats and mice exposed to 0.6 or 1.2 mg Al/kg/day as Al potassium sulfate in drinking water for 24 months (Schroeder and Mitchener, 1975a, b), or mice exposed to 979 mg Al/kg/day as Al potassium sulfate in food for 20 months (Oneda *et al.*, 1994). However, Hasseeb *et al.* (2011) reported neutrophilic and mononuclear cell infiltrations of lung alveoli of rats administered 37 mg/kg/day of Al chloride in drinking water for 8 weeks. Congested blood vessels in inter-alveolar spaces were reported after administration of different concentrations of Al chloride via gavage for 8 weeks (Buraimoh and Ojo, 2013). Pulmonary lesions are rare and inconsistent in experimental animals where Al exposure is not through aerosol vehicles. Under natural conditions, the vehicular substances and the Al speciation may influence the stimulation of chronic pathologic reactions in the lung.

Cardiovascular effects

Toxic myocarditis, myocardial hypokinesia, left ventricular thrombosis and myocardial dysfunction were reported in a case of Al phosphide intoxication (Hangouche *et al.*, 2017). Ischemic stroke due to thrombosis in the right middle cerebral artery was reported as the delayed complication of Al phosphide poisoning (Abedini *et al.*, 2014). However, other Al compounds may not cause cardiovascular lesion. Cardiac teratogenessis was reported in embryonic chick heart where defects in ventricular septation and ventricular myocardium were reported (El Mazoudy and Bekhet, 2016). There was significant association between increased maternal hair Al contents and risk of total congenital heart defects in offspring, especially in subtypes such as septal defects, conotruncal defects and right ventricular outflow obstruction in female rats (Wang *et al.*, 2012). No histological changes were observed in the hearts of rats given 70 mg Al/kg/day as Al chloride in drinking water for 30, 60, or 90 days (Dixon *et al.*, 1979). Similarly, no effect on organ weight nor histological changes were found in the hearts of rats that ingested 133 or 284 mg Al/kg/day as Al nitrate in drinking water or base diet for 30 days (Gomez *et al.*, 1986) or 100 days, respectively (Domingo *et al.*, 1987). Organ weight and histological changes were not observed in the hearts of dogs that consumed 75 mg Al/kg/day (Katz *et al.*, 1984) or 88 mg Al/kg/day (Pettersen *et al.*, 1990) as sodium Al phosphate in the diet for 6 months. In summary, cardiovascular effects due to toxicosis are congenital heart defects, inflammation and dysfunction of the myocardium and cardiovascular thrombosis.

Gastrointestinal effects

In horses, Al was found in tissues, blood vessel walls and granulomatous lesions of the intestines associated with equine granulomatous enteritis (Fogarty *et al.*, 1998), and Al was demonstrated to have the capacity to induce granuloma formation in vitro (de Chambrun *et al.*, 2014). Oral intake of Al may affect the intestinal microbiota, permeability and immune response which influence the local inflammatory conditions (Vignal *et al.*, 2016). In individuals that are genetically susceptible to Crohn's disease, Al is linked to the induction and persistence of the chronic relapsing intestinal inflammation (Lerner, 2007). Inflammatory bowl diseases, consisting of disease entities like Crohn's disease and ulcerative colitis, are characterized by excessive intestinal inflammation and experimental evidence in mice indicates that Al promotes intestinal inflammation, thereby implicating Al in the pathogenesis of inflammatory bowl diseases (de Chambrun *et al.*, 2014). Chemically-induced acute colitis and chronic colitis in transgenic mice lacking interleukin 10 were aggravated by oral exposure to Al, because Al increased the intensity and duration of intestinal inflammation and decreased regeneration or renewal of the intestinal epithelial mucosal cells (de Chambrun *et al.*, 2014). Furthermore, intestinal barrier function was impaired by Al exposure under basal conditions; and there was a synergistic stimulation of pro-inflammatory cytokine expression by Al and lipopolysaccharides (de Chambrun *et al.*, 2014). Oral Al chloride exposure caused epithelial degeneration, goblet cell proliferation and lymphocyte infiltration in the mucosa of the small intestine of Wistar rats (Buraimoh and Ojo, 2012). Few experimental studies (Gomez *et al.*, 1986; Oneda *et al.*, 1994) did not report intestinal lesions after oral exposure to Al at 133 mg Al/kg/day as Al nitrate in drinking water to rats for 30 days and 979 mg Al/kg/day as Al potassium sulfate in the food of mice for 20 months. The acute and chronic inflammations in the intestine may induce poor intestinal digestion and absorption.

Hematologic effects

Al exposure has been associated with significant inhibition of colony forming units-erythroid (CFU-E) development in the bone marrow of mice exposed to 13 mg Al/kg as Al citrate or chloride administered via gavage for 5 days/week for 22 weeks (Garbossa *et al.*, 1996), rats exposed to 27 mg Al/kg as Al citrate administered via gavage 5 days/week for 15 weeks (Garbossa *et al.*, 1998), and rats exposed to 230 mg Al/kg/day as Al citrate in drinking water for 8 months (Vittori *et al.*, 1999). The effect of Al on erythroid progenitor cells and erythrocytes was associated with slow growth and increased degradation of membrane band 3 proteins, respectively (Vittori *et al.*, 2002). The genotoxicity from Al exposure in mice resulted in mitodepressive effect in the bone marrow (D'Souza *et al.*, 2014). Anemia caused by Al toxicity is not associated with adequate regenerative activity of the bone marrow and reticulocytosis (Chmielnicka *et al.*, 1994; Osman *et al.*, 2012). The additional causes of anemia appear to be multi-factorial and include defective hemoglobin production due to inhibition of the enzymes of heme synthesis, altered erythrocyte membrane structure and fragility, shortening of red blood cell life span due to eryptotic and oncotic injuries, and inadequate iron utilization (Zatta *et al.*, 1989; Perez *et al.*, 2001; Bazzoni *et al.*, 2005; Vittori *et al.*, 2002; Niemoeller *et al.*, 2006; Hernández *et al.*, 2008; Sadhana, 2011; Vota *et al.*, 2012; Lukyanenko *et al.*, 2013; Al-Qayim *et al.*, 2014; Oztürk and Ozdemir, 2015; Zhang *et al.*, 2016; Cheng *et al.*, 2018). Significant decreases in hemoglobin, hematocrit (packed cell volume) and erythrocyte osmotic fragility were reported after Al exposure (Garbossa *et al.*, 1996; Garbossa *et al.*, 1998; Vittori *et al.*, 1999; Farina *et al.*, 2005). The anemia is characterized by decreases in mean corpuscular volume (microcytosis) and mean corpuscular hemoglobin (hypochromia), but in chronic exposures, the erythrocyte parameters recover with persistence of microcytosis and hypochromia (Mahieu *et al.*, 2000). In rats loaded with Al, heme dyshomeostasis was reported with evidence of decreased activity of aminolevulinic acid dehydratase and increased activity of heme oxygenase in the rat liver associated with activation of JNK pathway, indicating an increase in heme degradation (Lin *et al.*, 2013). No alterations in hemoglobin, hematocrit and erythrocyte osmotic fragility were reported in a number of experimental Al exposures (Katz *et al.*, 1984; Gomez *et al.*, 1986; Domingo *et al.*, 1987; Pettersen *et al.*, 1990;

Table 3. Summary of haematologic effects of aluminium toxicosis.

Toxic effects	Toxic actions
Depressed erythropoiesis	Inhibition of CFU-E
	Slow growth of erythroid cells
	Inhibition of heme synthesis
	Increased heme degradation
	Dysregulated erythropoietin receptor function
Anaemia	Reduced erythrocyte life span
	Erythrocyte apoptosis (eryptosis)
	Altered erythrocyte fragility
	Decreased erythrocyte membrane fluidity
	Inhibition of erythrocyte membrane ATPase
	Altered erythrocyte shape: echinocytes, acanthocytes, stomatocytes, target cells

Oteiza *et al.*, 1993b; Garbossa *et al.*, 1996**).** Vittori *et al.* (1999) did not find significant alterations in plasma iron levels or total iron binding capacity in rats exposed to 230 mg Al/kg/day as Al citrate in drinking water for 8 months; however, they reported impaired iron uptake and decreased iron incorporation into heme in the bone marrow. Farina *et al.* (2005) found significant decreases in blood iron concentrations and no change in total iron binding capacity in rats exposed to 54.7 mg Al/kg/day as Al sulfate in a sodium citrate solution in drinking water for 18 months. Florence *et al.* (1994) reported decreases in serum iron levels, total iron binding capacity, and transferrin saturation in rats exposed to 75 mg Al/kg/day as Al citrate in the diet for 6 months. Chronic Al exposure in rats disrupted iron homeostasis (Zhang *et al.*, 2010). In summary, the hematologic effect of toxicosis consists of anemia due to erythrocyte and erythroid pathology with suppression of erythropoiesis (Table 3)

Neurologic effects

In humans, Al accumulation in the brain and scalp hairs has been associated with neurodegenerative diseases such as dialysis-associated encephalopathy, Alzheimer's disease, Parkinson's disease (dementia), amyotropic lateral sclerosis, multiple sclerosis and autism (King *et al.*, 1981; Savory *et al.*, 1996; Kawahara and Kato-Negishi, 2011; Arain *et al.*, 2015; Jones *et al.*, 2017; Mirza *et al.*, 2017; Mold *et al.*, 2018). The Al in brains of 5 out of 12 donors with familial Alzheimer's disease was > 10 µg/g dry weight (Mirza *et al.*, 2017). In autism, Al in parts of the brain was up to 19 µg/g dry weight (Mold *et al.*, 2018). There is a role for Al in multiple sclerosis because patients excrete high amounts of Al in urine, facilitated by drinking silicon-rich mineral water (Jones *et al.*, 2017). Subchronic exposure to Al was associated with reduced population of neural stem cells and hampered cell proliferation and neuroblast differentiation in the brain of mice (Nam *et al.*, 2014, 2016). Injection of Al, especially intra-cisternally, induced neurological changes in animal models (Wisniewski *et al.*, 1980; Anon, 2008c). Rats orally administered Al (100 mg/kg/day) for 90 days accumulated more Al in their brains, had increased brain acetyl cholinesterase activity and had decreased brain choline acetyltransferase activity (Bilkei-Gorzó, 1993). Mice fed high Al levels (1,000 mg/kg diet of Al as Al lactate) were less active, had decreased grip strength, and increased startle responses after 90 days when compared with control (Golub *et al.*, 1992). Oteiza *et al.* (1993b) reported that mice fed diets containing 1,000 mg/kg diet of Al (as Al chloride) with sodium citrate accumulated more Al in the brain nuclear fraction and spinal cord, had lower grip strength, and greater startle responsiveness after 5 and 7 weeks. Old (18 months of age) rats exposed to Al (100 mg/kg/day) in drinking water with citrate (356 mg/kg/day of citrate) had decreased numbers of synapses and a greater percentage of perforated synapses than controls, but no changes in behavior (Colomina *et al.*, 2002). Garruto *et al.* (1989) reported that cynomolgus monkeys fed a low calcium diet (3,200 mg/kg diet) with Al (125 mg/day) for 41 to 46 months had more degenerative changes that were consistent with early Alzheimer's disease or Parkinson's dementia in the central nervous system than control monkeys. Golub and Germann (2001) observed growth depression and poorer performance on standardized motor tests in mice offspring when dams were exposed to Al (1,000 mg/kg diet as Al lactate) with marginal levels of calcium and magnesium during pregnancy and lactation. Mice fed lower rather than recommended levels of calcium (2,500 versus 5,000 mg/kg diet of calcium) with Al (15,600 mg/kg diet as Al hydroxide) for 11 to 25 months accumulated more hyperphosphorylated tau protein in the cortical neurons and had more atrophic neurons in the central nervous system (Kihira et al,. 2002). Transgenic mice with over-expressed human amyloid precursor protein had increased brain isoprostane levels and more amyloid-β peptide formation and deposition when Al was added to their diets, but the effects of Al were reversed by additional dietary vitamin E (Pratico *et al.*, 2002), suggesting that Al could contribute to neurodegeneration by enhancing amyloid deposition and aggravating lesions by oxidative events (Campbell and Bondy, 2000; Yuan *et al.*, 2012; Chen and Zhong, 2014; Liaquat *et al.*, 2019). In a nutshell, Al exposure promotes oxidative stress and amyloid deposition in the nervous tissue which results in neurodegeneration, neuronal necrosis and dysneurogenesis, which constitute the basis for the neurological diseases associated with Al intoxication.

Musculoskeletal effects

The major myopathy induced by Al exposure is macrophagic myofasciitis (aluminic granuloma) associated with chronic arthromyalgia or myalgia and chronic fatigue syndrome (Exley *et al.*, 2009; Gherardi and Authier, 2012; Rigolet *et al.*, 2014; Gherardi *et al.*, 2016; Miller, 2016). Skeletal muscle necrosis occurred in the diaphragm and abdominal muscles of rats adjacent to the peritoneum after intraperitoneal injection of Al lactate (Levine *et al.*, 1992). Muscle fiber atrophy, with retardation of growth, was reported in growing pigs which was associated with hypophosphatemia induced by dietary Al

hydroxide supplementation (Haglin *et al.*, 1994). Smooth muscle contraction induced by K+ ion was inhibited by Al exposure (Nasu *et al.*, 1998). Myocardial function may be altered in diabetic individuals by Al exposure, in as much as Al toxicity potentiates the decline in calcium uptake into the sarcoplasmic reticulum of the myocardial fibers of such individuals (Levine *et al.*, 1990). In individuals where neurodegerative conditions affect the nerve supply to muscles, the muscles may undergo denervation atrophy and become dysfunctional as in multiple sclerosis or amyotropic lateral sclerosis. Taken together, Al toxicosis may cause muscle damage, inflammation and dysfunction

The bone diseases associated with Al exposure are osteoporosis, osteomalcia, rickets, exostosis, osteodystrophy and osteitis fibrosa (Sherrard *et al.*, 1985; Chappard *et al.*, 2016; Rodríguez and Mandalunis, 2018; Klein, 2019). There is increased risk of osteoporosis and low bone mineral density during Al exposure (Cao *et al.*, 2016; Sun *et al.*, 2016) because of disruption of bone formation, and inhibition of osteoblast proliferation, differentiation and mineralization (Li *et al.*, 2012, 2016; Cao *et al.*, 2016; Sun *et al.*, 2016; Yang *et al.*, 2016; Zhu *et al.*, 2016b; Song *et al.*, 2017; Sun *et al.*, 2017; Huang *et al.*, 2017). In individuals with Al overload, undecalcified bone matrix contains Al and bone conditions like exostosis and osteomalacia may occur in circumstances that increase Al uptake and colocalization as observed in celiac disease, hemochromatosis and sickle cell anemia (Chappard *et al.*, 2016). Osteoclastogenesis is promoted by low-dose exposure while osteoclast apoptosis is caused by high-dose exposure (Yang *et al.*, 2018). There are case reports of osteomalacia and rickets in infants and adults using Al-containing antacids for the treatment of gastrointestinal illnesses (Chines and Pacifici, 1990; Pivnick *et al.*, 1995; Woodson, 1998). The Al in antacids binds with dietary phosphorus and prevents its absorption resulting in hypophosphatemia and phosphate depletion (Woodson, 1998). Osteomalacia, characterized by bone softening, increased spontaneous fractures and pain, has been reported in dialyzed uremic adults and children exposed to Al-contaminated dialysate or orally administered Al-containing phosphate-binding agents (Mayor *et al.*, 1985; Wills and Savory, 1989; Andreoli, 1990). Low osseous remodeling rate and peripheral resistance to parathyroid hormone are associated with Al intoxication (Pun *et al.*, 1990). Decreased Al urinary excretion caused by impaired renal function with, possibly, an increase in gastrointestinal absorption of Al results in increased Al load leading to markedly increased bone Al levels and the presence of Al between the junction of calcified and non-calcified bones (Alfrey, 1993). Long-term oral exposure to Al results in an increase in Al levels in the bone (Ahn *et al.*, 1995; Konishi *et al.*, 1996) that is responsible for the bone disease.

In brief, the review has identified the following events to occur during Al exposure to disrupt bone morphology: (a) interference with the availability of calcium for bone formation at the level of intestinal absorption and hormonal control through parathyroid hormone; (b) inhibition of osteoid formation and mineralization through osteoblast dysregulation; and (c) destabilization of osteoclast functions with alteration in osteoclastogenesis and osteoclast apoptosis (Figure 7).

Reproductive and developmental effects

Human reproduction may be affected negatively by Al exposure (Klein *et al.*, 2014; Mouro *et al.*, 2017). Human semen and spermatozoa contain Al and patients with oligospermia had higher Al concentration than healthy individuals (Klein *et al.*, 2014). At human dietary level of Al and continuous exposure for 60 days, the rat testes accumulated low Al levels of 3.35 µg/g and it was associated with increased oxidative stress and inflammation, decreased daily sperm production, reduced sperm count and motility and increase in abnormal spermatozoa (Martinez *et al.*, 2017). In male rats, subchronic exposure to Al chloride did not result in elevated Al accumulation in the testes, but toxic effects reported in the testes included impairment of spermatogenesis and increase in sperm malformation rate (Zhu *et al.*, 2014b). Imbalance in trace mineral metabolism occurred in the testis with

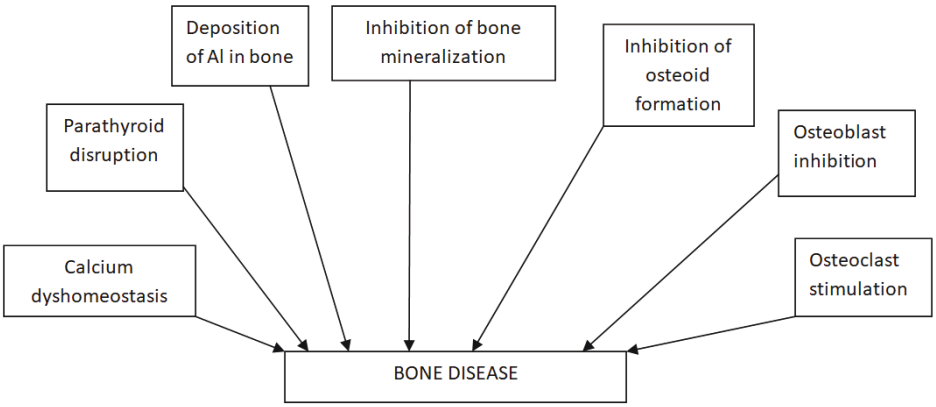

Figure 7. Pathogenesis of bone disease in aluminium toxicosis.

testicular levels of iron and zinc increasing and that of copper decreasing during exposure (Zhu *et al.*, 2014b). Furthermore, metabolic inhibition in the testis was reported with regard to the functions of acid phosphatase, succinate dehydrogenase, and lactate dehydrogenase and its isoenzymes (Zhu *et al.*, 2014b), alongside with testicular membrane dysfunction due to inhibition of membrane ATPase activities in Al-exposed rats (Sun *et al.*, 2018). The weights of the testes and epididymides were decreased by Al exposure in rats as serum testosterone levels dropped (Mouro *et al.*, 2018). In male rats, testicular development was impaired by Al exposure, associated with reduction in serum levels of testosterone and luteinizing hormone (LH) levels and decrease in androgen receptor protein expression without effect on serum follicle stimulating hormone (FSH) (Sun *et al.*, 2011, 2018). The offspring of exposed rats (F0) in a three-generation study belonging to F1 and F2 had decreased testosterone and LH levels, decreased testicular weight, and increase in the production of abnormal immobile spermatozoa, whereas the parental F0 group did not present with such reproductive abnormalities (Muselin *et al.*, 2016). In rats that were injected with Al chloride (4.125 pmole) in artificial cerebrospinal fluid via the lateral ventricle, there were significant decreases in serum FSH, LH and testosterone levels, and reduction in sperm count from the vas deferens and epididymides (Shahraki *et al.*, 2008). Bank voles (*Myodes glareolus*) exposed to Al produced lower quality and quantity of sperm than normal, but reproductive capacity was not significantly affected in females (Miska-Schramm *et al.*, 2017). After intraperitoneal treatment at 50 mg/kg for 20 days, blood testosterone and LH levels were increased in male rats, but FSH level was not affected (Resa and Palan, 2006). Khattab *et al.* (2010) reported that administration of Al chloride (20 mg/kg) to male rats via gavage for 70 days caused fertility disturbances and testicular dysfunction. Other reports showed that Al induced decrease in sperm counts, motility and viability, with increase in dead and abnormal sperm counts (Bataineh *et al.*, 1998; Guo *et al.*, 2005; Yousef *et al.*, 2007; Yousef and Salama, 2009; D'Souza *et al.*, 2014). Testicular and epididymal weights and serum testosterone and luteinizing hormone levels were reduced by Al exposure (Reza and Palan, 2006; Mouro *et al.*, 2017). In male and female gerbils (*Meriones unguiculatus*), Al exposure disrupted prostate development in neonates, with the consequence of adult offspring having elevated serum testosterone levels with low androgen receptor frequency associated with increased proliferation of cells of the prostate (Gomes *et al.*, 2019).

Chinoy and Patel (2001) exposed female mice to Al chloride at 200 mg/kg for 30 days and observed decreased steroidogenesis in the ovaries associated with decreased serum estradiol levels. Exposure to Al sulphate during gestation caused reduction in maternal body weight, reduction in fetal weight and crown rump length, and impairment of fetal bone development and preossification (Yassa *et al.*, 2017). In adult mice exposed to Al at 1000–1400 ppm in drinking water or 19–39 mg/kg intraperitoneally, pregnancy rate decreased with increased frequency of atretic follicles; after pregnancy, failure of pregnancy increased with increased rate of uterine resorption and decrease in the number of viable fetuses and implantation sites (Mohammed *et al.*, 2008). Fu *et al.* (2014) reported that Al exposure damaged ovarian structure, disrupted metabolism of iron, zinc and copper in the ovary and decreased the activities of ovarian ATPases and expressions of androgenic receptors for FSH and LH; and could consequently lead to infertility due to inhibition of ovulation and development of corpus luteum. Exposure to Al during mouse pregnancy resulted in reduced fetal weight and increased frequency of external anomalies in fetuses (Malekshah *et al.*, 2005) and fetal micronucleated erythrocytes (D'Souza *et al.*, 2014). Khalaf *et al.* (2007) reported perinatal and postnatal adverse effects of Al exposure on fetuses and neonates during gestation and lactation of female rats. The hepatic toxicity of Al chloride was also reported in pregnant rats and their offspring with observation of decreased fetal weight and size (Mestaghanmi *et al.*, 2003). Exposed embryonic chicks and rat fetuses developed congenital myocardial defects (Wang *et al.*, 2012; El Mazoudy and Bekhet, 2016).

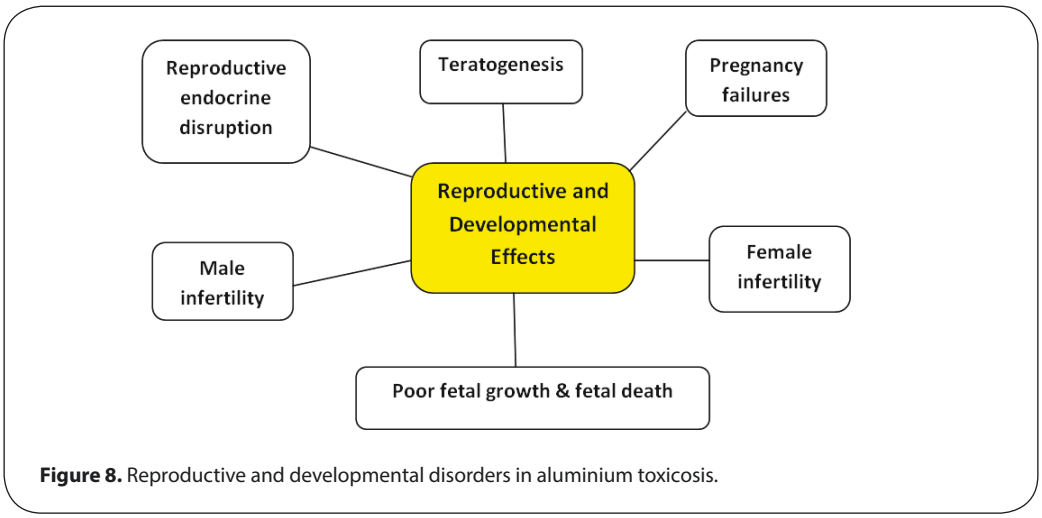

Figure 8. Reproductive and developmental disorders in aluminium toxicosis.

On the whole, Al toxicosis caused lesions in the testes and ovaries resulting in impairment of spermatogenesis and ovarian function related to ovulation, with the consequence of reproductive inefficiency associated with pregnancy failures and poor fetal development (Figure 8).

Hepato-renal and pancreatic effects

Al causes oxidative injuries to the kidney and liver leading to tissue degeneration and necrosis, and associated serum biochemical derangements (Nikolov *et al.*, 2010; Mailloux *et al.*, 2011; Bai *et al.*, 2012; Li *et al.*, 2015; Xu *et al.*, 2017). Abdel-Wahab (2012) reported a significant increase in the activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and total bilirubin, as well as increased serum urea and creatinine levels after oral administration of 20 mg/kg of Al chloride for 30 days in experimental rats. Ingestion of aluminium phosphide pellets was reported to induce acute pancreatitis in one patient (Verma *et al.*, 2007). Rats had moderate pancreatic islet necrosis after intermediate oral exposure (50 mg/kg for 28 days) to Al chloride (Figure 9) which was associated with impaired fasting blood glucose and impaired oral glucose tolerance (Igwenagu, 2017; Igwenagu *et al.*, 2019). Rats treated intra-peritoneally with Al chloride at 10 mg/kg for 30 days had significantly increased fasting blood glucose, serum insulin level and insulin resistance index on days 10 and 20 of treatment, but as treatment progressed to day 30, serum insulin level had decreased, indicating that pancreatic β-cell function decreased as pancreatic damage occurred with progression of treatment (Wei *et al.*, 2018). The hepatic and pancreatic lesions cause changes in metabolism (Figure 5) which result in hyperglycaemia, hypoproteinaemia, hyperlipidaemia, hypercholesterolaemia and hypertriglyceridaemia (Omar *et al.*, 2003; Kowalczyk *et al.*, 2004; Türkez *et al.*, 2011; Abdel-Wahab, 2012; Belaïd-Nouira *et al.*, 2013).

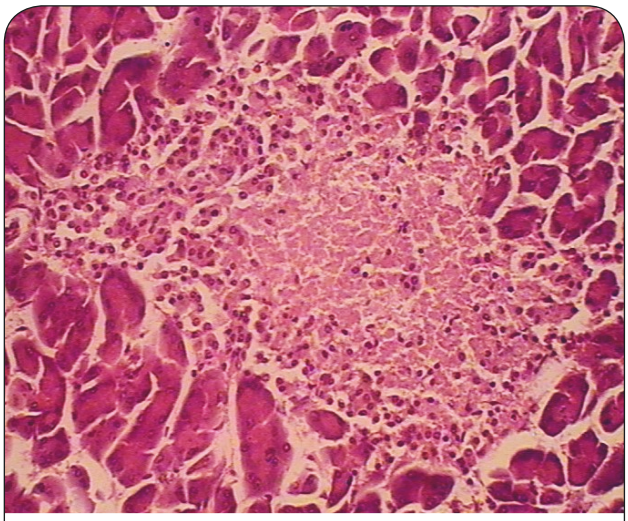

Figure 9. Photomicrograph of pancreas of aluminium chloride-treated rat showing coagulative necrosis of the pancreatic islet tissue with disorganization of its architecture (H & E, x 400) From Igwenagu *et al.* (2019).

Mammary gland or breast effects

Breast cancers and cysts are mammary gland conditions where emerging evidence are suggesting that Al may be involved in their causation (Darbre, 2016). Al chlorohydrate in antiperspirant cosmetics and other underarm cosmetic products may be an important source of Al exposure (Pineau *et al.*, 2014; Linhart *et al.*, 2017). In a case control study (Linhart *et al.*, 2017), the use of underarm cosmetic products containing Al was significantly associated with breast cancer incidence and the Al levels in breast tissues were significantly higher in breast cancer cases than controls (5.8 versus 3.8 nmol/g). Breast cancer patients had higher levels of Al in breast tissues than in blood serum (Darbre *et al.*, 2013b). There were higher levels of Al in nipple aspirates of cancer patients than healthy controls and higher Al levels in breast cyst fluid than serum or milk (Darbre *et al.*, 2011). The Al contents of nipple aspirates of breast cancer patients correlated with biomarkers of oxidative stress and inflammation in the breast microenvironment (Mannello *et al.*, 2013). The Al accumulating in the breast tissue may influence the biological characteristics of breast epithelial cells and carcinogenesis is considered a probable outcome (Pineau *et al.*, 2014). The content of Al in breast tissues from mastectomies are being efficiently and accurately estimated in order to properly assess the involvement of Al in the aetiology of breast cancer (House *et al.*, 2013). Current evidence suggests that Al can induce DNA damage in human breast epithelial cells and subsequently induce proliferation of the cells (Darbre *et al.*, 2013a, b). Thus, Al may increase the risk of breast cancer by acting as a metalloestogen (Darbre, 2016). The migratory and invasive properties of oestogen-responsive MCF-7 human breast cancer cells were increased in the presence of Al (Darbre *et al.*, 2013a). Long-term Al exposure also increased the migration of oestrogen-unresponsive MDA-MB-231 human breast cancer cells in culture where their expression of matrix metalloproteinases (MMP9/14) was increased (Bakir and Darbre, 2015).

Diagnosis and treatment of aluminium intoxication

Al can be measured in the blood, bone, urine, and feces to confirm Al load and association with toxicosis. A variety of analytical methods have been used to measure Al levels in biological materials and they include accelerator mass spectroscopy, graphite furnace atomic absorption spectrometry, flame atomic absorption spectrometry, electro-thermal atomic absorption spectrometry, neutron activation analysis, inductively coupled plasma atomic emission spectrometry, inductively coupled plasma mass spectrometry, and laser microprobe mass spectrometry (Maitani *et al.*, 1994; Owen *et al.*, 1994; Van Landeghem *et al.*, 1994; Razniewska and Trzcinka-Ochocka, 2003). Contamination is a major problem encountered in the

analysis of Al by all methods except that using radioactive ^{26}Al. When using the other methods, all items used during collection, preparation, and assay should be checked for Al contribution to the procedure.

Treatment of Al intoxication is done with the chelating agent, deferoxamine, which is a colourless crystalline base, produced by the bacterium, *Streptomyces pilosus*. Structurally, it is composed of one molecule of acetic acid, two molecules of succinic acid and three molecules of 1-amino-5 hydroxylamine pentane (Keberle, 1964). Deferoxamine is mainly used as an iron-chelating agent to treat iron overload. But due to the chemical similarity between Al and iron, it can also successfully mop-up excess Al from the body (Day, 1986; Martin *et al.*, 1987). Deferoxamine administered intravenously has been shown to reduce the body Al load and to ameliorate injury to the bone and brain in patients receiving hemodialysis and peritoneal dialysis (Malluche *et al.*, 1984). It has also been used successfully to treat Al toxicity in children (Warady *et al.*, 1986; Ogborn *et al.*, 1991). Deferoxamine therapy seems beneficial for those with established Al toxicity; however, this therapy is not without hazards. It may cause allergic reactions such as pruritus, wheals and anaphylaxis. Other adverse effects include dysuria, abdominal discomfort, diarrhea, fever, leg cramps, cataract, and tachycardia (Klaassen, 1990).

Malic acid is also a potent chelator of Al used in treatment of Al intoxication (Domingo *et al.*, 1988). Treatment with malic acid has been reported to greatly increase the fecal and urinary excretion of Al and reduce the concentration of Al present in various organs and tissues (Rim, 2007; Crisponi *et al.*, 2012; Al-Qayim *et al.*, 2014). Other chelating agents such as citric, malonic, oxalic, and succinic acids have been used experimentally to reduce aluminum load in rats and mice (Domingo *et al.*, 1988).

Antioxidants and free radical scavengers such as selenium, melatonin, boric acid and vitamin C have been employed experimentally to ameliorate the deleterious effects of free radicals produced as a result of Al intoxication (Omar *et al.*, 2003; Abubakar *et al.*, 2004; Fyiad, 2007; Turkez *et al.*, 2011). Other researchers have used plant extracts of fenugreek seed, grape seed, ginger, wheat grass powder, black tea, *Allium cepa, Caesalpinia crista, Arthrophytum* (*Hammada scoparia*), *Moringa oleifera* and *Celastrus paniculatus* to ameliorate the toxicosis caused by Al exposure (Khattab *et al.*, 2010; Hasseeb *et al.*, 2011; Osman *et al.*, 2012; Belaïd-Nouira *et al.*, 2013; Sumathi *et al.*, 2013; Bitra *et al.*, 2014; Osama *et al.*, 2014; Mathiyazahan *et al.*, 2015; Singh and Goel, 2015; Taïr *et al.*, 2016; Ravi *et al.*, 2018). The neuronal death in the hippocampus of the brain associated with neurodegeneration in rats caused by Al exposure was attenuated by quercetin (Sharma *et al.*, 2016). Ginsenoside Rb1 was reported to prevent Al-induced oxidative stress and reverse the osteoblast viability and growth after impairment by Al (Zhu *et al.*, 2016a). Chlorogenic acid was effective as a chelating agent and antioxidant in protection against the toxicity of Al (Wang *et al.*, 2018; Cheng *et al.*, 2017, 2019). Chenodeoxycholic acid ameliorated the neurotoxic effect of Al by improving insulin sensitivity (Bazzari *et al.*, 2019). Türkez *et al.* (2010) reported that propolis prevented the genetic and hepatic damages induced by Al intoxication.

On the whole, the approach to the treatment of Al toxicosis after diagnosis involves strategies that include the following: prevention of Al intake, reduction of Al absorption, increasing Al excretion, maintaining functional kidneys, reducing Al load by chelation with chelating agents and amelioration of toxic effects with antioxidants and other agents that reduce toxicity (Figure 10)

General perspective and conclusion

This review has provided an overview of the pathologic basis of Al toxicosis. The association of Al intoxications with various pathologic syndromes and pathogenic mechanisms linked to toxic actions may provide avenues for strategic interventions. The study of these pathologies

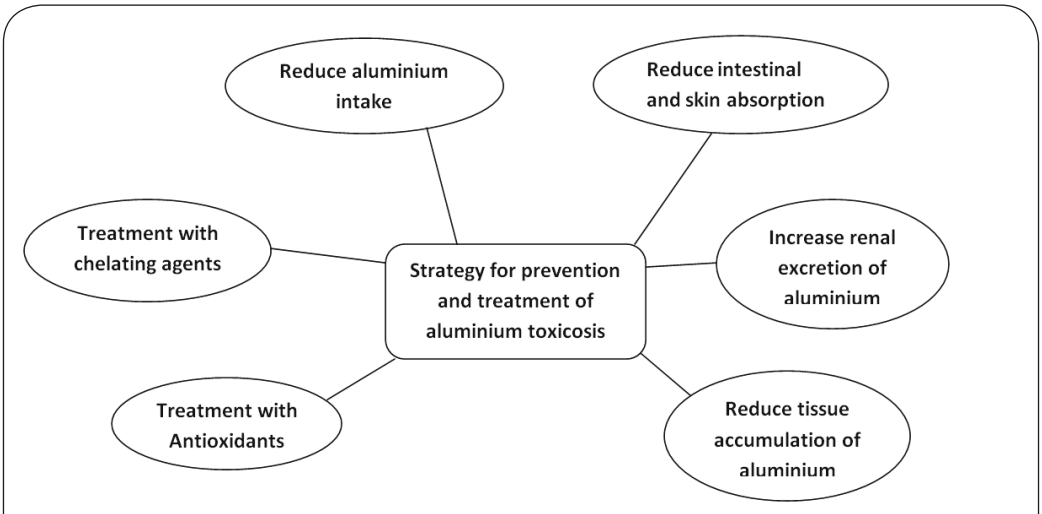

Figure 10. Prevention and treatment of aluminium toxicosis.

have received recent attention in epidemiological surveys in regard to some human diseases such as Alzheimer's disease, autism, osteoporosis, diabetes mellitus, inflammatory bowel disease and others mentioned in the review. We have reviewed the process of Al intoxication, toxic actions and systemic effects with an exploratory approach to provide the subsequent highlights of the review in this section.

After the intake of Al and its deposition in tissues, the cell is the primary target of the toxic action of Al, where the ion interacts with the plasma membrane moieties, cytoplasmic biomolecules, mitochondria and nuclear structures. The major toxic action of Al is to generate oxidative stress by producing reactive free radicals which can overwhelm the antioxidant defenses of the cell to perpetrate cellular injuries. The oxidative injuries emanate from the oxidation of proteins, lipids and nucleotides, which result in generation of altered functional biomolecules with defective operational capabilities towards cellular homeostasis. The disruption of homeostatic environments of the cell has the capacity to change the semi-permeability and receptor functions of the plasma membrane, alter the reactivity of metabolic intermediary molecules and the functions of enzymes and cofactors, and breach the energetic profile and synthetic infrastructure at transcriptional and post-transcriptional stages. In Al toxicosis, we identified the inhibition of cellular viability and function of neurons, osteoblasts, endocrine cells, lymphocytes, macrophages, erythrocytes, erythroid cells, enterocytes, myocytes, germinal cells, and pulmonary alveolar, hepatic, renal and pancreatic islet cells. Progenitor or blast cells were not able to proliferate, differentiate and function in accordance with their genetic resources. The secretory functions of specialized cells were impaired and several signaling pathways were recruited in abnormal chemico-biological settings. Cytokine expression was accelerated by oxidative cellular injuries to initiate inflammatory processes and alter immune responses that support inflammation and immunosuppression, respectively. There were Al-induced endocrine disruptions and altered sensitivities to hormones such as insulin, parathyroid hormone and hormonal vitamin D.

In Al toxicosis, the cellular structures were damaged by molecular mechanisms which cause degenerative changes (from lipid and amyloid depositions), cell death by apoptosis or necrosis, and dysplasia from genetically driven cell growth abnormalities. Cellular degeneration occurred in nervous tissue, liver and kidney. Apoptosis was associated with damage to immune cells, erythroid cells, erythrocytes, osteoblasts and germinal cells. Necrosis was encountered in pancreatic islet, liver, kidney, neurons and muscles. Dysplasia from chromosomal aberrations was associated with developmental defects, teratogenesis and growth abnormalities in fetuses and mammary epithelial cells. Mutagenesis, cell proliferation and impaired mitosis in Al toxicosis are gray areas requiring clarification because of the observed antithesis.

The systemic effects caused by Al toxicosis are diverse and multifaceted, but co-morbidities from multisystemic toxicosis are rarely reported in epidemiological cohorts. The convergence of toxic actions to engage multiple organ systems in an individual is often an observation in experimental animal models and lacks validity in observational studies in human or animal populations. The possible action of Al in the pathogenesis of diabetes mellitus and the concurrence of neurological disorders associated with Alzheimer's disease and other dementias (Arnold *et al.*, 2018) point to the common cellular basis of the pathogenesis of both metabolic and cognitive disorders, which can arise from toxic actions of Al. Longitudinal studies in the future may reveal co-morbidities and multisystemic toxicosis in Al-loaded individuals in locations where there is high risk of Al exposure.

Al-induced oxidative stress with the metabolic defects that accompanies it may incidentally be the crux of the toxicosis, to the extent that the use of antioxidant agents forms the fundamental basis for therapeutic interventions apart from chelating drugs. Chenodeoxycholic acid improved insulin sensitivity to ameliorate the neurotoxic effect of Al (Bazzari *et al.*, 2019). As new researches on the cellular mechanisms in the toxicosis continue to be further elucidated through in vitro and in vivo studies of the metallic toxicant, new therapies against the toxicosis should also focus on alleviating the known aberrations in signaling pathways, synthetic and secretory functions, cellular energetics and membrane integrity.

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REVIEW ARTICLE

World of earthworms with pesticides and insecticides

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ABSTRACT
Earthworms are important organisms in soil communities and are known for sustaining the life of the soil. They are used as a model organism in environmental risk assessment of chemicals and soil toxicology. Soil provides physical and nutritive support to agriculture system by regulating biogeochemical cycles, nutrient cycle, waste degradation, organic matter degradation *etc*. The biggest threat to soil health are pesticides and synthetic chemicals including fertilizers. Earthworms are most severely hit by these xenobiotic compounds leading to a sizeable reduction of their population and adversely affecting soil fertility. Earthworms are incredible soil organisms playing a crucial role in maintaining soil health. Pesticides used in crop management are known to be most over-purchased and irrationally used soil toxicants, simultaneously, used insecticides contribute to a quantum of damage to earthworms and other non-target organisms. LC_{50} and LD_{50} studies revealed that earthworms are highly susceptible to insecticides causing immobility, rigidity and also show a significant effect on biomass reduction, growth and reproduction by disrupting various physiological activities leading to loss of earthworm population and soil biodiversity.

KEY WORDS: earthworms; insecticide; non-target organism; soil macrofauna; xenobiotics

ABBREVIATIONS:

LC: Lethal Concentration; **LD:** Lethal Dose; **2,4-D:** 2,4-Dichlorophenoxyacetic acid; **2,4,5-T:** 2,4,5-Trichlorophenoxyacetic acid; **IRAC:** Insecticide Resistance Action Committee; **PAN:** Pesticide Action Network; **CCOHS:** Canadian center for occupational health and safety; **EPA:** Environment Protection Agency; **DDT:** Dichloro Diphenyl Trichloroethane; **B.C.:** Before Christ; **AChE:** Acetylchloineesterase; **nAChR:** Nicotinic Acetylcholine Receptor; **GABA:** gamma-Aminobutyric acid; **USD:** United States Dollar; **FICCI:** Federation of Indian chambers of commerce and Industry; **IOBC:** International Organization for Biological Control; **OECD:** Organization for Economic Cooperation and Development; **ISO:** International Standards Organizations; **GST:** Glutathione-S-transferase; **IPM:** Integrated Pest Management; **IGR:** Insect Growth Regulator

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Introduction

Agricultural expansion and indiscriminate use of pesticides often lead to affect soil ecosystem causing heavy population damage, toxicity and soil pollution (Hole *et al.,* 2005; Mangala *et al.,* 2009). An estimate has been made globally that $38 billion are spent on pesticides each year (Pan-Germany, 2012). The pesticides applied to the agricultural field should only be toxic to the target organisms, biodegradable and eco-friendly to some extent (Rosell *et al.,* 2008). But unfortunately most of the pesticides are non-specific and kill organisms that are harmless and very useful to the various ecosystems. This concern got attention just after the publication of Silent Spring by Rachel Carson in 1962, which brought environmental issues to concern to the general public. Along the developmental scale, the advance farming practices caused bioaccumulation in humans as well as in many other animals.

The pesticides used in agriculture land cause morphological, behavioral and physiological changes in reproductive, nervous, respiratory and osmoregulatory organs of many soil organisms and contaminate the soil which exerts a harmful impact on various invertebrates (Fingerman, 1984; Mangala *et al.,* 2009., De Silva P.M.C.S. 2009). Depending upon the chemical nature of pesticides and soil properties organs undergo a series of chemical pathways, transport, adsorption and desorption processes (Thapar *et al.,* 2015, Baishya, 2015). Among

the different classes of pesticides, insecticides are found to be most lethal toxic class of pesticides and pose risk to non-target organisms (Aktar *et al.,* 2009, Mahmood, 2016) The insecticide residues have been reported from agriculture systems along with many other ecotypes such as cropping fields, estuaries, oceans and even in the many urban settlements (Sánchez-Bayo, 2011; Guruge & Tanabe, 2001).

There are more than 8300 species in Oligochaetes, out of which more than half are terrestrial earthworms (Reynolds & Wetzel, 2004). The earthworm diversity of India represent 11.1% out of total earthworm diversity in the world. There are more than 505 species and subspecies of earthworms belonging to 67 genera and 10 families (Julka, 2001; Kathireswari, 2016). Earthworms are the supreme component of soil macrofauna and are the most important soil invertebrates responsible for developing and maintaining the nutritive value of soil by converting biodegradable material and organic waste into nutrient-rich vermicast (Kaushal *et al.,* 1995).

Vermicast obtained by modulation of organic waste through earthworm gut is different from its parental waste material and popularly known as black gold (Lim *et al.,* 2015b; Patangray, 2014). Earthworms are acknowledged as 'ecosystem engineers' as they extensively influence physical, chemical and biological properties of soil (Pelosi *et al.,* 2014). Earthworms boost soil physical properties such as hydraulic conductivity, porosity, bulk density, infiltrability, aggregate stability etc. (Devkota *et al.,* 2014). Earthworms improve nutrient availability by ingesting organic residues of different C:N ratios (Patnaik & Dash, 1990). Activities of earthworms also help in enhancing beneficial soil microbes. The gut mucus secretion and excretion from earthworm are known to enhance the activity of microorganisms (Bhaduria & Saxena 2010). The incredible services provided by the earthworms to the ecosystem are somehow at risk and recent research findings are now mainly focused on understanding earthworms and their responses to different pesticides.

World of pesticides

Historians have traced the use of pesticides to the time of Homer around 1000 B.C. but the earliest records of insecticides are associated with the burning of brimstone (Sulfur) as a fumigant. The insecticide selection was limited during the onset of World War II and by the end of it, it got a new concept of insect control with the modern era of chemicals. The first synthetic organic insecticide introduced was DDT. Traditionally, insecticides are chemical or biological agents meant for the control the insects. The control may be by killing of the insects or by preventing them from engaging in their destructive activities. Insecticides may be natural or manmade and applied to target pests by applying with various delivery systems such as spray, baits, slow-release, diffusion etc. (Ware & Whitacre, 2004).

Classification of pesticides

Pesticides are classified as insecticides, fungicides, herbicides, rodenticides, nematicides, molluscicides and plant growth regulators. Each group is specifically designed to target pests, but they put undesired toxic effects on non-target organisms. (Cortet *et al.,* 2002; Jänsch *et al.,* 2005; Lo, 2010; Zhang *et al.,* 2010; Yasmin & D'Souza, 2010; Wang *et al.,* 2012a; Milanovic *et al.,* 2014).

The major classes of pesticides are summarized in Table 1. Among the different classes of pesticides, insecticides are known as one of the major class that contributes greatly to pest control and are further divided into different groups. The insecticide groups are classified on the basis of their chemical nature as per Insecticide Resistance Action Committee IRAC 2016 (Table 2).

Toxicity and lethality of pesticides

The effect of toxic chemicals in a biological system is dose-related. The LC_{50} (Fifty percent lethal concentration) is the amount of pesticide dispersed in the air and the value is measured in milligrams per liter. The lower the LC_{50} value, the more lethal is the pesticide. Whereas LD_{50}

Table 1. Major classes of pesticides*.

Types of Pesticides	Use and Action	Examples
Insecticides	A substance used to control or eliminate or to prevent the attack of the insects that destroys/kill/mitigate plant/ animal.	DDT, Methyl Parathion, Phorate, Chloropyrifos, Imidacloprid, Cypermethrin, Dimethoate
Herbicides	Substances which are used to control the noxious weed and other vegetation that is growing with the desired species causing poor plant growth.	Acetochlor, Butachlor, Terbis, Glyphosate, 2,4-D, and 2,4,5-T.
Fungicides	Substances used to destroy or inhibit the growth of fungi/diseases that infect plants/animal.	Carbendazim, Ampropylfos, Carboxin
Rodenticides	Chemicals used to kill rodents i.e. mice, rat *etc.*	Warfarin, Arsenous oxide
Nematicides	Substances used to repel or inhibit the nematodes damaging various crops.	Aldicarb, Carbofuran
Molluscicides	Substances used to inhibit the growth and kills snails and slugs and small black sans-culottes.	Gardene, Fentin, Copper sulfate.
Plant growth regulators	A substance that causes the retardation or accelerates the rate of growth or rate of maturation.	Acibenzolar, Probenazole

*As per Pesticide Action Network 2010 (PAN 2010)

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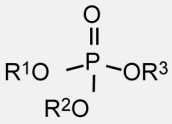
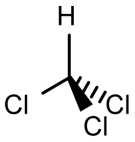
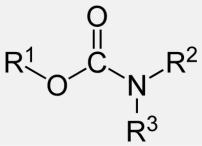
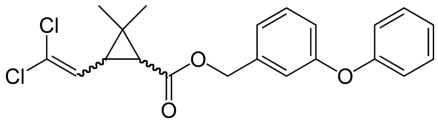
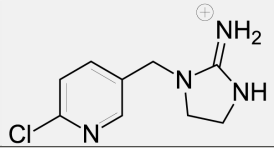
(Fifty percent lethal dose) is calculated under controlled laboratory conditions by administration of the specific dose within a particular time to estimate the toxicity of the pesticides to an organisms (Table 3 and Table 4). The LD_{50} values are expressed as milligram per kilogram of body weight (Canadian center for occupational health and safety 2018).

Production andconsumption of pesticides worldwide and Indian scenario

The rise in population has increased the demand for agricultural products and to meet the demand the agriculture practices are commercialized into agribusiness. This practice facilitated the growth of crop protection by formulating agrochemicals on a large scale. The pesticide market scenario seems to be export-oriented rather than import-oriented (Table 5). The pesticide market of India is expected to grow by 12% to 13% per annum to reach $6.8 billion by 2017 and export demand by 15% to 16% (Surana *et al.,* 2012). In the year 2018, as per India pesticide industry analysis, the CAGR (compound annual growth rate) observed 14.7% rendering the predicted size of the market at Rs. 2, 29, 800 million whereas on other side the global insecticide market is valued at USD 15.30 billion in 2016 and is likely to reach USD 20.82 Billion by 2022, at a CAGR of 5.27 from 2016 to 2022 respectively (Agro pages 2015).

The Indian pesticide industry is the biggest in Asia and the 12th in the world and ranks fourth among global suppliers and it is expected to increase its growth till 2026.

Table 2. Classification of insecticides based on their chemical nature (IRAC 2016)*

Main Groups	Action	Basic Structure	Examples
Organophosphates	Inhibit AChE in nervous system of target organisms		Chloropyrifos, Dichlorovos, Triazophos, Profenofos, Parathion, Phorate, Diazinon
Organochlorines	Binds at GABA site Inhibit chloride flow in the nervous system of target organisms		Chlordane, Endosulfan
Carbamates	Inhibit AChE in nervous system of target organisms		Aldicarb, Carbaryl, Carbofuran, Isoprocarb
Pyrethroids	Acts on Nervous system which cause changes in nerve membrane permeability to sodium and potassium ions		Acrinathrin, Allethrin, Bioallethrin, Cycloprothrin, beta-Cyfluthrin, Cyhalothrin, lambda- Cyhalothrin, gamma-Cyhalothrin, Cypermethrin, alpha-Cypermethrin, beta-Cypermethrin, theta cypermethrin, zeta-Cypermethrin, Pyrethrins (pyrethrum)
Neonicotinoids	Acts as an agonist of acetylcholine and is therefore effective on many insects		Acetamiprid, Clothianidin, Dinotefuran, Imidacloprid, Nitenpyram, Thiacloprid, Thiamethoxam.

* As per Insecticide Resistance Action Committee

Table 3. Toxicity range of pesticides (CCOHS 2018).

S.No.	Category	LD_{50} oral mg/kg (ppm)	Example
1	Extremely toxic	1 mg/kg(ppm) or less	Parathion, aldicarb
2	Highly toxic	1–50 mg/kg(ppm)	Endrin
3	Moderately toxic	50–500 mg/kg(ppm)	DDT, Carbofuran
4	Slightly toxic	500–1000 mg/kg(ppm)	Malathion
5	Non-toxic (practically)	1–5 gm/kg	–

Table 4. Acute toxicity range of pesticides according to the Environment Protection Agency (2009).

Class	Category	Rat as an animal model		
		Oral LD_{50} (mg/kg)	Dermal LD_{50} (mg/kg)	Inhalation LC_{50} (mg/l)
I	Danger	<50	<200	<0.2
II	Warning	50–500	200–2,000	0.2–2.0
III	Caution	500–5000	2,000–20,000	2.0–20
IV	Caution (Optional)	>5,000	>20,000	>20

The pesticide market is likely to display a CAGR of 7.04% in value terms by the year 2026 (Agro pages 2015). Among different classes of pesticides (Figure 1 and Figure 2) the insecticides dominate the other classes of pesticides and accounts for 60% of total market value and are used in major crops like rice and cotton, whereas herbicides and fungicides account for 16% and 18% respectively (FICCI 2015). Globally the consumption of herbicide is found to be highest followed by insecticides, fungicides and other pesticides (Arnab *et al.,* 2014). Consumption of agrochemicals in India is one of the lowest in the world with per hectare consumption of just 0.6 kg/ha compared to US (4.5 kg/ha) and Japan (11 kg/ha) (FICCI, 2014). This practice of pesticide usage in India needs to focus on high yield of bio-pesticides to promote eco-friendly and sustainable methods of agriculture.

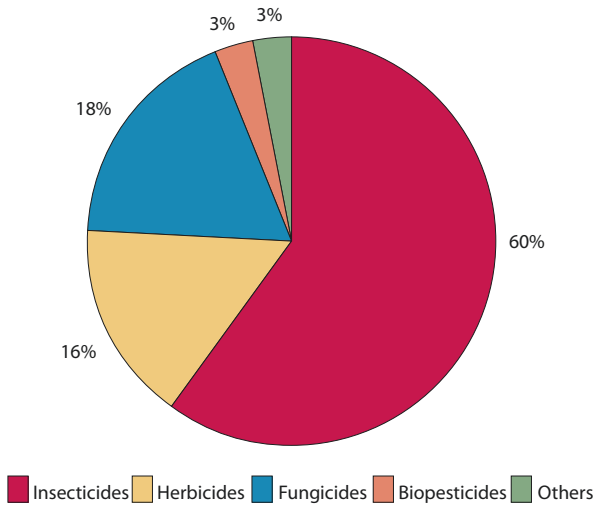


Figure 1. The consumption pattern of pesticides in India.

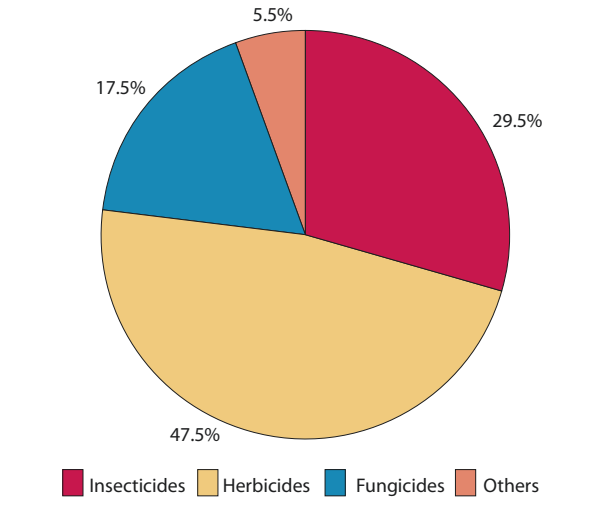


Figure 2. The consumption pattern of pesticides worldwide.

Globally, the pesticides cover only 25% of the cultivated land area and consumption of pesticide worldwide is 2 million tons per year including India while comparing with Korea and Japan where it is 6.6 and 12.0 kg/ha respectively whereas Indian consumption is 0.5 kg/ha.

Pesticides and soil environment

Soil has the center position for the existence of organisms and ensures their survival, the term soil health and soil environment are used to describe the soil property which holds soil physical, chemical, biological characteristics, those maintain productivity and environment quality which promote the health of plants and animals (Doran.,1994). Soil is a mandatory component for terrestrial environment and is acknowledged as "Biological engine of the earth" (Ritz *et al.,* 2004). Before the era of industrial revolution, i.e. early to mid-1900's, farming practices were environment-friendly and the connection between agriculture and ecology was very strong. Immediately after this, the ecology and farming linkage was ignored resulting in high productivity at the cost of the environmental quality. Therefore the agro-ecosystem safety becomes a daunting challenge and is adversely affected the soil health.

Use of pesticides has become an integral part of our modern life in order to meet the demand of a growing population which is expected to be 10 billion by 2050 (Saravi & Shokrzadeh, 2011). As per an estimate of the last decade nearly $38 billion was spent on pesticides globally (Pan-Germany, 2012). The major fraction of pesticides accumulated in the soil and further repeated use of pesticides may cause lethal effects. The accumulation of pesticides in organo-mineral components of complex structures greatly influence the processes like mobilization, immobilization, bioavailability and transport (Gevao *et al.,* 2003; Piccolo *et al.,* 1998). The degraded pesticides alter microbial diversity, biochemical reactions and enzymatic activity (Hussain *et al.,* 2009; Munoz-Leoz *et al.,* 2011). The enzymatic pool of soil comprises of free enzymes, immobilized extracellular enzymes and the enzymes secreted by the microorganism well known

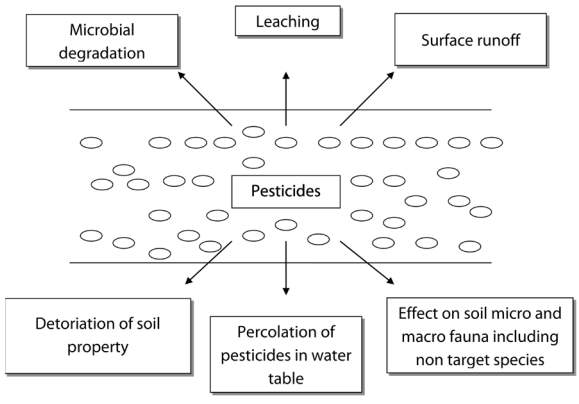


Figure 3. Behavior of pesticides in soil system.

Table 5. Import/Export of pesticides in India in recent years (metric tons of active ingredients), according to the Directorate General of Commercial Intelligence and Statistics, Kolkata, WB, Ministry of Commerce (DGCI&S, 2010-2017).

Import/Export	Category	Country	2010–11	2011–12	2012–13	2013–14	2014–15	2015–16	2016–17
Import	Pesticides	India	53996	58647	65018	77375	95361	71029	100238
Export	Pesticides	India	173171	207948	228790	252747	285209	307368	379852

*Q.T in Metric Tonnes (Technical Grade)

as bioindicators of soil health (Mayanglambam *et al.,* 2005; Hussain *et al.,* 2009). The change in enzymatic activity demonstrates the effect of pesticides on soil biological functions (Garcia *et al.,* 1997; Romero *et al.,* 2010). Pesticides channel themselves through various biophysical pathways in soil ecosystems (Figure 3).

Animals thriving in soil are always under the threat of various chemicals used in agricultural practices, more specifically the pesticides. It is well established that these xenobiotic products are usually difficult to degrade by soil microbes therefore there is always a chance of their entry to various food chains and food webs resulting bioaccumulation and bio-concentration (Maurya & Malik 2016; Dureja & Tanwar, 2012; Edward & Bolen, 1992; Paoletti, 1999). Earthworms bio-accumulate organic pollutant (Jager *et al.,* 2005), heavy metals (Nahmani *et al.,* 2007) and nanoparticles (Canesi & Prochazkova, 2014) through skin and via soil ingestion. The effect of these pesticides applied to soil has effect on earthworm mortality (Roberts & Dorough, 1984; Panda S & Sahu, 2002), reproduction (Senapati *et al.,* 1991; Schaefer 2004), metabolism (Brown *et al.,* 2004) and also enhance the mechanism of bio-amplification (Stephenson *et al.,* 1997; Johnson *et al.,* 1999). Earthworms experience inadvertent toxicity from terrestrially applied pesticides (Edward & Bolen, 1992) and this uptake of chemical increases bio-concentration of pesticides in earthworms.

Therefore the knowledge of the toxico-kinetics of terrestrially applied pesticides in earthworms is necessary to predict the risks of bio-concentration and bio-accumulation (Van Gestel & Weeks, 2004) on earthworm populations and ecological communities. The bio-accumulation of insecticides in earthworms may not lead to a significant effect on the animal to that extent but may produce serious damage to higher tropic level, but with long-term exposure to these pesticides, earthworms get acclimatized and accumulated (Huang & Iskandar, 1999). The increase in the concentration of pesticides and their non-biodegradable nature make them persist in the tissue of the organism at each successive level of food chain through the process of bio-amplification which cause greater harm to those of higher trophic level compared to those of lower levels. Several studies have been undertaken and demonstrated that at each trophic level the laethality of these pesticide increases (Gill & Garg, 2014).

Pesticide toxicity and non-target organism

The effect of pesticides on non-target organisms has been a matter of debate for researchers worldwide. There are many reports on the non-target killing of various species (Ware, 1980; Aktar *et al.,* 2009; Datta *et al.,* 2016; Dutta & Dutta, 2016; Stanley *et al.,* 2016). Pesticides show the extreme effect on the aquatic ecosystem, animal and plant biodiversity and terrestrial food webs. It is estimated that less than 0.1% of pesticides applied to crop reach to the target pest (Pimental, 1995) and more than 99% of applied pesticide have the potential to impact non-target organisms and it percolates deep into the soil ecosystems including the water-table.

In India, 76% of the pesticides used are insecticides whereas globally the insecticide consumption is 44% (Mathur, 1999). The insecticidal effects on non-target species are categorized as per Nasreen *et al.* (2000) harmless (<50% mortality), slightly harmful (50–79% mortality), moderately harmful (80–89% mortality) and harmful (>90% mortality) when tested as per the field recommended dose. The categorization standards are used by the International Organization for Biological Control, West Palaearctic Regional Section (IOBC/WPRS) working group, to assess the insecticidal effects on non-target organisms (Hassan, 1989). The insecticides also act as a potential neurotoxicant on non-target species as it inhibits the essential enzyme, acetylcholinesterase (AChE) in the nervous system of insects and other animal species (Gambi *et al.,* 2007; Caselli *et al.,* 2006). There are reports that toxicity of chemical pesticides used not only affects the target pests but also other species in different degrees (Sanchez-Bayo, 2012). Such as, natural insect enemies e.g., parasitoids and predators are most susceptible to insecticides and are severely affected (Aveling, 1981; Vickerman, 1988). Along with natural enemies, the population of soil arthropods is also drastically disturbed because of indiscriminate pesticide application in agricultural systems. Soil invertebrates are essential for the maintenance of soil structure, transformation, nutrient dynamics and mineralization of organic matter severally affecting the food chain and food webs.

In some cases, the concentrations of pesticides residue have been shown to be sufficiently high to affect many non-target species, including very important soil macrofauna, such as earthworms which are known to deliver ecosystem good and services (Frampton *et al.,* 2006; Daam *et al.,* 2011; Bertrand *et al.,*2015). Insecticides alter the eco-physiology of the earthworms (Liang *et al.,* 2007) and there are studies on toxicological effect of carbaryl in different earthworm species such as *Eisenia Andrei, P. Excavatus, Pheretima Posthuma and Metaphire Posthuma* (Lima *et al.,* 2015; Saxena *et al.,* 2014). Few studies have also shown the toxic effect of Imidacloprid, a common neonicotinoid insecticide, on earthworms

(Capowiez *et al*., 2005, 2006). Majority of work has been carried out on the potential risk of organophosphorus pesticides like fenitrothion, malathion, monocrotophos, phorate in tropical agro-ecosystem using earthworm as test organism (Panda & Sahu, 1999, 2004; Patnaik & Dash, 1990). Much more investigations are needed to study various insecticides and their level of toxicity to non-target soil macro-fauna including various earthworm species.

World of Earthworms

The earthworms thrive almost all soil types and are known as the indicator of soil health and toxicity including various soil pollutants and pesticides. Lee (1985) categorized earthworms based on their feeding habit as detrivores (feed near the surface on decomposing litter and on dead roots) and geophagous (remain on the subsurface which consumes large quantities of soil). According to Lavelle (1983), geophagous earthworms are further categorized into polyhumic (feed on topsoil and occupy different soil strata), oligohumic (feed on the soil of low organic matter) and mesohumic (feed on humus and soil) and are abundantly found in the tropical regions.

Edward & Bohlen (1992) reported that earthworms are highly susceptible to pesticides such as insecticides, therefore they are considered as a model organism to evaluate the effects of insecticides. There are certain pesticide families that are considered as harmful to earthworms i.e. neonicotinoids, strobilurins, sulfonylureas, triazoles, carbamates and organophosphates (Pelosi *et al.,* 2014). The pesticides affect mortality of earthworms by directly distressing them or by altering their physiology (Sabra & Mehana, 2015). Pesticides have a negative effect on the survival and reproduction of earthworms especially at higher concentration (>25mg/kg). Possible effects of pesticides and insecticides on earthworms in the soil are also depended on earthworm species, type of contaminant and its concentration, soil characteristics etc. (Roriguez-Campos *et al.,* 2014).

The organization for economic cooperation and development (OECD) proposed *Eisenia Fetida* (*Oligochaete*) as a reference earthworm species for toxicity testing because it can easily be cultivated in the laboratory, mature in few weeks and has a high reproductive rate (OECD, 1984, 2004, 2015; ISO, 1993). The different insecticides classes had different toxic effects on *Eisenia Fetida.* Earthworm growth, reproduction (cocoon production, number of hatchlings per cocoon and incubation period) is also influenced by use of pesticide in a dose-dependent manner (Yasmin & D'Souza, 2010).

Earthworms Morpho groups and their exposure to pesticides

Earthworms are classified into four ecological groups, each group is described by different traits in the soil system (Bouché, 1977; Edwards & Bohlen, 1996) including their exposure to various types of pesticides. **Epigeic** worms are represented by *Lumbricus rubellus, Dendrobaena octedra, Lumbricus castaneus* and usually found in upper 10–15 cm soil layer and feed on decaying organic matter present in the litter. The species that belong to this group are highly exposed to pesticides while ingesting litter. **Endogeic** worms are represented by *Aporrectodea caliginosa, Allolobophora chlorotica* or *Allolobophora icterica* and are of a bigger size ranging from 1 to 20 cm. They feed upon the organic matter which is incorporated and mixed with minerals in the soil where the pesticides have already reached and mixed with soil. **Anecic** worms include *Lumricus terrestis, Aporrectodea longa* and are usually bigger and pigmented. They reflect strong muscles with great burrowing activity and some species reaches to the giant size such as 10 to 110 cm. They feed upon the surface litter mainly during the night and create long sub-vertical burrows (1 to 6 m) and thus ingest more amount of soil and get exposed to pesticides by ingesting contaminated soil. **Compost** worm is represented by *Eisenia fetida* and *Dendrobaena veneta* commonly used in vermicomposting practices. Compost worms are bright red in color and stripy and are commonly called 'tiger worms'. These worms are usually kept in controlled soil pits therefore are less exposed to soil toxicants.

Effect of insecticides on earthworms

Solaimalai *et al.* (2004) investigated effect of various pesticides and their sub-lethal effect on earthworms and demonstrated that the sub-lethal effects cause rupturing of cuticle, oozing out of coelomic fluid, swelling, paling of body and softening of body tissues. Other studies include the cellular autolysis (Luo *et al.,* 1999), damage to male reproductive system (Sorour & Larnik, 2001), swelling (Bharathi & Subbarao, 1984) and coiling of tail (Espinoza-Navarro & Bustos-Obregon, 2004). The higher and the lower dose of insecticides cause physiological damage (cellular dysfunction and protein catabolism) to earthworms (Schreck *et al.,* 2008).

Temperature also plays an important role in degree of pesticide toxicity. Bindesbol *et al.* (2009) investigated effects of freezing temperatures on toxicities of abamectin and carbendazim. De Silva *et al.* (2009) investigated influence of temperature and soil type on the toxicities of chlorpyrifos and carbofuran. Lima *et al.* (2015) investigated effects of carbaryl under low and high temperatures and Garcia *et al.* (2008) assessed effects of three pesticides on the avoidance behavior under temperate and tropical conditions. These investigations showed that change in temperature may influence the pesticide toxicity, but the results obtained from these studies were not definite and substantiated by any other studies.

There are many studies on neurotoxicity caused by various insecticides namely neonicotinoid imidacloprid, oxadiazine indoxacarb, pyrethroids alpha-cypermethrin and lambda-cyhalothrin and the combination of organophosphate chlorpyrifos and pyrethroid cypermethrin. All these insecticides primarily affect nervous system – neonicotinoids interfere with the transmission of stimuli in the nervous system causing irreversible blockage of acetylcholine receptors, oxadiazines act as voltage-gated

sodium channel blockers, pyrethroids cause excitation of the sodium and potassium channels of neurons and the delay of closing of the channels during the phase of depolarization and organophosphates inhibit the action of enzyme acetylcholinesterase (AChE) leading to accumulation of acetylcholine, excessive stimulation of the cholinergic receptors and disruption of neural activity (Stenersen 2004; Casida, 2009; Ribera *et al.,* 2001; Gracia *et al.*,2011; Nasr & Badawy, 2015). Jeyanthi *et al.* (2016) reported that Carbaryl at higher concentration (50 kg/ha) decreases protein content and antioxidant enzymes glutathione-S-transferase (GST). The antibiotics, carbamates and organophosphates induced intermediate toxicity response to earthworms. Wang *et al.,* (2012) reported that the neonicotinoids are the most toxic to *Eisenia Foetida* among the six chemical classes followed by pyrethroids, while IGRs exhibited the lowest toxicity. Organophosphates are not very toxic to earthworms. Considering the high efficacy of neonicotinoids against target organisms, environmental managers should carefully evaluate the use of them in integrated pest management (IPM) programs to avoid serious damage to earthworms.

Impact of insecticides on earthworm growth and reproduction

Various reproductive parameters such as maturation, cocoon production, viability, hatching and sperms production were studied with reference to the genotoxicity when exposed to different types of insecticides and other chemical classes (Espinoza-Navaroo & Bustos, 2004; Govindarajan & Prabaharan, 2014). Pawar & Ahmad (2013) reported that the effect of Chlorpyriphos which is an organophosphate insecticide with the exposure period of 7, 14, 21, 28, and 35 days, the dose concentration of 0.1 and 0.2 showed less effect on growth with the exposure period of 7 and 14 days, but effected earthworms growth when exposed more than 14 days.

Booth & O'Halloran (2001) found significant reduction in growth of *A. Caliginosa* by exposure to two organophosphates, diazinon and chlorpyrifos, at 60 and 28 kg/ha dose. Rajshree *et al.* (2014) also found that Methyl parathion and phorate are very toxic to earthworms and showed progressive symptoms of toxicity such as coiling, curling and excessive mucous secretion with sluggish movements, swelling of the clitellum, degenerative changes in nervous system and loss of pigmentation which is elicited by organophosphorus insecticide.

Malathion, the organophosphate, showed a significant reduction in body weight and negative impact on the male reproductive organs that alter the cell proliferation and affect the DNA structure of spermatogonia of earthworms (Espinoza-Navarro, 2004). Sperm count is also a sensitive marker (Mosleh *et al.,* 2003; Venter & Reinecke 1985). Malathion could affect the sperm count, but in addition, its metabolites could affect the sperm quality (Espinoza-Navarro, 2004). Mosleh *et al.* (2003) assumed that the weight loss may indicate a reduced food intake, by which earthworms regulate intake of pesticides and leads to growth inhibition.

Mosleh *et al.* (2003) investigated that the toxicity of aldicarb, cypermethrin, profenofos, chlorfluazuron, atrazine, endosulfan and metalaxyl in the earthworms *Aporrectodea caliginosa* and *Lumbricus terrestris* causes a reduction in growth rate. Zhou *et al.* (2007) assessed and found that chlorpyrifos had an adverse effect on growth in earthworm exposed to 5 kg/ha chlorpyrifos after eight weeks. Some studies have shown that the growth of earthworms appeared to be more severely affected at juvenile stage than the adult stage.

Chlorpyrifos exposure had a significant effect on reproduction in earthworm as it shows the effect on fecundity when exposed to 5 kg/ha after eight weeks (Zohu *et al.,* 2006) According to Zohu *et al.,* 2008 reproduction of earthworm appeared to be more severely affected by cypermethrin at juvenile stage than at adult stage. Application of 20 kg/ha cypermethrin caused significant toxic effects in the reproduction of worms. Apart from the above mentioned facts there are many more effects and responses that have been studied by various researchers (Table 6).

Effect of insecticides on earthworm gut bacteria and cast production

In soil, earthworms explicate soil property and regulate biochemistry of terrestrial soil. The cast of earthworms contribute significantly to cyclic processes carried out in soil ecosystem by supplying nutrient to the plant roots and maintain pedological characteristics of the soil. The earthworms are voracious feeders and the nutrient-rich organic matter along with the soil flows through earthworms gut. The gut of earthworms is a straight tube bioreactor and maintains stable temperature by the regulatory mechanism (Karthikeyan *et al.,* 2004). The gut of earthworm is known as ideal habitat for many agriculturally important microbes (Wolter & Scheu, 1999) and mostly derives its energy and nutrient from gut-specific microbiota rather than from microbiota present in ingested soil (Sampredo *et al.,* 2006). Shi *et al.* (2007) examined that earthworm exposed to deltamethrin for 14 days exposure showed dose-dependent toxic effect on growth and cellulose activity. A decrease in cast production was found in *L. Terrestris* when exposed to methomyl, carbaryl, and imidacloprid respectively for 7 days (Capoweiz *et al.,* 2010).

Conclusions

The study highlights the use of pesticides in agriculture system results in many ecological problems. There is clear evidence that the population of earthworm and other non-target soil biota are influenced by pesticides and fertilizers use and the impact is wide-ranging and causing the unwanted shift in the community. Initially, pesticides were used for the benefit to human life by an increase in agricultural productivity and by controlling infectious diseases but their adverse effect on human health and environment were ignored. Multifarious and tremendous uses of pesticides are causing harm to the environment and its components. Some of the adverse effects emerged

Table 6. Response of various insecticides on earthworm species at different concentrations

Insecticide	Concentration of Insecticide/exposure	Species	Responses	References
Aldrinaldrin, Endrin, DDE, parathion and carbaryl	LD_{50} value 45 µg/g	*Lumbricus terrestris*	With drawl responses and discoloration of the skin	Cathey, 1982
Endrin	LD_{50} value45 µg/g	*Lumbricus terrestris*	With drawl responses and discoloration of the skin	Cathey, 1982
DDE	LD_{50} value 46 µg/g	*Lumbricus terrestris*	With drawl responses and discoloration of the skin	Cathey, 1982
Parathion	LD_{50} value 34 µg/g	*Lumbricus terrestris*	With drawl responses and discoloration of the skin	Cathey, 1982
Carbaryl	LD_{50} value28 µg/g	*Lumbricus terrestris*	With drawl responses and discoloration of the skin	Cathey, 1982
Chlorpyrifos	LC_{50} value 0.063 mg/cm^2	*Eisenia foetida*	Inhibition of acetylcholinesterase activity, Behavioral and morphological abnormalities	Rao *et al.*, 2003
Malathion	LD_{50} value 880 mg/kg soil	*Eisenia foetida*	Decreased the spermatic viability In spermatheca, altering the cell proliferation and modifying the DNA Structure of spermatogonia.	Espinoza-Navarro & Bustos-Obreg´on, 2004
Carbaryl		*Metaphire posthuma*	Sperm head abnormalities	Gupta & Saxena, 2003
Dieldrin	LC_{50} value 100 mg/kg	*Eisenia foetida (Juveniles)*	Clitellum development retarded,Influencing reproduction.Growth was retarded even at the agricultural dose of 5kg/ha	Venter & Reinecke, 1985
Imidacloprid	LC_{50} value 25.53 mg/kg	*Eisenia andrei*	Retarded development, reduced fertility, and teratogenic effects reveal qualitative and quantitative changes in earthworm population, mortality does not occur	Alves *et al.*, 2013
Dimethoate	LC_{50} value 28 mg/kg d.w.	*Eisenia foetida*	Significantly reducing earthworm weight and showing an avoidance response at soil concentrations	Rico *et al.*, 2016
Profenofos	LC_{50} value 4.56 and 3.55 µg/cm^2	*Eisenia foetida*	Body ruptures, bloody lesions, and internal excessive formation of glandular cell mass and disintegration of circular and longitudinal muscles, which failed to regulate the internal coelomic pressure, leading to fragmentation in earthworms	Reddy & Rao ,2008
Dichlorvos	LC_{50} value 76 mg/kg d.w	*Eisenia foetida*	The weight of earthworm decreases. Reproduction and avoidance behavior significantly affected.	Farrukh & Ali, 2011
Cypermethrin	LC_{50} value 0.008 mg/kg	*Perionyx excavatus*	Order of toxicity – cypermethrin> endosulfan> carbaryl> chlorpyrifos> aldicarb> monocrotophos	Gupta *et al.*, 2010
Endosulfan	LC_{50} value 0.03 mg/kg	*Perionyx excavatus*	Order of toxicity – cypermethrin> endosulfan> carbaryl> chlorpyrifos> aldicarb> monocrotophos	Gupta *et al.*, 2010
Carbaryl	LC_{50} value 6.07 mg/kg	*Perionyx excavatus*	Order of toxicity – cypermethrin> endosulfan> carbaryl> chlorpyrifos> aldicarb> monocrotophos	Gupta *et al.*, 2010
Chlorpyrifos	LC_{50} value 7.3 mg/kg	*Perionyx excavatus*	Order of toxicity – cypermethrin> endosulfan> carbaryl> chlorpyrifos> aldicarb> monocrotophos	Gupta *et al.*, 2010
Aldicarb	LC_{50} value 10.63 mg/kg	*Perionyx excavatus*	Order of toxicity – cypermethrin> endosulfan> carbaryl> chlorpyrifos> aldicarb> monocrotophos	Gupta *et al.*, 2010
Monocrotophos	LC50 Value 13.04 mg/kg	*Perionyx excavatus*	Order of toxicity – cypermethrin> endosulfan> carbaryl> chlorpyrifos> aldicarb> monocrotophos	Gupta *et al.*, 2010
Chlorpyrifos	LC_{50} value 0.5 mg/kg	*Eisenia foetida*	Effects on growth and weight of earthworms	Pawar & Shahzad, 2013
Lambda-cyhalothrin, Cypermethrin, Didcot, Termicot	LC_{50} ranging from 0.000 ml–0.002 ml	*Lumbricus terrestis*	Presents the highest number of mortality in all concentration	Yuguda *et al.*, 2015
Methyl Parathion and Phorate	Conc 0.05g/500 g of soil and Methyl parathion 0.12 g/500 g	*Edurilus eugeniae*	Coiling, curling and excessive mucous secretion with sluggish movements, Swelling of the clitellum, Extrusion of coelomic fluids resulting in bloody lesions. Earthworms also showed degenerative changes in the anterior part of the nervous system.The disappearance of metameric segmentations and loss of pigmentations.	Rajashree *et al.*, 2014
Dimethoate	LC_{50} value 300 mg/kg	*Eisenia foetida*	The decrease in cocoon production and coon viability	Pal & Patidar, 2013
Carbofuran	LC_{50} value 23.5 and 9.3 mg/kg	*Eisenia Andrei* and *Pontoscolex corethrunus*	After 7 days biomass reduction was observed only with E.andrei and after 14 days a biomass of both the species reduced significantly	Buch *et al.*, 2013
Chlorpyrifos (pure)	LC_{50} value 80 mg/kg soil	*Eisenia Fetida*	Adverse impact on growth and reproduction	Zhou *et al.*, 2007
Parathion	LC_{50} value 1478 mg/kg soil	*Eisenia foetida*	Adverse effect on cocoon production, cocoon viability and hatching success rate.	Bustos-Obreg & Goicochea, 2002

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Table 6. *Continued ...*

Insecticide	Concentration of Insecticide/exposure	Species	Responses	References
Imidacloprid	LC_{50} value 0.77 mg/kg dry soil	*Eisenia foetida*	Adult survival decreased significantly	Silva *et al.*, 2017
Thiacloprid	LC_{50} value 7.1 mg/kg dry soil	*Eisenia foetida*	Acting on sub-lethal endpoints leading to a reduction in the number of offspring.	Silva *et al.*, 2017
Cycloxaprid	LC_{50} of 10.21 mg/kg dry soil	*Eisenia fetida*	It induced tissue damage to the epidermis, gut, and neurochord at sublethal doses and also induce oxidative stress	Suzhen *et al.*, 2018

in the form of an increase in resistant pest population, decline in beneficial soil microorganisms, predators, pollinators and earthworms. Earthworm which is one of the important soil fauna is extremely at the edge of the exposure to pesticides. Such sensitivity of earthworms to pesticides, especially to the major class of pesticides i.e. the insecticides, is well documented in the present review. The toxicity of the insecticides to earthworms varies with the category of chemicals affecting the earthworm life cycle parameters. The persistent nature of pesticides has impacted our ecosystem too that have entered into various food chains and into the higher trophic levels such as that of humans and other large mammals. In order to reduce the effect of pesticides there should be input of sufficient organic manures instead of chemical fertilizers with minimal disturbances in soil and can be adapted for optimum activity of earthworms in the soil for healthy and fertile soil. A little effort has been made to provide a comprehensive review of the toxicity level of insecticide to one of the non-target taxa i.e. earthworm. Therefore farmers must be educated regarding the beneficial role of earthworms because of its importance and to reduce or minimize the use of pesticide to provide the threshold to the environment and biodiversity.

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ORIGINAL ARTICLE

Systematic review of the clinical consequences of butyrfentanyl and corresponding analogues

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ABSTRACT

Butyrfentanyl and its analogues are being increasingly used throughout the United States and Europe. Currently, lethal cases are emerging across the United States, England, and Europe without any end in sight. We therefore performed a systematic review of existing case reports on the literature of butyrfentanyl and similar analogs. We searched PubMed and Embase for articles (up until September 2018) using terms such as "butyrfentanyl" or "butyrylfentanyl." In total, our search found 271 articles and identified 10 for inclusion in this review. A total of 33 cases were found with 61% of those being fatal. The most common route of administration was intravenous, but other routes of administration were readily used such as oral, intranasal, and inhalation. Most cases reported use of concomitant licit and illicit pharmacological agents. The toxidrome was consistent with other opioid overdoses, and naloxone was successfully used in nine of 10 patients. We encourage toxicology screenings of novel fentanyl analogs such as butyrfentanyl or 4-fluorobutyrfentanyl when an opioid overdose of unknown nature presents.

KEY WORDS: opioids; street drugs; drug overdose; butyrfentanyl

Introduction

Opioid overdose is a problem for many worldwide. In 2015, approximately 118,000 individuals died due to an opioid use disorder. Both prescription and non-prescription (e.g. illicit) opioids are responsible for these deaths (World Health Organization, 2018). Mechanistically, opioids produce their analgesic effect by interacting with one of three major opioid receptor subtypes (i.e. μ, δ, and κ) found in the central nervous system (Prekupec *et al.*, 2017). However, opioids affect the part of the brain that regulates breathing and an overdose can lead to respiratory depression and death (World Health Organization, 2018). An increase in the number of opioid prescriptions dispensed has occurred in many developed countries including the United States, Canada, Australia, Germany, Norway, and the United Kingdom (Shipton *et al.*, 2018). Novel synthetic opioids (NSOs) are gaining momentum in changing the landscape of opioid use disorders. NSOs include a variety of fentanyl analogues and non-fentanyl agents (Prekupec *et al.*, 2017). Butyrfentanyl or butyrlfentanyl (BF), an analogue of fentanyl, is a NSO with growing use. BF's potency is seven times that of morphine (Armenian *et al.*, 2018). A number of analogues of BF have also been synthesized (Figure 1). It is currently a schedule I agent in the United States where it has no accepted medical use (Drug Enforcement Administration, Department of Justice, 2018). The scheduling of BF was due to the increasing number of deaths associated with its use. We performed a systematic review of the literature regarding BF and its analogues to understand the clinical consequences associated with this illicit agent.

Methods

A systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A literature search was conducted on PubMed and Embase for case reports, published until September 10, 2018, using keywords such as "butyrfentanyl" or "fentanyl analog." We utilized the following inclusion criteria for article selection: (1) involve humans using BF, or 4-fluorobutyrfentanyl, or

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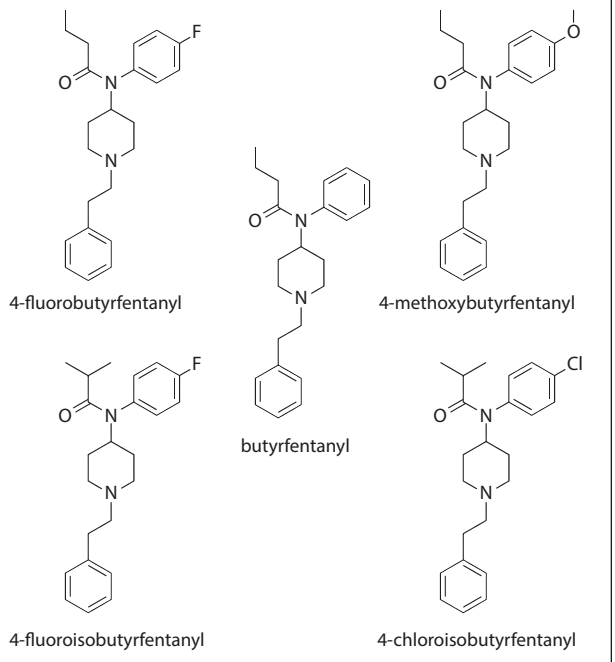

Figure 1. Chemical structure of butyrfentanyl and its analogues.

4-methoxybutyrfentanyl, or 4-chloroisobutyrfentanyl, or 4-fluoroisobutyrfentanyl; (2) be either a randomized controlled trial, prospective trial, retrospective analysis, case series or case report; (3) include clinical findings at presentation. Alex D. Le (ADL) formulated the search strategy. Saeed K. Alzghari (SKA) identified relevant articles reporting clinical and analytical outcomes. ADL extracted data from all articles. The extracted data were cross-reviewed by SKA.

Results

Our database search retrieved 271 articles, where 54 were screened as potentially relevant articles with 10 articles meeting inclusion criteria with publication dates between 2015 and 2018 (Figure 2) (Bäckberg *et al.*, 2015; Cole *et al.*, 2015; Farkas *et al.*, 2018; Helander *et al.*, 2016, 2017; Hikin *et al.*, 2018; McIntyre *et al.*, 2016; Poklis *et al.*, 2016; Rojkiewicz *et al.*, 2017; Staeheli *et al.*, 2016). The patients' characteristics are summarized in Table 1. We identified 33 patients that used BF or a similar analog. Of those cases, 82% were male with 26 of the 33 patients being from Europe (13 England, 10 Sweden, 2 Poland, and 1 Switzerland) and seven from the United States. Four cases involved oral exposure, four involved inhalation, four intranasal, and 11 involved intravenous route. All the others were of unknown exposure. Of the 33 cases, 61% were postmortem cases, while the others concerned patients that had recovered. According to the available autopsies, one patient had edematous/congested lungs, another had edema of the brain, one had mild atherosclerosis with left concentric ventricular myocardial hypertrophy, and one had mild left ventricular myocardial hypertrophy with mild nephrosclerosis.

Regarding the remaining patients who lived after exposure from BF and similar analogs, nine patients were from the Swedish STRIDA (**S**amverkansprojekt kring **T**oxicitetsutredning och **R**iskbedömning av **I**nternet**D**roger baserat på laboratorie**A**nalyser) Project. Bäckberg and colleagues had four patients that were monitored in the emergency department or the intensive care unit (ICU), with all patients being discharged either the same or the following day (Bäckberg *et al.*, 2015). Helander and colleagues reported four patients that required an ICU stay for 1–2 days with one patient in particular needing ventilator support due to pulmonary aspiration (Helander *et al.*, 2016). In another set of case series by Helander and colleagues, one patient was observed for 1–2 days at the hospital and another patient required intensive care support for five days (Helander *et al.*, 2017). Cole and colleagues published a detailed case of one patient overdosing on BF with diffuse alveolar hemorrhage (Cole *et al.*, 2015). The patient was transferred to the pediatric ICU, required intubation until day 4, and was discharged on day 7.

All but one case obtained analytical findings from blood and/or urine samples. The other case used high-performance gas chromatography and mass spectroscopy to identify the opioid in question.

Discussion

BF and its analogues are gaining use that is rivaling other licit and illicit opioids. Many of the cases presented in this review mimicked those of opioid overdoses associated with licit opioids such as respiratory depression and central nervous system depression (Schiller & Mechanic, 2018). Many patients who consumed BF and similar analogs survived as opposed to those taking other NSOs such as AH-7921 or U-47700 (Rambaran *et al.*, 2018, 2017). It is proposed that BF has 1/30th efficacy of that of fentanyl and is 7 times more potent than morphine. It is expected that other fluorinated analogs are even less efficacious (Higashikawa & Suzuki, 2008).

Many of the patients that survived originated from the Swedish STRIDA project, a program that monitors the occurrence and health hazards of newly emerging drugs in Sweden (Bäckberg *et al.*, 2015; Helander *et al.*, 2016, 2017). These patients used a number of different routes of administration including intranasal, inhalation, oral, and intravenous. Fentanyl analogs are being purchased through novel psychoactive substance (NPS) suppliers that are readily available via the internet. Bäckberg and colleagues noted that a simple search found acetylfentanyl, BF, and 4-fluorobutyrfentanyl readily marketed online for illicit use (Bäckberg *et al.*, 2015). Other formulations are available online for rectal and sublingual administration.

All of the cases from England were fatal (Hikin *et al.*, 2018). Markers for heroin use such as morphine,

6-monoacetylmorphine, noscapine and papaverine were found. Additionally, serum levels of carfentanil were found in blood or urine in all cases. Hikin and colleagues noted that BF and similar analogs were used more often in the first 3 months of 2017; however, the last 3 months of 2017 included patients that presented solely with serum levels of carefentanil (Hikin *et al.*, 2018). This could be due to a change in supply of illicit fentanyl analogs that are readily available in the immediate patient population in England.

There were two lethal case of 4-fluorobutyrfentanyl overdoses from Poland and one lethal overdose of BF from Switzerland (Rojkiewicz *et al.*, 2017; Staeheli *et al.*, 2016). One of the patients from Poland in particular had used an e-cigarette with e-liquid as the route of administration. The other patient from Poland had 4-fluorobutyrfentanyl in powder form (Rojkiewicz *et al.*, 2017). Interestingly, 4-fluorobutyrfentanyl was found in the blood, urine, liver, kidney, and stomach contents of the patient who had the e-liquid. This could be due to abuse of 4-fluorobutyrfentanyl through multiple routes of administration (Rojkiewicz *et al.*, 2017).

Of the seven cases originating from the United States, four patients survived (Farkas *et al.*, 2018; McIntyre *et al.*, 2016; Poklis *et al.*, 2016). Detailed autopsy findings were available for the deceased. Two patients had edematous lungs with a total weight of 1705g and 1250g respectively, while the other had lungs weighing 960g (McIntyre *et al.*, 2016; Poklis *et al.*, 2016). Overall, the autopsy findings were unremarkable. It should be noted that patients from the United States are using a number of routes of administration of BF formulations including intravenous, oral, and inhalation. One patient in particular that survived was an 18-year-old male who had a past medical history of intravenous heroin abuse (Cole *et al.*, 2015). The patient's mother found him unconscious with labored breathing. Once emergency medical services arrived, the patient was found obtunded with gurgling respirations. Naloxone was administered and the patient's mental status improved. At the emergency department, the patient reported to have purchased illicit fentanyl analogs from the internet. The patient complained of dyspnea and coughed up blood. His chest radiograph showed bilateral "batwing"–shaped perihilar opacities with diffuse interstitial markings. The patient was transferred to the pediatric intensive care unit. The patient became progressively more dyspneic requiring intubation. A bronchoscopy and a serial decline in hemoglobin were consistent with diffuse alveolar hemorrhage. The patient could not be extubated until day 4 and was discharged on day 7 after being treated for ventilator-associated pneumonia (Cole *et al.*, 2015).

There are limitations to this review. All of the cases reported in this review are from case reports or case series that are mostly from European reports of BF and similar analogs. The applicability of this review may not be generalizable to the entire population. Additionally, almost every patient had concomitant drug abuse that could have attributed to his or her overdose. There is only pre-clinical data as to the efficacy of BF and similar analogs. There is no way to ascertain BF's true effect from a clinical trial, only from observational cases. The route of administration varied between patients and specific doses were not stated.

Treating patients with BF or any similar analog should be similar to that of an opioid overdose. It is important to suspect a generalized opioid toxidrome such as respiratory depression, central nervous system depression, and pinpoint pupils. It is important to note that a majority of the patients who lived were transferred to the ICU, and intubation may be needed until patients have fully recovered (Lucyk & Nelson, 2017). Naloxone was successful in treating all but one patient in this review and should be considered when a patient is presenting with an opioid overdose. BF and similar analogs can show up as a false positive of fentanyl in urine and should be treated as such. Gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry may be useful in identifying the opioid in question (Bäckberg *et al.*, 2015; Cole *et al.*, 2015; Farkas *et al.*, 2018; Helander *et al.*, 2016, 2017; Hikin *et al.*, 2018; McIntyre *et al.*, 2016; Poklis *et al.*, 2016; Rojkiewicz *et al.*, 2017; Staeheli *et al.*, 2016).

An emerging concern is that a number of NPSs are readily available online including BF, 4-methoxybutyrylfentanyl, acetylfentanyl, U-47770, and AH-7921 (Alzghari *et al.*, 2017; Cole *et al.*, 2015; Helander *et al.*, 2016; Rambaran *et al.*, 2018, 2017; Solimini *et al.*, 2018). Many dosage forms are available and anecdotal accounts of experiences related to these NSOs are posted on internet forums (Alzghari *et al.*, 2017; Fort *et al.*, 2016; Rambaran *et al.*, 2018, 2017). Since 2013, there have been 40 fatal BF overdoses in the United States, a majority originating from New York, and as many as 88 reports of BF between 2014 and 2015 from California, Florida, Illinois, Indiana, Kansas, Minnesota, North Dakota, New York, Ohio, Oregon, Pennsylvania, Tennessee, Virginia, and Wisconsin (Drug Enforcement Administration, Department of Justice, 2016). As more NSOs become available, a growing public health crisis is

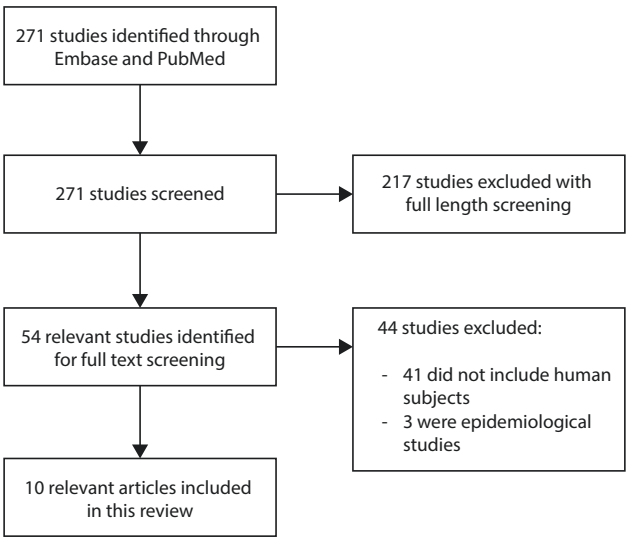

Figure 2. Search flow diagram.

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Table 1. Case reports involving the use of butyrfentanyl and its analogues.

Author (Year)	Agent	Country	Age	Sex	Peripheral blood concentration (ng/mL)	Heart blood concentration (ng/mL)	Route of administration	Presence of					Lung weight (g)	Autopsy findings	Death	Naloxone use
								Another opioid	BZD	Ethanol	Another illicit drug	Other drugs				
Bäckberg (2015)	BF	Sweden	23	M	0.9	–	Inhalation	X	–	–	X	X	–	–	–	–
Bäckberg (2015)	BF	Sweden	19	M	–	–	Intranasal	X	–	–	–	–	–	–	–	X
Bäckberg (2015)	BF	Sweden	23	M	0.6	–	Intranasal	–	–	–	X	X	–	–	–	X
Bäckberg (2015)	4-F-BF	Sweden	25	M	15	–	Oral	X	X	–	X	X	–	–	–	–
Cole (2015)	BF	USA	18	M	–	–	Inhalation	–	–	–	–	–	–	–	–	X
Farkas (2018)	4-F-BF	USA	39	M	–	–	–	X	–	–	X	–	–	–	–	X
Farkas (2018)	4-F-BF	USA	37	M	–	–	–	X	–	–	X	–	–	–	–	–
Farkas (2018)	4-F-BF	USA	38	F	–	–	Intranasal	X	–	–	X	–	–	–	–	X
Helander (2016)	BF; 4-MeO-BF	Sweden	29	F	1.3	–	–	X	–	–	X	–	–	–	–	–
Helander (2016)	4-MeO-BF	Sweden	28	M	3.1	–	–	X	X	–	X	X	–	–	–	X
Helander (2016)	4-MeO-BF	Sweden	34	M	–	–	Oral	–	X	–	–	X	–	–	–	X
Helander (2016)	4-MeO-BF	Sweden	22	M	11	–	Intranasal	X	X	–	–	X	–	–	–	X
Helander (2017)	4-Cl-IBF	Sweden	28	M	5.1	–	Intravenous	X	–	–	–	–	–	–	–	X
Helander (2017)	4-F-IBF	Sweden	27	M	38	–	Intravenous	–	–	–	–	–	–	–	X	X
Hikin (2018)	BF; 4-F-BF	England	39	M	–	–	Intravenous	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	47	M	–	–	Intravenous	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	41	M	–	–	Intravenous	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	44	M	–	–	Intravenous	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	47	M	–	–	Intravenous	–	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	39	M	–	–	–	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF	England	40	M	–	–	–	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	28	F	–	–	–	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	25	M	–	–	Intravenous	X	X	–	X	X	–	–	X	–

Abbreviations: 4-Cl-IBF: 4-chloroisobutyrfentanyl; 4-F-BF: 4-fluorobutyrfentanyl; 4-F-IBF: 4-fluoroisobutyrfentanyl; 4-MeO-BF: 4-methoxybutyrfentanyl; BF: butyrfentanyl; BZD: benzodiazepine; F: female; M: male

Table 1. Continued ...

Author (Year)	Agent	Country	Age	Sex	Peripheral blood concentration (ng/mL)	Heart blood concentration (ng/mL)	Route of administration	Presence of					Lung weight (g)	Autopsy findings	Death	Naloxone use
								Another opioid	BZD	Ethanol	Another illicit drug	Other drugs				
Hikin (2018)	BF; 4-F-BF	England	31	M	–	–	Intravenous	X	X	–	X	X	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	30	M	–	–	–	–	–	–	X	X	–	–	X	–
Hikin (2018)	4-F-BF	England	53	M	–	–	–	X	–	–	X	–	–	–	X	–
Hikin (2018)	BF; 4-F-BF	England	51	M	–	–	Intravenous	X	X	–	X	X	–	–	X	–
McIntyre (2016)	BF	USA	44	M	58	97	Intravenous	X	–	–	X	–	1705	X	X	–
Poklis (2016)	BF	USA	53	F	99	220	Oral	–	–	–	–	–	960	X	X	–
Poklis (2016)	BF	USA	45	F	3.7	9.2	Oral	X	–	X	X	X	1250	X	X	–
Rojkiewicz (2017)	4-F-BF	Poland	26	M	91	–	Inhalation	–	–	–	–	–	–	–	X	–
Rojkiewicz (2017)	4-F-BF	Poland	25	F	112	–	–	–	–	–	–	–	–	–	X	–
Staeheli (2016)	BF	Switzerland	23	M	66	39	Inhalation	X	–	–	–	–	–	X	X	–

Abbreviations: 4-Cl-IBF: 4-chloroisobutyrfentanyl; 4-F-BF: 4-fluorobutyrfentanyl; 4-F-IBF: 4-fluoroisobutyrfentanyl; 4-MeO-BF: 4-methoxybutyrfentanyl; BF: butyrfentanyl; BZD: benzodiazepine; F: female; M: male

unfolding. These NSOs are sold under the guise of "research chemicals" where one can easily purchase these substances online without fear of prosecution. Furthermore, detection of NSOs is limited to targeted spectrometry test because urine drug screens cannot detect the substance or the result shows up as a false positive for a different agent (Rambaran *et al.*, 2018, 2017). The ease of obtaining NSOs coupled with difficulty detecting these substances leads to the rising morbidity and mortality associated with this new class of drugs. Stricter regulation of "research chemicals", development of urine drug screens that can detect NSOs, and educating clinicians as well as the public are necessary measures to curtail this widespread problem (Alzghari *et al.*, 2017).

In conclusion, our systematic review shows the morbidity and mortality associated with BF and similar analogs. The most common route was intravenous administration. Patients could reverse the effects of overdose with naloxone. BF and its analogues are readily available on the Internet and governments across the globe need to consider ways to combat the spread of these agents in the midst of a growing public health crisis.

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ORIGINAL ARTICLE

Probiotics against alleviation of lead toxicity: recent advances

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ABSTRACT

Lead is a toxic heavy metal and there is no specific, safe and efficacious therapeutic management of lead toxicity. Scientific literature reported that some probiotic microorganisms alleviated experimentally induced lead toxicity. The present review attempts to collate the experimental studies on probiotics with ameliorative effects. Literature survey revealed that four (4) types of probiotic microorganisms exhibited significant protection from lead toxicity in experimental pre-clinical studies. No clinical study with significant outcome was found in the literature. From the outcomes of the preclinical studies it appears that probiotics are prospective for alleviation and treatment of lead toxicity.

KEY WORDS: probiotics; lead; lactobacilli; oxidative stress

Introduction

Lead (Pb) is a non-essential heavy metal of considerable toxicity with deleterious effect on most organ systems of humans and animals resulting in multisystem disease. It is considered as a potential worldwide threat to the environment. It undergoes biomagnification in the food chain. Environmental or occupational lead exposure in humans can produce chronic ill-health effects including hematological, hepatic, renal, pulmonary, nervous, cardiovascular, musculoskeletal and reproductive dysfunctions. Lead is also recognized as human carcinogen by the International Agency for Research on Cancer (IARC). Lead toxicity and its alleviation is a highly researched and recurrently published issue; nevertheless, complete control and prevention of lead exposure still appear far from being attained (Flora *et al*. 2012; Assi *et al*. 2016).

The most commonly used therapeutic strategy for heavy metal poisoning is chelation therapy to promote metal excretion. However, chelators for Pb toxicity are themselves reported to have a number of different safety and efficacy concerns. Chelating agents such as $CaNa_2EDTA$ and meso-2,3-dimercaptosuccinic acid (DMSA) have been reported to have protective effects against Pb toxicity. However, $CaNa_2EDTA$ can cause renal toxicity, especially during repeated high-dose treatment and in patients with renal diseases (Porru & Alessio, 1996). Because of its relative lack of selectivity, other essential metals such as zinc, iron and manganese are also reported to be simultaneously excreted and depleted following $CaNa_2EDTA$ therapy (Aposhian *et al*. 1995). DMSA also has side effects such as appetite loss, nausea and diarrhea (Liebelt & Shannon, 1994). Furthermore, most of the chelating agents are administrated subcutaneously or intraperitoneally, which might precipitate severe adverse effects. Hence, chelating agents are not suitable for high dose and long term treatment for chronic lead toxicity. Therefore, alternative options for counteracting lead toxicity appear necessary. Medicinal plants and constituents thereof (phytochemicals) have been reported to possess lead and other heavy metal toxicity ameliorative effects in pre-cinical studies (Kim *et al*. 2015; Bhattacharya, 2017; 2018a, b). There is a necessity to find safe and efficient dietary interventions against lead toxicity. Dietary strategies appear advantageous, as nutritional ingredients they can easily and affordably be incorporated into the regular diet and thus overcome the adverse effects of the chelation therapy.

Probiotics are living non-pathogenic microorganisms. When taken, they improve the intestinal microbial balance by preventing the growth of pathogens and thus they confer a health benefit to the host. Probiotics include species of *Bifidobacterium, Lactobacilli* as well as the yeast

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Saccharomyces (Foligné *et al*. 2013). The dairy products like curd, sour milk, yogurts and other fermented milk products contain these probiotics. Probiotic formulations containing these microorganisms are also available commercially as nutraceutical or functional food and are often prescribed for patients undergoing antimicrobial therapy. There is good number of studies and reviews indicating the benefits of probiotics in relation to antibiotic associated diarrhoea/constipation, allergy, lactose intolerance, reduction of cholesterol as well as development of the immune system and protection against gut pathogens (Jankovic *et al*. 2010; Rijkers *et al*. 2010; Feng *et al*. 2019). A literature survey reveals that it was from the last decade only that experimental pharmacological research has been started to assess probiotics against lead toxicity. So far there is no comprehensive account on studies on probiotics against lead toxicity. The objective of the present short review is to explore and summarize preclinical research findings in this sphere.

Review method

Internet assisted literature study was carried out by using Google, Scholar Google, Scopus, Web of Science, EMBASE and PubMed database search. Only the scientific journal articles published and/or abstracted in internet during the last decade (2009–2019) were considered. The experimental pre-clinical studies on probiotic microorganisms were selected. Clinical studies were also searched but no appreciable result was obtained. Combination of two or more natural microorganisms was regarded as a separate study.

Results

Four (4) types of probiotic microorganisms – three bacteria namely *Lactobacillus sp*., *Pediococcus pentosaceus*, *Bacillus sp*., and one yeast – *Saccharomyces cerevisiae* were reported to possess pre-clinical lead toxicity ameliorative potential. Except the first one, the rest three were used as a mixture with the first one. The details are summarized in Table 1.Two relevant clinical studies have been reported but their outcome was found to be indeterminate.

There is 1 (one) study *in vitro* in cell line. Out of the total of the 5 (five) *in vivo* studies in intact models, 3 (three) utilized mice, 1 (one) used broiler chicken and 1 (one) study used common carp fish (*Cyprinus caprio*). Most commonly studied parameters are hematological and tissue (liver, kidney,etc.) antioxidative parameters (biomarkers). Histopathological studies of these target vital organs and measurement of lead contents in concerned tissues were also performed in all *in vivo* studies. Urinary excretion study of lead or its metabolites was not performed. Fecal excretion study of lead was reported in 1 (one) case. Lead acetate was used as toxicant in most of the studies.

Discussion and conclusions

Chronic lead toxicity is considered a serious concern to biosphere. Apart from advising avoiding environmental or occupational lead exposure, relevant awareness; and certain acute and symptomatic treatments, there is no evidence based definitive treatment regimen prevailing to combat sub-chronic or chronic lead toxicity in humans. Notwithstanding, supplementation of antioxidants may be recommended (Kim *et al*. 2015; Lamidi & Akefe, 2017). Several mechanisms have been explained so far as mode of lead toxicity, including disruption of endogenous oxidant-antioxidant balance. Elicitation of oxidative stress by overproduction of oxidative and nitosative free radicals during the metabolism of lead in the body is considered to be pertinent event in exertion of lead toxicity (Patra *et al*. 2011; Lopes *et al*. 2016).

It is believed that, the beneficial properties of probiotic bacteria are related to their capacity to act by different mechanisms apart from modulation of gut microbiome, thus resulting in improved intestinal microbial balance and other benefits to the host (Monachese *et al.* 2012).

Present pre-clinical research work has demonstrated that probiotic microorganisms are able to protect animals/fish/cell line from lead toxicity principally by antioxidant effects. Intestinal lead sequestration *in vitro* is a less reported mode. Oral administration of these agents effectively reduced lead accumulation in tissue, alleviated tissue oxidative stress; reversed hepatic, renal and DNA damage and ameliorated the corresponding histopathological changes of lead-exposed tissues. The organisms of *Lactobacilli* are reported to be effective. The commercial formulation containing a mixture of different probiotic microorganisms also exhibited a similar outcome.

Previous researchers have noted a potential antioxidative property of probiotics *in vitro* and *in vivo,* including human subjects (Kullisaar *et al*. 2003; Ejtahed *et al*. 2012; Wang *et al*. 2017), presenting the mode of action of probiotics for lead toxicity amelioration *in vivo*. The cardinal mechanism is abrogation of lead-induced oxidative stress by probiotics, operative in systemic circulation (blood) followed by the organs of detoxification (liver), excretion (kidney) and other vital organs, thus alleviating tissue toxicity (Table 1). Further biochemical and mechanistic studies are necessary in this direction.

Two clinical studies have been reported so far. One randomized pilot study investigated the potential of *L. rhamnosus* GR-1 supplemented yogurt to lower lead levels in at-risk populations of pregnant women and in children in Tanzania. However, the strain *L. rhamnosus* GR-1 could not significantly reduce blood levels of Pb in pregnant women or children (Bisanz *et al*. 2014). Another recent randomized double blind study used long term oral supplementation of commercial formulation containing mixture of *Sreptococcus thermophilus* and strains of *Lactobacilli* and *Bifidobacteria* to pregnant woman in Italy to assess lead exposure in infants *via* breast milk but could not confirm whether prophylactic use of probiotics could reduce the absorption of lead (Astolfi *et al*. 2019).

Table 1. Protective effects of probiotics against lead toxicity.

Sl. No.	Probiotic microorganism(s)	Experimental model/cell line	Observed effects with proposed mechanisms	Reference(s)
1	*Lactobacillus plantarum* CCFM8661	Mice	Normalization of δ-aminolevulinic acid dehydratase (ALAD) and other antioxidative parameters and decreasing the lead levels in blood and tissues	Tian *et al*., 2012
2	Mixture of *Lactobacillus acidophilus, Lactobacillus plantarum, Bacillus subtilis, Bacillus licheniformis, Pediococcus pentosaceus,* and *Saccharomyces cerevisiae*	Broiler chicken (*Gallus domesticus*)	Decreased lead accumulation in tissues with normalized antioxidant parameters	Ghenioa *et al*., 2015
3	*Lactobacillus delbrueckii* subsp. *bulgaricus* KLDS1.0207	Mice	Alleviation of lead-induced hepatic and renal toxicity by excreting Pb in feces	Li *et al*., 2017
4	*Lactobacillus plantarum* CCFM8661	Mice	Alleviation of Pb toxicity by decreasing blood and tissue Pb concentration through abrogation of oxidative stress	Zhai *et al*., 2018
5	*Lactobacillus reuteri* P16	Common carp (*Cyprinus carpio*)	Improvement of growth and hematological parameters, modulation of oxidative stress and gene expression	Giri *et al*., 2018
6	*Lactobacillus rhamnosus* GR-1 (LGR-1)	Human intestinal epithelial cell line (Caco-2)	Pb absorption and immobilization reducing its translocation across the intestinal epithelium *in vitro*	Daisley *et al*., 2019

From the handful of demonstrated protective outcomes of the preclinical studies it appears that probiotics or probiotic mixtures have the prospect for alleviation and treatment of lead toxicity in humans. Nevertheless, this preclinical research appears to be in a quite initial stage. Definitive clinical studies are needed for due corroboration. The pre-clinically proven probiotics could be clinically tested alone or along with putative or newer chelating agents. Based on the outcome, probiotics may produce synergy, aid in disease reversal or may serve as auxiliary, complementary or disease modifying agents and thus could help in reducing the patient's adversities as palliative therapy.

Probiotics have long and widely been used as dietary supplement worldwide and are generally regarded as safe and well tolerated. Probiotic supplementation may be considered a new dietary therapeutic strategy against lead toxicity, concomitantly with conventional chelation, antioxidant, anti-inflammatory and other supportive therapy. Admissible research in this field could lead to development of a potentially useful functional food or therapeutic agent in management of lead toxicity in humans.

Declaration of interest: *The author reports no conflict of interest. The author alone is responsible for the content and writing of this paper.*

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ORIGINAL ARTICLE

Genotoxicity of cisplatin and carboplatin in cultured human lymphocytes: a comparative study

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ABSTRACT

Cisplatin and carboplatin are integral parts of many antineoplastic management regimens. Both platinum analogues are potent DNA alkylating agents that robustly induce genomic instability and promote apoptosis in tumor cells. Although the mechanism of action of both drugs is similar, cisplatin appears to be more cytotoxic. In this study, the genotoxic potential of cisplatin and carboplatin was compared using chromosomal aberrations (CAs) and sister-chromatid exchange (SCE) assays in cultured human lymphocytes. Results showed that cisplatin and carboplatin induced a significant increase in CAs and SCEs compared to the control group ($p<0.01$). Levels of induced CAs were similar in both drugs; however, the magnitude of SCEs induced by cisplatin was significantly higher than that induced by carboplatin ($p<0.01$). With respect to the mitotic and proliferative indices, both cisplatin and carboplatin significantly decreased mitotic index ($p<0.01$) without affecting the proliferative index ($p>0.05$). In conclusion, cisplatin was found to be more genotoxic than carboplatin in the SCE assay in cultured human lymphocytes, and that might explain the higher cytotoxicity of cisplatin.

KEY WORDS: cisplatin; carboplatin; DNA damage; chromosomal aberrations; sister-chromatid exchange

Introduction

Cisplatin is a widely used antineoplastic drug that belongs to the DNA alkylating family (Rjiba-Touati *et al.*, 2013). It binds to nucleophilic groups in DNA, introducing intrastrand and interstrand DNA cross-links which lead to growth inhibition and apoptosis. Cisplatin is first line in the treatment of several types of cancer, including ovarian, lung, head and neck, CNS, endometrial, esophageal, bladder, breast, and cervical cancers, as well as osteogenic sarcoma and neuroblastoma (de Castria *et al.*, 2013; Aguiar *et al.*, 2016; Castrellon *et al.*, 2017).

Carboplatin is a similar organoplatinum that possesses DNA alkylating properties and interferes with DNA duplication in a similar fashion to cisplatin (Bruning & Mylonas, 2011). Carboplatin is currently implicated in the treatment of several types of cancers, most of which overlap with those of cisplatin (Duan *et al.*, 2016; Fennell *et al.*, 2016). Carboplatin is also used in preparation for a stem cell or bone marrow transplantation (Agarwala *et al.*, 2011). Chemically, the two drugs are different in that carboplatin has a bidentate dicarboxylate in place of the two chloride ligand groups of cisplatin (Kralovanszky *et al.*, 1988).

While the two compounds share great similarity, cisplatin and carboplatin have a slightly different pharmacology. Carboplatin is relatively more stable inside the cells and is cleared more rapidly from the body (Duffull & Robinson, 1997). However, despite the lower toxicity profile, carboplatin can still cause myelosuppression which leads to neutropenia and consequently severe infections by opportunistic microbes (Pastor *et al.*, 2015).

More importantly, meta-analyses have shown a slight advantage of cisplatin-based therapy (Hotta *et al.*, 2004; Ardizzoni *et al.*, 2007) suggesting a different tumoricidal profile between the two drugs. Both drugs can equally induce platinum-DNA adducts but at different

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aquation rates (Hongo *et al.*, 1994). Furthermore, both drugs strongly activate components of the DNA damage response; however, phosphorylation of Chk1, H2AX and RPA2 is induced earlier by cisplatin than by carboplatin (Cruet-Hennequart *et al.*, 2009). Therefore, the aim of the current study is to further examine possible differences of the genotoxic effects of carboplatin and cisplatin at the chromosome level. Furthermore, the genotoxic effects of antineoplastic drugs in non-tumor cells are of special significance due to the risk of induction of secondary tumors in cancer patients.

To obtain relevant information, we utilized cultured human lymphocytes and sister-chromatid exchange (SCE) and chromosomal aberration (CA) assays. The SCE assay measures the exchange of genetic material between two identical sister chromatids and is greatly affected by mutagenic agents , while CA is used to measure chromosomal damage induced by clastogenic agents (Clare, 2012).

Material and methods

Blood donors

Five healthy subjects donated their blood for the study. Donors were recruited from the university campus using wall advertisements. All donors were non-smokers and non-alcoholic adults, aged 22–24 years. Donors had not been taking any medications or supplements for the past three months prior to blood donations. Ten milliliters of heparinized blood were collected from each donor in sterile vacutainer tubes. All donors gave written informed consents according to the Institutional Review Board prior to their participation in the study.

Drugs

Cisplatin (Ebewe Pharma) and carboplatin (Thymoorgan Pharmazie- Hikma) were dissolved in normal saline before beginning of the experiments. These drugs were initially in therapeutic concentrations and then they were serially diluted. The concentrations used in this study to evaluate the potential genotoxic side effects on normal lymphocytes were based on their known therapeutic half maximal inhibitory concentrations (IC50) on leukemia cell lines (CEM, HL60, U937, K562). The concentrations were evaluated on normal leukocytes. Procedures for SCE and CA analysis, described later, were carried out. The microscopic slides were divided using horizontal lines 1 mm apart and the number of dead versus live cells in each line was counted. A ratio of approximately 50% was used as a cutoff to accept the concentrations used for the purpose of this study. The chosen final concentrations of the drugs in cell culture were the mean concentrations used on different leukemia cell lines and these were 0.4 μg/mL for cisplatin and 6.2 μg/mL for carboplatin (Wu-Chou Su et al., 2000).

Cell cultures

Fresh blood was used to initiate lymphocytes cultures. About one mL of withdrawn blood was added to tissue-culture flasks containing nine ml of media (Chromosome Medium P – EuroClone, Italy: RPMI 1640 medium supplemented with suitable amount of fetal bovine serum, glutamine, penicillin-streptomycin and phytohemagglutinin).

Sister-chromatid exchange assay

The procedure that was followed to conduct SCEs in cultured human lymphocytes was described previously (Khabour *et al.*, 2011). In brief, a fresh solution of Bromodeoxyuridine (BrdU, Sigma-Aldrich, final concentration 20 μg/ml) was added to cultured human lymphocytes directly after culture initiation. Cultures were then incubated at 37 °C in dark CO2 incubator for 72 hours. Cisplatin/Carboplatin were added to cultures in the last 24 hours of incubation time (Al-Sweedan *et al.*, 2012). Before harvesting cultured lymphocytes, Colcemid (obtained from Euro clone, Italy, final concentration 10 μg/mL) was added to cultures for 90 minutes. Cultures were then centrifuged and the pellet was introduced to a hypotonic solution (0.075M KCl, Euro clone, Italy) and incubated at 37 °C for 20 min. Tubes were then centrifuged and the cellular pellet was fixed with three changes of ice-cold methanol:acetic acid (3:1, Carlo erba, China). Metaphase spreads were prepared on pre-chilled slides as previously described (Azab *et al.*, 2017). The slides were stained with the fluorescent-plus-Giemsa technique and SCEs were scored blindly using medical microscope at 1000× magnification. Fifty M2 metaphases were analyzed per each drug concentration/donor. In addition, M1, M3 and M4 metaphases were counted for analysis of mitotic and proliferative indices (Azab *et al.*, 2009; Alzoubi *et al.*, 2014).

Chromosomal aberrations (CAs) assay

CA assay cultures were prepared and treated similarly to those of SCE but without the addition of BrdU to the culture. After staining with 2% Giemsa solution (Medical Expertise House, Jordan, pH 6.8), 500 metaphase spreads (100 for each treatment/donor) were analyzed for CAs (Alzoubi *et al.*, 2012; Mhaidat *et al.*, 2016). Only breaks and exchanges were included in the analysis of CAs.

Cell kinetics analysis

The mitotic index and proliferative index were examined to reflect cytotoxicity of Cisplatin and Carboplatin. To determine the mitotic index for each concentration, at least 5,000 cells (1,000 cells from each donor) were included and the number of metaphases was counted. The mitotic index was calculated as the ratio of the number of metaphases seen vs. total number of intact cells seen. For the cell proliferation index the following calculation was used: (M1 +2*M2+3*M3)/100 for each slide, with a total of 500 metaphase cells used (Azab *et al.*, 2009).

Statistical analysis

Statistical comparisons were performed using GraphPad Prism statistical software (version 4) ANOVA, followed by Tukey *post hoc* test for analysis of the three

groups. A $p<0.05$ was used as a threshold for statistical significance.

Results

Chromosomal breaks and exchanges were scored using metaphases stained with Giemsa. Chrosomal/chromatid breaks and exchanges were included in the CAs assessment. First, both cisplatin and carboplatin significantly increased CAs by 3.0 and 2.3 fold, respectively ($p<0.01$, Figure 1). Interestingly, the level of the increase in CAs was slightly higher in the cisplatin-treated group than that in the carboplatin-treated group, despite lack of statistical significance ($p>0.05$).

Figure 2 shows the frequency of SCEs induced by each treatment. Cisplatin and carboplatin significantly increased SCEs 4.05 and 3.1 fold, respectively ($p<0.01$). The level of the increase in SCEs was significantly higher in the cisplatin-treated group than in the carboplatin-treated group ($p<0.01$). Thus cisplatin induced more genotoxicity in terms of SCEs than did carboplatin, a result that consistently agrees with previous observations (Shinkai *et al.*, 1988).

Finally, we wanted to examine if these genotoxic differences between cisplatin and carboplatin have variable outcomes on the proliferative potential of cultured cells. Both cisplatin and carboplatin significantly decreased the mitotic index ($p<0.01$, Figure 3) without affecting the proliferative index ($p>0.05$, Figure 4). However, mitotic and proliferative indices were similar in cisplatin and carboplatin groups ($p>0.05$), indicating that the discrepancy in the genotoxic profiles of the two compounds is not necessarily responsible for the difference in their cytotoxic effect.

Discussion

In the current study, the genotoxicity of cisplatin and carboplatin was compared in cultured human lymphocytes. Results showed that cisplatin was more genotoxic

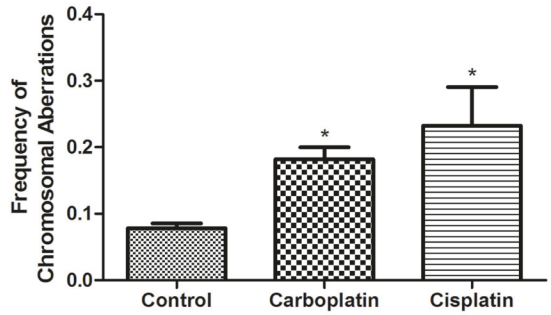

Figure 1. Chromosomal aberrations (CAs) induced by cisplatin and carboplatin. Cisplatin and Carboplatin significantly increased CAs frequency in cultured human lymphocytes ($p<0.01$). No significant differences were detected between the magnitude of CAs induced by Cisplatin and Carboplatin ($p>0.05$). * Indicates significant difference from control group.

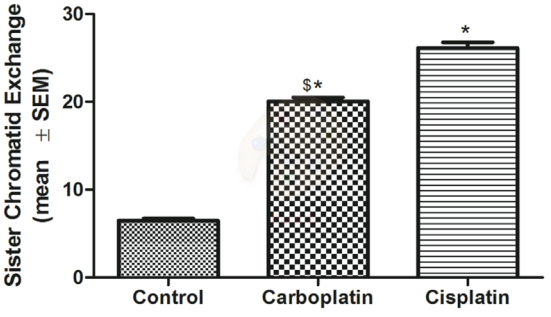

Figure 2. Sister-chromatid exchanges (SCEs) induced by cisplatin and carboplatin. Cisplatin and Carboplatin significantly increased SCEs frequency in cultured human lymphocytes ($p<0.01$). In addition, the magnitude of SCEs induced by Cisplatin was significantly higher than that induced by Carboplatin ($p<0.01$). * Indicates significant difference from control group. $ Indicates significant difference from Cisplatin group.

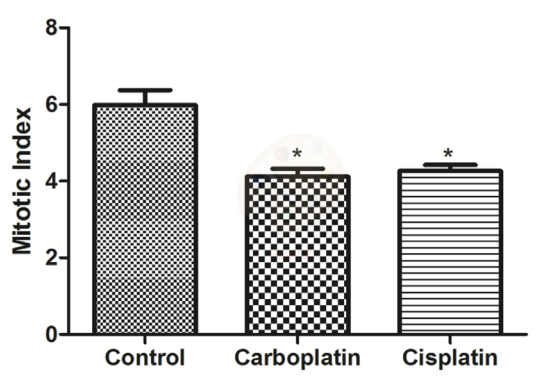

Figure 3. Effect of cisplatin and carboplatin on mitotic index. Treatment of cultured human lymphocytes with Cisplatin or Carboplatin significantly decreases mitotic index ($p<0.01$). No significant differences were detected between mitotic index of Cisplatin and Carboplatin ($p>0.05$). * Indicates significant difference from control group.

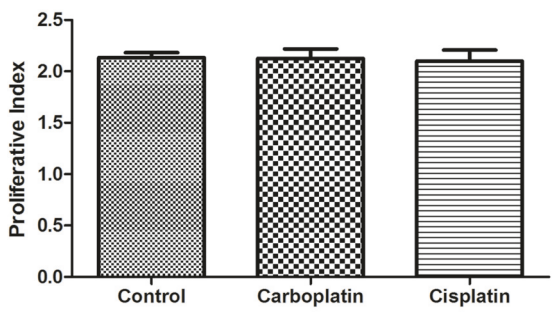

Figure 4. Effect of cisplatin and carboplatin on proliferative index. Treatment of cultured human lymphocytes with Cisplatin or Carboplatin did not affect proliferative index ($p>0.05$).

than carboplatin in the induction of SCEs. However, both drugs induced comparable levels of CAs, MI and PI.

The genotoxicity of cisplatin is well documented both *in vivo* and *in vitro* systems. This includes induction of SCEs, CAs, micronuclei and oxidative DNA damage in different models such as mice, rats, humans and cell lines (PC12, Ehrlich ascites tumor, HepG2) (Brozovic *et al.*, 2009; Desai & Gadhia, 2012; Ghosh *et al.*, 2013; Khabour *et al.*, 2014). Similarly, carboplatin was shown to induce chromosomal DNA damage in humans, animals and cell lines. For example, CAs and SCEs were shown to be induced by carboplatin in cultured human lymphocytes (Shinkai *et al.*, 1988), Ehrlich ascites tumor cells (Mylonaki-Charalambours *et al.*, 1998), and Chinese hamster ovarian cells (Gonzalez Cid *et al.*, 1995). The results that cisplatin is more potent in inducing SCEs is in agreement with a previous report conducted on Chinese hamster V 79 cells (Chibber & Ord, 1989). Thus the data presented in this study and those of other authors suggest that cisplatin is more genotoxic than carboplatin on normal cells. However, as previous works have shown, the genotoxic effects of cisplatin have not been correlated with increased incidence of cancer development in patients who received it. By extent, due to its lower genotoxic properties, carboplatin can be considered safe with regard to risk of secondary cancer development (Shinkai *et al.*, 1988).

The mechanisms for the induction of CAs and SCEs were suggested to be different. SCEs arise when damaged DNA induces the replicative bypass repair mechanisms during cell replication (Sasaki, 1980), whereas CAs are induced by damage repaired by post-replication repair processes. The differences in the ability of cisplatin and carboplatin in the induction of SCEs could be related to the type/magnitude of DNA damage they induced and how they interact with DNA. For example, carboplatin was shown to exhibit lower reactivity and slower DNA binding kinetics than cisplatin, although both form similar reaction products *in vitro* at equivalent doses (Hah *et al.*, 2006). With respect to treatment effectiveness, as noted earlier, it is well accepted that carboplatin has a relatively lower potency than cisplatin in treatment of some cancers (Moncharmont *et al.*, 2011). Therefore, all these mechanisms might contribute to the higher frequency of SCEs induced by cisplatin compared to carboplatin.

On the other hand, the comparable MI and PI can be attributed to the fact that the actual damage of the chromosomes measured by CA was similar between cisplatin and carboplatin. This means that the magnitude of damaged chromosomes in the two groups is comparable. Thus this translates to a comparable cytotoxic effect evaluated by PI and MI.

In the current investigation, only one dose of cisplatin and carboplatin were investigated and for one period. Future studies that examine a comprehensive concentration-effect of both drugs and for different periods are strongly recommended.

In conclusion, cisplatin was found to be more genotoxic than carboplatin in the SCE assay in cultured human lymphocytes.

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ORIGINAL ARTICLE

Nephroprotective effect of green tea, rosmarinic acid and rosemary on N-diethylnitrosamine initiated and ferric nitrilotriacetate promoted acute renal toxicity in Wistar rats

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ABSTRACT

The present study was designed to investigate the chemoprotective effect of green tea extract (GTE), rosmarinic acid (RA) and rosemary extract (RE) against diethylnitrosamine (DEN) initiated and ferric nitrilotriacetate (Fe-NTA) promoted nephrotoxicity in rats. Forty male rats were categorized into five: Group I included healthy rats, group II received DEN+Fe-NTA, group III received 200 mg/kg b.wt. of RE+DEN+Fe-NTA, group IV received 1 g/kg b.wt. of GTE+DEN+Fe-NTA and group V received 50 mg/kg b.wt. of RA+DEN+Fe-NTA. RE, GTE, RA were given orally for 14 days before single intraperitoneal administration of DEN (160 mg/kg) till the end of the experiment. Eighteen days after DEN, a single intraperitoneal dose of Fe-NTA (5 mg Fe/kg) was administrated to rats to promote nephrotoxicity. The biochemical parameters were analyzed in serum at time intervals while the malondialdehyde (MDA) and tumor necrosis factor-alpha (TNF-α) were assessed in both serum and renal tissues. Kidney from each group was histopathologically examined at time intervals. The administration of Fe-NTA after DEN dose to albino rats resulted in acute nephrotoxicity which was characterized by a highly significant elevation of serum urea, creatinine, uric acid (p=0.000), serum and renal MDA and TNF-α (p=0.000) with vacuolation of epithelial lining renal tubules. The administration of RE, GTE and RA prior to DEN+Fe-NTA treatment significantly ameliorated the observed increased levels of the above mentioned parameters. GTE, RA & RE exerted a protective effect against renal toxicity with GTE showing a more pronounced effect on renal function parameters while RA showed the best antioxidant impact.

KEY WORDS: vipoxin; phospholipase A_2; acidic component; acute toxicity

ABBREVIATIONS

DEN: diethylnitrosamine; **Fe-NTA:** ferric nitrilotriacetate; **RE:** rosemary extract; **GTE:** green tea extract; **RA:** rosmarinic acid; **ROS:** reactive oxygen species; **HO-1:** hemeoxygenase-1; **Nrf2:** nuclear factor erythroid 2; **Keap-1:** kelch like ECH-associated protein 1; **ARE:** antioxidant response element; **HSP90:** heat shock protein 90; **iNOS:** inducible nitric oxide synthase; **COX-2:** cyclooxygenase-2 enzyme; **CYP2E1:** cytochrome P450 2E1; **MDA:** malondialdehyde; **TNF-α:** tumor necrosis factor-alpha; **GA:** gallic acid; **GC:** (–)-gallocatechin; **EC:** (–)-epicatechin; **EGC:** (–)-epigallocatechin; **ECG:** (–)-epicatechin gallate; **EGCG:** (–)-epigallocatechin gallate; **CA:** p-coumaroylquinic; **GCG:** (–)-gallocatechin-3-gallate; **GTP:** green tea polyphenols; **NTA:** nitrilotriacetic acid disodium salt; **LPO:** lipid peroxidation; **SGLTI:** sodium-dependent glucose transporter I; **NF-κB:** nuclear factor kappa B.

Introduction

Renal toxicity is one of the most common problems that occur to the body upon exposure to a drug or a toxin. Free radicals are considered a potent factor for mediating oxidative damage and tissue impairments (Sundaram *et al.*, 2015). About 20% of nephrotoxicity is induced by medications, but for the elderly the nephrotoxicity incidence increases up to 66% because of the longer average life span.

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Chemotherapy and anticancer agents are used cautiously due to their nephrotoxic adverse effects. Nephrotoxicity takes place when detoxification and excretion functions of the kidney are disturbed due to kidney malfunction either by exogenous or endogenous toxins (Kim & Moon, 2012).

Diethylnitrosamine (DEN) is one of the well known hepato-carcinogenic nitrosamine in the environment that has many industrial and chemical applications. DEN is used as a solvent in the fiber industry, as an additive for lubricants and as a softener for co-polymers. It is also used in the synthesis of 1,1- diethylhydrazine and in condensers to elevate the dielectric constant (Koul *et al.* 2007). DEN can be produced in the human body as metabolic products of many drugs and found in processed meats, tobacco smoke, cheese, soybean, and many types of foods (Ahmed *et al.*, 2015).

Ferric nitrilotriacetate (Fe-NTA) is another potent hepato- and nephrotoxic agent. It has been used both in developing and developed countries as an alternative of polyphosphates in detergents for hospital and household use (Sundaram *et al.*, 2015). Nitrilotriacetic acid is also widely used in the treatment of boiler water to prevent mineral accumulation, in photography, paper, textile manufacture, cellulose production, cleaning operations and metal plating (Khan *et al.*, 2004). It induces subacute and acute necrosis of renal proximal tubules. It can also promote hepatic tumors effectively. It decreases the antioxidant content and induces oxidative stress (Kaur *et al.*, 2010). Fe-NTA is obtained when nitrilotriacetic acid reacts with iron at neutral pH forming a chelate complex which is soluble in water (Sundaram *et al.*, 2015). A single dose of Fe-NTA from 5 to 15 mg iron per kg body weight can induce acute nephrotoxicity, while repeated doses of Fe-NTA for 10–12 days of either 1 or 2 mg iron per kg body weight can induce sub-acute nephrotoxicity (Gupta *et al.*, 2007).

Reactive oxygen species (ROS) are one of the main causes leading to the progression of pathophysiological changes of kidney diseases. ROS have many physiological roles in cells; however, they can exert also deleterious effects such as lipid peroxidation, cellular protein oxidation and DNA damage. These effects may lead to altered integrity of plasma membrane and mitochondrial membrane. Also, the oxidative damage can result in impaired function of the protein and suppression of both cell repair and proliferation. In addition, the free iron has a very high catalytic activity, which leads to the generation of potent reactive free radicals that finally cause oxidative injury and tissue damage (Gupta *et al.*, 2009).

Herbal plants are becoming increasingly popular in modern society as natural remedies. It may be due to the antioxidant activity of the main constituents of many herbs that have the ability to inhibit the formation of ROS or to scavenge the formed ones. In addition, herb constituents can inhibit the production of ROS by the upregulation of vitagenes that protect the cells from various electrophiles and oxidants. Examples of such cytoprotective proteins are ferritin, glutathione reductase, hemeoxygenase-1 (HO-1) and repair enzymes such as 26S proteasome (Calabrese *et al.*, 2010).

Rosemary (*Rosmarinus officinalis*) is a well known spice and medicinal herb, belonging to the Lamiaceae family. Rosemary plant contains 2 active principle constituents; flavonoids, which include 6-methoxygenkwanin, diosmetin, apigenin, diosmin, hispidulin, genkwanin, sinensetin, luteolin, and di- and triterpenoids, which include carnosic acid, oleanolic acid, rosmariquinone, ursolic acid and picrosalvin (carnosol). At least, 3% of the main constituents of rosemary are represented by phenolic acids such as rosmarinic, chlorogenic and caffeic acids (Begum *et al.*, 2013). The predominant active compounds, carnosic acid and carnosol, and many other active polyphenolic compounds in rosemary, have the ability of both scavenging free radicals directly and increasing endogenous cellular antioxidant defenses indirectly via activation of the redox-sensitive system Nrf2/Keap-1/ARE transcriptional pathway, as well as potentially via multiple other mechanisms (Satoh *et al.*, 2013).

Rosemary was scientifically proved to possess antioxidant activity, anti-inflammatory activity, anti-nephrotoxic activity, anti-hepatotoxic activity, antimicrobial activity and anti-tumor activity (Begum *et al.*, 2013).

Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world (Feng, 2013). It is an evergreen tree (Ahmed & Stepp, 2013), belongs to the Theaceae family and from its leaves green tea is produced. Green tea (GT) includes more than 3000 phytochemical compounds and about one-third of these constituents are assorted as polyphenols (Shivashankara *et al.*, 2013). The most abundant and common polyphenolic compounds are gallic acid (GA), (–)-epicatechin (EC),(–)-gallocatechin (GC), (–)-epicatechingallate (ECG), (–)-epigallocatechin (EGC), (–)-gallocatechin-3-gallate (GCG), p-coumaroylquinic acid (CA) and (–)-epigallocatechin gallate (EGCG) which represents the highest concentrations of polyphenols compared to others (Forester & Lambert, 2014). Catechins of GTE possess antioxidant, anti-angiogenic and anti-proliferative activities that play a marked role in the treatment of various disorders such as stomach disorders, type 2 diabetes, headaches, atherosclerosis, hypotension and myocardial infarction. GTE also can reduce the risk of ovarian cancer, gastrointestinal cancer, breast cancer and lung cancer (Shivashankara *et al.*, 2013).

Green tea polyphenols can exert their antioxidant action by scavenging free radicals, suppressing inducible nitric oxide synthase activity (iNOS), down-regulating cyclooxygenase-2 enzyme (COX-2), upregulating the transcription of many cytoprotective (phase 2) genes and various detoxification enzymes thereby reducing ROS production and protecting cellular DNA from damage (Calabrese *et al.*, 2010). Smarinic acid (RA) is a caffeic acid ester with 3,4- dihydroxyphenyl lactic acid. It is a polyphenolic compound that is potentially used at the pharmaceutical/nutritional interface. RA is commonly found in several plants which belong to the Lamiaceae family, such as Rosmarinus officinalis, Perilla frutescens, Salvia officinalis and many other medicinal herbs (Ramalho *et*

al., 2014). These plant species, based on dry weight, contain more than 3% RA which has four phenolic hydrogens enabling it to control the oxidation of free radicals. RA improves its radical stability by intermolecular hydrogen bond formation between the hydrogen of its phenoxyl radical and that of its hydroxyl radical (Bhatt *et al.*, 2013).

RA exhibits a number of important biological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer and anti-neurodegenerative activities (Ramalho *et al.*, 2014). RA treatment might enhance the detoxification mechanisms by reducing the phase I metabolizing enzymes responsible for the conversion of chemicals into carcinogens such as cytochrome P450 2E1 and increasing the activity phase II endogenous antioxidant enzymes such as glutathione peroxidase and catalase (Ahmed, 2016). In addition, RA enhances DNA repair enzymes and induces endogenous cellular defense mechanism by activation of Nrf2 /HO-1 and caspase-3 pathways (Furtado *et al.*, 2015).

This study was designed to evaluate the possible nephroprotective effects of rosemary, green tea and rosmarinic acid on diethylnitrosamine initiated and ferric nitrilotriacetate promoted nephrotoxicity and increased oxidative stress in Wistar albino rats. This can be achieved through assessing the role of their extracts on kidney function parameters, lipid peroxidation status, proinflammatory marker levels and histopathological changes in the nephrotoxic rat model.

Materials and methods

Materials

Diethylnitrosamine, nitrilotriacetic acid disodium salt (NTA) and iron (III) nitrate nonahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA).Sodium bicarbonate and sodium hydroxide pellets were purchased from Loba Chemie, Mumbai, India. Rosmarinic acid 96% (Cat. No.536954) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rosemary was obtained from Herbarium of Cairo University, while green tea was obtained from local market in Cairo. Chemicals were used for the determination of lipid peroxide product (MDA); the thiobarbituric acid and the trichloroacetic acid were obtained from Merck (Germany) and BDH (England) Companies, respectively. Kits used for determination of urea, creatinine and uric acid in blood samples were purchased from Erba Diagnostics Mannheim GmbH (Friedrichsring 4, Mannheim, Germany). Kits used for determination of serum albumin and glucose were purchased from Bio-diagnostic Company, Cairo, Egypt. Kits for estimation of Rat Tumor Necrosis Factor Alpha (TNF-α) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methods

Preparation of rosemary water extract (RE)

Rosemary extract was prepared as described previously by Al-Attar & Shawush (2014) with some modifications. Fifty grams of rosemary powdered leaves were added into 2 L of hot water in a flask for 6 hours. Then, the solution was slowly boiled for 1 hour and cooled at room temperature. It was gently subjected to an electric mixer for 10 minutes and filtered. The extract was given fresh to the animals.

Preparation of green tea water extract (GTE)

GTE was prepared according to El-Beih *et al.* (2015) with some modifications by dissolving amount equivalent to 0.001 kg of tea/ kg body weight in glassware containing 5 ml boiling distilled water (equivalent to 5 cups for a 60-kg adult human), then covered and let stand for 10 minutes at room temperature. Finally, the extract was filtered and given fresh to the animals.

Preparation of Ferric Nitrilotriacetate (Fe-NTA)

Fe-NTA solution was freshly prepared immediately before use as described by Iqbal and Athar (1998). Ferric nitrate (0.16 mmol/5.0 ml) solution was mixed with a fourfold molar excess of disodium salt of nitrilotriacetic acid (0.64 mmol/5.0 ml) and the pH was adjusted to 7.4 with sodium bicarbonate. The concentration of iron in this solution was 9 mg Fe/10 ml.

HPLC method for determination of phenolic compounds

Phenolic compounds in the water extract of rosemary and green tea were determined according to Goupy *et al.* (1999). A high performance liquid chromatography system (HPLC) equipped with a variable wave length detector (Agilant, Germany) 1100 was used. The HPLC was equipped with auto sampler, quaternary pump degasser and column compartment. Analyses were performed on a C_{18} reverse phase (BDS 5 µm, Labio, Czech Republic) packed stainless-steel column (4×250mm, i.d.). Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

Experimental animals

Forty male adult Wistar albino rats of Sprague Dawley strain with a body weight ranging from 120–150 g were obtained from the Central Animal House of National Research Centre, Cairo, Egypt. Rats were housed in stainless steel cages in a room under controlled conditions of temperature and humidity. All rats were kept for adaptation to the environmental conditions for 2 weeks. Standard laboratory diet and water were allowed *ad-libitum* to animals during the experimental period. Animal experiments were carried out in compliance with the Guidelines for Institutional Animal Care and Use Committee of Cairo University, Egypt, and the study protocol was approved with the no: "CU-I-S-58-17".

Experimental design

Out of 40, 24 rats were given protective doses for 14 consecutive days of 200 mg/kg rosemary extract, 1 g/kg green tea extract and 50 mg/kg rosmarinic acid (Alnahdi, 2012; El-Beih *et al.*, 2015; Tavafi & Ahmadvand, 2011). The induction of toxicity was initiated with a single intraperitoneal injection of diethyl nitrosamine (DEN) at

a dose level of 160 mg/kg body weight (Becker *et al.*, 1985). After eighteen days of the initiation step, the animals were given a single intraperitoneal injection of Fe-NTA at a dose level of 5 mg Fe/kg body weight (Okada *et al.*, 2001). The animals continued to receive the extracts of medicinal plants and rosmarinic acid daily till the end of the experiment. The animals were divided into 5 groups, each of eight animals, as follows:

Group I (control negative)**:** received only standard diet and water.

Group II (control positive)**:** received a single i.p. injection of DEN in saline followed by a single i.p. injection of Fe NTA on the 18th day of DEN.

Group III: pretreated rats orally with rosemary extract at a dose 200 mg/kg b.wt. for 14 days followed by i.p. injection of DEN in saline and i.p. injection of Fe NTA on the 18th day of DEN.

Group IV: pretreated rats orally with green tea extract at a dose 1g/kg b.wt. for 14 days followed by i.p. injection of DEN in saline and i.p. injection of Fe NTA on the 18th day of DEN.

Group V: pretreated rats orally with pure rosmarinic acid in distilled water at a dose of 50 mg/kg b.wt. for 14 days followed by i.p. injection of DEN in saline and i.p. injection of Fe NTA on 18th day of DEN.

Changes in body weight were followed up twice a week throughout the experimental period. Blood samples were collected after 2 weeks of DEN injection, after 2 hours, 24 hours and after 48 hours of Fe-NTA injection to evaluate serum kidney function parameters. After 48 hours of Fe-NTA injection, all animals were sacrificed, blood was collected and serum was separated for further investigations of biochemical parameters. The kidneys of each rat were separated, washed with saline and weighed. Then one kidney of each animal was kept in 10% neutral buffered formalin for histopathological examination and the other one was stored in –20 °C for further estimation of malondialdehyde and tumor necrosis factor alpha.

Preparation of kidney homogenates for malondialdehyde and TNF-α assesment

A portion with known weight of the kidney of each animal was homogenized by homogenizer stirrer Wise Stir (HS-30E) in 5–10 mL cold buffer (50 mM potassium phosphate, pH 7.5) per gram tissue. Then, the homogenates were centrifuged at 4000 rpm for 15 minutes. Finally, the supernatant of tissue homogenates was separated and stored at –80 °C for malondialdehyde determination.

The other part of the kidney of each animal was rinsed in ice-cold phosphate buffer saline (PBS) (0.01mol/L, pH 7.0–7.2) to remove excess blood thoroughly and weighed before homogenization. The tissues were minced to 1–2 mm pieces and homogenized by homogenizer stirrer Wise Stir (HS-30E) in 5–10 mL of PBS with a glass homogenizer on ice. Then the homogenates were centrifuged for 5 minutes at 5000×g. The supernatants of tissue homogenates were separated and stored at –80 °C for estimation of TNF-α.

Biochemical analysis

Serum glucose was determined according to (Trinder, 1969). Serum urea was assessed according to (Talke & Schubert, 1965). Serum creatinine was determined by Modified Jaffe's reaction as described by (Bowers, 1980). Uric acid was estimated by Trinder's enzymatic reaction as described by (Trinder & Barham, 1972). Albumin was determined according to Doumas *et al.* (1971).

Estimation of lipid peroxidation level

The rate of production of TBARS (thiobarbituric acid reactive species) was measured in serum and renal tissues according to Draper & Hadley (1990). 2.5 ml of trichloroacetic acid (10% w/v) was added to 0.5 ml of serum and renal tissue homogenates in a boiling water bath for 15 minutes, cooled rapidly under tap water and finally centrifuged for 10 minutes at 3000 rpm. 2 ml of separated supernatant of serum and tissue homogenate samples was added to 1 ml of thiobarbituric acid (0.67% w/v) in a test tube and placed in a boiling water bath for 15 minutes then cooled again rapidly under tap water. The absorbance was measured at 532 nm against air. The results were calculated using a molar extinction coefficient of 1.53×10^5 $M^{-1}.cm^{-1}$.

Estimation of tumor necrosis factor-alpha

Tumor necrosis factor alpha (TNF-α) was assessed in serum and kidney tissue homogenates using a standard ELISA method. The assessment was done by sandwich ELISA technique according to the manufacturer's instructions (R&D Systems).

Histopathological examination

The kidneys were cleared in xylol, embedded in paraffin wax and longitudinally sectioned with a microtome into sections with 4–6 micron thickness. These sections were stained by hematoxylin and eosin and finally they were observed under an Olympus microscope with a camera.

Statistical analysis

Differences between groups were analyzed using one way analysis of variance (ANOVA), followed by Duncan multiple comparisons test using SPSS package version "20" for Windows. Values are represented as mean ± S.E. and $p<0.05$ was considered statistically significant, $p<0.01$ was considered highly significant and $p<0.001$ was considered very highly significant.

Results

Phenolic acid content of water extract of rosemary and green tea as determined by HPLC

Analysis by HPLC to water extract of rosemary and green tea revealed the presence of different phenolic compounds. Using 19 standard phenolic compounds, eighteen and seventeen different phenols were detected in RE and GTE, respectively (Figures 1 & 2). A high amount of rosmarinic acid, catechol, ellagic acid, 3,4,5-methoxycinnamic acid, vanillic acid, pyrogallol, catechins, p-hydroxybenzoic

acid, caffein, ferulic acid and protocatechic acid were found in the water extract of rosemary. In addition, a high amount of pyrogallol, ellagic acid, catechins, vanillic acid, p-hydroxybenzoic acid, catechol, protocatechic acid and 3-hydroxytyrosol were detected in the water extract of green tea (Table 1).

Effect of RE, GTE & RA on the change of body weight and kidney weight percent in DEN initiated and Fe-NTA treated rats

As compared to control negative group, the control positive group treated with DEN+Fe-NTA exhibited a marked decrease of body weight (p=0.026) and an insignificant increase of kidney weight percent (p>0.05) (Table 2). RE and GTE treated groups showed no change in body weight as compared to control negative group and that is a good response (p>0.05). Kidney weight percent of DEN+Fe-NTA group showed an insignificant change when compared to RE, RA and control negative group (p>0.05). It is important to mention that RE+DEN+Fe-NTA treated group showed a highly significant increase in kidney weight percent as compared to GT and RA pretreated rats (p=0.002 & 0.002, respectively) (Table 2).

Effect of RE, GTE & RA on biochemical parameters of renal functions at time intervals

Serum levels of urea, creatinine and uric acid are clinical markers for renal function. As shown in Figures 3 & 4, after 2 weeks of DEN administration, a highly significant increase of urea and creatinine concentration of DEN treated group was observed when compared to negative control group (p<0.001). However, oral administration of RE & GTE prevented increase of urea and creatinine levels, while RA prevented only the increase of creatinine level.

At time intervals of Fe-NTA injection, the biochemical studies revealed a very highly significant increase of serum urea and creatinine concentration of DEN+Fe-NTA group when compared to control negative group (p=0.000). However, the administration of protective doses of GTE and RA alleviated the increase of urea and creatinine concentration highly significantly when compared to DEN+Fe-NTA treated group at time intervals. The effect of RE took more time to exert its action and that was noticed after 2 days of Fe-NTA exposure when a highly significant decrease of creatinine (p=0.000) and only a significant decrease of urea concentration (p=0.032) were observed as compared to DEN+Fe-NTA group (Figures 3 & 4).

Uric acid concentration showed an insignificant difference between all groups after 2 weeks of DEN injection (Figure 5). While after Fe-NTA injection, uric acid level of DEN+Fe-NTA group showed a highly significant elevation at time intervals as compared to normal control group (p=0.000). However, the increase of uric acid levels was prevented by RA, RE and GTE administration showing a very high significant decrease at time intervals as compared to DEN+Fe-NTA group (Figure 5).

Albumin concentration showed an insignificant difference between all groups after 2 weeks of DEN, 2 hours & 24 hours of Fe-NTA injection (p>0.05). However, after 48 hours, albumin concentration of control positive group showed a significant decrease as compared to control negative group (p=0.016). With respect to groups that were pretreated with RE, GTE and RA, they exhibited an insignificant difference in their albumin levels as compared to control negative after 48 hours of Fe-NTA injection (p>0.05) (Figure 6).

Table 1. Differential pattern of different phenolic compounds identified in water extracts of rosemary and green tea as detected by HPLC

Phenolic compounds	Rosemary extract (ppm)	Green tea extract (ppm)
Gallic acid	86.07	57.43
Pyrogallol	955.40	7745.85
3-hydroxytyrosol	142.29	480.98
Protocatechuic acid	203.69	171.95
Catechins	731.80	1142.82
Catechol	1846.47	400.45
P-hydroxybenzoic	540.57	549.48
Caffeine	692.73	65.80
Vanillic acid	1269.73	321.09
P-coumaric acid	125.96	47.42
Ferulic acid	223.04	29.35
Iso-ferulic acid	99.48	16.66
Alpha-coumaric	34.31	5.85
Ellagic acid	1729.22	1583.35
Coumarin	105.49	21.13
3,4,5-methoxycinnamic acid	1663.55	30.86
Cinnamic acid	28.29	4.44
Rosmarinic acid	2664.52	–

Table 2. Body weight change and kidney weight % after 48 hours of Fe-NTA injection, of control and treated groups.

Group	B. wt. change (g)	Kidney Wt. %
Control	17.14±2.38^{a}	0.84±0.03^{ab}
DEN+Fe-NTA	4.50±4.08^{bc}	0.88±0.03^{ab}
RE+DEN+Fe-NTA	14.13±0.05^{ab}	0.95±0.06^{a}
GTE+DEN+Fe-NTA	10.38±5.77^{ab}	0.77±0.02^{b}
RA+ DEN+Fe-NTA	-2.00±3.77^{c}	0.78±0.03^{b}

*RM = rosemary, GTE = green tea extract, RA = rosmarinic acid, DEN= diethylnitrosamine, Fe-NTA = ferric nitrilotriacetate, b. wt. change = body weight change, kidney wt. % = kidney weight percentage.
*Data are represented as Mean±SE. Values that share the same letter at the same column are not significant. Values that share different letters at the same column are significant.

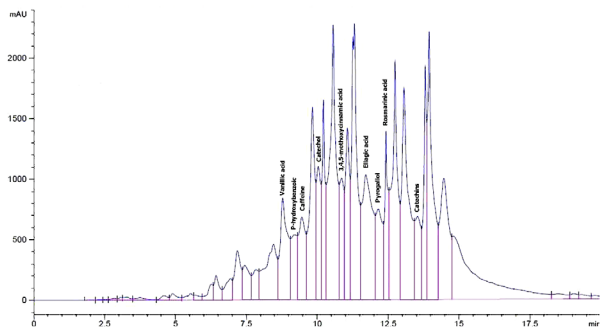

Figure 1. HPLC profile of phenolic compounds of water extract of rosemary as standards at 280 nm. (1) Rosmarinic acid, (2) Catechol, (3) Ellagic acid, (4) 3,4,5-methoxycinnamic acid, (5) Vanillic acid, (6) Pyrogallol, (7) Catechins, (8) Caffeine, and (9) P-hydroxybenzoic.

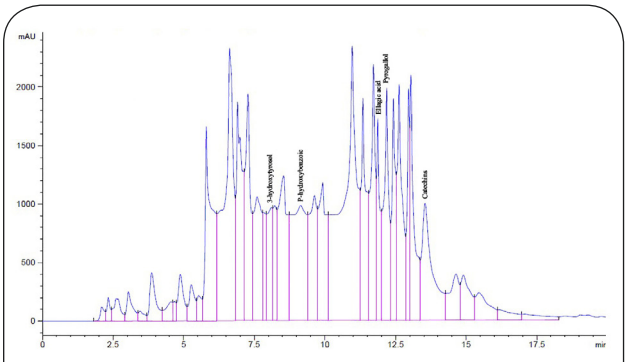

Figure 2. HPLC profile of phenolic compounds of water extract of green tea as standards at 280 nm. (1) Pyrogallol, (2) Ellagic acid, (3) Catechins, (4) P-hydroxybenzoic acid, and (5) 3-hydroxytyrosol.

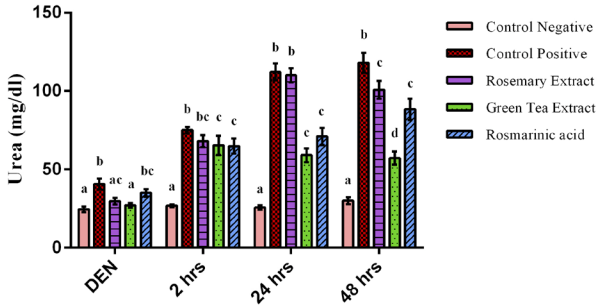

Figure 3. Urea concentration after 2 weeks of diethylnitrosamine (DEN) injection, after 2 hours, 24 hours & 48 hours of ferric nitrilotriacetate injection. Data are represented as Mean±SE. Bars that share the same letters within one series are not significant. Bars that share different letters within one series are significant.

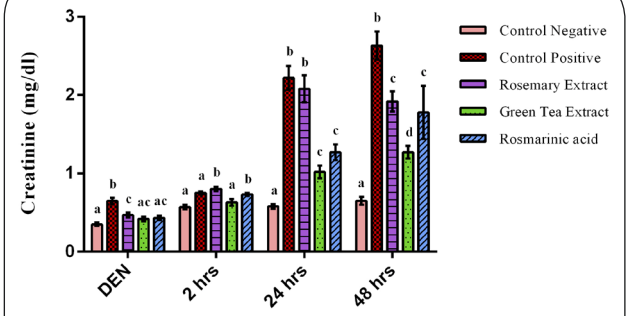

Figure 4. Creatinine concentration after 2 weeks of diethylnitrosamine (DEN) injection, after 2 hours, 24 hours & 48 hours of ferric nitrilotriacetate injection. Data are represented as Mean±SE. Bars that share the same letters within one series are not significant. Bars that share different letters within one series are significant.

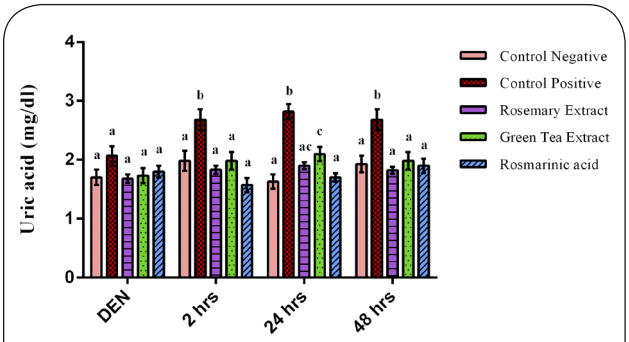

Figure 5. Uric acid concentration after 2 weeks of diethylnitrosamine (DEN) injection, after 2 hours, 24 hours & 48 hours of ferric nitrilotriacetate injection. Data are represented as Mean±SE. Bars that share the same letters within one series are not significant. Bars that share different letters within one series are significant.

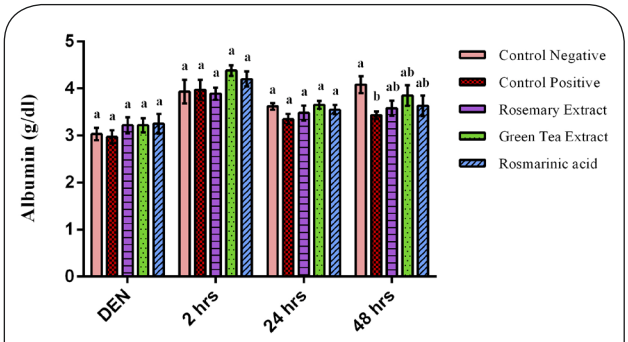

Figure 6. Albumin concentration after 2 weeks of diethylnitrosamine (DEN) injection, after 2 hours, 24 hours & 48 hours of ferric nitrilotriacetate injection. Data are represented as Mean±SE. Bars that share the same letters within one series are not significant. Bars that share different letters within one series are significant.

Effect of RE, GTE & RA on blood glucose concentration at time intervals

Glucose concentration showed an insignificant difference between all groups after DEN injection (p>0.05). However, Fe-NTA showed a hyperglycemic effect after 2 hours of its injection represented by a highly significant increase of serum glucose level of DEN+Fe-NTA treated group when compared to the control negative group with an increase of 47% (p=0.003).

After 24 hours, there was a slight significant elevation in the level of serum glucose of DEN+Fe-NTA group when compared to control negative group with an increase of 20% (p=0.018) (Figure 7). After 2 days of Fe-NTA injection, glucose concentration of the control positive group returned to the normal level showing an insignificant difference when compared to the control negative group and all treated groups (p>0.05). RE showed a hypoglycemic

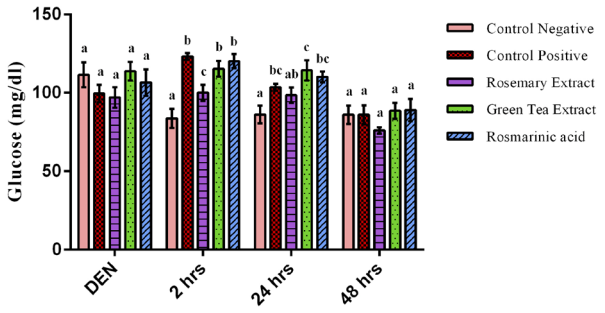

Figure 7. Glucose concentration after 2 weeks of diethylnitrosamine (DEN) injection, after 2 hours, 24 hours & 48 hours of ferric nitrilotriacetate injection. Data are represented as Mean±SE. Bars that share the same letters within one series are not significant. Bars that share different letters within one series are significant.

effect at time intervals reporting a highly significant decline in glucose concentration after 2 hours of Fe-NTA dose when compared to DEN+Fe-NTA treated group with a decrease of 19% (p=0.020). Both the GTE and RA treated groups showed no significant difference in their glucose concentration at time intervals when compared to control positive group (p>0.05).

Effect of RE, GTE & RA on production of serum and renal malondialdehyde in DEN initiated and Fe-NTA treated rats

After 48 hrs of Fe-NTA injection, a very high significant increase was observed in serum and renal malondialdehyde levels (MDA) of the group that was administrated DEN+Fe-NTA (p<0.001, p<0.001) with a percentage increase of 65 % and 72 %, respectively, as compared to the control negative group. However, the administration of protective doses of RE, GTE and RA at the mentioned doses significantly prevented increase of serum and renal MDA levels (p<0.001, p<0.001 & p<0.001) for serum and renal MDA. They reduced the serum MDA level by 28%, 36% & 41% and decreased the renal MDA level by 29%, 39% & 44%, respectively (Figures 8A & 8B).

Effect of RE, GTE & RA on production of serum and renal TNF- α in DEN initiated and Fe-NTA treated rats

The exposure of rats to an acute dose of Fe-NTA increased serum and renal TNF-α levels highly significantly as compared to the control negative one (p<0.001, p<0.001) with percentage increase of 386.7 % and 126.2 %. With respect to groups that were pretreated by protective doses of RE, GTE and RA, it was obvious that these natural extracts alleviated the increasing level of serum and renal TNF-α highly significantly as compared to control positive group

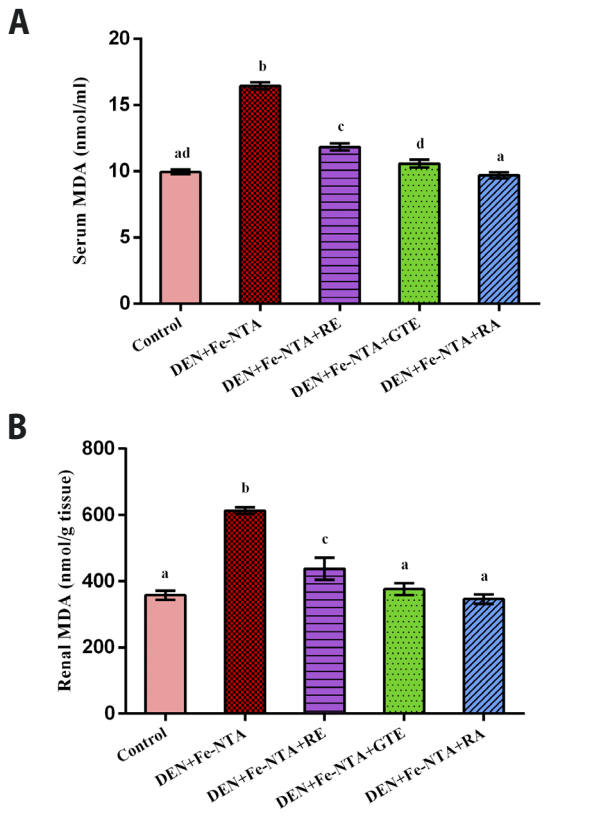

Figure 8. Malondialdehyde (MDA) concentration after 48 hours of ferric nitrilotriacetate injection (A) in serum samples, (B) in renal tissue homogenates. Data are represented as Mean±SE.

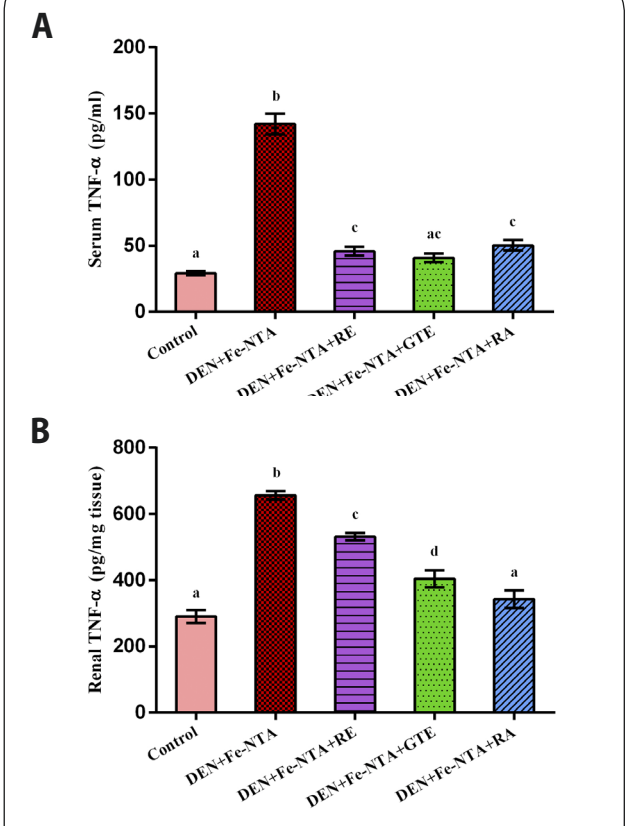

Figure 9. Tumor necrosis factor alpha (TNF-α) concentration after 48 hours of ferric nitrilotriacetate injection (A) in serum samples, (B) in renal tissue homogenates. Data are represented as Mean±SE.

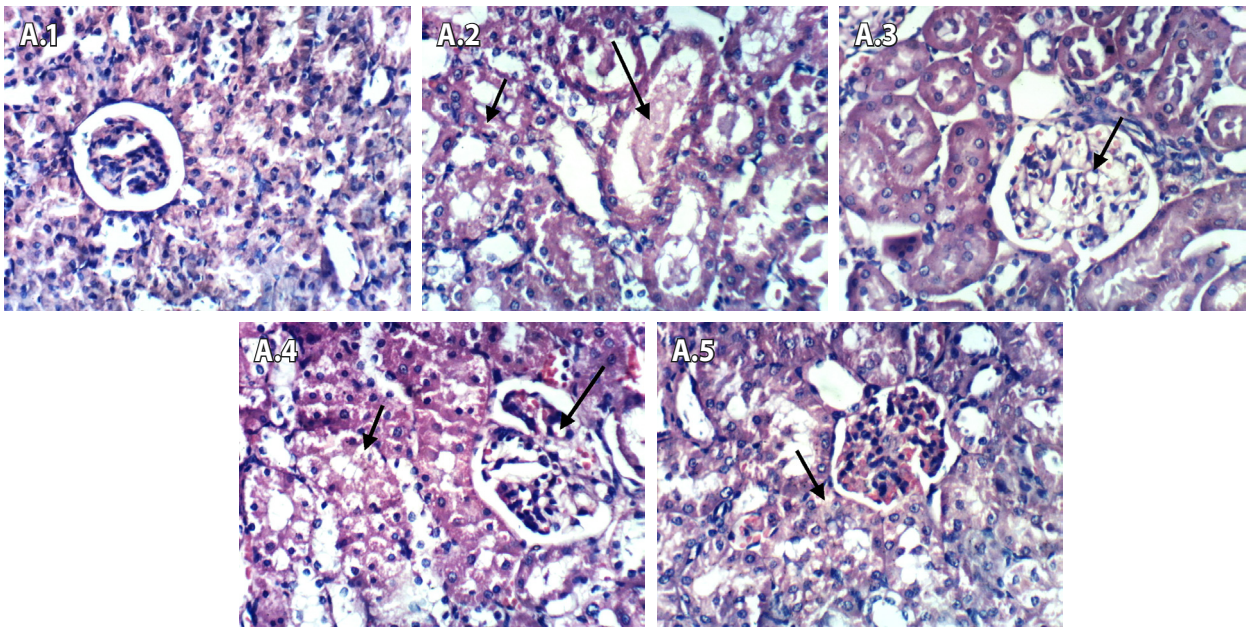

Figure 10. Light photomicrographs of kidney sections in rats after 2 hours of Fe-NTA injection (Series A; 1–5). (A.1) Control negative showed no pathological changes in renal parenchyma, (A.2) DEN+Fe-NTA group showed vacuolation of epithelial lining renal tubules, (A.3) DEN+Fe-NTA+RE group showed vacuolation of endothelial lining glomerular tuft, (A.4) DEN+Fe-NTA+GTE group showed vacuolation of epithelial lining renal tubules and endothelial lining glomerular tuft and (A.5) DEN+Fe-NTA+RA group showed vacuolation of epithelial lining of some renal tubules.

(p<0.001) with a percentage decrease of 68%, 71% and 65%, respectively for serum TNF-α level and 19%, 38% and 48%, respectively for renal TNF-α level (Figures 9A & 9B).

Effect of RE, GTE & RA on histopathological examination of kidney in acute nephrotoxicity rat model

After 2 hours of Fe-NTA injection (Figure 10 (A.1–A.5)), the control negative group showed no histopathological changes in renal parenchyma (A.1). Renal tissues of control positive group (DEN+Fe-NTA) showed vacuolation of the epithelial lining renal tubules and eosinophilic proteinaceous material in the lumen of renal tubules (A.2). Renal tissues of rosemary treated group (DEN+Fe-NTA+RE) showed vacuolation of endothelial lining glomerular tuft (A.3). In case of green tea treated group (DEN+Fe-NTA+GTE), renal tissues showed vacuolation of epithelial lining of renal tubules and endothelial lining glomerular tuft (A.4). Renal tissues of rosmarinic acid treated group (DEN+Fe-NTA+RA) showed vacuolation of epithelial lining of some renal tubules (A.5).

After 24 hours of Fe-NTA injection (Figures 11 (B.1–B.5)), the control negative group showed no histopathological changes in renal parenchyma (B.1). Kidneys of control positive group (DEN+Fe-NTA) showed coagulative necrosis of epithelial lining of renal tubules, vacuolation of renal tubular epithelium, hypertrophy and hypercellularity of glomerular tuft (B.2). Kidneys of the group that received rosemary extract (DEN+Fe-NTA+RE) showed focal coagulative necrosis of epithelial lining of renal tubule (B.3). As shown in B.4, green tea treated group (DEN+Fe-NTA+GTE) showed focal coagulative necrosis of epithelial lining renal tubules. Kidneys of rosmarinic acid group (DEN+Fe-NTA+RA) showed vacuolation of epithelial lining of some renal tubules and slight congestion of glomerular tuft (B.5).

Figures 12 (C.1–C.5) shows the histopathological changes after 48 hours of Fe-NTA injection. Figure 12 (C.1) of the control negative group shows no histopathological changes in renal parenchyma. As shown in C.2, kidneys of control positive group (DEN+Fe-NTA) showed vacuolation of epithelial lining of renal tubules, karyomegaly of the nuclei of renal epithelium and periglomerular inflammatory cell infiltration. Kidneys of rosemary treated group (DEN+Fe-NTA+RE) showed focal coagulative necrosis of epithelial lining of renal tubules, hypertrophy and congestion of glomerular tufts (C.3). Kidneys of the group treated with green tea (DEN+Fe-NTA+GTE) showed necrobiosis of epithelial lining of some renal tubules (C.4). Kidneys of the group treated with a pure extract of rosmarinic acid (DEN+Fe-NTA+RA) showed focal necrosis and apoptosis of epithelial lining of some renal tubules (C.5).

Discussion

Nephrotoxicity is one of the most common issues that occurs upon exposure to toxins or medications (Sundaram *et al.* 2015). Oxidative stress plays a fundamental role in the pathogenesis of nephrotoxicity and tumorigenesis caused by DEN and Fe-NTA (Rashid *et al.*, 2013). Fe-NTA is absorbed into portal vein through mesothelium upon intraperitoneal administration, and then passes into blood circulation through the liver. Because Fe-NTA has a low molecular weight, it can be

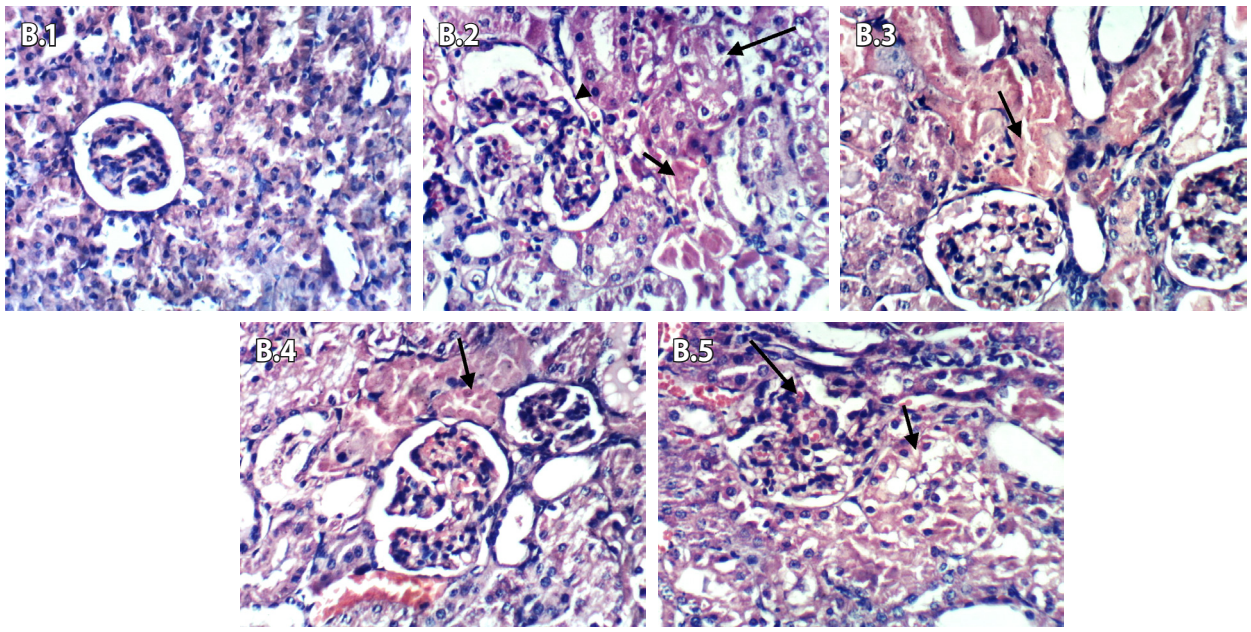

Figure 11. Light photomicrographs of kidney sections in rats after 24 hours of Fe-NTA injection (Series B; 1–5). (B.1) Control negative showed no pathological changes in renal parenchyma, (B.2) DEN+Fe-NTA group showed necrosis of epithelial lining renal tubules, (B.3) DEN+Fe-NTA+RE group showed focal coagulative necrosis of epithelial lining renal tubule, (B.4) DEN+Fe-NTA+GTE group showed focal coagulative necrosis of epithelial lining renal tubules, and (B.5) DEN+Fe-NTA+RA group showed vacuolation of epithelial lining öf some renal tubules.

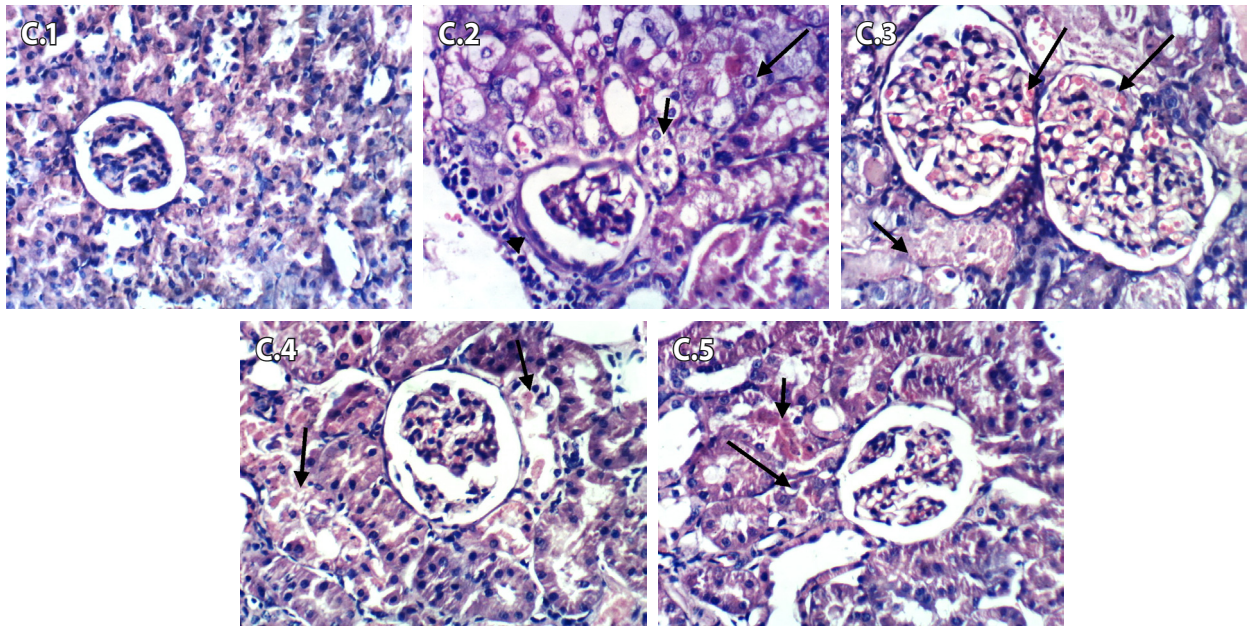

Figure 12. Light photomicrographs of kidney sections in rats after 48 hours of Fe-NTA injection (Series C; 1–5). (C.1) Control negative showed no pathological changes in renal parenchyma, (C.2) DEN+Fe-NTA group showed karyomegally of the nuclei of renal epithelium and periglomerular inflammatory cells infiltration, (C.3) DEN+Fe-NTA+RE group showed hypertrophy and congestion of glomerular tufts, (C.4) DEN+Fe-NTA+GTE group showed necrobiosis of epithelial lining of some renal tubules and (C.5) DEN+Fe-NTA+RA group showed focal necrosis and apoptosis.

easily filtered through the glomeruli into renal proximal tubules where Fe^{3+}-NTA is reduced to Fe^{2+}-NTA by two main reducing compounds, cysteine or cysteine glycine. This results in the generation of superoxide anion radicals ($O_2^{\bullet -}$) which induce the iron to catalyze Haber-Weiss reaction forming hydroxyl radical ($OH^{\bullet}$) that leads to the progression of lipid peroxidation, thereby to renal tissue damage (Rehman & Sultana 2011). Many herbal plants have been highlighted recently for their potent antioxidant activities among which are green tea and rosemary. They possess also anti-inflammatory activities (Begum *et al.*, 2013; Anand *et al.*, 2015).

In the current study, HPLC for water extract of rosemary and green tea revealed the presence of many antioxidant phenolic compounds such as catechins, catechol, vanillic acid, caffeine, ellagic acid, coumarin, pyrogallol, 3-hydroxytyrosol, protocatechuic acid, 3,4,5-methoxy cinnamic acid and rosmarinic acid detected in rosemary. These bioactive constituents have a strong antioxidant impact in inhibiting the production of free radicals, thereby preventing cells from the expected damage. These results are in agreement with other authors (Begum *et al.*, 2013; Anand *et al.*, 2015).

The polyphenols, including catechins and epicatechins, from green tea, carnosic acid, carnosol, caffeic acid and rosmarinic acid from rosemary and many polyphenolic compounds are oxidized into electrophilic hydroquinones or quinines during their reaction with free radicals. These electrophilic compounds have the ability to activate Nrf2 transcription factor through a modification of a specific cysteine in Keap1; the inhibitor of Nrf2. Such modification converts Keap1 to its inactive form thereby activating Nrf2 transcriptional pathway. After that, Nrf2 binds to antioxidant response element (ARE) resulting in upregulation of the transcription of genes for phase II detoxification enzymes (cytoprotective genes) such as glutathione reductase, thioredoxin reductase, repair enzymes and proteasome (Forman *et al.*, 2014).

In the present study, the rats exposed to acute doses of DEN and Fe-NTA had an obvious decline in their body weight, suggesting certain metabolic disorders (Olvera *et al.*, 2012). In addition, the observed increase of the relative kidney weight of DEN+ Fe-NTA treated rats may be due to the inflammatory action of Fe-NTA which induces the kidney to swell. The histopathological examination was in agreement with these findings, since the renal tissues of DEN+ Fe-NTA treated group showed a vacuolation of epithelial lining renal tubules which developed into necrosis of epithelial lining renal tubules, karyomegaly of the nuclei of renal epithelium and periglomerular inflammatory cells infiltration.

However, the daily administration of RE prevented any adverse effect on body weight and that may be due to its role in controlling wasting of muscles by increasing glucose metabolism (El-Boshy *et al.*, 2015). The supplementation of GTE could also restore the body weight and that may be attributed to the positive anabolic effect of GTE on decreasing the degeneration of adipocytes and muscle tissues through glucose metabolism improvement (Ramadan *et al.*, 2009). On the other hand, pretreatment of GTE and RA exhibited a protective effect on renal tissues, since the deterioration of renal histology was less than that observed in DEN+ Fe-NTA treated group and that was in agreement with the obtained biochemical analysis. These findings were in accordance with Eybl *et al.* (2008).

Urea, creatinine and uric acid are important clinical parameters for renal injury and their significant elevation upon DEN and Fe-NTA treatment indicates an acute reduction in the glomerular filtration rate which resulted in morphological alterations of renal tissues such as tubular brush border loss that makes them impermeable to creatinine and urea thereby causing their elevation in the blood (Abd El Kader *et al.*, 2012; Gupta *et al.*, 2009). These results seem to be in accordance with (Rashid *et al.* 2013; Sharmila *et al.*, 2016). Green tea polyphenols such as EC, ECG, EGC and EGCG are able to chelate metal ions specially iron and copper, which subsequently reduce iron deposition, inhibit hydroxyl radical generation, suppress lipid hydroperoxide degradation and cause reactive aldehyde formation, ameliorating renal functions (Weinreb *et al.*, 2009). These results were documented previously by (Rehman *et al.*, 2013). The oral administration of RA causes improvement in glomerular function and renal injuries and that may be due to the action of RA on the intracellular mechanisms responsible for DNA repair, instead of exerting a direct ROS-scavenging effect. On the other hand, RA enhanced an endogenous cellular antioxidant defense mechanism by activating Nrf2/HO-1 pathway resulting in upregulation of expression of many protective genes. These findings were reported previously by (Tavafi *et al.*, 2011; Forman *et al.*, 2014). Rosmarinic acid, carotenoids, diterpenoids and alpha-tocopherol have been documented as principal antioxidant constituents of rosemary aqueous extract which enables it to inhibit lipid peroxidation and free radical generation and improves the renal functions (Abd El-Ghany *et al.*, 2012).

The elevation of uric acid level in DEN+Fe-NTA treated group is an indicator of its increase in joints and various tissues. Such elevation gradually leads to permanent damage of tissue and acute inflammation (Mohamed & Al-Okbi, 2008; Sundaram *et al.*, 2015). Caffeic acid, one of phenolic phytochemical constituents of RE that have anti-gout activity, has been reported to be an inhibitor of xanthine oxidase which catalyzes the oxidation of hypoxanthine to xanthine and finally to uric acid. In addition, RA, which is the ester of caffeic acid, has the ability to inhibit xanthine oxidase and the reabsorption of renal urate, thereby reducing uric acid level (Mohamed & Al-Okbi, 2008).

The cause of the decrease of albumin concentration in the DEN+Fe-NTA treated group may be due to reabsorption impairment of albumin in the proximal tubules resulting in renal injuries which cause the remarkable release of serum protein into urine or also to the incident liver injuries and hepatocellular damage that lead to a decrease of albumin biosynthesis in the liver (Khouja & Al Nahari, 2015). The antioxidant constituents of RE such as carnosic acid, carnosol, rosmarinic acid, ursolic acid and caffeic acid play an important role in preventing lipid peroxidation, necrosis and the damage of hepatocytes (Al-Attar & Shawush, 2014). GT increases the intravascular movement of albumin, suppresses protein nitration, decreases the hepatocellular damage and improves hepatocyte functions, thereby improving the albumin concentration (Butterfield *et al.*, 2014).

The hyperglycemic effect of Fe-NTA may be explained by its role in the induction of ROS and the overloading of iron (II) which could impair pancreatic insulin secretion, participate in the impairment of insulin signaling

and interfere with insulin action and glucose uptake in adipocytes leading to an increase of glucose concentration (Al-Yousuf, 2015). The hypoglycemic effect of RE may be attributed to its role in increasing the production of insulin by regenerating the pancreatic β-cells or inhibiting the absorption of glucose in the intestine by suppressing α-glucosidase enzyme or intestinal amylase enzyme (Al-Attar & Shawush, 2014; Alnahdi, 2012).

The toxic effect of DEN and Fe-NTA compounds consists in elevation of the redox active iron levels inducing ROS formation able to attack the biomolecules of the cells causing membrane deterioration, DNA damage and mutation reported by the formation of 8-hydroxy guanosine in DNA molecules (Kaur *et al.*, 2010). These findings correlate with (Gupta *et al.* 2009). However, the administration of protective doses of RE diminished the level of serum and renal MDA and this may be attributed to its antioxidant property that maintains the lipid structure of cell membrane, neutralizes the reactive free radicals protecting various biomolecules, such as proteins and DNA in such biological systems (Abd El Kader *et al.*, 2012). Most of the antioxidant property of RE is contributed to carnosic acid and carnosol compounds which play an important role in attenuating the MDA levels in serum and renal tissues (Al-Attar & Shawush, 2014).

Green tea and rosmarinic acid extracts showed the best antioxidant activity against iron-induced oxidative stress, restoring the normal value of serum and renal MDA to that of the control negative group, considered as a good improvement. The polyphenolic constituents of GTE and polyphenolic structure of RA play a main role in redox homeostasis control in the cell by upregulating the redox-sensitive system Nrf2/Keap-1/ARE pathway leading to activation of many cytoprotective genes, activating HSF-1/Hsp90 pathways that might be involved in upregulation of protective stress response genes and inducing many other protective and detoxification mechanisms. In addition, the main GTE constituents, EGCG and ECG, have powerful antioxidant activity against DNA damage, LPO of phospholipid bilayers of cells and tumorigenesis (Weinreb *et al.*, 2009). The formed intramolecular hydrogen bond after the abstraction of hydrogen in the chemical structure of RA increases its antioxidant activity (Bhatt *et al.*, 2013). These investigations are in accordance with findings of other authors (Tavafi & Ahmadvand, 2011; Satoh *et al.*, 2013).

Proinflammatory cytokine marker, tumor necrosis factor alpha (TNF-α), plays a vital role in several diseases such as cancer owing to its mutagenicity and proliferative ability. Also, it has the ability to stimulate the production of other inflammatory mediators such as ROS through oxidative stress-responsive genes which promotes and develops inflammation (Rashid *et al.*, 2013). Previous reports stated that Fe-NTA induced the production of TNF-α which regulates the transcription of NF-κB directly (Rehman *et al.*, 2013). It has been demonstrated that ROS act as second messengers; they are able to activate NF-κB through TNF-α and other proinflammatory cytokines (Mannaa *et al.*, 2013). These cytokines have an essential role in vascular permeability, inflammation and cell proliferation (Rehman *et al.*, 2013). These findings are in agreement with (Rehman *et al.*, 2013) who reported a marked elevation in serum and renal TNF- α in DEN+Fe-NTA treated rats. RE, GTE and RA treated groups were observed to have an approximate effect on reducing serum TNF-α level near to normal range. The protective anti-inflammatory effect of rosemary, green tea and rosmarinic acid may be attributed to the redox property of polyphenolic constituents of their extracts and polyphenolic structure of RA which can modulate redox cellular homeostasis by activating the redox system Nrf2/Keap-1/ARE leading to overexpression of phase II detoxification enzymes such as glutathione reductase, glutathione peroxidases, thioredoxin reductase and thioredoxin peroxidase (Forman *et al.*, 2014). The redox property of polyphenols can also modulate redox homeostasis in the cell by inhibiting the activity of COX-2, interfering with Nf-kB binding to DNA and suppressing the phosphorylation of kinases (Calabrese *et al.*, 2010).

The anti-inflammatory effect of RE may be also attributed to its free radical quenching property due to its rich contents of polyphenolic constituents which inhibit the release of TNF-α (Bogdanski *et al.*, 2012). In addition, the anti-inflammatory effect of GTE may be attributed to the ability of its polyphenolic compounds to scavenge free radicals directly through hydrogen and electron donation, suppressing the propagation of LPO. Rosmarinic acid could restore the level of serum and renal TNF-α to the range of control negative group and that may be due to the anti-inflammatory impact of RA that may have the potentiality to reduce inflammation of injured tissue in many organs like liver, kidney and lung by down-regulating of TNF-α and inhibiting the NF-kB pathway. Our findings are compatible with those of (Rocha *et al.*, 2014).

In conclusion, the present investigations proved that green tea, rosmarinic acid and rosemary extracts are promising as nephroprotective agents against DEN and Fe-NTA induced nephrotoxicity in Wistar rats. Green tea can restore urea, creatinine and uric acid near to normal range, while rosmarinic acid showed the best antioxidant and anti-inflammatory effect in this rat toxicity model and so did rosemary but with lower extent. It can thus be stated that rosemary, green tea and rosmarinic acid rich substances are highly recommended specially for those who are more exposed to drug intoxication, environmental toxins and pollutants, such as workers in laboratories, chemical industries and factories, to obtain protection from renal toxicity. Since each item used has its own mechanisms of action resulting in improving some of the tested parameters, a combination of these plant extracts in one supplement may provie a more potent effect for detoxification of the kidney.

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