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EXPERIMENTAL INVESTIGATION ON THE RELATIONSHIP BETWEEN LEAD EXPOSURE AND THYROIDS IN DIABETES

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This dissertation is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Abdu Adem

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Declaration of Original Work

I, Salah Sumar Ali Al-Zadjali, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Experimental Investigation on the Relationship between Lead Exposure and Thyroids in Diabetes*", hereby, solemnly declare that this dissertation is an original research work that has been done and prepared by me under the supervision of Professor Abdu Adem, in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my dissertation have been properly cited and acknowledged.

Student's Signature: _____ Date: 26/ 05/ 2015

Approval of the Doctorate Dissertation

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Abstract

Lead exposure can cause multiple systemic toxicities, particularly affecting the hematopoietic, nervous and renal systems. However, its effects on the thyroid functions are not well elucidated and the published studies are controversial. In addition, although there are several experimental thyroid models, each one of them has its own limitations. Accordingly, in this dissertation, we investigated the possible relationship between lead exposure, thyroid functions and short-term systemic toxicity in two animal models, namely normal (non-diabetic) and diabetic animals. We also investigated the possibility of developing a hormonal thyroid model.

In the non-diabetic model, Wistar rats were divided into five groups and treated for five days. The four treatment groups received 1, 25, 50, or 100 mg/ kg of lead acetate trihydrate intraperitoneally (i.p.), respectively. The control group received i.p. injections of distilled water. In the diabetic model, diabetes was induced with an i.p. injection of 60 mg/ kg streptozocin (STZ). Six weeks later, lead exposure experiments started. Here, four groups were studied: a control; and 25, 50 and 100 mg/ kg lead acetate groups. In each model, the measured blood lead levels correlated very well with the administered doses of lead acetate. Treatment of the animals with lead acetate resulted in significant weight loss in both models. Lead exposure caused a dose-related increase in thyroid stimulating hormone (TSH) in non-diabetic and diabetic animals. Although, thyroxine (T4) and triiodothyronine (T3) levels remained within normal range in non-diabetic animals, their levels were reduced in diabetic animals. The highest dose of lead (100 mg/ kg) significantly increased white blood cell counts and caused a significant decrease in the number of platelets in non-diabetic animals. In addition, C-reactive protein levels

increased significantly in response to lead exposure in this model. Moreover, there was a significant increase in lactate dehydrogenase (LDH), aspartate aminotransferase, total bilirubin, and urea levels; following lead exposure in non-diabetic animals. In comparison, lead exposure in diabetic animals increased urea levels and caused a significant decrease in creatinine levels in plasma. While the concentrations of malondialdehyde were not affected, glutathione stores were depleted in response to lead exposure in the diabetic animals.

In the last stage, we tried to develop a new experimental thyroid model, based on the use of hormones. In this experiment, animals were treated for five days with either thyrotropin-releasing hormone (TRH) or octreotide (OCT) to induce hyperthyroidism or hypothyroidism, respectively. Although there were no effects on T4 and T3 levels, TRH was effective in causing an increase in TSH levels. However, TRH also elevated LDH levels. The use of TRH did not cause any other side effects on the tested parameters, which included weight change, oxidative stress markers and renal and hepatic functions. In comparison, OCT failed to affect TSH, T4 and T3 levels, at the dose and treatment duration that we used.

In conclusion, short-term lead exposure in healthy and diabetic animal models affected the functions of the anterior pituitary and thyroid glands, caused oxidative stress, liver and kidneys toxicity and induced systemic inflammation. In addition, we found that TRH has a potential to induce hyperthyroidism in experimental animals.

Keywords: lead, rat, diabetes, thyroid, experimental model and systemic toxicity.

Title and Abstract (in Arabic)

بحث مختبري تجريبي عن العلاقة بين التعرض للرصاص والغدد الدرقية في حالة مرض السكري

الملخص

إنه من الثابت علميا أن التعرض للرصاص يسبب أعراضا سمية في مختلف أنظمة الجسم، ويشمل هذا تأثيرات الرصاص على الجهاز الدموي والعصبي والكلى، وبالرغم من ذلك فإن آثاره على وظائف الغدة الدرقية ليست واضحة بشكل جلي، والدراسات المنشورة حاليا مثيرة للجدل، وبالإضافة إلى ذلك وعلى الرغم من وجود العديد من النماذج التجريبية للغدة الدرقية في حيوانات المختبرات، إلا أن كل واحد من هذه النماذج له مساوئه الخاصة به، ولذلك فإننا في هذا البحث ندرس العلاقة المحتملة بين التعرض للرصاص ووظائف الغدة الدرقية و باقي اجهزة الجسم على المدى القصير في الحيوانات السليمة والحيوانات المصابة بداء السكري، كما أننا قمنا بمحاولة تطوير نموذج جديد لمشاكل الغدة الدرقية في الحيوانات يعتمد على استخدام الهرمونات.

لقد تم تقسيم فئران التجارب من فصيلة "ويستار" في نوذج الحيوانات السليمة إلى خمس مجموعات واستغرقت التجارب 5 أيام، حيث تلقت مجموعات العلاج الأربعة 1، 25، 50 أو 100 ملغ/ كغم من الرصاص، على التوالي، على شكل حقن في البطن، بينما تم حقن مجموعة التحكم بالماء المقطر، أما بالنسبة لنموذج مرض السكري فقد تم حقن الجرذان بجرعة مقدارها 60 ملغ /كغ من مركب streptozocin (STZ) في البطن، وبعد ستة أسابيع بدأت تجارب التعرض للرصاص، وفي هذا النموذج تمت دراسة أربع مجموعات وهي مجموعة التحكم و 25 و 50 و 100 ملغم / كغم من الرصاص، ولقد اوضحت نتائج البحث في كلا النموذجين أن مستويات الرصاص في الدم تتناسب بشكل مباشر مع جرعات الرصاص التي اعطيت للجرذان، كما أن التعرض للرصاص أحدث نقصا ملحوظا في وزن الحيوانات وزيادة طردية في مستوى هرمون (TSH) في الحيوانات السليمة والمصابة بالسكري، وعلى الرغم من أن مستويات هرمون الغدة الدرقية (T4) و ثلاثي ايودو ثيرونين (T3) ظلت ضمن المعدل الطبيعي في الحيوانات السليمة، إلا أن مستوى هذه الهرمونات قد نقص في الجرذان المصابة بالسكري، ولقد سبب استخدام أعلى

جرعة من الرصاص (100 ملغ / كلغ) في نوذج الحيوانات السليمة زيادة كبيرة في عدد خلايا الدم البيضاء، كما أنها سببت انخفاضا كبيرا في عدد الصفائح الدموية، وبالإضافة إلى ذلك فلقد سبب التعرض للرصاص زيادة في مستويات بروتين (C-reactive protein) في هذه الحيوانات، وعلاوة على ذلك كان هناك زيادة كبيرة في مستوى انزيمات (lactate dehydrogenase) و (aspartate aminotransferase) وصفار الدم واليوريا بعد التعرض للرصاص، ومن ناحية أخرى سبب التعرض للرصاص في الجرذان المصابة بالسكري زيادة في مستوى اليوريا ونقصا في مستوى مادة الكرياتينين في الدم، وعلى الرغم من أن مستوى مادة (malondialdehyde) لم تتأثر بالتعرض للرصاص إلا أن مخزون مادة الجلوتاثيون (glutathione) قد انخفض بشكل ملحوظ .

وفي المرحلة الأخيرة من البحث حاولنا تطوير نموذج تجريبي جديد للغدة الدرقية في الحيوانات، ويستند هذا النموذج على استخدام الهرمونات لمدة خمسة أيام، وفي هذا الصدد تم إعطاء المجموعة الأولى من الحيوانات هرمون (TRH) لتحفيز نشاط الغدة الدرقية، بينما تم إعطاء المجموعة الثانية هرمون اوكتريوتيد (OCT) لتحبيط نشاط الغدة الدرقية، وعلى الرغم من عدم تأثيره على مستوى هرمونات (T4) و(T3)، كان هرمون (TRH) فعالا في إحداث زيادة في مستويات هرمون (TSH)، إلا أن استعمال هرمون (TRH) نتج عنه أيضا ارتفاع في مستويات انزيم (LDH)، ولم يسبب استخدام هرمون (TRH) أي آثار جانبية أخرى على وزن الحيوانات وعلامات الأكسدة ووظائف الكلى والكبد، وفي المقابل لم يستطع هرمون (OCT) حسب الجرعة المعطاة لمدة خسمة أيام في احداث أي تأثير على مستويات (TSH) و (T4) و(T3).

ونستنتج من هذا بأن التعرض للرصاص على المدى القصير في الحيوانات السليمة والمصابة بداء السكري يؤثر على وظائف الغدة النخامية الأمامية والغدة الدرقية وعلامات الأكسدة، ويسبب تسمما في الكبد والكلى، كما أنه قد يسبب التهابات عامة في الجسم، وبالإضافة إلى ذلك، فإن لهرمون (TRH) المقدرة على احداث زيادة في نشاط الغدة الدرقية في حيوانات التجارب.

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Dedication

To my beloved family and friends, and my home country Oman

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List of Abbreviations

ALA: 5-Aminolaevulinic Acid

ALB: Albumin

ALP: Alkaline Phosphatase

ALT: Alanine Aminotransferase

AST: Aspartate Aminotransferase

ATSDR: Agency for Toxic Substances and Disease Registry

BB: Bio Breeding

BBB: Blood Brain Barrier

BLL: Blood Lead Levels

CAP: Criteria Air Pollutants

CAT: Catalase Enzyme

CBC: Complete Blood Count

CDC: U.S. Centers for Disease Control and Prevention

CPK: Creatine Phosphokinase

CRP: C-reactive Protein

CSF: Cerebrospinal Fluid

CVS: Cardiovascular System

DE&OH: Department of Environmental and Occupational Health, in Oman

DM: Diabetes Mellitus

DW: Distilled Water

EPA: U.S. Environmental Protection Agency

EU: European Union

G6PD: Glucose 6 Phosphate Dehydrogenase

GFR: Glomerular Filtration Rate

GGT: Gamma-glutamyl Transpeptidase

GIT: Gastrointestinal Tract

HEPA: High Efficiency Particulate Air

IARC: International Agency for Research on Cancer

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

ILO: International Labor Organization

i.p.: Intraperitoneal Injection

IQ: Intelligence Quotient

i.v.: Intravenous Injection

LDH: Lactate Dehydrogenase

MOH: Ministry of Health

NMDAR: N-Methyl D-Aspartate Receptor

NOD: Non-obese Diabetic

OCT: Octreotide

OSHA: U.S. Occupational Safety and Health Administration

PIH: Pregnancy Induced Hypertension

RBC: Red Blood Cells

RIE: Al-Rusail Industrial Estate

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

SD: Sprague-Dawley Rats

SOD: Superoxide Dismutase

SQU: Sultan Qaboos University

STZ: Streptozotocin

s.c.: Subcutaneous Injection

t½: Half-life

T3: Triiodothyronine

T4: Thyroxine

TSH: Thyroid Stimulating Hormone

WBC: White Blood Cell

WHO: World Health Organization

XRF: X-ray Fluorescence

Chapter 1: Introduction

1.1. General Overview of Lead Exposure and Toxicity

As an element that has been used widely throughout history and until today, lead has proven itself as a useful heavy metal and as a toxin [1]. Consequently, it became one of the most commonly studied heavy metals [2, 3]. The latter are broadly defined as those that have a potential to cause toxicity and adverse effects either to humans or the environment [4]. Unlike some other metals such as zinc and manganese which are required as essential nutrients, lead has no known beneficial human physiologic function [5]. In addition, it can neither biodegrade i.e. it cannot break down, nor can it be detoxified by living organisms [6, 7]. Two types of lead are known, namely, organic and inorganic. Organic lead is much more toxic, particularly to the nervous system, than the inorganic type [8]. It's worth mentioning that the majority of poisoning cases with lead are caused by inorganic lead [9]. Examples of inorganic lead are lead carbonate, lead chromate, lead monoxide, lead tetraoxide and lead acetate [8]. According to the U.S. Centers for Disease Control and Prevention (CDC), lead is the main environmental toxin in children [1] and as such it results in one of 'the most prevalent diseases of environmental origin among children' [10]. Lead ranks second on the priority list of hazardous substances published by the U.S. Agency for Toxic Substances and Disease Registry (ATSDR) [11]. This list includes several chemicals e.g. lead, arsenic, benzene and cadmium. These chemicals are labeled as hazardous substances and were added to the list because of their "frequency, toxicity and potential for human exposure at National Priorities List (NPL)" [11].

1.1.1. Exposure among the General Population

Lead is ubiquitous in the environment [12]. The main route of exposure in the general population is ingestion [8, 13]. According to World Health Organization (WHO), over 80 % of the oral intake of lead is through food, dirt, and dust [8]. Other sources of lead exposure are lead-based paints especially in children; consumption of contaminated food, fish kidney and liver, drinking polluted water running in old lead pipes, or soldered joints [8, 14]. On a regional level, data from Saudi Arabia showed that 'vegetables' and 'cereals and cereal products' are the major dietary sources for lead exposure; as each one of these two groups represent about 25 % of the total dietary consumption [15]. In comparison, in a recent study in 15 European countries, a total of 140,000 results of measurable lead levels were found in food and tap water samples [16]. In Mexican immigrants in the U.S., tamarind and chili powder are known sources of lead exposure [17]. Besides food and water, another important source of lead exposure is the use of traditional remedies e.g. 'Bint Al-dhahab khol or Indian surma', which is particularly common in Middle Eastern countries e.g. Oman and UAE [17-23]. In a study conducted in Belgium, traditional teapots were found to be contaminated with different metals including lead, which can leach and thus might result in lead poisoning [24]. A recent study carried out in Saudi Arabia demonstrated that lead-paints are still being manufactured locally with lead levels reaching as high as 755 ppm, which exceeds the U.S. Environmental Protection Agency's (EPA) permissible value of 600 ppm [25].

In the U.S., lead is considered one of the main constituents of Criteria Air Pollutants (CAP). These represent contaminants in the ambient air that have a potential to cause

adverse health effects to the general public. Thus, the levels of CAPs are regulated, monitored and should not exceed the limits set by federal agencies such as EPA and local states [26, 27].

1.1.2. Occupational Lead Exposures

In the past, the use of leaded gasoline in automobiles, which was composed of organic lead compounds such as tetraethyl and tetramethyl lead [28], was a major cause for environmental lead exposures. These compounds were used in order to improve the performance of automobile engines [8]. This practice has been phased out in many countries since the 1970s [28], although around 9 countries are still using leaded gasoline [8]. In Korea, the initiatives to phase out leaded gasoline started in 1986 and by the year 2000 there was a tremendous reduction in lead levels [14]. Oman started using unleaded gasoline in October 2001 [29]. In comparison, the UAE started to use unleaded gasoline in 2003 [30]. This involved major changes such as converting 500 filling stations to unleaded gasoline, training of staff, and conducting educational and awareness programs throughout the country [30].

Examples of occupational sources of lead exposure are car batteries' manufacturing, radiator repair shops, welding and cutting operations, solid waste burning, and construction [28, 31]. There has been a continuous increase in lead's utilization in storage batteries and electronics; particularly in Asia [26]. The vast majority (about 97%) of batteries worldwide are recycled, often in underdeveloped countries [8]. At present, 70% of lead is used in storage batteries [3, 32], where the metal grids are made up of lead and the paste in the batteries is lead oxide [28]. As an example of

occupational exposures in the Arabian Peninsula, 12 % of a total group of 89 servicemen and 69 public transport drivers in Saudi Arabia had BLL of more than 40µg/ dL [33].

Occupational exposures typically take place through inhalation of lead oxide [8, 32]. In addition, they are often chronic in nature and might result in more serious consequences. One of the major problems with occupational exposures in general is the limitation of adequate surveillance systems for injuries and diseases, despite the fact that at present various work-related diseases cause more than 5000 deaths everyday worldwide [34].

1.1.3. Exposure Cut Levels

Health organizations such as CDC have continuously and progressively decreased the minimal action values for BLL. In the past, the BLL action level in children was set at 10 µg/ dL. In 2012, a new 'reference level' of 5 µg/ dL was established [35]. The corresponding level in occupational exposures should not exceed 25 µg/ dL [36]; however, by now, it is clear that no lower threshold for BLL could be established [37]. Perhaps, that is why no safety level for lead exposure has been established so far [14]. A study conducted in 2006 showed that BLL even below 10 µg/ dL in adults are associated with cardiovascular fatality [38].

Since lead-based paints are a major cause of lead exposure, particularly in children, the USA banned the use of lead in paints in 1977. Accordingly, lead levels in paint should not exceed 0.07 mg/ cm^2 [17]. Despite such strict regulations, around 40 million

houses in the USA were estimated to have lead-related problems in the year 2000 [17]. The majority of these houses are probably built before 1950.

1.2. Lead's Toxicokinetics

1.2.1. Absorption

The extent of inorganic lead absorption by the gastrointestinal tract (GIT) is mediated by several factors such as the physiological state of a person and the particular compound being ingested [13]. In comparison to larger pieces e.g. paint chips, lead particles that are smaller in size such as those in dust are more completely absorbed [2, 3, 9]. Other factors that facilitate the GIT absorption of lead are poor caloric intake, the consumption of diets rich in fats [2], and fasting conditions [2, 39]. In addition, there is more absorption of lead when it is dissolved in water than when it is attached to food or solid matter e.g. soil [13, 40]. Consequently, lead oxide, a very soluble compound that is utilized in battery manufacturing plants, can be easily absorbed [41].

There are three transporters involved in the GIT absorption of lead. The first one is the active process mediated by the calcium transporter [2]. Lead also competes for absorption as well as for binding to the 'mobilferrin' protein [12], which usually transports ferric iron (Fe^{3+}) [42]. Competition with the divalent metal transporter (DMT), required for the transportation of ferrous iron (Fe^{2+}) [42], is the third postulated mechanism for its absorption [43]. There is an inverse relationship between vitamin D intake, iron and calcium stores in the body and lead absorption in the GIT [44]. Consequently, dietary deficiencies of these minerals e.g. iron or calcium enhance lead's absorption [9, 43].

In contrast to ingestion, 95% of inhaled inorganic lead is absorbed [13, 45, 46]. Factors favoring the absorption of inhaled lead include smaller particle size, higher concentrations [47], and increased respiratory rate e.g. in children and in physically very active workers [12]. On the other hand, dermal absorption of organic lead, which is often encountered in occupational settings, is also possible. For instance, tetraethyl lead can be easily absorbed through the skin [8, 13, 40, 48]. Furthermore, both types of lead and particularly the organic type can cross the placenta and their ultimate levels in the fetus are positively associated with maternal BLL [48]. Lead is also found in milk and can pass from lactating mothers to their babies [49].

1.2.2. Distribution

In the blood stream, the vast majority of lead (99%) is bound to hemoglobin in the RBCs, so only 1% circulates in plasma. This is the amount available for eventual distribution to various soft tissues [43, 47, 48]. Although transferrin is responsible about the transportation of ferric iron, its C2 variant might also have a role in lead poisoning [14]. Lead has a decreasing affinity for the following organs: liver, kidneys, lungs and brain. Characteristically, the penetration rate of lead into the CNS is higher in children than in adults [48]. In the 3 compartment model, which are blood, soft tissues and bone, lead mainly targets the second compartment i.e. soft tissues [2, 12].

Ultimately, 94% of absorbed lead in an adult is stored in bones and teeth; in comparison to around 73% in children [13, 45]. Although it gets deposited in both, lead has a higher preference for bones than for teeth. In calcium deficiency states, more lead is absorbed and fixed into the bones [48], where it is found as tertiary lead phosphate.

This is why diets rich in phosphate encourage the storage of lead in bones [43]. After deposition in bones, lead is thought to be relatively inert [12, 48].

The half-life (t½) of inorganic lead in blood is relatively very short, only about 30 days, whereas, in bones, its t½ is 27 years [2]. Over time, lead is mobilized and is slowly released back into the circulation. Any stress on bones e.g. fractures, infections, menopause, osteoporosis or even pregnancy and lactation can release lead from bones and may cause toxic effects; even in the absence of recent exposures [2, 3, 40, 47, 48]. Because of this mobility, up to 50 % of lead in the blood might represent endogenous release from bones [12, 47].

1.2.3. Metabolism

Organic lead e.g. tetraethyl lead, which is a lipid soluble compound, was added to petroleum in many countries in the past. After exposure e.g. sniffing by adolescents, this compound is metabolized in the liver and converted into another organic substance called triethyl lead, thereby exerting its toxic effects. In addition, organic lead can also get converted into the inorganic form [12, 48], which in living organisms cannot be metabolized any further [26].

1.2.4. Elimination

Regardless of the route of exposure, eventually lead is mainly excreted in either urine or bile [13]. Renal excretion is by glomerular filtration where lead appears in the urine unchanged [47, 48]. Interestingly, the half-life (t½) of renal excretion of lead is

dependent upon and very comparable to where lead is e.g. renal excretion t½ is 25 days for blood lead, in contrast to being about 25 years for lead in bones [48].

Since only about 1% of blood lead is available for renal filtration (because the rest is bound to RBCs), chronic lead toxicity can develop fast if a subject's total exposure and intake of lead is increased even if only by a small amount [43]. Besides renal excretion, about a third of absorbed lead is excreted in bile [12, 47]. In comparison to adults, children have a lower lead excretion rate [12]. Lead can also be eliminated through milk [12] and sweat; and lead deposits can be found in hair and nails [39, 43].

1.3. Lead's Toxic Effects

Lead causes multiple systemic toxicities; and the organs that are most vulnerable to lead deposition are the nervous system, kidneys and liver [49, 50]. Lead exposure and hence its toxicity can be either acute or chronic in nature. In the initial stages and at minimal levels of exposure, clinical presentation is extremely vague and nonspecific and can easily pass unrecognized [2]. More specific and systemic features become evident at higher levels of exposure and cause renal failure and encephalopathy. In the coming sections, and in addition to systemic toxicity, specific effects related to children, the elderly and people with chronic diseases such as diabetes and hypertension will be highlighted and discussed in more detail.

1.3.1. Hematologic Effects

Effects on the hemopoietic system are a sensitive indicator of the toxic effects of lead [47]. It is well known that lead exposure can result in anemia [51, 52]. In

developing countries, another common problem is iron deficiency [53]. Shah et al. provided a list of several studies that showed an association between lead exposure and iron deficiency [53]. As such, these two conditions i.e. lead exposure and iron deficiency are interrelated and can eventually cause and worsen existing anemias [53].

There are several mechanisms that are involved in lead-induced anemia, the most important of which are the following. The first mechanism is lead's competition for binding to the proteins that are required for the GIT transportation of iron, which is an essential component for heme synthesis. The second mechanism is related to lead's ability to form covalent bonds with sulfhydryl groups; particularly those that are present in metalloenzymes such as delta-aminolevulinic acid dehydratase, coproporphyrinogen oxidase and ferrochelatase [2, 43, 48]. Related to heme, which is a cornerstone in the structure of hemoglobin, myoglobin, cytochromes and catalases [43]; five out of the seven enzymes that are involved in its biosynthesis are inhibited by lead [54] as shown in (Figure 1). The suppression of both δ- aminolevulinic acid dehydratase (ALAD) and ferrochelatase represents the most important inhibitory effects of lead on the hemopoietic system [43, 47, 54, 55]. Ferrochelatase enzyme is also referred to as heme synthetase in some resources [54].

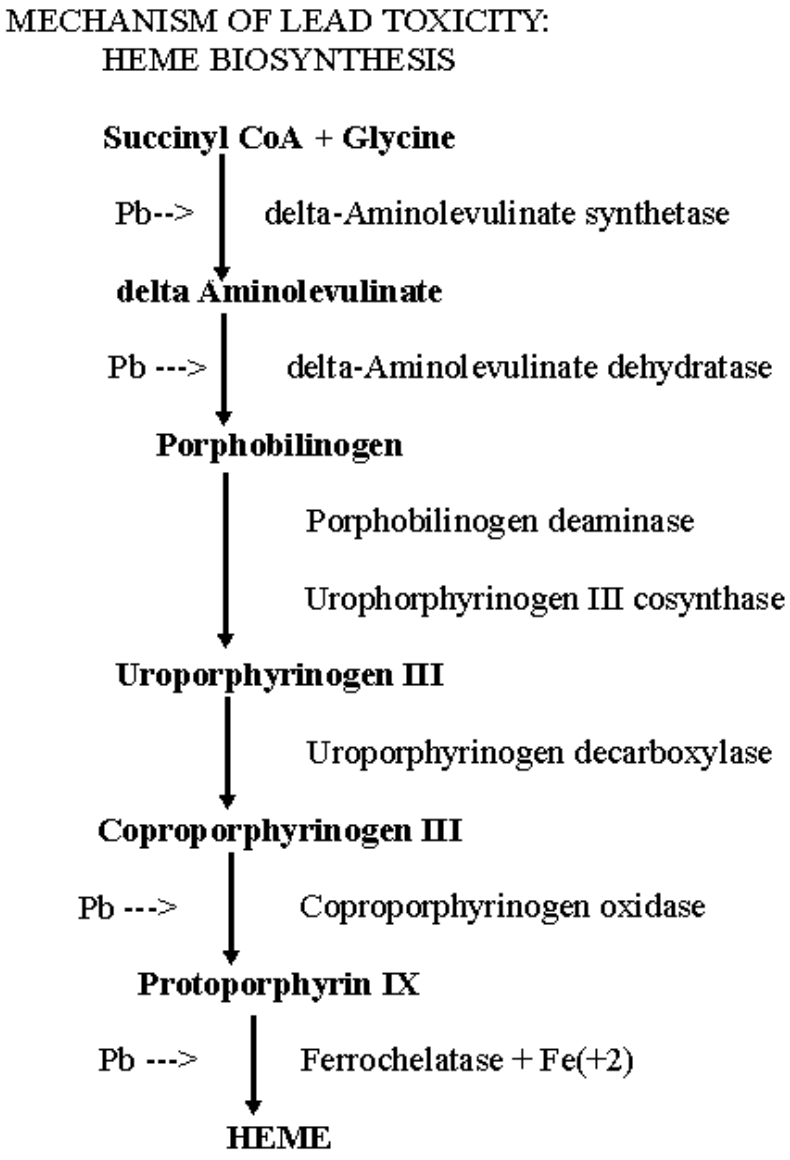

Figure 1: Mechanism of lead toxicity on heme biosynthesis [56].

Because of these inhibitory effects, instead of having heme, circulating red blood cells (RBCs) have protoporphyrin IX [43]. These RBCs get chelated by zinc at the site of iron [43, 47, 55]. This results in the formation of zincprotoporphyrin, which fluoresces, therefore; it is used as a diagnostic test for lead toxicity. Besides zincprotoporphyrin, the inhibition of the previously described enzymes causes an

elevation in the urinary levels of porphyrins, coproporphyrins and δ-aminolevulinic acid (dALA) [43, 47, 55, 57]. In addition, reduced heme concentration exerts a negative feedback effect and stimulates the synthesis of dALA, thereby leading to further increases in dALA levels [55] (Figure 1).

Apart from decreasing iron absorption and inhibiting heme-synthesis enzymes [53], lead-induced anemia might also result from splenic sequestration and the phagocytosis of RBCs, which were shown to be accompanied by an increase in the weight of spleen in lead-exposed rats [51]. In addition, lead exposure tends to shorten the lifespan of RBCs; mainly through increasing their membranes' instability and fragility [55]. The ultimate anemia, caused by all of the previously described mechanisms, is very similar to iron-deficiency anemia i.e. being microcytic and hypochromic [43, 47, 54]. This late manifestation of lead poisoning [41] is particularly important in those subjects who already had iron deficiency anemia before any lead exposures; since after such exposures, the anemia can obviously get worse. Besides causing anemia and a reduction in RBC levels [58], chronic lead exposure also decreases hematocrit counts [51, 59].

In addition, the breakdown of the remnants of ribonucleic acid (RNA) by pyrimidine-5-nucleotidase is inhibited by lead. This results in the basophilic stippling of RBCs due to the accumulation of RNA aggregates [12]. Although a very characteristic feature, basophilic stippling is not pathognomonic of lead toxicity [43].

1.3.2. CNS Effects

In medical literature, a lot of emphasis has been placed on the toxic effects of lead exposure on the nervous system, where interference with the metabolism of divalent

cations e.g. calcium and zinc [55, 60] underlies the basis of lead's toxic effects. Lead has a 'higher affinity than calcium to the calcium-binding protein calmodulin' [61]. Suppression of calmodulin, pyruvate kinase and other enzymes in the CNS affect the functioning and disturbs the homeostasis of several neurotransmitters e.g. glutamatergic, dopaminergic and cholinergic systems [2, 47, 55, 57, 62]. In addition, acute lead exposures have been shown to increase brain catalase (CAT) levels [63-65].

Lead exposure is a well-known cause for cerebral edema. A previous study demonstrated that there was a difference in the various regions of the brain that became edematous and this depended on the particular doses of lead acetate that were administered to rats, where, in comparison to cerebral cortex, the cerebellum was more sensitive to develop lead-induced edema [66]. In this regard, several mechanisms have been postulated to be the cause for blood brain barrier (BBB) disruption. Lead was reported to stimulate the 'vascular endothelial growth factor/vascular permeability factor in cultured astrocytes' and to cause cerebellar vasogenic edema and hemorrhage [67]. 'Occludin' is one of the important proteins that make up the tight junctions of BBB [68]. Lead exposure in experimental studies has been shown to cause a reduction in the level of this protein (down-regulation) [69] as well as in the level of the cytoplasmic protein ZO-1 [68], and consequently resulted in increasing the permeability of the BBB. Lead also disrupts the normal functioning of cerebral capillaries by inducing protein kinase C, which is a calcium-dependent enzyme that phosphorylates many proteins [3, 55]. With increased permeability, albumin, ions, and water enter the cerebrospinal fluid (CSF), causing edema in the surrounding areas [57]. Consequently, the increase in intracranial pressure results in the death of neurons and the proliferation

of glial cells [47, 54, 55, 57]. In contrast to adults, the incompletely developed BBB in children is much more vulnerable to the toxic effects of lead exposure [70]. This could be due to lead's effect on claudin-1, which is one of the important cellular proteins that are needed for the integrity of the tight junctions [71]. Lead was also shown to cause a reduction in mRNA and protein levels of claudin-1, thereby increasing BBB's permeability [71].

Synaptic plasticity refers to the strength of connections between two neurons [72]. By inhibiting the expression of the N-methyl D-aspartate receptor (NMDAR) in the hippocampus, lead impairs long term potentiation and de-potentiation [69] and thus affects synaptic plasticity and results in impairments in learning abilities and memory [70]. On the other hand, there are various psycho-neurological diseases that are either well-confirmed or at least associated with lead exposure. Lead encephalopathy is one of the most serious complications of lead toxicity, of which several pediatric cases have been reported in Oman [19, 73, 74]. In 2011, autism in 25 Saudi children was found to be associated with having high blood lead levels [75]. Recently, it has been shown that chronic lead exposure carries an increased risk of two-fold for the development of Parkinson's disease [76]. Besides, epigenetic studies showed that exposure to lead in early life caused an increase in protein and mRNA levels of tau, thus leading to an increase in tau phosphorylation in older mice [77]. This might eventually result in developing Alzheimer's disease [14].

1.3.3. Renal Effects

In the kidneys, the main site of action of lead is in the proximal tubules [54, 55], where one of the most characteristic features of lead exposure is revealed. This is the appearance of inclusion bodies, which are an inert form of lead and protein complexes [12] in the nuclei of renal cells [43, 47, 54, 55]. Metallothionein has been demonstrated on the outer layer of inclusion bodies, which suggests its protective role and involvement in their formation [47, 78-80]. Metallothioneins have low molecular weight and are dense with cysteine residues and thiol groups [14]. Among their many functions, metallothioneins have a major role in the various steps of absorption, transportation, and elimination of metals leading eventually to the detoxification of several metals including lead and mercury [14].

One of the most important tests for the diagnosis of renal functions and its failure is the glomerular filtration rate (GFR), which is an early indicator of renal injury [81]. Exposure to lead in early life can impair the growth of glomeruli, leading at the end to renal malformation and hypertension [81] i.e. the exposure to low levels of lead is considered a risk factor for developing chronic renal disease [82]. In such cases, GFR becomes a useful tool for assessing renal function. Additional manifestations of lead toxicity include the elevation in urea and creatinine levels [83]. Besides, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining is frequently used to look for apoptosis, which is defined as programmed cell death. Such studies have demonstrated that there is a significant increase in TUNEL-positive cells in lead exposed animals i.e. lead exposure is a cause of cellular death [83].

Lead exposure has two additional effects on the kidneys. Under normal physiological conditions, 25-vitamin D is converted into 1, 25-vitamin D through the action of a hydroxylase enzyme, which is a target for inhibition by lead [2]. Thus, leading to a reduction in the generation of the active form of vitamin D. Lead is also associated with a higher rate of hyperuricemia and gouty attacks [12, 43, 47].

1.3.4. Cardiovascular Effects

Related to the cardiovascular system (CVS), numerous studies showed a positive relationship between lead exposure and hypertension [84]. Systolic as well as mean blood pressure were found to be higher in occupationally lead-exposed workers, in comparison to a control group [85]. High readings of systolic and mean blood pressure were also reported in hypertensive subjects with lead exposure who were compared to hypertensive patients with no history of lead exposure [86]. In another study, acute exposure to low levels of lead was shown to result in increasing cardiac muscle contractility and increasing ventricular systolic pressure, which are among the predisposing factors that can lead to the development of hypertension [87]. Additional data from experimental animal studies support this relationship as after two months of lead exposure, there was an increase in systolic blood pressure in rats [88], whereas, in another study there was a positive effect on both systolic and diastolic arterial pressure as well as on cardiac contractility [89]. Furthermore, lead exposure even during fetal life and through maternal exposure to lead in drinking water during lactation resulted in an increase in systolic blood pressure in rat pups [90].

Lead-induced hypertension and the elevated systolic blood pressure [25, 86] are believed to be mediated through several mechanisms. The first is by stimulating the renin-angiotensin system, which elevates blood pressure [12] in both adults and children [47]. The second important mechanism is mediated through lead's effects on the vascular smooth muscles, where it inhibits Na+/K+ ATPase and activates Na+/Ca++ transporter [12], therefore, increasing the concentration of calcium within cells [3, 57]. In addition, lead can increase the levels of endothelin and thromboxane as well as changing the elasticity of arteries [47]. Lead can also activate the sympathetic nervous system, resulting subsequently in the release of catecholamines [84]. On the other hand, lead is also believed to inhibit nitric oxide [12] and cGMP [47], both of which relax vascular smooth muscles.

In addition, lead exposure widens pulse pressure [91], which represents the difference between systolic and diastolic blood pressures and gives an indication about the level of stiffness of arteries. High pulse pressure is also a risk factor for left ventricular hypertrophy, which is a predisposing factor for hypertension [86] and cardiovascular complications [84]. Lead also caused an enhanced responsiveness to norepinephrine in animal studies and consequently resulted in more dysrhythmias [12].

The European-developed SCORE system uses several risk factors such as gender, age, total cholesterol, systolic blood pressure, and smoking status in order to systematically evaluate the risk for developing coronary events. Based on this method, lead-exposed workers in Poland were found to have a higher incidence of fatal heart events than non-exposed workers [92]. It is also believed that lead can induce the development of pro-atherosclerotic changes because of its effects on lipid profile, which

include having increases in the levels of total cholesterol, LDL, and triglycerides [84]. It is postulated that these abnormalities are caused by several mechanisms such as the stimulation of lipid synthesis and lipid peroxidation, and by the alterations in the metabolism of polyunsaturated fatty acids [84].

Homocysteine is considered ‘a novel biochemical marker’ [81] and a risk factor for cardiovascular and neurodegenerative diseases [89]. A previous study has shown that there is a positive association between lead exposure and homocysteine levels [89]. The same study showed that vitamin B6 and folate could minimize homocysteine levels in lead-exposed people, thereby exerting a protective effect.

1.3.5. Skeletal Effects

As mentioned earlier, the vast majority of lead is eventually deposited in bones; and a lesser concentration is stored in teeth. In these tissues, lead can replace calcium, resulting in the formation of tertiary lead phosphate. Calcium deficiency promotes the storage of lead. The skeletal manifestations are also mediated through lead’s effects on the parathyroid glands, where it interferes with the homeostasis of calcium and vitamin D [12, 47]. In the past, it was thought that lead in the bones is inert [12, 48]. However, it is now believed that lead exposure, even at ‘environmentally relevant levels’ i.e. at relatively low exposure levels; can postpone the healing of fractures. Furthermore, higher exposure levels were shown to result in fibrous non-unions [93]. Similarly, accumulation of lead in the teeth inhibits mineralization and can subsequently lead to an increased rate of dental caries [47]. In a clinical case report, lead poisoning (BLL 125μg/ dL) through the use of old utensils caused chronic pain in both knees and

muscular fibrillations [94]. Radiographic tests showed pathognomonic features of osteonecrosis (e.g. bone infarction) in the knee joint [94].

1.3.6. Carcinogenicity

Lead is considered to be a ‘facilitative or permissive carcinogen’ i.e. it has the ability to enhance the carcinogenic effects of other agents [95]. In addition, there is conclusive evidence from experimental studies that lead is a carcinogen; and at the same time, associations from epidemiological studies are growing [96]. Consequently, EPA considers inorganic lead a probable human carcinogen (Group B2) [32, 97], whereas, the International Agency for Research on Cancer (IARC) has upgraded it from (Group 2B) to (Group 2A) [98-100].

Regarding its mechanisms of action, lead is believed to cause indirect genetic damage through DNA fragmentation [101], suppression of the formation and the repair of DNA, causing oxidative stress [102], and by interfering with DNA binding proteins and tumor-suppressor proteins [96, 103]. Lead can also change the DNA methylation of several important molecules e.g. ALAD [14] and cause chromosomal aberrations in vitro and in vivo [100]. In addition, the stimulation of protein kinase C increases the synthesis of DNA, which can subsequently induce replication and hyperplasia [55]. Lead can also cause an acceleration in the growth rate of tumors [104] and it can counteract the protective actions of the anticarcinogenic agent selenium, and thus, lead has a potential to promote carcinogenicity [105].

The most common sites for lead-induced tumors are the kidneys, brain, lung, and prostate [96]. In humans, chronic occupational lead exposures have been associated

with renal and pulmonary cancers in epidemiological studies [106]. The assessment of more than 4000 lead-exposed workers in printing industry revealed a strong association between renal and pancreatic cancers in both genders and inorganic lead exposure [107]. Another occupational health study estimated exposure levels to lead dusts and fumes and concluded that lead exposure increases the risk for developing meningiomas [108]. In another study, patients with ductal breast carcinoma had a significantly higher level of lead in blood and hair samples, in comparison to a control group [104]. At the same time, lead levels in their hair samples were positively correlated with tumor volume, whereas, selenium levels had a negative correlation with tumor volume [104].

1.3.7. Immunological Effects

Lead exposure is implicated in causing stimulatory as well as suppressive effects on the immune system. For instance, lead can cause an increase in the levels of immunoglobulin E (IgE), thus leading to type I hypersensitivity reactions and enhancing the likelihood for the development of allergic conditions e.g. asthma [109]. Respiratory sensitization and inflammatory changes, similar to asthmatic manifestations, were further demonstrated in guinea pigs that were exposed to lead acetate inhalation. These animals developed an increase in WBC counts, interleukin (IL)-4, interferon (IFN), histamine and IgE levels [110]. In addition, lead exposure was associated with an increase in IL-10 levels in occupationally exposed workers, in comparison to a control group, perhaps due to having an increase in tumor necrosis factor (TNF)-alpha levels [111]. In certain areas of Zambia, soil is contaminated with lead. In-vitro studies in this country showed that exposure of cultured WBCs from cattle to lead for 24 hours caused

an increase in the expression of mRNA of metallothionein-2 (MT-2), TNF-alpha, IL-1 beta, IL-6 and inducible nitric oxide synthase (iNOS) [112].

On the other hand, in another occupational health study, lead was found to have some suppressive effects as it caused a significant reduction in CD3(+), CD4(+)/ CD8(+) ratio, IL4, TNF-alpha and IFN-gamma levels, together with having significant increases in CD8(+) and IL-10 levels [113]. In another study, CRP levels were significantly high, whereas, IgA and IgT levels were low in lead exposed workers [114]. In addition, in an in-vitro study, lead was shown to inhibit the phagocytic activity and cellular adhesion of peritoneal macrophages, taken from mice [115], again demonstrating the depressive nature of lead exposure on the immune system. As such, these two studies concluded that lead might eventually weaken the immune response [113, 114], and thus increase the vulnerability to develop infectious and inflammatory diseases and cancer [114].

1.3.8. Gastrointestinal Effects

Lead poisoning in clinical cases can cause GIT disturbance e.g. nausea, vomiting, abdominal pain, and constipation [116]. In an experimental study in rats, there was an increase in basal and acetylcholine-stimulated gastric motility; and this was suggested to be attributed to an increase in nitric oxide (NO) levels [116]. The same authors also showed that NO in low-dose lead-exposed rats decreased gastric acid secretion, whereas, high lead-exposure levels increased acid secretion [117]. Oxidative damage is often associated with pro-ulcerative factors and is believed to be a predisposing factor for gastric ulcers [118]. Examination of the gastric mucosa of rats that have been

exposed to lead acetate in drinking water for 15 weeks and then exposed to an oral mixture of HCl and ethanol revealed lipid peroxidation, a reduction in catalase, superoxide dismutase and nitrite levels [118]. This demonstrates lead's ability to aggravate the ulcerative effects of HCl and ethanol, possibly through causing oxidative stress in the exposed animals [118].

Furthermore, lead exposure causes a wide range of abnormalities in the liver. This is because the liver is one of the favorite sites for lead's deposition, for example, even relatively short term exposures in rats for two weeks resulted in lead's accumulation in the liver [119]. Lead can cause a disruption in the hepatic oxidative parameters e.g. it elevates the levels of hepatic transaminases and superoxide dismutase (SOD) [59]. Lead also caused significant reductions in two steroid metabolizing enzymes, namely 17-beta hydroxysteroid oxidoreductase and uridine diphosphate-glucuronyltransferase, besides causing a reduction in hepatic DNA levels [119]. There was also an increase in acid phosphate levels in response to lead exposure [58]. A recent study in Wistar rats showed that chronic exposure over 4 months to low lead levels (0.06 %) in drinking water could initiate fibrogenic changes in the liver [50]. In addition, lead can inhibit the synthetic functions of the liver, causing a reduction in total protein and albumin levels [58]. Histologically, it causes degenerative and inflammatory changes and portal edema in the liver [120].

1.3.9. Reproductive Effects

In July 2003, the European Union (EU) classified lead as a category 1 agent because of its toxicity to the reproductive system (embryo-toxic). Based on such classifications,

it banned the utilization of lead, mercury, or cadmium in electronic gadgets [26]. Males exposed to lead might present with hypospermia and impaired sperm morphology and mobility e.g. in a study on 60 males, lead levels were correlated with impaired sperm motility, sperm concentration, and DNA damage [121], whereas, in patients with no occupational exposure, increased lead levels in semen were associated with reduced sperm counts [122]. Another study showed that maternal cord lead levels were associated with reduced height and small head circumference in their school-age children [123].

In comparison, several experimental studies documented the reproductive toxic effects of lead exposure. For example, a previous experimental study in rabbits that were exposed to lead in drinking water for 12 weeks showed several reproductive abnormalities in the testes and seminiferous tubules e.g. abnormal sperms, degenerative changes and necrosis [124]. Diminished spermatogenesis leading to impaired fertility was also demonstrated in mice exposed to lead orally over 2 months [125]. In these animals, lead deposits were found in the testes [125]. Another study that was conducted in mice has shown that maternal lead exposure during lactation resulted in significant effects in male pups, which had a reduction in the weight of their testis, low levels of serum and testicular testosterone, low numbers of spermatozoa, and reduced expression of several important enzymes that are required for the synthesis of testosterone [49]. On the other hand, the oral exposure of female rabbits to lead over 8 weeks resulted in degenerative changes in the ovarian follicles and apoptotic changes in the endometrial lining tissues [101].

1.3.10. Lead Exposure and Oxidative Stress

Lead exposure is also associated with oxidative damage [126, 127] such as the production of ROS and the overconsumption of intracellular glutathione stores [127, 128], which is one of the main antioxidant defense mechanisms in the human body. A related chemical is the glutathione-S-transferase enzyme, which is a phase II enzyme that is required for the detoxification process of many agents including heavy metals [129]. Lead can inhibit several antioxidant enzymes including glutathione-S-transferase, hepatic and brain glutathione reductase, and the selenium-dependent glutathione peroxidase enzyme [130, 131]. In comparison, it causes a reduction in the hepatic copper-dependent-SOD levels [126]. Lead exposure also caused a reduction in the levels of SOD and glutathione peroxidase; and an increase in MDA levels in renal tissue homogenates [83].

In order to counteract the toxic effects of lead exposure, mice were given milk by gavage. Milk was further fortified with the antioxidant bamboo leaves, vitamin C, calcium lactate, ferrous sulfate, and zinc sulfate for a total duration of 7 weeks [132]. The use of this formula resulted in decreasing BLL, lead's distribution to soft tissues including liver, kidneys and brain, together with ameliorating lead-induced oxidative damage [132]. The effects of oxidative stress will be discussed in more detail in chapter 4: Diabetic Animal Model.

1.3.11. Effects on Children

The two most important causes for lead poisoning in the 20th century were leaded gasoline and lead-based paints [8]. As with other subgroups in the general population,

children are exposed to lead mainly through ingestion [17]. In addition, parents may chronically expose their offspring to low lead levels through their contaminated bodies and clothes [12] or through maternal-fetal route in occupationally exposed women as lead easily crosses the placenta [17, 45, 46].

The effects of lead exposure in children might be different than its effects on adults [81]. Children are particularly vulnerable to lead poisoning since they carry several risk factors that predispose them to higher levels of toxicity once they are exposed to lead [17]. In fact, children's GITs absorb around 40 - 50% of ingested lead [12] compared to only about 3 - 10% in adults [13]. After absorption, children retain higher amounts of lead (about one third), whereas, adults retain less than 5% [47, 48]. Also, children tend to deposit more lead in soft tissues, rather than in bones, and their BBB is still undergoing development. In addition, the levels of lead exposure become much higher if a child is used to pica [8]. Since poverty is associated with malnutrition, poor children are often deficient in iron and calcium; and this will ultimately further predispose them to the toxic effects of lead exposure [8].

The peak age for lead poisoning in children is between 18 to 30 months of age [17]. Low levels of exposure (BLL even below 10 µg/ dL) may ultimately damage various organs; even if the initial presentation was asymptomatic [8]. Furthermore, BLL of less than 5 µg/ dL can cause brain damage [8]. In addition, gender may play a role in lead exposure cases. For instance, in 108 Indonesian children who were 6-7 years old; males were found to have significantly higher BLL than females (6.8 ± 2.0 µg/ dL versus 5.9 ± 1.9 µg/ dL) [133]. This was attributed possibly to behavioral differences between the two genders [133].

As mentioned earlier, the effects of lead exposure are worse in children and this is related to the total body burden with lead. Affected babies might present with low birth weight, failure to thrive and various forms of mental impairment e.g. having learning difficulty or low intelligence quotient (IQ) [2, 3, 97]. Other features of lead toxicity in children include effects on the mineralization of cartilage and inhibition of the renal conversion of vitamin D to its active form, both of which lead to reduced bone density and skeletal abnormalities [17]. When the results from 7 international cohort studies that examined a total number of 1333 children were compared after making adjustments for several variables, it was found out that low blood lead levels even below 7.5 µg/ dL were associated with intellectual impairment [134]. The survivors of acute and severe lead poisoning in childhood often suffer from life-long sequelae, especially neurological and behavioral abnormalities [8]. However, despite the wide range of neurobehavioral abnormalities seen in lead poisoning cases, no single feature has been identified as a pathognomonic indicator of lead toxicity [17].

Based on the previous discussion, both the American Academy of Pediatrics and CDC have made a recommendation to screen children, particularly those from underprivileged families, for lead levels at the age of 1 and 2 years [17]. In addition, preventive public health programs that tackle lead exposure can be very effective and productive e.g. reports from the U.S. show that the cost-benefit ratio for reducing lead exposure is US $ 1 to 17–220. This ratio is said to be even better than the ratio that is obtained from childhood vaccinations [8].

1.3.12. Effects on Women

At present, it is suggested that the lower bone lead levels and the higher blood lead levels in women are a possible reflection of the differences in the metabolism of lead between males and females. These levels might also reflect continuous endogenous release of lead from bones in women [135]. A study conducted in 2009 concluded that the higher blood lead levels found in 106 pregnant women during their 24^{th} to 28^{th} weeks of gestation might be an etiological factor for the pregnancy induced hypertension (PIH) that they developed [136]. There is also an association between gestational lead exposure and miscarriages and preterm deliveries [17]. In addition, cumulative lead exposure measured by K-shell X-ray fluorescence was associated with an earlier onset of menopause [137]. The chronic (12 months) exposure of female Wistar rats to lead acetate resulted in a significant increase in serum luteinizing hormone and testosterone levels, and at the same time, caused significant reductions in the levels of estradiol and progesterone [138].

The effects of lead may vary, depending on the sex of the exposed species. This was demonstrated in a behavioral study, in which rats were exposed to 50 mg/ L of lead acetate in drinking water [139]. Compared to females, male rats became hyperactive and their spatial memory deteriorated [139]. Lead can also have different effects on the progeny of exposed individuals. The exposure of rats in late pregnancy (days 15-21) resulted in differential effects on their male and female offspring; as the adult females had a suppressed level of type 4 (or delayed type) hypersensitivity reactions and higher levels of IL-10, in comparison to the adult males, which had higher IL-12 and lower IL-10 levels [140]. In addition, when rat pups were exposed to lead in chow for a month,

BLL between males and females remained comparable. However, there was a differential expression of more than a hundred genes in the hippocampus of these animals. This result might demonstrate the importance of sex in determining the deleterious effects of lead exposure [141].

1.3.13. Effects on the Elderly

In old age, lead exposure can result in more deleterious effects; given the fragility and susceptibility of this vulnerable age group. In this subpopulation, several studies such as the normative aging study in the U.S. [142] assessed the cumulative lead levels in bones using K-shell X-ray fluorescence. In Korea, researchers identified a positive relationship between the exposure to low lead levels in the environment (BLL 2.32 ± 1.35 μg/ dL) and high blood pressure among the elderly [143].

As mentioned earlier, lead competes with both calcium and iron for absorption in the GIT. In a related medical condition, known as hemochromatosis, affected subjects have increased body burden of iron. A previous study discovered that the elderly, who had the hemochromatosis variant genes (C282Y and/or H63D hemochromatosis gene (HFE)), had lower levels of lead in the patella [144]. Accordingly, there is a possibility that these genes might be responsible for modifying lead's toxicokinetics. In relationship to the nervous system, environmental exposure in the elderly may impair cognitive parameters, especially those related to visuospatial and visuomotor areas [142]. In addition, in premenopausal women, a positive association was observed between cumulative low level lead exposure, measured in bones, and depression and anxiety [145].

1.4. Mechanisms of Toxicity

Lead toxicity is dependent upon several factors including the total dose and both of the route as well as the duration of exposure. Various mechanisms might be involved in lead toxicity e.g. enzymatic suppression; especially for zinc-dependent enzymes [2]. The ultimate toxicity may vary from general nonspecific symptoms and signs up to fatality [47, 48]. These toxic effects of lead exposure might also be influenced by genetic polymorphism [12].

The main mechanism underlying lead toxicity is the interference with the normal physiology of calcium [48]. As it was mentioned earlier, lead and calcium compete with each other for GIT absorption. In normal situations, calcium's concentration in the extracellular fluid is 10,000 times higher than intracellular levels [146]. In lead poisoning, this homeostasis is disrupted. Lead targets anion exchange channels and calcium dependent transporters and pumps. Lead also competes with the transportation of calcium. Eventually, it replaces calcium [62, 147], thereby exerting its toxic effects. Lead can also induce calcium's release from the mitochondria [147], thus increasing the intracellular concentration of calcium. Additionally, lead has a 100,000 times a stronger binding capacity than calcium to calcium-activated proteins [2]. Another mechanism by which lead interferes with calcium metabolism is through interacting with vitamin D receptor, where polymorphisms of this receptor were found to be associated with high lead levels [148].

1.5. Measurement of Lead Levels

BLLs can be measured using an anodic stripping voltammetry machine, however, this equipment has several major problems as it is insensitive, very time consuming, not user-friendly, and yields inconsistent results as it is operator-dependent. In comparison, the inductively coupled plasma mass spectrometry (ICP-MS) is the state-of-art equipment for measuring heavy metals' levels whether for clinical practice or environmental studies [53]. This method usually requires the use of 'sample digestion techniques e.g. microwave digestion' [53]. ICP-MS has the following advantages [149-151]:

- High detection level sensitivity, which is about 10^{-12} (being better than atomic absorption). It is also faster than the latter and can detect many metals in one sample in just a few minutes.
- Its ability to analyze blood, serum, urine and water samples.
- Detecting a wide range of heavy metals e.g. mercury, arsenic and cadmium, thus, being useful for the diagnosis and monitoring of many clinical conditions.
- It is a computerized system and can analyze hundreds of samples per 24 hours.
- Many international occupational health organizations such as occupational safety and health administration (OSHA) in the U.S. mandate periodic and regular medical checkups for workers in hazardous jobs, including the ones that involve dealing with heavy metals such as lead. So, ICP-MS helps in complying with these agencies' mandatory requirements.

Recently, newer methods for the measurement of chronic lead exposures have been utilized in various studies. One of these most popular tests in epidemiological studies is the use of K-shell X-ray fluorescence for the noninvasive measurement of lead levels in bones [152, 153].

1.6. Occupational Lead Exposure in Oman

Lead poisoning burdens health facilities and this burden becomes bigger, when affected subjects present with chronic complications; particularly if the outcomes are serious. According to WHO, lead poisoning contributes 0.6% of the total burden of diseases worldwide [154], whereas, Fewtrell [155] estimated that lead exposure is responsible for about 1 % of the global burden; mainly in the form of causing mild mental retardation and cardiovascular effects. It is estimated that lead exposure-related health-issues cost the USA $ 43.5 billion per year [17]. As such, lead toxicity places heavy strains on both the financial as well as the human resources of health agencies. In Oman, various injuries and poisonings, among which is lead poisoning, resulted in a morbidity rate of 883/ 10,000 population in 2009 [156]. In addition, the total expenditure of the Omani Ministry of Health in 2008 was about 269 million Rials, while the overall cost for services was around 27 million [63].

Statistics, published by the Ministry of National Economy in Oman, showed that the total number of employees in the subgroup of ‘manufacturing’ and the number of expatriate employees under ‘industrial, chemical and food industries occupations’ in 2008 were about 85,000 and 42,000 workers, respectively [157, 158]. Furthermore, comprehensive occupational health and safety studies ranked number 19 among 30

important domains in the Seventh Five Year Health Development and Research Plans (2006 – 2010) in the Ministry of Health (MOH) in Oman [159]. All of these facts illustrate the importance of occupational health studies in the country so as to protect our most valuable resources, the people.

So far, there is no data regarding the prevalence of lead exposure among adult Omanis in the general population. In collaboration with WHO in 2003, the Department of Environmental and Occupational Health (DE&OH) at the MOH examined lead levels among 334 school children. The prevalence of lead exposure (BLL ≥ 10 µg/ dL) in this age group was found to be 6.58 % [160].

Regarding occupational health in Oman, Al-Rusail Industrial Estate (RIE) was established in 1983 and since then six more sites were developed throughout the country [161]. These are home for many manufacturing companies which deal with various metals, for example, lead in Al-Rusail and aluminum in Sohar. Two studies conducted at the College of Engineering at Sultan Qaboos University (SQU) and published in 2003 could not find significant levels of heavy metals including lead in total suspended particulate matter (air samples) that were collected from 23 locations in RIE [162], 19 sites in Sohar Industrial Estate and 12 homes in Sohar's neighborhood [162, 163]. The highest lead level was measured at an automobile battery factory (15.09 µg/ m^3) in Al-Rusail. This was within the acceptable level (an average level of 50 µg/ m^3 per 8 hour working shift) as set by the U.S. OSHA [162].

However, these results do not definitely exclude lead exposure because of several reasons. First, these projects neither studied any human subjects nor did they assess the relevance of these levels in causing adverse health effects in the exposed workers.

Second, lead's toxic effects might not necessarily result from acute exposures, but from chronic ones that lead to cumulative effects. Within any factory, there is a difference in the total concentration between the various workstations, a point that is not specifically addressed in the previous studies. In addition, air particles are dispersed over time, and thus, their concentrations decrease. This explains the importance of continuous measurement and the need for the establishment of surveillance systems. In addition, the examined areas in Sohar might have not involved industries related to lead exposures.

In addition, some data that were obtained directly from the batteries' manufacturing factory in Oman contradict the findings from the previous studies. These data provide clear evidence that lead exposure is still a serious problem at this establishment as they showed alarming levels (Tables 1, 2 & 3):

Table 1: Blood lead levels among Omani employees in Reem Batteries Company.

Blood Lead Levels (µg/ dL)	**2008**		**2010**	
	Number	**Percent (%)**	**Number**	**Percent (%)**
< 13	2	1.9	13	10.9
13 - 39	48	45.7	58	48.7
40 - 59	39	37.1	36	30.3
60 - 79	14	13.3	12	10.1
≥ 80	2	0.9	0	0.0
Total	105	100	119	100

Table 2: Blood lead levels among Reem Batteries Company employees in 2010.

Blood Lead Levels (µg/ dL)	**Number of Workers**		**Total**
	Omani	**Expatriates**	
< 10	11	0	11
10 – 19	12	1	13
≥ 20	96	123	219
Total	119	124	243

As mentioned earlier, according to CDC, the cut value of BLL for occupational exposures should not exceed 25 µg/ dL [2, 31]. It is medically recommended to relocate the workers, who have a BLL of > 60 µg/ dL, to other workstations in order to prevent them from being exposed to any more lead [26].

Table 3: Airborne lead levels by section in Reem Batteries Company in 2010.

Section	**Airborne Lead Level (µg/ m^3)**
Grid casting	94
Spreading (loading)	260
Forming & Polishing	264
Assembling (packaging)	12
Others	28

From Table 3, it is clearly seen that the employees at the first three work sections are exposed to airborne lead levels greater than the permissible values set by OSHA, which

should not exceed 50 µg/ m³ per 8 hour working shift. In addition to the manufacturing of batteries, occupational lead exposure is an ongoing problem in several other occupations e.g. construction and painting. Furthermore, some industries such as radiator repair shops are very common and scattered throughout Oman. All these workers are exposed to only one type of lead (inorganic lead) and this exposure often takes place through the same common route i.e. inhalation.

Occupational health nowadays rests heavily on having a solid basis and a strong background in ergonomics. However, when fifty managers from different industries in Oman participated in a study that was published in 2003, either a lack of knowledge or not having informational resources related to ergonomics were reported as the main obstacles facing 88 % of the participants [164]. As such, managers should have proper training in health and safety.

1.7. Lead Exposure in UAE

In the UAE, there are a few published studies that addressed lead exposure issues, mainly in Abu Dhabi Emirate. In the general population, a study on school children demonstrated an association between high lead levels and attention deficit hyperactivity disorder [165]. For occupational exposures, in one cross-sectional pilot study, Bener et al. examined a hundred occupationally lead-exposed workers in Al-Ain city and found the following results [166]. The exposed group complained of several general and non-specific symptoms and signs such as GIT disturbance, fatigue, irritability, and memory problems, which could be ultimately attributed to lead exposure. The mean BLL in the study group was high (77.5 ± 42.8 µg/ dL) and significantly different from the level in

the control group (19.8 ± 12.3 µg/ dL). In addition, ALP and LDH levels were significantly higher in the workers, in comparison to the control group [167]. Another similar study from the UAE reported a mean BLL of 81 µg/ dL among 110 workers, in comparison to 11 µg/ dL in age, sex and nationality-matched control group of 110 people [168]. This study also reported a significant association between BLL and systolic and diastolic blood pressures, fasting blood glucose levels, plasma levels of total cholesterol, LDH, and uric acid. Thus, it demonstrated a link between lead exposure and the risk for developing hypertension and DM. Based on these results, there is obviously an ongoing lead exposure problem in the country; and accordingly there is a big opportunity to conduct related high quality research and to develop practical guidelines and policies in order to deal with this issue successfully.

1.8. Diabetic Animal Models

There are many types of diabetic animal models; and each one has its own pros and cons. Chemically-induced models have several advantages, and are frequently used. These models are usually simple, inexpensive and can be used in rodents as well as in higher animals [169]. The two compounds that are most commonly used for this purpose are streptozotocin and alloxan [169] and they both can be administered as i.p., i.v. or s.c injections [170]. These chemicals are structurally similar to glucose; and therefore, they become more effective when they are administered to fasting animals [169]. However, alloxan has two main problems. First, it is toxic to the kidneys; and second, the animals can recover over time and ultimately become non-diabetic [171]. As for STZ, it has a higher level of selectivity to induce DM and is a better agent to be

used for the induction of DM in rats [171]. STZ has to be wrapped in aluminum foil in order to protect it from light. Because of its instability, it needs to be freshly prepared and utilized [169]. STZ model will be discussed in more detail in chapter 4: Diabetic Animal Model.

On the other hand, the most commonly-used animal models that can spontaneously develop type 1 diabetes are the non-obese diabetic (NOD) mouse and bio breeding (BB) models [172]. In these models, the individual animals that are more susceptible to develop type 1 DM have been carefully selected and bred over and over [171]. Such models are helpful in understanding the genetic aspects and the pathogenesis of type 1 DM [171].

In NOD, insulitis starts at around the 3^{rd} or 4^{th} postnatal week, however, full blown DM becomes clearly evident around 10 - 14 weeks of age; and it is characterized by CD4+ and CD8+ lymphocytic infiltration [169]. Although the NOD model is useful for studying the therapeutic potential of new drugs, some medications that were proved to be effective in mice were of no use in people [169]. In addition, this model has some other disadvantages, for example, it is quite expensive as the mice need to be kept free from infections and there is a variability in the incidence of DM in each batch of animals [169]. An additional factor to consider is that the incidence of DM in this model is higher in female animals than in males [173]. This model is also associated with the development of other autoimmune disorders such as thyroiditis [173].

In comparison, among the BB model, bio-breeding-diabetes-prone model is suggested to be the best type 1 diabetic animal model [171]. This model closely mimics human DM on several levels. It develops around adolescence (8-16 weeks in rats), has

an equal incidence in male and female rats and leads to severe ketoacidosis that requires treatment with insulin, just like in people [171].

Other animal models are based on surgical procedures or genetic modifications. However, such models require highly advanced techniques, are expensive, and might result in the loss of a large number of animals [170]. More recent models involve molecular and biological techniques such as the use of knock-in, generalized knock-out, and tissue-specific knockout animal models [172].

1.9. Research Problem and Rationale for the Study

In brief, lead is a useful heavy metal that has been used in various industries for centuries. Because of its widespread distribution in the environment and due to manmade activities, exposure to lead has been common and easy. Consequently, this resulted in the clinical manifestation of a wide range of poisoning cases. In the past, the majority of experimental studies exposed laboratory animals to large doses of lead. Also, the vast majority of literature concentrated on studying lead's toxic effects mainly on the hematopoietic, nervous, renal and cardiovascular systems. However, over time, it became evident that lead can basically target all body organs and that no safe level to lead exposure could be established. As such, recent studies addressed other problems e.g. lead's effects on the pituitary-thyroid pathway. In addition, there is an increasing interest in the immunological and allergenic potential of heavy metals, which has been recently reported in a few published studies. Besides determining lead's toxic effects, addressing its mechanisms of actions that yield such effects is obviously also worth examining.

There are some cross-sectional studies that looked at the relationship between lead exposure and its effects on the thyroid glands, however, the results from such studies have several limitations related to sample size, total duration of exposure and other cofounders e.g. smoking habits of the participants. In order to have firm answers, some animal studies also addressed this issue. Until today, however, this topic remains controversial. Accordingly, the lack of adequate data and the inconsistency in literature in regard with the relationship between lead exposure and its effects on the pituitary-thyroid pathway stimulated us to conduct this study. In addition, the U.S. ATSDR stated that 'the information regarding effects of lead on the liver in humans and animals is scarce and does not allow for generalizations' [174]. Besides, little is known about the association between lead exposure and inflammatory markers [175, 176]. Therefore, experimental studies that explore the effect of lead exposure on thyroid functions, inflammation, and hepatic and systemic toxicity are much needed.

In this thesis, we determined the toxic effects of lead exposure in normal rats and diabetic rats. Diabetes was selected because of several reasons. First, according to our knowledge, the relationship between these two conditions (i.e. lead exposure and DM) has not been addressed before in experimental work. Second, DM is a major global health problem. Third, there is a large prevalence of this condition in the UAE. In addition, there is a close association between thyroid disorders and DM in clinical and epidemiological studies. Although there are several diabetic animal models, we studied the effects of lead in STZ-induced diabetic Wistar rats; since this model is well established and it has been used successfully in our laboratory.

1.10. Aims and Objectives

The main aim of this thesis was to determine the toxic effects of exposure to several doses of lead acetate trihydrate on the pituitary-thyroid pathway in normal and diabetic rats. We also documented systemic effects on other major organs. The specific objectives were as follows:

- Studying the toxic effects of lead exposure in healthy Wistar rats
- Investigating the toxic effects of lead exposure in STZ-induced diabetic rats
- Developing a new experimental thyroid model based on the use of hormones, where TRH is used to induce hyperthyroidism and OCT is utilized for the induction of hypothyroidism

Chapter 2: Materials and Methods

This study was approved by the Research Ethics Committee (Institutional Review Board) at the College of Medicine and Health Sciences, UAE University.

2.1. Animals

Young male Wistar rats were used in all experiments. They were housed in polypropylene plastic cages and kept in 12 hour cycles of light and dark at 22 ± 2 ^{O}C. They were supplied with chow and tap drinking water ad libitum. The animals were handled in a humane way to minimize suffering and pain. The weight of all animals was assessed on the first and last days of experiments.

2.2. Study Design

2.2.1. Non-Diabetic Model

2.2.1.1. Treatment Protocol

Young male Wistar rats, with a weight range of 208 to 253 grams, were used per the following treatment protocol, as shown in (Table 4). The daily treatments of either distilled water (DW) or the various doses of lead acetate trihydrate were given as i.p. injections for a total duration of five days. Animals in the control group received DW, whereas, the animals in the four treatment groups A, B, C and D were given 1, 25, 50, and 100 mg/ kg of lead acetate trihydrate, respectively. These doses are among the standard and effective doses that have been used in lead studies [64, 177].

Table 4: Treatment protocol in non-diabetic animals.
All animals were treated with i.p. injections for five days. The control group received DW, whereas, the animals in treatment groups A, B, C and D were treated with 1, 25, 50, and 100 mg/ kg of lead acetate trihydrate, respectively.

Groups	5 Days Treatment Lead Dose (mg/ kg), i.p.
Control	DW
A	1
B	25
C	50
D	100

The LD_{50} through the i.p. route in rats is 150 mg/ kg for lead acetate and 200 mg/ kg for lead acetate trihydrate [178], which is a more soluble form of the compound and is miscible with water [179]. Since the LD_{50} of lead acetate trihydrate is in the range of 50 - 500 mg/ kg, this chemical is considered to be moderately toxic [180]. Accordingly, the doses that we used in our experiments represent the following percentages of LD_{50} of lead acetate trihydrate (Table 5).

Table 5: The relationship between lead acetate trihydrate doses and LD_{50} (%).

Dose (mg/ kg)	Percentage of LD_{50} (%)
1	0.5
25	12.50
50	25.00
100	50.00

2.2.2. Diabetic Model

2.2.2.1. Induction of Diabetes Mellitus

Type I diabetes was induced in one month old rats by treatment with a single dose of 60 mg/ kg STZ freshly prepared in citrate buffer, given as i.p. injections. The animals were monitored and their weight was recorded on a weekly basis. Six weeks later and just before the start of experiments, blood samples from the tail were examined for random blood sugar (RBS) levels using an Accucheck performa glucometer (Roche Diagnostics, NSW, Australia). The cut value for diagnosing diabetes was set at 300 mg/ dL and the animals that had not become diabetic were excluded. The weight of the animals was monitored before the induction of DM and throughout until the last day of experiments.

2.2.2.2. Treatment Protocol

The treatment protocol that we used in STZ-induced diabetic rats was similar to the one used for non-diabetic animals, except that we did not have the treatment group of 1 mg/ kg lead acetate as is shown in (Table 6). We excluded this group because we did not get significant results in the 1 mg/ kg lead acetate non-diabetic group. Six weeks post DM induction with STZ, the animals were divided into four groups i.e. the control group was also a diabetic group. All groups were treated with daily i.p. injections for five days. Control group animals were given DW. The three treatment groups received 25, 50, and 100 mg/ kg of lead acetate trihydrate, respectively.

Table 6: Treatment protocol in diabetic animals.
The control group was treated with daily i.p. injections of DW for five days. Treatment groups A, B and C were given 25, 50, and 100 mg/ kg of lead acetate trihydrate, respectively.

Groups	5 Days Treatment Lead Dose (mg/ kg), i.p.
Control	DW
A	25
B	50
C	100

2.2.3. Hormonal Thyroid Model

2.2.3.1. Treatment Protocol

These animals were treated for five days with i.p. injections (Table 7). DW was administered to the control group. The TRH group received 5 µg of TRH / 100 g body weight, whereas, OCT group was treated with 10 µg of OCT/ kg body weight.

Table 7: Treatment protocol for hormonal thyroid model.
All animals were treated for five days. The control group received i.p. DW, TRH group was treated with TRH at a dose of 5 µg/ 100 g body weight i.p., and OCT group was given 10 µg of OCT/ kg, i.p., respectively.

Groups	5 Days Treatment, i.p.
Control	DW
TRH	TRH
OCT	OCT

2.3. Chemicals

Lead acetate trihydrate was purchased from Sigma, Aldrich (Missouri, USA). For determination of BLL by using an ICP-MS, the following chemicals were utilized. Double distilled nitric acid, tetra methyl ammonium hydroxide (TMAH), triton X-100,

and EDTA disodium salt; all were TraceSelect® grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA). Lead, the primary standard and Rhodium, the internal standard, were PE Pure® grade and obtained from PerkinElmer (CT, USA). Standard reference materials (SRM) Sero Whole Blood level 2 were obtained from Seronorm® (Sero, Norway). Pure water was supplied through our lab resources and equipped with MilliQ Millipore® laboratory pure water (>18 MOhms).

2.4. Blood Samples

Blood samples were collected within half an hour to one hour after injecting the animals with the last dose on day 5 between 9.00 a.m. to 1.00 p.m. Before collecting the samples and sacrificing of the animals, sodium pentobarbital (70 mg/ kg) was administered as a general anesthetic. Following laparotomy, the samples were collected from the inferior vena cava in syringes containing 4% sodium citrate as an anticoagulant. Fresh whole blood samples were used to determine complete blood counts (CBC). The rest of blood samples were centrifuged at 3000 g for 10 minutes. The separated plasma and a portion of whole blood samples, that were used to determine BLL, were stored at -80.0 ºC pending analysis.

2.5. Assessment of BLL

BLL in whole blood samples were determined by using a modified version of the ICP-MS method, described by Nunes, J.A., et al. [181]. All the analyses were performed on a PerkinElmer Nexion 300® equipped with nickel sampler and skimmer cones, standard Ryton Double-Pass Scott-type spray chamber, standard ceramic injector, and

Meinhard® Concentric nebulizer. The instrument was equipped with an S-10 autosampler (PerkinElmer), and software version is Nexion®. Lead is monitored at its three major isotopes: m/ z 208, 207 and 206.

Lead Calibration Standards: Lead calibration standards were prepared from dilution of 1000 mg/ L lead primary standard (PE Pure, PerkinElmer) at 10, 50, 100 and 500 ppb (µg/ L) levels. For instrumental calibration, these stock standards were diluted 1:100 in the above diluent solution. The calibration blank was made up of the diluent solution.

Diluent: The blood diluent consists of 0.005% w/v Triton-X-100 and 0.05% EDTA. Both of TraceSelect® and Sigma Chemicals were prepared in MilliQ Millipore® laboratory pure water (> 18 MOhms).

Sample Preparation: All calibration standards, quality control materials (QCs), standard reference materials (SRMs), blanks, and unknown blood samples were equilibrated to room temperature prior to sampling. Blood samples (200 µL), were pipetted into (15 mL) conical tubes. Then, 500 µL of 10% v/v TMAH solution was added to the samples, incubated at room temperature for 10 min, and then the volume was made up to 10 mL with a solution containing 0.05% w/v EDTA + 0.005% v/v Triton X-100. Rhodium was added as internal standard to obtain a final concentration of 10 µg/ L. After that, samples were directly analyzed by ICP-MS.

2.6. Assessment of Hormonal Levels

TSH ELISA kits for rats were purchased from USCN Life Science Inc. (Wuhan, China), whereas total T3 and total T4 ELISA kits for rats were obtained from MyBioSource Company (California, USA). The plasma samples were analyzed

according to the relevant company's manuals.

2.7. Assessment of GSH

Glutathione levels in plasma were determined, using a kit from Sigma Aldrich (Missouri, USA) and following the manufacturer's instructions as described before [182].

2.8. Assessment of Complete Blood Counts (CBC)

CBC parameters in whole blood samples were determined using ABX VET ABC Hematology Analyzer machine from ABX Diagnostics (Montpellier, France), with a special analysis card for rats.

2.9. Assessment of Biochemistry Parameters

A total of 10 renal and hepatic function parameters in plasma were determined using Cobas Integra 400 biochemistry analyzer from Roche (Indianapolis, USA). These parameters are blood urea, creatinine (CRE), albumin (ALB), alkaline phosphatase (ALP), alanine Aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), C-reactive protein (CRP), total bilirubin, and total proteins.

2.10. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 for Windows, version 5.01, 2007. The various treatment groups i.e. lead doses, represent the independent variable, which was compared with the resultant and dependent variables e.g. levels of

thyroid hormones and biochemistry parameters. In the hormonal model, two-way ANOVA was used to analyze weight changes during the five days of treatment with lead acetate, whereas, for all the remaining parameters, one way ANOVA was used. This was followed with Bonferroni post-hoc test for selected groups to compare the control group with each treatment group in sequence. Results are presented as mean ± standard error of the mean (SEM).

Chapter 3: Lead's Effect in Non-Diabetic Model

3.1. Background

Chronic lead exposure causes multiple systemic toxicities, particularly affecting the hematopoietic, nervous, and renal systems [1, 183]. These effects are related to the total body burden with lead and are worse in children. In the initial stages and at minimal levels of exposure, clinical presentation is vague and nonspecific and can easily pass unrecognized [1]. Systemic and more specific features become evident at higher levels of exposure. For example, inhibition of the enzymes that are involved in the heme synthesis pathway elevate the levels of 5-aminolaevulinic acid in urine and zinc protoporphyrin in blood [184], both of which might be used as diagnostic parameters for lead poisoning. The resultant anemia is usually a late manifestation of lead poisoning [185]. Acute lead exposure can also result in elevated blood pressure, with a particular increase of systolic blood pressure [186, 187], which might be either a cause for or a sequela of renal damage. Lead encephalopathy is one of the most dreaded serious complications of acute lead toxicity [19, 73, 74]. In regard with lead toxicity, the nervous system is considered to be the 'critical organ' that is at risk when the blood lead level (BLL) is at a 'critical level' of 10.7 – 17.5 µg/ dL [188].

Despite the widespread multi-organ nature of lead toxicity, evidence of effects on the thyroid gland is still not elucidated since no definitive relationship has been confirmed yet as the limited studies that have been previously conducted showed contradicting and inconclusive results [189]. For example, among occupationally exposed workers, elevated BLL of more than 80 µg/ dL were associated with significantly high levels of

thyroid stimulating hormone (TSH), whereas, in the lower BLL groups (40-59 and 60-79 µg/ dL groups), TSH levels were lower and comparable between the two groups [190]. In another occupational study, in which 137 lead-exposed workers were compared with 83 controls, no statistical difference in (thyroxine) T4 levels was found between the two groups [191].

Although these cross-sectional studies addressed the relationship between lead exposure and thyroid effects, such studies cannot differentiate between causality and effect. In addition, there might be other confounding factors in these studies e.g. age of participants, variable durations of cumulative occupational exposures and smoking habits.

Experimental animal studies also showed variable results e.g. the daily administration of 5 mg/ kg of lead acetate orally to sheep over 8 weeks resulted in a reduction in all thyroid-related main parameters i.e. TSH, T4 and triiodothyronine (T3) [192]. Similar results were also obtained in lead-exposed rats [193]. In comparison, there were no effects on T3 and T4 levels when rats were exposed to lead acetate in drinking water for three months [194].

3.2.Results

3.2.1. Weight Monitoring

Animals in the control group, as expected, gained some weight (Figure 2), whereas, in the group that was treated with 1 mg/ kg of lead acetate, weight loss was slight and insignificant. However, there was a significant amount of weight loss in the remaining groups i.e. the ones that were treated with 25, 50 and 100 mg/ kg of lead acetate; and the highest amount of weight loss was observed in the group that was treated with 100 mg/ kg of lead acetate.

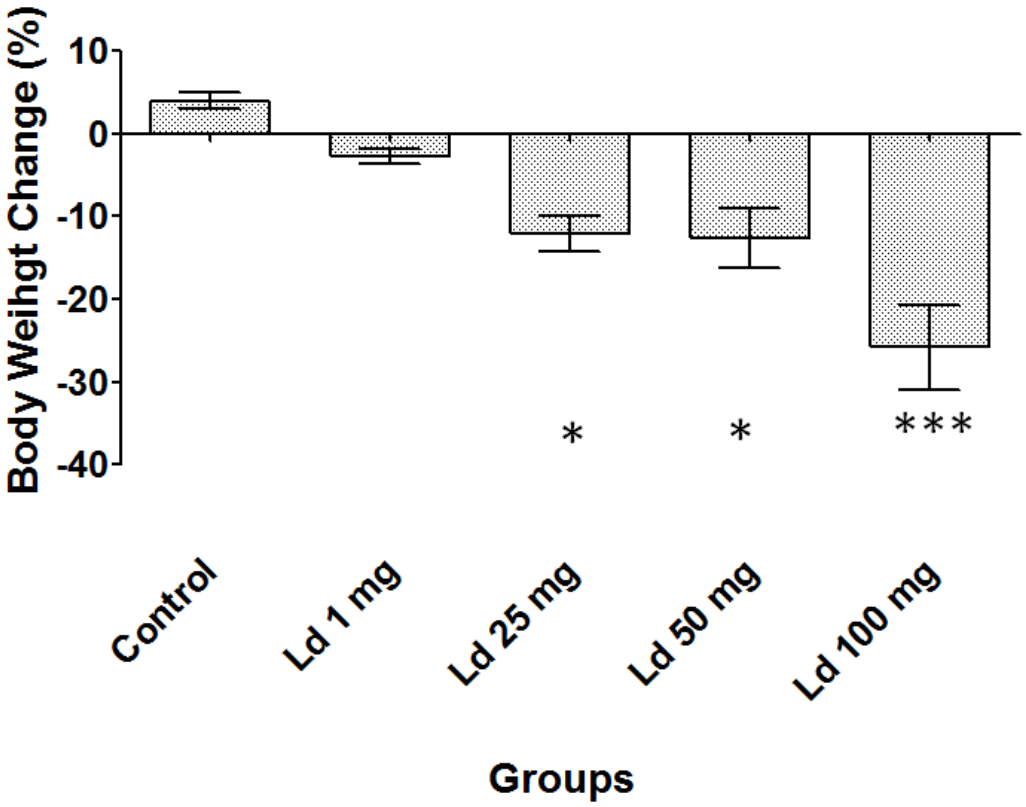

Figure 2: Weight change in non-diabetic animals.
Percentage of body weight change over five days of treatment. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 4-10), *: $p < 0.05$, and ***: $p < 0.001$

3.2.2. BLL Results

No measurable BLL were detected in the control group. BLL in the animals, which were exposed to 1 mg/ kg of lead acetate, were not significantly affected compared with the control group (Figure 3). However, animals receiving 25, 50, 100 mg/ kg of lead acetate had significantly high BLL, in comparison to the control group.

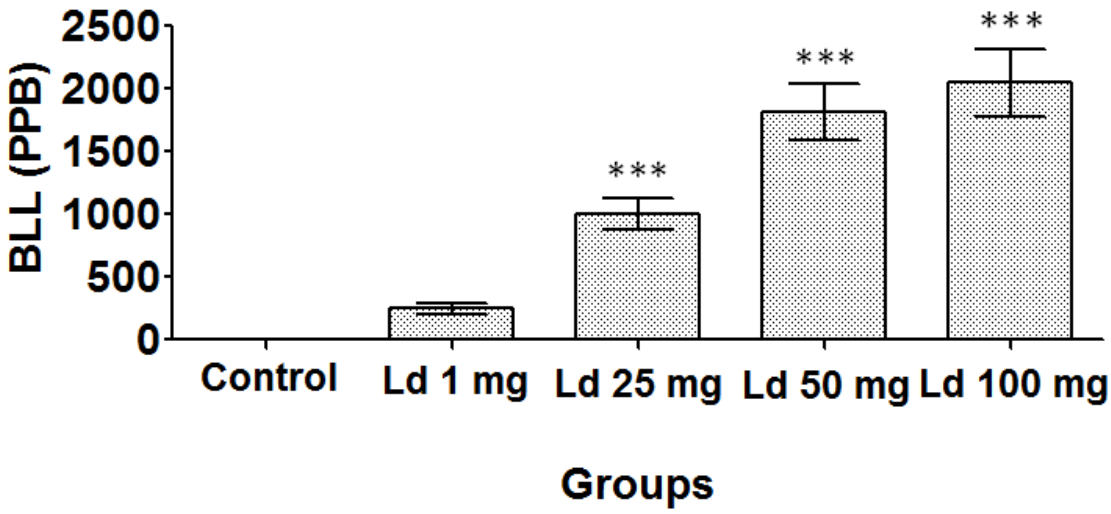

Figure 3: Blood lead levels in don-diabetic animals.
One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 3-5), and ***: $p < 0.001$

3.2.3. Thyroid Function Tests

Compared with the control group, TSH concentrations in the group, which was treated with 1 mg/ kg of lead acetate, were statistically not affected (Figure 4 A). The groups that received higher doses of lead acetate (25, 50, 100 mg/ kg) had a significant increase in TSH concentrations. However, with regard to thyroid hormones, no significant effects on T4 and T3 levels were detected at any used dose of lead (Figure 4 B & C).

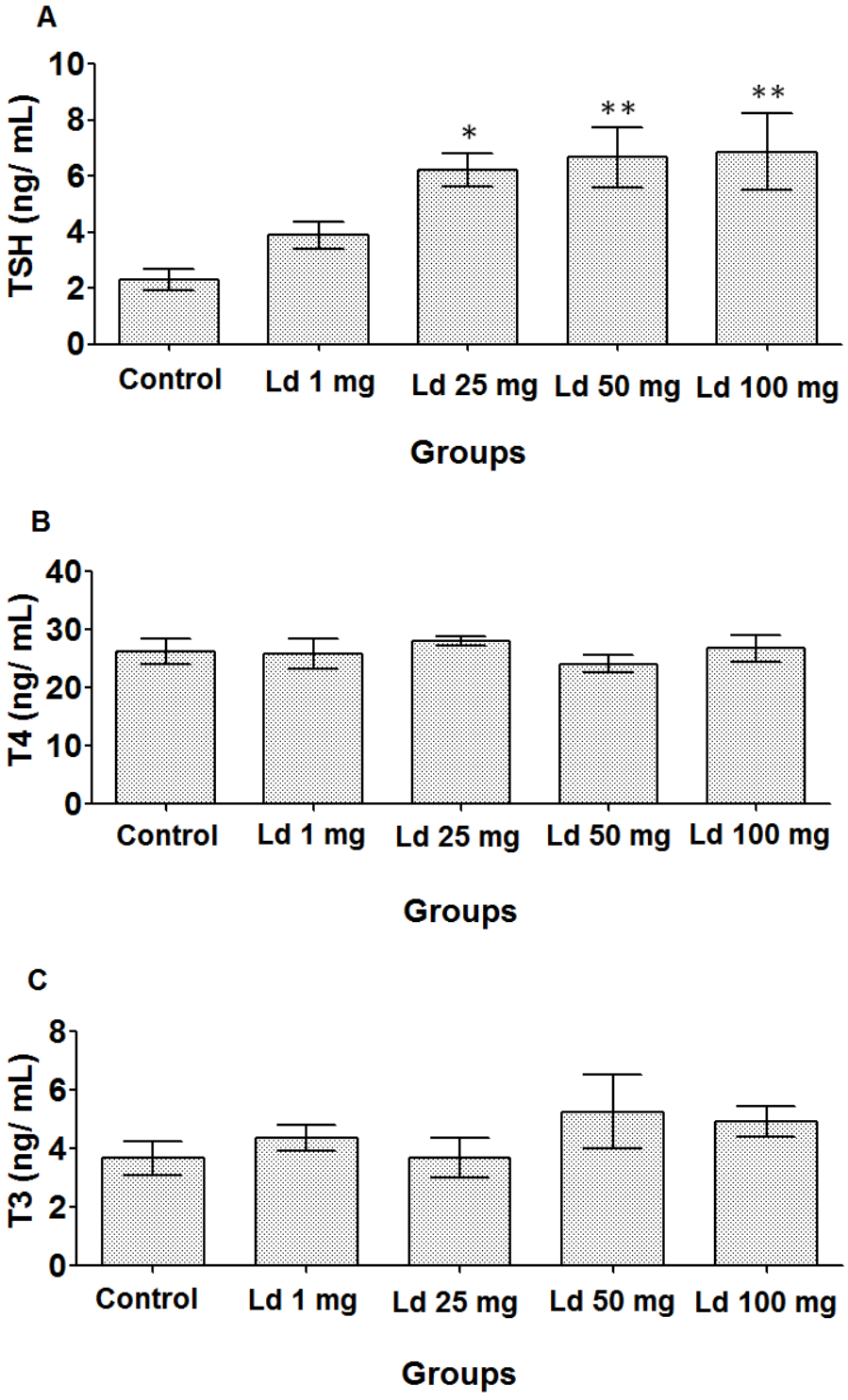

Figure 4: Thyroid function tests in non-diabetic animals.
Levels of (A) TSH. (B) T4. (C) T3. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 5-10), and *: $p < 0.05$, **: $p < 0.01$

3.2.4. GSH Levels in Plasma

Rats exposed to 1 mg/ kg of lead acetate showed a significant increase in GSH levels, compared to the control group; however, no significant effects on GSH were observed at higher doses of lead acetate exposure (Figure 5).

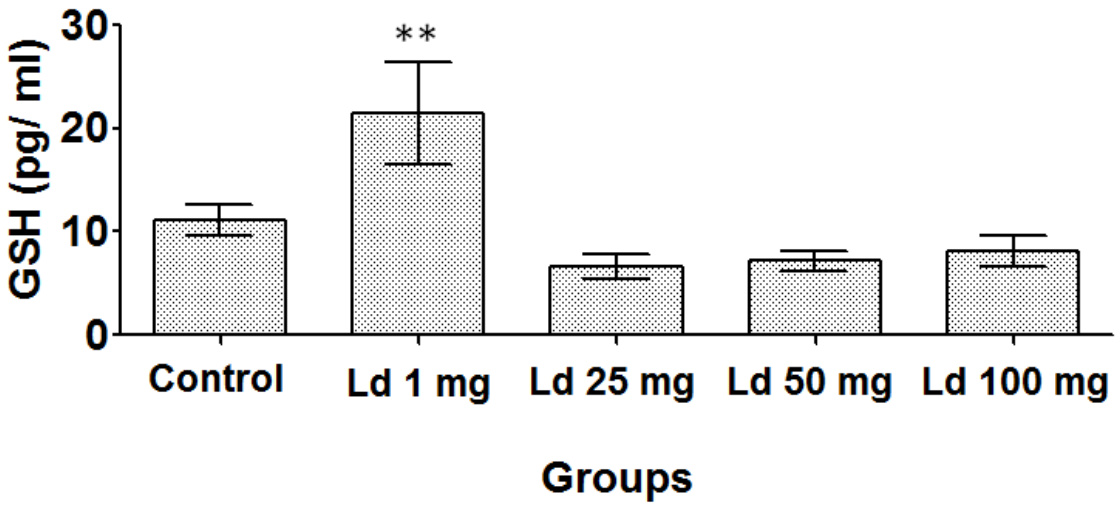

Figure 5: GSH levels in non-diabetic animals.
One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 5-9), and **: $p < 0.01$

3.2.5. CBC Results

We observed a gradual increase in WBC counts with increasing doses of lead acetate; however, the result was statistically significant only in the group receiving 100 mg/ kg of lead acetate (Figure 6 A). On the other hand, platelet counts were significantly reduced in rats that were treated with 100 mg/ kg of lead acetate (Figure 6 B). The current protocol did not affect hemoglobin levels, hematocrit percentage, or RBC counts (Figure 7 A-C).

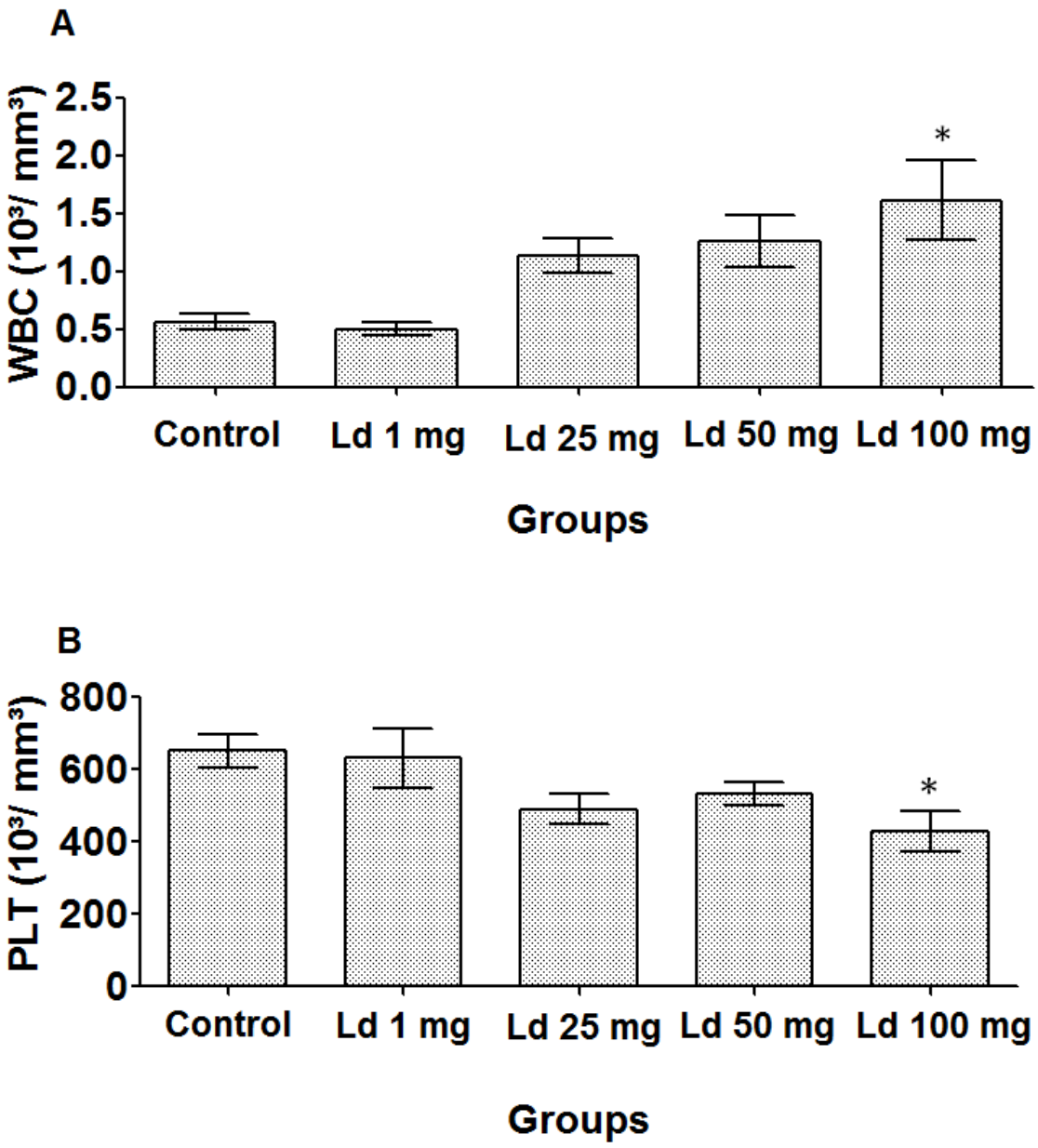

Figure 6: WBC and platelet counts in non-diabetic animals.
(A) WBC counts. (B) Platelet counts. One-way ANOVA test, and Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 5-10), and *: $p < 0.05$

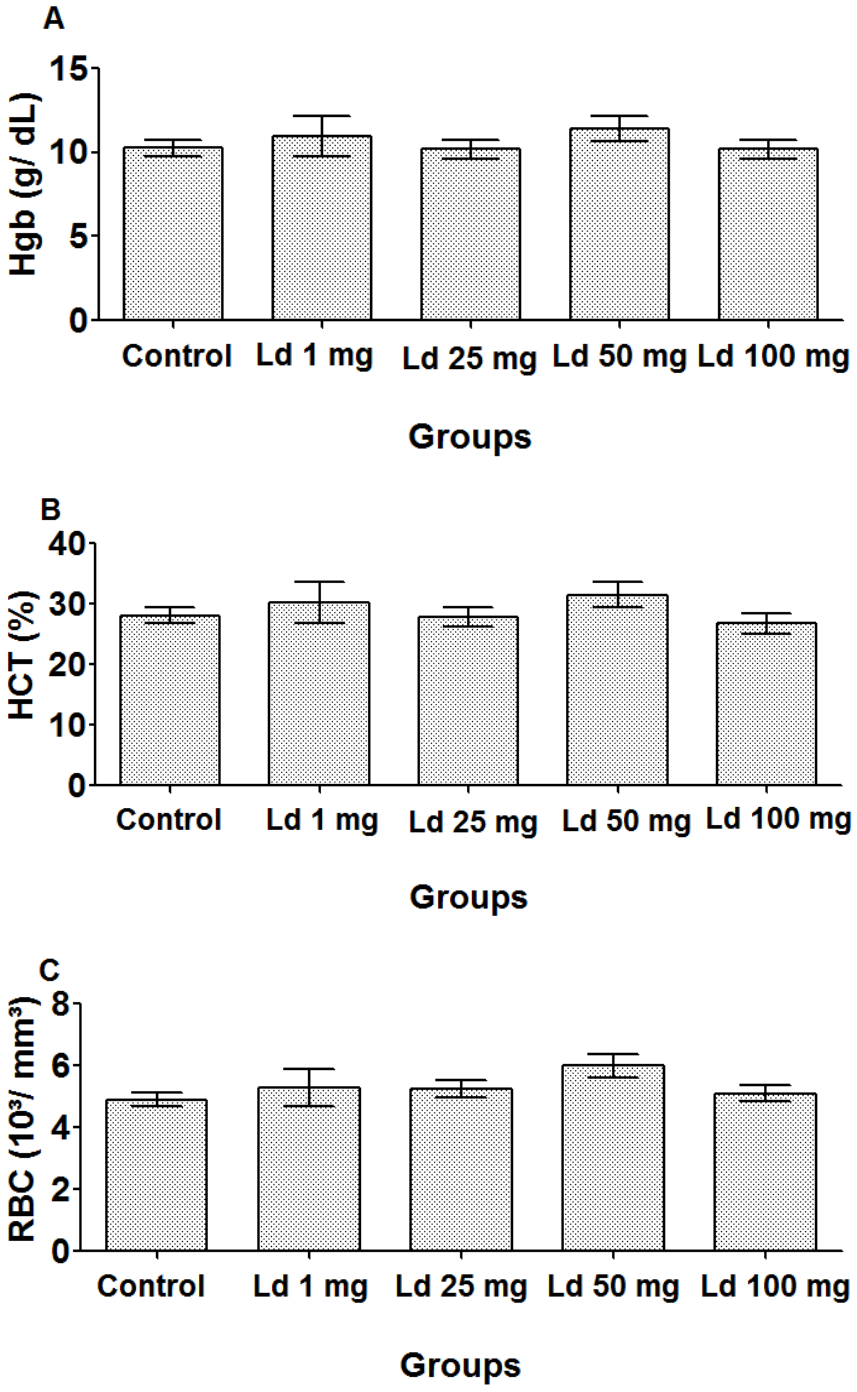

Figure 7: Hemoglobin, hematocrit and RBC counts in non-diabetic animals.
(A) Hemoglobin levels (B) Hematocrit percentage. (C) RBC counts. One-way ANOVA test, and Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 5-10)

3.2.6. Biochemistry Parameters

Treatment with increasing doses of lead acetate resulted in significant effects on several biochemical parameters (Figure 8) and (Figure 9). LDH activity was significantly raised in rats exposed to 50 and 100 mg/ kg of lead acetate (Figure 8 A). Both AST and total bilirubin were elevated in the group that was treated with 100 mg/ kg of lead acetate (Figure 8 B & C). CRP concentrations were significantly high in the groups that received 25 and 50 mg/ kg of lead acetate (Figure 8 D). Although the groups that received lead doses of 25 mg/ kg or higher had lower levels of ALP, the results were not significant (Figure 9 A). ALT levels were not affected and neither albumin nor total protein levels were affected by lead exposure for five days (Figure 9 B - D).

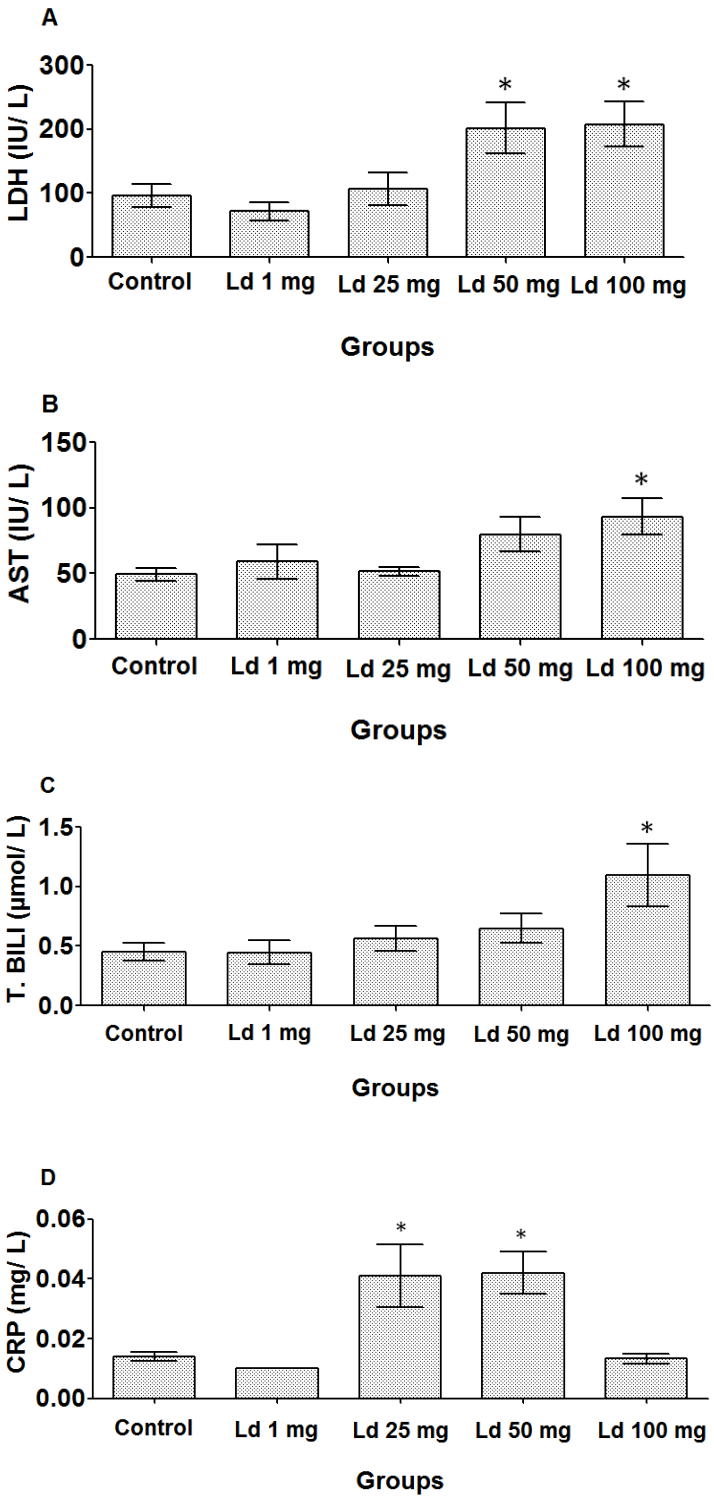

Figure 8: Levels of LDH, AST, total bilirubin and CRP in non-diabetic animals.
Levels of (A) LDH. (B) AST. (C) Total bilirubin. (D) CRP. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 4-11), and *: $p < 0.05$

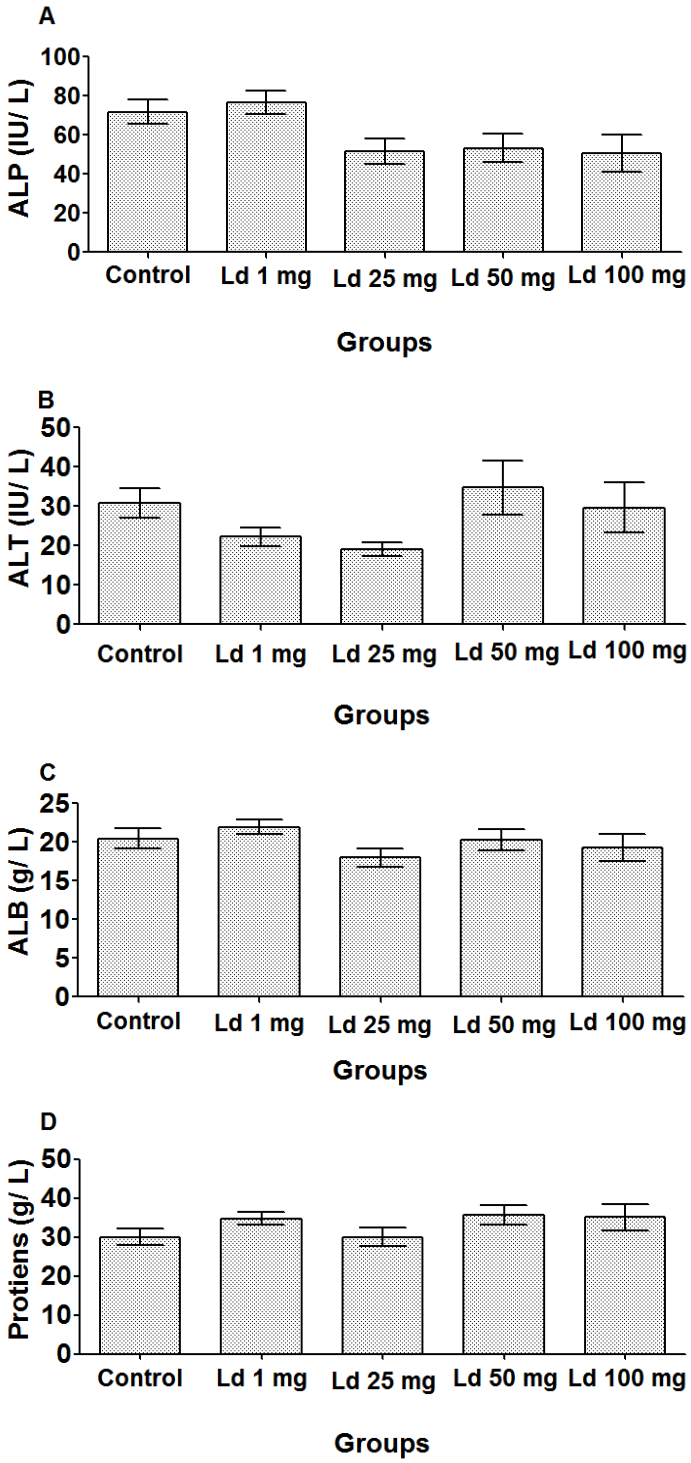

Figure 9: Levels of ALP, ALT, albumin and total proteins in non-diabetic animals. Levels of (A) ALP. (B) ALT. (C) Albumin. (D) Total Proteins. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 4-11)

Relative to renal functions, urea levels increased significantly in the group treated with 100 mg/ kg of lead acetate (Figure 10 A). Compared with the control group, creatinine levels remained statistically insignificant (Figure 10 B).

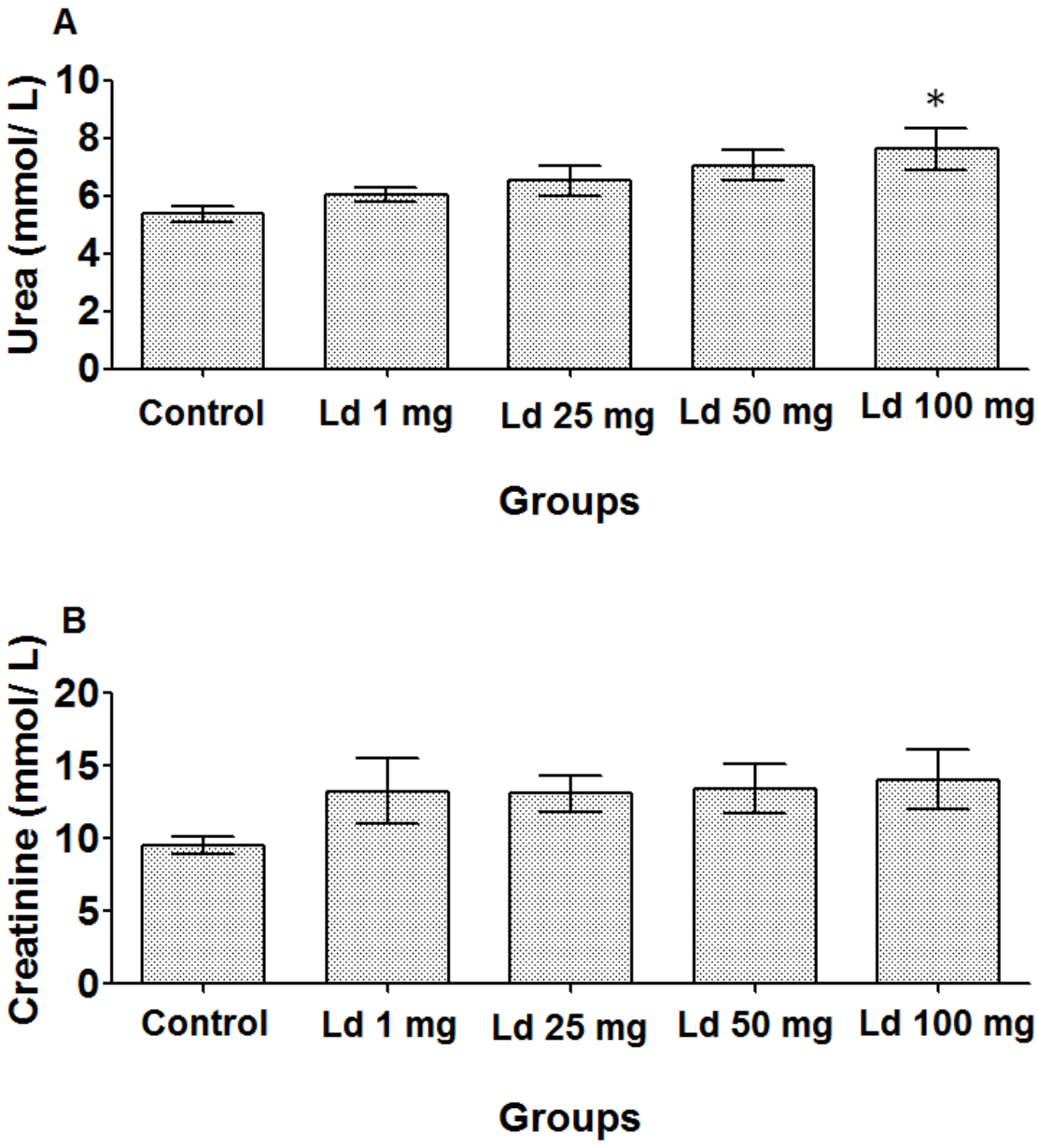

Figure 10: Urea and creatinine levels in non-diabetic animals.
(A) Urea levels. (B) Creatinine levels. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 6-11), and *: $p < 0.05$

3.3. Discussion

In this model, we demonstrated that lead acetate exposure increased the secretion of TSH from the anterior pituitary gland. It also caused impairments in renal and hepatic functions. Moreover, markers of oxidative stress (GSH) and inflammation (WBC and CRP) increased in response to lead exposure.

As the dose of lead increased, evident from BLL, animals lost more and more weight. Weight loss might be attributed to several factors e.g. the multisystemic toxic effects of lead exposure, and muscle and fat wastage. In addition, sick animals often lose appetite and have low food intake. Previous occupational-exposure studies have shown that parental lead exposure, both maternal [195] and paternal [196], is associated a reduction in the birth-weight of their infants. In comparison, when rats were exposed for one month to either 50 mg/ L of lead acetate in drinking water given ad libitum [197] or treated with daily intramuscular injections of lead acetate (first dose 4 mg/ 100 g, later doses 0.05 mg/100 g) [198], no significant effects on weight were observed.

In our study, BLL corresponded directly with the administered doses of lead acetate. The increases in BLL in a dose-response manner demonstrate the effectiveness and usefulness of i.p. route administration and the level of absorption of lead acetate through this route. Although this is not the natural route of exposure to lead in humans, the advantage of using i.p. injections is that it eliminates the differences in exposure levels compared to ad libitum oral exposures.

In order to convert the measured BLL in ppb to µg/ dL, which is the unit of measurement for BLL in clinical settings, we used the conversion factor: 1 µg/ dL = 10 ppb [199, 200]. As such, the measured BLL (in ppb) in the four treatment groups

correspond to the following clinically equivalent values: 25, 100, 181, and 205 µg/ dL, respectively. For diagnostic purposes, 25 µg/ dL of BLL is considered a mild condition, whereas, any value exceeding 80 µg/ dL represents a severe condition and might result in life threatening medical emergencies e.g. encephalopathy [201].

The hypothalamus-pituitary-thyroid axis is controlled by three mechanisms. The first one is the long loop i.e. thyroid hormones acting on the hypothalamus. There is also some control from the pituitary to the hypothalamus through the short loop. The last mechanism is within either the hypothalamus or pituitary (ultra-short loop) [202].

Our data show that lead exposure resulted in a clear dose-response effect on the anterior pituitary, causing an elevation in TSH levels. On the other hand, thyroid hormones, T4 and T3, were not affected. These findings, the combination of high TSH and normal T4 and T3 levels, are in line with a common clinical presentation, known as ‘subclinical hypothyroidism’ [203], which is considered to be a ‘compensatory stage’ that maintains thyroid hormones within normal levels [204]. This condition, which is often associated with autoimmune abnormalities or the use of exogenous drugs, is the early stage and a risk factor for developing full-blown hypothyroidism [31, 203].

The increasing TSH levels observed might demonstrate a direct stimulatory effect of lead on the anterior pituitary. High TSH levels might also indicate a disturbance in the mechanisms that control the long feedback loop process, whereby the hypothalamus and the pituitary fine-tune and decrease TSH secretion when T4 and T3 levels are normal [205]. Alternatively, there might be an interruption in the signaling process of the downward pathway from the pituitary; that is why high TSH levels could not induce an increased secretion of T4 and T3 [205]. In addition, the feedback control system is

relatively slow and can take some time until the features and signs of overt hypothyroidism are seen, and this gives rise to the biochemical presentation of subclinical hypothyroidism [206]. It has been also reported that TSH is secreted as a mixture of isoforms and this might result in having variable biological effects [207]. Apart from the stimulatory effects of TSH on the thyroid, other factors e.g. thyroglobulin can exert a negative feedback effect on the thyroid gland [208].

In regard to thyroid parameters, previous studies showed variable results. For instance, in an occupational-health cross-sectional study, 58 male workers with an average BLL of 51.9 µg/ dL were found to have high TSH levels and no effects on T4 and T3 levels [209]. In another occupational setting, high BLL of more than 60 µg/ dL were associated with a reduction in total and free T3 levels [210]. Yet, other studies in similar environments could not demonstrate any associations at all in workers with BLL of up to 75 µg/ dL [211, 212]. Finally, when Sprague Dawley rats were exposed to 25, 50 and 100 mg/ kg of lead acetate, the results showed a significant increase in TSH and a reduction in both of T3 and T4 levels in serum [213].

Glutathione (GSH) is one of the main defense mechanisms against reactive oxygen species (ROS) in our bodies. Our results showed that a low dose of lead treatment i.e. 1 mg/ kg stimulated the animals to increase the production of GSH, thereby counteracting the toxic effects of lead exposure and avoiding any damage to the tissues. Higher doses of lead acetate (25, 50, 100 mg/ kg) resulted in a reduction in GSH levels, albeit not statistically significant. Dewanjee, S., et al. [214] has shown that longer periods (40 days) of lead exposure caused significant depletions of GSH stores in several organs including the kidneys, liver, heart, and brain. The cause for this discrepancy might be

related to the difference in exposure periods (in our case 5 days of treatment) and to the fact that we measured GSH levels in plasma and not in tissues. As such, additional studies are needed to clarify this issue.

Our data showed an increase in WBC counts and CRP levels, which is a clear indication of the possibility of an inflammatory reaction in response to lead exposure. The increased level of CRP observed in our study is consistent with a few, recently-published studies, which were conducted in the general population [176] and in occupational settings [215]. Taken together, these findings suggest that inflammation might represent a possible mechanism for the adverse health effects of lead toxicity.

In this study, lead exposure resulted in a general trend of low platelet levels. This could be due to an inhibitory effect on the bone marrow, leading to decreased platelet production [216]. It can also indicate platelet activation in vivo. Moreover, it has been reported that exposure to lead caused an increase in pro-thrombotic effects in mice [217, 218]. A very recent study on occupationally exposed workers to lead-acid batteries demonstrated a similar reduction in platelet counts [219]. In addition, it has been previously demonstrated that exposure to other environmental pollutants such as ultrafine particles also caused a decrease in platelet numbers in patients with cardiovascular disease [220]. Furthermore, the intra-tracheal instillation of a single dose of diesel exhaust particles decreased platelet numbers in type 1 diabetic mice [221].

In the present study, we assessed the effects of lead exposure on the liver and kidneys. This is because among soft tissues, the liver is the most important storage site for lead [174]. Regardless of the route of exposure, inorganic lead is eventually excreted unchanged mainly in either urine or bile [175]. Also, it has been reported that a

significant amount of T4 is converted into T3 in these organs [222].

Higher doses of lead acetate caused an elevation in the levels of two hepatic enzymes, namely LDH and AST. These markers are released into the bloodstream whenever there is tissue breakdown [223, 224]. Similarly, the increase in total bilirubin levels also results from hepatocytes' damage [224, 225]. Since conjugated bilirubin is water-soluble and will be eventually excreted in urine, high levels of total bilirubin can indicate a failure in the glucuronidation and conversion process of unconjugated bilirubin to the conjugated one [226]. Thereby, total bilirubin levels build up in the circulation. In normal situations, heme oxygenase converts heme into bilirubin. Exposure to lead in animal studies activated this reaction i.e. lead exposure increases bilirubin levels [47, 227]. Previous studies of occupationally exposed workers and animal experiments observed a similar increase in AST, ALT, and total bilirubin levels [192, 228, 229]. In contrast, another occupational health study could not find any effects on cholesterol, LDH, and ALP levels [230].

In contrast, the synthetic abilities of the liver, manifested by albumin and total protein levels, are still well preserved at this stage. These functions might deteriorate only in chronic lead exposures, which are often accompanied by severe malnutrition or advanced disease status. As such, chronic exposures in Indian workers were associated with low total protein and albumin levels [228]. Similarly, chronic lead exposure of laboratory animals led to a reduction in total proteins [214].

After circulating in the body, a significant amount of lead is filtrated in the glomeruli and excreted unchanged in urine [47]. The increase in urea levels, observed in our study, is an indication of renal dysfunction. Regarding creatinine, its total blood levels

represent the overall levels, released from various tissues including kidneys, heart and skeletal muscles. Although renal impairment is often associated with an increase in creatinine levels, the latter remained insignificant in this study. This might be due to reduced muscle bulk, seen as weight loss. Similar results of no effects on creatinine levels were obtained when rats were treated with 8 mg/ kg of lead acetate i.p. for two weeks [231].

3.4. Conclusion

In this model, our results showed that lead exposure had significant effects on many systemic parameters. These included causing an elevation in TSH secretion from the anterior pituitary gland. In addition, lead exposure negatively affected the functions of the liver and kidneys. In addition, oxidative stress markers e.g. GSH and inflammatory parameters i.e. WBC and CRP were increased.

Chapter 4: Lead's Effects in Diabetic Model

4.1. Background

Being a ubiquitous element in the environment, lead causes toxic effects to various organ systems. Although environmental lead exposures are common, the most important sources currently are occupational settings [2], which usually involve exposures to inorganic lead [3, 32].

Over the last few decades, modern lifestyles have increased the prevalence of several non-communicable multisystemic diseases including diabetes mellitus (DM). In 2008, there were about 347 million people with diabetes [169]. According to WHO, the worldwide prevalence of DM in 2014 was 9% in adults aged 18 years or older, and 90% of these cases are type 2 diabetes [232]. WHO also estimated that DM was a direct cause for 1.5 million fatalities in 2012, and the majority (80%) of these deaths took place in low to middle income regions of the world [232]. Diabetes is a risk factor for cardiovascular diseases, neuropathies, and is also a main cause for blindness, amputations and renal failure [232]. In addition, the risk of death in diabetics is twice in comparison to the risk in non-diabetic people [232]. In the UAE, the prevalence of DM among expatriates ranges from 13 to 19%, obviously depending on their genetic backgrounds. On the other hand, 25% of Emiratis are diabetic [233]. With such high levels, the UAE has the second highest global prevalence of DM [234]. Because of the seriousness of this condition, WHO and in collaboration with the international diabetes federation designated the 14th of November of each year as 'world diabetes day' in order to raise awareness and to advocate for the prevention and the proper management of DM [232].

It has been estimated that thyroid disorders would eventually develop in about one third of type I diabetic patients [235], indicating that there is a strong association between these two conditions. In Spain, the annual incidence of thyroid dysfunction in patients with Type II diabetes was reported to be around 10%, whereas its prevalence was about 32% [236].

In experimental animal research, streptozocin (STZ), which is an alkylating agent that causes DNA damage, cross-linking [237], DNA strand breaks [172], and eventually DNA destruction and thus cell death [170], is frequently used for studying type I (insulin dependent) diabetes mellitus [238]. STZ also causes depletion of NAD and ATP, which will at the end lead to a cessation in insulin secretion and the development of type 1 DM [169]. Diabetes will develop within few days post STZ-injection; usually 4 days in mice and 7 days in rats [170]. STZ can be administered either as a single or multiple doses [169]. Being similar to the natural disease, this model can also induce an immune-mediated reaction [172] and can affect the thyroid functions of the treated animals. Because of the oncogenic features of STZ, it can lead to the development of hepatic and renal tumors [170]. Other STZ disadvantages include the possibility of spontaneous regeneration of beta cells in the pancreas and having low lymphocyte counts [169].

When 80 mg/ kg of STZ was given through sublingual injections, T3 levels in Sprague-Dawley (SD) rats had a statistically significant drop only during the first two weeks of induced DM, whereas, T4 levels remained low throughout the 7 weeks of assessment i.e. the levels of only the biologically active hormone, namely T3, were maintained [239].

Histological studies of thyroid glands showed that exposure to lead acetate can cause hypertrophy of the follicular epithelial cells, enlargement in the volume of follicles and reduced staining, which indicates low thyroglobulin levels [240, 241]. There might also be excessive fibrotic tissue formation due to necrosis [240].

Currently, oxidative stress is correlated with a variety of pathological conditions and complications including type 1 and 2 of DM [242, 243]. In addition, because of the intrinsic nature of the synthesis of thyroid hormones since iodide has to be oxidized first, interactions with reactive oxygen species (ROS) such as the oxidizing agent hydrogen peroxide (H_2O_2) may obviously result in glandular pathologies [244]. Many thyroid abnormalities such as overt hypothyroidism, Graves' disease, and Hashimoto thyroiditis have been associated with oxidative stress as the levels of SOD were found to be elevated [245, 246].

4.2. Results

4.2.1. Weight Monitoring

The initial weight (before the induction of DM) of the adult male Wistar rats, used in this experiment, ranged from 216 to 288 grams. A week later, there was a significant drop in the weight of these animals (Figure 11). Then over the next few weeks, the animals regained some weight.

Figure 11: Weight change post DM induction in diabetic animals.
The mean change in weight of all animals over the six weeks post DM induction, the initial weight in week 1 versus the weight in each subsequent week are compared. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 45), *: $p < 0.05$

During the experiments, the diabetic control group maintained its weight throughout the five days of treatment. However, treatment of the diabetic rats with various doses of lead acetate (25, 50, and 100 mg/ kg) resulted in significant reductions in the weight of these animals (Figure 12). The group that was treated with 100 mg/ kg of lead acetate had the highest amount of weight loss.

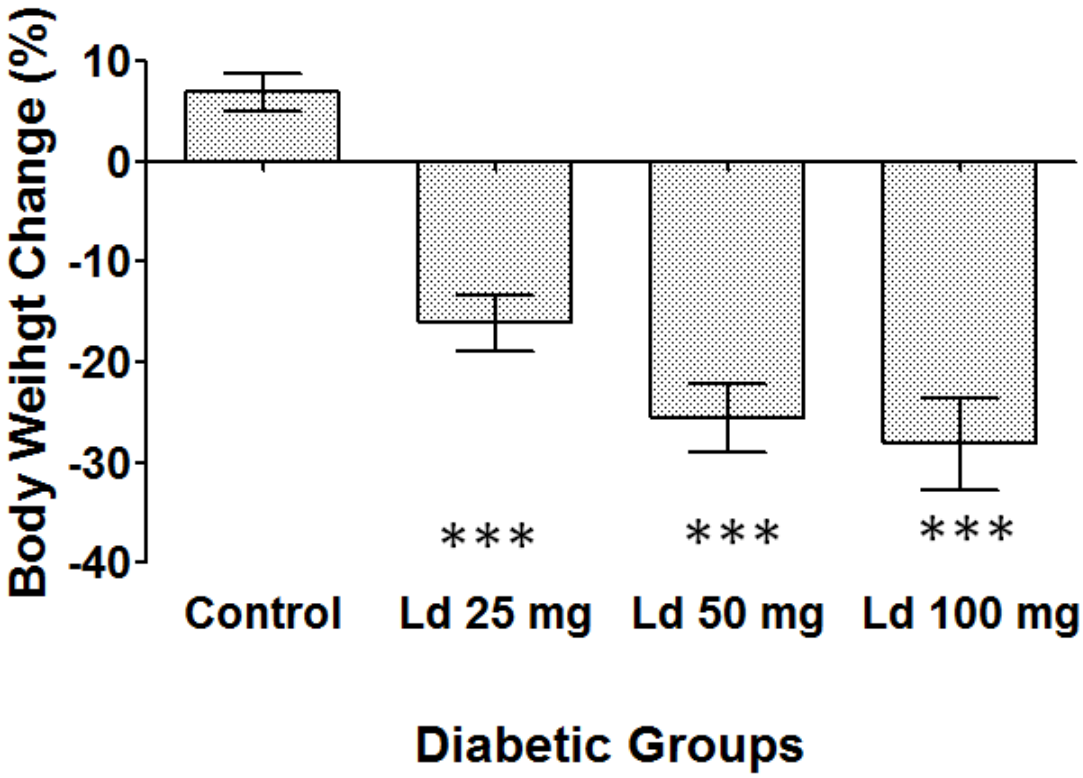

Figure 12: Weight change over five days of treatment in diabetic animals.
Percentage of body weight change over five days of treatment. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 9), and ***: $p < 0.001$

4.2.2. RBS Results:

Six weeks post DM induction; the animals had an RBS level ranging from 394.0 to 600.0 mg/ dL with a mean level of 546.3 ± 8.9 mg/ dL.

4.2.3. BLL Results

The control group did not have any detectable BLL (Figure 13). In comparison, the groups that were treated with lead acetate had increasing and statistically significant

BLL. Treatment of the rats with 25, 50, and 100 mg/ kg of lead acetate caused BLL of 1228, 1498, and 2330 ppb, respectively.

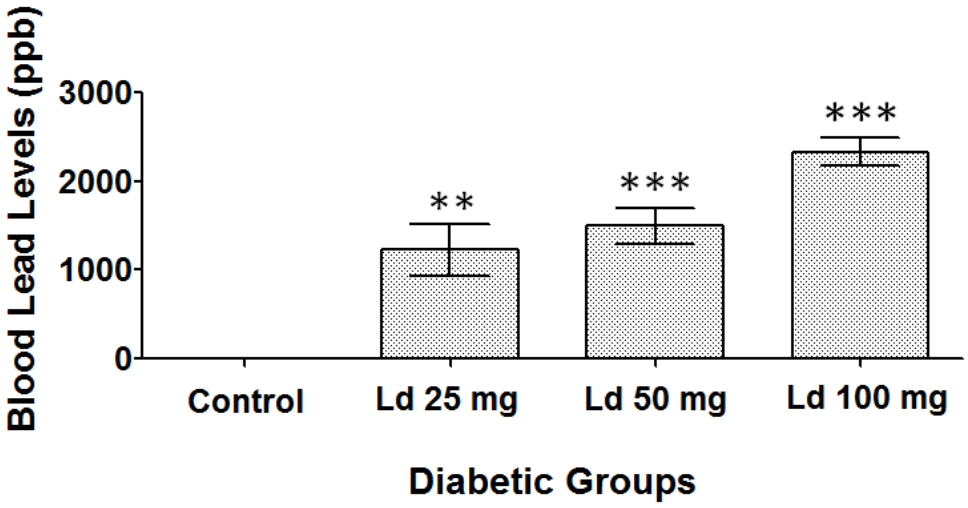

Figure 13: Blood lead levels in diabetic animals.
Blood lead levels (BLL). One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 4-7), and **: $p < 0.01$, ***: $p < 0.001$

4.2.4. Thyroid Function Tests

There was an increase in TSH levels in the groups that were treated with 50 and 100 mg/ kg of lead acetate, compared with the control group (Figure 14 A). However, the level was statistically significant only in the group that received the highest dose. On the other hand, treatment with 50 and 100 mg/ kg of lead acetate caused a statistically significant reduction in T4 levels (Figure 14 B). Similarly, the decrease in T3 levels was significant only in these same two groups i.e. 50 and 100 mg/ kg of lead acetate (Figure 14 C).

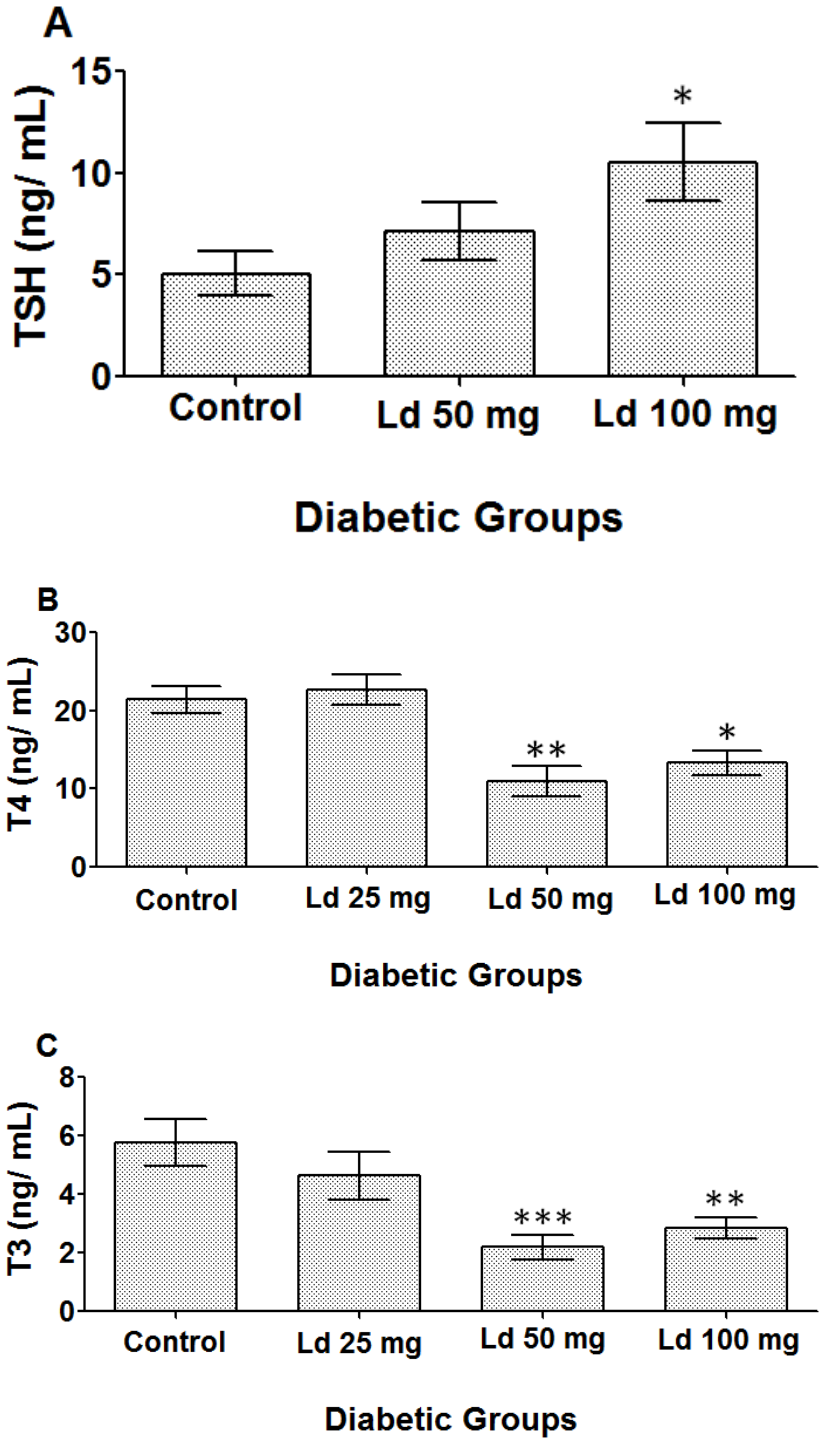

Figure 14: Thyroid function tests in diabetic animals.
Levels of (A) TSH. (B) T4. (C) T3. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 5-10), and *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$

4.2.5. Oxidative Stress Markers in Plasma

Compared to the control group, there was a reduction in GSH levels in all treated groups; however, the low levels were statistically significant only in the groups that received high lead acetate doses (50 and 100 mg/ kg) (Figure 15 A).

On the other hand, regarding MDA levels, no statistically significant results were observed in the various treatment groups (Figure 15 B).

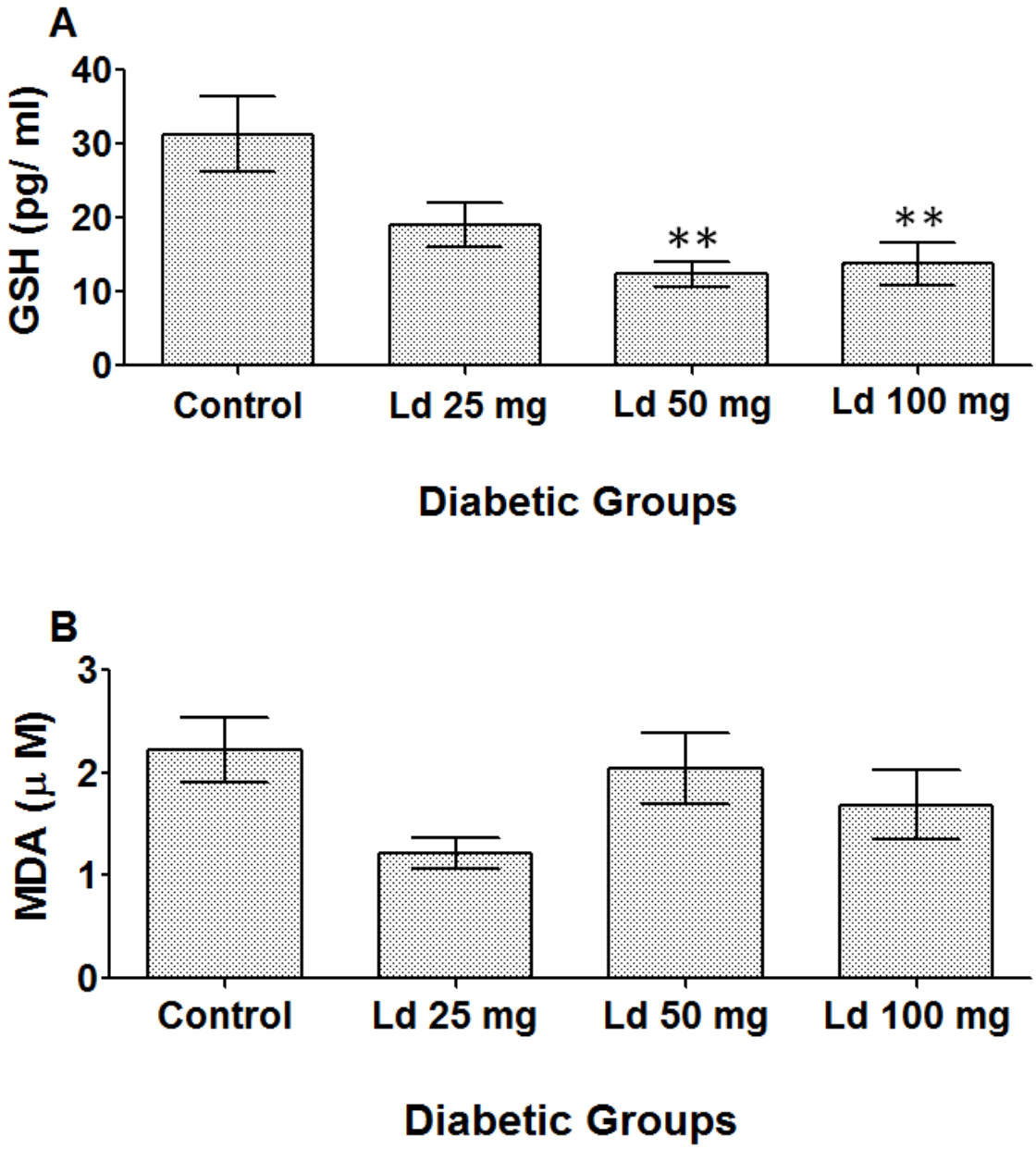

Figure 15: Levels of GSH and MDA in diabetic animals.
Levels of (A) GSH. (B) MDA. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 6-12), and **: $p < 0.01$

4.2.6. CBC Results

At the given treatment doses and exposure durations, CBC parameters including WBC counts and platelet counts were well preserved and no significant effects were detected (Figure 16 A & B). Similarly, no significant effects were observed regarding hemoglobin levels, RBC counts and hematocrit percentage (Figure 17 A - C).

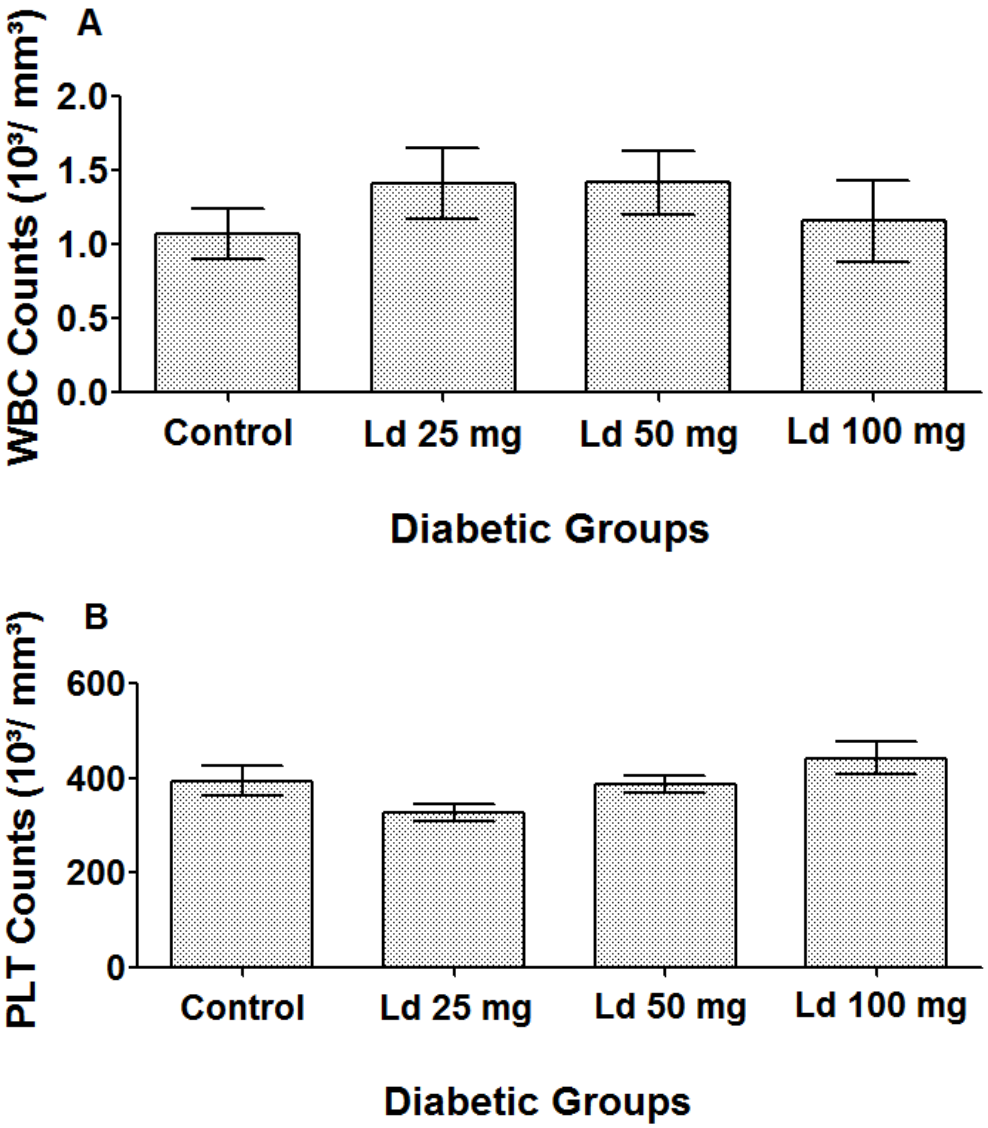

Figure 16: WBC and platelet counts in diabetic animals.
(A) WBC counts. (B) Platelet counts. One-way ANOVA test, and Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 7-10)

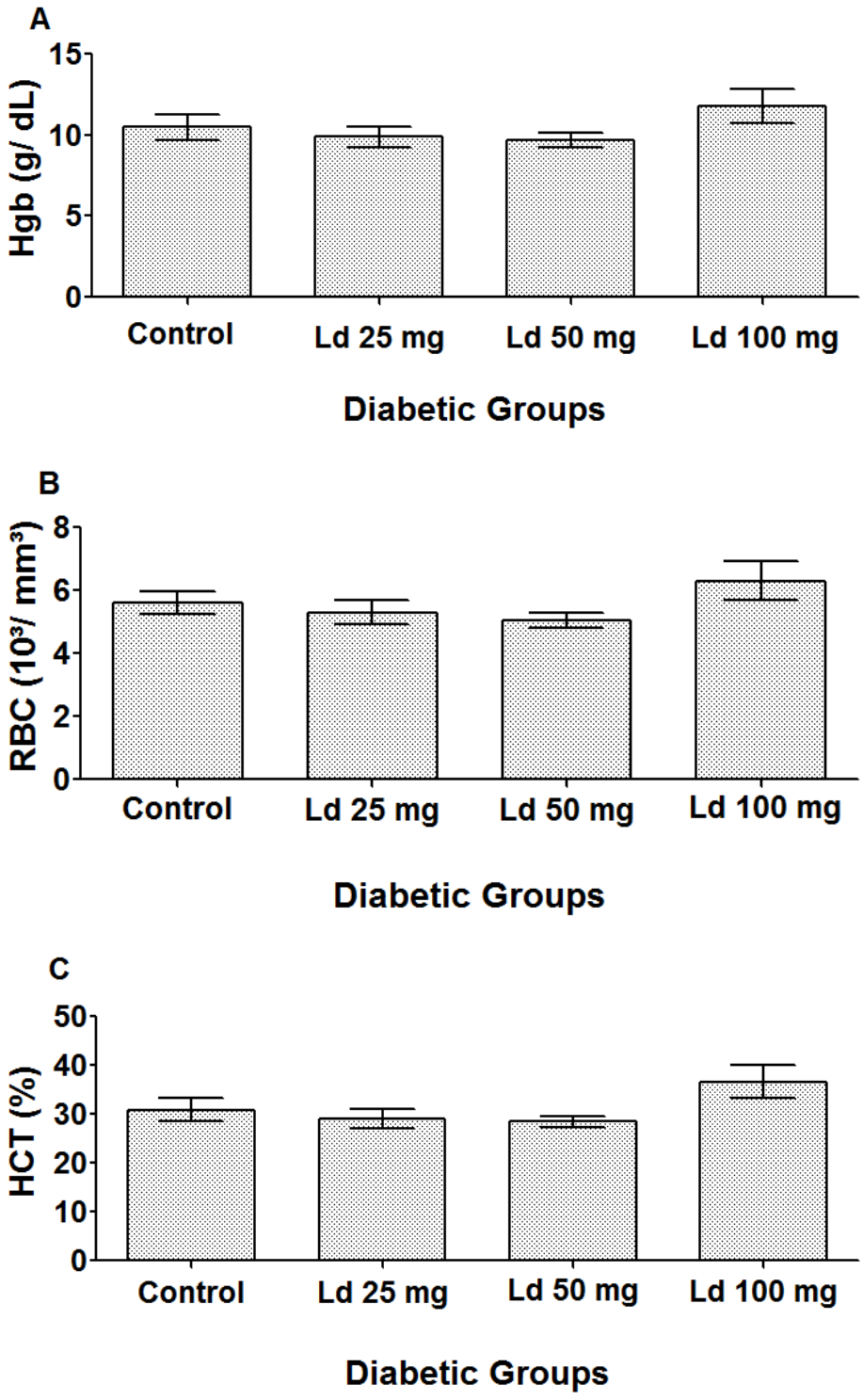

Figure 17: Hemoglobin, hematocrit and RBC counts in diabetic animals.
(A) Hemoglobin levels. (B) RBC counts. (C) Hematocrit percentage. One-way ANOVA test, and Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 7-10)

4.2.7. Biochemistry Parameters

Lead exposure resulted in significant effects on renal and hepatic functions. In comparison to the control group, treatment with high doses of lead acetate i.e. 50 and 100 mg/ kg caused a statistically significant increase in urea levels (Figure 18 A) and at the same time a significant reduction in creatinine levels (Figure 18 B). ALT levels were significantly low in all the groups that were exposed to lead acetate (Figure 19 A). As for ALP, treatment with increasing doses of lead acetate caused a gradual and significant decrease in its levels in the three treatment groups (Figure 19 B). On the other hand, lead exposure did not have any significant effect on AST (Figure 19 C). Likewise, differences in the levels of CRP, ALB, and total proteins were statistically insignificant (Figure 20 A - C).

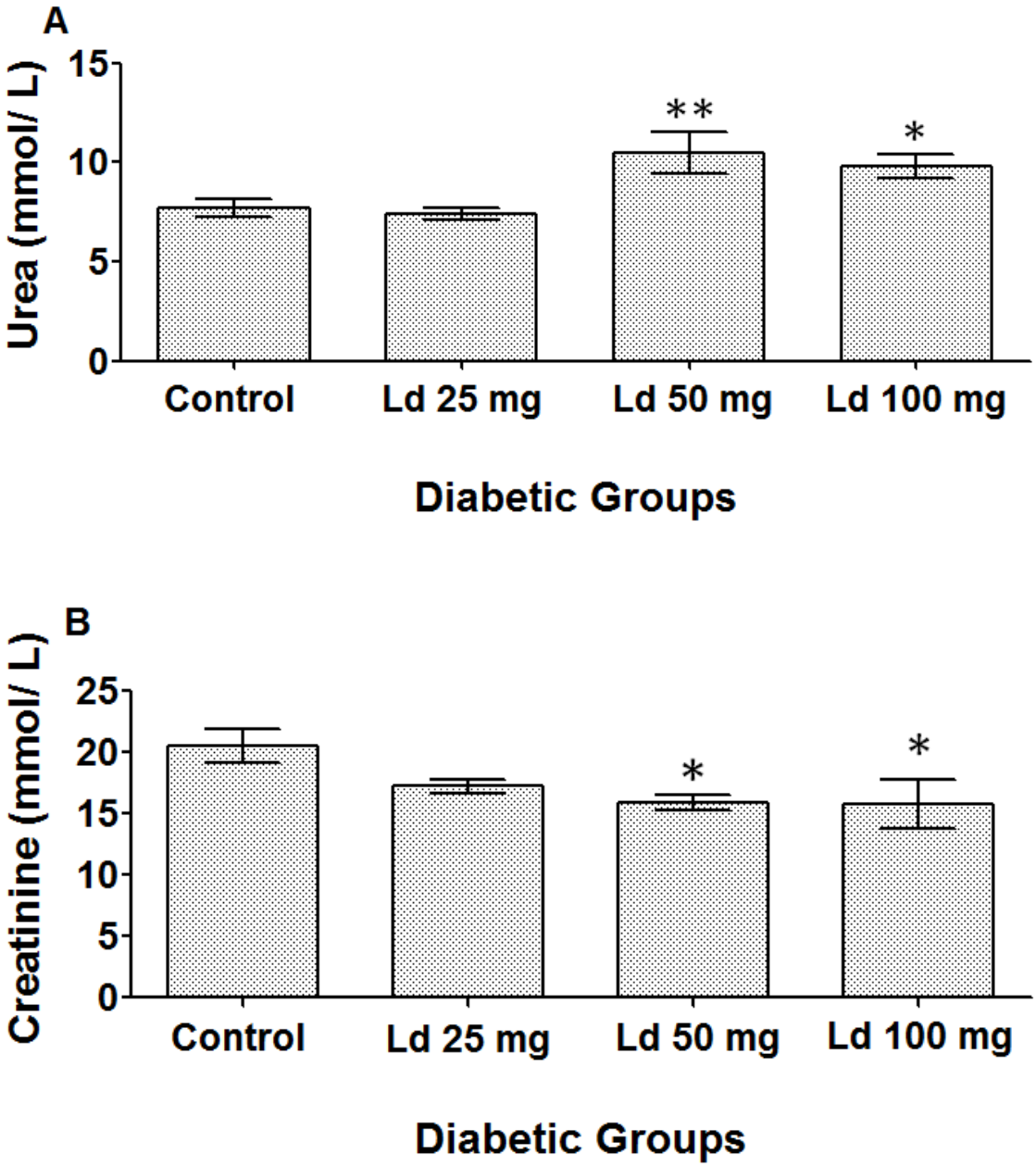

Figure 18: Urea levels and creatinine levels in diabetic animals.
(A) Urea levels. (B) Creatinine levels. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 7-14), and *: $p < 0.05$ and **: $p < 0.01$

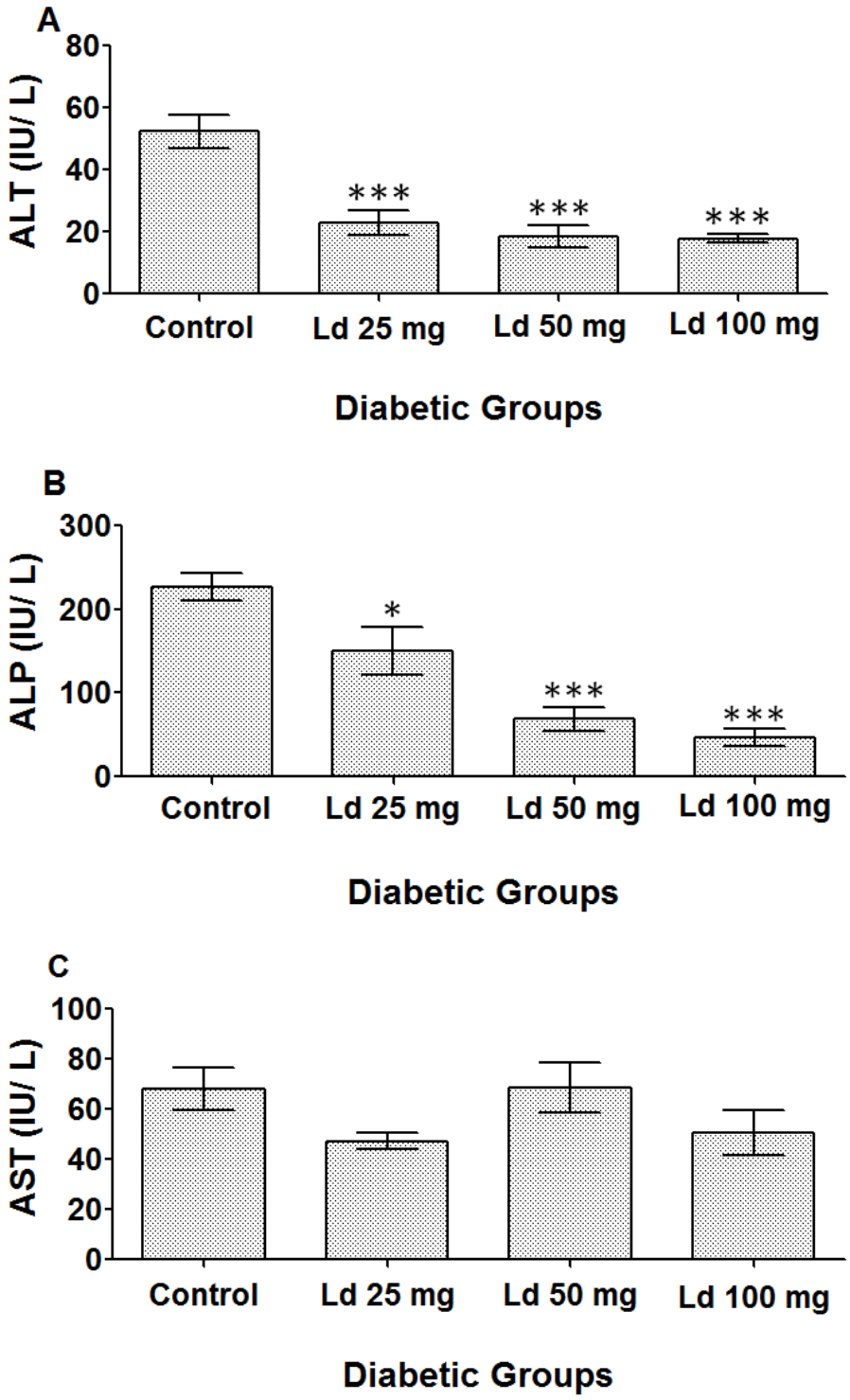

Figure 19: Levels of ALT and ALP in diabetic animals.
Levels of (A) ALT. (B) ALP. (C) AST. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 7-13), and *: $p < 0.05$, and ***: $p < 0.001$

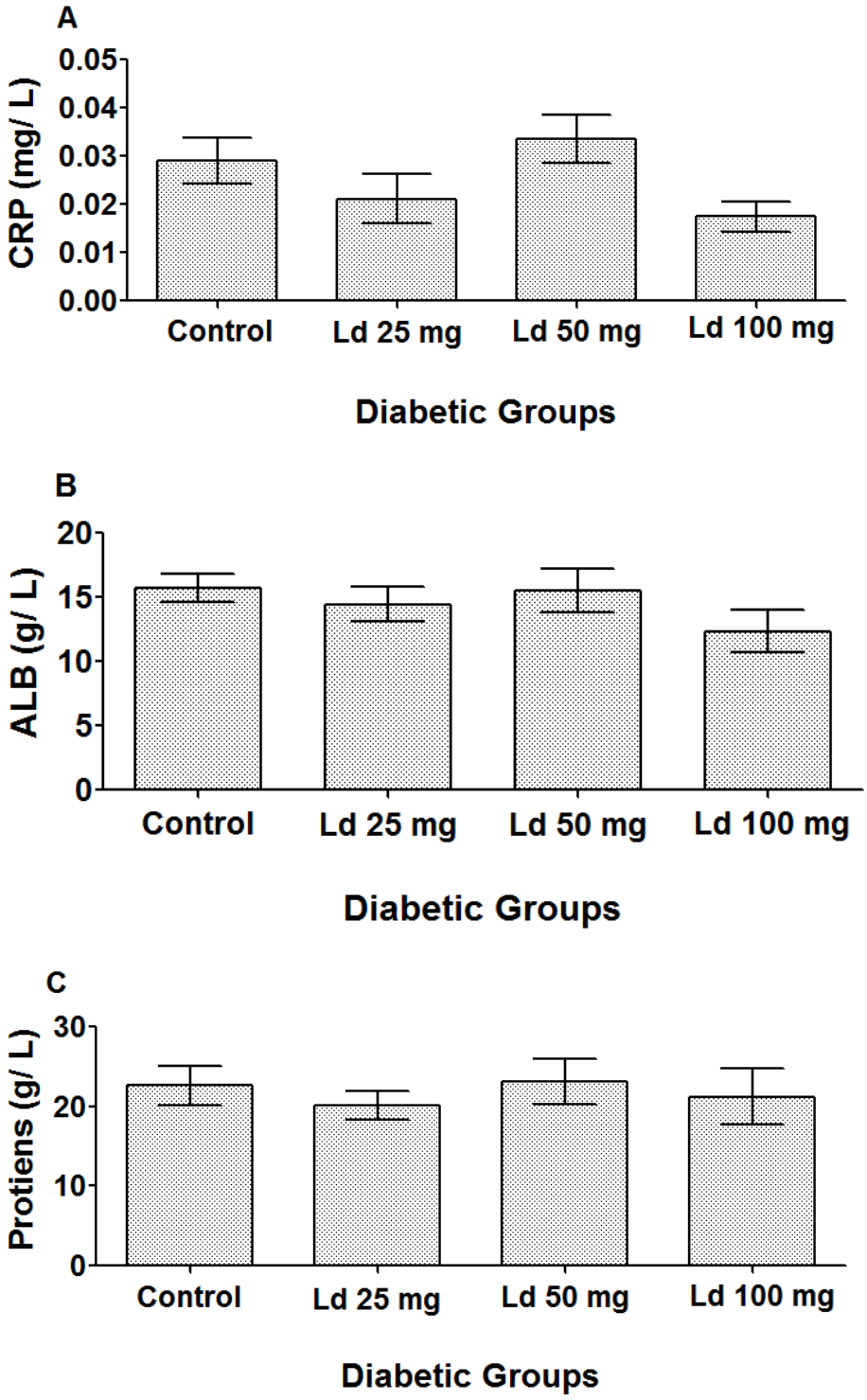

Figure 20: Levels of CRP, ALB and total proteins in diabetic animals.
Levels of (A) CRP. (B) ALB. (C) Total proteins. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 7-14)

4.3. Discussion

In this model, we investigated the effects of lead on the thyroid glands in STZ-induced diabetic rats. Lead exposure resulted in significant effects on the pituitary-thyroid pathway, leading to an increase in TSH levels and reductions in T4 and T3 levels. Our results demonstrated that lead exposure made the animals emaciated. Moreover, when the effect of lead on other systems was studied, our results showed a depletion of GSH stores and deleterious effects on renal parameters.

Our data showed that the rats lost weight during the first week of DM induction. This could be attributed to the toxic effects of STZ and the development of DM. However, over time, these animals recovered and regained some weight. In comparison, normal non-diabetic animals usually gain a significant amount of weight over time. However, our data showed that these diabetic rats maintained nearly the same weight throughout the six weeks post DM induction. The lack of normal increase in weight in these diabetic animals is most probably due to STZ exposure. A previous study showed that in comparison to a control group, weight was not affected 12 weeks post-injection with 60 mg/ kg of STZ [247]. However, in another study it was shown that six weeks after the administration of 50 mg/ kg of STZ to Wistar rats, there was a significant amount of weight loss in the STZ-induced diabetic animals in comparison to the control group [248].

Although the diabetic control rats maintained their weight during the five days of treatment, lead acetate treated groups lost significant amounts of weight. This was most significant for the group that was treated with 100 mg/ kg of lead acetate as these animals lost around 28% of their initial weight during the treatment period. A previous

study showed a similar effect of weight loss when animals were treated with 100 µL of 20 mg/ kg of lead acetate i.p. for 5 days [249].

There was a very positive relationship in a dose-response manner between the administered doses of lead acetate and the measured BLL in plasma. The exposure to 25, 50, and 100 mg/ kg of lead acetate resulted in BLL of 1228, 1498, and 2330 ppb, respectively. Using the conversion factor: 1 µg/ dL = 10 ppb [199, 200], the measured BLL in ppb in the three treatment groups equals 123, 150, and 233 µg/ dL, respectively. In clinical settings, such BLL of more than 100 µg/ dL are considered very high and would most probably result in severe lead poisoning [250].

In this study, we have demonstrated that lead exposure caused an elevation in TSH levels, which was statistically significant in the group that was treated with the highest dose i.e. 100 mg/ kg of lead acetate. Determination of TSH levels in clinical settings is the first step for the diagnosis of thyroid disorders since it is considered to be a more precise marker for identifying thyroid malfunctioning than the thyroid hormones themselves [251]. Any abnormalities in TSH levels are followed by measuring T4 and T3 levels, which in our study were found to be significantly reduced in these diabetic animals. Apparently, lead exposure decreased T4 and T3 levels, and this in turn led to a positive feedback effect on the anterior pituitary gland increasing TSH secretion. The combination of high TSH and low T4 and T3 levels in our study are highly suggestive of a clinical hypothyroid state.

Both Type I and Type II Diabetes Mellitus are considered to be risk factors for developing thyroid disorders [252]. A longitudinal clinical trial has shown that among 58 patients with Type I DM who were followed up for 18 years, 18 subjects developed

hypothyroidism [253]. So is hypothyroidism in our study caused by DM or lead exposure? It has been previously demonstrated that injecting rats with 100 mg/ kg of STZ could cause a reduction in T4 and T3 levels 12 weeks later. However, the administration of smaller doses of 40, 60, and 80 mg/ kg of STZ did not affect T4 and T3 levels [247]. Accordingly, the observed reduction in T4 and T3 levels in our study is most probably a direct result of lead exposure. Although the animals in our study had DM for only 6 weeks, which is relatively a short duration of time, the contribution of DM cannot be completely ruled out.

Our data show a clear effect of lead exposure on GSH levels (a marker of oxidative stress), which were significantly reduced. This can be explained by the fact that GSH protects our bodies from reactive oxygen species (ROS) through its sulfhydryl (SH) group, to which many reactive species and heavy metals e.g. lead can bind. This would eventually lead to a depletion of GSH stores and cause the various systemic toxicities of lead exposure such as hepatic and renal damage [254]. On the other hand, MDA, which is a ‘reliable marker’ and the ‘most mutagenic product’ of lipid peroxidation [255], was not affected in our study. However, in another study of a longer duration of exposure (six weeks), the treated Wistar rats with either 10 or 40 mg/ kg of lead acetate by gavage had significantly high MDA levels [256].

Our results show that lead exposure had a direct toxic effect on renal functions leading to an increase in urea levels. Urea is an end-product of protein degradation and is eventually filtered by the kidneys [257]. As such, our data are consistent with a recently published study which showed that treatment of mice with 40 mg/ kg of lead acetate for 10 days resulted in an increase in urea levels [258]. Along with creatinine

levels, urea is a commonly tested marker for renal functions [259]. On the other hand, the vast majority of creatinine comes from skeletal muscles and the heart [260]. Since creatinine levels are 'roughly proportional to muscle mass' [259], the most probable cause for the significantly low creatinine levels in these diabetic animals in our study is emaciation as the animals lost significant amounts of their weight and muscle bulk. In another study, when non-diabetic mice were exposed to 40 mg/ kg of lead acetate over 10 days, this treatment resulted in an elevation in their blood creatinine levels [258].

In this study, short-term treatment of diabetic animals with various doses of lead acetate over five days caused significant reductions in two biochemical markers i.e. ALT and ALP. In contrast to our findings, a previous study showed that lead exposure caused liver damage and resulted in elevations in ALT and ALP levels in non-diabetic Wistar rats that were exposed to 50 mg/ L of lead acetate in drinking water over 40 days [261]. However, in these animals, there were no significant effects on weight. Hence, the main cause for the low ALT and ALP levels in our study could probably be due to weight loss. It has been previously shown that the intake of low calorie diet over 8 weeks resulted in significant weight loss, determined as body-mass index (BMI), and induced a statistically significant reduction in ALT levels in men [262]. Another study showed that among a population of 862 people, the group with normal weight had a higher ALT level than the underweight group, and the overweight people had higher ALT levels than the group with normal weight [263]. In addition, elevated ALT levels have already been associated with metabolic syndrome, central obesity and insulin resistance [159].

4.4. Conclusion

Lead exposure in diabetic animals resulted in several deleterious effects e.g. the rats became emaciated. The pituitary-thyroid pathway was significantly affected; as TSH levels were elevated and T4 and T3 levels were reduced. In addition, there was a significant consumption of GSH stores and impairments in renal and hepatic functions, in response to lead exposure.

Chapter 5: Hormonal Thyroid Model

5.1. Background

5.1.1. Regulation of Thyroid Glands

The physiological regulation and the hormonal functions of the thyroid gland are controlled by higher centers, namely, the hypothalamus and the anterior pituitary gland. TRH from the hypothalamus stimulates the anterior pituitary to release TSH, which, in turn, induces the thyroid glands to secrete T3 and T4. Iodothyronine deiodinases type 1 and type 2 will then convert T4 to the more active form i.e. 3,5,3'-triiodothyronine (T3) [264]. Despite its extensive use for various purposes, the administration of TRH was usually limited to a total duration of less than two weeks in most previous studies since its repeated use led to a diminished response i.e. repeated administration of TRH failed to sustain TSH concentrations at high levels [265-267]. In addition, it has been shown previously that the maximum response to TRH could be achieved on the fifth day of treatment [266].

Somatostatin inhibits the release of TSH [268-270], T4 and T3 [271]. In animal models, a single dose of 1 µg of somatostatin injected either intravenously (i.v.) to pregnant rats or as i.p. injections to pups caused a reduction in serum TSH levels [272]. In comparison to somatostatin, the half-life of its synthetic analogue, octreotide (OCT), is 80 times longer and it is also much more potent than somatostatin (16). That is the reason why we selected this hormone to induce hypothyroidism in our project. Administration of 50 µg of OCT subcutaneously (s.c.) to healthy volunteers inhibited TSH release for at least 3 hours (16). In addition, OCT showed an ability to decrease the blood supply and vascularity of induced goiters in animal studies [273]. However, in a

randomized human study, the administration of 400 µg of OCT as a daily s.c. infusion to 7 patients with type I diabetes mellitus for a year resulted in only mild and transient hypothyroidism [274].

5.1.2. Induction of Hypothyroidism

Various agents have been used for the induction of hypothyroidism in experimental animal studies. Some of these that are most commonly used are the antithyroid treatment modalities such as propylthiouracil (PTU) [275], methimazole [276] and radiation [277]. Additional methods are the use of iodine [278], lithium [279] and hemi-thyroid electrocauterisation [280]. In clinical medicine, hypothyroidism is a common complication that is often seen after the administration of anti-thyroid drugs [281] and the irradiation of thyroid glands [282]. In addition, both deficiency states and excessive amounts of iodine can result in hypothyroidism [278]. On the other hand, treatment of bipolar disorders with lithium can result in subclinical hypothyroidism [283]. Although it commonly causes hypothyroidism, lithium administration in clinical settings has been reported to also induce hyperthyroidism [284].

The use of these agents has been shown to result in several adverse health effects. For instance, PTU-induced hypothyroidism in rats caused glucose intolerance since the islets' cells are probably under the control of T3 and T4 [285]. PTU also causes brain changes e.g. tau hyperphosphorylation and an elevation in the levels of proinflammatory cytokines, which represent some of the early features of Alzheimer's disease [275]. In addition, the administration of 0.05% PTU in drinking water to rats over 6 weeks caused an increase in urea, creatinine, homocysteine and MDA levels, in

comparison to the control group [286]. Maternal PTU-induced hypothyroidism during pregnancy in rats also resulted in a significant reduction in neonatal acetylcholinesterase levels, which is one of the most important enzymes in the brain [287]. Similar effects have been also seen in clinical practice as maternal treatment with antithyroid medications can result in hypothyroidism in their offspring [281]. As such, monitoring of maternal free T4 levels is of crucial importance [281].

The use of radioactive iodine in male Fisher rats caused a reduction in the weight and volume of thyroid glands as well as several histopathological changes [288]. The thyroid glands showed signs of inflammatory reactions, necrotic follicles, and thickened blood vessels [288]. In another study, the adverse effects of exposure to iodine were not limited to the exposed female rats, but also reached their offspring [289]. Histological examination of the thyroid glands of the pups showed hyperplasia and hyperthrophy of calcitonin-positive cells, which might predispose these animals to develop endocrine diseases in the future [289]. The administration of lithium to experimental rats caused oxidative damage, leading to an increase in SOD and MDA levels and a reduction in GSH levels [279]. There was also an increase in the osmotic fragility of RBCs, which might impair the stability of their membranes [279].

5.1.3. Induction of Hyperthyroidism

In comparison, hyperthyroidism in animal studies is often induced by using L-thyroxine (T4) [290] and triiodothyronine (T3) [291], whereas, in clinical cases, it can be the result of exposure to excessive doses of iodine and amiodarone [292]. The use of L-thyroxine in experimental studies has undoubtedly explained and confirmed many

hyperthyroid symptoms including cognitive and memory impairments [293]. However, this model has its own problems. When rats were treated with 500 mg/ kg, L-thyroxine s.c. for a total duration of 12 days, the levels of AST, ALT, LDH, and MDA increased, whereas, SOD, CAT, and GSH levels decreased [294]. In addition, treatment of rats with 0.3 mg/ kg L-thyroxine i.p. for 3 weeks resulted in an increase in zinc, selenium and calcium levels [290]. The induction of hyperthyroidism in rats by using 12.5 μg/ 100 g body weight of T3 for 10 days resulted in increased apoptosis in the hepatic tissues [291].

In this project, our aim was to develop an experimental thyroid model through the use of TRH to induce hyperthyroidism and OCT to induce hypothyroidism. Our study protocol was based on administering these hormones for five days since TRH was shown to have its maximum effect on TSH on day five [266].

5.2. Results

5.2.1. Weight Monitoring

As was expected in normal animals, the rats in the control group gained weight over the five days of treatment with DW. For the other two groups that were treated with TRH and OCT, there was statistically no significant weight change (Figure 21).

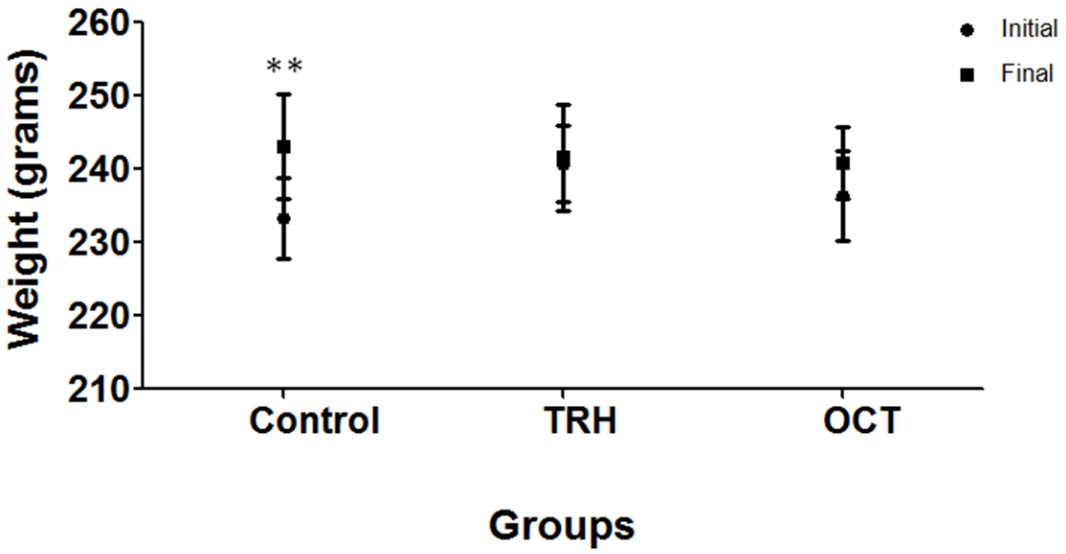

Figure 21: Weight change in hormonal thyroid model.
The change in weight over the five days of treatment, comparing the initial weight on day 1 versus final weight on day 5 within each group. Two-way ANOVA test is used. Results represent mean ± SEM, (n= 10-14), and **: $p < 0.01$

5.2.2. Thyroid Function Tests

Treatment of the animals with TRH for five days resulted in an increase in TSH levels (Figure 22 A). However, OCT treatment did not have any significant effect on TSH levels. On the other hand, the levels of both thyroid hormones, T3 and T4, remained statistically insignificant (Figure 22 B & C).

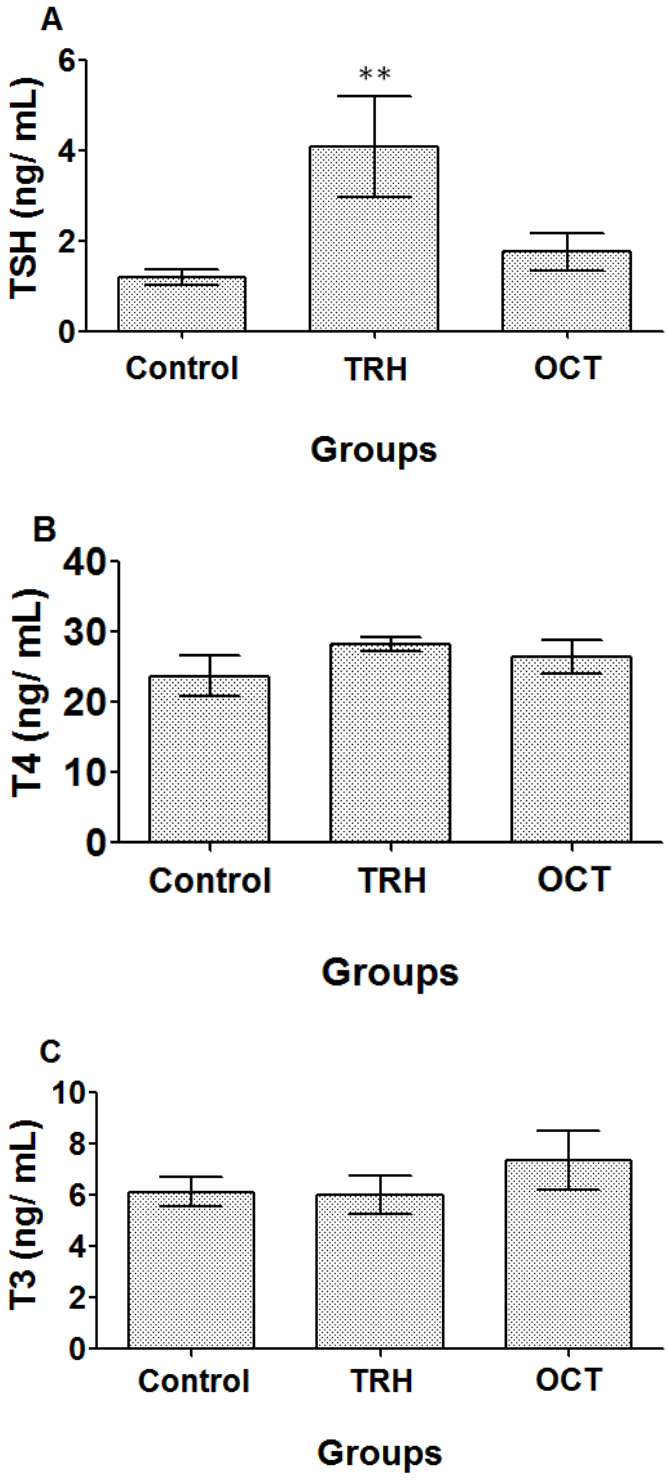

Figure 22: Thyroid function tests in hormonal thyroid model.
Levels of (A) TSH. (B) T4. (C) T3. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 5-9), and **: $p < 0.01$

5.2.3. GSH Results

The treatment protocol did not result in any significant effects on GSH levels in the various treatment groups as shown in (Figure 23).

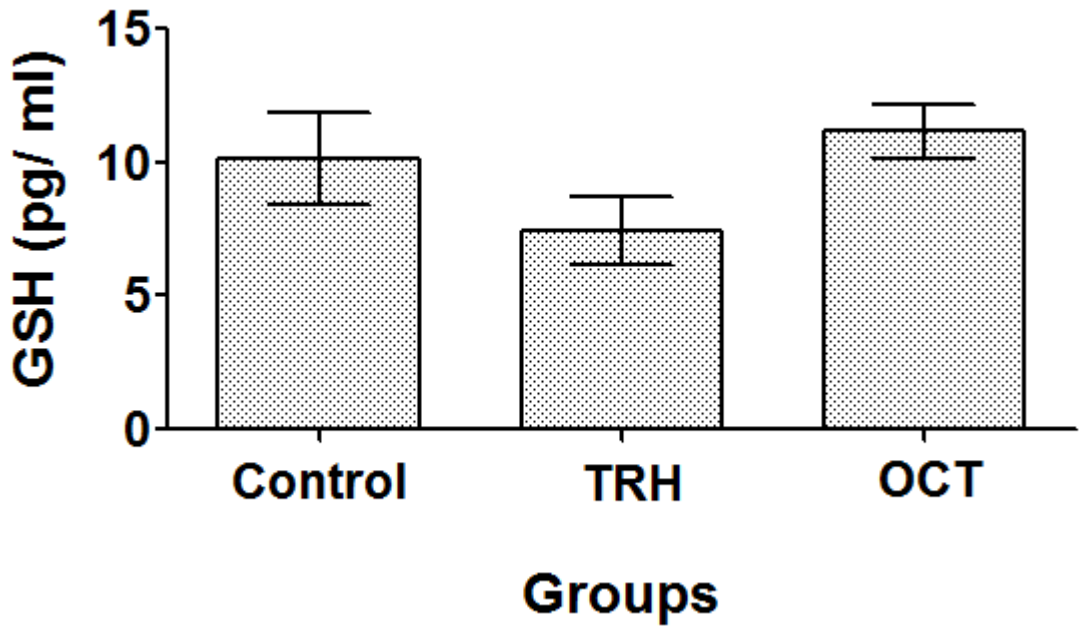

Figure 23: GSH levels in hormonal thyroid model.
One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 8-10)

5.2.4. CBC Results

Neither one of the two treatment modalities i.e. TRH or OCT, had any statistically significant effect on CBC parameters, which included WBC counts, platelet counts, hemoglobin levels, hematocrit percentage and RBC counts (Figures 24 and 25).

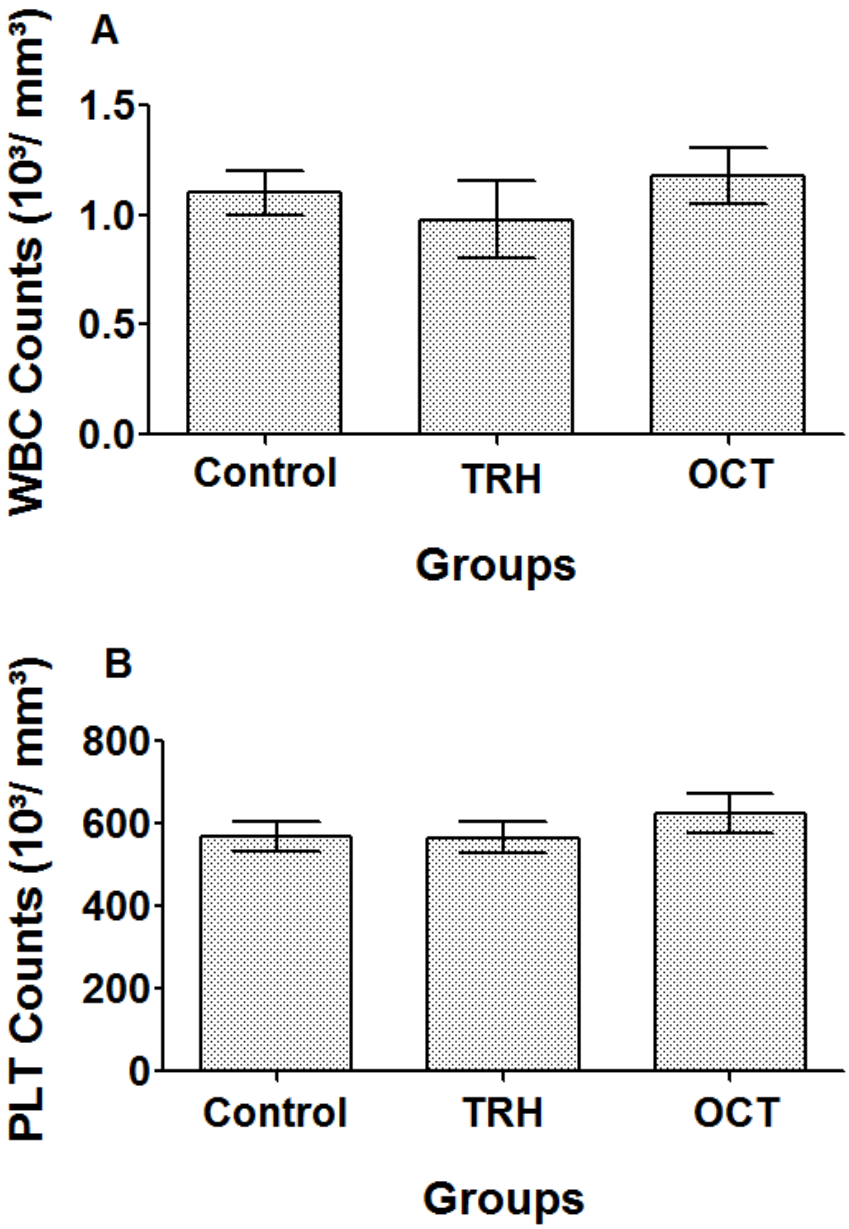

Figure 24: WBC and platelet counts in hormonal thyroid model.
(A) WBC counts. (B) Platelet counts. One-way ANOVA test, and Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 8-10)

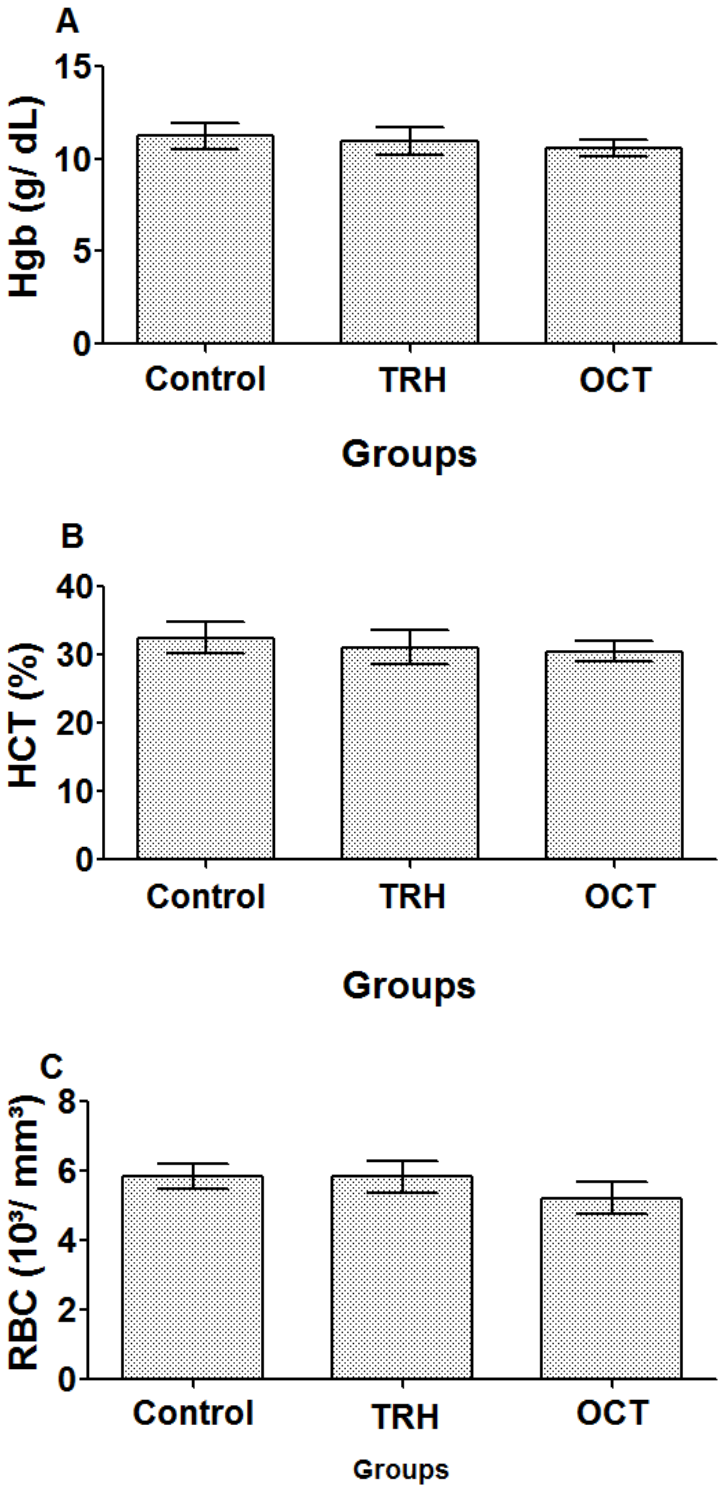

Figure 25: Hemoglobin, hematocrit and RBC counts in hormonal thyroid model.
(A) Hemoglobin levels (B) Hematocrit percentage. (C) RBC counts. One-way ANOVA test, and Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 5-12)

5.2.5. Biochemistry Parameters

Treatment of these animals with TRH for five days resulted in an increase in LDH levels, in comparison to the control group (Figure 26). However, none of the other eight biochemistry parameters that were investigated in these rats was statistically affected by exposure to either TRH or OCT (Figures 27 and 28). These parameters included AST, ALT, ALP, total bilirubin, albumin, total proteins, urea, and creatinine.

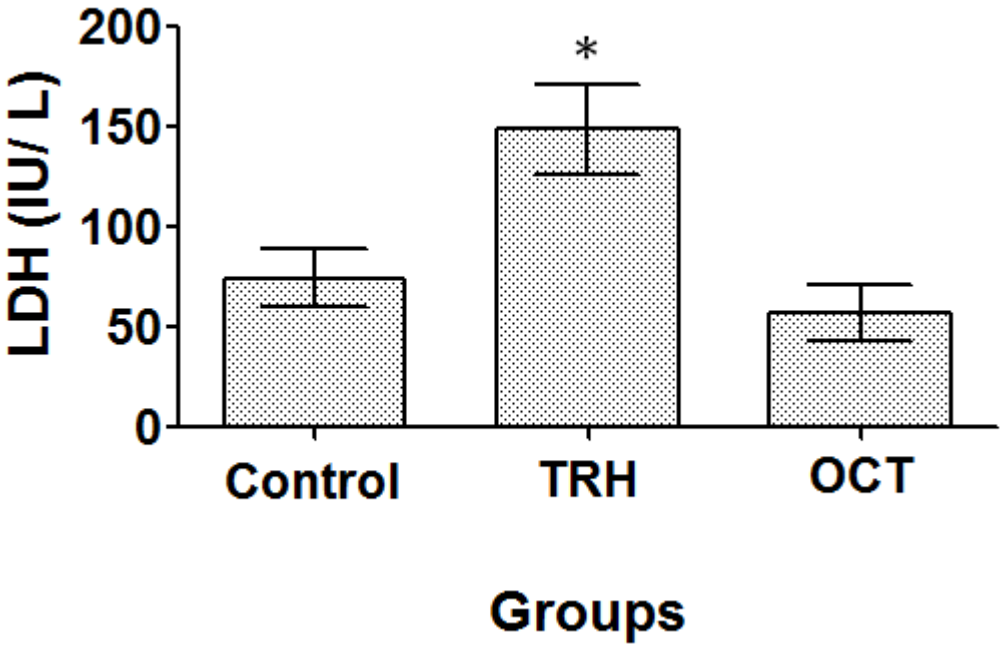

Figure 26: LDH levels in hormonal thyroid model.
One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 7-13), and *: $p < 0.05$

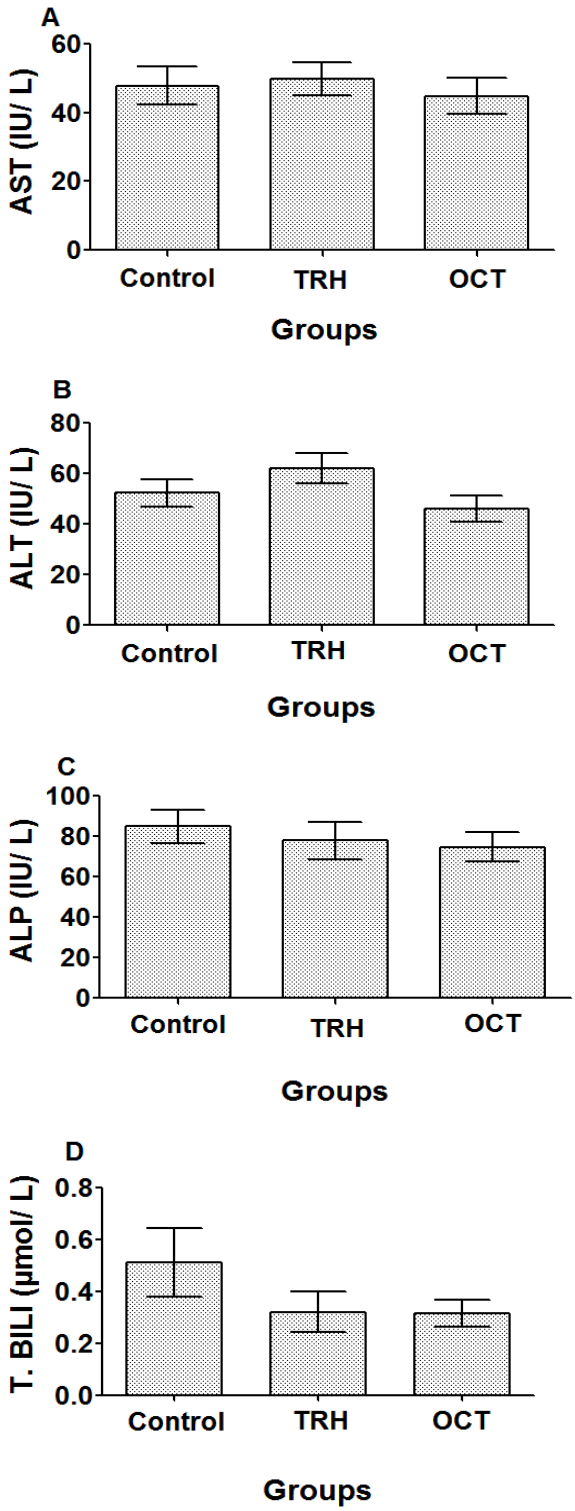

Figure 27: Levels of AST, ALT, ALP and total bilirubin in hormonal thyroid model.
Levels of (A) AST. (B) ALT. (C) ALP. (D) Total Bilirubin. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 6-10)

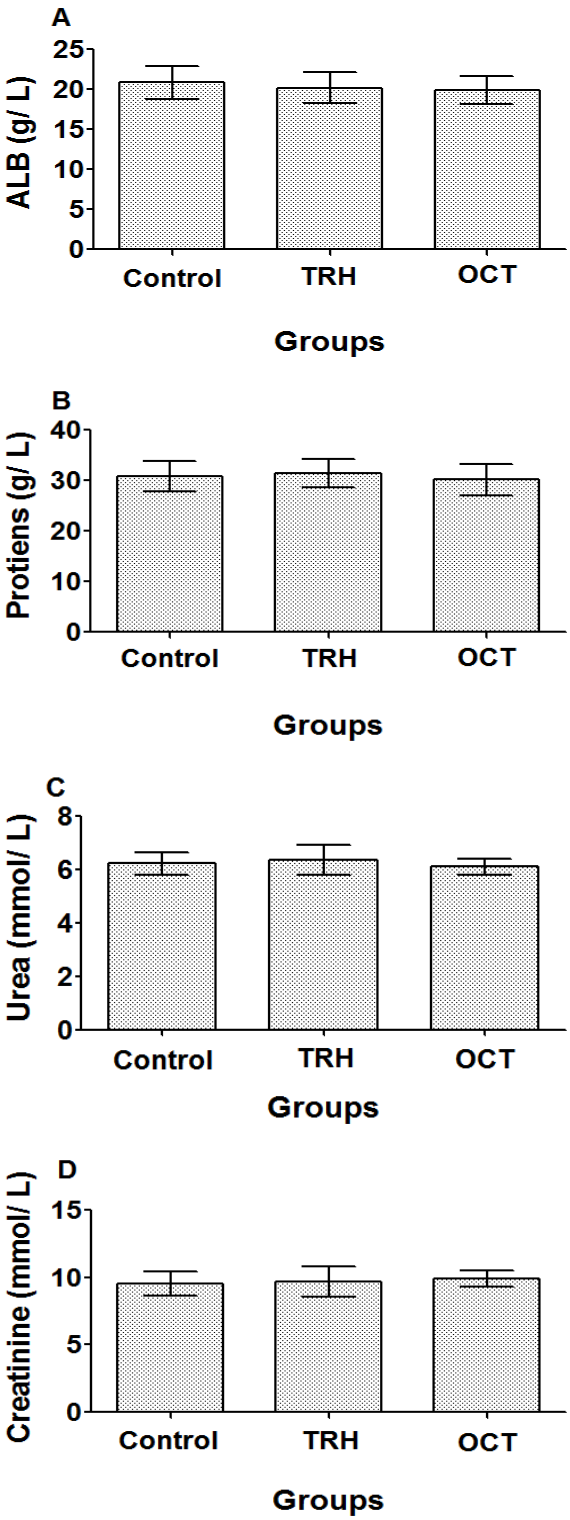

Figure 28: Levels of ALB, proteins, urea and creatinine in hormonal thyroid model. Levels of (A) ALB. (B) Total Proteins. (C) Urea. (D) Creatinine. One-way ANOVA test, followed by Bonferroni post-hoc test for selected groups are used. Results represent mean ± SEM, (n= 8-10)

5.3. Discussion

In this study, we tried to develop an experimental thyroid model using hormones in order to mimic the natural physiological process. Although T4 and T3 levels remained within normal range, TRH was effective in causing an increase in TSH levels, without having other major side effects. However, the use of OCT was not effective at the dose and treatment duration that we applied.

Animals in the control group gained some weight over time; however, neither TRH nor OCT caused any significant changes in weight over the five days of treatment. In a previous study, it was concluded that 'TRH stimulated resting metabolic rate, but inhibited food intake so that the rate of weight gain was reduced in obese (fa/fa) Zucker rats' [295]. Another study showed that the treatment of female rats with oral TRH for 30 days resulted in a reduction in their weight [296]. Similarly, it was found out that injecting high-fat fed SD rats with 40 μg/ kg octreotide, s.c., twice per day over a total duration of 8 days resulted also in weight loss, in comparison to an obese control group [297]. In addition, data from clinical studies also support the role of OCT in causing weight loss in obese subjects [298, 299]. The difference in the effect on weight between our study and these previous studies is probably related to the use of different doses and treatment durations.

Our data show that TRH treatment resulted in a statistically significant increase in TSH levels, although both of T4 and T3 were not increased. In this respect, it has been shown previously that the administration of TRH orally over 30 days caused an increase in T3 levels in female rats [296]. Similarly, injecting cattle with a single dose of 1 μg/ kg of TRH i.v. resulted in a slight elevation in T4 and T3 levels [300]. The use of OCT

in our study did not have any effect on TSH, T4 and T3 levels, although OCT is known to suppress TSH levels and has been used successfully in several medical cases for the treatment of pituitary adenomas [301, 302].

For systemic parameters, our experiments resulted in only an increase in LDH levels in response to TRH treatment, whereas, none of the other biochemical parameters was affected. The lack of systemic effects after TRH administration was also demonstrated in a previous study, where, treatment of cattle with 1 µg/ kg of TRH, i.v. did not have any effect on several biochemical parameters that were tested including ALT, creatine phosphokinase (CPK), gamma-glutamyl transpeptidase (GGT), LDH, cholesterol, albumin and blood minerals including calcium, phosphorus, and magnesium [300]. In addition, the administration of TRH in our experiments had no significant effect on hemoglobin concentrations, hematocrit, or RBC counts. Likewise, WBC and PLT counts were not affected.

Similarly, the use of OCT in our study did not affect the various systemic parameters that we tested. These included CBC, oxidative stress markers, and biochemical parameters. In a previous clinical study, 43 patients with acute pancreatitis were divided into a control group and an OCT treatment group, where the patients in this group received 0.5 µg/ kg/ hr OCT as continuous i.v. infusion. No significant difference was noticed between the two groups in regard with fasting blood glucose, hemoglobin, hematocrit, white blood cell count, calcium, LDH, AST, albumin, and urea levels neither at the time of presentation nor two days later [303]. As such, the use of OCT over short durations of time of few days did not cause any major systemic complications.

5.4. Conclusion

In this model, we established an increase in TSH levels in response to TRH administration, although it did not cause a corresponding increase in T4 and T3 levels. Apart from an increase in LDH levels, the animals tolerated this protocol very well as all the other parameters were within normal range. Taken together, these findings indicate that the administration of exogenous TRH is apparently safe and does not cause major adverse health effects.

Chapter 6: General Conclusions and Future Directions

6.1. Conclusion

In the first model, which involved the use of healthy animals, we have demonstrated that lead exposure resulted in several multisystemic effects. The most important effect was subclinical hypothyroidism i.e. high TSH and normal T4 and T3 levels. The other effects are related to hepatic and renal damage, which are clearly seen as several abnormal parameters in their respective panels. Since thyroid hormones were not affected at this stage, these abnormalities are probably a direct result of lead toxicity through oxidative damage and inflammation.

In the diabetic model, treatment with lead acetate caused clearer effects on the pituitary-thyroid axis. Here, the hormonal changes were consistent with a clinical hypothyroid state as the animals had high TSH and low T4 and T3 levels. Lead exposure in STZ-induced diabetic rats caused oxidative damage in the form of having a reduced level of the essential antioxidant, GSH. Lead exposure also resulted in renal damage leading to an elevation in urea levels. On the other hand, creatinine levels were reduced; perhaps due to muscle wastage.

The hormonal thyroid model resulted in a partial effect. TRH was effective in causing an increase in TSH levels, without having any major effects on other organ systems, apart from increasing LDH levels. However, OCT could not affect the pituitary-thyroid pathway since the various involved hormones (TSH, T4 and T3) remained within normal ranges.

In summary, short-term lead exposure in healthy and diabetic animal models affected the functions of the anterior pituitary and thyroid glands, caused oxidative stress, liver and kidneys toxicity and induced systemic inflammation.

6.2. Future Directions

In this research, we were able to demonstrate clear effects of lead exposure on the pituitary and thyroid glands, in both healthy and diabetic rats. As such, it is of paramount importance to uncover the mechanism of action of lead on these vital organs i.e. in addition to descriptive studies, mechanistic toxicity studies that target the molecular details on the pituitary-thyroid pathway need to be carried out. Furthermore, since lead exposure only caused subclinical hypothyroidism in the healthy animals, additional studies over longer periods of time are needed; since such exposures might eventually proceed to full-blown hypothyroidism. Since the group of 1 mg/ kg lead acetate did not produce significant effects in the non-diabetic animals, this group can be eliminated in future studies. In this project, STZ was used to induce DM, whereas, for future studies, other diabetic models e.g. NOD or BB models need to be considered and utilized. Here, the data from the healthy and diabetic animals were analyzed separately i.e. within each model. It would also be a good idea to compare the two models to each other.

There were several limitations in the newly developed experimental thyroid model. The first one is the total lack of effects of OCT on TSH, T4 and T3 levels. The second limitation is our inability to induce an increase in T4 and T3 levels in response to TRH administration, despite having a significant increase in TSH levels. In order to

overcome these issues and to optimize the hormonal responses, we recommend carrying out additional experiments where different doses of TRH and OCT are used. These experiments need to be tried over different time-points i.e. shorter and longer durations until the optimal response is achieved.

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List of Publications

Al Zadjali S, Nemmar A, Fahim M, Azimullah S, Subramanian D, Yasin J, Amir N., Hasan M, Adem A. (2015). Lead exposure causes thyroid abnormalities in diabetic rats. International Journal of Clinical and Experimental Medicine. [Forthcoming].

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