Landwirtschaftliche
Fakultät der
Universität Bonn

UNIVERSITÄT BONN
Rheinische
Friedrich-Wilhelms-
Universität Bonn

Arbeiten aus dem

Institut für Tierwissenschaften

Abteilung Tierzucht und Tierhaltung

Rheinische Friedrich-Wilhelms-Universität Bonn

Hoda Samir Badr Aglan

In vitro and in vivo assessment of lead toxicity on mammalian female reproduction and effect of antioxidants

Heft: 191

Institut für Tierwissenschaften

In vitro and in vivo assessment of lead toxicity in mammalian female reproduction and effect of antioxidants

D i s s e r t a t i o n

Zur Erlangung des Grades

Doktorin der Agrarwissenschaft (Dr. agr.)

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich–Wilhelms–Universität Bonn

von

Hoda Samir Badr Aglan

aus

Kairo, Ägypten

Bonn, 2020

Referent: Prof. Dr. Karl Schellander

Korreferent: Prof. Dr. Peter Stehle

Tag der mündlichen Prüfung: 06.12.2019

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

Dedicated to my parents, my brothers, my beloved husband and my son,

This work would not have been possible without you

Abstract

Heavy metals are among the environmental toxicants incurring great concern for human and animals because of their toxicity even at low concentrations. Lead (Pb) has been shown to induce severe and long lasting effects on female fertility and pregnancy outcomes in many animal species. The aim of this work was to investigate the effect of Pb on bovine granulosa cells (GCs) and preimplantation embryos and its association with dysregulation of Nrf2 and NF-κB and their downstream genes. We further aimed to study the role of *in utero* Pb exposure and the possibility of using antioxidant as prophylactic agent against Pb toxicity using rat model. For this, three experiments were conducted. First, *in vitro* cultured GCs were exposed to Pb toxicity which subsequently attenuated GCs proliferation and altered the cell cycle progression. Lead exposure suppressed the expression of both Nrf2 and NF-κB and their downstream genes. Additionally, Pb challenge on GCs increased the expression of endoplasmic reticulum stress marker genes (GRP78 and CHOP) and the pro-apoptotic gene (caspase-3), while the anti-apoptotic gene (BCL-2) was reduced.

Furthermore, treatment of bovine preimplantation embryos with Pb in a stage specific manner resulted in similar phenotypes. Blastocysts derived from different treatment groups exhibited aberrant developmental phenotypes regardless of the exposure stage. Exposure to Pb caused higher accumulation of ROS and reduced blastocyst cell number. Besides, the mRNA and protein levels of NF-κB were elevated with Pb treatment along with TNF-α level. On the contrary, the expression of Nrf2 protein showed significant reduction in all treatment groups. Apoptosis under Pb exposure was manifested by the higher ratio of BAX/BCL-2 and the number of TUNEL positive nuclei as compared to the control blastocysts. Moreover, Pb significantly upregulated DNMT1, a gene involved in maintenance of DNA methylation.

In order to investigate the effect of Pb toxicity *in vivo*, pregnant rats were orally ingested by Pb during the period of organogenesis, while the natural antioxidant, taurine (TA) was given throughout the gestation period. The dams and their fetuses were checked for morphological, biochemical and histopatholgical parameters. Results showed that, Pb caused a significant decline in the maternal body weight gain and an increase in the rate of abortion. Fetuses maternally-received Pb showed growth retardation and malformations in their skeleton. Additionally, Pb induced hematological and biochemical impairments in both dams and fetuses. Histopathological examination of the placenta and hepatic DNA fragmentation revealed the

toxicity of Pb. However, these events have been alleviated by TA pretreatment without affecting the normal course of pregnancy.

The present work demonstrates that Pb-induced oxidative stress displayed direct deleterious effect on bovine GCs proliferation and preimplantation embryo development. This effect may be in part through disrupting the Nrf2/NF-κB interaction and could vary according to the dose, the period of exposure and the type of cells. It is quite evident that even small doses of Pb are reprotoxic where the soundest approach is to minimize Pb exposure *in vivo* rather than treatment. Administration of antioxidants such as taurine could be promising approach to be used as a prophylactic agent against environmental heavy metals.

Kurzfassung

Schwermetalle gehören zu den Umweltgiften, die aufgrund ihrer Toxizität bereits in geringen Konzentrationen für Mensch und Tier von großer Bedeutung sind. So verursacht Blei (Pb) bei vielen Tierarten schwerwiegende und langanhaltende Auswirkungen auf die weibliche Fruchtbarkeit und die Schwangerschaft. Das Ziel dieser Arbeit war es, die Wirkung von Pb auf Rindergranulosazellen (GCs) und Präimplantationsembryonen und ihre Assoziation mit der Regulation von Nrf2 und NF-κB und ihren nachfolgenden Genen zu untersuchen. Es sollte ferner die Rolle der utero-Pb-Exposition und die Möglichkeit der Verwendung eines Antioxidants als Prophylaxe gegen die Pb-Toxizität unter Verwendung eines Rattenmodells analysiert werden. Hierzu wurden drei Versuche durchgeführt. Zunächst wurden in vitro kultivierte GCs einer Pb-Toxizität ausgesetzt, die anschließend die Proliferation der GCs abschwächte und das Fortschreiten des Zellzyklus veränderte. Die Exposition von Blei unterdrückte die Expression von Nrf2 und NF-κB sowie ihrer nachgeschalteten Gene. Zusätzlich erhöhte die Pb-Belastung auf GCs die Expression der Stressmarkergene des endoplasmatischen Retikulums (GRP78 und CHOP) und des proapoptotischen Gens Caspase-3, während das antiapoptotische Gen BCL-2 reduziert wurde. Darüber hinaus führte die Behandlung von Rinder-Präimplantationsembryonen mit Pb in einer Stadien spezifischen Weise zu ähnlichen Phänotypen. Von verschiedenen Behandlungsgruppen stammende Blastozysten zeigten ungeachtet des Expositionsstadiums aberrante Entwicklungsphänotypen. Die Exposition gegenüber Pb verursachte eine höhere Akkumulation von ROS und verringerte die Anzahl der Blastozysten. Außerdem wurden die mRNA- und der Proteinspiegel von NF-κB mit Pb-Behandlung zusammen mit dem TNF-α Spiegel erhöht. Im Gegensatz dazu, zeigte die Expression von Nrf2-Protein in allen Behandlungsgruppen eine signifikante Reduktion. Apoptose unter Pb-Exposition äußerte sich in einem höheren Verhältnis von BAX/BCL-2 und der Anzahl der TUNEL-positiven Kerne im Vergleich zu den Kontrollblastozysten. Darüber hinaus hat Pb DNMT1, ein Gen, das an der Aufrechterhaltung der DNA-Methylierung beteiligt ist, signifikant hochreguliert.

Um die Wirkung der Pb-Toxizität in vivo zu untersuchen, wurden trächtige Ratten während der Organogenese oral mit Pb versorgt, während parallel das natürliche Antioxidant Taurin (TA) während der gesamten Tragezeit verabreicht wurde. Die Muttertiere und ihre Feten wurden auf morphologische, biochemische und histopathologische Parameter untersucht. Die Ergebnisse zeigten, dass Pb eine signifikante Abnahme der Gewichtszunahme des mütterlichen Körpers und

eine Erhöhung der Arbortrate verursachte. Föten, die maternal Pb erhielten, zeigten Wachstumsverzögerungen und Missbildungen in ihrem Skelett. Zusätzlich induzierte Pb hämatologische und biochemische Beeinträchtigungen sowohl bei Muttertieren als auch bei Feten. Die histopathologische Untersuchung der Plazenta und der hepatischen DNA-Fragmentierung ergab die Toxizität von Pb. Diese Ereignisse wurden jedoch durch die TA-Vorbehandlung gelindert, ohne den normalen Schwangerschaftsverlauf zu beeinträchtigen. Die vorliegende Arbeit zeigt, dass Pb-induzierter oxidativer Stress eine direkte schädliche Wirkung auf die Proliferation von Rinder-GCs und die Embryonalentwicklung vor der Implantation hat. Dieser Effekt kann teilweise durch die Störung der Nrf2/NF-κB-Wechselwirkung verursacht werden und kann je nach Dosis, Expositionsdauer und Zelltyp variieren. Es ist evident, dass selbst kleine Dosen von Pb reprotoxisch sind, sodass die Pb-Exposition in vivo zu minimieren ist. Die Verabreichung von Antioxidantien wie Taurin könnte ein vielversprechender Ansatz sein als Prophylaxe gegen umweltbedingte Schwermetalle eingesetzt zu werden.

List of contents

List of abbreviations

A	Adenine
ALAD	Aminolevulinic acid dehydratase
ALAS	Aminolevulinic acid synthase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APC	Allophycocyanin
ARE	Antioxidant response element
As	Arsenic
AST	Aspartate aminotransferase
AT	Adenine-Thymine
BAX	Bcl-2-associated X protein
BCL-10	B-cell lymphoma/leukemia 10
BCL-2	B-cell lymphoma 2
BCL2L1	BCL2 like 1
BCL-xl	B-cell lymphoma-extra large
BER	Bair excision repair
BIM	Pro-apoptotic member of the BCL-2 family
BLL	Blood lead level
kbp	Kilo base pair
BSA	Bovine serum albumin
C	Cytosine
Ca	Calcium
CA	Cytosine-adenine
Caspase 3	Apoptosis-related cysteine peptidas
CAT	Catalase
CBP	CREB binding protein
CC	Cytosine-cytosine
CCDN2	Cyclin-D2
CCK-8	Cell counting kit
cDNA	Complementray DNA

Cd	Cadmium
CDC	Center for drug control and research
CHOP	C/EBP homologous protein
Co	Cobalt
CO_2	Carbon dioxide
COCs	Cumulus oocyte complexes
CPG	5'-Cytosine-phosphate-Guanine-3'
Cr	Chromium
Ct	Threshold cycle
Cu	Copper
DAPI	4’,6-Diamidin-2’-phenylindoldihydrochlorid
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMR	Differentially methylated regions
DMSO	Dimethyl Sulfoxide
DMT-1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
DNPH	2,4-Dinitrophenylhydrazine
dNTPs	Deoxyribonucleoside triphosphate
EDTA	Ethelenediamine tetra acetic acid
EGA	Embryonic genome activation
ER	Enoplasmic reticulum
ERK	Extracellular signal-regulated kinases
ERV	Endogenous retrovirus
EVs	Extracellular vesicles
FBS	Fetal bovine serum
Fe	Iron
FOXO	Forkhead box protein O
FSH	Follicle-stimulating hormone

G	Guanosine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenaseg
GCs	Granulosa cells
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GnRH	Gonadotropin-releasing hormone
GPx	Glutathione peroxidase
GRP78	78 kDa glucose-regulated protein
GRx	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S transfrase
H_2DCFDA	2′, 7′- Dichlorofluorescin diacetate
H_2O	Water
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HDAC3	Histone deacetylase 3
Hg	Mercury
HO-1	Heme oxygenase 1
HPG	Hypothalamic–pituitary–gonadal axis
HSCs	Hemopoitic stem cells
IAP	Murine intracisternal A-particle
IGF	Insulin like growth factor
IKK	Inhibitor of nuclear factor kappa-B kinase
IL-1β	Interleukin-1beta
IQ	Intelligence quotient
IUGR	Intra-uterine growth retardation
IVF	In vitro fertilization
IVM	In vitro maturation
IκB	inhibitor of nuclear factor kappa-B
JNK	c-Jun N-terminal kinases

kDa	Kilo Dalton
Keap-1	Kelch like ECH associated protein 1
Kpb	Kilo base pair
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LINEs (L1)	Long interspersed nuclear elements
LPO	Lipoxygenase
LPS	Lippopolysaccharides
LTR	Long terminal repeat
M	Gynemed (GM501) basic medium
MAPK	Mitogen-activated protein kinases
MDA	Malondialdehyde
Mg	Magnesium
miRNA	Micro RNAs
Mn	Manganese
mRNA	Messenger RNA
MSCs	Bone marrow stromal cells
MT	Metallothionine
NaCl	Sodium chloride
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
Ni	Nickel
Nrf2	Nuclear factor erythroid 2-related factor 2
OD	Optical density
ORF	Open reading frames
P4	Progesterone 4
P450	Cytochrome P450
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with tween 20
PCNA	Proliferating cell nuclear antigen
PCO	Polycystic ovarian syndrome

PCR	Polymerase chain reaction
Pd	Lead
PEG1/MEST	Paternally expressed gene1 (mesoderm specific transcript)
PG	Prostaglandine
PI	Propidium iodide
PRDX1	Peroxiredoxin
PVA	Poly vinyl alcohol
RBCs	Red blood cells
RHP	Rel homology domain
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revoulution per minute
qRT-PCR	Quantitative real-time polymerase chain reaction
SDS	Sodium dodecyl sulfate
Se	Selenium
SEM	Standard error mean
SINEs	Short interspersed nuclear elements
sMAF	Small MAF protein
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
TAD	Transactivation domain
TBST	Tris- buffered saline with tween 20
TEs	Transposable elements
TF	Transferrin
TFs	Transcriptional factors
Thrx	Thioredoxin
TNF-α	Tumor necrosis factor alpha
UPR	Unfolded protein response
VDR	Vitamin D Receptor
WBCs	White blood cells
Zn	Zinc

List of tables

List of figures

Chapter I

General Overview

1.1 Heavy metals

Heavy metals are among the environmental toxicants that have great concern for humans and animals because low concentrations, even at trace levels, can seriously impair health (Korashy & El-Kadi 2006). Exposure to heavy metals such as cadmium (Cd), lead, (Pb), chromium (Cr), arsenic (As) and mercury (Hg) continues to increase due to anthropogenic activities (Nagajyoti *et al.* 2010). Although some heavy metals such as iron (Fe), zinc (Zn), selenium (Se), manganese (Mn) and copper (Cu) are considered essential micronutrients and are vital to sustain normal body functions such as the synthesis of metallo-proteins, excess intake of these metal ions is considered toxic and linked to many pathological conditions such as the deposition of iron oxides in Parkinson's disease (Wintz *et al.* 2002; Mudgal *et al.* 2010). Since they are pervasive, their incorporation into the body has potential health risk to human and animal populations (Peretiatko & D'Souza 2002).

1.1.1 Sources of heavy metals

Different environmental sources of heavy metals include (1) natural sources such as the geologic parent material or the rock (He *et al.* 2005), (2) agricultural sources: here heavy metals could accumulate in soils and consequently in plants from the use of inorganic and organic fertilizers pesticide and fungicides (Gimeno-García *et al.* 1996), (3) industrial sources such as, mining, melting, welding, plating, incineration, refinement and recycling of metals (Mason *et al.* 1999; Jarup 2003; Wieloch *et al.* 2012), (4) domestic effluent (Yadav *et al.* 2002), (5) atmospheric sources, for example the metal containing airborne particulates which can be either transported by wind over great areas or precipitated with rain (Lu 2003) and (6) other sources such as automobiles, diesel-powered vehicles, aircraft coal burning and the corrosion of commercial waste products (Arruti *et al.* 2010).

Metals can bypass cellular control mechanisms such as homeostasis, transport, and compartmentalization, so they can exert toxic or even lethal effects (Jan *et al.* 2015). Moreover, their toxicity relies on the type and form of the metal, the mode and duration of exposure in addition to the individual susceptibility (Jan *et al.* 2011). They have the ability to induce the production of free radicals that can attack and damage nucleic acids, proteins and lipids. They can also form stable covalent complexes with these macromolecules leading to loss of their function (Flora *et al.* 2008). Besides, they can lead to disruption of many cellular processes and

signaling pathways via displacement of essential metals from their active sites (Leonard *et al.* 2004). Some of these heavy metals are bioaccumulative, since they neither break down in the environment nor are easily metabolized (Jan *et al.* 2015). The top position on the list of health hazards is occupied by Hg, Cd and Pb. They are called "the big three" due to their sever impact and they are considered systemic toxicants even at lower levels of exposure (Hegazy *et al.* 2010).

1.1.2 Lead

Lead (Pb) is one of the highly distributed natural substances in the environment. Due to its unique physical properties such as, high malleability and low melting point, it has a broad spectrum of industrial applications. It is involved in car batteries, fuel additives, paints, ceramics, solders, shielding for x-ray devices, water pipes, sound absorbers in addition to fertilizers and pesticides (Sanders *et al.* 2009). So the sources of Pb exposure are mainly industrial processes, food and smoking, drinking water and domestic sources. Extensive efforts have been made over the past decades to reduce Pb exposure (Karimooy *et al.* 2010).

1.1.3 Mode of lead exposure

Human and animals generally are exposed to the Pb through the respiratory tract, the skin or the digestive tract. The major route of Pb exposure is ingestion of food or drinking water contaminated with Pb (Levallois *et al.* 2014). Lead could be inhaled especially in Pb industries and this is dependent mainly on the particle size. Smoking is also related to the high body burdens of Pb in smoker population and even those exposed to secondhand smoke (Richter *et al.* 2013). Although absorption of Pb through the skin was believed to be non-significant or less efficient, a study of (Pan *et al.* 2010) demonstrated skin toxicity under topical Pb administration. Moreover, (Fang *et al.* 2014) further showed that Pb can accumulate and induce oxidative damage in the liver of rats via skin exposure.

1.1.4 Lead kinetics

After absorption through respiratory or digestive tract, 99 % of Pb content is bound to RBCs for approximately 30 days then it is either excreted in urine and through biliary clearance or accumulated in soft and mineralized tissues (Patrick 2006b). The half-life of Pb in brain could be for years while it could reside in teeth and bones for decades long (Verstraeten *et al.* 2008).

About 33 % of Pb accumulates in the liver tissue being the largest repository for Pb followed by the kidney (Mudipalli 2007). Bone releases Pb slowly to the circulation and exhibit an endogenous and persistent source for Pb exposure. The rate of release of Pb from the skeleton is increased in stressful conditions associated with high bone reabsorption as pregnancy, lactation, menopause, osteoporosis, immobilization, and hyperthyroidism (Vaziri 2008). Consequently, bone Pb level could be a biomarker for Pb-induced chronic impact than blood or urinary Pb which reflect only recent Pb exposure (Hu *et al.* 2007).

In children, the absorption of Pb occurs more than in adults, making children at high risk for Pb intoxication (Landrigan *et al.* 2002). It has been also reported that, Pb absorption is highly species specific, since from its intake, about 50 % is absorbed in human, 90 % is absorbed in bovine while only 2 % in ovine (Georgescu *et al.* 2011). In bovines, ingestion of contaminated fodder increases Pb levels in the liver to a 20-fold and in milk to a 3-4- fold where the milk content persists for 120 days (Georgescu *et al.* 2011). During pregnancy, Pb can eventually pass through the placental barrier by passive diffusion (Goyer 1990) where the ratio of fetal to maternal blood Pb is 0.7–0.9 (Rudge *et al.* 2009). Additionally, Pb has been assumed to induce metallothionein, small proteins with unusual high cysteine content and metal binding affinity that bind and retain Pb in the placenta (Ma *et al.* 2006; Gundacker & Hengstschlager 2012).
The nutritional status of the individual can further influence an individual's response to Pb. Deficiency of vitamin D, Ca, Fe or trace metals such as Zn and Cu may aggravate Pb absorption and hence its toxic hazards (Woolf *et al.* 2007; Rolston 2011).

1.1.5 Symptoms of lead toxicity

Despite the elimination of Pb from paints and gasoline in the USA, Pb exposure persists since Pb does not degrade in the environment, remaining strongly absorbed to soil (Sanders *et al.* 2009). According to the Center for Disease Control and Prevention (CDC), the maximum endurable blood lead level (BLL) is 10 μg/dL, however adverse effects have been reported with lower levels especially in pregnant females, children and developing embryos; suggesting no safe level of lead exposure (Chandramouli *et al.* 2009; Taylor *et al.* 2013). The problem of Pb pervasiveness is hence more severe in developing countries especially with the absence of preventive measures and public education (Jarosinska *et al.* 2004; Meyer *et al.* 2008). Lead

toxicity is considered as a silent environmental disease because of its long term adverse consequences (Karrari *et al.* 2012). It is also assumed Pb is one of the main causes of workplace illness (Needleman 2004).

The manifestation of Pb in human is usually vague until BLL reach 40 µg/dL and can be encountered in other diseases (Pearce 2007). There are also many studies showing no symptoms of Pb poisoning even with elevated BLL, and symptoms may arise only once irreparable damage occurs (Leech *et al.* 2016). However the mechanism beyond such differences in response to Pb toxicity is yet to be defined and is an issue of concern (Wani *et al.* 2015).

At low BLL, neurobehavioral deterioration may occur, including reduced cognitive functions and irritability and other nonspecific symptoms (Xu *et al.* 2009). As Pb accumulates in the body reaching the level of 40 µg/dl, more obvious symptoms may appear, such as headache, abdominal colic, anemia, and vomiting (Jacobs *et al.* 2002). Higher levels of Pb from 70 to 100 µg/dL have been associated with encephalopathy, delirium and coma. Even without symptoms, high Pb levels could induce permanent neurologic damage. A fatal dose of soluble Pb salts such as lead acetate could be about 20 grams (Smith *et al.* 2008). Lead is nearly affecting every organ in the body. Moreover, it can induce acute and chronic adverse effects ranging from subclinical impairments in function to symptomatic, life-threatening poisoning (Bandyopadhyay *et al.* 2014).

Several epidemiological studies showed higher blood levels in children than in adult probably due to their hand-to-mouth behavior or eating disorder (pica) (Stromberg *et al.* 2003; Ahamed *et al.* 2007). Because of their soft tissues and not fully developed organs, children are highly sensitive to Pb detrimental effects than adults even with lower threshold levels (Brochin *et al.* 2008).

1.1.6 The supralinear dose-response relationship in lead toxicity

It was observed that the dose-response relationship between BLLs and some of its toxic consequences is not linear, but supralinear or non-monotonic (Bowers & Beck 2006). For example, the decline rate in IQ scores of children is greater when BLLs is less than 10 µg/dL than at levels greater than 10 µg/dL (Cory-Slechta 2012). The CDC further stated that pregnant

women with blood lead level of 2-5 µg/dL could make the fetus at high risk of anomalies, so follow-up testing and patient education to minimize Pb exposure are required (Bellinger 2013).

1.1.7 Reproductive and developmental toxicity of lead

Environmental and occupational exposures to Pb have been associated with detrimental consequences affecting almost all aspects of the reproductive system in both males and females (Pant *et al.* 2003; Patrick 2006a; Ahmed *et al.* 2012). Lead can accumulate in testes, epididymis, seminal vesicle and seminal ejaculate inducing adverse effects on spermatogenesis, sperm count and motility, prostatic function and serum testosterone (Chowdhury 2009; Shan *et al.* 2009). The impact of Pb on female reproduction is more profound, where it has been detected in all compartment of female reproductive system in many species (Saleh *et al.* 2009). The impact of Pb with regard to female reproduction could exceed other environmental toxins (Mendola *et al.* 2008). Lead toxicity has been documented to cause infertility, miscarriage, pregnancy hypertension, premature membrane rupture, premature delivery and preeclampsia (Seyom *et al.* 2015; Bayat *et al.* 2016).

The effect of Pb as endocrine disruptor was documented, prenatal and later life exposures to Pb induced disruption of gonadal function and reproductive hormones (Pillai *et al.* 2010). Lead further interrupts several points along the hypothalamic–pituitary–gonadal (HPG) axis, for example GH, and FSH/LH responses to GHRH, and GnRH stimulation (Doumouchtsis *et al.* 2009). On in vitro study using Pb-exposed human ovarian granulosa cells showed lower levels of p450 aromatase, cytochrome p450 aromatase mRNA, and estrogen receptor β proteins (Taupeau *et al.* 2003). Ovarian accumulation of Pb irreversibly impaired folliculogenesis, with high rate of atresia, decreasing the number of the primary follicles in mice (Taupeau *et al.* 2001). In another study, Pb caused decreased ovarian response and ovulation rate in rabbits (Ahmed *et al.* 2012). Selevan *et al.* (2003) showed that exposure of 8- to 18-year-old girls to Pb was associated with delayed pubertal development. Moreover, occupationally Pb-exposed women exhibited menstrual abnormalities, including hypermenorrhea and spontaneous abortion (Tang & Zhu 2003). An association between chronic Pb exposure and early menopause was also reported by (Eum *et al.* 2014).

It has been reported that women with BLLs above 10 µg/dL, had higher likelihood of not achieving pregnancy compared with women with BLLs less than 10 µg/dL (Guerra-Tamayo *et*

al. 2003). The study of Lamadrid-Figueroa *et al.* (2007) showed the positive association between BLL and the incidence of spontaneous abortion. Adverse pregnancy outcomes like preterm birth and growth retardation were found to be induced even by low BLLs (Vigeh *et al.* 2011; Zhang *et al.* 2015). Because of the free placental transfer of Pb (Rudge *et al.* 2009), relatively low BLL may greatly harm the developing fetus without notable impact on the mother (Basha & Reddy 2015).

1.1.8 Ionic mechanism of lead toxicity

Literature data revealed the interactions between Pb and other toxic and essential trace metals (Goyer 1997). The most recognized are those between Pb and Ca, Fe and Zn which are most important nutritional elements for humans. A high BLL was reported to be associated with diet deficient in those elements (Rahil-Khazen *et al.* 2002; Kwong *et al.* 2004). At the physiological levels of essential metals, Pb has the ability to displace several bivalent cations like Ca, Mg, Fe, Zn and monovalent cations like Na, in their binding site within the molecular machinery of living organisms. This in turn affects various biological processes such as cellular signaling, cell adhesion, protein folding and apoptosis (Lidsky & Schneider 2003; Garza *et al.* 2006). For example, Ca and Zn are structural components of some proteins being vital for their activities with high affinity and selectivity that ensure optimum protein-ion interactions (Katz *et al.* 1995). However, this selectivity is only restricted to those ions that are physiologically relevant in the cell while exogenous toxic metals could be a serious problem difficult to be handled by the cellular machinery (Garza *et al.* 2006). Because of their ionic mimicry, Pb can compete and substitute these cations inducing pathological impact (Florea *et al.* 2013). Neal & Guilarte (2010) showed that Pb can potentially pass through the blood-brain barrier, due in large part to its ability to substitute for Ca ions and further block the Ca ion channels. Even in picomolar levels, Pb can displace Ca affecting important neurotransmitters as protein kinase (Bressler *et al.* 1999). Lead induces damage in many regions within the brain such as hippocampus and cerebellum (Sanders *et al.* 2009).

Similarly, Pb competes with Zn for transport proteins like metallothionein in the gastrointestinal tract reducing Zn absorption (Ahamed *et al.* 2007). Since Zn is a key element of over 300 enzymes and proteins, deficiency of Zn affects physical and mental growth while its

supplementation can reduce the availability of binding sites for Pb and mitigate its toxicity (Cengiz *et al.* 2004; Prasanthi *et al.* 2010; Malekirad *et al.* 2010).

Lead is known to be a noxious element to Fe metabolism, since both metals are absorbed intestinally by the common divalent metal transporter-1(DMT-1) (Bressler *et al.* 2004). That is why, Fe deficiency is usually a risk factor for Pb toxicity (Park *et al.* 2014). Furthermore, Pb interferes with a number of important Fe dependent metabolic steps such as heme biosynthesis. (Hegazy *et al.* 2010) showed that BLLs ≥ 10 μg/dL were accompanied by low serum Fe and ferritin in children.

A lead-selenium interaction has been also observed (Yuan & Tang 2001). Selenium (Se) is a cofactor of glutathione peroxidase and plays an important role as an antioxidant (Hsu & Guo 2002). Several studies showed the antagonistic relationship between Pb and Se, where Se can reduce Pb-induced oxidative damage (Nehru *et al.* 1997; Li *et al.* 2005; Diouf *et al.* 2006). This is probably due to the formation of inactive Pb-Se complexes which hinder the absorption and tissue distribution of Pb (Li *et al.* 2005).

Moreover, competition between Pb and Cu for binding sites on proteins, such as the ATPase complex was demonstrated by (Qian *et al.* 2005). Herein, the free displaced Cu could induce ROS generation by Fenton reaction and hence aggravate Pb driven-oxidative stress. A study of (Kasperczyk *et al.* 2012) revealed positive correlation between both elements in occupationally exposed workers. Whereas the studies by Mehdi *et al.* (2000) and Wasowicz *et al.* (2001) reported no association between Pb and Cu levels.

1.1.9 Molecular mechanism of lead toxicity

The susceptibility of an individual toward the harmful effects of Pb toxicity has further been linked with the genetic makeup constituting the molecular basis of Pb toxicity (Kim *et al.* 2014). It is quite important to study the influence of gene-environment interactions in the risk assessment of an individual. Differences in allele frequencies of genes known to modify body Pb levels across populations may explain inter-individual variations in response to Pb toxicity. Several single nucleotide polymorphisms (SNPs) and mutations in various genes have been stated in literature which believed to play a plausible role in the susceptibility towards Pb toxicity (Mitra *et al.* 2017).

Delta-aminolevulinic acid dehydratase (ALAD) enzyme catalyzes the second step of heme synthesis (Kelada *et al.* 2001). ALAD polymorphism allows Pb to bind tightly to ALAD enzyme and keeps Pb retained in the blood and tissues increasing the susceptibility to Pb induced oxidative stress (Zhao *et al.* 2007; Scinicariello *et al.* 2007). Furthermore, divalent metal transporter1 (DMT1), one of the proton-coupled metal ion transporter family (Cellier *et al.* 1995), which plays a pivotal role in iron uptake and recovery in most cell types besides its role in transport of divalent cations like Zn, Mn, Ni, Co and Pb (Garrick *et al.* 2003; Garrick *et al.* 2006). A SNP in DMT1 gene has been reported to be associated with the levels of blood Pb and Fe in Turkish people, where individuals with CC genotype had higher BLL and more susceptibility to Pb toxicity compared to AA and CA genotypes (Kayaalti *et al.* 2015). Similarly, metallothioneins (MT) are a group of low molecular weight cysteine rich metal-binding proteins involved in the hemostasis of essential metals like Cu and Zn and detoxification of toxic heavy metals inside the body (Ngu & Stillman 2009). They are ideal candidates in susceptibility to Pb toxicity (Raudenska *et al.* 2014). A SNP in MT (A to G nucleotide transversion) in pregnant women with AG genotype resulted in significantly higher maternal Pb levels than those with AA genotype.

Other significant relation between gene polymorphisms and modification of body Pb burden were documented such as the transferrin (TF) gene in Mexican children (Hopkins *et al.* 2008). The SNPs in the glutathione S-transferases (GST) gene produce enzymes with variation in their catalytic activity and thus also may play a role towards susceptibility to Pb toxicity (Lee *et al.* 2012a; Eum *et al.* 2013). The SNP in Vitamin D receptor (VDR) gene alter bone mineralization activity by affecting the amount of VDR protein which may further interact with Pb (Krieg *et al.* 2010).

1.2 Lead and oxidative stress

1.2.1 Oxidative stress

Oxidative stress is caused by the shift in balance between oxidants and antioxidants in favor of oxidants and compromising the system's ability to potentially detoxify the body of those oxidants resulting in cellular damage (Flora *et al.* 2007). Reactive oxygen species (ROS), including free radicals, are generated in the living organisms from either endogenous or exogenous sources (Willcox *et al.* 2004). Endogenous free radicals are generated from immune

cell activation, inflammation, mental stress, ischemia, infection, cancer, aging (Pacher *et al.* 2007). Whereas exogenous ROS result from pollution of air and water, smoking, alcohol, heavy metals (Cd, Pb, Hg, Cr, Fe, As), certain drugs, industrial solvents, and radiation (Droge 2002). Free radicals, since containing one or more unpaired electrons in their atomic orbital, need stability either by donating their unpaired electron to other molecules or by acquiring an extra electron from adjacent molecules (Pham-Huy *et al.* 2008; Salvemini & Botting 1993). As a result, these are highly reactive molecules and can damage the cell structures such as carbohydrates, nucleic acids, lipids, and proteins and alter their functions (Flora *et al.* 2004).

Reactive oxygen species are well documented for playing a twofold job as both deleterious and beneficial species, where a delicate balance between the two antagonistic effects is an important aspect of life (Valko *et al.* 2007; Genestra 2007). ROS have been shown to be important signaling molecules especially hydrogen peroxide and superoxide in which low levels (usually submicromolar concentrations) induce growth but higher concentrations (usually >10–30 μM) induce cellular death (Stone & Yang 2006). Cell surface receptors produce ROS upon activation; for example, receptors for epidermal growth factor, vascular endothelial growth factor, platelet-derived growth factor, insulin-like growth factor and various cytokines (Vardatsikos *et al.* 2009). However, higher ROS levels are damaging to organelles, particularly mitochondria (Lee *et al.* 2012b), which may result in energy depletion, accumulation of cytotoxic mediators and cell death. Oxidative stress plays a major part in the development of chronic and degenerative disorders such as cancer (Valko *et al.* 2004), arthritis (Mahajan & Tandon 2004), aging, autoimmune disorders, cardiovascular (Ceriello 2008), and neurodegenerative diseases (Uttara *et al.* 2009).

1.2.2 Reactive oxygen species and reproduction

The dual role of ROS in the reproductive system has been justified depending on the nature and the concentration of the ROS as well as the location and length of exposure to ROS (Agarwal *et al.* 2005; Agarwal *et al.* 2006). Free radicals can act as key signal molecules modulating various reproductive functions and can influence the oocytes, sperm, and embryos in their microenvironments (Valko *et al.* 2007; Agarwal *et al.* 2008). The work of Shkolnik *et al.* (2011) demonstrated that reactive species produced in the preovulatory follicle of mice are indispensible for ovulation, and inhibition of ROS by antioxidants has been found to disturb ovulation,

suggesting a possible harmful effect of antioxidants on ovulation. Moreover, a balance between ROS and antioxidants is obligatory for oocyte development and maturation (Kala *et al.* 2017). Oxidative stress has been implicated in male and female infertility. Increased ROS levels correlate negatively with sperm concentration and sperm motility (Choudhary *et al.* 2010). Excess ROS production was reported to induce single- and double stranded DNA fragmentation in sperms (Dalzell *et al.* 2004). Fertilization of the oocyte by a spermatozoon with unrepaired DNA damage may cause implantation failure, embryo development arrest, pregnancy loss, or birth defects (Rubes *et al.* 2005).

Oxidative stress has also been implicated in many of the causes of female infertility, such as endometriosis, unexplained infertility, tubal infertility, and recurrent pregnancy loss (Bedaiwy *et al.* 2002; Madazli *et al.* 2002; Tamura *et al.* 2008; Matsubara *et al.* 2015). The placenta experiences a heightened level of ROS in certain pathologic conditions of pregnancies, including gestational diabetes, fetal growth restriction, preeclampsia and miscarriage (Sbrana *et al.* 2011; Smith *et al.* 2013). Oxidative stress is also considered as one of the important factors beyond the limited success rate of in vitro fertilization (IVF) and fertilization outcomes (Wojsiat *et al.* 2017). Excess generation of ROS in the human GCs of women with polycystic ovarian (PCO) syndrome adversely affected IVF success rates (Karuputhula *et al.* 2013). Pregnancy itself is a state of oxidative stress arising from the increased metabolic activity in the placental mitochondria and increased ROS production due to the higher metabolic demand of the growing fetus (Myatt & Cui 2004). Oxidative stress imbalance has a detrimental effect on pregnancy outcome (Jana *et al.* 2010). The developing embryo is susceptible to high levels of ROS because of its weak antioxidant defense; especially in the early stages of organogenesis (Zaken *et al.* 2000). Many neonatal disorders are correlated with oxidative stress and poor antioxidant status, in particular, intra-uterine growth retardation (IUGR) (Hracsko *et al.* 2008). IUGR is associated with high rate of neonatal morbidity and mortality, and deformation of the umbilical cord (Biri *et al.* 2007). Furthermore, oxidative stress-based pregnancy complications may be a contributing factor in adulthood consequences like the increased incidence of hypertension, type II diabetes, insulin resistance, metabolic syndrome, and obesity (Thompson & Al-Hasan 2012).

1.2. 3 Lead-induced oxidative stress

Although Pb-induced toxicity cannot be linked to a single defined mechanism, oxidative stress could be a potential mechanism beyond the pathophysiology of lead toxicity (Flora *et al.* 2012) (Figure 1.1).

Unlike redox active metals such as Fe and Cu where Fenton-like reactions, appear to play a major role in their toxicity in addition to their ability to act as catalyst that to form more free radicals (Fisher & Naughton 2003), Pb is a redox-inert metal unable to perform redox reactions and the mechanism underlying Pb-induced oxidative stress is not clear or easily understood (Solliway *et al.* 1996). Koedrith & Seo (2011) reported that Pb induces its oxidative stress effects by binding to sulfhydryl groups of proteins, which results in depletion of glutathione. Lead shows a strong electron sharing activity that allow the formation of covalent bonds between Pb and the sulfhydryl moiety (SH) in the antioxidant enzymes, making them highly susceptible to Pb attack and rendering them inactive (Jomova & Valko 2011). This mechanism accordingly, highlights the impact of Pb exposure on glutathione metabolism. Glutathione, an important antioxidant in mammalian tissues to nullify free radicals, is a cysteine-based tripeptide having sulfhydryl groups (Mates 2000; Mari *et al.* 2009). Glutathione exists in cells in two states: reduced (GSH) and oxidized (GSSG), where the ratio of both states determines the redox status of cells. Under physiological healthy conditions, cells have a GSH/GSSG ratio >100 while the ratio decline to 1 to 10 under oxidant stress (Pizzorno 2014). Additionally, glutathione is involved in the metabolism of certain drugs and toxins via complex formation (glutathione conjugation) in the liver and facilitate their excretion. Lead inactivates glutathione by binding to its sulfhydryl groups and in the same way it inactivates other sulfhydryl-containing enzymes like δ-amino levulinic acid dehydratase (ALAD), glutathione reductase (GRx), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), which further decreases the bioavailability of glutathione (Hunaiti *et al.* 1995; Ahamed & Siddiqui 2007a). Knowing that Pb-inhibited ALAD increases the level of δ-amino levulinic acid which is further oxidized generating more ROS and contributing to the Pb-dependent DNA carcinogenicity (Hiraku & Kawanishi 1996).

Other antioxidant enzymes inhibited by Pb include super oxide dismutase (SOD) and catalase (CAT). Apart from targeting their sulfhydryl groups, Pb is able to replace the Zn ions which are important co-factors for these antioxidant enzymes. Consequently, decreased level of SOD

reduces the clearance of superoxide radical, while reduced CAT hinders the decomposition of hydrogen peroxide (Flora *et al.* 2007). However, the studies of Rico *et al.* (2009) and Paul *et al.* (2013) revealed that both redox active and non-active metals generate ROS, especially superoxide and peroxides. According to Flora (2009) and Gurer-Orhan *et al.* (2004), the onset of Pb induced-oxidative stress could start in two simultaneous pathways: first, the generation of ROS such as hydro peroxides, singlet oxygen, and hydrogen peroxide, second, the depletion of endogenous antioxidant reserves.

Another serious biomarker of Pb-driven oxidative stress is lipid peroxidation which reveals the negative impact of ROS on lipid membranes (Yiin & Lin 1995; Sandhir & Gill 1995; Adonaylo & Oteiza 1999). Lead can bind to phospholipids (mainly to phosphatidylcholine) in the cellular membrane and induce changes in the membrane biophysical properties (Adonaylo & Oteiza 1999). Moreover, the presence of double bonds in the fatty acid weakens the C–H bonds on the carbon atom adjacent to the double bonds and making the removal of H removal easier. Therefore, in the cell membrane, polyunsaturated fatty acids containing more than two double bonds are highly susceptible to Pb-induced oxidation than fatty acids containing zero to two double bonds (Yiin & Lin 1995). Moreover, an early study showed that Pb can alter the membrane lipid composition, it induces arachidonic acid elongation increasing the rate of peroxidation, since fatty acid chain length and unsaturation are linked to membrane vulnerability to peroxidation (Lawton & Donaldson 1991). Taken together, Pb-induced lipid peroxidation perturb different membrane- related functions such as signal transduction processes, the activity of membrane enzymes, the solutes transport in addition to endo- and exocytosis (Adonaylo & Oteiza 1999; Ahamed & Siddiqui 2007b). This effect is beyond the increased rate of erythrocyte hemolysis under Pb exposure (Shafiq-ur-Rehman 2013). Hemolysis is the end step of ROS-mediated lipid peroxidation and loss of cell membrane fluidity in the erythrocytes and these results in anemia (Vij 2009). Lipid peroxidation of cell membranes has also been demonstrated in various regions of the brain of Pb-exposed rats (Flora & Seth 2000). It is noteworthy to mention that, malondialdehyde (MDA) which is a by-product of lipid peroxidation, is an active aldehyde attacking cell constituents such as proteins and inactivates them by oxidation (Levine 2002). This events end by loss of protein function; this could be a further consequence of Pb-induced ROS and damage in various organs (Flora *et al.* 2003; El-Nekeety *et al.* 2009).

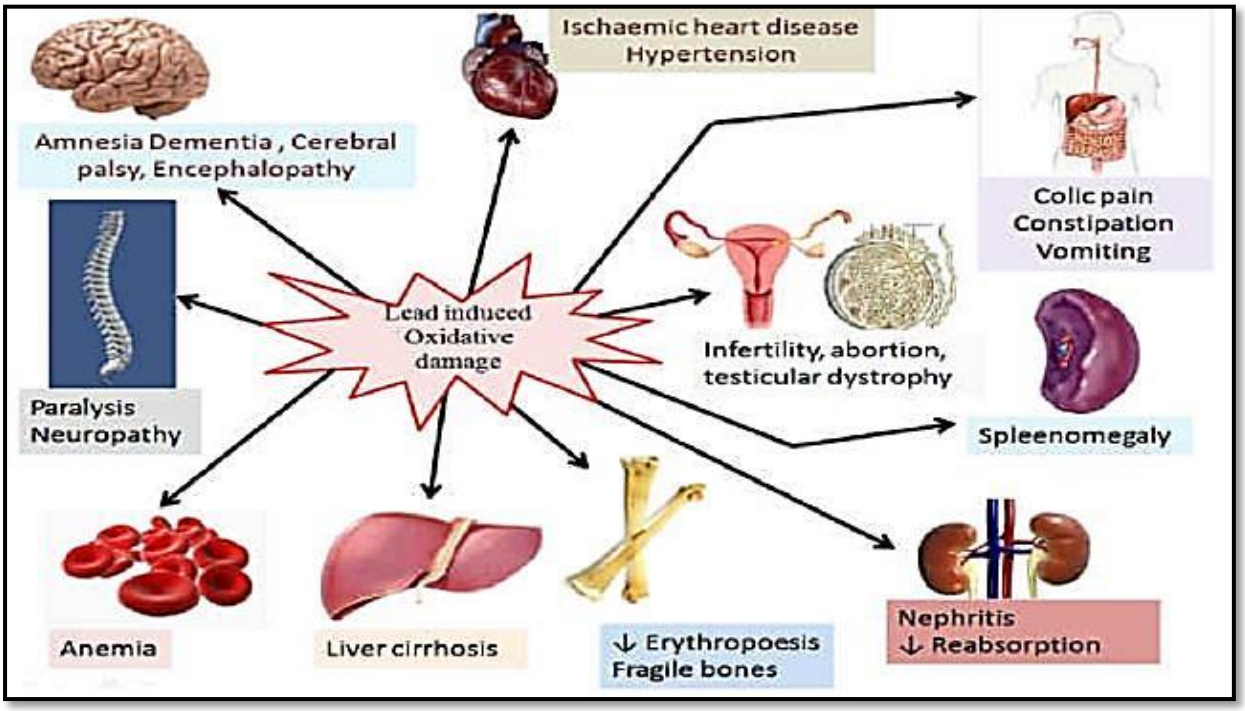


Figure 1.1: Lead induced oxidative damage in different organs and organ systems (Bandyopadhyay *et al.* 2014).

1.2.4 Management of lead toxicity by antioxidants

Lead exposure often occurs with little or no distinct symptoms, particularly in lower-level chronic exposure, manifesting only once irreparable damage has been done (Leech *et al.* 2016). While acute symptoms of exposure can be treated, there is no treatment for the underlying damage which is usually irreversible (Flora *et al.* 2012). So the soundest approach is to consider preventive or prophylactic measures rather than treatment (Guidotti & Ragain 2007). Since Pb induces its toxic effects mostly by oxidative stress, several studies suggested targeting oxidative stress by the use of naturally occurring antioxidants (Patrick 2006a; Antonio-Garcia & Masso-Gonzalez 2008). Although chelating agents such as EDTA and D-penicillamine can bind to Pb forming nontoxic complex so decrease the Pb body burden (Flora & Pachauri 2010), they still have side effects including redistribution of toxic metal, binding to essential metals, pro-oxidant effects, increased blood pressure, hepatotoxicity and nephrotoxicity (Kianoush *et al.* 2015). So the use of antioxidants as adjuvant or alternative therapy could have beneficial outcome in the management of Pb poisoning.

The role of vitamin C and thiamine (vitamin B1) against Pb-induced hepatotoxicity in mice was documented by Wang *et al.* (2007a). The flavonoid, quercetin has been confirmed to protect against Pb-induced histopathological alterations in the rat kidney (Liu *et al.* 2010). Sulfur-containing antioxidants like N-acetylcysteine, alpha-lipoic acid, methionine and homocysteine could mitigate the damaging effect of Pb on red blood cells (RBCs) in rats in addition to replenish other endogenous antioxidants (Caylak *et al.* 2008). Kianoush *et al.* (2012) reported the benefit of herbal antioxidants like garlic powder for workers with occupational Pb poisoning. Similarly, the curative role of curcumin with Pb neurotoxicity in male rats was documented by Dairam *et al.* (2007). Nevertheless, uncontrolled administration of antioxidants could render them as pro-oxidants especially when using a combination of antioxidants (Flora *et al.* 2012). Taken together, further studies are warranted to assess the safety and efficacy of these antioxidants with regard to reproduction.

1.3 The signaling molecules between oxidative stress and reproduction

Oxidative stress arises from the imbalance between oxidants and antioxidants. This lead to the activation of a variety of signaling pathways, resulting in crosstalk between many transcriptional factors (TFs) in the body. These signaling molecules proved to have great significance in the etiology of reproductive diseases (Lu *et al.* 2018).

Figure 1.2 shows the main ROS-induced signaling pathways associated with reproductive diseases, encompassing the p38 MAPK pathway, the Nrf2/Keap1 pathway, the JNK pathway, FOXO family, and apoptosis. According to Lu *et al.* (2018), each of the above-mentioned signaling molecules is not independently working but they will integrate forming various networks and conferring more complexity of signal molecule research. Oxidative stress during pregnancy, in addition to changes in TNF-α and progesterone levels, trigger a series of signaling pathways in cells through cAMP messanger, such as stimulation of the Keap1-Nrf2 signaling pathway, NF-κB signaling pathway, extracellular signal-regulated kinases (ERK) signaling pathway, then promoting an increase in cytokines and changes in antioxidant-related genes. Stress-activated c-Jun N-terminal kinase (JNK) phosphorylates cytoplasmic FOXO1 and promotes nuclear localization (Essers *et al.* 2004). FOXO1 induced apoptosis is achieved via the pro-apoptotic factor BIM (Dijkers *et al.* 2000).

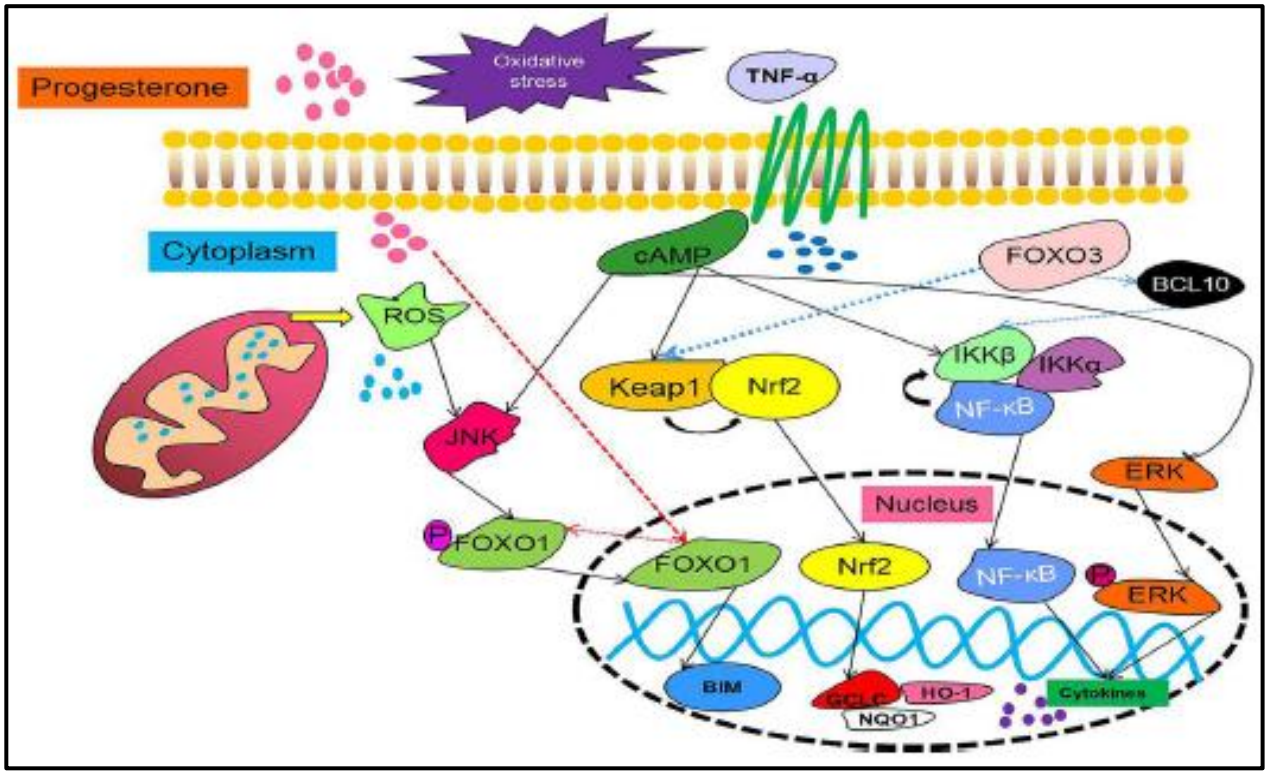


Figure 1.2: The main ROS-induced signaling pathways associated with reproductive diseases (Lu *et al.* 2018).

When progesterone is reduced, nuclear translocation occurs in FOXO1, and it is phosphorylated so excluded from the nucleus, where it is then ubiquitinated and degraded. Meanwhile, FOXO3 is involved in these signaling pathways; When FOXO3 is increased, it promotes the binding of Keap-1/Nrf2, lowering the level of antioxidants (Guan *et al.* 2016). A study also revealed that FOXO3 is a positive regulator of NF-κB signaling pathway and found that FOXO3 activated NF-κB by inducing expression of B-cell lymphoma/leukemia 10 (BCL10), an upstream regulator of IKK/NF-κB signaling, thereby enhancing cytokines production and apoptosis (Li *et al.* 2012). However the early study of Hu *et al.* (2004) showed that, IKKβ-mediated phosphorylation promotes nuclear exclusion and degradation of FOXO3 (Hu *et al.* 2004).

In the next part we focused on two signaling molecules, involved in reproduction, to discuss profoundly:

1.3.1 Nuclear factor erythroid 2-related factor 2 (Nrf2)

Nrf2 is considered the master regulator of antioxidant, detoxification, and cell defense against different oxidative insults (Kobayashi *et al.* 2004). Under unstressed conditions, Nrf2 resides in

the cytoplasm sequestered by the Kelch-like ECH-associated protein 1 (Keap-1) which facilitates Nrf2 ubquitination and proteosomal degradation (Dinkova-Kostova *et al.* 2002). However, exposure to ROS mediates oxidation of critical cysteine residues contained in Keap1 enhancing the dissociation of the Keap-1/Nrf2 complex and the nuclear accumulation of Nrf2 (Kansanen *et al.* 2013). Afterwards, Nrf2 associates with small Maf proteins (sMaf) and binds to a cis-element called the antioxidant-response element (ARE) that are located in the promoter region of genes encoding a battery of cytoprotective enzymes namely phase II antioxidant enzymes, like superoxide dismutase (SOD), catalase, thioredoxin (Trx), heme oxygenase-1 (HO-1), glutathione peroxidase (GPx) and glutathione S-transferase (GST), which buffer the ROS, providing protection against the accumulation of toxic metabolites (Thimmulappa *et al.* 2002; Dinkova-Kostova & Talalay 2008; Hirotsu *et al.* 2012). Nrf2-knockout mice were more vulnerable the toxicity of many chemicals and carcinogens such as acetaminophen, benzo[a]pyrene, diesel exhaust, and N-nitrosobutyl (4-hydroxybutyl) amine (Enomoto *et al.* 2001; Aoki *et al.* 2001; Iida *et al.* 2004; Aoki *et al.* 2007).

1.3.1.1 Nrf2 and heavy metals

Exposure to environmental toxic metals has been reported to induce ROS which are supposed to activate the Nrf2 pathway; the protective role of Nrf2 against many heavy metals as Cd and Mn has been shown (Casalino *et al.* 2007). The data from Massrieh *et al.* (2006); Wang *et al.* (2007b) and Lau *et al.* (2013) showed the upregulation of Nrf2 pathway in response to As-induced stress in different human cell lines. Nrf2 also plays an important role in reduction of methyl mercury toxicity (Kumagai *et al.* 2013). Nrf2 nuclear translocation and activation was demonstrated after exposure to Cr in mouse Hepa1c1c7 cells (He *et al.* 2007). Moreover, Lewis *et al.* (2006) showed that Ni exposure increased Nrf2 nuclear accumulation in human THP1 monocyte. On the contrary, a study of Simmons *et al.* (2011) revealed that Cr, Mn, and Ni did not stimulate Nrf2 activity in any cell model tested; however, at high concentrations, they induced apparent cytotoxicity that could be through a mechanism other than oxidative stress. Such differences in responses between cell models could be according to tissue, cell type, culture conditions and the experimental endpoint in addition to the pharmacokinetic of metals (Stohs & Bagchi 1995). Moreover, metals exert multiple effects on the Nrf2 pathway, including reduction of sulfhydryl groups in Keap-1, inhibition of proteasomal pathways and activation of MAPK and hence Nrf2 phosphorylation and upregulation of anti-oxidant genes (Simmons *et al.* 2011).

1.3.1.2 Nrf2 and reproduction

The significant role of Nrf2 in pregnancy and in protecting the embryo from oxidative stress in the womb has been highlighted (Cheng *et al.* 2013). In normal pregnancy, the level of Nrf2 declines only after term vaginal delivery revealing its importance for fetal survival. However, it significantly decreases and its binding to Keap-1 becomes stronger when the uterus is infected, since Nrf2 is sensitive to the maternal immunological status (Lim *et al.* 2015b; Sussan *et al.* 2017). In preeclampsia, several studies described the decreased nuclear localization and hence activity of Nrf2 in placentas of both human and rats (Chigusa *et al.* 2012; Acar *et al.* 2014). This consequently assists the impact of ROS which impair the proliferation of placental trophoblasts and increase the risk of intrauterine growth retardation (IUGR) (Kweider *et al.* 2012). Deficiency of Nrf2 caused fetal DNA damage and neurological deficits, and also contributes to inflammation-induced trophoblastic apoptosis (Ishii *et al.* 2000; Cho *et al.* 2005).

The importance of Nrf2 in maintaining the ovarian homeostasis and function was demonstrated by (Hu *et al.* 2006). It acts as a chemical sensor that protects the cells from ovotoxicants such as chemicals, carcinogens and pathogens (Dinkova-Kostova *et al.* 2002; Nguyen *et al.* 2004). Exposure to environmental or occupational toxicants could underlie ovarian destruction in animals or premature ovarian failure in human which is commonly associated with infertility (Hoyer & Sipes 1996). Deletion of Nrf2 was found to reduce the number of ovarian follicles and accelerates ovarian ageing in mice (Lim *et al.* 2015a). A recent study of Akino *et al.* (2018) showed that Nrf2 activation alleviated oxidative stress in human granulosa cells and raised the possibility to therapeutically target Nrf2 to combat the oxidative stress-induced fertility disorders.

1.3.1.3 The dark side of Nrf2

The dark side of Nrf2 activation was discovered in many cancerous cells including ovarian cancer (Marchan & Bolt 2013; Grossman & Ram 2013; van der Wijst *et al.* 2014). Preliminary studies demonstrate that deactivation of Nrf2 is as important as activation of Nrf2 (Niture *et al.* 2014). Uncontrolled activation of Nrf2 detected in cancerous cells, provides an ideal environment for cellular growth against oxidative stress and chemotherapy (Wang *et al.* 2008). Although the cause is not fully understood, it is widely accepted that the sustained activity of Nrf2 in cancer is due to Keap-1 mutation that induces overactivation of Nrf2 and its downstream

genes in addition to inhibition of Nrf2 degradation (Ganan-Gomez *et al.* 2013; No *et al.* 2014). Besides, epigenetic modification in both Nrf2 and Keap1 may also activate Nrf2 as reported in various human malignancies (Muscarella *et al.* 2011; Barbano *et al.* 2013).

1.3.2 Nuclear factor kappa B (NF-κB)

NF-κB is a family of transcription factors composed of five subunits; RelA (p65), RelB, c-rel, p50 and p52, however, the term NF-κB is usually used to refer specifically to the 'classical' p50/RelA (p65) heterodimer (Baldwin, JR 1996; Hayden & Ghosh 2004). The class I (p65, RelB, c-Rel) which contains a transactivation domain (TAD), has intrinsic ability to activate transcription. The class II (p50 and p52) lacks TAD so their homodimers generally repress transcription of target genes but their heterodimers with class I have transcriptional activity (Perkins 2007). It is known as pleiotropic agent that regulates immunity and inflammatory responses, angiogenesis, apoptosis, cell cycle, and cell proliferation (Shishodia & Aggarwal 2004; Perkins 2004; Sethi *et al.* 2008).

In the cytoplasm, NF-κB is bound to its inhibitory protein, inhibitors of NF-κB (IκBs) (Whiteside & Israel 1997). Upon stimulation with pro-inflammatory cytokines as interleukin IL-1β and tumor necrosis factor TNF-α, viral infection, bacterial lipopolysaccharide (LPS) or ROS, IκB is phosphorylated by kinases (IKKs) or MAP kinases and degraded by proteasomes (Karin & Ben-Neriah 2000). This allows NF-κB translocation to the nucleus where it binds to a κB element located in the promoter regions of proinflammatory genes inducing their expression. Activated NF-κB also causes production of IκBα, which enters the nucleus, captures freed NF-κB, and facilitates its export to the cytoplasm for termination of transcription (Hayden & Ghosh 2004; Ferreiro & Komives 2010). The transcriptional activation of NF-κB depends also on the phosphorylation of its active subunit p65/RelA by IKK, along with the activation of a variety of other kinases (Viatour *et al.* 2005). Furthermore, the cysteine residue in position 62 in p50 which is essential for DNA binding is sensitive to oxidative modifications. While NF-κB activation and nuclear translocation are stimulated by oxidizing conditions, reducing condition is necessary for DNA binding (Bowie & O'Neill 2000). The complexity of NF-κB response arose from the fact that, NF-κB subunits contain 300 amino-acid N-terminal domains, called the Rel homology domain (RHD), which is required for dimerization, DNA binding, nuclear translocation and IκB binding.

The composition of the NF-κB dimer (Figure 1.3) can even vary according to the cell type and the nature of activator. Since NF-κB is expressed in all mammalian cell types and there are diverse stimulants, the contradictory functions of NF-κB may vary, dependent on the cell type, the context and the onset of its activation (Muenchen *et al.* 2000; Saccani *et al.* 2003; Perkins & Gilmore 2006; Mincheva-Tasheva & Soler 2013).

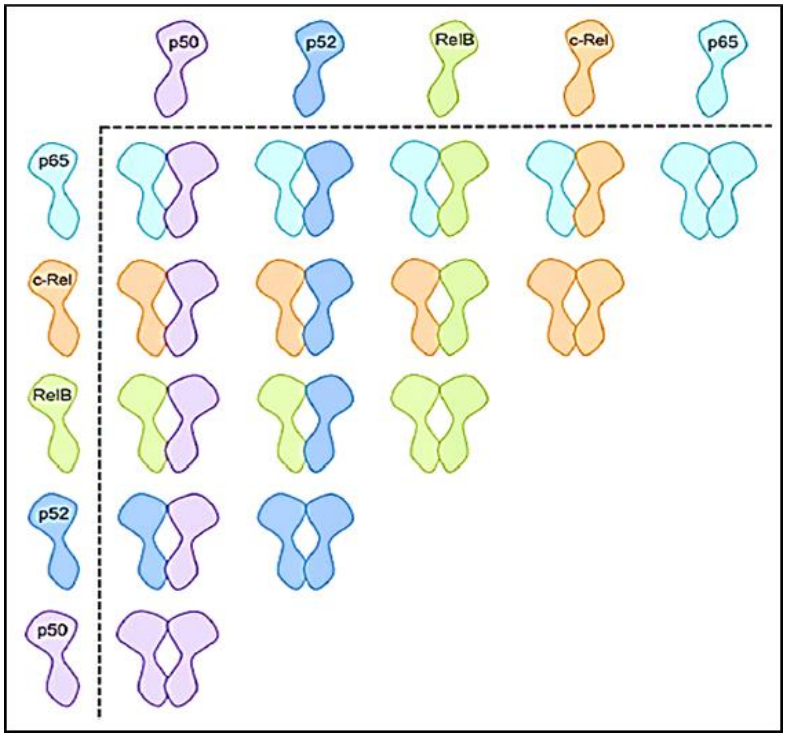


Figure 1.3: Nuclear factor NF-κB dimer pairs (Herrington *et al.* 2016)

1.3.2.1 NF-κB and heavy metals

NF-κB signaling with metals has different attitudes; Zn has been previously reported to inhibit the NF-κB pathway by binding directly to IKKβ (Liu *et al.* 2013).

It is well established that acute ROS production can stimulate the NF-κB pathway. Knowing that, Cd can disrupt the oxidative balance within cells through generation of reactive oxygen species (ROS). However, Cd has been shown to inhibit the NF-κB pathway (Xie & Shaikh 2006; Cox *et al.* 2016). In contrast, Cd has been found to be an activator of NF-κB in acute exposure models that utilized higher Cd concentration (Freitas & Fernandes 2011). Nickel was shown to activate NF-κB in mice (Liu *et al.* 2015). Mercuric chloride was reported to enhance NF-κB signaling pathway in rat's liver (Zhang *et al.* 2017).

1.3.2.2 NF-κB and reproduction

NF-κB is highly expressed in sertoli cells of murine testis where it regulates many genes involved in spermatogenesis (Delfino *et al.* 2003) but can also inhibit steroidogenesis (Hong *et al.* 2004). The role of NF-κB in ovarian cells has been observed, it has anti-apoptotic effects on rat corpus luteum and inhibits progesterone (P4) catabolism (Wang *et al.* 2002; Telleria *et al.* 2004) but has no effect on P4 secretion in human luteal cells (Gonzalez-Navarrete *et al.* 2007). (Pavlova *et al.* 2011) reported that, NF-κB along with FSH regulates porcine ovarian functions including proliferation, secretory activities and apoptosis. It has been observed that aberrant NF-κB/ IκBa signaling is involved in both murine and bovine oocyte ageing, suggesting a role for age-related fertility decline (Patel *et al.* 2007; Tatone *et al.* 2008). NF-κB is involved in implantation, the menstrual cycle regulation and parturition in human (Cookson & Chapman 2010). NF-κB can also regulate the endocrine functions in non-ovarian cells. It can induce prostaglandin (PG) production via upregulation of cyclooxygenase and prostaglandin E synthesis in human amnion mesenchymal cells (Sugino *et al.* 2004; Ackerman *et al.* 2008). NF-κB inhibited the expression of insulin-like growth factor-I (IGF-I) in hepatocytes (Buzzelli *et al.* 2008).

NF-κB is critical for embryonic development since p65 knockout mice died on E15 and p65/p50 double knockout mice died on E13 due to the defects in hemopoietic liver progenitors (Grossmann *et al.* 1999). Almost all the NF-κB pathway members are detected in mouse embryonic, trophoblastic, and uterine cells. It was supposed that NF-κB, possibly through its anti-apoptotic effect, may guard the embryos against embryopathic stressors during organogenesis (Torchinsky & Toder 2004). Furthermore, the role of IκBα/NF-κB in bovine oocyte maturation and early embryogenesis has been shown, where NF-κB/p65-binding activity decrease during oocyte maturation then increases during EGA, suggesting a similar pattern of oocyte-to-embryo transition in human (Paciolla *et al.* 2011). NF-κB signaling is also important in regulating the early differentiation of stem cells including embryonic stem cells (Yang *et al.* 2010), hematopoietic stem cell (Montano-Almendras *et al.* 2012), and mesenchymal stem cells (Cho *et al.* 2010).

1.3.2.3 The dark side of NF-κB

Like Nrf2, several lines of evidence demonstrated that NF-κB signaling acts as a double- edge sword (Perkins & Gilmore 2006; Yang *et al.* 2010; Mukherjee *et al.* 2015). Extensive studies have shown that nuclear transcription factor signaling pathway involving NF-κB could be responsible for either term or preterm labor since they have been implicated in the formation of pro-inflammatory and pro-labour mediators in human placenta (Lappas & Rice 2009; Lappas 2012). NF-κB is also involved in many pregnancy maladies like pre-eclampsia and IUGR via its proapoptotic activity in the placental tropoblast (Aban *et al.* 2004; Vaughan & Walsh 2012). NF-κB signaling is always activated whenever reproductive stress exist (Cindrova-Davies *et al.* 2007). In endometriosis, the increased expression of NF-κB has been reported in vitro and in vivo (Sakamoto *et al.* 2003; Lousse *et al.* 2008).

Another discrepancy has been observed in the role of NF-κB in cancer (Mukherjee *et al.* 2015). NF-κB induces a crosstalk between inflammation and cancer since the elevated NF-κB activity mediates pro-inflammatory cytokines and chemokines at the tumor site which contribute to the tumorigenic microenvironment (Luo *et al.* 2005; Terzic *et al.* 2010). Herein the role of NF-κB in cancer cells as a master regulator of cell proliferation, apoptosis, angiogenesis and metastasis was anticipated (Kim *et al.* 2006). In contrast, other experimental data suggest that NF-κB can have tumor suppressor effect in some circumstances which are not as fully understood as its tumor promoting roles cellular survival (Perkins & Gilmore 2006; Qin *et al.* 2007).

Great efforts have been done by pharmaceutical companies to develop inhibitors of IKK and NF-κB to treat inflammation, cancer and resistance to chemotherapeutic agents (Kim *et al.* 2006; Duncan *et al.* 2008; Lounnas *et al.* 2009; Umezawa 2011). Although about 700 of different NF-κB inhibitors have been in vitro studied, very few of them has been present in the clinic (Herrington *et al.* 2016). This can be ascribed to the vast and complex range of biological activities of NF-κB making its therapeutic targeting a challenge (DiDonato *et al.* 2012). (Camandola & Mattson 2007) suggested the development of drugs targeting NF-κB in a cell-specific manner. So, NF-κB inhibition should be specific, transient and highly reversible to avoid long-term immunosuppression (Baud & Karin 2009). It has been demonstrated that, murine macrophages and dendritic cells which are the central mediators of immunity, showed massive apoptosis after treatment with several IKK/NF-κB inhibitors. This was induced by ROS accumulation, which induces a loss of mitochondrial membrane potential and activation of

caspase signaling (Tilstra *et al.* 2014). While NF-κB inhibitors-mediated suppression of these cells is desired in cancer and inflammation management, prolonged NF-κB inhibition can be detrimental due to its important role in innate immunity and cellular hemostasis (Gupta *et al.* 2010). Moreover, one should consider the complex molecular mechanisms that regulate either the oncologic or the therapeutic role of NF-κB (Herrington *et al.* 2016).

1.3.3 Nrf2 and NF-κB cross-talk

As mentioned before, each signaling molecule is not working independently but they will form networks and interactions between them, leading to the complexity of signal molecule research. Oxidative stress that occurs during reproduction activates many molecules, however the interaction among them is not fully understood (Lu *et al.* 2018).

Though accumulating studies have been conducted to unravel the interplay between Nrf2 and NF-κB, discrepant results still remain. It was found that, the absence of Nrf2 can exacerbate NF-κB activity leading to increased cytokine production and aggressive inflammation in murine astrocytes (Pan *et al.* 2012). Usually, phytochemicals like curcumin and quercetin activate Nrf2 by inhibiting NF-κB and its downregulated genes (Liu *et al.* 2015; Sahin *et al.* 2016). In contrast, many factors as ischemia, LPS and cigarette smoke have been found to increase both Nrf2 and NF-κB activity (Wakabayashi *et al.* 2010, 2010; Meng *et al.* 2017). In addition, in vivo studies have showed that NF-κB activity is decreased in livers isolated from Nrf2−/− mice and NF-κB binding activity is lower in Nrf2−/− than in Nrf2+/+ mice (Yang *et al.* 2005). According to (Ganesh Yerra *et al.* 2013), Nrf2 and NF-κB pathways inhibit each other at their transcription level via protein–protein interactions or through secondary messenger effects. It is currently well-established that both positive and negative regulations exist between NF-kB and Nrf2/ARE pathways (Wardyn *et al.* 2015).

As shown in Figure 1.4, Keap-1 was reported to suppress IKK activity either by ubiquitination or inhibiting its phosphorylation, thus inhibiting the activity of NF-κB (Lee *et al.* 2009) whereas, p65 helps to increase the abundance of nuclear Keap1 levels and diminish Nrf2/ARE activity (Yu *et al.* 2011). NF-κB also competes with Nrf2 for the transcriptional co-activator CBP which shows higher preference to NF-κB. Moreover, p65 suppress ARE by recruitment of the co-repressor, HDAC3 allowing its interaction with CBP resulting in histone hypoacetylation (Liu *et al.* 2008). Meanwhile, HO-1 which is one of Nrf2- induced genes was found to inhibit NF-κB

related pathways (Soares *et al.* 2004). Nrf2 has many κB sites in its proximal promoter, which are subjected to binding and transcription initiation by p65 (Rushworth *et al.* 2012). It has been found also that the N-terminal region of p65 subunit of NF-κB is physically associated with Keap-1, and NF-κB not only interacted with cytosolic Keap-1 but also enhanced its nuclear translocation.

This provides further mechanism for Nrf2-ARE inhibition by NF-κB (Yu *et al.* 2011). The role of Nrf2/ NF-κB cross-talk is to maintain redox homeostasis in healthy cells (Wardyn *et al.* 2015). However, this regulation is disturbed during pathological conditions giving the opportunity for therapeutic intervention (Ganesh Yerra *et al.* 2013; Wardyn *et al.* 2015; Ahmed *et al.* 2017; Khurana & Sikka 2018).

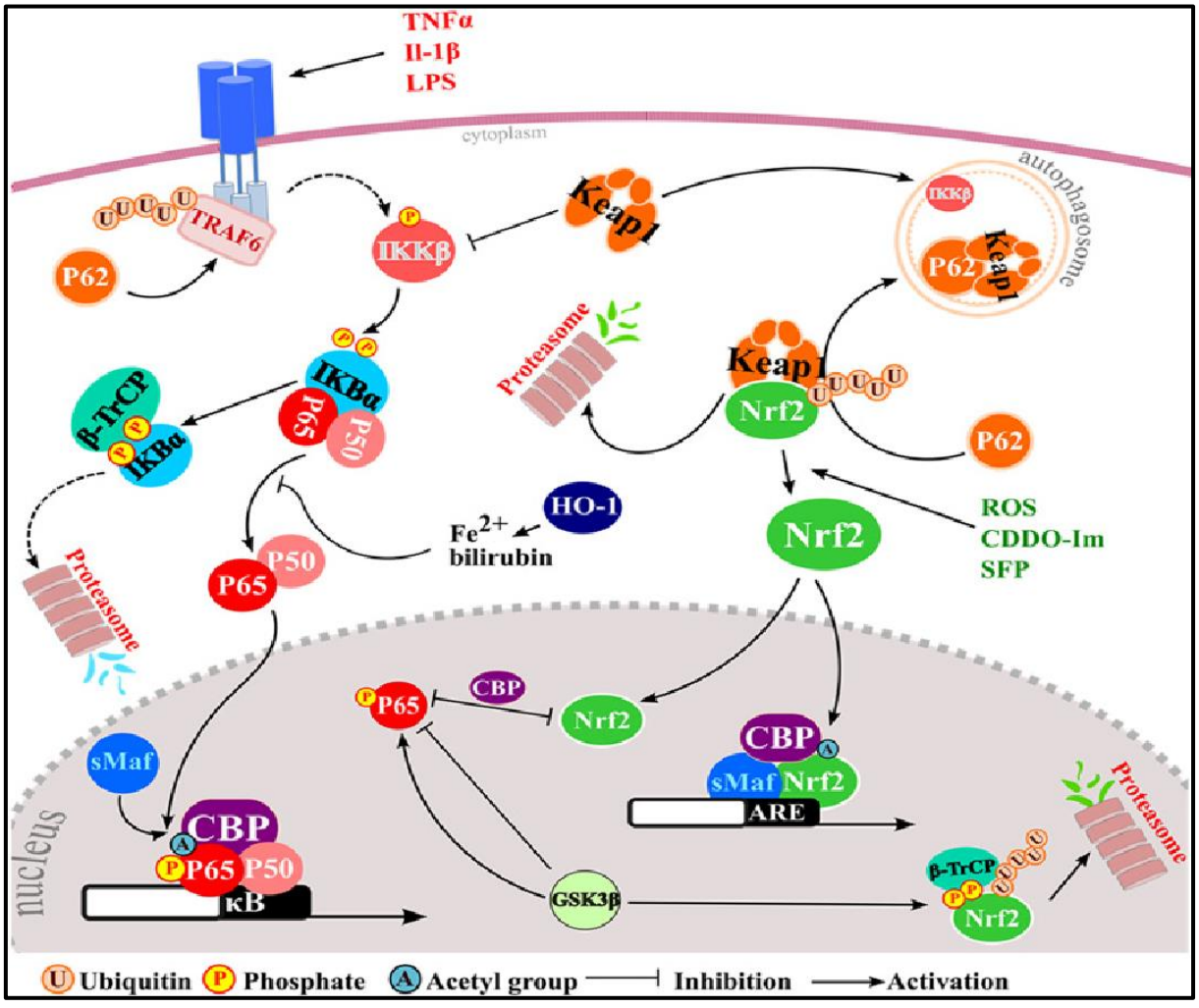


Figure 1.4: Molecular cross-talk between Nrf2 and NF-κB signaling pathways (Wardyn *et al.* 2015).

1.4 Epigenetic modification by lead exposure

1.4.1 Epigenetics and environment

Epigenetics, or “on top of genetics” as a literal meaning, is the study of heritable changes in the genetic expression without alterations in the underlying DNA sequence of a gene (Wolffe & Guschin 2000; Kanherkar *et al.* 2014).

It is well-known that, the effect of environment on the cellular functions may occur on different levels. Environmental factors may disrupt the cellular functions on the level of signaling mechanisms, protein functions or apoptotic pathway, where these events are mainly due to ROS production as reported in previous studies (Flora *et al.* 2004; Avery 2011). Furthermore, the environmental agents may affect the genome directly inducing mutations that could result in disruptions in the cell function or could pass without any phenotypic alterations (Crow 2000). Although such mutations are responsible for genetic diseases affecting human, their incidence is relatively low (Wallace 2010). McCarrey (2012) showed that not all environmental toxins are able to induce genetic mutation suggesting that there should be other molecular mechanisms explaining the gene-environment interactions far from the alterations in genomic sequence. So epigenetic processes are often the mechanistic link by which a phenotypic change could exist in response to the environment and the change in gene expression (Bollati & Baccarelli 2010). Abnormal epigenetic mechanisms can result in alterations in gene expression patterns, these epigenetic aberrations can lead to pathological consequences (Crews *et al.* 2014). Epigenetic traits, including modifications of DNA methylation, histone proteins, and the action of noncoding RNAs, all contribute to regulating activation or repression of gene expression (Lewis *et al.* 2005; Richards 2006).

1.4.2 Lead as epigenetic modifier

Several experimental and epidemiological studies have identified gene–environment interactions and epigenetic regulations to have a plausible role in modification of the harmful and long-lasting effects of Pb (Zheng *et al.* 2011; Mitra *et al.* 2017). The studies of Bihaqi *et al.* (2011); Dosunmu *et al.* (2012) and Eid & Zawia (2016) reported Pb as epigenetic modifier responsible for the latent incidence of neurobehavioral disorders like Alzheimer's disease in both animal models and human. Fetal Pb exposure has been found to inversely associate with global DNA methylation in the cord blood (Pilsner *et al.* 2009). Lead has been also reported to inhibit TET

enzyme family by oxidative damage (Senut *et al.* 2012). Similarly, (Schneider *et al.* 2013) reported that hippocampal DNMTs where affected by developmental Pb exposure in animal model. Moreover, Pb has showed to modify the methylation of some genes, for example, it induces hypermethylation of ALAD gene and downregulation of ALAD transcription (Li *et al.* 2011). *In vitro* Pb exposure of human embryonic kidney cell line showed change in the differentially methylated region (DMR) regulating some imprinted genes such as IGF-2 and PEG1/MEST (Nye *et al.* 2015).

Luo *et al.* (2014) demonstrated an increase in the level of histone acetylation in brain of Pb exposed rats, and concluded that this perturbation in histone proteins could be the underlying cause for Pb mediated attention deficit hyperactivity disorder. However, the alteration of histone modification under Pb exposure has not been observed in human yet (Mitra *et al.* 2017).

Recently, Sanders *et al.* (2015) showed several miRNA in cervical swabs from 60 pregnant women and identified their association with bone and BLL. While the expression of miR-297 and miR-188 increased in association with BLLs, six miRNAs (miR-320e, miR-22, miR-93, miR-769, miR-425, and miR- 361) decreased and miR-297 increased associated with patellar Pb level. In the rat model, a set of miRNA related to neurodegenerative diseases has been changed due to chronic Pb exposure, for example, increased expression of miR-211, miR-204, miR-448, miR-34b, miR-449a, and miR-34c with decreased expression of miR-49 (An *et al.* 2014).

1.4.3 Transposable elements

DNA methylation could be investigated either in specific genes or using genome wide methylation markers such as methylation within transposable elements (TEs) (Roman-Gomez *et al.* 2005; Bollati *et al.* 2007; Pilsner *et al.* 2007). TEs are highly repetitive DNA sequences, occupying at least half of the human genome (Koning *et al.* 2011), derived from remnants of either viral DNA (transposons) or viral RNA (retrotransposons) that were incorporated into the human genome over evolutionary history (Carnell & Goodman 2003; Wallace *et al.* 2008). They have been called jumping genes because of their ability to move through the host genome (McClintock 1984; Hancks & Kazazian 2012). Unlike DNA-transposons that move by “cut and paste mechanism” occupying about 3.2 % of the human genome, retrotransposons move by “copy and paste mechanism” and they are the most abundant TEs in the mammalian genomes.

(Chalopin *et al.* 2015). Retrotransposons are further divided into autonomous long terminal repeat (LTR) and the non-LTR containing retrotransposons. LTR are endogenous retro virus (ERVs) ranging from 100 bp to 5kb comprising 8.6% of the human genome and are considered fossils of ancient retroviruses (Bannert & Kurth 2006; Jern & Coffin 2008). ERVs are transcriptionally active in mice but not in human except under some pathological conditions (Munoz-Lopez *et al.* 2016). Non-LTR retrotransposons include the autonomous long interspersed nuclear elements (LINEs) which comprise 17-25% of the human genome and the non- autonomous short interspersed nuclear elements (SINEs), occupying 12.8 % of the human genome (Kazazian 2004). The term autonomous here indicates the property of self-sufficiency for transposition (Carnell & Goodman 2003).

LINE-1 (L1) is the prime non-LTR retrotransposon since it occupies a substantial proportion of the genome, it is also active in both mouse and human, and mediates the transposition of SINEs by LINE-1 encoded proteins (Dewannieux *et al.* 2003). The human genome encompasses more than 100,000 copies of LINE-1 elements most of them are defective due to truncation or mutation, however about 3000 elements are complete of which 300 are transcriptionally active "hot" (Brouha et al. 2003). It was believed that L1 are just "junk DNA" with no biological functions until their critical roles have been established for human development, genome stability, tissue differentiation and gene expression (Belancio *et al.* 2009). Furthermore, some newly inserted L1s were found to be highly polymorphic and specific to a few individuals, suggesting that L1 elements may contribute to the susceptibility of individuals to develop disease over other (Beck *et al.* 2010; Huang *et al.* 2010).

As shown in Figure 1.5, a typical L1 element has two internal promoters at their 5' end, two non-overlapping open reading frames (ORF1 and ORF2), and a 3′ untranslated region that ends with a poly (A) tail (Becker *et al.* 1993; Beck *et al.* 2011). TEs are known to be harmful to the host genome due to their transposition which may induce DNA breakage, mutational insertions and chromosomal rearrangement that contribute to tumor initiation and progression (Iskow *et al.* 2010; Criscione *et al.* 2014). Consequently, the host strives to regulate their expression by many mechanisms at DNA, RNA and protein levels. However, the central mechanism to govern transposition is the DNA methylation at TEs promoters (Bourc'his & Bestor 2004; Barchitta *et al.* 2014).

The L1 promoter contains numerous CpG islands which are extensively methylated to hinder their expression and maintain the genome integrity (Liang *et al.* 2002). The loss of methylation has been associated with reactivation of retrotransposons and consequently with altered chromosome integrity and modified gene expression by insertion events which characterizes a large percentage of human neoplasms (Ross *et al.* 2010; Jones 2012). So studying DNA methylation patterns of L1 could be an informative and cost effective method to evaluate the relation between methylation profile and exposure to environmental factors (Wang *et al.* 2010). Moreover, the role of L1 repeats as a surrogate marker for global DNA methylation in human genome has been proposed by many authors (Yang *et al.* 2004; Cordaux & Batzer 2009; Huen *et al.* 2016).

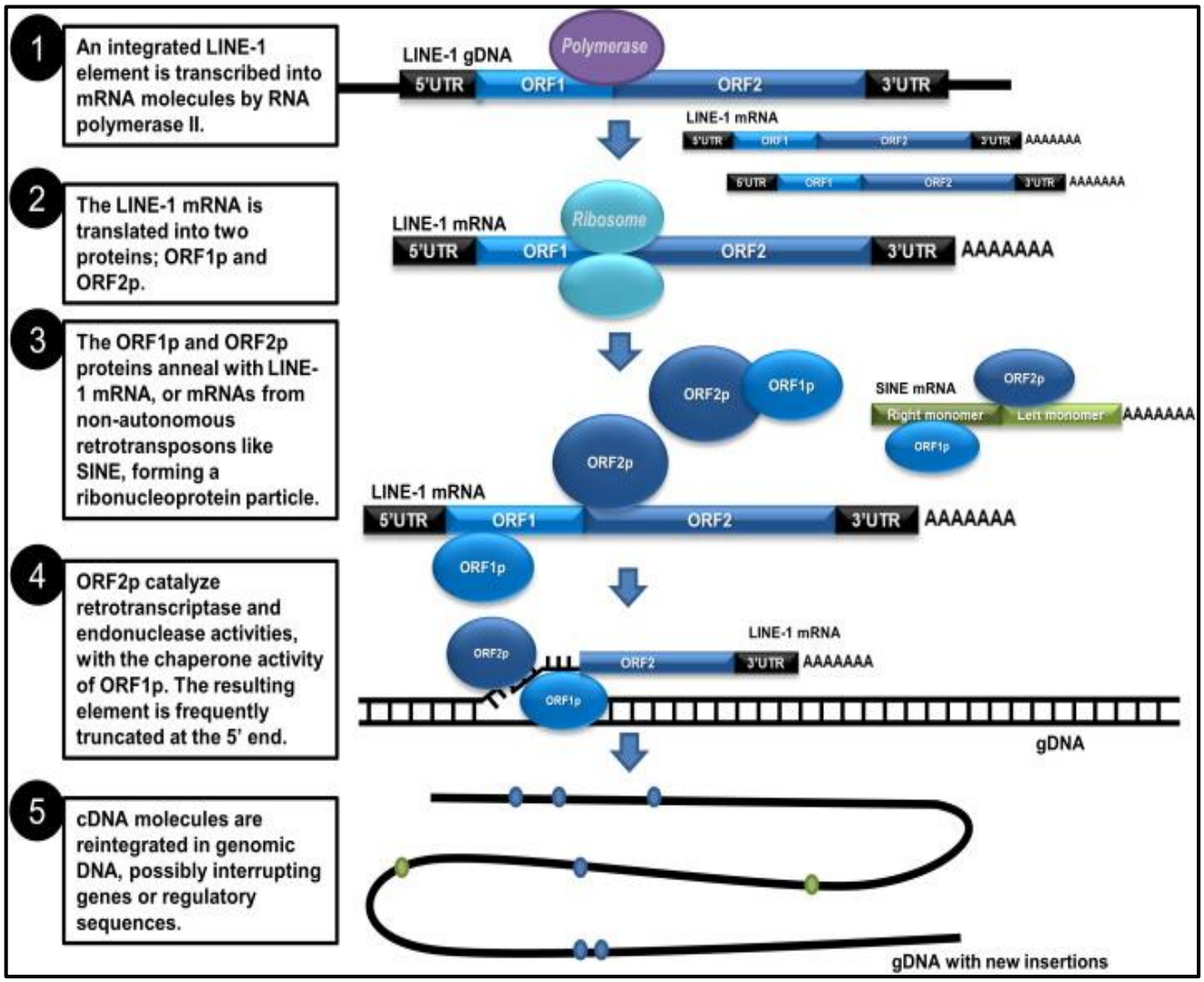


Figure 1.5: The mechanism of LINE-1 (L1) transposition (Miousse *et al.* 2015)

1.4.4 Lead impact on the expression of transposable elements

The early study of (Pilsner *et al.* 2009) showed a decrease in TEs (LINE-1 and SINE) methylation in cord blood DNA associated with maternal exposure to Pb. Similarly, (Wright *et al.* 2010) found that exposure to Pb has also been associated with alterations in DNA methylation in peripheral blood specially L1 element. They considered changes in L1 methylation as a biomarker of past Pb exposure. In vitro exposure of human embryonic kidney HEK293 cells to Pb for 24 and 48 hours elicited a significant loss of L1 methylation (Li *et al.* 2013). These results were further confirmed in a case-control study involving workers occupationally exposed to Pb in a battery plant that reported a significant hypomethylation of blood L1 as compared to healthy volunteers (Li *et al.* 2013). A recent study revealed the ability of Pb to induce alteration in DNA methylation of a member of ERVs family, IAP in different tissues of a murine model (Montrose *et al.* 2017).

1.5 Bovine as model for human

In the field of reproductive toxicology, most of data are obtained from case studies, clinical analyses and in vivo tests with rodents (Bremer *et al.* 2007). In human, studies of environmental exposure are usually not experimental but observational which lack the significant number of samples (Bellinger 2004). The use of in vivo assays studies to test the safety of many thousands of chemicals, requires large numbers of animals making an unsolved debate many years ago (Lorenzetti *et al.* 2011), since about 70 % of animals used in toxicological studies are only for reproductive toxicological assays (Spielmann 2009). Moreover, rat and mice are not the most suitable models for human, especially when considering oocyte maturation and fertilization (Menezo & Herubel 2002). Although, alternative and novel assays have been developed including computational and integrative in vitro tests using embryonic stem cells and cell lines, they are still unsatisfactory (Piersma *et al.* 2013). Due to the complicated nature of reproductive cells such as oocytes, they cannot be easily mimicked by somatic cells (Li & Albertini 2013). The oocytes with their surrounding cumulus cells have to undergo maturation, which is critical process involving oocyte meiosis. Furthermore, oocytes are susceptible to epigenetic modifications that may interfere with fertilization and subsequent early embryo development (Sirard 2012). A recent use of humanized mice to study many human-specific pathogens has been encouraged; however translational studies in these animals are not indicative when considering female reproduction. Beside, oocyte maturation and fertilization in mice are difficult

to be genetically changed to be humanized (Brehm *et al.* 2013). A growing body of evidence from different studies revealed the possibility to apply reliable toxicity screening methods, by using bovine or porcine as models for human in vitro IVM, IVF as well as embryo production (Campagna *et al.* 2007; van Beker Woudenberg *et al.* 2012). The ovaries of both bovine and porcine models are just leftover organs obtained from slaughterhouse as animals enter the food production chain. So the problem of using large number of animals for reprotoxic testing can be diminished (Magnusson 2005). In addition, these farm animals are supposed to rear on grass or locally made fodder, so they can serve as good biomonitor for environmental contamination (Lopez Alonso *et al.* 2004).

The early work of (Malhi *et al.* 2005), validated the use of the bovine model to study reproductive aging in women and concluded that cow is a sound model to investigate conserved ovarian and endocrine functions since the obtained results were similar to those reported for women approaching menopause. Oocyte maturation process differs greatly among species, where it takes several weeks in mice (Wassarman & Litscher 2012), but several months in bovine, porcine and human (Driancourt 2001, 2001; Baerwald *et al.* 2012). When comparing the oocyte diameter and the time to reach the 2-cell stage and blastocyst, they were found to be similar between human, porcine and bovine, but shorter in mice (Santos *et al.* 2014). The interactions between embryo and the corpus luteum are similar in cows and humans (Menezo & Herubel 2002). The process of DNA methylation which is indispensable for human embryo development shows a higher degree of structural similarity with bovine than mouse (Vassena *et al.* 2005; Rodriguez-Osorio *et al.* 2010). With regard to intrinsic paternal and maternal regulatory processes, bovine and human preimplantation embryos look similar (Bilodeau-Goeseels 2011).

1.6 Objective and rational of the study

The female reproductive system is greatly affected by exposure to environmental toxicants which impairs different aspect of reproduction starting from gametes formation till embryo development. Lead being one of the reproductive toxicants, is believed to induce oxidative stress resulting in its pathophysiology. However our knowledge of its impact on Nrf2 and NF-κB signaling molecules is still low. The role of Pb as epigenetic modifier has been recently clarified by many authors that could further be added to its mode of actions. In addition, the data on the effect of Pb on bovine model are scarce.

Finally, the possibility to protect the pregnant female from Pb exposure using antioxidant prophylactic therapy could be a matter of concern to guard against Pb toxicity especially in Pb exposed areas.

1.6.1 Hypothesis

Lead-induced oxidative stress may affect the Nrf2 and NF-κB dynamic cross-talk which subsequently perturb embryo development.

1.6.2 Objectives of the study

- To study the effect of Pb on bovine granulosa cells *in vitro* and its association with Nrf2 and NF-κB pathways (Chapter 2).

- To study the response of bovine preimplantation embryo to stage specific exposure to Pb (Chapter 3).

- To study the effect of *in utero* Pb exposure and the possibility to use antioxidant (taurine) as prophylactic agent against Pb toxicity using rat model (Chapter 4)**.**

Chapter 2

Regulation of Nrf2 and NF-κB During Lead Toxicity in Bovine Granulosa Cells

Hoda Samir Aglan[1,4], Samuel Gebremedhn[1], Dessie Salilew-Wondim[1], Christiane Neuhof[1], Ernst Tholen[1], Michael Hölker[1, 2], Karl Schellander[1,3] and Dawit Tesfaye[1,3] *

[1]Institute of Animal Science, Department of Animal Breeding and Husbandry, University of Bonn, Bonn, Germany

[2]Teaching and Research Station Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany

[3]Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

[4]Department of Pharmacology, National Organization for Drug Control and Research, Giza, Egypt

*Corresponding author

(Manusript accepted in Cell and Tissue Research)

2.1 Abstract

Lead (Pb), one of the pervasive and protracted environmental heavy metals, has been believed to affect the female reproductive system by inducing oxidative stress. The Nrf2 and NF-κB are the two key transcriptional factors (TFs) regulating cellular redox status and response against stress and inflammation, respectively. The aim of this study was to investigate the impact of Pb on bovine granulosa cells (GCs) and its association with the cross-talk between Nrf2 and NF-κB. For this, bovine GCs were cultured in vitro and exposed to different doses of lead acetate (1, 2 or 3 µg/ml) for 2 hours. Results showed that exposure of GCs to Pb induced ROS accumulation and protein carbonylation, in addition to cell cycle arrest at G_0/G_1 phase, reduction in cell viability and decrease in the expression of cell proliferation marker genes (CCND2 and PCNA). Moreover, Pb elicited downregulation of both Nrf2 and NF-κB and their downstream genes. Lead increased the expression of endoplasmic reticulum (ER) stress marker genes (GRP78 and CHOP) and the pro-apoptotic gene (caspase-3) while the anti-apoptotic gene (BCL-2) was reduced. Our findings suggest that Pb driven-oxidative stress affected GCs proliferation, enhances ER stress, induces cell cycle arrest and mediates apoptosis probably via disruption of Nrf2/NF-κB cross-talk

Keywords:
Lead, granulosa cells, Nrf2, NF-κB, ROS, endoplasmic reticulum, apoptosis.

2.2 Introduction

Lead (Pb) is a widespread naturally occurring toxic heavy metal that has a broad spectrum of industrial applications (Sanders *et al.* 2009). Due to its non-biodegradable nature, Pb extends its abundance in the environment and constitutes a persistent public health problem throughout the world, despite extensive efforts to reduce its use (Ahamed & Siddiqui 2007). Reproductive and developmental toxicity of Pb have been demonstrated in both males and females of experimental animals and human (Pant *et al.* 2003; Qureshi *et al.* 2010; Vigeh *et al.* 2011). Lead can accumulate in testes, epididymis and seminal vesicle inducing adverse effects on spermatogenesis, prostatic function, sperm count and motility (Shan *et al.* 2009; Chowdhury 2009). Moreover, the impact of Pb on female reproduction is more profound and even could exceed other environmental toxins, it has been documented to cause infertility, miscarriage, pregnancy hypertension, premature delivery and preeclampsia (Seyom *et al.* 2015; Bayat *et al.*

2016). Experimental studies have detected Pb in the follicular fluid of many species, in human (Paksy *et al.* 2001), in cattle (Swarup *et al.* 2005), in sheeps (Bires *et al.* 1995) and in mouse (Taupeau *et al.* 2001). Ovarian accumulation of Pb irreversibly impaired folliculogenesis, with high rate of atresia, decreasing the number of the primary follicles in mice (Taupeau *et al.* 2001, 2001; Sharma *et al.* 2012). The effect of Pb as endocrine disruptor was also documented, as it could interrupt several points along the hypothalamic–pituitary–gonadal (HPG) axis, inducing disruption of the gonadal function and reproductive hormones (Doumouchtsis *et al.* 2009; Pillai *et al.* 2010). Moreover, occupationally Pb-exposed women exhibited menstrual abnormalities, including hypermenorrhea and early menopause (Eum *et al.* 2014).

Although Pb toxicity cannot be linked to a single mechanism, oxidative stress could be implicated in the pathophysiology of Pb toxicity (Flora *et al.* 2012). According to (Sharma *et al.* 2012), Pb-induced oxidative stress is responsible for ovarian dysfunction and poor fertility outcomes. Moreover, oxidative stress arises when the balance between pro-oxidants and antioxidants is disrupted (Luderer 2014). This in turn activates a variety of transcriptional factors (TFs) which proved to have great significance in the etiology of reproductive diseases (Bolisetty & Jaimes 2013; Lu *et al.* 2018). The Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and Nuclear factor-kappa B (NF-κB) are the two key redox-sensitive TFs regulating the cellular redox status in response to intracellular and environmental signals (Wardyn *et al.* 2015). The Nrf2 mediates transcription of phase II antioxidant genes, which are responsible for the clearance of ROS, providing protection against the accumulation of toxic metabolites (Kang *et al.* 2005; Zhang *et al.* 2013). Deletion of Nrf2 was reported to reduce the number of ovarian follicles and accelerate ovarian ageing in mice (Lim *et al.* 2015).

Moreover, the NF-κB is a pleiotropic factor, which plays important roles in immunity and inflammation. In addition, it is involved in regulating other processes, including development, cell growth, survival, and proliferation (Vallabhapurapu & Karin 2009). The role of NF-κB in ovarian cells has been observed, it has anti-apoptotic effects on rat corpus luteum and inhibits progesterone (P4) catabolism (Wang *et al.* 2002; Telleria *et al.* 2004). Pavlova *et al.* (2011) reported that, NF-κB along with FSH regulates porcine ovarian functions such as proliferation, secretory activities and apoptosis. Furthermore, pharmacological animal studies showed a

potential crosstalk between Nrf2 and NF-κB pathways, this crosstalk defines the cellular response to different environmental insults (Li *et al.* 2008; Wardyn *et al.* 2015; Lu *et al.* 2018). Ovarian granulsa cells (GCs) are sensitive to reactive oxygen species (ROS) and can protect oocytes from oxidative damage through their own antioxidant system during maturation of oocytes (Tripathi *et al.* 2013). The integrity of GCs is hence crucial for oocytes maturation, competency and response to gonadotropins (Jahromi *et al.* 2015). Although the properties of Pb have been extensively studied, little is known about the effect of Pb on bovine GCs, with regard to role of both Nrf2 and NF-κB. Meanwhile; the Center for Disease Control and Prevention (CDC), stated that there is no safe level for Pb exposure especially in case of pregnancy or developing embryos (Chandramouli *et al.* 2009; Taylor *et al.* 2013). So in the current study, we exposed bovine GCs to Pb once for just 2 hours, and 24 hours after changing Pb-containg media, we investigated whether GCs recovered from Pb insult or the effect of Pb persisted to distress GCs. We postulate that Pb exposure affects in vitro cultured bovine GCs proliferation via perturbation of Nrf2/ NF-κB.

2.3 Materials and methods

2.3.1 Collection of ovaries and granulosa cells

Bovine ovaries were collected from a local slaughter house and transported to the laboratory in thermo flask containing warm physiologic saline solution (0.9 % NaCl) within 2 hours of collection. Ovaries were processed as described previously by (Gebremedhn *et al.* 2015). Upon arrival, ovaries were first washed three times with pre-warmed 0.9 % NaCl solution. Then they were rinsed in 70 % warm ethanol for 30 seconds, followed by additional washing three times with warm 0.9 % NaCl. Aspiration of follicular contents was done from small healthy follicles (with a diameter of 3-5 mm) using 18-gauge sterile needle (B-Braun, Germany) and transferred into a 15 ml sterilized Falcon tube (Thermo Fisher Scientific, Germany) containing warm PBS (without Ca and Mg). The cumulus oocyte-complexes (COCs) and cellular debris were allowed to settle down while the upper suspension containing the GCs was carefully transferred into 15 ml tubes and centrifuged at 750 rpm for 7 minutes. The supernatant follicular fluid was discarded, and GCs pellets were re-suspended in 1 mL red blood cell (RBC) lysis buffer for 1 minute. The pellets were then washed twice with DMEM/F-12 Ham (Roth, Karlsruhe, Germany) culture medium supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and centrifuged. The GCs were re-suspended in DMEM/F-12 Ham (Roth, Karlsruhe, Germany)

culture media supplemented with 10 % FBS (Sigma-Aldrich, Germany). Cell viability was determined via trypan blue exclusion method.

2.3.2 Granulosa cell culture

Granulosa cells ($2x10^5$ cells per well) were seeded in a 24-well plate (Starlab, Hamburg, Germany) with 600 µl of culture media per well comprised of DMEM/F-12 Ham (Roth, Karlsruhe, Germany) supplemented with (10 % FBS, 1 % penicillin-streptomycin, and 1 % Amphotericin, Sigma-Aldrich, Germany). After the cells were grown to 70 % confluency at 37° C under 5 % CO_2 in humidified air, cells were exposed to different concentration of lead acetate (1, 2, 3, 5, 10 µg/ml) (Sigma-Aldrich, Germany) for 2 hours under culture condition. Based on phenotypic evaluation of treated cells, three concentrations (1, 2 and 3 µg/ml) were chosen for further investigations in addition to untreated cells (control). Cultured GCs were harvested using 0.25 % trypsin-EDTA (Sigma-Aldrich, Germany) 24 hours after treatment and kept under -80°C for further use.

2.3.3 Cell proliferation assay

$2x10^4$ of GCs were cultured in 96-well plate containing 100 µl of medium and after sub-confluency, cells were treated with lead acetate. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8) (Dojindo EU GmbH, Germany) for both treated and untreated cells (control). Briefly, 24 hours post treatment, 10 µl of CCK-8 solution was added to each well, and the plates were incubated for 4 hours at 37° C in 5 % CO_2 in humidified air. The optical density (OD) of released formazan dye, which is proportional to the number of living cells was measured at a wave length of 450 nm using Synergy™ H1 Multi- Mode Reader (BioTek Germany, Germany). Blank measurements were obtained from wells containing only culture medium and used for normalization.

2.3.4 Detection of intracellular ROS accumulation

The intracellular levels of ROS were measured by loading GCs with H_2-dichlorofluorescein diacetate (H_2DCF-DA) (Life Technologies, Germany). Dichlorofluorescein diacetate (DCF-DA) is taken up by cells, is cleaved to DCF by intracellular esterases, and is oxidized in the presence of ROS to DCF. The H_2DCF-DA probe was freshly reconstituted in DMSO before loading. In brief, $2x10^4$ cells per well were cultured in 96-well plate till confluency The cells were loaded with 50 µl of freshly prepared 75 µM H_2DCF-DA in serum free medium for 20 min at 37° C in

dark. Then the H_2DCF-DA containing medium was removed, cells were washed twice with PBS and imaged using Leica DM IRB inverted microscope (Leica, Bensheim, Germany). Images were analyzed using imageJ 1.48v (National Institutes of Health, Bethesda, MD, USA).

2.3.5 Analysis of cell cycle using flow cytometry

Cultured granulosa cells were harvested 24 hours after treatment in a 15 ml Falcon tube (Thermo Fisher Scientific, Germany), and washed twice with 1x PBS. A minimum of ~$1x10^6$ cells were fixed in ice-cold 70 % and ethanol and kept at 4^o C overnight. Ethanol was discarded after centrifugation, and the cell pellets were washed twice with 500 µl of 1x PBS then re-suspended in 500 µl of 1x PBS containing 50 µg/ml of propidium iodide (PI, ab14083) and 50 µg/ml of RNase for 30 minutes at 37^o C. GCs relative number in different phases was determined by LSRFortessa™ Flow cytometer (BD Biosciences, USA) using ModFit LT software (http://www.vsh.com/products/mflt/index.asp) and the percentages of cells calculated in G_0/G_1, S and G_2/M phases of the cell cycle.

2.3.6 Annexin V and propidium iodide (PI) staining

The apoptotic cells were distinguished from viable or necrotic cells by the combined application of annexinV-APC and propidium iodide (PI) using Cell Meter™ APC-Annexin V Binding Apoptosis Assay Kit (Biomol, USA), according to the manufacturer's recommendations with modifications. Annexin V binds phosphatidylserine residues, which are translocated from the inner to the outer leaflet of the plasma membrane early in apoptosis (van Engeland *et al.* 1996). However, PI will stain double-stranded nucleic acids from cells lacking intact plasma membranes (necrotic nonviable). Detached cells of all treatments were collected with the supernatant media, pelleted by centrifugation and washed with cold PBS. The adherent cells were then harvested via trypsnization centrifuged and washed with PBS. Detached and adherent cells pellets were pooled and resuspended in 200 µl Assay Buffer. Afterward, 2 µl of 100X APC-annexin V and 2 µl 100X PI were added into the cells for 20 minutes at room temperature in dark. Additional 200 µl of Assay Buffer were added to increase the volume of cells before analyzing with LSRFortessa™ Flow cytometer (BD Biosciences, USA). Data was processed using FACSDiva 6.1.3 software (Becton Dickinson).

2.3.7 Extraction of total RNA and cDNA synthesis

Total RNA was isolated using miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Genomic DNA contamination was removed by on-column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany). The purity and concentration of total RNA were assessed by a Nanodrop ND-8000 (NanoDrop technologies). The RNA was then reverse transcribed using cDNA Synthesis Kit (Thermo Fisher scientific, Germany) according to the manufacturer's instruction. The concentration of RNA was adjusted using RNase free water to a final volume of 10 µl. Briefly, oligo $(dT)_{18}$ and Random primer (0.5 µl each) were added to each RNA sample and incubated for 5 min at 65° C. Then other components were added to the mixture, RiboLock RNase Inhibitor (1 µl); 5× RT Buffer (4 µl); dNTP mix (2 µl), Revert Aid Reverse Transcriptase Enzyme mix (2 µl) and incubated for 5 min at 25° C followed by 60 min at 37° C and the reaction was terminated by heating at 70° C for 5 min. The resulting cDNA samples were stored in –20° C until further use.

2.3.8 Real time quantitative PCR

The qRT-PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Germany) in Applied Biosystem® StepOnePlus™ (Applied biosystems, CA, USA). All samples were run in triplicates. The qRT-PCR were performed in a reaction volume of 20 µl with 7.4 µl of dd H_2O, 0.3 µl of forward and reverse primers, 10 µl of 1x SYBR Green master mix (Bio-Rad Laboratories GmbH, Germany), and 2 µl of cDNA template. The thermal cycling conditions were: pre-heating for 3 minutes at 95^{o} C, then 40 cycles of amplification at 95^{o} C for 15 s, and 1 minute at 60^{o} C. Gene-specific primers were designed using Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/ Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/) and Primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 2.1). A total of 16 genes: oxidative stress response genes (Nrf2, Keap-1, SOD, CAT, HO-1, Thrx, NF-κB, IKK, TNF-α), Cell proliferation related genes (CCDN2 and PCNA), Endoplasmic reticulum related genes (GRP78 and CHOP) proapoptotic (BAX and caspase-3) and antiapoptotic (BCL-2) related genes were quantified in GCs. The expression of mRNA data was analyzed by using comparative Ct ($2^{-\Delta\Delta Ct}$) methods (Livak & Schmittgen 2001) and the expression levels of β- Actin and GAPDH housekeeping genes were used for normalization.

Table 2.1: Details of primers used in qPCR:

Gene	Primer sequence	Size (bp)	Accession Number
BAX	F: 5´- ACTCTCCCCGAGAGGTCTTT-3´ R: 5´- GGGTGTCCCAAAGTAGGAGA-3´	248	NM_173894.1
BCL-2	F: 5´- TCTTTGAGTTCGGAGGGGTC-3´ R: 5´- ATGCTAGGGCCATACAGCTC-3´	169	NM_001166486.1
Caspase-3	F: 5´- TGCCACTGTATCAGGGAACA-3´ R: 5´- TGCTCAGCACAAACATCACA-3´	185	NM_001077840.1
CAT	F: 5´-TGGGACCCAACTATCTCCAG-3´ R: 5´-AAGTGGGTCCTGTGTTCCAG-3´	178	NM_001035386.1
CCND2	F: 5´-CGACTTCATCGAACACATCC-3´ R: 5´-ATCTTTGCCAGGAGATCCAC-3´	234	NM_001076372.1
CHOP	F: 5´- GCCAAAATCAAAGCCGGAAC-3´ R: 5´- GTCCTCATACCAGGCTTCCA-3´	172	NM_001078163.1
GAPDH	F: 5´-CCCAGAATATCATCCCTGCT-3´ R: 5´-CTGCTTCACCACCTTCTTGA-3´	185	NM_001034034

GRP78	F: 5´- AACACAGTCTTCGACGCCAA-3´ R: 5´- TTGCCCACCTCCAACATCAA-3´	141	XM_024998380.1
HO-1	F: 5´- GCTATGTTCAGCGACTCCAG-3´ R: 5´- GGGGTCATCTCCAGAGTGTT -3´	241	NM_001014912.1
IKK	F: 5´-AGAGCGAGATGGACATTGTG-3´ R: 5´- TGTTCAGGACATGAAGCAGC-3´	237	NM_174353.2
Keap-1	F: 5´-TCACCAGGGAAGGATCTACG-3´ R: 5´-AGCGGCTCAACAGGTACAGT-3´	199	NM_001101142.1
NF-κB	F: 5´-AATTTGGGAAGGATTTGGAG-3´ R: 5´- CTGTCGTTTCCTTTGCACTT-3´	217	NM_001076409.1
Nrf2	F: 5´-CCCAGTCTTCACTGCTCCTC-3´ R: 5´-TCAGCCAGCTTGTCATTTTG-3´	165	NM_001011678
PCNA	F: 5´-CACCAGCATGTCCAAAATAC-3´ R: 5´-CTGAGATCTCGGCATATACG-3´	240	NM_001034494.1
SOD	F: 5´-TGCCATCGTGGATATTGTAG-3´ R: 5´-GCAATTCCAATTACACCACA-3´	174	NM_174615

Thrx	F: 5´-AGCTGCCAAGATGGTGAAAC-3´ R: 5´-ACTCTGCAGCAACATCCTGA-3´	215	NM_173968.3
TNF-α	F: 5´-CTTCCACCCCCTTGTTCCT-3´ R: 5´- AGGCGATCTCCCTTCTCCA-3´	187	NM_173966.3
β -Actin	F: 5´-TGTCCACCTTCCAGCAGAT-3´ R: 5´-TCACCTTCACCGTTCCAGT-3´	249	NM_173979

2.3.9 Western blot analysis

Whole cell protein lysate was prepared from bovine granulosa cell samples using 1x passive lysis buffer (Promega GmbH, Mannheim, Germany). Protein concentration was verified using the Bradford method and equal amounts of protein lysate (30 µg) were resolved with 4–18 % gradient SDS-polyacrilamide gel and transferred onto a nitrocellulose membrane (Whatman-protran, Germany). Ponceau S staining was used to confirm consistent transfer of proteins. To block the non-specific binding sites, the membranes were incubated in 1x Roti-Block blocking solution (Carl Roth, Germany) for 1 hour at room temperature. They were then incubated overnight at 4^{o} C with diluted primary antibodies of anti-PCNA rabbit polyclonal antibody (1:200 dilution; sc-7907, Santa Cruz Biotechnology, Germany), anti-BAX rabbit polyclonal antibody (1:200 dilution; sc-493, Santa Cruz Biotechnology, Germany), anti-GRP78 goat polyclonal antibody (1:250 dilution; sc-1050, Santa Cruz Biotechnology, Germany), anti-catalase rabbit polyclonal antibody (1:500 dilution; LS-B1441) and anti-β-Actin mouse monoclonal antibody (1:500 dilution; Santa Cruz Biotechnology, Germany), as an internal control. After washing with Tris-Buffered Saline with Tween 20 (1x TBST), the membranes were incubated with horseradish peroxidase (1:5000 dilution; Santa Cruz Biotechnology, Germany), goat anti-rabbit, donkey anti-goat or goat anti-mouse secondary antibodies for 1 hour at room temperature. Afterwards, membranes were washed with 1x TBST and protein bands were visualized with

enhanced chemiluminescence using Clarity Western ECL Substrate (Bio-Rad, USA) and acquired using Gel Doc XRS+ imaging system (Bio-Rad, Germany).

2.3.10 Oxyblot procedure

Oxidized Protein Western Blot Detection Kit (ab178020) was used for detection of carbonyl groups introduced into proteins by oxidative reactions, according to the manufacturer's instructions with minor modifications as described elsewhere (Kriebardis et al., 2006). Briefly, DNPH derivatization was carried out on equal amounts (5 μg) of proteins, for 10 min at room temperature. Following neutralization, the sample mixtures were separated by SDS-PAGE then transferred to nitrocellulose membranes. The nonspecific protein binding was blocked by incubation with 5 % non-fat milk dissolved in PBS containing 0.1 % Tween 20. Thereafter, the proteins were incubated with a primary anti-DNP rabbit antibody (1:5000 dilution) specific for the 2,4-dinitrophenol group (DNP moiety of the proteins) and a peroxidase-labelled secondary antibody goat anti-rabbit (1:5000 dilution), appropriately diluted in blocking buffer, for 1 hour each. After the blots were developed using enhanced chemiluminescence reagent kit, the images were acquired using Gel Doc XRS+ imaging system (Bio-Rad, Germany). Due to the sample processing procedure for protein carbonylation analysis, it was not possible to re-probe membranes for a loading control. Therefore equal protein loading of samples was verified with use of Panceau S stain.

2.3.11 Immunofluorescence detection of Nrf2 and NF-κB proteins

Immunohistochemistry was performed to detect and localize Nrf2 and NF-κB proteins in GCs. Briefly; cells were washed three times in phosphate- buffered saline (PBS), and then fixed overnight at 4^{o} C in 4 % (w/v) paraformaldehyde in PBS. Fixed cells were washed twice with PBS Tween 20 (PBST), then permeabilized with 0.5 % (v/v) Triton-X100 (Sigma-Aldrich) in PBS for 15 min at room temperature. The samples were incubated in 3 % bovine serum albumin (BSA) (Sigma-Aldrich) in PBST for 1 hour at room temperature, followed by incubation with specific primary antibodies against Nrf2 (1:100 dilution, orb11165, Biorbyt) or NF-κB (1:100 dilution, orb11118, Biorbyt, UK) overnight at 4^{o} C. Then, cells were washed three times with PBS and further incubated at room temperature for 2 hours in the dark with Alexa Fluor 568-conjugated goat anti-rabbit secondary antibody (1:300 dilution, A-11011, Life Technologies). The secondary antibody without the primary antibody was used as negative control. A droplet of

Vectashield mounting medium (Vector Laboratories, USA) containing 4', 6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Finally the slides were mounted, and samples were visualized under a CLSM LSM-780 confocal laser scanning microscope (Zeiss, Germany; magnification 20 x; Scale bar 50 µm) and analyzed using imageJ 1.48v.

2.3.12 Statistical analysis

Data obtained from all groups were compared statistically by ANOVA Test followed by Tukey-Kramer multiple comparison test. Statistical analysis was performed using GraphPad Prism Software (version 5, San Diego, CA, USA). Values of $P < 0.05$ were considered for statistical significance. All values represent the mean ± SEM from three independent experiments.

2.4 Results

In order to investigate the dose dependent effect of Pb treatment on GCs, different doses of Pb namely, 1, 2, 3, 5 and 10 µg/ml were used to treat the GCs for 2 hours. Morphological observation, 24 hours post treatment, showed that there was no significant difference between doses of 1, 2, 3 µg/ml and the control untreated cells. However, cells exposed to 5 µg/ml and 10 µg/ml showed cellular shrinkage and detachment from the plate, respectively (Figure 2.S1).

2.4.1 Exposure of bovine GCs to lead elevated intracellular ROS accumulation

The relative fluorescence emissions were higher within GCs exposed to Pb than the control group counterparts, confirming that Pb provoked intracellular ROS in GCs (Figure 2.1A & 2.1B). However, the doses 5 and 10 µg/ml showed less fluorescence signals revealing that these doses may cause cytotoxicity (Figure 2.S1). Accordingly, the doses 1, 2 and 3 µg/ml were chosen to investigate cellular behavior under Pb- induced oxidative stress.

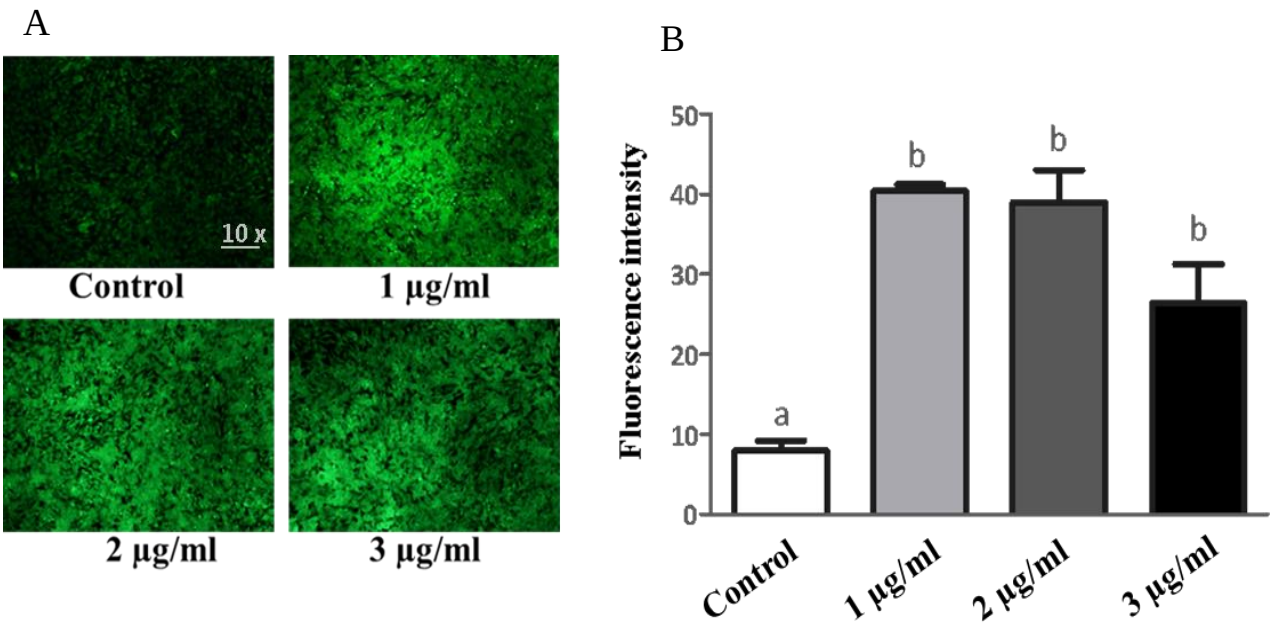


Figure 2.1: Lead induced intracellular reactive oxygen species (ROS) accumulation in bovine granulosa cells: Fluorescent photomicrographs of bovine GCs stained with 2′, 7′-dichlorofluorescin diacetate (H_2DCFDA) were shown in untreated control and Pb-exposed groups (A). Quantitative analysis of relative fluorescence emission (B). Values are expressed as mean ± SEM of n = 3. a, b indicate statistically significant differences ($P < 0.05$). Magnification: 10 x.

2.4.2 Lead inhibited proliferation of bovine granulosa cells in vitro

The impact of Pb on GCs proliferation was assessed using cell proliferation assay and it was observed that all doses of Pb significantly reduced GCs viability compared to the control ones (Figure 2.2A). Cell proliferation markers (PCNA and CCND2) were further investigated and it was found that the mRNA expression of both markers was downregulated under Pb exposure (Figure 2.2C & 2.2D). Protein level of PCNA showed almost parallel expression patterns (Figure 2.2B).

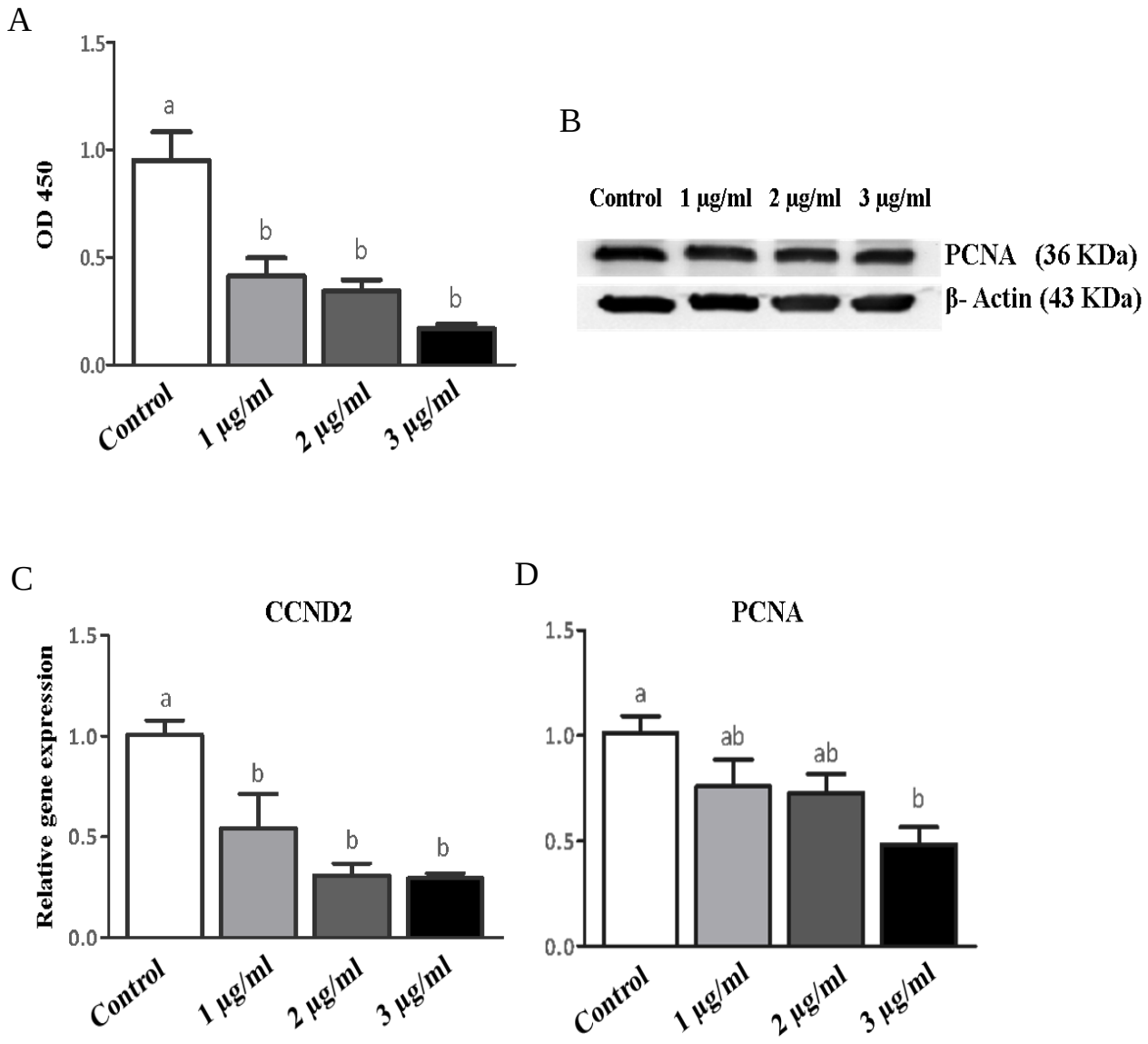


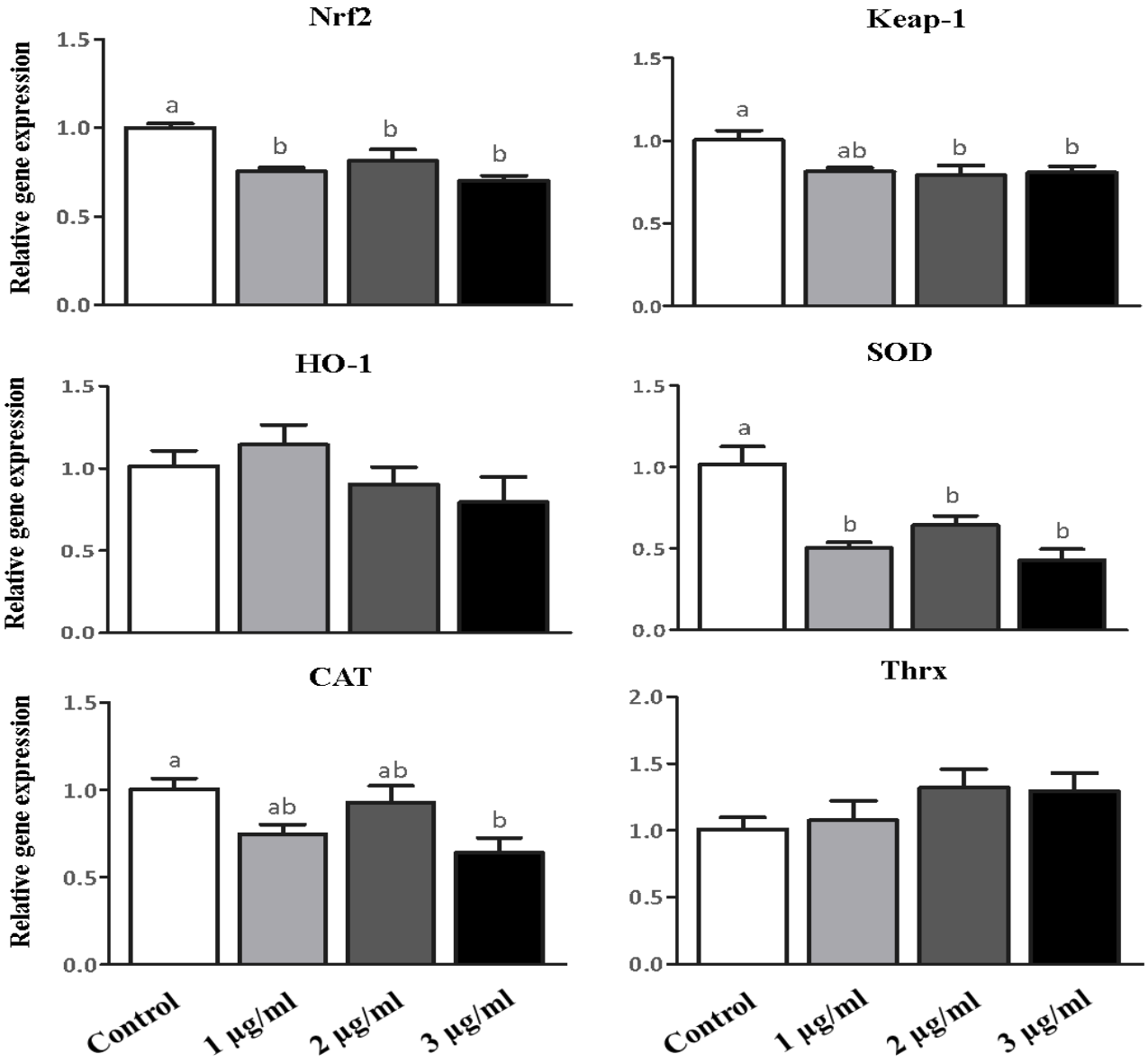
Figure 2.2: Effect of lead on bovine granulosa cell proliferation and expression of proliferation marker genes: Cell proliferation assay (A), protein expression of PCNA (B), mRNA expression of CCND2 (C) and PCNA (D) in bovine GCs co-cultured with Pb and their corresponding control. β-Actin and GAPDH were used to normalize the expression of targets genes while β-Actin was used as internal control for protein expression of PCNA. Data shown as means ± SEM, n=3. a, b indicate statistically significant differences ($P < 0.05$).

2.4.3 Exposure of cells to lead altered the expression of Nrf2 and NF-κB

QPCR results in Figure 3A showed that there was significant attenuation of Nrf2 and Keap-1 genes in GCs exposed to the three doses of Pb (1, 2, 3 µg/ml) compared to control group

Concerning Nrf2 downstream antioxidant genes namely SOD, CAT, Thrx, and HO-1, it was observed that the expression of SOD was significantly reduced (Figure 2.3A) Additionally, both mRNA and protein analysis of CAT showed marked reduction in its expression (Figure 2.3B). However, there were no significant differences in the expression of other genes. Other striking finding was that, there was significant decrease in the expression of NF-κB concomitantly with no change in the expression of IKK (Figure 2.4A & 2.4B). We further investigate TNF-α gene to validate if the inflammatory pathway involved in this dosing paradigm and we found a significant reduction in TNF-α expression (Figure 2.4C).

A



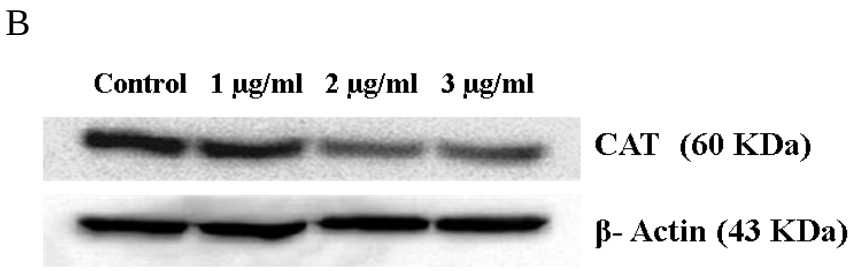


Figure 2.3: Expression levels of genes associated with the Nrf2 pathway: mRNA expression level of Nrf2, its inhibitor Keap-1, and its downstream candidate genes: HO-1, SOD, CAT and Thrx (A) protein expression of CAT (B) in bovine GCs exposed to Pb and their corresponding control. β-Actin and GAPDH were used to normalize the expression of targets genes, values are expressed as mean ± SEM of n = 3. a, b indicate statistically significant differences ($P < 0.05$).

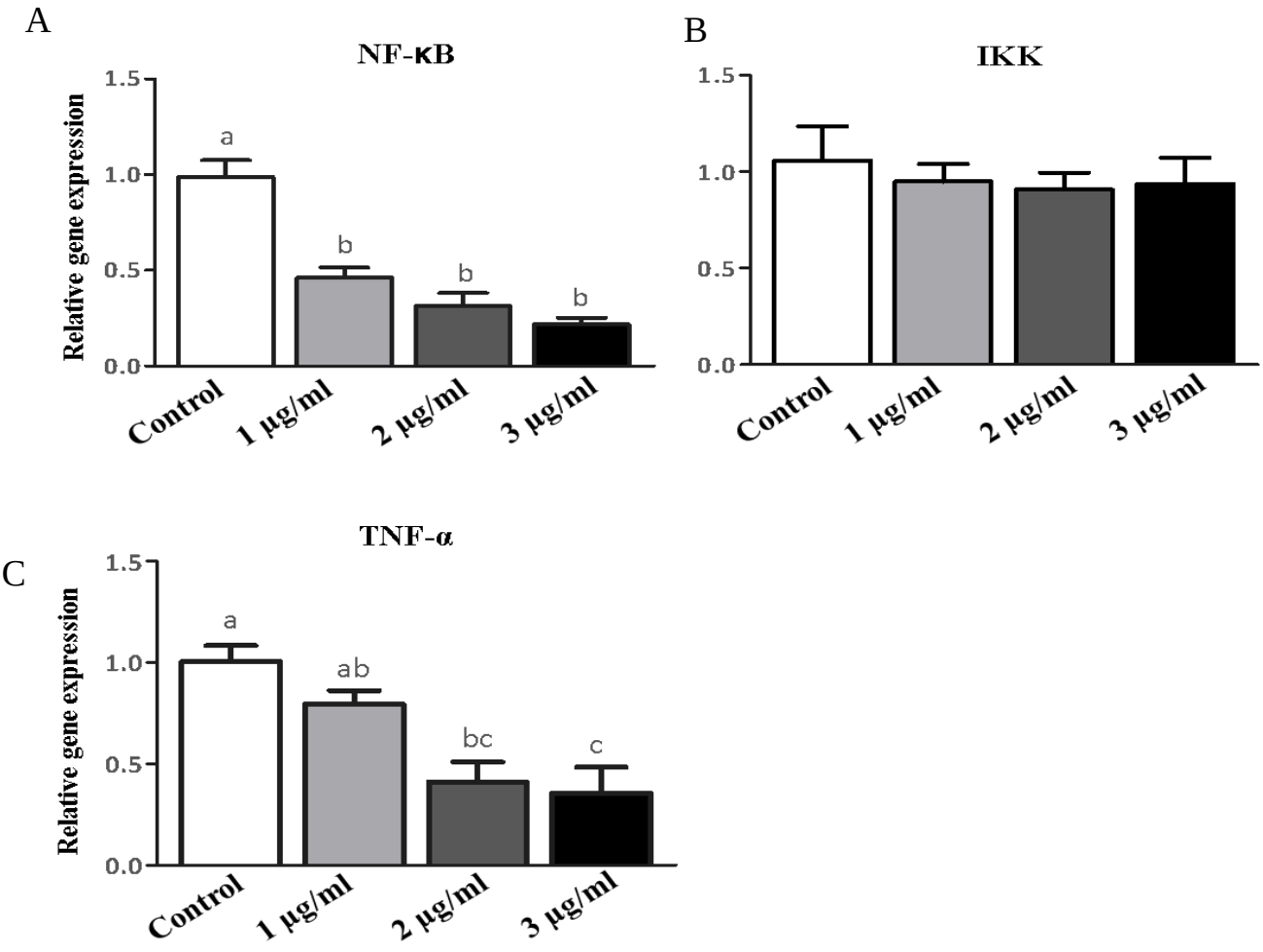
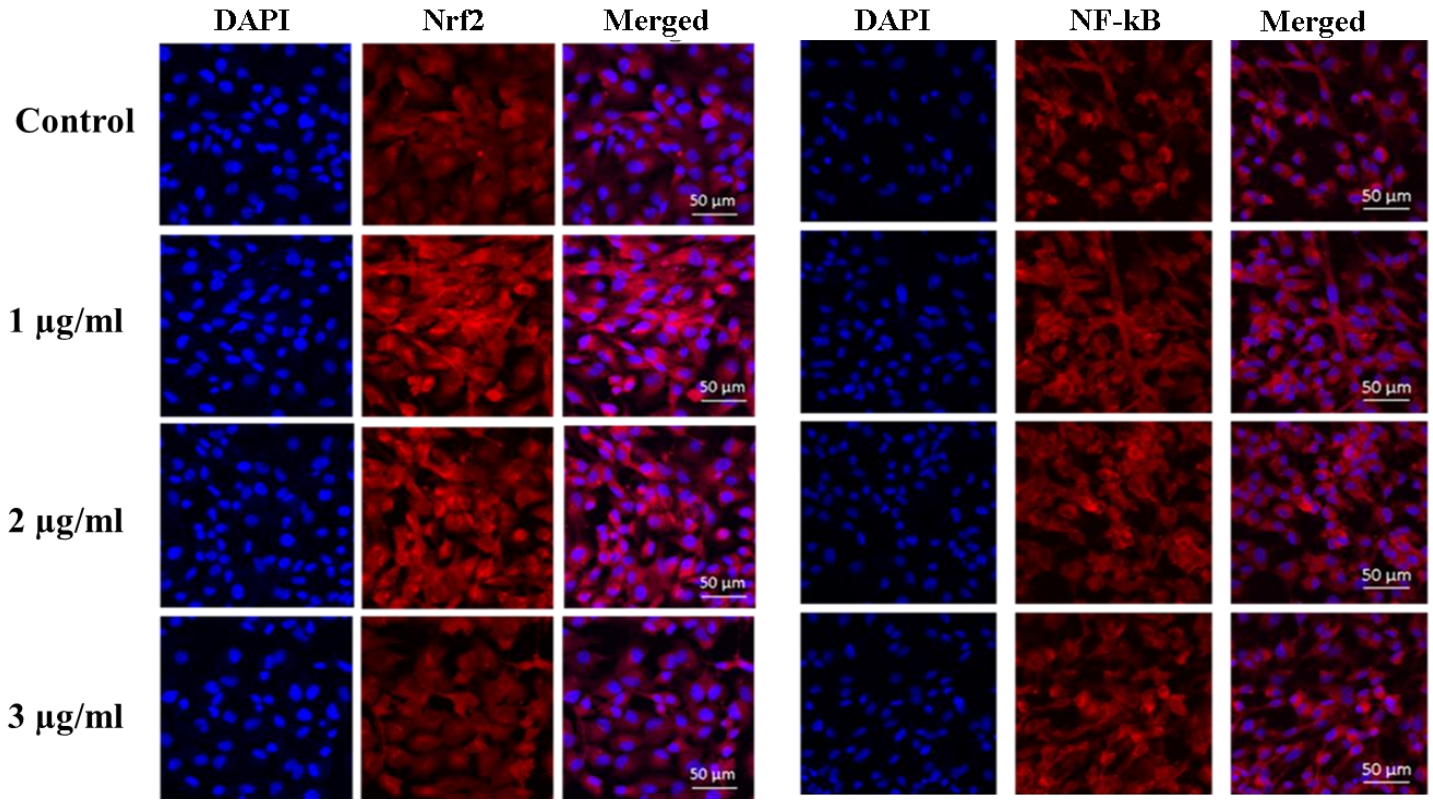


Figure 2.4: Expression levels of genes associated with the NF-κB pathway: mRNA expression level of NF-κB (A), IKK (B), and TNF-α (C) in bovine GCs exposed to Pb for 2 hours and their corresponding control. β-Actin and GAPDH were used to normalize the expression of targets genes, values are expressed as mean ± SEM of n = 3. a, b & c indicate statistically significant differences ($P < 0.05$).

2.4.4 Immunofluorescence staining for Nrf2 and NF-κB

The protein expression level of NF-κB tend to be high in Pb exposed groups being significant at the highest dose, while the level of Nrf2 showed significant elevation only in 1 and 2 µg/ml compared to the untreated control counterparts (Figure 4.5A, 4.5B & 4.5C).

A



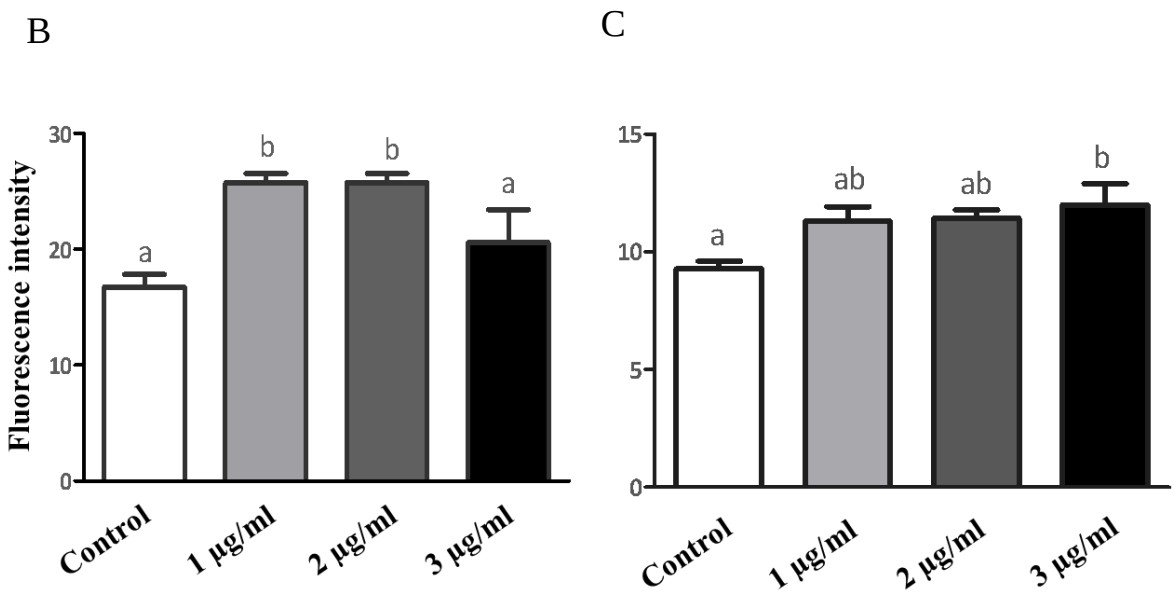


Figure 2.5: Protein expression of Nrf2 and NF-κB: Bovine GCs cultured under Pb challenge and their corresponding control. Red fluorescence signals reveal Nrf2 and NF-κB protein localization, while blue fluorescence showed nuclear staining using Dapi (A). Scale bars 50 µm. Mean values of fluorescence intensity of Nrf2 (B) and NF-κB (C) as analyzed by imageJ software. a, b indicate statistically significant differences ($P < 0.05$).

2.4.5 Effect of lead on the protein carbonyl content

The introduction of carbonyl groups (aldehydes and ketones) to protein side chains could be studied as a sign of oxidative stress and is also characterized by their stable and irreversible nature (Kriebardis *et al.* 2006). In comparison with untreated control group, Pb -treated samples displayed an evident increment in the intensity of the carbonylated protein bands as shown in the immunostained gel, ranging from about MW 180 KDa to 17 KDa (Figure 2.6A & 2.6B).

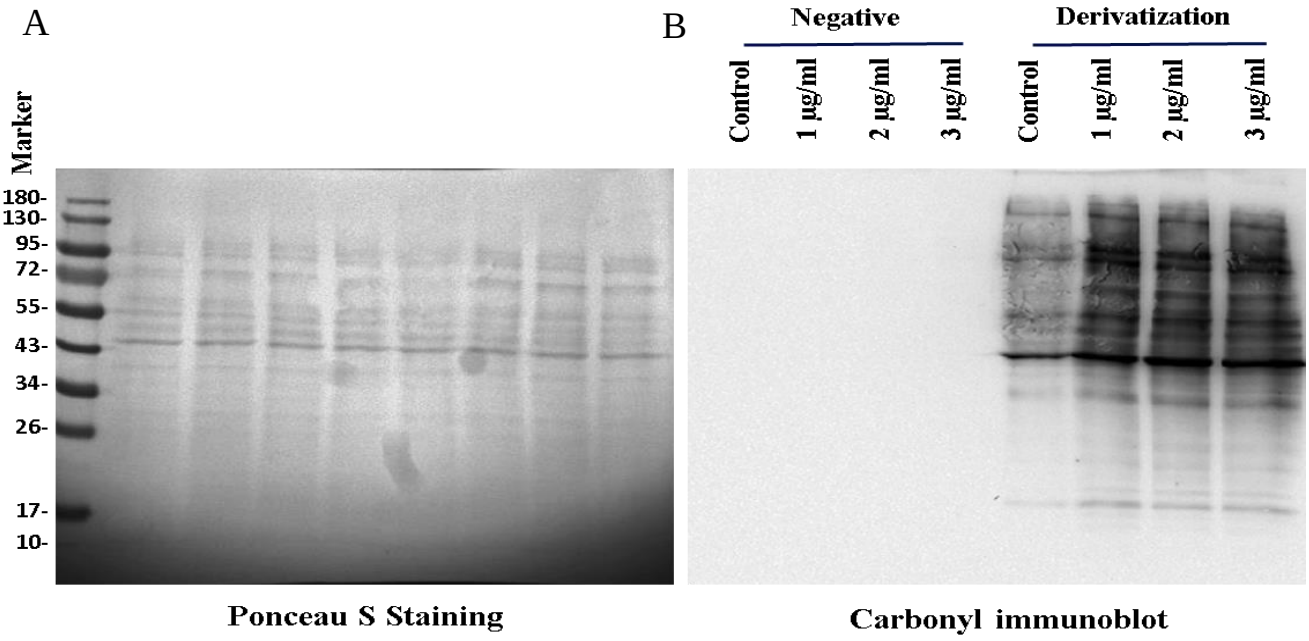


Figure 2.6: Lead induced protein carbonylation in bovine granulosa cells: Carbonylated proteins in cell lysates were investigated using OxyBlot protein oxidation detection kit then subjected to western blotting**.** Panceu S staining shows equal protein loading for each sample (A). Carbonylation level in the control and Pb-exposed groups (B).

2.4.6 Lead exposure induced apoptosis in bovine granulosa cells

We determined whether apoptosis was induced in GCs due to Pb exposure, the expression of proapoptotic (BAX and caspase-3) and antiapoptotic (BCL-2) related genes were investigated. Results indicated that the transcript level of BCL-2 was significantly downregulated under Pb exposure as compared to control untreated group (Figure 2.7A) with non-significant increase in BAX level which was further confirmed by western blot (Figure 2.7B & 2.7D). This in turn resulted in a substantial decrease of BAX/BCL-2 ratio. However, caspase-3 showed upregulation with the dose 3 µg/ml in comparison to the untreated group (Figure 2.7C).

Apoptosis was further assessed by flow cytometric analysis using annexin V-APC/PI stain. As shown in Figure 2.8, the percentage of apoptotic granulosa cells (early and late apoptosis as determined by the Annexin V+/PI- (Quadrant 4) or Annexin V+/PI+ (Quadrant 2) cells respectively) in the groups subjected to Pb were higher than the basal levels of Annexin V+/PI- (Quadrant 4) or Annexin V+/PI+ (Quadrant 2) cells in the control untreated group.

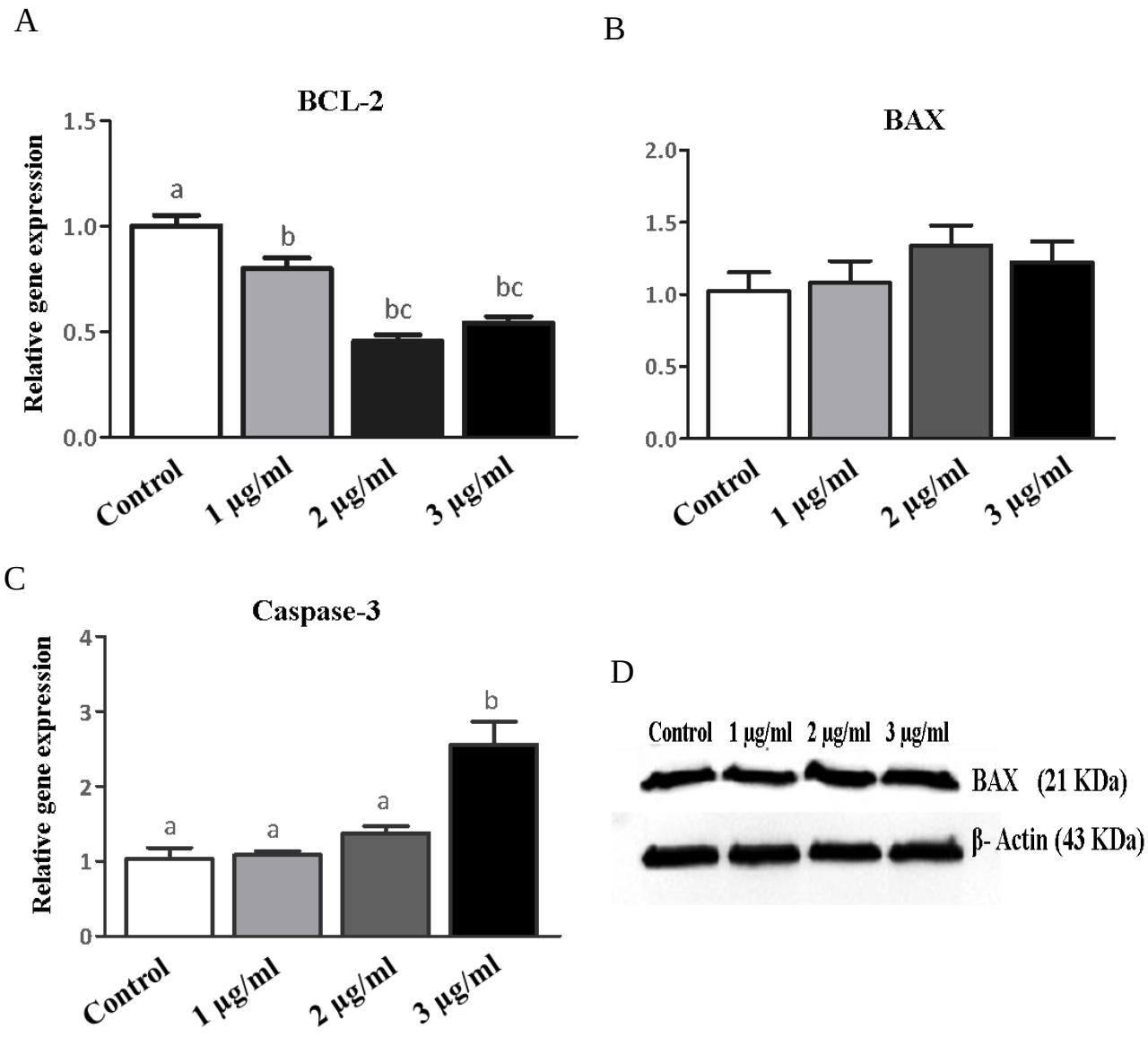


Figure 2.7: Lead induced apoptosis in bovine granulosa cells: mRNA expression levels of BCL-2 (A) BAX (B) and caspase-3 (C) as well as protein level of BAX (D) in bovine granulosa cells exposed to Pb and their corresponding control. Data shown as means ± SEM, n=3. a, b & c indicate statistically significant differences ($P < 0.05$). Expression of β-Actin and GAPDH acted as internal control for gene expression while β-Actin was used for protein analysis.

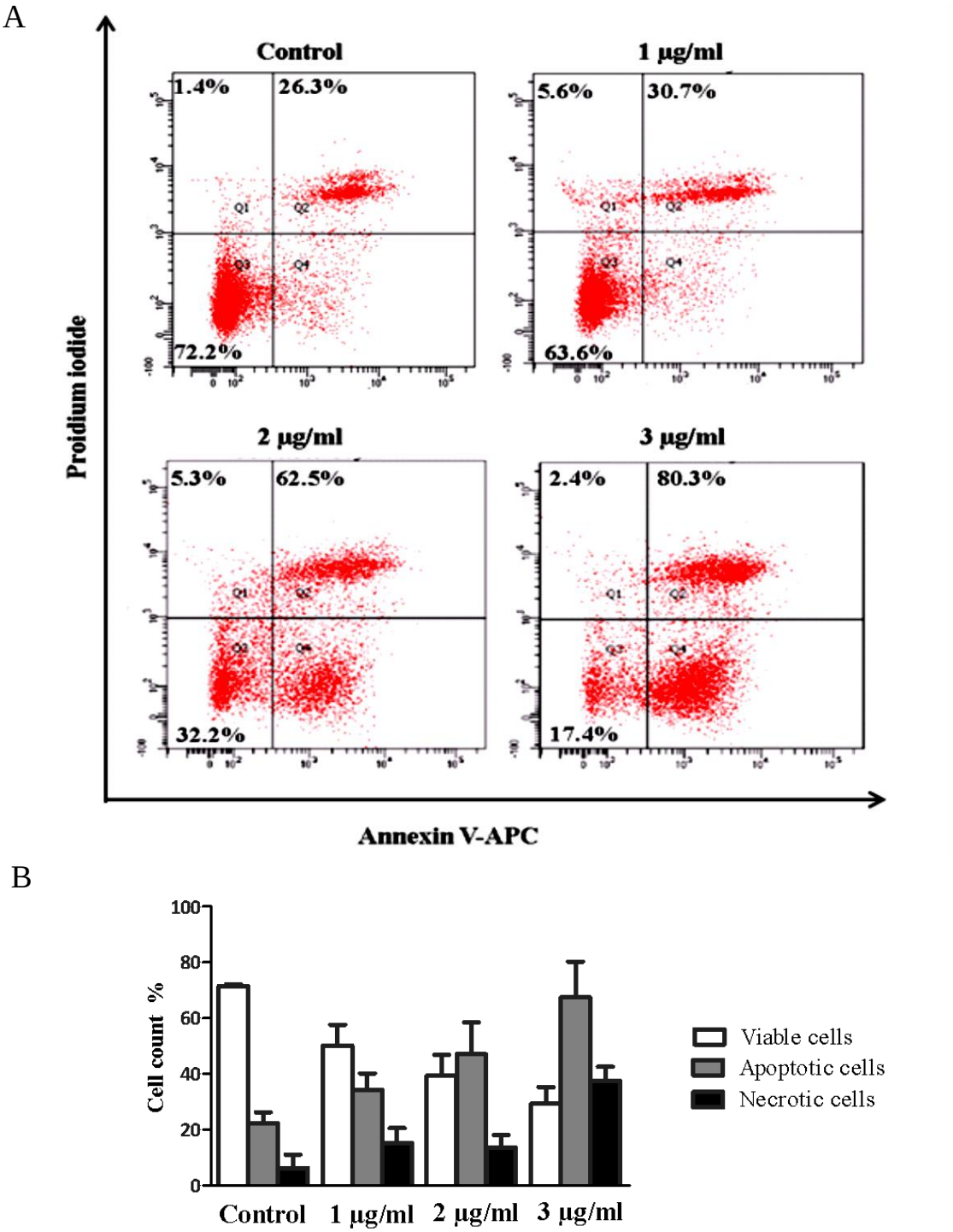


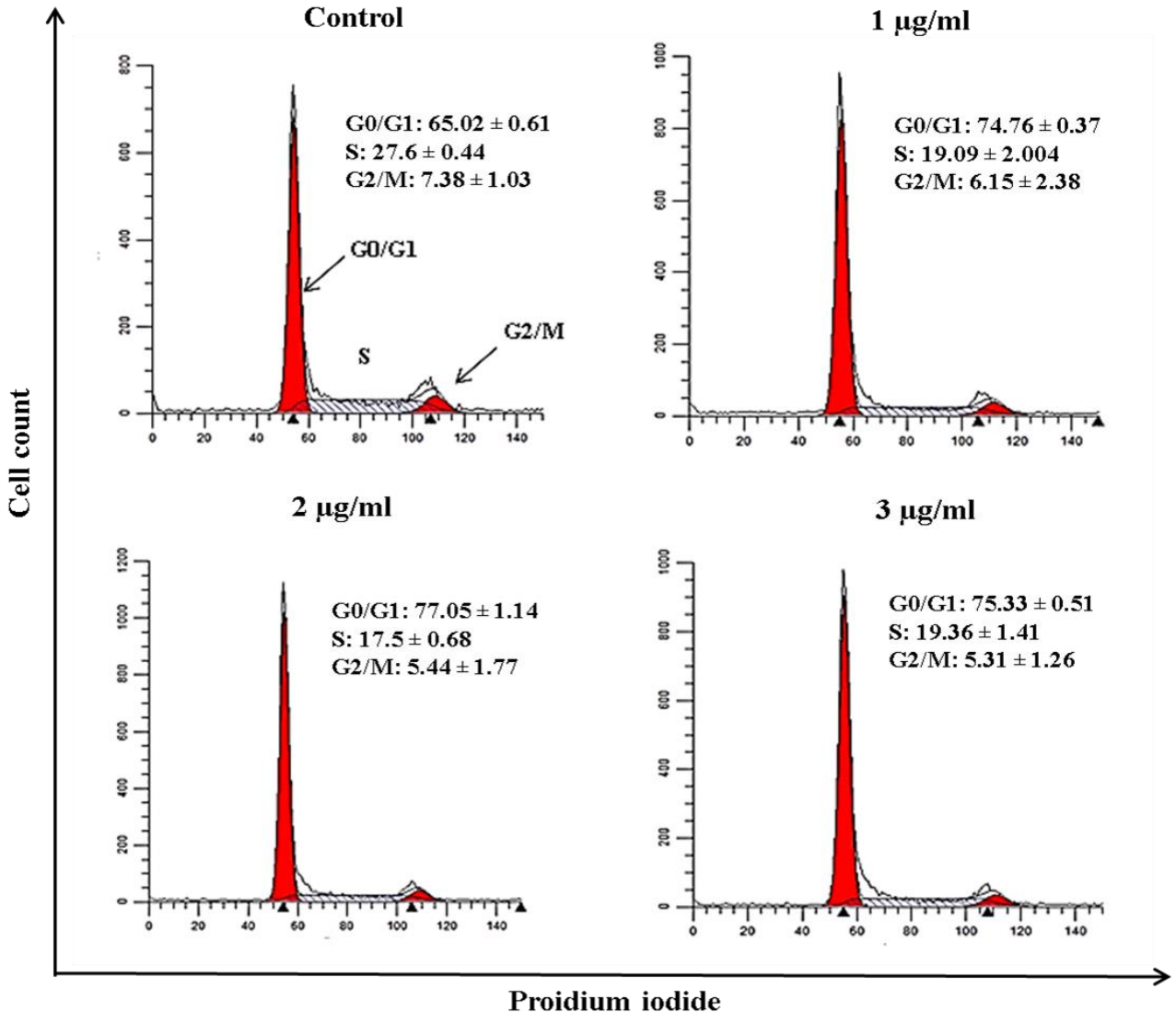
Figure 2.8: Flow cytometric analysis of annexin V-APC/PI- stained bovine GCs cultured under Pb exposure and their corresponding control. The lower left quadrant (Q3) shows viable cells, the lower right quadrant (Q4) shows early apoptotic cells, the upper right quadrant (Q2) shows late apoptotic cells, and the upper left quadrant (Q1) shows necrotic cells. Representative histograms

from one typical experiment are shown. APC, allophycocyanin; PI, propidium iodide. The percentage of apoptotic cells presented on histograms (A) and on the graph (B) is the sum of cells in early and late stages of apoptosis. Data from three independent experiments are expressed as average percentages of viable, apoptotic (early plus late), and necrotic cells. Each value on the graph represents the mean value ± SEM, n = 3 independent experiments.

2.4.7 Lead altered cell cycle transition in bovine granulosa cells

Cell cycle was evaluated in GCs by FACScan flow cytometry. As depicted in (Figure 2.9A & 2.9B.), the exposure of GCs to Pb increases the percentage of cell populations in the G_0/G_1 phase while reduces the cell populations in S phase (DNA synthesis) as compared with the untreated control groups.

A



B

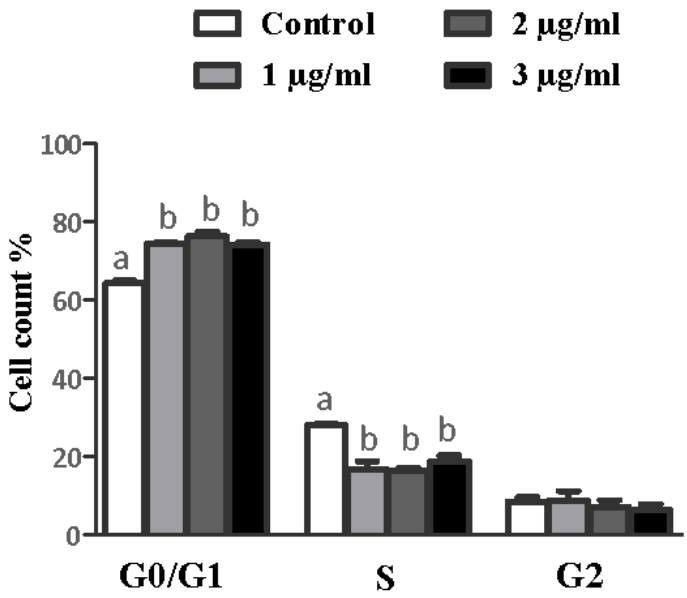


Figure 2.9: Lead changed cell cycle transition in granulosa cells: Flow cytometric analysis of bovine GCs cultured under Pb exposure and their corresponding control. The analyzed cell counts are shown on the Y-Axis and the DNA content of cells detected by PI staining is indicated on the X-axis (A). Quantitative analysis of cell populations at different cell cycle stages (B). Data shown as means ± SEM, n=3. a, b indicate statistically significant differences ($P < 0.05$).

2.4.8 Lead exposure upregulated GRP78 and CHOP expression

To determine the potential effect of Pb in ER stress response of GCs, the expression of GRP78 and CHOP was investigated in GCs exposed to Pb. The results showed that Pb triggered both GRP78 and CHOP expression in dose dependent manner (Figure 2.10A & 2.10B). Moreover, similar expression pattern of GRP78 protein was detectable as indicated by western blot analysis (Figure 2.10C).

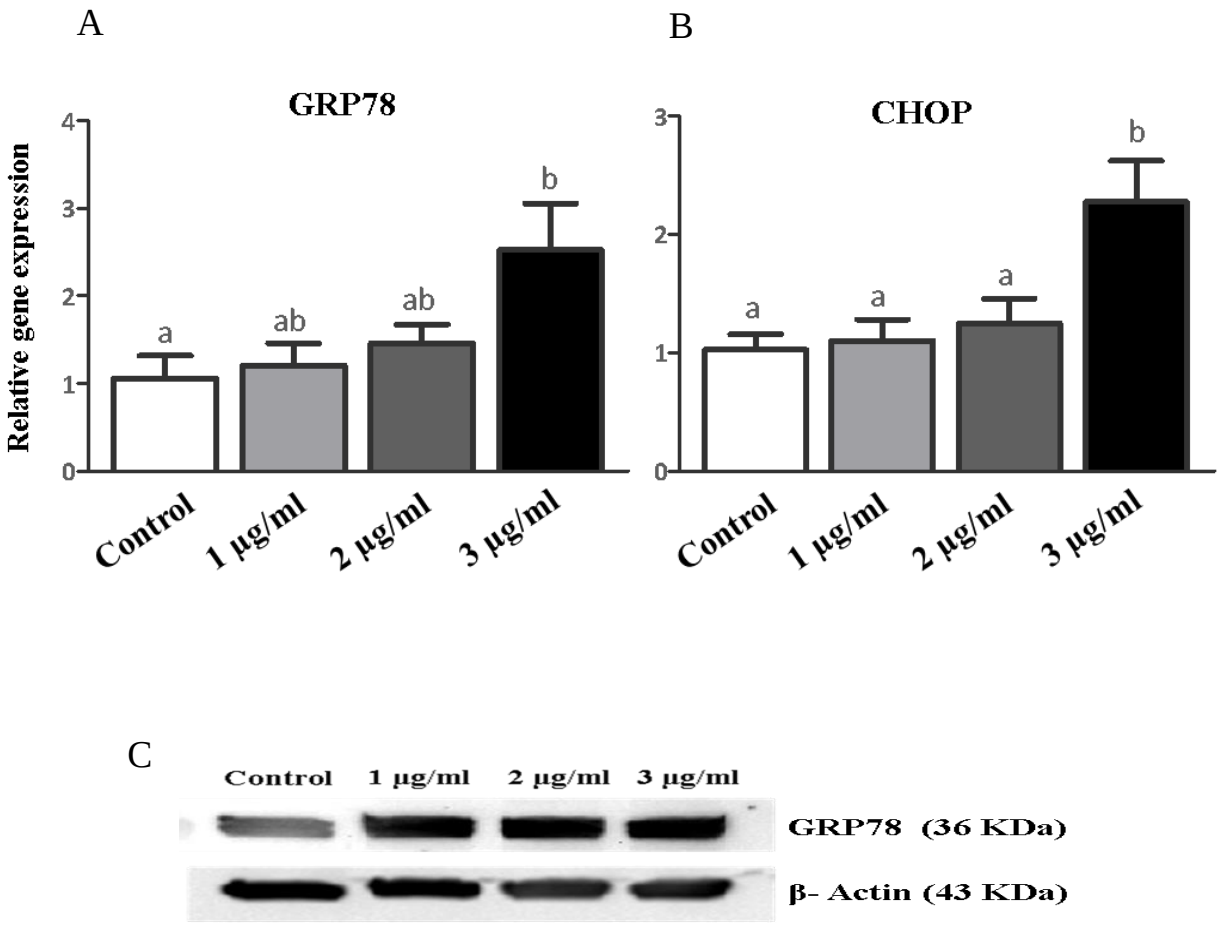


Figure 2.10: Lead triggered expression of unfolded protein response (UPR) marker genes: mRNA expression level of GRP78 (A), CHOP (B) and protein level of GRP78 (C) in bovine GCs cultured under Pb exposure and their corresponding control. β-Actin and GAPDH were used to normalize the expression of targets genes while β-Actin as internal control for protein expression of GRP78. The results are expressed as the mean ± SEM of n = 3. a, b indicate statistically significant differences ($P < 0.05$).

2.5 Discussion

Lead exposure is known to affect female fertility in human and animals (Qureshi & Sharma 2012). The reproductive impact of Pb is complicated and seems to involve multiple pathways, most of which are not fully understood. It has been suggested that, ROS-mediated cellular damage could be one major mechanism implicated in Pb pathogenesis (Flora *et al.* 2007). In this context, our results demonstrated that, Pb exposure induced significant ROS accumulation in bovine GCs, which remain prominent even 24 hours after changing the Pb-containing media.

The integrity of ovarian GCs, being the key marker of oocyte quality and viability, is critical to protect the oocytes from oxidative stress damage (Tripathi *et al.* 2013). Increased ROS can induce oxidative stress when the balance between oxidation and reduction-regulated cellular processes is disturbed hence, causes GCs dysfunction (Luderer 2014). It was also reported that Pb-treated mice exhibited reduced number of developing follicles and increased number of atretic follicles (Sharma *et al.* 2012; Nakade *et al.* 2014).

In the current work we have attempted to investigate the effect of Pb on the fine-tuned events induced by two major redox-sensitive transcription factors, NF-κB and Nrf2, which are crucial to maintain cellular hemostasis. The Nrf2/ARE pathway is a pivotal protective self-defense mechanism against oxidative stress within the cell by activating an array of downstream detoxifying genes (Kaspar *et al.* 2009). We showed here significantly lower Nrf2 and Keap-1 transcript abundance under Pb challenge in addition to downregulation of both SOD and CAT levels. A previous report of Liu *et al.* (2017) showed that Pb decreased the levels of Nrf2 and Keap-1 in the rat kidney. They further demonstrated the ability of Pb to activate miR-153 that target Nrf2 mRNA suggesting the probable effect of Pb as epigenetic modifier. This may explain the transient elevation then decline of Nrf2 protein level along with the decreased transcript abundance, however further investigations are required with different Pb doses and exposure periods.

The NF-κB, is an inducible, pleiotropic transcription factor involved in many physiological and pathological processes, efficiently regulating an array of gene expression (Bellezza *et al.* 2010). The activity of NF-κB was markedly reduced in the present work in Pb-treated groups, without significant difference in mRNA level of its regulator IKK, while NF-κB protein level, being mostly cytoplasmic, increased only in 3 µg/ml treatment group. Though accumulating studies have been conducted to unravel the interplay between Nrf2 and NF-κB, discrepant results still remain. It was reported that the absence of Nrf2 can exacerbate NF-κB activity leading to increased cytokine production (Pan *et al.* 2012). Usually, phytochemicals like curcumin and quercetin activate Nrf2 by inhibiting NF-κB and its downregulated genes (Liu *et al.* 2015; Sahin *et al.* 2016). In contrast, many factors as ischemia, LPS and cigarette smoke have been found to increase both Nrf2 and NF-κB activity (Wakabayashi *et al.* 2010; Meng *et al.* 2017). The striking finding in our work was the downregulation of both transcriptional factors under metal exposure

at the transcriptional level. There is growing consensus that the ROS level may define the cellular fate by modulating these redox-sensitive TFs. According to Bolisetty & Jaimes (2013), low ROS levels are neutralized by Nrf2 activation and its downstream signaling, while NF-κB is activated with moderate ROS levels. However, apoptosis is induced with persistent ROS accumulation in the cell. It is widely accepted that NF-κB signaling pathways may be triggered to protect against increased cellular stress by inducing a list of anti-oxidant genes such as SOD, CAT, Thrx, Ferritin, HO-1, glutathione peroxidase and many others (Perkins & Gilmore 2006). Conversely, NF-κB plays also an important role in inflammation through upregulation of pro-ROS enzymes like NOS, NADPH oxidase, and cyclooxygenases (Deng *et al.* 2003; Nakata *et al.* 2007). Furthermore, while reports showed that ROS can mediate NF-κB activation in many cell types (Morgan and Liu 2011). Recent studies suggested that ROS can potentially repress NF-κB activity (Nakajima & Kitamura 2013).

Another intriguing point is that many metals are able to affect NF-κB signaling mediating both activation and inhibition of NF-κB even without the ROS effect (Chen & Shi 2002). The early work of Xie & Shaikh (2006) showed the involvement of ROS in apoptosis induction through the suppression of NF-κB signaling under cadmium challenge. They attributed this to the oxidation of IKK and hence inhibition of IκB and NF-κB phosphorylation in addition to interference with its DNA binding. Furthermore, no associated decrease was observed in IKK protein suggesting that the metal inhibited IKK activity but not its protein level. Thus, it appears that suppression of NF-κB and its downstream pathway is an underlying mechanism drive to apoptosis by a number of divalent metals (Dieguez-Acuna *et al.* 2004), where the binding of metals to the sulfhydryl groups of target protein molecules and the oxidative stress caused by these metals could be plausible mechanisms beyond their effects on the NF-κB activity. Similarly, it has been reported that thiol groups in the enzymes and proteins are a target for Pb, and further its irreversible binding might alter their function, being a major underling cause of Pb-derived oxidative stress (Surh 2008; Haleagrahara *et al.* 2010; Hasanein *et al.* 2017). Furthermore, many studies reported the existence of the auto-regulatory loop between NF-κB and proinflammatory cytokines like TNF-α and IL-1β, where NF-κB activation leads to the induction of these cytokines, which in turn further induce NF-κB activation (Fan *et al.* 2015). Based on our results, it is inferred that inflammatory pathway may not be involved in the time or the dosing paradigm used in this study.

Proteins can undergo post-translational modifications by a large number of reactions involving ROS. Among these reactions, carbonylation has attracted a proufound attention owing to its stable and irreversible nature (Nyström 2005). It is also assumed that, the elevated level of protein carbonyls is generally a marker of oxidative stress as well as protein dysfunction (Kriebardis *et al.* 2006). Protein carbonylation can be emerged through either oxidative cleavage of proteins or by secondary reaction of protein side chain at cysteine, lysine, and histidine through lipid peroxidation by-products like malondialdehyde (MDA) (Levine 2002). Indeed, Pb is unable to induce ROS directly; however, it could affect the process of lipid peroxidation by antioxidant exhaustion resulting in elevated free radicals and protein carbonyls hence loss of plasma membrane integrity structure and function which are partially responsible for Pb accumulation (Sivaprasad *et al.* 2004; Haleagrahara *et al.* 2010; Ashafaq *et al.* 2016). In this context, our oxyblot results showed that the level of protein carbonyl was significantly higher in Pb-exposed groups than in their control counterpart.

The cell cycle is a fine regulated process that regulate cellular growth and differentiation (Darzynkiewicz *et al.* 2012). The major check points of the cell cycle, G_1 and G_2 phases, possess a critical role in cell cycle transition (Chakravarti *et al.* 2012). Previous study reported that, Pb increased the cell population at the G_0/G_1 yet reduced the cell population at the S phase in human leukemia cells (Yedjou *et al.* 2015). Likewise, our results revealed cell cycle arrest at the G_0/G_1 checkpoint with decreased cell population at S phase in Pb-exposed bovine GCs. This was further confirmed by investigating two markers of proliferation (PCNA and CCDN2) which showed downregulation under Pb challenge suggesting that the cells were driven to apoptosis. Moreover, our findings showed that the level of BCL-2 mRNA was significantly downregulated in all Pb- exposed groups, while there was no change in BAX level, however the expression levels of caspase-3 was significantly up-regulated only in 3 µg/ml Pb-exposed group. Our results demonstrated that Pb- induced apoptosis in GCs by enhancing BAX/BCL-2 ratio as compared to control group and this was further confirmed by results obtained by flow cytometry. These data were consistent with previous results that hepatic apoptosis induced by low-dose of Pb, was accompanied by changes in levels of apoptogenic proteins, such as BCL-2, BAX, and caspase-3 (Yuan *et al.* 2014; Abdel Moneim 2016).

NF-κB has been suggested to control the proliferation and secretory function of porcine ovarian cells accumulating PCNA and inhibiting nuclear apoptosis (Pavlova *et al.* 2011). Additionally, Nrf2 has been recently shown to control the expression of several antiapoptotic proteins, as BCL-2 and BCL-xl, since Nrf2 binds to the antioxidant response element (ARE) located in the promoter region of these genes (Niture & Jaiswal 2012). So we could speculate that, alteration in both Nrf2 and NF-κB may underline the mechanism of Pb-induced apoptosis.

Previous studies showed that Pb could induce protein misfolding and endoplasmic reticulum (ER) stress responses in the liver tissue of rats (Liu *et al.* 2013; Fang *et al.* 2014). In consistence with these data, our results displayed that Pb induced ER stress in bovine GCs by upregulating ER stress-related protein GRP78 and CHOP. Under ER stress and accumulation of unfolded proteins, cells trigger a cascade of protective signaling pathways, named the unfolded protein response (UPR). These include transient inhibition of translation to reduce the protein folding load, activation of chaperone proteins (such as GRP78) to elevate the folding capacity of the ER, degradation of misfolded proteins via proteosomes, and induction of apoptosis (via pro-apoptotic genes as CHOP) in case of severe ER mess-up (Ron & Walter 2007; Olzmann *et al.* 2013; Iurlaro & Munoz-Pinedo 2016). According to Luo *et al.*(2006), GRP78 is a known stress induced ER chaperone and is necessary in early mouse embryo for cell proliferation and guard the inner cell mass against apoptosis. Moreover, ER stress is the main signaling pathway entangled in goat ovarian GCs apoptosis and follicular atresia (Lin *et al.* 2012). The early studies of Qian *et al.* (2000); Zhang *et al.* (2008) suggested that, Pb even at low concentration could bind firmly to GRP78 to stimulate its removal from the cell or sequester it in a nontoxic site. Accordingly, the up-regulation of GRP78 suggests a defense mechanism of cells against the accumulation of intracellular Pb. Meanwhile, this up-regulation may be an adaptive response to the potential inactivation of GRP78 by its binding to Pb and this may also reveal the paradox of upregulation of CHOP simultaneously with GRP78. According to Lee (2014), GRP78 depletion triggers not only the pro-apoptotic CHOP but also intrinsic apoptosis. Moreover, it was reported that, in the absence of Nrf2, UPR is compromised and CHOP has also been found to be upregulated (Meakin *et al.* 2014; Tebay *et al.* 2015).

It is noteworthy to mention that, the toxicity of Pb is highly cell and species specific. For example, from its intake, about 50 % is absorbed in human, 90 % is absorbed in bovine while

only 2 % in ovine (Georgescu *et al.* 2011). Moreover, Taupeau *et al.* (2003) demonstrated that Pb could accumulate in human granulosa cells after 5 hours of exposure affecting the level of p450 aromatase without affecting the cellular viability. While, Gargouri *et al.* (2013) showed that exposure of kidney cell line to different doses of Pb for 4 hours did not affect the viability but induced ROS accumulation. Although, prolonged time of Pb exposure may induce great cellular damage, the effect of short term exposure cannot be neglected and could intervene with important cellular signaling pathways. To the best of our knowledge, no sufficient data was found in the literature concerning Pb impact on bovine GCs with regard to Nrf2/NF-κB signaling interplay. In the present work we demonstrated that exposure to Pb induces oxidative stress that attenuates bovine GCs proliferation and alter cell cycle progression exposing the cells to apoptosis may be in part through disrupting the Nrf2/NF-κB interaction. However, it is not easy to draw conclusions about the mechanisms involved in this interaction, since the cross-talk between Nrf2 and NF-κB involves wide range of complicated molecular interaction that may be also dependent on cell type and tissue context. Therefore, further functional investigations are required to understand different aspects of Nrf2 and NF-κB interactions and their potential role in development of strategies aiming at modulating the resistance against environmental toxins.

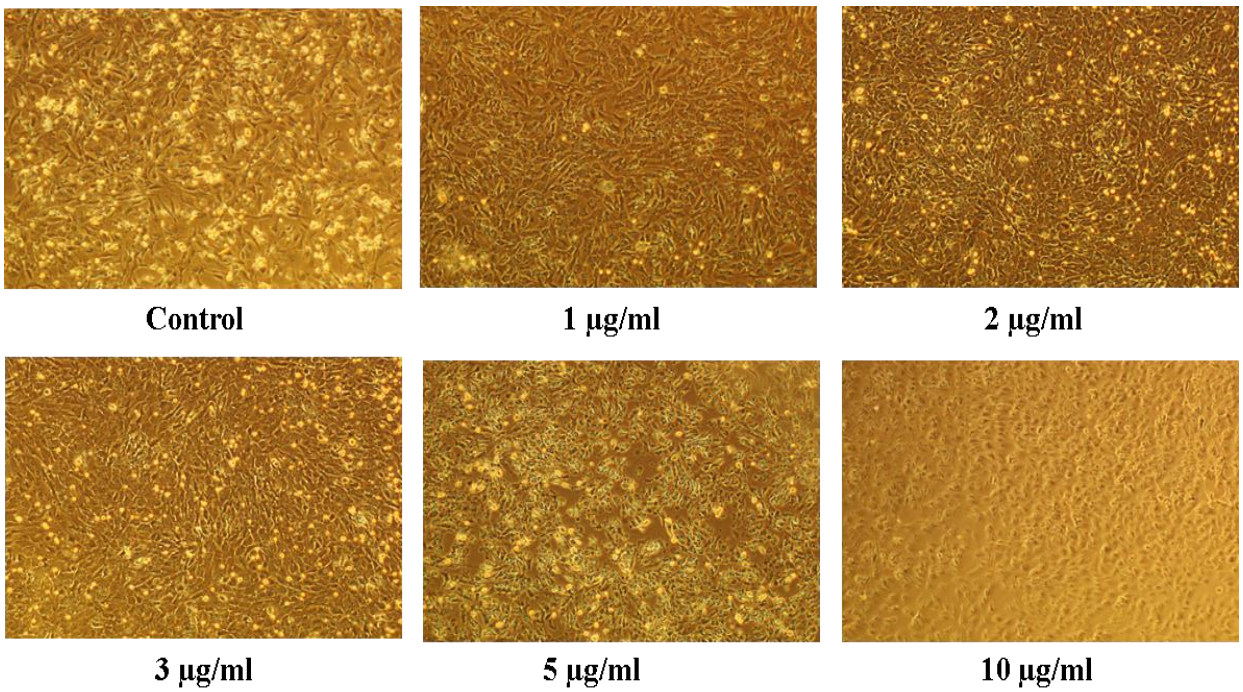


Figure 2.S1: Effect of lead on the morphology of bovine granulosa cells: Cell were exposed to Pb at concentrations of (1, 2, 3, 5, 10 µg/ml) for 2 hours then observed 24 hours after exposure. The confluency of the monolayer and cellular contacts was still kept at lower doses as compared to untreated control while, at higher doses, shrinkage of cells and detachment from the plate were noted. Magnification: 10 x.

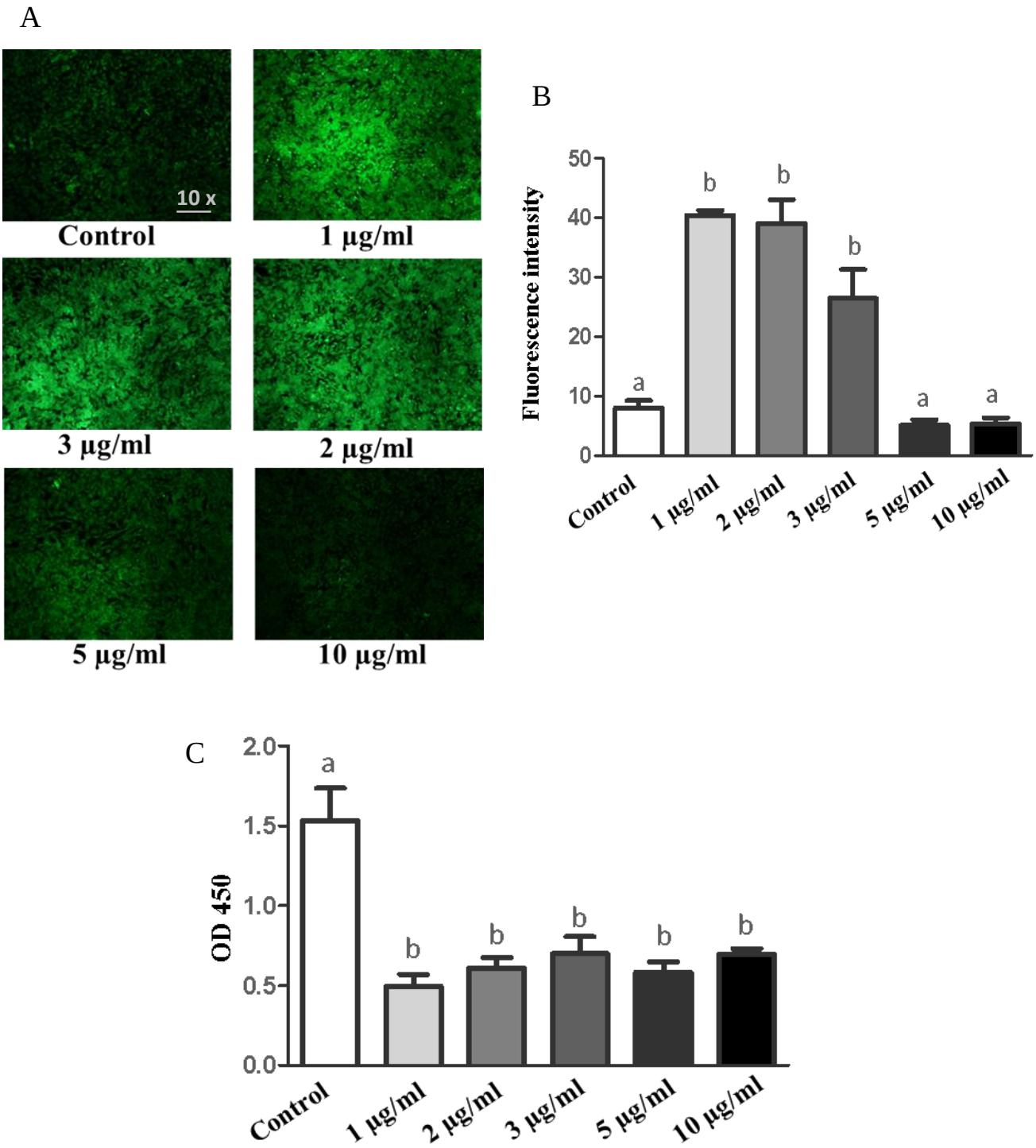


Figure 2.S2: Cytotoxic effect of lead on bovine granulosa cells**:** ROS accumulation (A), quantitative analysis of relative fluorescence emission (B) and Cell proliferation assay (C) in bovine GCs exposed to different concentrations of Pb for 2 hours then investigated 24 hours post treatment along with their corresponding control. Data shown are mean ± SEM, n = 3. a, b indicate statistically significant differences ($P < 0.05$).

2.6 References

Abdel Moneim AE 2016 Indigofera oblongifolia prevents lead acetate-induced hepatotoxicity, oxidative stress, fibrosis and apoptosis in rats. *PloS One* **11** (7) e0158965.

Ahamed M & Siddiqui MKJ 2007 Low level lead exposure and oxidative stress: Current opinions. *Clinica Chimica Acta* **383** (1-2) 57–64.

Ashafaq M, Tabassum H, Vishnoi S, Salman M, Raisuddin S & Parvez S 2016 Tannic acid alleviates lead acetate-induced neurochemical perturbations in rat brain. *Neuroscience Letters* **617** 94–100.

Bayat F, Akbari SAA, Dabirioskoei A, Nasiri M & Mellati A 2016 The relationship between blood lead level and preeclampsia. *Electronic Physician* **8** (12) 3450–3455.

Bellezza I, Mierla AL & Minelli A 2010 Nrf2 and NF-κB and their concerted modulation in cancer pathogenesis and progression. *Cancers* **2** (2) 483–497.

Bires J, Maracek I, Bartko P, Biresova M & Weissova T 1995 Accumulation of trace elements in sheep and the effects upon qualitative and quantitative ovarian changes. *Veterinary & Human Toxicology* **37** (4) 349–356.

Bolisetty S & Jaimes EA 2013 Mitochondria and reactive oxygen species: Physiology and pathophysiology. *International Journal of Molecular Sciences* **14** (3) 6306–6344.

Chakravarti B, Maurya R, Siddiqui JA, Bid HK, Rajendran SM, Yadav PP & Konwar R 2012 In vitro anti-breast cancer activity of ethanolic extract of Wrightia tomentosa: Role of pro-apoptotic effects of oleanolic acid and urosolic acid. *Journal of Ethnopharmacology* **142** (1) 72–79.

Chandramouli K, Steer CD, Ellis M & Emond AM 2009 Effects of early childhood lead exposure on academic performance and behaviour of school age children. *Archives of Disease in Childhood* **94** (11) 844–848.

Chen F & Shi X 2002 Signaling from toxic metals to NF-kappaB and beyond: Not just a matter of reactive oxygen species. *Environmental Health Perspectives* **110** (Suppl 5) 807–811.

Chowdhury AR 2009 Recent advances in heavy metals induced effect on male reproductive function-A retrospective. *Al Ameen Journal of Medical Science* **2** (2) 37–42.

Darzynkiewicz Z, Zhao H, Halicka HD, Rybak P, Dobrucki J & Wlodkowic D 2012 DNA damage signaling assessed in individual cells in relation to the cell cycle phase and induction of apoptosis. *Critical Reviews in Clinical Laboratory Sciences* **49** (5-6) 199–217.

Deng WG, Zhu Y & Wu KK 2003 Up-regulation of p300 binding and p50 acetylation in tumor necrosis factor-alpha-induced cyclooxygenase-2 promoter activation. *The Journal of Biological Chemistry* **278** (7) 4770–4777.

Dieguez-Acuna FJ, Polk WW, Ellis ME, Simmonds PL, Kushleika JV & Woods JS 2004 Nuclear factor kappa B activity determines the sensitivity of kidney epithelial cells to apoptosis: Implications for mercury-induced renal failure. *Toxicological Sciences* **82** (1) 114–123.

Doumouchtsis KK, Doumouchtsis SK, Doumouchtsis EK & Perrea DN 2009 The effect of lead intoxication on endocrine functions. *Journal of Endocrinological Investigation* **32** (2) 175–183.

Eum KD, Weisskopf MG, Nie LH, Hu H & Korrick SA 2014 Cumulative lead exposure and age at menopause in the Nurses' Health Study cohort. *Environmental Health Perspectives* **122** (3) 229–234.

Fan B, Dun SH, Gu JQ, Guo Y & Ikuyama S 2015 Pycnogenol Attenuates the Release of Proinflammatory Cytokines and Expression of Perilipin 2 in Lipopolysaccharide-Stimulated Microglia in Part via Inhibition of NF-kappa B and AP-1 Activation. *PloS One* **10** (9) e0137837.

Fang JY, Wang PW, Huang CH, Hung YY & Pan TL 2014 Evaluation of the hepatotoxic risk caused by lead acetate via skin exposure using a proteomic approach. *Proteomics* **14** (21-22) 2588–2599.

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

Flora SJS, Saxena G & Mehta A 2007 Reversal of lead-induced neuronal apoptosis by chelation treatment in rats: Role of reactive oxygen species and intracellular (Ca^{2+}). *The Journal of Pharmacology & Experimental Therapeutics* **322** (1) 108–116.

Gargouri M, Magne C, Dauvergne X, Ksouri R, El Feki A, Metges MAG & Talarmin H 2013 Cytoprotective and antioxidant effects of the edible halophyte Sarcocornia perennis L. (swampfire) against lead-induced toxicity in renal cells. *Ecotoxicology & Environmental Safety* **95** 44–51.

Gebremedhn S, Salilew-Wondim D, Ahmad I, Sahadevan S, Hossain MM, Hoelker M, Rings F, Neuhoff C, Tholen E, Looft C, Schellander K & Tesfaye D 2015 MicroRNA Expression profile in bovine granulosa cells of preovulatory dominant and subordinate follicles during the late follicular phase of the estrous cycle. *PloS One* **10** (5) e0125912.

Georgescu B, Georgescu C, Dărăban S, Bouaru A & Paşcalău S 2011 Heavy metals acting as endocrine disrupters. *ScientificP: Animal Science & Biotechnologies* **44** (2) 89–93.

Gurer-Orhan H, Sabir HU & Ozgunes H 2004 Correlation between clinical indicators of lead poisoning and oxidative stress parameters in controls and lead-exposed workers. *Toxicology* **195** (2-3) 147–154.

Haleagrahara N, Jackie T, Chakravarthi S, Rao M & Kulur A 2010 Protective effect of Etlingera elatior (torch ginger) extract on lead acetate-induced hepatotoxicity in rats. *The Journal of Toxicological Sciences* **35** (5) 663–671.

Hasanein P, Ghafari-Vahed M & Khodadadi I 2017 Effects of isoquinoline alkaloid berberine on lipid peroxidation, antioxidant defense system, and liver damage induced by lead acetate in rats. *Redox Report* **22** (1) 42–50.

Iurlaro R & Munoz-Pinedo C 2016 Cell death induced by endoplasmic reticulum stress. *The FEBS Journal* **283** (14) 2640–2652.

Jahromi BN, Mosallanezhad Z, Matloob N, Davari M & Ghobadifar MA 2015 The potential role of granulosa cells in the maturation rate of immature human oocytes and embryo development: A co-culture study. *Clinical & Experimental Reproductive Medicine* **42** (3) 111–117.

Kang KW, Lee SJ & Kim SG 2005 Molecular mechanism of Nrf2 activation by oxidative stress. *Antioxidants & Redox Signaling* **7** (11-12) 1664–1673.

Kaspar JW, Niture SK & Jaiswal AK 2009 Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radical Biology & Medicine* **47** (9) 1304–1309.

Kriebardis AG, Antonelou MH, Stamoulis KE, Economou-Petersen E, Margaritis LH & Papassideri IS 2006 Membrane protein carbonylation in non-leukodepleted CPDA-preserved red blood cells. *Blood Cells, Molecules & Diseases* **36** (2) 279–282.

Lee AS 2014 Glucose regulated proteins in cancer: Molecular mechanisms and therapeutic potential. *Nature reviews. Cancer* **14** (4) 263–276.

Levine RL 2002 Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radical Biology & Medicine* **32** (9) 790–796.

Li W, Khor TO, Xu C, Shen G, Jeong WS, Yu S & Kong AN 2008 Activation of Nrf2-antioxidant signaling attenuates NF-kappa B-inflammatory response and elicits apoptosis. *Biochemical Pharmacology* **76** (11) 1485–1489.

Lim J, Ortiz L, Nakamura BN, Hoang YD, Banuelos J, Flores VN, Chan JY & Luderer U 2015 Effects of deletion of the transcription factor Nrf2 and benzo apyrene treatment on ovarian follicles and ovarian surface epithelial cells in mice. *Reproductive Toxicology* **58** 24–32.

Lin P, Yang Y, Li X, Chen F, Cui C, Hu L, Li Q, Liu W & Jin Y 2012 Endoplasmic reticulum stress is involved in granulosa cell apoptosis during follicular atresia in goat ovaries. *Molecular Reproduction & Development* **79** (6) 423–432.

Liu B, Zhang H, Tan X, Yang D, Lv Z, Jiang H, Lu J, Baiyun R & Zhang Z 2017 GSPE reduces lead-induced oxidative stress by activating the Nrf2 pathway and suppressing miR153 and GSK-3beta in rat kidney. *Oncotarget* **8** (26) 42226–42237.

Liu CM, Ma JQ, Xie WR, Liu SS, Feng ZJ, Zheng GH & Wang AM 2015 Quercetin protects mouse liver against nickel-induced DNA methylation and inflammation associated with the Nrf2/HO-1 and p38/STAT1/NF-κB pathway. *Food & Chemical Toxicology* **82** 19–26.

Liu C-M, Zheng GH, Ming QL, Sun JM & Cheng C 2013 Protective effect of quercetin on lead-induced oxidative stress and endoplasmic reticulum stress in rat liver via the IRE1/JNK and PI3K/Akt pathway. *Free Radical Rresearch* **47** (3) 192–201.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. *Methods* **25** (4) 402–408.

Lu J, Wang Z, Cao J, Chen Y & Dong Y 2018 A novel and compact review on the role of oxidative stress in female reproduction. *Reproductive Biology & Endocrinology* **16** (1) 80.

Luderer U 2014 Ovarian toxicity from reactive oxygen species. *Vitamins & Hormones* **94** 99–127.

Luo S, Mao C, Lee B & Lee AS 2006 GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. *Molecular & Cellular Biology* **26** (15) 5688–5697.

Meakin PJ, Chowdhry S, Sharma RS, Ashford FB, Walsh SV, McCrimmon RJ, Dinkova-Kostova AT, Dillon JF, Hayes JD & Ashford MLJ 2014 Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance. *Molecular & Cellular Biology* **34** (17) 3305–3320.

Meng QT, Chen R, Chen C, Su K, Li W, Tang LH, Liu HM, Xue R, Sun Q, Leng Y, Hou JB, Wu Y, Xia ZY 2017 Transcription factors Nrf2 and NF-κB contribute to inflammation and apoptosis induced by intestinal ischemia-reperfusion in mice. *International Journal of Molecular Medicine* **40** (6) 1731–1740.

Morgan MJ & Liu ZG 2011 Crosstalk of reactive oxygen species and NF-kappa B signaling. *Cell Research* **21** (1) 103–115.

Nakade UP, Garg SK, Sharma A, Choudhury S, Yadav RS, Gupta K & Sood N 2014 Lead-induced adverse effects on the reproductive system of rats with particular reference to histopathological changes in uterus. *Indian Journal of Pharmacology* **47** (1) 22–26.

Nakajima S & Kitamura M 2013 Bidirectional regulation of NF-kappa B by reactive oxygen species: A role of unfolded protein response. *Free Radical Biology & Medicine* **65** 162–174.

Nakata S, Tsutsui M, Shimokawa H, Yamashita T, Tanimoto A, Tasaki H, Ozumi K, Sabanai K, Morishita T, Suda O, Hirano H, Sasaguri Y, Nakashima Y & Yanagihara N 2007 Statin treatment upregulates vascular neuronal nitric oxide synthase through Akt/NF-kappa B pathway. *Arteriosclerosis, Thrombosis & Vascular Biology* **27** (1) 92–98.

Niture SK & Jaiswal AK 2012 Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *The Journal of Biological Chemistry* **287** (13) 9873–9886.

Nyström T 2005 Role of oxidative carbonylation in protein quality control and senescence. *The EMBO Journal* **24** (7) 1311–1317.

Olzmann JA, Kopito RR & Christianson JC 2013 The mammalian endoplasmic reticulum-associated degradation system. *Cold Spring Harbor Perspectives in Biology* **5** (9) a013185.

Paksy K, Gati I, Naray M & Rajczy K 2001 Lead accumulation in human ovarian follicular fluid, and in vitro effect of lead on progesterone production by cultured human ovarian granulosa cells. *Journal of Toxicology & Environmental Health* **62** (5) 359–366.

Pan H, Wang H, Wang X, Zhu L & Mao L 2012 The absence of Nrf2 enhances NF-kappa B-dependent inflammation following scratch injury in mouse primary cultured astrocytes. *Mediators of Inflammation* **2012** 217580.

Pant N, Upadhyay G, Pandey S, Mathur N, Saxena DK & Srivastava SP 2003 Lead and cadmium concentration in the seminal plasma of men in the general population: Correlation with sperm quality. *Reproductive Toxicology* **17** (4) 447–450.

Pavlova S, Klucska K, Vasicek D, Kotwica J & Sirotkin AV 2011 Transcription factor NF-kappa B (p50/p50, p65/p65) controls porcine ovarian cells functions. *Animal Reproduction Science* **128** (1-4) 73–84.

Perkins ND & Gilmore TD 2006 Good cop, bad cop: The different faces of NF-kappa B. *Cell Death & Differentiation* **13** (5) 759–772.

Pillai P, Pandya C, Gupta S & Gupta S 2010 Biochemical and molecular effects of gestational and lactational coexposure to lead and cadmium on ovarian steroidogenesis are associated with oxidative stress in F1 generation rats. *Journal of Biochemical & Molecular Toxicology* **24** (6) 384–394.

Qian Y, Harris ED, Zheng Y & Tiffany-Castiglioni E 2000 Lead targets GRP78, a molecular chaperone, in C6 rat glioma cells. *Toxicology & Applied Pharmacology* **163** (3) 260–266.

Qureshi N & Sharma R 2012 Lead toxicity and infertility in female Swiss mice: A review. *Journal of Chemical, Biological & Physical Sciences* **2** (4) 1849.

Qureshi N, Sharma R, Mogra S & Panwar K 2010 Protective effects of combined treatment of vitamin E and C on lead induced folliculogenesis in swiss mice. *Journal of Herbal Medical & Toxicology* **4** (2) 207–213.

Ron D & Walter P 2007 Signal integration in the endoplasmic reticulum unfolded protein response. *Nature reviews. Molecular Cell Biology* **8** (7) 519–529.

Sahin K, Pala R, Tuzcu M, Ozdemir O, Orhan C, Sahin N & Juturu V 2016 Curcumin prevents muscle damage by regulating NF-kappa B and Nrf2 pathways and improves performance: An in vivo model. *Journal of Inflammation Research* **9** 147–154.

Sanders T, Liu Y, Buchner V & Tchounwou PB 2009 Neurotoxic effects and biomarkers of lead exposure: A review. *Reviews on Environmental Health* **24** (1) 15–45.

Seyom E, Abera M, Tesfaye M & Fentahun N 2015 Maternal and fetal outcome of pregnancy related hypertension in Mettu Karl Referral Hospital, Ethiopia. *Journal of Ovarian Research* **8** 10.

Shan G, Tang T & Zhang X 2009 The protective effect of ascorbic acid and thiamine supplementation against damage caused by lead in the testes of mice. *Journal of Huazhong University of Science & Technology*. **29** (1) 68–72.

Sharma R, Qureshi N, Mogra S & Panwar K 2012 Lead Induced Infertility in Swiss Mice and Role of Antioxidants. *Universal Journal of Environmental Research & Technology* **2** (2).

Sivaprasad R, Nagaraj M & Varalakshmi P 2004 Combined efficacies of lipoic acid and 2,3-dimercaptosuccinic acid against lead-induced lipid peroxidation in rat liver. *The Journal of Nutritional Biochemistry* **15** (1) 18–23.

Surh YJ 2008 NF-kappa B and Nrf2 as potential chemopreventive targets of some anti-inflammatory and antioxidative phytonutrients with anti-inflammatory and antioxidative activities. *Asia Pacific Journal of Clinical Nutrition* **17 Suppl 1** 269–272.

Swarup D, Patra RC, Naresh R, Kumar P & Shekhar P 2005 Blood lead levels in lactating cows reared around polluted localities; transfer of lead into milk. *The Science of the Total Environment* **347** (1-3) 106–110.

Tang N & Zhu ZQ 2003 Adverse reproductive effects in female workers of lead battery plants. *International Journal of Occupational Medicine & Environmental Health* **16** (4) 359–361.

Taupeau C, Poupon J, Nome F & Lefevre B 2001 Lead accumulation in the mouse ovary after treatment-induced follicular atresia. *Reproductive Toxicology* **15** (4) 385–391.

Taupeau C, Poupon J, Treton D, Brosse A, Richard Y & Machelon V 2003 Lead reduces messenger RNA and protein levels of cytochrome p450 aromatase and estrogen receptor beta in human ovarian granulosa cells. *Biology of Reproduction* **68** (6) 1982–1988.

Taylor CM, Golding J, Hibbeln J & Emond AM 2013 Environmental factors predicting blood lead levels in pregnant women in the UK: The ALSPAC study. *PloS One* **8** (9) e72371-e72371.

Tebay LE, Robertson H, Durant ST, Vitale SR, Penning TM, Dinkova-Kostova AT & Hayes JD 2015 Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Radical Biology & Medicine* **88** (0 0) 108–146.

Telleria CM, Goyeneche AA, Stocco CO & Gibori G 2004 Involvement of nuclear factor kappa B in the regulation of rat luteal function: Potential roles as survival factor and inhibitor of 20alpha-hydroxysteroid dehydrogenase. *Journal of Molecular Endocrinology* **32** (2) 365–383.

Tripathi A, Shrivastav TG & Chaube SK 2013 An increase of granulosa cell apoptosis mediates aqueous neem (Azadirachta indica) leaf extract-induced oocyte apoptosis in rat. *International Journal of Applied & Basic Medical Research* **3** (1) 27–36.

Vallabhapurapu S & Karin M 2009 Regulation and function of NF-kappa B transcription factors in the immune system. *Annual Review of Immunology* **27** 693–733.

van Engeland M, Ramaekers FC, Schutte B & Reutelingsperger CP 1996 A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. *Cytometry* **24** (2) 131–139.

Vigeh M, Yokoyama K, Seyedaghamiri Z, Shinohara A, Matsukawa T, Chiba M & Yunesian M 2011 Blood lead at currently acceptable levels may cause preterm labour. *Occupational & Environmental Medicine* **68** (3) 231–234.

Wakabayashi N, Slocum SL, Skoko JJ, Shin S & Kensler TW 2010 When Nrf2 talks, who's listening? *Antioxidants & Redox Signaling* **13** (11) 1649–1663.

Wang Y, Chan S & Tsang BK 2002 Involvement of inhibitory nuclear factor-kappa B (NF kappa B)-independent NFkappa B activation in the gonadotropic regulation of X-linked inhibitor of apoptosis expression during ovarian follicular development in vitro. *Endocrinology* **143** (7) 2732–2740.

Wardyn JD, Ponsford AH & Sanderson CM 2015 Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochemical Society Transactions* **43** (4) 621–626.

Xie J & Shaikh ZA 2006 Cadmium-induced apoptosis in rat kidney epithelial cells involves decrease in nuclear factor-kappa B activity. *Toxicological Sciences* **91** (1) 299–308.

Yedjou CG, Tchounwou HM & Tchounwou PB 2015 DNA damage, cell cycle arrest, and apoptosis induction caused by lead in human leukemia cells. *International Journal of Environmental Research & Public Health* **13** (1) 56.

Yuan G, Dai S, Yin Z, Lu H, Jia R, Xu J, Song X, Li L, Shu Y & Zhao X, Chen Z, Fan Q, Liang X, He C, Yin L, Lv C, Lei Q, Wang L, Mi Y, Yu X & Zhang M 2014 Sub-chronic lead and cadmium co-induce apoptosis protein expression in liver and kidney of rats. *International Journal of Clinical & Experimental Pathology* **7** (6) 2905–2914.

Zhang M, An C, Gao Y, Leak RK, Chen J & Zhang F 2013 Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Progress in Neurobiology* **100** 30–47.

Zhang Y, Sun LG, Ye LP, Wang B & Li Y 2008 Lead-induced stress response in endoplasmic reticulum of astrocytes in CNS. *Toxicology Mechanisms & Methods* **18** (9) 751–757.

Chapter 3

Response of Bovine Embryo to Stage Specific Exposure to Lead Acetate

Hoda Samir Aglan[1,4], Samuel Gebremedhn[1], Dessie Salilew-Wondim[1], Franca Rings[1], Michael Hölker[1, 2], Christiane Neuhof[1], Ernst Tholen[1], Karl Schellander[1,3] and Dawit Tesfaye[1,3] *

[1]Institute of Animal Science, Department of Animal Breeding and Husbandry, University of Bonn, Bonn, Germany

[2]Teaching and Research Station Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany

[3]Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

[4]Department of Pharmacology, National Organization for Drug Control and Research, Giza, Egypt

*Corresponding author

(Manuscript under preparation)

3.1 Abstract

Lead (Pb), a widespread environmental heavy metal, has been shown to be embryo-toxic in a variety of animal species. However, little is known about the mechanism beyond Pb toxicity in bovine model. In the current study, we aimed to investigate the effect of Pb on *in vitro* early embryonic development. Oocytes were aspirated from bovine ovaries and matured *in vitro* in the absence (Control) or presence of Pb (10 µg/ml) followed by *in vitro* fertilization. The resultant zygotes were divided into two groups, those cultured *in vitro* without Pb (Control), while Pb-exposed zygotes were further randomly divided into three groups, cultured in the absence of Pb (Oocyte only**)** or presence of Pb until the 16 cell (1-16 cell) stage and those cultured until the blastocyst stage (All stages). Results showed that Pb impaired the embryonic cleavage, blastocyst rates and blastocyst cell number. Moreover, higher ROS accumulation was observed in all Pb-exposed groups. Pb induced downregulation of Nrf2 protein level accompanied by downregulation of Keap-1 and CAT and no change in Nrf2, SOD and HO-1 at mRNA level. Additionally, NF-κB showed significant increment in both mRNA and protein levels with upregulation of TNF-α. Apoptosis was induced by Pb as manifested by elevation of BAX/BCL-2 ratio and the number of positive TUNEL apoptotic cells. Our results suggest the detrimental effect of Pb on early embryonic development could originate from its effect on oocyte maturation which perturbs subsequent embryo development probably via altered Nrf2/ NF-κB signaling.

3.2 Introduction

Heavy metals are widespread environmental pollutants due to their integration in different industries in our modern life. They are highly toxic and can cause damaging effects even at very low concentrations (Korashy & El-Kadi 2006). Of these, lead (Pb) is one of the most common natural substances in the environment. It is present in cigarette smoke, cutlery, hair dyes, lead pipes, car exhaust, and paints. Over the past decades, efforts have been made to reduce Pb exposure (Karimooy *et al.* 2010). However, due to its non-biodegradable nature, it has accumulated in the soil, entered the food chain and resided in animal and human bodies. Environmental and occupational exposures to Pb have been associated with detrimental consequences affecting almost all aspects of the female reproductive system in mammals (Pant *et al.* 2003; Patrick 2006; Ahmed *et al.* 2012). Lead has been reported to cause wide array of reproductive pathologies such as infertility, miscarriage, pregnancy hypertension, premature

membrane rupture, premature delivery and preeclampsia (Seyom *et al.* 2015; Bayat *et al.* 2016). Experimental studies have detected Pb in the follicular fluid of many species including human (Paksy *et al.* 2001), cattle (Swarup *et al.* 2005), sheeps (Bires *et al.* 1995) and mouse (Taupeau *et al.* 2001). Moreover, the accumulation of Pb in the ovaries could impair folliculogenesis even damage the ovarian follicle and the oocytes (Avazeri *et al.* 2006) and hence compromises pregnancy (Silberstein *et al.* 2006). Lead disrupts the pituitary-gonadal axis which in turn inhibit ovulation and pregnancy (Choi *et al.* 2004; Nampoothiri *et al.* 2007). The decline in female fertility worldwide has prompted the assisted reproductive techniques (ART) (Mouzon *et al.* 2010). Meanwhile, the exposure to environmental toxic metals might affect oocyte maturation and fertilization during *in vitro* fertilization (IVF) (Bloom *et al.* 2010). Blood lead level (BLL) was found to negatively affect human fertilization outcome (Al-Saleh *et al.* 2008). An inverse association was also detected between Pb level in the follicular fluid and fertilization (Bloom *et al.* 2012). The relation between Pb exposure and pregnancy outcome is still controversial. In a prospective cohort study, an inverse association of BLL and oocyte fertilization was detected but this effect did not extend to pregnancy (Al-Saleh *et al.* 2008). A report by Nandi *et al.* (2010) showed significant decline in buffalo embryo cleavage rate following the culture of oocytes in Pb-containing media. In contrary, many studies on occupationally Pb-exposed women reported no association between BLL and the probability to conceive (Sallmen *et al.* 1995; Cole *et al.* 2006; Bloom *et al.* 2011a). The differential susceptibility of developing human conceptus to Pb impact is still unresolved question (Bloom *et al.* 2011b).

Although the mechanisms underlying Pb-induced toxicity are complex, oxidative stress could be a potential mechanism beyond the pathophysiology of Pb toxicity (Flora *et al.* 2012). Oxidative stress arises from the imbalance between oxidants and antioxidants. This leads to the activation of a variety of transcriptional factors (TFs) which proved to have great significance in the etiology of reproductive diseases (Lu *et al.* 2018a). In this context, we focused on two relevant redox-sensitive transcription factors: nuclear factor erythroid 2-related factor 2 (Nrf2), and nuclear factor κB (NF-κB) which play an important role in antioxidant defense mechanism and inflammation, respectively (Pedruzzi *et al.* 2012). Additionally, these TFs do not work individually or independently but a functional cross-talk between them has been revealed in previous studies (Li *et al.* 2008; Wardyn *et al.* 2015; Sivandzade *et al.* 2018). Very limited studies have been conducted on the effect of Pb on mammalian oocytes maturation and early

embryo development. Therefore, we aimed to investigate the effect of Pb on the bovine *in vitro* oocyte maturation (IVM), fertilization (IVF) and subsequent embryo development.

3.3 Materials and methods

3.3.1 Oocyte collection, *in vitro* maturation, fertilization and embryo culture.

Bovine ovaries were obtained from a local slaughterhouse, maintained in saline at 37° C, and transported to the laboratory. After washing the ovaries twice, follicles that were 3–8 mm in diameter were aspirated. Cumulus-oocyte complexes (COCs) surrounded by more than three layers of cumulus cells were washed three times with TL-HEPES then assigned into two groups (control and Pb groups). In the control group, COCs were cultured in modified TCM 199 culture medium (Sigma-Aldrich, Munich, Germany) with 12 % heat inactivated oestrus cow serum, 0.02 U/mL FSH and 50 mg/ml gentamicin at 38.5° C in a humidified atmosphere of 5 % CO2. The COCs of Pb group were cultured in the same previous medium but supplemented with 10 µg/ml lead acetate (Sigma-Aldrich, Germany). After 24 hours, matured COCs of both groups (control and Pb groups) were *in vitro* inseminated with frozen thawed bull sperm at a concentration of $2x10^6$ sperm/ ml and cultured for 18 hours in Tyrode's albumin-lactate-pyruvate media with 0.6 % (w/v) fatty acid-free bovine serum albumin (BSA), 50 mg/ml gentamicin, 1 mg/ml heparin and 10 mM hypotaurine and 2 mM noradrenaline.

After fertilization, the control presumptive zygotes (without Pb) were *in vitro* cultured in GM501 basic medium (M) (Gynemed, Lensahn, Germany) supplemented with 3 mg/ml BSA, kept under mineral oil at 38.5° C in an atmosphere of 5 % CO2, 5 % O2 and 90 % N2 and allowed to proceed to blastocyst stage. While Pb-exposed zygotes were randomly divided into three experimental groups (Figure 3.1); Oocyte only group: COCs that exposed to Pb till fertilization, continued division to the blastocyst stage in M medium without Pb, 1-16 cell group: zygots exposed to Pb till 16 cell stage then continued division without Pb until the blastocyst stage and All stages group: Pb-exposed zygots until the blastocyst stage. The cleavage and blastocyst rates were determined for each group. Expanding blastocysts (Day 7–8) were harvested, frozen in liquid nitrogen and stored at −80° C.

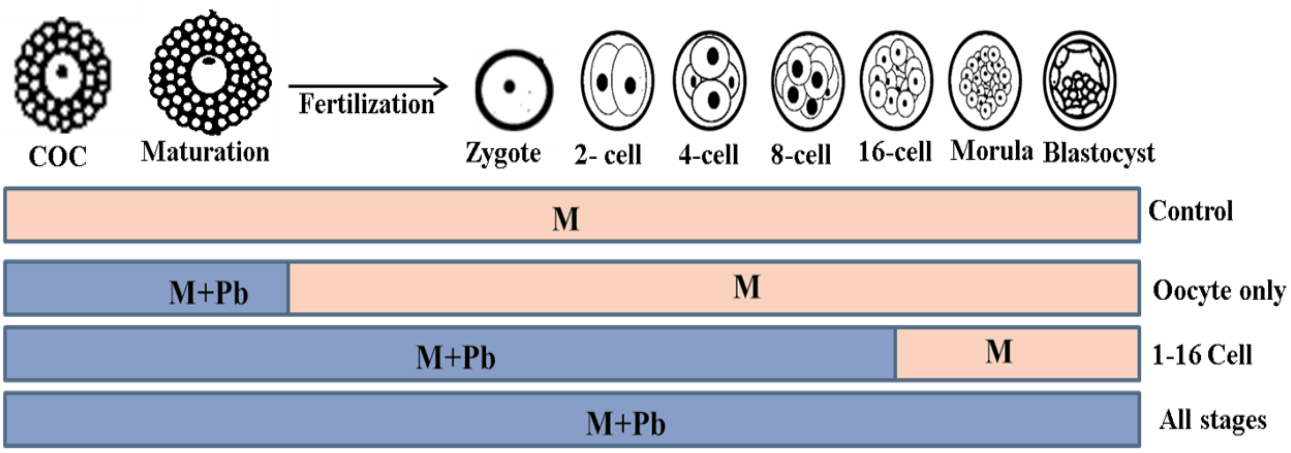


Figure 3.1: Diagram of the experimental design. Bovine embryos were subjected to *in vitro* culture under four experimental groups: (1) Control group: GM501 basic medium (M), (2) Oocyte only group Pb added to the maturation media only, (3) 1–16 Cell group: Pb added to the media from the oocyte maturation until 16-cell stage and (4) All stages group: Pb added to the media from the oocyte maturation until the blastocyst stage.

3.3.2 Reactive oxygen species (ROS) detection

The level of ROS was assessed using H_2-dichlorofluorescein (H_2DCFDA) (Life Technologies, Germany). Fifteen blastocysts from each group were incubated with 400 µl of (M) medium containing 5 mM H_2DCFDA for 20 min in dark at 37^o C. Embryos were then washed twice with PBS and imaged using Leica DM IRB inverted microscope (Leica, Bensheim, Germany).

3.3.3 Assessment of blastocyst cell number

Total blastocyst cell number was quantified using nuclear fluorescence staining with the glycerol-based Hoechst 33342 according to the manufacturer's recommendation. 15 blastocysts derived from each experimental group were fixed for 5 min in a solution containing 2 % formalin and 0.25 % gluteraldehyde. The fixed blastocysts were stained for 10 min with Hoechst 33342 solution on clean glass slides and visualized using Leica DM IRB inverted microscope (Leica, Bensheim, Germany). The cell number was scored for individual blastocysts from each culture group.

3.3.4 RNA isolation and cDNA synthesis

Four biological replicates each containing 15 embryos (day 8 blastocysts) from each group were used for RNA isolation. Total RNA was isolated using Pico Pure RNA isolation kit (Arcturs,

Munich, Germany) with some modifications (Norhazlin *et al.* 2015). Embryos were incubated with 100 µl extraction buffer at 42° C for 30 min. The lysates were homogenized and vortexed for 30 s, then centrifuged at 3000 *g* for 2 min. A total of 100 µl 70 % ethanol was added and mixed by pipetting then the mixture was transferred into the preconditioned column and centrifuged at 100 *g* for 2 min, followed by 1 min at 16,000 *g*. Wash buffer 1 (100 µl) was added into the column and centrifuged at 8000 *g* for 1 min. On-column DNase treatment was done using RNase-free DNase enzyme (Qiagen GmbH, Hilden, Germany) by incubating 5 µl RNase-free DNase I with 35 µl RDD buffer for 15 min at room temperature. After two times washing, RNA was recovered in 20 µl elution buffer, checked for quality using nanodrop ND-8000 (NanoDrop technologies). The RNA from each blastocyst group was then reverse transcribed to cDNA using cDNA Synthesis Kit (Thermo Fisher scientific, Germany) according to the manufacturer's instruction. Briefly, oligo $(dT)_{18}$ and Random primer (0.5 µl each) were added to each RNA sample and incubated for 5 min at 65° C. Then other components were added to the mixture, RiboLock RNase Inhibitor (1 µl); 5× RT Buffer (4 µl); dNTP mix (2 µl), Revert Aid Reverse Trancriptase Enzyme mix (2 µl) and incubated for 5 min at 25° C followed by 60 min at 37° C and finally 70° C for 5 min. The resulting cDNA samples were stored in –20° C till further use

3.3.5 Real time quantitative PCR

A total of 13 genes Nrf2, Keap1, Nrf2-target antioxidant genes (SOD, CAT, HO-1), NF-κB, TNF-α, apoptosis relarted genes (BAX and BCL-2), DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B), were investigated for their expression using sequence-specific primers (previously mentioned in Table 2.1 and Table 3.1). Quantitative PCRs were performed in 20 µl reaction volumes containing 10 µl SYBR green master mix (Eppendorf, Hamburg, Germany), 2 µl cDNA, and an optimized amount of forward and reverse primers diluted in water to a volume of 8 µl. Following initial denaturation step at 95° C for 3 min, then 40 cycles of 15 sec at 95° C and 45 sec at 60° C was used as thermal cycling program and the relative quantity was normalized using β- Actin and GAPDH.

Table 3.1: Details of primers used in qPCR:

Gene	Primer sequence	Size (bp)	Accession Number
DNMT1	F: 5´-AGCAATGGGCAGATGTTCCA-3´ R: 5´-ATCTCGCGTAGTCTTGGTCG-3´	268	XM_015471995.2
DNMT3A	F: 5´-GTGCTGAAGACAGGAAAAGGA-3´ F: 5´-TTGGACGGGGGAGAATAAGTA-3´	189	NM_001206502.2
DNMT3B	F: 5´-CTGCTGAATTACACTCGCCC-3´ F: 5´-CCAGAAGTATCGGGCTCTGT-3´	177	XR_003037672.1

3.3.6 Immunofluorescence detection of Nrf2 and NF-κB proteins

Immunohistochemistry was performed to localize Nrf2 and NF-κB proteins in (day 8) blastocysts. Briefly, 10 blastocyst from each group were fixed overnight at 4^{o} C in 4 % (w/v) paraformaldehyde in PBS. Fixed blastocysts were washed and permeabilized with 0.5 % (v/v) Triton-X 100 (Sigma-Aldrich) in PBS (PBSX) for 2 hours at room temperature. The samples were washed and blocked with 3 % BSA (Sigma-Aldrich) in PBS for 1 hour at 37^{o} C, followed by incubation with primary antibodies against Nrf2 (1:100 dilution, orb11165, Biorbyt; UK) or NF-κB (1:200 dilution, orb11118, Biorbyt, UK) overnight at 4^{o} C. After washing, embryos were further incubated at room temperature for 1 hour in the dark with Alexa Fluor 568-conjugated goat anti-rabbit secondary antibody (1:300 dilution, A-11011, Life Technologies). Blastocysts were stained and mounted into slide with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Dabco; Acros, Geel, Belgium) then visualized using CLSM LSM-780 confocal laser scanning microscope (Zeiss, Germany; Scale bar 50 µm).

3.3.7 Immunofluorescence detection of 5mC

Day 8 blastocysts were fixed overnight in 4 % (w/v) paraformaldehyde in PBS. This was followed by denaturation with 1 N HCl at room temperature for 30 min and neutralization with 0.1 M Tris-HCl, pH 8.0 for 15 min. Subsequently, blastocysts were incubated in PBS containing 1% BSA, and then incubated overnight at 4 °C with 5mC antibody (1:250 dilution, orb499937, Biorbyt; UK). After washing three times with PBS/PVA, the oocytes and embryos were incubated at 37 °C for 1 h with goat anti-rabbit IgG (A11011, 1:200, Invitrogen). The embryos were then stained with DAPI, mounted onto slides, and examined using a confocal microscope (Zeiss LSM 780, Germany).

3.3.8 TUNEL assay (Terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate (dUTP) nick-end labeling)

Blastocysts (n =10) were fixed in 4 % paraformaldehyde for 15 min at room temperature then permeabilized by incubation in 0.5 % Triton X-100 for 30 min at 37 °C. The embryos were incubated with TUNEL reagent (*In Situ* Cell Death Detection Kit, Roche-11684795910) for 1 hour at 37 °C, then washed three times with PBS/PVA. Samples used as positive control were treated with DNase (Promega, WI; USA) and the negative controls were incubated only with working solution. After mounting into slide with Vectashield mounting medium containing DAPI, the embryos were visualized using confocal laser microscope.

3.3.9 Statistical Analysis

Data obtained from different experimental groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by a multiple pairwise comparison (Tukey test). Differences were considered significant at $P < 0.05$. All values represent the mean ± SEM from three independent experiments.

3.4 Results

3.4.1 Effect of lead exposure on *in vitro* development of bovine embryos

We examined the effects of Pb exposure on the cleavage and blastocyst developmental rates at days 7, 8 and 9 of bovine embryos. Post fertilization cleavage rate in presumptive zygotes and blastocyst development (day 7 and day 8) in all Pb-exposed groups were significantly lower than ($P<0.05$) the control group (Figure 3.2).

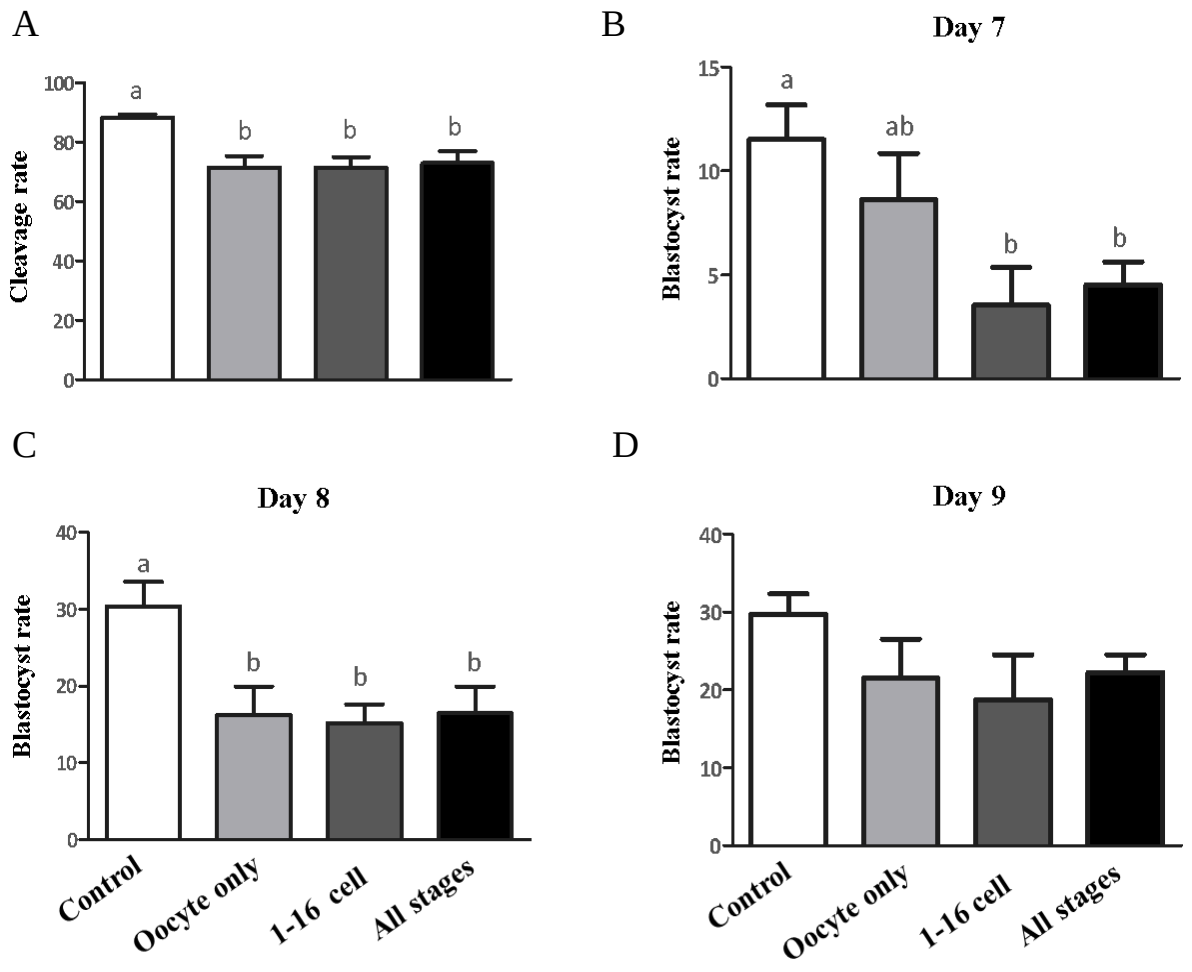


Figure 3.2: Effect of lead on developmental rates of *in vitro* cultured bovine embryos at different time points: Percentage of cleaved embryos (A), blastocyst rate at day 7 (B), blastocyst rate at day 8 (C), and blastocyst rate at day 9 (D). Values are expressed as mean ± SEM. a, b indicate statistically significant differences ($P < 0.05$).

3.4.2 Effect of lead exposure on reactive oxygen species (ROS) accumulation and blastocyst total cell number in bovine embryos

As shown in Figure 3.3A, Pb induced ROS accumulation in the treated group in addition to significant decline in the blastocyst cell number (Figure 3.3B).

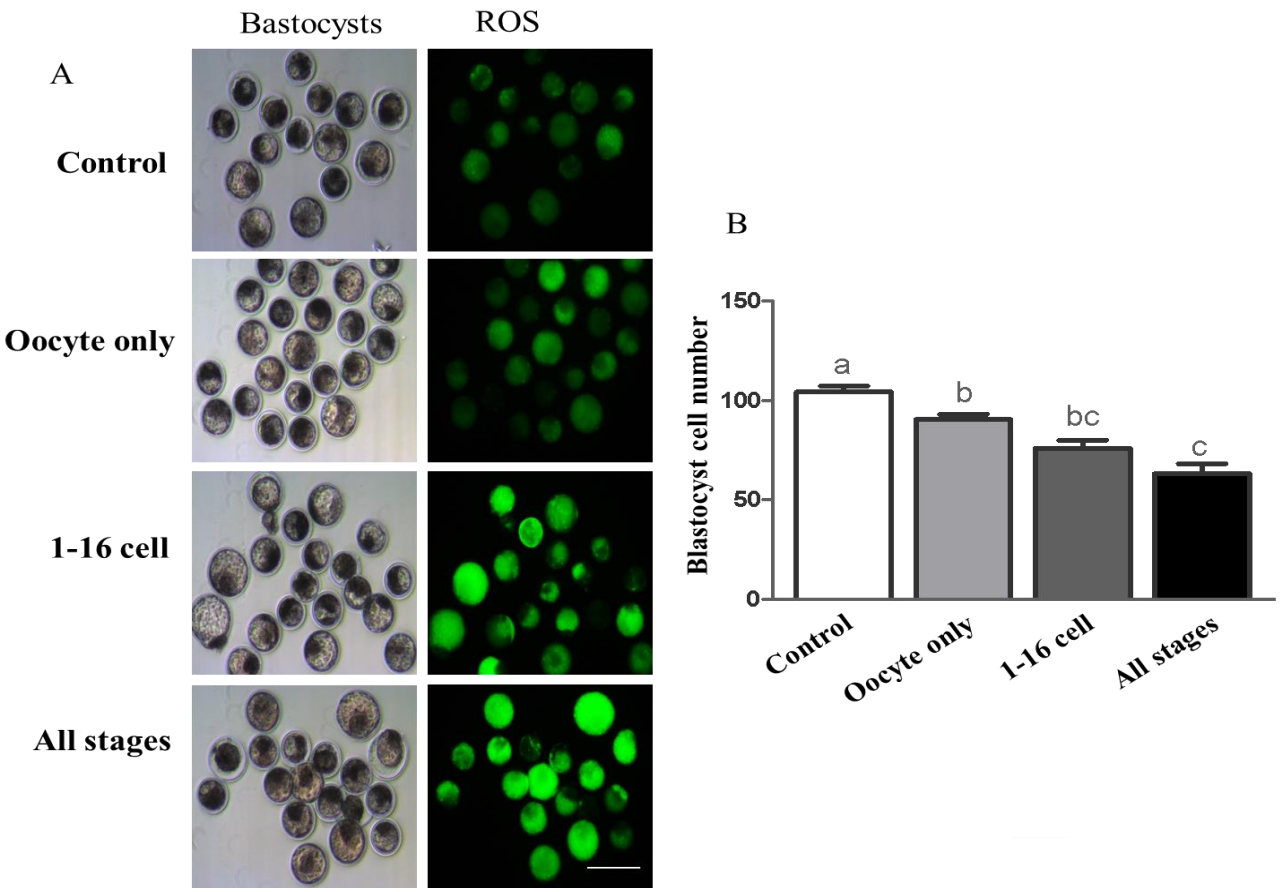


Figure 3.3: Lead induced intracellular reactive oxygen species (ROS) accumulation and decrease in blastocyst cell number: Fluorescent photomicrographs of bovine blastocysts *in vitro* cultured with or without Pb at different time points during early embryo development, stained with 2′, 7′-dichlorofluorescin diacetate (H_2DCFDA) (A). Blastocyst total cell number (B). Scale bar: 50 µm. Values are expressed as mean ± SEM. a, b & c indicate statistically significant differences ($P < 0.05$).

3.4.3 Effect of lead exposure on Nrf2 and NF-κB pathways in bovine embryos

As shown in Figure 3.4, there was no significant difference in the gene expression level of Nrf2 in all Pb-exposed groups with significant downregulation of its inhibitor Keap-1 ($P<0.05$). However, we observed significant downregulation of CAT without significant change in SOD and HO-1 levels, as downstream genes for Nrf2. The NF-κB gene expression level showed significant increase in Pb- exposed groups in addition to upregulation of TNF-α (Figure 3.5).

The co-localization of Nrf2 and NF-κB was further assessed using immunofluorescence staining. The result showed significant reduction ($P < 0.05$) in the level of Nrf2 in day-8 blastocysts

derived from all Pb-exposed groups compared to the non-treated control ones. Moreover, embryos groups of (16-cell) and (All stages) showed significant upregulation of NF-κB compared to control.

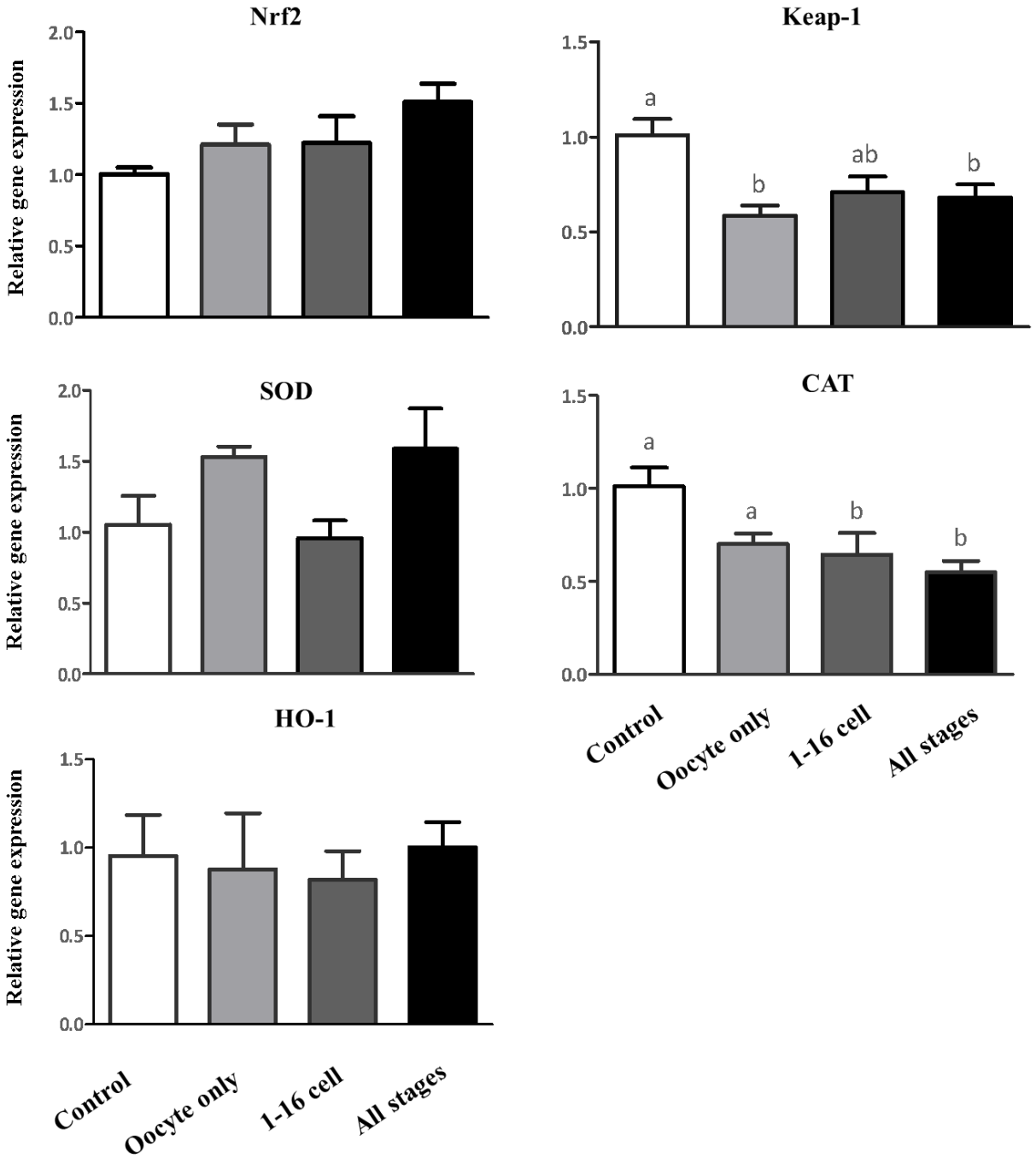


Figure 3.4: Expression levels of genes associated with the Nrf2 pathway: mRNA expression level of Nrf2, its inhibitor Keap-1, and its downstream candidate genes: SOD, CAT and HO-1 in

bovine blastocysts *in vitro* cultured with or without Pb at different time points during early embryo development. β-Actin and GAPDH were used to normalize the expression of targets genes. Values are expressed as mean ± SEM. Data are obtained from four replicates of independent groups of 15 blastocysts (day 8). a, b indicate statistically significant differences ($P < 0.05$).

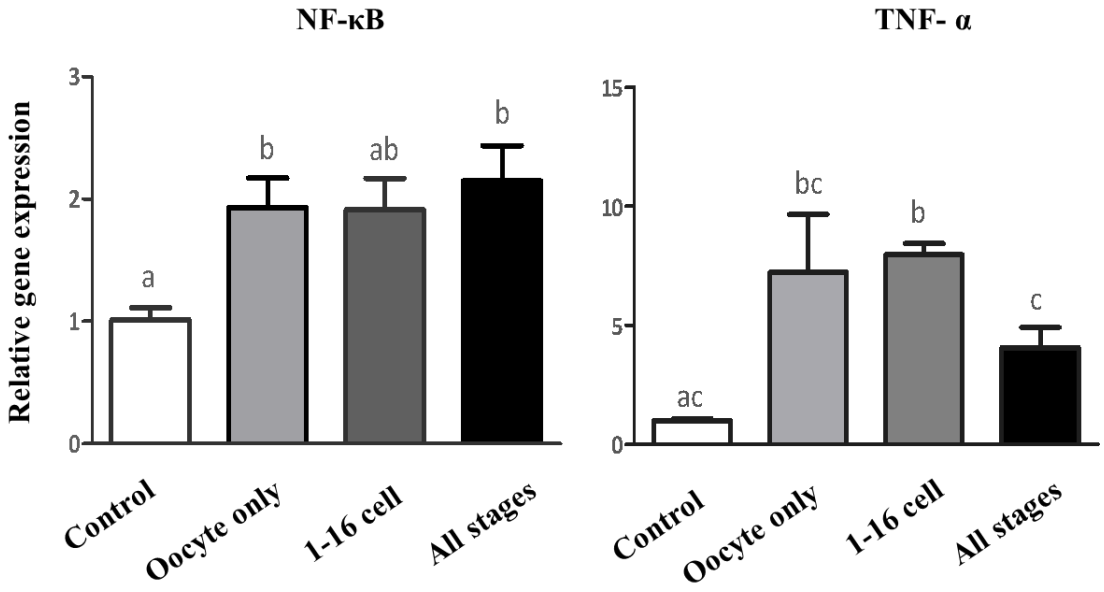


Figure 3.5: Expression levels of NF-κB and TNF-α mRNA in bovine blastocysts *in vitro* cultured with or without Pb at different time points during early embryo development. β-Actin and GAPDH were used to normalize the expression of targets genes. Values are expressed as mean ± SEM. Data are obtained from four replicates of independent groups of 15 blastocysts (day 8). a, b & c indicate statistically significant differences ($P < 0.05$).

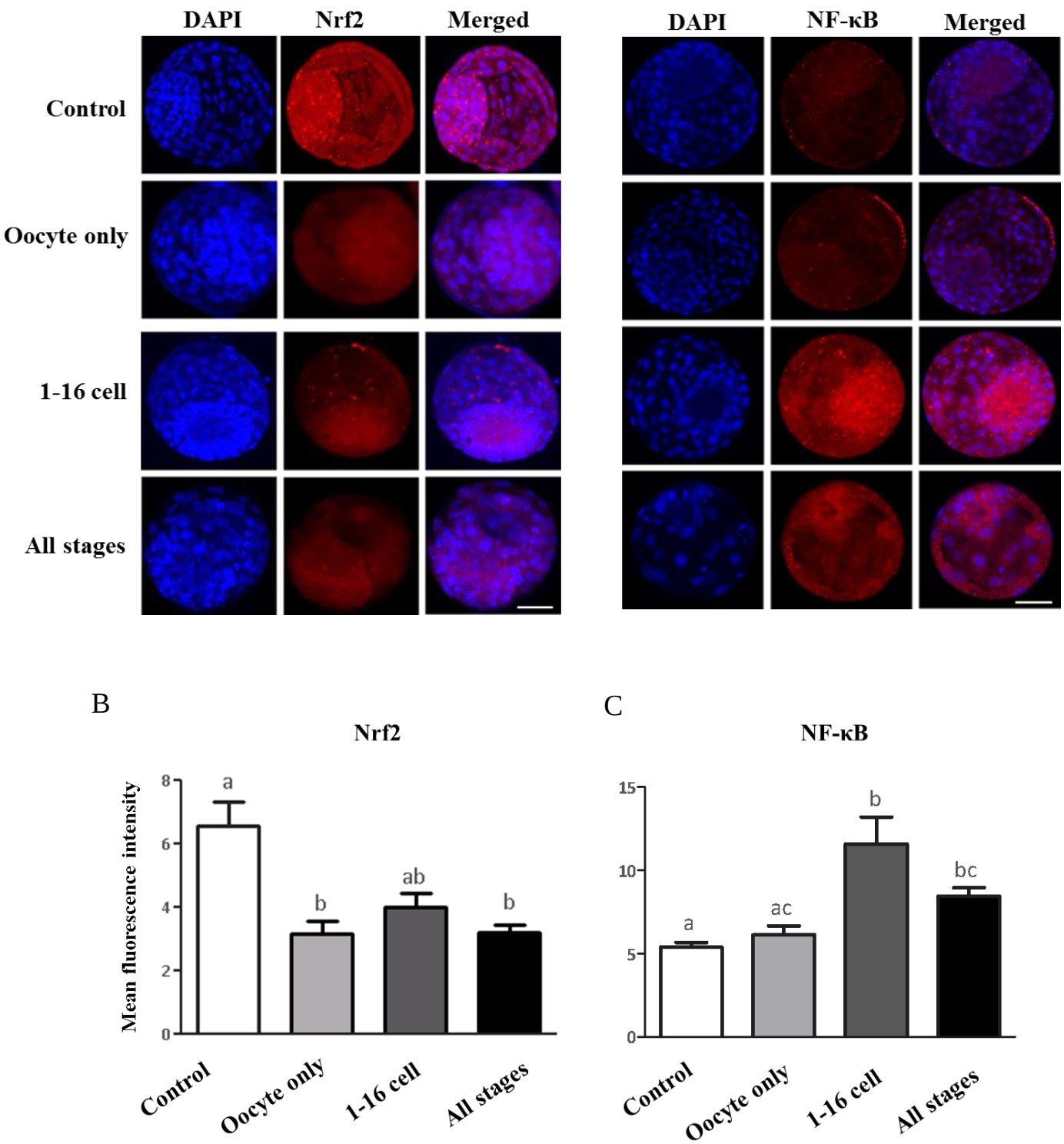


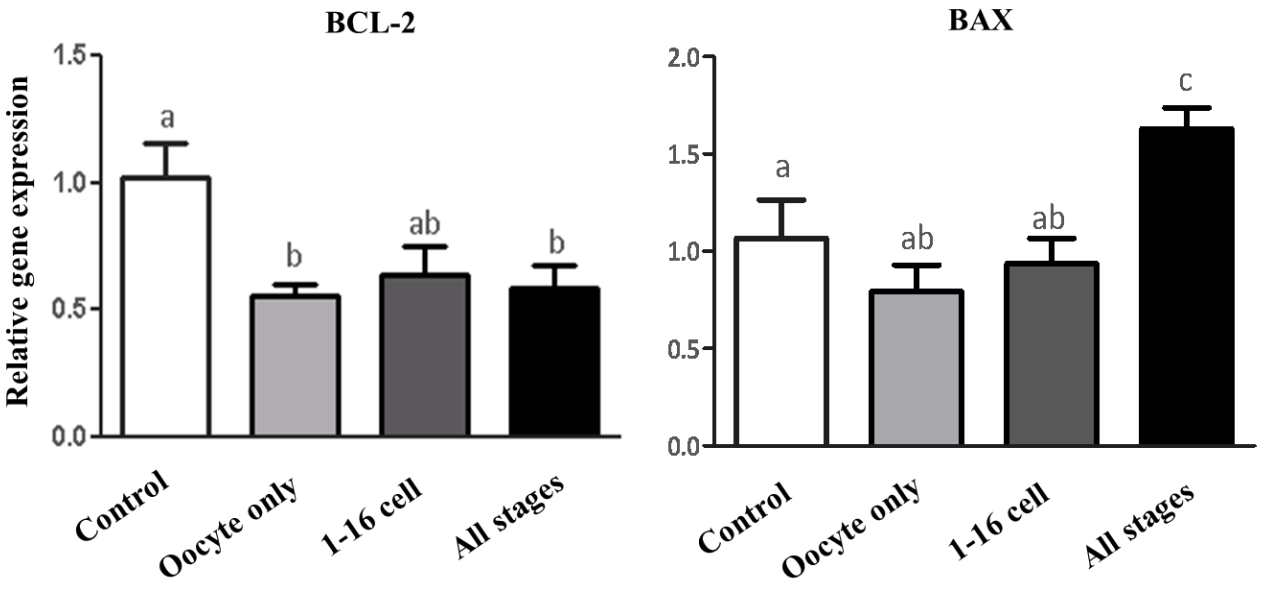
Figure 3.6: Effect of lead exposure on Nrf2 and NF-κB protein expression: Bovine blastocysts *in vitro* cultured with or without Pb at different time points during early embryo development. Red fluorescence signals reveal Nrf2 and NF-κB protein localization, while blue fluorescence

showed nuclear staining with Dapi (A). Scale bars: 50 µm. Mean values of fluorescence intensity of Nrf2 (B) and NF-κB (C) as analyzed by imageJ software. a, b & c indicate statistically significant differences ($P < 0.05$).

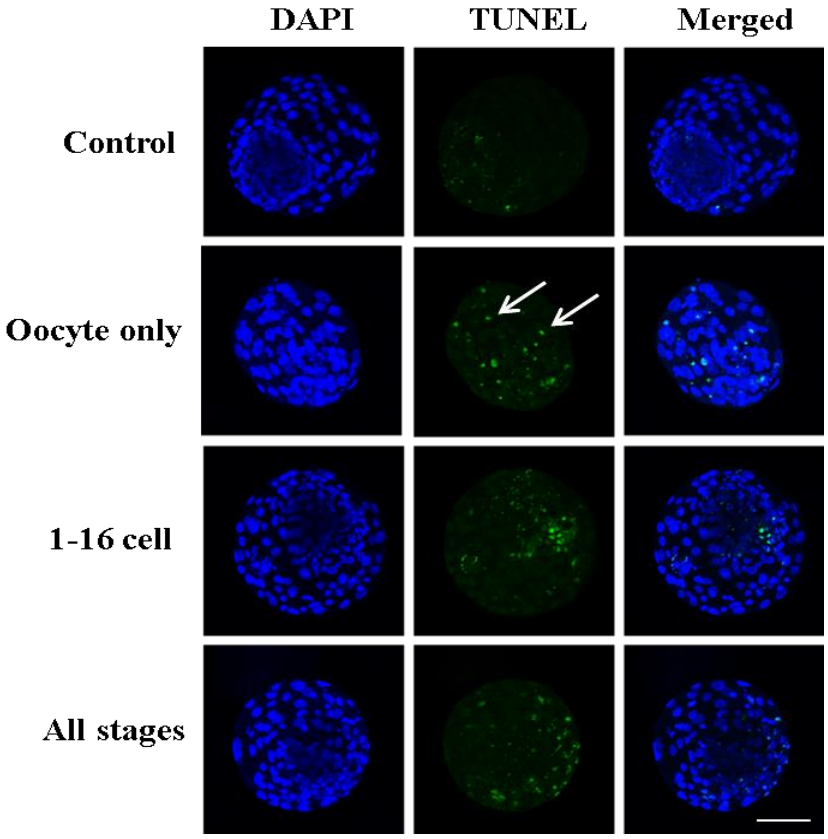
3.4.4 Lead exposure induced apoptosis in bovine embryos

The mRNA level of the anti-apoptoic gene BCL-2 showed significant decrease in (Oocyte only) and (All stages) groups compared to control ones (Figure 3.7A). However, gene expression level of BAX showed no significant variation between (Oocyte only) and (1-16 Cell) groups compared to control, while it was higher in (All stages) group ($P < 0.05$). Moreover, the number of positive apoptotic cells in individual blastocysts exposed to Pb was significantly higher as compared to control group (Figure 3.7B & 3.7C).

A



B



C

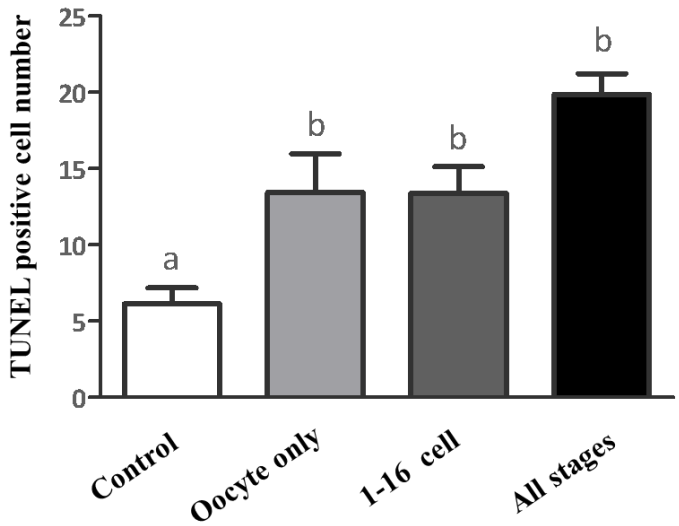


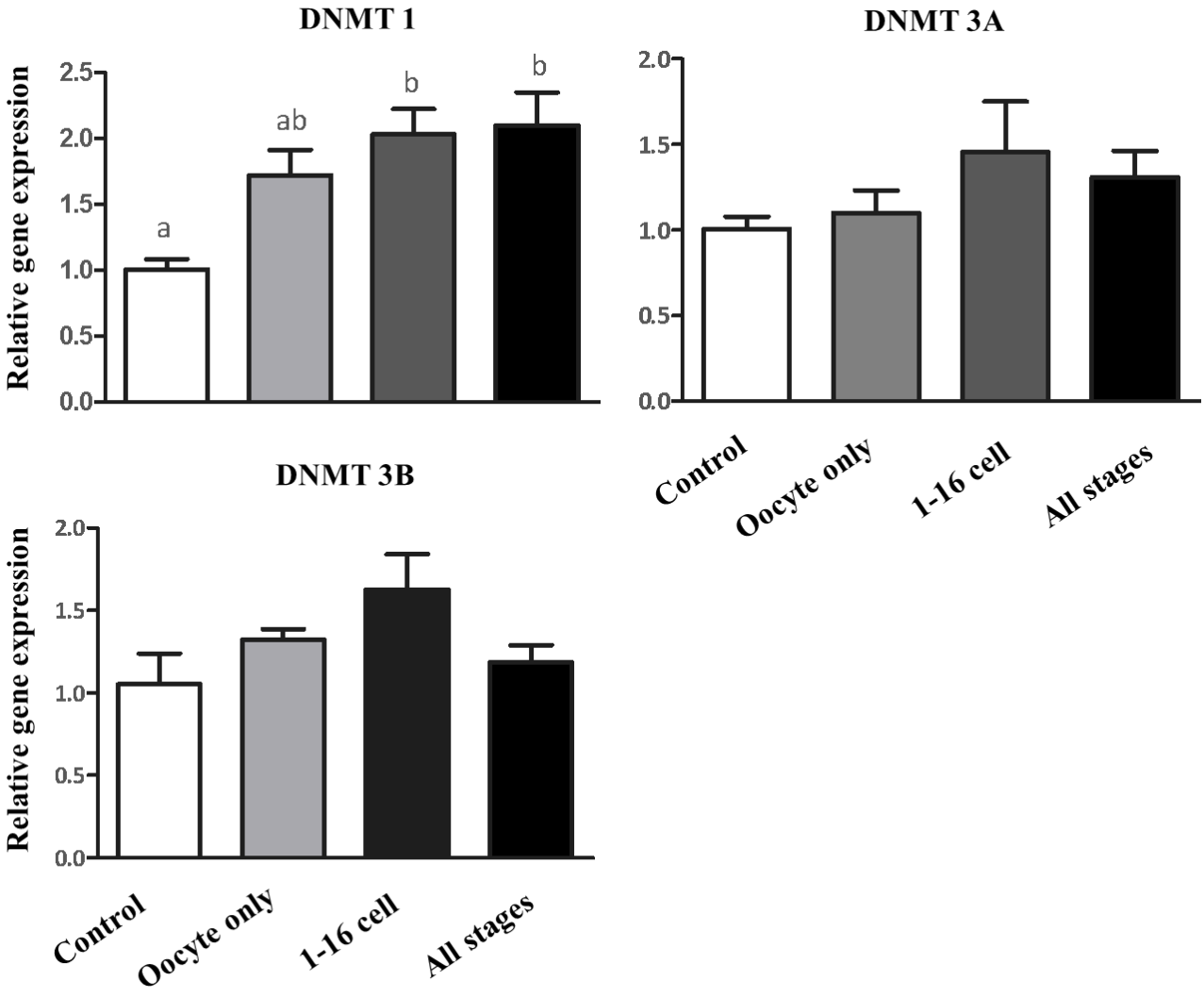
Figure 3.7: Effect of lead exposure on apoptosis: Relative gene expression level of BAX and BCL-2 (A). Representative image of TUNEL positive cells (arrows) (B). The average number of TUNEL positive cells (C) in bovine blastocyst *in vitro* cultured with or without Pb at different

time points during early embryo development. Scale bars: 50 µm. Values are expressed as mean ± SEM. Data are obtained from four replicates of independent groups of 15 blastocysts (day 8). a, b & c indicate statistically significant differences ($P < 0.05$).

3.4.5 Lead exposure induced alteration in DNA methylation

To investigate the probable impact of embryo exposure to Pb on DNA methylation, we analyzed the mRNA levels of DNA methyltransferase (DNMT1, DNMT1A and DNMT1B). Result showed that there was significant ($P < 0.05$) increase of DNMT1 in both (1-16 cell) and (All stages) groups compared with control group (Figure 3.8A). We further assessed the level of 5-methyl cytosine (5-mC), as shown in figure 3.8B, the relative intensity of 5-mC decreased compared with that in the control blastocysts.

A



B

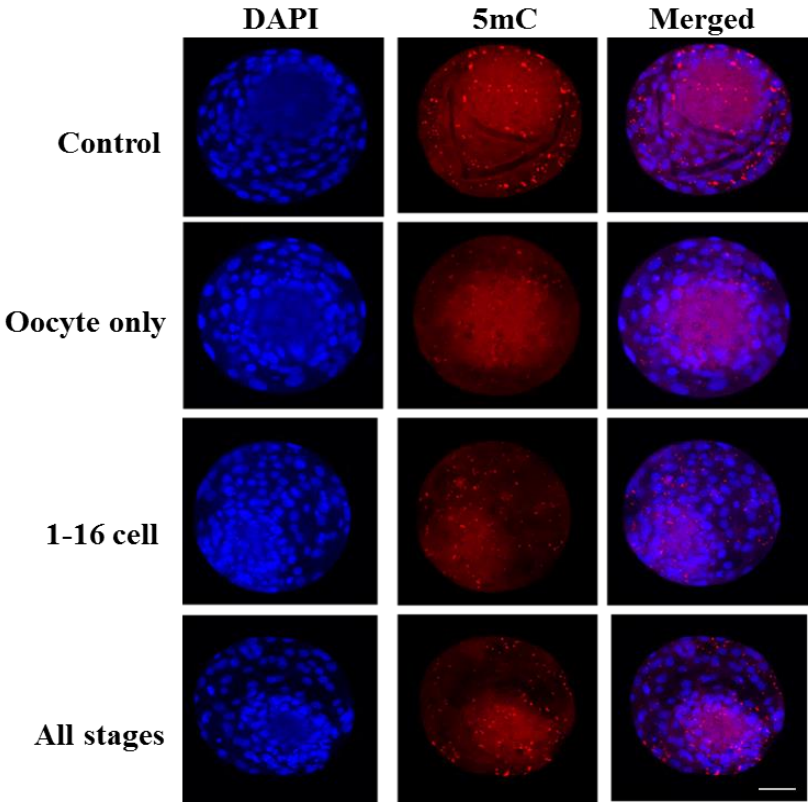


Figure 3.8: Lead exposure modifies DNA methylation: Relative mRNA expression of DNMTs in bovine blastocyst *in vitro* cultured with or without Pb at different time points during early embryo development (A) Immunostaining detection of 5mC (B). Values are expressed as mean ± SEM. Data are obtained from four replicates of independent groups of 15 blastocysts (day 8). a, b & c indicate statistically significant differences ($P < 0.05$).

3.5 Discussion

Lead (Pb) is one of the prevalent heavy metals that has high environmental concern due to its substantial impact on human and animals (Nandi *et al.* 2010). The exposure to environmental toxic metals might affect oocyte maturation and fertilization during *in vitro* fertilization (IVF) (Bloom *et al.* 2010). Results of the present study demonstrated a significant adverse effect of Pb on *in vitro* bovine oocyte maturation and embryo development. Because oocyte maturation determines the success of *in vitro* fertilization (IVF) and embryo development (Revelli *et al.* 2009), we propose that the reprotoxic effect of Pb results, in part, from its interference with oocyte maturation and its ability to support the subsequent steps of development until the embryonic genome activation (EGA). Our results were in agreement with earlier work of Avazeri *et al.* (2006) which showed that, Pb could interfere with oocyte meiotic arrest and meiotic resumption propapely by disrupting protein kinase (PKC) pathway. A report of Nandi *et al.* (2010) showed significant decrease in buffalo embryo cleavage rate and subsequent blastocyst development following the culture of oocytes in Pb-containing media. There could be more than one mechanism beyond the effect of Pb on oocyte maturation. According to Taupeau *et al.* (2003) and Nampoothiri *et al*. (2007), Pb being an endocrine disruptor can alter the steroidogenic activity of granulosa cells which impair its function to support oocyte maturation. In addition, Pd can compete and displace many nutritive metals, such as Ca, Zn, Fe, Cu and Mn, because of their ionic mimicry. This in turn affects various biological processes such as cellular signaling, cell adhesion, and apoptosis (Lidsky & Schneider 2003; Garza *et al.* 2006). Alternatively, Pb possess a strong electron sharing activity that allow the formation of covalent bonds with the sulfhydryl moiety (SH) of the cellular protein reserves such as antioxidant enzymes rendering them inactive (Jomova & Valko 2011).

In the present work, Pb exposure resulted in significant decrease in the blastocyst cell number along with reactive oxygen species (ROS) accumulation in all exposed groups compared to the control group. Cell number is one of the most critical indicators for development potential, since it could directly reflect an embryo's ability for cell cycle progression (Stylianou *et al.* 2012). Lower or higher embryo cell numbers are considered morphological anomalies associated with reduced developmental potential and poor implantation (Alikani *et al.* 2000; Kochhar *et al.* 2002). Moreover, exposure to ROS is always a challenge during *in vitro* embryo production and could be an underling cause for the poorly developed *in vitro* preimplantation embryos when

compared to their *in vivo* counterparts (Takahashi 2012). Lead has been shown to induce ROS in animals as well as in cell cultures (Stacchiotti *et al.* 2009; Abdel Moneim *et al.* 2011). Besides, oxidative stress could be a potential mechanism beyond the pathophysiology of Pb toxicity (Flora *et al.* 2012).

Oxidative stress triggers the activation of a variety of signaling pathways, resulting in crosstalk between many transcriptional factors (TFs). These signaling molecules proved to be significantly implicated in the etiology of reproductive diseases (Lu *et al.* 2018a). Herein we investigate the effect of Pb on Nrf2 and NF-κB pathways and the results revealed that Pb induced its deleterious effect by targeting Nrf2/Keap-1 signaling. Typically, ROS elevation induces the dissociation of Nrf2 from its inhibitor Keap-1 and allows Nrf2 nuclear translocation where it binds to the antioxidant responsive elements (ARE) and enhances the expression an array of downstream antioxidant genes like CAT, SOD and HO-1 (Kansanen *et al.* 2013). Although we demonstrated that Keap-1 expression level significantly decreased under Pb exposure, we expected upregulation of Nrf2. However, our results showed almost no change in mRNA of Nrf2 and further downregulation of its protein level. Similar data were obtained by Liu *et al.* (2017) who showed that Pb decreased the levels of both Nrf2 and Keap-1 in murine kidney. They attributed this to the ability of Pb to activate miR153 that target Nrf2 mRNA and suggested the probable effect of Pb as epigenetic modifier. The importance of Nrf2 in maintaining the ovarian homeostasis and function was demonstrated earlier by Hu *et al.* (2006). It acts as a chemical sensor that protects the cells from ovo-toxicants such as chemicals, carcinogens and pathogens (Dinkova-Kostova *et al.* 2002; Nguyen *et al.* 2004). Deletion of Nrf2 results in reduction of the number of ovarian follicles and accelerates ovarian ageing in mice (Lim *et al.* 2015). A recent study (Akino *et al.* 2018) showed that Nrf2 activation combat oxidative stress in human granulosa cells and this raises the possibility to therapeutically target Nrf2 to manage oxidative stress-related fertility disorders.

In the present work, upregulation of NF-κB has been found on both mRNA and protein levels in Pb-exposed groups. Similar results were obtained by Lu *et al.* (2018b). The nuclear factor kappa B, NF-κB is a pleiotropic transcription factor that regulates immunity and inflammatory responses, angiogenesis, apoptosis, cell cycle, and cell proliferation (Sethi *et al.* 2008; Hayden & Ghosh 2011). The role of NF-κB during embryonic development have been documented

(Espin-Palazon & Traver 2016). This was emphasized earlier by the embryo lethality of knockout mice with disruption of any member of the NF-κB pathway such as p65 (Beg *et al.* 1995), IκBa (Klement *et al.* 1996) or IKK (Li *et al.* 1999). It has been observed that aberrant NF-κB/IκB signaling are involved in both murine and bovine oocyte ageing (Patel *et al.* 2007; Tatone *et al.* 2008). NF-κB signaling is reported to be activated whenever reproductive stress exist which necessitates its targeting by novel therapeutic intervention (Giridharan & Srinivasan 2018). In the present study, upregulation of TNF-α in Pb-exposed groups was observed and this was in accordance with (Hossain *et al.* 2016). Studies reported the existence of the auto-regulatory loop between NF-κB and proinflammatory cytokines like TNF-α, where NF-κB activation leads to the induction of these cytokines, which in turn further induce NF-κB activation (Fan *et al.* 2015). Moreover, a functional interaction between Nrf2 and NF-κB, being mostly antagonistic, has been reported before (Wardyn *et al.* 2015). Kobayashi et al. (2016) has also shown that, Nrf2 has the ability to block inflammatory cytokines production, independent on its redox control activity. Consequently, in the present work we may suggest that NF-κB activated by Pb and subsequent ROS accumulation, induces inflammatory responses and can antagonize the activity of Nrf2 at their transcriotional levels (Sivandzade *et al.* 2018).

We assessed the expression of apoptosis related genes and we demonstrated that Pb triggered embryonic apoptosis which was manifested by downregulation of the pro-apoptotic gene BCL-2 along with upregulation of BAX. This has been confirmed by the high number of TUNEL positive cells in the blastocysts derived from all exposed groups. A direct link between Nrf2 and the anti-apoptotic proteins, BCL-2 and BCL-xl (and not the apoptotic protein BAX) expression was previously reported by Ariza *et al.* (2016), since Nrf2 binds to the ARE located in the promoter region of both BCL-2 and BCL-xl genes and control their expression. So attenuation of Nrf2 pathway triggers apoptotic pathway as observed in our study.

Several experimental and epidemiological studies have correlated gene- environment interactions and epigenetic regulations with the harmful and long-lasting effects of Pb (Zheng *et al.* 2011; Mitra *et al.* 2017). DNA methylation, the major epigenetic mechanism, is a reversible event essential for normal embryo development since it modifies the genome function, chromosomal stability, cell cycle and differentiation (Golding *et al.* 2011; Messerschmidt *et al.* 2014). Lead exposure was reported to alter the epigenome by inducing aberrant DNA methylation (Bihaqi *et*

al. 2011; Dosunmu *et al.* 2012; Eid & Zawia 2016). We found in the present work modification in the expression of DNMTs with significant elevation of DNMT1, gene involved in maintenance of DNA methylation. Overexpression of DNMT1 was reported to causes genomic hypermethylation, loss of imprinting, and embryonic lethality (Biniszkiewicz *et al.* 2002). Similarly, Schneider *et al.* (2013) reported that hippocampal DNMTs where affected by developmental Pb exposure in animal model. *In vitro* Pb exposure of human embryonic kidney cell line showed change in the differentially methylated region (DMR) regulating some imprinted genes such as IGF-2 (Nye *et al.* 2015).

In summary, we conclude that Pb displayed a harmful effect on bovine early embryonic development, regardless the stage of exposure, by inducing oxidative and inflammatory stress. This effect may be in part through disrupting the Nrf2/NF-κB interaction. The reprotoxic effect of Pb originates from its interference with oocyte maturation and its ability to support the subsequent steps of development until the embryonic genome activation. Further investigations are required to evaluate DNA methylation dynamics in bovine embryos under Pb exposure and if this mechanism contributes to Nrf2/NF-κB perturbation.

3.6 References

Abdel Moneim AE, Dkhil MA & Al-Quraishy S 2011 The protective effect of flaxseed oil on lead acetate-induced renal toxicity in rats. *Journal of Hazardous Materials* **194** 250–255.

Ahmed YF, Eldebaky HAA, Karima KGH & Nawito M 2012 Effects of lead exposure on DNA damage and apoptosis in reproductive and vital organs in female rabbits. *Global Veterinaria* **9** (4) 401–408.

Akino N, Wada-Hiraike O, Terao H, Honjoh H, Isono W, Fu H, Hirano M, Miyamoto Y, Tanikawa M, Harada M, Hirata T, Hirota Y, Koga K, Oda K, Kawana K, Fujii T &Osuga Y 2018 Activation of Nrf2 might reduce oxidative stress in human granulosa cells. *Molecular & Cellular Endocrinology* **470** 96–104.

Alikani M, Calderon G, Tomkin G, Garrisi J, Kokot M & Cohen J 2000 Cleavage anomalies in early human embryos and survival after prolonged culture in-vitro. *Human Reproduction***15** (12) 2634–2643.

Al-Saleh I, Coskun S, Mashhour A, Shinwari N, El-Doush I, Billedo G, Jaroudi K, Al-Shahrani A, Al-Kabra M & El Din Mohamed G 2008 Exposure to heavy metals (lead,

cadmium and mercury) and its effect on the outcome of in-vitro fertilization treatment. *International Journal of Hygiene & Environmental Health* **211** (5-6) 560–579.

Ariza J, Gonzalez-Reyes JA, Jodar L, Diaz-Ruiz A, Cabo R de & **Villalba JM** 2016 Mitochondrial permeabilization without caspase activation mediates the increase of basal apoptosis in cells lacking Nrf2. *Free Radical Biology & Medicine* **95** 82–95.

Avazeri N, Denys A & Lefevre B 2006 Lead cations affect the control of both meiosis arrest and meiosis resumption of the mouse oocyte in vitro at least via the PKC pathway. *Biochimie* **88** (11) 1823–1829.

Bayat F, Akbari SAA, Dabirioskoei A, Nasiri M & Mellati A 2016 The relationship between blood lead level and preeclampsia. *Electronic Physician* **8** (12) 3450–3455.

Beg AA, Sha WC, Bronson RT, Ghosh S & Baltimore D 1995 Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* **376** (6536) 167–170.

Bihaqi SW, Huang H, Wu J & Zawia NH 2011 Infant exposure to lead (Pb) and epigenetic modifications in the aging primate brain: Implications for Alzheimer's disease. *Journal of Alzheimer's Disease* **27** (4) 819–833.

Biniszkiewicz D, Gribnau J, Ramsahoye B, Gaudet F, Eggan K, Humpherys D, Mastrangelo M-A, Jun Z, Walter J & Jaenisch R 2002 Dnmt1 overexpression causes genomic hypermethylation, loss of imprinting, and embryonic lethality. *Molecular & Cellular Biology* **22** (7) 2124–2135.

Bires J, Maracek I, Bartko P, Biresova M & Weissova T 1995 Accumulation of trace elements in sheep and the effects upon qualitative and quantitative ovarian changes. *Veterinary & Human Toxicology* **37** (4) 349–356.

Bloom MS, Kim K, Kruger PC, Parsons PJ, Arnason JG, Steuerwald AJ & Fujimoto VY 2012 Associations between toxic metals in follicular fluid and in vitro fertilization (IVF) outcomes. *Journal of Assisted Reproduction & Genetics* **29** (12) 1369–1379.

Bloom MS, Louis GMB, Sundaram R, Kostyniak PJ & Jain J 2011a Associations between blood metals and fecundity among women residing in New York State. *Reproductive Toxicology* **31** (2) 158–163.

Bloom MS, Parsons PJ, Kim D, Steuerwald AJ, Vaccari S, Cheng G & Fujimoto VY 2011b Toxic trace metals and embryo quality indicators during in vitro fertilization (IVF). *Reproductive Toxicology* **31** (2) 164–170.

Bloom MS, Parsons PJ, Steuerwald AJ, Schisterman EF, Browne RW, Kim K, Coccaro GA, Conti GC, Narayan N & Fujimoto VY 2010 Toxic trace metals and human oocytes during in vitro fertilization (IVF). *Reproductive Toxicology* **29** (3) 298–305.

Choi SM, Yoo SD & Lee BM 2004 Toxicological characteristics of endocrine-disrupting chemicals: Developmental toxicity, carcinogenicity, and mutagenicity. *Journal of Toxicology & Environmental Health* **7** (1) 1–23.

Cole DC, Wainman B, Sanin LH, Weber J-P, Muggah H & Ibrahim S 2006 Environmental contaminant levels and fecundability among non-smoking couples. *Reproductive Toxicology* **22** (1) 13–19.

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M & Talalay P 2002 Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences* **99** (18) 11908.

Dosunmu R, Alashwal H & Zawia NH 2012 Genome-wide expression and methylation profiling in the aged rodent brain due to early-life Pb exposure and its relevance to aging. *Mechanisms of Ageing & Development* **133** (6) 435–443.

Eid A & Zawia N 2016 Consequences of lead exposure, and it's emerging role as an epigenetic modifier in the aging brain. *Neurotoxicology* **56** 254–261.

Espin-Palazon R & Traver D 2016 The NF-kappa B family: Key players during embryonic development and HSC emergence. *Experimental Hematology* **44** (7) 519–52.

Fan B, Dun S-H, Gu J-Q, Guo Y & Ikuyama S 2015 Pycnogenol attenuates the release of proinflammatory cytokines and expression of perilipin 2 in lipopolysaccharide-stimulated microglia in part via inhibition of NF-kappa B and AP-1 activation. *PLoS One* **10** (9) e0137837.

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

Garza A, Vega R & Soto E 2006 Cellular mechanisms of lead neurotoxicity. *Medical Science Monitor* **12** (3) RA57-65.

Giridharan S & Srinivasan M 2018 Mechanisms of NF-κB p65 and strategies for therapeutic manipulation. *Journal of Inflammation Research* **11** 407–419.

Golding MC, Williamson GL, Stroud TK, Westhusin ME & Long CR 2011 Examination of DNA methyltransferase expression in cloned embryos reveals an essential role for Dnmt1 in bovine development. *Molecular Reproduction & Development* **78** (5) 306–317.

Guo J, Zhao MH, Shin KT, Niu YJ, Ahn YD, Kim NH & Cui XS 2017 The possible molecular mechanisms of bisphenol A action on porcine early embryonic development. *Scientific Reports* **7** (1) 8632.

Hayden MS & Ghosh S 2011 NF-kappa B in immunobiology. *Cell Research* **21** (2) 223–244.

Hossain S, Bhowmick S, Jahan S, Rozario L, Sarkar M, Islam S, Basunia MA, Rahman A, Choudhury BK & Shahjalal H 2016 Maternal lead exposure decreases the levels of brain development and cognition-related proteins with concomitant upsurges of oxidative stress, inflammatory response and apoptosis in the offspring rats. *Neurotoxicology* **56** 150–158.

Hu X, Roberts JR, Apopa PL, Kan YW & Ma Q 2006 Accelerated ovarian failure induced by 4-vinyl cyclohexene diepoxide in Nrf2 null mice. *Molecular & Cellular Biology* **26** (3) 940–954.

Jomova K & Valko M 2011 Advances in metal-induced oxidative stress and human disease. *Toxicology* **283** (2-3) 65–87.

Kansanen E, Kuosmanen SM, Leinonen H & Levonen A-L 2013 The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biology* **1** 45–49.

Karimooy HN, Mood MB, Hosseini M & Shadmanfar S 2010 Effects of occupational lead exposure on renal and nervous system of workers of traditional tile factories in Mashhad (northeast of Iran). *Toxicology & Industrial health* **26** (9) 633–638.

Klement JF, Rice NR, Car BD, Abbondanzo SJ, Powers GD, Bhatt PH, Chen CH, Rosen CA & Stewart CL 1996 Ikappa Balpha deficiency results in a sustained NF-kappa B response and severe widespread dermatitis in mice. *Molecular & Cellular Biology* **16** (5) 2341–2349.

Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, Tanaka N, Moriguchi T, Motohashi H, Nakayama K & Yamamoto M 2016 Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription.Nature Communication **7** 11624.

Kochhar HPS, Rao KBCA, Luciano AM, Totey SM, Gandolfi F, Basrur PK & King WA 2002 In vitro production of cattle-water buffalo (Bos taurus-Bubalus bubalis) hybrid embryos. *Zygote* **10** (2) 155–162.

Korashy HM & El-Kadi AOS 2006 The role of aryl hydrocarbon receptor and the reactive oxygen species in the modulation of glutathione transferase by heavy metals in murine hepatoma cell lines. *Chemico-Biological Interactions* **162** (3) 237–248.

Li Q, van Antwerp D, Mercurio F, Lee KF & Verma IM 1999 Severe liver degeneration in mice lacking the Ikappa B kinase 2 gene. *Science* **284** (5412) 321–325.

Li W, Khor TO, Xu C, Shen G, Jeong W-S, Yu S & Kong A-N 2008 Activation of Nrf2-antioxidant signaling attenuates NFkappa B- inflammatory response and elicits apoptosis. *Biochemical Pharmacology* **76** (11) 1485–1489.

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

Lim J, Ortiz L, Nakamura BN, Hoang YD, Banuelos J, Flores VN, Chan JY & Luderer U 2015 Effects of deletion of the transcription factor Nrf2 and benzo apyrene treatment on ovarian follicles and ovarian surface epithelial cells in mice. *Reproductive Toxicology* **58** 24–32.

Lingappan K 2018 NF-κB in Oxidative Stress. *Current Opinion in Toxicology* **7** 81–86.

Liu B, Zhang H, Tan X, Yang D, Lv Z, Jiang H, Lu J, Baiyun R & Zhang Z 2017 GSPE reduces lead-induced oxidative stress by activating the Nrf2 pathway and suppressing miR153 and GSK-3beta in rat kidney. *Oncotarget* **8** (26) 42226–42237.

Liu G-H, Qu J & Shen X 2008 NF-kappa B/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochimica et Biophysica Acta* **1783** (5) 713–727.

Lu J, Wang Z, Cao J, Chen Y & Dong Y 2018a A novel and compact review on the role of oxidative stress in female reproduction. *Reproductive Biology & Endocrinology* **16** (1) 80.

Lu J, Jiang H, Liu B, Baiyun R, Li S, Lv Y, Li D, Qiao S, Tan X & Zhang Z 2018b Grape seed procyanidin extract protects against Pb-induced lung toxicity by activating the AMPK/Nrf2/p62 signaling axis. *Food & Chemical Toxicology* **116** (Pt B) 59–69.

Messerschmidt DM, Knowles BB & Solter D 2014 DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes & Development* **28** (8) 812–828.

Mitra P, Sharma S, Purohit P & Sharma P 2017 Clinical and molecular aspects of lead toxicity: An update. *Critical Reviews in Clinical Laboratory Sciences* **54** (7-8) 506–528.

Mouzon J, **Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, Kupka M, Nygren KG & Nyboe Andersen A** 2010 Assisted reproductive technology in Europe, 2006: Results generated from European registers by ESHRE. *Human Reproduction* **25** (8) 1851–1862.

Nampoothiri LP, Agarwal A & Gupta S 2007 Effect of co-exposure to lead and cadmium on antioxidant status in rat ovarian granulose cells. *Archives of Toxicology* **81** (3) 145–150.

Nandi S, Gupta PSP, Selvaraju S, Roy SC & Ravindra JP 2010 Effects of exposure to heavy metals on viability, maturation, fertilization, and embryonic development of buffalo (Bubalus bubalis) oocytes in vitro. *Archives of Environmental Contamination & Toxicology* **58** (1) 194–204.

Nguyen T, Yang CS & Pickett CB 2004 The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radical Biology & Medicine* **37** (4) 433–441.

Norhazlin J, Nor-Ashikin MNK, Hoh BP, Sheikh Abdul Kadir SH, Norita S, Mohd-Fazirul M, Wan-Hafizah WJ, Razif D, Rajikin MH & Abdullah B 2015 Effect of DNase treatment on RNA extraction from preimplantation murine embryos. *Genetics & Molecular Research* **14** (3) 10172–10184.

Nye MD, Hoyo C & Murphy SK 2015 In vitro lead exposure changes DNA methylation and expression of IGF2 and PEG1/MEST. *Toxicology in Vitro* **29** (3) 544–550.

Paksy K, Gati I, Naray M & Rajczy K 2001 Lead accumulation in human ovarian follicular fluid, and in vitro effect of lead on progesterone production by cultured human ovarian granulosa cells. *Journal of Toxicology & Environmental Health* **62** (5) 359–366.

Pant N, Upadhyay G, Pandey S, Mathur N, Saxena DK & Srivastava SP 2003 Lead and cadmium concentration in the seminal plasma of men in the general population: Correlation with sperm quality. *Reproductive Toxicology* **17** (4) 447–450.

Patel OV, Bettegowda A, Ireland JJ, Coussens PM, Lonergan P & Smith GW 2007 Functional genomics studies of oocyte competence: Evidence that reduced transcript abundance for follistatin is associated with poor developmental competence of bovine oocytes. *Reproduction* **133** (1) 95–106.

Patrick L 2006 Lead toxicity part II: The role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. *Alternative Medicine Review* **11** (2) 114–127.

Pedruzzi LM, Stockler-Pinto MB, Leite M, JR & Mafra D 2012 Nrf2-keap1 system versus NF-kappaB: The good and the evil in chronic kidney disease? *Biochimie* **94** (12) 2461–2466.

Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M & Rinaudo P 2009 Follicular fluid content and oocyte quality: From single biochemical markers to metabolomics. *Reproductive Biology & Endocrinology* **7** 40.

Sallmen M, Anttila A, Lindbohm ML, Kyyronen P, Taskinen H & Hemminki K 1995 Time to pregnancy among women occupationally exposed to lead. *Journal of Occupational & Environmental Medicine* **37** (8) 931–934.

Santos RR, Schoevers EJ & Roelen BAJ 2014 Usefulness of bovine and porcine IVM/IVF models for reproductive toxicology. *Reproductive Biology & Endocrinology* **12** 117.

Schneider JS, Kidd SK & Anderson DW 2013 Influence of developmental lead exposure on expression of DNA methyltransferases and methyl cytosine-binding proteins in hippocampus. *Toxicology Letters* **217** (1) 75–81.

Sethi G, Sung B & Aggarwal BB 2008 Nuclear factor-kappa B activation: From bench to bedside. *Experimental Biology & Medicine* **233** (1) 21–31.

Seyom E, Abera M, Tesfaye M & Fentahun N 2015 Maternal and fetal outcome of pregnancy related hypertension in Mettu Karl Referral Hospital, Ethiopia. *Journal of Ovarian Research* **8** 10.

Silberstein T, Saphier O, Paz-Tal O, Trimarchi JR, Gonzalez L & Keefe DL 2006 Lead concentrates in ovarian follicle compromises pregnancy. *Journal of Trace Elements in Medicine & Biology* **20** (3) 205–207.

Sivandzade F, Prasad S, Bhalerao A & Cucullo L 2018 Nrf2 and NF-κB interplay in cerebrovascular and neurodegenerative disorders: Molecular mechanisms and possible therapeutic approaches. *Redox Biology* **21** 101059.

Stacchiotti A, Morandini F, Bettoni F, Schena I, Lavazza A, Grigolato PG, Apostoli P, Rezzani R & Aleo MF 2009 Stress proteins and oxidative damage in a renal derived cell line exposed to inorganic mercury and lead. *Toxicology* **264** (3) 215–224.

Stylianou C, Critchlow D, Brison DR & Roberts SA 2012 Embryo morphology as a predictor of IVF success: An evaluation of the proposed UK ACE grading scheme for cleavage stage embryos. *Human Fertility* **15** (1) 11–17.

Swarup D, Patra RC, Naresh R, Kumar P & Shekhar P 2005 Blood lead levels in lactating cows reared around polluted localities; transfer of lead into milk. *The Science of the Total Environment* **347** (1-3) 106–110.

Takahashi M 2012 Oxidative stress and redox regulation on in vitro development of mammalian embryos. *The Journal of Reproduction & Development* **58** (1) 1–9.

Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P & Focarelli R 2008 Cellular and molecular aspects of ovarian follicle ageing. *Human Reproduction Update* **14** (2) 131–142.

Taupeau C, Poupon J, Nome F & Lefevre B 2001 Lead accumulation in the mouse ovary after treatment-induced follicular atresia. *Reproductive Toxicology* **15** (4) 385–391.

Taupeau C, Poupon J, Treton D, Brosse A, Richard Y & Machelon V 2003 Lead reduces messenger RNA and protein levels of cytochrome p450 aromatase and estrogen receptor beta in human ovarian granulosa cells. *Biology of Reproduction* **68** (6) 1982–1988.

van Beker Woudenberg A, Grollers-Mulderij M, Snel C, Jeurissen N, Stierum R & Wolterbeek A 2012 The bovine oocyte in vitro maturation model: A potential tool for reproductive toxicology screening. *Reproductive Toxicology* **34** (2) 251–260.

Wardyn JD, Ponsford AH & Sanderson CM 2015 Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochemical Society Transactions* **43** (4) 621–626.

Yu M, Li H, Liu Q, Liu F, Tang L, Li C, Yuan Y, Zhan Y, Xu W, Li W Yuan Y, Zhan Y, Xu W, Li W, Chen H, Ge C, Wang J & Yang X 2011 Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. *Cellular Signalling* **23** (5) 883–892.

Zheng G, Tian L, Liang Y, Broberg K, Lei L, Guo W, Nilsson J, Bergdahl IA, Skerfving S & Jin T 2011 delta-Aminolevulinic acid dehydratase genotype predicts toxic effects of lead on workers' peripheral nervous system. *Neurotoxicology* **32** (4) 374–382.

Chapter 4

Developmental Toxicity of Lead in Rats after Gestational Exposure and Protective Role of Taurine

Hoda Samir Aglan[1,4*], Marwa Mohamed Safar[5], Afaf Abdel-Moniem Ain-Shoka[5], Asmaa Munir Kandil[4], Samuel Gebremedhn[1], Karl Schellander[1,3], Dawit Tesfaye[1,3]

[1]Institute of Animal Science, Department of Animal Breeding and Husbandry, University of Bonn, Bonn, Germany

[2]Teaching and Research Station Frankenforst, Faculty of Agriculture, University of Bonn, Königswinter, Germany

[3]Center of Integrated Dairy Research, University of Bonn, Bonn, Germany

[4]Department of Pharmacology, National Organization for Drug Control and Research, Giza, Egypt

[5]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Cairo, Egypt

*Corresponding author

(Manuscript submitted to: Journal of Biochemical and Molecular Toxicology)

4.1 Abstract

Exposure to lead (Pb) during *in utero* period has been proven to be detrimental to human and animal offspring. So the aim of the current study is to evaluate the role of taurine (TA), a natural antioxidant with various cytoprotective activities, against Pb-induced toxicity in pregnant albino rats and their fetuses. Pregnant rats were divided into four groups (10 rats of each) where group 1 was given distilled water and served as control. Group 2 was gavaged lead acetate (250 mg/L) from day 7 to 16 of gestation (the period of organogenesis). Groups 3 and 4 received taurine (50 mg/kg) orally throughout the gestation period, while group 4 received also lead acetate from day 7 to 16 of gestation. Dams were sacrificed on the 20th day of gestation and fetuses were removed by cesarean section, where implantation sites, fetal mortality, morphological examination, body weight and length were recorded. Blood samples were collected from both dams and fetuses for hematological and biochemical parameters assessment. Hepatic malondialdehyde, reduced glutathione (GSH) and catalase (CAT) were also analyzed. Results showed that Pb caused a reduction in the maternal body weight gain and an increase in the rate of abortion, accompanied with fetal growth retardation and skeletal malformations. Moreover, Pb induced severe hematological and biochemical alterations in both dams and fetuses. The toxicity of Pb was further emphasized by histopathological examination of the placenta and hepatic DNA fragmentation. However, TA pretreatment revealed potential prophylaxis against Pb toxicity.

Keywords: lead toxicity, taurine, *in utero*, rat, fetus, skeleton, DNA fragmentation.

4.2 Introduction

Lead (Pb) is a prevalent environmental heavy metal that has a great impact on human health and it is mainly conveyed to humans through dietary or occupational sources (Garza *et al.* 2006; Flora *et al.* 2006; Payne 2008). Due to its unique properties like malleability, ductility, softness, low melting point and resistance to corrosion, it is nearly available in all aspects of human life and results in contamination of the air, water and soils. Besides, the non-biodegradable nature of Pb, its compounds are abundantly present in the environment (Florea & Busselberg 2006; Ahamed & Siddiqui 2007). After absorption, Pb is transported by blood to liver, kidney, brain, hemopoietic system and finally stored in bone and teeth throughout life (Monir *et al.* 2010; Renner 2010). Regardless of concentration, evidences are accumulated showing that, Pb can cause wider array of behavioral, hematological, gastrointestinal, cardiovascular, immunological

and reproductive disorders (Shannon 2003; Ahamed *et al.* 2005; Navas-Acien *et al.* 2007; Sanders *et al.* 2009; Jangid *et al.* 2012). Moreover, the *in utero* period is considered the most critical period affected by Pb toxicity consequences and remains a matter of concern. According to Gulson *et al.* (2003) and Vaziri (2008), Pb is released from bone due to the elevated bone turnover during pregnancy and crosses the placenta by diffusion into the developing embryo, which is endowed with low defense mechanism. Consequently, the fetus is vulnerable to Pb-induced stress, especially in early organogenesis. Al-Saleh *et al.* (2011) reported also that, Pb was detected in the placenta, umbilical cord and maternal blood, reflecting its tranasplacental transfer. Studies have elucidated that exposure to Pb are linked with gestational hypertension, miscarriage, stillbirth, premature membrane rupture and preterm delivery (Shannon 2003; Troesken 2008; Saleh *et al.* 2009; Edwards 2014). It is important to mention that, the period and dose of Pb exposure play a role in fetal growth (Cantonwine *et al.* 2010). In addition, placental blood Pb levels at or even below 10 μg/dL were associated with adverse pregnancy outcomes (Mennick 2006; Zhu *et al.* 2010). Furthermore, reports claim that there is no safe level of Pb exposure and it is quite evident that even small doses have adverse consequences on both humans and animals, where the soundest approach is to minimize Pb exposure rather than treatment (Guidotti & Ragain 2007; Iavicoli & Calabrese 2011).

Taurine, 2-aminoethanesulfonic acid (TA), an antioxidant that is distributed in almost all animal tissues especially brain, heart, retina, leukocytes and muscles and it is essential for the normal functioning of different organs (Wojcik *et al.* 2010). The main source of TA in the body is either biosynthesis from methionine and serine or dietary intake of meat and seafood (Schuller-Levis & Park 2006). Various biological functions of TA comprise regulation of cell volume, calcium (Ca) mobilization and cholesterol excretion. In addition to its role as neurotransmitter, potent antioxidant and antiapoptotic agent where its function starts at conception and continue throughout life (Verner *et al.* 2007; Nishimura *et al.* 2010; Roysommuti & Wyss 2014; Roy & Sil 2012). Taurine administration has been shown to be pharmacologically effective against many disorders in human and animal studies (Rahman *et al.* 2012; Ito *et al.* 2012; Han & Chesney 2012; Menzie *et al.* 2014; Miyazaki & Matsuzaki 2014). However, cellular depletion of TA is accompanied by many pathologies as cardiomyopathy (Zulli 2011) renal dysfunction (Yamori *et al.* 2010), degeneration of retinal neurons (Froger *et al.* 2013), cellular senescence and aging (Ito *et al.* 2014). Rather, the beneficial role of TA in development was documented in

the literature, where it protects against toxemia during pregnancy in rats (El-Agousa *et al.* 2009). During pregnancy and lactation, taurine insufficiency causes growth retardation, malformation, blindness, myocardial damage and disorders of the central nervous system (Franconi *et al.* 2006). Limited data are available on the protective effect of TA against Pb toxicity on female reproduction. Thus, the current work was carried out to investigate the protective effect of TA against lead insult consequences on pregnant albino rats and their fetuses.

4.3 Material and methods

4.3.1 Experimental animals

Adult virgin (Sprague-Dawley) female rats weighing 180–200 g were left for two weeks of acclimatization period under standard laboratory conditions with the temperature at 23±2^{o} C, and 12:12 light / dark cycle. The standard guideline of National Organization for Drug Control and Research (NODCAR) was used in handling the experimental animals and complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The reproductive cycles of every rat were monitored for 15 days as described in (Cora *et al.* 2015). Every two female rats determined to be in proestrus were caged with one male for one day and those which had spermatozoa in their vaginal smears were considered to be at day 0 of gestation period (Zhou *et al.* 2016).

4.3.2 Treatment and doses

Lead acetate (Loba Chemie, India) was dissolved in distilled water and orally given at a dose of (250 mg/L) as described in (Oyagbemi *et al.* 2015) from day 7 to 16 of the gestation period. Similarly, taurine (Sigma Aldrich, USA) was dissolved in distilled water and was administered orally at a dose of 50 mg/kg/day (Akande *et al.* 2014) from the first to the 20th day of the gestation period.

4.3.3 Experimental design

Forty pregnant rats were divided into four groups (10 each), the first (i) group received distilled water and served as control, group (ii) received lead acetate. Taurine was given to group (iii) while group (iv) was treated as the same way like group (iii) plus lead acetate from 7th to 16th day of the gestation period. Water and food were supplied *ad libitum* during all the experiment. Pregnant dams were observed daily throughout the gestation period for mortality and general

appearance. On the 20th day of the gestation period, blood samples were collected from dams, using orbital sinus technique (Parasuraman *et al.* 2010). Concerning fetuses, blood samples were collected by the decapitation methods (Beuret *et al.* 2005). Few drops of blood were collected in heparinized tubes for hematological studies. The other portion of blood was allowed to clot, serum was separated by centrifugation at 3000 rpm for 20 min then, the clear non hemolysed serum was collected, divided into aliquots and stored at -20° C until further use. Dams were euthanized by anesthesia and after hysterectomy; each fetus was examined and weighed. Total implantation sites, fetal mortality rate (resorbed or stillbirth) and living fetuses were recorded. Placentas were harvested for histopathological examination.

Fetal growth parameters: body weight (gm), crown-rump length (cm), and tail length (cm) were measured. 50 % of the fetuses were fixed in 96 % ethanol and stained with Alcian blue and Alizarin Red-S combined technique (Takarada *et al.* 2013). The skeletal system was examined under dissecting microscope.

Maternal and fetal livers were extirpated, homogenized in 50 mM phosphate buffer (pH 7.4) using electronic homogenizer (Ezister Daihan, Korea) to prepare 10 % w/v homogenate and stored at -80º C. The liver homogenates were divided into aliquots after 3000 rpm centrifugation for 15 min at 4 °C using cooling centrifuge (Hermile Labortechnik, Germany). Liver homogenates were used for the determination of malonaldehyde (MDA) according to the method described by (Rajasekaran & Kalaivani 2011), reduced glutathione (GSH) content and catalase (CAT) activity (Biodiagnostic, Egypt) according to (Lukaszewicz-Hussain 2011) and (Aebi 1984) respectively. Another portion of the liver of dams and their fetuses was used to isolate the DNA using the Wizard genomic purification kit (Promega, USA). The DNA was analyzed using 1.5 % agarose gel electrophoresis (Lee *et al.* 2012).

Hematological parameters of dams and fetuses including hemoglobin level (Hb) using the kits of (Biodiagnostic, Egypt) according to the methods adopted by (Lewis *et al.* 2006), erythrocyte count (RBCs) and total leucocytes count were measured using "Improved Neuberger hemocytometer" (Viana *et al.* 2012). Serum aspartate amino transaminase (AST) and alanine aminotransaminase (ALT) were determined colorimetrically (Huang *et al.* 2006) using kits from (Diamond, Diagnostics, Egypt) and alkaline phosphatase (ALP) activity was measured using

(Stanbio, USA) kits. Serum urea and creatinine were also measured by (Diamond, Diagnostics, Egypt) kits (Romero *et al.* 2016).

Placenta samples were kept in 10 % neutral buffered formalin for 24 hours and were dehydrated through alcohol, cleared in xylene and then embedded in paraffin wax at 56° C in a hot air oven for 24 hours. Sections (4 µm thick) were stained with hematoxylin and eosin and visualized using light microscopy (Bancroft & Stevens 1996).

4.3.4 Statistical analysis

Data obtained from all groups were compared statistically by ANOVA Test followed by Tukey-Kramer multiple comparison test. Statistical analysis was performed using GraphPad Prism Software (version 5, San Diego, CA, USA). All values represent the mean ± SEM.

4.4 Results

4.4.1 Morphological parameters

There were no major or minor abnormalities of any system in the fetuses of the control group. However, morphological and skeletal examination of Pb-treated fetuses showed high incidence of fetal malformation. There was significant decrease ($P < 0.05$) in the body weight gain, weights of uteri and placentas of Pb-treated dams by (61 %, 39 % and 47 %) respectively than that of the control group. The rate of abortion (40 %) and partial resorption (33 %) increased in Pb-treated group compared to the control. On the other hand, no abortion or resorption cases were recorded in the taurine (TA) treated groups (Tables 4.1 & 4.2, Figures 4.1 & 4.2). The body weight and length of fetuses maternally received Pb was significantly decreased by 37.5 % and 13.5 % respectively compared to that of control group. These effects were significantly improved by TA pretreatment (Figure 4.3).

Fetuses maternally received Pb had different abnormalities; hematoma, growth retardation and contraction in the fore limb (Figure 4.4 & 4.5). In fetuses maternally received Pb, skeletal system abnormalities included lack of ossification of the skull bones. Also, incomplete ossification of vertebral column was observed in lumber, sacral and caudal vertebrae. The major skeletal defects were observed mainly in pelvic and hind limb bones in the form of shortness and lack of ossification of ilium, ischium, pubis, femur, tibia and fibula. The major skeletal defects observed in fore and hind limb bones were lack of ossification of long bones, asymmetric ossification of

both fore limbs and absent of digits (Figure 4.6). Taurine protection against Pb effects was observed in the form of complete ossification of skull bones and improvement of other skeletal elements compared to Pb group as compared to control fetuses.

Table 4.1: Effect of taurine (50 mg/Kg) and/or lead (250 mg/L) on the uteri of pregnant rats

Parameters / Treatments	No. of pregnant rats	No. of abortion (%)	No. of sacrificed rats	No. of uteri without resorption	No. of uteri with partial resorption (%)	Average uterine weight (gm)
Control	10	--------- (0%)	10	9	1 (10 %)	49.4±1.72[a]
TA	10	--------- (0%)	10	10	-------- (0 %)	50.2±2.49[a]
Pb	10	4 (40%)	6	4	2 (33.33 %)	30.1±1.6[b]
TA + Pb	10	--------- (0%)	10	7	2 (20 %)	42.1±0.6[a]

Data represent mean ± SEM of 10 animals.

a, b indicate statistically significant differences ($P < 0.05$).

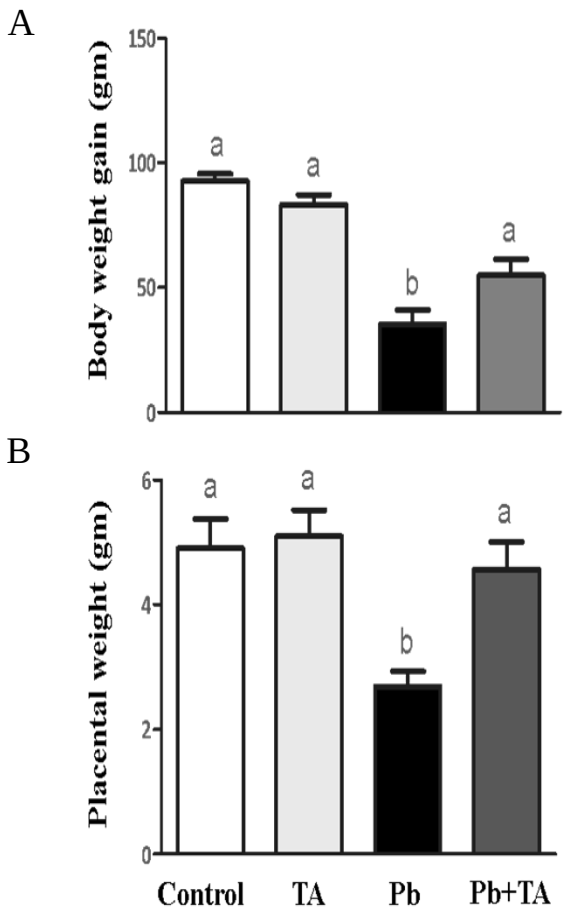


Figure 4.1: Effect of taurine (50 mg/ kg) and/or lead (250 mg/L) on body weight gain (A) and placental weight (B) of dams. Data represent mean ± SEM of 10 animals. a, b indicate statistically significant differences ($P < 0.05$).

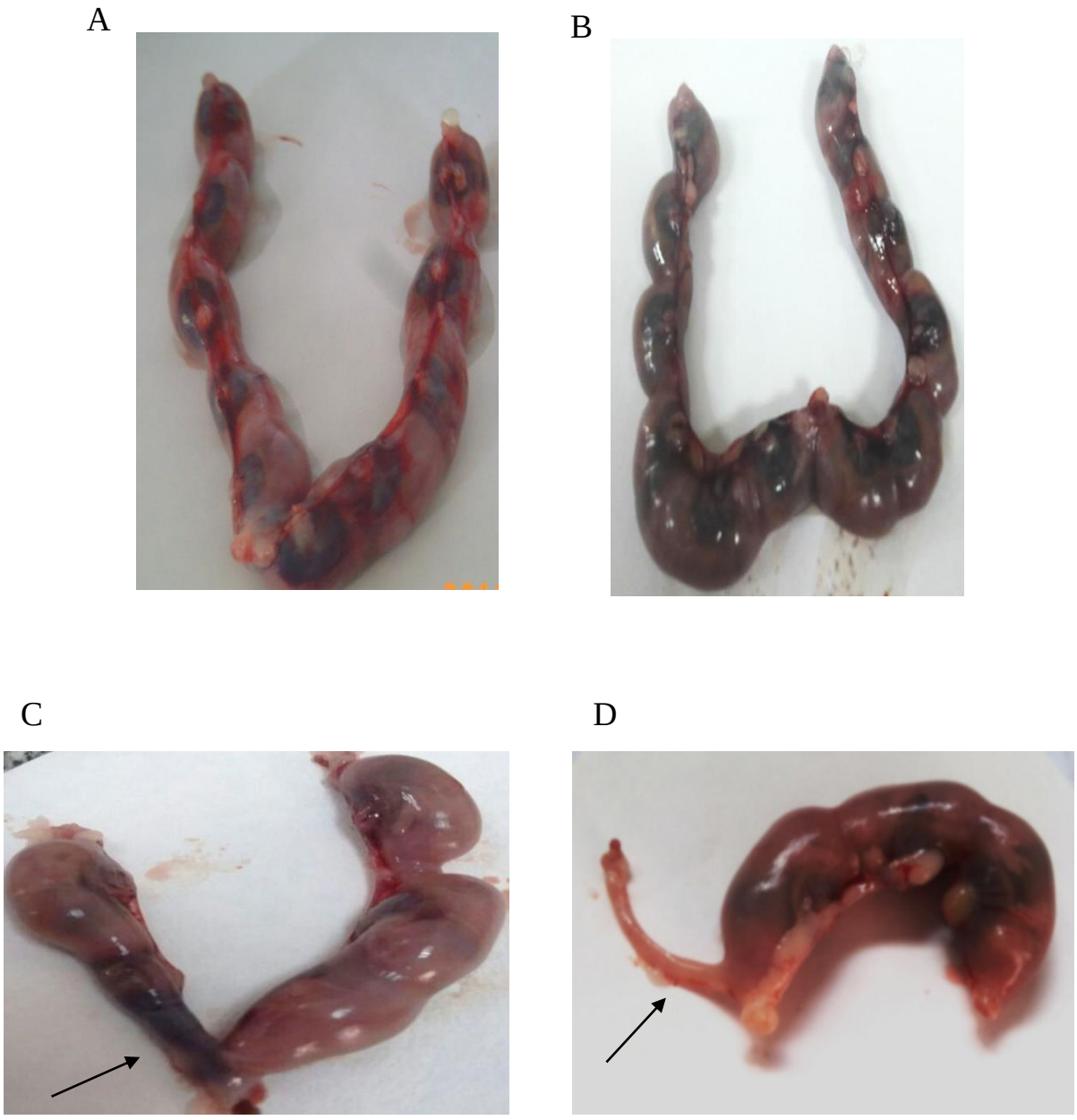


Figure 4.2: Uteri of pregnant rats on the 20th day of gestation, maternally receiving: distilled water (control) (A), taurine (50 mg/ kg) (B) and lead (250 mg/L) (C, D); showing symmetrical distribution of fetuses on horns (A, B), partial resorption (arrow) (C) and partial abortion of fetuses (arrow) (D).

Table 4.2: Effect of taurine (50 mg/kg) and/or lead (250 mg/L) on the mortality of fetuses

Parameters / Treatments	Number of sacrificed dams	Noumber of implantation sites	Number of resorbed fetuses	(%) of resorbed fetuses	Number of live fetuses
Control	10	62	1	1.6 %	61
TA	10	50	---------	0 %	50
Pb	6	27	2	7.4 %	25
TA+ Pb	10	51	2	3.9 %	49

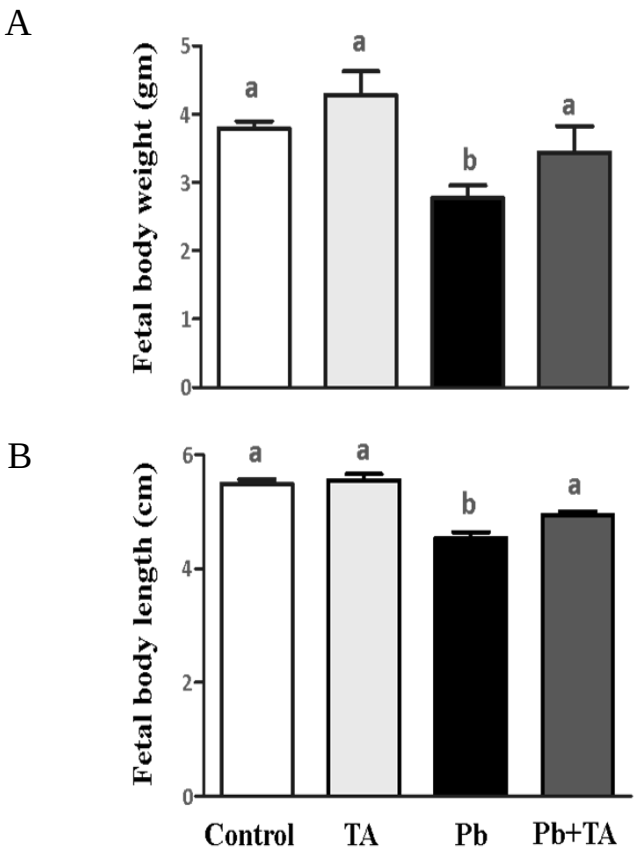


Figure 4.3: Effect of taurine (50 mg/ kg) and/or lead (250 mg/L) on body weight (A) and length (B) of fetuses. Data represent mean ± SEM of 10 animals. a, b indicate statistically significant differences ($P < 0.05$).

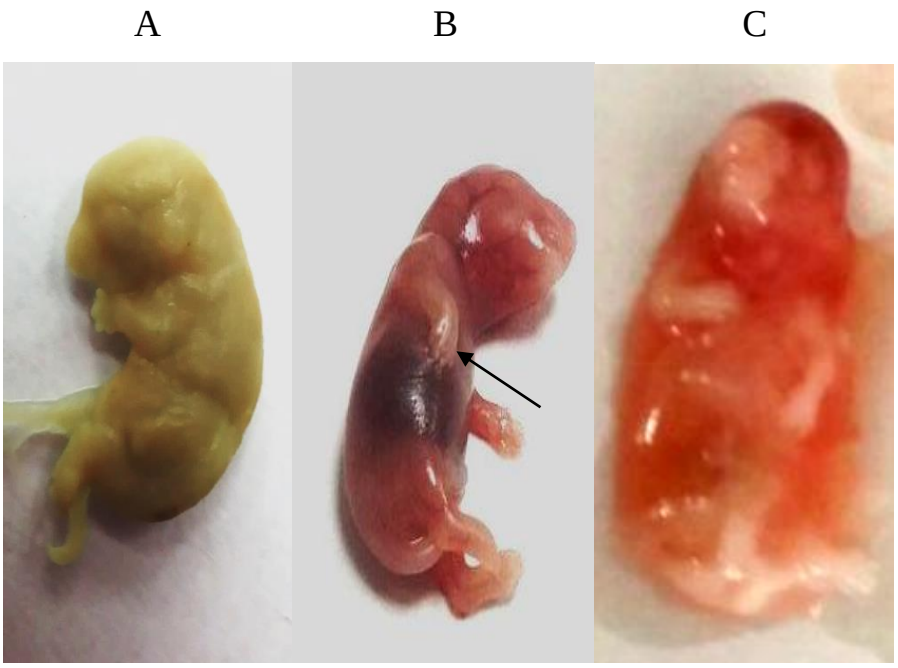


Figure 4.4: Full term fetuses on the 20th day of gestation, maternally receiving: lead (250 mg/L); showing severe growth retardation (A), contraction on the right fore limb (arrow) and hematoma (B), and gelatinous malformed body (C).

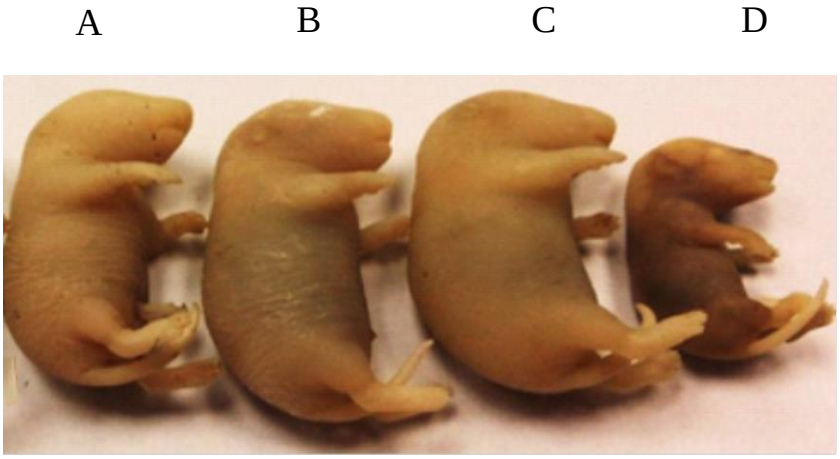


Figure 4.5: Full term fetuses on the 20th day of gestation, maternally receiving: distilled water (control) (A), taurine (50 mg/ kg) (B), taurine+ lead (C) and lead (250 mg/L) (D); showing normal structure of fetuses (A, B & C) and growth retardation with congestion all over the body (D).

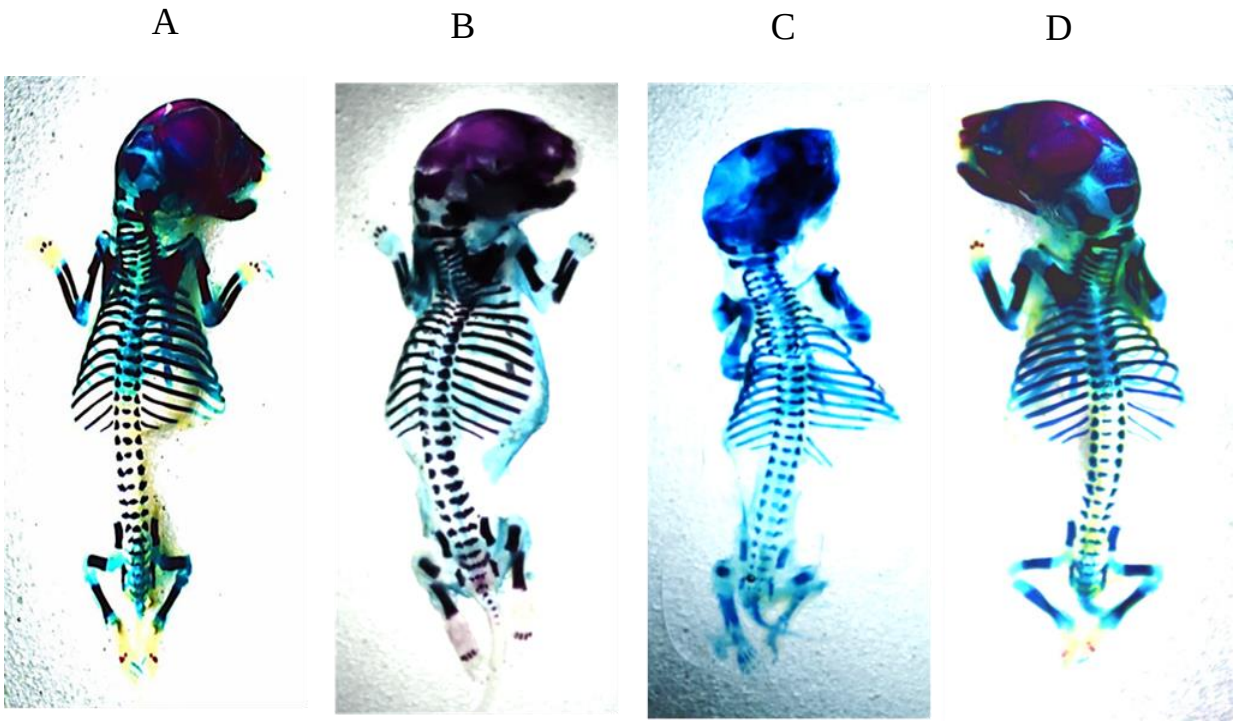


Figure 4.6: Skeletons of fetuses on the 20th day of gestation maternally receiving: distilled water (control) (A), taurine (50 mg/ kg) (B), lead (250 mg/L) (C) and taurine+lead (D); showing normal skeletal system formation (A & B), severe lack of ossification (C) and improvement of skeletal elements (D). Magnification 4 x.

4.4.2 Hematological parameters

The results revealed that exposure of dams to Pb from the 7th to 16th day of the gestation period caused significant reduction ($P < 0.05$) in Hb content and RBCs count and significant increase in WBCs count as compared to control group and the same effects were observed in their fetuses. Upon administration of TA all over the period of gestation, the deleterious effects of Pb on dams were significantly improved ($P < 0.05$) regarding Hb and RBCs while there was insignificant change in fetal parameters compared to Pb group (Table 4.3).

Table 4.3: Effect of taurine (50 mg/kg/day) and/or lead (250 mg/L) on hematological parameters

Groups	Dams			Fetuses		
	Hb (g/dL)	**RBCs** ($*10^6$/cmm)	**WBCs** ($*10^3$/cmm)	**Hb** (g/dL)	**RBCs** ($*10^6$/cmm)	**WBCs** ($*10^3$/cmm)
Control	14.9±0.58[a]	5.3±0.21 [a]	7,4±0.49 [a]	12.8±0.67[a]	4.6±0.38 [a]	8.6±0.05 [a]
TA	15±0.9 [a]	5.7±0.35 [a]	9.1±0.22 [a]	12.7±1.1 [a]	4.2±0.48 [a]	8.9±0.35 [a]
Pb	10±0.33 [b]	3.3±0.11 [b]	14.6±1.4 [b]	6.6±0.78[b]	2.3±0.26 [b]	12.2±0.83 [b]
TA+Pb	12.9±0.8 [a]	4.3±0.26 [a]	11.3±0.8 [b]	9.3±0.88 [b]	3.3±0.23 [b]	10.9±0.58 [b]

Data represent mean ± SEM of 10 animals.

a, b indicate statistically significant differences ($P < 0.05$).

4.4.3 Oxidative stress markers

Lead administration to dams induced a marked elevation ($P < 0.05$) of tissue MDA level and depletion in both GSH content and catalase activity in the dams and their fetuses. On the other hand, TA administration significantly ($P < 0.05$) augmented GSH and CAT while reduced MDA in dams and fetuses compared to Pb group (Table 4.4).

Table 4.4: Effect of taurine (50 mg/kg/day) and/or lead (250 mg/L) on lipid peroxidation and antioxidant enzymes

Groups	Dams			Fetuses		
	MDA (nmol/g)	**GSH** (mg/g)	**CAT** (u/g)	**MDA** (nmol/g)	**GSH** (mg/g)	**CAT** (u/g)
Control	28.3±1.4 [a]	45.7±2.4 [a]	3±0.15 [a]	26.3±1.1 [a]	38.9±1.3 [a]	1.3±0.1 [a]
TA	24.9±0.1 [a]	47.9±2.2 [a]	3.2±0.19 [a]	26.5±2.6 [a]	42.6±1.4 [a]	1.4±0.08 [a]
Pb	68.8±1.5 [b]	26.9±2.1 [b]	2.1±0.12 [b]	58.8±2.8 [b]	25.8±1.5 [b]	0.8±0.06 [b]
TA+Pb	41.4±2.3 [a]	37.1±2.2 [a]	3.1±0.11 [a]	46.4±1.1 [c]	37.9±2.3 [a]	1.3±0.09 [a]

Data represent mean ± SEM of 10 animals.

a, b indicate statistically significant differences ($P < 0.05$).

4.4.4 Liver and kidney function

Exposure of dams to Pb from day 7 to 16 of gestation caused significant increase ($P < 0.05$) in serum AST, ALT and ALP. However, significant reduction ($P < 0.05$) in the activities of these enzymes was observed in dams administered TA and their fetuses compared to Pb group (Table 4.5). Additionally, Pb evoked significant elevation ($P < 0.05$) in serum urea and creatinine in dams and their fetuses compared to control groups. Treatment with TA recovered the levels of urea and creatinine below the normal values (Table 4.6).

Table 4.5: Effect of taurine (50 mg/kg/day) and/or lead (250 mg/L) on some liver function tests

Groups	Dams			Fetuses		
	AST (U/L)	**ALT** (U/L)	**ALP** (U/L)	**AST** (U/L)	**ALT** (U/L)	**ALP** (U/L)
Control	38.2±1.8 [a]	39.8±1.2 [a]	38.6±1.1 [a]	44.3±0.33 [a]	43.7±0.89 [a]	42.5±1.2 [a]
TA	38.4±2.22 [a]	39.5±2.26 [a]	30.4±2.1 [a]	41±0.58 [a]	42±1.5 [a]	35.5±0.53 [a]
Pb	71.8±2.8 [b]	68.7±1.7 [b]	60.1±2.9 [b]	56±0.52 [b]	80.7±2.9 [b]	66.9±0.97 [b]
TA+Pb	47.1±2.45 [a]	48.4±2.8 [a]	41.9±3.4 [a]	47±0.5 [a]	50.3±0.8 [a]	47.9±0.98 [a]

Data represent mean ± SEM of 10 animals.

a, b indicate statistically significant differences ($P < 0.05$).

Table 4.6: Effect of taurine (50 mg/kg/day) and/or lead (250 mg/L) on kidney function tests

Groups	Dams		Fetuses	
	Urea (mg/dL)	**Creatinine** (mg/dL)	**Urea** (mg/dL)	**Creatinine** (mg/dL)
Control	29.6±1.9 [a]	0.5±0.13 [a]	38.8±0.52 [a]	0.5±0.09 [a]
TA	35.2±1.8 [a]	0.5±0.11 [a]	37.9±0.09 [a]	0.3±0.02 [a]
Pb	58.1±2.5 [b]	2.1±0.25 [b]	59.2±0.28 [b]	2.4±0.09 [b]
TA+Pb	41.3±0.4 [a]	1.2±0.28 [ab]	42.2±2.1 [a]	0.9±0.24 [c]

Data represent mean ± SEM of 10 animals.

a, b indicate statistically significant differences ($P < 0.05$).

4.4.5 DNA fragmentation via agarose gel electrophoresis

In the absence of Pb (control and TA groups), no ladder was observed and the fragmentation of the DNA remained negligible (Figure 4.7). Lead induced DNA fragmentation in livers of dams and their fetuses, which were clearly indicated on the agarose gel and detected by UV fluorescence after staining with ethidium bromide. Pretreatment with TA, protected the cells and prevented Pb-induced DNA degradation.

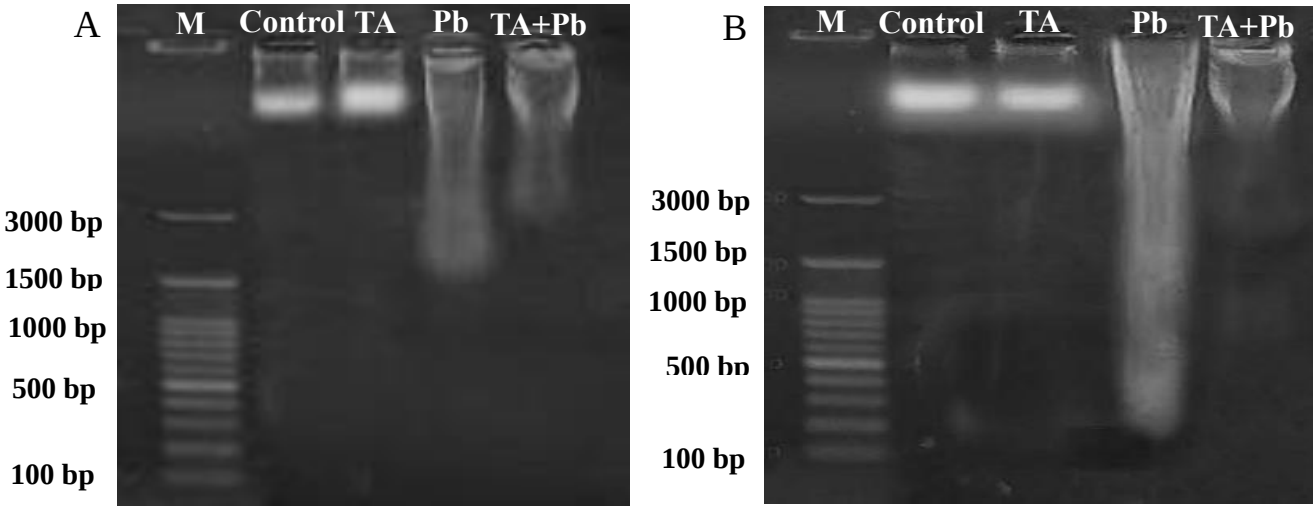


Figure 4.7: DNA isolated from the liver of dams (A) and fetuses (B), on agarose gel 1.5 %. Receiving: distilled water (control), taurine (50 mg/ kg) (TA), lead (250 mg/L) (Pb) and taurine +lead (TA+Pb); showing significant DNA fragmentation (Pb) and (TA+Pb). (M) Marker (3 kbp).

4.4.6 Histopathological examination of placenta

Normal histological structure of the chorioallantoic membrane with trophoblast and giant cells as well as the underlying labyrinth zone was observed in placentas of the control and TA groups (Figure 4.8). However, in dams of the Pb group, placenta revealed vascular congestion, severe focal hemorrhage, degeneration, inflammatory infiltration and fibrin deposition in the trophoblast of the labyrinth zone. However, the administration of TA along with Pb showed improvement in the placental architecture as compared to group subjected only to Pb.

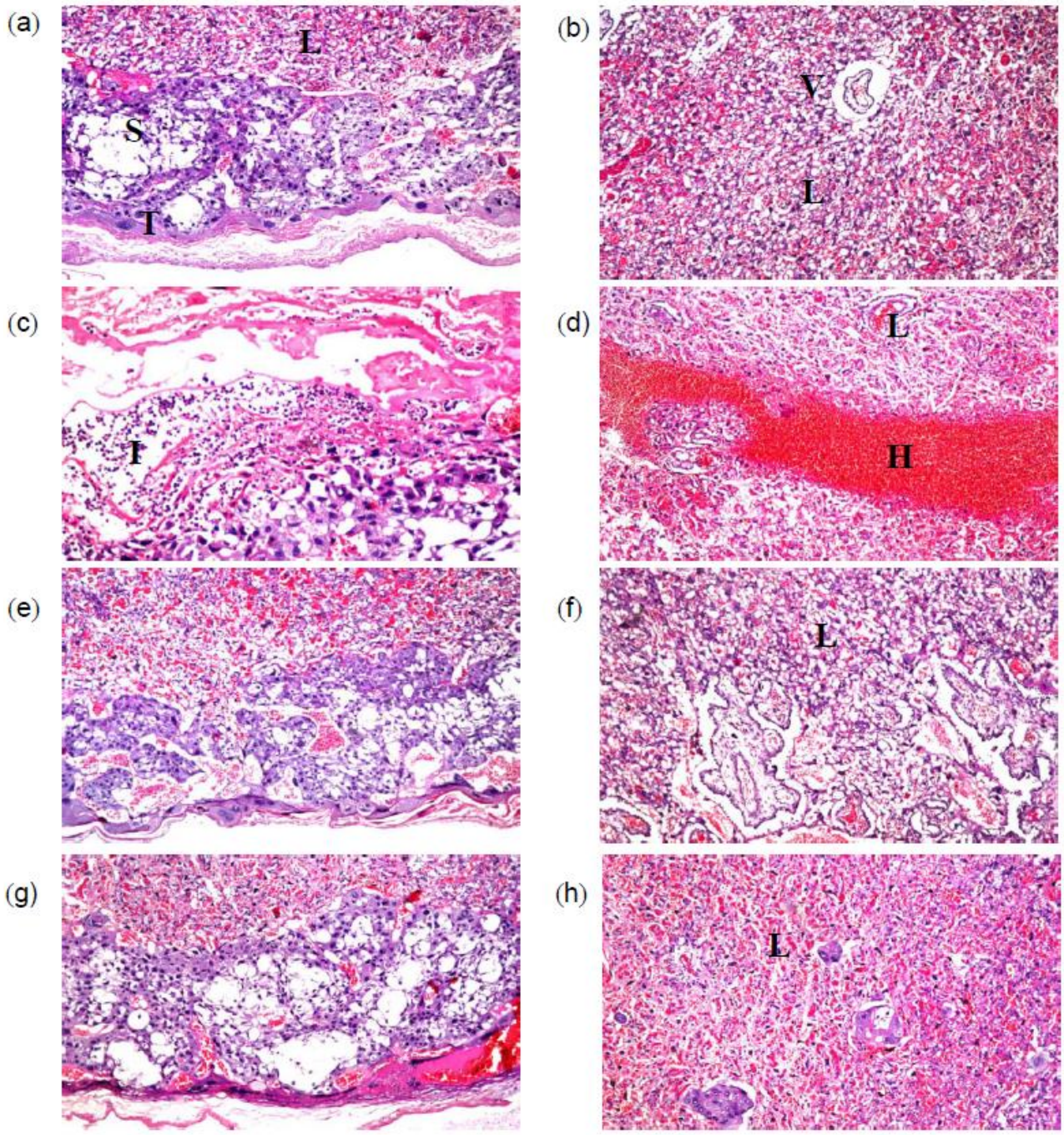


Figure 4.8: Histopathological findings in rat placenta (Haematoxylin and Eosin ×100): Control group showing (a) normal architecture of trophoblast (**T**) and giant cell in chorioallantoic membrane, (b) normal histology of labyrinth zone (**L**) and fetal villi (**V**). Lead group: showing (c) lymphocytic infiltration (**I**) and loss of architecture, (d) focal hemorrhage in labyrinth (**H**).

Taurine group: showing (e) normal histology of chorioallantoic membrane, (f) normal histology of labyrinth zone. Taurine + lead group: showing (g) slight congestion in chorioallantoic blood vessels, (h) slight congestion and hemorrhage of labyrinth.

4.5 Discussion

Lead is a naturally occurring heavy metal and systemic toxicant that has a broad spectrum of toxic effects in human and animal systems (Chang *et al.* 2012; Flora *et al.* 2012). It has been shown that reproductive consequences of Pb toxicity have drastic reproductive outcomes and nearly all compartments of reproductive system are target organs (Anjum 2012). In the present study, teratogenicity was induced in rats by administration of Pb in a dose of 250 mg/L orally from day 7 to 16 of gestation. The results showed a decrease in body weight gain during pregnancy and a high percentage of abortion as well as some alteration in the gross morphological and anatomical features compared to control group. These results were in agreement with the findings of Sharma & Mogra (2013) and Edwards (2014), which stated that Pb exposure during pregnancy results in post-implantation losses, growth retardation and high incidence of fetal mortality. The present work also revealed that prenatal Pb exposure caused significant decline in fetal body weight, crown-rump length and fetal tail length and this is in accordance with Saleh *et al.* (2009), Rahman *et al.* (2012) and Al-Saleh *et al.* (2014), in which they concluded that Pb exposure in the very early stages of pregnancy hinders normal organogenesis where the placental and maternal blood Pb levels are predictors of birth height and weight.

It is known that, for appropriate fetal development and growth, normal placental function is mandatory (Azpurua *et al.* 2010) and previous reports showed that low placental weight increase the risk of fetal distress at birth (Husslein *et al.* 2012). As shown in the present work, there was significant reduction in placental weight of Pb-treated dams compared to the control group. Moreover, histopathological examination of placental trophoblasts in the present work showed vascular congestion, hemorrhage, degeneration, inflammatory infiltration and fibrin deposition in Pb-exposed dams compared to the control group. These finding consequently lead to reduced fetal-maternal nutrition and oxygen exchanges in addition to the impaired fetal development and increased neonatal mortality incidences (Heazell & Crocker 2008; Redline 2008). Studies reported that Pb exposure resulted in ROS-mediated endoplasmic reticulum stress via caspase-12

activation which in turn triggers procaspase-9 and caspase-3 leading to apoptosis and the pathophysiology of the placenta (Xu *et al.* 2012; Wang *et al.* 2014).

In the present study, the skeletal systems of fetuses maternally exposed to Pb showed severe lack of ossification in most components of the skeleton including the skull, fore-limbs and hind-limbs, shortness of bones of limbs as well as missed ossification of vertebrae. These results coincide with Li *et al.* (2013), Figueiredo *et al.* (2014) and Grizlova & Yakimova (2014). In this context, many hypotheses have been shown to explain Pb impact on bone development. Due to ionic mimicry, Pb was found to displace Ca in the mineral bone matrix and consequently disturb the hormonal regulation of Ca absorption (Conti *et al.* 2012). In addition, Pb has direct effects on osteoblast function including inhibition of ALP; the enzyme marker of osteoblast activity as well as the vitamin D3-stimulated synthesis of osteocalcin; a major non-collagen constituent of bone and important for mineralization (Gangoso *et al.* 2009; Saleh *et al.* 2009). Several studies showing that ROS stimulate osteoclast differentiation and bone resorption through uncoupling of the delicate balance between resorption and formation, and this will lead to osteoporosis and fracture risk (Baek *et al.* 2010; Cervellati *et al.* 2014).

In the present study, Pb significantly decreased hemoglobin (Hb) content and red blood cell count (RBCs), meanwhile total white blood cells (WBCs) was significantly higher in dams and fetuses of Pb-exposed group in comparison to control group. These findings are consistent with previous studies of Sharma *et al.* (2012), Kianoush *et al.* (2013) and Liu *et al.* (2015). They attributed leukocytosis to the ability of Pb to elicit ROS that can damage cell membrane and in turn trigger the cascades of inflammatory process. Various studies have also reported that perinatal exposure to Pb decreases the fetal and adult Hb synthesis in rats due to the inhibition of enzymatic activities involved in heme biosynthesis as aminolevulinic acid dehydratase (ALAD), aminolevulinic acid synthetase (ALAS) and ferrochelatase (Liao *et al.* 2008; Barber *et al.* 2011; Jangid *et al.* 2012). It was demonstrated that Pb can also induce anemia through induction of erythrophagocytosis and increase the rate of RBCs clearance (Jang *et al.* 2011). Besides, Pb-induced ROS showed a detrimental effect on hematopoiesis homeostasis of HSC (hematopoietic stem cell) by activation of apoptosis or premature senescence of adult HSCs (Liu *et al.* 2015). Herein, following Pb administration, MDA levels in dams and fetuses livers significantly increased which confirmed the involvement of oxidative stress in Pb poisoning since the major

aldehyde product of lipid peroxidation is MDA (Demir *et al.* 2011). Furthermore, there was a significant decrement in the activities of hepatic antioxidant enzymes (GSH and CAT) of both dams and fetuses in Pb group. These results are in accordance with Jackie *et al.* (2011), Chander *et al.* (2014), Wang *et al.* (2013) and (Dewanjee *et al.* 2015). It is worth to mention that, Pb inactivates glutathione by forming covalent bond with sulfhydryl groups which are the most potential target of Pb (Flora *et al.* 2012). Besides, it can replace Zn ion that serves as important cofactors for CAT activity involved in superoxide radical overwhelming (Flora *et al.* 2007).
In the present study, plasma AST and ALT, ALP activities in Pb-treated dams and their fetuses significantly increased. This may be attributed to Pb-induced ROS which elicit lipid peroxidation and cell membrane damage and consequently leakage of these enzymes into peripheral circulation (Dewanjee *et al.* 2013; Thenmozhi *et al.* 2013).

There was also elevation in urea and creatinine levels in Pb-exposed rats and their fetuses in the current study. These results were previously investigated by Nisar *et al.* (2011), Oyagbemi *et al.* (2015) and Hammed (2015) who proved that environmental Pb-mediated oxidative stress induces significant pathological lesions in the kidney reflected by interstitial nephritis, tubular necrosis and decreased glomerular filtration rate, hence reduction in renal clearance of urea and creatinine. Additionally, Ahmed *et al.* (2013) reported that apoptosis and activation of caspase-8 and -9 and the pro-apoptotic marker; BAX are involved in the mechanism of renal Pb toxicity.
In the current study, agarose gel analysis of DNA isolated from livers of both dams and fetuses revealed the impact of Pb toxicity as it has been reported by Moniem *et al.* (2010), Dewanjee *et al.* (2013) and Abdou & Hassan (2014). It was also shown that Pb exposure increases ROS and lipid peroxidation that cause DNA damage and affect the synthesis of both DNA and RNA in the liver (Ahmed *et al.* 2012). Lead also can hamper DNA repair by interfering with base excision repair (BER) and nucleotide excision repair (NER) mechanisms as well as replacing Zn in DNA binding proteins (Garcia-Leston *et al.* 2012). While Zhang *et al.* (2014) demonstrated two mechanisms of Pb-induced DNA damage in mice liver; either by binding to phosphate backbone of DNA through electrostatic forces forming Pb-DNA complex or entering into the minor grooves of DNA by combinations with purines and pyrimidines, resulting in damage of the double helix structure of DNA. Another recent study by Li *et al.* (2016) showed that there is an association between maternal Pb levels and DNA hypomethylation in newborns and adults. Although the biological significance of reduced DNA methylation in Pb-exposed humans is still

unknown, this epigenetic change is known to alter disease susceptibility in adulthood (Bernal *et al.* 2013; Nilsson & Skinner 2015; Nilsson *et al.* 2018).

In the current study, concomitant administration of taurine (TA) along with Pb showed neither teratogenic nor embryotoxic effects. Taurine, a conditionally essential amino acid, can be synthesized from methionine and serine. However, during pregnancy, TA is essential due to the lack of synthetic enzymes in both fetus and placenta. Therefore, this demand for TA must be covered by its transport from maternal plasma into placenta (Nishimura *et al.* 2010; Roysommuti & Wyss 2014).

Administration of TA with Pb insult revealed normal histology of placenta. In this regard, many researches highlighted the cytoprotective role of TA in placental trophoplasts and its importance for cell differentiation and survival. Desforges *et al.* (2013b; 2015) and Nishimura *et al.* (2015) demonstrated that TA depletion compromised placental cell differentiation and increased the susceptibility to ROS-mediated DNA damage and apoptosis in vitro. Reduced TA also was reported as contributing factor in pregnancy complications as pre-eclampsia and fetal growth restriction compared with normal pregnancies (Desforges *et al.* 2013a). It was demonstrated also that in both placental and nonplacental cells, TA is a key osmoregulator, important for cell volume regulation and consequently; growth, differentiation (Lambert 2004; Nishimura *et al.* 2010). Furthermore, TA pretreatment improved fetal body growth and bone formation in the current study compared to Pb-exposed group. Also, Liu *et al.* (2012) concluded that perinatal TA supplementation may reduce neuronal apoptosis in intrauterine growth retardation (IUGR) rat fetuses via upregulating BCL2/BAX ratio and downregulating the expression of caspase-3.

The beneficial role of TA in bone has been also demonstrated in many studies: Wang *et al.* (2011) found that TA significantly reduces synovial inflammation, cartilage damage and bone erosion by inhibiting lymphocyte proliferation and osteoclast formation in mice with collagen-induced arthritis. TA was found to inhibit the expression of inflammatory cytokines, such as TNF-α, IL-1 and IL-6, which are crucial for bone destruction (Marcinkiewicz & Kontny 2014; Kim & Cha 2014). Roman-Garcia *et al.* (2014) reported that TA restores osteoblast function and protects against growth retardation and osteoporosis in the offspring of vitamin B_{12} deficient mice. In addition, Choi & Chang (2013) showed that TA increases bone mineral density bone

makers in rats. Rather, it was reported that, TA transporter knockout mouse exhibited short lifespan and skeletal muscle defects (Ito *et al.* 2014).

In the present study, administration of TA to dams throughout gestation significantly augmented Hb content and RBCs count and reduced WBCs count in both dams and fetuses as compared to Pb-received group. Moreover, there was attenuation of lipid peroxidation in the livers of dams and their fetuses in TA+Pb group in addition to improvement in hepatic GSH content and CAT activity. This was in agreement with Shivananjappa & Muralidhara (2012) who demonstrated that, TA mitigates maternal and embryonic oxidative stress in a streptozotocin-diabetic rat model. According to Sevin *et al.* (2013) and Taziki *et al.* (2013), TA is capable of functioning as an indirect antioxidant, either by reducing ROS production or by augmenting the antioxidant defense system. It is noteworthy that, TA forms chloramines with hypochlorous acid, thus inhibits its direct attack on cell membranes of organs. TA also replaces glutathione (GSH) in biological systems during oxidative stress (Akande *et al.* 2014).

In the present work, TA pretreatment along with Pb alleviated the alterations in the activities of the enzymes (AST, ALT and ALP) in dams and fetuses. It was reported that TA reverse the liver injury in rat with alcholoic fatty liver (Wu *et al.* 2015). Similarly, we found significant reduction in the levels of urea and creatinine in TA+Pb group as compared to Pb only group. Consistent with these results, the previous work of Koh *et al.* (2014) and Sirdah (2015) showed that TA delayed the onset of nephropathy in diabetic rats through its antioxidant, antiapoptotic and membrane stabilizing effects. TA also exhibits nephroprotection by regulating blood flow in the renal vasculature and Na transport in the proximal tubules (Karbalay-Doust *et al.* 2012; Han & Chesney 2012). Furthermore, pretreatment with TA alleviates DNA fragmentation in dams and their fetuses. Matching with the current results, TA was found to reduce DNA damage and DNA- protein cross-links as well as single- strand breaks induced by potassium bromate in rat intestine (Ahmad *et al.* 2015). TA also inhibited DNA fragmentation in the testes of aged rats since it potentially suppressed lipid peroxidation and reduced BAX, cytochrome C and caspase-3 expressions (Yang *et al.* 2015). This may suggest the chemoprotective and antimutagenic effects of TA (Turkez & Aydin 2012).

In summary, this study emphasizes the detrimental effects of Pb on the pregnant rats as well as the developing fetuses and highlights the ameliorative role of TA as a potential antioxidant

against Pb toxicity *in vivo*. However, the mechanisms implicated in TA therapeutic activity against metal toxicity; needs to be investigated in depth. So future work is warranted considering TA safety and pharmacokinetics during pregnancy.

4.6 References

Abdou HM & Hassan MA 2014 Protective role of omega-3 polyunsaturated fatty acid against lead acetate-induced toxicity in liver and kidney of female rats. *Biomedical Research International* **2014** 435857.

Aebi H 1984 Catalase in vitro. *Methods in Enzymology* **105** 121–126.

Ahamed M & Siddiqui MKJ 2007 Environmental lead toxicity and nutritional factors. *Clinical Nutrition* **26** (4) 400–408.

Ahamed M, Verma S, Kumar A & Siddiqui MKJ 2005 Environmental exposure to lead and its correlation with biochemical indices in children. *The Science of the Total Environment* **346** (1-3) 48–55.

Ahmad MK, Khan AA, Ali SN & Mahmood R 2015 Chemoprotective effect of taurine on potassium bromate-induced DNA damage, DNA-protein cross-linking and oxidative stress in rat intestine. *PloS One* **10** (3) e0119137.

Ahmed MB, Ahmed MI, Meki A-R & Abdraboh N 2013 Neurotoxic effect of lead on rats: Relationship to apoptosis. *International Journal of Health Sciences* **7** (2) 192–199.

Ahmed YF, Eldebaky HAA, Mahmoud KGM & Nawito M 2012 Effects of lead exposure on DNA damage and apoptosis in reproductive and vital organs in female rabbits. *Global Veterinaria* **9** (4) 401–408.

Akande MG, Aliu YO, Ambali SF & Ayo JO 2014 Taurine alleviated biochemical alterations in male Wistar rats co-exposed to chlorpyrifos and lead. *Journal of Toxicology and Environmental Health Sciences* **6** (2) 13–25.

Al-Saleh I, Shinwari N, Mashhour A, Mohamed GED & Rabah A 2011 Heavy metals (lead, cadmium and mercury) in maternal, cord blood and placenta of healthy women. *International Journal of Hygiene & Environmental Health* **214** (2) 79–101.

Al-Saleh I, Shinwari N, Mashhour A & Rabah A 2014 Birth outcome measures and maternal exposure to heavy metals (lead, cadmium and mercury) in Saudi Arabian population. *International Journal of Hygiene & Environmental Health* **217** (2-3) 205–218.

Anjum MR 2012 Supplementation of testosterone restores the suppressed fertility in male rats exposed to lead during perinatal period. *IOSR Journal of Pharmacy* **2** (6) 49–53.

Azpurua H, Funai EF, Coraluzzi LM, Doherty LF, Sasson IE, Kliman M & Kliman HJ 2010 Determination of placental weight using two-dimensional sonography and volumetric mathematic modeling. *American Journal of Perinatology* **27** (2) 151–155.

Baek KH, Oh KW, Lee WY, Lee SS, Kim MK, Kwon HS, Rhee EJ, Han JH, Song KH, Cha BY Lee KW & Kang MI 2010 Association of oxidative stress with postmenopausal osteoporosis and the effects of hydrogen peroxide on osteoclast formation in human bone marrow cell cultures. *Calcified Tissue International* **87** (3) 226–235.

Bancroft JD & Stevens A 1996 *Theory and practice of histological techniques,* edn 4. New York, Edinburgh: Churchill Livingstone.

Barber I, Sharma R, Morga S, Panwar K & Garu U 2011 Lead induced alterations in blood cell counts and hemoglobin during gestation and lactation in Swiss albino mice. *Journal of Cell & Molecular Biology* **9** (1).

Bernal AJ, Dolinoy DC, Huang D, Skaar DA, Weinhouse C & Jirtle RL 2013 Adaptive radiation-induced epigenetic alterations mitigated by antioxidants. *FASEB Journal* **27** (2) 665–671.

Beuret CJ, Zirulnik F & Gimenez MS 2005 Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. *Reproductive Toxicology* **19** (4) 501–504.

Cantonwine D, Hu H, Sanchez BN, Lamadrid-Figueroa H, Smith D, Ettinger AS, Mercado-Garcia A, Hernandez-Avila M, Wright RO & Tellez-Rojo MM 2010 Critical windows of fetal lead exposure: adverse impacts on length of gestation and risk of premature delivery. *Journal of Occupational & Environmental Medicine* **52** (11) 1106–1111.

Cervellati C, Bonaccorsi G, Cremonini E, Romani A, Fila E, Castaldini MC, Ferrazzini S, Giganti M & Massari L 2014 Oxidative stress and bone resorption interplay as a possible trigger for postmenopausal osteoporosis. *Biomedical Research International* **2014** 569563.

Chander K, Vaibhav K, Ejaz Ahmed M, Javed H, Tabassum R, Khan A, Kumar M, Katyal A, Islam F & Siddiqui MS 2014 Quercetin mitigates lead acetate-induced behavioral and histological alterations via suppression of oxidative stress, Hsp-70, Bak and upregulation of Bcl-2. *Food & Chemical Toxicology* **68** 297–306.

Chang BJ, Jang BJ, Son TG, Cho IH, Quan FS, Choe NH, Nahm SS & Lee JH 2012 Ascorbic acid ameliorates oxidative damage induced by maternal low-level lead exposure in the hippocampus of rat pups during gestation and lactation. *Food & Chemical Toxicology* **50** (2) 104–108.

Choi MJ & Chang KJ 2013 Effect of dietary taurine and arginine supplementation on bone mineral density in growing female rats. *Advances in Experimental Medicine & Biology* **776** 335–345.

Conti MI, Terrizzi AR, Lee CM, Mandalunis PM, Bozzini C, Pineiro AE & Martinez MdP 2012 Effects of lead exposure on growth and bone biology in growing rats exposed to simulated high altitude. *Bulletin of Environmental Contamination and Toxicology* **88** (6) 1033–1037.

Cora MC, Kooistra L & Travlos G 2015 Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicologic Pathology* **43** (6) 776–793.

Demir F, Uzun FG, Durak D & Kalender Y 2011 Subacute chlorpyrifos-induced oxidative stress in rat erythrocytes and the protective effects of catechin and quercetin. *Pesticide Biochemistry & Physiology* **99** (1) 77–81.

Desforges M, Ditchfield A, Hirst CR, Pegorie C, Martyn-Smith K, Sibley CP & Greenwood SL 2013a Reduced placental taurine transporter (TauT) activity in pregnancies complicated by pre-eclampsia and maternal obesity. *Advances in Experimental Medicine & Biology* **776** 81–91.

Desforges M, Parsons L, Westwood M, Sibley CP & Greenwood SL 2013b Taurine transport in human placental trophoblast is important for regulation of cell differentiation and survival. *Cell death & Disease* **4** e559.

Desforges M, Whittaker H, Farmer E, Sibley CP & Greenwood SL 2015 Effects of taurine depletion on human placental syncytiotrophoblast renewal and susceptibility to oxidative stress. *Advances in Experimental Medicine & Biology* **803** 63–73.

Dewanjee S, Dua TK, Khanra R, Das S, Barma S, Joardar S, Bhattacharjee N, Zia-Ul-Haq M & Jaafar HZE 2015 Water Spinach, Ipomoea aquatic (Convolvulaceae), ameliorates lead toxicity by inhibiting oxidative stress and apoptosis. *PloS One* **10** (10).

Dewanjee S, Sahu R, Karmakar S & Gangopadhyay M 2013 Toxic effects of lead exposure in Wistar rats: involvement of oxidative stress and the beneficial role of edible jute (Corchorus olitorius) leaves. *Food & Chemical Toxicology* **55** 78–91.

Edwards M 2014 Fetal death and reduced birth rates associated with exposure to lead-contaminated drinking water. *Environmental Science & Technology* **48** (1) 739–746.

El-Agousa I, El-nashar D, Eissa S & Sharoud M 2009 Possible ameliorative effect of antioxidant (Taurine) in pregnant toxemic female Rats. *Open Hypertension Journal* **2** 1–15.

Figueiredo FAT de, **Gerlach RF, da Veiga MAMS, Nakadi FV, Ramos J, Kawakita ER, Guerra CdS & Issa JPM** 2014 Reduced bone and body mass in young male rats exposed to lead. *Biomedical Research International* **2014** 571065.

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

Flora SJS, Flora G & Saxena G 2006 Environmental occurrence, health effects and management of lead poisoning. *Chemistry, Analytical Aspects, Environmental Impacts & Health Effects* 158-228.

Flora SJS, Flora G, Saxena G & Mishra M 2007 Arsenic and lead induced free radical generation and their reversibility following chelation. *Cellular & Molecular Biology* 53 (1) 26–47.

Florea AM & Busselberg D 2006 Occurrence, use and potential toxic effects of metals and metal compounds. *Biometals* **19** (4) 419–427.

Franconi F, Loizzo A, Ghirlanda G & Seghieri G 2006 Taurine supplementation and diabetes mellitus. *Current Opinion in Clinical Nutrition & Metabolic Care* **9** (1) 32–36.

Froger N, Jammoul F, Gaucher D, Cadetti L, Lorach H, Degardin J, Pain D, Dubus E, Forster V & Ivkovic I, Simonutti M, Sahel JA &Picaud S 2013 Taurine is a crucial factor to

preserve retinal ganglion cell survival. *Advances in Experimental Medicine & Biology* **775** 69–83.

Gangoso L, Alvarez-Lloret P, Rodriguez-Navarro AAB, Mateo R, Hiraldo F & Donazar JA 2009 Long-term effects of lead poisoning on bone mineralization in vultures exposed to ammunition sources. *Environmental Pollution* **157** (2) 569–574.

Garcia-Leston J, Roma-Torres J, Vilares M, Pinto R, Prista J, Teixeira JP, Mayan O, Conde J, Pingarilho M, Gaspar JF, Pásaro E, Méndez J & Laffon B 2012 Genotoxic effects of occupational exposure to lead and influence of polymorphisms in genes involved in lead toxicokinetics and in DNA repair. *Environment International* **43** 29–36.

Garza A, Vega R & Soto E 2006 Cellular mechanisms of lead neurotoxicity. *Medical Science Monitor* **12** (3) RA57-65.

Grizlova LV & Yakimova EA 2014 Effect of lead acetate on the placental barrier and the development of bone tissue in the early ontogenesis. *Advances in Environmental Biology* 938–943.

Guidotti TL & Ragain L 2007 Protecting children from toxic exposure: three strategies. *Pediatric Clinics of North America* **54** (2) 227-235.

Gulson BL, Mizon KJ, Korsch MJ, Palmer JM & Donnelly JB 2003 Mobilization of lead from human bone tissue during pregnancy and lactation-a summary of long-term research. *The Science of the Total Environment* **303** (1-2) 79–104.

Hammed MS 2015 Evaluation of performance of date palm pollen on urea and creatinine levels in adult female rats exposed to lead acetate intoxication. *International Journal of Biomedical & Advance Research* **6** 20–24.

Han X & Chesney RW 2012 The role of taurine in renal disorders. *Amino Acids* **43** (6) 2249–2263.

Heazell AEP & Crocker IP 2008 Live and let die-regulation of villous trophoblast apoptosis in normal and abnormal pregnancies. *Placenta* **29** (9) 772–783.

Huang XJ, Choi YK, Im HS, Yarimaga O, Yoon E & Kim HS 2006 Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) detection techniques. *Sensors* **6** (7) 756–782.

Husslein H, Moswitzer B, Leipold H, Moertl M & Worda C 2012 Low placental weight and risk for fetal distress at birth. *Journal of Perinatal Medicine* **40** (6) 693–695.

Iavicoli I & Calabrese EJ 2011 Redefining low lead levels. *Environmental Health Perspectives* **119** (5) A202.

Ito T, Schaffer SW & Azuma J 2012 The potential usefulness of taurine on diabetes mellitus and its complications. *Amino Acids* **42** (5) 1529–1539.

Ito T, Yoshikawa N, Inui T, Miyazaki N, Schaffer SW & Azuma J 2014 Tissue depletion of taurine accelerates skeletal muscle senescence and leads to early death in mice. *PloS One* **9** (9) e107409.

Jackie T, Haleagrahara N & Chakravarthi S 2011 Antioxidant effects of Etlingera elatior flower extract against lead acetate - induced perturbations in free radical scavenging enzymes and lipid peroxidation in rats. *BMC Research Notes* **4** 67.

Jang WH, Lim KM, Kim K, Noh JY, Kang S, Chang YK & Chung JH 2011 Low level of lead can induce phosphatidylserine exposure and erythrophagocytosis: a new mechanism underlying lead-associated anemia. *Toxicological Sciences* **122** (1) 177–184.

Jangid AP, John PJ, Yadav D, Mishra S & Sharma P 2012 Impact of chronic lead exposure on selected biological markers. *Indian Journal of Clinical Biochemistry* **27** (1) 83–89.

Karbalay-Doust S, Noorafshan A & Pourshahid S-M 2012 Taxol and taurine protect the renal tissue of rats after unilateral ureteral obstruction: a stereological survey. *Korean Journal of Urology* **53** (5) 360–367.

Kianoush S, Balali-Mood M, Mousavi SR, Shakeri MT, Dadpour B, Moradi V & Sadeghi M 2013 Clinical, toxicological, biochemical, and hematologic parameters in lead exposed workers of a car battery industry. *Iranian Journal of Medical Sciences* **38** (1) 30–37.

Kim C & Cha YN 2014 Taurine chloramine produced from taurine under inflammation provides anti-inflammatory and cytoprotective effects. *Amino Acids* **46** (1) 89–100.

Koh JH, Lee ES, Hyun M, Kim HM, Choi YJ, Lee EY, Yadav D & Chung CH 2014 Taurine alleviates the progression of diabetic nephropathy in type 2 diabetic rat model. *International Journal of Endocrinology* **2014** 397307.

Lambert IH 2004 Regulation of the cellular content of the organic osmolyte taurine in mammalian cells. *Neurochemical Research* **29** (1) 27–63.

Lee PY, Costumbrado J, Hsu CY & Kim YH 2012 Agarose gel electrophoresis for the separation of DNA fragments. *Journal of Visualized Experiments* (62) 3923.

Lewis SM, Bain BJ, Bates I & Dacie JV 2006 *Dacie and Lewis practical haematology*, edn 10. Philadelphia: Churchill Livingstone/Elsevier.

Li HW, Deng JG, Du ZC, Yan MS, Long ZX, Pham Thi PT & Yang KD 2013 Protective effects of mangiferin in subchronic developmental lead-exposed rats. *Biological Trace Element Research* **152** (2) 233–242.

Li Y, Xie C, Murphy SK, Skaar D, Nye M, Vidal AC, Cecil KM, Dietrich KN, Puga A & Jirtle RL *et al.* 2016 Lead exposure during early human development and DNA methylation of imprinted gene regulatory elements in adulthood. *Environmental Health Perspectives* **124** (5) 666–673.

Liao Y, Yu F, Jin Y, Lu C, Li G, Zhi X, An L & Yang J 2008 Selection of micronutrients used along with DMSA in the treatment of moderately lead intoxicated mice. *Archives of Toxicology* **82** (1) 37–43.

Liu C, Huo X, Lin P, Zhang Y, Li W & Xu X 2015 Association between blood erythrocyte lead concentrations and hemoglobin levels in preschool children. *Environmental Science & Pollution Research International* **22** (12) 9233–9240.

Liu J, Liu L, Wang X-F, Teng H-Y & Yang N 2012 Antenatal supplementation of taurine for protection of fetal rat brain with intrauterine growth restriction from injury by reducing neuronal apoptosis. *Neuropediatrics* **43** (5) 258–263.

Lukaszewicz-Hussain A 2011 Liver and serum glutathione concentration and liver hydrogen peroxide in rats subchronically intoxicated with chlorfenvinphos-organophosphate insecticide. *Medycyna Pracy* **62** (1) 23–29.

Marcinkiewicz J & Kontny E 2014 Taurine and inflammatory diseases. *Amino Acids* **46** (1) 7–20.

Mennick F 2006 Two Expanded Cautions for Pregnant Women: Even low levels of lead exposure- and ACE inhibitors in the first trimester- may harm fetal development. *The American Journal of Nursing* **106** (9) 22.

Menzie J, Pan C, Prentice H & Wu J-Y 2014 Taurine and central nervous system disorders. *Amino Acids* **46** (1) 31–46.

Miyazaki T & Matsuzaki Y 2014 Taurine and liver diseases: a focus on the heterogeneous protective properties of taurine. *Amino Acids* **46** (1) 101–110.

Moniem AEA, Dkhil MA & Al-Quraishy S 2010 Protective role of flaxseed oil against lead acetate induced oxidative stress in testes of adult rats. *African Journal of Biotechnology* **9** (42) 7216–7223.

Monir AU, Gundberg CM, Yagerman SE, van der Meulen MCH, Budell WC, Boskey AL & Dowd TL 2010 The effect of lead on bone mineral properties from female adult C57/BL6 mice. *Bone* **47** (5) 888–894.

Navas-Acien A, Guallar E, Silbergeld EK & Rothenberg SJ 2007 Lead exposure and cardiovascular disease—a systematic review. *Environmental Health Perspectives* **115** (3) 472–482.

Nilsson EE, Sadler-Riggleman I & Skinner MK 2018 Environmentally induced epigenetic transgenerational inheritance of disease. *Environmental Epigenetics* **4** (2) dvy016.

Nilsson EE & Skinner MK 2015 Environmentally induced epigenetic transgenerational inheritance of reproductive disease. *Biology of Reproduction* **93** (6) 145.

Nisar MF, Nasir I, Shaheen S, Khalid A & Tazeen N 2011 Chronic lead acetate nephrotoxicity: A histological study on albino rats. *Annals of King Edward Medical University* **17** (3) 239.

Nishimura T, Duereh M, Sugita Y, Yoshida Y, Higuchi K, Tomi M & Nakashima E 2015 Protective effect of hypotaurine against oxidative stress-induced cytotoxicity in rat placental trophoblasts. *Placenta* **36** (6) 693–698.

Nishimura T, Sai Y, Fujii J, Muta M, Iizasa H, Tomi M, Deureh M, Kose N & Nakashima E 2010 Roles of TauT and system A in cytoprotection of rat syncytiotrophoblast cell line exposed to hypertonic stress. *Placenta* **31** (11) 1003–1009.

Oyagbemi AA, Omobowale TO, Akinrinde AS, Saba AB, Ogunpolu BS & Daramola O 2015 Lack of reversal of oxidative damage in renal tissues of lead acetate-treated rats. *Environmental Toxicology* **30** (11) 1235–1243.

Parasuraman S, Raveendran R & Kesavan R 2010 Blood sample collection in small laboratory animals. *Journal of Pharmacology & Pharmacotherapeutics* **1** (2) 87–93

Payne M 2008 Lead in drinking water. *Canadian Medical Association Journal* **179** (3) 253–254.

Rahman A, Al-Rashidi HAG & Khan A-R 2012 Association of maternal blood lead level during pregnancy with child blood lead level and pregnancy outcome in Kuwait. *Ecology of Food & Nutrition* **51** (1) 40–57.

Rajasekaran A & Kalaivani M 2011 Antioxidant activity of aqueous extract of Monascus fermented Indian variety of rice in high cholesterol diet fed-streptozotocin diabetic rats, an in vivo study. *International Journal of Current Scientific Research* **1** (2) 35–38.

Redline RW 2008 Placental pathology: a systematic approach with clinical correlations. *Placenta* **29 Suppl A** S86-91.

Renner R 2010 Exposure on tap: drinking water as an overlooked source of lead. *Environmental Health Perspectives* **118** (2) A68-72.

Roman-Garcia P, Quiros-Gonzalez I, Mottram L, Lieben L, Sharan K, Wangwiwatsin A, Tubio J, Lewis K, Wilkinson D & Santhanam B, Sarper N, Clare S, Vassiliou GS, Velagapudi VR, Dougan G & Yadav VK 2014 Vitamin B(1)(2)-dependent taurine synthesis regulates growth and bone mass. *The Journal of Clinical Investigation* 124 (7) 2988–3002.

Romero AC, Bergamaschi CT, Souza DN de & **Nogueira FN** 2016 Salivary alterations in rats with experimental chronic kidney disease. *PloS One* **11** (2) e0148742.

Roysommuti S & Wyss JM 2014 Perinatal taurine exposure affects adult arterial pressure control. *Amino Acids* **46** (1) 57–72.

Saleh HA, El-Aziz GA, El-Fark MM & El-Gohary M 2009 Effect of maternal lead exposure on craniofacial ossification in rat fetuses and the role of antioxidant therapy. *Anatomia, Histologia, Embryologia* **38** (5) 392–399.

Sanders T, Liu Y, Buchner V & Tchounwou PB 2009 Neurotoxic effects and biomarkers of lead exposure: a review. *Reviews on Environmental Health* **24** (1) 15–45.

Schuller-Levis G & Park E 2006 Is Taurine A Biomarker? *Advances in Clinical Chemistry* **41** 1–21.

Sevin G, Ozsarlak-Sozer G, Keles D, Gokce G, Reel B, Ozgur HH, Oktay G & Kerry Z 2013 Taurine inhibits increased MMP-2 expression in a model of oxidative stress induced by glutathione depletion in rabbit heart. *European Journal of Pharmacology* **706** (1-3) 98–106.

Shannon M 2003 Severe lead poisoning in pregnancy. *Ambulatory Pediatrics* **3** (1) 37–39.

Sharma R & Mogra S 2013 Effects of gestational exposure to lead acetate on implantation and neonatal mice. *Journal of Cell & Molecular Biology* **11** (1/2) 47.

Sharma R, Panwar K & Mogra S 2012 Effects of prenatal and neonatal exposure to lead on white blood cells in Swiss mice. *Journal of Cell & Molecular Biology* **10** (1).

Shivananjappa MM & Muralidhara 2012 Taurine attenuates maternal and embryonic oxidative stress in a streptozotocin-diabetic rat model. *Reproductive Biomedicine Online* **24** (5) 558–566.

Sirdah MM 2015 Protective and therapeutic effectiveness of taurine in diabetes mellitus: a rationale for antioxidant supplementation. *Diabetes & Metabolic Syndrome* **9** (1) 55–64.

Takarada T, Hinoi E, Nakazato R, Ochi H, Xu C, Tsuchikane A, Takeda S, Karsenty G, Abe T, Kiyonari H & Yoneda Y 2013 An analysis of skeletal development in osteoblast-specific and chondrocyte-specific runt-related transcription factor-2 (Runx2) knockout mice. *Journal of Bone & Mineral Research* **28** (10) 2064–2069.

Taziki S, Sattari MR & Eghbal MA 2013 Mechanisms of trazodone-induced cytotoxicity and the protective effects of melatonin and/or taurine toward freshly isolated rat hepatocytes. *Journal of Biochemical & Molecular Toxicology* **27** (10) 457–462.

Thenmozhi M, Dhanalakshmi M, Devi KM, Sushila K & Thenmozhi S 2013 Evaluation of hepatoprotective activity of Leucas aspera hydroalcoholic leaf extract during exposure to lead acetate in male albino Wistar rats. *Asian Journal of Pharmaceutical & Clinical Research* **6** (1) 78–81.

Troesken W 2008 Lead water pipes and infant mortality at the turn of the twentieth century. *Journal of Human Resources* **43** (3) 553–575.

Turkez H & Aydin E 2012 The effects of taurine on permethrin induced cytogenetic and oxidative damage in cultured human lymphocytes. *Arhiv Higijenu Rada Toksikologiju* **63** (1) 27–34.

Vaziri ND 2008 Mechanisms of lead-induced hypertension and cardiovascular disease. *American journal of physiology. Heart & Circulatory Physiology* **295** (2) H454-465.

Verner A, Craig S & McGuire W 2007 Effect of taurine supplementation on growth and development in preterm or low birth weight infants. *The Cochrane Database of Systematic Reviews* (4) CD006072.

Viana MT, Perez MC, Ribas VR, Martins GdF & Castro CMMB de 2012 Leukocyte, red blood cell and morphological adaptation to moderate physical training in rats undernourished in the neonatal period. *Revista Brasileira de Hematologia e Hemoterapia* **34** (4) 285–291.

Wang J, Zhu H, Yang Z & Liu Z 2013 Antioxidative effects of hesperetin against lead acetate-induced oxidative stress in rats. *Indian Journal of Pharmacology* **45** (4) 395–398.

Wang Y, Cha Y-N, Kim KS & Kim C 2011 Taurine chloramine inhibits osteoclastogenesis and splenic lymphocyte proliferation in mice with collagen-induced arthritis. *European Journal of Pharmacology* **668** (1-2) 325–330.

Wang Y, Hu H, Li H, Ma H, Xu F & Qu B 2014 Effects of lead exposure on placental cellular apoptosis and endoplasmic reticulum stress in rats. *Chinese Medical Journal* **127** (9) 1744–1748.

Wojcik OP, Koenig KL, Zeleniuch-Jacquotte A, Costa M & Chen Y 2010 The potential protective effects of taurine on coronary heart disease. *Atherosclerosis* **208** (1) 19–25.

Wu G, Tang R, Yang J, Tao Y, Liu Z, Feng Y, Lin S, Yang Q, Lv Q & Hu J 2015 Taurine accelerates alcohol and fat metabolism of rats with alcoholic Fatty liver disease. *Advances in Experimental Medicine & Biology* **803** 793–805.

Xu X, Liu T, Zhang A, Huo X, Luo Q, Chen Z, Yu L, Li Q, Liu L & Lun ZR & Shen J 2012 Reactive oxygen species-triggered trophoblast apoptosis is initiated by endoplasmic reticulum stress via activation of caspase-12, CHOP, and the JNK pathway in Toxoplasma gondii infection in mice. *Infection & Immunity* **80** (6) 2121–2132.

Yamori Y, Taguchi T, Hamada A, Kunimasa K, Mori H & Mori M 2010 Taurine in health and diseases: consistent evidence from experimental and epidemiological studies. *Journal of Biomedical Science* **17 Suppl 1** S6.

Yang J, Zong X, Wu G, Lin S, Feng Y & Hu J 2015 Taurine increases testicular function in aged rats by inhibiting oxidative stress and apoptosis. *Amino Acids* **47** (8) 1549–1558.

Zhang H, Wei K, Zhang M, Liu R & Chen Y 2014 Assessing the mechanism of DNA damage induced by lead through direct and indirect interactions. *Journal of Photochemistry & Photobiology B, Biology* **136** 46–53.

Zhou L, Huang Q, Wang R, Zhou J, Ma A, Chong L, Wu Y, Wang Y, Xu L, Chen Y, Jia Y, Gui B & Sun Z 2016 Reproductive toxicity of Zishen Yutai pill in rats: The fertility and early embryonic development study (Segment I). *Evidence-Based Complementary & Alternative Medicine* **2016** 3175902.

Zhu M, Fitzgerald EF, Gelberg KH, Lin S & Druschel CM 2010 Maternal low-level lead exposure and fetal growth. *Environmental Health Perspectives* **118** (10) 1471–1475.

Zulli A 2011 Taurine in cardiovascular disease. *Current Opinion in Clinical Nutrition & Metabolic Care* **14** (1) 57–60.

Chapter 5

General Discussion

5.1 General discussion

Heavy metals are among the environmental toxicants incurring great concern for human and animals because low concentrations, even at trace levels, can seriously impair health (Korashy & El-Kadi 2006). Of these, lead (Pb) is a naturally occurring heavy metal and systemic toxicant that has a broad spectrum of harmful effects in human and animal systems (Chang et al. 2012; Flora et al. 2012). The impact of Pb on female reproduction is more profound, where it has been detected in all compartments of female reproductive system in many species (Saleh et al. 2009). Additionally, Pb has been shown to be both embryo-toxic and teratogenic in animal studies. The aim of this PhD work was to investigate the direct effect of Pb on bovine granulosa cells (GCs), oocyte maturation and subsequent embryo development *in vitro*. Moreover, the influence of Pb on Nrf2 and NF-κB, two transcriptional factors involved in maintaining cellular hemostasis against oxidative stress and inflammation respectively, was also investigated. Thereafter, the possibility to use antioxidant regimen as prophylaxis against Pb impact during the course of pregnancy was assessed using rat model.

The reproductive impact of Pb is complicated and seems to involve multiple pathways, most of which are not fully understood. It has been suggested that, ROS-mediated cellular damage could be a major mechanism implicated in Pb pathogenesis (Flora *et al.* 2007). In this context, results revealed that exposure of GCs to Pb induced significant ROS accumulation which remained prominent 24 hours after changing the Pb-containing media. The viability of GCs showed marked decline with Pb exposure as compared to the control group. Since the integrity of ovarian GCs is a key marker of oocyte quality and viability, being critical to protect the oocytes from oxidative stress damage (Tripathi *et al.* 2013), we speculate that Pb-induced ROS can intervene with GCs function and hence folliculogenesis. It was also reported that Pb-treated mice exhibited reduced number of developing follicles and increased number of atretic follicles (Sharma *et al.* 2012; Nakade *et al.* 2014). Moreover, the ROS level may define the cellular fate by modulating the redox-sensitive transcriptional factors such as Nrf2 and NF-κB. The Nrf2 pathway is a pivotal protective self-defense mechanism against oxidative stress within the cell by activating an array of downstream detoxifying genes (Kaspar *et al.* 2009). We showed here significantly lower Nrf2 and Keap-1 transcript abundance with Pb challenge in addition to downregulation of

both SOD and CAT levels. A previous report of Liu *et al.* (2017) showed that Pb decreased the levels of Nrf2 and Keap-1 in the rat kidney.

The NF-κB, is an inducible, pleiotropic transcription factor involved in many physiological and pathological processes, efficiently regulating an array of gene expression (Bellezza *et al.* 2010). The activity of NF-κB was markedly reduced in the present work in Pb-treated groups. According to Bolisetty & Jaimes (2013), low ROS levels are neutralized by Nrf2 activation and its downstream signaling, while NF-κB is activated with moderate ROS levels. However, apoptosis is induced with persistent ROS accumulation in the cell. Although reports showed the activation of NF-κB by ROS in many cell types (Gloire *et al*. 2006), other studies suggested that ROS can potentially repress NF-κB activity (Nakajima & Kitamura 2013). The effect of different metals on NF-κB is even controversial; either activating or suppressing (Liu *et al*. 2013; Xie & Shaikh 2006; Cox *et al*. 2016; Liu *et al*. 2015). Moreover, suppression of NF-κB and its downstream pathway is an underlying mechanism drive to apoptosis by a number of divalent metals (Chen & Shi 2002; Dieguez-Acuna *et al*. 2004), where the binding of metals to the sulfhydryl groups of target protein molecules and the oxidative stress caused by these metals could be plausible mechanisms beyond their effects on the NF-κB activity. Similarly, it has been reported that thiol groups in the enzymes and proteins are target of Pb, and its irreversible binding might alter their function, being a major underlying cause of Pb-derived oxidative stress (Haleagrahara *et al*. 2010; Hasanein *et al*. 2017). This was confirmed by our oxyblot results, which showed a high level of carbonylated protein in Pb-exposed groups.

In the present work, Pb induced cell cycle arrest at the G_0/G_1 checkpoint with decreased cell population at the S phase (DNA synthesis), and this was in line with the work of Yedjou *et al*. (2015). This was further confirmed by investigating two cell proliferation marker genes (PCNA and CCDN2), which showed downregulation under Pb challenge, suggesting that the cells were driven to apoptosis. Our results demonstrated that Pb induced apoptosis in GCs by enhancing the BAX/BCL-2 ratio as compared to the control group, and this was further confirmed by the results obtained by flow cytometry analysis. These finding were consistent with previous results of Yuan *et al*. (2014) and Abdel Moneim (2016). Lead also enhanced endoplasmic reticulum (ER) stress and unfolded protein response (UPR), which was manifested by the upregulation of the chaperon GRP78, simultaneously with elevation of the pro-apoptotic factor CHOP at

transcriptional level. Meanwhile, we observed an elevated protein level of GRP78, suggesting an adaptive response to the potential inactivation of GRP78 by its binding to Pb as reported by Qian *et al*. (2000) and Zhang *et al*. (2008). According to Lee (2014), GRP78 depletion triggers not only the pro-apoptotic CHOP but also intrinsic apoptosis. Moreover, it was reported that, in the absence of Nrf2, UPR is compromised and CHOP has also been found to be upregulated (Meakin *et al*. 2014; Tebay *et al*. 2015). As described before, there is a possible interplay between ROS and NF-κB activation, a similar phenomenon is also observed in the regulation of NF-κB by the UPR. ROS have the potential to induce ER stress, and the consequent UPR can suppress the activation of NF-κB (Kitamura & Hiramatsu 2010). In the early phase, ER stress activates NF-κB transiently, whereas in the later phase, ER stress acts as an inhibitor of NF-κB (Kitamura 2009). However, an early study reported that GRP78 can promote cell survival by activating Akt and NF-κB signaling cascades (Misra *et al*. 2006). This further confirms our suggestion that the upregulated GRP78 may not work properly under Pb exposure. Our findings suggest that Pb-induced oxidative stress dysregulates GCs viability and proliferation, enhances ER stress, induces cell cycle arrest and mediates apoptosis, probably via disruption of the Nrf2/NF-κB cross-talk.

In the second experiment, we investigated the effect of Pb exposure on IVF bovine preimplantation embryos, starting from oocyte maturation (Oocyte only) till 16 cell stage (1-16 cell) or blastocyst stage (All stages). Results revealed similar phenotypes as in GCs, manifested by the impairment of embryonic developmental parameters in all treated groups regardless of the stage of exposure. The cleavage and blastocyst rates were significantly lower as compared to the control group. Exposure to Pb increased ROS accumulation and caused decline in blastocyst cell number. This was in agreement with the early studies of Nandi *et al*. (2010) and Avazeri *et al*. (2006), where it has been suggested that, the reprotoxic effect of Pb results from its interference with oocyte maturation and its ability to support the following steps of development until the embryonic genome activation (EGA).

In the present work, Pb induced significant downregulation of Keap-1 gene expression. Meanwhile, there was no change in the expression of Nrf2 and its downsrtream genes; SOD and HO-1, but there was reduced abundance of CAT transcript. The protein level of Nrf2 showed downregulation in Pb-exposed groups as compared to control group. Moreover, NF-κB and

TNF-α were found to be upregulated in Pb-treated groups. This was consistent with the report by Liu *et al*. (2017), which showed that Pb decreased the levels of Nrf2 and Keap-1 while inducing NF-κB in rat kidney. Here, we demonstrate the induction of NF-κB expression with Pb treatment which is different from the phenotype observed in bovine GCs. This can be attributed to the metal-mediated ROS behavior, which can both induce or supress NF-κB in a phase and context dependent manner (Lingappan 2018; Cox *et al*. 2016; Liu *et al*. 2015). Many studies demonstrated the interaction between NF-κB and Nrf2, being predominantly antagonisitic (Wardyn *et al*. 2015; Sivandzade *et al*. 2018). The role of Nrf2 as upstream regulator of inflammatory cytokines, independent on its redox control function, has been displayed by Kobayashi *et al.* (2016). Therefore, we may conclude that NF-κB activated by Pb exposure and subsequent ROS accumulation, triggers inflammatory responses and can antagonize the activity of Nrf2 at the transcriptional level (Sivandzade *et al*. 2018).

Blastocysts derived from Pb-exposed groups exhibited downregulation of the pro-apototic BCL-2 along with upregulation of the apoptotic gene BAX. This was confirmed by elevation of the number of TUNEL positive nuclei after Pb treatment. We further evaluated the effect of Pb on the mRNA expression of DNA methyltransferases (DNMT3A, DNMT3B and DNMT1) and results showed the upregulation of DNMT1, the gene involved in maintenance of DNA methylation. Similar results were reported by Schneider *et al*. (2013) where hippocampal DNMTs were affected by developmental Pb exposure in an animal model. *In vitro* Pb exposure of human embryonic kidney cell line showed change in differentially methylated region (DMR) regulating some imprinted genes such as IGF-2 (Nye *et al*. 2015). From the previous results we conclude that Pb displayed a harmful effect on bovine early embryonic development through oxidative and inflammatory stress probably via Nrf2/NF-κB signaling pathways. However, further investigations are required to evaluate DNA methylation dynamics in bovine embryos under Pb exposure and verify wheather this mechanism contributes to Nrf2/NF-κB perturbation.

In the third experiment, we investigated the effect of Pb *in vivo*, using rat model, and the possibility to guard against its deleterious effects during the course of pregnancy using antioxidant regimen. Taurine (TA), a conditionally essential amino acid, can be synthesized in mammals from methionine and serine. However, during pregnancy, TA is essential due to the shortage of necessary enzymes in both fetus and placenta. Therefore, the demand for TA must be

covered by its transport from maternal plasma to the placenta where it is critical for growth and development throughout life (Verner *et al*. 2007; Nishimura *et al*. 2010; Roysommuti & Wyss 2014).

In this experiment, pregnant rats were orally ingested by Pb during the period of organogenesis, while TA was given throughout the gestation period. Results showed a decrease in the maternal body weight gain during pregnancy and a high percentage of abortion as well as some alteration in the gross morphological and anatomical features of fetuses compared to control group. These results were in agreement with the findings of Sharma & Mogra (2013) and Edwards (2014), which showed that Pb exposure during pregnancy results in post-implantation losses, growth retardation and high incidence of fetal mortality. The present work also revealed that prenatal Pb exposure caused significant decline in fetal body weight, crown-rump length and fetal tail length. This was in accordance with Saleh *et al.* (2009), Rahman *et al.* (2012) and Al-Saleh *et al.* (2014), where Pb exposure in very early stages of pregnancy hindered normal organogenesis.

As shown in the present work, there was significant reduction in placental weight of Pb-treated dams compared to the control group. Moreover, histopathological examination of placenta showed vascular congestion, hemorrhage, degeneration and inflammatory infiltration in Pb-exposed dams compared to the control group. Oxidative stress has been reported to be a major mechanism of Pb toxicity (Flora *et al*. 2012). Furthermore, Studies reported that Pb exposure resulted in ROS-mediated ER stress via caspase-12 activation which in turn triggers procaspase-9 and caspase-3 leading to apoptosis and the pathophysiology of the placenta (Xu *et al.* 2012; Wang *et al.* 2014).

Fetuses maternally exposed to Pb showed severe lack of ossification in most components of the skeletal system and these results coincide with Li *et al.* (2013), Figueiredo *et al.* (2014) and Grizlova & Yakimova (2014). Many hypotheses have been reported to explain the impact of Pb on bone development. Due to ionic mimicry, Pb was found to displace Ca in the mineral bone matrix and consequently disturb the hormonal regulation of Ca absorption (Conti *et al.* 2012). Several studies showing that ROS stimulate osteoclast differentiation and bone resorption through uncoupling of the delicate balance between resorption and formation, and this will result in osteoporosis and high fracture risk (Baek *et al.* 2010; Cervellati *et al.* 2014). On the contrary, TA pretreatment improved the maternal parameters, the fetal body growth and bone formation in

the current study compared to Pb-exposed group. Liu *et al*. (2012) concluded that perinatal TA supplementation may reduce neuronal apoptosis in rat fetuses via up-regulating the ratio of BCL2/BAX and down-regulating the expression of caspase-3. Additionally, the cytoprotective role of TA in placenta was revealed in the present study and this was previously documented by the studies of Desforges *et al.* (2015) and Nishimura *et al.* (2015). Pretreatment with TA showed also enhancement of the hematological parameters like hemoglobin content, RBCs count and reduced WBCs count in both dams and fetuses as compared to Pb group. These events could be assigned to the enhanced stability of RBCs and preservation of the normal levels of the antioxidant enzymes and metabolites of the erythrocytes as found in the results of Roy & Sil (2012) and Sirdah *et al*. (2013).

Unlike Pb-exposed rats, there was attenuation of lipid peroxidation in the livers of dams and their fetuses with TA pretreatment, in addition to the improvement in hepatic GSH content and CAT activity. This effect was in agreement with Shivananjappa & Muralidhara (2012), who demonstrated that, TA mitigates maternal and embryonic oxidative stress in a streptozotocin-diabetic rat model. Furthermore, TA protects the livers of dams and their fetuses from liver injury since our results showed normalization of the activities of the enzymes AST, ALT and ALP in TA+Pb group. This was in agreement with the work of Wu *et al*. (2015), which reported the protective role of TA in rats with alcoholic fatty liver. This could be attributed to the ability of TA to decrease oxidative stress, enhance mitochondrial function, modulate Ca homeostasis and inhibit hepatocytes apoptosis (Asha & Devadasan 2013; Zhang *et al*. 2014).

Similarly, a significant reduction in the levels of serum urea and creatinine was observed in TA+ Pb group compared to the Pb group. The previous work of Das & Sil (2012), Koh *et al*. (2014) and Sirdah (2015) showed that TA delayed the onset of nephropathy in diabetic rats through its antioxidant, antiapoptotic and membrane stabilizing effects. TA also exhibits nephroprotection by regulating blood flow in the renal vasculature and Na transport in the proximal tubules, thus maintaining osmoregulation and scavenging ROS in the glomerulus (Karbalay-Doust *et al*. 2012; Han & Chesney 2012). Furthermore, concomitant administration of TA along with Pb alleviated DNA fragmentation in dams and their fetuses. Matching with these results, TA was found to reduce DNA damage and DNA- protein cross-links as well as single strand breaks induced by potassium bromate in rat intestine (Ahmad *et al*. 2015).

It is noteworthy to mention that TA has been reported to activate Nrf2 signaling pathway (Gebara *et al*. 2015; Lu *et al*. 2019) in addition to be an anti-inflammatory agent suppressing NF-κB *in vivo* (Gebara *et al*. 2015), making TA a good candidate against Pb toxicity. In summary, this study emphasizes the detrimental effect of Pb on the pregnant rats as well as the developing fetuses and highlights the role of TA as a potential prophylactic antioxidant against Pb insult.

5.2 Future prespects

Compared to other pathological conditions, research in the reproductive disorders is complicated especially in females during pregnancy. ROS-mediated signaling molecules like Nrf2, NF-κB and FOXO-1 proved to have great significance in both preventing and contributing to diseases, making their therapeutic targeting a challenge. Additionally, epigenetics has emerged as another layer for regulation of these molecules (Cheng *et al*. 2016). Therefore, further studies are required to understand the dual role of these signaling molecules in oocyte maturation and early embryo development considering the role of epigenetics in their regulation and interaction.

Furthermore, robust evidence showed that reproductive disease and infertility may be, in part, due to the ancestral environmental exposures through epigenetic transgenerational inheritance mechanisms. This in turn support the hypothesis "Developmental Origins of Health and Disease" (DOHaD) (Nilsson *et al*. 2018). Epigenetic alterations with toxic metal exposure have been demonstrated, suggesting that heavy metals could be epigenetic modifiers and this may also describe the differential susceptibility of individuals to metal toxicity. Therefore, epigenetic screening may confer a diagnostic tool to explore the ancestral toxicant exposures, so actions can be taken to help mitigate possible disease onset. Unlike the static genetic mutations, epigenetic alterations such as aberrant DNA methylation are potentially reversible and could be targeted paving the way for novel epigenetic-based therapy. A better understanding of miRNA signature in the ovary under heavy metal exposure should also be considered.

Moreover, the interest in extracellular vesicles (EVs) research and their role in cell-cell communication have increased in the past few years. However, the link between exposure to toxins, altered EVs cargo and disease progression are not fully understood (Harischandra *et al*. 2017). So future work is needed to reveal how environmental toxins trigger EVs biogenesis and

define their contents and how the released cargo could reprogram the cellular response leading to disease progression.

Chapter 6

Summary

Preconceptional and prenatal exposure to environmental heavy metals induce severe and long lasting effects on fertility, in general, and pregnancy outcomes in particular. Of these, lead (Pb) has been shown to be both embryo-toxic and teratogenic in variety of animal species. However, limited data is available about the direct effect of Pb on bovine granulosa cells (GCs) and embryo development. The aim of this thesis was to investigate the effect of Pb on bovine GCs, oocyte maturation and subsequent embryo development *in vitro* and *in utero* during pregnancy with potential application of antioxidants against Pb toxicity. Moreover, the influence of Pb on Nrf2 and NF-κB, two transcriptional factors (TFs) involved in maintaining cellular hemostasis against oxidative stress and inflammation respectively, was also investigated.

In the first experiment, bovine ovarian GCs aspirated from small growing follicles (3-5 mm of diameter) were *in vitro* cultured and, after confluency, they were challenged with three doses of lead acetate (1, 2 or 3 µg/ml) for 2 hours. The cell viability and the accumulation of intracellular reactive oxygen species (ROS) were assessed 24 hours later. Moreover, cells were harvested and subjected to total RNA isolation, cDNA synthesis and the gene expression analysis. The protein lysate from GCs was used to assess carbonylated protein. Results showed that, exposure of GCs to Pb evoked intracellular ROS accumulation and protein carbonylation. This was accompanied by cell cycle arrest at G_0/G_1 phase, reduction in cell viability and decrease of the expression of cell proliferation marker genes (CCND2 and PCNA). Moreover, Pb downregulated both Nrf2 and NF-κB and their downstream antioxidant genes, SOD and CAT, while no significant differences were shown in the level of other genes involved in both TFs pathways (Keap-1, HO-1, Thrx, IKK and TNF-α). In addition, Pb challenge increased the expression of endoplasmic reticulum stress marker genes, GRP78 and CHOP and the pro-apoptotic gene, caspase-3 while reduced the anti-apoptotic gene, BCL-2.

In order to investigate the effect of Pb toxicity on bovine oocyte maturation and preimplantation embryo development, *in vitro* cultured oocytes and embryos were used in the absence (Control) or presence of Pb (10 µg/ml). Oocytes were aspirated from bovine ovaries and matured. Following IVF using frozen-thawed spermatozoa, the resultant Pb-exposed zygotes were further randomly divided into three groups cultured in the absence of Pb (Oocyte only) or in presence of Pb either until the 16 cell (1-16 cell) stage or blastocyst stage (All stages). Cleavage rates and blastocyst developmental rates were recorded. Following this, the day 8 blastocysts of different

experimental groups were analyzed for ROS accumulation and subjected to RNA isolation, cDNA synthesis and the gene expression. The protein levels of Nrf2 and NF-κB were detected using immunostaining. The results showed a reduction in the cleavage and blastocyst rates in all Pb-exposed groups. In addition, Pb toxicity reduced blastocyst cell number along with ROS accumulation. We further displayed upregulation of NF-κB on both transcriptional and protein levels along with downregulation of the protein level of Nrf2 and this was accompanied by elevation of TNF-α. Apoptosis under Pb exposure has been shown by the higher ratio of BAX/BCL-2 and the number of TUNEL positive nuclei as compared to control group. Moreover, Pb significantly induced the expression of DNMT1, a gene involved in maintenance of DNA methylation.

In the third experiment, we evaluated the role of taurine (TA) against Pb-induced toxicity in pregnant albino rats and their fetuses. For this, pregnant rats at day 1 of gestation were divided into four groups (10 rats of each), where group 1 was given distilled water and served as control. The second group received Pb (233.25 mg/kg) orally from day 7 to 16 of gestation (the organogenesis period). The 3rd group received TA (50 mg/kg) orally throughout the gestation period, while the 4th group received both TA throughout the gestation period and Pb from day 7 to 16 of gestation. Dams were sacrificed on the 20th day of gestation period and fetuses were removed by cesarean section. Fetal mortality, morphological examination, body weight and length were recorded. Blood samples were collected for hematological and biochemical parameter assessment. Moreover, hepatic malondialdehyde (MDA), reduced glutathione (GSH) and CAT were also analyzed. Results revealed that, Pb caused a reduction in the maternal body weight gain, increase in the rate of abortion, as well as fetal growth retardation and skeletal malformations. Additionally, Pb induced hematological and biochemical alterations in both dams and fetuses. The toxicity of Pb was further emphasized by histopathological examination of the placenta and hepatic DNA fragmentation. However, these events have been mitigated by TA pretreatment without affecting the normal course of pregnancy.

All in all, the present work demonstrates that exposure to Pb-induced oxidative stress attenuates bovine GCs proliferation and alters cell cycle progression, exposing the cells to apoptosis. This was further confirmed in preimplantation embryo development, which exhibited aberrant developmental parameters regardless the Pb exposure stage, suggesting that the reprotoxic effect of Pb originates from its interference with oocyte maturation and its ability to support the

subsequent development until the embryonic genome activation (EGA). The deleterious effect of Pb-induced ROS may be through disruption of the Nrf2/NF-κB interaction and this effect could vary according to the dose, the period of exposure and the type of cells tested. Moreover, antioxidants such as taurine could be a reassuring approach to be used as prophylactic agents against environmental heavy metal exposure during pregnancy.

Chapter 7

References

References of chapter 1

Aban M, Cinel L, Arslan M, Dilek U, Kaplanoglu M, Arpaci R & Dilek S 2004 Expression of nuclear factor-kappa B and placental apoptosis in pregnancies complicated with intrauterine growth restriction and preeclampsia: An immunohistochemical study. *The Tohoku Journal of Experimental Medicine* **204** (3) 195–202.

Acar N, Soylu H, Edizer I, Ozbey O, Er H, Akkoyunlu G, Gemici B & Ustunel I 2014 Expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and peroxiredoxin 6 (Prdx6) proteins in healthy and pathologic placentas of human and rat. *Acta Histochemica* **116** (8) 1289–1300.

Ackerman WE, 4th, Summerfield TLS, Vandre DD, Robinson JM & Kniss DA 2008 Nuclear factor-kappa B regulates inducible prostaglandin E synthase expression in human amnion mesenchymal cells. *Biology of Reproduction* **78** (1) 68–76.

Adonaylo VN & Oteiza PI 1999 Pb^{2+} promotes lipid oxidation and alterations in membrane physical properties. *Toxicology* **132** (1) 19–32.

Agarwal A, Gupta S & Sharma RK 2005 Role of oxidative stress in female reproduction. *Reproductive Biology & Endocrinology* **3** 28.

Agarwal A, Gupta S & Sikka S 2006 The role of free radicals and antioxidants in reproduction. *Current Opinion in Obstetrics & Gynecology* **18** (3) 325–332.

Agarwal A, Gupta S, Sekhon L & Shah R 2008 Redox considerations in female reproductive function and assisted reproduction: From molecular mechanisms to health implications. *Antioxidants & Redox Signaling* **10** (8) 1375–1403.

Ahamed M & Siddiqui MKJ 2007a Environmental lead toxicity and nutritional factors. *Clinical Nutrition* **26** (4) 400–408.

Ahamed M & Siddiqui MKJ 2007b Low level lead exposure and oxidative stress: Current opinions. *Clinica Chimica Acta* **383** (1-2) 57–64.

Ahamed M, Singh S, Behari JR, Kumar A & Siddiqui MKJ 2007 Interaction of lead with some essential trace metals in the blood of anemic children from Lucknow, India. *Clinica Chimica Acta* **377** (1-2) 92–97.

Ahmed SMU, Luo L, Namani A, Wang XJ & Tang X 2017 Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochimica et biophysica acta. Molecular Basis of Disease* **1863** (2) 585–597.

Ahmed, YF, Eldebaky HAA, Mahmoud KGM & Nawito M 2012 Effects of lead exposure on DNA damage and apoptosis in reproductive and vital organs in female rabbit. *Global Veterinaria* **9** (4) 401–408.

Akino N, Wada-Hiraike O, Terao H, Honjoh H, Isono W, Fu H, Hirano M, Miyamoto Y, Tanikawa M & Harada, M Hirata T, Hirota Y, Koga K, Oda K, Kawana K, Fujii T & Osuga Y 2018 Activation of Nrf2 might reduce oxidative stress in human granulosa cells. *Molecular & Cellular Endocrinology* **470** 96–104.

An J, Cai T, Che H, Yu T, Cao Z, Liu X, Zhao F, Jing J, Shen X, Liu M, Du K, Chen J & Luo W 2014 The changes of miRNA expression in rat hippocampus following chronic lead exposure. *Toxicology Letters* **229** (1) 158–166.

Antonio-Garcia MT & Masso-Gonzalez EL 2008 Toxic effects of perinatal lead exposure on the brain of rats: Involvement of oxidative stress and the beneficial role of antioxidants. *Food & Chemical Toxicology* **46** (6) 2089–2095.

Aoki Y, Hashimoto AH, Amanuma K, Matsumoto M, Hiyoshi K, Takano H, Masumura K-i, Itoh K, Nohmi T & Yamamoto M 2007 Enhanced spontaneous and benzo(a)pyrene-induced mutations in the lung of Nrf2-deficient gpt delta mice. *Cancer Research* **67** (12) 5643–5648.

Aoki Y, Sato H, Nishimura N, Takahashi S, Itoh K & Yamamoto M 2001 Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicology & Applied Pharmacology* **173** (3) 154–160.

Arruti A, Fernandez-Olmo I & Irabien A 2010 Evaluation of the contribution of local sources to trace metals levels in urban PM2.5 and PM10 in the Cantabria region (Northern Spain). *Journal of Environmental Monitoring* **12** (7) 1451–1458.

Avery SV 2011 Molecular targets of oxidative stress. *The Biochemical Journal* **434** (2) 201–210.

Baerwald AR, Adams GP & Pierson RA 2012 Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Human Reproduction Update* **18** (1) 73–9.

Baldwin AS, JR 1996 The NF-kappa B and I kappa B proteins: New discoveries and insights. *Annual Review of Immunology* **14** 649–683.

Bandyopadhyay D, Ghosh D, Chattopadhyay A, Firdaus SB, Ghosh AK, Paul S, Bhowmik D, Mishra S & Dalui K 2014 Lead induced oxidative stress: A health issue of global concern. *Pharmacy Research* **8** 1198–1207.

Bannert N & Kurth R 2006 The evolutionary dynamics of human endogenous retroviral families. *Annual Review of Genomics & Human Genetics* **7** 149–173.

Barbano R, Muscarella LA, Pasculli B, Valori VM, Fontana A, Coco M, La Torre A, Balsamo T, Poeta ML, Marangi GF, Maiello E, Castelvetere M, Pellegrini F, Murgo R, Fazio VM & Parrella P 2013 Aberrant Keap1 methylation in breast cancer and association with clinicopathological features. *Epigenetics* **8** (1) 105–112.

Barchitta M, Quattrocchi A, Maugeri A, Vinciguerra M & Agodi A 2014 LINE-1 hypomethylation in blood and tissue samples as an epigenetic marker for cancer risk: A systematic review and meta-analysis. *PloS One* **9** (10) e109478.

Basha CD & Reddy RG 2015 Long-term changes in brain cholinergic system and behavior in rats following gestational exposure to lead: Protective effect of calcium supplement. *Interdisciplinary Toxicology* **8** (4) 159–168.

Baud V & Karin M 2009 Is NF-κB a good target for cancer therapy? Hopes and pitfalls. *Nature Reviews. Drug Discovery* **8** (1) 33–40.

Bayat F, Akbari SAA, Dabirioskoei A, Nasiri M & Mellati A 2016 The Relationship between blood lead level and preeclampsia. *Electronic Physician* **8** (12) 3450–3455.

Beck CR, Collier P, Macfarlane C, Malig M, Kidd JM, Eichler EE, Badge RM & Moran JV 2010 LINE-1 retrotransposition activity in human genomes. *Cell* **141** (7) 1159–1170.

Beck CR, Garcia-Perez JL, Badge RM & Moran JV 2011 LINE-1 elements in structural variation and disease. *Annual Review of Genomics & Human Genetics* **12** 187–215.

Becker KG, Swergold GD, Ozato K & Thayer RE 1993 Binding of the ubiquitous nuclear transcription factor YY1 to a cis regulatory sequence in the human LINE-1 transposable element. *Human Molecular Genetics* **2** (10) 1697–1702.

Bedaiwy MA, Falcone T, Sharma RK, Goldberg JM, Attaran M, Nelson DR & Agarwal A 2002 Prediction of endometriosis with serum and peritoneal fluid markers: A prospective controlled trial. *Human Reproduction* **17** (2) 426–431.

Belancio VP, Deininger PL & Roy-Engel AM 2009 LINE dancing in the human genome: Transposable elements and disease. *Genome Medicine* **1** (10) 97.

Bellinger DC 2004 Lead. *Pediatrics* **113** (4 Suppl) 1016–1022.

Bellinger DC 2013 Prenatal exposures to environmental chemicals and children's neurodevelopment: An Update. *Safety & Health at Work* **4** (1) 1–11.

Bihaqi SW, Huang H, Wu J & Zawia NH 2011 Infant exposure to lead (Pb) and epigenetic modifications in the aging primate brain: Implications for Alzheimer's disease. *Journal of Alzheimer's Disease* **27** (4) 819–833.

Bilodeau-Goeseels S 2011 Cows are not mice: The role of cyclic AMP, phosphodiesterases, and adenosine monophosphate-activated protein kinase in the maintenance of meiotic arrest in bovine oocytes. *Molecular Reproduction & Development* **78** (10-11) 734–743.

Biri A, Bozkurt N, Turp A, Kavutcu M, Himmetoglu O & Durak I 2007 Role of oxidative stress in intrauterine growth restriction. *Gynecologic & Obstetric Investigation* **64** (4) 187–192.

Bollati V & Baccarelli A 2010 Environmental epigenetics. *Heredity* **105** (1) 105–112.

Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D, Byun H-M, Jiang J, Marinelli B, Pesatori AC, Bertazzi PA & Yang AS 2007 Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Research* **67** (3) 876–880.

Bourc'his D & Bestor TH 2004 Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature* **431** (7004) 96–99.

Bowers TS & Beck BD 2006 What is the meaning of non-linear dose-response relationships between blood lead concentrations and IQ? *Neurotoxicology* **27** (4) 520–524.

Bowie A & O'Neill LAJ 2000 Oxidative stress and nuclear factor-κB activation-Bowie A and O'Neill LAJ, unpublished results: A reassessment of the evidence in the light of recent discoveries. *Biochemical Pharmacology* **59** (1) 13–23.

Brehm MA, Jouvet N, Greiner DL & Shultz LD 2013 Humanized mice for the study of infectious diseases. *Current Opinion in Immunology* **25** (4) 428–435.

Bremer S, Pellizzer C, Hoffmann S, Seidle T & Hartung T 2007 The development of new concepts for assessing reproductive toxicity applicable to large scale toxicological programmes. *Current Pharmaceutical Design* **13** (29) 3047–3058.

Bressler J, Kim KA, Chakraborti T & Goldstein G 1999 Molecular mechanisms of lead neurotoxicity. *Neurochemical Research* **24** (4) 595–600.

Bressler JP, Olivi L, Cheong JH, Kim Y & Bannona D 2004 Divalent metal transporter 1 in lead and cadmium transport. *Annals of the New York Academy of Sciences* **1012** 142–152.

Brochin R, Leone S, Phillips D, Shepard N, Zisa D & Angerio A 2008 The cellular effect of lead poisioning and its clinical picture. *Issues* **5** (2).

Brouha B, Schustak J, Badge RM, Lutz-Prigge S, Farley AH, Moran JV & Kazazian HH Jr 2003 Hot L1s account for the bulk of retrotransposition in the human population. *PNAS* **100** (9) 5280–5285.

Buzzelli MD, Nagarajan M, Radtka JF, Shumate ML, Navaratnarajah M, Lang CH & Cooney RN 2008 Nuclear factor-kappa B mediates the inhibitory effects of tumor necrosis factor-alpha on growth hormone-inducible gene expression in liver. *Endocrinology* **149** (12) 6378–6388.

Camandola S & Mattson MP 2007 NF-kappa B as a therapeutic target in neurodegenerative diseases. *Expert Opinion on Therapeutic Targets* **11** (2) 123–132.

Campagna C, Ayotte P, Sirard M-A, Arsenault G, Laforest J-P & Bailey JL 2007 Effect of an environmentally relevant metabolized organochlorine mixture on porcine cumulus-oocyte complexes. *Reproductive Toxicology* **23** (2) 145–152.

Carnell AN & Goodman JI 2003 The long (LINEs) and the short (SINEs) of it: Altered methylation as a precursor to toxicity. *Toxicological Sciences* **75** (2) 229–235.

Casalino E, Calzaretti G, Landriscina M, Sblano C, Fabiano A & Landriscina C 2007 The Nrf2 transcription factor contributes to the induction of alpha-class GST isoenzymes in liver of acute cadmium or manganese intoxicated rats: Comparison with the toxic effect on NAD(P)H:quinone reductase. *Toxicology* **237** (1-3) 24–34.

Caylak E, Aytekin M & Halifeoglu I 2008 Antioxidant effects of methionine, alpha-lipoic acid, N-acetylcysteine and homocysteine on lead-induced oxidative stress to erythrocytes in rats. *Experimental & Toxicologic Pathology* **60** (4-5) 289–294.

Cellier M, Prive G, Belouchi A, Kwan T, Rodrigues V, Chia W & Gros P 1995 Nramp defines a family of membrane proteins. *Proceedings of the National Academy of Sciences* **92** (22) 10089–10093.

Cengiz B, Soylemez F, Ozturk E & Cavdar AO 2004 Serum zinc, selenium, copper, and lead levels in women with second-trimester induced abortion resulting from neural tube defects: A preliminary study. *Biological Trace Element Research* **97** (3) 225–235.

Ceriello A 2008 Possible role of oxidative stress in the pathogenesis of hypertension. *Diabetes Care* **31 Suppl 2** S181-S184.

Chalopin D, Naville M, Plard F, Galiana D & Volff JN 2015 Comparative analysis of transposable elements highlights mobilome diversity and evolution in vertebrates. *Genome Biology & Evolution* **7** (2) 567–580.

Chandramouli K, Steer CD, Ellis M & Emond AM 2009 Effects of early childhood lead exposure on academic performance and behaviour of school age children. *Archives of Disease in Childhood* **94** (11) 844–848.

Cheng X, Chapple SJ, Patel B, Puszyk W, Sugden D, Yin X, Mayr M, Siow RCM & Mann GE 2013 Gestational diabetes mellitus impairs Nrf2-mediated adaptive antioxidant defenses and redox signaling in fetal endothelial cells in utero. *Diabetes* **62** (12) 4088–4097.

Chigusa Y, Tatsumi K, Kondoh E, Fujita K, Nishimura F, Mogami H & Konishi I 2012 Decreased lectin-like oxidized LDL receptor 1 (LOX-1) and low Nrf2 activation in placenta are involved in preeclampsia. *Journal of Clinical Endocrinology & Metabolism* **97** (10) E1862-E1870.

Cho HH, Shin KK, Kim YJ, Song JS, Kim JM, Bae YC, Kim CD & Jung JS 2010 NF-kappaB activation stimulates osteogenic differentiation of mesenchymal stem cells derived from human adipose tissue by increasing TAZ expression. *Journal of Cellular Physiology* **223** (1) 168–177.

Cho H-Y, Reddy SP, Debiase A, Yamamoto M & Kleeberger SR 2005 Gene expression profiling of NRF2-mediated protection against oxidative injury. *Free Radical Biology & Medicine* **38** (3) 325–343.

Choudhary R, Chawala VK, Soni ND, Kumar J & Vyas RK 2010 Oxidative stress and role of antioxidants in male infertility. *Pakistan Journal of Physiology* **6** (2) 54–59.

Chowdhury AR 2009 Recent advances in heavy metals induced effect on male reproductive function-A retrospective. *Al Ameen Journal of Medical Science* **2** (2) 37–42.

Cindrova-Davies T, Yung HW, Johns J, Spasic-Boskovic O, Korolchuk S, Jauniaux E, Burton GJ & Charnock-Jones DS 2007 Oxidative stress, gene expression, and protein changes induced in the human placenta during labor. *The American Journal of Pathology* **171** (4) 1168–1179.

Cookson VJ & Chapman NR 2010 NF-kappa B function in the human myometrium during pregnancy and parturition. *Histology & Histopathology* **25** (7) 945–956.

Cordaux R & Batzer MA 2009 The impact of retrotransposons on human genome evolution. *Nature reviews. Genetics* **10** (10) 691–703.

Cory-Slechta DA 2012 Low level lead exposure harms children: A Renewed call for primary prevention.

Cox JN, Rahman MA, Bao S, Liu M, Wheeler SE & Knoell DL 2016 Cadmium attenuates the macrophage response to LPS through inhibition of the NF-κB pathway. *American Journal of Physiology. Lung Cellular & Molecular Physiology* **311** (4) L754-L765.

Crews D, Gillette R, Miller-Crews I, Gore AC & Skinner MK 2014 Nature, nurture and epigenetics. *Molecular & Cellular Endocrinology* **398** (1-2) 42–52.

Criscione SW, Zhang Y, Thompson W, Sedivy JM & Neretti N 2014 Transcriptional landscape of repetitive elements in normal and cancer human cells. *BMC Genomics* **15** 583.

Crow JF 2000 The origins, patterns and implications of human spontaneous mutation. *Nature reviews. Genetics* **1** (1) 40–47.

Dairam A, Limson JL, Watkins GM, Antunes E & Daya S 2007 Curcuminoids, curcumin, and demethoxycurcumin reduce lead-induced memory deficits in male Wistar rats. *Journal of Agricultural & Food Chemistry* **55** (3) 1039–1044.

Dalzell LH, McVicar CM, McClure N, Lutton D & Lewis SEM 2004 Effects of short and long incubations on DNA fragmentation of testicular sperm. *Fertility & Sterility* **82** (5) 1443–1445.

Delfino FJ, Boustead JN, Fix C & Walker WH 2003 NF-kappa B and TNF-alpha stimulate androgen receptor expression in Sertoli cells. *Molecular & Cellular Endocrinology* **201** (1-2) 1–12.

Dewannieux M, Esnault C & Heidmann T 2003 LINE-mediated retrotransposition of marked Alu sequences. *Nature Genetics* **35** (1) 41–48.

DiDonato JA, Mercurio F & Karin M 2012 NF-kappaB and the link between inflammation and cancer. *Immunological Reviews* **246** (1) 379–400.

Dijkers PF, Medema RH, Lammers JW, Koenderman L & Coffer PJ 2000 Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Current Biology* **10** (19) 1201–1204.

Dinkova-Kostova AT & Talalay P 2008 Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Molecular Nutrition & Food Research* **52 Suppl 1** S128-S138.

Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, Katoh Y, Yamamoto M & Talalay P 2002 Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences* **99** (18) 11908.

Diouf A, Garcon G, Diop Y, Ndiaye B, Thiaw C, Fall M, Kane-Barry O, Ba D, Haguenoer JM & Shirali P 2006 Environmental lead exposure and its relationship to traffic density among Senegalese children: A cross-sectional study. *Human & Experimental Toxicology* **25** (11) 637–644.

Dosunmu R, Alashwal H & Zawia NH 2012 Genome-wide expression and methylation profiling in the aged rodent brain due to early-life Pb exposure and its relevance to aging. *Mechanisms of Ageing & Development* **133** (6) 435–443.

Doumouchtsis KK, Doumouchtsis SK, Doumouchtsis EK & Perrea DN 2009 The effect of lead intoxication on endocrine functions. *Journal of Endocrinological Investigation* **32** (2) 175–183.

Driancourt MA 2001 Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology* **55** (6) 1211–1239.

Droge W 2002 Free radicals in the physiological control of cell function. *Physiological Reviews* **82** (1) 47–95.

Duncan EA, Goetz CA, Stein SJ, Mayo KJ, Skaggs BJ, Ziegelbauer K, Sawyers CL & Baldwin AS 2008 IkappaB kinase beta inhibition induces cell death in Imatinib-resistant and T315I Dasatinib-resistant BCR-ABL+ cells. *Molecular Cancer Therapeutics* **7** (2) 391–397.

Eid A & Zawia N 2016 Consequences of lead exposure, and it's emerging role as an epigenetic modifier in the aging brain. *Neurotoxicology* **56** 254–261.

El-Nekeety AA, El-Kady AA, Soliman MS, Hassan NS & Abdel-Wahhab MA 2009 Protective effect of Aquilegia vulgaris (L.) against lead acetate-induced oxidative stress in rats. *Food & Chemical Toxicology* **47** (9) 2209–2215.

Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, Harada T & Yamamoto M 2001 High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. *Toxicological Sciences* **59** (1) 169–177.

Essers MAG, Weijzen S, Vries-Smits AMM, **Saarloos I, Ruiter ND**, **Bos JL & Burgering BMT** 2004 FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *The EMBO Journal* **23** (24) 4802–4812.

Eum K-D, Wang FT, Schwartz J, Hersh CP, Kelsey K, Wright RO, Spiro A, Sparrow D, Hu H & Weisskopf MG 2013 Modifying roles of glutathione S-transferase polymorphisms on the association between cumulative lead exposure and cognitive function. *Neurotoxicology* **39** 65–71.

Eum K-D, Weisskopf MG, Nie LH, Hu H & Korrick SA 2014 Cumulative lead exposure and age at menopause in the Nurses' Health Study cohort. *Environmental Health Perspectives* **122** (3) 229–234.

Fang JY, Wang PW, Huang CH, Hung YY & Pan TL 2014 Evaluation of the hepatotoxic risk caused by lead acetate via skin exposure using a proteomic approach. *Proteomics* **14** (21-22) 2588–2599.

Ferreiro DU & Komives EA 2010 Molecular mechanisms of system control of NF-kappa B signaling by I kappa B alpha. *Biochemistry* **49** (8) 1560–1567.

Fisher, Anna E. O. & Naughton DP 2003 Redox-active metal ions and oxidative stress: Therapeutic implications. *Proceedings-Indian National Science* **69** (4) 453–460.

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

Flora GJ & Seth PK 2000 Alterations in some membrane properties in rat brain following exposure to lead. *Cytobios* **103** (403) 103–109.

Flora SJS & Pachauri V 2010 Chelation in metal intoxication. *International Journal of Environmental Research & Public Health* **7** (7) 2745–2788.

Flora SJS 2009 Nutritional Components Modify Metal Absorption, Toxic Response and Chelation Therapy. *Journal of Nutritional & Environmental Medicine* **12** (1) 53–67.

Flora SJS, Flora G, Saxena G & Mishra M 2007 Arsenic and lead induced free radical generation and their reversibility following chelation. *Cellular & Molecular Biology* **53** (1) 26–47.

Flora SJS, Mittal M & Mehta A 2008 Heavy metal induced oxidative stress & its possible reversal by chelation therapy. *The Indian Journal of Medical Research* **128** (4) 501–523.

Flora SJS, Pande M & Mehta A 2003 Beneficial effect of combined administration of some naturally occurring antioxidants (vitamins) and thiol chelators in the treatment of chronic lead intoxication. *Chemico-Biological Interactions* **145** (3) 267–280.

Flora SJS, Pande M, Kannan GM & Mehta A 2004 Lead induced oxidative stress and its recovery following co-administration of melatonin or N-acetylcysteine during chelation with succimer in male rats. *Cellular & Molecular Biology* **50 Online Pub** OL543-51.

Florea A-M, Taban J, Varghese E, Alost BT, Moreno S & Büsselberg D 2013 Lead (Pb^{2+}) neurotoxicity: Ion-mimicry with calcium (Ca^{2+}) impairs synaptic transmission. A review with animated illustrations of the pre- and post-synaptic effects of lead. *Journal of Local & Global Health Science* **12** (2013) 4.

Freitas M & Fernandes E 2011 Zinc, cadmium and nickel increase the activation of NF-kappaB and the release of cytokines from THP-1 monocytic cells. *Metallomics Integrated Biometal Science* **3** (11) 1238–1243.

Ganan-Gomez I, Wei Y, Yang H, Boyano-Adanez MC & Garcia-Manero G 2013 Oncogenic functions of the transcription factor Nrf2. *Free Radical Biology & Medicine* **65** 750–764.

Ganesh Yerra V, Negi G, Sharma SS & Kumar A 2013 Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-kappa B pathways in diabetic neuropathy. *Redox Biology* **1** 394–397.

Garrick MD, Dolan KG, Horbinski C, Ghio AJ, Higgins D, Porubcin M, Moore EG, Hainsworth LN, Umbreit JN, Conrad ME, Feng L, Lis A, Roth JA, Singleton S & Garrick LM 2003 DMT1: A mammalian transporter for multiple metals. *Biometals* **16** (1) 41–54.

Garrick MD, Singleton ST, Vargas F, Kuo H-C, Zhao L, Knopfel M, Davidson T, Costa M, Paradkar P, Roth JA & Garrick LM 2006 DMT1: Which metals does it transport? *Biological Research* **39** (1) 79–85.

Garza A, Vega R & Soto E 2006 Cellular mechanisms of lead neurotoxicity. *Medical Science Monitor* **12** (3) RA57-65.

Genestra M 2007 Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cellular Signalling* **19** (9) 1807–1819.

Georgescu B, Georgescu C, Dărăban S, Bouaru A & Paşcalău S 2011 Heavy metals acting as endocrine disrupters. *Scientific Papers: Animal Science & Biotechnologies* **44** (2) 89–93.

Gimeno-García E, Andreu V & Boluda R 1996 Heavy metals incidence in the application of inorganic fertilizers and pesticides to rice farming soils. *Environmental Pollution* **92** (1) 19–25.

Gonzalez-Navarrete F, Eisner V, Morales P, Castro O, Pommer R, Quiroga C, Lavandero S & Devoto L 2007 Tumor necrosis factor-alpha activates nuclear factor-kappa B but does not regulate progesterone production in cultured human granulosa luteal cells. *Gynecological Endocrinology* **23** (7) 377–384.

Goyer RA 1990 Transplacental transport of lead. *Environmental Health Perspectives* **89** 101–105.

Goyer RA 1997 Toxic and essential metal interactions. *Annual Review of Nutrition* **17** 37–50.

Grossman R & Ram Z 2013 The dark side of Nrf2. *World Neurosurgery* **80** (3-4) 284–286.

Grossmann M, Metcalf D, Merryfull J, Beg A, Baltimore D & Gerondakis S 1999 The combined absence of the transcription factors Rel and RelA leads to multiple hemopoietic cell defects. *Proceedings of the National Academy of Sciences* **96** (21) 11848–11853.

Guan L, Zhang L, Gong Z, Hou X, Xu Y, Feng X, Wang H & You H 2016 FoxO3 inactivation promotes human cholangiocarcinoma tumorigenesis and chemoresistance through Keap1-Nrf2 signaling. *Hepatology* **63** (6) 1914–1927.

Guerra-Tamayo JL, Hernandez-Cadena L, Tellez-Rojo MM, Mercado-Garcia AdS, Solano-Gonzalez M, Hernandez-Avila M & Hu H 2003 Time to pregnancy and lead exposure. *Salud Publica de Mexico* **45 Suppl 2** S189-195.

Guidotti TL & Ragain L 2007 Protecting children from toxic exposure: Three strategies. *Pediatric Clinics of North America* **54** (2) 227-235.

Gundacker C & Hengstschlager M 2012 The role of the placenta in fetal exposure to heavy metals. *Wiener medizinische Wochenschrift* **162** (9-10) 201–206.

Gupta SC, Sundaram C, Reuter S & Aggarwal BB 2010 Inhibiting NF-κB activation by small molecules as a therapeutic strategy. *Biochimica et Biophysica Acta* **1799** (10-12) 775–787.

Gurer-Orhan H, Sabir HU & Ozgunes H 2004 Correlation between clinical indicators of lead poisoning and oxidative stress parameters in controls and lead-exposed workers. *Toxicology* **195** (2-3) 147–154.

Hancks DC & Kazazian HH, JR 2012 Active human retrotransposons: Variation and disease. *Current Opinion in Genetics & Development* **22** (3) 191–203.

Hayden MS & Ghosh S 2004 Signaling to NF-kappa B. *Genes & Development* **18** (18) 2195–2224.

He X, Lin GX, Chen MG, Zhang JX & Ma Q 2007 Protection against chromium (VI)-induced oxidative stress and apoptosis by Nrf2. Recruiting Nrf2 into the nucleus and disrupting the nuclear Nrf2/Keap1 association. *Toxicological Sciences* **98** (1) 298–309.

He ZL, Yang XE & Stoffella PJ 2005 Trace elements in agroecosystems and impacts on the environment. *Journal of Trace Elements in Medicine & Biology* **19** (2-3) 125–140.

Hegazy AA, Zaher MM, Abd El-Hafez MA, Morsy AA & Saleh RA 2010 Relation between anemia and blood levels of lead, copper, zinc and iron among children. *BMC Research Notes* **3** 133.

Herrington FD, Carmody RJ & Goodyear CS 2016 Modulation of NF-κB signaling as a therapeutic target in autoimmunity. *Journal of Biomolecular Screening* **21** (3) 223–242

Hiraku Y & Kawanishi S 1996 Mechanism of oxidative DNA damage induced by delta-aminolevulinic acid in the presence of copper ion. *Cancer Research* **56** (8) 1786–1793.

Hirotsu Y, Katsuoka F, Funayama R, Nagashima T, Nishida Y, Nakayama K, Engel JD & Yamamoto M 2012 Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. *Nucleic Acids Research* **40** (20) 10228–10239.

Hong CY, Park JH, Ahn RS, Im SY, Choi H-S, Soh J, Mellon SH & Lee K 2004 Molecular mechanism of suppression of testicular steroidogenesis by proinflammatory cytokine tumor necrosis factor alpha. *Molecular & Cellular Biology* **24** (7) 2593–2604.

Hopkins MR, Ettinger AS, Hernandez-Avila M, Schwartz J, Tellez-Rojo MM, Lamadrid-Figueroa H, Bellinger D, Hu H & Wright RO 2008 Variants in iron metabolism genes predict higher blood lead levels in young children. *Environmental Health Perspectives* **116** (9) 1261–1266.

Hoyer PB & Sipes IG 1996 Assessment of follicle destruction in chemical-induced ovarian toxicity. *Annual Review of Pharmacology & Toxicology* **36** 307–331.

Hracsko Z, Orvos H, Novak Z, Pal A & Varga IS 2008 Evaluation of oxidative stress markers in neonates with intra-uterine growth retardation. *Redox Report* **13** (1) 11–16.

Hsu P-C & Guo YL 2002 Antioxidant nutrients and lead toxicity. *Toxicology* **180** (1) 33–44.

Hu H, Shih R, Rothenberg S & Schwartz BS 2007 The epidemiology of lead toxicity in adults: Measuring dose and consideration of other methodologic issues. *Environmental Health Perspectives* **115** (3) 455–462.

Hu MCT, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H Kobayashi R & Hung MC 2004 Ikappa B kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* **117** (2) 225–237.

Hu X, Roberts JR, Apopa PL, Kan YW & Ma Q 2006 Accelerated ovarian failure induced by 4-vinyl cyclohexene diepoxide in Nrf2 null mice. *Molecular & Cellular Biology* **26** (3) 940–954.

Huang CRL, Schneider AM, Lu Y, Niranjan T, Shen P, Robinson MA, Steranka JP, Valle D, Civin CI & Wang T, Wheelan SJ, Ji H, Boeke JD & Burns KH 2010 Mobile interspersed repeats are major structural variants in the human genome. *Cell* **141** (7) 1171–1182.

Huen K, Harley K, Kogut K, Rauch S, Eskenazi B & Holland N 2016 DNA methylation of LINE-1 and Alu repetitive elements in relation to sex hormones and pubertal timing in Mexican-American children. *Pediatric Research* **79** (6) 855–862.

Hunaiti A, Soud M & Khalil A 1995 Lead concentration and the level of glutathione, glutathione S-transferase, reductase and peroxidase in the blood of some occupational workers from Irbid City, Jordan. *The Science of the Total Environment* **170** (1-2) 95–100.

Iida K, Itoh K, Kumagai Y, Oyasu R, Hattori K, Kawai K, Shimazui T, Akaza H & Yamamoto M 2004 Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Research* **64** (18) 6424–6431.

Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S & Yamamoto M 2000 Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *The Journal of Biological Chemistry* **275** (21) 16023–16029.

Iskow RC, McCabe MT, Mills RE, Torene S, Pittard WS, Neuwald AF, van Meir EG, Vertino PM & Devine SE 2010 Natural mutagenesis of human genomes by endogenous retrotransposons. *Cell* **141** (7) 1253–1261.

Jacobs DE, Clickner RP, Zhou JY, Viet SM, Marker DA, Rogers JW, Zeldin DC, Broene P & Friedman W 2002 The prevalence of lead-based paint hazards in U.S. housing. *Environmental Health Perspectives* **110** (10) A599-606.

Jan AT, Ali A & Haq Q 2011 Glutathione as an antioxidant in inorganic mercury induced nephrotoxicity. *Journal of Postgraduate Medicine* **57** (1) 72–77.

Jan AT, Azam M, Siddiqui K, Ali A, Choi I & Haq QMR 2015 Heavy metals and human health: Mechanistic insight into toxicity and counter defense system of antioxidants. *International Journal of Molecular Sciences* **16** (12) 29592–29630.

Jana SK, K NB, Chattopadhyay R, Chakravarty B & Chaudhury K 2010 Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. *Reproductive Toxicology* **29** (4) 447–451.

Jarosinska D, Peddada S & Rogan WJ 2004 Assessment of lead exposure and associated risk factors in urban children in Silesia, Poland. *Environmental Research* **95** (2) 133–142.

Jarup L 2003 Hazards of heavy metal contamination. *British medical bulletin* **68** 167–182.

Jern P & Coffin JM 2008 Effects of retroviruses on host genome function. *Annual Review of Genetics* **42** 709–732.

Jomova K & Valko M 2011 Advances in metal-induced oxidative stress and human disease. *Toxicology* **283** (2-3) 65–87.

Jones PA 2012 Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nature reviews. Genetics* **13** (7) 484–492.

Kala M, Shaikh MV & Nivsarkar M 2017 Equilibrium between anti-oxidants and reactive oxygen species: A requisite for oocyte development and maturation. *Reproductive Medicine & Biology* **16** (1) 28–35.

Kanherkar RR, Bhatia-Dey N & Csoka AB 2014 Epigenetics across the human lifespan. *Frontiers in Cell & Developmental Biology* **2** 49.

Kansanen E, Kuosmanen SM, Leinonen H & Levonen AL 2013 The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. *Redox Biology* **1** 45–49.

Karimooy HN, Mood MB, Hosseini M & Shadmanfar S 2010 Effects of occupational lead exposure on renal and nervous system of workers of traditional tile factories in Mashhad (northeast of Iran). *Toxicology & Industrial Health* **26** (9) 633–638.

Karin M & Ben-Neriah Y 2000 Phosphorylation meets ubiquitination: The control of NF-kappaB activity. *Annual Review of Immunology* **18** 621–663.

Karrari P, Mehrpour O & Abdollahi M 2012 A systematic review on status of lead pollution and toxicity in Iran; Guidance for preventive measures. *Daru Journal of Pharmaceutical Science* **20** (1) 2.

Karuputhula NB, Chattopadhyay R, Chakravarty B & Chaudhury K 2013 Oxidative status in granulosa cells of infertile women undergoing IVF. *Systems Biology in Reproductive Medicine* **59** (2) 91–98.

Kasperczyk A, Prokopowicz A, Dobrakowski M, Pawlas N & Kasperczyk S 2012 The effect of occupational lead exposure on blood levels of zinc, iron, copper, selenium and related proteins. *Biological Trace Element Research* **150** (1-3) 49–55.

Katz AK, Glusker JP, Beebe SA &Bock CW 1995 Calcium Ion Coordination: A Comparison with That of Beryllium, Magnesium, and Zinc. *American Chemical Society* **118** (245) 752-5763.

Kayaalti Z, Akyuzlu DK & Soylemezoglu T 2015 Evaluation of the effect of divalent metal transporter 1 gene polymorphism on blood iron, lead and cadmium levels. *Environmental Research* **137** 8–13.

Kazazian HH, JR 2004 Mobile elements: Drivers of genome evolution. *Science* **303** (5664) 1626–1632.

Kelada SN, Shelton E, Kaufmann RB & Khoury MJ 2001 Delta-aminolevulinic acid dehydratase genotype and lead toxicity: A HuGE review. *American journal of epidemiology* **154** (1) 1–13.

Khurana N & Sikka SC 2018 Targeting Crosstalk between Nrf-2, NF-κB and androgen receptor signaling in prostate cancer. *Cancers* **10** (10) 352.

Kianoush S, Balali-Mood M, Mousavi SR, Moradi V, Sadeghi M, Dadpour B, Rajabi O & Shakeri MT 2012 Comparison of therapeutic effects of garlic and d-Penicillamine in patients with chronic occupational lead poisoning. *Basic & Clinical Pharmacology & Toxicology* **110** (5) 476–481.

Kianoush S, Sadeghi M & Balali-Mood M 2015 Recent advances in the clinical management of lead poisoning. *Acta Medica Iranica* **53** (6) 327–336.

Kim HJ, Hawke N & Baldwin AS 2006 NF-kappa B and IKK as therapeutic targets in cancer. *Cell Death & Differentiation* **13** (5) 738–747.

Kim J, Lee Y & Yang M 2014 Environmental exposure to lead (Pb) and variations in its susceptibility. *Journal of Environmental Science & Health, Environmental Carcinogenesis & Ecotoxicology Reviews* **32** (2) 159–185.

Kobayashi A, Ohta T & Yamamoto M 2004 Unique function of the Nrf2-Keap1 pathway in the inducible expression of antioxidant and detoxifying enzymes. *Methods in Enzymology* **378** 273–286.

Koedrith P & Seo YR 2011 Advances in carcinogenic metal toxicity and potential molecular markers. *International Journal of Molecular Sciences* **12** (12) 9576–9595.

Koning APJ de, **Gu W, Castoe TA, Batzer MA & Pollock DD** 2011 Repetitive elements may comprise over two-thirds of the human genome. *PloS Genetics* **7** (12) e1002384.

Korashy HM & El-Kadi AOS 2006 The role of aryl hydrocarbon receptor and the reactive oxygen species in the modulation of glutathione transferase by heavy metals in murine hepatoma cell lines. *Chemico-Biological Interactions* **162** (3) 237–248.

Krieg EF, JR, Butler MA, Chang M-H, Liu T, Yesupriya A, Dowling N & Lindegren ML 2010 Lead and cognitive function in VDR genotypes in the third National Health and Nutrition Examination Survey. *Neurotoxicology & Teratology* **32** (2) 262–272.

Kumagai Y, Kanda H, Shinkai Y & Toyama T 2013 The role of the Keap1/Nrf2 pathway in the cellular response to methylmercury. *Oxidative Medicine & Cellular Longevity* **2013** 848279.

Kweider N, Huppertz B, Wruck CJ, Beckmann R, Rath W, Pufe T & Kadyrov M 2012 A role for Nrf2 in redox signalling of the invasive extravillous trophoblast in severe early onset IUGR associated with preeclampsia. *PloS One* **7** (10) e47055.

Kwong WT, Friello P & Semba RD 2004 Interactions between iron deficiency and lead poisoning: Epidemiology and pathogenesis. *The Science of the Total Environment* **330** (1-3) 21–37.

Lamadrid-Figueroa H, Tellez-Rojo MM, Hernandez-Avila M, Trejo-Valdivia B, Solano-Gonzalez M, Mercado-Garcia A, Smith D, Hu H & Wright RO 2007 Association between the plasma/whole blood lead ratio and history of spontaneous abortion: A nested cross-sectional study. *BMC Pregnancy & Childbirth* **7** 22.

Landrigan PJ, Schechter CB, Lipton JM, Fahs MC & Schwartz J 2002 Environmental pollutants and disease in American children: Estimates of morbidity, mortality, and costs for lead poisoning, asthma, cancer, and developmental disabilities. *Environmental Health Perspectives* **110** (7) 721–728.

Lappas M & Rice GE 2009 Transcriptional regulation of the processes of human labour and delivery. *Placenta* **30 Suppl A** S90-95.

Lappas M 2012 Nuclear factor-kappa B mediates placental growth factor induced pro-labour mediators in human placenta. *Molecular Human Reproduction* **18** (7) 354–361.

Lau A, Whitman SA, Jaramillo MC & Zhang DD 2013 Arsenic-mediated activation of the Nrf2-Keap1 antioxidant pathway. *Journal of Biochemical & Molecular Toxicology* **27** (2) 99–105.

Lawton LJ & Donaldson WE 1991 Lead-induced tissue fatty acid alterations and lipid peroxidation. *Biological Trace Element Research* **28** (2) 83–97.

Lee BK, Lee SJ, Joo JS, Cho KS, Kim NS & Kim HJ 2012a Association of Glutathione S-transferase genes (GSTM1 and GSTT1) polymorphisms with hypertension in lead-exposed workers. *Molecular & Cellular Toxicology* **8** (2) 203–208.

Lee DF, Kuo HP, Liu M, Chou CK, Xia W, Du Y, Shen J, Chen CT, Huo L, Hsu MC Li CW, Ding Q, Liao TL, Lai CC, Lin AC, Chang YH, Tsai SF, Li LY & Hung MC 2009 KEAP1 E3 ligase-mediated downregulation of NF-kappa B signaling by targeting IKKbeta. *Molecular Cell* **36** (1) 131–140.

Lee J, Giordano S & Zhang J 2012b Autophagy, mitochondria and oxidative stress: Cross-talk and redox signalling. *The Biochemical Journal* **441** (2) 523–540.

Leech TGJ, Adams EA, Weathers TD, Staten LK & Filippelli GM 2016 Inequitable chronic lead exposure: A dual legacy of social and environmental injustice. *Family & Community Health* **39** (3) 151–159.

Leonard SS, Harris GK & Shi X 2004 Metal-induced oxidative stress and signal transduction. *Free Radical Biology & Medicine* **37** (12) 1921–1942.

Levallois P, St-Laurent J, Gauvin D, Courteau M, Prevost M, Campagna C, Lemieux F, Nour S, D'Amour M & Rasmussen PE 2014 The impact of drinking water, indoor dust and paint on blood lead levels of children aged 1-5 years in Montreal (Quebec, Canada). *Journal of Exposure Science & Environmental Epidemiology* **24** (2) 185–191.

Levine RL 2002 Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radical Biology & Medicine* **32** (9) 790–796.

Lewis BP, Burge CB & Bartel DP 2005 Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120** (1) 15–20.

Lewis JB, Messer RL, McCloud VV, Lockwood PE, Hsu SD & Wataha JC 2006 Ni(II) activates the Nrf2 signaling pathway in human monocytic cells. *Biomaterials* **27** (31) 5348–5356.

Li C, Xu M, Wang S, Yang X, Zhou S, Zhang J, Liu Q & Sun Y 2011 Lead exposure suppressed ALAD transcription by increasing methylation level of the promoter CpG islands. *Toxicology Letters* **203** (1) 48–53.

Li C, Yang X, Xu M, Zhang J & Sun N 2013 Epigenetic marker (LINE-1 promoter) methylation level was associated with occupational lead exposure. *Clinical Toxicology* **51** (4) 225–229.

Li M, Gao J-Q & Li X-W 2005 Antagonistic action of selenium against the toxicity of lead. *Journal of Hygiene Research* **34** (3) 375–377.

Li R & Albertini DF 2013 The road to maturation: Somatic cell interaction and self-organization of the mammalian oocyte. *Nature Reviews, Molecular Cell Biology* **14** (3) 141–152.

Li Z, Zhang H, Chen Y, Fan L & Fang J 2012 Forkhead transcription factor FOXO3a protein activates nuclear factor kappaB through B-cell lymphoma/leukemia 10 (BCL10) protein and promotes tumor cell survival in serum deprivation. *The Journal of Biological Chemistry* **287** (21) 17737–17745.

Liang G, Chan MF, Tomigahara Y, Tsai YC, Gonzales FA, Li E, Laird PW & Jones PA 2002 Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Molecular & Cellular Biology* **22** (2) 480–491.

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

Lim J, Ortiz L, Nakamura BN, Hoang YD, Banuelos J, Flores VN, Chan JY & Luderer U 2015a Effects of deletion of the transcription factor Nrf2 and benzo apyrene treatment on ovarian follicles and ovarian surface epithelial cells in mice. *Reproductive Toxicology* **58** 24–32.

Lim R, Barker G & Lappas M 2015b The transcription factor Nrf2 is decreased after spontaneous term labour in human fetal membranes where it exerts anti-inflammatory properties. *Placenta* **36** (1) 7–17.

Liu C-M, Ma J-Q & Sun Y-Z 2010 Quercetin protects the rat kidney against oxidative stress-mediated DNA damage and apoptosis induced by lead. *Environmental Toxicology & Pharmacology* **30** (3) 264–271.

Liu C-M, Ma J-Q, Xie W-R, Liu S-S, Feng Z-J, Zheng G-H & Wang A-M 2015 Quercetin protects mouse liver against nickel-induced DNA methylation and inflammation associated with the Nrf2/HO-1 and p38/STAT1/NF-kappaB pathway. *Food & Chemical Toxicology* **82** 19–26.

Liu G-H, Qu J & Shen X 2008 NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochimica et Biophysica Acta* **1783** (5) 713–727.

Liu M-J, Bao S, Gálvez-Peralta M, Pyle CJ, Rudawsky AC, Pavlovicz RE, Killilea DW, Li C, Nebert DW, Wewers MD & Knoell DL 2013 ZIP8 regulates host defense through zinc-mediated inhibition of NF-κB. *Cell Reports* **3** (2) 386–400.

Lopez Alonso M, Prieto Montana F, Miranda M, Castillo C, Hernandez J & Luis Benedito J 2004 Interactions between toxic (As, Cd, Hg and Pb) and nutritional essential (Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn) elements in the tissues of cattle from NW Spain. *Biometals* **17** (4) 389–397.

Lorenzetti S, Altieri I, Arabi S, Balduzzi D, Bechi N, Cordelli E, Galli C, Ietta F, Modina SC & Narciso L, Pacchierotti F, Villani P, Galli A, Lazzari G, Luciano AM, Paulesu L, Spanò M & Mantovani A 2011 Innovative non-animal testing strategies for reproductive toxicology: The contribution of Italian partners within the EU project ReProTect. *Annali Dell'Istituto Superiore Di Sanita* **47** (4) 429–444.

Lounnas N, Frelin C, Gonthier N, Colosetti P, Sirvent A, Cassuto JP, Berthier F, Sirvent N, Rousselot P, Dreano M, Peyron JF, Imbert V 2009 NF-kappaB inhibition triggers death of imatinib-sensitive and imatinib-resistant chronic myeloid leukemia cells including T315I Bcr-Abl mutants. *International Journal of Cancer* **125** (2) 308–317.

Lousse J-C, van Langendonckt A, Gonzalez-Ramos R, Defrere S, Renkin E & Donnez J 2008 Increased activation of nuclear factor-kappa B (NF-kappaB) in isolated peritoneal macrophages of patients with endometriosis. *Fertility & Sterility* **90** (1) 217–220.

Lu J, Wang Z, Cao J, Chen Y & Dong Y 2018 A novel and compact review on the role of oxidative stress in female reproduction. *Reproductive Biology & Endocrinology* **16** (1) 80.

Lu R 2003 Dry deposition of airborne trace metals on the Los Angeles Basin and adjacent coastal waters. *Journal of Geophysical Research* **108** (D2) 321.

Luo JL, Kamata H & Karin M 2005 IKK/NF-kappa B signaling: Balancing life and death-a new approach to cancer therapy. *The Journal of Clinical Investigation* **115** (10) 2625–2632.

Luo M, Xu Y, Cai R, Tang Y, Ge MM, Liu ZH, Xu L, Hu F, Ruan DY & Wang HL 2014 Epigenetic histone modification regulates developmental lead exposure induced hyperactivity in rats. *Toxicology Letters* **225** (1) 78–85.

Ma Hy, Li H, Wang Jc & Xu Fs 2006 Expression and significance of metallothionein in the placenta of women with low level lead exposure during pregnancy. *Zhonghua fu Chan ke za zhi* **41** (10) 676–679.

Madazli R, Benian A, Aydin S, Uzun H & Tolun N 2002 The plasma and placental levels of malondialdehyde, glutathione and superoxide dismutase in pre-eclampsia. *Journal of Obstetrics & Gynaecology* **22** (5) 477–480.

Magnusson U 2005 Can farm animals help to study endocrine disruption? *Domestic Animal Endocrinology* **29** (2) 430–435.

Mahajan A & Tandon VR 2004 Antioxidants and rheumatoid arthritis. *Journal of Indian Rheumatology Association* **12** 139–142.

Malekirad AA, Oryan S, Fani A, Babapor V, Hashemi M, Baeeri M, Bayrami Z & Abdollahi M 2010 Study on clinical and biochemical toxicity biomarkers in a zinc-lead mine workers. *Toxicology & Industrial Health* **26** (6) 331–337.

Malhi PS, Adams GP & Singh J 2005 Bovine model for the study of reproductive aging in women: Follicular, luteal, and endocrine characteristics. *Biology of Reproduction* **73** (1) 45–53.

Marchan R & Bolt HM 2013 The cytoprotective and the dark side of Nrf2. *Archives of Toxicology* **87** (12) 2047–2050.

Mari M, Morales A, Colell A, Garcia-Ruiz C & Fernandez-Checa JC 2009 Mitochondrial glutathione, a key survival antioxidant. *Antioxidants & Redox signaling* **11** (11) 2685–2700.

Mason HJ, Williams N, Armitage S, Morgan M, Green S, Perrin B & Morgan WD 1999 Follow up of workers previously exposed to silver solder containing cadmium. *Occupational & Environmental Medicine* **56** (8) 553–558.

Massrieh W, Derjuga A & Blank V 2006 Induction of endogenous Nrf2/small maf heterodimers by arsenic-mediated stress in placental choriocarcinoma cells. *Antioxidants & Redox Signaling* **8** (1-2) 53–59.

Mates JM 2000 Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology* **153** (1-3) 83–104.

Matsubara K, Higaki T, Matsubara Y & Nawa A 2015 Nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. *International Journal of Molecular Sciences* **16** (3) 4600–4614.

McCarrey JR 2012 The epigenome as a target for heritable environmental disruptions of cellular function. *Molecular & Cellular Endocrinology* **354** (1-2) 9–15.

McClintock B 1984 The significance of responses of the genome to challenge. *Science* **226** (4676) 792–801.

Mehdi JK, Al-Imarah FJ & Al-Suhail AA 2000 Levels of some trace metals and related enzymes in workers at storage-battery factories in Iraq. *Eastern Mediterranean Health* **6** (1) 76–82.

Mendola P, Messer LC & Rappazzo K 2008 Science linking environmental contaminant exposures with fertility and reproductive health impacts in the adult female. *Fertility & Sterility* **89** (2 Suppl) e81-94.

Menezo YJR & Herubel F 2002 Mouse and bovine models for human IVF. *Reproductive Biomedicine Online* **4** (2) 170–175.

Meng Q-T, Chen R, Chen C, Su K, Li W, Tang L-H, Liu H-M, Xue R, Sun Q , Leng Y, Hou JB, Wu Y& Xia ZY 2017 Transcription factors Nrf2 and NF-κB contribute to inflammation and apoptosis induced by intestinal ischemia-reperfusion in mice. *International Journal of Molecular Medicine* **40** (6) 1731–1740.

Meyer PA, Brown MJ & Falk H 2008 Global approach to reducing lead exposure and poisoning. *Mutation Research* **659** (1-2) 166–175.

Mincheva-Tasheva S & Soler RM 2013 NF-kappa B signaling pathways: Role in nervous system physiology and pathology. *Neuroscientist* **19** (2) 175–194.

Miousse IR, Chalbot M-CG, Lumen A, Ferguson A, Kavouras IG & Koturbash I 2015 Response of transposable elements to environmental stressors. *Mutation Research* **765** 19–39.

Mitra P, Sharma S, Purohit P & Sharma P 2017 Clinical and molecular aspects of lead toxicity: An update. *Critical Reviews in Clinical Laboratory Sciences* **54** (7-8) 506–528.

Montano-Almendras CP, Essaghir A, Schoemans H, Varis I, Noel LA, Velghe AI, Latinne D, Knoops L & Demoulin JB 2012 ETV6-PDGFRB and FIP1L1-PDGFRA stimulate human hematopoietic progenitor cell proliferation and differentiation into eosinophils: The role of nuclear factor-kappa B. *Haematologica* **97** (7) 1064–1072.

Montrose L, Faulk C, Francis J & Dolinoy DC 2017 Perinatal lead (Pb) exposure results in sex and tissue-dependent adult DNA methylation alterations in murine IAP transposons. *Environmental & Molecular Mutagenesis* **58** (8) 540–550.

Mudgal V, Madaan N, Mudgal A, Singh RB & Mishra S 2010 Effect of toxic metals on human health. *Open Nutraceuticals Journal* **3** (1) 94–99.

Mudipalli A 2007 Lead hepatotoxicity & potential health effects. *The Indian Journal of Medical Research* **126** (6) 518–527.

Muenchen HJ, Lin DL, Walsh MA, Keller ET & Pienta KJ 2000 Tumor necrosis factor-alpha-induced apoptosis in prostate cancer cells through inhibition of nuclear factor-kappa B by an IkappaBalpha "super-repressor". *Clinical Cancer Research* **6** (5) 1969–1977.

Mukherjee N, Houston TJ, Cardenas E & Ghosh R 2015 To be an ally or an adversary in bladder cancer: The NF-kappa B story has not unfolded. *Carcinogenesis* **36** (3) 299–306

Munoz-Lopez M, Vilar-Astasio R, Tristan-Ramos P, Lopez-Ruiz C & Garcia-Perez JL 2016 Study of Transposable Elements and Their Genomic Impact. *Methods in Molecular Biology* **1400** 1–19.

Muscarella LA, Barbano R, D'Angelo V, Copetti M, Coco M, Balsamo T, La Torre A, Notarangelo A, Troiano M, Parisi S, Icolaro N, Catapano D, Valori VM, Pellegrini F, Merla G, Carella M, Fazio VM & Parrella P 2011 Regulation of KEAP1 expression by promoter methylation in malignant gliomas and association with patient's outcome. *Epigenetics* **6** (3) 317–325.

Myatt L & Cui X 2004 Oxidative stress in the placenta. *Histochemistry & Cell Biology* **122** (4) 369–382.

Nagajyoti PC, Lee KD & Sreekanth TVM 2010 Heavy metals, occurrence and toxicity for plants: A review. *Environmental Chemistry Letters* **8** (3) 199–216.

Neal AP & Guilarte TR 2010 Molecular neurobiology of lead (Pb^{2+}): Effects on synaptic function. *Molecular Neurobiology* **42** (3) 151–160.

Needleman H 2004 Lead poisoning. *Annual Review of Medicine* **55** 209–222.

Nehru B, Dua R & Iyer A 1997 Effect of selenium on lead-induced alterations in rat brain. *Biological Trace Element Rresearch* **57** (3) 251–258.

Ngu TT & Stillman MJ 2009 Metalation of metallothioneins. *IUBMB Life* **61** (4) 438–446.

Nguyen T, Yang CS & Pickett CB 2004 The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radical Biology & Medicine* **37** (4) 433–441.

Niture SK, Khatri R & Jaiswal AK 2014 Regulation of Nrf2-an update. *Free Radical Biology & Medicine* **66** 36–44.

No JH, Kim Y-B & Song YS 2014 Targeting nrf2 signaling to combat chemoresistance. *Journal of Cancer Prevention* **19** (2) 111–117.

Nye MD, Hoyo C & Murphy SK 2015 In vitro lead exposure changes DNA methylation and expression of IGF2 and PEG1/MEST. *Toxicology in Vitro* **29** (3) 544–550.

Pacher P, Beckman JS & Liaudet L 2007 Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews* **87** (1) 315–424.

Paciolla M, Boni R, Fusco F, Pescatore A, Poeta L, Ursini MV, Lioi MB & Miano MG 2011 Nuclear factor-kappa-B-inhibitor alpha (NFKBIA) is a developmental marker of NF-kappa B/p65 activation during in vitro oocyte maturation and early embryogenesis. *Human Reproduction* **26** (5) 1191–1201.

Pan H, Wang H, Wang X, Zhu L & Mao L 2012 The absence of Nrf2 enhances NF-kappa B-dependent inflammation following scratch injury in mouse primary cultured astrocytes. *Mediators of Inflammation* **2012** 217580.

Pan TL, Wang PW, Al-Suwayeh SA, Chen CC & Fang JY 2010 Skin toxicology of lead species evaluated by their permeability and proteomic profiles: A comparison of organic and inorganic lead. *Toxicology Letters* **197** (1) 19–28.

Pant N, Upadhyay G, Pandey S, Mathur N, Saxena DK & Srivastava SP 2003 Lead and cadmium concentration in the seminal plasma of men in the general population: Correlation with sperm quality. *Reproductive toxicology* **17** (4) 447–450.

Park S, Sim CS, Lee H & Kim Y 2014 Effects of iron therapy on blood lead concentrations in infants. *Journal of Trace Elements in Medicine & Biology* **28** (1) 56–59.

Patel OV, Bettegowda A, Ireland JJ, Coussens PM, Lonergan P & Smith GW 2007 Functional genomics studies of oocyte competence: Evidence that reduced transcript abundance for follistatin is associated with poor developmental competence of bovine oocytes. *Reproduction* **133** (1) 95–106.

Patrick L 2006a Lead toxicity part II: The role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. *Alternative Medicine Review* **11** (2) 114–127.

Patrick L 2006b Lead toxicity, a review of the literature. Part 1: Exposure, evaluation, and treatment. *Alternative Medicine* **11** (1) 2–22.

Paul S, Ghosh D, Ghosh AK, Mira E, Dey M, Chattopadhyay A & Bandyopadhyay D 2013 Lead induces oxidative stress in rats heart and liver tissue homgenates: an in vitro study. *Journal of Cell & Tissue Research* **13** (3) 3829–3837.

Pavlova S, Klucska K, Vasicek D, Kotwica J & Sirotkin AV 2011 Transcription factor NF-kappaB (p50/p50, p65/p65) controls porcine ovarian cells functions. *Animal Reproduction Science* **128** (1-4) 73–84.

Pearce JMS 2007 Burton's line in lead poisoning. *European neurology* **57** (2) 118–119.

Peretiatko R & D'Souza C 2002 The nexus between industrialization and environment: A case study of Indian enterprises. *Environmental Management & Health* **13** (1) 80–97.

Perkins ND & Gilmore TD 2006 Good cop, bad cop: The different faces of NF-kappa B. *Cell Death & Differentiation* **13** (5) 759–772.

Perkins ND 2004 NF-kappa B: Tumor promoter or suppressor? *Trends in Cell Biology* **14** (2) 64–69.

Perkins ND 2007 Integrating cell-signalling pathways with NF-kappa B and IKK function. *Nature reviews. Molecular Cell Biology* **8** (1) 49–62.

Pham-Huy LA, He H & Pham-Huy C 2008 Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science* **4** (2) 89–96.

Piersma AH, Bosgra S, van Duursen MBM, Hermsen SAB, Jonker LRA, Kroese ED, van der Linden SC, Man H, Roelofs MJE, Schulpen SHW Schwarz M, Uibel F, van Vugt-Lussenburg BM, Wolterbeek AP & van der Burg B 2013 Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. *Reproductive Toxicology* **38** 53–64.

Pillai P, Pandya C, Gupta S & Gupta S 2010 Biochemical and molecular effects of gestational and lactational coexposure to lead and cadmium on ovarian steroidogenesis are associated with oxidative stress in F1 generation rats. *Journal of Biochemical & Molecular Toxicology* **24** (6) 384–394.

Pilsner JR, Hu H, Ettinger A, Sanchez BN, Wright RO, Cantonwine D, Lazarus A, Lamadrid-Figueroa H, Mercado-Garcia A, Tellez-Rojo MM & Hernández-Avila M 2009 Influence of prenatal lead exposure on genomic methylation of cord blood DNA. *Environmental Health Perspectives* **117** (9) 1466–1471.

Pilsner JR, Liu X, Ahsan H, Ilievski V, Slavkovich V, Levy D, Factor-Litvak P, Graziano JH & Gamble MV 2007 Genomic methylation of peripheral blood leukocyte DNA: Influences of arsenic and folate in Bangladeshi adults. *The American Journal of Clinical Nutrition* **86** (4) 1179–1186.

Pizzorno J 2014 Glutathione! *Integrative Medicine* **13** (1) 8–12.

Prasanthi RPJ, Devi CB, Basha DC, Reddy NS & Reddy GR 2010 Calcium and zinc supplementation protects lead (Pb)-induced perturbations in antioxidant enzymes and lipid peroxidation in developing mouse brain. *International Journal of Developmental Neuroscience* **28** (2) 161–167.

Qian Y, Zheng Y, Ramos KS & Tiffany-Castiglioni E 2005 The involvement of copper transporter in lead-induced oxidative stress in astroglia. *Neurochemical Research* **30** (4) 429–438.

Qin Z-h, Tao L-y & Chen X 2007 Dual roles of NF-kappa B in cell survival and implications of NF-kappa B inhibitors in neuroprotective therapy. *Acta Pharmacologica Sinica* **28** (12) 1859–1872.

Rahil-Khazen R, Bolann BJ & Ulvik RJ 2002 Correlations of trace element levels within and between different normal autopsy tissues analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES). *Biometals* **15** (1) 87–98.

Raudenska M, Gumulec J, Podlaha O, Sztalmachova M, Babula P, Eckschlager T, Adam V, Kizek R & Masarik M 2014 Metallothionein polymorphisms in pathological processes. *Metallomics integrated biometal science* **6** (1) 55–68.

Richards EJ 2006 Inherited epigenetic variation-revisiting soft inheritance. *Nature Reviews. Genetics* **7** (5) 395–401.

Richter PA, Bishop EE, Wang J & Kaufmann R 2013 Trends in tobacco smoke exposure and blood lead levels among youths and adults in the United States: The National Health and Nutrition Examination Survey, 1999-2008. *Preventing Chronic Disease* **10** E213.

Rico D, Martin-Gonzalez A, Diaz S, Lucas P de & **Gutierrez J-C** 2009 Heavy metals generate reactive oxygen species in terrestrial and aquatic ciliated protozoa. *Comparative Biochemistry & Physiology. Toxicology & Pharmacology CBP* **149** (1) 90–96.

Rodriguez-Osorio N, Wang H, Rupinski J, Bridges SM & Memili E 2010 Comparative functional genomics of mammalian DNA methyltransferases. *Reproductive Biomedicine Online* **20** (2) 243–255.

Rolston DDK 2011 Uncommon sources and some unsual manifestations of lead poisoning in a tropical developing country. *Tropical Medicine & Health* **39** (4) 127–132.

Roman-Gomez J, Jimenez-Velasco A, Agirre X, Cervantes F, Sanchez J, Garate L, Barrios M, Castillejo JA, Navarro G, Colomer D Prosper F, Heiniger A & Torres A 2005 Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene* **24** (48) 7213–7223.

Ross JP, Rand KN & Molloy PL 2010 Hypomethylation of repeated DNA sequences in cancer. *Epigenomics* **2** (2) 245–269.

Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, Robbins WA & Perreault SD 2005 Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. *Human reproduction* **20** (10) 2776–2783.

Rudge CV, Rollin HB, Nogueira CM, Thomassen Y, Rudge MC & Odland JO 2009 The placenta as a barrier for toxic and essential elements in paired maternal and cord blood samples of South African delivering women. *Journal of Environmental Monitoring* **11** (7) 1322–1330.

Rushworth SA, Zaitseva L, Murray MY, Shah NM, Bowles KM & MacEwan DJ 2012 The high Nrf2 expression in human acute myeloid leukemia is driven by NF-kappa B and underlies its chemo-resistance. *Blood* **120** (26) 5188–5198.

Saccani S, Pantano S & Natoli G 2003 Modulation of NF-kappa B activity by exchange of dimers. *Molecular Cell* **11** (6) 1563–1574.

Sahin K, Pala R, Tuzcu M, Ozdemir O, Orhan C, Sahin N & Juturu V 2016 Curcumin prevents muscle damage by regulating NF-kappa B and Nrf2 pathways and improves performance: An in vivo model. *Journal of Inflammation Research* **9** 147–154.

Sakamoto Y, Harada T, Horie S, Iba Y, Taniguchi F, Yoshida S, Iwabe T & Terakawa N 2003 Tumor necrosis factor-alpha-induced interleukin-8 (IL-8) expression in endometriotic

stromal cells, probably through nuclear factor-kappa B activation: Gonadotropin-releasing hormone agonist treatment reduced IL-8 expression. *The Journal of Clinical Endocrinology & Metabolism* **88** (2) 730–735.

Saleh HA, El-Aziz GA, El-Fark MM & El-Gohary M 2009 Effect of maternal lead exposure on craniofacial ossification in rat fetuses and the role of antioxidant therapy. *Anatomia, Histologia, Embryologia* **38** (5) 392–399.

Salvemini D & Botting R 1993 Modulation of platelet function by free radicals and free-radical scavengers. *Trends in Pharmacological Sciences* **14** (2) 36–42.

Sanders AP, Burris HH, Just AC, Motta V, Amarasiriwardena C, Svensson K, Oken E, Solano-Gonzalez M, Mercado-Garcia A, Pantic I, Schwartz J, Tellez-Rojo MM, Baccarelli AA & Wright RO 2015 Altered miRNA expression in the cervix during pregnancy associated with lead and mercury exposure. *Epigenomics* **7** (6) 885–896.

Sanders T, Liu Y, Buchner V & Tchounwou PB 2009 Neurotoxic effects and biomarkers of lead exposure: A review. *Reviews on Environmental Health* **24** (1) 15–45.

Sandhir R & Gill KD 1995 Effect of lead on lipid peroxidation in liver of rats. *Biological Trace Element Research* **48** (1) 91–97.

Santos RR, Schoevers EJ & Roelen BAJ 2014 Usefulness of bovine and porcine IVM/IVF models for reproductive toxicology. *Reproductive Biology & Endocrinology* **12** 117.

Sbrana E, Suter MA, Abramovici AR, Hawkins HK, Moss JE, Patterson L, Shope C & Aagaard-Tillery K 2011 Maternal tobacco use is associated with increased markers of oxidative stress in the placenta. *American Journal of Obstetrics & Gynecology* **205** (3) 246.e1-7.

Schneider JS, Kidd SK & Anderson DW 2013 Influence of developmental lead exposure on expression of DNA methyltransferases and methyl cytosine-binding proteins in hippocampus. *Toxicology Letters* **217** (1) 75–81.

Scinicariello F, Murray HE, Moffett DB, Abadin HG, Sexton MJ & Fowler BA 2007 Lead and delta-aminolevulinic acid dehydratase polymorphism: Where does it lead? A meta-analysis. *Environmental Health Perspectives* **115** (1) 35–41.

Selevan SG, Rice DC, Hogan KA, Euling SY, Pfahles-Hutchens A & Bethel J 2003 Blood lead concentration and delayed puberty in girls. *The New England Journal of Medicine* **348** (16) 1527–1536.

Senut M-C, Cingolani P, Sen A, Kruger A, Shaik A, Hirsch H, Suhr ST & Ruden D 2012 Epigenetics of early-life lead exposure and effects on brain development. *Epigenomics* **4** (6) 665–674.

Sethi G, Sung B & Aggarwal BB 2008 Nuclear factor-kappa B activation: From bench to bedside. *Experimental Biology & Medicine* **233** (1) 21–31.

Seyom E, Abera M, Tesfaye M & Fentahun N 2015 Maternal and fetal outcome of pregnancy related hypertension in Mettu Karl Referral Hospital, Ethiopia. *Journal of Ovarian Research* **8** 10.

Shafiq-ur-Rehman 2013 Effect of lead on lipid peroxidation, phospholipids composition, and methylation in erythrocyte of human. *Biological Trace Element Research* **154** (3) 433–439.

Shan G, Tang T & Zhang X 2009 The protective effect of ascorbic acid and thiamine supplementation against damage caused by lead in the testes of mice. *Journal of Huazhong University of Science &Technology. Medical Sciences* **29** (1) 68–72.

Shishodia S & Aggarwal BB 2004 Nuclear factor-kappa B: A friend or a foe in cancer? *Biochemical Pharmacology* **68** (6) 1071–1080.

Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D & Dekel N 2011 Reactive oxygen species are indispensable in ovulation. *Proceedings of the National Academy of Sciences of the United States of America* **108** (4) 1462–1467.

Simmons SO, Fan CY, Yeoman K, Wakefield J & Ramabhadran R 2011 Nrf2 Oxidative stress induced by heavy metals is cell type dependent. *Current Chemical Genomics* **5** 1–12.

Sirard MA 2012 Factors affecting oocyte and embryo transcriptomes. *Reproduction in Domestic Animals* **47 Suppl 4** 148–155.

Smith DM Jr, Mielke HW & Heneghan JB 2008 Subchronic lead feeding study in male rats. *Archives of Environmental Contamination & Toxicology* **55** (3) 518–528.

Smith R, Maiti K & Aitken RJ 2013 Unexplained antepartum stillbirth: A consequence of placental aging? *Placenta* **34** (4) 310–313.

Soares MP, Seldon MP, Gregoire IP, Vassilevskaia T, Berberat PO, Yu J, Tsui TY & Bach FH 2004 Heme oxygenase-1 modulates the expression of adhesion molecules associated with endothelial cell activation. *Journal of Immunology* **172** (6) 3553–3563.

Solliway BM, Schaffer A, Pratt H & Yannai S 1996 Effects of exposure to lead on selected biochemical and haematological variables. *Pharmacology & Toxicology* **78** (1) 18–22.

Spielmann H 2009 The way forward in reproductive/developmental toxicity testing. *Alternatives to Laboratory Animals* **37** (6) 641–656.

Stohs SJ & Bagchi D 1995 Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology & Medicine* **18** (2) 321–336.

Stone JR & Yang S 2006 Hydrogen peroxide: A signaling messenger. *Antioxidants & Redox Signaling* **8** (3-4) 243–270.

Stromberg U, Lundh T, Schutz A & Skerfving S 2003 Yearly measurements of blood lead in Swedish children since 1978: An update focusing on the petrol lead free period 1995-2001. *Occupational & Environmental Medicine* **60** (5) 370–372.

Sugino N, Karube-Harada A, Taketani T, Sakata A & Nakamura Y 2004 Withdrawal of ovarian steroids stimulates prostaglandin F2alpha production through nuclear factor-kappa B activation via oxygen radicals in human endometrial stromal cells: Potential relevance to menstruation. *The Journal of Reproduction & Development* **50** (2) 215–225.

Sussan TE, Sudini K, Talbot CC, JR, Wang X, Wills-Karp M, Burd I & Biswal S 2017 Nrf2 regulates gene-environment interactions in an animal model of intrauterine inflammation: Implications for preterm birth and prematurity. *Scientific Reports* **7** 40194.

Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ & Sugino N 2008 Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *Journal of Pineal Research* **44** (3) 280–287.

Tang N & Zhu ZQ 2003 Adverse reproductive effects in female workers of lead battery plants. *International Journal of Occupational Medicine & Environmental Health* **16** (4) 359–361.

Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P & Focarelli R 2008 Cellular and molecular aspects of ovarian follicle ageing. *Human Reproduction Update* **14** (2) 131–142.

Taupeau C, Poupon J, Nome F & Lefevre B 2001 Lead accumulation in the mouse ovary after treatment-induced follicular atresia. *Reproductive Toxicology* **15** (4) 385–391.

Taupeau C, Poupon J, Treton D, Brosse A, Richard Y & Machelon V 2003 Lead reduces messenger RNA and protein levels of cytochrome p450 aromatase and estrogen receptor beta in human ovarian granulosa cells. *Biology of Reproduction* **68** (6) 1982–1988.

Taylor CM, Golding J, Hibbeln J & Emond AM 2013 Environmental factors predicting blood lead levels in pregnant women in the UK: The ALSPAC study. *PloS One* **8** (9) e72371.

Telleria CM, Goyeneche AA, Stocco CO & Gibori G 2004 Involvement of nuclear factor kappa B in the regulation of rat luteal function: Potential roles as survival factor and inhibitor of 20alpha-hydroxysteroid dehydrogenase. *Journal of Molecular Endocrinology* **32** (2) 365–383.

Terzic J, Grivennikov S, Karin E & Karin M 2010 Inflammation and colon cancer. *Gastroenterology* **138** (6) 2101-2114.

Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M & Biswal S 2002 Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Research* **62** (18) 5196–5203.

Thompson LP & Al-Hasan Y 2012 Impact of oxidative stress in fetal programming. *Journal of Pregnancy* **2012** 582748.

Tilstra JS, Gaddy DF, Zhao J, Davé SH, Niedernhofer LJ, Plevy SE & Robbins PD 2014 Pharmacologic IKK/NF-κB inhibition causes antigen presenting cells to undergo TNFα dependent ROS-mediated programmed cell death. *Scientific Reports* **4** 3631.

Torchinsky A & Toder V 2004 To die or not to die: The function of the transcription factor NF-kappa B in embryos exposed to stress. *American Journal of Reproductive Immunology* **51** (2) 138–143.

Umezawa K 2011 Possible role of peritoneal NF-kappa B in peripheral inflammation and cancer: Lessons from the inhibitor DHMEQ. *Biomedicine & Pharmacotherapy* **65** (4) 252–259.

Uttara B, Singh AV, Zamboni P & Mahajan RT 2009 Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology* **7** (1) 65–74.

Valko M, Izakovic M, Mazur M, Rhodes CJ & Telser J 2004 Role of oxygen radicals in DNA damage and cancer incidence. *Molecular & Cellular Biochemistry* **266** (1-2) 37–56.

Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M & Telser J 2007 Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell biology* **39** (1) 44–84.

van Beker Woudenberg A, Grollers-Mulderij M, Snel C, Jeurissen N, Stierum R & Wolterbeek A 2012 The bovine oocyte in vitro maturation model: A potential tool for reproductive toxicology screening. *Reproductive Toxicology* **34** (2) 251–260.

van der Wijst MGP, Brown R & Rots MG 2014 Nrf2, the master redox switch: The Achilles' heel of ovarian cancer? *Biochimica et Biophysica Acta* **1846** (2) 494–509.

Vardatsikos G, Sahu A & Srivastava AK 2009 The insulin-like growth factor family: Molecular mechanisms, redox regulation, and clinical implications. *Antioxidants & Redox Signaling* **11** (5) 1165–1190.

Vassena R, Dee Schramm R & Latham KE 2005 Species-dependent expression patterns of DNA methyltransferase genes in mammalian oocytes and preimplantation embryos. *Molecular Reproduction & Development* **72** (4) 430–436.

Vaughan JE & Walsh SW 2012 Activation of NF-kappa B in placentas of women with preeclampsia. *Hypertension in Pregnancy* **31** (2) 243–251.

Vaziri ND 2008 Mechanisms of lead-induced hypertension and cardiovascular disease. *American journal of physiology. Heart & Circulatory Physiology* **295** (2) H454-65.

Verstraeten SV, Aimo L & Oteiza PI 2008 Aluminium and lead: Molecular mechanisms of brain toxicity. *Archives of Toxicology* **82** (11) 789–802.

Viatour P, Merville M-P, Bours V & Chariot A 2005 Phosphorylation of NF-kappa B and IkappaB proteins: Implications in cancer and inflammation. *Trends in Biochemical Sciences* **30** (1) 43–52.

Vigeh M, Yokoyama K, Seyedaghamiri Z, Shinohara A, Matsukawa T, Chiba M & Yunesian M 2011 Blood lead at currently acceptable levels may cause preterm labour. *Occupational & environmental medicine* **68** (3) 231–234.

Vij AG 2009 Hemopoietic, hemostatic and mutagenic effects of lead and possible prevention by zinc and vitamin C. *Al Ameen Journal of Medical Sceince* **2** (2).

Wakabayashi N, Slocum SL, Skoko JJ, Shin S & Kensler TW 2010 When Nrf2 talks, who's listening? *Antioxidants & Redox Signaling* **13** (11) 1649–1663.

Wallace DC 2010 Bioenergetics and the epigenome: Interface between the environment and genes in common diseases. *Developmental Disabilities Research Reviews* **16** (2) 114–119.

Wallace NA, Belancio VP & Deininger PL 2008 L1 mobile element expression causes multiple types of toxicity. *Gene* **419** (1-2) 75–81.

Wang C, Liang J, Zhang C, Bi Y, Shi X & Shi Q 2007a Effect of ascorbic Acid and thiamine supplementation at different concentrations on lead toxicity in liver. *The Annals of Occupational Hygiene* **51** (6) 563–569.

Wang L, Wang F, Guan J, Le J, Wu L, Zou J, Zhao H, Pei L, Zheng X & Zhang T 2010 Relation between hypomethylation of long interspersed nucleotide elements and risk of neural tube defects. *The American Journal of Clinical Nutrition* **91** (5) 1359–1367.

Wang XJ, Sun Z, Chen W, Eblin KE, Gandolfi JA & Zhang DD 2007b Nrf2 protects human bladder urothelial cells from arsenite and monomethylarsonous acid toxicity. *Toxicology & Applied Pharmacology* **225** (2) 206–213.

Wang XJ, Sun Z, Villeneuve NF, Zhang S, Zhao F, Li Y, Chen W, Yi X, Zheng W & Wondrak GT, Wong PK & Zhang DD 2008 Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs, the dark side of Nrf2. *Carcinogenesis* **29** (6) 1235–1243.

Wang Y, Chan S & Tsang BK 2002 Involvement of inhibitory nuclear factor-kappa B (NFkappa B)-independent NFkappa B activation in the gonadotropic regulation of X-linked inhibitor of apoptosis expression during ovarian follicular development in vitro. *Endocrinology* **143** (7) 2732–2740.

Wani AL, Ara A & Usmani JA 2015 Lead toxicity: A review. *Interdisciplinary Toxicology* **8** (2) 55–64.

Wardyn JD, Ponsford AH & Sanderson CM 2015 Dissecting molecular cross-talk between Nrf2 and NF-kappa B response pathways. *Biochemical Society Transactions* **43** (4) 621–626.

Wasowicz W, Gromadzinska J & Rydzynski K 2001 Blood concentration of essential trace elements and heavy metals in workers exposed to lead and cadmium. *International Journal of Occupational Medicine & Environmental health* **14** (3) 223–229.

Wassarman PM & Litscher ES 2012 Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility. *The International Journal of Developmental Biology* **56** (10-12) 833–839.

Whiteside ST & Israel A 1997 I kappa B proteins: Structure, function and regulation. *Seminars in Cancer Biology* **8** (2) 75–82.

Wieloch M, Kaminski P, Ossowska A, Koim-Puchowska B, Stuczynski T, Kuligowska-Prusinska M, Dymek G, Mankowska A & Odrowaz-Sypniewska G 2012 Do toxic heavy metals affect antioxidant defense mechanisms in humans? *Ecotoxicology & Environmental Safety* **78** 195–205.

Willcox JK, Ash SL & Catignani GL 2004 Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science & Nutrition* **44** (4) 275–295.

Wintz H, Fox T & Vulpe C 2002 Responses of plants to iron, zinc and copper deficiencies: Portland Press Limited.

Wojsiat J, Korczynski J, Borowiecka M & Zbikowska HM 2017 The role of oxidative stress in female infertility and in vitro fertilization. *Advances in Hygiene & Experimental Medicine* **71** (0) 359–366.

Wolffe AP & Guschin D 2000 Review: Chromatin structural features and targets that regulate transcription. *Journal of Structural Biology* **129** (2-3) 102–122.

Woolf AD, Goldman R & Bellinger DC 2007 Update on the clinical management of childhood lead poisoning. *Pediatric Clinics of North America* **54** (2) 271-94.

Wright RO, Schwartz J, Wright RJ, Bollati V, Tarantini L, Park SK, Hu H, Sparrow D, Vokonas P & Baccarelli A 2010 Biomarkers of lead exposure and DNA methylation within retrotransposons. *Environmental Health Perspectives* **118** (6) 790–795.

Xie J & Shaikh ZA 2006 Cadmium-induced apoptosis in rat kidney epithelial cells involves decrease in nuclear factor-kappa B activity. *Toxicological Sciences* **91** (1) 299–308.

Xu J, Yan HC, Yang B, Tong LS, Zou YX & Tian Y 2009 Effects of lead exposure on hippocampal metabotropic glutamate receptor subtype 3 and 7 in developmental rats. *Journal of Negative Results in Biomedicine* **8** 5.

Yadav RK, Goyal B, Sharma RK, Dubey SK & Minhas PS 2002 Post-irrigation impact of domestic sewage effluent on composition of soils, crops and ground water-a case study. *Environment International* **28** (6) 481–486.

Yang AS, Estécio MRH, Doshi K, Kondo Y, Tajara EH & Issa J-PJ 2004 A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Research* **32** (3) e38.

Yang C, Atkinson SP, Vilella F, Lloret M, Armstrong L, Mann DA & Lako M 2010 Opposing putative roles for canonical and noncanonical NFkappa B signaling on the survival, proliferation, and differentiation potential of human embryonic stem cells. *Stem Cells* **28** (11) 1970–1980.

Yang H, Magilnick N, Lee C, Kalmaz D, Ou X, Chan JY & Lu SC 2005 Nrf1 and Nrf2 regulate rat glutamate-cysteine ligase catalytic subunit transcription indirectly via NF-kappa B and AP-1. *Molecular & Cellular Biology* **25** (14) 5933–5946.

Yiin SJ & Lin TH 1995 Lead-catalyzed peroxidation of essential unsaturated fatty acid. *Biological Trace Element Research* **50** (2) 167–172.

Yu M, Li H, Liu Q, Liu F, Tang L, Li C, Yuan Y, Zhan Y, Xu W, Li W Chen H, Ge C, Wang J & Yang X 2011 Nuclear factor p65 interacts with Keap1 to repress the Nrf2-ARE pathway. *Cellular Signalling* **23** (5) 883–892.

Yuan X & Tang C 2001 The accumulation effect of lead on DNA damage in mice blood cells of three generations and the protection of selenium. *Journal of Environmental Science & Health* **36** (4) 501–508.

Zaken V, Kohen R & Ornoy A 2000 The development of antioxidant defense mechanism in young rat embryos in vivo and in vitro. *Early Pregnancy (Online)* **4** (2) 110–123.

Zhang B, Xia W, Li Y, Bassig BA, Zhou A, Wang Y, Li Z, Yao Y, Hu J, Du X Zhou Y, Liu J, Xue W, Ma Y, Pan X, Peng Y, Zheng T & Xu S 2015 Prenatal exposure to lead in relation to risk of preterm low birth weight: A matched case-control study in China. *Reproductive Toxicology* **57** 190–195.

Zhang H, Tan X, Yang D, Lu J, Liu B, Baiyun R & Zhang Z 2017 Dietary luteolin attenuates chronic liver injury induced by mercuric chloride via the Nrf2/NF-κB/P53 signaling pathway in rats. *Oncotarget* **8** (25) 40982–40993.

Zhao Y, Wang L, Shen HB, Wang ZX, Wei QY & Chen F 2007 Association between delta-aminolevulinic acid dehydratase (ALAD) polymorphism and blood lead levels: A meta-regression analysis. *Journal of Toxicology & Environmental Health* **70** (23) 1986–1994.

Zheng G, Tian L, Liang Y, Broberg K, Lei L, Guo W, Nilsson J, Bergdahl IA, Skerfving S & Jin T 2011 delta-Aminolevulinic acid dehydratase genotype predicts toxic effects of lead on workers' peripheral nervous system. *Neurotoxicology* **32** (4) 374–382.

References of chapter 5

Abdel Moneim AE 2016 Indigofera oblongifolia prevents lead acetate-induced hepatotoxicity, oxidative stress, fibrosis and apoptosis in rats. *PloS One* **11** (7) e0158965.

Ahmad MK, Khan AA, Ali SN & Mahmood R 2015 Chemoprotective effect of taurine on potassium bromate-induced DNA damage, DNA-protein cross-linking and oxidative stress in rat intestine. *PloS One* **10** (3) e0119137.

Al-Saleh I, Shinwari N, Mashhour A & Rabah A 2014 Birth outcome measures and maternal exposure to heavy metals (lead, cadmium and mercury) in Saudi Arabian population. *International Journal of Hygiene & Environmental Health* **217** (2-3) 205–218.

Asha KK & Devadasan K 2013 Protective effect of taurine on the mitochondria of albino rats induced with fulminant hepatic failure. *Biomedicine & Preventive Nutrition* **3** (3) 279–283.

Avazeri N, Denys A & Lefevre B 2006 Lead cations affect the control of both meiosis arrest and meiosis resumption of the mouse oocyte in vitro at least via the PKC pathway. *Biochimie* **88** (11) 1823–1829.

Baek KH, Oh KW, Lee WY, Lee SS, Kim MK, Kwon HS, Rhee EJ, Han JH, Song KH, Cha BY Lee KW & Kang MI 2010 Association of oxidative stress with postmenopausal osteoporosis and the effects of hydrogen peroxide on osteoclast formation in human bone marrow cell cultures. *Calcified Tissue International* **87** (3) 226–235.

Bellezza I, Mierla AL & Minelli A 2010 Nrf2 and NF-κB and their concerted modulation in Cancer Pathogenesis & Progression. *Cancers* **2** (2) 483–497.

Bolisetty S & Jaimes EA 2013 Mitochondria and reactive oxygen species: Physiology and pathophysiology. *International Journal of Molecular Sciences* **14** (3) 6306–6344.

Cervellati C, Bonaccorsi G, Cremonini E, Romani A, Fila E, Castaldini MC, Ferrazzini S, Giganti M & Massari L 2014 Oxidative stress and bone resorption interplay as a possible trigger for postmenopausal osteoporosis. *Biomedical Research International* **2014** 569563.

Chang B-J, Jang B-J, Son TG, Cho I-H, Quan F-S, Choe N-H, Nahm S-S & Lee J-H 2012 Ascorbic acid ameliorates oxidative damage induced by maternal low-level lead exposure in the hippocampus of rat pups during gestation and lactation. *Food & Chemical Toxicology* **50** (2) 104–108.

Chen F & Shi X 2002 Signaling from toxic metals to NF-kappa B and beyond: Not just a matter of reactive oxygen species. *Environmental Health Perspectives* **110** (Suppl 5) 807–811.

Cheng D, Wu R, Guo Y & Kong A-NT 2016 Regulation of Keap1-Nrf2 signaling: The role of epigenetics. *Current Opinion in Toxicology* **1** 134–138.

Conti MI, Terrizzi AR, Lee CM, Mandalunis PM, Bozzini C, Pineiro AE & Martinez MdP 2012 Effects of lead exposure on growth and bone biology in growing rats exposed to simulated high altitude. *Bulletin of Environmental Contamination & Toxicology* **88** (6) 1033–1037.

Cox JN, Rahman MA, Bao S, Liu M, Wheeler SE & Knoell DL 2016 Cadmium attenuates the macrophage response to LPS through inhibition of the NF-κB pathway. *American Journal of Physiology. Lung Cellular & Molecular Physiology* **311** (4) L754-L765.

Das J & Sil PC 2012 Taurine ameliorates alloxan-induced diabetic renal injury, oxidative stress-related signaling pathways and apoptosis in rats. *Amino Acids* **43** (4) 1509–1523.

Desforges M, Ditchfield A, Hirst CR, Pegorie C, Martyn-Smith K, Sibley CP & Greenwood SL 2013a Reduced placental taurine transporter (TauT) activity in pregnancies complicated by pre-eclampsia and maternal obesity. *Advances in Experimental Medicine & Biology* **776** 81–91.

Dieguez-Acuna FJ, Polk WW, Ellis ME, Simmonds PL, Kushleika JV & Woods JS 2004 Nuclear factor kappa B activity determines the sensitivity of kidney epithelial cells to apoptosis: Implications for mercury-induced renal failure. *Toxicological Sciences* **82** (1) 114–123.

Figueiredo FAT , **Gerlach RF, da Veiga MAMS, Nakadi FV, Ramos J, Kawakita ER, Guerra CdS & Issa JPM** 2014 Reduced bone and body mass in young male rats exposed to lead. *Biomedical Research International* **2014** 571065.

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

Flora SJS, Saxena G & Mehta A 2007 Reversal of lead-induced neuronal apoptosis by chelation treatment in rats: Role of reactive oxygen species and intracellular Ca^{2+} . *The Journal of pharmacology & Experimental Therapeutics* **322** (1) 108–116:

Gebara E, Udry F, Sultan S & Toni N 2015 Taurine increases hippocampal neurogenesis in aging mice. *Stem Cell Research* **14** (3) 369–379.

Gloire G, Legrand-Poels S & Piette J 2006 NF-kappa B activation by reactive oxygen species: Fifteen years later. *Biochemical Pharmacology* **72** (11) 1493–1505.

Grizlova LV & Yakimova EA 2014 Effect of lead acetate on the placental barrier and the development of bone tissue in the early ontogenesis. *Advances in Environmental Biology* 938–943.

Haleagrahara N, Jackie T, Chakravarthi S, Rao M & Kulur A 2010 Protective effect of Etlingera elatior (torch ginger) extract on lead acetate—induced hepatotoxicity in rats. *The Journal of Toxicological Sciences* **35** (5) 663–671.

Han X & Chesney RW 2012 The role of taurine in renal disorders. *Amino Acids* **43** (6) 2249–2263.

Harischandra DS, Ghaisas S, Rokad D & Kanthasamy AG 2017 Exosomes in toxicology: relevance to chemical exposure and pathogenesis of environmentally linked diseases. *Toxicological Sciences* **158** (1) 3–13.

Hasanein P, Ghafari-Vahed M & Khodadadi I 2017 Effects of isoquinoline alkaloid berberine on lipid peroxidation, antioxidant defense system, and liver damage induced by lead acetate in rats. *Redox Report* **22** (1) 42–50.

Karbalay-Doust S, Noorafshan A & Pourshahid SM 2012 Taxol and taurine protect the renal tissue of rats after unilateral ureteral obstruction: a stereological survey. *Korean Journal of Urology* **53** (5) 360–367.

Kaspar JW, Niture SK & Jaiswal AK 2009 Nrf2:INrf2 (Keap1) signaling in oxidative stress. In *Free Radical Biology & Medicine* 47 (9) 1304–1309.

Kitamura M & Hiramatsu N 2010 The oxidative stress: Endoplasmic reticulum stress axis in cadmium toxicity. *Biometals* **23** (5) 941–950.

Kitamura M 2009 Biphasic, bidirectional regulation of NF-kappa B by endoplasmic reticulum stress. *Antioxidants & Redox Signaling* **11** (9) 2353–2364.

Koh JH, Lee ES, Hyun M, Kim HM, Choi YJ, Lee EY, Yadav D & Chung CH 2014 Taurine alleviates the progression of diabetic nephropathy in type 2 diabetic rat model. *International Journal of Endocrinology* **2014** 397307.

Korashy HM & El-Kadi AOS 2006 The role of aryl hydrocarbon receptor and the reactive oxygen species in the modulation of glutathione transferase by heavy metals in murine hepatoma cell lines. *Chemico-Biological Interactions* 162 (3) 237–248.

Lee AS 2014 Glucose regulated proteins in cancer: Molecular mechanisms and therapeutic potential. *Nature reviews. Cancer* **14** (4) 263–276.

Li C, Yang X, Xu M, Zhang J & Sun N 2013 Epigenetic marker (LINE-1promoter) methylation level was associated with occupational lead exposure. *Clinical Toxicology* **51** (4) 225–229.

Li HW, Deng JG, Du ZC, Yan MS, Long ZX, Pham Thi PT & Yang KD 2013 Protective effects of mangiferin in subchronic developmental lead-exposed rats. *Biological Trace Element Research* **152** (2) 233–242.

Lingappan K 2018 NF-κB in oxidative stress. *Current Opinion in Toxicology* **7** 81–86.

Liu B, Zhang H, Tan X, Yang D, Lv Z, Jiang H, Lu J, Baiyun R & Zhang Z 2017 GSPE reduces lead-induced oxidative stress by activating the Nrf2 pathway and suppressing miR153 and GSK-3beta in rat kidney. *Oncotarget* **8** (26) 42226–42237.

Liu CM, Ma JQ, Xie WR, Liu SS, Feng ZJ, Zheng GH & Wang AM 2015 Quercetin protects mouse liver against nickel-induced DNA methylation and inflammation associated with the Nrf2/HO-1 and p38/STAT1/NF-kappa B pathway. *Food & Chemical Toxicology* **82** 19–26.

Liu MJ, Bao S, Gálvez-Peralta M, Pyle CJ, Rudawsky AC, Pavlovicz RE, Killilea DW, Li C, Nebert DW & Wewers MD & Knoell DL 2013 ZIP8 regulates host defense through zinc-mediated inhibition of NF-κB. *Cell Reports* **3** (2) 386–400.

Lu Z, He X, Ma B, Zhang L, Li J, Jiang Y, Zhou G & Gao F 2019 Dietary taurine supplementation improves breast meat quality in chronic heat-stressed broilers via activating the Nrf2 pathway and protecting mitochondria from oxidative attack. *Journal of the Science of Food & Agriculture* **99** (3) 1066–1072.

Meakin PJ, Chowdhry S, Sharma RS, Ashford FB, Walsh SV, Mc-Crimmon RJ, Dinkova-Kostova AT, Dillon JF, Hayes JD & Ashford MLJ 2014 Susceptibility of Nrf2-null mice to steatohepatitis and cirrhosis upon consumption of a high-fat diet is associated with oxidative stress, perturbation of the unfolded protein response, and disturbance in the expression of metabolic enzymes but not with insulin resistance. *Molecular & Cellular Biology* **34** (17) 3305–3320.

Misra UK, Deedwania R & Pizzo SV 2006 Activation and cross-talk between Akt, NF-kappa B, and unfolded protein response signaling in 1-LN prostate cancer cells consequent to ligation of cell surface-associated GRP78. *The Journal of Biological Chemistry* **281** (19) 13694–13707.

Nakade UP, Garg SK, Sharma A, Choudhury S, Yadav RS, Gupta K & Sood N 2014 Lead-induced adverse effects on the reproductive system of rats with particular reference to histopathological changes in uterus. *Indian Journal of Pharmacology* 47 (1) 22–26.

Nakajima S & Kitamura M 2013 Bidirectional regulation of NF-kappa B by reactive oxygen species: A role of unfolded protein response. *Free Radical Biology & Medicine* **65** 162–174.

Nandi S, Gupta PSP, Selvaraju S, Roy SC & Ravindra JP 2010 Effects of exposure to heavy metals on viability, maturation, fertilization, and embryonic development of buffalo (Bubalus bubalis) oocytes in vitro. *Archives of Environmental Contamination & Toxicology* **58** (1) 194–204.

Nilsson EE, Sadler-Riggleman I & Skinner MK 2018 Environmentally induced epigenetic transgenerational inheritance of disease. *Environmental Epigenetics* **4** (2) dvy016.

Nishimura T, Duereh M, Sugita Y, Yoshida Y, Higuchi K, Tomi M & Nakashima E 2015 Protective effect of hypotaurine against oxidative stress-induced cytotoxicity in rat placental trophoblasts. *Placenta* **36** (6) 693–698.

Nishimura T, Sai Y, Fujii J, Muta M, Iizasa H, Tomi M, Deureh M, Kose N & Nakashima E 2010 Roles of TauT and system A in cytoprotection of rat syncytiotrophoblast cell line exposed to hypertonic stress. *Placenta* **31** (11) 1003–1009.

Nye MD, Hoyo C & Murphy SK 2015 In vitro lead exposure changes DNA methylation and expression of IGF2 and PEG1/MEST. *Toxicology in Vitro* **29** (3) 544–550.

Qian Y, Harris ED, Zheng Y & Tiffany-Castiglioni E 2000 Lead targets GRP78, a molecular chaperone, in C6 rat glioma cells. *Toxicology & Applied Pharmacology* **163** (3) 260–266.

Rahman A, Al-Rashidi HAG & Khan A-R 2012 Association of maternal blood lead level during pregnancy with child blood lead level and pregnancy outcome in Kuwait. *Ecology of Food & Nutrition* **51** (1) 40–57.

Roy A & Sil PC 2012 Tertiary butyl hydroperoxide induced oxidative damage in mice erythrocytes: Protection by taurine. *Pathophysiology* **19** (2) 137–148.

Roysommuti S & Wyss JM 2014 Perinatal taurine exposure affects adult arterial pressure control. *Amino Acids* **46** (1) 57–72.

Saleh HA, El-Aziz GA, El-Fark MM & El-Gohary M 2009 Effect of maternal lead exposure on craniofacial ossification in rat fetuses and the role of antioxidant therapy. *Anatomia, Histologia Embryologia* **38** (5) 392–399.

Schneider JS, Kidd SK & Anderson DW 2013 Influence of developmental lead exposure on expression of DNA methyltransferases and methyl cytosine-binding proteins in hippocampus. *Toxicology Letters* **217** (1) 75–81.

Sharma R, Qureshi N, Mogra S & Panwar K 2012 Lead induced infertility in swiss mice and role of antioxidants. *Universal Journal of Environmental Research & Technology* **2** (2).

Shivananjappa MM & Muralidhara 2012 Taurine attenuates maternal and embryonic oxidative stress in a streptozotocin-diabetic rat model. *Reproductive Biomedicine Online* **24** (5) 558–566.

Sirdah MM 2015 Protective and therapeutic effectiveness of taurine in diabetes mellitus: a rationale for antioxidant supplementation. *Diabetes & Metabolic Syndrome* **9** (1) 55–64.

Sirdah MM, Abushahla AK & Al-Sarraj HAA 2013 Effect of the addition of the antioxidant taurine on the complete blood count of whole blood stored at room temperature and at 4 masculineC for up to 7 days. *Revista Brasileira de Hematologia e Hemoterapia* **35** (1) 44–51.

Sivandzade F, Prasad S, Bhalerao A & Cucullo L 2018 Nrf2 and NF-κB interplay in cerebrovascular and neurodegenerative disorders: Molecular mechanisms and possible therapeutic approaches. *Redox Biology* **21** 101059.

Tebay LE, Robertson H, Durant ST, Vitale SR, Penning TM, Dinkova-Kostova AT & Hayes JD 2015 Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. *Free Radical Biology & Medicine* **88** (0 0) 108–146.

Tripathi A, Shrivastav TG & Chaube SK 2013 An increase of granulosa cell apoptosis mediates aqueous neem (Azadirachta indica) leaf extract-induced oocyte apoptosis in rat. *International Journal of Applied & Basic Medical Research* **3** (1) 27–36.

Verner A, Craig S & McGuire W 2007 Effect of taurine supplementation on growth and development in preterm or low birth weight infants. *The Cochrane database of systematic reviews* (4) CD006072.

Wang Y, Hu H, Li H, Ma H, Xu F & Qu B 2014 Effects of lead exposure on placental cellular apoptosis and endoplasmic reticulum stress in rats. *Chinese medical journal* **127** (9) 1744–1748.

Wardyn JD, Ponsford AH & Sanderson CM 2015 Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochemical Society Transactions* **43** (4) 621–626.

Wu G, Tang R, Yang J, Tao Y, Liu Z, Feng Y, Lin S, Yang Q, Lv Q & Hu J 2015 Taurine accelerates alcohol and fat metabolism of rats with alcoholic Fatty liver disease. *Advances in Experimental Medicine & Biology* **803** 793–805.

Xie J & Shaikh ZA 2006 Cadmium-induced apoptosis in rat kidney epithelial cells involves decrease in nuclear factor-kappa B activity. *Toxicological Sciences* **91** (1) 299–308.

Xu X, Liu T, Zhang A, Huo X, Luo Q, Chen Z, Yu L, Li Q, Liu L & Lun ZR & Shen J 2012 Reactive oxygen species-triggered trophoblast apoptosis is initiated by endoplasmic reticulum stress via activation of caspase-12, CHOP, and the JNK pathway in Toxoplasma gondii infection in mice. *Infection & Immunity* **80** (6) 2121–2132.

Yedjou CG, Tchounwou HM & Tchounwou PB 2015 DNA damage, cell cycle arrest, and apoptosis induction caused by lead in human leukemia cells. *International Journal of Environmental Research & Public Health* **13** (1) ijerph13010056.

Yuan G, Dai S, Yin Z, Lu H, Jia R, Xu J, Song X, Li L, Shu Y & Zhao X, Chen Z, Fan Q, Liang X, He C, Yin L, Lv C, Lei Q, Wang L, Mi Y, Yu X & Zhang M 2014 Sub-chronic lead and cadmium co-induce apoptosis protein expression in liver and kidney of rats. *International Journal of Clinical & Experimental Pathology* **7** (6) 2905–2914.

Zhang Y, Sun LG, Ye LP, Wang B & Li Y 2008 Lead-induced stress response in endoplasmic reticulum of astrocytes in CNS. *Toxicology Mechanisms & Methods* **18** (9) 751–757.

Zhang Z, Liu D, Yi B, Liao Z, Tang L, Yin D & He M 2014 Taurine supplementation reduces oxidative stress and protects the liver in an iron-overload murine model. *Molecular Medicine Reports* **10** (5) 2255–2262.

First of all, my great thanks to ALLAH, the most merciful, the most gracious, who helps me in all my life events, and provides me the persistence and strength to accomplish this work.

No word can express my deepest appreciation to my supervisor, Prof. Dr. Karl Schellander, for everything that he has done for me during the steps of the current study. For giving me the chance to complete my PhD in ITW and for making my life so pleasant that I didn't have to worry during my work. For his confidence in me to the extent that I realized many aspects of power in me that I never experienced before. For his deep encouragement and support to me and my family. For converting my fears and furstrations into strengths. For being an inspiring father and giving me great confidence that my hard work will pay off. I have been honored being one of his students.

I would like to thank Prof. Dr. Peter Stehle for his willingness to co-supervise my thesis and for his evaluations and insightful comments. Special thanks go to Prof. Dr. Karl-Heinz Südekum for his kind and encouraging words appretiating his time he took to advise and encourage me.

I wish to express thanks and deepest gratitude to PD. Dr. Dawit Tesfaye for his efforts and leadership. His support, understanding, thoughtfulness and constructive comments have empowered me and his confidence in me has encouraged me always to do my best.

I respect and thank PD. Dr. Michael Hölker, head of Frankenforst research for his understanding and giving me such precious opportunity to achieve the IVF part of my thesis in Frankenforst in spite of his busy schedule. I would like to take a moment to thank Mrs Franca Rings, for her commitment and great assistance in the IVF part of my thesis and for teaching and training me sincerely the details of IVF and immunostaining. I would like also to thank Dr. Christine Neuhoff for their help with the German translation of the abstract of the thesis.

I am grateful to Dr. Dessie Salilew-Wondim for the kind help, insights and for being like a grand brother during my study. My special thanks to Dr. Samuel Gebremedhn for his indispensable advice, constant encouragement, kind guidance, efforts in revising my thesis and for standing by my side when times went hard.

My deep thanks and wishes to Dr. Ernst Tholen for his kindness, sense of humor and encouragement to do my best in my work and to learn the German language. And my thanks to his amazing cooperative group; Dr. Christine Große-Brinkhaus, Katharina Roth, Esther Heuß, Ines Brinke and Christina Dauben.

Great thanks to Mrs Biance Peters for her administrative support and help. I would like to thank the technical staff; Helga Brodeßer, Birgit Koch, Nadine Leyer, Julia Lindlar and Michel Posanski for their generous help and active participation to accomplish my laboratory work and for being my second family during the three years of my study. I appreciate Mr. Heinz Biörnsen for providing me the ovarian samples from the slaughterhaus.

I have received a great deal of support and assistance from my dear colleagues, Dr. Mikhael Poirier, Dr. Lucia Martinez-Fresneda, Mr. Haiko Hofmann, Dr. Hari Om Pandey, Mr. Teshome Alemu and Dr. Omar Khadrawy, Dr. Aboubakr Taqi. I heartily thank you all and I will keep remembering our sweet and fruitful moments. Special thanks to my dear colleague Dr. Tsige Hailay for being my close friend and sister during my study and for every step we shared together during the laboratorywork. I sincerely thank my dear friend, Mr. Dennis Miskel for assisting me while working in Frankenforst and while writing my thesis. Further, I would like in particular to thank my former colleague, Dr.Ashraf El-sayed for his assistance sceintifically and morally.

Special thanks to the staff members of Flowcytometry Core Facility, Mr. Peter Wurst, Mr. Andreas Dolf and Mr. Maximilian Germer at the Institute of Molecular Medicine for their training and deep explanation of basics of flow cytometer. My great appreciations to my kind friend, Dr. Philippe Fuchs in INRES Institute, Chemical signaling Department, for his generous assistance with the confocal microscope facility.

On the Egyptian side of my thesis, I wish to express my thanks and gratitude to Dr. Asmaa Munir Kandil, Professor of Embryology, National Organization for Drug Control and Research, Cairo, Egypt. Since I was a fresh graduate, she taught me sincerely the basics of teratology until I became mature in this interesting field with her ongoing support. She introduced me to Professor Schellander and assisted me to get this chance in his institution. I am grateful to her effort and time she sacrificed to help me and my family.

I want to express my deep appreciation to Dr. Afaf Ain Shoka, Professor of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, for her generosity, kindness, keen advice and understanding. For all the things she has done for me since I was a master student till now. I would like also to thank Dr. Marwa Safar, Associate Professor of Pharmacology and Toxicology,

Faculty of Pharmacy, Cairo University, for enriching me with her vast experience and valuable comments on my thesis part achieved in Egypt.

I would like to express my thank all staff members in the Pharmacology Department, National Organization for Drug Control and Research, Egypt, and to all my colleagues for their support and kind wishes.

I am grateful to The Egyptian Higher Education Ministry, Cultural Affairs and Mission Sector, for the financial support of my scholarship.I would like also to thank The Egytian Cultural Office in Berlin, for their care and administrative help during my stay in Germany.

My grateful thanks to my parents, who put me on the first way of science, for their unconditional love, for giving me a continuous boost when I have fallen down and calming me down when I needed it. I cannot thank my brothers (Mohammed and Osama) enough for their care, moral support and encouragement. My journey would not have been possible without my life partner, my beloved husband, Dr. Diaa Hamama. I am indebted to him for his love, understanding and support, for listening to my frustrations, brightening up my life and celebrating my accomplishments. Thank you for making my heart filled with love and peace whatever I met in this world. "*Mein Schatz; Wie Lily zwischen Dornen, bist du zwischen Männern. Ich bin stolz auf dich. Nur jetzt, konnen wir unser Erfolg feiern. Wie ich getraumt, werde ich alle Straßen in Bonn umarmen, kein Weinen, Keine Sorge and keine Angst*". Finally, I like to thank the precious gift of my life, my little hero, my son, Nooreldeen for giving me unlimited happiness and pleasure; he had inspired me in ways that he may never understand.