CHAPTER 5

DISCUSSION

DISCUSSION

Oceanography monitoring

A decisive step in the development of human technology and culture was the discovery of metals below the surface of our planet, their excavation, extraction and use as tools to fulfill human needs. Nowadays, we recognize that the wastes of metals are distributed over the soils and waters of the earth's surface and exert detrimental effects on life in the environment and human health. Unlike organic waste, metals and their compounds are not degraded by living organisms and may accumulate up to harmful levels [255].

Heavy metal contamination in marine ecosystems is of global concern. Metals generally enter the aquatic environment via atmospheric deposition, geological matrix erosion or due to anthropogenic impacts caused by industrial effluents, domestic sewage, mining wastes, and agricultural activities [256]. It will be toxic to physiological and behavioral effects on the aquatic biota which results in adverse effects on humans. Many investigations are going on to detect and reduce the effects of heavy metals on ecosystem, aquaculture and humans and to quantify its presence in coastal waters and tissues of aquatic animals [257, 258].

The accumulation patterns of heavy metals in fish and other aquatic organisms depend on their uptake and elimination rates [259]. The contamination levels of the aquatic environment by heavy metals can be estimated by analyzing water, sediments and marine organisms. The levels of heavy metals in mollusks and other invertebrates are often considerably higher than in other constituents of marine environment due to their habitat and their feeding habits [259-261]. Compared to sediments, mollusks exhibit greater spatial sensitivity and therefore, are the most reliable tool for identifying sources of biologically available heavy metal contamination [262-265].

Some studies have been developed to assess certain biological parameters as specific markers or indicators which help to examine the existence or quantity of heavy metals. Biomarkers offer a qualitative measure of exposure to toxic chemicals or environmental stresses [266]. In this aspect mussels are used as test organisms for biomarker survey because they are widely distributed geographically, sessile and resistant to a wide range of metal concentrations [267]. Among the most used biomarker for pollution in marine environment, metallothioneins have been particularly useful as monitoring device, namely as a contaminant specific biochemical indicator of metal exposure [268]. Therefore, the primary purpose of the present study was to obtain quantitative estimation of metallothionein concentrations in mussels as a biomarker of exposure to heavy metals, to monitoring the pollution of Abu Qir bay (El-Maadiya region).

The present results indicated that the studied area was contaminated with some heavy metals as cadmium, lead, copper, chromium, and zinc which were in agreement with many previous studies, which proved that Abu Qir bay contaminated with many heavy metals as cadmium, nickel, cobalt, and aluminum [259,262,263,265]. Meanwhile, the present study proved the presence of measurable amounts of metallothionein in mussel samples collected from the studied area. The variations of metallothionein concentrations in different mussel samples are due to a random mussel samples were collected from studied area.

Metallothioneins are inducible proteins. Heavy metal cations accumulated within the cells stimulate metalloprotein neosynthesis by enhancing metallothionein gene transcription. The MT mRNA is translated by cytosolic free ribosome, leading to an increase of apo-metallothionein that will rapidly react with free metal cations present in the cytosol [269]. Due to their biochemical and functional characteristics, metallothioneins are able to protect cell structures from non-specific interactions with heavy metal cations and to detoxify metal excess penetrating into the cell. Due to their inducibility to heavy metals, metallothioneins are usually considered an important specific biomarker to detect organism response to inorganic pollutants such as Cd, Hg, Cu, Zn present in the aquatic environment. Bioindicator organisms that have been commonly employed in the application of metallothioneins as biomarkers are fish, mollusks and crustaceans. The importance of metallothioneins as a tool for biomonitoring activities is increased by the fact that they are ubiquitary proteins and therefore can be studied in most living organisms [269].

Metallothioneins (MTs) have been implicated in the homeostasis of essential metals such as Cu and Zn, and in the detoxification of excess levels of essential and nonessential metals in marine invertebrates [270]. Several researches demonstrated that metallothioneins protect organisms from metal toxicity due to the ability of MT to bind metals (eg. Cd, Cu, Zn and Hg), and the ability to increase the metallothionein expression with the raise of metal concentrations in tissues [271-275]. Furthermore, different studies suggested that metallothioneins can be a useful surrogate for the toxicity assessment of metals in aquatic organisms before they experience sublethal and lethal damage [258, 276], as reported previously by several authors. Amiard et al. (2006) [271] stated that metallothioneins play roles both in the routine metabolic handling of essential Cu and Zn, but also in the detoxification of intracellular excess amounts of these metals and of non-essential Cd, Ag and Hg. Perceval et al. (2006) [272] showed that metallothioneins induction was correlated to environment and organisms Cd levels. Etelvina et al (2012) [277] showed that exposure to heavy metals as Cd, Cu and Zn induce the synthesis of metallothioneins, for any concentration of the metal used, metallothioneins increased significantly compared to the control, these results confirmed that metals induce the expression of metallothioneins, even when organisms apparently are not in stress, and that induction increased with the concentration of metal. Thus metallothioneins may serve as a very sensitive biomarker when bivalves are exposed to cadmium stress. Paul-Pont et al. (2010b) [278] demonstrated that the concentration of metallothioneins increased with the progressive accumulation of cadmium. Freitas et al (2012a, 2012b) [279, 280], demonstrated the high sensitivity of metallothioneins in *C. edule* exposed to different levels of environmental metal contamination (Cd, As, Hg, Pb, Zn, Cr, Ni, and Cu). Works performed by Smaoui-Damak et al (2004) [281], demonstrated the potential use of metallothioneins as a biomarker of Cd contamination in *Ruditapes decussates*. Recently Serafim and Bebiano (2010) [282] described the response of MTs to a mixture of sub-lethal Cd, Cu and Zn concentrations in *R. decussates*. The potential of MTs, as a biomarker of metal exposure (Cd, Cu and Zn) was shown in *R. decussatus* from the environment [283, 284]. Working with *Ruditapes philippinarum*, Ng and Wang (2004) [285] demonstrated that MTs play an important role in the detoxification of Cd. The sensitivity of MTs was further demonstrated when R. *phillipinarum* was subjected to Cd [286]. Figueira et al (2012) [287], while studying the responses of *R. decussatus* and *R. philippinarum* to Cd exposure, also showed the induction of MTs synthesis with increasing Cd levels.

Biological monitoring

Living around polluted areas is one of the most common sources of exposure to environmental toxicants. Of these toxicants, heavy metals are widely used in foundries, mining, and manufacturing industries. Once heavy metals accumulate in the ecosystem components, such as air, soil, and water the risk of human exposure increases among industrial workers, as well as, the people who live near polluted areas [288-291]. Heavy metals adversely affect a variety of body systems such as the cardiovascular, the respiratory, the endocrine, the immune, and the reproductive systems [292,293]. In addition, long-term exposure and accumulation of heavy metals in the body may disturb oxidative stress genes and thus increase the susceptibility to various diseases [294].

Abu Qir Bay is a shallow semi-circular tideless basin east of Alexandria. The bay is adjacent to one of the most populous, most industrialized and most commercialized coastal metropolitan areas in Egypt. The people of the region put the Bay to a wide variety of recreational, commercial, and industrial uses. The Bay contributes about 10 % of the fish and shrimp catch from the Egyptian Mediterranean waters. The bay is subjected to multiple pollution from two main point sources *(i)* Tabia Pumping Station, the station pumps to the bay (1.5–2.0) x 10^6 m^3 d^{-1} of industrial waste water containing "Black liquor" from 20 different factories, mostly textile, food processing and canning, and mixed agricultural as well as domestic waste water and *(ii)* Lake Edku Outlet [241].

In the light of information described above, residents of El Maadiya region face immediate environmental impact of heavy metals pollution; however, no single study in the available literature has addressed the impact of heavy metals contamination to human resident in study area. Therefore, the second purpose of the present study was conducted to study the risk assessment of the environmental pollution in Abu Qir bay on human health through the determination of some metals in blood of all studied subjects, metallothionein and the detection of oxidative stress through the estimation of malondialdehyde (MDA), glutathione content (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), and their impact on the gene expression of insulin-like growth factor 2 (IGF-2).

The present study was conducted on (56) human subjects who were divided to two groups: group I contained (12) control subjects and group II comprised of (44) fishermen.

The results of the current study proved the presence of high concentrations of some heavy metals as cadmium, chromium, lead, copper and Zinc in the blood of fishermen group, associated with striking significant high levels of metallothionein ($p \leq 0.009$) in their erythrocytes.

The most conspicuous feature of metallothionein (MT) is the inducibility of MT-1 and MT-2 genes by a variety of agents and conditions. The regulation of MT biosynthesis occurs primarily at the level of transcription, where *cis*-acting DNA elements respond to transacting transcriptional regulatory proteins. MT-1 and MT-2 genes in higher species are rapidly induced in vitro and in vivo by a variety of stimuli including metals, hormones, cytokines, oxidants, stress and irradiation [294].

Owing to their induction by a variety of stimuli, metallothioneins are considered valid biomarker in medical and environmental field [295]. Metallothionein is a remarkable protein, with a variety of cellular roles to play in homeostasis and during the management of toxicant exposure [296]. This small, cysteine-rich protein can complex with divalent heavy metal cations

via cysteine associated thiols. Under normal circumstances, metallothionein is associated with zinc and copper cations, and thus serves as an important reservoir of these essential metals, donating them to apoenzymes and other apoproteins that require these metals to function [297]. Other metals (e.g., mercury and cadmium) can also complex with metallothionein, thereby limiting the toxic effects of these metals. Metallothionein's cysteine-associated thiols can also serve as anti-oxidant moieties that protect DNA and other macromolecules from the damaging effects of reactive oxygen species (ROS). This regulation of ROS levels can indirectly regulate NF-κB activity, but metallothionein has also been found to directly interact with NF-κB and may regulate NF-κB-responsive genes via that direct interaction [298,299].

The upstream regulatory regions that control metallothionein biosynthesis are quite complex. Inducers of metallothionein include metals, organic solvents and other environmental pollutants, irradiation, endotoxin, glucocorticoids, acute phase cytokines, and ROS, each of which is associated with immunomodulatory activity [297].

Metallothionein (MT) is thought to be involved in homeostasis of the essential metals, copper, and zinc, as it is the major zinc and copper binding protein in many tissues, and there is a close relationship between tissue MT and zinc content. The importance of zinc is evident from its role as a major component of many enzymes and cell processes and from the severe pathology observed in response to zinc deficiency in skin, neurological, immune, and reproductive tissues. Indeed, second to iron, zinc is the most abundant trace element in the body [300].

A hypothesis has proposed that metallothionein acts as a chaperone during synthesis and modulation of metalloproteins and metallothionein appears to be stabilized at high cellular GSH concentrations. Metal-requiring apoenzymes can abstract metals from MT as demonstrated *in vitro*. GSH can form a complex with MT; release of zinc from MT mediated by interactions with GSH and GSSG through *S*-thiolation has been reported [300,301]. Evidence suggests that zinc release from MT is facilitated by direct coupled interaction of GSH and GSSG with MT [301] via the thiolate ligands that confer redox activity on zinc clusters resulting in an oxidoreductive MT-Zn complex [300]. The aforementioned finding confirm the results of herein study which proved the presence of negative correlation coefficient ($r = -0.321$, $p< 0.030$) between metallothionein and glutathione values. MT therefore may be acting as a sensor of the localized intracellular redox balance and may itself influence redox balance through GSH and the known antioxidant properties of zinc. It has been suggested that "the control of cellular zinc distribution as a function of the energy state of the cell is the long sought role of MT" [301]. In cells in culture MT can donate Cu to Cu-Zn superoxide dismutase and at least *in vitro* the Cu–MT interaction is also under redox control. These facts support the hypothesis that MTs act as physiological transporters of essential metals (such as Cu and Zn) and that their distribution is regulated in a redox-sensitive manner [302].

Metallothioneins may provide mammalian cells with a primitive antioxidant defense mechanism. Metallothionein is induced by treatments (hyperoxia, ionising radiation, exercise, or cold exposure) and substances (ethanol, paraquat, or tert-butyl hydroperoxide) that cause oxidative stress, as well as agents involved in inflammatory processes (interleukins, interferon, and tumor necrosis factor alpha). This suggests that MT may protect against reactive oxygen and nitrogen species [277]. Moreover, animals or cells in culture that are deficient in metallothionein isoforms exhibit greater susceptibility to oxidative stress caused by electrophilic mutagens, antineoplastic drugs, nitric oxide as well as cadmium [303,304]. Over expression of metallothionein reduces the sensitivity of cells and tissues to free radical

damage and metallothionein genes are transcriptionally activated in cells and tissues in response to oxidative stress [300].

It has been suggested that the thiolate clusters of metallothionein are primary targets for the reaction of hydroxyl radicals. Dithiothreitol and GSH only cause a limited reduction in peroxidation compared with that of MT. At least *in vitro,* hydrogen peroxide, superoxide anions, and peroxyl radicals interact with MT and result in oxidative modification of the protein, thus suggesting that *in vivo* this may affect the capacity of MT to bind metals [305]. Some authors therefore favor MT-released zinc as the primary element of protection via interaction and stabilization of membranes [306]. It has been postulated that the free metals (cadmium and zinc) interact with the cell membrane and interfere with iron redox reactions (by either competing for binding sites or causing structural changes which reduced binding), thereby reducing the conversion of H_2O_2 to $OH^{\cdot}$ radicals. A similar effect has been proposed in relation to Zn- and Cd, Zn-MT, where free radicals were thought to interact with the MT to release the metals and influence redox events [307]. However, Cu-MT enhances lipid peroxidation initiated by organic hydroperoxides, suggesting that MT may act as an antioxidant or a pro-oxidant depending on its association with metals [308]. MT is also induced in tissues subjected to a rapid increase in metabolic activity, such as brown adipose tissue during thermogenesis, and may indicate a physiological role for this protein in scavenging free radicals, the levels of which are elevated under these conditions [300].

Metallothionein plays a major role in metal detoxification, a fact supported by extensive evidence from both *in vivo* and *in vitro* studies. After exposure to various metals there is a significant increase of MT in tissues such as kidney, liver and intestine. Likewise, various cell types have been shown to accumulate MT after metal exposure [309-311]. Some metals (such as lead) are known to induce and bind to other intracellular proteins, which may also play a role in their detoxification [300].

GSH has also been implicated in metal detoxification. Indeed, there are several studies both *in vivo* and *in vitro* that have reported increased sensitivities towards the toxic effects of mercury and cadmium following depletion of GSH levels. GSH, however, appears to be the first line of defense against cadmium toxicity preceding MT induction [300].

There are many examples in the literature demonstrating both *in vivo* and *in vitro,* that exposure to a non-toxic, low dose of metal allows a higher, toxic dose to be tolerated. This has been attributed to the priming of MT induction by low doses of metal which facilitates subsequent MT expression and sequestration of higher metal doses [312].

Similarly, protection against the toxic effects of several heavy metals via increased metallothionein expression has been detected after pretreatment of rat hepatocytes with zinc [386]. Pretreatment with non-metal inducers of MT such as vitamin A have also been shown to reduce the hepatotoxic effects of cadmium [313].

In addition to metal sequestration, metallothionein may reduce the toxic effects of metals by reducing the uptake of metal into cells [300]. Indeed, metal-induced metal resistance also occurs without any increase in intracellular MT. This has been demonstrated in bovine endothelial cell cultures after simultaneous administration of toxic doses of cadmium with zinc or copper. These protective effects appear to be due to a decrease in the uptake of cadmium into the cells since less intracellular accumulation of cadmium and more of zinc or copper were found on exposure to both metals. Likewise, pretreatment of these endothelial cells with zinc for 24 hours protects against cadmium cytotoxicity, without the induction of

MT by decreasing the accumulation of cadmium within the zinc-pretreated cells, indicating that uptake of cadmium is inhibited by the increase in intracellular zinc [300,314]. Hence, it is thought that this characteristic, which appears to be unique to MT, aids the transfer of metal ions and is fundamental to its biological role [305]. Proposed functions for MTs include metal absorption/excretion, metal homeostasis and metabolism, free radical scavenging, metal detoxification, apoptosis, and modulation of the intracellular redox balance [300].

The results of a number of investigations suggest that MT plays an important role in cell proliferation. For instance, in mammals, high levels of MT expression have been detected during the late stages of gestation and neonatal periods [19,200,218], although significant changes in MT levels in several organs have been noted during rat fetal development [300]. MT levels are also transiently elevated during liver regeneration, as well as in liver and kidney of uninephrectomized rats [315]. Increased MT levels have also been reported *in vitro* during exponential cell growth [316]. MT is mainly a cytoplasmic protein but it is also present in the nucleus during cell proliferation and development [317]. For example, MT has been detected in both the nucleus and cytoplasm of human fetal hepatocytes, and the levels of MT increase with gestational age [317]. MT is also found in the nucleus of regenerating hepatocytes as well as in rat fetal hepatocytes but in opposition to what occurs in human cells, the protein redistributed to the cytoplasm 2 to 3 weeks postpartum [315]. Although the mechanism behind the redistribution of MT is unclear, it is believed that nuclear retention is ATP dependent. Cellular localization of MT appears to be cell cycle specific because it peaks in the nucleus during S, and G2/M phases while maximal expression in the cytoplasm occurs during G0 and G1 phases [316]. MT is located mainly in the cytoplasm of both human fetal and adult renal proximal tubule cells, but no correlation between gestational age, levels of MT or cellular localization in the kidney has been found, suggesting that the distribution and expression on MT during development may be organ specific. Thus, while cytoplasmic MT seems to protect the cells against toxic insult caused by metals, reactive oxygen species and oxidizing agents, nuclear expression of the protein may have an anti-mutagenic role [19].

A number of metallothionein inducers, such as glucocorticoids, lipopolysaccharides, steroid hormones, cytokines and tumor necrosis factors, among others, can influence apoptosis in certain cells [19]. This and other experimental data would seem to suggest that metallothionein plays a role during the apoptotic process. Cells from MT-null mice have been found to be more susceptible to apoptosis after exposure to tert-butyl hydroperoxide and anticancer agents than normal MT-expressing cells [223,224].

Over the years the functions of MT-I+II have slowly been unrevealed, and they have been shown to be multi-purpose proteins involved in abroad range of functions, including ROS scavenging, metal homeostasis, regulation of Zn-containing proteins (e.g. p53), immune defense responses, angiogenesis, cell cycle progression, and cell differentiation[215, 300, 318-320]. The immunomodulatory actions of MT-I+II in the central nervous system consist of reducing leukocyte and macrophage activation, transendothelial migration and chemoattraction, hereby reducing inflammatory cell infiltrates and synthesis of proinflammatory mediators (e.g. cytokines, proteases, and adhesion molecules) [318]. In addition, MT-I+II increase expression of anti-inflammatory and regenerative factors, such as IL-10, fibroblast growth factor (FGF),transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), neurotrophin (NT), and their receptors; hereby mediating repair, angiogenesis, and vascular remodeling [318]. Finally, MT-I+II may potentially affect the activity of certain transcriptional factors by donating Zn, and hereby regulate cell proliferation and differentiation [321]. Thus, MT-I+II has been shown to play an important role in regulating inflammation, oxidative stress, DNA damage, and cell death following a number of

pathological conditions, such as multiple sclerosis and traumatic brain injury [318], as well as for protecting tissues against various forms of stress; such as oxidative injury, radiation, chemotherapy, and metal toxicity (e.g. Cd, Zn, Hg) [322,323].

Due to their involvement in cell proliferation and survival, increased MT-I+II levels in human neoplasms have been associated with poor prognosis [320]. The transcriptional control of MT-I+II and its nuclear/cytoplasmic localization changes during cell proliferation and differentiation. In non-pathological tissues MT-I+II are mainly cytoplasmic proteins, while in dividing cells MT-I+II localization varies with cell cycle progression and is expressed in the nucleus during the S and G2 phase [300,315,324], suggesting that altered levels of MT-I+II could be expected to contribute to abnormal cell growth, as seen in cancer, as well as in the acquisition of therapy resistance. So far the studies have primarily focused on investigating the potential of MT-I+II expression as a prognostic marker for survival and response to treatment. However, the results show great discrepancies. Moreover, several authors draw a parallel between MT-I+II expression as a potential marker for prognosis, and MT-I+II's role as oncogenic factors [325].

In relation to cancer, the major focus has been devoted to the interaction between MT-I+II and p53 [326]. Mutation of the p53 gene is found in most human cancers, resulting in p53 inactivation ('p53 null state'), which ultimately prevents apoptosis regulation [4]. *In vitro* and *in vivo* studies have demonstrated a strong positive correlation between mutated p53 and increased MT-I+II levels in tumors [319,326], and MT-I+II null cells have been shown to be more sensitive to apoptotic stimuli and to have approximately 3–4-fold higher basal levels of functional p53 than wild-type cells. Zn ions are essential for the maintenance and stability of p53 and for its affinity for DNA [327]. The removal of Zn ions from p53 results in a highly aggregation prone state, which results in instability and inactivation of p53 [319]. Metal-free apo-MT regulates folding of several Zn metalloprotein including p53 [328], and apo-MT removes Zn from p53 and inactivates it [326-329]. Persistent expression of apo-MT in tumor cells hereby promotes accelerated growth and survival through induction of a 'p53-null state' [320,324,327,330,331]. Furthermore, a direct interaction between apo-MT (but not Zn-MT-I+II) with p53 has been observed in breast cancer epithelial cells with both wild-type and inactive p53 in vitro [329,330], suggesting a possibility of MT-I+II directly preventing binding of p53 to DNA in addition to its Zn-chelating function, here by directly modulating gene transcription and apoptosis in tumor cells, and functioning as an oncogenic factor [325].

In addition, a correlation between MT-I+II and functional p53 has been found (i.e. higher MT-I+II induction was observed in functional p53-positive cells) [320,332]. These studies suggests that even though apo-MT can remove Zn from p53 and inactivate it, Zn-containing MT-I+II might work as tumor suppressor proteins that are important for supplying Zn to p53 for its optimum activity and stability. This highlights the importance of time, doses, isoform, subcellular location, and form of MT (i.e. apo-MT versus metal-containing MT) expressed in the malignant cells [325].

Metals are small entities when compared to organic materials and their reactions with living matter, are seemingly simple to evaluate. However, the picture emerging today shows a very complex pattern of metal interactions with cellular macromolecules, metabolic and signal transduction pathways and genetic processes. Some metal compounds even undergo metabolic transformation, such as reduction to lower oxidation state or alkylation. Hence, the toxicological assessment of metal effects is by no means simple, which is especially true for mechanisms of metal carcinogenicity. Even for single metal species, the hitherto revealed mechanisms involved in their carcinogenic action are multiple. An especial feature of metal

biology is the fact that even metals that are essential for the sustainment of life (such as iron and copper) may become toxic depending on the oxidation state, complex form, dose and mode of exposure [255].

Therefore, the current results elucidated that fishermen group exposed to various types of heavy metals which was evident by the presence of cadmium, chromium, copper, lead and zinc in their blood which generated a sever oxidative stress which manifested by the presence of highly levels of malondialdehyde, accompanied by severe decrease in the antioxidant defense in their blood i.e. decrease in the glutathione content and decrease in the antioxidant enzymes activities of glutathione peroxidase and catalase, but the results of the current study showed there are on significant changes in the enzymatic activities of superoxide dismutase between the two studied groups.

Metals may be carcinogenic in the form of free ions, metal complexes, or particles of metals and poorly soluble compounds. The toxicity of metals and their compounds is governed by their physicochemical properties. Regarding metal ions, oxidation state, charge and ionic radii are crucial. With metal complexes, the coordination number, the geometry and the type of ligands (e.g., their lipophilicity) are important for toxic interactions. Regarding metals and their poorly soluble compounds, particle size and crystal structure are important. Not only toxic metal cations, but also essential transition metal ions bind to biological ligands of opposite charge, such as acidic amino acid side chains of proteins and phosphate groups of nucleotides and nucleic acids, and form complexes with oxygen, sulfur and nitrogen groups of proteins, nucleic acids and other biomolecules. If toxic metal ions have similar physiochemical properties such as charge and size as those of essential ions, they may compete for the biological binding sites of the latter and cause structural perturbations resulting in aberrant function of biochemical macromolecules [255]. Some examples are discussed in more detail here. Cd^{2+} ions have ionic radii very similar to those of Ca^{2+} (in hexacoordination 0.95 and 1.00 Å, respectively, in octacoordination 1.10 and 1.12 Å, respectively). Although the preferred ligand of Ca^{2+} is oxygen, whereas Cd^{2+} prefers sulfur, Cd^{2+} also accepts oxygen and is able to substitute Ca^{2+} in protein binding sites. Cd^{2+} interferes with the functions of numerous Ca^{2+}-transport and Ca^{2+}-dependent signaling proteins. Cd^{2+} ions have an analogous electron configuration with Zn^{2+} (4d10 vs. 3d10). Despite its larger radius (0.95 vs. 0.74 Å), Cd^{2+} can often substitute for Zn^{2+} in zinc enzymes and transcription factors and disturb or abolish the biochemical functions of such proteins. Pb^{2+} has ionic radii of 1.19 Å in hexacoordination and 1.29 Å in octacoordination. These are sufficiently close to that of Ca^{2+}, and Pb^{2+} interferes with many types of Ca^{2+}-regulated physiological processes, especially the Ca^{2+}-signaling system [255].

The induction of oxidative stress is an attractive hypothesis to explain mutagenic and carcinogenic effects of metals. Ions of the carcinogenic metals, such as antimony, arsenic, chromium, cobalt, nickel and vanadium, are capable of performing redox reactions in biological systems. They have been shown to induce the formation of reactive oxygen and nitrogen species in vivo and in vitro in mammalian cells. Frequently the formation of hydroxyl radicals, most probably by Fenton-and Haber–Weiss-type reactions, has been detected. These radicals are known to cause oxidative damage to lipids, proteins and DNA (Figure 37) [255].

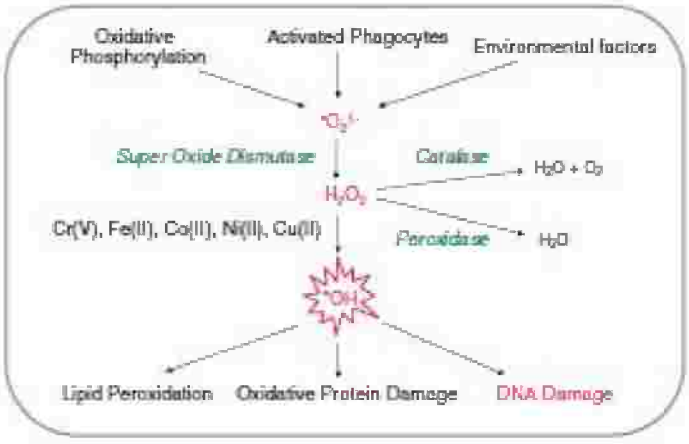

Figure 37: Metal ions and oxidative stress [255].

Although the ions of the carcinogenic metal cadmium are not capable of exerting redox reactions in biological systems, they have been found to generate oxidative stress too. The reason for this property of cadmium seems to be the inhibition of antioxidative enzymes *in vitro* and *in vivo*. Cadmium has been shown to inhibit catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase [255].

These findings cast light and represented excellent interpretation to herein results which demonstrated the presence of high significant decrease in the enzymatic activities of glutathione peroxidase ($p \leq 0.0$) and catalase ($p \leq 0.0$) in blood of fishermen group than that of control group.

In addition to the metals classified as carcinogens, iron and copper are also effective catalysts for Fenton and Fenton-type reactions. Nevertheless, in living systems iron and copper are tightly regulated with respect to uptake, transport, storage, mobilization, transfer to target molecules and excretion, ensuring that increased deliberation of free ions is restricted to conditions of extreme overload, genetic defects in metal homeostasis and/or metabolic stress. Besides generating DNA damage directly, reactive oxygen species at low concentrations function as mitogenic signals and activate redox-sensitive transcription factors [333]. Hence, oxidative stress may not only initiate tumor development by mutagenesis but also deregulate cell growth and promote tumor growth depending on extent and time of interference [255].

Several metals and metalloids have been rated as proven or probable carcinogens by the International Agency for Research on Cancer (IARC). For instance, exposure to chromium or nickel is associated with nasopharyngeal carcinoma, exposure to lead or mercury with brain tumors, exposure to lead or cadmium with kidney cancer and exposure to cadmium with prostate cancer [334, 335]. Metals modulate gene expression by interfering with signal transduction pathways that play important roles in cell growth and development [6, 336]. The underlying mechanism involves formation of the superoxide radical, hydroxyl radical, and finally the production of mutagenic and carcinogenic malondialdehyde (MDA), 4-hydroxynonenal (HNE), and exocyclic DNA adducts [6]. Carcinogenic metals and metalloids, such as As, Cd, Ni and Co can also, inhibit zinc finger-containing DNA repair proteins [337,338].

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H2O2), and hydroxyl radical (HO•) are by-products of cellular respiration, generated by incomplete reduction of oxygen to H2O. To enable the use of oxygen for energy product ion and yet to minimize oxygen-derived toxicity, a complex antioxidant net work has evolved including the scavenging of reactive species by glutathione and vitamins; the enzymatic conversion of highly reactive oxygen species to less harmful ones by superoxide dismutase, catalase, and glutathione peroxidase; and finally the repair or elimination of damaged macromolecules (339). Oxidative stress occurs if the equilibrium between the generation of ROS and the efficiency of detoxification is disrupted. Nevertheless, even under normal cellular conditions, protection is not complete and a measurable amount of oxidatively damaged macromolecules exists in mammalian cells (4). Especially the generation of elevated levels of DNA damage has been implicated in carcinogenicity. Oxidatively generated DNA damage includes a range of lesions such as DNA base modifications, sugar lesions, DNA single-and double-strand breaks, DNA–protein cross-links, DNA–DNA cross-links, and abasic sites. The main ROS identified so far that lead to DNA damage are $HO^{\bullet}$, singlet oxygen ($^1O_2$), and one-electron oxidants. Among these, only $HO^{\bullet}$ is able to generate DNA single strand breaks as a consequence of initial hydrogen abstraction from the 2-deoxyribosemoieties, with different probabilities of hydrogen abstraction in different positions [340-342]. Concerning DNA single-base damage, $^1O_2$ reacts specifically with guanine, producing 8-oxo-7,8-dihydroguanine (8-oxo-Gua) without further reaction products [343]. Furthermore, 8-oxoGua, as well as 13 other singly oxidized purine and pyrimidine bases, has been detected in cellular DNA, mediated by $HO^{\bullet}$ or high intensity UVC laser pulses [344]. In addition to single-base DNA damage, $HO^{\bullet}$ and one-electron oxidants have been shown to generate organic radicals such as radical cations, carbon centered or peroxyl radicals, which are able to react further with other DNA constituents or proteins, giving rise to more complex DNA lesions such as intra-and inter strand DNA cross-links as well as DNA–protein cross-links. Finally, DNA double-strand breaks (DSBs) arise from one nick in each DNA strand within one or two helix turns; they may, however, also be generated, for example, by replication of damaged DNA due to collapse of stalled replication forks [345, 346]. Among these, several oxidatively generated DNA base modifications such as 8-oxo-Gua have miscoding and thus premutagenic properties and therefore may act as initiators in carcinogenesis [347]. Especially transition metal ions play an important role in the induction of oxidatively damaged DNA (Figure 38). Where as neither superoxide radical anion nor hydrogen peroxide is able to react with DNA directly, in the presence of transition metals such as iron, copper, cobalt, or nickel H_2O_2 is converted into the highly reactive $HO^{\bullet}$ by Fenton-type reactions. Therefore, in the case of essential elements such as iron and copper, the controlled uptake, protein-bound transport, and intracellular sequestration of redox-active metal ions by metal-binding proteins are one important prerequisite to protect from elevated levels of oxidatively generated DNA damage. However, this protection will be overwhelmed under conditions of cellular over load by transition metals because of elevated exposure and/or non-physiological uptake routes such as inhalation [4, 6].

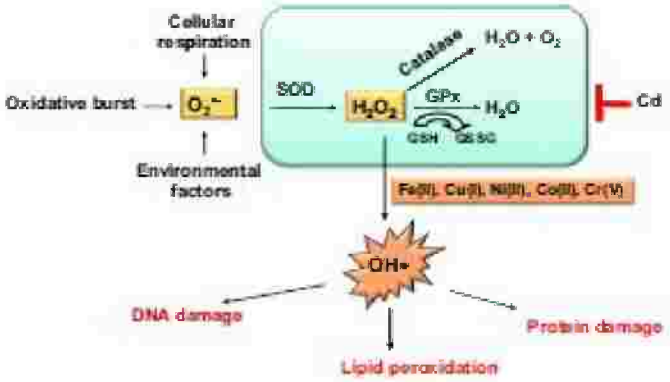

Figure 38: Role of metal ions in the generation of cellular damage by reactive oxygen species (ROS). Where as redox-active transition metal ions or reactive intermediates such as Cr(V) may convert H_2O_2 into highly reactive HO•, redox -inactive cadmium ions may increase the formation of ROS by inhibiting cellular defense enzymes such as superoxide dismutase (SOD), catalase, and/or glutathione peroxidase (GPx) [339].

One unique example is Cr (VI). Under physiological conditions, Cr (VI) enters the cell as the anionic tetrahedral species chromate, Cr $(O_4)^{2-}$, via anion transport systems such as the sulfate carrier, and is intracellularly reduced to Cr (III), described by the so-called ‘‘uptake–reduction’’ model [339, 348]. Within the cell, reduction does not require enzymatic steps but is mediated by direct electron transfer from ascorbate and non protein thiols such as glutathione and cysteine; during this process, potentially toxic intermediates such as oxygen and sulfur radicals are generated, dependent on the intracellular reductants [349]. DNA lesions generated after exposure to Cr (VI) consist of two categories, namely oxidatively induced DNA damage and DNA lesions resulting from Cr (III)–DNA interactions. With respect to the formation of ROS during the intracellular reduction process, several pathways have been proposed, including there action of Cr (V)–glutathione complexes with hydrogen peroxide and the formation of $HO^{\bullet}$ and/or a one-electron reduction of Cr (VI) to Cr (V) by NADPH-dependent flavoenzymes [338]. Cr (V) may either react with hydrogen peroxide in a Fenton-type reaction to yield again $HO^{\bullet}$, and thus induce DNA strand breaks as well as oxidized purine and pyrimidine bases, or directly oxidize guanine DNA bases [339]. With respect to the latter, the resulting guanine radical is able to undergo hydration to give rise to 8-oxo-Gua and 2, 6-diamino-4-hydroxy-5-formamidopyrimidine. Furthermore, the guanine radical has been proposed to be the key intermediate in oxidatively generated DNA–protein and DNA–DNA interstrand cross-links [346]. Nevertheless, the induction of oxidatively damaged DNA by Cr (VI) appears to be restricted to high exposure concentrations [350]. Although the relevance of oxidatively induced DNA lesions under physiological conditions has been questioned [339], especially ternary Cr–DNA adducts may be of special importance for chromate-induced carcinogenicity, in which Cr bridges DNA and small molecules such as cysteine, histidine, glutathione, or ascorbate, presumably arising from preformed Cr–ligand complexes during the reduction process. Under physiological conditions, ascorbate appears to be the major reductant, and especially ternary adducts formed from Cr–ascorbate are potent premutagenic DNA lesions [351]. Furthermore, these lesions lead to aberrant mismatch repair (MMR) and upon chronic exposure to toxic doses of Cr (VI) the selective outgrowth of MMR-deficient clones exerting a high degree of genomic instability has been postulated [339,352]. Finally, metal compounds exerting no redox chemistry such as cadmium may also contribute to elevated levels of oxidatively damaged DNA, which may be attributed to an inhibition of ROS-detoxifying enzymes such as superoxide dismutase [4,339,353]. Last but not least, an inhibition

of DNA repair systems involved in the removal of oxidatively generated DNA lesions may increase their steady-state levels upon chronic exposure to metal compounds. Because these inhibitions have frequently been observed at far lower concentrations compared to the induction of considerable amounts of oxidatively generated DNA lesions, as for example, in the case of nickel and cadmium compounds, this may be particularly relevant for metal-induced carcinogenicity [339].

In our laboratory, Aziza et al (2010) [340] found that pollution of Abo Qir bay (El Maadiya region) with aromatic amines induced a panic oxidative stress and high frequencies of chromosomal aberrations in the peripheral blood of fishermen. In addition, the results of the present study revealed that there was a high significant increase in the gene expression of insulin-like growth factor-2 (IGF-2) ($p \leq 0.0$) in the blood of the aforementioned fishermen group.

Insulin-like growth factor-2 (IGF-2) is involved in the regulation of liver cell growth and metabolism. IGF-2 is structurally related to proinsulin, IGF-1, and relaxin. The mitogenic and antiapoptotic properties of both IGF peptides as well as differentiation-related signaling are mediated primarily through IGF-1 receptor (IGF-1R). IGF-2 is physiologically expressed at high levels in various human and rodent fetal tissues such as liver, kidney, and skeletal muscle. In contrast, it is down regulated or virtually absent in the corresponding adult organs [355].

The parenchymal cells of the adult liver synthesize and secrete IGF-I and -II. However, whereas hepatocytes are the main contributors in liver IGF-I synthesis and secretion, hepatocytes as well as resident macrophages (Küpffer cells), endothelial cells and hepatic stellate cells do synthesize IGF-II. Liver IGF-I and -II are secreted in the serum and stand for the ''endocrine'' pool of the IGFs [189].

In addition, circulating IGF-2 arises in part from liver, its concentration having been reported to reflect hepatic integrity [356]. Liver disease could, therefore, confound interpretation of the concentration. Gene expression and plasma protein signatures may enable early diagnosis of cancer in the future [195].

In a study of Baddour et al (2011) [357], a significant positive correlation was observed between IGF-2 expression and the grade of inflammatory activity, this is in accordance with the findings of Grisham et al (2001) [358] that; upregulation of IGF-2 in chronic hepatitis results from the combined actions by cytokines produced by chronic inflammatory cells that infiltrate damaged livers and viral transactivation. In this respect, from the findings of the present study it may be suggested that the investigated fishermen group may suffered from insidiously hepatitis with a consequential serious effect on fishermen health. It has been demonstrated that upregulation of IGF-2 in the course of HCV related chronic hepatitis and in normal livers, IGF-2 is expressed minimally [357, 359].

Recent research findings suggest much potential clinical utility for IGF-2 testing in the context of liver cancer. The possibility of predicting hepatocarcinogenesis by genetic testing is perhaps the most exciting [360]. Genomic assays that provide molecular signatures for multiple genes, including IGF-2, may also predict cancer risk [361]. The Collaborative Oncological Gene environment study has already detected more than 80 gene variants associated with increased risk of breast, prostate and ovarian cancers. This is a rational approach to prediction because IGF-2 is only one of many genes working together to determine risk. These techniques could potentially be combined with traditional screening

approaches to increase efficacy in disease detection. It is increasingly recognized that methylation patterns can be used as biomarkers for disease or predisposition to disease [362]. The Human Epigenome Project is underway to identify methylation patterns throughout the genome [195,363].

A significant (40–100) fold increase in IGF-2 gene expression was observed in human cirrhotic liver, in liver cancers and in human hepatoma cell lines, when compared to that of normal adult liver [364, 365]. High focal expression of IGF-2 has also been observed in chronic active hepatitis, in persistent hepatitis and in HCCs of hepatitis B and C infected liver [189, 355]. In some instances, increased IGF-2 gene expression *(i)* has been correlated with increased rates of cell mitotic activity, as estimated by proliferating cell nuclear antigen (PCNA) expression [359] and *(ii)* may contribute to tumoural angiogenesis [189].

Similarly, IGF-2 gene expression was reactivated during hepatocarcinogenesis in transgenic mice and was associated with high replicative activity, but not with changes in apoptosis [189]. Interestingly, re-expression and overexpression of the IGF-2 gene in mouse and human HCCs, respectively, was concomitant *(i)* with the re-activation of a fetal pattern of gene expression [364, 365], and *(ii)* with silencing of the liver-specific promoter P1 in human HCCs [189]. In addition, overexpression of the IGF-2 gene in human preneoplastic foci and HCCs has also been reported to be associated with the restoration of an allelic imbalance at the IGF-2 locus [189]. That overexpression of IGF-2 may be involved in the hepatocarcinogenetic process, or in HCC cell proliferation could be deduced from ex vivo experiments [189]. HuH-7 and HepG2 human hepatoma cells produced five-fold more intracellular IGF-2 than other cell lines. When the production of IGF-2 was suppressed by specific antisense oligodeoxynucleotides, the decrease in IGF-2 peptide resulted in growth inhibition of HuH-7 and HepG2 cells [189]. Accordingly, we have shown that IGF-I-stimulated proliferation of HepG2 cells is enhanced when endogenous IGF-2 production has previously been suppressed, targeting IGF-2 transcripts with small interfering RNA [189].

On the one hand, an elegant study by Eriksson et al. (2001) [366] has evidenced a close correlation between IGF-2 gene expression from P3 and the methylation status of the promoter, with expression being linked to promoter hypomethylation. On the other hand, the hepatitis B virus X protein (HBx) has been shown to up-regulate IGF-2 gene expression from P4 via increased Sp1 (or Egr-1 or -2) binding to bona fide Sp1 cis-elements [189]. HBx does not activate Sp1 through direct protein-protein interaction, but rather via an indirect mechanism that requires the activation of both protein kinase C (PKC) and ERK-1/ERK-2 signaling [367]. Interestingly, the protein phosphatase activity of the tumor suppressor gene PTEN (phosphatase and tensin homologue on chromosome 10)/ MMAC1/TEP1 blocks Sp1 phosphorylation in response to HBx, by inactivating PKC and ERK-1/ERK-2 activities [368]. In this connection, it should be stressed that PTEN is frequently inactivated by mutation or deleted in human HCC [189].

Finally in some cases, accumulation of IGF-2 in HCCs tissue could be due to up-regulation of IGF-2 gene transcription by p53mt249, a gain-of-function mutant of p53 frequently observed in patients that have developed HCCs after prolonged exposure to aflatoxin B1 [369]. p53mt249 enhances transcription from the fetal IGF-2 promoter P4 [189].

Tumor development is characterized by a deregulation of cell growth and differentiation. Carcinogenic metal compounds may alter cell growth by several distinct mechanisms, either affecting the expression of growth stimulating factors or inactivating growth control mechanisms. With respect to the former, some metal ions are found to activate mitogenic

signaling pathways and induce the expression of cellular proto-oncogenes. Furthermore, epigenetic mechanisms, such as hypo- or hyper-methylation of DNA or disturbed histone acetylation, may contribute to modified patterns of gene expression. Changes in gene regulation are observed prior to manifestation of tumors. Initially, they are not fixed by mutation, and the agent must be present for an extended time period to cause persistent modifications, which can be genetically fixed during tumor development. Concerning the interference with cellular growth control, some metal carcinogens have been shown to inactivate the tumor suppressor proteins p53 and/or down regulate the expression of tumor suppressor genes Fhit, p16, p53 and of senescence genes. Finally, metal ions may deregulate cell proliferation by inactivating apoptotic processes resulting in adaptation to the cytotoxicity of the metal [195].

There is increasing evidence for interaction between IGF-2 and p53 in cancer development. Normally, IGF-2 transcription is repressed by the tumor suppressor p53, which also increases IGFBP3 and suppresses IGF1R expression [195]. Decreased activity of p53 in tumors, therefore, increases both IGF-2 expression and action. Recent data suggest that increased IGF-2 signaling favors tumor development by suppressing activity of the p53 pathway [370].

The aforementioned finding represented a good interpretation to the herein results which elucidated that severe oxidative stress in the blood of fishermen group predisposed in up-regulation of IGF-2 gene by hypomethylation of DNA.

Chemical-modified gene activation may involve the ordered cascade of epigenetic events that begin with histone modifications and finalize with alterations in DNA methylation in promoter CpG islands [371-373]. A general hypothesis of environmental chemicals as lifelong modulators of DNA hypomethylation is those xenobiotics, including metals, influences one-carbon metabolism directly or indirectly [374, 375]. This may explain the population cohort studies that exhibit significant inverse linear relationships between POPs or metals exposures and blood global DNA methylation [376-378]. Patients with atherosclerotic vascular disease often exhibit higher homocysteine and S-adenosyl homocysteine (SAHC) and lower genomic DNA methylation status [379, 380], which is directly connected with one-carbon pathways. Indirectly, oxidative stress mechanisms generated by xenobiotics may also involve aberrant epigenetic modification of DNA [374], and histones [381], via the depletion of glutathione (GSH) and changing the ratio of reduced GSH and its oxidized form, GSSG (i.e. GSH disulphide). Oxidative stress may also alter epigenetic modification via mitochondrial dysfunction [382-384]. To be inhibitors, isoflavones, polyphenol, zinc and cadmium may inhibit DNA Methyl transferases (DNMTs) directly and indirectly and further inhibit methylation of candidate genes [371, 385-387].

Coinciding with gene-specific aberrant methylation following exposure to endocrine disrupting chemicals, DNMTs were abnormally expressed in some cases [382-390]. Endocrine disrupting chemicals induced aberrant methylation of estrogen-regulated genes [369,391,392]; steroid hormone interacts with chromatin-modifying enzymes by binding the receptors [393]. May suggest other pathways by which chemicals alter epigenetic markers, i.e. they may involve the expression of target genes by modifying their epigenetic regulators directly [371].

Conclusively, the present study elucidated that El Maadiya region is polluted with heavy metals. At the same time another two studies in the same laboratory proved the pollution of El Maadyia region with some aromatic amines [340] and some polycyclic aromatic hydrocarbons [394]. The pollution induces a panic oxidative stress in fishermen in the vicinity of this area.

The risk will persist because the high increase levels of malondialdehyde coincide with high decrease in the levels of the antioxidant glutathione, and the enzymatic activities of catalase and glutathione peroxidase. It was proved that carcinogenic metal compounds often are comutagenic, that is, they enhance the mutagenicity of other genotoxic agent. Indeed, many carcinogenic metal compounds at low concentration have been identified as inhibitors of the repair of DNA damage that is caused either by other xenobiotics or by endogenous factors. Inhibition of repair and persistent DNA damage results in genomic instability which may become especially deleterious under conditions of acceleration cell proliferation and/or impair apoptosis [(255)]. The present results exhibited the presence of high significant level of metallothionein in fishermen blood ($p \leq 0.009$) and there is a positive correlation between metallothionein and malondialdehyde, while there are negative correlation between metallothionein and the antioxidant glutathione, glutathione peroxidase, and catalase, the present finding is in agreement with other studies which elucidated that increasing the level of ROS and oxidative stress induce increase expression of MT mRNA and protein levels, which can increase tumor cell survival and viability due to their antioxidative and antiapoptotic effects [(320,321,327,331,395,396)].

Meanwhile, the present data emphasized that there was a high significant increase in the gene expression of IGF-2 ($p \leq 0.0$), and there is a positive correlation between expression of IGF-2 and metallothionein ($r = 0.442$, $p = 0.002$). In addition a negative correlation between gene expression of IGF-2 and the level of glutathione ($r = - 0.422$, $p = 0.007$) as well the enzymatic activities of glutathione peroxidase ($r = - 0.366$, $p = 0.017$) and catalase ($r = - 0.385$, $p = 0.013$), furthermore, Aziza et al (2010) [(340)] found high frequencies of chromosomal aberrations in lymphocytes of peripheral blood of the same group of fishermen in the same area.

Oxidative stress mechanisms generated by xenobiotics may also involve aberrant epigenetic modification of DNA [(374)] and histones [(381)] via the depletion of glutathione and changing the ratio of reduced GSH and its oxidized form, GSSG. Oxidative stress may also alter epigenetic modification via mitochondrial dysfunction [(382-384)]. To be inhibitors, isoflavones, polyphenol, zinc and cadmium may inhibit DNA methyl transferases (DNMTs) directly and indirectly and further inhibit methylation of candidate genes [(371,385-387)].

Up-regulation of IGF-2 in some hepatocytes may lead to high focal IGF-2 levels sufficient to saturate local IGF-2 binding capacities, and may result in an increased susceptibility to cellular dedifferentiation and, ultimately, liver cancer. Down regulation of hepatocellular M6P/IGF-2R and upregulation of IGF-2 seem to be early events in hepatocarcinogenesis prior to the appearance of morphologically distinct dysplastic lesions. Elevated focal IGF-2 transcript levels may therefore indicate an increased risk for hepatocellular and cholangiocellular carcinomas [(355)].

In addition, circulating IGF2 arises in part from liver, its concentration having been reported to reflect hepatic integrity. Liver disease could, therefore, confound interpretation of the concentration. Gene expression and plasma protein signatures may enable early diagnosis of cancer in the future [(195)].

Then, the coexistence of urinary metabolites of aromatic amines and polycyclic aromatic hydrocarbons with heavy metals in the blood of fishermen group a long with the coincidence of oxidative stress concomitant with increase metallothionein levels, chromosomal aberrations, and overexpression of IGF-2 gene let the fishermen of El Maadiya region are under high risk of cancer.