CHAPTER I

INTRODUCTION

I.1. X-rays and ionizing radiation:

Ionizing radiation consists of energetic particles and electromagnetic radiations, which can penetrate living tissues or cells and result in the transfer of radiation energy to biological material [(1)]. Scientific and technological advancements have increased radiation burden in humans, since frequent exposure to low level of radiation has become common during medical diagnosis procedures, air travel and the use of certain electronic equipment. Besides, with the increasingly successful experience in achieving larger survival in cancer patients receiving radiotherapy, the threat of late effects of radiation exposure, such as reduced fertility, inheritable genetic damage and carcinogenesis has assumed greater importance [(2)].

X-rays are usually produced by allowing accelerated electrons, generated in a linear accelerator, to hit a thin target made of material with high atomic number. They are described as photons or electromagnetic waves, on the short wavelength side of the electromagnetic spectrum, which act like energetic particles. X-rays are considered indirectly ionizing radiation since their interactions with matter result in liberation of orbital electrons. These liberated electrons are responsible for the ionization of the target materials or the macromolecular components of biological tissues [(3,4)].

The measurements of x-rays include its intensity, exposure or flux. In dealing with the biological effects of x-rays it is most reliable to use the absorbed dose, which is defined as the amount of energy dissipated per unit mass of the matter. In other words; the dose is the amount of radiation absorbed by an object. In this respect two units are used; the radiation absorbed dose (rad) and the Gray (Gy). The rad is defined as the dose causing 100 ergs of energy to be absorbed by one gram of matter, while the Gray is the absorption of one joule per one kilogram of matter [(5)]. One Gy is equivalent to 100 rad. The Gy measures the absorbed energy of radiation, but the biological effects vary by the type and energy of the radiation and the organism and tissues involved. This is expressed by the equivalent dose with its unit the Sievert (Sv). For x-ray radiation, exposure measured in Gray or Sievert is equal [(6)].

I.2. Biochemical effects of ionizing radiation:

Cellular exposure to ionizing radiation can directly disrupt atomic structures, producing chemical and biological changes. It can also act indirectly through radiolysis of water, thereby generating reactive chemical species that may damage nucleic acids, proteins and lipids. Ionizing radiation activates many signal transduction pathways. Together, the direct and indirect effects of radiation initiate a series of biochemical and molecular signaling events that may repair the damage or culminate in permanent physiological changes or cell death [(7)].The early biochemical modifications, which occur during or shortly after the radiation exposure, were thought to be responsible for most of the effects of ionizing radiation in mammalian cells. However, oxidative changes may continue to increase for days or months after the initial exposure presumably because of

continuous generation of reactive oxygen (ROS) and reactive nitrogen (RNS) species [8]. These processes occur not only in the irradiated cells but also in their progeny [9, 10].

I.2.1. Relationship of oxidative stress and radiation:

I.2.1.1.Oxidative stress:

Molecular oxygen is essential for the survival of all aerobic organisms. Aerobic energy metabolism is dependent on oxidative phosphorylation, a process by which the oxidoreduction energy of mitochondrial electron transport is converted to the high energy phosphate bond of ATP. Molecular oxygen (O_2) serves as the final electron acceptor. Partially reduced and highly reactive metabolites of oxygen may be formed during electron transfer reactions. These oxygen metabolites include superoxide anion (O_2•−) and hydrogen peroxide formed by one- and two-electron reduction of O_2 respectively. In the presence of transition metal ions, the even more reactive hydroxyl radical ($HO^{\bullet}$) can be formed. These partially reduced metabolites of O_2 are often referred to as reactive oxygen species (ROS) due to their higher reactivity relative to molecular oxygen [11,12]. The harmful effects and biological damage caused by reactive oxygen-and reactive nitrogen species are termed oxidative stress and nitrosative stress [13].

Reactive oxygen species can be produced from both endogenous and exogenous cellular substances. Potential endogenous sources include mitochondria, cytochrome P450, peroxisomes, and inflammatory cells activation [14]. Because free radicals contain an unpaired electron, they are extremely reactive and short lived [15]. Overproduction of reactive oxygen or reactive nitrogen species can damage and inhibit the normal functions of lipids, proteins and DNA [16].

Oxidative stress arises from an imbalance between free radical production and the capability of opposing antioxidant forces. Generally, ROS and RNS play dual roles of deleterious and beneficial effects. While ROS can be involved in beneficial processes, for example, by acting as second messengers in signal transduction pathways**,** unfavorable side effects occur when there is an imbalance between overproduction of ROS/RNS and decrease of antioxidant molecules in body [17,18].

A serious effect of oxidative stress is its action on DNA. Oxidative stress can perturb several cellular processes of which a combination may be disrupted in concert to destabilize the genome. On a basic level, ROS induce direct damage to cellular structures that may ultimately have carcinogenic potential, e.g. DNA strand breaks, base modifications and DNA-protein cross linkages[19]. Furthermore, DNA repair mechanisms, such as mismatch repair, are disrupted by ROS-mediated modifications to crucial repair proteins [20].

I.2.1.2 Oxidative stress and lipid peroxidation:

Of the many biological targets of oxidative stress, lipids are the most involved class of molecules. Lipid oxidation gives rise to a number of secondary products, mainly aldehydes, with the ability to exacerbate oxidative damage [21]. These molecules act inside and outside the cells interacting with biomolecules such as nucleic acids and proteins, often irreversibly, damaging the delicate mechanisms involved in cell functionality [22]. Lipid

peroxidation occurring within the cellular and mitochondrial membranes can trigger apoptosis [23].

Malondialdehyde (MDA) is the principal product, which may be derived by lipid peroxidation, but it can also be generated by enzymatic processes from metabolism of various prostaglandins [22,24]. It has been suggested that as MDA can be detected with relative ease, it can be used as a marker of lipid peroxidaion and oxidative stress in general[25]. However, MDA should not be considered only as a simple marker for lipid peroxidation, but a clear alarm of high risk of mutation[22,24]. In its physiological state, at neutral pH, MDA is of low chemical reactivity[26]. However, different potential genotoxic activities have been proposed for MDA, which may lead to mutations. This molecule is able to interact with nucleic acid bases to form several different adducts[27]. The main product of this reaction is able to induce sequence-dependent frameshift mutations and base-pair substitutions in different cell types[24]. MDA is also able to generate DNA-protein (e.g., histones) cross links under physiological conditions [27].

I.2.2. Defense mechanisms against free radicals:

In the normal and healthy cells, there is a precise balance between free radicals production and the level of antioxidant molecules, but under oxidative stress condition, the balance is tilted towards excessive of oxidative radicals. For neutralizing the threat of free radicals to the tissues and cells, a wide variety of antioxidant and repair systems has been evolved. Defense mechanisms against oxidative stress can be divided into: antioxidant, preventive and repair mechanisms, and physical defenses. To cope with ROS damage, organisms possess comprehensive and integrated endogenous repair systems. Many enzymes participate in free radical neutralizing processes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase. The endogenous non-enzymatic antioxidants that participate in oxidative stress defense include glutathione (GSH), ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), carotenoids, and flavonoids [12,17].

I.2.2.1 Glutathione:

Glutathione is synthesized in the body from three amino acids: cysteine, glutamine and glycine. Reduced glutathione (GSH) is an important cellular antioxidant molecule. It is considered to be the most powerful, versatile and important antioxidant in the body. When an electron of the GSH is lost it becomes oxidized and when the level of oxidized form of this molecule is increased two molecules are linked with each other by a disulfide bridge to form glutathione disulfide or oxidized glutathione (GSSG)[28]. The centrality of glutathione in cellular redox homeostasis is well-recognized. Glutathione, as the most abundant free thiol in eukaryotic cells, maintains an optimal intracellular redox environment for proper function of cellular proteins. Reduced glutathione is the biological active form that is oxidized during oxidative stress. The ratio of GSH/GSSG thus offers a simple and convenient expression of cellular oxidative stress[29]. Accumulated evidence suggests that in addition to its antioxidant functions, glutathione participates in cell signaling processes through alteration in both the levels of total glutathione[30-32], and in the ratio of the oxidized to reduced forms [33]. Cellular GSH depletion has been found to be associated with proliferation in vascular endothelial cell[34,35] and increased proliferation of fibroblasts[36].

Typically, greater than 90% of total cellular glutathione is maintained in the reduced form through cytosolic *de novo* synthesis, enzymatic reduction of GSSG and exogenous GSH uptake [37]. Specificity and targeted redox control are achieved, in part, through the existence of distinct intracellular redox compartments that exhibit a unique distribution of GSH and other redox couples[38]. The cellular ratio of GSH-to-GSSG under physiological conditions highly favors the reduced form (around 100 to 1 in the liver) and is decreased during oxidative stress and apoptosis [39].

The main protective roles of glutathione against oxidative stress are: [i] glutathione is a cofactor for other detoxifying enzymes like glutathione peroxidase (GPx), and glutathione transferase; [ii] GSH participates in amino acid transport through the plasma membrane; [iii] GSH scavenges hydroxyl radicals and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; [iv] Glutathione is able to reduce oxidized vitamin C and vitamin E back to their unoxidized state. Moreover, GSH in the nucleus is involved in mechanisms that are necessary for DNA repair and expression [28].

I.3 Prostaglandin E_2

Prostaglandins (PGs) are unsaturated carboxylic acids, consisting of a 20-carbon skeleton with a five-member (cyclopentane) ring, synthesized biochemically from arachidonic acid. Their biosynthesis is limited by substrate availability and depends on the release of arachidonic acid from cell membrane phospholipids. Cyclooxygenases are the primary enzymes that metabolize the liberated arachidonic acid into prostaglandin H_2. Isomerases and PG synthases effect the transformation of PGH_2 into terminal prostanoids distinguished by substitutions on their cyclopentane ring[40,41]. There are two known isoforms of cyclooxygenases; cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is constitutively expressed in nearly all tissues and mediates physiological responses, COX-2 on the other hand is induced by inflammatory cytokines, growth factors, transcription factors and mitogens[42]. It is generally considered that COX-1 is involved in physiological protection functions, whereas COX-2 is involved in pathophysiological conditions. However, this general statement represents oversimplification of the situation because COX-2 has many other functions depending on age and the tissue involved[43 44].

There are 4 principal bioactive PGs generated *in vivo*: PGE_2, prostacyclin (PGI_2), PGD_2 and $PGF_{2\alpha}$. They are ubiquitously produced; usually each cell type generates 1 or 2 dominant products and act as autocrine and paracrine lipid mediators to maintain local homeostasis in the body. The production of PGs is generally very low in uninflammed tissues, but increases immediately in acute inflammation[40]. The biologically active prostaglandins are further metabolized into decomposition products that are subsequently eliminated from the body by excretion into the urine. As with their biosynthesis, PGs metabolism and degradation occur rapidly and to different extents in different tissues [45,46]. Although PGs can cross membranes by simple diffusion, the estimated flow rate is too low to exert a biological function. Specific transporters facilitate central and compartmental transport of PGs. Such transporters mediate both the efflux of newly synthesized PGs to effect their biological actions, and their influx from the extracellular milieu for their inactivation[46,47].

Prostaglandin E_2 (PGE_2) is one of the most abundant PGs synthesized in the body and produces a variety of versatile biological activities. It does not exist preformed in any cellular reservoir. When cells are activated, PGE_2 is *de novo* synthesized and released into the extracellular space. It is rapidly converted to inactive metabolites with a biological half-life in the circulatory system of approximately 30 seconds and normal plasma levels are 3-12 pg/ml [48]. Prostaglandin E_2 exerts its effect through G-protein coupled receptors (EP_1 to EP_4). Both EP_2 and EP_4 receptors are coupled to adenylate cyclase producing cyclic adenosine monophosohate (cAMP) that in turn activates the protein kinase A signaling pathway. EP_1 is coupled to phospholipase C generating two second messangers; inositol triphosphate and diacylglycerol. EP_3 receptors exhibit diverse activities ranging from inhibition of cAMP production to increases in calcium and inositol triphosphate [49,50].

Under physiological conditions, PGE_2 is an important mediator of many biological functions, including regulation of immune responses, blood pressure, gastrointestinal integrity and fertility. Deregulated PGE_2 synthesis or degradation has been associated with a wide range of pathological conditions [51] and is deeply involved in the pathogenesis of inflammation.

I.4 Cytokines:

Cytokines are essentially low molecular weight proteins or glycoproteins, mostly between 8 and 30 kDa, that mediate interactions between immunocompetent and haematopoietic cells and between the immune and the neuroendocrine systems. They are hormone-like proteins that serve in autocrine, paracrine and at times endocrine fashion, as intercellular messengers[52]. Cellular sources for cytokines are widely distributed throughout the body. Virtually all nucleated cells, but especially endothelial and epithelial cells as well as resident macrophages, are potent producers of interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF-α). Most cytokines are synthesized for a short period after a mitogenic or antigenic stimulus. They are produced directly under the form that will allow their secretion in the cellular milieu. However, two cytokines do not follow this rule. IL-1 β and TNF-α, produced as precursors, have to be cleaved to allow the secretion of the mature, active, soluble cytokine[53].

A characteristic feature of cytokines is their redundancy. Cytokines are redundant in that the same biological function can be executed by several distinct cytokines. Cytokine redundancy is a biologically important feature because it provides a measure of safety for the process of regulation of normal cellular functions. Should one cytokine be absent or its level limited, an option for the substitution by another cytokine exists[54]. They act sequentially and may synergize or inhibit each other. They also may induce the production of other cytokines. Another characteristic feature of cytokines is their pleiotropy (one cytokine may exhibit many biological activities). A number of cytokines including interleukin-1 (IL-1), tumor necrosis factor (TNF), and interleukin-6 (IL-6) stimulate a broad range of cell and tissue activities[55]. For example, upregulation or the lack of upregulation of the nuclear factor kappa-B (NFκB) leads to a seemingly paradox observation; that TNF may lead to apoptosis of some cells, yet induce proliferation or differentiation of others[56]. Once released by the producing cell, cytokines have a short half-life, often not exceeding a few minutes. After induction, their concentrations may rapidly and differentially peak and then decrease to undetectable levels[57]. This means that very small quantities of cytokines present in the microenvironment for a short period of

time are sufficient to induce functional changes in responding cells[58]. They are considered as powerful physiological mediators with a potential for inducing tissue damage. However, in normal tissues, cytokine-mediated tissue damages do not occur, largely because of carefully regulated cytokine secretion, while in pathological conditions, excessive cytokine release from activated cells not infrequently results in a local tissue injury[59,60]. Cytokine levels produced as a result of activation can vary widely between individuals, and this trend may be genetically controlled and the existing levels of cytokines might determine susceptibility or resistance to disease [60].

Some cytokines clearly promote inflammation and are called proinflammatory cytokines, whereas other cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines. Proinflammatory cytokines include IL-1 (both α and β isoforms) and TNF-α. Systemically, these cytokines raise the thermoregulatory set point (causing fever) and, via differential influences on the expression of iron binding proteins, mediate a redistribution of iron from extracellular to intracellular sites. Within a few minutes following binding to cells, IL-1 induces several biochemical events[61]. Some of the biochemical changes associated with signal transduction is likely to be cell-specific. Such events include multiple protein phosphorylations and activation of phosphatases[62] and some are thought to be initiated by the release of lipid mediators. In some cells IL-1 acts as a growth factor[63].

Locally, the cytokines stimulate leukocyte proliferation, cytotoxicity, release of proteolytic enzymes, and synthesis of prostaglandins and initiate a cascade of "secondary" cytokine synthesis and secretion. One of these secondary cytokines, IL-6, is often called an inflammatory cytokine because of its temporal association with the proinflammatory processes. However, many actions of IL-6 (including downregulation of IL-1 and TNF-α synthesis) are counter-inflammatory, acting to keep potentially destructive inflammatory responses from overshooting [64]. Proinflammatory cytokine-mediated inflammation is a cascade of gene products. The expression of these genes is stimulated by IL-1 and TNF-α and in some cases interferon (IFN)-gamma. IL-1 and TNF-α act synergistically in this process, while the anti-inflammatory cytokines block or at least suppress the intensity of the cascade.

IL-6 is a multifunctional cytokine produced by a variety of cells, including macrophages and epithelial cells. In addition to promoting differentiation of monocytes toward macrophages, IL-6 stimulates monocytes to release macrophage chemotactic protein-1, a chemokine involved in mediating tissue injury by activating monocytes. This leads to increased production of reactive oxygen intermediates and expression of cell adhesion molecules [65,66]. IL-6 is a pleiotropic cytokine and substantial evidence exists about its inflammatory effects such as induction of acute phase proteins by the liver [66]. However, IL-6 has also been considered as an anti-inflammatory cytokine because of its ability to induce antagonists of IL-1 and TNF-α [67].

Interleukin 10 (IL-10), also known as a human cytokine synthesis Inhibitory factor, is an anti-inflammatory cytokine produced by nearly all leukocytes, including macrophages, dendritic cells (DC), natural killer (NK) cells, neutrophils, eosinophils, mast cells, B cells and a number of CD4+ T-cell subsets[68]. The liver is considered to be the main source of IL-10 production[69]. IL-10 is capable of inhibiting the synthesis of proinflammatory cytokines, such as interferon-γ, TNF-α, IL-1β, and IL-6, from activated

macrophages or monocytes. It also possesses a hepatic protective effect on proliferation and fibrosis [70,71].

IL-10 is produced by nearly all leukocytes, including macrophages, which secrete IL-10 in response to different stimuli, thus providing the immune system with an exquisitely regulated mechanism to control the effects of IL-10 in a tissue-specific and temporal manner[68].

I.5 Compatible solutes and ectoine:

To ensure survival, simple organisms in hostile environments have developed a special mechanism. To maintain cell stability they produce a certain group of active substances, the so-called compatible solutes, which are also referred to as extremolites. This group was first identified in bacteria living in the hostile environment in the salt lakes of Wadi Elnatrun, Egypt[72]. Compatible solutes are small organic osmolytes including, but not limited to, sugars, polyols, amino acids and their derivatives, betaines, ectoines and occasionally peptides suitably altered to remove charges[73]. A key feature of these molecules is that they do not inhibit overall cellular functions, although they may modulate individual enzyme activities. This behavior led to labeling them as "compatible solutes"[74]. They are compatible with cell metabolism even at molar concentrations. A variety of organisms synthesizes or takes-up compatible solutes for adaptation to extreme environments. In addition to their protective action on whole cells, compatible solutes display significant effects on bio-molecules *in vitro*, including stabilization of native protein and nucleic acid structures[75]. Compatible solutes are found in microorganisms from all three domains: Archaea, Bacteria and Eucarya. They are also present in higher organisms[76] and as a natural component of food traditionally processed by microorganisms[76,77].

In addition to their function as osmoprotectants, compatible solutes are characterized by being effective stabilizers of biomolecules including proteins, nucleic acids and biomembranes. These properties, in addition to the fact that compatible solutes are biologically inert and do not interfere with overall cellular functions, even though they accumulate in high concentrations in the cytoplasm, make them potential candidates for cellular protection [78]. Beneficial effects of compatible solutes on proteins *in vitro* [79], as well as the effects on protein expression [80], and stabilization of whole cells [81,82] have been studied. Such effects extend to nucleic acid stabilization [83], and improvement of protein/NA formation [84]. They do not directly interact with the protein surface, but they slow down the diffusion of solvent molecules by strongly interacting with water molecules and consequently enhance the stability by making denaturation thermodynamically less favorable.

The compatible solute Ectoine Figure (1); a cyclic tetrahydropyremidine (1,4,5,6-tetrahydro-2-methyl-4 pyremidine carboxylic acid) is a natural zwitterionic, low molecular weight, strong water binding organic molecule. It was first isolated from *Ectithiorhodospia holochloris*. Later it was also found in several aerobic chemoheterophic and halophilic/halotolerant bacteria. It is synthesized in response to extreme environmental conditions and acts to protect biopolymers (e.g., proteins, nucleic acids and lipids) from dehydration and other stress conditions which could lead to conformational changes and loss of biological activity. Nowadays commercially available ectoine is produced by biotechnological techniques in high purity using the "bacterial milking" fermentation technology followed by downstream purification[85]. Like other compatible solutes, ectoine confers resistance towards environmental stress conditions such as high temperature, freezing, extreme dryness and high salinity[79,86], which could lead to conformational changes and loss of biological activity. Ectoine and other compatible solutes are believed to exert their protective effects by stabilizing the native conformation of biological macromolecules through the so-called "preferential exclusion" phenomenon, whereby these agents are excluded as such from the immediate hydration shell of biomolecules, such as proteins, and preferentially hydrate the protein surface through their property of being strong water structure formers [87-89]. When added to mammalian cells, ectoine was shown to possess many protective effects including stabilization of cell membranes [90] and cytoprotection of human keratinocytes [91].

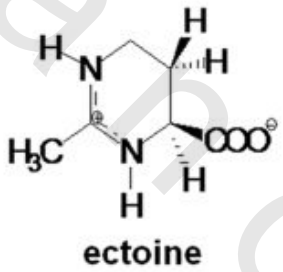

Figure (1): Structural formula of ectoine