# V.DISCUSSION

The exposure of mammals to ionizing radiation leads to the development of a complex dose-dependent series of physiological and pathological changes [97]. Most of the deleterious effects are the result of hydroxyl and superoxide radicals as well as hydrogen peroxide produced via the radiolysis of water, imparting severe oxidative stress to all of the cell's components [98]. Increasing evidence also supports the role of chronic oxidative stress in the progression of radiation-induced late tissue injury [16]. A founding concept of radiobiology that deals with x-rays is that this type of radiation indiscriminately damages cellular macromolecules. This concept has been challenged. The lethal effects of radiation appear to be governed by oxidative protein damage, which inactivates enzymes including those needed to repair and replicate DNA[99]. Experimental evidence, led to the conclusion that proteins are more probable initial targets of cellular radiation damage than DNA. The ability of cells to protect their proteins from oxidation by scavenging ionizing radiation-induced ROS has been proposed as the key mechanism for survival of ionizing radiation-resistant microorganisms [98]. This concept led to considering the possible protective effect of a compatible solute like ectoine in this respect since it is known to protect both hydration and folding of proteins[90]. So far the compatible solute ectoine has been shown to prevent signaling events triggered by UVA irradiation in skin epithelial cells. This kind of environmental stress initiates proinflammatory signaling via the induction of ROS in keratinocytes [100]. Therefore, a better understanding of the mechanism by which ectoine confers radioprotection will enable its use more effectively for protection in patients after radiation therapy or in those suffering from overdose or accidental irradiation.

Opposing hypotheses on the potential risks of low-dose radiations have been advanced. One hypothesis proposes that there is no dose of radiation that can be considered completely safe and that the use of radiation must always be determined on the basis of risk and benefit. Another hypothesis suggests that the health risks of diagnostic doses less than 10Gy are not measurable [101], and radiation doses less than 2Gy were reported not to cause significant damage[102]. However at 2-8 Gy, radiation syndrome develops proportional to radiation dose. This was the basis for the choice of the relatively low radiation doses of 2 and 6 Gy used in the present work.

The organs most sensitive to radiation are the hematopoietic, lymphoid, gastrointestinal, reproductive, vascular, and cutaneous systems [103]. The liver has been reported to be a highly radiosensitive hematopoietic organ. It is the primary organ responsible for drug metabolism, detoxifying damaging electrophiles generated during oxidative stress[104,105]. When compared with other organs in the body, the CNS is susceptible to ROS-mediated damage due to its biochemical, physiological and anatomic characteristics. The severity of the effects depends on the radiation dose, frequency and duration of the exposure as well as the size of the exposed area [106]. The most serious complication of radiation exposure to the brain is tissue damage that develops in the form of edema and necrosis, which have been reported to be mediated by free radicals[107].

The normal adult testis is known to be extremely sensitive to the effects of radiation[108]. The relationship between radiation dosage and length of time to recovery has been well-established after single dose irradiation in adult men. Renal involvement [chronic renal failure] may be responsible for marked Leydig cell dysfunction1[109]. In a series of experiments using a rat model Delic and co-workers [110-112] provided evidence

that the pubertal status modified the testicular response to radiation injury. The threshold dose for induction of Leydig cell dysfunction in prepubertal, pubertal and adult rat was about 5 Gy; however, the younger animals appeared to be more vulnerable to persistent Leydig cell damage[113].

Accordingly, these three organs; liver, brain and testicles, were chosen in the present study for their greater risk; although other organs like skin, heart, kidney, intestine and lung are just as important and are at similar risk.

The early biochemical modifications may continue to rise for days and months after the initial exposure to irradiation presumably because of continuous production of ROS[8]. These toxic products initiate a cascade of events on the molecular level, which alter the cytokine content of the microenvironment and affect the balance of antioxidant systems such as glutathione and enzymatic antioxidant defense systems[114-116]. In the present study, this phenomenon was observed in most of the biochemical changes in the tested organs following irradiation by either of the two low x-ray doses. Continued increases in the values of all determined parameters were observed with both doses used between day one and day 7 of irradiation with the exception of MDA. Whether the decrease in MDA observed in day seven may be taken as an early sign of recovery or it may be due to excessive damage of cellular membranes causing depletion of fatty acids, particularly arachidonic acid, because of extensive production of interleukins and $PGE_2$ or due to decreased activity of the oxidative enzymes, needs to be clarified.

The reported effects of cytokines in case of irradiation are conflicting. Several possible mechanisms of radioprotection have emerged including reduction of oxidation damage through induction of such enzymes as manganese superoxide dismutase (MnSOD) and other scavenging proteins, as well as reduction of apoptosis. It has been suggested that natural levels of IL-1 and TNF-α contribute to radio-resistance of normal mice, and their cooperative interaction is necessary to achieve successful radioprotection [117] and to enhance the ability of the host to recover from lethal radiation[118]. In contrast, sensitizing mechanisms may include increased oxidative damage, which may occur in absence of scavenger induction, as well as enhanced apoptosis and arrest of cells in the G1 phase of the cell cycle at the time of exposure to radiation, which may promote apoptosis [102].

Cytokine measurements in tissue or in the peripheral circulation have been an important part of the process of defining the role various cytokines play in health and disease. It has been suggested that local cytokine levels and activity are of considerably greater value for monitoring of pathological events in a target tissue than are systemic serum cytokine levels [117]. In the present study, the x-irradiated mice showed elevated interleukins in all tested tissues. The magnitude of elevation was dependent on the radiation dose and the time after irradiation. Some of the biochemical changes associated with signal transduction is likely to be cell-specific. This could be seen in the present work in the differences in the magnitude of the changes in the levels of the different interleukins in the tissues tested. The levels in the liver were much higher than in brain or testicles.

In the present study, increases in the concentration of IL-1β were accompanied by concomitant increases in IL-6 in the three organs tested. IL-1β has been reported to induce IL-6 [119], which is considered to be an essential contributor to natural resistance to lethal irradiation. However, by itself IL-6 was shown not afford any protection, but obligatory interaction of IL-1 or TNF-α with IL-6 may be a prerequisite for some of the biological

effects of these inflammatory cytokines . Induction of IL-6 and/or its receptors has been reported to vary in different tissues and many of the actions of IL-1β can be mimicked by IL-6 [120].

It is noteworthy that the increases of the pro-inflammatory cytokines, in the present study, were also accompanied by significant increases in the anti-inflammatory IL-10, which followed the same pattern. High expression of IL-10 has been reported in the thymus on day 5 following irradiation [121,122]. Probably such increase represents a defense mechanism against the high levels of IL-1β and other pro-inflammatory mediators caused by irradiation. It is generally recognized that counteraction of the inflammatory response to radiation is important to attenuate acute radiation effects and prevent consequences [123]. It has been proposed that the biological activities of IL-10 in modulating inflammation in this case may be caused, in part, by down-regulation of pro-inflammatory cytokines and the expression of their receptors and up-regulation of cytokine inhibitors [124]. The down-regulation of IL-6 by IL-10 has been shown and it has been proposed that limiting one step of the inflammatory process cascade might control the progression of the inflammatory reactions. This can be of benefit since it has been widely shown that the inflammatory reaction is intrinsically destructive for surrounding tissues. In addition an early release of cytokines could be responsible for the damage leading to the hypothesis of perpetual cascade of cytokines initiating radiation-induced late effects [125].

The intracellular increase in the level of IL-1 is followed rapidly by induction of several biochemical events[60], some of which are associated with signal transduction and are likely to be cell-specific and some are thought to be initiated by the release of lipid mediators. IL-1 preferentially stimulates new transcripts for the inducible type II form of $PLA_2$, which cleaves the fatty acid in the number 2 position of cell membrane phospholipids, resulting in most cases in the release of arachidonic acid, which is the rate limiting step in the synthesis of PGs and leukotrienes. Besides, IL-1 induces the transcription of COX-2. Once triggered, COX-2 production is elevated and large amounts of $PGE_2$ are produced in cells stimulated with IL-1. Therefore, many of the biological activities of IL-1 are proposed to be due to increased $PGE_2$ production [126].

Prostaglandin $E_2$, in the present study, increased in all three organs tested proportional to the radiation dose and the time after irradiation. Increased prostaglandin-like activity in most tissues of mice exposed to whole body irradiation was previously reported[127]. The underlying mechanisms through which prostaglandins may be cytoprotective are unknown. It was reported that too little $PGE_2$ in the early period post-irradiation reduced positive anti-apoptotic and self-renewal effects, while too much $PGE_2$ signaling at later time points inhibits hepatopoietic progenitor cells expansion and reduces hematopoietic recovery[101]. A broad spectrum of mediators regulates the expression of COX-2. Whereas proinflammatory cytokines such as IL-1β and IL-6 among other factors induce COX-2, the anti-inflammatory cytokine IL-10 inhibits the expression of this enzyme[42,128]. Therefore it seems that the balance between the effects of proinflammatory and anti-inflammatory cytokines may determine the short-term or long-term outcome of irradiation effects.

In the present study indicators of oxidative stress and damage due to increased production of free radicals were highest after one day of exposure to low levels of x-irradiation followed by attenuation on day seven in all tested organs. After gamma

radiation exposure, levels of the lipid peroxidation indicator MDA have been shown to increase in brain [107, 129], liver [130-134], lens[135], serum[136], and skeletal muscle [137] of rats. Ionizing radiation-induced lipid peroxidation reactions can occur at both the cell membrane and the mitochondria membranes, and either can subsequently trigger cell death through apoptosis and/or autophagy [138]. Autophagy is a catabolic process involving the bulk degradation of cellular constituents in lysosomes[139]. Therefore, blockade of lipid peroxidation could be a useful approach to prevent radiation injury.

The data obtained in the present work point out to that the low dose x-irradiation has stronger effect on interleukins and prostaglandin $E_2$ than on oxidative stress as measured by changes in different forms of glutathione and MDA. The magnitude of effect appears to be radiation dose and organ specific. The effect of low-dose x-irradiation on glutathione was not as strong as may be expected. There were no statistically significant differences in the results of total glutathione among all the groups of mice as compared to control values. However, there were significant differences among the values of the reduced and oxidized forms. The effect was relatively higher after one day of irradiation with a tendency toward recovery and going back to near control. Because of the relatively small initial reduced and oxidized levels in the control group, the percentage changes in the irradiated groups were apparently large. Current concepts of the mechanism of action of ROS include alteration in intracellular redox state and oxidative modification of proteins. Cellular cytosol is normally maintained under strong reducing conditions, which is accomplished by the action of intracellular redox-buffering systems[11]. This could be reflected in the calculated values of redox potential, which showed some variations in the different groups, but they were all within the values indicating that the cells are in the proliferative phase and far from the apoptotic phase [29].

A number of natural and synthetic compounds of diverse structures have displayed significant protection against radiation[140,141]. All of them are presumably working through different mechanisms of action. However, if the main effect of radiation is on protein[99] and the key to cell survival is through conserving the integrity of the antioxidant enzymes [98,142], then compounds known to possess such properties would be good candidates for the protective action. Compatible solutes, including ectoine, are characterized by being effective stabilizers of biomolecules including proteins and nucleic acids as well as biomembranes[91]. These properties make them potential candidates for cellular protection. It was shown that ectoine increases the hydration of a model biological membrane resulting in higher membrane fluidity[90]. The increased hydration and fluidization of the cell membrane may help to withstand membrane damaging stressors and might also accelerate repair mechanisms. Ectoine was also found to block nuclear translocation of NF-κB to down-regulate the expression of the proinflammatory cytokines IL-1, IL-6, IL-8 and TNF-α[91]. These effects might be partially mediated by ectoine's impact on membrane fluidity leading to interference with membrane-coupled proinflammatory signaling [143]. From the results of the present work it could be seen that the effect of ectoine on day seven in all three examined organs; i.e., liver, brain and testicles, was much more prominent than on day one. This probably implies that ectoine gives stronger effects after it accumulates in these organs following multiple dosing.

Results of the present study demonstrated that the 2Gy and 6Gy whole-body irradiation caused a significant increase in the MDA level, whereas the antioxidant levels of GSH were markedly decreased in the livers, brains and testes of irradiated mice.

Administration of ectoine effectively decreased MDA levels in the livers, brains and testes of all irradiated animals. GSH, as an antioxidant, has been considered as the most accurate single indicator of cell health, as GSH depletion represents vulnerability to oxidant attack[144]. Significantly elevated levels of GSH were observed in the livers, brains and testes of mice treated with ectoine, which may be a factor responsible for the inhibition of MDA generated from lipid peroxidation. In addition, the significant increase in GSH protects cellular proteins against oxidative damage through the glutathione redox cycle and also directly detoxifies ROS induced by irradiation[145]. Therefore, ectoine administration can effectively mitigate oxidative stress in the liver, brain and testis. Whether the protective mechanism of ectoine may be due to its ROS scavenging activity or to regulating the activity of antioxidant enzymes needs further investigation.

The ability of ectoine to evoke a proinflammatory process was previously investigated [91]. When keratinocytes were pretreated with ectoine before stimulation with a well-characterized proinflammatory stimulus lipopolysaccharide (LPS), the up regulation of the proinflammatory cytokines IL-1 β, IL-6, IL-8, and TNF-α was not observed as occurred in the LPS-treated cells. However, the sole ectoine treatment did not modify the level of expression for the determined cytokines. It was concluded that ectoine does not have the ability to induce a proinflammatory process; moreover, this compatible solute probably acts as an anti-inflammatory molecule.

In conclusion, protection of biological systems from ionizing radiation is of paramount importance in planned as well as unplanned accidental exposures to radiation. Development of novel and effective agents to combat radiation damages using nontoxic radioprotectors is of considerable interest in health care, particularly in radiodiagnostics and therapy. Despite the lack of clinical studies, results of the present study suggest that ectoine has the potential to protect tissues from radiation injury and is a candidate for further development as a radiation countermeasure. Further experiments and clinical trials are necessary to validate this.