Background Information
2 BACKGROUND INFORMATION

2.1 Calcium and radiation damage

The unique role of Ca\(^{2+}\) as an activator and regulator of a variety of biological processes is now well established. It sends out electrical and chemical signals that reach out to the cell surfaces and alter the metabolic activities. In order to control cellular processes effectively, the levels of Ca\(^{2+}\) are immaculately regulated (Carafoli 1987). Intracellular concentration of free Ca\(^{2+}\) is always kept very low so as to avoid any conflict between the breakdown and resynthesis of ATP, not letting them interfere with the normal phosphorylation/dephosphorylation processes of the cell. Under normal conditions, the intracellular concentrations of Ca\(^{2+}\) is around 0.1\(\mu\)M. In conditions of cellular stress, these concentrations may be increased to 0.5 – 2.0 \(\mu\)M. The calcium concentration of the extracellular fluids is about 1 mM. Thus the plasma membrane maintains a low intracellular calcium against a chemical gradient of about 10\(^{-4}\).

Calcium concentrations of the cell are regulated by a precise control of the membrane permeability for Ca\(^{2+}\). It is also regulated by the action of Ca\(^{2+}\)-ATPases which extrude Ca\(^{2+}\) from the cell using metabolic energy. Calcium ions are sequestered in the endoplasmic reticulum using ATP-driven pumps. Mitochondria also take up Ca\(^{2+}\) by utilizing the electric potential, which develops across their inner membrane. All these mechanisms help maintain a very low intracellular calcium concentration against a high extracellular concentration.

As mentioned above, Ca\(^{2+}\) regulated functions are huge in number. Ca\(^{2+}\) microgradients can act as selective switches for certain cellular functions and thus activate several processes synchronously. Calcium ions are also known to control gene transcription, for instance in the neurons, where dendritic calcium signals induce gene transcription (Bading 2000). Calcium has an important role in the skeletal muscle activity. Moreover, cellular calcium overload has been suggested to be the
final common pathway of cell death. The flexibility in coordination number, its suitable size and weak polarizing power make Ca\(^{2+}\) a versatile candidate and potent biological regulator over other cations (Williams 1977). Ionizing radiation is known to cause cell damage and death. However, the involvement of calcium in the radiolytic processes is not well understood.

The biological effects of radiation are the end products of a long series of phenomenon, which are set in motion by the passage of radiation through the medium. Harmful effects of radiation are brought about through chemical changes in the cell caused by the ionization, excitation and atomic displacement. Severity and nature of damage and time at which they appear depends on spatial and temporal distribution of radiation energy (Goodhead 1988). Radiation damage to biological system involves either direct or indirect action depending on whether the energy is absorbed by the tissue biomolecules or by the surrounding water. Since cells consist of about 60-80% of water, to a greater extent the biological effects are mediated through the action of radiation on water.

\[
\text{H}_2\text{O} \rightarrow \text{H}^+, \text{HO}^+, \text{O}_2^-, \text{H}_2\text{O}_2, \text{H}_2, \text{etc.}
\]

Radiolytically formed free radicals and molecular species react with biomolecules and bring about the changes in structure and function. Thus, free radical processes are involved in radiation-induced cellular lethality (Redpath 1981).

2.1.1 Radiation induced DNA damage

For the past 30 years, DNA is believed to be one of the main cellular targets of the biological action of ionizing radiation (Ward 1988, Baverstock and Will 1989, Close et al. 1988, Teoule and Cadet 1978, Cadet and Teoule 1978). If one or more genes are involved, modification of the genetic message can have disastrous consequences on heredity. The damage is known to occur, particularly by formation of DNA double strand breaks. Attack by \text{HO}^+ radicals is mainly responsible for this damage (Chapman et al. 1973, Udovicic et al. 1991, Bump et al. 1988). None of the
other diffusible radicals produced by radiation are suggested to participate in DNA damage because they diffuse away from the site before they have a chance to react with the DNA (Bump et al., 1988). Other major classes of radiation-induced lesions include base damage (Close et al. 1988, von Sonntag 1987, Cadet and Berger 1985, Maccubbin et al. 1992) and DNA-protein cross-links (von Sonntag 1987). There is an extreme variety of radiation-induced base modifications, the formation of which depend on the oxygen concentration (Cadet and Teoule, 1978, Cadet 1994, Piette 1991, Boon et al. 1984). Studies on mutagenesis and DNA replication have indicated that oxidative DNA base damage may have deleterious effects (Ide et al. 1994, Wood et al. 1992, Grollman and Moria 1993, Turk et al. 1995), over and above their contribution to the clustered DNA lesions that are believed to be important in classical clonogenic cell killing.

2.1.2 Radiation-induced membrane damage

Apart from DNA, membranes are also considered to be critical targets of ionizing radiation effect. Bacq and Alexander (1964) had proposed that a significant contribution to radiobiological effect of ionizing radiation is due to cell membrane damage. Several studies have supported the idea that membrane damage induced by radiation is the critical event (Alper 1971, 1977, 1979, 1968, Dutta et al. 1976, Obioha et al. 1984). Kerr et al. have suggested that damage to membrane organization is an initial step in triggering cell death. As mentioned earlier, a correlation has been observed between unrepaird membrane damage and loss of colony forming ability in cells (Sato et al. 1972, 1975, 1981), the breakdown of nuclear membrane and chromosomal condensation and damage to the organization of mitotic spindles (Sato et al., 1983), interphase death in non-proliferating cells and the disorganization of membrane system (Sato and Yonei 1987). Ramakrishnan et al. (1993) showed that lipid peroxidation occurs in irradiated lymphocytes and Trolox, a vitamin E analog, can protect against radiation-induced apoptosis in these cells. Haimovitz-Friedman et al. (1994) have reported that radiation-induced ceramide formation, which is implicated in the induction of apoptosis in endothelial cells by
radiation, can occur in isolated plasma membrane preparations and therefore does not require radiation-induced DNA damage.

Membrane damage is initiated on the unsaturated lipids, which are particularly susceptible to free radical attack (Kappus 1985). The weakness of the hydrogen-carbon bond at a bisallylic carbon (carbon atom that is flanked by carbon atoms that are participating in double bonds) facilitates hydrogen abstraction from that position. This hydrogen abstraction, which initiates a chain reaction, is most commonly the result of attack by relatively stable secondary radicals. The resulting lipid radical rearranges and reacts with oxygen to form a peroxy radical. The peroxy radical can abstract a hydrogen from another lipid molecule (propagation of the chain reaction). The resulting hydroperoxide can decompose to form an alkoxyl radical that again abstracts a hydrogen from another lipid molecule. Termination of the chain reaction occurs when one of the intermediate radicals reacts with another radical or with a chain breaking antioxidant such as vitamin E.

It is possible that lipid peroxidation could play a role in effects of ionizing radiation that are mechanistically different from DNA-damage-induced classical reproductive cell death.

2.1.2.1 Role of calcium in membrane damage

It is known that influx of calcium through the membranes is one of the key events in cell damage and death, and integrity of the membranes is essential to maintain Ca\(^{2+}\) homeostasis. Also, in radiolytic systems, membranes are considered to be critical targets of detrimental effects. Thus membrane damage seems to be an initial event both in Ca\(^{2+}\) mediated toxicity as well as radiation action and the two are expected to be interlinked. Lipid peroxidation, as discussed above, is one of the important effects of ionizing radiation on biological membranes. Non-radiolytical studies on the role of lipid peroxidation in cell death implicated a concurrent involvement of divalent calcium (Casini and Farber 1981, Casini et al. 1982, Barsachhi et al. 1983). In several reports, Ca\(^{2+}\) was shown to modify the extent of lipid peroxidation (Braughler 1988, Singh and Kale 1994) and enhance lipid peroxidation-mediated damage in different systems. Phenothiazines were shown to
modulate radiation lipid peroxidation (Kale and Sitasawad 1990, Varshney and Kale 1990). However, although they are known to be calmodulin antagonists, the direct involvement of \(\text{Ca}^{2+}\) in radiation-induced peroxidation process has not been studied. Indirect studies suggest a role for calcium in membrane damage. Direct evidence and mechanism for the process are still lacking.

2.1.3 Radiation-induced apoptosis

Apoptosis is a distinct mode of cell death that plays a significant role in many different aspects of biology and medicine. Apoptosis can be induced by a variety of extracellular and intracellular stimuli, one of which is radiation. It appears that the stimuli activate various signal transduction pathways that converge at a common point to trigger the apoptotic death of the cell. It was first noted in 1952 that distinctive morphological changes are associated with interphase cell death (Trowell 1952, 1961). One of the earliest visible postirradiation changes during apoptosis is clumping of chromatin, followed by nuclear vacuolization, pyknotic degeneration and nuclear disintegration. These morphological features led Kerr et al. (1972) to define the term apoptosis. Cell shrinkage, cell surface blebbing, formation of apoptotic bodies are characteristics of apoptosis. Fragmentation of DNA into oligonucleosomal subunits is typical of radiation-induced apoptosis.

Recent data suggests that membrane processes may be important in the induction of apoptosis by radiation, in contrast to the widely acknowledged role of DNA damage in inducing cell death. Apoptosis can be induced with stimuli that trigger specific receptors in the plasma membrane which in turn activate signal transduction. An important component of the signaling process in apoptosis induced by radiation or oxidative damage appears to be the early generation of free radicals in the plasma membrane. These cause peroxidation of the membrane. It has been shown that by 2 min postirradiation, the levels of peroxides in irradiated murine thymocytes are approximately 10-11 times greater than those in the same cells before irradiation and levels continue to increase with time (Ramakrishnan et al. 1996). This peroxidation is shown to occur much earlier that DNA fragmentation and loss of cell viability (Hockenbery et al. 1993). Moreover, Trolox, a water-soluble analog of
vitamin E and an inhibitor of membrane lipid peroxidation (Doba et al. 1985, Castle and Perlins 1986, Mickle et al. 1989, Wu et al. 1990, Casini et al. 1985) inhibits internucleosomal DNA fragmentation in thymocytes (Ramakrishnan et al. 1993) and MOLT-4 human leukemic cells (McClain et al. 1995) exposed to gamma radiation. In view of these, membrane lipid peroxidation seems to be an early biochemical event in irradiated cells that could contribute to apoptotic signaling.

Apoptosis involves the expression of several oncogenes and suppressor genes. The modulation of expression of such genes is mediated by transcription factors. AP-1 and NF-κB are two transcription factors that have been shown to be activated by free radicals (Schreck et al. 1992, Karin and Smeal 1992). These transcription factors are stored in the cytoplasm as inactive complexes, and their activation involves cytoplasmic signals. Studies with enucleated cells indicate that the signaling cascade responsible for activating both NF-κB and AP-1 is initiated at or near the plasma membrane and is not elicited by DNA damage in the nucleus (Devary et al. 1993).

In non-stimulated cells, NF-κB occurs in association with the I-κB polypeptide. Nuclear uptake and DNA binding of NF-κB require release of I-κB (Schreck et al. 1992). NF-κB is activated in human lymphoid cells following low doses of ionizing radiation (Prasad et al. 1994). Several antioxidants are known to inhibit the activation of NF-κB (Schreck et al. 1992). It is possible that radiation-induced membrane damage might signal PKC to activate NF-κB, which in turn could activate the transcription of specific death genes involved in apoptosis.

AP-1 is induced by a wide spectrum of inducers, including ionizing radiation. AP-1 consists of a collection of structurally related transcription factors belonging to the jun and fos families. Ionizing radiation stimulates the expression of c-fos and c-jun genes.

One of the genes that appear to exert direct control over radiation-induced apoptosis is the tumor suppressor gene p53. Lowe et al. (1993) and Clarke et al. (1993) showed that thymocytes with wild-type p53 were very sensitive to ionizing
radiation-induced apoptosis, while thymocytes with two defective alleles were resistant. An important function of p53 is to block the cell cycle in G1 to allow necessary repair to be carried out following DNA damage (Lane 1992, Maltzman and Czyzyk 1984, Kastan et al. 1991, Szumiel 1994). P53 has also been reported recently to regulate the expression of the bax gene and indirectly bcl-2 (Zhan et al. 1994).

2.1.3.1 Role of calcium in apoptosis

Calcium is a very important second messenger involved in the apoptotic death program. Cellular Ca\(^{2+}\) overload has been suggested to be the final common pathway of cell death (Yu et al. 2001). Large increase in Ca\(^{2+}\) influx inevitably accompanies the collapse of the plasma membrane, which occurs in necrosis. In several cases, which probably share a relatively slow rate of progression, this increase in Ca\(^{2+}\) is responsible for the ensuing cell death. For example, membrane toxin-induced death of hepatocytes requires extracellular Ca\(^{2+}\) (Schanne et al. 1979). Similarly breaching the plasma membrane by sustained activation of agonist-gated Ca\(^{2+}\)-permeable channels (e.g., nicotinic acetylcholine receptors on skeletal muscle (Leonard and Salpeter 1979)) induces extracellular Ca\(^{2+}\) dependent necrosis. There are many reasons why large elevations in Ca\(^{2+}\) are cytotoxic, including a subsequent derangement in signaling and mitochondrial function, as well as the destruction of cellular components by Ca\(^{2+}\) activated catabolic enzymes and free radicals (Yu et al. 2001). In some cases, an early increase in Ca\(^{2+}\) may induce the cellular injury that then triggers apoptosis. This early Ca\(^{2+}\) insult pattern occurs, for example, in neurons undergoing apoptosis after low level NMDA receptor activation or in rat prostate cancer cells undergoing apoptosis after exposure to a Ca\(^{2+}\) ionophore (Martikainen et al. 1991). In other cases, an early increase in Ca\(^{2+}\) may not per se induce cellular damage, but rather it may serve as a component of a signaling cascade culminating in triggering apoptosis. Immortalized granulosa cells subjected to serum withdrawal exhibit an almost immediate progressive increase in Ca\(^{2+}\), reaching four times the resting levels within 10 minutes and then become apoptotic over the ensuing hours; cell death can be abrogated by chelating intracellular Ca\(^{2+}\) with the calcium chelator BAPTA (Lynch et al. 2000). A similar moderate early increase in Ca\(^{2+}\) has been observed in PC12
cells (Krumlan et al. 1998) and cochlear neurons (Zirpel et al. 1998) exposed to the broad spectrum kinase inhibitor, staurosporine, and in thymocytes exposed to thapsigargin, suggesting that moderate increases in Ca$^{2+}$ might be a common mechanism. Late elevations in Ca$^{2+}$ have been implicated in the execution of apoptosis. A late elevation has been observed in thymocytes (Shen et al. 2001). Possible targets of a late rise in Ca$^{2+}$ during apoptosis include key Ca$^{2+}$-activated proteases (Vanags et al. 1996) and endonucleases, such as NUC18 (Gaido and Cidlowski 1991) as well as perhaps caspase-3 (Juin et al. 1998). The magnitude of the increase in intracellular calcium may be a critical factor in determining whether it would lead to apoptosis or necrosis.

The importance of calcium in the radiation-induced apoptotic pathway has also been suggested. Irradiation results in the production of reactive oxygen species and induction of membrane peroxidation. This leads to activation of phospholipase C. Mobilization of calcium is stimulated in irradiated cells by Inositol 1,4,5-triphosphate produced by the breakdown of phosphatidylinositol by phospholipase C. Zhivotovsky et al. (1993) have shown that 1h after exposure of rat thymocytes to 6 Gy X-rays, cytosolic Ca$^{2+}$ increases to 122 +/- 7% of the value in unirradiated controls. Calcium may also participate in the signal transduction process leading to apoptosis. Cellular processes that could be affected by alterations in Ca$^{2+}$ homeostasis are numerous. There are atleast two Ca$^{2+}$ dependent enzymes that are commonly activated during apoptosis- the endonuclease that cleaves chromatin at internucleosomal sites (Martin et al. 1990) and tissue transglutaminase that participate in the formation of apoptotic bodies. Endonucleases are responsible for the characteristic feature of apoptosis, the ladder. The radiation-induced influx of calcium, the activation of endonucleases and the induction of DNA fragmentation occur concurrently in irradiated thymocytes (Ramakrishnan et al. 1993).

In view of the above, the following model has been proposed for radiation-induced apoptosis (Ramakrishnan et al. 1998). Radiation exposure results in the production of reactive oxygen intermediates and the induction of membrane lipid
Schematic representation of radiation-induced apoptosis

**Ca**

**Ca INFLUX**

**Ca mobilization**

**Ca INFLUX**

**IP3**

**Ca mobilization**

**PI**

**PLC**

**PTK**

**PM**

**SMase**

**ANTIOXIDANTS**

**SANS MEmBRANE**

**PLASMA MEMBRANE**

**ROS / PEROXIDATION**

**RADIATION**

**DAG**

**PKC**

**p53**

**NF-κB**

**CAM**

**Ca mobilization**

**p53**

**Transcriptional activation of death genes**

**Apoptosis**

**ENDONUCLEASE ACTIVATION**

**NUCLEAR MEMBRANE**
peroxidation chain reactions. These reactions activate phospholipase C either directly or through increased protein tyrosine kinase activity. Phospholipase C catalyses the breakdown of phosphatidylinositol to diacylglycerol and inositol 1,4,5-triphosphate and the conversion of phosphatidylcholine to diacylglycerol. Diacylglycerol can activate PKC directly or be further degraded to arachidonic acid, which also stimulates PKC. IP3 production stimulates the mobilization of intracellular calcium and the uptake of extracellular calcium through calcium channels located in the plasma membrane. Ceramide can activate a variety of messengers, including protein kinases, protein phosphatase, and NF-xB. Ceramide can also be processed further to sphingosine-1-phosphate, which can activate the transcription factor AP-1 and mobilize intracellular calcium. The protein kinases stimulated by diacylglycerol, arachidonic acid or ceramide may trigger the import of p53 into the nucleus where it can act as a G1-blocker and/or enhance transcription of "death" genes. The transcription factors NF-xB and AP-1 may also function in the activation of the death genes, and NF-kB can stimulate the production of p53. The elevated intracellular calcium mobilized from intracellular stores as a result of inositol 1,4,5-triphosphate or sphingosine-1-phosphate production, and calcium imported from the extracellular medium, can bind to calmodulin and be transported to the nucleus of the cell. There, the calcium could activate the endonucleases responsible for the internucleosomal cleavage of DNA. The ultimate result of the convergence of these distinct signal transduction pathways is apoptosis.

Although the role of Ca^{2+} and membrane damage in the radiation-induced apoptotic pathway is reasonably well illustrated and Ca^{2+} ions are known to interact with membrane lipids, the modulation of radiation-induced damage by Ca^{2+} ions is not well understood. This aspect is one of the questions addressed in the present work.

2.2 Radiation protection

In view of the various damaging effects described above, protection against the radiation effect has been a long-standing challenge. The search for chemical agents
able to protect against the deleterious effects of ionizing radiation has been undertaken for at least two main reasons. One is related to the protection of individuals against accidental exposure. The other is to reduce the damage to normal surrounding tissues during radiation therapy of cancer (Livesey et al. 1985, Turrisi et al. 1983, Dillon et al. 1988, Bedwell et al. 1991).

2.2.1 Potential mechanisms of radioprotection

A radioprotector is an agent that when applied before or during the radiation exposure evokes a significant reduction in radiation injury. Development of effective and non-toxic radioprotective agents is of considerable interest for radiation medicine, space flights, nuclear industries and emergencies. The radioprotection ability may be ascribed to the following mechanisms.

2.2.1.1 Free radical scavenging

Radiation damage is known to be mediated mainly through free radicals. Therefore, chemical agents which can interact with these free radicals and scavenge them, would be effective in protecting against radiation damage. Large number of chemicals have been tested for this ability. Free radical scavengers act via various models. Nitroxides have been shown to react with a variety of biological oxidants including oxygen free radicals. They terminate free radical chain reactions in a catalytic manner. Micronutrients like vitamin E and its analogues are chain-breaking antioxidants and stoichiometrically inhibit lipid peroxidation. Radioprotection by thiols is ascribed to restoration of damaged target molecules by $\cdot H$ or electron transport.

2.2.1.2 Suppression of protracted oxidative stress

Apart from free radicals and secondary radicals produced radiolytically, they may be produced in some other manner as a consequence of irradiation. A possible source of oxidative stress following irradiation is the activation of signal transduction pathways that result in the production of reactive oxygen species over a protracted period. For example, ionizing radiation can cause increased production of TNFα
Hallahan et al. 1991) and reactive oxygen species have been implicated in its mechanism (Zimmerman et al. 1989). Activation of immunological processes that involve the generation of reactive oxygen species over a prolonged period may explain the radioprotective effects of agents such as superoxide dismutase that are effective when administered after irradiation.

2.2.1.3 Decreasing Oxygen concentration

It is now well-established that the extent of radiation damage in a tissue is directly related to the degree of oxygenation of that tissue. Tumors are known to have a portion of hypoxic cells due to the rapid fall-off in the concentration of oxygen with increase in distance from blood capillaries. The presence of this hypoxic region makes the tumors highly radioresistant. Since hypoxic cells are radioresistant, treatments that decrease microenvironmental oxygen concentrations can be radioprotective. The general ability of radioprotectors to reduce electron-deficient free radicals can also lead to oxygen depletion since molecular oxygen is a diradical. Under some conditions, it is possible to demonstrate thiol radioprotection by oxygen depletion (Durand et al. 1983). Oxygen depletion or impairment of oxygen delivery may thus be considered as a radioprotective strategy in the design of new therapeutic agents.

2.2.1.4 Enhancement of DNA repair

DNA repair appears to be one of the major factors in determining radiosensitivity. It is estimated that about 100 DNA double-strand breaks are produced by a therapeutic dose of ionizing radiation (1.5 Gy) (Ward 1990), but most of these lesions are repaired (Sakai and Okawa 1984) and only one unrepaired double-strand break, as reflected in the number of chromosome aberrations, appears to be sufficient to kill a cell (Nagasawa et al. 1990). Thus, modulation of repair capacity could be a potential protective strategy. Repair deficient cell lines can be considerably more sensitive than their wild-type counterparts and radiosensitivity can be increased by treatment with repair inhibitors (Okayasu and Iliakis 1994). However,
ample evidence does not exist to show that radioprotection can be achieved by enhancing repair capacity.

2.2.1.5 Decreasing intracellular Ca\(^{2+}\) concentration

Since the elevated levels of calcium are known to induce apoptosis, blocking a rise in Ca\(^{2+}\) concentration could inhibit this process of cell death. Ca\(^{2+}\) can be significantly reduced in both irradiated and unirradiated thymocytes by incubating cells for 2h in Ca\(^{2+}\) free medium containing 3 mM EGTA. Decreasing Ca\(^{2+}\) by such techniques is shown to block DNA fragmentation and prevent apoptosis in both unirradiated and irradiated lymphocytes (Zhivotovsky et al. 1993), indicating that Ca\(^{2+}\) plays an important role. Loading cells with 50 mM BAPTA-AM, a highly specific intracellular Ca\(^{2+}\) chelator, prevents both DNA fragmentation and loss of viability in irradiated lymphocytes (Story et al. 1992). Membrane located calcium channels, which are responsible for voltage dependent influx of calcium into the cytosol upon irradiation, are also potentially important targets. Blocking or inactivating such channels could potentially lead to a decrease in the intracellular calcium concentration and perhaps protection against radiation damage.

2.2.2 Differential radiation protection of normal tissues over tumor tissues

The ability to protect normal tissues from radiation injury has been a long sought-after goal both in the field of protection from accidental overexposures and in the use of radiation to treat malignant disease. In the treatment of malignant diseases, radiation is directed only at the tumor and great care is taken to expose as little normal tissue as possible. However, normal tissues in intimate association with the tumor and in the path of the treatment beam are unavoidably irradiated. The goal of radiotherapy is to deliver the maximum dose possible to the tumor to achieve a high probability of tumor cure while delivering a minimal dose to the normal tissues in the treatment field to avoid unacceptable reactions in these tissues.

This situation is different when the tissues of healthy individuals are accidentally exposed to radiation. Unlike in the treatment of malignant tumors with
radiation, where radiation is intentionally given and agents can be given before or after irradiation to protect the normal tissue, individuals are unintentionally exposed in these accidents and thus agents that reduce radiation damage in normal tissues can only be given after exposure. Radioprotection of unavoidably irradiated normal tissues in radiotherapy is further complicated by the fact that this protection must be selective; i.e., it must protect the normal tissue with no protection of the tumor, thus providing a therapeutic benefit. Some thiol-containing compounds were shown to selectively protect normal tissue against radiation while offering little tumor protection (Denakamp et al. 1983). However thiol radioprotectors were not widely used because of the associated unacceptable toxicities. As a result of the increasing knowledge of the molecular processes that govern tissue damage, repair and recovery, new nonthiol chemical protectors such as antioxidant vitamins have been developed (Seifter et al. 1988). Various other radioprotective substances have also been discovered that have a differential action. Phenothiazines have been shown to act differentially based on tissue oxygenation. Euoxic radioprotection and hypoxic radiosensitization is afforded by this class of drugs. Selective radiomodifiers such as these are likely to be more beneficial clinically rather than those that protect all tissues.

2.2.3 Radioprotective Compounds

2.2.3.1 Sulfhydryl Compounds

As early as 1942, a decrease was observed in the inactivation of enzymes by X-rays on addition of several substances, including colloidal sulfur and thiourea, to aqueous solutions of the enzymes (Dale et al. 1949). The significance of sulfur containing molecules for radioprotection was shown from the very earliest experiments with living systems, although the reasons for selection of sulfur compounds were not apparent.

The importance of the mercapto (or thiol) function was first demonstrated in 1952 by Bacq, who removed the carboxyl group of cysteine and obtained 2-
mercaptoethylamine (MEA) which is still regarded as the most potent of the whole-body radioprotective agents. The presence of the amino group was also considered essential for effective radioprotection, and the bulk of the mercaptans and other sulfur-containing molecules later synthesized also contained an amino or other basic function. The most effective compound of this type, tested against a lethal dose of X-rays or gamma rays in mice became known as WR2721 (Piper et al. 1969).

Aminothiols have been proposed to radioprotect by scavenging of free radicals, restoration of damaged target molecules by H* donation or electron (e-) transfer reactions, induction of hypoxia, chelation of metal ions and formation of mixed disulfides. (Klayman and Copeland 1982). WR2721 was reported to protect normal tissues to a greater extent than tumors at relatively nontoxic concentrations (Yuhas et al. 1980). Also, clinical experience suggests little protection of human tumors by WR2721 (Schuchter and Glick 1993). However, the achievable concentration of WR2721 is limited by its toxicity. Transient side effects include nausea, vomiting, a metal taste in the mouth, sneezing, hypocalcemia and occasional allergic reactions. The most clinically significant and potentially dose limiting toxicity is hypotension, which is rapidly reversed after discontinuation of WR-2721. Improved radioprotection and reduced drug toxicity may ultimately be achievable by combining low, nontoxic doses of agents that protect by different, but complementary mechanisms.

2.2.3.2 Physiologically active substances

A number of physiologically active agents exert some radiation protection. Serotonin (5-hydroxytryptamine), has been reported equal in activity to MEA (Langendorff and Koch 1957). It is effective at a dose well below the toxic level (Doull et al. 1961), in contrast to the aminothiols. Its activity has been attributed to its vasoconstrictor effect, causing hypoxia in radiosensitive tissues (van der Meer and van Bekkum 1961).
A number of enzymes involved in free radical metabolism are known to have radioprotective properties. Various intracellular enzymes, involved in radioprotection are described below:

Superoxide dismutase catalyzes the conversion of $O_2^+$ to $H_2O_2$ in the presence of any substrate that provides protons (Harris 1992; Baez et al., 1994). There are at least three SOD isozymes present in the mammalian cells: copper zinc-superoxide dismutase (CuZnSOD) in the cytosol of cells, manganese-superoxide dismutase (MnSOD) in the mitochondrial matrix and the extracellular —superoxide dismutase (EC-SOD) in the extracellular space (Marklund, 1984). SOD is present in all oxygen metabolizing cells to provide them with an endogenous defense against $O_2^+$ generated in aerobic biological system. Since $O_2^+$ is one of the several reactive species produced by ionizing radiation, the potential of SOD to function as a radioprotector has been investigated in a number of experimental systems. Considerable evidence supports a role for SOD in protection against reperfusion injury, hyperbaric oxygen, photosensitization and ionizing radiation.

The pharmaceutical version of Cu,Zn-SOD (orgotein) has been tested clinically and has been claimed to be an effective, slow acting, nonanalgesic anti-inflammatory drug. It is believed that superoxide anion-induced DNA strand breaks are inhibited by SOD (Bimboim 1986). However, SOD would be most effective in protecting the cell if it could enter the cell, since the biological half-life of $O_2^+$ is short and its dismutation should take place near the site of its formation. Being a large protein, SOD cannot readily traverse the membrane. Also many investigators have been unable to observe the radioprotective effect of SOD (Petkau 1987). Orgotein evoked hypersensitivity responses in both in vitro and in vivo tests (Corominas et al. 1990). These negative results warrant a cautious approach to the clinical use of SOD and further clinical testing is needed to establish the therapeutic benefits of SOD.

In addition to SOD, catalase, glutathione peroxidase and vitamins A, C and E are known to be low-toxicity natural antioxidants and it is possible that antioxidants,
other than SOD may also be radioprotective. (Jones et al. 1990, Gee et al. 1985, Malick et al. 1978).

*Catalase* serves two functions: a) decomposition of hydrogen peroxide and b) oxidation of hydrogen donor, methanol, ethanol, formic acid, phenols, with the consumption of one molecule of peroxide. The major damage from \( \text{H}_2\text{O}_2 \) arises from the highly reactive hydroxyl (\(^\cdot\)OH) radical, generated in the Fenton reaction between \( \text{H}_2\text{O}_2 \) and metal ions such as \( \text{Fe}^{2+} \), which can react with various components of cells, making it desirable for most aerobic organisms to have catalases or peroxidases to circumvent the damage.

*Glutathione-S-transferase* is found in most aerobic microorganisms, plants and animals; and has selenium independent peroxidase activity using organic peroxides, but can not reduce \( \text{H}_2\text{O}_2 \) (Batist et al. 1986). The primary function of the enzyme, particularly in higher organisms is generally considered to be the detoxification of both endogenous and xenobiotic alkylating agents such as epoxides, \( \alpha, \beta \)-unsaturated aldehydes and ketones, alkyl and aryl halides and others. The co-factor for reactions catalyzed by this enzyme is the tripeptide glutathione (GSH). The importance of GSH in the protective mechanism is now well established. Thiols act as protective agents against electrophiles, radical damage and oxidative stress. GST catalyzes many antioxidant processes of thiols (Choudhary et al. 1997; Dixon et al. 1998).

*DT-diaphorase* catalyzes the reduction of quinones, quinone epoxides, quinoneimines, certain aromatic nitro compounds, aromatic C-nitroso compounds, azo dyes and hexavalent chromium (Lind et al. 1990; Cadenas et al. 1992). Although, some metabolites generated from the DTD catalyzed reaction could be cytotoxic, this enzyme is also shown to have an antioxidant property. The antioxidant functions of DTD is attributed to its ability to maintain membrane bound Coenzyme Q (CoQ) in reduced antioxidant state and provide protection to membrane components against free radical damage (Beyer et al., 1996, Landi et al.; 1997). DTD belongs to the
family of phase II detoxification enzymes which includes GST and glutathione peroxidase along with other transferases and reductases (Nebert 1994) known for their function to divert potentially active electrophiles from damaging interactions with nucleophilic groups of DNA and in turn protect tissues against carcinogenic and mutagenic compounds (Talalay and Benson, 1982; Riley and Workman, 1992; Ross et al., 1993).

DTD activity is reported to increase with the activity of other antioxidant enzymes such as SOD, catalase and glutathione peroxidase (Agrawal et al. 2001a, Prestera et al. 1993; Whitney and Frank 1993).

Glutathione peroxidases catalyze the oxidation of GSH to GSSG at the expense of H$_2$O$_2$. In animal cells, glutathione peroxidases (GPx) are selenoenzymes, which catalyze the reduction of hydroperoxides at the expense of GSH (Flohe 1989; Ursini et al., 1995). In this process, hydrogen peroxide is reduced to water whereas organic hydroperoxides are reduced to alcohols.

An important feature of the antioxidant network is that its components act in synergy to destroy activated oxygen species. Such synergistic interactions are reinforced by mutual protections of antioxidant enzymes. For example, SODs protect Se-GPx and catalase from inactivation by superoxide (Kono and Fridovich, 1982; Shimizu et al., 1984; Blum and Fridovich, 1985), while Se-GPx and/or catalase protect SODs from inactivation by hydroperoxides (Sinet and Garber, 1981). In addition it seems that the regeneration of most, if not all, reducing cofactors involved is coupled to glutathione and/or NADPH metabolism.

The possible radioprotective ability of these antioxidant enzymes is further supported by the fact that their specific activity is increased upon irradiation (Agrawal et al. 2001b). The specific activities decrease at relatively high doses of radiation (beyond 3 Gy), which might be due to a direct damaging effect of radiation on the enzymes. However, apart from SOD, other antioxidants have not been tested by
exogenous addition. Therefore, although they are known important intracellular protectors, clinical use is limited by lack of knowledge of effects of exogenous administration.

2.2.3.3 Polymeric substances

A synthetic polymer prepared from N-vinylpyrrolidone and S-vinyl-(2,2-dimethylthiazolidyl)-N-monothiol carbamate (IX) are shown to have protective ability, which has been ascribed to liberation of thiol groups in vivo by opening of the thiazolidine ring (Overberger et al. 1965). Other copolymers containing thiol-liberating functions, such as isothiuronium salts, thiosulfates and dithiocarbamates, gave appreciable protection to mice when administered 24 to 48 h prior to irradiation (Barnes et al. 1975). Both poly (vinyl sulfate) (Kharitanovich et al. 1975) and heparin (Brueckner 1973), a sulfated mucopolysaccharide, increased survival rates, possibly by affecting deoxyribonuclease activity.

Other naturally occurring polymeric substances, such as glucan (B, 1-3 polyglucose) (Patchen et al. 1988) polysaccharides isolated from \textit{S. cerevisiae}, dextran sulfate (Ross and Peeks 1986) and bacterial endotoxins (Rehling 1983), lipopolysaccharides with molecular weights of approximately 1,000,000 have provided varying degrees of radiation protection in animals.

2.2.3.4 Cytokines

Cytokines have been found to protect cells from the damaging effects of ionizing radiation. Interleukin-1 and tumor necrosis factor-\(\alpha\) protect mice from the lethal doses of radiation, given before irradiation. At lower doses of radiation, hemopoetic growth factors, interleukins-1,4 and 6, tumor necrosis factor, interferon and leukemia inhibitory factor promote recovery when administered after radiation, possibly by initiating autocrine/paracrine recovery and repair pathways (Neta and Oppenheim 1991).
However, cytokines are shown to cause dose dependent side effects. In clinical trials, patients experienced flu like symptoms, including chills, fever, nausea, fatigue, decreased serum cholesterol. The maximum tolerated dose in cancer patients was determined to be relatively low. The effects of cytokines can depend on dose, schedule of exposure and type of normal tissue. More work at the molecular, cellular and whole animal level is needed before cytokines can be used to maximum advantage.

2.2.3.5 DNA binding ligands

Hoechst 33258 and Hoechst 33342 are two commercially available DNA ligands in current use as fluorescent dyes. They have been shown to be sequence-selective minor groove binders (Zimmer and Wahnert 1986). Radioprotection by Hoechst 33342, was first reported by Smith and Anderson (Smith and Anderson 1984). Studies suggest that this radioprotection might be mediated by reduction of transient radiation-induced species on DNA, by H-atom or electron transfer from the ligand. A potential important advantage of the DNA-binding radioprotectors compared with the classical aminothiol compounds is their limited penetration through cell layers. This may provide the opportunity for preferential protection of normal epithelia in some radiotherapy settings. For example, in topical applications to skin, high concentrations of the radioprotector will be delivered to the germinal layer of the skin, but penetration through to the dermis an systemic uptake (and hence delivery to the tumor) will be inefficient. However, on the other hand, the fact that these drugs are subject to P-glycoprotein pump activity may limit or even preclude radioprotection of some types of cells.

2.2.3.6 Calcium channel blockers

Calcium channel blockers (CCBs) or calcium antagonists are a diverse group of compounds with the general property of uncoupling calcium-mediated cellular processes by blocking the uptake of this ion through the calcium channels of the plasma membrane (Greenberg 1987). These drugs bind reversibly and stereospecifically and with high affinity to both membrane-bound and the purified
receptor complex. This characteristic distinguishes them from other drugs such as sodium nitroprusside, papaverine, hydralazine and diazoxide which interfere with the availability of calcium ions for their physiological functions by acting at sites other than the calcium channels.

CCBs are particularly effective on vascular smooth muscle and for this reason have found widespread use in the treatment of cardiovascular disease (Godfraind et al. 1986). They are well accepted in the prevention of ischaemia in patients with chronic stable angina, unstable angina, variant angina and silent ischaemia and in the treatment of hypertension. CCBs are potent vasodilators and have important effects on several vascular beds including the coronary, cerebral, mesenteric, renal and skeletal muscle beds. In various animal models, calcium overload initiates lesions of an arteriosclerotic character. Adequate treatment with calcium channel blockers prevents calcium overload and can thereby protect arteries and arterioles from functional disturbances and structural damage. In spontaneously hypertensive rats, specific calcium antagonists normalize blood pressure by reducing transmembrane calcium influx into vascular smooth muscle cells. Mechanisms underlying an anti-atherosclerotic effect may include attenuation of endothelial dysfunction, prevention of LDL peroxidation, stimulation of LDL receptor activity, inhibition of superoxide radical generation and inhibition of vascular smooth muscle cell growth. Calcium entry blockers are potentially protective in various tissue injuries. Since calcium overload may be a universal contributor to cell death, CCBs may be cytoprotective agents in general, and in tissue not commonly regarded as sites for CCB action. The majority of calcium antagonists used clinically belong to three distinct chemical classes based on their chemical structure and preferential site of activity.

**Phenylalkylamines**

Verapamil is an example of this category. These primarily target the cardiac conduction mechanism and coronary blood vessels, but have activity at the large systemic blood vessels at higher doses. They attenuate heart rate increases in response to stress. Binding site is located internally on the membrane and is facilitated by the
repetitive depolarization of atrioventricular and cardiac tissue, a phenomenon known as use dependence. Therefore these drugs are not highly selective.

**Dihydropyridines**

Nifedipine is a dihydropyridine CCB. They target the large systemic blood vessels and cause reflex increases in heart rate. Binding sites are located externally on the cell. Dihydropyridine binding is voltage dependent.

**Benzothiazepines**

Diltiazem, belongs to this class of calcium channel blockers. They have activity only on coronary vessels and attenuate heart rate increases in response to stress. Binding site is located in the cytosolic part of the membrane and is use-dependent and hence non-selective.

Membrane damage is a key event caused by lipid peroxidation of cell membrane through the action of oxygen derived free radicals generated upon irradiation. This damage may allow the entry of excess calcium into cells with subsequent biochemical and microanatomical cellular deregulation and cell death. Hence since calcium antagonists act on ion-conducting cell membrane channels, they might attenuate radiation-induced injury by inhibiting cellular calcium overload, subsequent to cell membrane damage caused by radiation-generated free radicals. Therefore, this class of drugs was included in a search for radioprotectors with a more favorable therapeutic index than that of aminothiol radioprotectors (Floersheim 1992).

In earlier work, CCBs such as nifedipine, diltiazem and nimodipine have been shown to enhance the survival of female C3H mice irradiated with lethal gamma radiation (Floersheim 1992). The survival was further enhanced when mice were treated with a combination of CCBs with other radioprotectors such as zinc aspartate, DMSO etc, indicating a synergistic effect of these compounds (Floershiem 1993). Calmodulin antagonists phenothiazines enhanced the antioxidant potential of
irradiated mice, indicating their importance in protection against radiation-induced oxidative stress (Chandra et al.). With combined-agent regimens, the therapeutic index of radioprotective protocols may be improved by increasing radioprotection and minimizing side effects because of presumably independent toxicities. Although these studies suggest effective radioprotection by CCBs, the mechanisms underlying the radioprotective action remain yet to be understood.

2.2.4 Diltiazem

Diltiazem hydrochloride is a benzothiazepine derivative calcium channel blocker with proven antianginal and antihypertensive capabilities. It interacts with the transmembrane segments IIIS6 and IVS6 in the alpha1 subunit of L-type Ca\(^{2+}\) channels. Studies suggest that diltiazem inhibits calcium ion influx across receptor-operated calcium channels and may also inhibit calcium ion release from intracellular structures (ref).

DTZ has been reported to protect mice against lethal damage induced by ionizing radiation (Floersheim 1992, 1993, Goel et al. 1996). It is also shown to prevent radiation-induced conditional taste aversion in rats (Mukherjee et al. 1997). At cellular level, protective action of DTZ was seen in terms of reduction of cytogenetic damage (Goel et al. 1996). DTZ does not adversely affect electrolytes or carbohydrates or lipid metabolism, and it may have beneficial effects on the heart and kidneys. DTZ reduces myocardial hypertrophy and exerts antianginal effects on the heart through coronary vasodilatation and reduction in the blood pressure double product. DTZ improves renal perfusion and attenuates protein urea. These effects may be helpful in limiting the progression of renal injury. Moreover, DTZ is known to be relatively more effective and less toxic than other calcium channel blockers (Floersheim 1992). As thiol radioprotectors are thought to act principally by scavenging radiation-induced free radicals by the formation of disulfides, diltiazem is likely to be an optimal protector. Importantly, DTZ is currently in use to treat cardiovascular disorders. Since the use of other known radioprotectors such as WR-2721 are limited by their toxicities, the fact that Diltiazem is a proven non-toxic drug, may be significant. However, for the wide acceptability at clinical level as a
Chemical structure of Diltiazem: a benzothiazepine calcium channel blocker
radioprotector, further work needs to be done particularly to understand its mode of action in attenuation of radiation damage.

In view of the above, the present study was undertaken to understand the mechanism of action of Diltiazem in irradiated murine splenocytes. An attempt has also been made to examine the ability of DTZ to act as a differential radioprotector. For this purpose, its effect on the clonogenic survival of some human tumor cell lines and normal untransformed cell lines was evaluated. The work is likely to be significant from the radiation therapy of cancer point of view.

2.3 Aims and Objectives

The interaction of Ca\(^{2+}\) ions with membranes is likely to bring about conformational changes in their structure and in turn influence the radiosensitivity of cell. Ca\(^{2+}\) is also likely to alter the radioresponse through its involvement in the radiation-induced signal transduction pathways. Therefore, the present work attempts to:

- study the effect of radiation dose on the specific activities of antioxidants like Superoxide Dismutase (SOD), DT-Diaphorase (DTD), Glutathione S-Transferase (GST), and levels of glutathione (GSH), membrane peroxidation in terms of TBARS, membrane fluidity, specific activity of lactate dehydrogenase (LDH), apoptosis, NF-κB DNA binding activity, and levels of NO\(^*\) in splenocytes of Swiss Albino mice.

- investigate the possible interaction between radiation induced membrane damage and Ca\(^{2+}\) in murine splenocytes. An attempt has also been made to examine the influence of Ca\(^{2+}\) on apoptosis, DNA binding activity of an oxidative stress responsive transcription factor NF-κB, nitric oxide, lactate dehydrogenase as well as antioxidant status in irradiated splenocytes.

- to understand the importance of calcium channels in radiomodulation. For this purpose, the modification of the radioresponse of splenocytes by a calcium channel blocker, Diltiazem (DTZ) and ionophore A23187 was examined, in terms of changes in membrane peroxidation, membrane fluidity, antioxidant potential, nucleosomal ladder formation for apoptosis and NF-κB DNA binding activity. In
addition, the specific activities of LDH and levels of NO\(^*\) were examined in irradiated splenocytes.

- determine whether DTZ has a differential protective ability on irradiated human normal and tumor cell lines using the colony formation assays and activation of Caspase-3, an important component of the apoptotic pathway.
- reveal some basic mechanistic aspects of radiation-induced damage, involvement of Ca\(^{2+}\) in radiosensitivity and the radioprotective potential of DTZ.
- show the close link between membrane damage and calcium related biochemical events in cell damage and death.