CHAPTER II

REVIEW OF RELATED LITERATURE

Dental radiation is one type of x-radiation used in dental clinic for diagnostic purpose. It is used not only intra-oral examinations but also extra-oral examinations. There are several techniques for dental radiography, of which periapical radiograph are commonly used because this technique provides several advantages superior to other imaging modalities as follows the least distortion, the highest resolution and the lowest radiation dose patient received (Vivek and Byron, 2000). According to United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) in 2000, doses in the order of 1–10 mGy are typically delivered with diagnostic x-ray exposures and represent the range of doses received by individuals per year due to environmental background radiation. Patient dose from dental radiograph is usually reported as the patient entrance dose which is defined as the absorbed dose (to air) measured at the end of the spacer/director 'cone' for a typical examination without backscatter from the patient. The third quartile patient entrance dose for an adult mandibular molar intra-oral radiograph is 3.9 mGy, while a standard adult panoramic radiograph is 66.7 mGy (Napier, 1999) (Table 1).

Table 1 Patient entrance dose distributions for intra-oral and panoramic x-ray sets.

X-ray sets in sample	Sample size	Mean dose (mGy)	Highest dose (mGy)	Lowest dose (mGy)	Third quartile dose (mGy)
All intra-oral sets	6,344	3.3	45.7	0.14	3.9
45 to 55 kV sets	2,175	5.0	45.7	0.64	5.9
60 to 70 kV sets	3,105	2.2	9.6	0.22	2.5
Sets using E speed film	1,577	2.6	20.5	0.14	3.0
45 to 55 kV sets using E speed film	471	4.1	20.5	0.64	5.1
60 to 70 kV sets using E speed film	839	1.8	7.7	0.22	2.1
All panoramic sets	387	57.4	328	1.7	66.7

Thus, dental radiation is classified as low dose radiation in a milligray range which the absorbed dose below 1 Gray. Although low-dose radiation has no immediately noticeable effects on human, there is great interest in its long-term biological effects, which may include cancer in exposed individuals and genetic defects in their progeny.

Biological effects of ionizing radiation

Biological effects of ionizing radiation may be divided into two categories: deterministic effects and stochastic effects. Deterministic effects are those effects in which the severity of response proportional to the dose. These effects occur in all people when the dose is large enough. Deterministic effects have a dose threshold

below which the response is not seen. Examples of deterministic effects include oral change after radiation therapy and radiation sickness after whole-body irradiation. In contrast, stochastic effects are those for which the probability of occurrence of the change, rather than its severity, is dose dependent. Stochastic effects are all-or-none: a person either has or does not have the condition. For example, radiation-induced cancer (White and Pharoah, 2004).

Radiation acts on living organism through direct and indirect effects (Fig. 2.1). When the energy of a photon or secondary electron ionized biological macromolecules such as the atom of DNA molecules, or some other cellular component critical to the survival of the cell, it is referred to as a direct effect. The other effect is indirect effect resulting from the action of radiation on water (water radiolysis). Ionizing radiation produces a variety of reactive oxygen species (ROS) from water. Each of these will avidly attack nearby material to make strange compounds and atomic fragments (free radicals) which can change the structure of DNA (Riley, 1994).

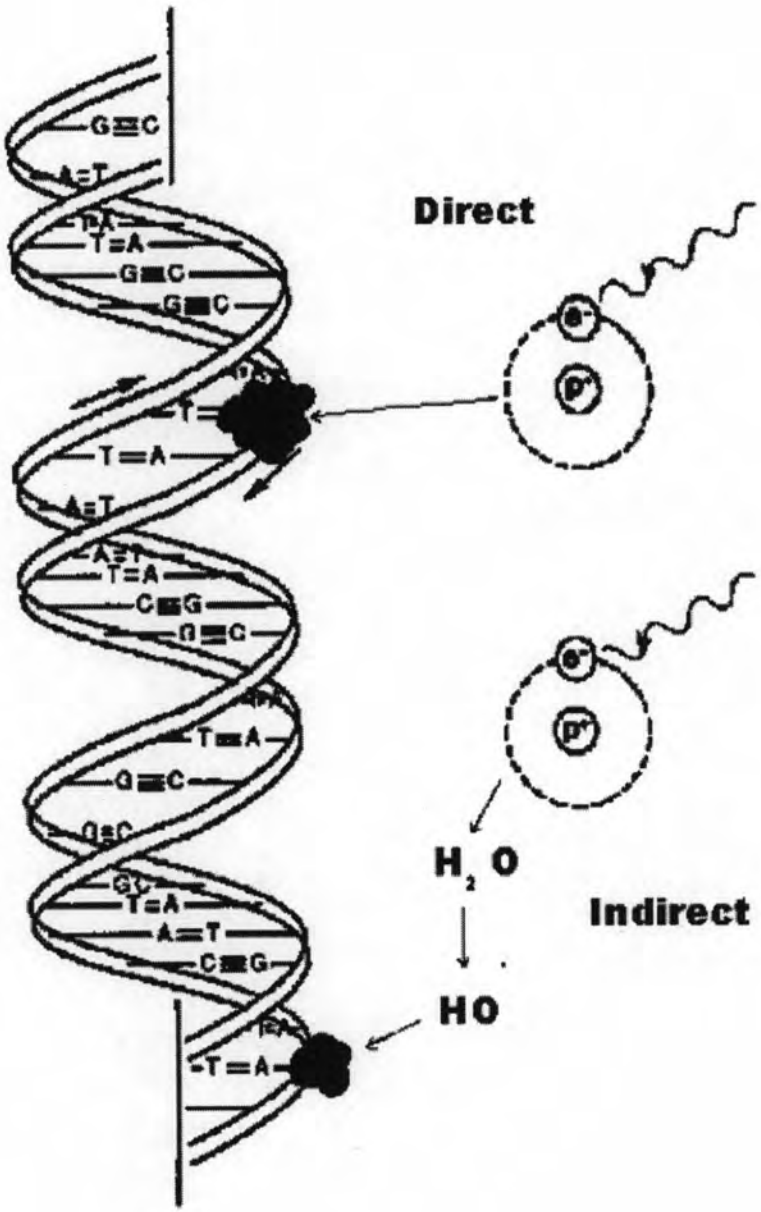

Figure2.1 Mechanisms of Strand Breakage Linked to Ionizing Radiation. Available from: http://www.chem.kuleuven.ac.be/safety/liab4.html

Therefore, exposure to ionizing radiation cause cellular damage primarily through a spectrum of DNA lesions (Rakhorst *et al.*, 2006) including single or double strand breaks, cross-linking of DNA strands within the helix, to other DNA strands, or to proteins, change or loss of a base and disruption of hydrogen bonds between DNA strands (Fig. 2.2). Among these, double-strand breakage represents the most lethal form of damage (Hoeijmakers, 2001b) especially when the damage occur at the same site of both strands or within a few base pair because accurate repair may be prevented and the linear continuity of mitotic chromosomes necessary for accurate transfer of genetic information to daughter cells is destroyed (Bonner, 2003)

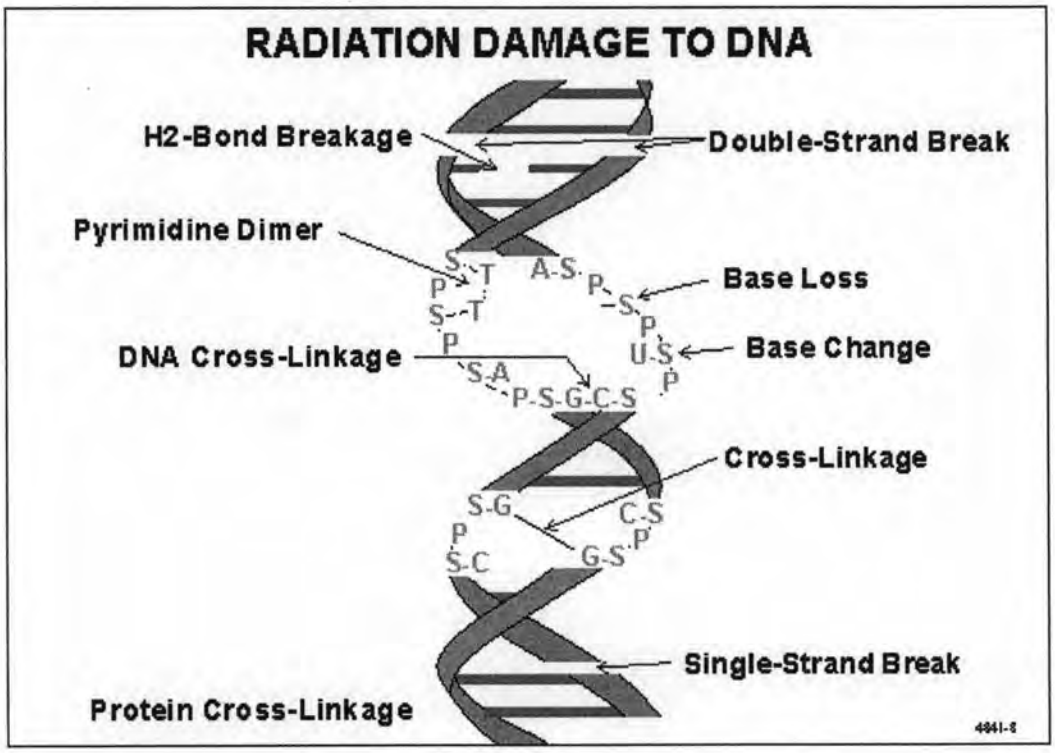

Figure 2.2 The various type of radiation-induced DNA damage. Available from: http://www.radiation-scott.org/radsource/3-0.htm

Currently, more sensitive assay to detect the DNA double-strand break (DSB) have been developed. This assay is based on the detection of histone proteins that package the DNA into chromatin, H2AX, becomes phosphorylated at the sites of nascent DSB (Rogakou *et al.*, 1998). Using a fluorescent antibody specific for the phosphorylated form of H2AX (γ-H2AX), discrete nuclear foci can be visualized at sites of DSBs, either induced by exogenous agents such as ionizing radiation (Rogakou *et al.*, 1999).

The previous study revealed that double-strand breaks can be detected in fibroblast cell culture after irradiation with radiation dose as low as 1 mGy and provide evidence of a linear relationship between DSB induction and dose between 1 mGy and 100 Gy by using immunofluorescent method to detect γ-H2AX foci (Fig. 2.3). This study illustrated expected numbers of DSBs were found at 3 min. Over 24 h the numbers decreased, they did not return to the pre-exposure average of 0.05 DSB per cell but stabilized at 0.1 DSB per cell for at least 14 days independent of dose. Thus 5% of the cells in the unirradiated cultures contained DSBs compared with 10% in the exposed cultures. However, when the quiescent cultures were exposed daily to 1.2 or 5 mGy for 10 days, the percentage of cells sustaining DSBs returned in each case to 10% within 24 h of the last irradiation. Thus only the first exposure increased the percentage of cells with DSBs after 24 h; subsequent exposures had no effect (Rothkamm and Lobrich, 2003).

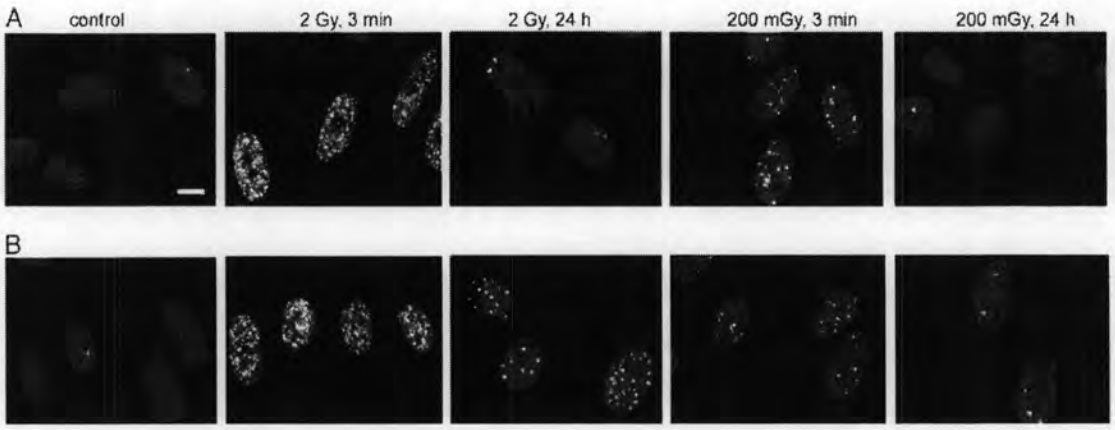

Figure 2.3 Illustration of DSB induction and repair after irradiation in various dose. Repair-proficient (MRC-5) and repair-deficient (180BR) primary human fibroblasts after irradiation in various dose. (*A*) γ-H2AX foci (white dot) in MRC-5 cells; nuclei were stained with 4,6 diamidino-2-phenylindole (gray); scale bar =10 nm. (*B*) γ-H2AX foci in 180BR cells (Rothkamm and Lobrich, 2003).

Factors influencing the effects of irradiation

Several factors affect the response of ionizing radiation as follows (Hall, 2000; Pizzarello and Witcofski, 1967; White and Pharoah, 2004)

1. The quality of radiation

The quality of radiation is determined by linear energy transfer (LET). Depending upon its energy, the high LET radiation are alpha rays (nuclei of the element e.g., helium), beta rays (electrons), protons (hydrogen with only one electron) and neutrons. While, the low LET include ultraviolet rays, x-rays and gamma rays. The higher-LET radiations are more efficient in damaging biological systems because

their high ionization density is more likely than x-rays to induced double strand breakage in DNA.

2. Dose of radiation

Radiation induced DNA damage and genomic instability increases with absorbed dose. The previous study revealed that double-strand breaks can be detected in fibroblast cell culture after irradiation with doses as low as 1 mGy and provide evidence of a linear relationship between DSB induction and dose between 1 mGy and 100 Gy.(Rothkamm and Lobrich, 2003).

3. Dose rate

The term 'dose rate' indicates the rate of exposure. For example, a total dose of 5 Gy may be given at a high dose rate (5 Gy/min) or a low dose rate (5 mGy/min). Giving a high dose rate exposure causes more damage than exposure to the same total given dose at a lower dose rates, a greater opportunity exists for repair of damage, thereby resulting in less net damage.

4. Oxygen pressure

The tension of oxygen in cells at the time of irradiation is an important determinant of the degree of severity of radiation damage. The greater cell damage sustained in the presence of oxygen is related to the increased amounts of hydrogen peroxide and hydroperoxyl free radicals formed due to water radiolysis. Both peroxyl radicals and hydrogen peroxide are oxidizing agents that can significantly alter

biologic molecules and cause cell destruction. They are considered to be major toxins produced in the tissues by ionizing radiation.

5. Cell radiosensitivity

Radiosensitivity is the relative susceptibility of cells, tissues, organs, organisms, or other substances to the injurious action of radiation. In general, it has been found that cell radiosensitivity is directly proportional to the rate of cell division and inversely proportional to the degree of cell differentiation. Cells are least sensitive when in the S phase, then the G_1 phase, then G_2 phase and the most sensitive in the M phase of the cell cycle. In 1906, the French biologist **Bergonie and Tribondeau** (Bergonie and Tribondeau, 1906) observed the most radiosensitive cells are those that 1) have a high mitotic rate, 2) undergo many future mitoses, and 3) are most primitive in differentiation. Therefore, mammalian cells are divided into five categories according to their radiosensitivity (Table 2).

Table 2 Classification of radiosensitivity and cell type

CELL TYPE	CHARACTERISTICS	EXAMPLES	RADIOSENSITIVITY
Vegetative intermitotic (VIM)	Rapidly dividing; undifferentiated; do not differentiate between divisions	Type A spermatogonia Erythroblasts. Crypt cells of intestines Basal cells of epidermis	Most radiosensitive
Differentiating intermitotic (DIN)	Actively dividing; more differentiated than VIMs: differentiate between divisions	Intermediate spermatogonia Myelocytes	Relatively radiosensitive
Multipotential connective tissue (MCT)	Irregularly dividing; more differentiated than VIMs or DIMs	Endothelial cells Mesenchymal cells Fibroblasts	Intermediate in radiosensitivity
Reverting Postmitotic (RPM)	Do not normally divide but retain capability of division: differentiated	Parenchymal cells of liver. Lymphocytes*	Relatively radioresistant
Fixed post mitotic (FPM)	Do not divide; differentiated	Some nerve cells Muscle cells Erythrocytes (RBCs) Spermatozoa	Most radioresistant

Thus, bone cells derived from mesenchymal stem cells are classified as intermediate in radiosensitivity. Apoptosis is the end point used to determine radiosensitivity of cell (Olive and Durand, 1997). The most recent study investigated

the effect of radiation between 2 and 16 Gy in multiple cell lines varying in clonogenic radiosensitivity and expression of specific genes. It was found that clonal radiosensitivity is genotype dependent and wildtype TP53 and mutated TP53 which are the template gene for tumor protein p53 associated with clonal radiosensitivity (Williams *et al.*, 2008).

6. The physical status of the irradiated organism

6.1 Age

In general, radiosensitivity decreases as age increases; older organisms are more radioresistant than young ones. This concept is supported by the most recent study that apoptotic cell death of lymphoid cells resulting from radiation-induced DNA damage is significantly reduced with increasing age.(Camplejohn *et al.*, 2006) Very old organisms become relatively more radiosensitive than younger ones. This, however, may be a reflection of loss of resistance to any form of insult (not merely radiation) on the part of the very old. During the period of rapid growth to maturity, most organisms will be more radiosensitive than those having adult status. Puberty is an especially sensitive period.

6.2 Sex

In mammals, females appear generally to be somewhat more resistant to radiation than males, but the differences are not great.

6.3 Body Weight

The heavier organisms are more resistant to radiation than are lighter ones of the same kind.

Cellular Responses to low dose radiation

Low dose radiation in the mGy range causes a dual effect on cellular DNA. One is a relatively low probability of DNA damage per energy deposition event and increases in proportion to the dose. The other effect at comparable doses is adaptive protection against DNA damage depending on cell type, species and metabolism. Adaptive protection causes DNA damage prevention and repair and immune stimulation. It develops with a delay of hours, may last for days to months, decreases steadily at doses above about 100 mGy to 200 mGy and is not observed any more after acute exposures of more than about 500 mGy (Feinendegen, 2005). These protective responses may be grouped into four categories:

1) Damage prevention by temporarily stimulated detoxification of free radical species from radiation effect. (Feinendegen, 2005) The process involves the increase level of antioxidant such as glutathione, superoxide dismutase (Kojima *et al.*, 2002) (Ayala *et al.*) with decreased lipid peroxidation lasting for weeks in some tissues after low dose irradiation. This response accompanied a change in enzyme activities such as of thymidine kinase in mouse bone marrow in vivo to some 70% of control. Moreover, ROS detoxification has been linked to gene activation such as transcriptional regulation of the gamma- glutamylcysteine synthetase gene, predominantly through the AP-1 binding site in its promoter (Feinendegen, 2005).

2) Damage repair by temporary stimulation of repair mechanisms. Low dose x-irradiation stimulated the reduction of chromosomal aberrations that occur in cultures of human lymphocytes following large challenging doses (Shadley *et al.*, 1987; Shadley and Wiencke, 1989). The degree of protection varied from zero to a

maximum with individual donors of these cells. Protection was seen when the challenging dose of 2 Gy was given between about 4 and 70 hours after the conditioning dose of 0.005 - 0.01 Gy. It was not seen when the conditioning dose of 0.01 Gy was given at the very low dose rate of 0.0064 Gy/minute, or when the conditioning dose exceeded 0.1 Gy (Shadley *et al.*, 1987; Shadley and Wolff, 1987), or when the challenging dose was 4 Gy instead of 2 Gy (Shadley and Wiencke, 1989).

3) Damage removal by induction of apoptosis (Norimura *et al.*, 1996). Apoptosis is cell death in response mainly to DNA alterations; it is triggered by intracellular signals and eliminates damaged cells from tissue. At low doses of x-radiation, removal of damaged cells outweighed the induction of tissue damage from lost cell. In one study, the incidence of this protective cell death in cultures of human lymphocytes rose up to day 4 after exposure to low LET radiation; it appeared linear with dose between about 0.1 and 2 Gy with a slope of 0.08 per 0.1 Gy (Menz, 1996). Damaged cells may be induced into apoptosis by intracellular and intercellular cellular signalling. Apoptosis may also occur within hours after high-dose irradiation. Low-dose induced apoptosis of pre-damaged cells with replacement by healthy cells may be a major route of in vivo removal of oncogenically transformed cells. Low-dose induced apoptosis is also assumed to operate through intercellular signalling from normal cells, which may also be activated by transformed cells in culture. Non-growing human fibroblasts in culture with DSBs from low-dose low-LET irradiation readily lost this damage to the level of DSBs in non-irradiated control cells after induction of proliferation; this damage removal was mainly due to apoptosis. Low-dose induced enhancement of DNA repair may be responsible for the observation in rat thymocytes, where the incidence of radiation-induced apoptosis first declined at

low doses and only rose with higher doses. The induction of apoptosis apparently requires a certain level of DNA damage (Feinendegen, 2005).

4) Damage removal by stimulated immune response (Anderson, 1992). Previous studies have demonstrated the induction of a hormetic effect in peripheral immune cells, such as lymphocytes, thymocytes and splenocytes after low-dose irradiation (Wang and Cai, 2000). The maximum response to acute whole-body gamma-irradiation was at doses between 0.1 - 0.3 Gy. This reponse improved surveillance of damaged cells over periods of weeks, and eliminated cancerous cells (Anderson, 1992). An improved immune response may result in an increased resistance to common infections and prolong life.

Models of low-dose radiation effect

According to Feinendegen (Feinendegen, 2005), low dose radiation causes both biopositive and bionegative effects on cells. Therefore, biological effect of low dose radiation have been debated and remains unclear. Bonner (Bonner, 2003) purposed several different models of these effects as follows

Linear no threshold model

National Council on Radiation Protection (NCRP) 1993 postulates that low-dose radiation is just as harmful per gray as high-dose radiation; thus any dose no matter how small is potentially harmful. Generating a wide margin of safety but

perhaps entailing unnecessarily large expenditures for radiation safety, the linear non-threshold model is subject to considerable discussion and controversy.

Threshold model

This model postulates that low-dose radiation is harmless below a certain level. Analysis of the epidemiological data, mainly of the life-span study of atomic-bomb survivors, seems to indicate in some cases a linearity between dose and risk at low doses but also cannot exclude a threshold at 60 mSv (Azzam *et al.*, 2001; Preston and Pierce, 1988). Moreover, using the parameters of cancer mortality rates or mean lifespan in humans, no scientifically acceptable study was found which showed that less than 10 cGy was harmful (Luckey, 2006).

Adaptive-response model

This model postulates that certain doses of low-dose radiation may even be beneficial. It is described as the reduced damaging effect of a challenging radiation dose when induced by a previous low priming dose. This model was first proposed in 1984 to explain the finding that cultures of human lymphocytes growing in low concentrations of radioactive thymidine developed fewer chromosomal aberrations than cultures of non-radioactive lymphocytes when both were challenged with high-dose radiation (Olivieri *et al.*, 1984)

Adaptive responses have been observed in vitro and in vivo using various indicators of cellular damage, such as cell lethality, chromosomal aberrations, mutation induction, radiosensitivity, and DNA repair. Adaptive response can be

divided into three successive biological phenomena, the intracellular response, the extracellular signal, and the maintenance. The intracellular response leading to adaptation of a single cell is a complex biological process including induction or suppression of gene groups. An extracellular signal, the nature of which is unknown, may be sent by the affected cell to neighbouring cells causing them to adapt as well. This occurs either by a release of diffusible signalling molecules or by gap-junction intercellular communication. Adaptive response can be maintained for periods ranging from of a few hours to several months (Tapio and Jacob, 2007). Typically, the adaptive response is induced with 1–100 mGy of γ-rays, doses 100–10,000 times larger than the natural background of ≈0.01 mSv/day. Other studies also seem to support this model (Ikushima *et al.*, 1996; Ikushima, 1999; Okazaki *et al.*, 2007; Shadley, 1994).

Hormesis model

Hormesis is the stimulation of any system by low doses of any agent. Large and small doses of most agents elicit opposite responses. A dose that elicits a response which separates positive from negative effects is the threshold dose; it is the "zero equivalent point" (ZEP) for that specific parameter. Low dose is any dose below ZEP. Dose rate is also important. Taking one pill per day may be life-saving; taking 365 of most pills in one day would be lethal (Luckey, 2006).

Radiation hormesis is the stimulation, often considered to be beneficial, from low doses of ionizing radiation (generally in the range of 1-50 cGy of low-LET radiation) (Macklis and Beresford, 1991). Luckey (Luckey, 1980) demonstrated that

low dose radiation could stimulate the metabolic activities, such as DNA and protein synthesis. Several recent studies revealed the induction of proliferation in various cell types after irradiation with low dose radiation (<1Gy) such as human lung fibroblasts, normal human diploid cells, Chinese hamster fibroblasts, neuron cells, hematopoietic cells (Kang *et al.*, 2006; Kim *et al.*, 2007; Korystov *et al.*, 1996; Wang and Cai, 2000; Watanabe *et al.*, 2002) which was determined as hormetic effect from low dose irradiation.

Although radiation hormesis and adaptive response have similarities in beneficial effect of low dose radiation, the difference between radiation hormesis and adaptive response model is that adaptive response will occur only when the adapted organism are irradiated by a relative large dose, but radiation hormesis dose not need large doses for its appearance.

Bystander effect model

This model postulates that low-dose radiation may be even more damaging than that predicted by the linear non-threshold model. These effects suggest that irradiated cells may signal their distress to other cells, perhaps by direct cell-to-cell interaction or by molecules secreted into the medium. The latter form of cellular communication is supported by findings showing that the bystander effect could be induced in non-irradiated cell cultures incubated with conditioned medium from irradiated cultures (Azzam *et al.*, 2001; Mothersill and Seymour, 2001; Seymour *et al.*, 1986) . One possible candidate for the signal is IL-8 (Narayanan *et al.*, 1999), a cytokine with important roles as a chemoattractant and activator of

polymorphonuclear leukocytes that is up-regulated and secreted in a variety of cell types during oxidative stress.

Apoptosis

Apoptosis is a fundamental biochemical cell death pathway essential for normal tissue homeostasis. The characteristics of the apoptotic cell include chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing, and cell shrinkage. Eventually, the cells breaks into small membrane-surrounded fragments (apoptotic bodies), which are cleared by phagocytosis without inciting an inflammatory response. The release of apoptotic bodies is what inspired the term "apoptosis" from the Greek, meaning "to fall away from" and conjuring notions of the falling of leaves in the autumn from deciduous trees (Kerr *et al.*, 1972). There are two types of apoptotic inducing pathway, the intrinsic pathway and the extrinsic pathway. The extrinsic pathway can be induced by members of the tumor necrotic factor receptor family, such as Fas and tumor necrosis factor-related apoptosis-inducing ligand receptors. The intrinsic pathway is induced by various apoptotic stimuli, including growth factor deprivation, hypoxia, anti-cancer drugs and radiation, and regulated by Bcl-2 family proteins which operately upstream of mitochondria (Reed, 2000).

Necrosis vs apoptosis

Apoptotic cell death can be distinguished from necrotic cell death. Necrotic cell death is a pathologic form of cell death resulting form acute cellular injury, which is typified by rapid cell swelling and lysis. On the other hand, apoptotic cell death is characterized by controlled autodigestion of the cell (Fig. 2.4). Activation of endogeneous proteases which is called caspases initiate their own apoptosis death (Dive *et al.*, 1992) The main morphological changes of a cell undergoing apoptosis consist of cell shrinkage, membrane blebbing, condensation of the nuclear chromatin, cleavage of chromosomal DNA at internucleosomal sites, and fragmentation of the nucleus. The consequence of the cell membrane blebbing is the release of the membrane-bound apoptotic bodies. Phosphatidylserine, which is normally located on the inner face of the plasma membrane, becomes exposed on the outer surface where provides a recognition signal for the uptake by phagocytes. Thus, apoptosis results in the rapid and efficient removal of cells. The radiation-induced cell death was different by the dose. In Molt-4 cells irradiated with very high dose radiation (> 100 Gy), radiation-induced cell death caused by necrosis. While irradiation with 2-30 Gy had features of both apoptosis and necrosis (Akagi *et al.*, 1993).

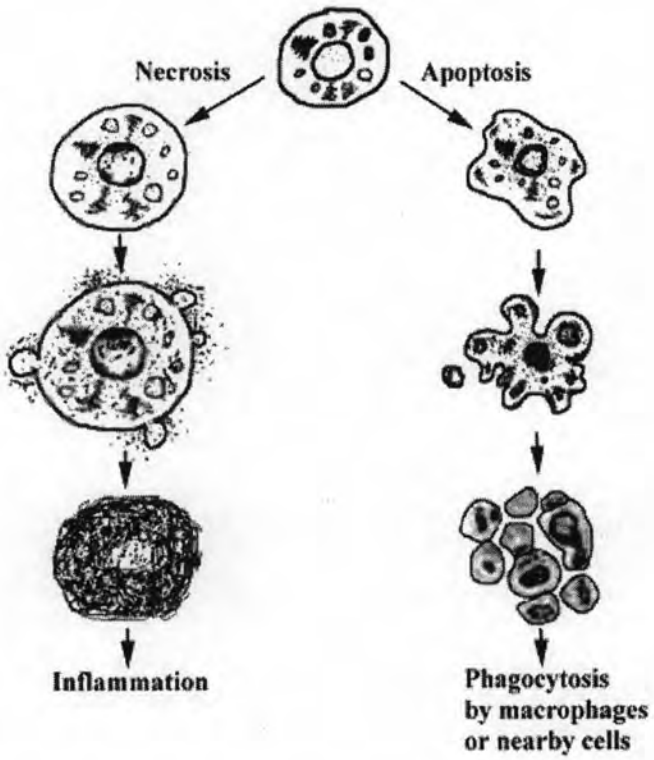

Figure 2.4 Two cell death pathways, necrosis and apoptosis. Necrosis involves breakdown of the cellular membrane, which leads to leakage of intracellular proteins to the extracellular space and subsequently, inflammation. Necrosis usually affects large groups of cells while apoptosis typically involves single cells that undergo organised destruction of the cellular cytoskeleton and formation of apoptotic bodies, which are phagocytosed without an inflammatory reaction.

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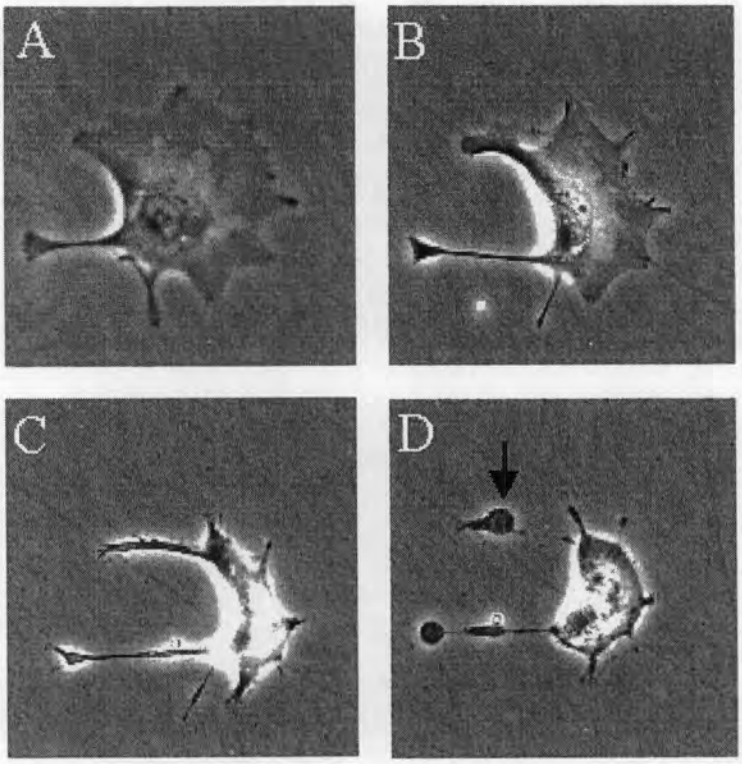

Figure 2.5 Illustration of apoptotic cell death. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton (A). The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance (B). Cells continue to shrink (C), packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often ungergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface. The end stages of apoptosis are often characterised by the appearance of membrane blebs (D) or blisters process. Small vesicles called apoptotic bodies are also sometimes observed (D,arrow). Available from: http://www.sgul.ac.uk/depts/immunology/~dash/apoptosis/

Role of Caspase-3 on apoptotic process

Caspases are the group of cysteine proteases crutially involved in both the initiation and the final execution of apoptosis (Datta *et al.*, 1997; Earnshaw *et al.*, 1999; Tepper and Seldin, 1999). Caspases are synthesized as inactive pro-enzymes and can themselves be activated by proteolytic cleavage at specific aspartate residues, generating large and small subunits to form the active heterotetramer. Caspases are classified into initiator and effector caspases. Initiator caspases (e.g. caspases 8, 9 and 10) act upstream in the protease hierarchy and activate effector caspases (e.g. caspase 3, 6 and 7), which are considered to be the executioners of the apoptotic program (Bartelink *et al.*, 2000). Some effector caspases cleave the nuclear laminin causing the irreversible breakdown of the nuclear laminin; the others cleave the protein holding a DNAse which cut up the DNA. Among effector caspases, caspase-3 is a frequently activated death protease. Pathway of caspase-3 activation can be initiated by two distinct mechanisms; one mediated by cell surface receptors such as Fas (CD95) or the members of tumor necrosis factor receptor (TNF-R) (Savitsky *et al.*, 1995) and the other by mitochondria-dependent activation (Fig. 2.6), via cytochrome c (cyt c) release from mitochondria (Green and Reed, 1998) Caspase-3 cleaves the inhibitor of caspase activated DNase (ICAD), leading to DNA degradation or fragmentation (Enari *et al.*, 1998).

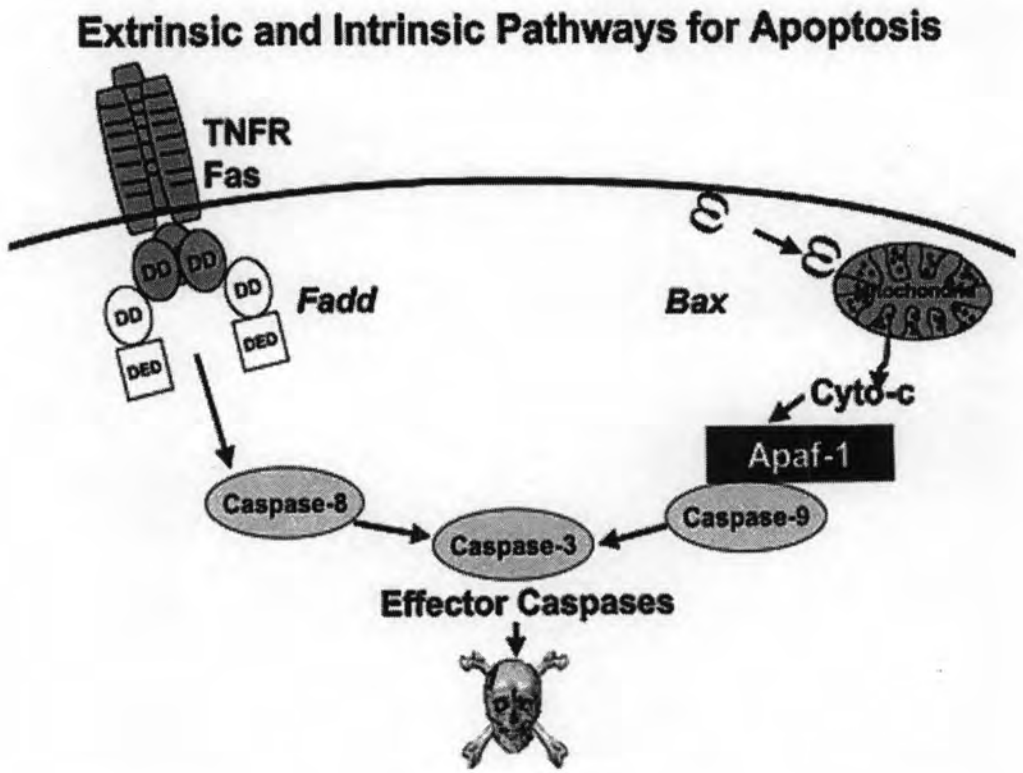

Figure 2.6 Pathways for caspase activation. Two of the major pathways for caspase activation in mammalian cells are presented, the extrinsic (left) and intrinsic (right) . The extrinsic pathway can be induced by members of the TNF family of cytokine receptors, such as TNFR1 and Fas. These proteins recruit adapter proteins to their cytosolic DDs, including Fadd, which then binds DED-containing pro-caspases, particularly pro-caspase-8. The intrinsic pathway can be induced by release of cytochrome c from mitochondria, induced by various stimuli, including elevations in the levels of pore-forming pro-apoptotic Bcl-2 family proteins such as Bax. In the cytosol, cytochrome c binds and activates Apaf-1, allowing it to bind and activate pro-caspase-9. Active caspase-9 (intrinsic) and caspase-8 (extrinsic) have been shown to directly cleave and activate the effector protease, caspase-3 (Reed, 2000)

Role of Bcl-2 family proteins on apoptosis

Although the caspases represent a central role in apoptosis, their activation is regulated by a variety of other factors. Among these, Bcl-2 family plays an important role in caspases activation by the determination of the propensity to mitochondrial membrane permeability (Burlacu, 2003; Smith *et al.*, 2008). The Bcl-2-regulated changes in mitochondrial membrane barrier function responsible for deciding sequestration *versus* release of cytochrome c dictate cell life-death decisions (clonigenic survival). Once mitochondrial membrane permeability has been induced, it cause the release of cytochrome c from mitochondria into the cytosol, this in turn leads to activation of caspase cleavage. In addition to cytochrome c, Bcl-2 family proteins have been reported to control the release of other proteins from mitochondria. These proteins include certain caspases (caspase-2, -3, and -9) which reportedly are sequestered inside mitochondria in some types of cells (Mancini *et al.*, 1998; Susin *et al.*, 1999). In such instances where caspases are inhibited, cell death occurs via necrosis but is nevertheless regulated by members of the Bcl-2 family (Reed, 2000).

The Bcl-2 family consists of both pro-apoptotic (Bax, Bak, Bcl-Xs and Bad) and anti-apoptotic (Bcl-2, Bcl-Xl, Mcl-1, Bcl-W and A1) members. Many Bcl-2 family proteins are constitutively localized to the membranes of mitochondria, whereas others are induced to target these organelles in response to specific stimuli. Anti-apoptotic members such as Bcl-2, Bcl-XL, and many other members of the Bcl-2 family have a hydrophobic stretch of amino acids near their C-termini (transmembrane domains) that anchors them in the outer mitochondrial membrane.

Some of these proteins also insert into endoplasmic reticulum and nuclear envelope, though their effects on cell death regulation in these compartments are poorly understood compared to those of mitochondria (Green and Reed, 1998). In contrast to Bcl-2, Bcl-XL, and many of the Bcl-2 family proteins, the pro-apoptotic proteins Bid, Bim, and BAD lack C-terminal transmembrane domains. The large majority of the pro-apoptotic proteins is localized to the cytosol, but following a death signal, it appears that they undergo a conformational change that enables them to target and integrate into mitochondria outer membrane and to function as pro-apoptotic proteins (Wolter et al. 1997; Hsu et al. 1997; Goping et al. 1998). Thus, anti-apoptotic proteins prevent the release of cytochrome c (Gross *et al.*, 1999) whereas pro-apoptotic members increase mitochondrial membrane permeability as a result cytochrome c is released from mitochondria (Chai *et al.*, 2008; Childs *et al.*, 2008; Kawakami *et al.*, 2008).

All Bcl-2 family members are characterized by containing at least one of four conserved Bcl-2 homology domains, designated BH1-BH4, which correspond to -helical segments (Adams and Cory, 1998). In general, the anti-apoptotic members show sequence conservation in all four domains, while all pro-apoptotic members contain only BH3 domain-an interacting domain that is both necessary and sufficient for the killing action without BH4 domain (Fig. 2.7). Thus, The BH3 domain is presumed as a critical death domain in the pro-apoptotic members (Puthalakath et al. 2002). Bcl-2 and its closest homologs promote cell survival, but two other factions promote apoptosis. The BH3-only proteins sense and relay stress signals, but commitment to apoptosis requires Bax or Bak. The BH3-only proteins appear to

activate Bax and Bak indirectly, by engaging and neutralizing their pro-survival relatives, which otherwise constrain Bax and Bak from permeabilizing mitochondria.

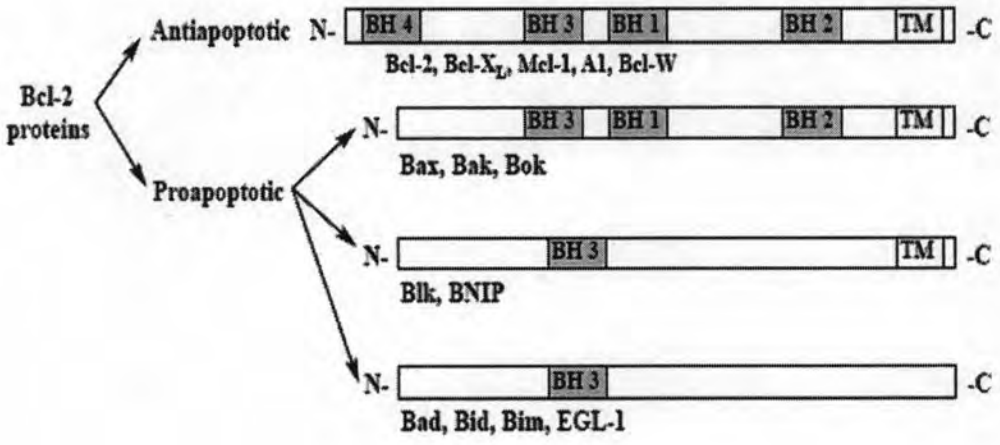

Figure 2.7 Schematic representation of the structural features of anti-apoptotic and pro-apoptotic Bcl-2 proteins. The structures of proteins are presented in linear forms, BH1-4 indicate the homologue domains and TM the carboxy-terminal hydrophobic transmembrane domain. The Bcl-2 proteins include: anti-apoptotic proteins (Bcl-2, Bcl-XL, Mcl-1, A1, Bcl-W) with all four BH and the TM domains, pro-apoptotic proteins (Bax, Bak, Bok) having TM, and BH1-3 domains but without BH4 domain, and pro-apoptotic ligands possesing only the BH3 domain with (Bik, BNIP) or without (Bad, Bid, Bim, EGL-1) TM. (Burlacu, 2003)

An important feature of the members of Bcl-2 family is their ability to form homo- as well as heterodimers, suggesting neutralizing competition between these proteins (Burlacu, 2003). Thus, the relative ratio of pro- and anti-apoptotic proteins determine the sensitivity or resistance of cells to a variety of apoptotic stimuli. Bcl-2 (a 239 amminoacids protein, 26 kDa) forms heterodimers with Bax, a proapoptotic protein with ~ 21% amminoacid identity with Bcl-2 (Adams and Cory, 1998). The increased ratio of Bax/Bcl2 in a given cell determines its susceptibility to apoptosis (Kaseta *et al.*, 2008; Oltvai *et al.*, 1993; Wiren *et al.*, 2006). It has been suggested that the bax/bcl-2 ratio may be more important than either promoter alone in determining apoptosis (Green and Reed, 1998; Stoetzer *et al.*, 1996). However, in the case of high expression of Bcl-XL protein, it has been report that the Bax/Bcl-2 ratio might not be used as apoptotic indicator (Lee *et al.*, 1999). The Bax/Bcl-2 ratio may up-regulate caspase-3 expression and modulate apoptosis (Salakou *et al.*, 2007) In addition to bax, several other genes have been reported to encode proteins having sequence homology with Bcl-2 and capacity to form heterodimers with it. Among these, bcl-X is able to generate two proteins through an alternative splicing mechanism: Bcl-XL (longer form) and Bcl-XS (shorter form). Bcl-XL is a 241 amminoacids protein that suppresses the cell death, having 43% sequence identity with Bcl-2, while Bcl-XS, in which a 63-aa region (aa 126-188) found in Bcl-XL is missing, functions as a proapoptotic protein (Adams and Cory, 1998). The BH3 domain of Bad protein is required for heterodimerization with Bcl-2 and Bcl-XL (Fig. 2.8). Bad strongly heterodimerized with Bcl-XL, less strongly to Bcl-2, and did not bind to other members of the Bcl-2 family (Yang *et al.*, 1995). When Bad heterodimerization with Bcl-XL may cause Bcl-XL to release Apaf-1 (apoptotic

protease activating factor), resulting in caspase 9-initiated cascade of proteolysis and induction of apoptosis (Gross *et al.*, 1999).The Bad mutants showed reduced binding with Bcl-2 and Bcl-XL (Lee *et al.*, 2004). Moreover, Bcl-xL, like Bcl-2, could also heterodimerize with the death-promoting molecule Bax (Chao and Korsmeyer, 1998).

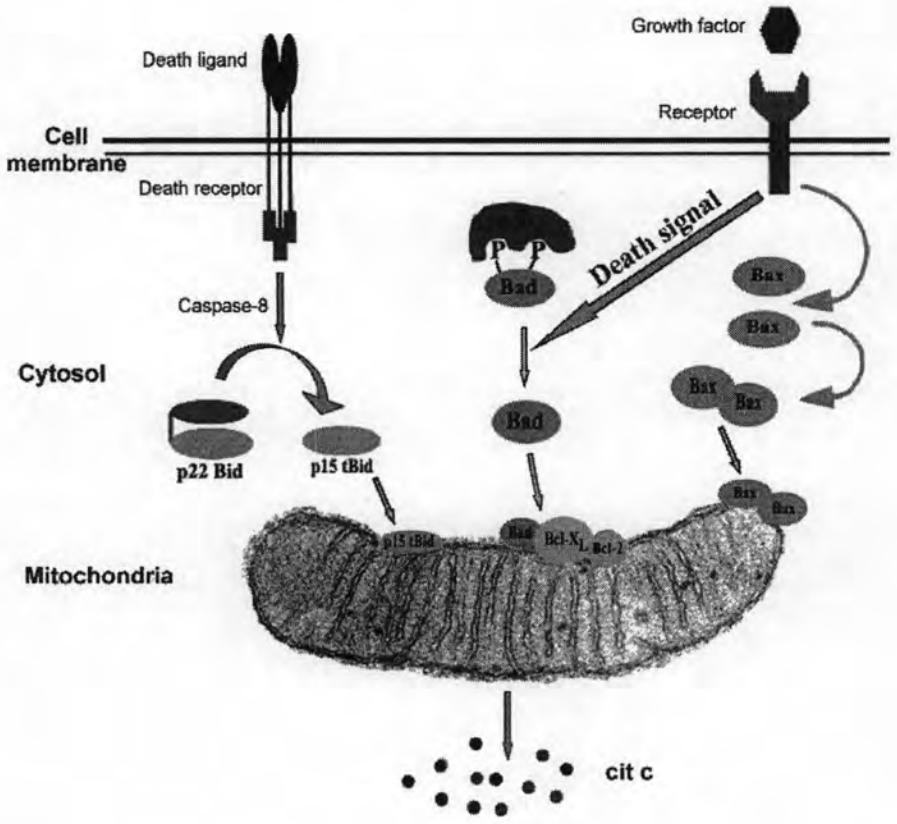

Figure 2.8 Schematic model of apoptosis signaling pathways involving pro-apoptotic Bcl-2 members. Activation of death receptors by their trimerization leads to cleavage of Bid by activated caspase-8 (not shown), generating a p15 tBid that translocates to the mitochondria. Growth factor deprivation induces Bax dimerization and translocation to the mitochondria membrane. The same kind of stimulus can inhibit the kinases that phosphorilate Bad and inactive it sequestered in the cytosol by a 14-3-3 molecule. This results in Bad dephosphorilation, its translocation in the

mitochondria and its association with Bcl-XL Bcl-2. Each of these proapoptotic proteins, once translocated in the mitochondria, can induce the release of cytochrome c, which will activates the caspase cascade (Burlacu, 2003).

Radiation-induced apoptosis

Ionizing radiation provokes a variety of cellular and DNA damages as a result cell death through apoptosis pathway (Borges *et al.*, 2008; Terato *et al.*, 2007). Changes in genes related to apoptosis, cell cycle, and DNA have been reported after irradiation (Amundson *et al.*, 1999). X-ray usually produce single- and double- strand DNA breaks and nucleotide modifications (Olive, 1998). Among the various types of DNA lesion, the DNA double-strand break (DSBs) is determined the most serious and potentially lethal type of cellular damage (Dugle *et al.*, 1976; Hoeijmakers, 2001a; Radford, 1985; Radford, 1986). DSBs are more likely to be lethal and mutagenic than other DNA lesions for two reasons: both broken DNA strands may have lost the same genetic information, preventing accurate repair, and the linear continuity of mitotic chromosomes necessary for accurate transfer of genetic information to daughter cells is destroyed (Bonner, 2003). DNA-damaged cells do not necessarily die immediately but may undergo several cycles of cell division before reaching a critical level of genomic instability (Seymour *et al.*, 1986). Moreover, kinetic patterns for apoptosis and redistribution show a common change at approximately 24 hours (Williams *et al.*, 2008).

The tumour suppressor protein p53 plays an important role in the cellular response to DNA damage (Fig. 2.9)(Csuka *et al.*, 1997). Radiation-induced p53 activation causes a delay in cell cycle progression, predominantly at the G1-S transition, allowing the damaged DNA to be repaired before replication and mitosis occur (Kastan *et al.*, 1991; Kastan *et al.*, 1995). Cell cycle arrest triggered by ionizing radiation occurs largely through the transactivation of p21 by p53 (el-Deiry *et al.*, 1993). After irradiation cells with high dose radiation (2 Gy), Accumulated p53 is activated as a transcription factor and stimulates transcription of a group of genes, including the p21 gene. In contrast, The phosphorylation of p53 and accumulation of p53 and p21 did not occur with low dose radiation (0.05 Gy) (Kim *et al.*, 2007). If repair fails, p53 may provoke the deletion of cells through apoptotic process. The involvement of p53 in radiation-induced apoptosis is demonstrated by several observations. Thymocytes lacking p53 fail to undergo radiation-induced apoptosis, whereas wild-type p53 thymocytes are extremely radiosensitive and die by apoptosis after doses as low as 1 Gy (Mayneord and Clarke, 1975). Furthermore, in p53-deficient mice, no significant apoptosis is observed in the gastrointestinal tract following irradiation, in contrast to p53-proficient mice (Merritt *et al.*, 1994). Finally, overexpression of wild-type p53 has been shown to induce a radiosensitive apoptotic phenotype in leukemic and colonic tumour cells (Shaw *et al.*, 1992). Whereas these studies provide support for an important role of p53 in mediating radiation-induced apoptosis, p53-independent mechanisms of apoptosis also exist. For example, radioresistant p53-deficient thymocytes retain the ability to undergo apoptosis after treatment with corticosteroids (Clarke *et al.*, 1993). Furthermore, in the absence of functional p53, certain cell types are still capable of dying by apoptosis after

irradiation (Strasser *et al.*, 1994). The mechanisms by which p53 mediates the apoptotic response following radiation-induced DNA damage relates to the ability of p53 to regulate the expression of pro- and anti-apoptotic members of the Bcl-2 family (Miyashita *et al.*, 1994; Miyashita and Reed, 1995). Bax, the pro-apoptotic protein, has been shown to contain p53-binding sites and is upregulated in response to DNA damage and increased p53 (Miyashita and Reed, 1995). By contrast, the antiapoptotic gene Bcl-2 is transcriptionally repressed by p53 (Miyashita *et al.*, 1994). Although the way in which these Bcl-2 family members govern apoptosis is not completely understood, direct regulation of mitochondrial activity has emerged as a major mechanism of action (Adams and Cory, 1998; Green and Reed, 1998). In certain cell types, DNA damaging agents, such as ionizing radiation, have been shown to induce the expression of death receptors and/or their ligands in a p53-dependent fashion (Kastan, 1997; Muller *et al.*, 1997), causing an autocrine or paracrine type of apoptosis. Finally, it should also be noted that other proteins may regulate p53 function. It has recently been shown that the product of the gene that is mutated in the human autosomal recessive disorder ataxia telangiectasia (ATM) normally contributes to radiation-induced stabilization of p53 and cell-cycle delay, placing ATM as a sensor for DNA damage upstream of p53 (Kastan, 1997; Kastan *et al.*, 1992). In addition to DNA-dependent protein kinase (DNA-PK), which plays an essential role in the repair of double-stranded breaks, may also regulate p53 function. Thus, radiation-induced DNA damage elicits a variety of cellular responses, including DNA repair, cell cycle arrest and apoptosis. The significance of p53 in radiation-induced apoptosis remains complex and depends on the existence of other pathways of cell cycle control and response to injury.

Moreover, a low level of p53 expression in bone cells after irradiation with high dose radiation could not activate osteoblast cell death.(Szymczyk *et al.*, 2004) The most recent study illustrated that p53 is one of the four specific genes associated with clonal radiosensitivity (Williams *et al.*, 2008).

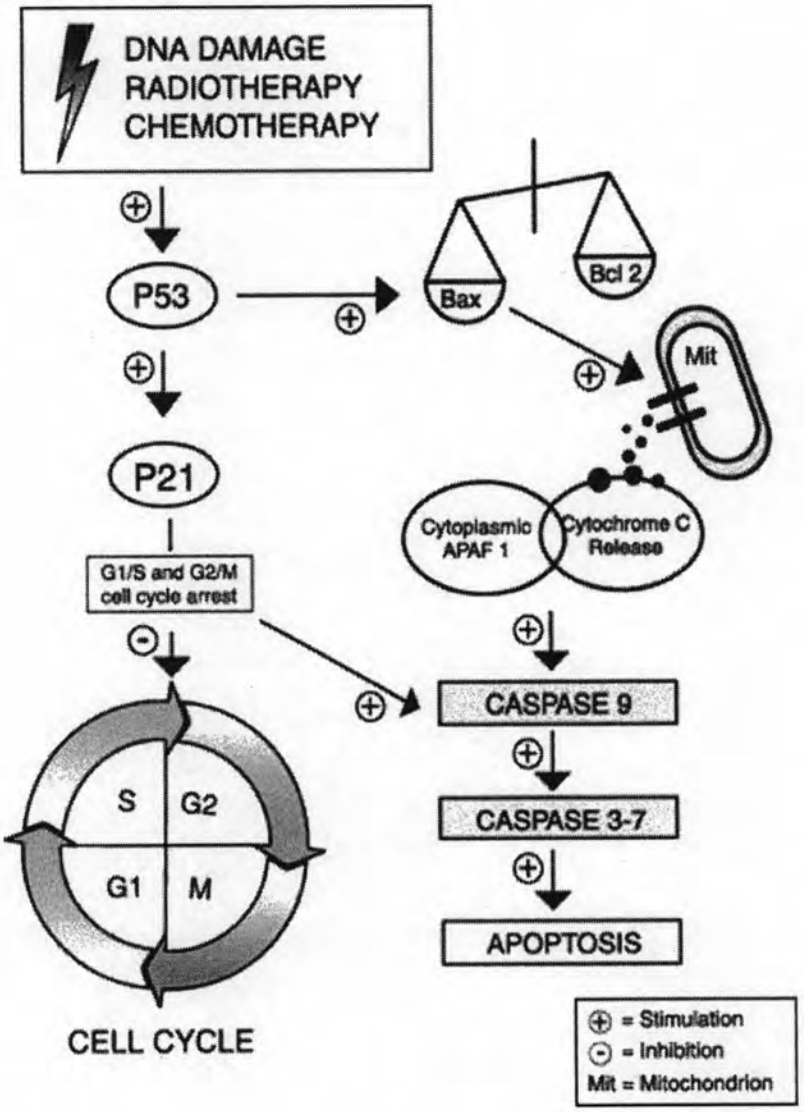

Figure 2.9 Scheme of the p53-dependent apoptosis pathway.

Available from: http://www.wjso.com/content/figures/1477-7819-5-97-2.jpg

Radiation effects on bone cells

Limited information is available on the effects of low doses of ionizing radiation on the healing and remodeling of bone tissue. Previous studies reported damage to immature bone and healing bone exposed to ionizing irradiation (2 to 5 Gy) but no significant effect on mature bone tissue (Jacobsson *et al.*, 1985a; Jacobsson *et al.*, 1985b). This observation suggested that irradiation affects immature more than mature osteoblasts, supporting the classic proposal that pre-osteoblasts are extremely radiosensitive (Tonna and Pavelec, 1970).

A number of studies revealed the effect of high dose radiation (>1 Gy) on several cell lines apoptosis (Akagi *et al.*, 1993; Park *et al.*, 1998; Zhivotovsky *et al.*, 1999). However, Szymczyk and associates (Szymczyk *et al.*, 2004) revealed that high dose radiation caused inhibition of bone cell proliferation rather than killing osteoblasts from the observation of the decrease of MTT activity without the increase of the number of TUNEL positive cells by TUNEL staining. Flow cytometric analysis indicated that the reduction of cell proliferation was caused by G2M cell cycle arrest. Linked to cell cycle arrest, there was an increase in the expression of p53.

A number of studies revealed the effects of high dose radiation alter bone cell behaviour such as the reduction of bone cell proliferation and cell synthetic activity as well as the increase of cell cytotoxicity, several markers of cellular apoptosis and cellular differentiation (Dare *et al.*, 1997; Gal *et al.*, 2000; Margulies *et al.*, 2006). There is no sciencetific study illustrate the effect of low dose radiation affect bone cells. However, dental irradiation is not recommended immediately after dental implantation because of the possibility of the detrimental effect of ionizing radiation on the healing and remodeling of bone (Branemark *et al.*, 1990). Moreover,

the recent study of Rothkamm and Lobrich (Rothkamm and Lobrich, 2003) showed that 1 mGy dose of radiation could induce DNA-double strand breaks of human fibroblasts which classified as intermediate radiosensitivity as osteoblast. Therefore, dental radiation in a milligray range might affect bone cell behaviour.