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Effects of hTERT on genomic instability caused by either metals or radiation or combined exposure

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Effects of hTERT on Genomic Instability Caused by either Metals or Radiation or Combined Exposure

Submitted by

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For the Degree of Philosophiae Doctor to the Dublin Institute of Technology

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Abstract

There is currently a great interest in delayed chromosomal damage and other damaging effects of low-dose exposure to a variety of agents, which appear collectively to act through induction of stress-response pathways related to oxidative stress (and ageing). These agents have been studied mostly in the radiation field but evidence is accumulating that chemicals, especially heavy metals, can also act in the same manner. Therefore, this work investigated the effects of metals and/or radiation in human fibroblasts *in vitro*.

Humans are exposed to **metals**, including chromium (Cr) (VI) and vanadium (V) (V), from the environment, industry and surgical implants. Thus, the impact of low-dose stress responses may be greater than expected from individual toxicity projections. In this study, a short (24 hours) exposure of human fibroblasts to low doses of Cr (VI) and V (V) caused both acute chromosome damage and genomic instability in the progeny of exposed cells for at least 30 days after exposure. Acutely, Cr (VI) caused chromatid breaks without aneuploidy while V (V) caused aneuploidy without chromatid breaks. The long-term genomic instability was similar but depended on hTERT positivity. In telomerase-negative hTERT- cells, Cr (VI) and V (V) caused a long lasting and transmissible induction of dicentric chromosomes, nucleoplasmic bridges, micronuclei and aneuploidy. There was also a long term and transmissible reduction of clonogenic survival, with an increased β-galactosidase staining and apoptosis. This instability was not present in telomerase positive hTERT+ cells. In contrast, in hTERT+ cells the metals caused a persistent induction of tetraploidy, which was not noted in hTERT- cells.

Interestingly, the clonogenic assay demonstrated that **radiation** induced genomic instability in hTERT+ cells, and to a lesser extent, in hTERT- cells. This showed that the telomerase activity

in hTERT+ cells did not provide protection against genomic instability caused by the radiation insult. Furthermore, neither 0.05 Gy nor 0.5 Gy doses of radiation induced chromosomal instability in either types of cells used (hTERT- and hTERT+ cells). However, hTERT+ cells had a slight higher incidence of micronuclei, immediately after radiation exposure of 0.5 Gy compared to hTERT- cells. Similarly to the metal only experiments, there was a higher level of tetraploidy in the hTERT+ cells compared to the hTERT- cells, although it only reached a level of statistical significance immediately after the radiation exposure of the 0.05 Gy dose. This finding was different to what was seen for the metal only treated [Cr (VI)] cells, where hTERT- cells showed significant cell damage and this damage was less compared to hTERT+ cells.

Combined exposure caused loss of clonogenic survival and therefore genomic instability in both types of cells (hTERT- and hTERT+ cells). This genomic instability was more pronounced in hTERT+ cells after *Metal Followed by Radiation*, and it was more pronounced in hTERT- cells after *Radiation Followed by Metal*. Similarly, cytogenetic damage was higher in hTERT+ cells after *Metal Followed by Radiation*, and higher in hTERT- cells after *Radiation Followed by Metal*. Similar to the metal only experiments, there was a higher level of tetraploidy in the hTERT+ cells compared to the hTERT- cells, although it did not reach a level of statistical significance. It appears that the biological effects provoked by combined exposure of metal and radiation has led to a synergistic action in both types of cells, compared to metal treatment only or radiation exposure only. In fact, in most of the significant results, the damage caused by the combination of metal and radiation was higher than the damage induced by either metal itself or radiation itself. Similarly to the radiation only experiments, it was interesting to observe that ectopic hTERT expression had no effect in preventing the loss of clonogenic survival, as well as the formation of cell damage after combined exposure. This was

in contrast to metal only treated [Cr (VI)] cells, where hTERT- cells showed cell damage, which was less compared to that observed in hTERT+ cells.

This study suggests that the type of genomic instability caused by metals in human cells may depend critically on whether they are telomerase-positive or –negative. However, the type of genomic instability caused by either radiation or combined exposure to metals and radiation in human cells appears to be not prevented by telomerase activity.

Declaration

I declare that the work presented in this thesis for the award of PhD is my own work, except for STELA Analysis, which was performed by Dr Duncan Baird (University of Cardiff), and Statistical Analysis, which was performed by Dr Roger Newson (King's College London).

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute or University.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Signature Antonino Glaviano Date 26/07/2007

Antonino Glaviano BSc, MSc

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Dedication

Alla Nonna Iolanda
e alla Zia Maria

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Abbreviations

AC	Number of Apoptotic Cells
BNC	Number of Binucleated Cells
Cr (VI)	Chromium (VI)
DMEM	Dulbecco's Modified Eagle's Medium
DSBs	Double Strand Breaks
ETA	Exchange Type of Aberration
FBS	Fetal Bovine Serum
HRR	Homologous Recombination Repair
hTERT	Human Telomerase Reverse Transcriptase
LET	Linear Energy Transfer
MC	Mean Counts
MNi	Micronuclei
MNC	Number of Mononucleated Cells
M + SI	Metal + Sham Irradiation
M + 0.05 Gy	Metal + 0.05 Gy
M + 0.5 Gy	Metal + 0.5 Gy
0.05 Gy + M	0.05 Gy + Metal
0.5 Gy + M	0.5 Gy + Metal
N	Total Number of Cells Scored
NC	Number of Necrotic Cells
NDI	Nuclear Division Index
NDCI	Nuclear Division Cytotoxicity Index
NHC	Number of Harvested Cells

NHEJ	Non-Homologous End-Joining
NPB	Nucleoplasmic Bridges
NPC	Number of Plated Cells
PBS	Phosphate Buffered Saline
PE	Plating Efficiency
RIBE	Radiation-Induced Bystander Effects
RIGI	Radiation-Induced Genomic Instability
RNC	Number of Tetranucleated Cells
ROS	Reactive Oxygen Species
SCE	Sister Chromatid Exchanges
TFI	Telomere Fluorescence Intensity
TNC	Number of Trinucleated Cells
TPG	Total Product Generated
TRAP	Telomeric Repeat Amplification Protocol
SI + M	Sham Irradiation + Metal
SSBs	Single Strand Breaks
STELA	Single Telomere Length Analysis
V (V)	Vanadium (V)
VC + 0.05 Gy	Vehicle Control + 0.05 Gy
VC + 0.5 Gy	Vehicle Control + 0.5 Gy
0.05 Gy + VC	0.05 Gy + Vehicle Control
0.5 Gy + VC	0.5 Gy + Vehicle Control

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CHAPTER 1

INTRODUCTION

1.1 Metals

1.2 Radiation

1.3 Combined Exposure

1.4 Genomic Instability

1.5 Telomeres and Telomerase

1.6 Aims

1.1 Metals

1.1.1 General Information

A large number of human beings are exposed to metals from industry, the environment and surgical implants. Potential sources of metal exposure include groundwater contamination, leather tanning, and mining (Steenland *et al* 1996, Mahata *et al* 2003). Their intensive use in ever growing numbers of industrial processes, as well as their wide natural distribution in the planet, results in the common occurrence of metals in the environment (IARC 1990, 1993, Ayres 1992). Metals are a class of agents, which are often present in the workplace (Goyer *et al* 1995, Butler and Howe 1999). Even though metals, such as iron, are required in small amounts for the normal function of living organisms, extensive exposure to certain metals has been linked to inflammation, cellular damage, and cancer, particularly of the lung and skin (Wang and Shi 2001, Desurmont 1983). Metals have been documented as an important emerging class of human carcinogens (Waalkes and Rehm 1994, Wang and Shi 2001). Metals are a diversified group of agents, but they share some general characteristics when considered as toxins or carcinogens (Wang and Shi 2001). Even though some metals are regarded as essential nutrients, they are potentially considered to be able to induce adverse reactions in biological systems (Goyer *et al* 1995). Metals are indestructible and have the potential for accumulation in the body leading to chronic effects, since they cannot be eliminated like the majority of organic chemicals from tissues by metabolic degradation (IARC 1990, 1993, Goyer *et al* 1995). Once metals reach the cell interior they will react with various cell constituents and they are likely to become integrated within the cells (Costa *et al* 1981, Goyer *et al* 1995). For example, this phenomenon occurs when cadmium binds to an intracellular protein called metallothionein (Friberg *et al* 1986, Simpkins 2000). It is known that for some metals, intracellular binding appears to provide a mechanism for detoxification (Goyer *et al* 1995).

However, it can also occur that the toxic effects of some non-essential metals such as lead, cadmium, and mercury, may be mediated or even enhanced by interactions or deficiencies of nutritionally essential metals (Goyer 1995). Some metals are able to react with various nucleophillic centres in the cell, such as nucleic acids and proteins, and this interaction could eventually lead to cytogenetic damage (Gunshin *et al* 1997, Simpkins 2000). There are metals that appear to be direct acting carcinogens, whereas others may act as promoters by influencing epigenetic mechanisms or by altering cellular susceptibility to other carcinogens (Kasprzak *et al* 1997). However, the molecular mechanism of metal carcinogenesis is not yet completely understood (Wang and Shi 2001).

1.1.2 Metal Toxicity

Metals have become a more important category of human carcinogens in the late 1800. Since then, arsenic was one of the first agents recognised as a human carcinogen (Hutchinson 1888). At least five metals, in one form or another, as human carcinogens, have been accepted by the International Agency for Research on Cancer (IARC). These comprise arsenic and arsenic compounds (IARC 1987), beryllium and beryllium compounds (IARC 1993), cadmium and cadmium compounds (IARC 1993), chromium and chromium compounds (IARC 1990) and nickel and nickel compounds (IARC 1990). Furthermore, iron has been considered to be a metal with carcinogenic properties (Park *et al* 2005). Several types of occupation involving possible exposure to metals are also considered to be human carcinogenic hazards (IARC 1987, Goyer 1995). Activities that potentially involve metal exposure and show clear evidence of a relationship with human cancer include iron and steel founding, as well as exposure to welding fumes (IARC 1987). Iron and steel founding are known to expose humans to various mixtures of potentially carcinogenic metals, while welding fumes often contain chromium and nickel, which are established carcinogens (IARC 1987). The incidence of local tumour at the site of an

implanted metallic prosthetic device may be considered a special case of metal carcinogenesis (Sunderman 1986). There have been several clinical cases in which tumours have clearly arisen at the site of a metallic orthopaedic device (Langkamer *et al* 1997). These tumours are often associated with the deterioration or corrosion of the implant (Case *et al* 1994). This occurs because devices, such as medical prosthesis, associated with these tumours are frequently made up of alloys containing chromium and occasionally nickel (Black and Hastings 1998). Some of the metal alloys used in orthopaedic surgery, such as nickel, chromium and cobalt have been documented to be carcinogenic (Goyer 1995, Wang and Shi 2001). Unfortunately, there are still several metals where the carcinogenic potential is not known (Friberg *et al* 1986, Goyer *et al* 1995), among which there is vanadium (V) (NIOSH, 1997).

1.1.3 Evaluation of Metal Toxicity

There have been several problems trying to evaluate the carcinogenic effects of a single toxic agent, such as a metal.

1. Human exposures to a single toxic metal either in the environment or in other contexts are very uncommon (Butler and Howe 1999).

2. The carcinogenic effects and results of a particular metal, when present in the environment with other agents and therefore with toxicological consequences of multiple exposures, could be due to reductive, additive or synergistic effects (Waalks *et al* 1992).

3. The life-long exposure of an individual to various compounds and the interactions between different agents undoubtedly complicates the assessment of carcinogenic potential of a single metal (Waalks *et al* 1992, Black 1999).

4. When data on a metal carcinogen are from either high-level exposures in humans or single high-dose studies in animals it would be difficult to try to assess the carcinogenic potential of that particular metal (Butler and Howe 1999). Usually, chronic low-level exposures are

predominant occurring in both the workplace and in the environment (Nordberg 1989). Therefore, the assessment of single-dose carcinogenesis would fail to see (if there are) any physiological adaptive responses, continuous low-level accumulative effects and the definitive elimination of threshold effects (Waalks *et al* 1992).

Normally, the most direct connection between metal exposure and effect is determined at the cellular level. Therefore, *in vitro* techniques can provide an insight into the specific interactions between metals and cells, providing possible mechanisms of action (Kjellstrom *et al* 1984).

1.1.4 Chromium (VI) Toxicity

Concerns have been raised about Cr (VI) toxicity. Several compounds containing chromium (VI) are recognised occupational human lung carcinogens and are likely to cause environmental health risk. Several hundred thousand workers are potentially exposed to Chromium (VI) from industry alone (WHO 2001, O'Brien *et al* 2003). The International Agency for Research on Cancer (IARC) classifies Cr (VI)-containing compounds as Group 1 human carcinogens and the limit of exposure has been set to 0.5 mg/m^3 (IARC, 1990). The genotoxicities of Cr (VI) are complex both in terms of the lesions that are induced and of the cellular response that they induce (Cheng *et al* 2003, Koizumi and Yamada 2003). In the short term Cr (VI) causes both clastogenic and aneugenic damage (Sugden and Stearns 2000, Seone and Dulout 2001).

Soluble chromium (VI) oxyanions in the immediate cellular microenvironment traverse the cell membrane, by non-specific anion transporters, and are reductively metabolised within the cell by some agents including ascorbic acid, glutathione and cysteine (Quievryn *et al* 2003). During chromium (VI) reduction, a diverse range of genetic lesions can be generated including chromium-DNA binary adducts, chromium-DNA ternary adducts, chromium-DNA protein crosslink, bi-functional DNA interstrand crosslink adducts (DNA-Cr-DNA interstrand

crosslink), DNA single-strand breaks, DNA double-strand breaks and oxidised bases (O'Brien *et al* 2003), Cr-DNA base/phosphate adducts and abasic sites (O'Brien *et al* 2003, Ha *et al* 2004). It has been observed that chromium-induced bi-functional DNA interstrand crosslink adducts present physical barriers to DNA replication or transcription and, thus likely promote a terminal cell fate such as apoptosis or terminal growth arrest (O'Brien *et al* 2003). Furthermore, chromium (VI) exposure can provoke a classical DNA damage response within cells including activation of the p53 signaling pathway and cell cycle arrest of apoptosis. Moreover, chromium (VI) also induces the ATM-dependent DNA damage response pathway, which is required for both apoptosis and survival after the chromium (VI) insult (O'Brien *et al* 2003). Consistent with their ability to generate DNA strand breaks, chromium (VI)-containing compounds are well-documented clastogens (Bagchi *et al* 2002). In most cases, damaged metaphases have been observed for both water-soluble and insoluble chromates (Wise *et al* 2002). Evidence also indicates that chromosomal abnormalities and genomic instability are possibly involved in the induction of human lung cancer by chromium (VI) (Hirose *et al* 2002). The production of single strand breaks by chromium (VI) reduction, either directly as a consequence of chromium-DNA interactions or as a result of oxygen/carbon radical generation or by the replication post/repair of DNA lesions, represents one of the most commonly reported lesions arising from chromium (VI) treatment. Chromosomal aberrations such as single chromatid gaps, breaks, interchanges, dicentric chromosomes, isochromatid breaks, chromosome/chromatid rings and sister-chromatid exchanges have been observed in chinese hamster cells treated *in vitro* with chromium compounds ($K_2Cr_2O_7$ and Na_2CrO_7) (Majone *et al* 1979). It has been shown that potassium dichromate ($K_2Cr_2O_7$) (Figure 1.1 and Figure 1.2) dissolved in water increases the frequency of micronuclei in the crayfish Procambarus clarkii (De La Sienra *et al* 2003). Significant increase in mean comet tail length, indicating DNA damage, has been documented with potassium dichromate in mice using alkaline single-cell gel

electrophoresis (SCGE)/comet assay (Dana Devi *et al* 2001). Several other forms of chromate can induce a variety of genetic effects including DNA damage, gene mutation, sister chromatid exchanges, chromosomal aberrations, cell transformation and dominant lethal mutations in a number of targets including animal cells *in vivo* and human cells *in vitro* (IARC 1990). Furthermore, Benova *et al* (2002) showed that Cr (VI) appears to have aneugenic effects, as well as clastogenic, in humans. Other authors, working with lead chromate, have recently suggested that one possible mechanism for lead chromate-induced carcinogenesis is through centrosome dysfunction, leading to the induction of aneuploidy (Holmes *et al* 2006).

Figure 1.1. Potassium Dichromate (molecular formula $K_2Cr_2O_7$) has a red-orange crystalline solid appearance. As with other Cr (VI) products, potassium dichromate is carcinogenic. It is a powerful oxidizing agent and is the preferred compound for cleaning laboratory glassware of any possible organics (http://upload.wikimedia.org/wikipedia/commons/2/23/Potassium_dichromate.jpg).

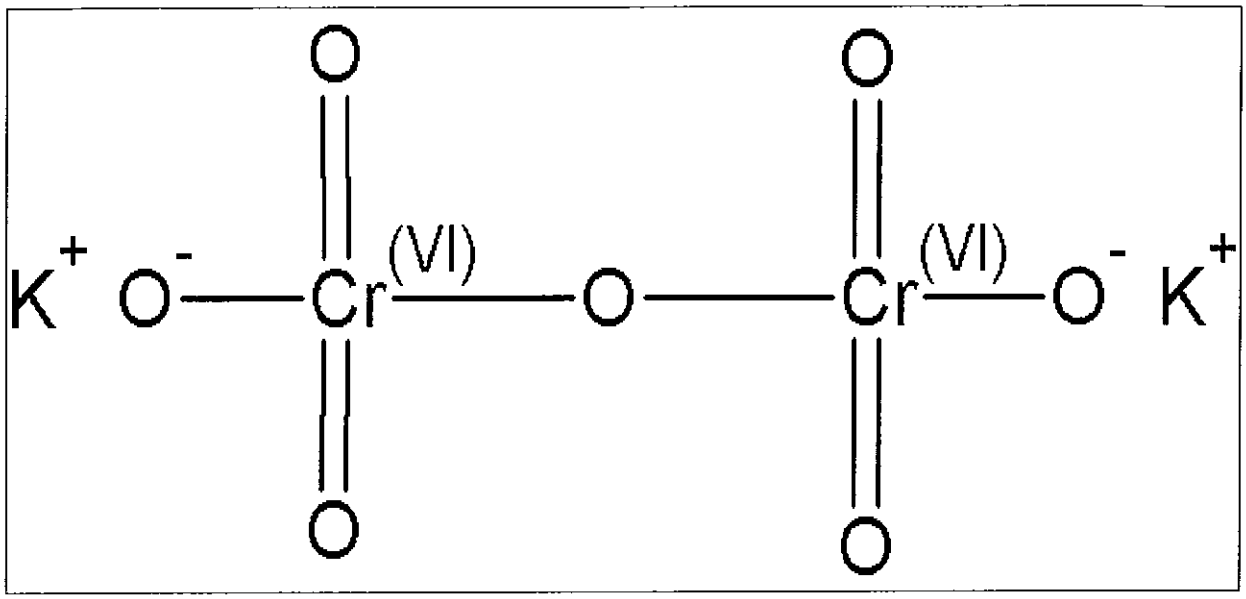

Figure 1.2. Structural formula of Potassium Dichromate (molecular formula $K_2Cr_2O_7$)

1.1.5 Vanadium (V) Toxicity

Vanadium is a transition metal that is emitted into the atmosphere during the combustion of fossil fuels (Hope 1994). Vanadium (V) has been shown to be toxic, but its carcinogenic potential has not yet been fully explored. However, owing to respiratory tract toxicity the permissible exposure limit set by the Occupational Safety and Health Administration (OSHA) is 0.5 mg/m^3 (NIOSH 1997). In the environment vanadium occurs in the (V) oxidation state, but in the body it is found exclusively in the (IV) oxidized form (Redher 1995). Vanadium tetraoxide is an inorganic chemical species in (IV) oxidized form that has been shown to induce toxic effects *in vitro* and *in vivo* models (Aragón and Altamirano-Lozano 2001). Aragón and Altamirano-Lozano have shown that sperm and testicular modifications are induced by subchronic treatments with vanadium (IV) in CD-1 mice. The toxic effects of vanadium in the reproductive system are of concern because they are involved with systems related to the formation of gametes, and such effects can affect the next generation (Aragón and Altamirano-Lozano 2001, Domingo *et al* 1986). Aragón *et al* (2005) have demonstrated that vanadium can induce ultrastructural changes and apoptosis in male germ cells. In their study the reproductive

toxicity of vanadium in males was analysed through monitoring germ cell apoptosis during spermatogenesis. They analyzed ultrastructural damage after vanadium tetraoxide administration to male mice for two months. Spermatogenesis stages I–III and X–XII often showed apoptotic germ cells in control and treated animals and vanadium tetraoxide treatment caused an increase in the number of germ cell apoptosis in stages I–III and XII (Aragón *et al* 2005).

Some compounds containing vanadium (V) are recognised to cause environmental health risk. Inhalation of dust containing vanadium pentoxide (V_2O_5) (Figure 1.3 and Figure 1.4) has been reported to cause lung diseases. However, information related to the genotoxicity and potential carcinogenicity of vanadium pentoxide is still limited (Zhong *et al* 1994). It has been observed in chinese hamster V79 cells *in vitro* that vanadium pentoxide induces cytotoxic damage and aneuploidy. Since vanadium pentoxide-induced micronucleated cells contained kinetochore-positive micronuclei, their induction appears to be due to damage to the spindle apparatus (Zhong *et al* 1994). Ciranni *et al* (1995) have also demonstrated the genotoxicity of vanadium in *vivo*. Vanadyl sulfate (SVO_5), sodium orthovanadate (Na_3VO_4) and ammonium metavanadate (NH_4VO_3) were found to cause micronuclei formation in bone marrow of mice following intragastric treatment. Furthermore, these three compounds caused an increasing number of chromosomal aberrations, which support the finding of a significant presence of types of micronuclei that are probably aneuploidy-related. The frequency of hypodiploid and hyperdiploid cells was also higher than the control value (Ciranni *et al* 1995). Vanadium (V)-compounds such as vanadate has been shown to increase DNA single strand bleaks in cultured human fibroblasts in a dose-dependent manner (Ivancsits *et al* 2002). Vanadium (V) was found to increase the frequency of sister chromatid exchanges, DNA single strand breaks and DNA-protein crosslinks (Ivancsits *et al* 2002). Vanadium (V) also interferes with microtubule assembly and spindle formation to create aneuploidy (Ramirez *et al* 1997). Roldan and

Altamirano (1990) observed that vanadium pentoxide significantly increased the frequency of polyploid cells. Furthermore, the average generation time, the frequency of cells with satellite associations and the frequency of chromosome association increased in treated cultures (Roldan and Altamirano 1990).

Figure 1.3. Vanadium Pentoxide (molecular formula V_2O_5), has a orange-yellow crystalline solid appearance. It is extremely toxic if inhaled, and dangerous for the environment. It is used as a catalyst, dye and color-fixer. Because of its high oxidation state, is both an amphoteric oxide and an oxidising agent (http://upload.wikimedia.org/wikipedia/commons/e/eb/Vanadium%28V%29_oxide.jpg).

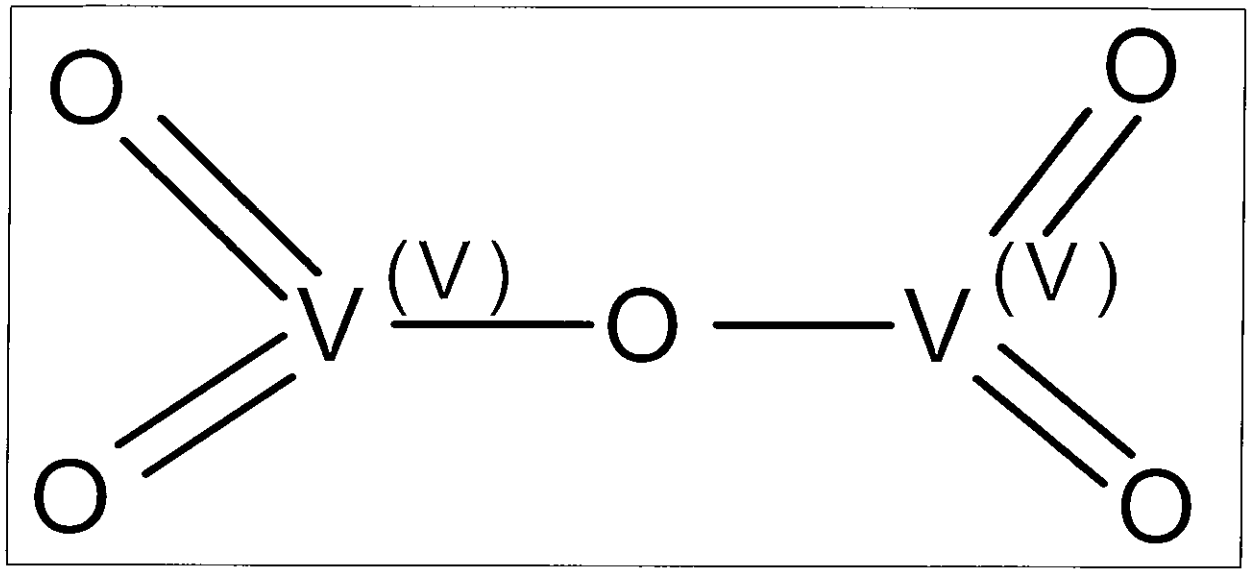

Figure 1.4. Structural formula of Vanadium Pentoxide (molecular formula V_2O_5).

1.1.6 Metal Toxicity in Surgical Implants

Orthopaedic surgery, through the utilization of joint replacement, has alleviated the suffering of millions of patients who would otherwise be crippled by rheumatoid arthritis or osteoarthritis (Figure 1.5). Whatever are the uncertainties about the effect of this procedure, the benefits are very real. The average life span of an implant is only usually 15 years and about 10 percent of implanted prostheses require revision within 10 years (Sargeant and Goswami 2006). This is a remarkable performance record considering the harsh biomechanical and biochemical environment of the body (Breen and Stoker 1993). This short life span has created several problems for the growing number of people receiving implants, and for this reason, this procedure is being extended to younger more active patients, where a service life of 30 years or more is desirable for the implant (Black and Hastings 1998, Black 1999). The need for a more durable prosthesis to meet physical requirements is necessary but also the question of long-term effects needs to be addressed.

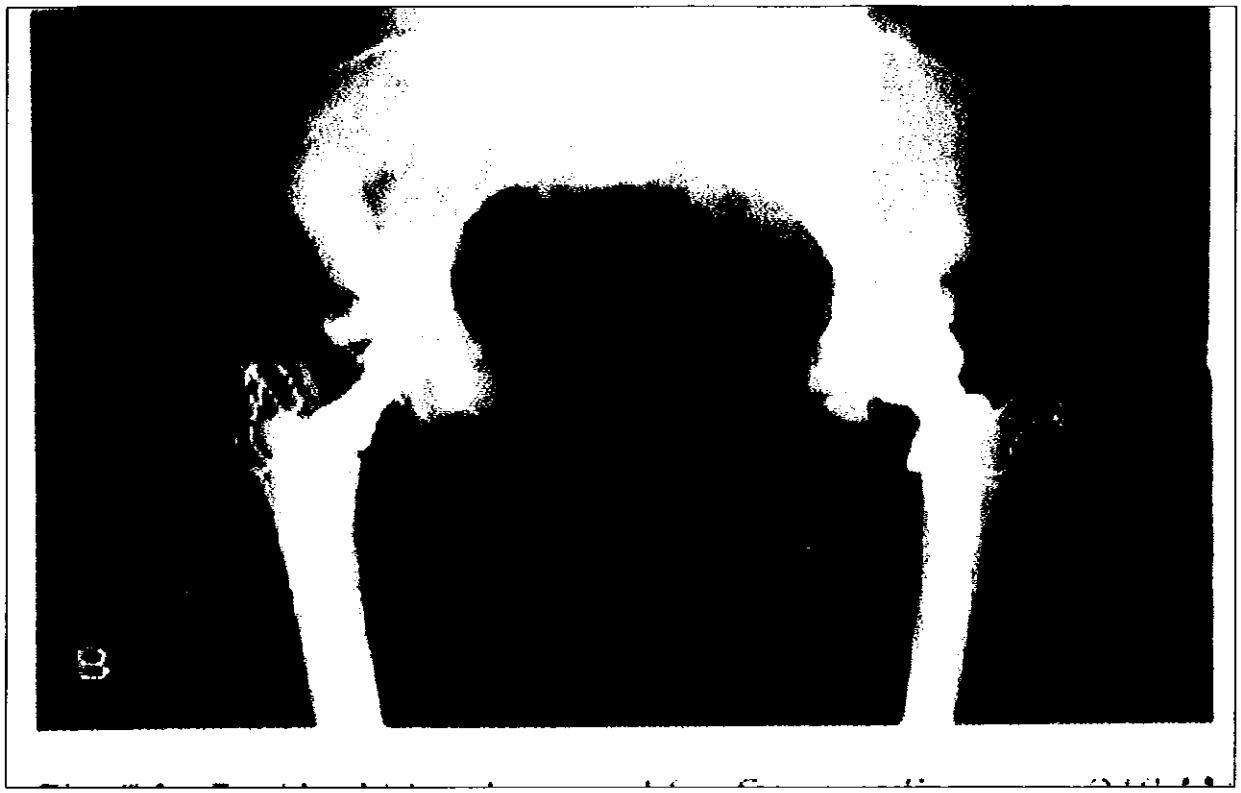

Figure 1.5. Hip replacement in orthopaedic surgery.

1.1.6.1 Biofunctionality and Biocompatibility of the Metal Prostheses

The material used in the prosthesis plays an important role in the success of the implant. The biofunctionality and biocompatibility of the material determine the success of any material, which is introduced in the body (Gotman 1997). The biofunctionality can be easily established by analyzing its physical and mechanical characteristics, while biocompatibility is difficult to determine, because of the many substances that interact with the material in the body. The material therefore determines the biocompatibility, rigidity, corrosion characteristics and tissue receptivity of the implant (Simon and Fabry 1991). Biocompatibility refers to the totality of the interfacial reactions between biomaterial and tissues and to their consequences (Black 1999).

1.1.6.2 Metal Composition of the Prostheses

The most common biomaterials being used for prosthesis are drawn from the stainless steels, the cobalt-based alloys and from the titanium/titanium-base alloy system (Black 1999). In total hip replacements, cobalt based alloys have been used successfully, as they have shown adequate strength and corrosion resistance for total joint replacement applications (Merritt and Brown 1996, Black 1999). The major cobalt-chromium alloys in orthopaedic use are cast cobalt-chromium-molybdenum with a nominal composition of 27%-30% chromium, 1% nickel, 5%-7% molybdenum and 59%-60% cobalt (Merritt and Brown 1996). Other types of cobalt alloys contain at least 11% nickel in their alloy composition (Merritt and Brown 1996). Titanium alloys comprise 90% titanium, 6% aluminium and 4% vanadium (Breen and Stoker 1993). Titanium is widely used for joint prosthesis because of its superior mechanical properties and biocompatibility (Breen and Stoker 1993, Black 1999). The inertness of titanium alloys is greatly enhanced by the "*in vivo*" formation of a stable oxide layer (Hughes Wassell and Embery 1996). However, micromotion at the cement-prosthesis and cement-bone interfaces may result in titanium oxide and titanium alloy particles (Breen and Stoker 1993). Titanium is generally considered to belong to a group of metals with low carcinogenicity (Friberg *et al* 1986). The toxicity of titanium essentially deals with the local effects of orthopaedic implants (Takamura *et al* 1994). Some of the metals alloys such as nickel, chromium and cobalt have been documented to be carcinogenic (Goyer 1995, Wang and Shi 2001). Many types of implant models have been produced using different combinations of articulating surfaces. Metal-on-metal implants have become popular since 2002, when they were first introduced, because they have a very slow wear rate. Usually, the anatomic medullary locking stem is made of cobalt–chromium and the socket is made of titanium alloy (Sargeant and Goswami 2006).

1.1.6.3 Corrosion of the Metal Prostheses

Corrosion of the metal prosthesis and the resulting release of metal ions is the source of many adverse effects (Figure 1.6 and Figure 1.7). The susceptibility of a material to corrosion and the effect of the corrosion debris on the tissues are used to determine the biocompatibility of the material (Gotman 1997). The occurrence of metallic wear debris in relation to titanium prostheses, and less frequently cobalt-chromium prostheses, is a well-documented phenomenon (Breen and Stoker 1993). The release of wear debris occurs when surface strains accumulate and form ripples in the lining of the joint. Later on fibrils are created from the rippled protrusions and a wear particle is formed when the fibril ruptures (Breen and Stoker 1993).

The production of a wear particle is a process of three steps:

1. Formation of surface nodules,

2. Formation of surface fibrils,

3. Release of debris particle from the partially detached fibril.

The size and morphology of the particle depend on the integrity of the materials that are used in the implant (Yamamoto *et al* 2001). The most common events by which undergo metal prostheses on implantation are bending, scratching and pounding, which may disrupt the passive oxide layer favouring the release of metal ions (Friedman *et al* 1993, Merritt and Brown 1996, Donati *et al* 1998, Jacobs *et al* 1998). Metal in contact with fluids, including body fluids that are saline based, undergo uniform attack causing the dissolution of the metal (Hennig *et al* 1992, Merritt and Brown 1996, Liu *et al* 1998). It is estimated that 30 μg/day and 11mg/year of metal ions for a total hip replacement may elute from the prostheses (Merritt and Brown 1996).

There are generally five main types of corrosion occurring in metal prostheses: galvanic corrosion, crevice corrosion, pitting corrosion, fretting and intergranular corrosion (Lopez 1993).

1. Galvanic corrosion occurs when two different metals are in physical contact and result in being immersed in an ionic conducting fluid such as serum (Black and Hastings 1998, Black 1999). There could be a preferential dissolution of one metal and passivation of the other component. Different metals are alloyed to make different metal devices in orthopaedic surgery making this type of corrosion a concern.

2. Crevice corrosion is related to structural details such as the presence of a crevice on the prosthesis (Merritt and Brown 1996, Black and Hastings 1998) and it is chemically due to sulphur in amino acids (Traisnel *et al* 1981). Because of these conditions, a significant amount of metal can be released because of this localised phenomenon (Traisnel *et al* 1981).

3. Pitting is a more isolated symmetrical form of corrosion (Rea 1981, Merritt and Brown 1996), where scratches or handling damage usually begin this type of corrosion (Friedman *et al* 1993, Black 1999).

4. Fretting is a type of corrosion generally associated with micromotions between components, causing disruptions to the passive film (Merritt and Brown 1996, Black and Hastings 1998, Black 1999, Brown *et al* 1988). This disruption to the passive film lead to consumption and consequently lack of oxygen at the tight junctions, that would cause over time massive pitting and crevice corrosion (Merritt and Brown 1996, Black 1999). This is probably a major cause of metal release into the tissues.

5. Intergranular corrosion is the result of mechanical and heat treatments (Traisnel *et al* 1981).

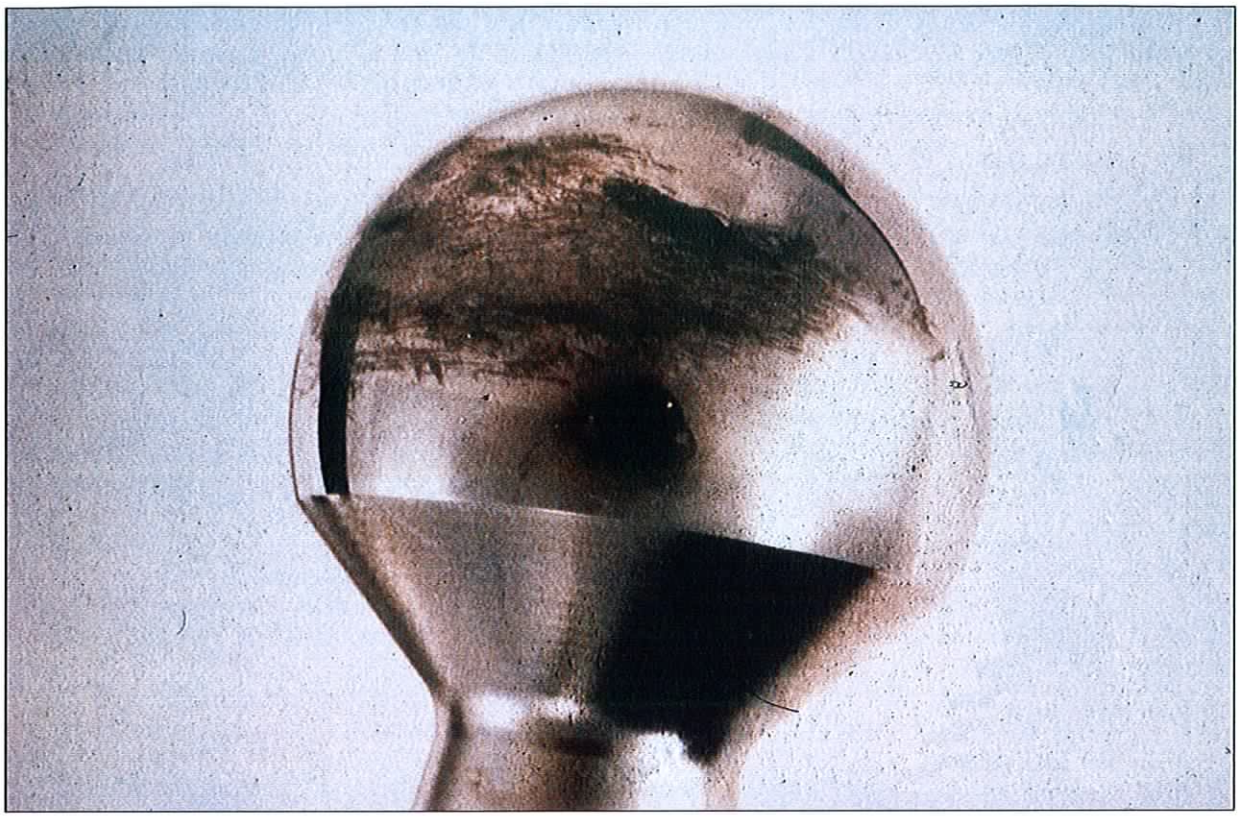

Figure 1.6. Metal prosthesis showing corrosion on the anatomic medullary locking stem.

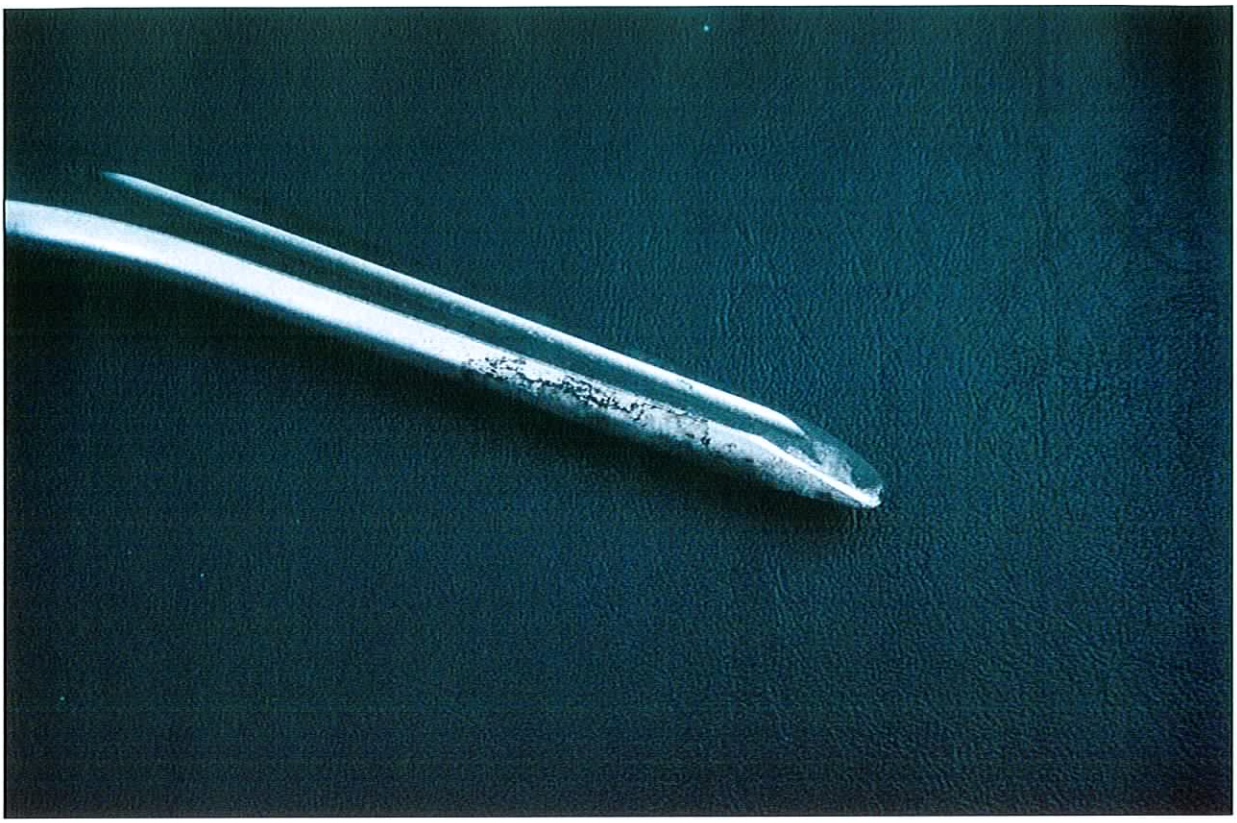

Figure 1.7. Metal prosthesis showing corrosion on the socket.

1.1.6.4 Metal Accumulation in the Body

As corrosion of the metal prostheses progresses, materials accumulate in the tissues surrounding the implant (Darby 1990, Langkamer *et al* 1992, Case *et al* 1994). These materials include membrane-bound ions, particulate debris released by corrosion and insoluble reaction products such as metal hydroxides (Galante *et al* 1991, Black 1999). It is essential that particulate debris from the implant possess a certain degree of asymmetry in order to move through soft tissue (Merritt and Brown 1996). Large material particles may also become involved in blood circulation and particulate debris can easily reach the lymphatic drainage system (Hennig *et al* 1992, Liu *et al* 1998, Ueda 1998, Schaffer *et al* 1999, Hallab *et al* 2000). Phagocytic cells are known to ingest wear debris and transport the particles by passing either into the blood, lymphatic circulation or lymph nodes (Donati *et al* 1998). Metal ions can also bind to proteins to form organometallic complexes. Proteins are Zwitter ions, or ions that can

become either positively or negatively charged. Based on their isoelectric points, most proteins are negatively charged in the human body, with a pH of 7.4. It is likely that positively charged metal ions, including cobalt, chromium, and nickel, therefore, bind to proteins, changing the pH of albumin solutions. Metal ions such as cobalt and chromium dissolve more in the presence of proteins, which consequently increases the corrosion rate of the implant (Merritt and Brown 1988, Clark and Williams 1982). Even though cobalt and chromium have the same affinity for proteins, nickel significantly competes for cobalt and chromium binding areas (Yang and Black 1994). When a metal binds to a protein, it can be either transported within the body or excreted. Cobalt is transported from tissues to the blood and eliminated in the urine within 48 hours, while chromium builds up in the tissues and red blood cells (Schaffer *et al* 1999). Metal-bound protein complex can also be transported to a site where the metal may compete with essential metals and thus disrupt the metabolic pathways (Williams *et al* 1992). Cr (VI) is the only ion that can be taken up intracellularly by red blood cells following corrosion of stainless steel. Once it reaches the intracellular environment, it is then rapidly converted to Cr (III). Intracellular Cr (III) exerts mutagenic and carcinogenic properties by interacting with the DNA molecule (Schaffer *et al* 1999, Merritt and Brown 1995). It has also been found that chromium and nickel are retained in bone marrow (Morais *et al* 1999). Cobalt is also mutagenic and carcinogenic. Cobalt binds to both red blood cells and white blood cells (Merritt and Brown 1988, Clark and Williams 1982, Schaffer *et al* 1999). Even though only very small quantities of Cr (III) bind to cells, Cr (VI) binds very strongly to red blood cells and white blood cells (Merritt *et al* 1984). Nickel is very small and has a low affinity for blood cells. A difference in the cellular response to different types of metal-alloy particles of the same size has been demonstrated in several studies (Sargeant and Goswami 2006). It has been demonstrated that cobalt–chromium particles are the most toxic to tissue, but it is also true that particles from less toxic metal-alloys may be worse because of their ability to cause the release

of inflammatory mediators (Sergeant and Goswami 2006). Even though many studies have shown potentially adverse pathophysiological effects related to metal ions including Co, Cr, Ti, Al, and Ni in the human body, the definite effects have yet to be determined. Toxicity, carcinogenicity, and metal allergy are the most significant concerns (Sergeant and Goswami 2006). This degradation of metal prostheses is the counterpart to the local host response, both being contributory to the biocompatibility of the system (Williams *et al* 1992). The term biodegradation is often used to describe degradation, which occurs in such situations. Biodegradation is defined as the breakdown of a material by a biological environment (Williams *et al* 1992).

1.1.6.5 Metal Effects in the Body

The movement of particles increases the possibility of local as well as systemic effects (Black and Hastings 1998). Denatured molecules, bound to wear debris are involved by either active or passive transport in the body. They are likely to induce remote effects either by directly or indirectly impairing the immune system (Case *et al* 1994, Donati *et al* 1998). Ions released from the metallic surface of the prostheses are correlated with a depressed immune system by causing significant changes in the lymphocyte populations (Donati *et al* 1998). It is likely that the impaired immune system leads to an allergic response to the prostheses. The main sign of the inflammatory response is the accumulation of cells at the implant site such as macrophages (Manlapaz *et al* 1996, Rogers *et al* 1997, Overgaard *et al* 1998). Macrophages are monocytes, which are a type of leukocyte, that mature and settle in tissues instead of remaining in the bloodstream and are components of the immune system (Howling *et al* 2001). These cells ingest the foreign particles, which stimulates the release of cytokines (Tengvall *et al* 1989, Rogers *et al* 1997, Wang *et al* 1997b). Cytokines are cellular proteins that mediate inflammation and communication between cells of the immune system (Abbas *et al* 2000).

Particles must be a critical size to cause macrophages to activate cytokines (Green *et al* 1998). The biologically active particle size falls in the range of 0.1–10 μm and macrophages only ingest particles in this specific size range (Germain *et al* 1999). Later on the cytokines promote the osteoclasts, which are large phagocytic cells. These osteoclasts remove the bone around the prosthesis, resulting in bone loss, with subsequent loss of integrity of the implant-bone interface, implant loosening and failure (Darby 1990, Yao *et al* 1995, Wang *et al* 1997a, Wang *et al* 1997b). This phenomenon is medically called osteolysis. The degradation of the bone, due to osteoclasts, occurs by attaching via a membrane extension to create a sealing zone between the bone and the osteoclasts that acts as a lysosome. The pumping of protons creates an acidic pH that causes the dissolution of hydroxyapatite and collagen, which make up the bone. This consequently leads to the break down of the bone (Ganong 2003). After the bone has been resorbed, the osteoclasts cease their activity until they attach to a different bone resorptive site (Lassus *et al* 1998). It may be possible to prevent osteolysis following particular accuracy.

The process of osteolysis has commonly been considered a chronic inflammatory response that has an insidious beginning. However, it has been shown that particulate debris may instigate an acute inflammatory response apart from, or in addition to, the chronic inflammation that results. Therefore, it may be useful to prevent the development of an acute inflammatory process in future cases. As already mentioned before, metal-on-metal total hip replacements offer lower wear rates than metal-on-polyethylene total hip replacements because of less wear debris generated, and thus, less risk for osteolysis (Sargeant and Goswami 2006). The implantation of hip prostheses is often carried out in inflamed osteoarthritic joint cavities. In these cavities the enzymatic substances and reactive oxygen species produced by the inflamed synoviocytes can cause a breaking of the polymer chains and increase the level of free radicals at the onset of implantation. This tissue response can cause the failure of the implant by promoting osteolysis at the time of the initial surgery. Therefore, a synoviectomy may be

necessary at the time of surgery in order to eliminate inflammatory products and to prevent the spread of inflamed synovial tissue outside the joint cavity (Sergeant and Goswami 2006). It may be also possible to inhibit or reduce osteolysis by using particular drugs called biophosphanates. These biophosphanates are a class of drugs that inhibit osteolysis by becoming incorporated into the crystal structure of hydroxyapatite on new bone surfaces. As soon as the osteoclasts dissolve the hydroxyapatite crystals, they release the biophosphanates that prevent further degradation by the osteoclasts (Wang *et al* 1999).

1.2 Radiation

1.2.1 General Information

In 1895, Wilhelm Roentgen was the first to generate radiation in the form of X-rays, emitted from a gas discharge jar. At that time, X-rays were used as a diagnostic tool to visualise fractures in bones.

In 1927 Muller showed the effects of X-rays on the mutation rates of Drosophila Melanogaster (a fruit fly) and he found, for the first time, a dose-response relationship between radiation exposure and incidence of mutation (Muller 1927).

In 1956 Puck and Marcus investigated the dose-response relationship by exposing cells in culture to radiation and showed the effects of radiation on the proliferating cell population (Puck and Marcus 1956).

In 1959, Elkind and Sutton observed that after radiation-induced damage, cells have more time to repair their DNA, as they have longer cell cycles (Elkind and Sutton 1959). Since then, radiation was considered the first mutagenic agent known.

Radiation is currently defined as the physical transfer of energy from one place to another by mean of particles or waves.

1.2.2 The Electromagnetic Spectrum

Solar radiation has made possible life on the earth for millions of years. There are different forms of radiation, which vary in their wavelength and their consequential effects. The term used to describe the entire range of light radiation, from gamma rays to radio waves, is the electromagnetic spectrum. The rainbow of colours that make up visible light is only a tiny part of a much broader range of light energy. There are no hard breaks in this distribution of light energy, although for convenience, there have been assigned different names to various

sections, as shown in the diagram below (Figure 1.8). These main sections, ranging from gamma rays to radio waves, are listed and described below.

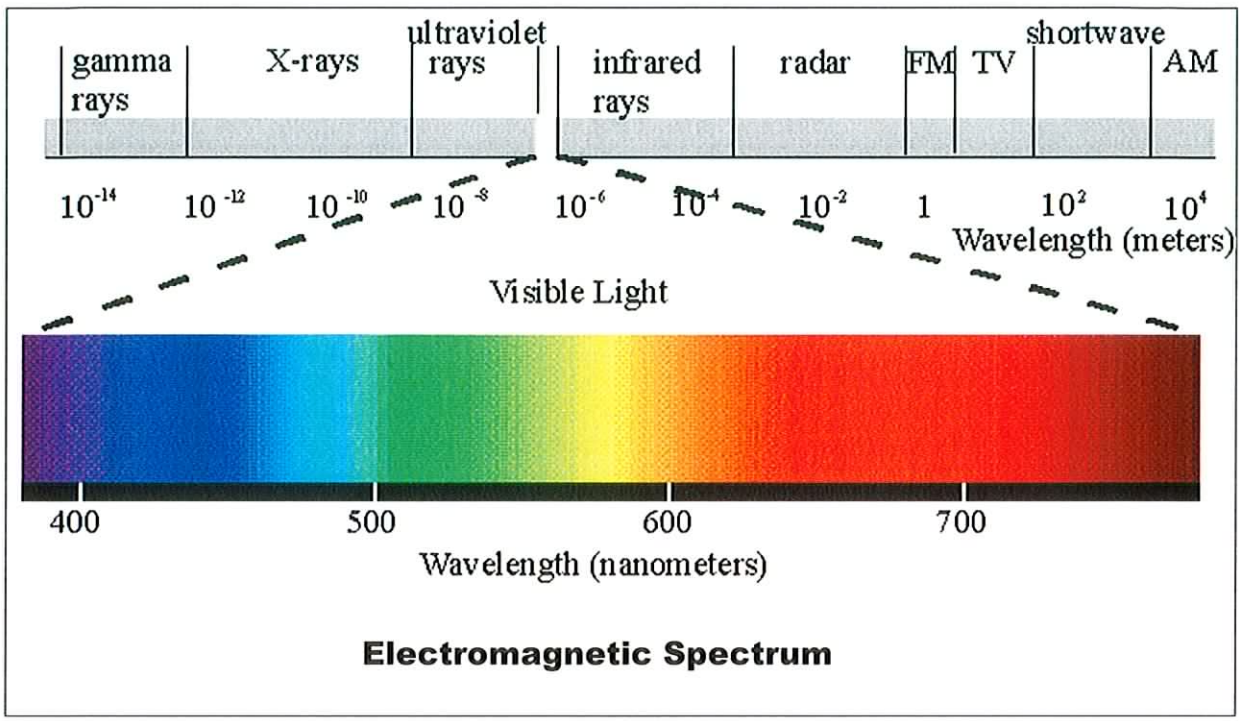

Figure 1.8. The electromagnetic spectrum describes the entire range of light radiation, from gamma rays to radio waves (www.yorku.ca/eye/spectrum.gif).

1. Gamma Rays

They have very high energy and can even pass through metals. For that reason, γ-rays can be used for finding tiny cracks in metals. Some radioactive materials produce gamma rays and they can cause cancer, but gamma rays can also be used to destroy cancer cells through a medical treatment called radiotherapy. At shorter wavelengths, in the case of γ-rays, the orbital electrons are removed from the surface atoms of the skin and further penetration is possible.

2. X-Rays

X-Rays have so much energy and such a short wavelength that they can go through objects or living organisms. However, they cannot pass through bone as easily as they can pass through

muscle, as bone contains a lot of calcium. Thus, X-Rays are commonly used in hospitals, in order to detect broken bones. X-Rays have been implicated in carcinogenesis.

3. Ultra Violet

These waves have very high energy and very short wavelengths. These wavelengths are shorter than visible light. UV radiation does not penetrate the skin but causes atomic excitation on the surface of the organ. Atomic excitation in this context is where orbital electrons, within atoms, are raised to higher energy levels. Studies have shown that UV radiation causes skin cancer.

4. The Visible Spectrum

Red, orange, yellow, green, blue, indigo, and violet are the colours of the visible spectrum. Red light has a longer wavelength (less energy) than blue light. UV light has a shorter wavelength (more energy) than visible light.

5. Infra Red

These radio/light waves have a longer wavelength than visible light.

6. Microwaves

Microwaves have short wavelengths and they are very easily absorbed by water. In microwave ovens, the water in the food absorbs the microwaves and the energy of the microwaves is converted into heat.

7. Radio Waves

These waves are used for radio, television, and mobile phones. Different parts of the radio spectrum have been allocated to the various services. Radio waves have a much longer wavelength than light waves. The longest waves are several kilometres in length, and the shortest ones are only millimetres long.

All forms of radiation have similar features. They all travel in straight lines and their intensity falls off with distance travelled. The intensity follows the inverse square law: radiation will have a quarter of its intensity after it has travelled twice the distance.

1.2.3 Radiation Units

In 1928, the Roentgen (R) unit became the first dosimetric unit used to measure radiation. This unit was based on radiation-induced ionization in the air, and is defined as "the quantity of X-Ray or γ-radiation such that the associated secondary electrons emitted produce ions of 2.58 x 10^{-4} Coulombos per kilogram of air". The R unit is a unit of exposure, which is the amount of radiation directed at the material, not the amount absorbed by the material. In 1956, the Rad (radiation absorbed dose) was accepted as a unit of absorbed dose. One Rad was defined as the absorption of 10^{-2} Joules of radiation energy per kilogram of material. The energy absorbed in tissue following exposure to 1 R was found to measure 0.95 Rad. Consequently, the Rad and the Roentgen are sometimes considered equivalent in physics literature. Nowadays, the accepted unit of absorbed dose is the Gray (Gy), which is defined as 1 $J.Kg^{-1}$. Therefore, 1 Rad is equal to 100 cGy.

The Sievert (Sv) unit is a unit used to derive a quantity called equivalent dose. The Sv unit relates the absorbed dose in human tissue to the effective biological damage of the radiation. Different types of radiation do not have the same biological effects, even for the same amount of absorbed dose. Normally, equivalent dose is expressed in terms of millionths of a Sievert, or micro-Sievert. In order to determine equivalent dose (Sv), the absorbed dose (Gy) is multiplied by a quality factor (Q), which is exclusive to the type of incident radiation. In this work, γ-radiation is used which has a quality factor of 1.

1.2.4 Ionizing Radiation and Non-Ionizing Radiation

Radiation can be distinguished in two types: non-ionizing radiation (e.g. UV and microwaves) and ionizing radiation (e.g. gamma rays and X-rays).

1. Non-ionizing radiation, such as UV and microwaves, are found at the other end of the electromagnetic spectrum. This type of radiation does not ionise atoms (Grossweiner 2004).

2. Ionising radiation, such as gamma rays (γ-ray) and X-rays, has sufficient kinetic energy to eliminate bound electrons from their orbits, leading an atom to become charged or ionised. Linear energy transfer (LET) is the term used to describe the density of ionization in particle tracks. LET is the average energy in keV given up by a charged particle, which traverse a distance of 1 μm. Alpha particles are considered as high LET ionizing radiation, whereas gamma rays and X-rays are low LET ionizing radiation.

The wavelength of the γ-ray depends on both the energy of the electrons and the atomic number of a radioactive isotope (for example, Cobalt-60, ^{60}Co). Atoms, in biological tissues that may be hit by ionizing radiation, have varying atomic number and would react in different ways to the same radiation dose. γ-rays deposit their energy in different ways, depending on the intensity of the delivered dose.

1. For γ-rays with energy of less than 0.5 MeV the predominant method of interaction is by the *Photoelectric Effect*, in which the photon is completely absorbed by the target atom, an electron is emitted and characteristic radiation is produced.

2. When the energy is between 0.5 MeV and 5 MeV *Compton Scattering* occurs, whereby the incident photon hits an orbital electron, leading to the expulsion of an electron. Subsequently, the incident photon interacts with additional target atoms to produce either a photoelectric effect or another Compton scattering. The production of another Compton scattering can only occur if the incident photon (that interacts with additional target atoms) possesses sufficient energy. The expulsion of an electron and the resultant unstable positively charged atom is termed ionization.

3. When the energy is higher than 5 MeV, photons are converted into positrons and electrons. Positrons are positive electrons that are turned into two photons on collision with electrons. This phenomenon is called *Pair Production*.

1.2.5 Radiation-Induced Cell Damage

If ionizing radiation interacts with matter, it can activate several physical and chemical effects, for example heat generation and breakage of chemical bonds, depending on the type of radiation and target (Grossweiner 2004).

Studies on radiobiology have focussed on the effects that ionizing radiation can have in the nucleus of the cells, and therefore in the DNA molecule. Zirkle and Bloom (1953) showed that DNA is more susceptible to ionizing radiation than the cytoplasm.

During the last 50 years, several researchers have studied the link between radiation and DNA damage. In radiobiology, point mutations, such as changes in the basis of nucleic acid, are quite frequent, and these usually occur at a rate that is directly proportional to the dose of radiation administered. But the most lethal damage, caused by ionizing radiation is double strand breaks (DSB), where there is a break in both DNA strands, at the same position. Single strand breaks (SSB) are less harmful to the cells, as the remaining DNA strand can be used as a template for repair (Zirkle and Bloom 1953, Munro 1970). DNA has been considered to be the main target for the biological effects of radiation (Zirkle and Bloom 1953). In fact, if cells are irradiated with γ rays, they show susceptibility to several breaks in either of the single strands of the DNA molecule. If two single breaks may be opposite in the DNA, it means that a double strand break (DSB) has occurred, and therefore it can lead to mutation or even cell death. It has been shown that the number of DSB following irradiation of cells is approximately 0.04 times that of single strand breaks (SSB) (Munro 1970). It has been demonstrated that all the DNA content of a cell is likely to be vulnerable to strand breaks and a DSB is essential for a lethal event in the cell (Kellerer and Rossi 1972). This has suggested that a low dose of radiation normally causes SSB, while higher doses are likely to induce DSB. It has been reported that the position of the cell in the cell cycle determines its potential of repairing post-irradiation damage (Sinclair 1973).

Damage to the nucleus after radiation exposure is believed as the main cause of radiogenic cell death (Zirkle and Bloom 1953). Nucleus and nucleolar lesions, increases in nuclear diameter, micronucleation, marginated chromatin and nuclear fragmentation are the main endpoints of damage resulting from exposure to radiation, which normally lead to the death of the cell (Mc Clain *et al* 1990, Cornforth and Goodwin 1991).

Moreover, ionizing radiation can induce damage not only to the nucleus and DNA, but also to membrane and protein structure. Several biological effects are provoked by ionizing radiation through direct interaction with nucleic acid and production of free radical species, or dysfunction of cellular organelles (Kantak *et al* 1993, Kasper *et al* 1993, Somosy *et al* 1995, Singh and Vadasz 1983).

1.2.5.1 DNA Repair

The process by which a cell identifies and corrects damage to the DNA molecules that encode its genome is called DNA repair. Structural damage to the DNA molecule can alter or eliminate the ability of the cell to transcribe the gene that the affected DNA encodes. Furthermore, potentially harmful mutations in the genome of the cell are likely to affect the survival of its daughter cells (Lodish *et al* 2004).

DNA damage can be divided into two main sources:

1. Endogenous damage, which is induced by reactive oxygen species produced from normal metabolic byproducts (spontaneous mutation), such as the process of oxidative phosphorylation.

2. Exogenous damage, which is caused by external agents such as ultraviolet [UV 200-300nm] radiation from the sun, x-rays, gamma rays and aromatic compounds that act as DNA intercalating agents (Lodish *et al* 2004).

Mechanisms of DNA Repair

Damage to the DNA molecule alters the spatial configuration of the helix and such alterations are normally detected by the cell. Depending on the type of damage caused on the double helical structure of the DNA, there are four main types of repair mechanisms, which may restore the lost information.

1. Direct Reversal

These mechanisms do not require a template, since the types of damage they counteract can only occur in one of the four bases and are specific to the type of damage incurred. The formation of thymine dimers (a common type of cyclobutyl dimer) after irradiation with UV light results in an abnormal covalent bond between adjacent thymidine bases. In bacteria, the photoreactivation process directly reverses this damage by the action of the enzyme photolyase, which uses energy absorbed from UV light to promote catalysis (Watson *et al* 2004).

2. Single Strand Break

When only one of the two strands of a double helix has a defect, the other strand can be used as a template to guide the correction of the damaged strand. There are three main excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide, which is complementary to that found in the undamaged DNA strand (Watson *et al* 2004).

a) Base excision repair, which repairs damage due to a single nucleotide caused by oxidation, alkylation, hydrolysis, or deamination.

b) Nucleotide excision repair, which repairs damage affecting longer strands of 2-30 bases. This process recognizes bulky, helix-distorting changes such as thymine dimers as well as single-strand breaks.

c) Mismatch repair, which corrects errors of DNA replication and recombination that result in mispaired nucleotides following DNA replication.

3. Double Strand Breaks

This type of DNA damage occurs when there is a break to both strands in the double helix. There are two main mechanisms, which can repair this damage (Watson *et al* 2004).

a) The Non-Homologous End-Joining (NHEJ) mechanism acts when the cell has not yet replicated the region of DNA on which the lesion has occurred. This process directly joins the two ends of the broken DNA strands without a template, losing sequence information in the process. Therefore, this repair mechanism is necessarily mutagenic. The NHEJ relies on chance pairings, or microhomologies, between the single-stranded tails of the two DNA fragments to be joined (Wang *et al* 2003, Watson *et al* 2004).

b) The homologous Recombination Repair (HRR) requires the presence of an identical, or nearly identical sequence, to be used as a template for repair of the break. This mechanism allows a damaged chromosome to be repaired using the newly created sister chromatid as a template (an identical copy that is also linked to the damaged region through the centromere). Double-stranded breaks repaired by HRR are normally caused by the replication machinery that attempts to synthesise across a single-strand break, which results in collapse of the replication fork (Wang *et al* 2003, Watson *et al* 2004).

4. Translesion Synthesis

This mechanism is an error-prone last-resort method of repairing a DNA lesion that has not been repaired by any other mechanism. Since the DNA replication machinery cannot continue replicating past a site of DNA damage, the advancing replication fork will stall whenever it encounters a damaged base. The translesion synthesis pathway is mediated by specific DNA polymerases that insert extra bases at the site of damage and therefore permits the replication to bypass the damaged base to continue with chomosome duplication (Watson *et al* 2004).

1.2.5.2 Radiation-Induced Oxidative Damage

The damage to the DNA caused by oxidative stress is detected in the bases and sugar-phospate in the structure of DNA, as well as SSB and DSB. SSB and DSB can be induced from direct ionizing radiation. However, indirect damage can be caused by radicals generated from radiation and lead to base damage. The majority of these indirect effects occur by free radicals in water, since this comprises 70-80% of mammalian cells. The free radicals react with other molecules to form reactive oxygen species (ROS). The most important ROS are the superoxide radical, the hydroxyl radical and hydrogen peroxide (Horsman and Overgaard 1997). There are more than twenty different types of base damage, which are induced by oxidative stress. The most frequent oxidative damage to purines is 7,8-dihydro-8-oxoguanine (8-oxodGuo), where the conformation is capable to mispair with adenine determining a transversion of G to T (Martinez *et al* 2003). The most common oxidative damage to the pyrimidines is the formation of thymine glycol (Tg) (Slupphaug *et al* 2003).

1.2.6 Evaluation of Radiation Damage by Linear No Threshold Model (LNT)

In 1960 the Linear No Threshold (LNT) model was introduced to quantify radiation damage. The LNT model allows the evaluation of risk of radiation exposure at any dose. This model was derived from the observation that no threshold dose had been recognised and that "even the smallest doses involve a proportionately small risk". The discovery of oncogenes gave support to the LNT model, as one single mutation could potentially turn a proto-oncogene into an oncogene. However, there are still some doubts about the LNT model, especially with low doses of radiation, but these uncertainties could be due to insufficient statistical power (Tubiana 2003).

However, the incidence of mutation depends not only on the DNA lesions, but also on the efficiency of DNA repair. The estimation of the DSB can be displayed in survival curves,

which have the typical shoulder region. This is the point at which the repair mechanism of the cell repairs the DNA breaks. Cells, which present defects in DSB repair mechanisms, do not display this particular shoulder region in survival curves (Hall 2000).

Joiner *et al* (2001) have shown that cells are hypersensitive at doses lower than 0.3 Gy. When the dose increases, the cells become less sensitive displaying the standard survival curves. Animal studies have shown that at equal doses, low dose rate irradiation is more likely to induce cancer, compared to high dose rate irradiation. This feature is inconsistent with the LNT model. Furthermore, in about 40% of whole animal studies there is a negative slope below 0.5 Gy for tumour appearance, which is not predicted by the LNT model. This highlights the deficiency of using the LNT model to extrapolate to low-dose damage (Tubiana 2003).

1.2.7 Nontargeted Effects of Radiation

The three most important nontargeted effects of radiation, described below are:

1. Bystander Effect
2. Adaptive Response
3. Genomic Instability

1.2.7.1 Bystander Effect

The bystander effect is the effect that describes the ability of cells, affected by an agent (in this case radiation), to convey manifestations of damage to other cells, not directly targeted by the agent or necessarily susceptible to it themselves (Djordjevic 2000).

Parsons *et al* (1954) observed that unirradiated tissues showed radiation-induced clastogenic effects when plasma from irradiated animals was injected into them. It was then noted that this effect of radiation was present in cells and tissues, which were not irradiated, but were exposed

to plasma or ultrafiltrates from irradiated humans or animals (Hollowell and Littlefield 1968, Goh and Summer 1968).

Nagasawa and Little (1992) examined the induction of sister chromatid exchanges (SCE) in Chinese hamster ovary cells irradiated in the G1 phase of the cell cycle with alpha-particles from a plutonium-238 source. They observed a significant increase in the frequency of SCE with doses as low as 0.31 mGy (31 millirads). Although 30% of the cells showed an increased frequency of SCE at this dose, less than 1% of cell nuclei were actually traversed by an alpha-particle. A dose of approximately 2.0 Gy was necessary to produce a similar increase in SCE by X-rays. Their work showed that genetic damage might be induced by low doses of alpha-radiation in cell nuclei not actually traversed by an alpha-particle. This phenomenon may have important implications in the estimation of risks of such exposures.

The charged particle microbeam is a useful tool in the investigation of the radiation-induced bystander effects. This tool can pass an exact number of particles through particular areas of a single cell (Prise *et al* 1998). When a single α particle was passed through the nucleus of 10% of the cells, in a confluent mammalian cell population, the mutation rate was found to be similar to that detected when 100% of the cells were irradiated in the same way. This shows that one α particle, the lowest possible dose, passing through the nucleus of a cell, elicits a response in cells that were not irradiated (Zhou *et al* 2002). Formation of micronulei and the importance of gap junction intracellular communication and reactive oxygen species (ROS) in the mechanism of the bystander effect have been shown (Shao *et al* 2003).

Mothersill and Seymour (1997, 1998) showed that γ irradiation of cells *in vitro* release a "factor" into the culture medium. This medium can promote apoptosis and necrosis and loss of clonogenic survival. Furthermore, genomic instability was induced when this medium was transferred to *in vitro* cultures of non-irradiated cells (Mothersill and Seymour 1997, Mothersill and Seymour 1998). Not all cells are capable of generating a bystander factor, nor are all cells

capable of receiving and reacting to the factor (Mothersill and Seymour 1997). Lyng *et al* (2000) demonstrated that one of the first endpoints caused by medium containing this bystander factor on unirradiated mammalian cells is a calcium pulse, followed by changes in mitochondrial membrane permeability and induction of ROS. Brenner *et al* (2001) have suggested that at low doses, up to 0.5 Gy, most of the effects detectable in the cells might be due to bystander factors.

All this work showing bystander effect *in vitro*, have led to reconsideration of the existing concept of *in vivo* radiation dose and target size. If bystander factors can be elicited in monolayer cultures, they could also be present in whole animal tissues following exposure. Furthermore, Brooks *et al* (1974) observed that irradiation of defined areas of Chinese hamster liver led to chromosome damage in the entire liver. The number of tumours and time to tumour formation, following irradiation, did not depend on the number of irradiated cells, as there was no sign of effect with increased exposure to radiation (Brooks *et al* 1983). Their work demonstrated the existence of a bystander effect in tissue, and also showed that, in some circumstances, the signal released might be specific only for the cell type in which it is created (Morgan 2003).

Mothersill *et al* (2005) have shown that irradiated C57BL/6, but not CBA/Ca mice produce bystander signals that induce apoptosis and loss of clonogenic survival in reporter HPV-G keratinocytes, suggesting that genetic factors can influence *in vivo* induction of bystander signals. Watson *et al* (2000) have demonstrated the presence of chromosomal instability in the progeny of non-irradiated haemopoietic stem cells *in vivo*, by using a mouse bone marrow transplantation protocol. This protocol used a mixture of irradiated and non-irradiated bone marrow, distinguished by a cytogenetic marker, which were then transplanted into recipients. Xue *et al* (2002) showed that human colon LS174T adenocarcinoma cells, prelabelled with lethal doses of the radionuclide 5-[(125)I]iodo-2'-deoxyuridine, co-injected with unlabelled

cells into nude mice, can exert an inhibitory effect on the growth of the tumour derived from unlabelled cells, suggesting that the effects are a consequence of bystander signalling produced *in vivo* by factors present within or released from the radiolabeled cells.

There has been much interest in the *in vivo* bystander effects in tissue histologically distinct from the irradiated cells, which are called abscopal effects. Kahn *et al* (1963), have demonstrated this feature *in vivo* by using rat lung cells, and is validated by *in vitro* medium transfer between cells of different histological lineage. Other authors have shown *in vivo* abscopal effects (Hahn and Feingold 1973), although others have argued that there is not sufficient evidence yet to give a defined conclusion about this topic (Goldberg and Lehnert 2002).

1.2.7.2 Adaptive Response

The adaptive response is another low-level radiation effect, which is characteristic of an increased resistance in cells or organisms exposed to a priming dose, followed by exposure to a challenging dose. Olivieri *et al* (1984) exposed cultured human lymphocytes to low doses of chronic radiation. Later these cells were exposed to a high dose of X-rays, and surprisingly they observed approximately 50% less chromosomal abnormalities, compared to cells that had not been pre-irradiated.

It was then suggested that the "adaptation" was due to the induction of an efficient chromosome repair mechanism, that when activated, by the priming radiation dose, stayed active at the time of the high dose, reducing the damage (Olivieri *et al* 1984, Wiencke *et al* 1986). Shadley and Wolff (1987) concluded that the priming dose did not have to be chronic, and a single dose, as low as 1 cGy, was considered to be sufficient to prime the cells. It was then shown that the protein synthesis inhibitor cycloheximide could inhibit the adaptive response (Youngblom and Wiencke *et al* 1989). Reduced radiosensitivity in cells cultured in

medium, which was harvested from other cells previously exposed to 2.5 Gy of X-radiation has been shown (Matsumoto *et al* 2001). Medium from cells exposed to low doses of γ-radiation, such as 1 cGy, resulted in an adaptation (reduced damage) of non-irradiated cells, to exposure of 2 and 4 Gy direct radiation (Iyer and Lehert 2002). Smith and Raaphorst (2003) have shown that the adaptive response leads to an increase in clonogenic survival.

There are still many doubts about the mechanism by which the adaptive response acts. Youngblom *et al* (1989) observed that adaptive response depends on protein synthesis, but radiation-induced genomic instability and bystander effects do not. Kadhim *et al* (2004) have shown that cells primed with direct irradiation have a higher level of TP53, an elevated level of reactive oxygen species and uncharged apurinic endonuclease (APE) protein levels. Furthermore, the protein kinase C mediated signalling pathways may be an essential step in the adaptive response, where the priming dose is from direct irradiation (Kadhim *et al* 2004). However, Iyer and Lehnert (2002) have demonstrated that, following a priming exposure with irradiated cell culture medium (ICCM), there is a decrease in TP53 levels and a more noticeable increase in ROS. Also APE protein has been found to increase. APE protein is upregulated in response to sublethal levels of ROS and is important in the repair of damaged DNA. This suggests that the bystander adaptive response might be more competent at repairing DNA damage than the direct bystander response (Iyer and Lehnert 2002). Another mechanism of bystander adaptive response was also postulated, in which the priming dose of radiation provokes the cell to bypass normal cell cycle checkpoints. This, coupled with DNA repair, no longer suppressed by TP53, may lead to the adaptive response observed (Iyer and Lehnert 2002).

1.2.7.3 Genomic Instability

Puck and Marcus (1956) demonstrated that DNA damage is associated with alterations in its structure, immediately after radiation exposure. Therefore, a cell with DNA alterations is capable of passing on these changes to its daughter cells in a clonal manner. Radiation-induced genomic instability is characterised by non-clonal effects in the descendants of irradiated cells, leading to the possibility of cell death, many population doublings post irradiation in cell lines. Lyng *et al* (1996) have shown that non-clonal morphological abnormalities can occur many replication post irradiation, whereas Mothersill *et al* (1998) observed that non-clonal toxicity and lethal mutations can take place many replication post irradiation. Furthermore, non-clonal chromosome changes *in vivo* up to two years post irradiation have been detected (Pampfer and Streffer 1989, Kadhim *et al* 1992, Lorimore *et al* 1998, Watson *et al* 2000, Watson *et al* 2001). Genomic instability comprehends several endpoints, among which there are chromosomal rearrangements and alterations, micronuclei, sister chromatid exchange, mutations at target loci and apoptosis (Clutton *et al* 1996). It is not clear yet if all these individual endpoints of genomic instability results from a single mechanism. Many authors have suggested that genomic instability may be the consequence of epigenetic processes, maintained through successive generations by reactive oxygen species (ROS), cytokines, transforming growth factor β1 or altered methylation, acetylation and phosphorylation patterns (Clutton *et al* 1996, Barcellos-Hoff and Brooks 2001, Nagar *et al* 2003, El-Osta 2004, Hake *et al* 2004). It has also been shown that other agents, and not only radiation, can cause genomic instability. All these agents are capable of inducing oxidative stress (Mothersill *et al* 1998, Coen *et al* 2001). It has been suggested that post irradiation, during clonal growth, low levels of oxidative stress can lead to new point mutations and DNA strand breaks (Clutton *et al* 1996, Kadhim *et al* 2004). This feature could ultimately result in a "mutator phenotype", increasing the rate at which mutations accumulate (Loeb *et al* 2003).

It has been hypothesised that non-targeted effects of radiation might be important factors in the induction of cancer, but unfortunately the understanding of genomic instability, bystander effects and adaptive responses is not sufficiently developed to postulate a theory which would explain the effects of low dose radiation (Goldberg and Lehnert 2002, Lorimore *et al* 2003).

1.2.8 Radiation-Induced Cell Death on Human Cell Lines

Mothersill *et al* (1997) observed that radiation is capable of causing several morphological and biochemical abnormalities in human cells, including nuclear fragmentation and other signs of programmed cell death, apoptosis, necrosis, persistent abnormal growth and function, growth and cell cycle control and hit shock responses. All these endpoints can be detected in the progeny of irradiated cells.

Therefore, post-irradiation, cells can mainly die in three different ways.

1. Through apoptosis (programmed cell death), whereby the contents of the cells are degraded and delimited by membranes, in order to form apoptotic bodies prior to non-toxic phagocytosis. Apoptotic cells, or their residue, are phagocytosed by either macrophages or neighbouring cells. Apoptotic bodies then undergo secondary necrosis and may appear as lysosomal residual bodies within macrophages. During apoptosis, the cell and eventually the fragments of the cell, maintain their ion-gradients and energy levels. ATP levels remain high, and Na^{2+} and Ca^{2+} levels remain low in the apoptotic bodies, because membrane ion pumps continue to function. As soon as a cell enters apoptosis, the principal morphological change is shrinkage, and the cell constituents form apoptotic bodies. Nuclear chromatin condensation, cytoplasmic shrinking and membrane blebbing are characteristic of apoptosis. Furthermore, DNA is fragmented, organelles are dispersed and they form membrane bound apoptotic bodies that can later be phagocytosed (Birge 2004)

2. Through necrosis, which normally occurs when a cell is extremely damaged, and it is a less ordered process. Necrotic cells, or their residue, are also phagocytosed by macrophages. When an insult damages the cell, it also causes a drop in the ATP levels and a loss of control of ion gradients, causing Na^{2+} and Ca^{2+} to enter the cell, resulting to necrosis. During necrosis, there is also a loss of proton gradient across the membrane of the mitochondria. During necrosis, potentially cytotoxic contents are released from dying cells. Usually, toxic compounds or oxidation can be harmful to the mitochondria or membrane ion pumps and cause a drop in the energy levels in the cells (Birge 2004).

3. Cells not dying immediately after an initial exposure to radiation *in vitro*, die via mitotic death, as such cells die in their attempt to divide. Following irradiation, the cell may divide many times before damaged chromosomes cause a death during the process of division. Mitotic death, in the majority of tumour cells, is at least as important as cell death occurring through apoptosis, and sometimes it can be the only cause of death (Hall 2000). Erenpreisa and Cragg (2001), as well as many other authors, have suggested that chromosomal aberrations represent the main mechanism for radiation induced mitotic death in mammalian cells. Cells having an asymmetrical exchange type of aberration (ETA) are likely to lose their reproductive integrity. It has been observed that ETAs require two chromosomal breaks. With low doses, these two chromosomal breaks might occur from the passage of a single electron that was set in the motion by absorption of a photon of γ-radiation (Erenpreisa and Cragg 2001).

The death of a cell due to mitotic death has been commonly named "mitotic catastrophe". Mitotic catastrophe has been linked with incomplete DNA synthesis and premature chromosome condensation with features in common with apoptosis (Mackey *et al* 1988, Ianzini and Mackey 1998). Mitotic catastrophe results in a divergence from the mitotic cycle and entrance into the endocycle, which is a variant of the normal cell cycle, where the cells are able to duplicate their DNA without cell division (Miranda *et al* 1996, King and Cidlowski

1995). There is still a lack of knowledge regarding the transition from a normal mitotic cycle to the endocycle. Erenpreisa *et al* (2000) observed that multinucleated giant cells are generated by entrance into the endocycle, and these giant cells are temporarily viable but reproductively dead. They also suggested that polyploid giant cells could be the terminal manifestation of mitotic death.

1.3 Combined Exposure

1.3.1 General Information

Many efforts have been made to measure the combined effects of different toxic agents. Normally, research into the deleterious effects from toxicants are directed mainly towards single agents, whereas real world environmental and occupational exposures to natural and anthropogenic agents quite often involve the associated presence of several toxicants. These combined exposures may result in health risks that differ from those expected from simple addition of the individual risks. For example, combined exposures to physical and chemical agents such as radon and smoking or asbestos and smoking leads to over-additive effects, at exposure levels typical in certain workplaces (Burkart *et al* 1997).

However, it is difficult to determine if interactions occurring at high exposure levels are also important at the low exposure levels relevant for the public and for modern workplaces. Unfortunately, this information is not available, since the existing database on combined effects is elementary and it does not cover sufficiently large exposure ranges to make a direct suggestion of "low-dose exposure" situations, which normally occur. Since there are thousands of possible interactions between the large numbers of potentially harmful agents in the human environment, new approaches should be complemented with the use of mechanistic models for critical health endpoints such as cancer. Therefore, in order to predict the effects of combined exposures, agents have to be grouped depending on their physical or chemical mode of action on the molecular and cellular level. Moreover, this grouping has to be directed by specific mechanistic studies designed to inspect the fundamental hypothesis about the mechanism of interaction among various classes of agents (Burkart *et al* 1997).

1.3.2 Different Outcomes of Combined Exposure

The effects of a particular agent, when present in the environment with other agents and therefore with toxicological consequences of multiple exposures, could be due to three different outcomes:

1. "Additive Effects" is the case when the toxicity effects of a mixture are roughly equal to the summation of the toxicity values of its individual agents. Therefore, when there are additive effects, each agent expresses roughly the same toxicity in a mixture as it would be when it is tested alone, and the estimation of the toxicity of a mixture can be made by adding together the expected contributions from each of its agents (Burkart 2001, Walker *et al* 2001, Waalks *et al* 1992).

2. "Synergistic Effects" (Potentiation) is the case when the toxic effects should be greater than the sum of the toxicity of the single agents (Burkart 2001, Walker *et al* 2001, Waalks *et al* 1992).

3. "Reductive Effects" (Antagonism), is the case when the toxic effects should be less than the sum of the toxicity of the single agents (Burkart 2001, Walker *et al* 2001, Waalks *et al* 1992).

Figure 1.9 illustrates an example of combined effects, where two agents (compounds) *A* and *B* are under consideration. The maximum dose of ether *Agent A* or *Agent B* gives the same degree of toxicity response (X). Doses for either *Agent A* or *Agent B* are from 0% to 100% of the maximum dose. The summation of the contribution of *Agent A* and *Agent B* is always 100% (for example, the maximal dose of *Agent A* is 40% and the maximal dose of *Agent B* is 70%). Different outcomes can be observed from the combined effects of these two agents (*A* and *B*).

1. If the effects are simply *additive*, all of these combinations should give the same response (X), as the maximal dose of *Agent A* or *Agent B*.

2. If the effects are *synergistic* (potentiation), the toxic effect should be greater than expected.

3. If the effects are *reductive* (antagonism), the toxic effect should be less than expected.

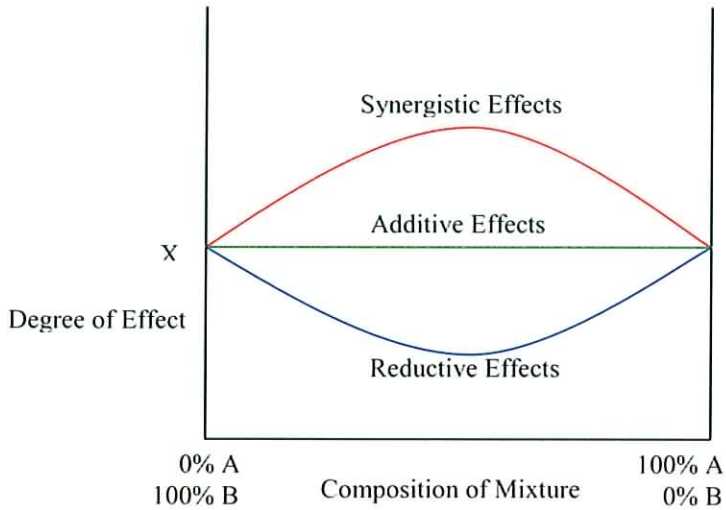

Figure 1.9. Combined Effects of two agents, *A* and *B*. The vertical axis indicates the degree of toxic effect and the horizontal axis represents composition of the mixture. The maximum dose of *Agent A* and *Agent B* both give the same degree of toxicity response X.
Synergistic Effects (potentiation) (red line) occur when the toxicity of a mixture of two agents (*A* + *B*) exceeds the summation of toxicities of the individual agent.
Additive Effects (green line) is the case when the toxicity effects of a mixture are roughly equal to the summation of the toxicity values of its individual agents.
Reductive Effects (antagonism) (blue line) occur when the toxicity of a mixture of two agents (*A* + *B*) is less than the summation of toxicities of the individual agents (Walker *et al* 2001).

1.3.2.1 Biochemical Features of Synergistic Effects

The identification of combinations of toxic agents, which present problems of synergistic effects (potentiation), is a difficult task to solve. However, there are procedures, which allow the recognition of such combined effects. Recent rapid advances in toxicology have given more insight into the potentiation of toxicity due to interactions at the toxicokinetic level, where either one agent inhibits the detoxification of another or one agent increases the rate of activation of another (Walker *et al* 2001).

For example, when an *Agent A* causes a change in the metabolism of an *Agent B*, two types of interaction are recognised as follows.

1. *Agent A* inhibits an enzyme system that detoxifies *Agent B*. In this case, the rate of detoxification of *Agent B* is reduced because of the action of *Agent A*.

2. *Agent A* induces an enzyme system, which activates *Agent B*. As a result, the rate of activation of *Agent B* is enhanced because of the action of *Agent A* (Walker *et al* 2001).

1.3.3 Additive Effects

There are some important examples of combined exposures leading to additive effects. Muller and Streffer (1987) analysed the risk to preimplantation mouse embryos of combined exposure of heavy metals (arsenic, cadmium or lead) and radiation. Morphological development and cell proliferation were used for evaluation of risk after combined exposure to these metals and X-rays. The effects of arsenic were merely additives without alteration of the radiation risk. Cadmium acted similarly, suggesting that morphological development was extra-damaged after combined exposure, than expected from the addition of the single effects. Lead had an additive effect only in the case of morphological development and cell proliferation (Muller and Streffer 1987).

Sahu *et al* (1989) studied the effects of nickel sulfate, lead sulfate, and sodium arsenite alone and with UV light on sister chromatid exchanges in cultured human lymphocytes. Sister chromatid exchanges (SCE) were examined in human lymphocytes following *in vitro* treatments with nickel sulfate, lead sulfate and sodium arsenite. All these metal salts resulted in significant increases of the SCE frequencies. The SCE frequencies were also measured for metal treatments combined with ultraviolet light (200 ergs/mm^2). Combined exposure of lead sulfate and UV, gave an additive SCE response, compared to the SCE responses for UV or metal alone. However, combined exposure of either nickel sulfate or sodium arsenite with UV produced a less than additive SCE response for most concentrations tested (Sahu *et al* 1989).

Besselink *et al* (2003) have shown that photodynamic treatment (PDT) of red blood cell (RBC) suspensions results in virus inactivation, but unluckily it also causes worsening of cell quality. Unfortunately, the protection that dipyridamole can confer to RBC against long-term PDT is

incomplete. But when dipyridamole was applied in combination with Trolox (a hydrophilic vitamin E analogue), in order to supplement RBC protection, there were additive protective effects, suggesting different protection mechanisms for the two scavengers. Therefore, combined inclusion of dipyridamole and Trolox resulted in substantially improved protection of RBC suspensions from PDT (Besselink *et al* 2003).

Karmouty Quintana *et al* (2005) studied the combined effects of allergen and endotoxin in order to detect if this combination would result in additive effects or synergistic effects, with respect to increasing the sensitivity of the airways of the Brown Norway rat to adenosine. They observed that the interaction between allergen and endotoxin showed additive effects, rather than synergistic effects. Furthermore, it was suggested that allergen and endotoxin acting together could play a role in up-regulating the response of the human asthmatic airway to adenosine (Karmouty Quintana 2005).

1.3.4 Synergistic Effects

Consideration based on carcinogenesis, indicate the potential for highly damaging interactions if two or more consecutive rate-limiting steps are specifically effected by different agents. However, low specificity toward molecular structure or DNA sequence of many genotoxic agents, indicate little functional specificity and therefore little susceptibility toward synergism in most context of environmental and occupational exposure (Burkart 2001). There are several important examples of combined exposures leading to synergistic health effect risks that differ from those expected from simple addition. For example, combined exposures to physical and chemical agents such as asbestos and smoking on asbestos-induced lung cancer, or ethanol and smoking on ethanol-induced esophageal cancer (Burkart 2001, Kamp *et al* 2001).

Brooks *et al* (1989) conducted studies to determine the effects of combined exposure of beryllium ($BeSO_4$) and radiation (X rays), in Chinese hamster ovary cells. The frequency of

chromatid-type exchanges and total aberrations was significantly higher in the radiation plus beryllium-exposed cells, compared to cells exposed either to beryllium or to X-rays only. Furthermore, when beryllium was combined with the X-ray exposure, the interactive response, in term of chromosome aberrations, was predicted by a multiplicative model and was significantly higher than predicted by an additive model (Brooks *et al* 1989).

It has been observed that $CuCl_2$ alone is not mutagenic in Escherichia Coli or in Chinese hamster cells, but exposure of Escherichia Coli to $CuCl_2$ during UV-irradiation results in enhancement of UV-mutagenesis. This co-mutagenic effect seems to be due to increased DNA damage by the combined treatment of UV and Cu (II), compared with UV or Cu (II) alone. These results suggested that UV-irradiation of Cu (II) causes a photoactivation, enabling it to produce free radicals, possibly by reacting with dissolved oxygen. It was unlikely that the co-mutagenic effect of Cu (II) plus UV could be due to hydroxyl radical (formed via a Fenton reaction) involving Cu (II) and UV-generated H_2O_2, since no H_2O_2 was detectable in aqueous medium after UV irradiation, and catalase did not block the DNA damage (Rossman 1989).

Vitvitskii *et al* (1996) demonstrated that combined exposure of gamma rays and Cr (VI) can have enhancing effects on the outcome, compared to the single agent (either gamma rays or metal alone). In their studies, the effect of chromium ions (VI) on the mutagenic activity of gamma rays were assessed by a micronucleus test in mouse bone marrow polychromatocytes. They observed that chromium ions (VI) enhanced mutagenic effects of gamma rays in both acute and chronic experiments (Vitvitskii *et al* 1996).

Hanna *et al* (1997) studied the combined effects of zinc and radiation. Their work was based on the fact that in mammals transitory zinc deficiency during embryogenesis has a negative influence on foetal development, and similar to Zn deficiency, maternal exposure to high doses of maternal irradiation during late stages of embryogenesis results in malformations. They showed that maternal zinc deficiency and exposure of high doses of ^{137}Cs gamma-irradiation,

during late stages of embryogenesis, had an enhancing effect on the frequency of fetal malformations in mice *in vivo* (Hanna *et al* 1997).

It has been shown that neither exposure of mice to lead (in the drinking water) nor chronic gamma-irradiation of animals induces single-stranded DNA breaks in thymocytes (Chernikov *et al* 1998). Interestingly, combined exposure of acute gamma-irradiation (1 and 4 Gy) of mice, previously treated with lead, caused an inhibition of repair of radiation-degraded DNA in thymocytes, and an increase in the level of DNA lesions detected in erythroblasts of bone marrow, detected by the micronuclear test method. This work reinforces the findings that combinations of metals and radiation are likely to induce DNA damage, due to their synergistic combined effects (Chernikov *et al* 1998).

In order to clarify the mechanism underlying the combined effects of metals and radiation, Takahashi *et al* (2000) studied the effect of arsenite or nickel (II) on the repair of DNA double-strand breaks (DSBs) induced by gamma-irradiation in Chinese hamster ovary (CHO) cells, using pulsed-field gel electrophoresis. After arsenite or nickel chloride exposure for 2 hours, cells were irradiated with gamma rays at a dose of 40 Gy, and the numbers of DNA DSBs were calculated immediately after irradiation, as well as at 30 minutes post irradiation. Both arsenite and nickel (II) repressed the repair system of DNA DSBs, such as rejoining of DSBs, in a dose dependent manner. Their work has provided another example of combined exposure underlying the effects of arsenite and ionizing radiation in our environment (Takahashi *et al* 2000).

Anan'eva *et al* (2000) showed that combined exposure of rats to low-dose irradiation and heavy metal (Cu^{2+}) ions caused significant accumulation of the free-radical products proportional to exhausting antioxidant and oxidizing-reduction potential in various organs and tissues, compared to either radiation or metal only. Radiation was observed to be the primary harmful

factor for the brain, spleen, lungs, and blood plasma, whereas the liver and heart muscle were affected with Cu^{2+} at first (Anan'eva *et al* 2000).

Osipov *et al* (2000) studied the combined effects of long-term low-dose gamma radiation and/or cadmium for 20, 40 and 80 days in mouse in mice. The maximal level of DNA-protein cross-links in cells of lymphoid organs of mice upon exposure to gamma radiation or cadmium was recorded on the 40^{th} day, and under combined exposure on the 20^{th} day of the experiment. Therefore, combined effects, in their experiments, reduced the number of days at which the maximal damage level was observed, compared to either radiation exposure, or metal exposure only (Osipov *et al* 2000).

Zaichkina *et al* (2001) studied the induction of cytogenetic damage by combined action of heavy metal (lead and cadmium) salts and chronic/acute gamma irradiation in bone marrow cells of mice and rats. They showed that the chronic exposure of rats and mice *in vivo* to gamma-irradiation induced an adaptive response. The salts of heavy metals, given as a supplement to the diet of the rats, increased the cytogenetic damage to the non-irradiated animals and enhanced the effect of chronic and acute gamma-irradiation. However, the salts of heavy metals surprisingly decreased the cytogenetic adaptive response provoked by chronic gamma-irradiation (Zaichkina *et al* 2001).

Liess *et al* (2001) studied the combined effects of ultraviolet-B (UV-B) radiation and food shortage on the sensitivity of the Antarctic amphipod Paramoera Walkeri to copper. They exposed the Antarctic amphipod Paramoera Walkeri to environmentally realistic levels of copper, UV-B radiation, and food shortage. Exposure to copper for 21 days in the absence of any additional stressors (food present, no UV-B) showed a lowest observable effective concentration (LOEC) of greater than 100 µg Cu/L. Exposure to copper and UV-B in combination, with no shortage of food, resulted in a LOEC of 45 µg Cu/L. Exposed to copper and UV-B, with shortage of food, gave a LOEC of 3 µg Cu/L. Therefore, the combination of

environmental stress from exposure to UV-B radiation and shortage of food increases the sensitivity of Paramoera Walkeri to copper more than 30-fold. A possible explanation of this phenomenon could be due to the increased metabolic energy that the Paramoera Walkeri would require for defence mechanisms, in response to the concomitant presence of toxicants agents (Liess *et al* 2001).

It has been shown that combined exposure of UV-B radiation and metals can exert a synergistic (negative) action on the growth of wheat seedlings. In these studies, the dual stress of UV-B and Cd^{2+} exposure on the growth of wheat (Triticum Aestivum L.) seedlings was observed. The combined stress of UV-B and Cd^{2+} resulted in reduction of biomass yield, growth, chlorophyll content, changes in protein, changes in free amino acid content, changes in starch content and total reducing sugar content. This supports the assumption that UV-B may have a regulatory role apart from a damaging effect and that an increased UV-B environment is likely to increase this regulatory influence of UV-B radiation, and the adverse effects of one stress (Cd^{2+}) could be modulated in the presence of other stresses (UV-B radiation) (Shukla *et al* 2002).

Chiu *et al* (2003) investigated the combined effect of germanium oxide (GeO_2) and X-radiation on cell viability. Cells were exposed to a range of GeO_2 concentrations for 12 hours, followed by 1 Gy X irradiation. They observed a synergistic cytotoxic effect for the combined treatment with a dose-dependent reduction in cell viability. Survival curves displayed a 2.3- and 2.75-fold increase in radiosensitivity, for 50% cell death, respectively in the presence of 5 and 15 mM GeO_2. Interestingly, the combined treatment dramatically increased the production of reactive oxygen species (ROS) in the cells. Furthermore, the combination of GeO_2 and X radiation significantly increased the frequency of DNA double-strand breaks (DSBs), and reduced the efficiency of DNA repair. Therefore, it can be concluded that combined exposure of GeO_2 and X irradiation increases DNA DSBs and cell death (Chiu *et al* 2003).

In another experiment, they treated CHO cells with several metal salts, such as $NiCl_2$, $NaAsO_2$, $ZnCl_2$, $CdCl_2$, $CuCl_2$ and potassium antimonyl tartrate for 2 hours, and irradiated with γ-rays at the same dose of 40 Gy in order to induce DNA-DSB. The incidence of DNA-DSB was determined by an electrophoresis technique, immediately after irradiation and following a 30-minute repair period. The DNA-DSB repair was significantly inhibited by exposure to Ni, Cu, Zn, As, Sb, and Cd compounds at concentrations of 200, 2.0, 0.4, 0.08, 0.55, and 1.0 mM, respectively. At these concentrations, the cell viability was over 50% for all the chemicals, suggesting that all of these compounds inhibited the repair of radiation-induced DNA-DSB at the concentrations where the acute cytotoxicity was relatively low. Furthermore, the plating efficiencies for As, Sb, and Zn compounds, at these concentrations, were higher than 10%, suggesting that these chemicals inhibited DNA-DSB repair at relatively low concentrations where some of the cells were capable of proliferation (Takahashi *et al* 2003).

Burns *et al* (2004) established the dose-response relationship for dietary sodium arsenite as a co-carcinogen with ultraviolet radiation (UVR) in a mouse skin model. Hairless mice, at 21 days of age, were continuously fed with sodium arsenite, at concentrations of 0.0, 1.25, 2.5, 5.0, and 10 mg/L in drinking water. When mice were 42 days of age, solar spectrum UVR exposures were applied three times weekly to the dorsal skin of the mice, until the experiment ended at 182 days. There was no development of tumors in untreated mice and mice fed only with arsenite. The carcinoma yield in mice exposed only to UVR was 2.4 ± 0.5 cancers/mouse at 182 days. Dietary arsenite clearly enhanced the UVR-induced cancer yield in a pattern consistent with linearity up to a peak of 11.1 ± 1.0 cancers/mouse at 5.0 mg/L arsenite. These results demonstrated that arsenite is a cancer-enhancing agent, when combined with UVR exposure, and this enhancement clearly follows a pattern consistent with linearity (Burns *et al* 2004).

1.3.5 Reductive Effects

Lotareva (1990) demonstrated a clear example of antagonistic effect, due to the combined action of chemicals and radiation on bacteria. The mutagenic interaction between the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and ultraviolet irradiation was studied in repair-competent and excision-deficient strains of Bacillus Subtilis. Pre-exposure to low doses of MNNG followed by treatment with low and intermediate doses of UV light increase the resistance of Bacillus Subtilis to UV radiation, therefore showing an antagonistic effect. It is likely that pre-exposure to MNNG leads to induction of repairing enzymes, with UV damage being controlled by adaptive response genes (Lotareva 1990).

Vitvitskii *et al* (1996) showed that combined exposure of gamma rays and metals can have enhancing effects as well as reducing effects on the outcome, compared to the single agent (either gamma rays or metal alone). In their studies, the effects of lead ions (II) or (III) on the mutagenic activity of gamma rays were assessed by a micronucleus test in mouse bone marrow polychromatocytes. Acute and chronic multiple actions of the combined effects were investigated. In the acute experiments lead ions (II) below 15 mg/kg body weight decreased the number of gamma-ray-induced micronuclei, while higher doses increased it. Chronic combined action of lead ions (III) and gamma rays inhibited mutagenic activity of radiation. Furthermore, this work has also provided strong evidence that metal ions, such as lead ions (II), can have a different behaviour, when combined with radiation, at different doses used (Vitvitskii *et al* 1996).

Privezentsev *et al* (1996) provided another example of protective effect due to combined action of radiation and metals. They detected the combined effects of Cd and gamma-irradiation on DNA damage and repair in lymphoid tissues of mice, using single-cell gel assay. Single injection of $CdCl_2$, 2 hours prior to irradiation, resulted in an increase of DNA lesions in peripheral blood lymphocytes, when compared to non-injected animals. However, the same

treatment 48 hours prior to irradiation, decreased DNA damage in peripheral blood lymphocytes and splenocytes, compared to untreated mice. In thymocytes a maximal protective effect of Cd was observed when mice were irradiated 24 hours after injection. The protective effect observed was due to a decrease in the initial level of DNA damage in thymocytes, as well as acceleration of DNA repair in peripheral blood lymphocytes and splenocytes (Privezentsev *et al* 1996).

Katsifis *et al* (1996) showed another example of reductive effects. In their studies, individual treatments of human lymphocytes with Ni (II) (0.5-25 μM), Cr (VI) (0.65-1.30 μM), UV-light or X-rays induced sister chromatid exchanges (SCEs) in a dose-dependent manner, and combined exposure of Ni (II) with Cr (VI), UV-light or X-rays acted together antagonistically.

Selezneva *et al* (2004) detected an example of time-dependent antagonism, by exposing grain to a long-term combined action of medium-wave UV-radiation and cadmium. The influence of cadmium on the UV-radiation induced inhibition of the vegetative mass development of grain. This was observed to be dependent on the duration of stress: with increase in the exposure time, the ability of cadmium to reduce the negative effects of irradiation decreases.

Recent studies focussed on radiotherapy, have demonstrated that metals, such as zinc, can provide radioprotection *in vivo* (Dani and Dhawan 2005). Even though irradiation with radioiodine (^{131}I) is used for the treatment of various thyroid disorders, it is likely that ^{131}I might cause some unfavourable effects on antioxidative enzymes present in red blood cells (RBCs). Zinc has also provided protection, as it preserves the integrity of cells under certain toxic conditions. An increase in the activity of glutathione reductase (GR), superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) in the lysates of erythrocytes of rats was seen after two days of exposure from ^{131}I radiation. Interestingly, zinc supplemented to ^{131}I-exposed rats, reduced the adverse effects caused by ^{131}I on the levels of antioxidative enzymes. This work proved that ^{131}I induces significant oxidant/antioxidant

changes in RBCs and that zinc could be a good candidate for radioprotection (Dani and Dhawan 2005).

1.3.6 Adaptive Response

The adaptive response is a phenomenon characteristic of an increased resistance in cells or organisms exposed to a priming dose, followed by exposure to a challenging dose. Several authors have shown that combined exposure of two agents, in different times, can lead to an adaptive response.

Cortes *et al* (1990) showed that adaptive responses can occur in human lymphocytes conditioned with hydrogen peroxide before irradiation with X-rays. In their work, cultured human lymphocytes were first exposed to a low "conditioning" dose of hydrogen peroxide and later irradiated with a "challenge" dose of 1.5 Gy of X-rays, in order to analyse the induction of an adaptive response to oxidative damage. There were two different observable behaviours of the human lymphocytes, depending on the experiment schedule.

1. Cells that underwent a repeated exposure to H_2O_2 before irradiation, showed the expected yield of aberrations from the combined exposure of H_2O_2 and X-rays.

2. In contrast, a significant reduction in X-ray-induced chromosome damage was observed, when H_2O_2 was given as a single 30-minute pulse 24 hours after preparing the cultures, and the lymphocytes were then exposed to X-rays at 48 hours (Cortes *et al* 1990).

Perminova *et al* (2000) demonstrated that increased viability of a human rhabdomyosarcoma cell line to challenge doses of $NiSO_4$ ($10^{(-5)}$-$10^{(-3)}$ M) was produced when cells were pre-irradiated with low doses of gamma-radiation (10-14 cGy). The observed adaptive response was similar to the radio-adaptive response in human fibroblasts, pre-treated with low doses of gamma-radiation and challenge doses of the same mutagen. Pre-treatment with low concentration of $NiSO_4$ induced in human fibroblasts an increased resistance of DNA to the

treatment with challenge doses of gamma-radiation and stimulated DNA repair synthesis after treatment with $NiSO_4$ and 4-nitroquinoline-1-oxide. This study confirmed the existence of "cross-adaptation" in experiments using $NiSO_4$ (Perminova *et al* 2000).

1.4 Genomic Instability

1.4.1 General Information

It has been shown that cells surviving radiation may produce descendants, which have a high incidence of genetic aberrations arising "*de novo*", many generation later. The most common types of damage usually observed are gene mutation, delayed cell death/lethal mutations, micronucleus formation and cellular transformation. These types of alterations have been named as "Radiation-Induced Genomic Instability" (RIGI) (Seymour and Mothersill 1986, Kadhim *et al* 1992, 1995, Mothersill *et al* 1996, Little 1998). These effects are regarded as delayed effects resulting from transmissible genomic instability, which is induced at frequencies greater than mutations that spontaneously occur in nature (Morgan *et al* 1996, Wright 1998). The endpoints of RIGI are phenotypically different, but they all occur in unirradiated progeny of irradiated cells and they are permanent (Morgan *et al* 1996, Little 1998, Mothersill and Seymour 1998, Wright 1998). RIGI was a surprising finding for two main reasons: 1) RIGI occurs many generations after the initial insult and this damage cannot be attributed to the direct action of radiation, but as a consequence of radiation (Mothersill and Seymour 1998); 2) RIGI is a relatively high frequency event occurring in 10-50% of cells (Ullrich and Davis 1999).

1.4.2 Endpoints of Genomic Instability

The most common endpoints of genomic instability are listed below and will be separately described.

1. Lethal Mutations or Delayed Cell Death
2. Chromosomal Instability
3. Gene Mutation

4. Gene Amplification

5. Micronuclei

6. Malignant Transformation

1.4.2.1 Lethal Mutations

The term of lethal mutations was given, by Seymour and Mothersill (1986), who first detected them, because a cell that was able to proliferate for several generations and then suddenly failed to divide normally, was believed to have undergone a lethal mutation. Lethal mutations, which are also known as delayed reproductive (cell) death, are detected by a reduction in the ability of cells to form colonies after irradiation (Seymour and Mothersill 1986, Little *et al* 1990 and O'Reilly *et al* 1994). These authors demonstrated that such delayed death was maintained for 70-population doublings, after the cells had been exposed to radiation (Seymour and Mothersill 1986, Seymour and Mothersill 1992). Low dose radiation did not seem to cause damage to the clones, which appeared to be normal and healthy after exposure. These clones, which were then isolated and grown on multi-well dishes all showed higher than expected levels of division failure (Seymour and Mothersill 1992). Early investigations that examined the effects of high doses of radiation on cell populations derived from irradiated cells showed persistent abnormalities such as prolonged doubling time leading to the formation of small colonies and reduced plating efficiency (Nias and Lajtha 1965, Westra and Barendsen 1966).

1.4.2.1.1 Radiation-Induced Lethal Mutations

Lethal mutations have been demonstrated to occur in different types of cells and with both high and low liner energy transfer (LET) radiation (Seymour *et al* 1986, Chang and Little 1992, Seymour and Mothersill 1992, Kadhim *et al* 1992, O'Reilly *et al* 1994). It was shown that the initial expression of lethal mutations was dose-dependant but in later passages, once a

threshold has been exceeded, this dose dependency was lost (O'Reilly *et al* 1994). Furthermore, it was deduced that responses might be induced in surviving cells post-irradiation, which may lead them to apoptosis. It could also be possible that there is a lethal gene mutation, which can cause the progeny to reduce their divisions successfully, once it is activated (O'Reilly *et al* 1994). It was later suggested that when lethal mutations are induced they can remove damage from the genome, but this phenomenon does not occur in early carcinogenesis (Mothersill *et al* 1996). This was also deduced by the observation that neoplastically transformed cells and cells treated with nitrosamine, which is carcinogen, showed a reduction in the lethal mutation effect (Mothersill *et al* 1996). It was later postulated that there could be a mutation threshold in cellular systems whereby cells surviving irradiation, which also have some error/s, are flagged but not killed (Seymour and Mothersill 1997). If further DNA damage can alter this threshold, the mutational load is rearranged to a lower acceptance. This means that flagged cells may become apoptotic and this cell termination is reflected by the effect of lethal mutation (Seymour and Mothersill 1997).

Lyng *et al* (1996) showed that lethal mutations, which include all types of cell death, such as death due to chromosomal aberrations or rearrangements by a controlled apoptotic response, had a persistent expression of morphological abnormalities in the distant progeny of irradiated cells, which was consistent with the *de novo* manifestation of apoptosis many generations after initial irradiation. These changes occurred at a higher frequency in the irradiated cultures and many of these abnormalities were incompatible with further division of the cells (Lyng *et al* 1996; Mothersill *et al* 1998).

Also Limoli *et al* (1998) showed a positive and important correlation between lethal mutation effect and apoptosis, as in their study, the portion of cells dying by apoptosis or necrosis could completely account for the persistence of the lethal mutation effect. Anther study, carried out by examining tissue cultures of human ureter, demonstrated genetic variations in the human

response to the induction of apoptosis (Mothersill *et al* 1999). There was a hypersensitive response to low doses with considerable amounts of necrosis and apoptosis. This strongly suggested that genetic variation might play a role in determining cellular response to radiation. These delayed appearances of *de novo* apoptotic cells may be a protective mechanism eliminating the genome of potential carcinogenic initiation lesions, following the hypothesis that lethal mutations reflect a cleansing mechanism (Mothersill and Seymour 1998).

1.4.2.1.2 Chemical/Metal-Induced Lethal Mutations

It was also demonstrated that lethal mutations could be induced by ethyl methanesulfonate (Stamato *et al* 1987), cadmium (Lyons-Alcantara *et al* 1995), and cadmium and nickel (Mothersill *et al* 1998). Past studies have focused their attention on implant failure and inflammatory response, without taking into consideration the long-term consequences of wear debris on cellular viability and the eventual induction of genomic instability. It has been shown that genomic instability can be induced by heavy metals and other environmental toxins (Mothersill *et al* 1998, Coen *et al* 2001). Coen *et al* (2001) demonstrated that cadmium and nickel can produce delayed effects in human cells *in vitro*, which are characteristic of genomic instability. It was found that the effects of the chemicals occurred even at levels where no acute toxic effects can be observed. In their experiments, cell populations exposed for only 1 hour or 24 hours, to either cadmium or nickel, were expanded for several months, and the yield of chromosomal aberrations and cell loss due to lethal mutations did not decrease. Genomic instability was demonstrated by persistent induction of cytogenetic abnormalities and delayed cell death in progeny of cells many generations after exposure. The consequences of this genomic instability are not yet known but it is possible that many of the systemic symptoms associated with exposure to low concentrations of these metals could involve delayed

expression of cellular damage (Coen *et al* 2001). Once induced, genomic instability is permanent and it is well known that an unstable genome can lead to cancer (Emerit 1994).

Kilemade and Mothersill (2003) have also shown that chemicals can induce delayed reproductive cell death. The purpose of their work was to investigate if 2,4-Dichloroaniline (2,4-DCA) (CASRN: 554-00-7), a primary aromatic amine and suspected genotoxican, could induce delayed reproductive cell death (lethal mutations) in a teleost cell line, CHSE-214. It was found that CHSE-214 cells surviving 2,4-DCA exposure had heritable lethal defects, which were detected only after numerous apparently successful divisions. However, delayed cell death expression was not dose-dependent. Furthermore, CHSE-214 cell growth kinetics post-exposure showed that the apparent reduced growth rate of the cells was due to reduced numbers of reproductively viable cells in the population (Kilemade and Mothersill 2003).

These results proved that lethal mutations are also induced in the surviving progeny by a wide range of genotoxic agents, and not only by radiation.

1.4.2.2 Chromosomal Instability

Weissenborn and Streffer (1989) observed the first transmissible chromosomal instability in X-irradiated two-cell mouse embryos, whereby new aberrations were noticed in the subsequent mitoses post irradiation. Two types of radiation-induced chromosomal instability (RICI) have been characterised:

1) The first RICI is characterised by the presence of non-clonal aberrations that are usually lethal in the clonal descendants;

2) The second RICI is characterised by the appearance of chromosomal instability that is clonal and the aberrations are not usually lethal (Morgan *et al* 1996, Mothersill and Seymour 1998).

Exposure to alpha-particles, carried out in CFU-A derived colonies (produced by murine stem cells) showed that 40-60% of these colonies had karyotypic abnormalities and about 50% of

metaphases within a colony had single or multiple, non-identical aberrations. This non-clonality and high frequency of cells exhibiting chromatid aberrations arose *de novo* in cells that were not directly exposed by radiation, but were descendants of irradiated cells. Surprisingly, in this study X-rays did not produce the expected high frequency delayed aberrations (Kadhim *et al* 1992).

However, in contrast to these findings, other studies have shown that exposure of human dermal fibroblasts to irradiation with heavy ions revealed that chromosomal instability was clonal (Sabatier *et al* 1992). Since other researchers reported that X-rays induced genomic instability and that it was clonal, it is possible that these discrepancies were due to the measurement of different manifestations of chromosomal instability (Holmberg *et al* 1993, Marder and Morgan 1993). Holmberg *et al* (1993) examined X-irradiated human T-lymphocyte chromosomes by G-banding, which allows for the measurement of chromosomal rearrangements, whereas Marder and Morgan (1993) used fluorescent *in situ* hybridisation (FISH), which detects rearrangements accumulating over time, as well as arrangements arising *de novo* in their human-hamster hybrid cells. All these results demonstrated that chromosomal instability is detectable, in unexposed descendant cells, many generations after the radiation insult.

Morgan *et al* (1996) showed that there were differences involved in the induction of genomic instability due by different types of radiation. This could reflect the susceptibility of specific cell types to chromosomal instability, which may be genetically predetermined (Morgan *et al* 1996). Kadhim *et al* (1994) also observed that chromosomal instability in human bone marrow only occurred in half of the examined examples. It was suggested that this type of inter-individual variation was caused by genetically determined differences in the inducibility of chromosomal instability (Kadhim *et al* 1994).

Watson *et al* (1997) showed this variation both *in vitro* and *in vivo*. They examined *in vitro* chromosomal instability in haemopoietic stem cells, from three different strains of mice, *CBA/H*, *DBA/2* and *C57BL/6* mice. This study showed that *CBA/H* and *DBA/2* were sensitive to the induction of chromosomal instability, whereas *C57BL/6* mice were resistant to this induction. It was later determined which was the dominant phenotype through crossing the resistant strain of mouse with both sensitive strains. Cytogenetic analysis of stem cells derived from the F_1 hybrid bone marrow demonstrated that resistance was the dominant phenotype (Watson *et al* 1997). They also irradiated male haemopoietic stem cells *in vitro* and transplanted these cells into female recipients demonstrating the *in vivo* transmission of chromosomal instability (Watson *et al* 1996). Chromosomal instability was detected in the repopulated haemopoietic system for up to one year post-transplantation. It was deduced that the persistent production of unstable chromosome aberrations post-transplantation could reflect long-lived lesions that are transmitted from the irradiated repopulating stem cells and maintained in the new stem cell pool (Watson *et al* 1996).

The induction of long-term instability in human peripheral lymphocytes has also been proven recently (Anderson *et al* 2000 and Kadhim *et al* 2001). These studies showed increased complex aberrations observed using FISH techniques (Anderson *et al* 2000). Also a significant proportion of aberrant cells were observed to be mainly of the chromatid type aberration, 12-13 population doublings post exposure (Kadhim *et al* 2001). The induction of chromosome instability was also shown to be induced by dye-bromodeoxyuridine photolysis treatment and was shown to persist for several generations (Limoli *et al* 1998). Radiation-induced chromosomal instability is not unique to any particular cell line or quality of ionizing radiation and it has been also demonstrated both *in vitro* and *in vivo* by other authors (Morgan *et al* 1996 and Limoli *et al* 2000). Furthermore, Duesberg and Rasnick (2000) have recently shown the endpoint of aneuploidy and its relevance to the genetic instability of cellular cancer.

1.4.2.3 Other Endpoints of Genomic Instability

Genomic instability includes other endpoints, as well as lethal mutations and chromosomal instability, such as gene mutation and amplification, micronucleus formation and malignant transformation (Morgan *et al* 1996, Limoli *et al* 1997, Little 1998). Genomic instability, in either mentioned endpoints, has been associated with the progression of carcinogenesis (Loeb 1991).

1. Gene mutation. The hypoxanthineguanine-phospho-ribosyl-transferase (Hprt) X-chromosome locus was used as a marker gene for instability in order to assess its mutation frequency. Chang and Little (1992) showed that constantly high levels of spontaneous mutations at the Hprt locus occur up to 100 population doublings post-exposure to radiation (Chang and Little 1992, Harper *et al* 1997, Limoli *et al* 1997, Little 1998).

2. Gene amplification. This is an event normally observed in cells resistant to particular selective agents. Cells are generally analysed for their capacity to amplify the CAD gene in response to N-phosphonacetyl-L-aspartate (PALA) selection (Morgan *et al* 1996, Limoli *et al* 1997).

3. Micronuclei are created by either loss of whole chromosomes or a portion of chromosomes from daughter nuclei of a cell (Tucker and Preston 1996). An increased number of micronuclei was shown to occur in the progeny of irradiated cells (Jamali and Trott 1996, Manti *et al* 1997).

4. Malignant transformation. This phenomenon can be revealed by the identification of intestinal alkaline phosphase (IAP) as p57/150, where expression is linked with tumorigenicity (Mendonca *et al* 1993).

1.4.3 Correlation between Different Endpoints

It is still unclear if there is any link between the endpoints of genomic instability. Mothersill *et al* (1998) who looked at non-clonal aberrations, such as chromatid breaks, found no direct correlation between the direct production of chromosomal instability and lethal mutations. However, it was suggested that unstable aberrations that usually result in apoptotic cell death might underlie the lethal mutation occurrence (Mothersill *et al* 1998). In contrast to these results, Limoli *et al* (1997) who quantified clonal aberrations such as rearrangements instead, showed that chromosomal instability was significantly correlated to lethal mutations/reproductive death suggesting that the formation of abnormal chromosomal rearrangements may contribute to reproductive failure (Limoli *et al* 1997). These rearrangements were clonal and therefore were probably directly induced by radiation. The discrepancies between the results of these two groups could be due to the quantification of different manifestations of chromosomal instability. It has been observed that persistent increase in delayed mutations at the Hprt locus may be associated with the lethal mutation/delayed reproductive death phenotype (Chang and Little 1992). It was then hypothesised that chromosomal mechanisms, such as ploidy may change, or a persistent production of chromosomal aberrations may give rise to delayed mutations, as well as delayed reproductive death (Chang and Little 1992).

Lyng *et al* (1996) have shown that lethal mutation/delayed death is linked with the *de novo* production of apoptosis using electron microscopy. Mothersill *et al* (1999) also found increased expression of apoptotic cells in tissue cultures of human ureters. Kadhim *et al* (1995) also observed apoptotic cells in human bone marrow cells post irradiation. Lethal mutations/delayed reproductive death has also been positively related with the formation of micronuclei (Manti *et al* 1997).

Miura *et al* (2005) have shown that accumulated chromosomal instability in murine bone marrow mesenchymal stem cells (BMMSCs) results in malignant transformation. They tried to test a hypothesis that tumorigenesis may originate from spontaneous mutation of stem cells. In their study, they observed that murine BMMSCs, after many passages, had unlimited population doublings and progress to a malignant transformation state, leading in fibrosarcoma formation *in vivo*. Furthermore, this transformation system could provide an ideal system to elucidate the mechanisms of carcinogenesis in stem cells and try to screen anti-sarcoma drugs.

1.4.4 Induction of Genomic Instability

It has been suggested that double strand breaks or their mis-repair is the critical lesion, which leads to the induction of the genomic instability phenotype (Chang and Little 1992, Seymour and Mothersill 1992). Chinese hamster ovary (CHO) cells surviving radiation exhibited the delayed death phenotype (Chang and Little 1992). Exposure of CHO cells to a specialised restriction endonuclease (HinfI), which binds to DNA producing double strand breaks was also effective at inducing delayed death. This leads to the deduction that DNA double strand breaks were associated with the induction of the lethal mutation phenotype in CHO cells (Chang and Little 1992). Furthermore, DNA double strand breaks are known to induce chromosomal aberrations, such as chromatid breaks (Marder and Morgan 1993, Morgan *et al* 1996).

Limoli *et al* (1997) used five DNA strand-breaking agents to see if they were able to induce genomic instability in human-hamster hybrid cells, taking in to consideration chromosomal instability as an endpoint (Limoli *et al* 1997). These agents were bleomysin, neocarzinostatin, hydrogen peroxide, restriction endonucleases and ionizing radiation. Results showed that only bleomysin, neocarzinostatin and ionizing radiation induced delayed chromosomal instability. This suggested that agents able to produce DNA double strand breaks are also capable of inducing genomic instability, although double strand breaks might not necessarily be the most

important lesion involved in the induction of genomic instability. Morgan *et al* (1998) also demonstrated that DNA double strand breaks lead to delayed chromosomal rearrangements. Limoli *et al* (1999) showed that cells substituted with bromodeoxyuridine (Brd-U), which modifies cellular DNA and enhances the sensitivity of cells to the action of radiation, can increase the number of chromosomal instability, compared to unsubstituted control cells. This suggests that DNA is at least one of the critical targets important for the induction of delayed chromosomal instability.

Somodi *et al* (2006) have recently carried out a study to determine the role of single (SSB) and double strand break (DSB) repair in the induction and propagation of radiation-induced instability. They used two defined hamster cell lines with known DNA repair deficiencies in DSB repair (XR-C1) and base excision repair (EM-C11) and the parental wild-type line (CHO-9) were used. The rate of micronucleus formation, apoptosis and survival were measured at 0, 7 and 14 days after X-ray radiation. An enhanced rate of production of damaged cells was observed in wild type and the repair deficient mutants after X-ray irradiation. These results were cell type, dose and time-dependent. All cells demonstrated delayed death up to day 14 after irradiation as well as increased apoptosis. Micronuclei formation was not significantly increased in the wild-type cells, but it was in the mutant cells. They results showed that there was an increase in damage in the SSB deficient cell line, and SSB and/or oxidized base damage play a major role, rather than DSB, in radiation induced instability.

Telomeres have also been linked to genomic instability. Day *et al* (1998) showed that interstitial telomere repeat-like sequences contribute to the delayed induction of chromosomal instability in human-hamster hybrid cells post-exposure to X-rays. This study led to the conclusion that interstitial bands can function as hot spots for recombination, which promote chromosome rearrangements directly after exposure to DNA damaging agents and in the

progeny of treated cells. This work suggested that DNA breaks are necessary but not sufficient to induce the genomic instability phenotype.

Epigenetic mechanisms may be involved in the phenotype of genomic instability, which is pronounced at low doses, indicating the importance of non-nuclear targets, the local environment and neighbouring cells in determining genomic instability (Mendonca *et al* 1993, Mothersill and Seymour 1997, Watson *et al* 1997, Limoli *et al* 2000). Others have shown that cells exposed to superoxide, exhibit high levels of cytogenetic aberrations, especially of the chromatid type (Emerit 1994, Duell *et al* 1995). This suggests that oxy-radicals may be the epigenetic factor that initiates and perpetuates genomic instability.

Clutton *et al* (1996) supported the oxy-radical theory, by demonstrating that the production of oxidative stress was particularly active in cultures derived from irradiated (but not unirradiated) bone marrow. In this work, bone marrow stem cells were exposed to 12-O-tetradecanoylphorbal-13-acetate (TPA), a stimulator of a superoxide generating electron transport chain, to iron and ascorbate, which generate reactive oxygen species and elicits lipid peroxidation. There was an increase of superoxide production after exposure of cells to TPA, increased rates of lipid peroxidation in cells challenged with iron or ascorbate before irradiation and increased free radical activity, which compromise the integrity of the DNA. Watson *et al* later (1997) demonstrated that between different strains of murine haemopoietic stem cells there was a genetic predisposition to α-particle induced-chromosomal instability. The mice used in this work, which were sensitive to the induction of genomic instability, showed twice the rate of superoxide production than mice resistant to the induction of genomic instability (Watson *et al* 1997). Mothersill *et al* (1998) showed that agents causing oxidative stress such as cadmium and nickel can induce lethal mutations.

Rugo and Schiestl (2004) also supported the oxy-radical theory. In their work, intracellular levels of reactive oxygen species (ROS) in human lymphoblast clones derived from

individually X-irradiated cells were monitored for about 55 generations after exposure. It was found that some clones, derived from irradiated cells, had an increase in dichlorofluorescein (DCF) fluorescence at various times. Cells with abrogated TP53 expression had a reduced oxidant response and flow cytometry analysis of clones, with increased fluorescence, showed no increases in the sub-G1 fraction nor decreased cell viability, compared to nonirradiated clones, suggesting that increased levels of apoptosis and cell death were not present. The oxidative stress response protein heme oxygenase 1 (HO1) was induced in some cultures derived from X-irradiated cells but not in cultures derived from unirradiated cells. The expression of the dual specificity mitogen-activated protein (MAP) kinase phosphatase (MPK1/CL100), which is inducible by oxidative stress (and has a role in modulating ERK signaling pathways), was also increased in the progeny of some irradiated cells. There was also an increase in the phosphorylated tyrosine content of a prominent protein band of about 45 kDa. This work supports the hypothesis that increased oxy-radical activity is a persistent effect in X-irradiated mammalian cells. Furthermore, it suggests that this might lead to changes in the expression of proteins involved in signal transduction (Rugo and Schiestl 2004).

1.4.5 Bystander Factor-Induced Genomic Instability

Medium from irradiated cells exposed to low doses of radiation has been shown to reduce the clonogenic survival and increase the level of apoptosis in unirradiated cells (Mothersill and Seymour 1997, Seymour and Mothersill 1997, 2000). Lyng *et al* (2000) demonstrated that medium from irradiated human keratinocytes (HPV cells), which was transferred to unirradiated keratinocyte cells, caused a rapid calcium fluxes (within 30 seconds) and loss of mitochrondrial membrane potential, both associated with the initiation of apoptosis. A consistent reduction in clonogenic survival and an increase in the number of apoptotic cells 48 hours after the transfer of the medium were also observed (Lyng *et al* 2000). This work showed

that a factor is released in the medium by the irradiated cells, which is like an indicator for cell death. According to Limoli *et al* (1998), these dying cells give a source of oxidative stress. It was suggested that a "bystander factor", which is released by the cells directly damaged by radiation, initiates genomic instability in cells, which appear to be normal. The oxygen species generated from these dying cells may preserve this phenotype (Limoli *et al* 1998).

Watson *et al* (2000) demonstrated chromosomal instability in the progeny of nonirradiated hemopoietic stem cells, by using a bone marrow transplantation protocol in which they transplanted a mixture of irradiated and nonirradiated bone marrow cells that were distinguishable by a cytogenetic marker. This first demonstration of a link between a bystander effect of ionizing radiation and the induction of genomic instability *in vivo* clearly poses a major challenge to the current views of the mechanisms of radiation-induced DNA damage with mechanistic implications for the health consequences of radiation exposure, particularly in the context of the induction of malignancy.

Lorimore *et al* (2003) showed genetic alterations in cells not exposed to ionizing radiation, which exhibited responses typically associated with direct radiation exposure. These effects were demonstrated in cells that were the descendants of irradiated cells [radiation-induced genomic instability (RIGI)] as well as in cells that were in contact with irradiated cells or received certain signals from irradiated cells [radiation-induced bystander effects (RIBE)]. Their work gave evidence that RIGI may be a consequence of (and in some cell systems may also produce) bystander interactions involving intercellular signalling, production of cytokines and free-radical generation.

Koturbash *et al* (2006) have recently demonstrated bystander effects *in vivo*. In their study, mice were unilaterally exposed to X-irradiation and the levels of DNA damage, DNA methylation and protein expression were estimated in irradiated and bystander cutaneous tissue. They found that X-ray exposure to one side of the animal body induced DNA strand

breaks and caused an increase in the levels of Rad51 in unexposed bystander tissue. Unilateral radiation suppresses global methylation in directly irradiated tissue, but not in bystander tissue at given time-points studied. However, they observed a significant reduction in the levels of the *de novo* DNA methyltransferases DNMT3a and 3b and an increase in the levels of the maintenance DNA methyltransferase DNMT1 in bystander tissues. The levels of two methyl-binding proteins, known to be involved in transcriptional silencing, MeCP2 and MBD2, were also increased in bystander tissue. Therefore, this study showed that irradiation can induce DNA damage in bystander tissue more than a centimeter away from directly irradiated tissues, and imply that epigenetic transcriptional regulation could be involved in the radiation-induced bystander effects.

1.4.6 Genomic Instability and Carcinogenesis

The development of cancer is a complex process triggered by physical, chemical or biological carcinogenic factors (Popescu 1994). It has been suggested that the majority of cancers occur from a single cell that has undergone the process of malignant transformation and cancer is considered to be clonal in origin (Nowell 1976, Loeb 1991). The manifestations of genomic instability, such as chromosomal instability, gene amplification, gene mutation and lethal mutations, may increase the probability of the incidence of several genetic changes in a single cell, which is necessary for its conversion to a malignant cell (Caron *et al* 1997).

Evidence for a possible link between radiation-induced genomic instability and malignancy may be determined by considering the involvement of chromosomal instability syndromes in cancer predisposition. Such chromosomal instability syndromes include Ataxia telangiectasia (AT), Fanconi anaemia (FA) and Blooms syndrome (BS). Genetic instability is an intrinsic feature of the AT phenotype. This is characteristic of increased frequency of chromatid breaks in lymphocytes from children with AT. Chromosomal aberrations have been documented in

multiple cells from AT homozygotes such as T lymphocytes, keratinocytes, fibroblasts and hepatocytes (Meyn 1997). The most common chromosomal aberrations are: chromatid breaks, chromosome gaps, dicentric chromosomes, acentric chromosomes, fragments and aneuploidy (Meyn 1997). AT homozygotes have a 30-40% lifetime risk of malignancy, the most common form being lymphomas and acute lymphocytic leukaemia. Genomic instability has also been a defined characteristic of FA. The most common aberrations observed in FA homozygotes are: single chromatid breaks and gaps, and endoreduplication. The most common malignancy in FA homozygotes is myeloid leukaemia (Meyn 1997, Wright 1999). Increased frequency of chromosome breaks, chromosome gaps and micronuclei has also been found in BS homozygotes. The most common forms of cancer in BS homozygotes are leukaemia and lymphoma (Myen 1997). Chromosomal instability syndromes found in AT, FA and BS (such as chromatid breaks) share characteristics with radiation-induced genomic instability. These similar features suggest that genomic instability, if it is genetically determined or induced by ionising radiation or induced by genotoxic agents, may produce changes within an individual cell that could lead to the beginning of cancer.

1.5 Telomeres and Telomerase

1.5.1 Telomeres

1.5.1.1 Structure and Function

Telomeres are the G-rich DNA arrays and associated proteins that cap most eukaryotic chromosomes. Their main function is to protect chromosome ends from damage and degradation (Greider and Blackburn 1996). It is known that unprotected chromosome ends can trigger checkpoint responses and recombination events, but telomeres prevent DNA sequences from being recognised as double-stranded breaks (Blackburn 2001), and may make use of the cellular machinery involved in the DNA damage response in their maintenance (De Lange and Petrini 2000). The protective function of telomeres plays an essential role in regulating the integrity of the genome and other aspects of cell physiology. Therefore, this capping function of telomeres protects from chromosomal fusion (end-to-end joining) and its consequence, which is genomic instability (Levis 1989).

Subtelomeric sequences that follow the terminal telomeric repeats are often also tandemly repeated and shared between telomeres, but can differ greatly between species. Homogeneity of tandem subtelomeric repeats within a species is apparently maintained by unequal crossing-over events (Blackburn 2001).

There are also several other proteins, which play important roles in regulating telomere length, integrity, and function (Figure 1.10).

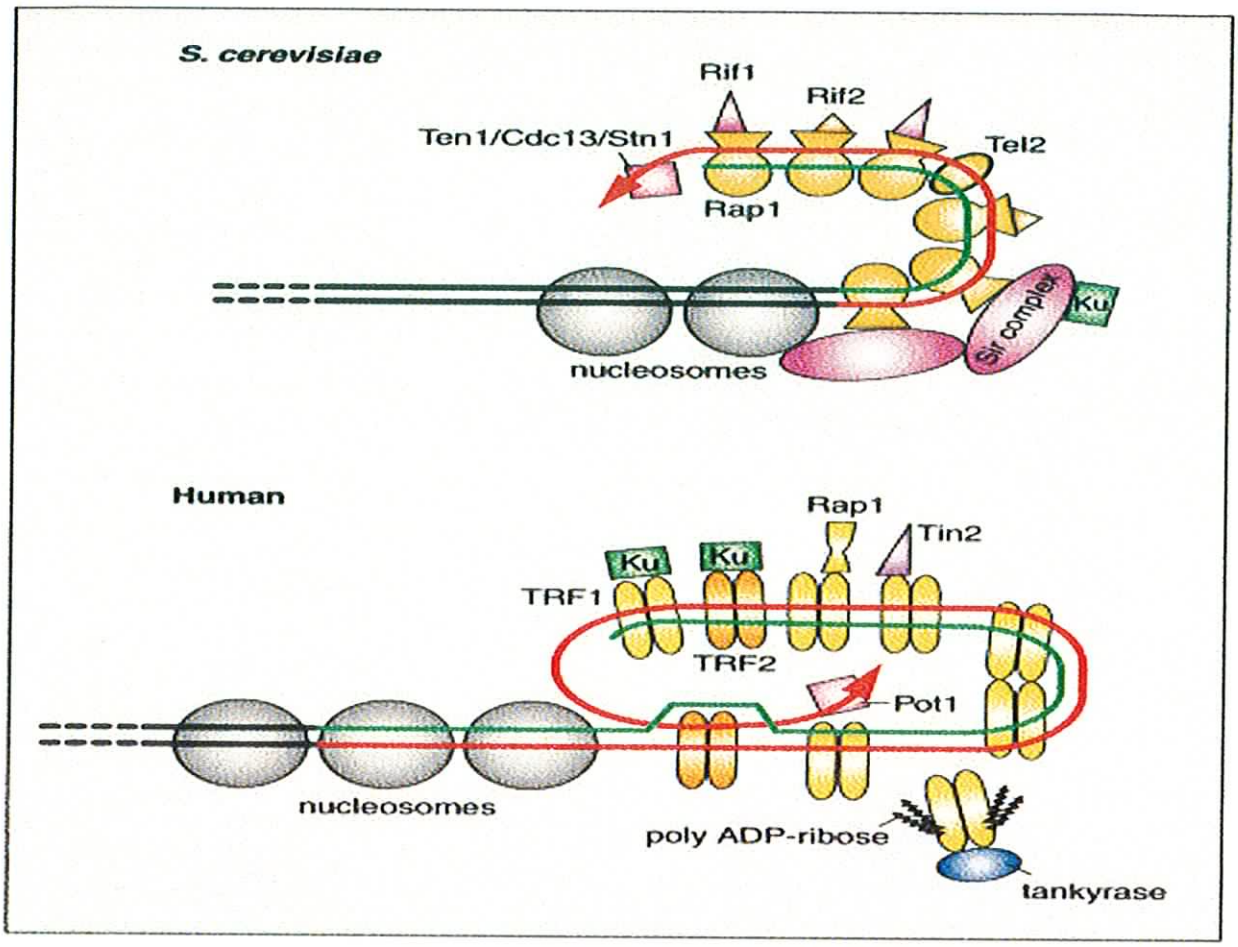

Figure 1.10. Two different examples of the Structural DNA-Protein Complexes.
Above, Telomeres in Budding Yeast, which include the following proteins: Sir (Silent Information Regulator) **Complex, Ku, Tel2** (Telomere Length Regulation Protein), **Rif2** (Telomere Binding Protein), **Rif1** (Rap1 Interacting Factor), **Rap1** (Telomere ds DNA Binding Protein), **Ten1** (Telomeric Protein Association with Stn1), **Cdc13** (Cell Division Control Protein) **and Stn1** (Suppressor of Cdc13) **(Blackburn 2001 and Grandin *et al* 2001).**
Below, Telomeres in Humans. which include the following proteins: Tankyrase [a Poly (ADP-ribose) Polymerase,TRF1-Interacting], **Tin2** (TRF1-Interacting Protein 2), **Rap1** (Protein-DNA complexes), **TRF2** (Transcription Factor 2), **Ku**, **TRF1** (Transcription Factor 1) and **Pot1** (Protecting-Telomere Protein).
The red line shows the G-rich telomeric repeat strand synthesized by telomerase (telomerase is not shown), which interacts with and extends the 3′ single-stranded overhang (indicated by the arrow). The green line shows the complementary C-rich strand of the telomeric repeats. The black lines indicate the subtelomeric DNA. The 3′ terminal single-stranded telomeric DNA in human telomeres maybe found in two alternative forms: either bound by Pot1 protein or engaged in T-loop formation (Blackburn 2001, Lei *et al* 2002).

In budding yeasts such as *S. cerevisiae*, the nonnucleosomal complex includes the proteins Rap1p and Tel2p, which bind sequence specifically to the double-stranded telomeric repeat DNA (Evans and Lundblad 2000).

In mammalian cells, the telomere repeat binding factors, TRF1 and TRF2, bind telomeric DNA and are important regulators of telomere structure and function (Van Steensel *et al* 1998, Van

Steensel and De Lange 1997). TRF2 protein can organise linear telomere ends into a duplex loop (t-loop) (Griffith *et al* 1999, Stansel *et al* 2001). It has been proposed that this binding helps protect the telomeric end *in vivo* (Griffith *et al* 1999). Both TRF1 and TRF2 proteins play a role in regulating telomere length and integrity (Van Steensel *et al* 1998, Van Steensel and De Lange 1997). TRF1 and TRF2 bind several other proteins, serving as critical attachment and recruitment factors for other proteins involved in telomere homeostasis (De Lange, 2002). Some of these proteins are quite important in preventing chromosome ends from being recognised as double stranded DNA breaks (De Lange and Petrini 2000, Karlseder 2003). In addition to TRF1 and TRF2, there is a third telomere binding protein, called POT1, which binds single stranded telomeric DNA and also plays an essential role in keeping telomere integrity and regulating telomere length (Baumann and Cech 2001, Loayza and De Lange 2003). TRF1-interacting factor, TIN2, and POT1 and TIN2 organizing protein (PTOP/PIP1/TINT1), play a role in the regulation of telomere length by regulating these telomere-associated factors (Liu *et al* 2004, Ye *et al* 2004).

There are also additional DNA sequence-specific proteins that can directly bind and protect the G-rich, single-stranded DNA 3′ overhang. These proteins consist of the α-protein homolog Pot1 in fission yeast and humans, the α/β-protein in ciliates, and Cdc13p in *S. cerevisiae* (Hemann and Greider 1999, Baumann and Cech 2001). Cdc13p has a double function, as it can bind the proteins Stn1p and Ten1p in order to protect the telomere from degradation *in vivo*, and can also recruit telomerase (see paragraph below) by a direct interaction with Est1p, a telomerase subunit protein (Pennock *et al* 2001)

1.5.1.2 Senescent Cells (hTERT- Cells)

Normal human cells have a limited replicative capacity when they are kept in culture (Hayflick and Moorhead 1961). After many passages in culture, the cells enter a growth arrest state

called replicative senescence (Wright and Shay 1992). The number of divisions that normal cells complete before they senesce depends on the cell type and the species, age, and genotype of the donor. This number can be large; for example, >80 population doublings (PD) for fetal or neonatal human fibroblast cultures (Rubio *et al* 2002, Stanulis-Praeger 1987, Campisi *et al* 1996).

Typical characteristics of replicative senescence are stereotypical morphological changes and the activation of P-galactosidase active at acidic pH (Dimri *et al* 1995). Recent studies have suggested that cells, when they reach replicative senescence, also show activation of the cellular machinery that responds to DNA damage (Bakkenist *et al* 2004, Herbig *et al* 2004). When the cells complete many divisions, mortal human cells exhibit telomere shortening while immortal human cells maintain stable telomere lengths (Harley *et al* 1990, Counter *et al* 1992). This implies that telomere shortening induces replicative senescence when telomeres reach a critical length. Even though these studies suggest that telomere length is the parameter that triggers replicative senescence, recent authors have shown that alterations in telomere state, such as the status of the 3′ telomeric overhang, rather than length, are associated with the start of replicative senescence (Wright and Shay 2001, Karlseder *et al* 2002, Stewart *et al* 2003). However, there are some important factors, which are linked to telomere shortening-induced senescence, such as telomere-associated proteins, telomere function and particular stimuli.

1. First, telomere-associated proteins have been identified that regulate telomere length indirectly. Some of these proteins appear to alter the telomeric structure and hence the ability of telomerase to access the telomere (McEachern *et al* 2000, Campisi *et al* 2001). One such protein is TIN2. TIN2 negatively regulates telomere length in a telomerase-dependent fashion but does not act directly on the enzyme (Kim *et al* 1999). Thus, telomerase expression alone may not be sufficient to prevent replicative senescence.

2. Second, short telomeres may be more prone than long telomeres to structural dysfunction, and telomere function, rather than length, may control cellular senescence. It has been suggested that telomerase can prevent cellular senescence by preferentially capping and acting on the shortest telomeres and that the senescence response is not induced by telomere length but, rather, by a dysfunctional telomere structure (Campisi *et al* 2001, Hemann *et al* 2001, Blackburn 2000). Furthermore, it has been demonstrated that loss of structure or function of telomeres may initiate DNA damage checkpoint responses leading to senescence (D'Adda Di Fagagna *et al* 2003, Takai *et al* 2003).

3. Third, both replicative and cellular senescence induce similar phenotypes, but cellular senescence can occur after very few doublings and in response to stimuli that most likely act independently of telomeres (Hemann *et al* 2001). Some of these stimuli, however, may damage telomeres. For example, human fibroblasts cultured under hyperoxia senesce very rapidly but accumulate single-strand breaks at the telomeres. Furthermore, there are several other stimuli, including DNA damage and oncogene expression, which can induce a state that shares functional and morphological likeness with replicative senescence (Wong *et al* 2000, Serrano *et al* 1997).

Conversely, telomeres may influence the sensitivity of cells and organisms to DNA-damaging agents such as ionizing radiation (IR). Wong *et al* (2000) demonstrated an inverse correlation between telomere length and chromosomal radiosensitivity in the lymphocytes of some breast cancer patients, and short telomeres enhanced IR-induced lethality in telomerase-deficient mice.

1.5.1.3 Genomic Instability in hTERT- Cells

As already discussed, the mechanism of initiation and perpetuation of genomic istability is currently unclear, but it has been postualted to occur as a result of epigenetic factors such as

persistent oxidative stress (Clutton *et al* 1996). This suggests that agents, such as oxy-radicals, which can produce DNA double strand breaks, are also capable of inducing genomic instability, although double strand breaks might not necessarily be the most important lesion involved in the induction of genomic instability (Clutton *et al* 1996). Somodi *et al* (2006) observed that single strand breaks and/or oxidized base damage play a major role, rather than DSB, in radiation induced instability. Mothersill *et al* (1998) showed that agents causing oxidative stress such as cadmium and nickel are able to induce lethal mutations. It has been shown that DNA double strand breaks lead to delayed chromosomal rearrangements (Morgan *et al* 1998). Cells substituted with bromodeoxyuridine (Brd-U), which modifies cellular DNA and enhances the sensitivity of cells to the action of radiation, increased chromosomal instability, compared to unsubstituted control cells (Limoli *et al* 1999). This suggests that DNA is at least one of the critical targets important for the induction of delayed chromosomal instability. Recent reports on cultured human cells suggest that telomeres and telomerase play an important role in genomic instability (Coen *et al* 2001, Blasco *et al* 1997, Lee *et al* 1998, Hande *et al* 1999, Rudolph *et al* 1999, Hackett *et al* 2001, Riha *et al* 2001). There has been considerable interest in the role of telomeres in DNA damage responses. It has been proven that telomeres stabilise DNA at the ends of chromosomes, preventing chromosome fusion and genetic instability (Lydall 2003). More evidence that telomeres are related to genomic instability has been shown by Day *et al* (1998). They showed that interstitial telomere repeat-like sequences contribute to the delayed induction of chromosomal instability in human-hamster hybrid cells post-exposure to x-rays. This study led to the conclusion that interstitial bands can function as hot spots for recombination, which promote chromosome rearrangements directly after exposure to DNA damaging agents and in the progeny of treated cells. This work has suggested that DNA breaks are necessary but may not be sufficient to induce the genomic instability phenotype. Loss of structure or function of telomeres has been

shown to initiate DNA damage checkpoint responses leading to senescence (D'Adda Di Fagagna *et al* 2003, Takai *et al* 2003).

Therefore, it is interesting to determine which role the telomeres play in genomic instability. For example in chromosomal instability such as chromosome end fusion, when they are insulted with agents which can produce oxyradicals and consequently DNA breaks.

1.5.1.3.1 Mechanisms for the Induction of Chromosome End Fusion

In theory, chromosome end fusion, such as dicentric chromosomes, could be caused by three different mecchanisms.

1. Two broken ends of chromosomes may fuse and then possibly be processed by homologous recombination repair (HRR) or non-homologous end joining (NHEJ). Double strand breaks could be converted by HRR to either reciprocal translocations or dicentrics and acentric fragments. Alternatively, double strands breaks may be converted by NHEJ to dicentric chromosomes and fragments (Obe *et al* 2002). HRR involves the members of the Rad52 gene group and needs regions of extensive sequence homology, whereas NHEJ depends on the products of the genes XRCC4-7 and does not necessarily require sequence homology (Pfeiffer *et al* 2000). The indispensable requirement of HRR for sequence homology is reflected by the fact that it occurs preferentially between sister chromatids, in cells undergoing mitotic cell cycles or between homologous chromosomes in cells undergoing meiotic cell cycles, where it could lead to gene conversion. HRR can also occur between homologous DNA sequences in different chromosomes (called ectopic HRR), which may lead to exchange type chromosomal aberrations such as dicentric chromosomes and translocations (already mentioned above). Normally, HRR is initiated by one single DSB in order to produce both correct intrachromosomal repair products and incorrect exchange type chromosomal aberrations. In this case, the two DNA ends of a single DSB interact with the two strands of a homologous

unbroken DNA duplex. NHEJ rejoins DSB in the deficiency of complete sequence homology and this means that it has a generally related pathway for the repair of DSB, which occurs within chromosome regions without sequence homology. In order to occur, NHEJ needs a single DSB to generate intrachromosomal repair products. In this case, the two DNA ends of a single DSB interact with each other. But to originate exchange type chromosomal aberrations, NHEJ requires at least two initial DSB. In this case, the four ends of two DSB interact cross-wise with each other (Obe *et al* 2002).

2. Telomeres could fuse with double strand breaks in cells lacking telomerase and/or with eroded telomeres (Chan and Blackburn 2002, 2003). These authors reported that telomeres fuse rapidly (within hours) to the HO-generated double-strand break in cells missing active telomerase. They observed that the number of fusions increased when either telomerase or Tel1p was deleted. The creation of telomere-DSB fusions occurred by the NHEJ pathway, since they were depending on DNA ligase IV. The telomere-DSB fusion was observed before critical telomere shortening caused any detectable cellular senescence, and it was even present in cultures where most of the telomeres were significantly elongated. This implies that significant loss of most of the telomeric DNA, and a resulting defect in chromosome end protection, could occur at a low incidence in cells lacking telomerase. These authors also studied whether telomere-DSB fusion could occur in cells with long telomeres. Their results confirmed that it is not the telomere length itself, but rather the absence of telomerase and Tel1p that caused rigorous telomere shortening and consequently telomere-DSB fusion.

3. Fusion between telomeres could occur in two ways:

a) If telomeres are dysfunctional, lacking TRF2 (Smorgorzewska *et al* 2002) and having DNA damage foci (Takai *et al* 2003); in this case telomeres are then processed as a NHEJ (Smorgorzewska *et al* 2002).

b) If telomeres were damaged by single strand breaks (Urushibara *et al* 2004).

a) Fusion between telomeres could occur if they were dysfunctional, lacking TRF2 (Smorgorzewska *et al* 2002) and having DNA damage foci (Takai *et al* 2003). Telomeres are required to prevent end-to-end chromosome fusions, but it was observed that end-to-end fusions of chromosomes occurred in cells with dysfunctional telomeres, due to reduced function of telomere-associated proteins, and in cells undergoing extensive erosion of telomeric DNA. Telomere fusions, resulting from inhibition of the telomere-protective factor TRF2, are produced by DNA ligase IV-dependent NHEJ. Therefore, NHEJ can results in covalent ligation of the C strand of one telomere to the G strand of another. The breakage of the resulting dicentric chromosomes results in nonreciprocal translocations. Telomere NHEJ takes place before and after DNA replication, and both sister telomeres contribute in the reaction. Telomere fusions are followed by active degradation of the 3′ telomeric overhangs. The main risk to dysfunctional telomeres is the degradation of the 3′ overhang and subsequent telomere end-joining by DNA ligase IV. The involvement of NHEJ in telomere fusions is paradoxical since the NHEJ factors Ku70/80 and DNA-PKcs are present at telomeres and protect chromosome ends from fusion. Several lines of evidence indicate that telomeres lacking TRF2 resemble the critically shortened telomeres of senescent cells (Smogorzewska and De Lange 2002, Karlseder *et al* 2002). Therefore, the conclusion that dysfunctional telomeres are treated like DNA breaks and repaired by NHEJ is also likely to be related to the shortened telomeres of primary human cells nearing replicative senescence or those of transformed cells undergoing telomere crisis (Smorgorzewska *et al* 2002).

b) Fusion between telomeres could occur if they were damaged by single strand breaks (Urushibara *et al* 2004). These authors demonstrated that a major type of delayed chromosome aberrations by ionizing radiation consists of dicentrics that might be triggered by end-to-end fusions. Interestingly, it was found that the induction of delayed formation of dicentric chromosomes that retained telomere sequences at the fused position (tel+ end-fusion) increased

in response to radiation dose, suggesting that ionizing radiation induces telomere dysfunction that might promote the tel+ end-fusions. These results suggest that telomeres are one of the critical targets for induction of delayed chromosomal instability by ionizing radiation. It has been observed that exposure to ionizing radiation induces enhanced and constant generation of reactive oxygen species (ROS) in irradiated progeny cells (Clutton *et al* 1996, Limoli *et al* 2003). This persistent generation of ROS in irradiated cells partially explains why delayed effects of radiation appeared over many cell divisions post-irradiation. Single-strand breaks or single-stranded regions induced by ROS are usually maintained in telomeric DNA, even in normal oxygen cell culture condition (Von Zglinicki *et al* 1995, Petersen *et al* 1998). This shows that telomeres are vulnerable to oxidative damage. Thus, ionizing radiation has the potential to induce persistent instability of telomeres mediated by ROS. It would be interesting to see how the oxidative damage in telomeres induces telomeric fusions. A recent study on telomere integrity post-replication indicated that mammalian telomeres require strand-specific post-replicative processing (Bailey *et al* 2001). A defect in TRF2 or DNA-PKcs gives rise to damage processing of leading strands of the DNA, resulting to the formation of end-to-end fusions. Thus, it might be possible that elevated oxidative stress post-irradiation may decrease the integrity of post-replicative processing of telomeres and this could be provoked by the gathering of single-strand breaks. In addition to an indirect effect of radiation on telomere integrity, it was also demonstrated that a defect in DNA-PKcs support the end-to-end fusions characterized by telomeric instability such as the tel+ end-fusions. This sustains the evidence that DNA-PKcs plays a protective role in telomeric end-capping, in addition to the task of repairing DNA double-strand breaks, as shown by other authors (Goytisolo *et al* 2001), (Bailey *et al* 1999).

1.5.2 Telomerase

1.5.2.1 Structure and Function

Telomerase (telomere terminal transferase) is an enzyme whose role is to extend the lagging strand of each chromosome, after incomplete replication of the 3′ end by the DNA polymerases during the cell division (Greider and Blackburn 1985). It is responsible for maintaining telomeres, and is detectable at high levels in most cancer cells and immortalized tumor cell lines in which telomerase is often upregulated (Kim *et al* 1994, Broccoli *et al* 1995). Therefore, in dividing cells containing the enzyme telomerase, the end of the telomeric DNA is continuously replaced from the gradual loss of chromosome termini, and turns over because of continuous additions and losses. McEachern and Blackburn (1995) demonstrated this process by the alteration of the template sequence within the telomerase RNA, in the budding yeast *Kluyveromyces Lactis*. This alteration determined the corresponding altered telomeric DNA sequence to be added to telomeric ends *in vivo*. By using a phenotypically silent template mutation, they were able to monitor the normal action of telomerase over many cell divisions and detect a gradual replacement of the terminal region of telomeres, at an average rate of 1–3 bp per generation (McEachern and Blackburn 1995, Roy *et al* 1998).

Telomerase enzyme is present in low levels in some human somatic cell lines, such as BJ and WI-38 fibroblasts, as well as peripheral blood leukocytes and bone marrow. In mammals cells telomerase synthesizes TTAGGG DNA repeats at the end of chromosomes (Moyzis *et al* 1988).

Human telomerase is composed of two core subunits (Figure 1.11):

1. A telomerase reverse transcriptase (TERT), which is a 127 kDa protein,
2. A RNA subunit composed of 451 nucleotides, which is the telomerase RNA component (TERC), containing a template for telomere elongation (Greider and Blackburn 1989, Lingner and Cech 1996).

These two components have been shown to be sufficient to reconstitute telomerase activity *in vitro* (Weinrich *et al* 1997, Beattie *et al* 1998).

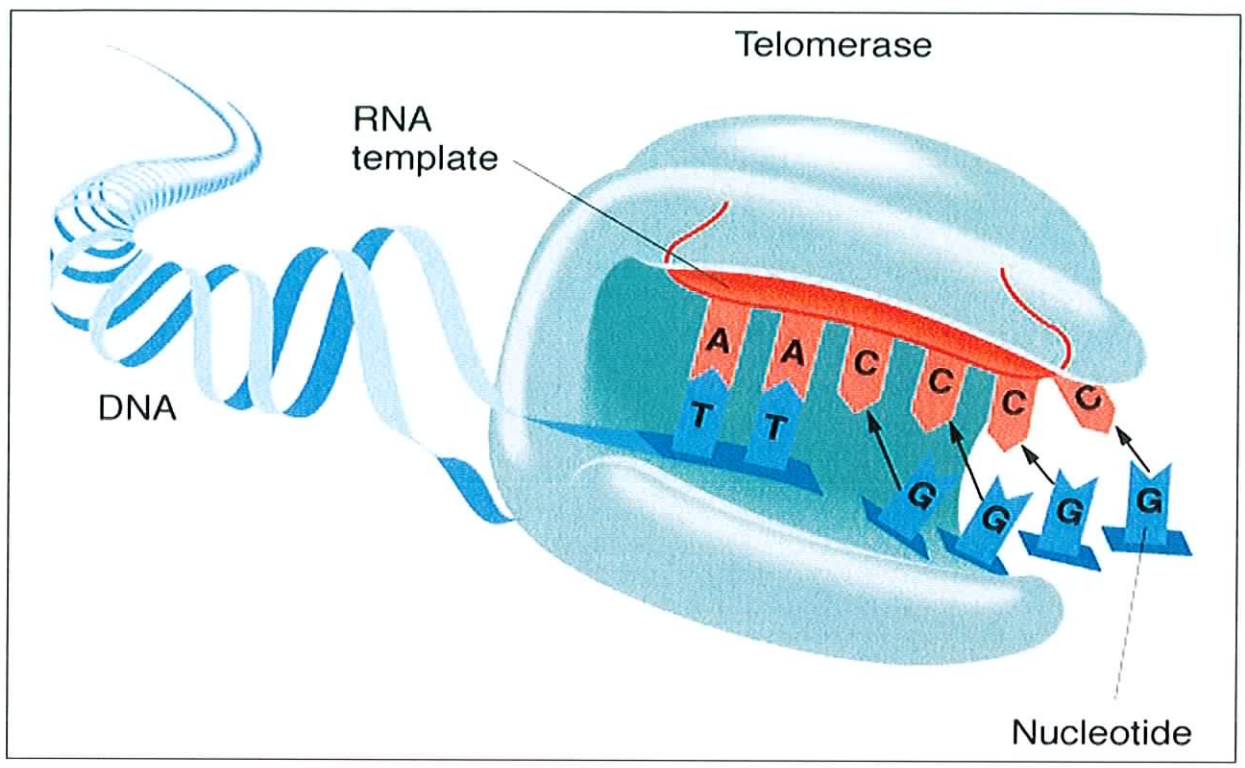

Figure 1.11. Human telomerase is composed of two core subunits: the telomerase reverse transcriptase (TERT) subunit (gray), and the telomerase RNA component (TERC), containing a template (complementary to the DNA sequences), for telomere elongation (red). DNA sequences are shown in blue (www.bio.miami.edu).

The human telomerase RNA is a PolII transcript and contains a small nucleolar RNP (snoRNP) domain. Like other snoRNP complexes, the assembly of telomerase take place in the nucleolus (Collins and Mitchell 2002). Telomerase assembly requires Hsp90 and p23 and involves interactions with dyskerin and other snoRNP proteins (Holt *et al* 1999, Mitchell *et al* 1999).

In addition to these core components, biochemical and genetic studies identify several other potential components of the telomerase holoenzyme complex. In *Saccharomyces Cerevisiae*, Estlp is essential for telomere maintenance *in vivo* and recruits telomerase to the chromosome end by binding to the telomerase RNA (Lundblad and Szostak 1989, Pennock *et al* 2001). The

human ortholog hESTl A can bind active telomerase and may also be involved in chromosome capping and telomere elongation (Reichenbach *et al* 2003, Snow *et al* 2003). The Ku protein, which is a heterodimer, is responsible for non-homologous end joining of broken chromosomes and binds to the RNA subunit of the telomerase. This interaction of the telomerase RNA and Ku protein promotes the addition of telomeres to broken chromosome termini (Stellwagen *et al* 2003). TEP1 protein is also associated with telomerase in mammalian cells (Harrington *et al* 1997), although TEP1 is not essential for telomerase activity (Liu *et al* 2000). The exact roles of these proteins in telomere and telomerase function *in vivo* are still unclear.

1.5.2.2 Mechanism for the Regulation of Telomerase

In human cells, hTERT is primarily regulated at the transcriptional level (Meyerson *et al* 1997). Cancer cells constitutively express hTERT, and therefore they can proliferate unlimitedly. The regulation of hTERT expression is complex, and both positive and negative regulators have been reported. The hTERT promoter contains the Myc/Mad binding site (E-box) and is a direct transcriptional target of c-Myc (Wang *et al* 1998). Overexpression of Mad 1, which is a c-Myc antagonist, has been found to suppress hTERT expression (Gunes *et al* 2000). Despite these findings, c-Myc overexpression cannot explain telomerase activation in all tumors, and a number of other transcriptional activators have been reported. Estrogen activates telomerase in mammary epithelial cells that express the estrogen receptor by both direct and indirect effects on the hTERT promoter and androgen signaling appears to upregulate hTERT expression (Kyo *et al* 1999, Guo *et al* 2003).

Hypoxia-inducible factor-1 (Yatabe *et al* 2004), the Ewing's sarcoma fusion protein EWS/ETS (Takahashi *et al* 2003) and oncogenic constituents of the RAS signaling pathway (Goueli and Janknecht 2004) have also been demonstrated to induce telomerase activity.

As well as these potential inducers of telomerase activity, there are also several negative regulators of hTERT transcription, which have also been found. The Mad 1/c-Myc pathway, the SIP1, and the Menin have been demonstrated to play a role as transcriptional repressors of hTERT expression. Particularly, suppression of Menin induced sufficient telomerase activity to immortalize human fibroblasts (Lin and Elledge 2003).

The complexity of hTERT transcriptional regulation in human cancer cells makes this study quite complicated. Furthermore, since telomerase is expressed at high levels in cells during embryonic development and only low levels of expression are detected in adult somatic tissue, it is comprehensible that hTERT is subject to several levels of transcriptional regulation (Masutomi *et al* 2003).

Protein interactions among TRF1, TRF2, TIN2, POT1, and PTOP/PIP1/TINT1 also play important roles in regulating telomere length and might be implicated in the regulation of the binding of the telomere to hTERT (Liu *et al* 2004, Ye *et al* 2004).

1.5.2.3 Immortal Cells (hTERT+ Cells)

Telomere length, in cells containing active telomerase, is normally regulated and kept within defined limits: this phenomenon has been called telomere homeostasis (Shampay and Blackburn 1988).

It has been demonstrated that telomeres (the higher-order telomeric DNA-protein complex) exist in two different physical states:

1. The first state (temporarily uncapped) accessible by telomerase (Figure 1.12) (Teixeira *et al* 2004);

2. The second state (capped) where telomere elongation cannot take place (Figure 1.12) (Teixeira *et al* 2004).

Alternate rounds of incomplete replication and/or degradation at chromosome ends determine telomere shortening, increasing the likelihood of uncapping. A shortened telomere temporarily uncaps and becomes accessible by the telomerase. When the telomere is elongated by the telomerase, the telomere has an increased chance of changing to the capped state. This dynamic cycle of uncapping and capping maintains the telomere length within certain defined limits (Blackburn 2001). Normally, in a cell with telomerase, such temporary uncapping obtains a response involving ATM (Tel1) kinase, which acts on the telomere to make it accessible for telomerase action. This is at least in part achieved through the phosphorylation of the Rad50/Mre11/Xrs2 (NBS1) complex (Blackburn 2001).

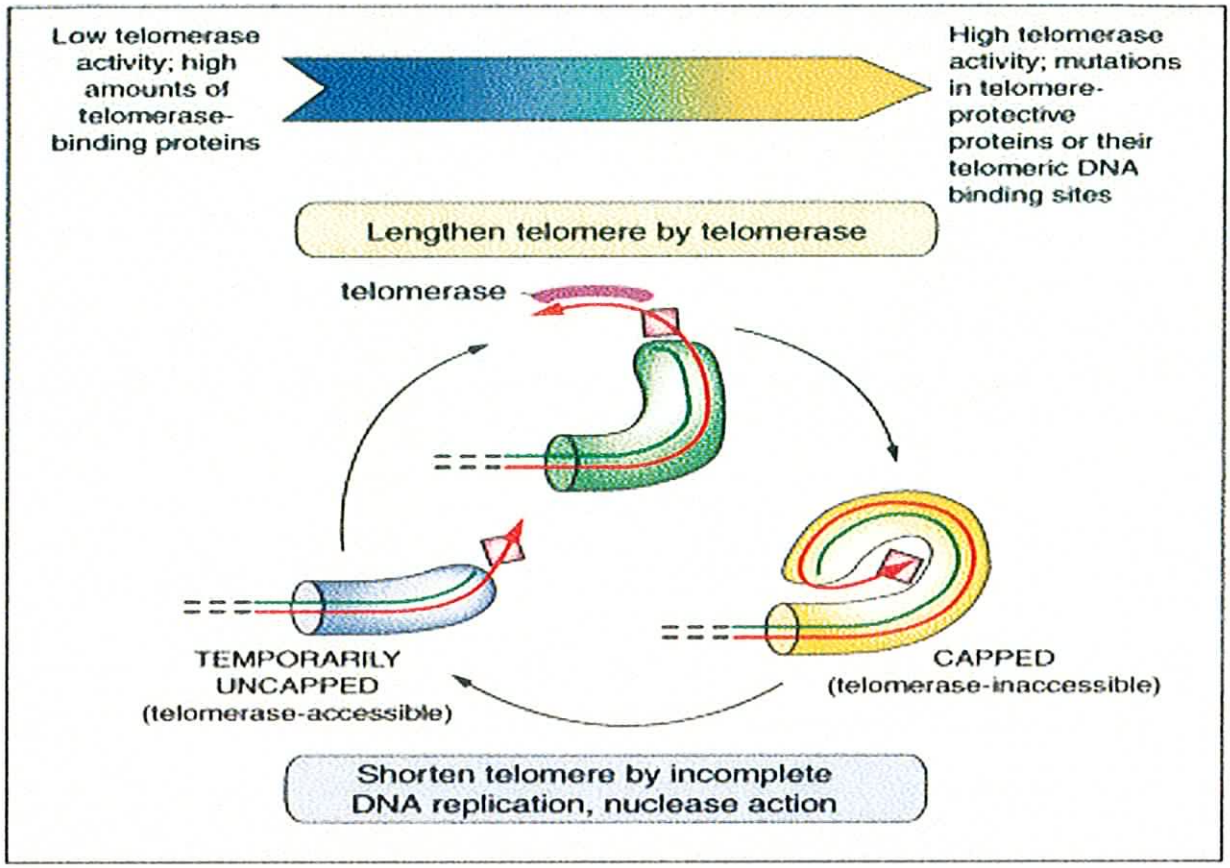

Figure 1.12. The figure shows the changes following the cycle of Capping (telomerase-inaccessible state) and Temporary Uncapping (telomerase-accessible state) of Telomeres in Dividing Cells with Telomerase. The Temporarily Uncapped telomeric DNA-protein complex (lower left), which is shortened, becomes capable for telomerase action (telomerase-accessible). Later, the telomeric complex becomes extended by the telomerase (top), until it has a telomeric structure that is capped, and consequently telomerase-inaccessible (lower right). The Capping (telomerase-inaccessible) state can endure for many cell divisions even with no further lengthening of the telomere by telomerase, until the telomere shortens enough to return to the Temporary Uncapping (telomerase-accessible) state again (McEachern and Blackburn 1995).

The balance between lengthening and shortening that determines the mean length and length distribution of telomeres is influenced by multiple factors: genetic, developmental (Blackburn 2000), and physiological (Von Zglinicki *et al* 2000). At least two kinds of factors, such as telomerase activity and components of the telomere itself, have been observed (Liu *et al* 2000). Ectopic overexpression of hTERT can cause telomere lengthening or shortening, and then rebalance to a new mean length, with telomeres becoming flexible to further length change, as the cells continue to divide (Bodnar *et al* 1998, Zhu *et al* 1999). Therefore, in dividing cells with telomerase, the telomere-telomerase complex has this dynamic feature, in which the

balance between lengthening and shortening, that determines the mean length, maintains the telomere length homeostasis.

Despite these new studies during the last years, the detailed knowledge regarding the hTERT regulation is still incomplete, and much future work will be required to establish the biochemical mechanisms that regulate the expression of hTERT and its binding to the telomere. According to this feature, constitutive expression of hTERT leading to telomerase activity in human cells stabilises telomere length and allows some telomerase-expressing cells to bypass the threshold of replicative senescence (Bodnar *et al* 1998, Vaziri and Benchimol 1998). But expression of hTERT fails to allow cells to bypass the replicative arrest provoked by these stimuli (Wei and Sedivy 1999). While hTERT expression facilitates the immortalization of all human cells, the status of retinoblastoma (RB) and p53 also play important roles in regulating replicative potential in human cells (Shay *et al* 1991). In fact, it has been proven that disruption of the retinoblastoma (RB) and p53 tumor suppressor pathways also permits human cells to avoid the senescence threshold, as well as the replicative arrest induced by these other stimuli (Shay *et al* 1991).

Moreover, telomerase has been reported to protect some cells from the lethality that results from severe damage to telomeres (Ludwig *et al* 2001). Rubio *et al* (2002) observed that telomere length influenced replicative senescence but that human fibroblasts can senesce with telomeres that are significantly shorter or longer than expected. They showed that telomerase normally acts on short telomeres and that telomere structure, rather than length, determines replicative senescence.

1.5.2.4 Prevention of Genomic Instability in hTERT+ Cells

Telomerase, which maintains telomere length, is thought to provide telomere protection as well as telomere elongation, therefore its absence has been suggested to exascerbate genomic

instability (Chan and Blackburn 2003). Cui *et al* (2002) have demonstrated a direct relationship between hTERT mRNA expression and karyotypic stability, apparently independent of telomere length. Expression of telomerase may prevent chromosome end joining/fusion, such as dicentrics and/or nucleoplasmic bridges (Cui *et al* 2002). Telomerase has been reported to protect some cells from the lethality that results from severe damage to telomeres (Ludwig *et al* 2001). To explore the relationships between telomere length, replicative senescence, and IR sensitivity, Rubio *et al* (2002) created essentially isogenic human cell populations that had varying average telomere lengths. They started with normal human fibroblasts and used genetically defined manipulations. They showed that telomere length does indeed influence replicative senescence but that human fibroblasts can senesce with telomeres that are significantly shorter or longer than expected. Their results supported the idea that telomerase acts preferentially on short telomeres and that telomere structure, rather than length, causes replicative senescence. But interestingly, they showed that telomere length, but not telomerase, influences the response of human cells to IR. This suggests that telomeres *per se* can be sufficient, and even independent of telomerase, in preventing cell damage.

Moreover, Rubio *et al* (2004) have shown that hTERT only protects cells with short telomeres from the clonogenically lethal effects of ionising radiation, bleomycin, hydrogen peroxide, and etoposide and did not protect cells with long telomeres. This suggests that variables such as telomere length or age of the cells at hTERT transfection are also important factors to be considered in this respect. This is the reason why, in this project, old cells with short telomeres were used, instead of young cells. Therefore, this would allow observe if telomerase *per se* is capable to prevent genomic instability in human fibroblasts.

1.5.2.4.1 Roles of Telomerase in Chromosome Stability

It has been suggested that telomerase plays two roles in chromosome stability.

1. First, telomerase can avoid the initiation of chromosomal instability by preventing telomere dysfunction.

2. Second, telomerase can change the spectrum of chromosome repair, since it allows *de novo* telomere addition.

Without the telomerase, a broken chromosome can either gain a telomere through a translocation event (Figure 1.13, section B) or form a dicentric chromosome that enters a breakage-fusion-bridge (BFB) cycle (Figure 1.13, section C). It might be possible, that telomerase allows the initiation of a BFB cycle to be avoided by permitting *de novo* telomere addition (Figure 1.13, section A). The distinguishable functions of telomerase activity and telomere dysfunction in the induction and manteinance of chromosomal instability could provide insight into the roles of telomeres and telomerase in tumorigenesis (Hackett *et al* 2001).

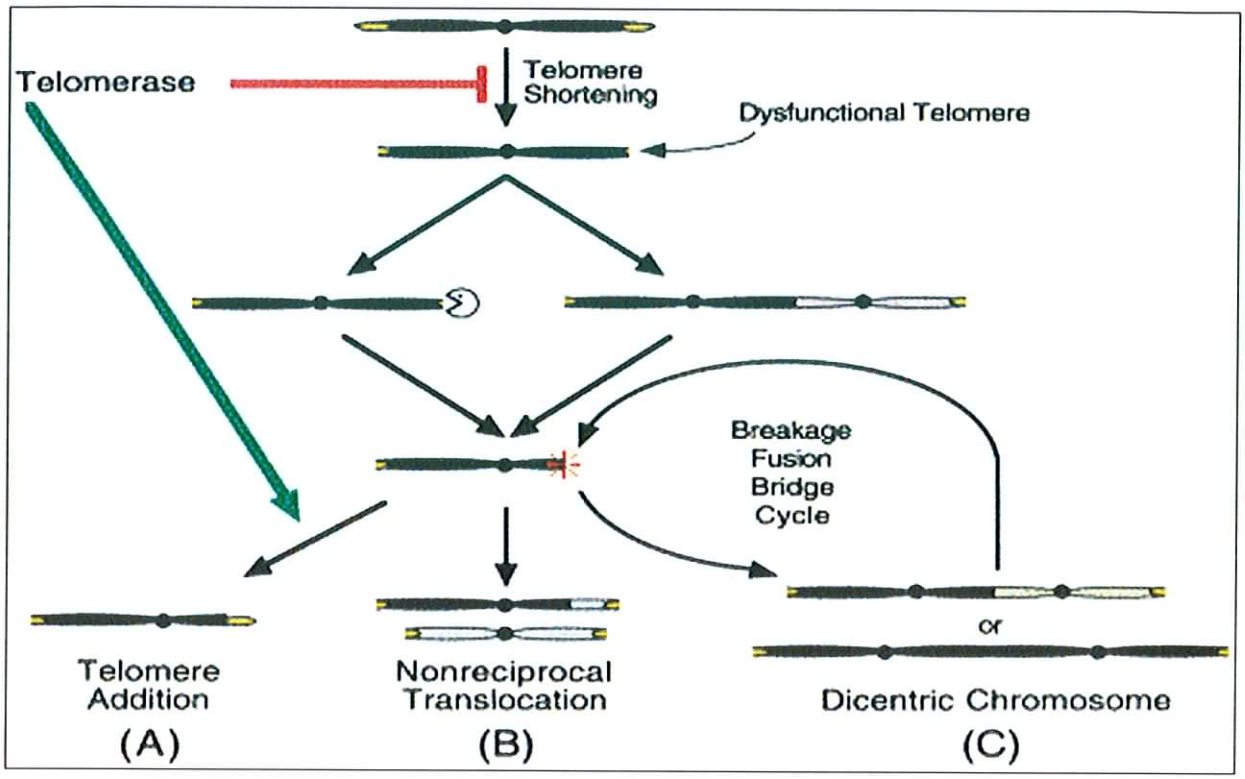

Figure 1.13. The Figure shows the Model for the Role of Telomerase and Telomere Dysfunction in Chromosomal Instability. When telomerase is not present, telomere shortening can produce dysfunctional telomeres. Dysfunctional telomeres can induce the formation of DNA breaks (orange star) through either direct telomere resection, or end-to-end chromosome fusion, or another mechanism. A broken chromosome can be healed by (A) *de novo* telomere addition, or by (B) nonreciprocal translocation through break-induced replication or another mechanism. Processing of the broken chromosome to produce a dicentric chromosome will result in subsequent DNA breaks through BFB (C) (Hackett *et al* 2001).

Hackett *et al* (2001) have shown that the absence of telomerase increases chromosomal instability and that telomerase can influence the structure of the resulting chromosomal changes. They observed chromosomal changes which involved interchromosomal rearrangements that were initiated by DNA breaks. Furthermore, the formation of dicentric chromosomes provided a mechanism whereby telomere shortening can cause both DNA breaks and chromosome gain or loss. This implies that telomere dysfunction may be one mechanism for the initiation of chromosomal instability in tumorigenesis. The chromosomal changes that they noticed were comparable to those normally observed in human tumors with loss of heterozygosity (Thiagalingam *et al* 2001).

1.5.2.5 Telomerase and Cancer

Even though functional disruption of the RB and p53 pathways allows human cells to bypass senescence, these cells do not become immortal. In fact, these post-senescent cells continue to display telomere shortening and eventually reach a second threshold, called crisis, which is stereotyped by cell death through apoptosis (Counter *et al* 1992, Vaziri and Benchimol 1999). At the stage of crisis, the mean telomere lengths are quite short, and cells show evidence of significant genomic instability (Counter *et al* 1992). This means that at crisis telomeres are not able to protect the terminal chromosomes any more (Counter *et al* 1992). There are rare types of cells which fail to express evident telomerase activity, but are able to maintain the telomere lengths by an alternative mechanism of telomere lengthening (ALT), therefore avoiding this crisis (Bryan *et al* 1995). For example, in yeast this mechanism involves recombination, and some authors have suggested that an analogous process might also take place in human cells (Lundblad and Blackburn 1993, Cerone *et al* 2001).

It has been shown that crisis does contribute to cancer development. Since mutations in the RB and p53 pathway occur in most human cancers, continued proliferation in the setting of these mutations results in shortened telomeres which do not guarantee protection of chromosome ends any more (Sellers and Kaelin 1997, Artandi and DePinho 2000). Therefore, this threshold of crisis drives genomic instability, which facilitates additional mutations important for cancer progression (DePinho 2000).

It has been shown that mice lacking both p53 and the murine telomerase RNA subunit (mTERC) show signs of enhanced tumorigenesis with a higher rate of carcinomas (Chin *et al* 1999). Cytogenetic analysis of these cells, which have no p53 and display short dysfunctional telomeres, have a higher percentage of chromosomal aberrations such as fusions, aneuploidy, complex nonreciprocal translocations, and chromosomal amplifications and deletions (Chin *et al* 1999, Artandi *et al* 2000, O'Hagan *et al* 2002).

In normal culture conditions, human cells that survive crisis exhibit stable telomere lengths, usually through re-activation of telomerase (Counter 1994). Inhibition of telomerase by genetic means induces telomere shortening and cell death (Hahn *et al* 1999). This implies that telomerase activation observed in the majority of human tumors plays an important role in the process of malignant transformation (Shay and Bacchetti 1997).

These studies indicate that the roles of telomere shortening and telomerase activation in cancer development are complex. For example, while telomere shortening limits cell lifespan, such shortening also drives genomic instability in the context of other mutations, promoting further genetic mutations. In human cells, the telomerase activation, achieved in part through this genomic instability, facilitates immortalization, and further tumor development. Therefore, alterations in telomere biology can both promote and suppress cancer development (DePinho 2000, Shay and Bacchetti 1997).

1.6 Aims

These were the aims of this research project.

1. To investigate if sub-lethal concentrations of either metals or radiation or combined exposure could cause genomic instability in human fibroblasts (hTERT- cells).

2. To determine if the same strain of human fibroblasts infected with a retrovirus engineered to express hTERT, which rendered the cells telomerase-positive and replicatively immortal (hTERT+ cells), showed genomic instability, caused by either metals or radiation or combined exposure.

3. To detect the telomerase activity in both types of cells, to confirm the expression of hTERT in hTERT+ cells, which rendered the cells telomerase-positive and replicatively immortal.

4. To measure the telomere length in both types of cells, to see if hTERT+ cells had longer telomeres compared to hTERT- cells (due to their acquired telomerase activity), which may have resulted in changes in genomic instability, caused by either metals or radiation or combined exposure.

CHAPTER 2

MATERIALS AND METHODS

2.1 Metal Exposure

2.2 Radiation Exposure

2.3 Combined Exposure

2.1. Metal Exposure

2.1.1 Cell Lines

Two types of BJ human foreskin fibroblasts were used for all the experiments: hTERT- human fibroblasts (normal cells) and hTERT+ human fibroblasts (immortalised cells). These cells were obtained from Miguel A. Rubio and Judith Campisi (Lawrence Berkeley National Laboratory, Berkeley CA, United States of America). BJ normal human foreskin fibroblasts were infected at population doubling (PD) 50 with pLXSN-hTERT retroviruses (Rubio *et al* 2002). The plasmid was created by introducing hTERT from pBABE-PURO-hTERT (Rubio *et al* 2002) in the pLXSN retroviral vector (Clontech). Cells were selected with G418, and the population (hTERT+) expanded before being frozen prior to use after PD 55. BJ hTERT- cells were also used after PD 50. These cells became senescent after PD 80. Both types of cells were grown at 70% confluency in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), supplemented with 20% of Medium 199 (Sigma), 10% FBS (Gibco), 20 mM Hepes Buffer (Sigma), 4 mM L-Glutamine (Gibco), 1 mM Sodium Pyruvate (Sigma) and 1X Antibiotics Antimycotic (Sigma). During each harvesting, cells were detached from the bottom surface of the flasks using Versene (Gibco) supplemented with 0.6X Trypsin (Sigma). The cells were trypsinised every three days. Both types of cells were cultured at 37°C, either in 25cm^2 or 75cm^2 flasks, depending on the experiment carried out. All the experiments were started with cells at approximately PD 60. All experiments were performed in triplicate.

2.1.2 Experimental Procedure

For all the experiments, 5 x 10^5 cells, plated in 75 cm^2 flasks were allowed to attach for 24 hours. Exactly a day later, these cells were treated for 24 hours with different doses of either Cr (VI) or V (V), depending on the experiment. Control flasks were exposed with the same

volume of 1X PBS only. 24 hours after metal exposure the cells were washed twice with 1X PBS, to remove thoroughly any traces of metal ions. After the double washing, the cells were harvested for the detection of damage immediately after the metal exposure (*Day 0*). The other cells, used for delayed effects of the metals (*Day 5* and *Day 30*), were also washed twice with 1X PBS and fresh medium was added into the flasks. The effects of metal treatment were studied immediately (*Day 0*), 5 days (*Day 5*) and 30 days (*Day 30*) after a single 24-hour exposure to metal ions. These cells were trypsinised every three days.

2.1.3 Metal Salts

Both types of cells were exposed to a maximum of three doses of Cr (VI) (0.04 μM, 0.4 μM and 4 μM), using potassium dichromate ($K_2Cr_2O_7$) (Sigma) and three doses of V(V) (0.5 μM, 5 μM and 50 μM), using vanadium pentoxide (V_2O_5) (Fluka) for 24 hours. Both metal salts were diluted in 1X PBS in order to reach the concentration used.

2.1.4 Cell Survival

In these experiments cells were treated for 24 hours with the following concentrations of metal salts: 0.04 μM Cr (VI), 0.4 μM Cr (VI), 4 μM Cr (VI), 0.5 μM V (V), 5 μM V (V) and 50 μM V (V). These metal doses were selected because higher doses were supposed to induce high toxicity, resulting in a low plating efficiency (PE) [PE (%) = (№ of Colonies Counted / № of Cells Plated) x 100].

2.1.4.1 Cell Viability

250 μl of cell suspension was added to 250 μl of trypan blue stain in a 1.5ml eppendorf, to obtain a 1:1 ratio, and mixed well. The suspension was placed on a haemocytometer (Figure

2.1) and at least 100 cells (viable and dead) for each dose point were counted to give a percentage of non-viable cells. The haemocytometer is made from two sections. Each section contains 9 squares, so there are 18 squares overall. The counting of the cells was carried out only in 10 squares (5 squares for each section) according to the standard method used for the viability assay. The counting of the cells was completed once 100 cells (viable and dead) were scored, independent of the number of the squares counted in the haemocytometer. At least 100 cells were counted for each dose point. Using trypan blue, cells that were alive, remained transparent. Dead cells stained blue because the trypan blue stain penetrated the damaged cell membrane.

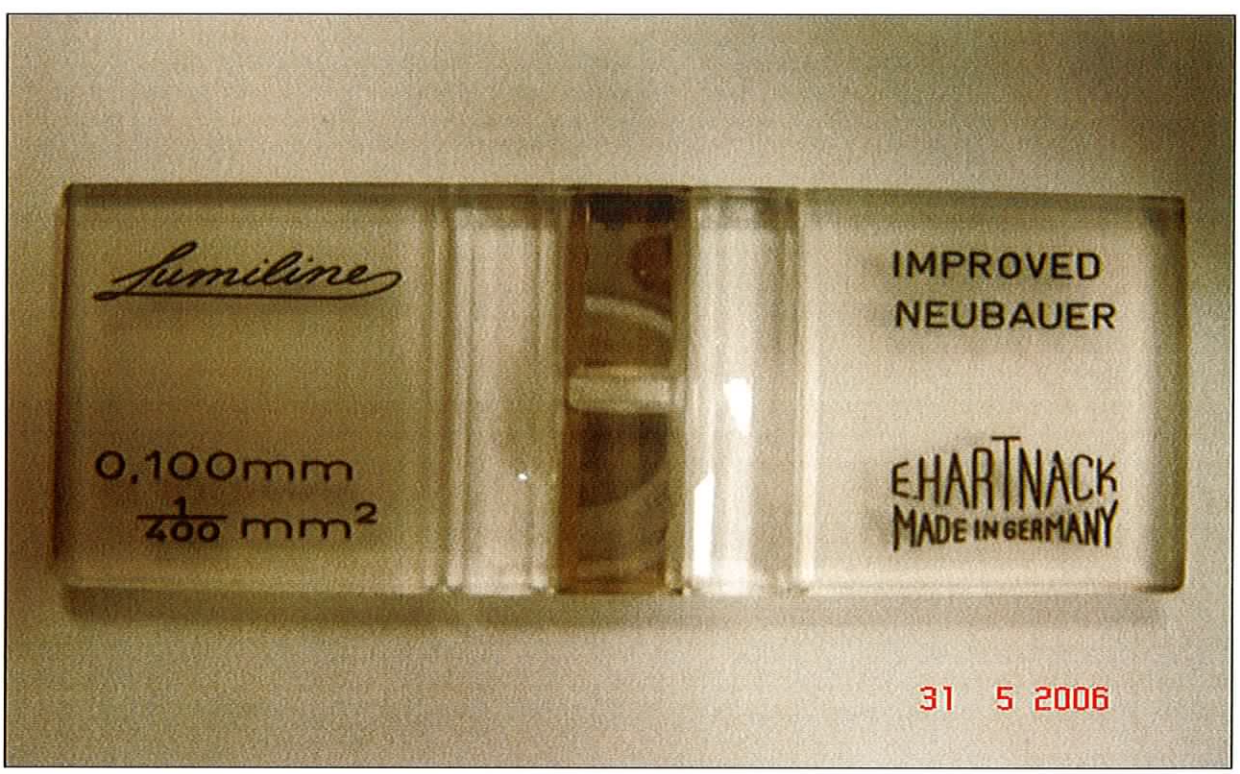

Figure 2.1. The haemocytometer is made from two sections. Each section contains 9 squares, therefore there are 18 squares overall. The counting of the cells was carried out only in 10 squares (5 squares for each section) according to the standard method used for the viability assay.

2.1.4.2 Mean Counts

250 µl of cell suspension was added to 250 µl of trypan blue in a 1.5ml eppendorf, in order to obtain 1:1 ratio, and mixed well. This suspension was placed in a haemocytometer and all of the viable cells, contained in the 10 squares (5 squares for each section of the haemocytometer) were counted. No dead cells were taken into consideration.

2.1.4.3 Number of Harvested Cells

The Number of Harvested Cells (NHC) from each dose point was calculated using a standard formula. For example, if 100 viable cells had been counted in the 10 squares of the haemocytometer:

100 cells / 10 squares = 10

10 x 2 (because of the 1:1 cell suspension/trypan blue dilution) = 20

20 x 10,000 (this is 10 squares multiplied by 1000µl of cell suspension) = 200,000

200,000 is the number of cells contained in a 1 ml of cell suspension.

200,000 x 6 ml (since the cell suspension collected during each harvesting was 6 ml) = 1,200,000

1,200,000 is the NHC for that particular dose point (the whole cell content of a $75cm^2$ flask).

2.1.4.4 Doubling Time

The doubling time (or cell cycle time), which was expressed as a number of hours, showed the length of time for the cells to complete their cell cycle (duplicate). The cells were kept in culture for 48 hours (from the plating to the harvesting stage).

The Number of Harvested Cells (NHC) and the Number of Plated Cells (NPC) was taken in consideration in order to determine the doubling time, which was calculated using the following formula: $48 \times \text{Log}_2 / \text{Log}_{(NHC / NPC)}$

2.1.4.5 Clonogenic Assay

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and then plated (2000 cells) in 25 cm^2 flasks at concentrations adjusted to yield approximately 100 colonies according to the method established by Puck and Marcus (1956). Six flasks for each dose point were used for this experiment. The cells were cultured for two weeks and then stained with carbol fuchsin (20%, Ziehl Niehlson) to assess the colony formation. Three flasks were used for each experimental group and results were expressed as mean of three results ± standard deviation.

2.1.4.6 Senescence

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and then plated as described for the clonogenic assay. The cells were cultured for two weeks and then fixed with the following procedure.

1. Media was discarded from the flasks.
2. Flasks were washed twice with 1X PBS.
3. Flasks were fixed with 10% Formalin for 5 minutes.
4. Flasks were washed three times with 1X PBS.
5. 5 ml of 1X PBS was kept in the flasks.
6. 5 ml of 1X PBS was discarded from the flasks.
7. The face of the flasks where the cells were adhering to was cut using a soldering iron.
8. The cells, on this cut segment, were stained with β-Galactosidase (Cell Signaling Technology), according to Dimri *et al* (1995) and kept in the incubator overnight.
9. Stained cells were then washed with 1X PBS.
10. A Nikon Eclipse E600 microscope was used for the scoring. Senescent cells stained light blue, whereas not-senescent cells remained transparent/very light brown.

Three flasks were used for each experimental group and results were expressed as mean of three results ± standard deviation.

2.1.4.7 Apoptosis

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and then plated as described for the clonogenic assay. The cells were cultured for two weeks and then harvested and fixed with the following procedure.

1. Media was discarded from the flasks.
2. Flasks were washed twice with 1X PBS.
3. Flasks were fixed with 10% Formalin for 5 minutes.
4. Flasks were washed three times with 1X PBS.
5. 5 ml of 1X PBS was kept in the flasks.
6. 5 ml of 1X PBS was discarded from the flasks.
7. Flasks were cut using pliers and only the faces of the flasks where the cells were adhering to were kept.
8. The cells, on these cut surfaces, were stained with Haematoxylin Harris' (mercury free) for 8 minutes.
9. Stained cells were then washed twice with 1X PBS.
10. A Nikon Eclipse E600 microscope was used for the scoring. Cells were defined as apoptotic if they displayed evidence of two or more of the following: cell volume shrinkage, pyknotic nucleus (chromatin condensation), blebbing of the cytoplasm, nuclear fragmentation and formation of apoptotic bodies (Collins *et al* 1991, Takano *et al* 1991). Three flasks were used for each experimental group and results were expressed as mean of three results ± standard deviation.

2.1.5 Cell Damage

In this experiment cells were treated for 24 hours with the following concentrations of metal salts: 0.04 μM Cr (VI), 0.4 μM Cr (VI), 0.5 μM V (V) and 5 μM V (V). These dilutions were selected because higher concentrations of metal were found to induce high cytotoxicity resulting in a) a low yield of binucleated cells in the Micronucleus Assay; and b) a very low yield of metaphase spread in the Chromosome Aberration Analysis. Control flasks were treated with the same volume of 1X PBS only.

2.1.5.1 Micronucleus Assay

Immediately after commencement of the metal treatment the cells were exposed to cytochalasin-B (Sigma) at concentration of 6 μg/ml for 24 hours. After 24 hours metal exposure, cells were first washed twice with 1X PBS solution to remove the metal ions, secondly harvested for the detection of micronuclei formation and other endpoints (see below) to assess the initial effects (*Day 0*) of the metal insult. The cells were then centrifuged (1200 rpm for 6 minutes), and resuspended in 1 ml of fresh media. Flasks containing cells used for the detection of delayed damage were washed twice with 1X PBS to remove the metal ions, and 20 ml of fresh growth medium was added. These cells were subcultured every three days. Delayed effects were assayed at *Day 5* and *Day 30* post-metal exposure. The micronucleus assay (in binucleated cells) was performed according to the method of Fenech and Morley (1985).

1. Each sample of cells were cytospun onto pre-cleaned slides using a Shandon Elliot Cytospin (500 rpm for 6 minutes), and allowed to air dry for 10 minutes only. Two spots for each slide were made.

2. The cells were fixed in absolute methanol (VWR) at –20°C for 10 minutes, and then let air dry overnight.

3. The cells were stained with freshly prepared filtered 5% Giemsa stain for 8 minutes. The two nuclei (in a binucleated cell) stained dark purple, whereas the cytoplasm of the cell stained light purple.

4. Slides were rinsed in deionised water to remove excess stain and allowed to air dry at room temperature.

5. Cover slips were mounted onto the slides using a small amount of DPX mountant for microscopy. Slides were allowed to set in the fume hood, and then stored until required.

Slides were coded and observed at 1000X magnification with an Olympus BX-50 microscope under oil immersion. For each dose point the following information was gathered:

- The number of binucleated cells that contained micronuclei (MNi) and nucleoplasmic bridges (NPB) was determined.

- The frequency of MNi and NPB per 1000 binucleated cells was calculated.

- The number of binucleated cells with no micronuclei, with one, two, or more micronuclei was determined.

- The distribution of mononucleated, binucleated, tri-nucleated, tetra-nucleated and multinucleated cells was evaluated. From this information the nuclear division index (NDI) was calculated. The NDI was useful in investigating any changes in the division rate of the cells. It was calculated according to the method of (Eastmond and Tuker 1989, Tucker and Eastmond 1990). Five hundred viable cells were scored to determine the frequency of the cells with different numbers of nuclei and the NDI was calculated.

NDI = (MNC) + 2 x (BNC) + 3 x (TNC) + 4 x (RNC) / N,

- NDI = nuclear division index,
- MNC = number of mononucleated cells,
- BNC = number of binucleated cells,
- TNC = number of tri-nucleated cells,

- RNC = number of tetra-nucleated cells,
- N = total number of cells scored (500).

A more accurate assessment of the nuclear division status of the cells was obtained when necrotic and apoptotic cells were included in the total number of cells scored, as higher doses of either Cr (VI) or V (V) might have caused a large proportion of cells to become non-viable. It was therefore important to note, that both the binucleate ratio and the NDI could have been overestimated if necrotic and/or apoptotic cells were not included during the scoring of the cells. A more accurate estimate of nuclear division status and cell division kinetics was obtained using the following modified equation, which took account of viable as well as non-viable (necrotic and apoptotic) cells:

NDCI = AC + NC (MNC) + 2 x (BNC) + 3 x (TNC) + 4 x (RNC) / N,

- NDCI = nuclear division cytotoxicity index,
- AC = number of apoptotic cells
- NC = number of necrotic cells
- MNC = number of mononucleated cells,
- BNC = number of binucleated cells,
- TNC = number of tri-nucleated cells,
- RNC = number of tetra-nucleated cells,
- N = total number of cells scored (500).

Micronuclei (MNi) had the following characteristics, if they were lacking any of these properties they were ignored and not scored as a MNi.

- They were not refractile and so could be easily differentiated from any crystals originating from the Giemsa stain.

- They were in no way linked or connected to either of the nuclei.

- Their nuclear boundary was clearly distinguishable.

- The diameter of the micronucleus was less than a third of the mean diameter of the main nuclei.

- MNi had the same staining intensity as the main nuclei.

Only MNi (and the other endpoints) that were in a binucleated cell were scored. It was imperative that binucleated cells were critically evaluated. The following criteria were used:

- There were two distinct nuclei.

- The two nuclei were of similar size, and staining pattern and intensity.

- The two nuclei had intact nuclear membranes and exist in the same cell.

- The two main nuclei sometimes touched, but they were clearly observed as being two separate nuclei.

- The plasma membrane of the binucleated cells was intact, and distinguishable from the membrane of adjacent cells.

Nucleoplasmic Bridges (NPB) had the following characteristics, if they were lacking any of these properties they were not scored.

- The width of a NPB was considerably variable but it did not exceed $1/4^{th}$ of the diameter of the nuclei within the cell.

- The NPB had the same staining characteristics of the main nuclei.

On very rare occasions, more than one NPB was observed within one binucleated cell. Sometimes, a binucleated cell with a NPB contained one or more MNi (Fenech 2000).

2.1.5.2 Chromosome Aberration Analysis

Chromosome preparations were made by accumulating metaphases in the presence of colchicine (Sigma) at concentration of 200 μg/ml for 6 hours. After 24 hours metal exposure,

cells were first washed twice with 1X PBS solution to remove the metal ions, secondly harvested for the detection of chromosomal aberrations to assess the initial effects (*Day 0*) of the metal insult. Flasks containing cells used for the detection of delayed damage were also washed twice with 1X PBS solution to remove the metal ions, and 20 ml of fresh growth medium was added. These cells were split every three days. Delayed effects were assayed at *Day 5* and *Day 30* after metal exposure. A score was taken of chromatid breaks, chromatid gaps, chromatid fragments, dicentric chromosomes, telomeric associations, ring chromosomes, tetraploid cells (those with 92 chromosomes), total aneuploidy (chromosome loss and gain), aneuploidy gain, aneuploidy, hypodiploidy, and hyperdiploidy. The mitotic index was also estimated.

The chromosome preparation was performed according to the method described by Coen *et al.* (2001).

1. Cells were harvested with trypsin/versene solution and spun down at 1000 rpm for 8 minutes and the supernatant poured off.

2. This was followed by treatment with 0.5% (w/v) potassium chloride for 20 minutes in the incubator after which cells were spun down at 1000 rpm for 8 minutes and the supernatant poured off. Before spinning down, 3 drops of methanol: acetic acid (3:1 v/v) fixative were added to the tubes.

3. Cells were then fixed twice in methanol: acetic acid (3: 1 v/v) first for 20 minutes and then for 15 minutes. After the last centrifugation a single cell suspension was made and the cells were dropped on the glass slides.

4. Before dropping the cell suspension, the slides were breathed on as condensation improved the metaphase spreads. The fixed cells were air dried overnight.

5. These cells were then stained using 5% Giemsa (VWR) for 10 minutes and air dried at room temperature.

6. Slides were rinsed in deionised water to remove excess stain and air dried at room temperature.

7. Cover slips were mounted onto the slides using a small amount of DPX mountant for microscopy (VWR). Slides were allowed to set in the fume hood, and stored until required. A minimum of 100 well spread metaphases was scored per dose point to determine the frequency of aberrations.

2.1.6 Cell Biology

In this experiment cells were treated for 24 hours with the following concentrations of metal salts: 0.4 µM Cr (VI) and 5 µM V (V). These dilutions were selected because they had given the most interesting results in cytogenetic analysis (Micronucleus Assay and Chromosomal Aberration Analysis). Control flasks were treated with the same volume of 1X PBS only.

2.1.6.1 Telomerase Activity

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and then used for the measurement of telomerase activity.

Telomerase activity was determined by the Telomeric Repeat Amplification Protocol (TRAP), using the TRAPeze® telomerase detection kit (Chemicon, CA).

1. Protein concentration was measured by Quick Start Bradford Protein Assay kit (Bio-Rad, USA).

2. Extension of the oligonucleotide substrate by telomerase was followed by PCR generating a ladder of products with 6 base increments starting at 50 nucleotides and amplification of a 36 bp internal standard for quantification of telomerase activity.

3. In each assay, 50 µl TRAP reaction mixture containing 1X TRAP buffer, 50 mM dNTPs, 1µl TS primer, 1µl TRAP primer mix, 0.1µg of the protein extract and 2U Taq DNA polymerase

(Sigma) was placed in a thermocycler block. This reaction mixture was preheated to 30°C for 30 minutes, 94°C for 1 minute, then 94°C for 30 seconds, 59°C for 30 seconds, for 30 and 36 cycles for hTERT+ and hTERT- cells respectively.

4. A 25µl aliquot of each PCR product was applied to a 12.5% non-denaturing polyacrylamide gel, which was then used for the electrophoresis.

5. The gels were stained by SYBR green 1 nucleic acid stain (Cambrex USA). Images were captured by Syngene software. The quantification of telomerase activity was carried out using the protocol described by Kim and Wu (1997). They showed advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). This assay has been reproduced in routine analyses and can be used to estimate the processivity of telomerase activity (Kim and Wu 1997).

2.1.6.2 Telomere Length

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and used for the measurement of the telomere length.

The telomere length measurement was performed by flow cytometry (Flow-FISH) as described by Cabuy *et al* (2004).

1. After each time interval the cells were washed in PBS and 5 x 10^5 cells were resuspended in 500 µl of hybridisation mixture, containing 70% deionised formamide, 20mM Tris buffer pH 7, 1% BSA and 0.3 µg/ml FITC (fluorescein isothiocyanate) conjugated $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe (Applied Biosystems, MA, USA).

2. Samples were heat-denatured for 10 minutes at 80°C and left to hybridise for two hours in the dark at room temperature.

3. Samples without a PNA probe were used as negative controls.

4. Any excess probe was then washed away twice in a wash solution containing 70% formamide, 10 mM Tris buffer (pH 7), 0.1% BSA and 0.1% Tween-20 and centrifuged for 5 min at room temperature (RT) at 3000 rpm

5. Again it was washed twice in PBS, 0.1% BSA and 0.1% Tween-20 and centrifuged for 5 minutes at RT at 2000 rpm.

6. The cells were incubated in 500 µl PBS, 0.1% BSA, 10 µg/ml RNAse A and 0.1 µg/ml propidium iodide (PI) for an hour at 4°C prior to the analysis.

The analysis was performed on fresh samples using FACS coulter EPICS XL (Becton Dickinson, USA). The FITC signal was detected in the FL1 channel and the PI fluorescence in the FL3 channel with no compensation set on the instrument. List mode data from approximately $2x10^4$ un-gated events were collected in each experiment and evaluated using Expo32 ADC Analysis software (Coulter corporation, USA). Mean telomere fluorescence intensity (TFI) was calculated as the difference between the mean fluorescence FL1 channel of electronically gated G0/G1 cells and the control samples. The performance of the instrument was monitored before analysis by using Flow-Check fluorospheres (Beckman Coulter). The conversion of TFI into bp was performed using the formula $y = 4.13x + 2.56$. This formula was obtained following telomere length correlation analysis in LY-R and LY-S cells, which have telomere lengths of 49 and 7 kb, respectively (McIlrath *et al* 2001), by two independent methods, flow-FISH and Q-FISH (quantitative-FISH).

2.1.6.3 STELA Analysis

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and used for the Single Telomere Length Amplification (STELA) analysis.

DNA extractions and STELA reactions were carried out as described by Baird *et al* (2003):

1. The cells were detached from the flasks with trypsin and washed in phosphate-buffered saline.

2. The genomic DNA was extracted by standard proteinase K, RNase A and phenol/chloroform protocols (Sambrook *et al* 1989).

3. The DNA was solubilized by digestion with *Eco*RI, and quantified by Hoechst 33258 fluorometry (BioRad) and diluted it to 10 ng μl^{-1} in 10 mM Tris-HCl (pH 7.5).

4. The DNA was ligated at 35 °C for 12 hours in a volume of 10 µl containing 10 ng of genomic DNA, 0.9 µM telorette linker and 0.5 U of T4 DNA ligase (Amersham Biosciences) in 1× manufacturer's ligation buffer. As a control, before the ligation step it was removed the 5' overhang by digesting 2 µg of genomic DNA with 40 U of mung bean nuclease (Amersham Biosciences) in 1X manufacturer's nuclease buffer.

5. After phenol/chloroform extraction, the DNA was precipitated in ethanol, washed it in 70% ethanol, resuspended it in 10 mM Tris-HCl (pH 8.0) and quantified it by Hoechst 33258 fluorometry.

6. The ligated DNA was diluted to 250 pg μl^{-1} in water.

7. Multiple PCRs were carried out (typically between 9 and 18 reactions per sample) for each test DNA in volumes of 10 µl containing 100–250 pg of ligated DNA, 0.5 µM telomere-adjacent and teltail primers, 1.2 mM NTPs, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgCl_2$ and 1 U of a 25:1 mixture of *Taq* (ABGene) and *Pwo* polymerase (Roche Molecular Biochemicals).

8. The reactions were cycled with an MJ PTC-225 thermocycler (MJ research) under the following conditions: 25 cycles of 94 °C for 15 s, 65 °C (XpYpE2) or 66.5 °C (XpYp-427G/415C and XpYp-427A/415T allele-specific primers) for 30 s, and 68 °C for 10 min.

9. The DNA fragments were resolved by 0.5% Tris-acetate-EDTA agarose gel electrophoresis, and detected them by Southern hybridization with a random-primed α-^{32}P-labeled (Amersham Biosciences) telomere-adjacent probe generated by PCR using primers XpYpE2 and XpYpB2 and a probe to detect the 1-kb molecular weight marker (Stratagene).

10. The hybridized fragments were detected by a Molecular Dynamics Storm 860 phosphorimager (Amersham Biosciences).

11. The molecular weights of the DNA fragments were calculated using the Phoretix 1D quantifier (Nonlinear Dynamics). When allele-specific STELA could not be carried out and a clear bimodal distribution was observed, the means of the upper and lower distributions was calculated by dividing the distributions on the basis of the overall mean and calculating the means of the separated distributions.

2.1.6.4 Cell Cycle Analysis

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and cell cycle distribution was measured. The cell cycle distribution, in the three different cell cycle phases (G0/G1, S and G2/M), was performed by flow cytometry (Flow-FISH) as described previously (see Section 2.1.6.2) by Cabuy *et al* (2004). Approximate numbers of cells in the G0/G1, S and G2/M cell cycle phases were measured on the FL3 (PI) linear channel.

2.1.6.5 Apoptosis

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and used for the detection of apoptotic cells. The level of apoptotic cells was performed by flow cytometry (Flow-FISH) as described previously (see Section 2.1.6.2) by Cabuy *et al* (2004). Apoptotic cells were identified and measured on the PI fluorescence histogram as hypodiploid peaks, as previously described (see Section 2.1.6.2) (Nicoletti *et al* 1991). Apoptosis was measured by

estimating the sub G1 peak. It was only counted when a clear sub-peak was visible, which was separate from the G0/G1 peak.

2.1.6.6 Necrosis

After each time interval (*Day 0*, *Day 5* or *Day 30*) the cells were harvested and then used for the detection of necrotic cells. The level of necrotic cells was performed by flow cytometry (Flow-FISH) as described previously (see Section 2.1.6.2) by Cabuy *et al* (2004). Necrotic cells were identified and measured on the PI fluorescence histogram as hypodiploid peaks, as previously described (see Section 2.1.6.2) (Nicoletti *et al* 1991). Necrosis was measured by estimating the sub-sub G1 peak. It was only counted when a clear sub-peak was visible, which was separate from the G0/G1 peak. The flow cytometer measured cell debris, which were either fragments of necrotic cells or whole necrotic cells.

2.1.7 Statistical Methods

Analyses used generalized linear models with quasi-likelihood standard errors (Hardin and Hilbe, 2001) and hypergeometric confidence intervals and Fisher exact tests for odds ratios.

For the TRAP assay data and telomere length assay data, a linear regression model was used, with the logs of the assay measurements as the outcomes, to calculate confidence intervals for geometric means and their Treated/Control and hTERT+/hTERT- Ratios.

For the assays of proportions of cells that were clonogenic, tetraploid, micronucleate, with nucleoplasmic bridges, binucleate, staining with Trypan Blue, apoptotic, necrotic, and in cell cycle phases G0/G1, S and G2/M, a generalized linear model was used, with a log link function, a binomial variance function with total equal to the number of cells in the sample assayed, and a common overdispersion parameter based on Pearson's chi-squared, to calculate

confidence intervals for population proportions of cells assayed as positive ("positivity rates") and their Treated/Control and hTERT+/hTERT- Ratios ("rate ratios").

The chromosome aberration types assayed were: breaks, dicentrics, tetraploidy, "total aneuploidy" (defined as aneuploidy, hypodiploidy or hyperdiploidy) and "aneuploidy gain" (defined as aneuploidy or hyperdiploidy). These were assayed in samples of cells, and some of these aberration types were expected to occur only in a small number of cells in the sample. For each aberration type, hypergeometric confidence intervals were calculated for the Treated/Control and hTERT+/hTERT- Odds Ratios, and Fisher exact tests were carried out for an odds ratio of one, as would be expected if treatment or cell type had no effect on the proportion of aberrant cells.

2.2 Radiation

2.2.1 Cell Lines

The cell lines used were the same as those described in Section 2.1.1.

2.2.2 Experimental Procedure

For all the experiments, 5 x 10^5 cells, plated in 75 cm^2 flasks were allowed to attach for 24 hours. These cells were then exposed to different doses of γ-radiation. Control flasks were not exposed to radiation. The effects of radiation exposure were studied immediately *(Day 0)* and at 30 days *(Day 30)* after a single exposure to radiation. These cells were trypsinised every three days.

2.2.3 Radiation

Both types of cells were exposed to two different doses of gamma radiation (either 0.05 Gy or 0.5 Gy). The cells were irradiated at room temperature using a Colbalt ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin). The dose administered from this unit is a function of the distance of the source from the culture flask/object, dose rate and size of radiation field. The standard distance from source to skin/flask (SSD) is 80cm, but in this study flasks were irradiated at a distance of 100cm and 170cm from the source. A correction factor was applied for the change in distance. The dose rate was approximately 1.8 Gy/ min during these experiments.

2.2.4 Cell Survival

In these experiments cells were exposed to either 0.05 Gy or 0.5 Gy doses of ionizing radiation. These radiation doses were selected because higher doses of radiation were supposed

to induce high toxicity, resulting in a low plating efficiency (PE) [PE (%) = (№ of Colonies Counted / № of Cells Plated) x 100].

2.2.4.1 Clonogenic Assay

The clonogenic assay was performed as described in Section 2.1.4.5.

2.2.5 Cell Damage

In these experiments cells were exposed to either 0.05 Gy or 0.5 Gy doses of ionizing radiation. These radiation doses were selected because higher doses of radiation were supposed to induce high cytotoxicity, resulting in a) a low yield of binucleated cells in the Micronucleus Assay; and b) a very low yield of metaphase spread in the Chromosome Aberration Analysis.

2.2.5.1 Micronucleus Assay

Immediately after the radiation exposure (24 hours before the harvesting) the cells were exposed to cytochalasin-B (Sigma) at a concentration of 6 μg/ml for 24 hours. After 24 hours of exposure, cells were harvested for the detection of micronuclei formation and other endpoints (see below) to assess the initial effects (*Day 0*) of the radiation insult. The cells were then centrifuged (1200 rpm for 6 minutes), and resuspended in 1 ml of fresh media. Flasks containing cells used for the detection of damage at *Day 1* and at *Day 30* were grown a day more and 30 days more respectively. These cells were subcultured every three days. The micronucleus assay (in binucleated cells) was performed according to the method of Fenech and Morley (1985).

The protocol and the scoring criteria were the same as described in Section 2.1.5.1.

2.2.5.2 Chromosome Aberration Analysis

The chromosome aberration analysis was performed as described in Section 2.1.5.2.

2.2.6 Statistical Methods

The statistical analysis was performed as described in Section 2.1.7.

2.3 Combined Exposure

2.3.1 Cell Lines

The cell lines used were the same as those described in Sections 2.1.1 and 2.2.1.

2.3.2 Experimental Procedure

For all the experiments, 5 x 10^5 cells, plated in 75 cm^2 flasks were allowed to attach for 24 hours. Exactly a day later, these cells followed a different experimental procedure, depending on the sequence of the different insults.

1) Cells were first treated for 24 hours with a single dose of metal, 0.4 μM Cr (VI), they were then exposed to a single dose of radiation (0.05 Gy or 0.5 Gy), and the next day they were harvested.

2) Cells were first exposed to a single dose of radiation (0.05 Gy or 0.5 Gy), the next day they were treated for 24 hours with a single dose of metal, 0.4 μM Cr (VI), and the next day they were harvested.

Therefore, in both experimental procedures, the cells were kept in culture for three days.

For each type of cells, there were eleven dose points, four of which (8, 9, 10 and 11) were combined exposure:

1. Control
2. Metal + *Sham Irradiation*
3. *Sham Irradiation* + Metal
4. Radiation (0.05 Gy) + *Vehicle Control*
5. *Vehicle Control* + Radiation (0.05 Gy)
6. Radiation (0.5 Gy) + *Vehicle Control*
7. *Vehicle Control* + Radiation (0.5 Gy)

8. Metal + Radiation (0.05 Gy)

9. Radiation (0.05 Gy) + Metal

10 Metal + Radiation (0.5 Gy)

11. Radiation (0.5 Gy) + Metal

Control flasks were exposed to neither metal nor radiation. 1X PBS was used as a control for the metal treatment with the same volume (*Vehicle Control*), whereas the flasks were treated identically to the irradiated cells without the actual irradiation (*Sham Irradiation*).

The effects of the insult/s on the cells were studied immediately (*Day 0)* and 30 days *(Day 30)* after either a single metal treatment, or a single radiation exposure, or a combined exposure (metal + radiation, or *viceversa*). These cells were trypsinised every three days. 24 hours after the metal treatment the *Day 0* cells were washed twice with 1X PBS, to remove thoroughly any traces of metal ions. The other cells, used for delayed effects *(Day 30)* of either only metal exposure or combined exposure, were also washed twice with 1X PBS and fresh medium was added into the flasks.

2.3.3 Metal

Both types of cells were exposed to a single dose of Cr (VI) (0.4 μM), using potassium dichromate ($K_2Cr_2O_7$) (Sigma) for 24 hours. The metal salt was diluted in 1X PBS in order to reach the concentration used.

2.3.4 Radiation

The radiation exposure was performed as described in Section 2.2.3.

2.3.5 Cell Survival

In these experiments cells were treated with 0.4 μM Cr (VI) for 24 hours and/or exposed to either 0.05 Gy or 0.5 Gy doses of ionizing radiation. The metal dose of 0.4 μM was chosen because it had given significant results in cell survival (See Chapter 9). These radiation doses were selected because higher doses of radiation were supposed to induce high toxicity, resulting in a low plating efficiency (PE) [PE (%) = (№ of Colonies Counted / № of Cells Plated) x 100].

2.3.5.1 Clonogenic Assay

The clonogenic assay was performed as described in Sections 2.1.4.5 and 2.2.4.1.

2.3.6 Cell Damage

In these experiments cells were treated with 0.4 μM Cr (VI) for 24 hours and/or exposed to either 0.05 Gy or 0.5 Gy doses of ionizing radiation. The metal dose of 0.4 μM was chosen because it had given significant results in previous cytogentic studies. The radiation doses of 0.05 Gy or 0.5 Gy were selected because higher doses of radiation were supposed to induce high cytotoxicity, resulting in a) a low yield of binucleated cells in the Micronucleus Assay; and b) a very low yield of metaphase spread in the Chromosome Aberration Analysis.

2.3.6.1 Micronucleus Assay

Immediately after either the metal exposure (in the experiment of *Radiation Followed by Metal*) or the radiation exposure (in the experiment of *Metal Followed by Radiation*), and therefore 24 hours before the harvesting, the cells were exposed to cytochalasin-B (Sigma) at concentration of 6 μg/ml for 24 hours. After 24 hours of exposure, cells were harvested for the detection detection of micronuclei formation and other endpoints (see below) to assess the

initial effects (*Day 0*) of either metal only, radiation only or combined exposure insult. The cells were then centrifuged (1200 rpm for 6 minutes), and resuspended in 1 ml of fresh media. Flasks containing cells used for the detection of damage at *Day 30* were grown 30 days more. These cells were subcultured every three days. The micronucleus assay (in binucleated cells) was performed according to the method of Fenech and Morley (1985).

The protocol and the scoring criteria were the same as described in Sections 2.1.5.1 and 2.2.5.1.

2.3.6.2 Chromosome Aberration Analysis

The chromosome aberration analysis was performed as described in Sections 2.1.5.2 and 2.2.5.2.

2.3.7 Statistical Methods

The statistical analysis was performed as described in Sections 2.1.7 and 2.2.6.

CHAPTER 3

RESULTS

3.1 Metal Exposure

3.2 Radiation Exposure

3.3 Combined Exposure

3.1 Metal Exposure

3.1.1 Cell Survival

hTERT- (wild type) and hTERT+ (immortalised) human BJ fibroblasts were exposed to three doses of Cr(VI) ions (0.04 μM, 0.4 μM and 4 μM) and three doses of V(V) ions (0.5 μM, 5 μM and 50 μM) for 24 hours. The cells were then examined for signs of cell survival at 0 (*Day 0*), 5 (*Day 5*) and 30 days (*Day 30*) after exposure.

3.1.1.1 Cell Viability

Both Cr (VI) and V (V) caused an increase in dead cells in a dose dependent manner up to 30 days after exposure (Figures 3.1). The increase was most pronounced immediately after exposure (*Day 0*) and then decreased thereafter (*Day 5* and *Day 30*). There was a loss of cell viability in hTERT+ cells (Figures 3.1b and 3.1d) but this was significantly less than in hTERT- cells (Figures 3.1a and 3.1c), especially with the highest dose of Cr (VI) (4 μM) and V (V) (50 μM). This difference was most pronounced at *Day 0* and diminished thereafter (Figure 3.2). Only the 5 μM and 50 μM doses of V (V) showed a persistent difference, in the hTERT+/hTERT- rate ratio, up to 30 days after metal exposure (Figure 3.2). There was no difference in cell viability of control values, at any time after the metal exposure, between hTERT- cells and hTERT+ cells. These results were expressed as a percentage of dead cells.

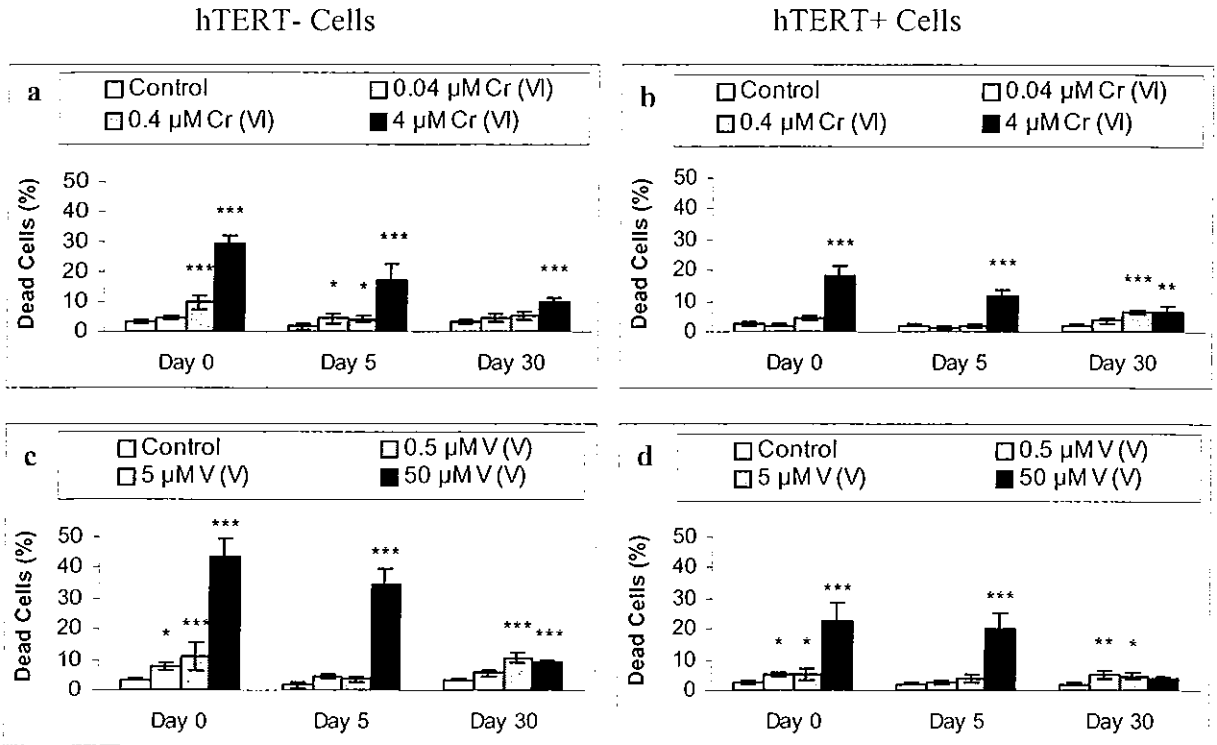

Figure 3.1. Cell Viability. The figures show the percentage of dead cells in hTERT– cells (Fig. 3.1a and 3.1c) and hTERT+ cells (Fig. 3.1b and 3.1d) after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

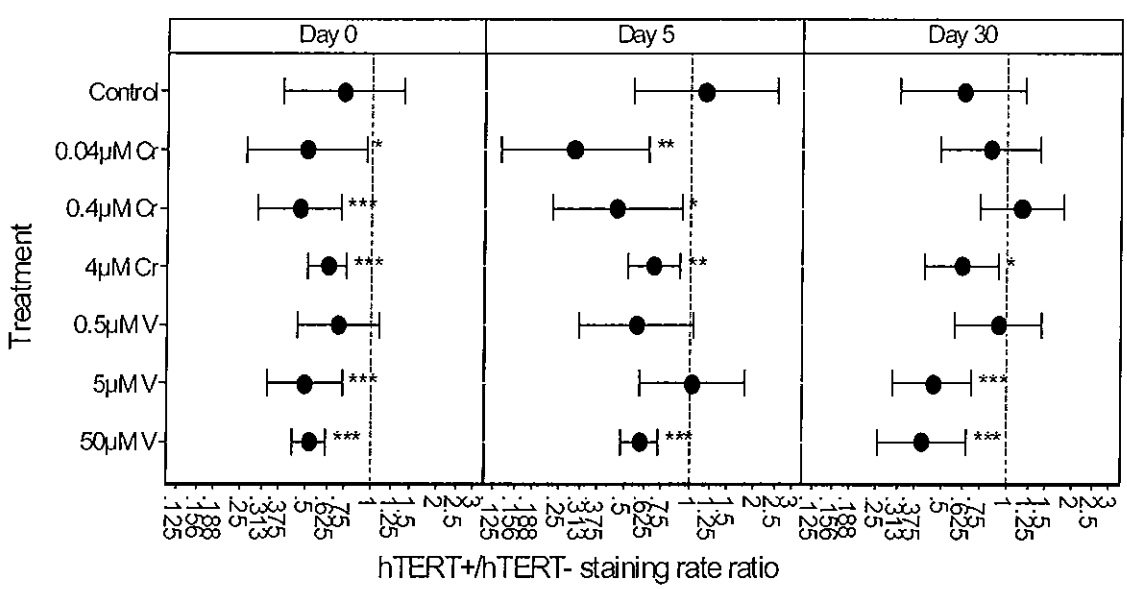

Figure 3.2. Cell Viability. The figure illustrates the rate ratio of hTERT+/hTERT- for trypan blue staining of dead cells, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.1.2 Mean Counts

In both types of cells, the highest dose of Cr (VI) (4 μM) and V (V) (50 μM) caused a significant decrease in the Mean Counts (MC) of viable cells at *Day 0*, which persisted up to *Day 30* (Figures 3.3). The dose of 5 μM V (V) also caused a significant decrease of the MC at *Day 0* in both types of cells, but it persisted up to *Day 30* only in hTERT+ cells. Furthermore, the lowest dose of V (V) (0.5 μM) caused a significant decrease of the MC only in hTERT+ cells, but from *Day 5* onwards. The hTERT- control cells gave a MC of 97,67 cells at *Day 0*, which decreased to 80,00 at *Day 30* (Figures 3.3a and 3.3c), whereas the hTERT+ control cells had a MC of 148,00 cells at *Day 0*, which slightly decreased to 140,33 at *Day 30* (Figures 3.3b and 3.3d). In all of the experiments, at all survival times, with or without metal treatment, there was a statistically significant difference in the MC, between hTERT- and hTERT+ cells (Figure 3.4). These results were expressed as a MC of viable cells.

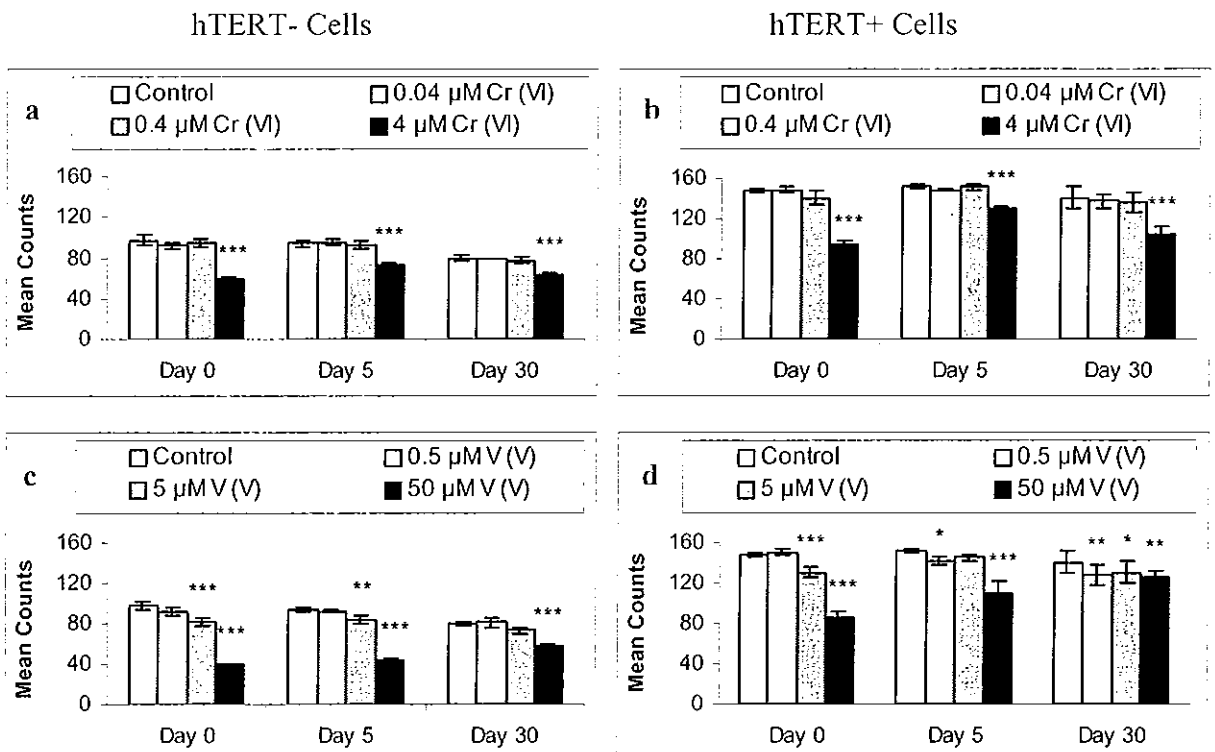

Figure 3.3. Mean Counts (MC). The figures show the MC of viable hTERT– cells (Fig. 3.3a and 3.3c) and viable hTERT+ cells (Fig. 3.3b and 3.3d) after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

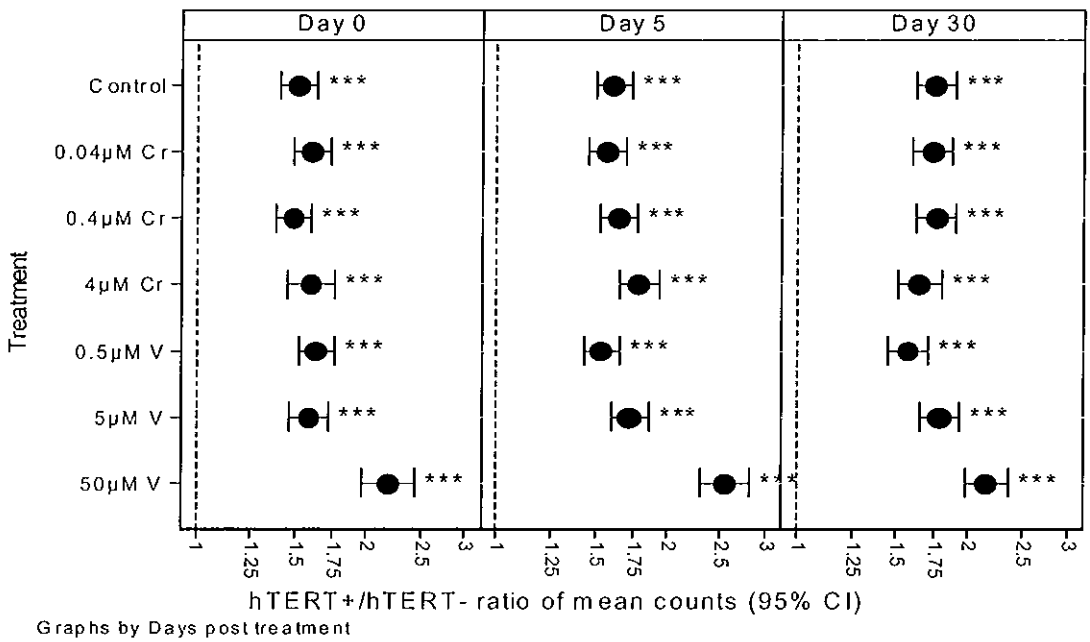

Figure 3.4. Mean Counts (MC). The figure illustrates the rate ratio of hTERT+/hTERT- for the MC of viable cells, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.1.3 Doubling Time

The hTERT+ cells grew faster than the hTERT- cells when cultured routinely at 70% confluency and there was a very slight slowing of growth in control hTERT- cells by *Day 30*. Only the highest dose of Cr (VI) (4 µM) and V (V) (50 µM) caused a significant decrease in cell proliferation (Figure 3.5). The decrease was most pronounced immediately after exposure and then decreased thereafter. The other concentrations of Cr (VI) (0.04 µM and 0.4 µM) and V (V) (0.5 µM and 5 µM) did not alter the division (or proliferation) of the cells over 48 hours. It took 24.42 hours for control hTERT- cells (Figures 3.5a and 3.5c) to complete their cell cycle doubling time (or cell cycle time) at *Day 0*, and 28.60 hours at *Day 30*. Due to a much higher cell proliferation, control hTERT+ cells (Figures 3.5b and 3.5d) completed their cell cycle in 18.71 hours at *Day 0*, and 19.29 hours at *Day 30*. The effect of the highest dose of Cr (VI) and V (V), in hTERT- cells, was persistent up to 30 days after the metal exposure. The 4

μM Cr (VI)-treated hTERT- cells showed a cell cycle time of 39.00 hours at *Day 0*, which slightly decreased to 35.59 at *Day 30*. The 50 μM V (V)-treated hTERT- cells showed a cell cycle time of 73.41 hours at *Day 0*, which decreased to 39.27 hours at *Day 30*. The effect of the highest dose of Cr (VI) (4 μM) and V (V) (50 μM) were much less pronounced in hTERT+ cells, both immediately after the metal exposure and 30 days after. In all of the experiments, at all survival times, with or without metal treatment, there was a statistically difference in doubling time, between hTERT- and hTERT+ cells (Figure 3.6). These results were expressed in hours.

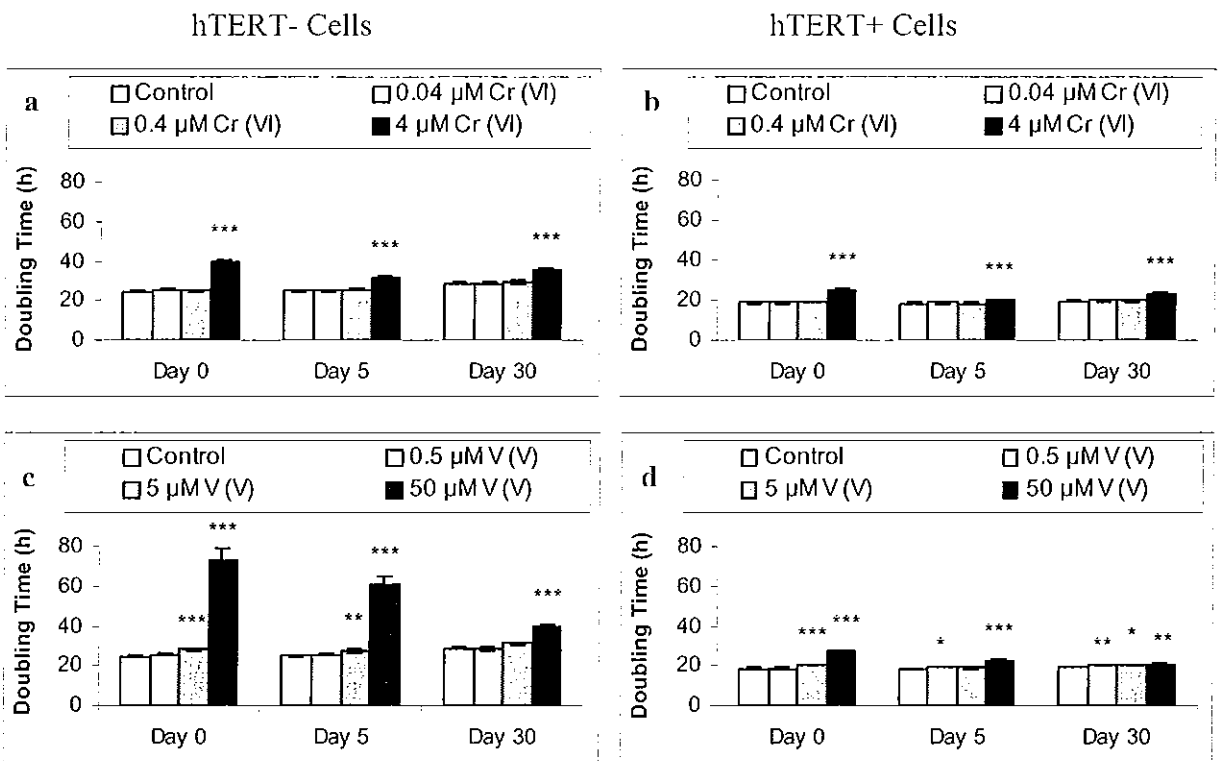

Figure 3.5. Doubling Time. The figures show the doubling time (hours) in hTERT– cells (Fig. 3.5a and 3.5c) and hTERT+ cells (Fig. 3.5b and 3.5d) after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

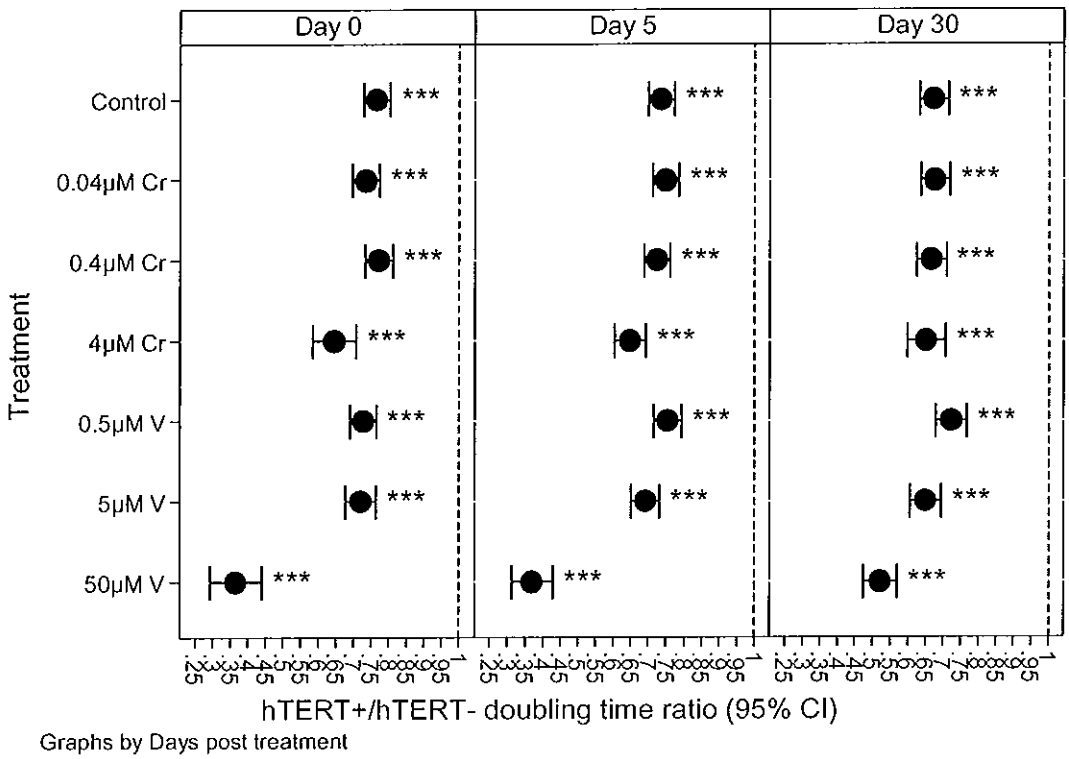

Figure 3.6. Doubling Time. The figure illustrates the rate ratio of hTERT+/hTERT- cells for the doubling time, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.1.4 Clonogenic Survival

Both Cr (VI) and V (V) caused a reduction in clonogenic survival of hTERT- cells in a dose dependent manner (Figures 3.7a and 3.7c). This reduction persisted up to 30 days after a single 24-hour exposure to metal. A different pattern was observed in hTERT+ cells (Figures 3.7b and 3.7d). There was no loss of survival in metal exposed hTERT+ cells, except at the highest doses of Cr (VI) (4μM) and V (V) (50μM). However, at these doses, the loss of clonogenic survival was less than in hTERT- cells. In all of the experiments, at all survival times, with or without metal exposure there were more colonies in hTERT+ cells compared to hTERT- cells ($p<0.01$) (Table A and Figure 3.8). These values were expressed as a percentage of control (PBS) and control was set to 100%.

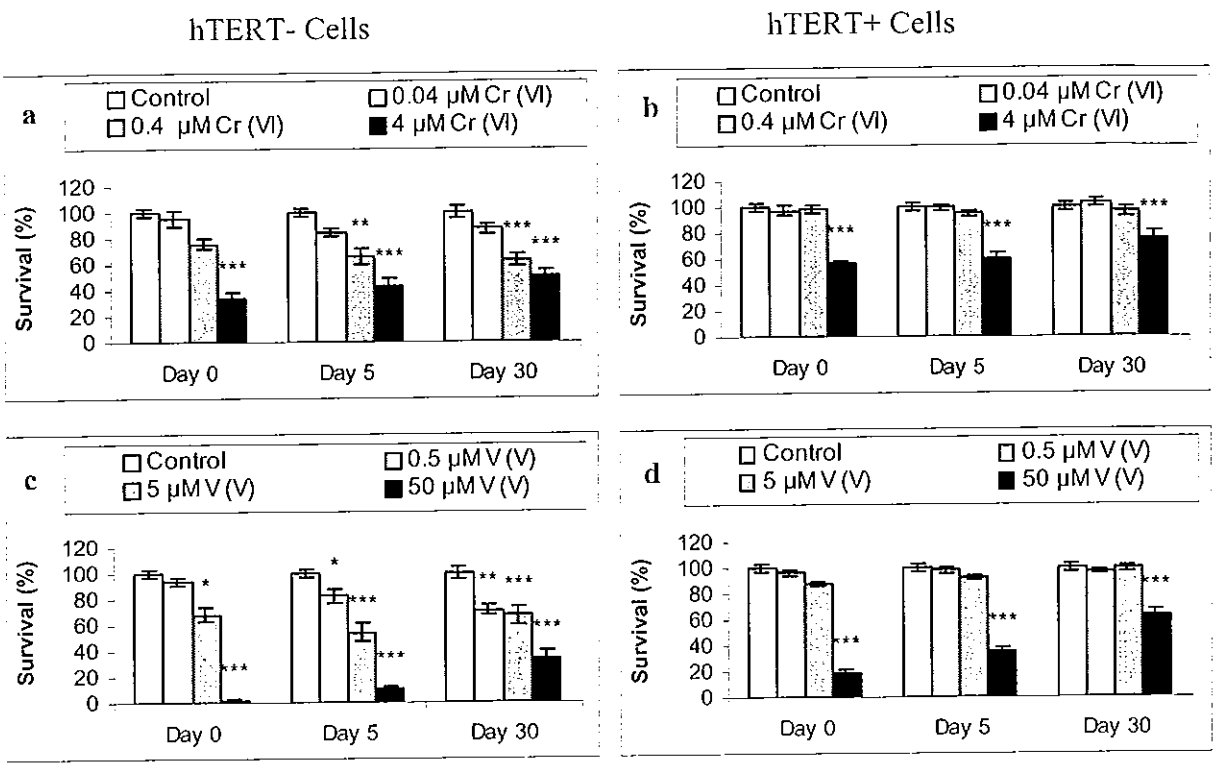

Figure 3.7. Clonogenic Survival. The figures show the clonogenic survival in hTERT– cells (Fig. 3.7a and 3.7c) and hTERT+ cells (Fig. 3.7b and 3.7d) after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure. These values were expressed as a percentage of control (PBS) and control was set to 100%. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

Table A. Clonogenic Survival in hTERT– cells and hTERT+ cells after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure. These values were expressed as a Number of Colonies

Treatment	Types of Cells					
	hTERT- Cells			hTERT+ Cells		
	Day Post Treatment			Day Post Treatment		
	Day 0	Day 5	Day 30	Day 0	Day 5	Day 30
Control	103.00	102.00	94.00	147.17	143.17	137.00
0.04 μM Cr (VI)	98.33	86.50	81.83	143.17	142.50	140.83
0.4 μM Cr (VI)	78.17	67.83	59.83	145.00	135.50	131.83
4 μM Cr (VI)	35.50	44.17	47.17	83.50	87.00	102.33
0.5 μM V (V)	96.67	84.17	67.00	142.33	140.00	132.83
5 μM V (V)	70.33	55.50	63.00	129.83	131.50	136.33
50 μM V (V)	2.33	11.17	31.17	27.50	50.17	86.00

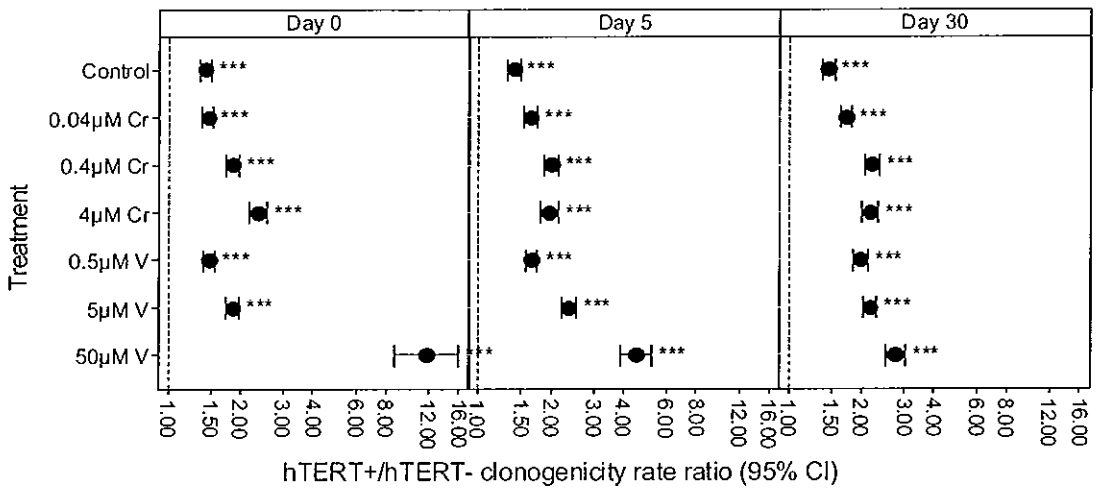

Figure 3.8. Clonogenic Survival. The figure illustrates the rate ratio of hTERT+/hTERT- cells for the clonogenic survival, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.1.5 Senescence

Despite little induction of senescence associated proteins in the clonogenic cells immediately after metal exposure, there was a progressive and dose dependent increase in β-galactosidase stained cells up to 30 days after metal treatment of hTERT- cells (Figures 3.9a and 3.9c). Minimal staining was seen without metal exposure. No increase was seen in the hTERT+ cells (Figures 3.9b and 3.9d). Both with Cr (VI) and V (V), at *Day 5* and *Day 30*, there was a statistically significant lower percentage of senescence in hTERT+ cells, compared to hTERT- cells (Figure 3.10). These results were expressed as a percentage of senescent cells. Figure 3.11 shows a senescent cell stained with β-galactosidase.

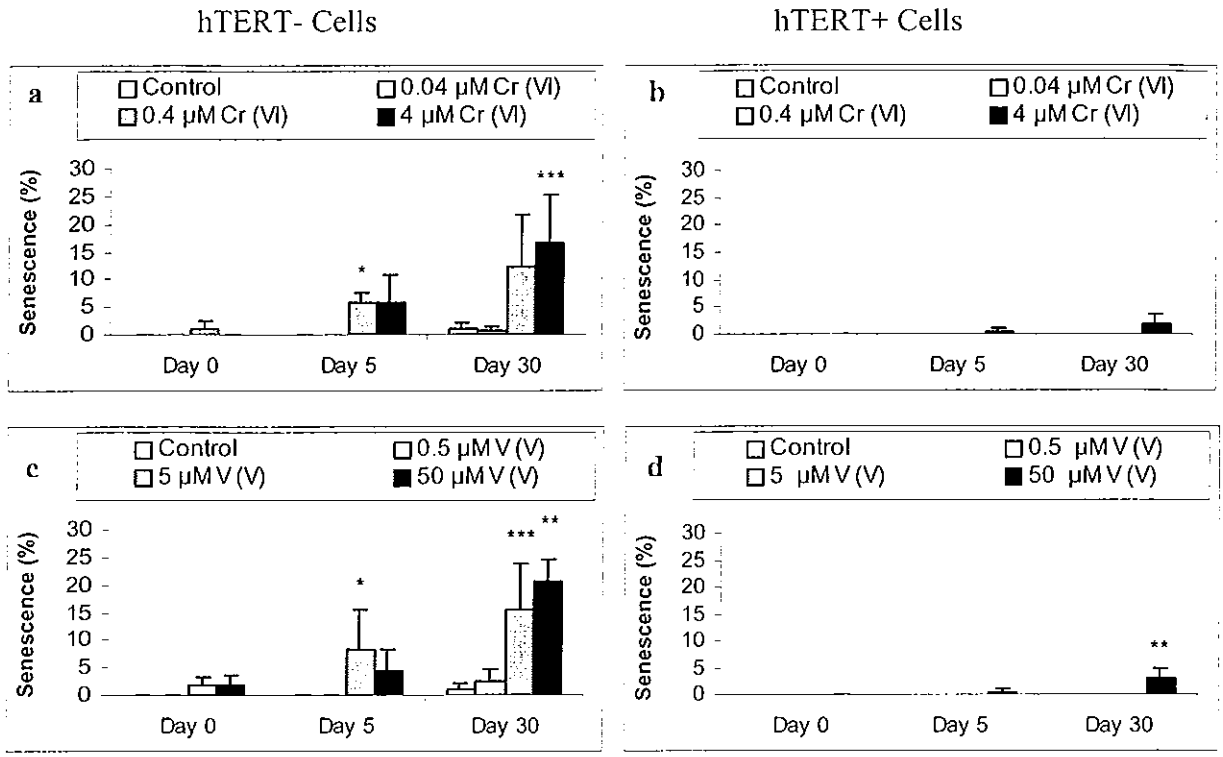

Figure 3.9. Senescent Cells. The figures show the percentage of senescence in hTERT– cells (Fig. 3.9a and 3.9c) and hTERT+ cells (Fig. 3.9b and 3.9d) after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control (PBS).

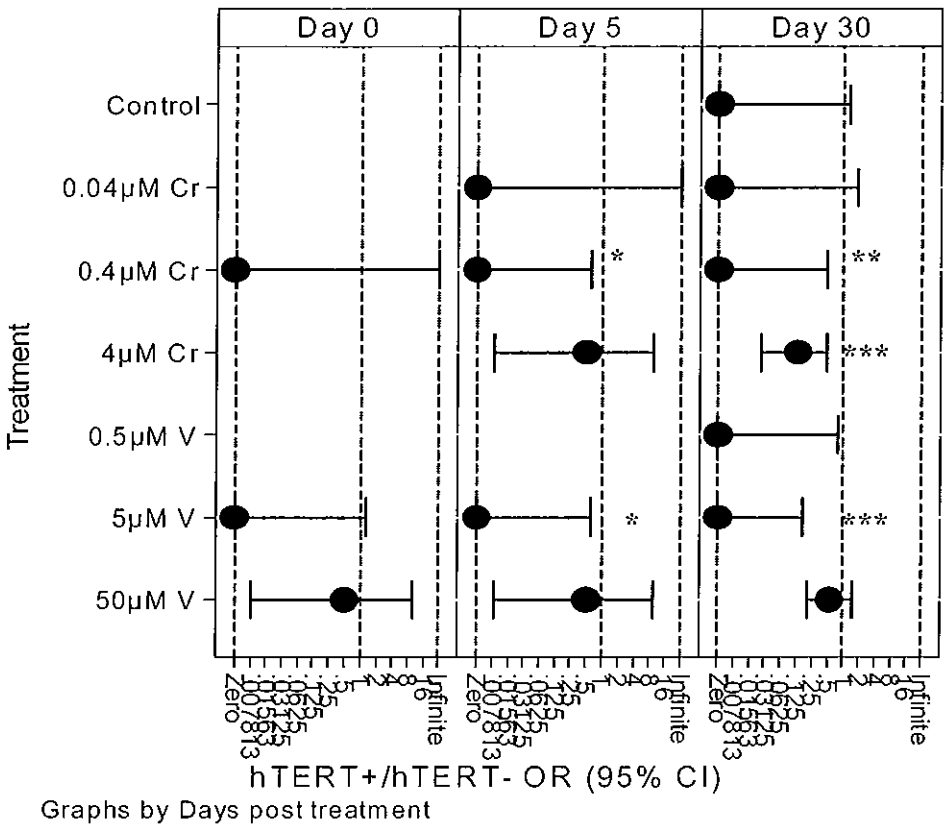

Figure 3.10. Senescence. The figure illustrates the odd ratio of hTERT+/hTERT- for the senescent cells, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to hTERT+/hTERT- = 1.

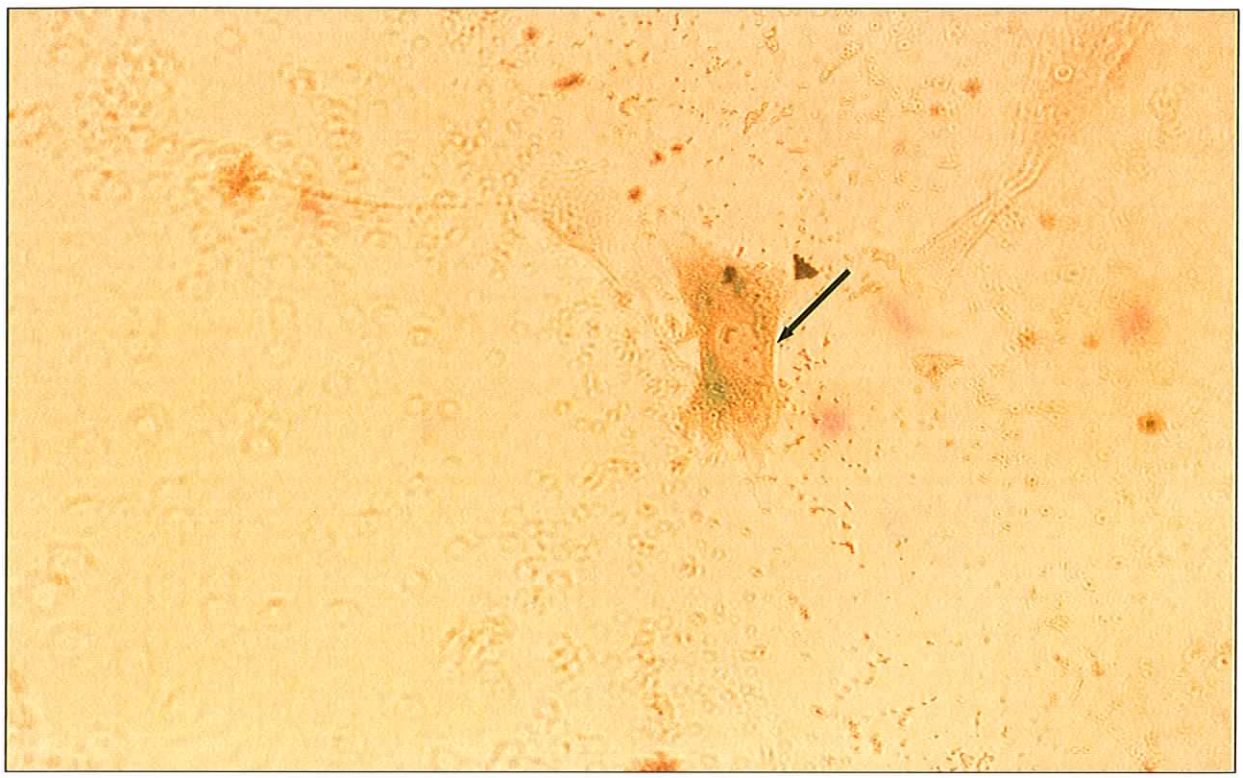

Figure 3.11. Senescent cell stained with β-Galactosidase

3.1.1.6 Apoptosis

The level of background apoptosis in the clonogenic cells increased slightly with time from 15% to 20% in hTERT- cells without metal treatment (Figures 3.12a and 3.12c). The level was higher in the control hTERT+ cells at 32% (Figures 3.12b and 3.12d). After metal exposure there was a persistent and dose dependent induction of apoptosis up to 30 days in hTERT- cells. The response of hTERT+ cells was different, since there was a small increase in apoptosis only with the highest dose of Cr (VI) (4 μM) at *Day 30* and with the highest dose of V (V) at *Day 0* and *Day 5*. In all of the experiments, at all survival times, with or without metal treatment, there was a lower percentage of apoptosis in hTERT- cells compared to hTERT+ cells (Figure 3.13). At *Day 0* and *Day 5*, there was a significant difference in control values and 0.04μM Cr (VI) values, between hTERT- and hTERT+ cells, which did not persist until *Day 30*. There was also significant difference, with 0.4μM Cr (VI) and 5 μM V (V) at *Day 0*,

between hTERT- and hTERT+ cells, which persisted until *Day 30*. These results were expressed as a percentage of apoptotic cells. Figure 3.14 shows apoptotic bodies (late stage of apoptosis).

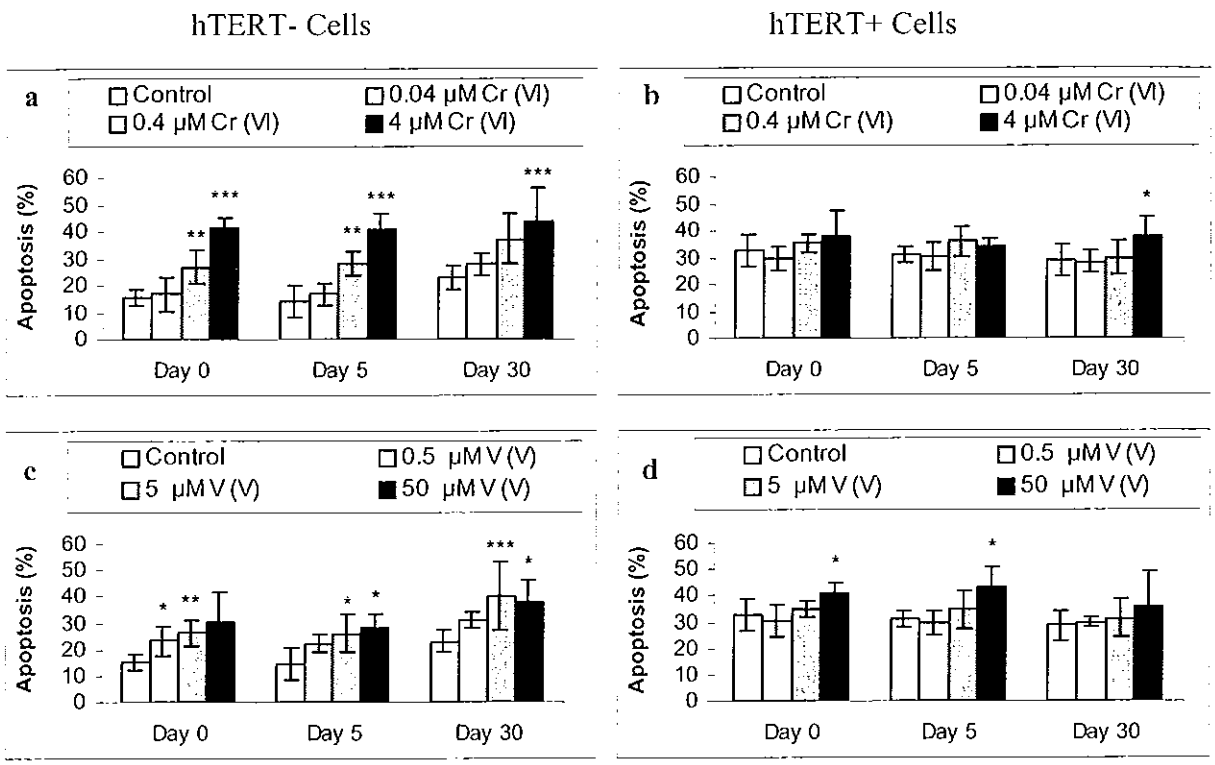

Figure 3.12. Apoptosis. The figures show the percentage of apoptosis in hTERT– cells (Fig. 3.12a and 3.12c) and hTERT+ cells (Fig. 3.12b and 3.12d) after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

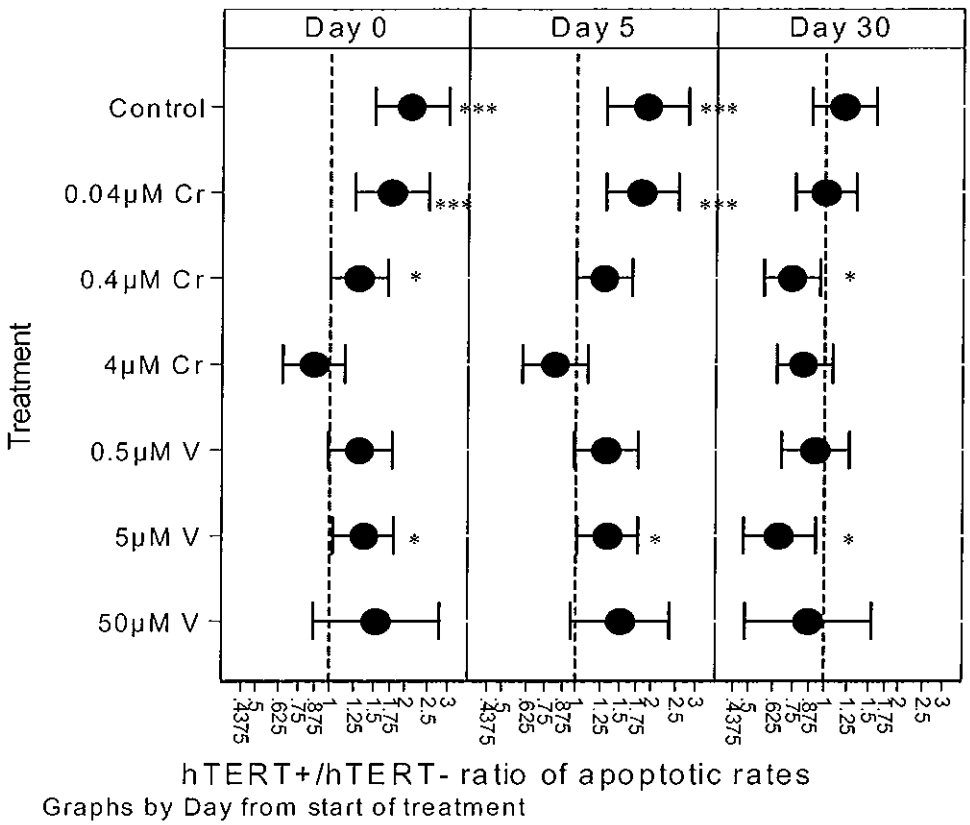

Figure 3.13. Apoptosis. The figure illustrates the rate ratio of hTERT+/hTERT- for apoptotic cells, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post treatment. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

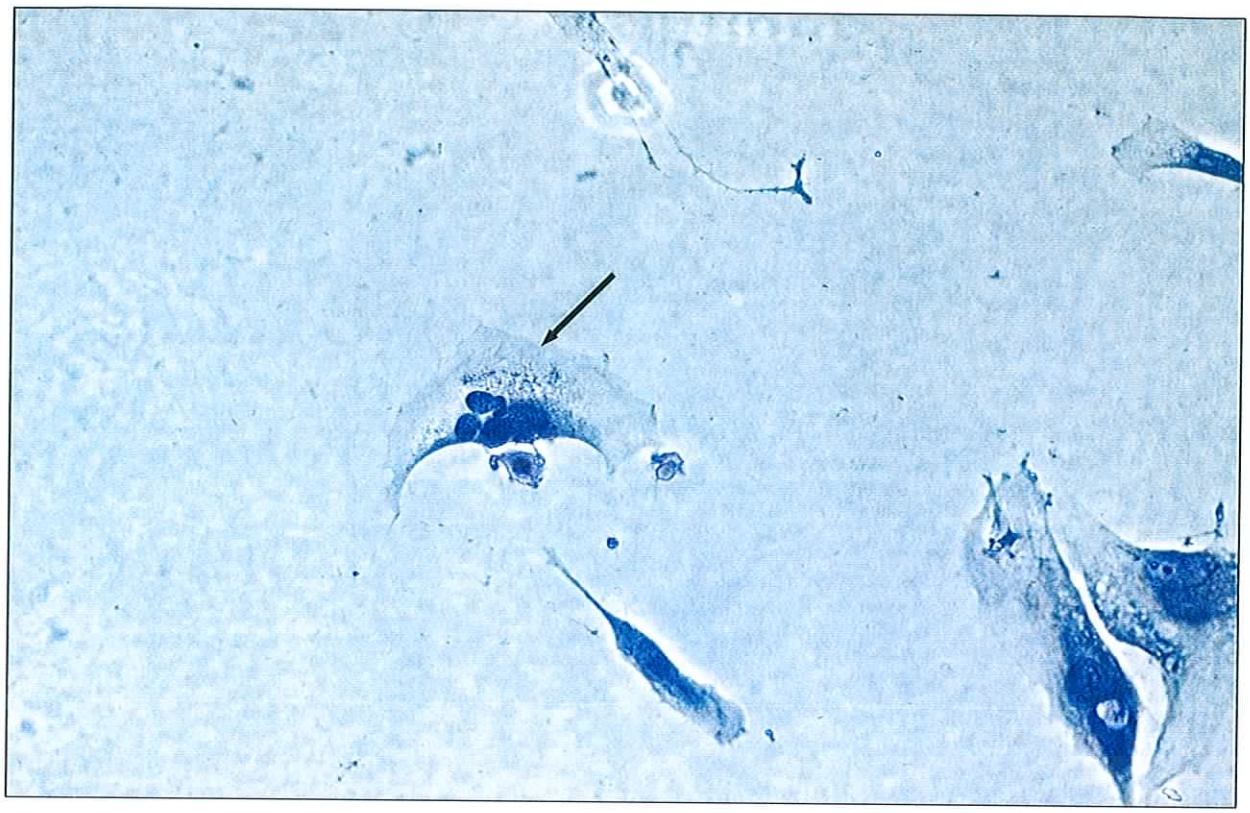

Figure 3.14. Apoptotic cell stained with Haematoxylin Harris' (mercury free) containing apoptotic bodies, which is a late sign of apoptosis.

3.1.1.7 Necrosis

Both Cr (VI) and V (V) caused a small induction of necrosis immediately after metal exposure. However, this induction persisted and slightly increased up to 30 days after metal treatment in hTERT- cells (Figures 3.15a and 3.15c) and hTERT+ cells (Figures 3.15b and 3.15d). hTERT- cells exposed to 50 μM V(V) had a higher percentage of necrosis than hTERT+ cells, at all times post treatment. These results were expressed as a percentage of necrotic cells.

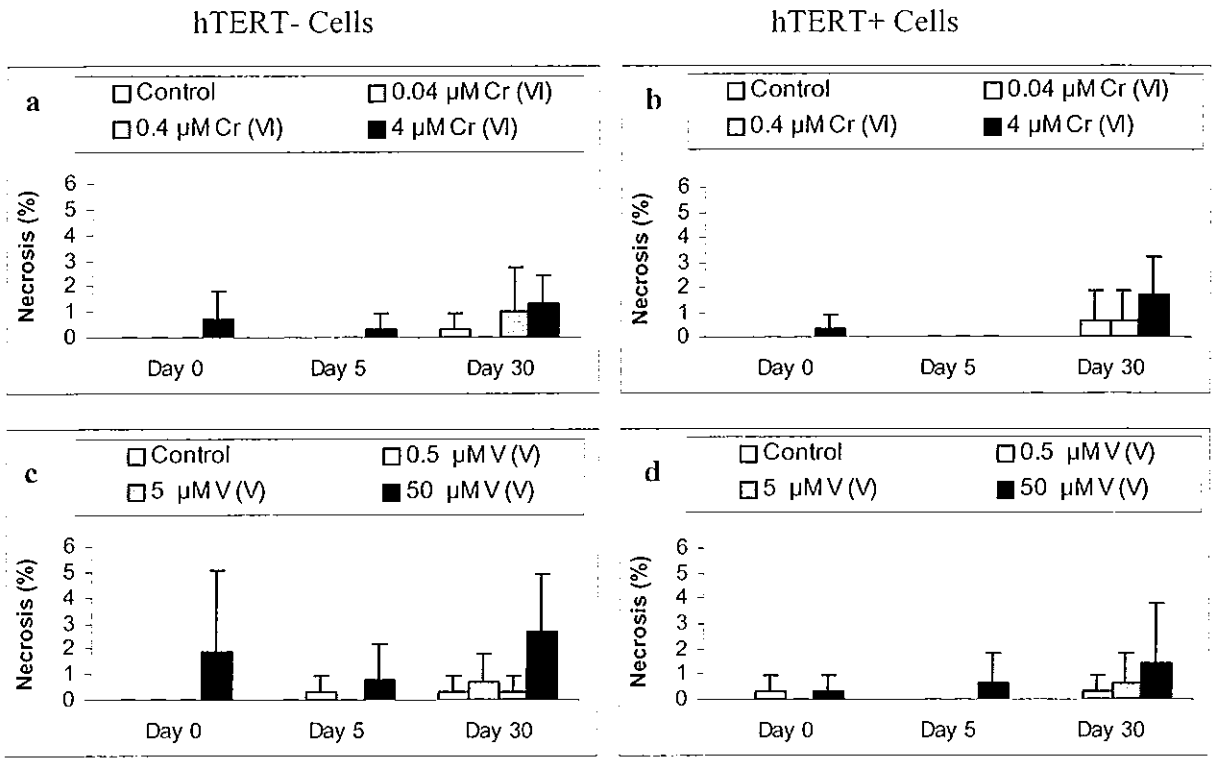

Figure 3.15. Necrosis. The figures show the percentage of necrosis in hTERT– cells (Fig. 3.15a and 3.15c) and hTERT+ cells (Fig. 3.15b and 3.15d) after a 24-hour exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure.

In view of the cell viability, cell cycle time and clonogenic data, the lower doses of Cr (VI) (0.04µM, 0.4µM) and V (V) (0.5µM, 5µM) were chosen in order to allow survival of more than 50% with minimal toxicity and permit a more detailed and practical study of genomic instability.

3.1.2 Cell Damage

hTERT- (wild type) and hTERT+ (immortalised) human BJ fibroblasts were exposed to two doses of Cr (VI) ions (0.04 µM and 0.4 µM) and two doses of V (V) ions (0.5 µM and 5 µM) for 24 hours. The cells were then examined for signs of cell damage at 0 (*Day 0*), 5 (*Day 5*) and 30 days (*Day 30*) after exposure.

3.1.2.1 Micronuclei

Both Cr (VI) and V (V) induced micronuclei (MNi) in hTERT- cells (Figures 3.16a and 3.16c). This induction of micronuclei persisted up to 30 days after a single 24-hour exposure to the metal. A different pattern was seen in hTERT+ cells (Figures 3.16b and 3.16d). There was only a small temporary increase in micronuclei 5 days after a 24-hour exposure and at the higher concentrations of metal. No increase in micronuclei was seen either immediately or at 30 days after metal exposure in hTERT+ cells. The induction of micronuclei by metal, where it was seen, followed a dose response in both hTERT- and hTERT+ cells. In all of the experiments, at all survival times, with or without metal treatment, there was a significantly lower level of micronuclei in the hTERT+ cells compared to the hTERT- cells. This was particularly true 30 days after exposure to metal (Figure 3.21). These results were expressed as a percentage of MNi. Figure 3.17 shows a binucleated cell containing two MNi.

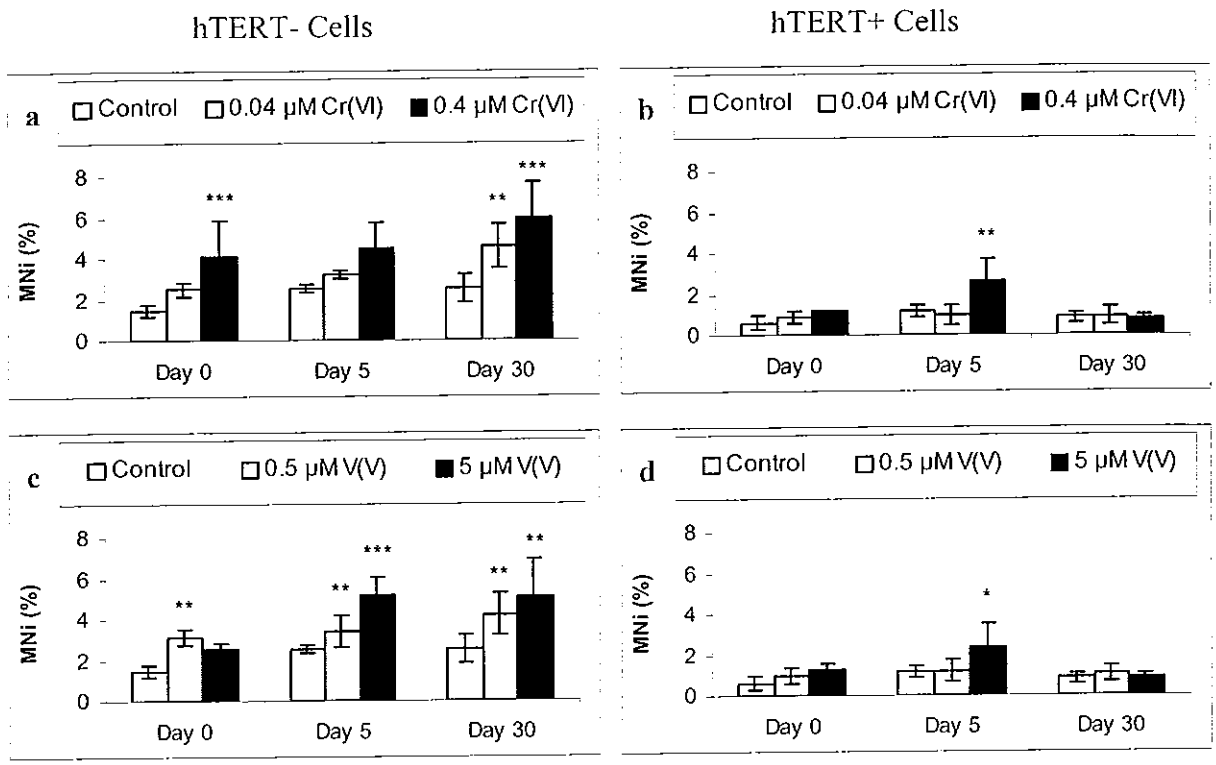

Figure 3.16. Micronuclei (MNi). The figures show the percentage of MNi in hTERT– cells (Fig. 3.16a and 3.16c) and hTERT+ cells (Fig. 3.16b and 3.16d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control (PBS).

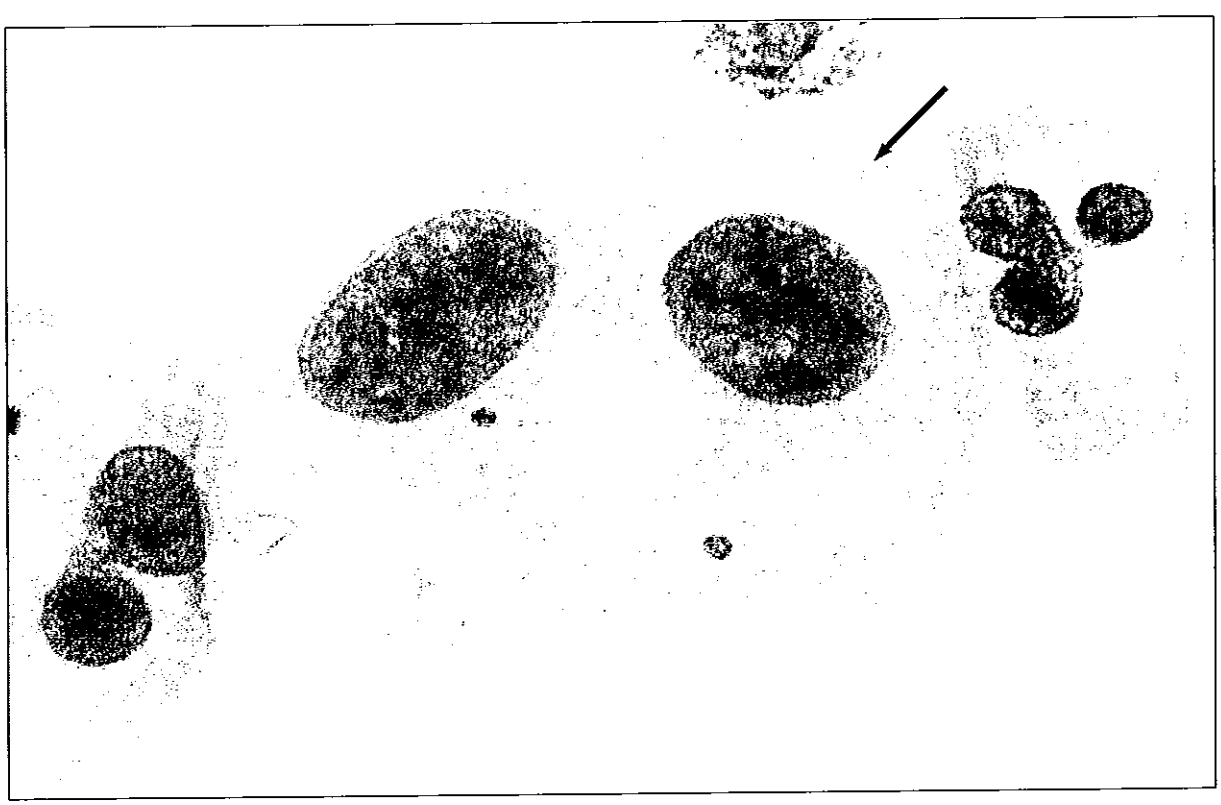

Figure 3.17. Binucleated cell stained with Giemsa containing two micronuclei.

3.1.2.2 Nucleoplasmic Bridges

Both Cr (VI) and V (V) caused an increase in nucleoplasmic bridges (NPB) in hTERT- cells as observed with the micronucleus assay (Figures 3.18a and 3.18c). However, the increase in NPB only reached the level of statistical significance 30 days after the initial 24-hour exposure. Unlike micronuclei, there was no significant increase in NPB by metals in hTERT+ cells (Figures 3.18b and 3.18d). In general, in all of the experiments, at all survival times, with or without metal treatment, there was a significantly lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was particularly true 30 days after metal exposure (Figure 3.21). These results were expressed as a percentage of NPB. Figure 3.19 shows a binucleated cell with a NPB.

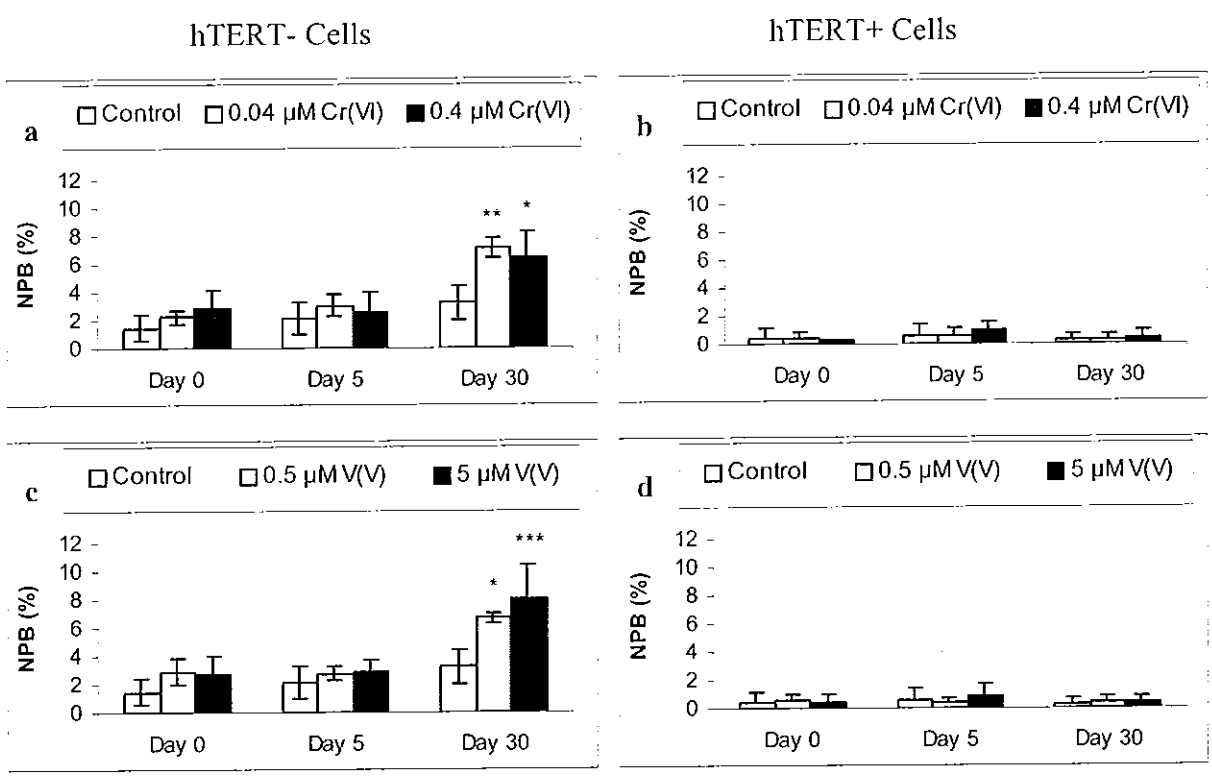

Figure 3.18. Nucleoplasmic Bridges (NPB). The figures show the percentage of NPB in hTERT– cells (Fig. 3.18a and 3.18c) and hTERT+ cells (Fig. 3.18b and 3.18d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

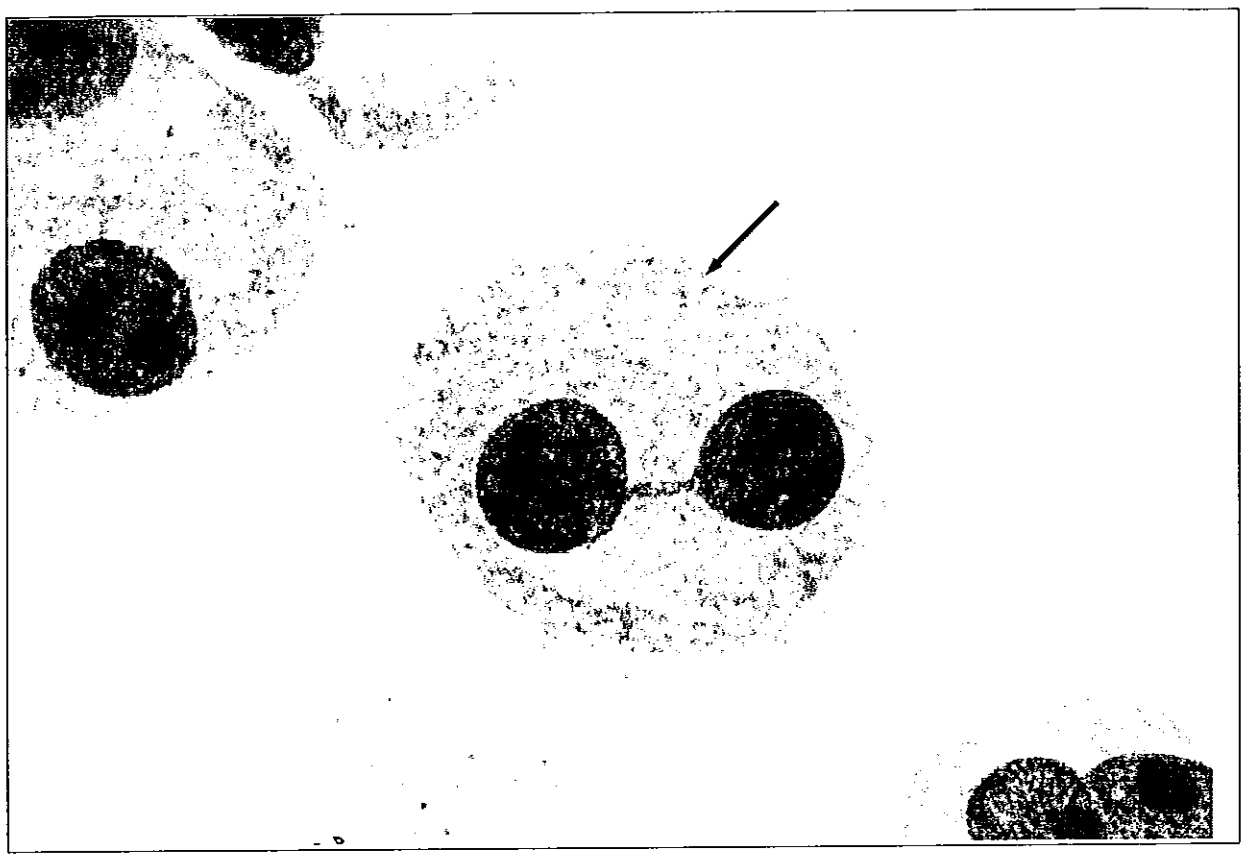

Figure 3.19. Binucleated cells stained with Giemsa having a nucleoplasmic bridge

3.1.2.3 Number of Binucleated Cells

Neither Cr (VI) nor V (V) caused a statistically significant reduction of binucleated cells (BNC). However, both doses of V (V) caused a slight decrease of BNC from *Day 5* onwards, in hTERT+ cells only. In all of the experiments, at all survival times, with or without metal treatment, there was a higher number of binucleated cells in hTERT+ cells (Figures 3.20b and 3.20d) compared to the hTERT- cells (Figures 3.20a and 3.20c). This was particularly true 30 days after exposure to metal, where the number of BNC in hTERT+ cells was double compared to hTERT- cells (Figure 3.21). These results were expressed as a number of BNC.

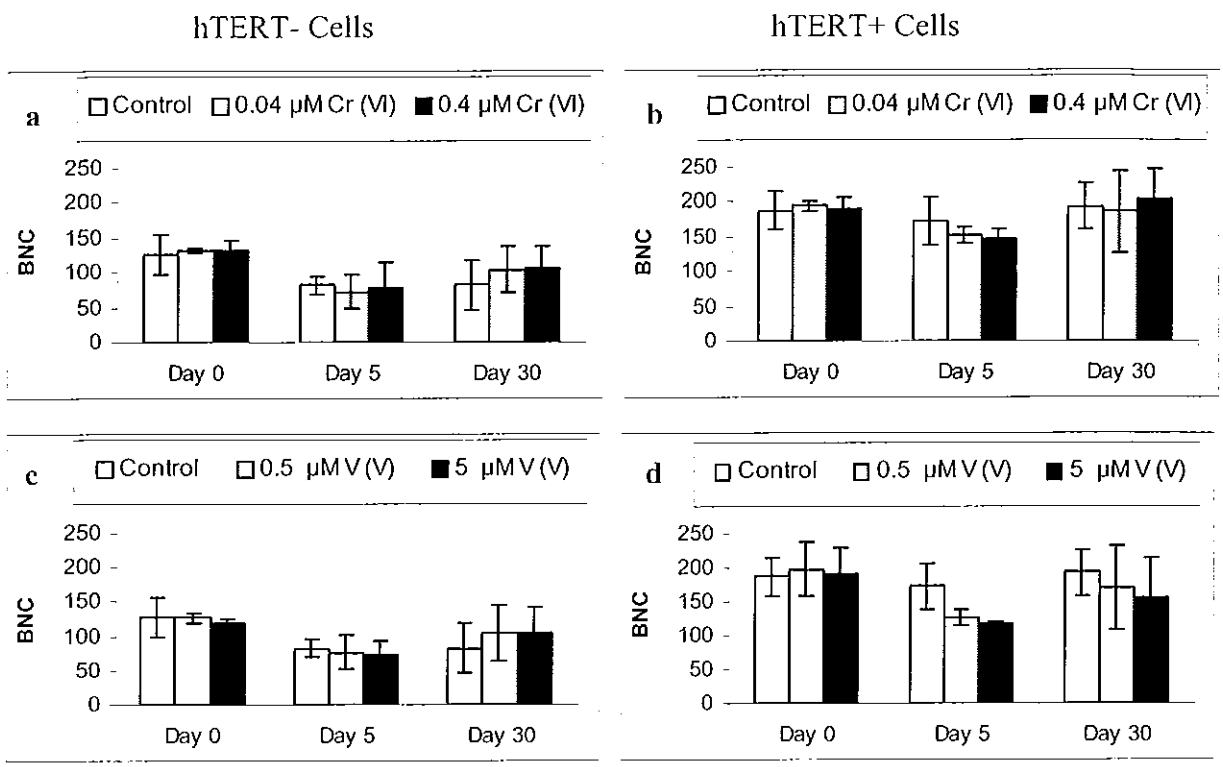

Figure 3.20. Binucleated Cells (BNC). The figures show the number of BNC in hTERT– cells (Fig. 3.20a and 3.20c) and hTERT+ cells (Fig. 3.20b and 3.20d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

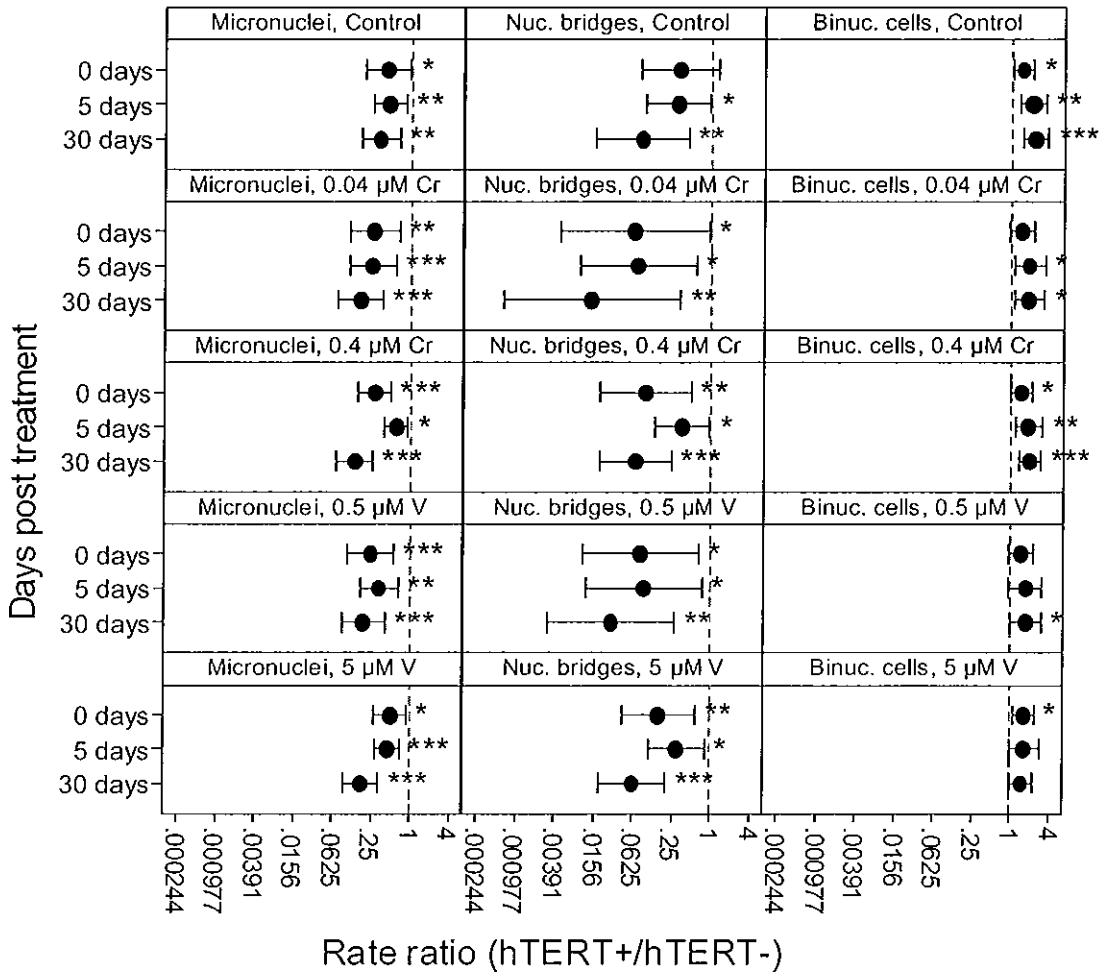

Figure 3.21. Micronuclei (MNi), Nucleoplasmic Bridges (NPB) and Binucleated Cells (BNC). The figure illustrates the rate ratio of hTERT+/hTERT- for MNi, NPB and BNC, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.2.4 Nuclear Division Index

The nuclear division index (NDI), an estimate of cell division, did not change after metal treatment. However, in general for every permutation of metal concentration and days post-exposure, with or without treatment, the number of binucleated cells and hence the nuclear division index was higher in the hTERT+ cells (Figures 3.22b and 3.22d) compared to the hTERT- cells (Figures 3.22a and 3.22c).

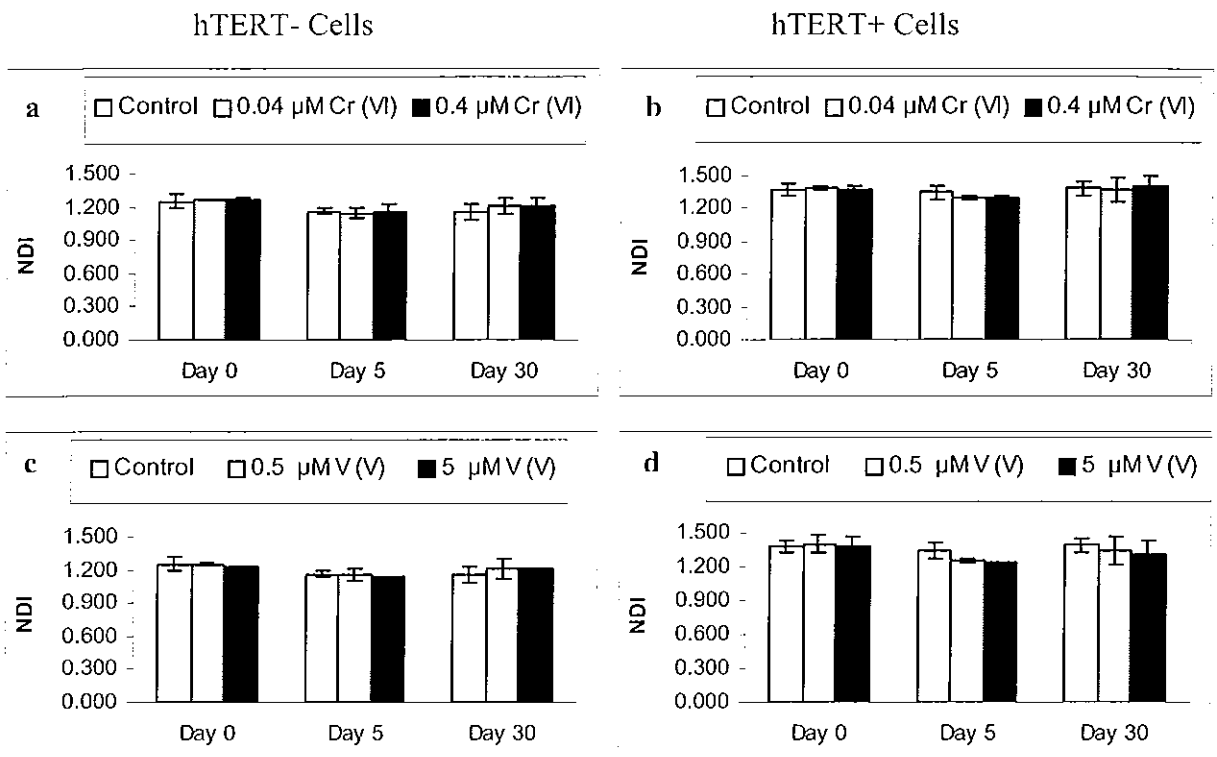

Figure 3.22. Nuclear Division Index (NDI). The figures show the NDI in hTERT– cells (Fig. 3.22a and 3.22c) and hTERT+ cells (Fig. 3.22b and 3.22d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

3.1.2.5 Nuclear Division Cytotoxicity Index

The Nuclear Division Cytotoxicity Index (NDCI), an estimate of cell division, which also takes into consideration the number of dead cells (necrotic and apoptotic cells), did not change after metal treatment. However, in general for every permutation of metal concentration and days post-exposure, with or without treatment, the number of necrotic cells, binucleated cells and hence the NDCI was higher in the hTERT+ cells (Figures 3.23b and 3.23d) compared to the hTERT- cells (Figures 3.23a and 3.23c).

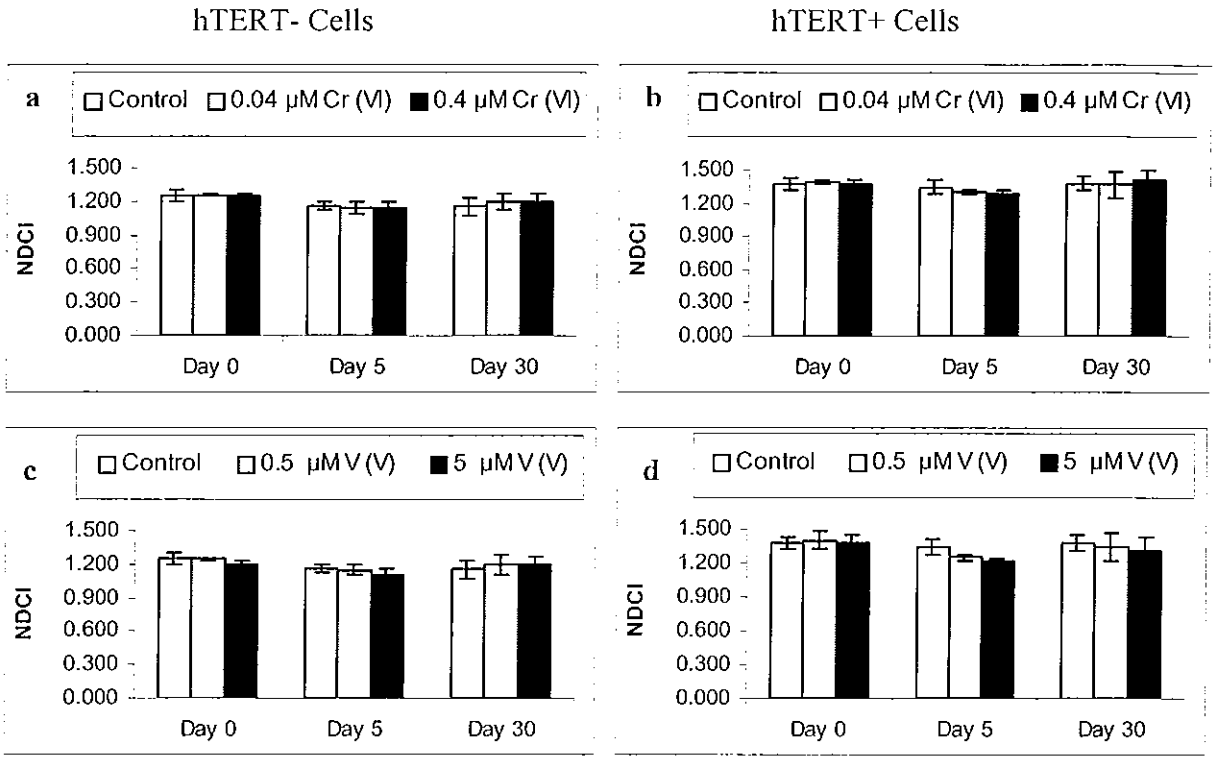

Figure 3.23. Nuclear Division Cytotoxicity Index (NDCI). The figures show the NDCI in hTERT– cells (Fig. 3.23a and 3.23c) and hTERT+ cells (Fig. 3.23b and 3.23d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

3.1.2.6 Chromatid Breaks

Cr (VI) caused a significant increase in chromatid breaks in the hTERT- cells at the higher dose (0.4µM) immediately after metal exposure (Figures 3.24a and 3.24c). No statistically significant change was seen immediately after V (V) treatment. The increase in breaks after Cr (VI) exposure decreased with time. There was a slight increase in chromatid breaks 30 days after treatment with the lower dose (0.5 µM) of V (V). In the hTERT+ cells, there was also a significant and temporary increase in chromatid breaks after Cr (VI) treatment with no statistically significant change after V (V) exposure (Figures 3.24b and 3.24d). However, the incidence of breaks induced by Cr (VI) was lower in the hTERT+ compared to the hTERT- cells and did not persist with time. A direct comparison of hTERT+ and hTERT- cells showed that there were significantly fewer breaks after Cr (VI) treatment in hTERT+ cells but

significantly more after 5 µM V (V) treatment (Figure 3.39). These results were expressed as a percentage of chromatid breaks. Figure 3.25 shows a chromosome with a chromatid break.

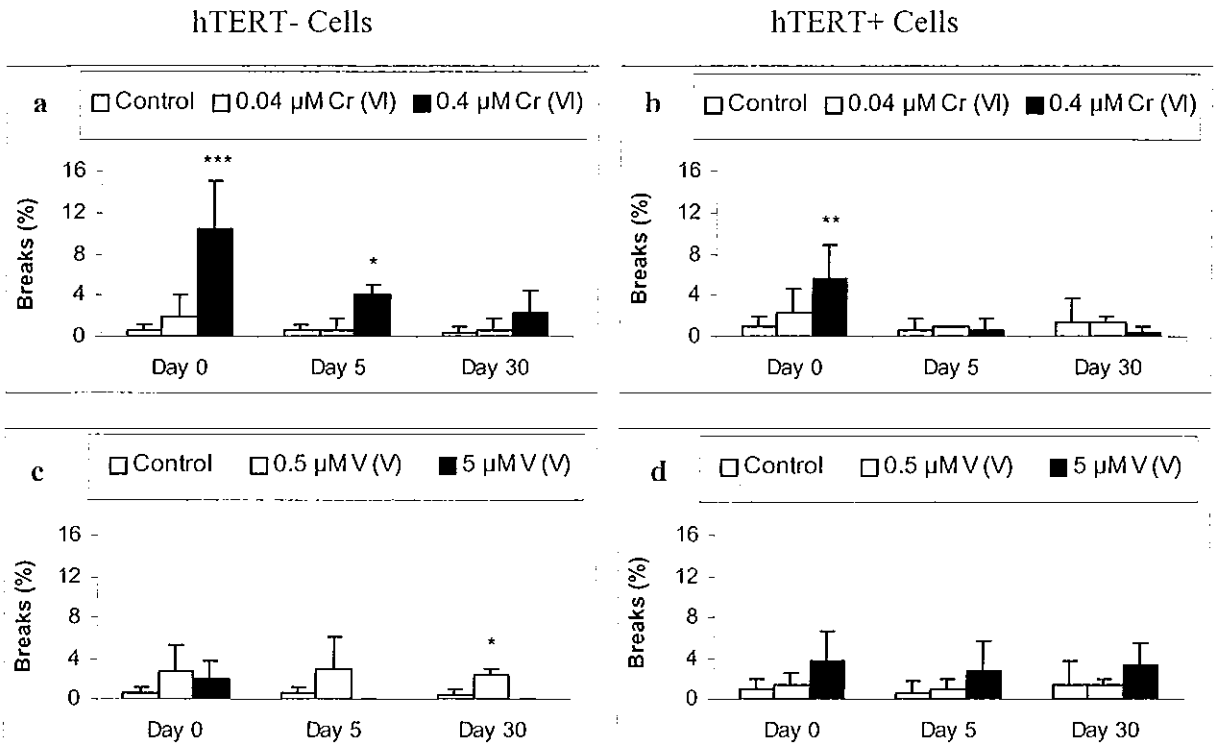

Figure 3.24. Chromatid Breaks. The figures show the percentage of chromatid breaks in hTERT– cells (Fig. 3.24a and 3.24c) and hTERT+ cells (Fig. 3.24b and 3.24d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

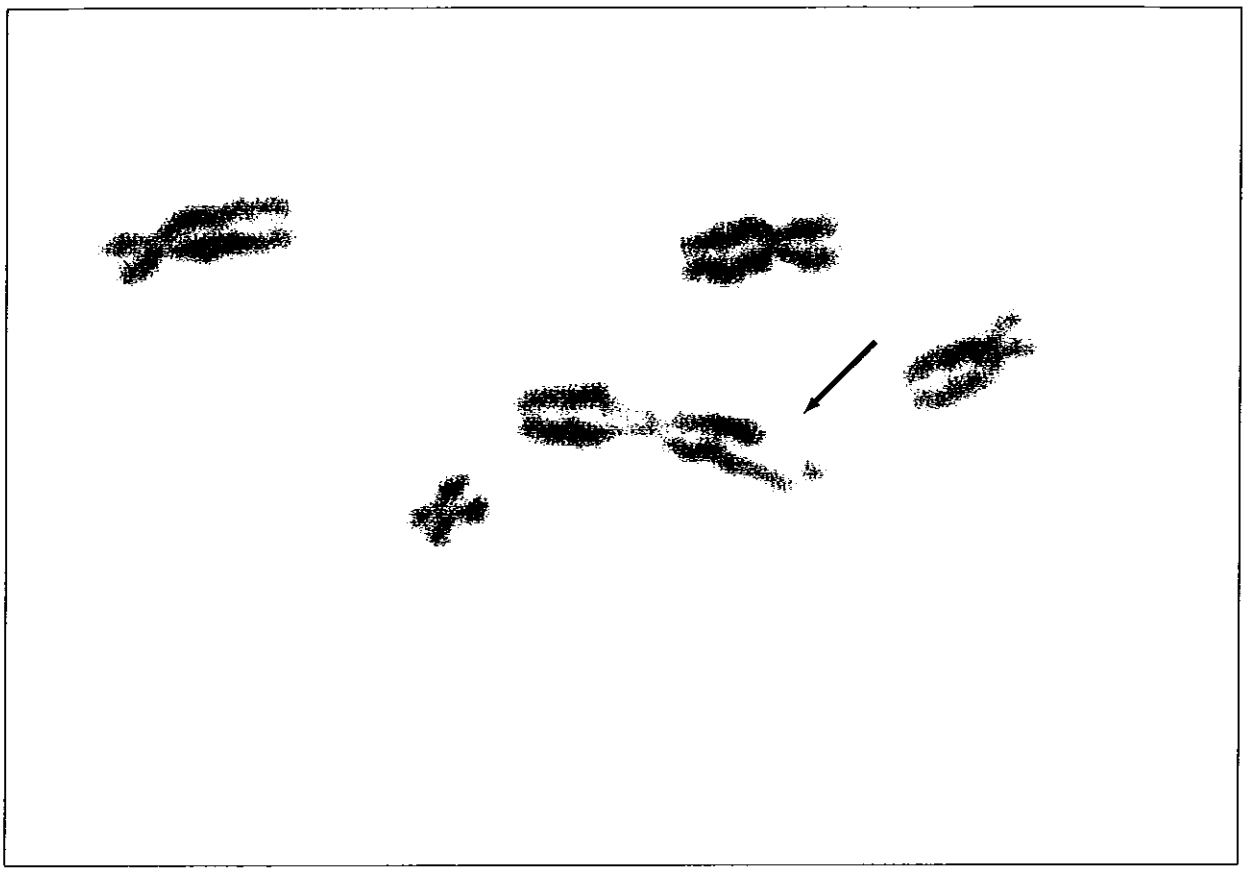

Figure 3.25. Chromosome stained with Giemsa having a chromatid break

3.1.2.7 Chromatid Gaps

No chromatid gaps were seen in either control hTERT- or hTERT+ cells without metal treatment. Cr (VI) caused a small incidence of gaps in the hTERT- cells at the lower dose (0.04 μM) immediately after metal exposure, which decreased with time. However, they were not statistically significant (Figures 3.26a and 3.26c). No change was seen immediately after V (V) treatment. In the hTERT+ cells, there was no increase in gaps after Cr (VI) treatment. There was a small incidence of gaps with 0.5 μM V (V) immediately after the metal exposure, which did not persist with time. However, they were not statistically significant (Figures 3.26b and 3.26d). A direct comparison of hTERT+ and hTERT- cells showed that there were fewer gaps after Cr (VI) treatment in hTERT+ cells, at all times after exposure, but more after 0.5

μM V (V) treatment, immediately after the metal treatment. These results were expressed as a percentage of chromatid gaps.

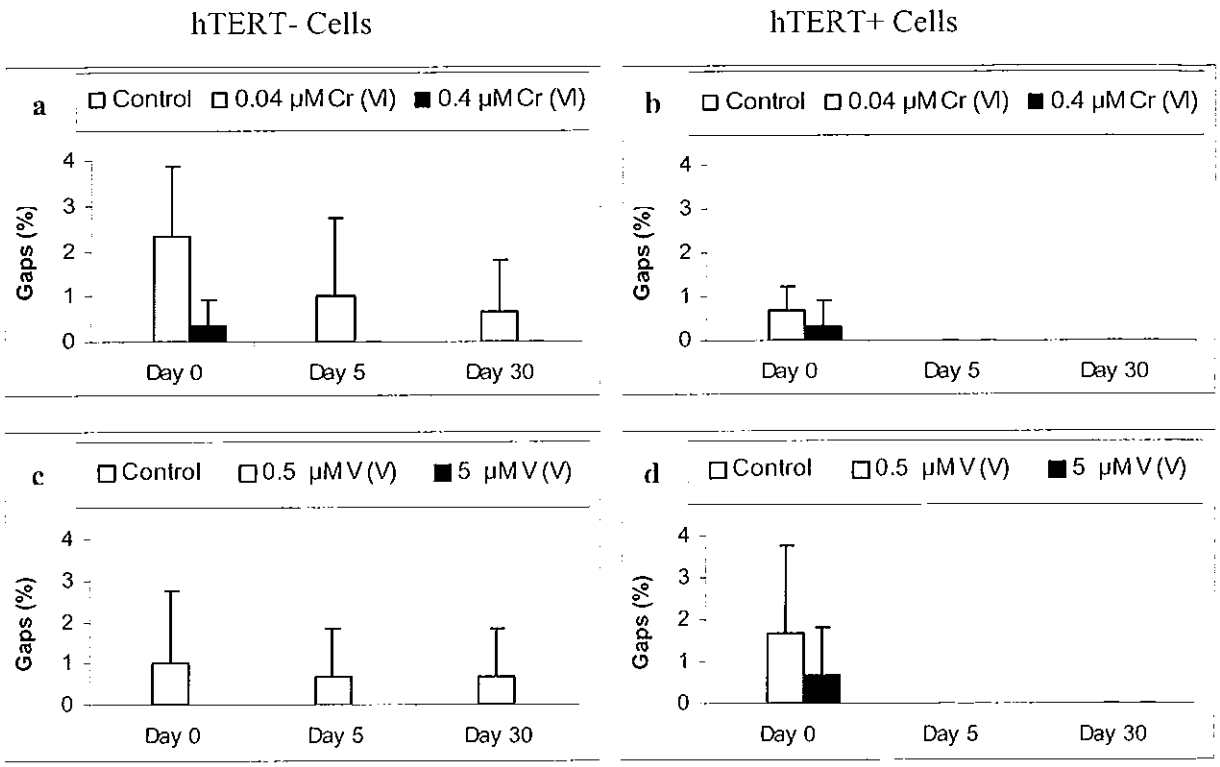

Figure 3.26. Chromatid Gaps. The figures show the percentage of chromatid gaps in hTERT– cells (Fig. 3.26a and 3.26c) and hTERT+ cells (Fig. 3.26b and 3.26d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

3.1.2.8 Chromatid Fragments

Cr (VI) caused an increase in chromatid fragments in the hTERT- cells only at the higher dose (0.4μM) immediately after metal exposure, but it was not statistically significant (Figures 3.27a and 3.27c). No change was seen immediately after V (V) treatment. In the hTERT+ cells, there was also a temporary increase in fragments after 0.4μM Cr (VI) treatment up to 5 days after metal exposure. There was no change after V (V) exposure (Figures 3.27b and 3.27d). A direct comparison of hTERT+ and hTERT- cells showed that there were fewer fragments after 0.4μM

Cr (VI) treatment in hTERT+ cells at *Day 0*, but significantly more at *Day 5*. These results were expressed as a percentage of chromatid fragments.

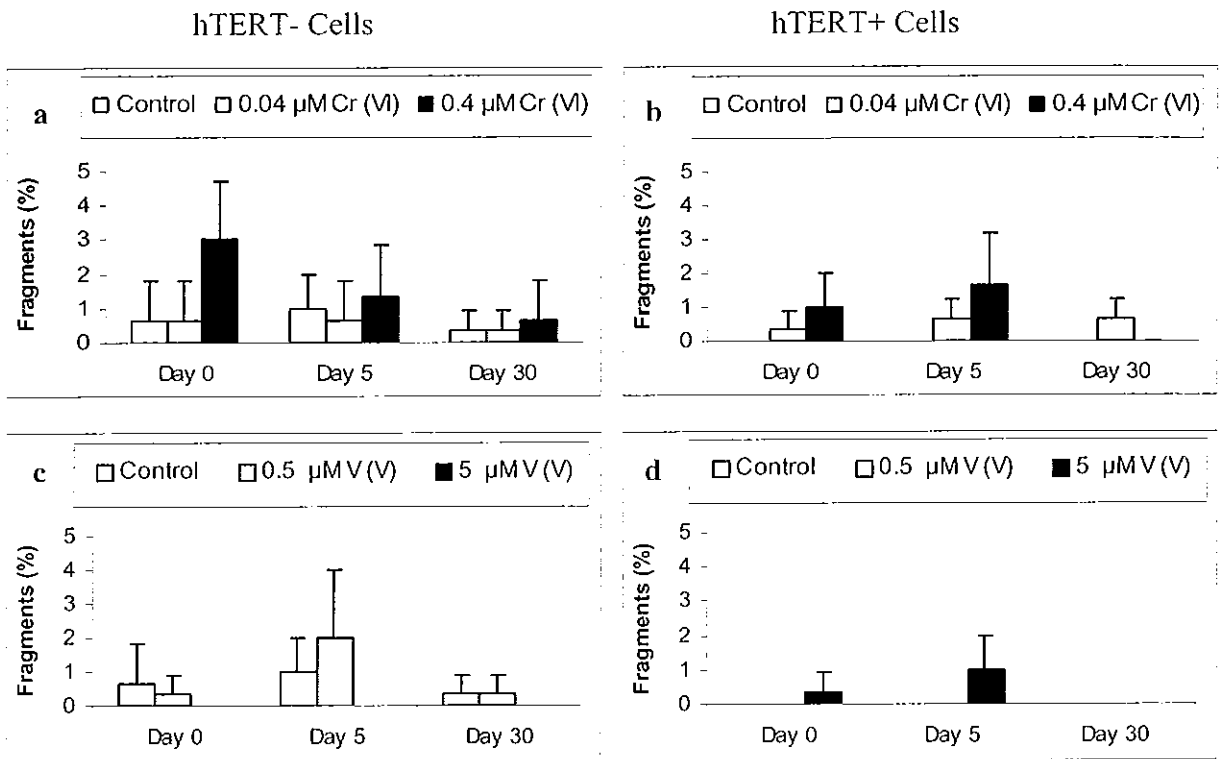

Figure 3.27. Chromatid Fragments. The figures show the percentage of chromatid fragments in hTERT– cells (Fig. 3.27a and 3.27c) and hTERT+ cells (Fig. 3.27b and 3.27d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

3.1.2.9 Dicentric Chromosomes

No dicentric chromosomes were seen in either control hTERT- or hTERT+ cells without metal treatment. Both Cr (VI) (particularly at the higher dose) and V (V) (particularly at the lower dose) induced dicentric chromosomes up to 30 days after metal exposure in hTERT- cells (Figures 3.28a and 3.28c). In contrast, neither Cr (VI) nor V (V) induced a significant level of dicentric chromosomes in hTERT+ cells (Figures 3.28b and 3.28d). A direct comparison of hTERT- and hTERT+ cells revealed that the excess of dicentrics in hTERT- cells after 0.4 µM Cr (VI) and 0.5 µM V (VI) treatments was statistically significant compared to that in the

hTERT+ cells (Figure 3.39). These results were expressed as a percentage of dicentric chromosomes.

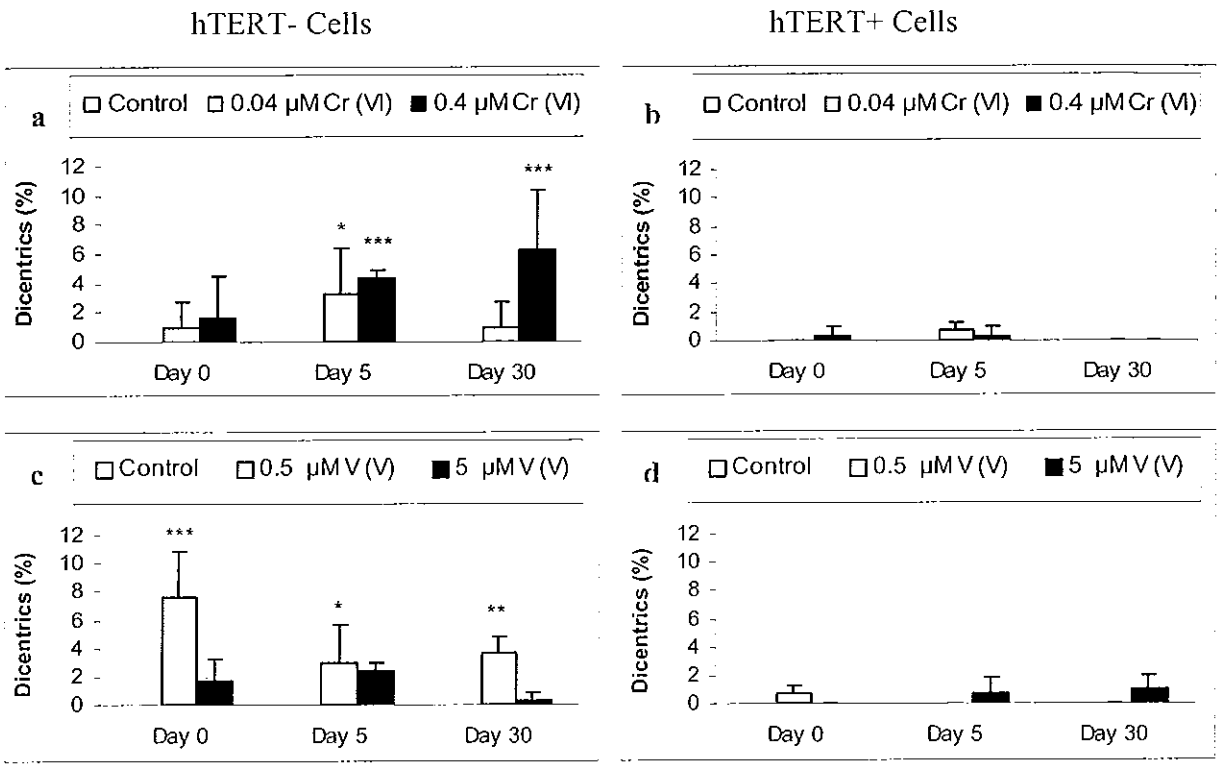

Figure 3.28. Dicentric Chromosomes. The figures show the percentage of dicentric chromosomes in hTERT– cells (Fig. 3.28a and 3.28c) and hTERT+ cells (Fig. 3.28b and 3.28d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

3.1.2.10 Telomeric Association

No telomeric associations were seen in either control hTERT- or hTERT+ cells without metal treatment. Both metals produced very few telomeric associations, which were not statistically significant. Cr (VI) caused a small increase in telomeric associations in the hTERT- cells (particularly at the lower dose) immediately after metal exposure (Figures 3.29a and 3.29c). 0.5 µM V (V) caused a slight increase in telomeric associations immediately after the treatment, which decreased with time. In the hTERT+ cells, only the higher dose of V (V) (5 µM) showed

a small increase in telomeric association (Figures 3.29b and 3.29d). These results were expressed as a percentage of telomeric associations.

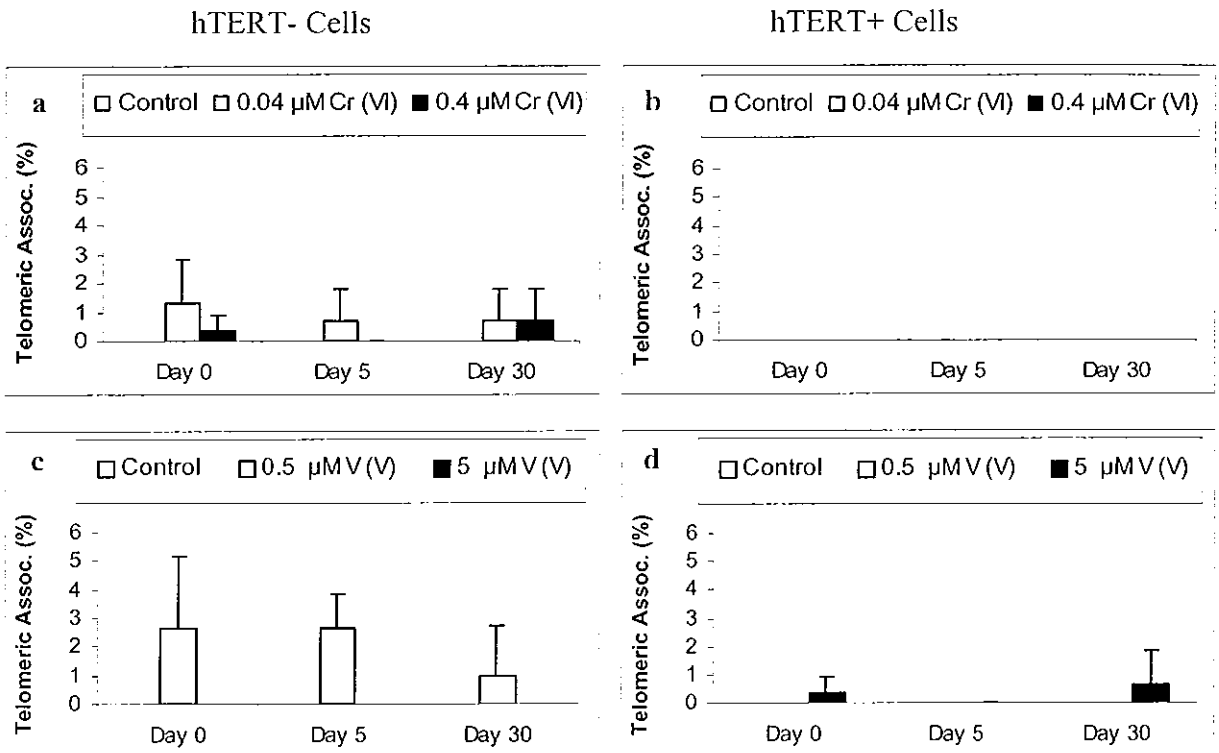

Figure 3.29. Telomeric Association. The figures are showing the percentage of telomeric association in hTERT– cells (Fig. 3.29a and 3.29c) and hTERT+ cells (Fig. 3.29b and 3.29d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

3.1.2.11 Ring Chromosomes

No ring chromosomes were seen in either control hTERT- or hTERT+ cells without metal treatment. Both metal treatments produced very few ring chromosomes in either hTERT- (Figures 3.30a and 3.30c) or hTERT + cells (Figures 3.30b and 3.30d), which were not statistically significant. However, Cr (VI) caused an increase in ring chromosomes in the hTERT- cells (particularly at the lower dose) immediately after metal exposure (Figures 3.30a and 3.30c), which persisted up to 30 days after the metal exposure. These results were expressed as a percentage of ring chromosomes.

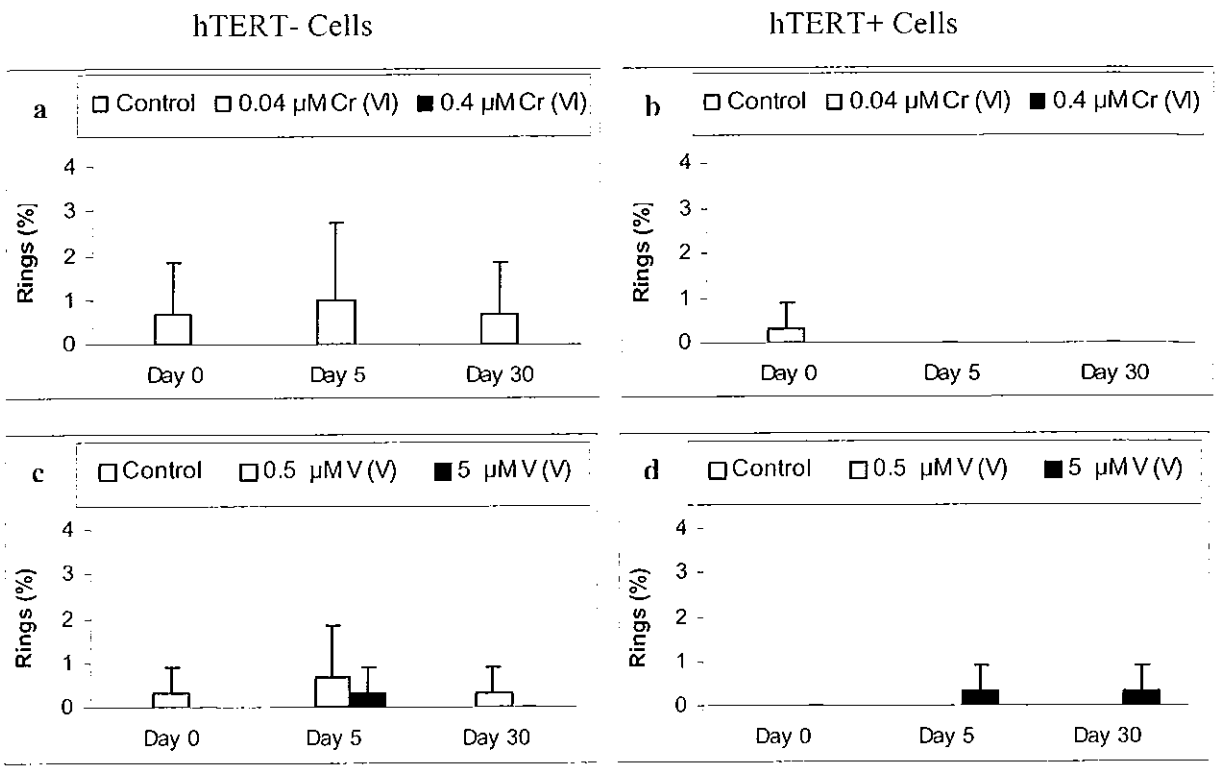

Figure 3.30. Ring Chromosomes. The figures show the percentage of ring chromosomes in hTERT– cells (Fig. 3.30a and 3.30c) and hTERT+ cells (Fig. 3.30b and 3.30d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

3.1.2.12 Tetraploidy

No significant increase in tetraploidy was seen in hTERT- cells up to 30 days after short term exposure to Cr (VI) or V (V) (Figures 3.31a and 3.31c). In contrast, in the hTERT+ cells there was a progressive and dose dependent increase in tetraploidy up to 30 days after a 24-hour exposure to Cr (VI) or V (V) (Figures 3.31b and 3.31d). A direct comparison of hTERT- and hTERT+ cells showed that there were significantly more tetraploid cells in the hTERT+ cells after treatment with the higher doses of Cr (VI) and V (V), but no difference without metal treatment (Figure 3.32 and Figure 3.39). These results were expressed as a percentage of tetraploid cells. Figure 3.33 shows a tetraploid cell, containing 92 chromosomes.

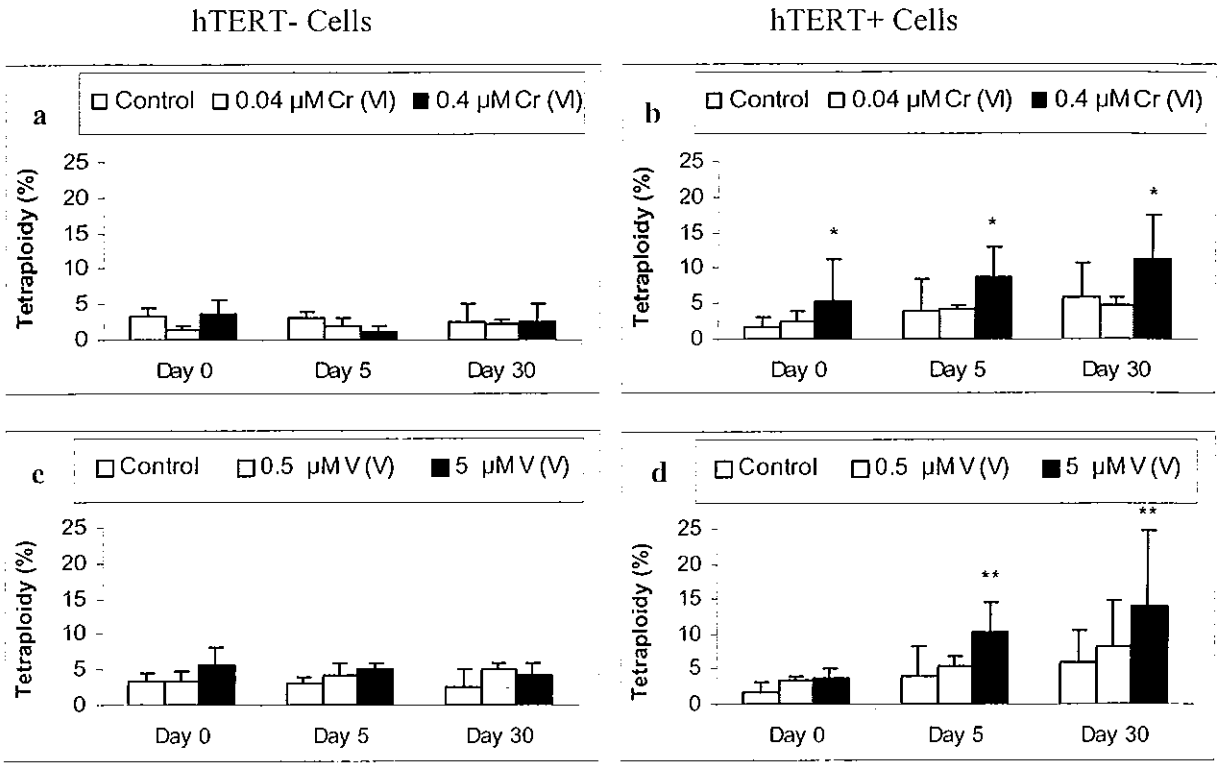

Figure 3.31. Tetraploidy. The figures show the percentage of tetraploidy in hTERT– cells (Fig. 3.31a and 3.31c) and hTERT+ cells (Fig. 3.31b and 3.31d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control (PBS).

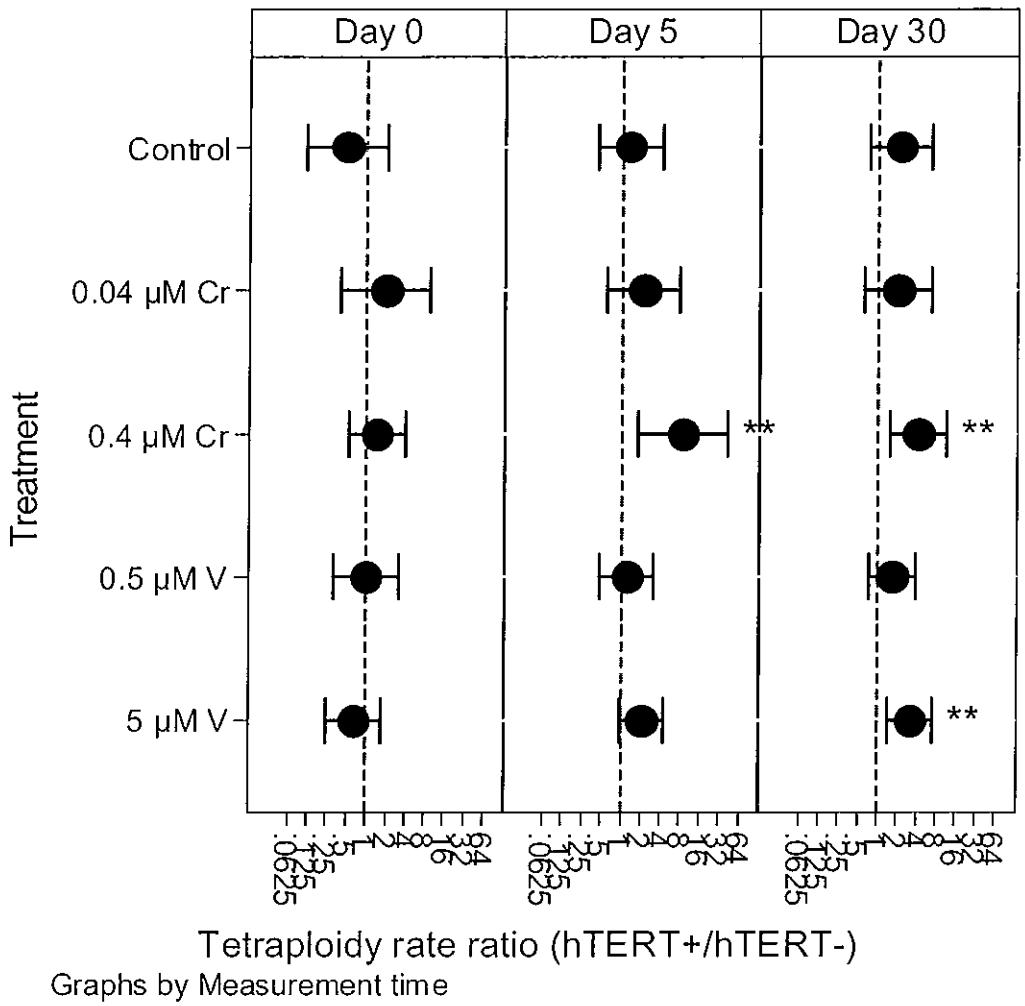

Figure 3.32. Tetraploidy. The figure illustrates the rate ratio of hTERT+/hTERT- for the tetraploid cells, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. ** $p<0.01$ compared to hTERT+/hTERT- = 1.

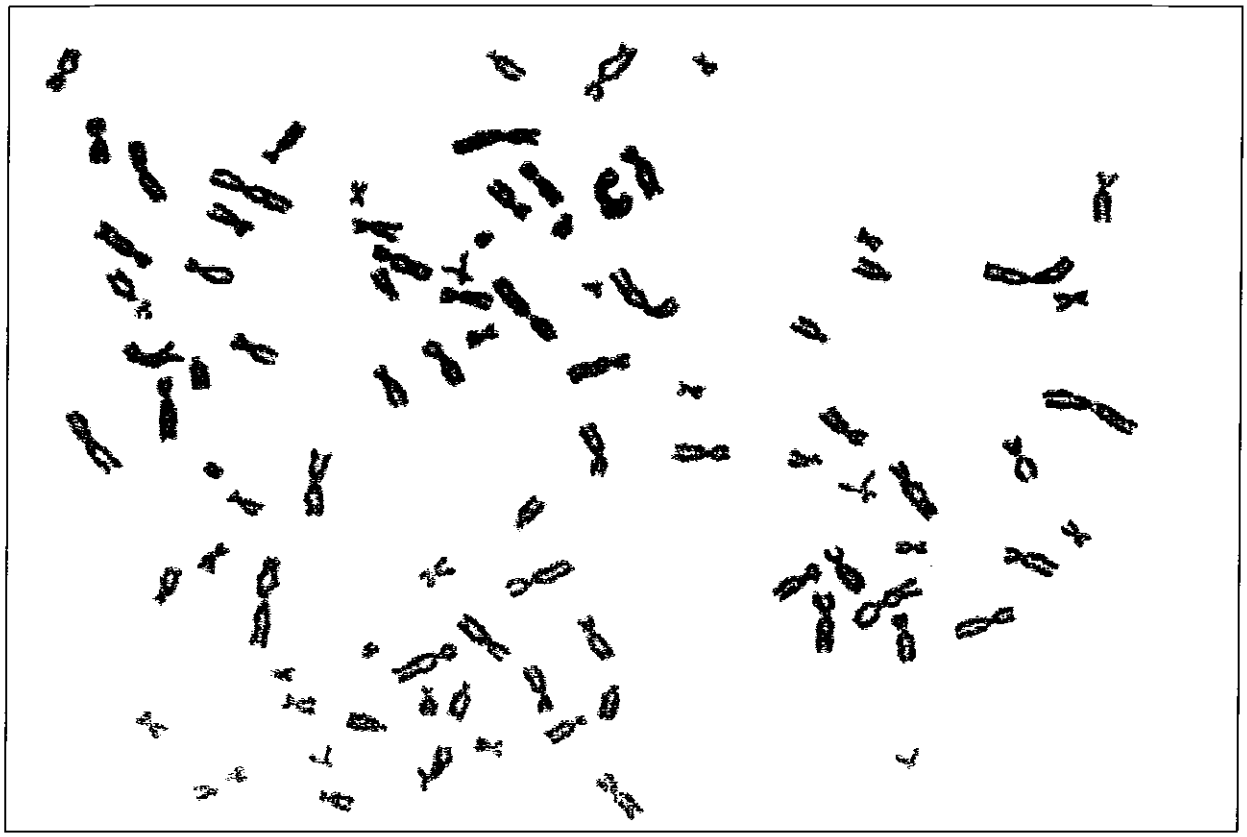

Figure 3.33. Tetraploid cell stained with Giemsa containing 92 chromosomes

3.1.2.13 Total Aneuploidy

No total aneuploidy (aneuploidy + hypodiploidy + hyperdiploidy) was seen in the control hTERT- cells (Figure 3.34a and 3.34c). There was a statistically significant induction of total aneuploidy by V (V), but not by Cr (VI), immediately after a 24-hour exposure of hTERT- cells. Both Cr (VI) and V (V), however, induced total aneuploidy at 5 days and 30 days after exposure. The level of total aneuploidy was lower in hTERT+ cells immediately after and 30 days after the metal exposure (Figure 3.34b and 3.34d). A direct comparison of hTERT- and hTERT+ cells showed that this was statistically significant (Figure 3.39). These results were expressed as a percentage of total aneuploidy.

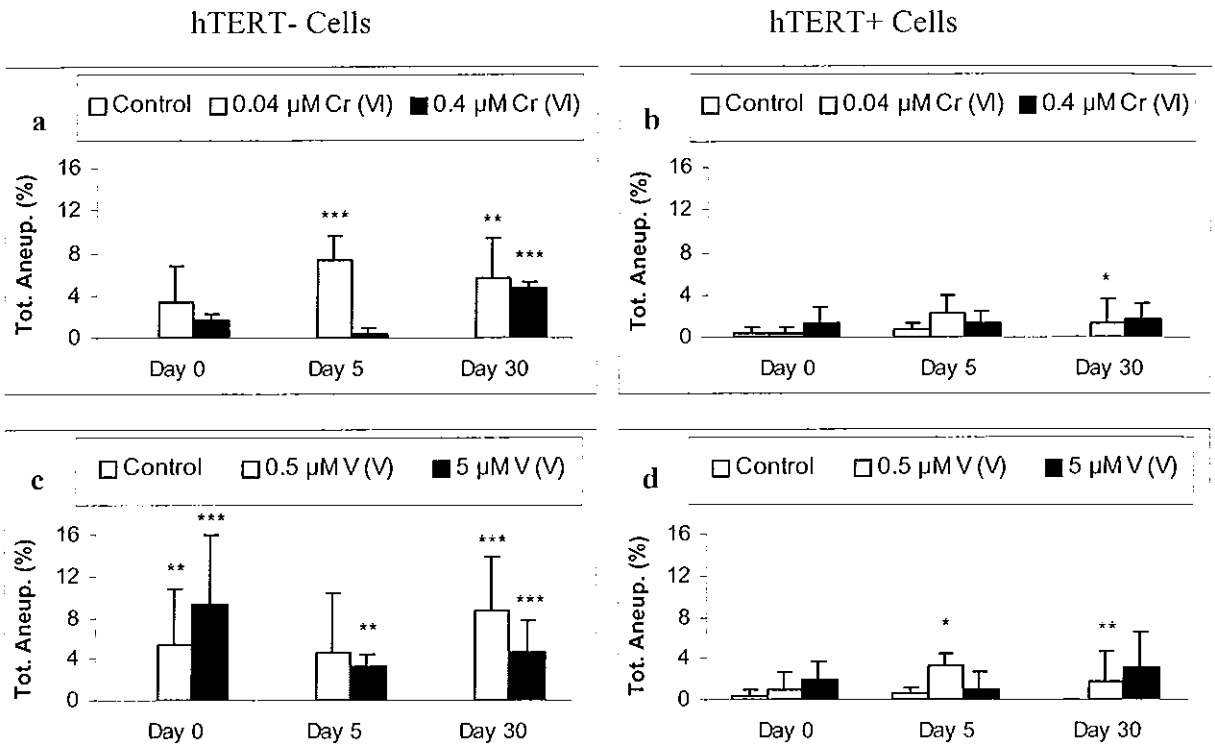

Figure 3.34. Total Aneuploidy. The figures show the percentage of total aneuploidy in hTERT– cells (Fig. 3.34a and 3.34c) and hTERT+ cells (Fig. 3.34b and 3.34d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

3.1.2.14 Aneuploidy Gain

No aneuploidy gain (aneuploidy + hyperdiploidy) was seen in the control hTERT- cells (Figure 3.35a and 3.35c). There was a statistically significant induction of aneuploidy gain by V (V) but not by Cr (VI) immediately after a 24-hour exposure of hTERT- cells. Both Cr (VI) and V (V), however, induced aneuploidy gain at 30 days after exposure. The level of aneuploidy gain, with both Cr (VI) and V (V), was lower in hTERT+ cells at *Day 0* and *Day 30* (Figure 3.35b and 3.35d). A direct comparison of hTERT- and hTERT+ cells showed that this was statistically significant (Figure 3.39). These results were expressed as a percentage of aneuploidy gain.

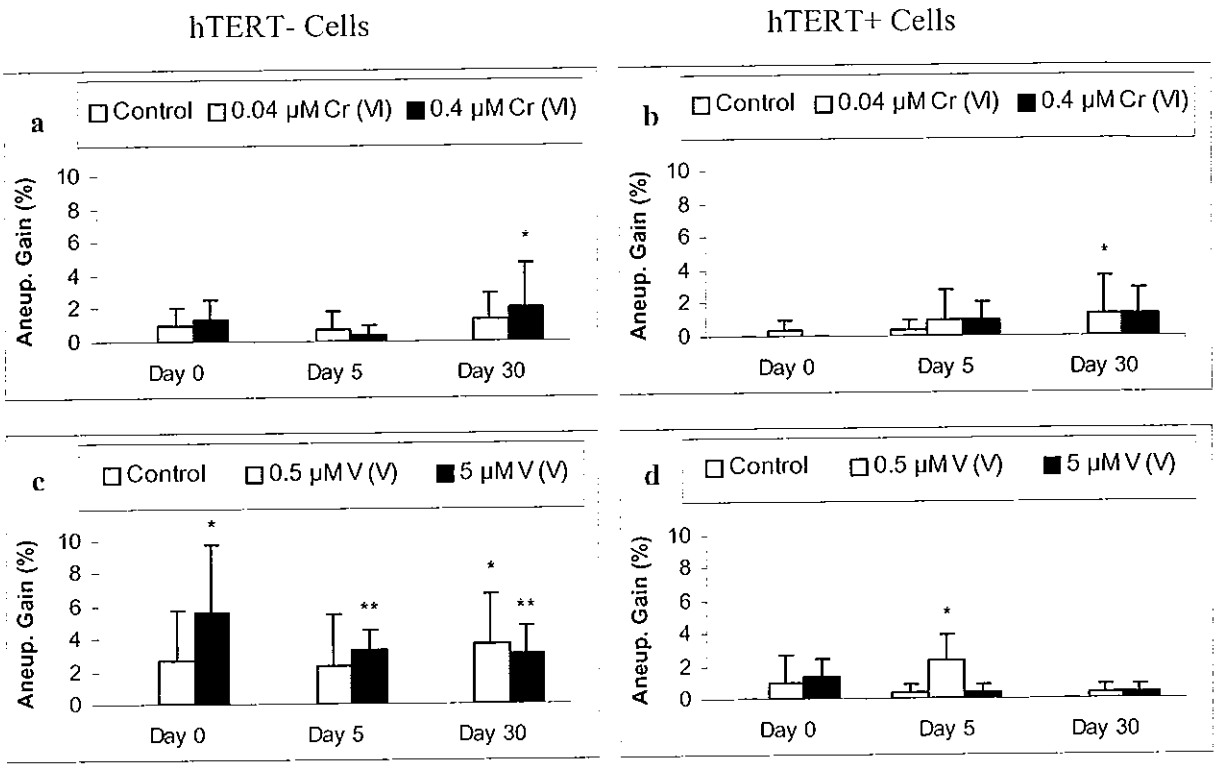

Figure 3.35. Aneuploidy Gain. The figures show the percentage of aneuploidy gain in hTERT– cells (Fig. 3.35a and 3.35c) and hTERT+ cells (Fig. 3.35b and 3.35d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. * $p<0.05$, ** $p<0.01$ compared to the control (PBS).

3.1.2.15 Aneuploidy

No aneuploidy was seen in the control hTERT- cells in this study (Figures 3.36a and 3.36c). V (V) (particularly at the highest dose) induced significant aneuploidy immediately after a 24-hour exposure, which persisted up to *Day 30* ($p<0.05$). Both Cr (VI) and V (V), however, induced aneuploidy 30 days after exposure. Both the acute and the long-term induction of aneuploidy by metals were reduced in hTERT+ cells (Figures 3.36b and 3.36d). This was statistically significant in a direct comparison of hTERT- and hTERT+ cells after the higher dose (5 μM) of V (V). These results were expressed as a percentage of aneuploid cells.

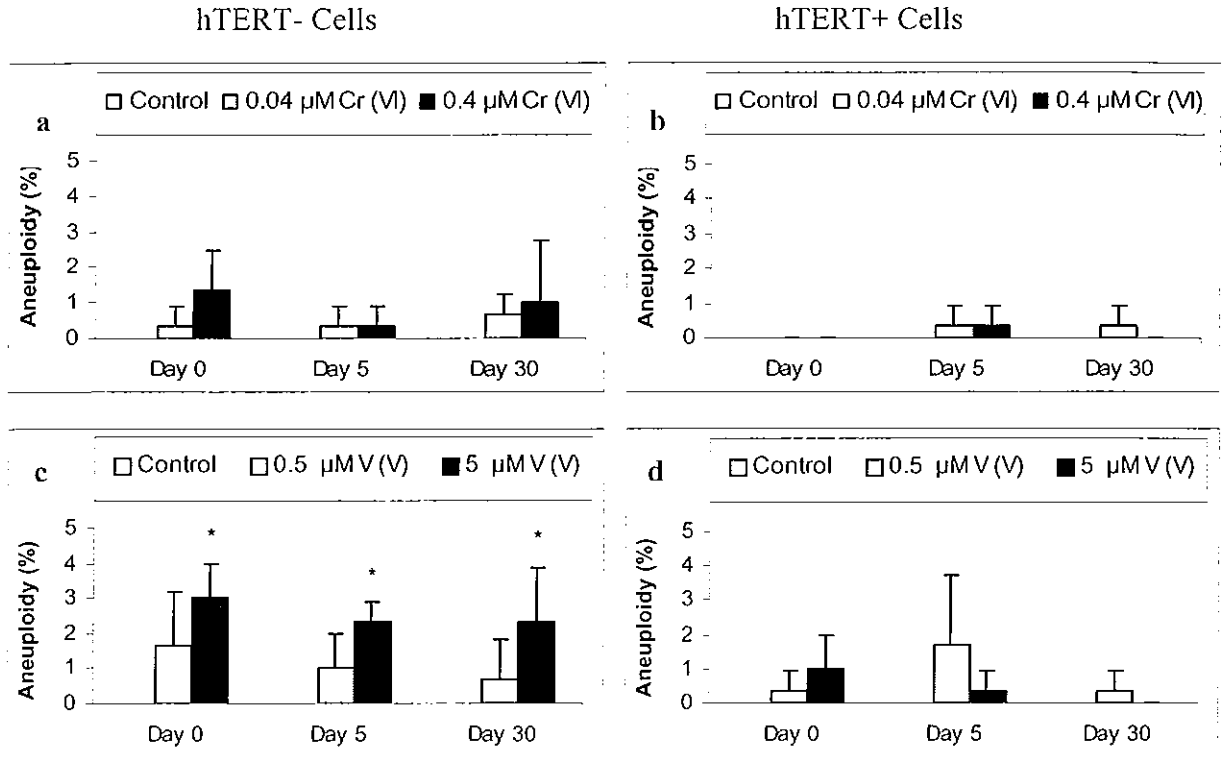

Figure 3.36. Aneuploidy. The figures show the percentage of aneuploidy in hTERT– cells (Fig. 3.36a and 3.36c) and hTERT+ cells (Fig. 3.36b and 3.36d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. Statistical Analysis: * $p<0.05$ compared to the control (PBS).

3.1.2.16 Hypodiploidy

No hypodiploidy was seen in the control hTERT- cells in this study. Cr (VI) at the lower doses, induced statistically significant hypodiploidy at *Day 5* ($p<0.01$) in hTERT- cells, which persisted (but decrease) up to 30 days after the metal treatment ($p<0.05$) (Figures 3.37a and 3.37c). V (V) induced statistically significant aneuploidy at *Day 0* with the higher dose (5 µM) ($p<0.05$) and at *Day 30* with the lower dose (0.5 µM) ($p<0.05$). The incidence of hypodiploidy induced by Cr (VI) and V (V) was lower in the hTERT+ (Figures 3.37b and 3.37d) and did not persist until *Day 30* with the 0.04 µM dose of Cr (VI). These results were expressed as a percentage of hypodiploidy.

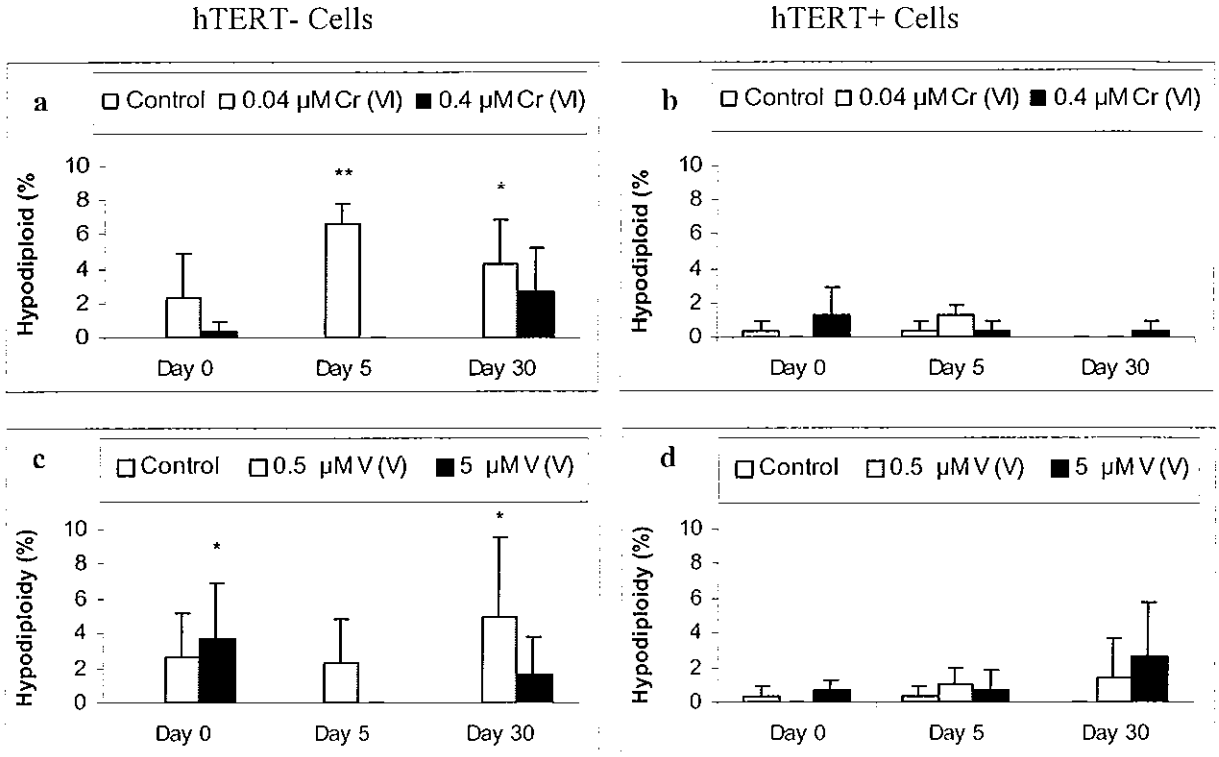

Figure 3.37. Hypodiploidy. The figures show the percentage of hypodiploidy in hTERT– cells (Fig. 3.37a and 3.37c) and hTERT+ cells (Fig. 3.37b and 3.37d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. Statistical Analysis: * $p<0.05$, ** $p<0.01$ compared to the control (PBS).

3.1.2.17 Hyperdiploidy

No statistically significant hyperdiploidy was seen in the control and Cr (VI)-treated hTERT- or hTERT+ cells in this study. The incidence of hyperdiploidy induced by V (V) was lower in the hTERT+ (Figures 3.38b and 3.38d) compared to hTERT- cells, but there was no statistical significance (Figures 3.38a and 3.38c). This was particularly true with 5 µM V (V) at *Day 0* and 0.5 µM V (V) at *Day 30*. These results were expressed as a percentage of hyperdiploidy.

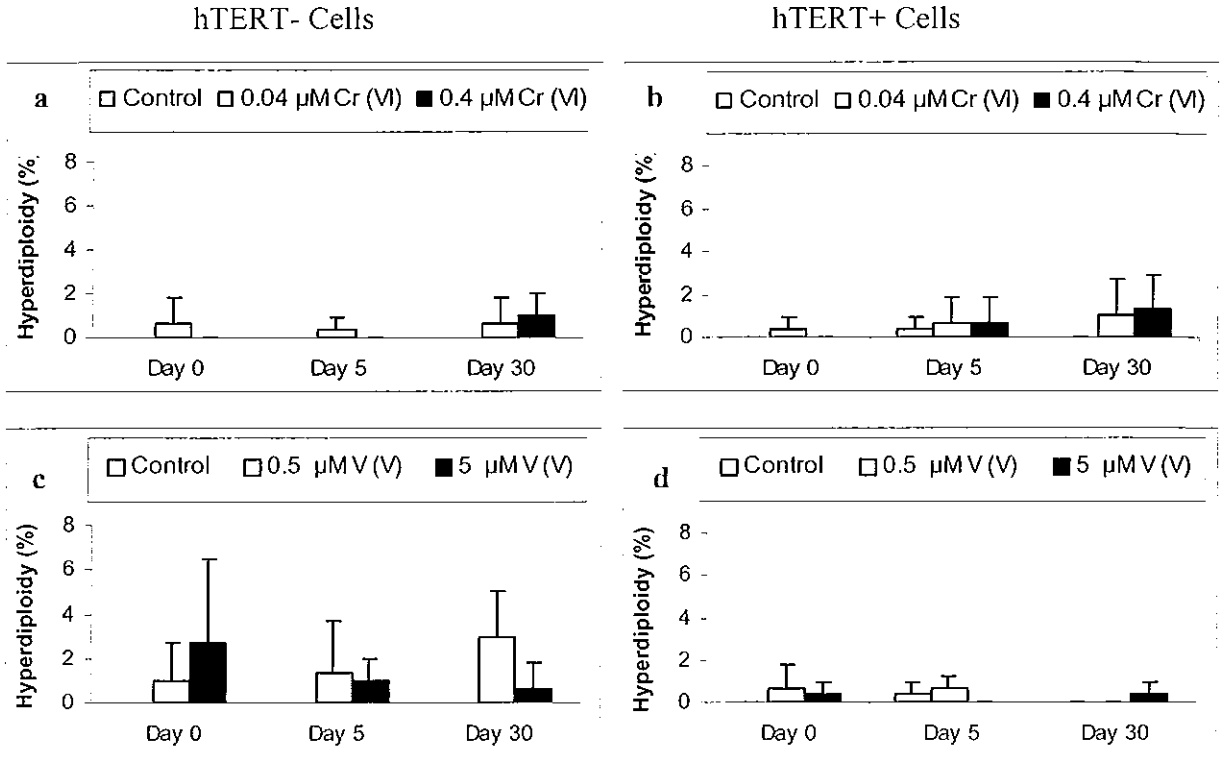

Figure 3.38. Hyperdiploidy. The figures show the percentage of hyperdiploidy in hTERT– cells (Fig. 3.38a and 3.38c) and hTERT+ cells (Fig. 3.38b and 3.38d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure.

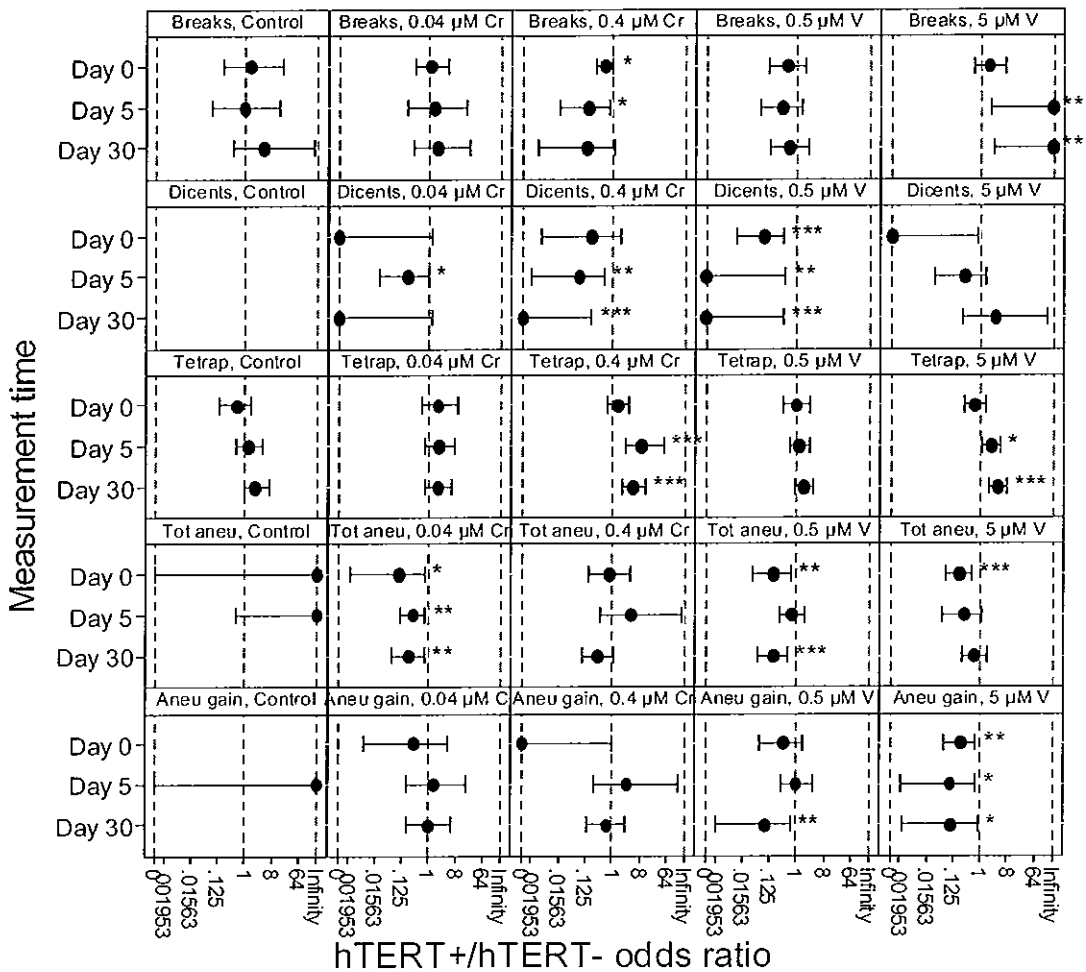

Figure 3.39. Breaks, Dicentrics, Tetraploidy, Total Aneuploidy and Aneuploidy Gain. The figure illustrates the rate ratio of hTERT+/hTERT- for breaks, dicentrics, tetraploidy, tot. aneuploidy and aneuploidy gain, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.2.18 Mitotic Index

The higher dose of Cr (VI) (0.4 µM) caused a statistical significant reduction of the mitotic index in hTERT- cells, immediately after the metal exposure ($p<0.001$). This reduction persisted up to 30 days after the single 24-hour exposure to Cr (VI) ($p<0.05$) (Figures 3.40a and 3.40c). Both doses of V (V), but particularly the higher dose (5 µM), caused a statistical significant reduction of the mitotic index in hTERT- cells, immediately after the metal

exposure ($p<0.01$ with 0.5 μM and $p<0.001$ with 5 μM). This reduction persisted up to 30 days after the single 24-hour exposure to V (V) ($p<0.05$ with 0.5 μM and $p<0.01$ with 5 μM) (Figures 3.40a and 3.40c). The hTERT+ cells followed a different pattern. The higher dose of Cr (VI) caused a temporary decrease of the mitotic index in hTERT+ cells up to 5 days after the 24-hour exposure, which was statistically significant (Figures 3.40b and 3.40d). V (V) with the higher dose (5 μM) caused also a temporary decrease of the mitotic index up to 5 days after the 24-hour exposure, which was statistically significant ($p<0.001$ at *Day 0* and $p<0.05$ at *Day 5*) (Figures 3.40b and 3.40d). Therefore, both metals induced a reduction of the mitotic index in hTERT+ cells, which did not persist up to 30 days after the metal exposure. The decrease of the mitotic index, caused by both metals, followed a dose response in both hTERT- and hTERT+ cells. In all of the experiments, at all survival times, with or without metal treatment, there was a lower percentage of metaphase spreads in the hTERT- cells compared to the hTERT+ cells. The results of the mitotic index were expressed as a percentage of metaphase spread.

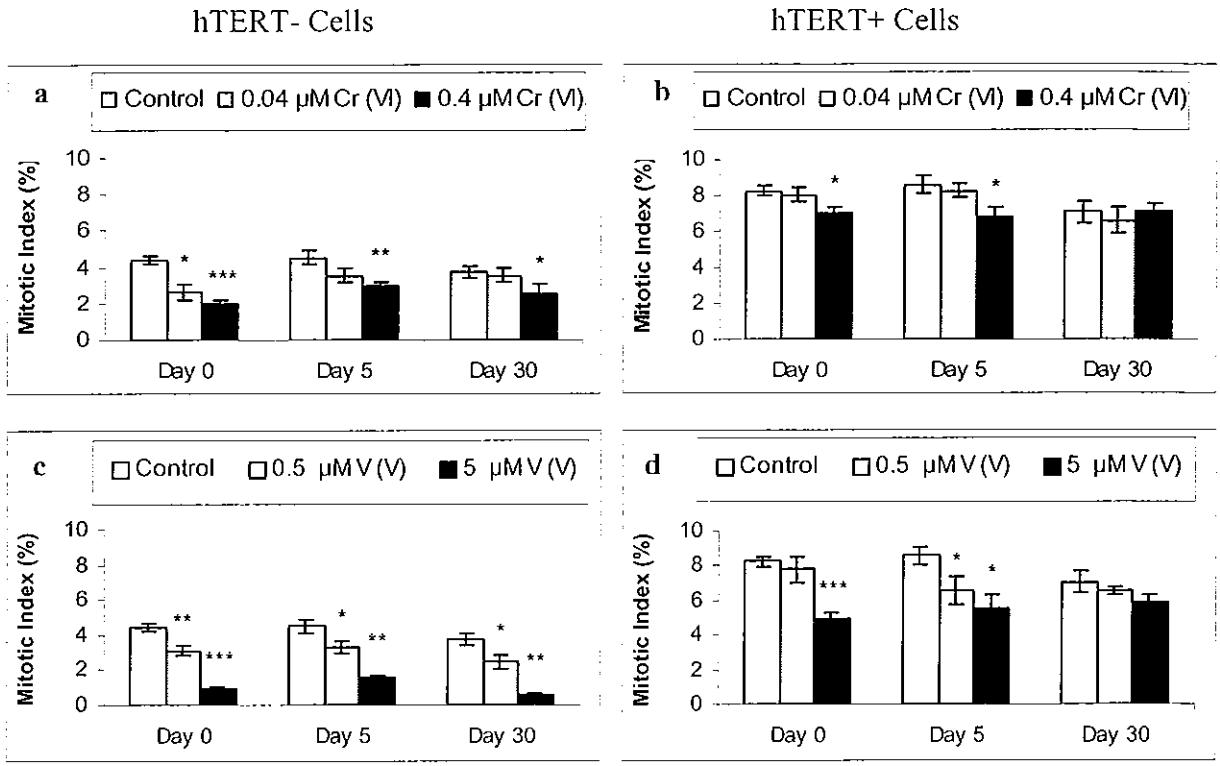

Figure 3.40. Mitotic Index. The figures show the percentage of metaphase spread in hTERT– cells (Fig. 3.40a and 3.40c) and hTERT+ cells (Fig. 3.40b and 3.40d) after a 24-hour exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure. Statistical Analysis: * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

3.1.3 Cell Biology

hTERT- (wild type) and hTERT+ (immortalised) human BJ fibroblasts were exposed to a 0.4 μM dose of Cr(VI) ions and a 5 μM dose of V(V) ions for 24 hours. The cells were then examined for cell biology, at 0 (*Day 0*), 5 (*Day 5*) and 30 days (*Day 30*) after exposure.

3.1.3.1 Telomerase Activity

The level of telomerase activity was much greater, as expected, in hTERT+ cells (Figures 3.41b and 3.41d) (2.04 ± 0.25 TPG units) compared to the hTERT – cells (Figures 3.41a and 3.41c) (0.01 ± 0.008 TPG units), when measured with the TRAP assay. There was a very slight increase of telomerase activity after V (V), but not Cr (VI) treatment of hTERT- cells. No significant change was noted after metal treatment of hTERT+ cells. However, in all of the

experiments, at all survival times, V (V) treatment caused a slight reduction of the telomerase activity in hTERT+ cells. This was particularly true immediately after metal exposure. These results were expressed as total product generated (TPG).

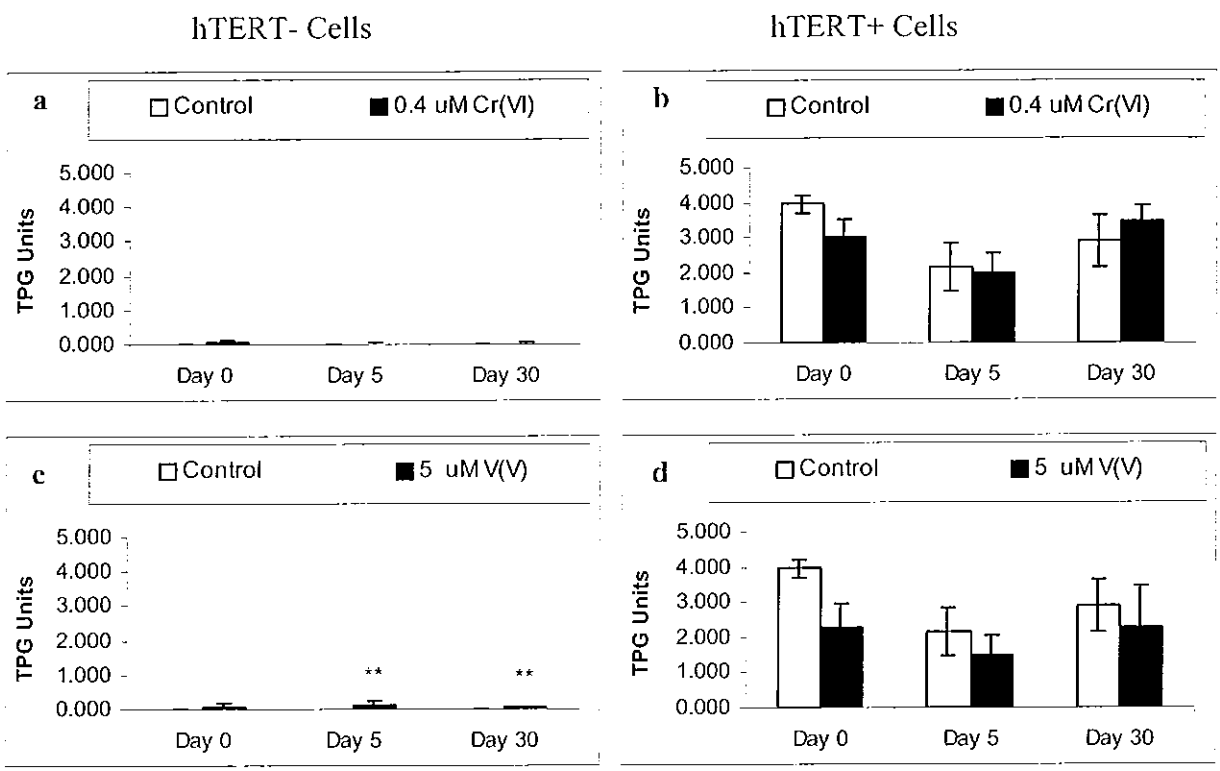

Figure 3.41. Telomerase Activity. The figures show the telomerase activity in hTERT– cells (Fig. 3.41a and 3.41c) and hTERT+ cells (Fig. 3.41b and 3.41d) after a 24-hour exposure to a dose of Cr (VI) and V (V) and at different times after the exposure. * $p<0.05$, ** $p<0.01$ compared to the control (PBS).

3.1.3.2 Telomere Length

The telomere lengths of untreated hTERT- cells (Figures 3.42a and 3.42c) and hTERT+ cells (Figures 3.42b and 3.42d) did not vary over the time course of this experiment, when measured with flow-FISH. In all the experiments, at all survival times, the telomere lengths were slightly longer in control (untreated) hTERT+ cells compared to control (untreated) hTERT- cells. Furthermore, telomere lengths were slightly shorter after metal treatment, either with Cr (VI) (with the only exception at *Day 0* in hTERT+ cells) or V (V), compared to control. Even though the telomere lengths were slightly longer in the control hTERT- or hTERT+ cells, and

slightly shorter after metal treatment, neither were statistically significant changes. Despite this, the telomere lengths in metal treated hTERT+ cells were significantly longer (approximately two fold) compared to metal treated hTERT- cells, 30 days after the 24-hour exposure (Figure 3.43). These results were expressed as telomere fluorescence intensity (TFI).

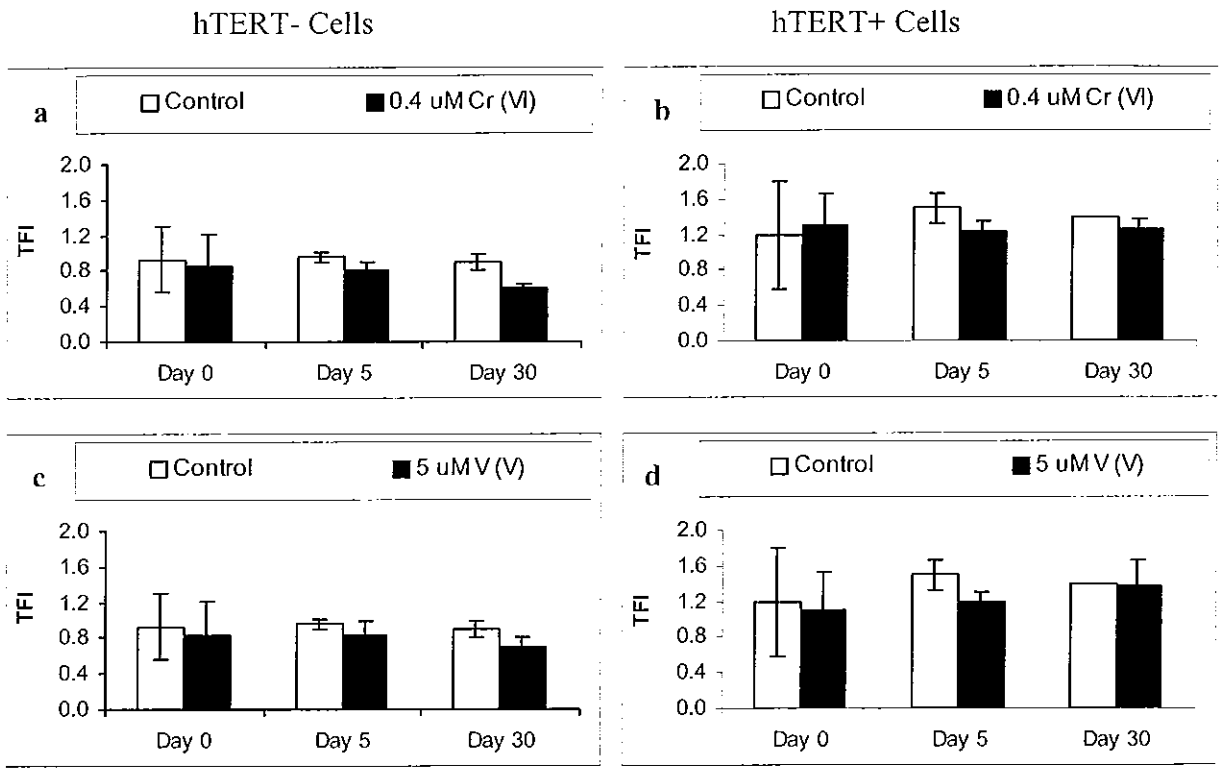

Figure 3.42. Telomere Length. The figures show the telomere length in hTERT– cells (Fig. 3.42a and 3.42c) and hTERT+ cells (Fig. 3.42b and 3.42d) after a 24-hour exposure to a dose of Cr (VI) and V (V) and at different times after the exposure.

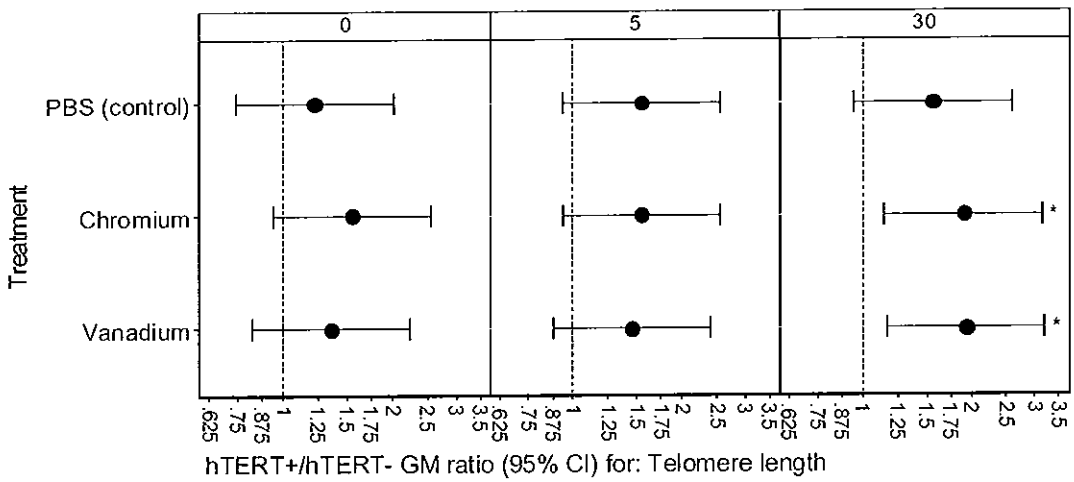

Figure 3.43. Telomere length. The figure illustrates the rate ratio of hTERT+/hTERT- for telomere length (TFI), showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * $p<0.05$ compared to hTERT+/hTERT- = 1.

3.1.3.3 STELA

A more detailed analysis of telomere lengths was carried out with the Single Telomere Length Analysis (STELA), using PCR (Baird et al 2003). Analysis of the XpYp telomere confirmed that hTERT+ cells had longer telomeres compared to hTERT- cells (Figures 3.44, 3.45 and 3.46).

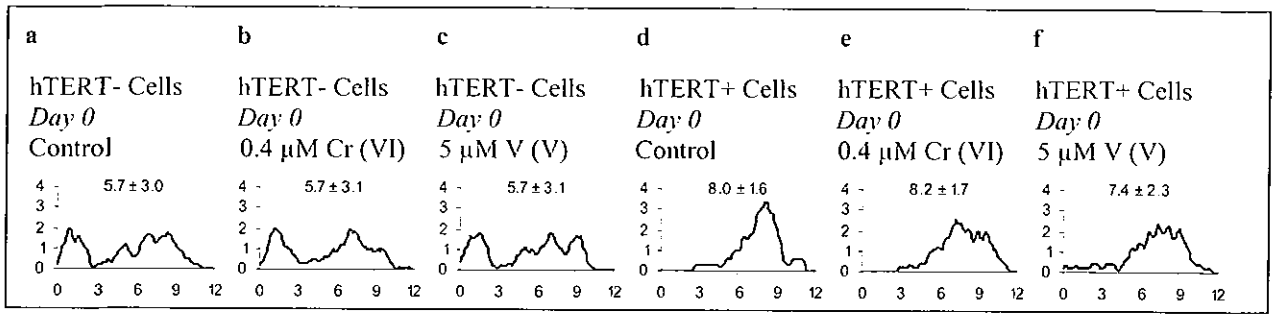

Figure 3.44. STELA (*Day 0*). The figure shows the STELA in hTERT– cells (Fig. 3.44a, 3.44b and 3.44c) and hTERT+ cells (3.44d, 3.44e and 3.44f) immediately after a 24-hour exposure to a dose of Cr (VI) and V (V).

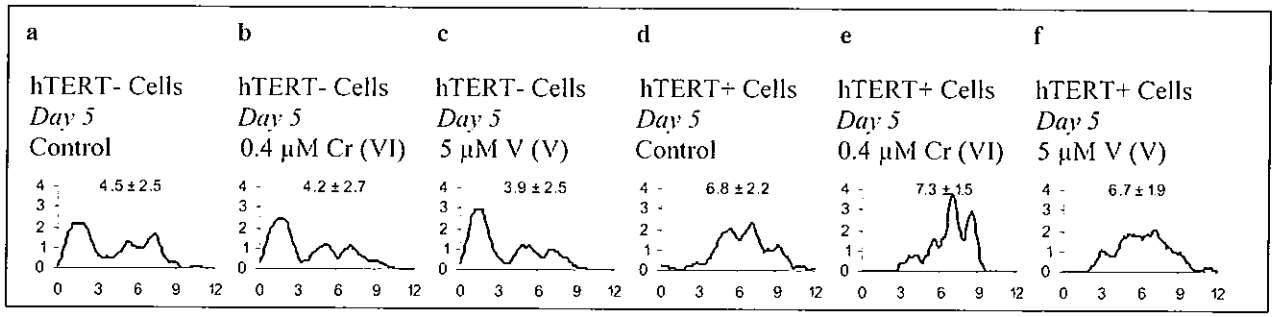

Figure 3.45. STELA (*Day 5*). The figure shows the STELA in hTERT– cells (Fig. 3.45a, 3.45b and 3.45c) and hTERT+ cells (3.45d, 3.45e and 3.45f) 5 days after a 24-hour exposure to a dose of Cr (VI) and V (V).

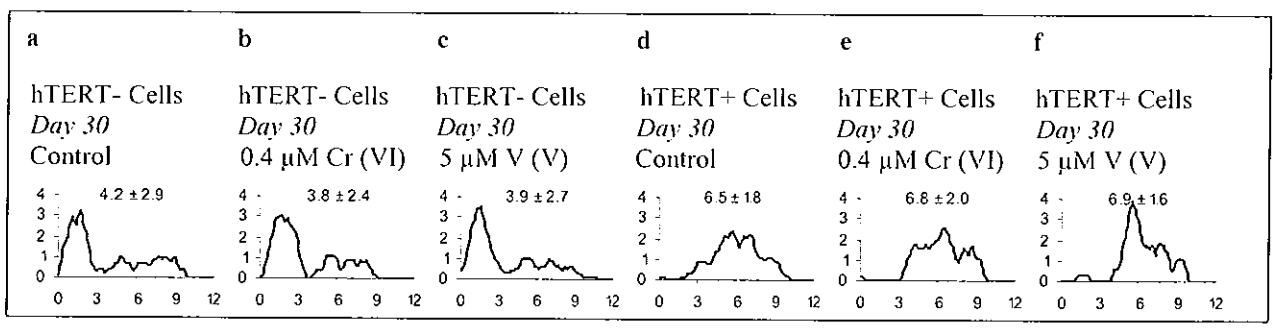

Figure 3.46. STELA (*Day 30*). The figure shows the STELA in hTERT– cells (Fig. 3.46a, 3.46b and 3.46c) and hTERT+ cells (3.46d, 3.46e and 3.46f) 30 days after a 24-hour exposure to a dose of Cr (VI) and V (V).

However this was not due to an overall increase in all telomere lengths. Rather, it was because the hTERT- cells had a neat bimodal allelic-like distribution of lengths, 0-3 Kb and 3-11 Kb, that was lacking in the hTERT+ cells, which have only the larger fraction 3-11 Kb. With increasing time, as expected, the telomere lengths were shorter on average. As in the flow-FISH analysis, there was no evidence that metals had significantly shortened the telomere lengths or changed their distributions.

3.1.3.4 Cell Cycle Distribution

3.1.3.4.1 G0/G1 Phase

The most striking change in the cell cycle, when measured with flow cytometry, was an increase in the proportion of cells in G0/G1 phase immediately after V (V) treatment of hTERT- cells (Figures 3.47a and 3.47c). No differences were seen at longer intervals (5 days or 30 days) after V (V) exposure, and no differences were seen after Cr (VI) treatment. This effect of V (V) was not noted in hTERT+ cells (Figures 3.47b and 3.47d). In all of the experiments, at *Day 0*, with or without metal treatment, there was a statistically significant higher proportion of hTERT- cells in G0/G1 phase, compared to hTERT+ cells. This was particularly true after the metal exposure (Figure 3.48). Furthermore, this difference was noticed, only in treated cells, up to 30 days after metal exposure. These results were expressed as a percentage of cells in G0/G1 phase.

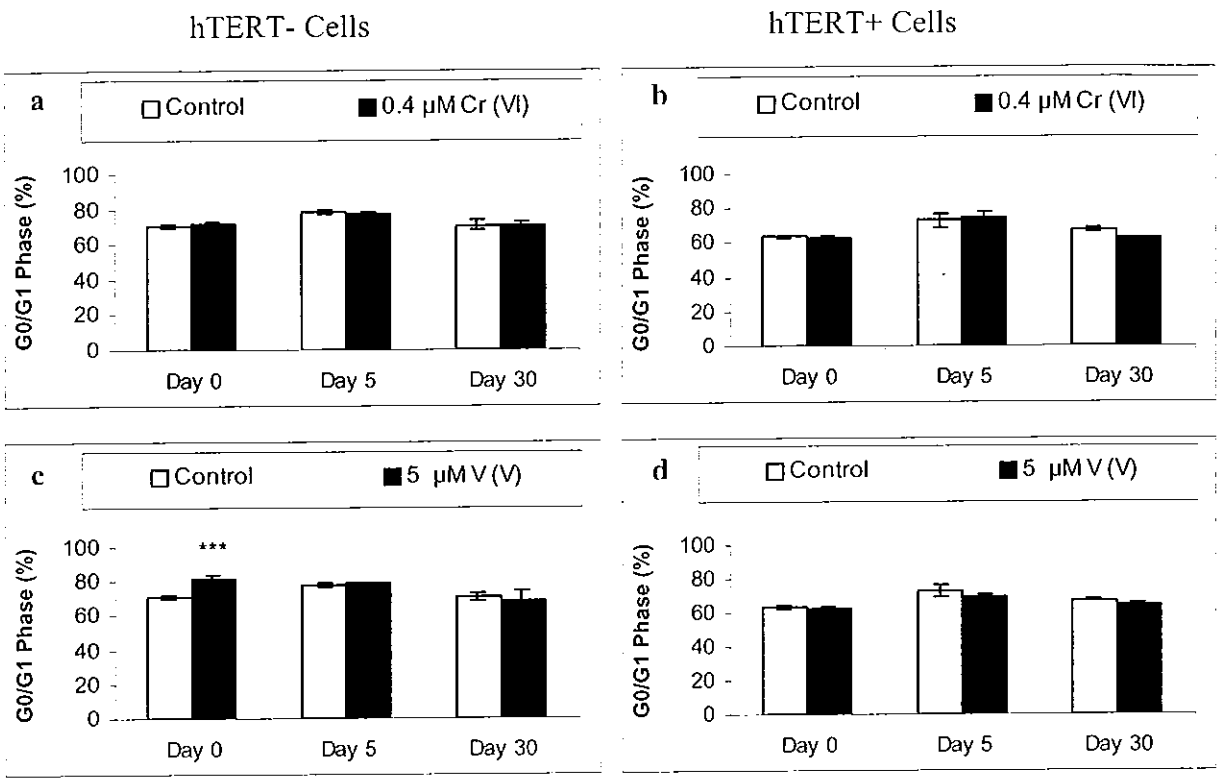

Figure 3.47. Cell Cycle Distribution (G0/G1 Phase). The figures show the percentage of G0/G1 phase in hTERT– cells (Fig. 3.47a and 3.47c) and hTERT+ cells (Fig. 3.47b and 3.47d) after a 24-hour exposure to a dose of Cr (VI) and V (V) and at different times after the exposure. *** p<0.001 compared to the control (PBS).

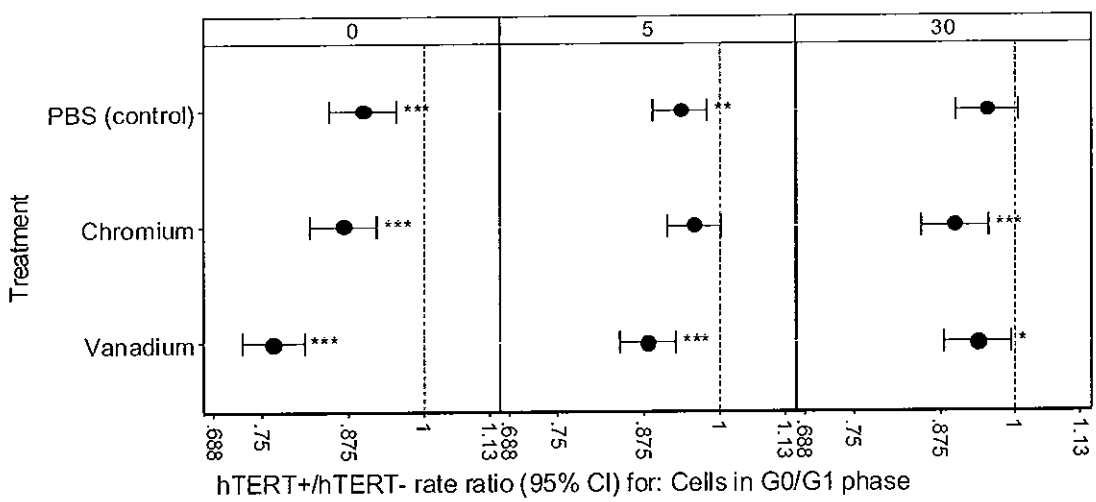

Figure 3.48. Cell Cycle Distribution (G0/G1 Phase). The figure illustrates the rate ratio of hTERT+/hTERT- for G0/G1 phase, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.3.4.2 S Phase

V (V) treatment caused a significant increase in the proportion of hTERT- cells in S phase 30 days after the metal exposure (Figures 3.49a and 3.49c). Furthermore, V (V) treatment caused a significant increase in the proportion of hTERT+ cells in S phase (Figures 3.49b and 3.49d) but only at *Day 5*, whereas Cr (VI) exposure slightly increased it at *Day 30*. In all of the experiments (with the only exception of V (V) at *Day 30*), at all survival times, with or without metal treatment, there was a statistically significant higher proportion of hTERT+ cells in S phase, compared to hTERT- cells. This was particularly true with both Cr (VI) and V (V), immediately after the metal exposure (Figure 3.50). These results were expressed as a percentage of cells in S phase.

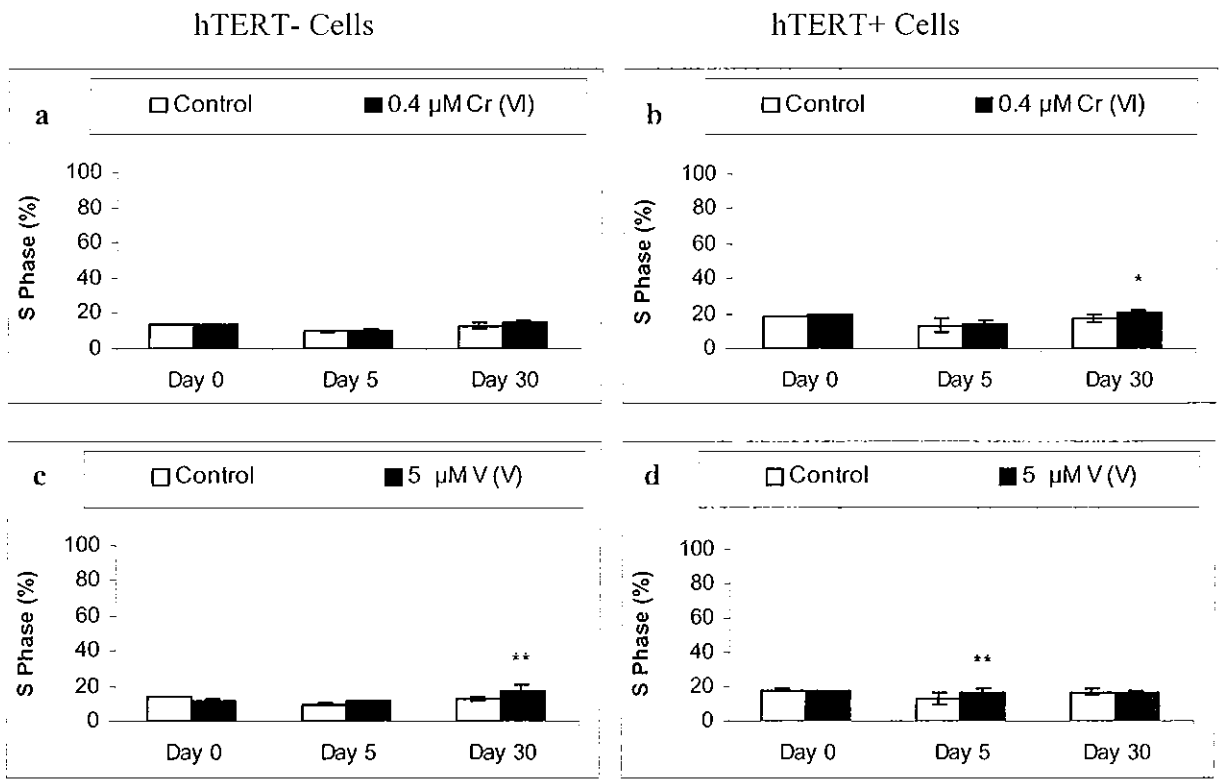

Figure 3.49. Cell Cycle Distribution (S Phase). The figures show the percentage of S phase in hTERT– cells (Fig. 3.49a and 3.49c) and hTERT+ cells (Fig. 3.49b and 3.49d) after a 24-hour exposure to a dose of Cr (VI) and V (V) and at different times after the exposure. * $p<0.05$, ** $p<0.01$ compared to the control (PBS).

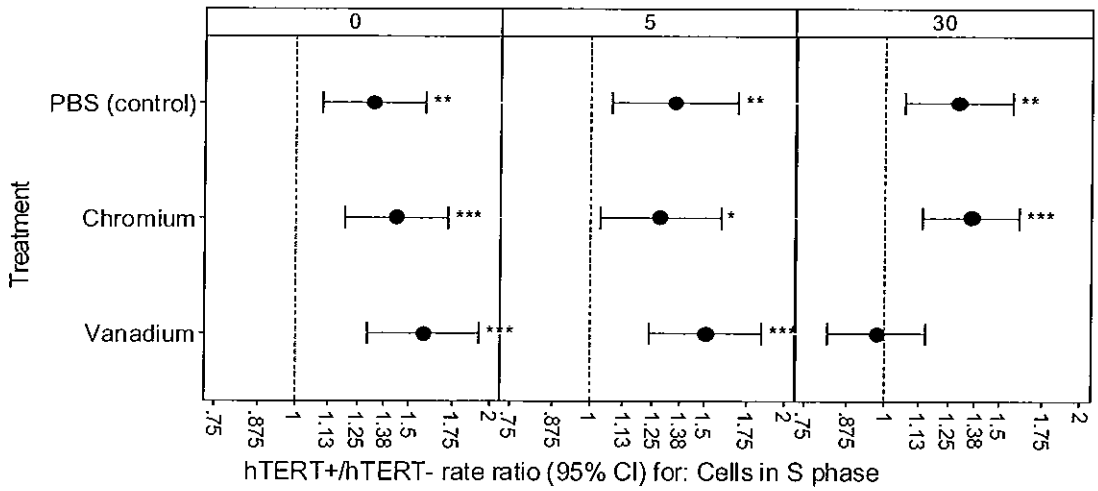

Figure 3.50. Cell Cycle Distribution (S Phase). The figure illustrates the rate ratio of hTERT+/hTERT- for S phase, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.3.4.3 G2/M Phase

The increase in the proportion of cells in G0/G1 phase was accompanied by a decrease of cells in G2/M phase after V (V) treatment of hTERT- cells (Figures 3.51a and 3.51c). Like the corresponding changes in G0/G1 phase, this decrease of hTERT- cells in G2/M phase after V (V) treatment was temporary. Furthermore, like the corresponding changes in G0/G1 phase, there was no acute change in cells in G2/M phase after V (V) treatment of hTERT+ cells (Figures 3.51b and 3.51d) and no change in either cell type after Cr (VI) treatment. There was a statistically significant difference between hTERT- and hTERT+ cells, especially after V (V) exposure (Figure 3.52), which persisted up to 30 days, and Cr (VI) immediately after metal exposure. These results were expressed as a percentage of cells in G2/M phase.

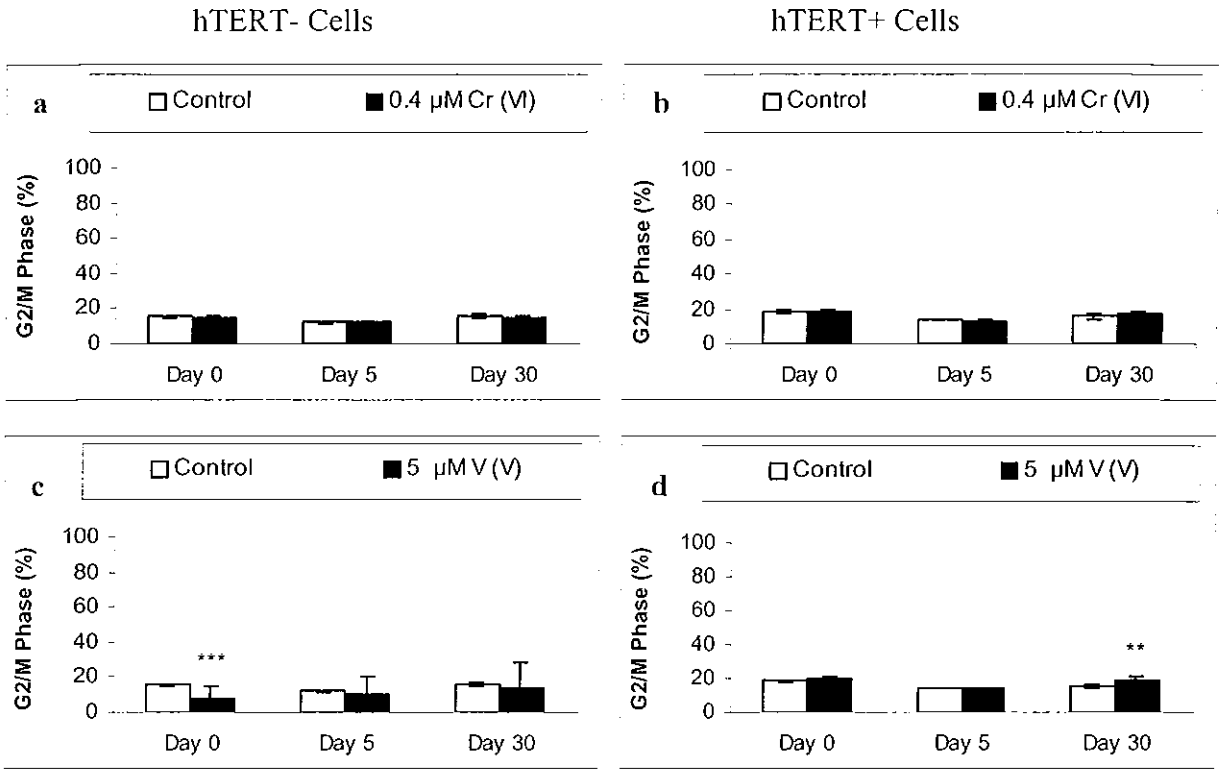

Figure 3.51. Cell Cycle Distribution (G2/M Phase). The figures show the percentage of G2/M phase in hTERT– cells (Fig. 3.51a and 3.51c) and hTERT+ cells (Fig. 3.51b and 3.51d) after a 24-hour exposure to a dose of Cr (VI) and V (V) and at different times after the exposure. ** p<0.01, *** p<0.001 compared to the control (PBS).

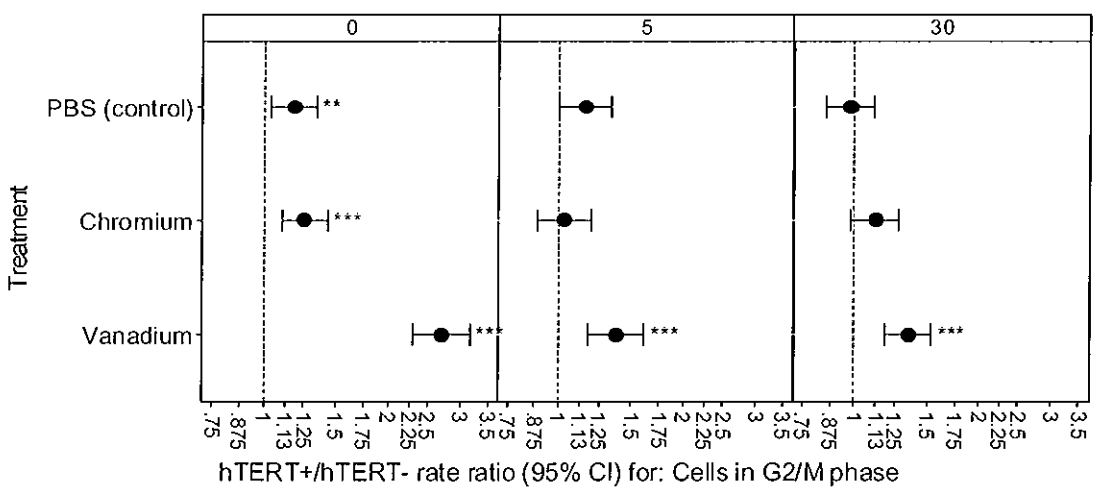

Figure 3.52. Cell Cycle Distribution (G2/M Phase). The figure illustrates the rate ratio of hTERT+/hTERT- for G2/M phase, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

As mentioned before, the proportion of cells in different phases of the cell cycle was different in hTERT- and hTERT+ cells. There were, in general, significantly fewer cells in G0/G1 phase and significantly more in S and G2/M phase in the hTERT+ cells compared to the hTERT- cells. The three different cell phases (G0/G2, S and G2/M) in hTERT- and hTERT+ cells were also compared at each survival time (*Day 0*, *Day 5* and *Day 30*) post exposure (Figures 3.53a, 3.53b and 3.53c for *Day 0* values, Figures 3.53d, 3.53e and 3.53f for *Day 5* values, and Figures 3.53g, 3.53h and 3.53i for *Day 30* values).

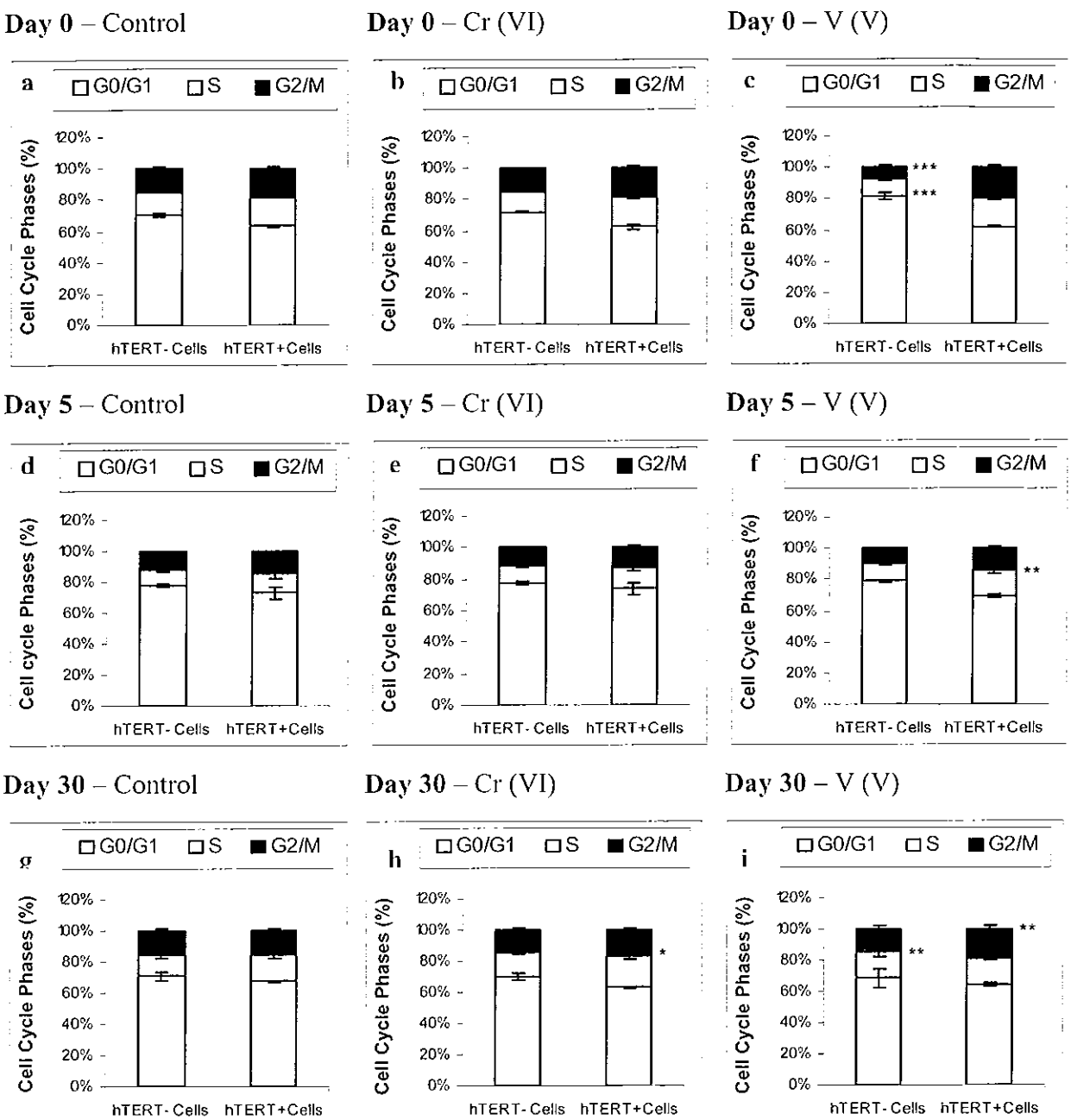

Figure 3.53. Cell Cycle Distribution. The figures show the percentage of G0/G1, S and G2/M phases in hTERT– cells and hTERT+ cells at *Day 0* (Fig. 3.53a, 3.53b and 3.53c), *Day 5* (Fig. 3.53d, 3.53e and 3.53f) and *Day 30* (Fig. 3.53g, 3.53h and 3.53i), respectively in the control, after a 24-hour exposure to a dose of Cr (VI) and after a 24-hour exposure to one dose of V (V). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control (PBS).

3.1.3.5 Apoptosis

The level of apoptosis in the cells, which were cultured routinely at 70% confluency, was measured with flow cytometry (flow-FISH) by estimating the sub G1 peak (Nicoletti et al 1991). This was only counted when a clear sub-peak was visible, which was separate from the G0/G1 peak. There was an increase in the sub G1 peak of flow cytometry immediately after either Cr (VI) (0.4μM) or V (V) (5μM) treatment of hTERT- cells (Figures 3.54a and 3.54c). This was interpreted as an indication that 24-hour metal exposure induced statistically significant apoptosis in hTERT- cells (Nicoletti *et al* 1991). No increase in this peak was seen at 5 days and 30 days after metal exposure. The sub G1 peak was significantly less in the hTERT+ cells (Figures 3.54b and 3.54d), and there was no short-term (*Day 0*) increase after metal exposure. In all of the experiments, up to 5 days after the exposure, with or without metal treatment, there was a statistically significant higher level of apoptosis in hTERT- compared to hTERT+ cells. This difference was most pronounced immediately after the metal exposure (Figure 3.55), and persisted, up to 30 days, in control cells. These results were expressed as a percentage of apoptotic cells.

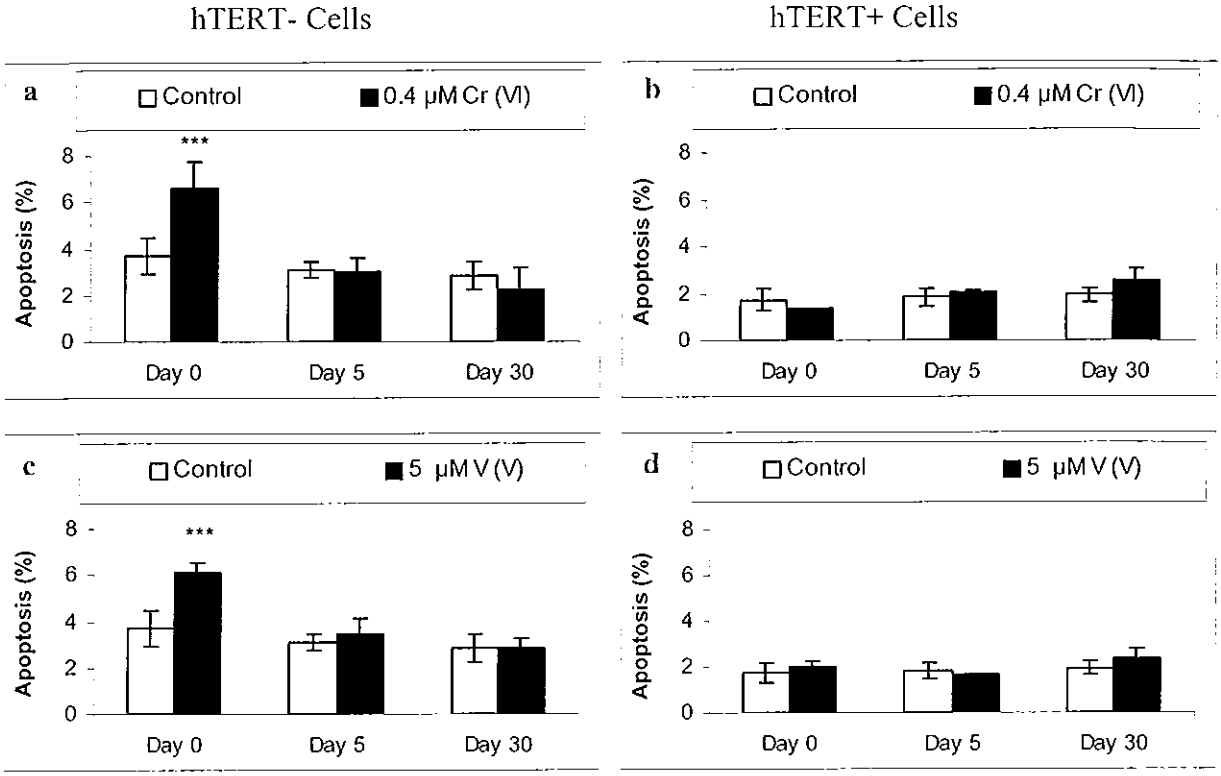

Figure 3.54. Apoptosis. The figures show the percentage of apoptosis in hTERT– cells (Fig. 3.54a and 3.54c) and hTERT+ cells (Fig. 3.54b and 3.54d) after a 24-hour exposure to a dose of Cr (VI) and V (V) and at different times after the exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

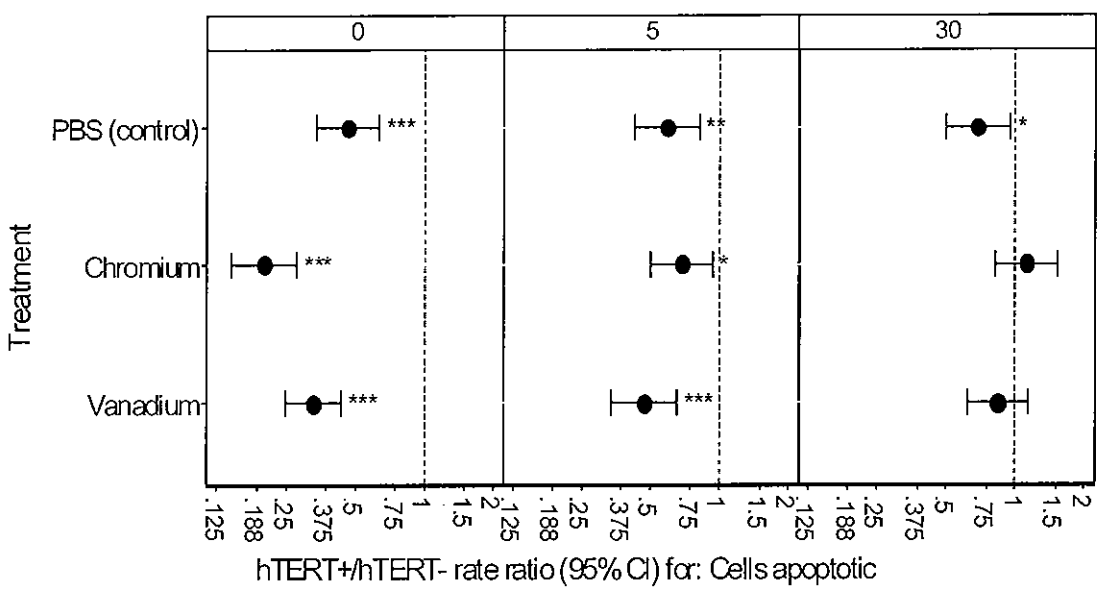

Figure 3.55. Apoptosis. The figure illustrates the rate ratio of hTERT+/hTERT- for apoptosis, showing combinations of treatments and days (*Day 0*, *Day 5* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

3.1.3.6 Necrosis

The level of necrosis in the cells, which were cultured routinely at 70% confluency, was measured with flow cytometry (flow-FISH) by estimating the sub sub G1 peak (Nicoletti *et al* 1991). This was only counted when a clear sub sub-peak was visible, which was separate from the G0/G1 peak. There was an increase in the sub sub G1 peak of flow cytometry (flow-FISH) immediately after either Cr (VI) (0.4μM) or V (V) (5μM) treatment of hTERT- cells (Figures 3.56a and 3.56c). This was interpreted as an indication that a 24-hour metal exposure induced necrosis in hTERT- cells (see Nicoletti *et al* 1991), but it did not reach a level of statistical significance. No increase in this peak was seen at longer times after the 24-hour metal exposure. The sub sub G1 peak was significantly less in the hTERT+ cells (Figures 3.56b and 3.56d) and there was no short-term (*Day 0*) increase after metal exposure. In all the experiments, at all survival times, with or without metal treatment, there was a higher level of necrosis in hTERT- cells compared to hTERT+ cells. These results were expressed as a percentage of necrotic cells.

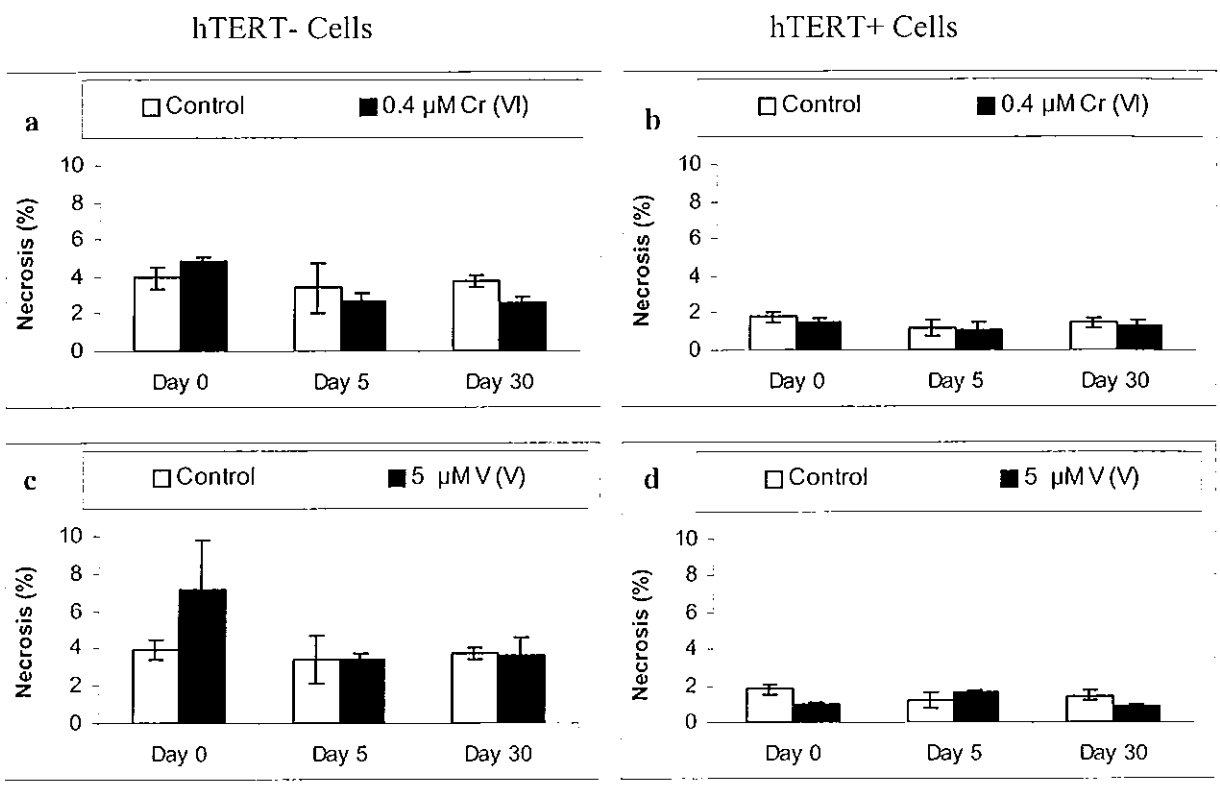

Figure 3.56. Necrosis. The figures show the percentage of necrosis in hTERT– cells (Fig. 3.56a and 3.56c) and hTERT+ cells (Fig. 3.56b and 3.56d) after a 24-hour exposure to a dose of Cr (VI) and V (V) and at different times after the exposure.

3.2 Radiation Exposure

3.2.1 Cell Survival

hTERT- (wild type) and hTERT+ (immortalised) human BJ fibroblasts were exposed to either 0.05 Gy or 0.5 Gy doses of gamma radiation. The cells were then examined for signs of cell survival at 0 (*Day 0*) and 30 days (*Day 30*) after exposure.

3.2.1.1 Clonogenic Survival

Neither 0.05 Gy nor 0.5 Gy caused a significant decrease of clonogenic survival in hTERT- cells (Figure 3.57a). The hTERT+ cells followed the same pattern with both doses of radiation (0.05 Gy and 0.5 Gy) immediately after the radiation exposure (*Day 0*). However, there was a slight increase (9.9%) of clonogenic survival with the lower dose of radiation (0.05 Gy) and a slight reduction (10%) of clonogenic survival with the higher dose of radiation (0.5 Gy) 30 days after the radiation exposure in the hTERT+ cells, which were statistically significant ($p<0.01$) (Figure 3.57b). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with radiation exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells (Table B). Furthermore, both types of cells showed more colonies at *Day 30* compared to *Day 0*. These values were expressed as a percentage of control (PBS) and control was set to 100%.

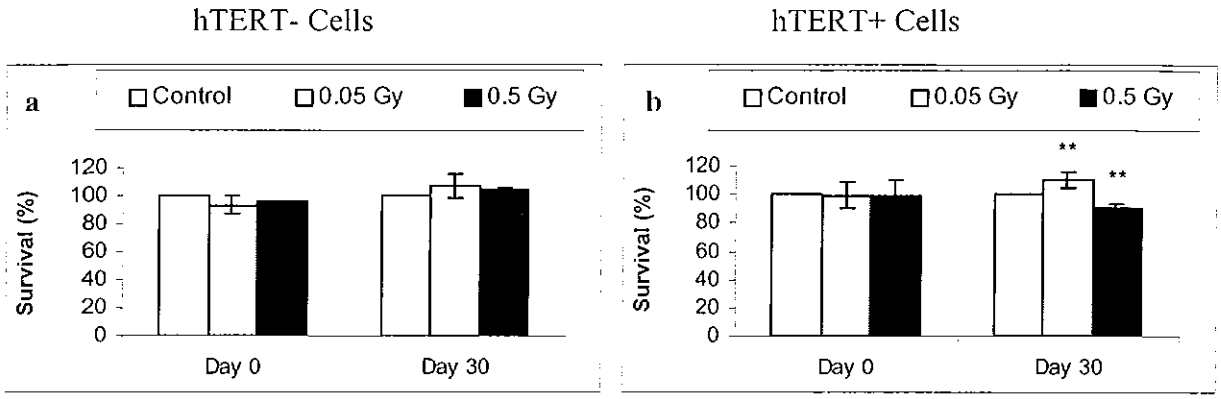

Figure 3.57. Clonogenic Survival. The figures show the clonogenic survival in hTERT– cells (Fig. 3.57a) and hTERT+ cells (Fig. 3.57b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure. These values were expressed as a percentage of control (PBS) and control was set to 100%. ** $p<0.01$ compared to the control (PBS).

Table B. Clonogenic Survival in hTERT– cells and hTERT+ cells after after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure These values were expressed as Number of Colonies

Treatment	Type of Cells			
	hTERT- Cells		hTERT+ Cells	
	Day Post Treatment		Day Post Treatment	
	Day 0	Day 30	Day 0	Day 30
Control	136.00	138.17	135.83	171.50
0.05 Gy	127.00	147.83	134.17	188.25
0.5 Gy	129.83	143.00	132.83	154.17

3.2.2 Cell Damage

hTERT- (wild type) and hTERT+ (immortalised) human BJ fibroblasts were exposed to either 0.05 Gy or 0.5 Gy doses of gamma radiation. The cells were then examined for signs of cell damage at 0 (*Day 0*) and 30 days (*Day 30*) after exposure.

3.2.2.1 Micronuclei

The radiation dose of 0.5 Gy induced micronuclei (MNi) in hTERT- cells (Figure 3.58a) at *Day 0*, but it was not statistically significant. This induction of micronuclei did not persist up to 30 days. The same pattern was seen in hTERT+ cells (Figure 3.58b) but this time the induction

of MNi at *Day 0* was statistically significant. No increase in MNi was seen up to 30 days after the radiation exposure. However, in all of the experiments, at both survival times, with or without radiation exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was particularly true 30 days after exposure to radiation, especially with the 0.5 Gy dose ($p<0.05$). These results were expressed as a percentage of MNi.

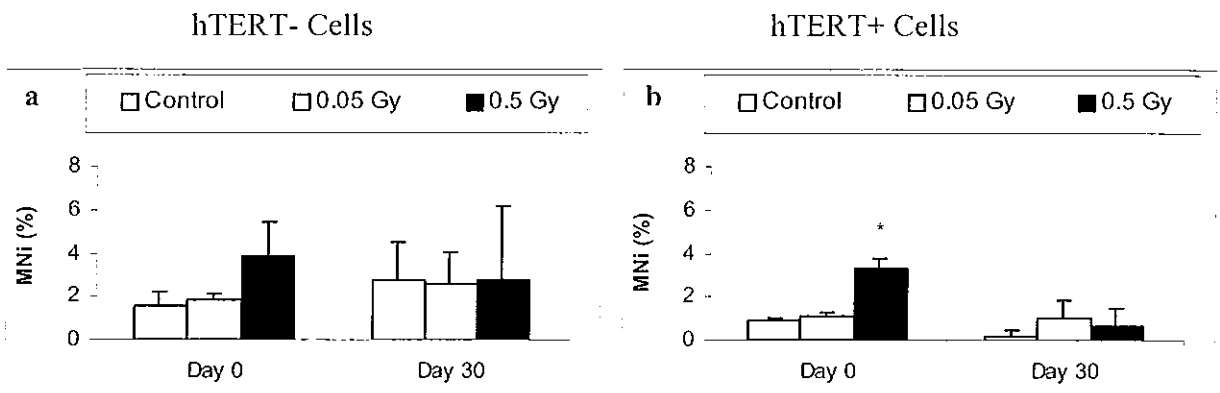

Figure 3.58. Micronuclei (MNi). The figures show the percentage of MNi in hTERT– cells (Fig. 3.58a) and hTERT+ cells (Fig. 3.58b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure. * $p<0.05$ compared to the control (PBS).

3.2.2.2 Nucleoplasmic Bridges

Neither 0.05 Gy nor 0.5 Gy doses of radiation resulted in a statistically significant increase in nucleoplasmic bridges (NPB) in hTERT- cells (Figure 3.59a). The hTERT+ cells followed the same pattern, as there was no significant increase in NPB caused by radiation (Figure 3.59b). In all of the experiments, at both survival times, with or without radiation exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was particularly true 30 days after radiation exposure, where both doses of radiation (0.05 Gy and 0.5 Gy) reached high level of significance ($p<0.01$) (Results shown in Chapter 3, Figure 3.76: 0.05 Gy corresponds to VC + 0.05 and 0.5 Gy corresponds to VC + 0.5). These results were expressed as a percentage of NPB.

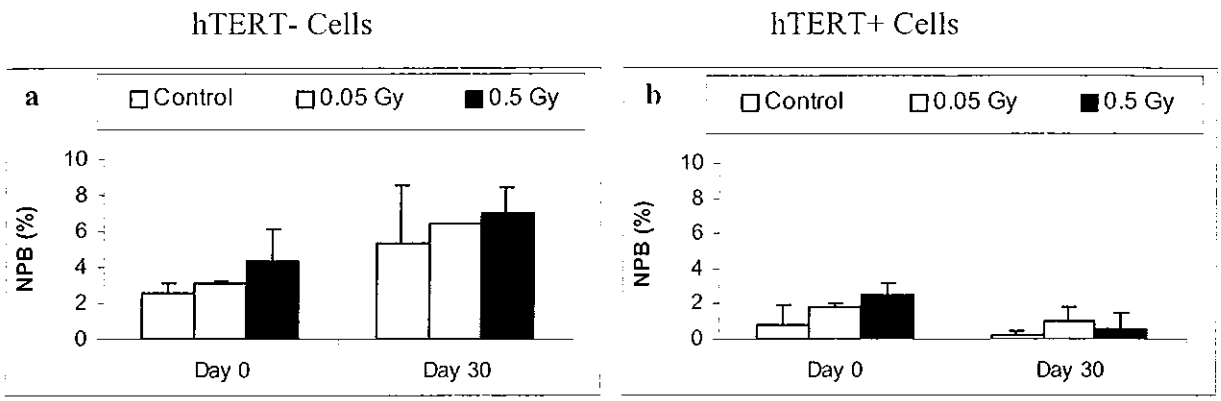

Figure 3.59. Nucleoplasmic Bridges (NPB). The figures show the percentage of NPB in hTERT– cells (Fig. 3.59a) and hTERT+ cells (Fig. 3.59b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure.

3.2.2.3 Chromatid Breaks

Radiation caused a small increase of chromatid breaks in the hTERT- cells at the lower dose (0.05 Gy) immediately after the exposure (*Day 0*), but it had no statistical significance (Figure 3.60a). This increase in breaks, after the exposure to 0.05 Gy radiation, slightly decreased with time. In the hTERT+ cells, there was also a small increase in chromatid breaks after both doses of radiation at *Day 0*, and the 0.5 Gy dose slightly increased up to 30 days after the radiation exposure (Figure 3.60b). However, none of these data were statistically significant. These results were expressed as a percentage of chromatid breaks.

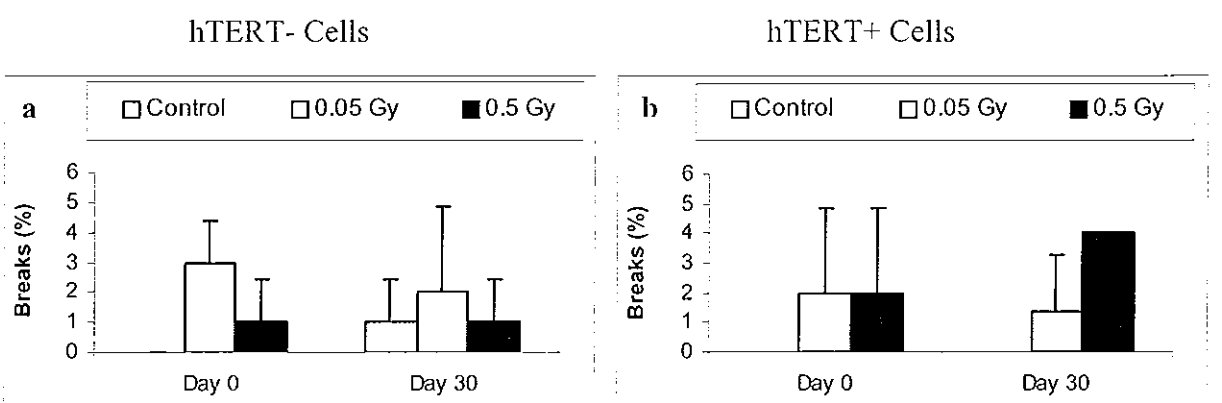

Figure 3.60. Chromatid Breaks. The figures show the percentage of chromatid breaks in hTERT– cells (Fig. 3.60a) and hTERT+ cells (Fig. 3.60b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure.

3.2.2.4 Chromatid Gaps

The radiation dose of 0.05 Gy caused very few chromatid gaps in hTERT- cells with no statistical significance (Figure 3.61a). In the hTERT+ cells, there was no significant increase in gaps after radiation, although the 0.5 Gy dose, and to a less extent the 0.05 Gy dose, produced some gaps at *Day 30* (Figure 3.61b). These results were expressed as a percentage of chromatid gaps.

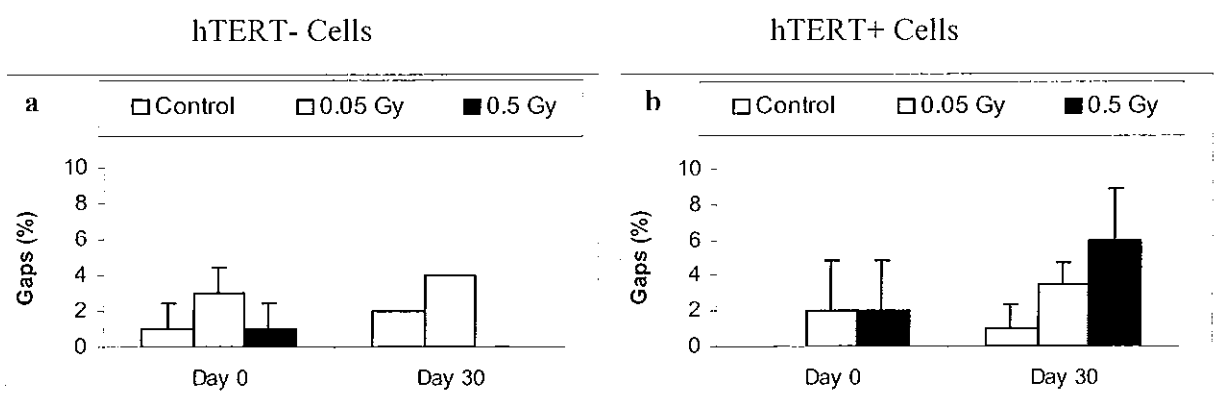

Figure 3.61. Chromatid Gaps. The figures show the percentage of chromatid gaps in hTERT– cells (Fig. 3.61a) and hTERT+ cells (Fig. 3.61b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure.

3.2.2.5 Chromatid Fragments

Radiation did not cause any increase in chromatid fragments in the hTERT- cells (Figure 3.62a). In the hTERT+ cells, there was a small temporary increase in fragments after 0.5 Gy immediately after radiation exposure, which was not statistically significant (Figure 3.62b). These results were expressed as a percentage of chromatid fragments.

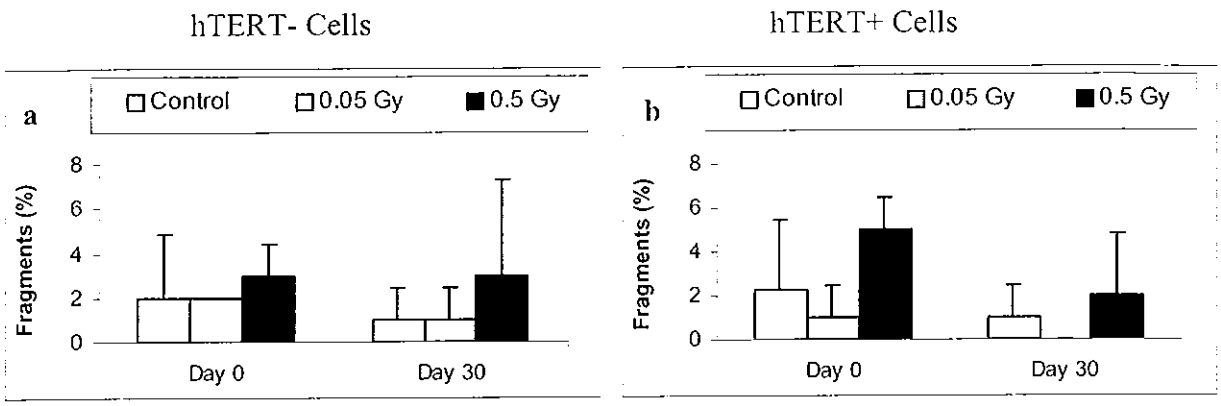

Figure 3.62. Chromatid Fragments. The figures show the percentage of chromatid fragments in hTERT– cells (Fig. 3.62a) and hTERT+ cells (Fig. 3.62b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure.

3.2.2.6 Dicentric Chromosomes

Both doses of radiation (particularly 0.5 Gy) induced dicentric chromosomes up to 30 days after radiation exposure in hTERT- cells, although this data was not statistically significant (Figure 3.63a). In contrast, neither 0.05 Gy nor 0.5 Gy induced dicentric chromosomes in hTERT+ cells (Figure 3.63b). In all of the experiments, at both survival times, with or without radiation exposure, there was a lower level of dicentric chromosomes in the hTERT+ cells compared to the hTERT- cells. This was statistically significant 30 days after radiation exposure to the 0.5 Gy dose ($p<0.05$) (Results shown in Chapter 3, Figure 3.88: 0.05 Gy corresponds to VC + 0.05 and 0.5 Gy corresponds to VC + 0.5). These results were expressed as a percentage of dicentric chromosomes.

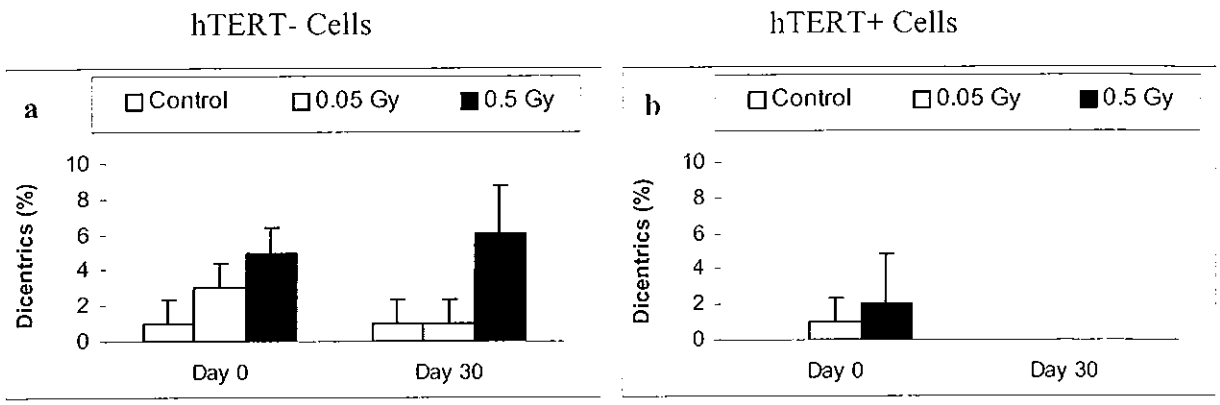

Figure 3.63. Dicentric Chromosomes. The figures show the percentage of dicentric chromosomes in hTERT– cells (Fig. 3.63a) and hTERT+ cells (Fig. 3.63b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure.

3.2.2.7 Tetraploidy

Both doses of radiation caused a very small increase in tetraploidy in hTERT- cells up to 30 days after a single radiation exposure (Figure 3.64a). The hTERT+ cells followed the same pattern (Figure 3.64b). However, none of this data was statistically significant. In all of the experiments, at both survival times, with or without radiation exposure, there was a higher level of tetraploid cells in the hTERT+ cells compared to the hTERT- cells. This was statistically significant immediately after the radiation exposure (*Day 0*) to the 0.05 Gy dose ($p<0.05$). These results were expressed as a percentage of tetraploid cells.

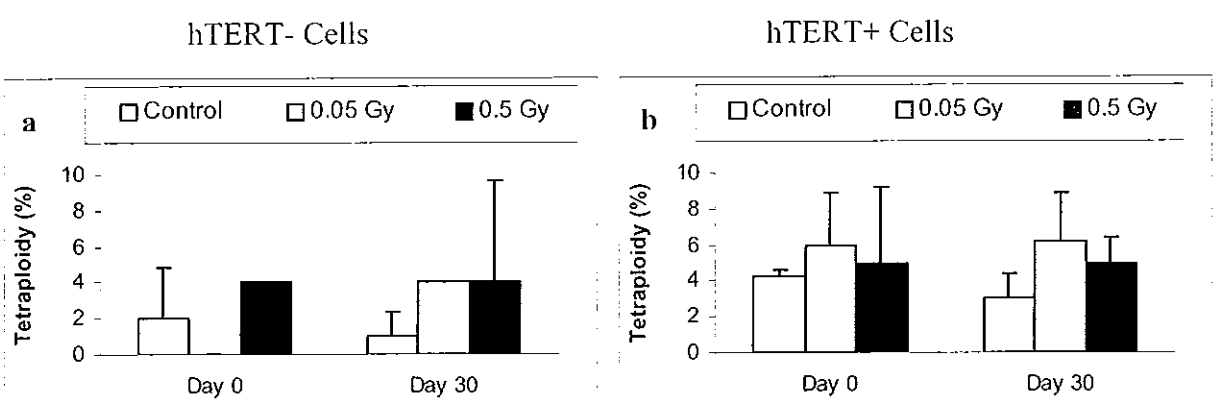

Figure 3.64. Tetraploidy. The figures show the percentage of tetraploidy in hTERT– cells (Fig. 3.64a) and hTERT+ cells (Fig. 3.64b) after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure.

3.3 Combined Exposure

3.3.1 Cell Survival

hTERT- (wild type) and hTERT+ (immortalised) human BJ fibroblasts were exposed to a combined dose of Cr (VI) ions (0.4 μM) + either 0.05 Gy or 0.5 Gy doses of gamma radiation and *viceversa*. Cells were also exposed to control, metal + sham irradiation (SI) and *viceversa*, 0.05 Gy + vehicle control (VC) and *viceversa*, 0.5 Gy + vehicle control and *viceversa*. The cells were then examined for signs of cell survival at 0 (*Day 0*) and 30 days (*Day 30*) after exposure.

3.3.1.1 Clonogenic Survival

Combined Exposure of M + 0.05 Gy

M + SI and M + 0.05 Gy caused a significant decrease in clonogenic survival (respectively $p<0.01$ and $p<0.05$) in hTERT- cells at *Day 0* (Figure 3.65a and Figure 3.67). The hTERT+ cells followed a different pattern since M + 0.05 Gy caused a statistically significant reduction of clonogenic survival ($p<0.05$) immediately after the combined exposure (*Day 0*), which increased up to 30 days after the combined exposure ($p<0.01$) (Figure 3.65b and Figure 3.67). Furthermore, there was a significant increase in clonogenic survival ($p<0.01$) 30 days after the radiation exposure (VC + 0.05 Gy). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with either metal treatment or radiation exposure or combined exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells (with the only exception of M + 0.05 Gy at *Day 0*). Furthermore, in all of the experiments the hTERT+ cells showed more colonies at *Day 30* compared to *Day 0* (Table C). The clonogenicity rate ratios of hTERT+/hTERT- cells were plotted with their confidence intervals in Figure 3.68, and tabulated with their confidence intervals and *P*-values in Table D. All the *P*-values were so

small as to be indistinguishable from zero, at the precision level used by the computer. Statistical analysis showed that the hTERT+ cells were significantly more capable of forming colonies compared to hTERT- cells. These values were expressed as a percentage of control (PBS) and control was set to 100%.

Combined Exposure of M + 0.5 Gy

M + SI and M + 0.5 Gy caused a significant decrease of clonogenic survival (respectively $p<0.01$ and $p<0.05$) in hTERT- cells at *Day 0*. This clonogenic reduction persisted only with M + 0.5 Gy ($p<0.01$) up to 30 days after the combined exposure (Figure 3.65c and Figure 3.67). The hTERT+ cells followed a different pattern, since M + 0.5 Gy caused a significant reduction in clonogenic survival only 30 days after the combined exposure ($p<0.001$). Furthermore, there was a 10% reduction in clonogenic survival 30 days after radiation exposure only (VC + 0.5 Gy) ($p<0.01$) (Figure 3.65d and Figure 3.67). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with either metal treatment, radiation exposure or combined exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells. Furthermore, in all of the experiments the hTERT+ cells showed more colonies at *Day 30* compared to *Day 0* (Table C). The clonogenicity rate ratios of hTERT+/hTERT- cells were plotted with their confidence intervals in Figure 3.68, and tabulated with their confidence intervals and *P*-values in Table D. All the *P*-values were so small as to be indistinguishable from zero, at the precision level used by the computer. Statistical analysis showed that the hTERT+ cells were significantly more capable of forming colonies compared to hTERT- cells. These values were expressed as a percentage of control (PBS) and control was set to 100%.

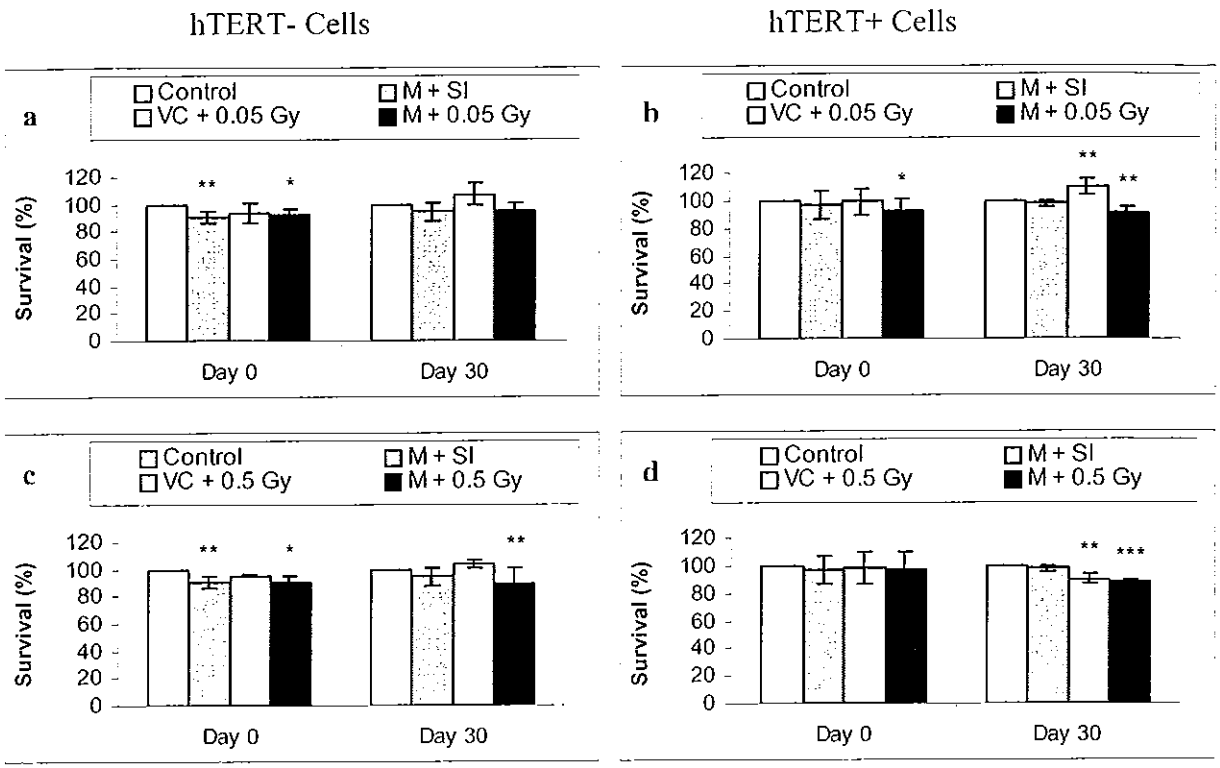

Figure 3.65. Clonogenic Survival. The figures show the clonogenic survival in hTERT– cells (Fig. 3.65a and 3.65c) and hTERT+ cells (Fig. 3.65b and 3.65d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS. These values were expressed as a percentage of control (PBS) and control was set to 100%. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the control (PBS).

Combined Exposure of 0.05 Gy + M

0.05 Gy + VC, SI + M and 0.05 Gy + M resulted in a significant decrease (approximately 13%) in clonogenic survival in hTERT- cells at *Day 0* ($p<0.05$). SI + M and 0.05 Gy + M caused a persistent decrease in clonogenic survival (respectively $p<0.01$ and $p<0.001$) up to 30 days after either the metal or the combined exposure (Figure 3.66a and Figure 3.67). The hTERT+ cells showed a reduction in clonogenic survival following radiation exposure (0.05 Gy + VC) at *Day 0* ($p<0.01$), and an increase in clonogenic survival 30 days after the metal exposure ($p<0.05$). 0.05 + M caused a significant reduction in clonogenic survival at *Day 0* ($p<0.05$), which persisted and became more significant up to 30 days after the combined exposure

($p<0.01$) (Figure 3.66b and Figure 3.67). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with either metal treatment or radiation exposure or combined exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells (with the only exception of 0.05 Gy + VC at *Day 0*). Furthermore, in all of the experiments the hTERT+ cells showed more colonies at *Day 30* compared to *Day 0* (Table C). The clonogenicity rate ratios of hTERT+/hTERT- cells were plotted with their confidence intervals in Figure 3.68, and tabulated with their confidence intervals and *P*-values in Table D. All the *P*-values were so small as to be indistinguishable from zero, at the precision level used by the computer. Statistical analysis showed that the hTERT+ cells were significantly more capable of forming colonies compared to hTERT- cells. These values were expressed as a percentage of control (PBS) and control was set to 100%.

Combined Exposure of 0.5 Gy + M

SI + M and 0.5 Gy + M caused a significant decrease (approximately 18%) in clonogenic survival in hTERT- cells ($p<0.05$) at *Day 0*. There was a further reduction in clonogenic survival with SI + M ($p<0.01$) and 0.5 Gy + M ($p<0.001$) up to 30 days after either the metal or the combined exposure (Figure 3.66c and Figure 3.67). Furthermore, there was a loss of clonogenic survival 30 days after the radiation exposure (0.5 Gy + VC) ($p<0.05$). The hTERT+ followed a different pattern, since only the radiation exposure (0.5 Gy + VC), but not the combined exposure (0.5 Gy + M), resulted in a decrease (approximately 14.5%) in clonogenic survival immediately after the radiation exposure (*Day 0*) ($p<0.001$), which persisted up to 30 days after the radiation exposure ($p<0.001$) (Figure 3.66d and Figures 3.67). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with either metal treatment or radiation exposure or combined exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells (with the only exception of 0.5 Gy + VC at *Day 0*). Furthermore, in all of the experiments the hTERT+ cells showed more colonies at *Day 30*

compared to *Day 0* (Table C). The clonogenicity rate ratios of hTERT+/hTERT- cells are plotted with their confidence intervals in Figure 3.68, and tabulated with their confidence intervals and *P*-values in Table D. All of the *P*-values were so small as to be indistinguishable from zero, at the precision level used by the computer. Statistical analysis showed that the hTERT+ cells were significantly more capable of forming colonies compared to hTERT- cells. These values were expressed as a percentage of control (PBS) and control was set to 100%.

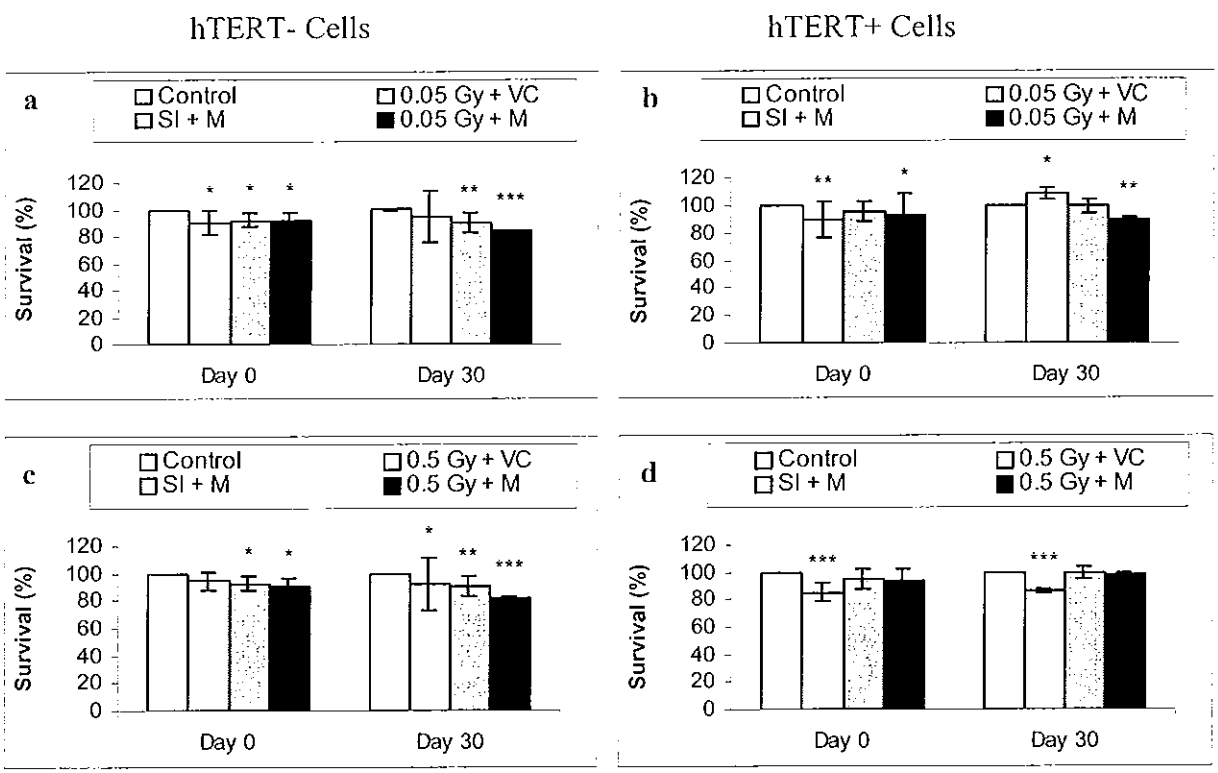

Figure 3.66. Clonogenic Survival. The figures show the clonogenic survival in hTERT– cells (Fig. 3.66a and 3.66c) and hTERT+ cells (Fig. 3.66b and 3.66d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS. These values were expressed as a percentage of control (PBS) and control was set to 100%. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

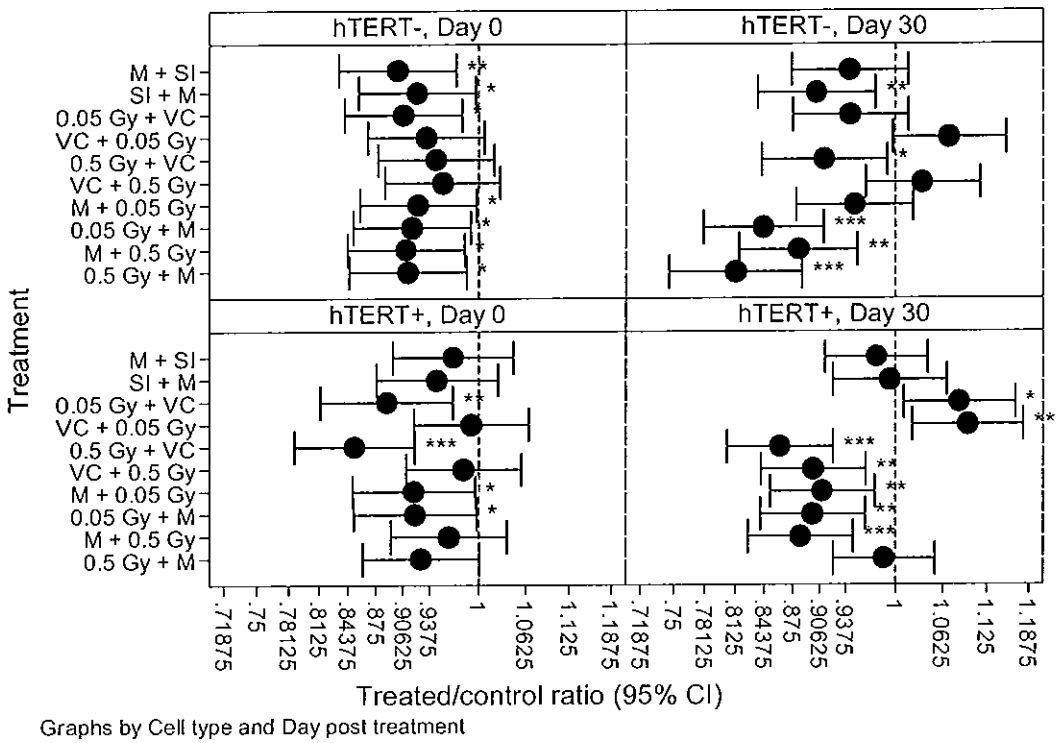

Figure 3.67. Clonogenic Survival. The figure shows the treated/control ratios of clonogenic survival rates, with 95% confidence intervals, for all the combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to the control (PBS).

Table C. Clonogenic Survival in hTERT– cells and hTERT+ cells after either a *Single Exposure* to Metal + Sham Irradiation (M + SI), Sham Irradiation + Metal (SI + M), 0.05 Gy + Vehicle Control (0.05 Gy + VC), Vehicle Control + 0.05 Gy (VC + 0.05 Gy), 0.5 Gy + Vehicle Control (VC + 0.5 Gy) and Vehicle Control + 0.5 Gy (VC + 0.5 Gy) or *Combined Exposure* to Metal + 0.05 Gy (M + 0.05 Gy), 0.05 Gy + Metal (0.05 Gy + M), Metal + 0.5 Gy (M + 0.5 Gy) and 0.5 Gy + Metal (0.5 Gy + M) and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS. These values were expressed as Number of Colonies

Treatment	Type of Cells			
	hTERT- Cells		hTERT+ Cells	
	Day Post Treatment		Day Post Treatment	
	Day 0	Day 30	Day 0	Day 30
Control	136.00	138.17	135.83	171.50
M + SI	122.33	130.00	131.08	167.50
SI + M	125.50	124.50	128.42	170.25
0.05 Gy + VC	123.33	130.50	120.25	186.25
VC + 0.05 Gy	127.00	147.83	134.17	188.25
0.5 Gy + VC	128.67	126.25	115.33	147.83
VC + 0.5 Gy	129.83	143.00	132.83	154.17
M + 0.05 Gy	125.67	130.83	124.50	156.00
0.05 Gy + M	124.67	116.50	124.83	154.17
M + 0.5 Gy	123.67	121.67	130.33	151.67
0.5 Gy + M	124.00	112.58	125.67	169.00

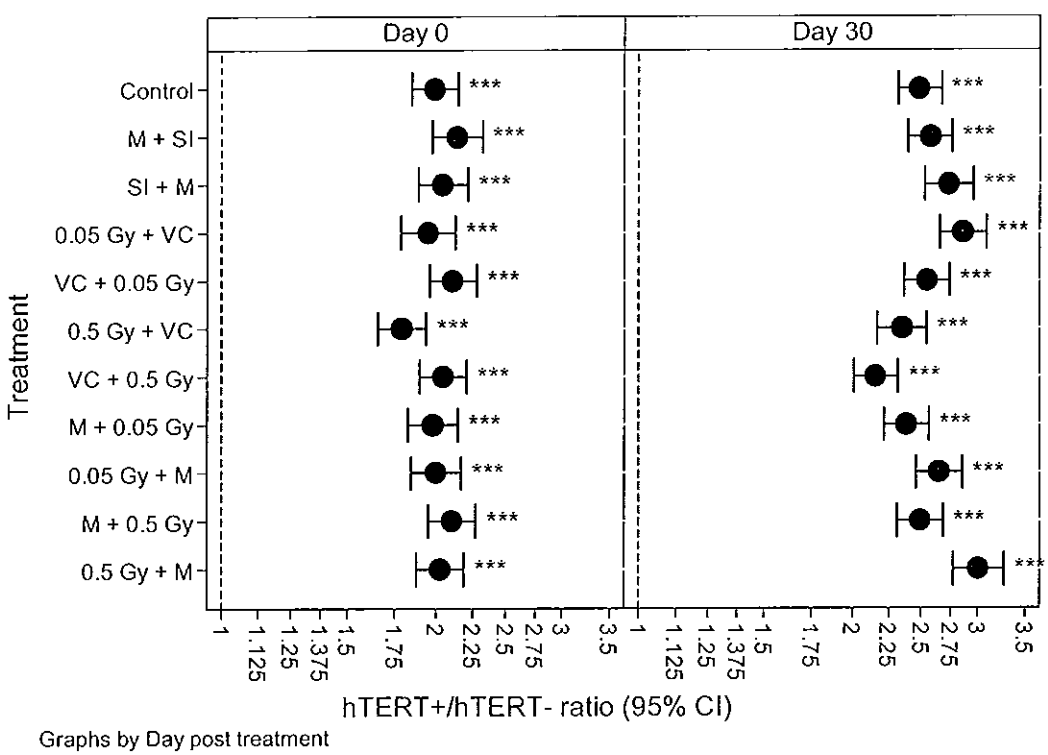

Figure 3.68. Clonogenic Survival. The figure illustrates the rate ratio of hTERT+/hTERT- for clonogenic survival, with 95% confidence intervals, showing combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure. *** p<0.001 compared to hTERT+/hTERT- = 1.

Table D. hTERT+/hTERT- cells clonogenicity rate ratios (with 95% confidence intervals and *P*-values) for each combination of cell type, day post treatment and treatment. All the *P*-values were so small as to be indistinguishable from zero, at the precision level used by the computer. Statistical analysis showed that the hTERT+ cells were significantly more capable of forming colonies compared to hTERT- cells. All the estimates and confidence intervals for clonogenicity rates are for rates per cell in the inoculums. Therefore, the hTERT- counts are based on twice the number of cells in the inoculums (8000 cells were plated for the hTERT- cells and 4000 cells were plated for the hTERT+ cells). ***P-values below a Simes corrected critical P-value of 0.05.**

Day Post Treatment	Treatment	Rate	(95%	CI)	P
Day 0	Control	1.99	(1.85,	2.15)	0*
	M + SI	2.14	(1.98,	2.32)	0*
	SI + M	2.05	(1.89,	2.22)	0*
	0.05 Gy + VC	1.95	(1.79,	2.13)	0*
	VC + 0.05 Gy	2.11	(1.96,	2.28)	0*
	0.5 Gy + VC	1.79	(1.66,	1.94)	0*
	VC + 0.5 Gy	2.05	(1.90,	2.21)	0*
	M + 0.05 Gy	1.98	(1.83,	2.15)	0*
	0.05 Gy + M	2.00	(1.85,	2.17)	0*
	M + 0.5 Gy	2.11	(1.95,	2.28)	0*
	0.5 Gy + M	2.03	(1.88,	2.19)	0*
Day 30	Control	2.49	(2.32,	2.67)	0*
	M + SI	2.57	(2.40,	2.77)	0*
	SI + M	2.74	(2.53,	2.96)	0*
	0.05 Gy + VC	2.86	(2.65,	3.09)	0*
	VC + 0.05 Gy	2.55	(2.37,	2.74)	0*
	0.5 Gy + VC	2.35	(2.17,	2.54)	0*
	VC + 0.5 Gy	2.16	(2.01,	2.32)	0*
	M + 0.05 Gy	2.39	(2.22,	2.56)	0*
	0.05 Gy + M	2.65	(2.45,	2.86)	0*
	M + 0.5 Gy	2.49	(2.31,	2.69)	0*
	0.5 Gy + M	3.01	(2.77,	3.27)	0*

3.3.2 Cell Damage

hTERT- (wild type) and hTERT+ (immortalised) human BJ fibroblasts were exposed to a combined dose of Cr (VI) ions (0.4 μM) plus either 0.05 Gy or 0.5 Gy doses of gamma radiation and *viceversa*. Cells were also exposed to control, metal + sham irradiation and *viceversa*, 0.05 Gy + vehicle control and *viceversa*, 0.5 Gy + vehicle control and *viceversa*. The cells were then examined for signs of cell damage at 0 (*Day 0*) and 30 days (*Day 30*) after exposure.

3.3.2.1 Micronuclei

Combined Exposure of M + 0.05 Gy

M + SI, VC + 0.05 and M + 0.05 Gy did not induce significant micronuclei (MNi) in hTERT- cells (Figure 3.69a and Figure 3.71). The hTERT+ cells followed the same pattern (Figure 3.69b and Figure 3.71). However, in all of the experiments at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was statistically significant in the control ($p<0.05$) and after metal exposure to M + SI ($p<0.01$) at *Day 30* only (Figure 3.72). These results were expressed as a percentage of MNi.

Combined Exposure of M + 0.5 Gy

M + 0.5 Gy caused a statistically significant increase in MNi immediately after the combined exposure (*Day 0*) in hTERT- cells, which did not persist up to *Day 30* (Figure 3.69c and Figure 3.71). The hTERT+ cells followed a similar pattern, but M + 0.5 Gy induced a significant persistence of MNi, since they were present at both survival times. Furthermore, VC + 0.5 Gy caused significant increase in MNi immediately after the radiation exposure (*Day 0*), which did not persist up to *Day 30* (Figure 3.69d and Figure 3.71). In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was particularly true at *Day 30*, where control ($p<0.05$), exposure to metal M + SI ($p<0.01$) and radiation VC + 0.5 ($p<0.05$) reached level of statistical significance (Figure 3.72). These results were expressed as a percentage of MNi.

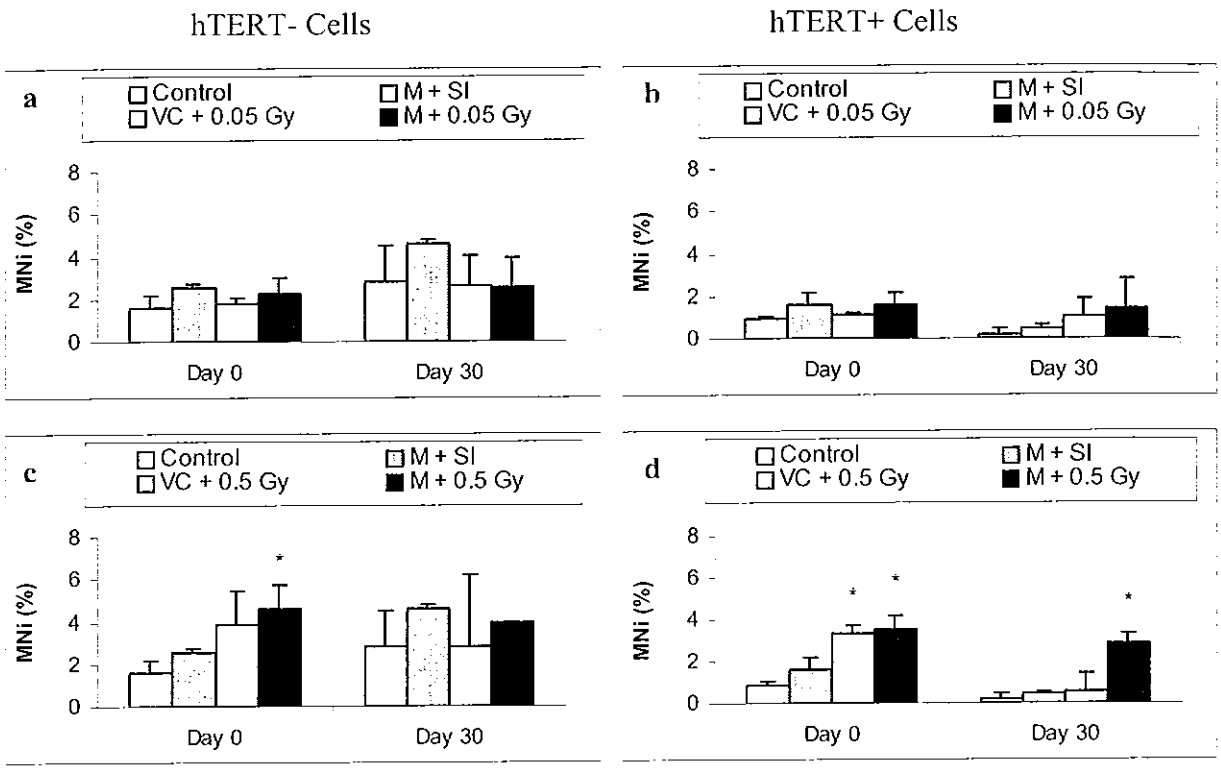

Figure 3.69. Micronuclei (MNi). The figures show the percentage of MNi in hTERT– cells (Fig. 3.69a and 3.69c) and hTERT+ cells (Fig. 3.69b and 3.69d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS. * $p<0.05$ compared to the control (PBS).

Combined Exposure of 0.05 Gy + M

0.05 + VC, SI + M and 0.05 Gy + M did not induce significant MNi in hTERT- cells, although SI + M and 0.05 Gy + M were higher than control (Figure 3.70a and Figure 3.71). The hTERT+ cells followed the same pattern (Figure 3.70b and Figure 3.71). In all of the experiments at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was statistically significant after exposure to SI + M at *Day 0* ($p<0.05$) and *Day 30* ($p<0.01$), and in the control ($p<0.05$), after radiation exposure to 0.05 + VC ($p<0.05$) and combined exposure to 0.05 Gy + M ($p<0.05$) at *Day 30* only (Figure 3.72). These results were expressed as a percentage of MNi.

Combined Exposure of 0.5 Gy + M

0.5 Gy + VC and 0.5 Gy + M caused a statistically significant increase in MNi immediately after the combined exposure (*Day 0*) in hTERT- cells, which had no persistence up to *Day 30* (Figure 3.70c and Figure 3.71). The response of hTERT+ cells was different, since there were no MNi. Only the combined exposure to 0.5 Gy + M induced some MNi at *Day 0*, but this was not significant (Figure 3.70d and Figure 3.71). In all of the experiments at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was statistically significant in the control at *Day 30* ($p<0.05$), after exposure to SI + M at *Day 0* ($p<0.05$) and *Day 30* ($p<0.01$), and after radiation exposure to 0.5 + VC at *Day 0* only ($p<0.05$) (Figure 3.72). These results were expressed as a percentage of MNi.

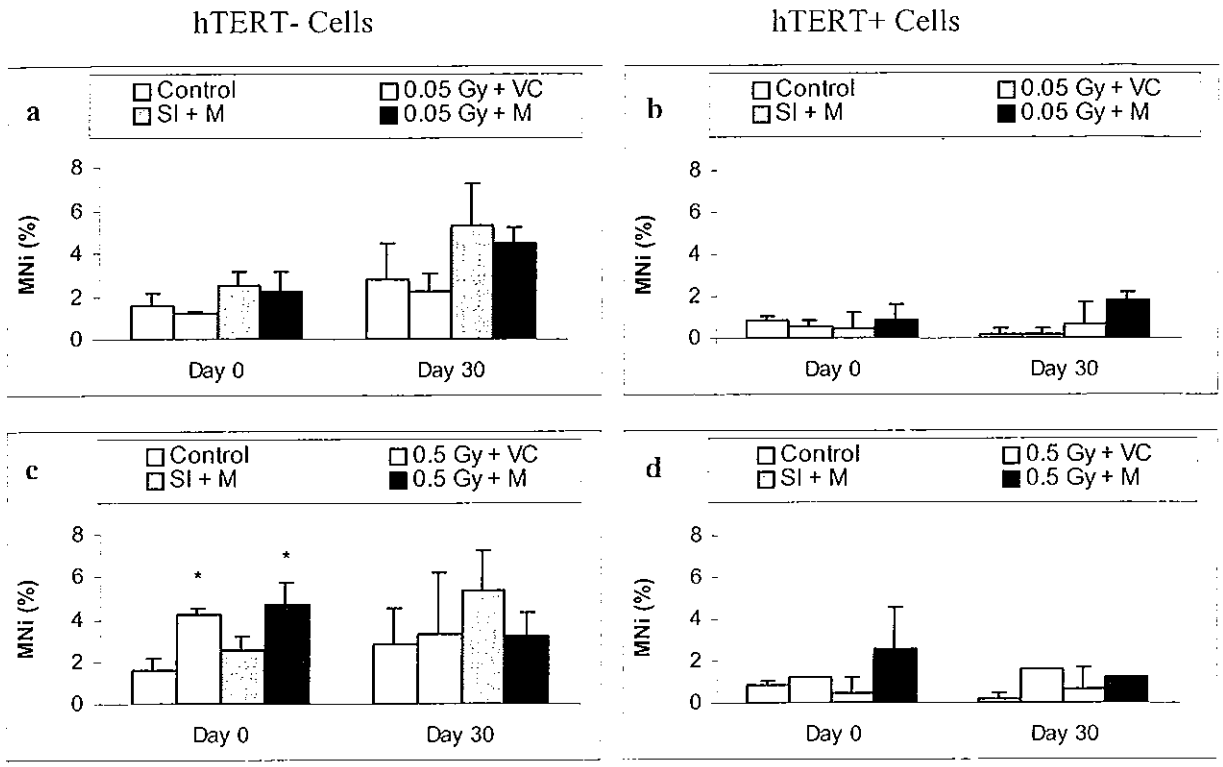

Figure 3.70. Micronuclei (MNi). The figures show the percentage of MNi in hTERT– cells (Fig. 3.70a and 3.70c) and hTERT+ cells (Fig. 3.70b and 3.70d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS. * $p<0.05$ compared to the control (PBS).

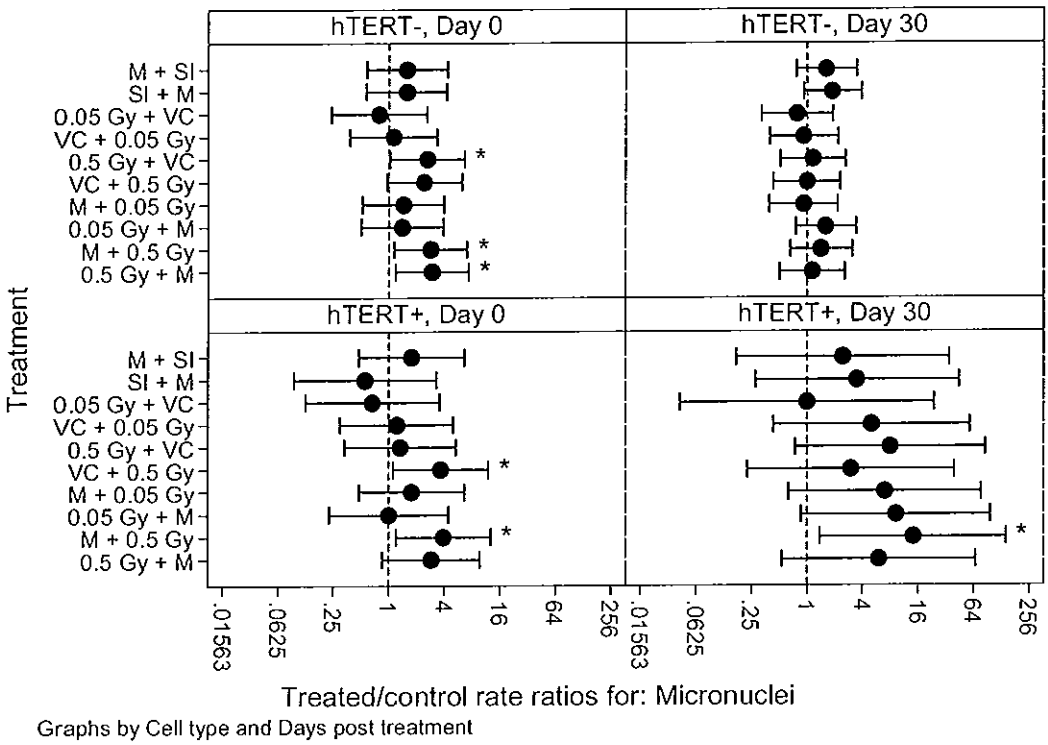

Figure 3.71. Micronuclei (MNi). The figure shows the treated/control ratios of MNi rates, with 95% confidence intervals, for all the combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure. * $p<0.05$ compared to the control (PBS).

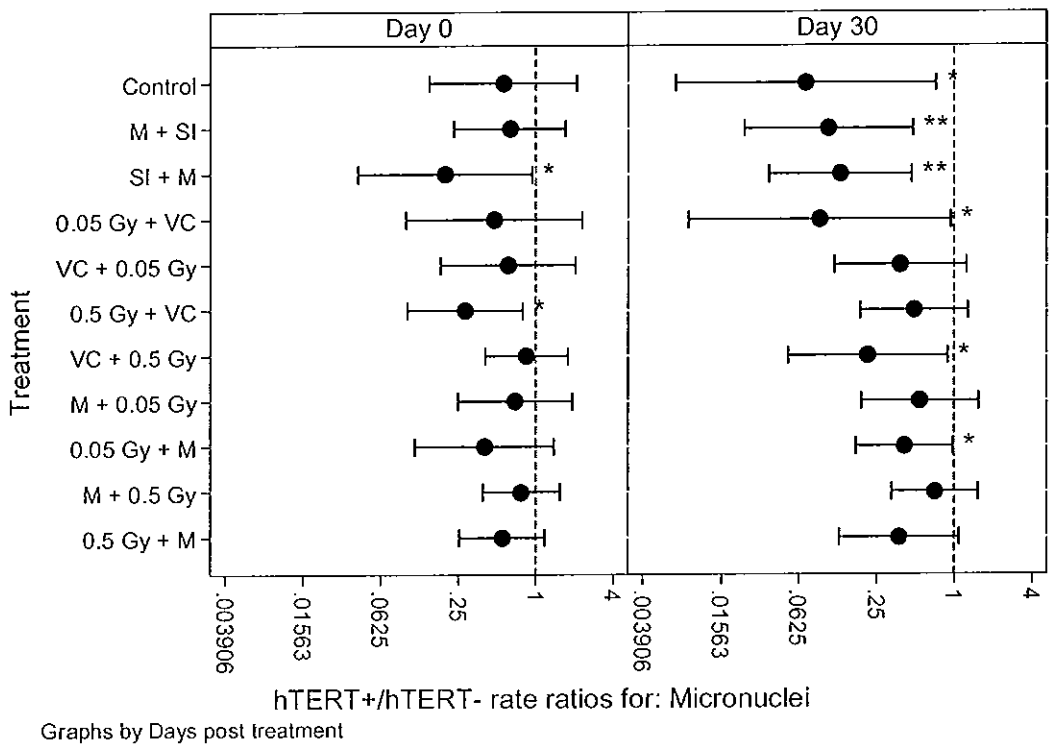

Figure 3.72. Micronuclei (MNi). The figure illustrates the rate ratio of hTERT+/hTERT- for MNi, with 95% confidence intervals, showing combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure. * p<0.05, ** p<0.01 compared to hTERT+/hTERT- = 1.

3.3.2.2 Nucleoplasmic Bridges

Combined Exposure of M + 0.05 Gy

M + SI, VC + 0.05 and M + 0.05 Gy did not induce nucleoplasmic bridges (NPB) in hTERT- cells (Figure 3.73a and Figure 3.75). The hTERT+ cells followed the same pattern (Figure 3.73b and Figure 3.75). However, in all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was statistically significant in the control ($p<0.05$), after metal exposure to M + SI ($p<0.001$), radiation exposure to VC + 0.05 ($p<0.01$) and combined exposure to M + 0.05 Gy ($p<0.001$) at *Day 30* only (Figure 3.76). These results were expressed as a percentage of NPB.

Combined Exposure of M + 0.5 Gy

M + SI, VC + 0.5 and M + 0.5 Gy did not cause nucleoplasmic bridges (NPB) in hTERT- cells (Figure 3.73c and Figure 3.75). The hTERT+ cells followed the same pattern (Figure 3.73d Figure 3.75). However, in all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was statistically significant in the control ($p<0.05$), after metal exposure to M + SI ($p<0.001$), radiation exposure to VC + 0.5 ($p<0.01$) and combined exposure to M + 0.5 Gy ($p<0.001$) at *Day 30* only (Figure 3.76). These results were expressed as a percentage of NPB.

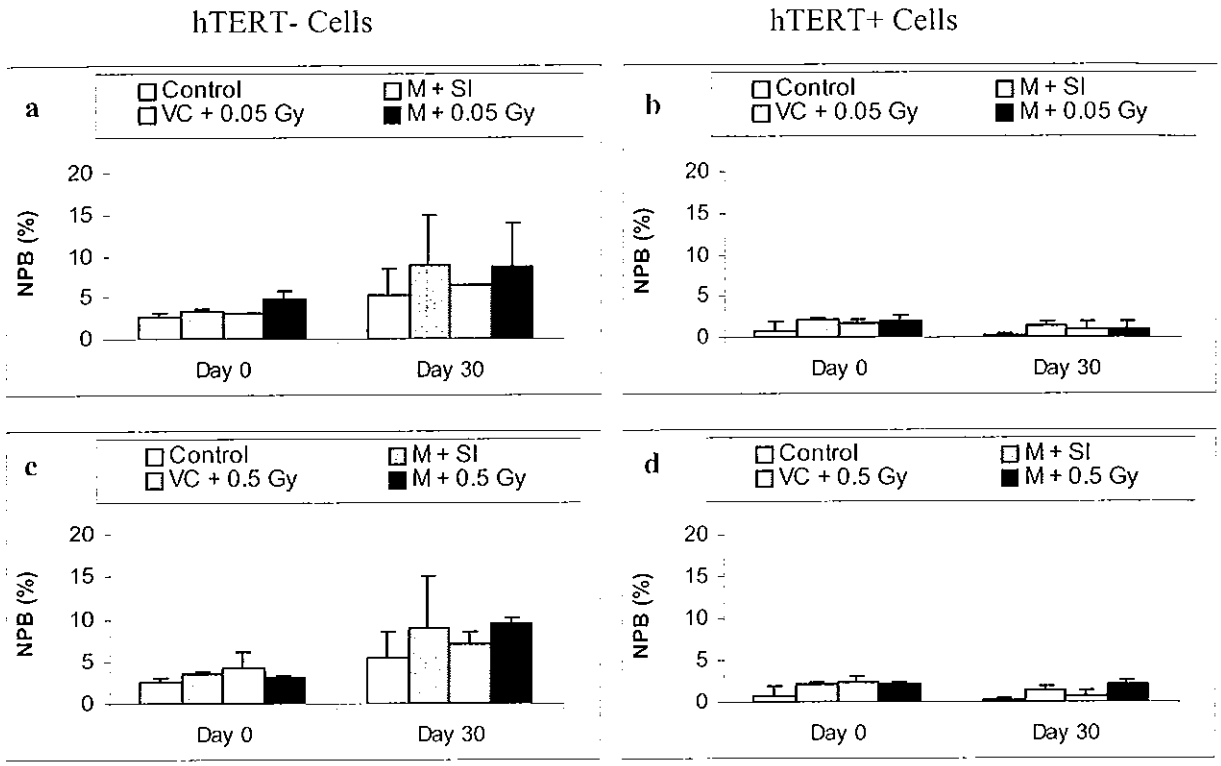

Figure 3.73. Nucleoplasmic Bridges (NPB). The figures show the percentage of NPB in hTERT– cells (Fig. 3.73a and 3.73c) and hTERT+ cells (Fig. 3.73b and 3.73d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS.

Combined Exposure of 0.05 Gy + M

0.05 + VC, SI + M and 0.05 Gy + M did not induce significant NPB in hTERT- cells (Figure 3.74a and Figure 3.75). The hTERT+ cells followed the same pattern (Figure 3.74b and Figure 3.75). In all the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was statistically significant in the control ($p<0.05$), after metal exposure to SI + M ($p<0.001$), radiation exposure to 0.05 Gy + VC ($p<0.01$), and to a lesser extent, after combined exposure to 0.05 Gy + M ($p<0.05$) at *Day 30* only (Figure 3.76). These results were expressed as a percentage of NPB.

Combined Exposure of 0.5 Gy + M

0.5 + VC, SI + M and 0.5 Gy + M did not cause significant NPB in hTERT- cells (Figure 3.74c and Figure 3.75). The hTERT+ cells followed the same pattern (Figure 3.74d and Figure 3.75). In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was statistically significant in the control ($p<0.05$), after metal exposure to SI + M ($p<0.001$), radiation exposure to 0.5 Gy + VC ($p<0.05$), and to a higher extent, after combined exposure to 0.5 Gy + M ($p<0.01$) at *Day 30* only (Figure 3.76). These results were expressed as a percentage of NPB.

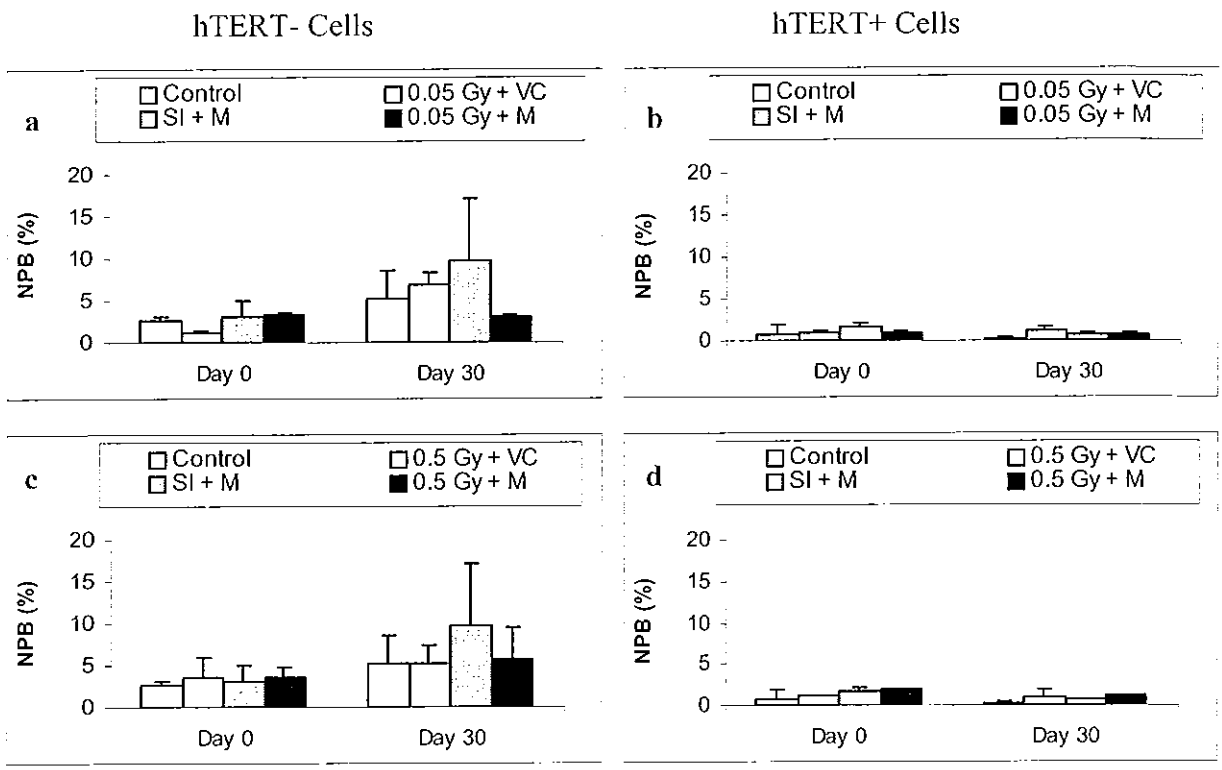

Figure 3.74. Nucleoplasmic Bridges (NPB). The figures show the percentage of NPB in hTERT– cells (Fig. 3.74a and 3.74c) and hTERT+ cells (Fig. 3.74b and 3.74d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS.

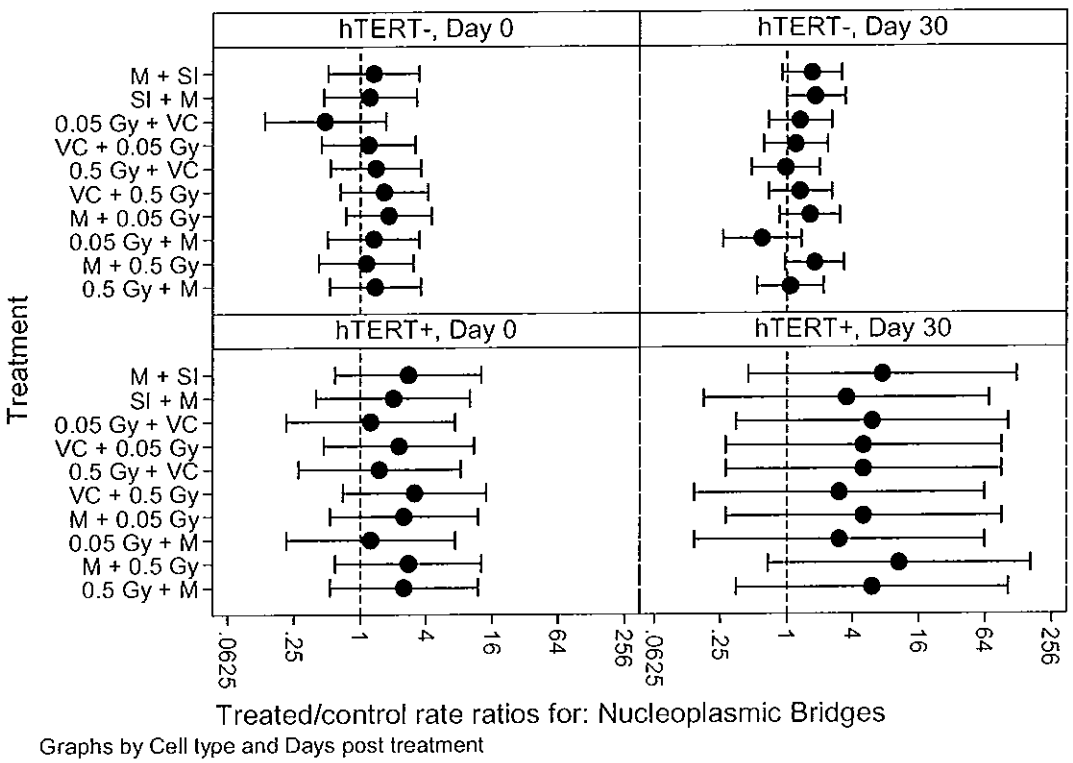

Figure 3.75. Nucleoplasmic Bridges (NPB). The figure shows the treated/control ratios of NPB rates, with 95% confidence intervals, for all the combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure.

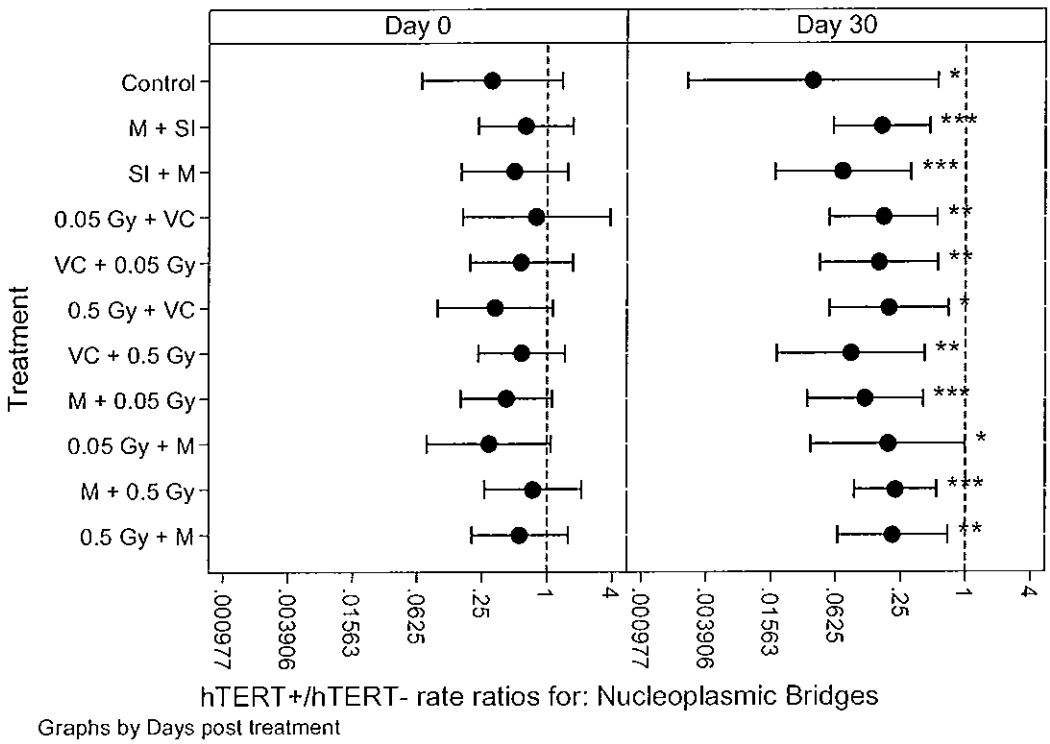

Figure 3.76. Nucleoplasmic Bridges (NPB). The figure illustrates the rate ratio of hTERT+/hTERT- for NPB, with 95% confidence intervals, showing combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure. * p<0.05, ** p<0.01, *** p<0.001 compared to hTERT+/hTERT- = 1.

3.3.2.3 Chromatid Breaks

Combined Exposure of M + 0.05 Gy

M + SI, VC + 0.05 and M + 0.05 Gy induced some chromatid breaks in hTERT- cells at both survival times, but they were not statistically significant (Figure 3.77a and Figure 3.87). The hTERT+ cells had very few breaks and only at *Day 0* (Figure 3.77b and Figure 3.87). In all of the experiments, at both survival times, with either metal treatment or radiation exposure or combined exposure, there was a lower level of breaks in the hTERT+ cells compared to the hTERT- cells. However, this was not statistically significant (Figure 3.88). These results were expressed as a percentage of chromatid breaks.

Combined Exposure of M + 0.5 Gy

M + SI (at both survival times) and M + 0.5 Gy (only at *Day 0*) induced some chromatid breaks in hTERT- cells, but they were not statistically significant (Figure 3.77c and Figure 3.87). In the hTERT+ cells, the combined exposure to M + 0.5 Gy followed the same pattern, but the breaks at *Day 0* reached a level of statistical significance ($p<0.05$). There was also a small increase in breaks, caused by VC + 0.5 Gy at *Day 30* only, which was not significant (Figure 3.77d and Figure 3.87). At both survival times, there was a lower lever of breaks after metal treatment in the hTERT+ cells compared to the hTERT- cells, whereas the level of breaks after combined exposure (M + 0.5 Gy) was slightly higher in the hTERT+ cells compared to the hTERT- cells. However, all of this data was not statistically significant (Figure 3.88). These results were expressed as a percentage of chromatid breaks.

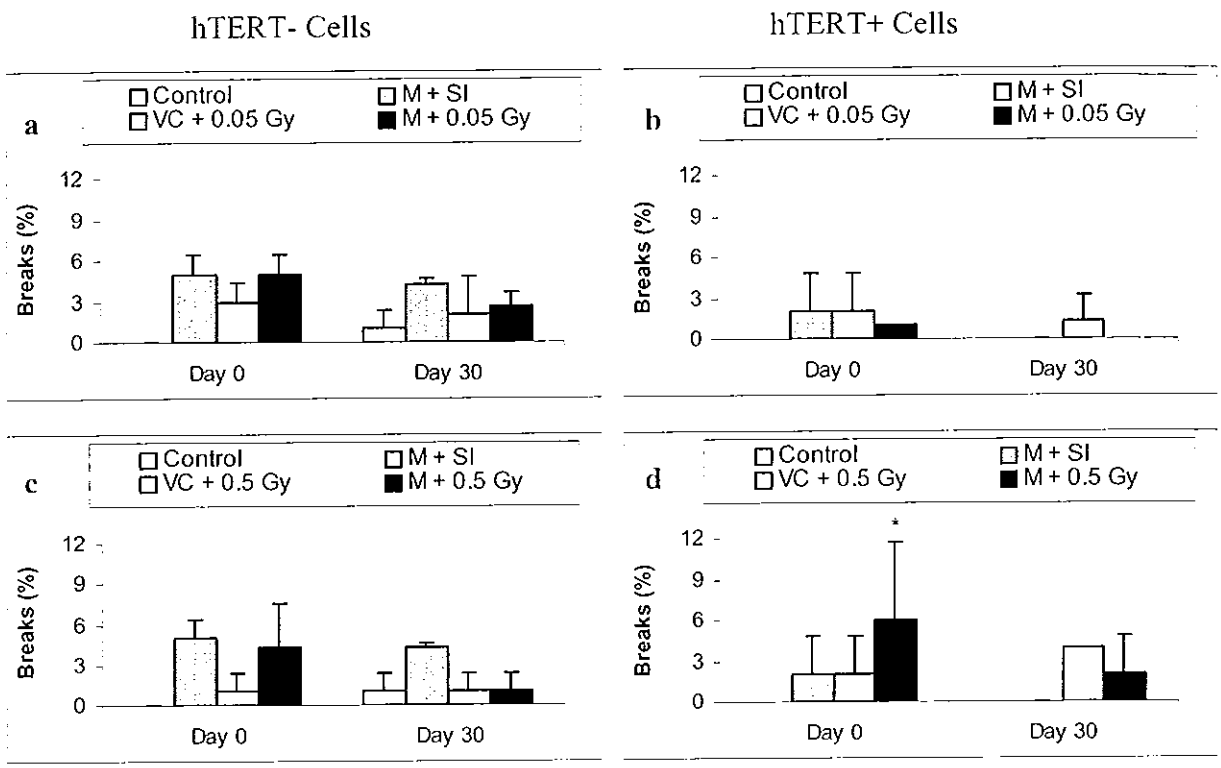

Figure 3.77. Chromatid Breaks. The figures show the percentage of chromatid breaks in hTERT– cells (Fig. 3.77a and 3.77c) and hTERT+ cells (Fig. 3.77b and 3.77d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS. * $p<0.05$ compared to the control (PBS).

Combined Exposure of 0.05 Gy + M

Metal treatment (SI + M) and combined exposure (0.05 Gy + M) induced chromatid breaks in hTERT- cells, but only 0.05 Gy + M reached a level of statistical significance ($p<0.05$) (Figure 3.78a and Figure 3.87). In the hTERT+ cells, the combined exposure to 0.5 Gy + M followed a similar pattern (Figure 3.78b and Figure 3.87). At both survival times, there was a lower level of breaks after metal treatment (SI + M) in the hTERT+ cells compared to the hTERT- cells, whereas level of breaks after combined exposure (0.5 Gy +M) was similar in both types of cells. However, all of this data was not statistically significant (Figure 3.88). These results were expressed as a percentage of chromatid breaks.

Combined Exposure of 0.5 Gy + M

SI + M and 0.5 Gy + VC induced few chromatid breaks in hTERT- cells, but they were not statistically significant (Figure 3.78c and Figure 3.87). The hTERT+ cells followed the same pattern (Figure 3.78d and Figure 3.87). At both survival times, there was a lower level of breaks, after metal treatment (SI + M) in the hTERT+ cells compared to the hTERT- cells. Furthermore, there were a higher percentage of breaks after radiation exposure (0.5 Gy + VC) in hTERT- cells, which was not present in the hTERT+ cells. However, all of this data was not statistically significant (Figure 3.88). These results were expressed as a percentage of chromatid breaks.

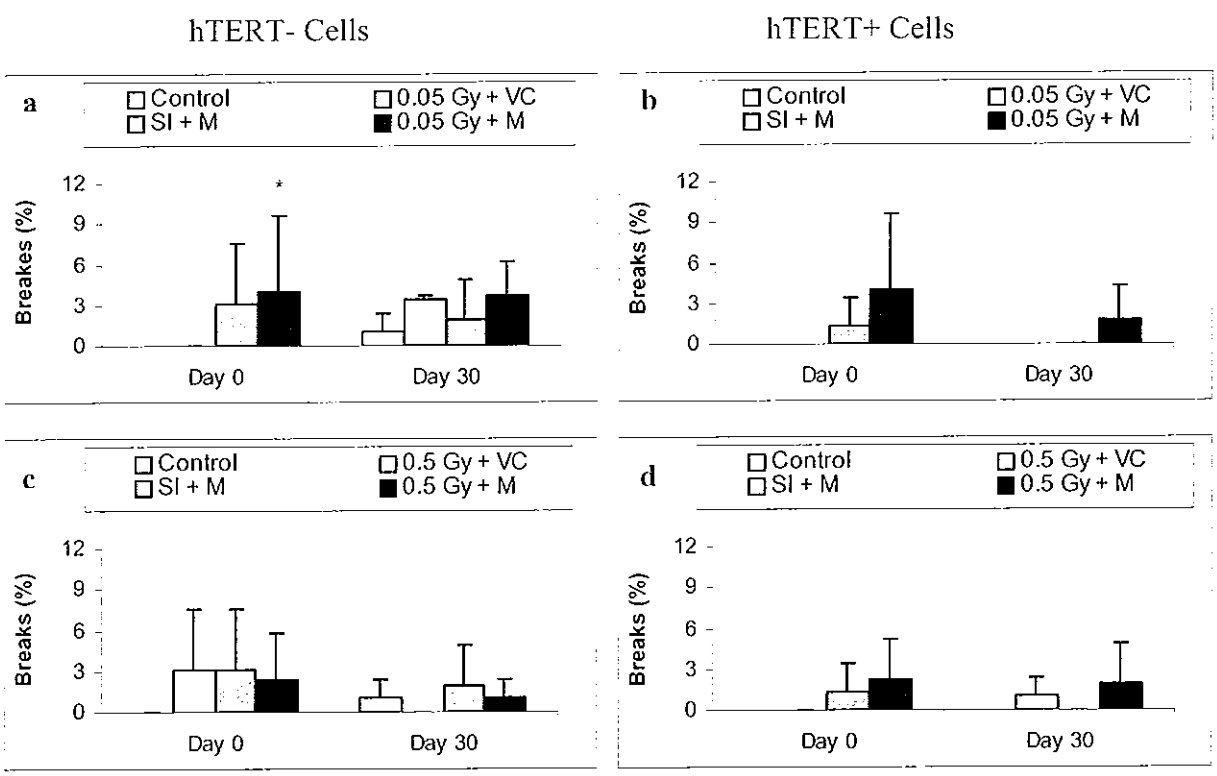

Figure 3.78. Chromatid Breaks. The figures show the percentage of chromatid breaks in hTERT– cells (Fig. 3.78a and 3.78c) and hTERT+ cells (Fig. 3.78b and 3.78d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS. * $p<0.05$ compared to the control (PBS).

3.3.2.4 Chromatid Gaps

Combined Exposure of M + 0.05 Gy

M + SI, VC + 0.05 and M + 0.05 Gy induced very few chromatid gaps in hTERT- cells, which were not significant (Figure 3.79a and Figure 3.87). The hTERT+ cells followed the same pattern (Figure 3.79b and Figure 3.87). The overall level of gaps was similar in both types of cells (Figure 3.88). These results were expressed as a percentage of chromatid gaps.

Combined Exposure of M + 0.5 Gy

M + 0.5 Gy (at both survival times) induced some chromatid gaps in hTERT- cells, but they were not statistically significant (Figure 3.79c and Figure 3.87). The hTERT+ cells had some gaps after radiation exposure (VC + 0.5 Gy) and combined exposure (M + 0.5 Gy) at *Day 30* only, but they were not significant either (Figure 3.79d and Figure 3.87). At both survival times, the level of gaps, after radiation exposure (VC + 0.5 Gy) was higher in the hTERT+ cells compared to hTERT- cells, but this was not significant (Figure 3.88). These results were expressed as a percentage of chromatid gaps.

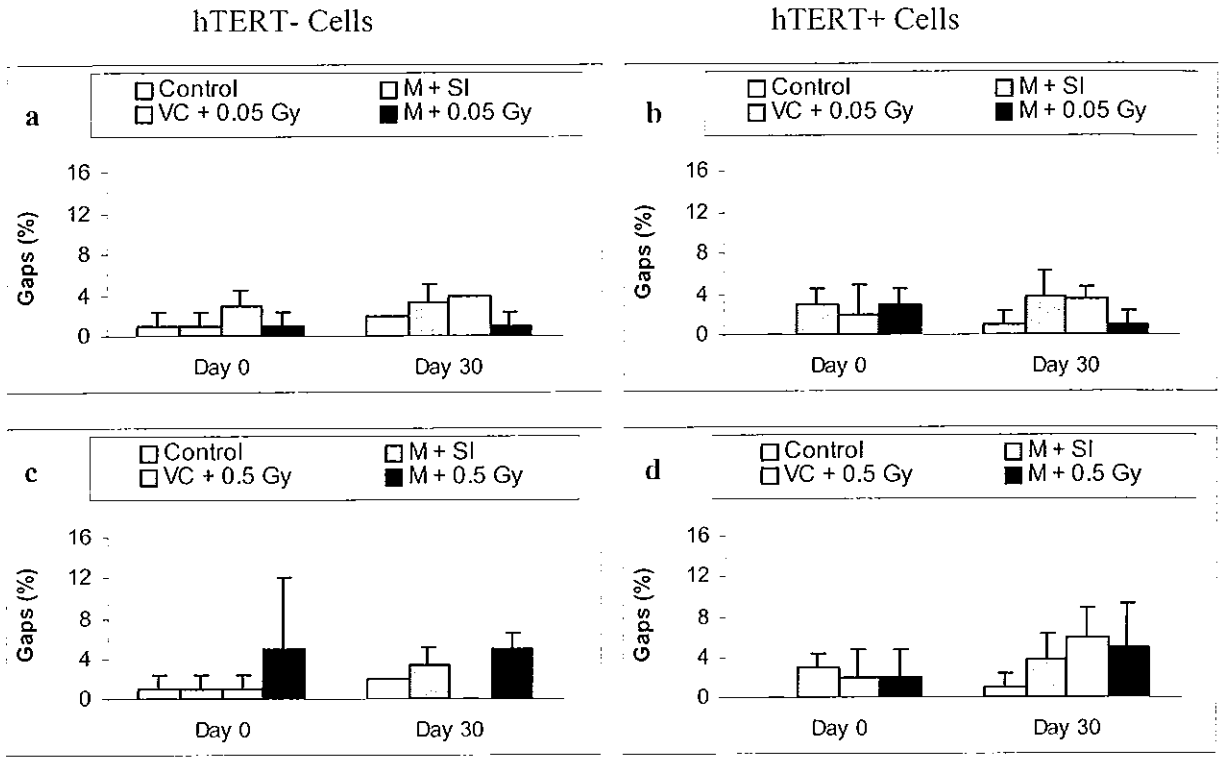

Figure 3.79. Chromatid Gaps. The figures show the percentage of chromatid gaps in hTERT– cells (Fig. 3.79a and 3.79c) and hTERT+ cells (Fig. 3.79b and 3.79d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS.

Combined Exposure of 0.05 Gy + M

0.05 Gy + M induced chromatid gaps in hTERT- cells, which decreased, but persisted, up to *Day 30*. However, this was not statistically significant (Figure 3.80a and Figure 3.87). The hTERT+ cells followed the same patter (Figure 3.80b and Figure 3.87). At both survival times, there was a lower level of gaps after combined exposure (0.05 Gy + M) in the hTERT+ cells compared to the hTERT- cells, but this had no statistical significance (Figure 3.88). These results were expressed as a percentage of chromatid gaps.

Combined Exposure of 0.5 Gy + M

SI + M, 0.5 Gy + VC and 0.5 Gy + M caused very few chromatid gaps in hTERT- cells (Figure 3.80c and Figure 3.87). The hTERT+ cells followed the same pattern (Figure 3.80d and Figure

3.87). At both survival times, the overall levels of gaps, was similar in both types of cells (Figure 3.88). These results were expressed as a percentage of chromatid gaps.

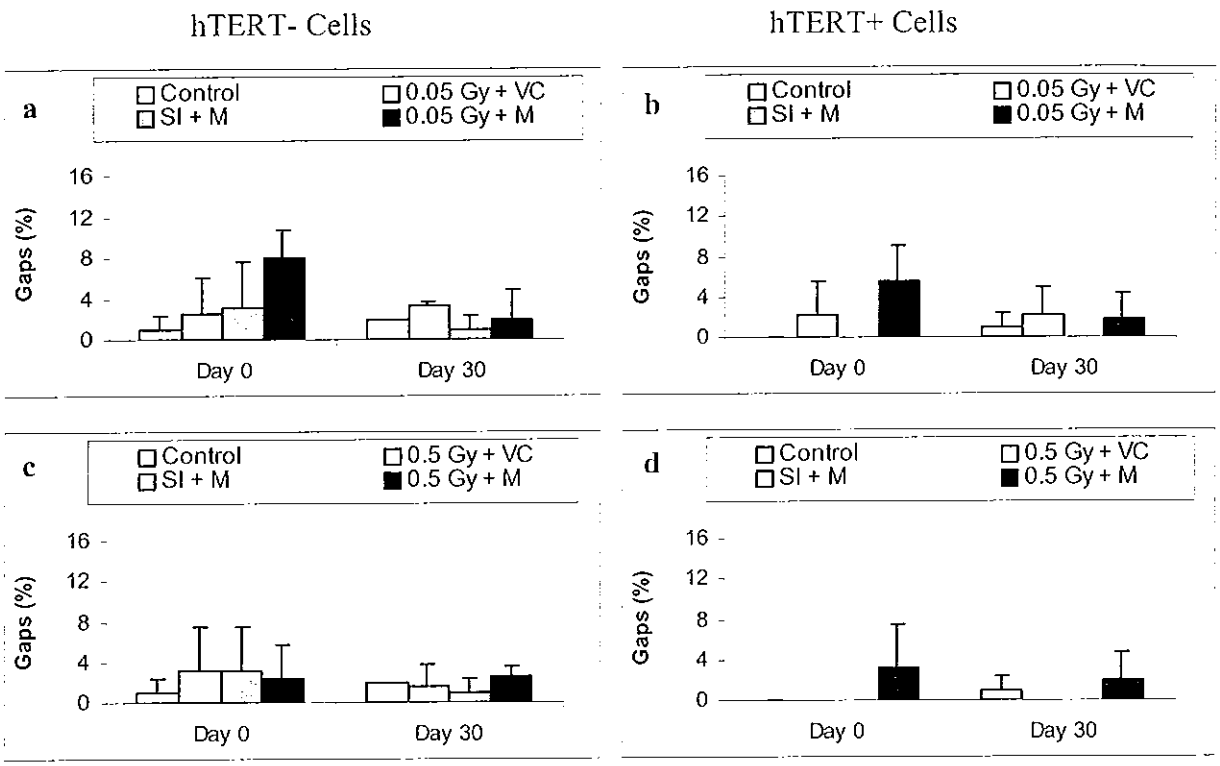

Figure 3.80. Chromatid Gaps. The figures show the percentage of chromatid gaps in hTERT– cells (Fig. 3.80a and 3.80c) and hTERT+ cells (Fig. 3.80b and 3.80d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS.

3.3.2.5 Chromatid Fragments

Combined Exposure of M + 0.05 Gy

M + SI, induced very few chromatid fragments up to 30 days after metal treatment in hTERT- cells, but they were not significant. M + 0.05 Gy caused very few fragments (Figure 3.81a and Figure 3.87). The hTERT+ cells had no significant fragments (Figure 3.81b and Figure 3.87). Therefore, at both survival times, the level of fragments, after either metal treatment, radiation exposure or combined exposure was lower in hTERT+ cells compared to hTERT- cells.

However, all of this data was not significant (Figure 3.88). These results were expressed as a percentage of chromatid fragmens.

Combined Exposure of M + 0.5 Gy

M + SI, induced very few chromatid fragments up to 30 days after metal treatment in hTERT- cells, but they were not significant. M + 0.5 Gy caused some fragments immediately after the combined exposure (*Day 0*), which were not significant either (Figure 3.81c and Figure 3.87). In the hTERT+ cells, VC + 0.5 Gy caused a small induction of fragments only at *Day 0*, but it was not significant (Figure 3.81d and Figure 3.87). At both survival times, the level of fragments, after either metal treatment or combined exposure, was lower in hTERT+ cells compared to hTERT- cells. However, all of this data was not significant (Figure 3.88). These results were expressed as a percentage of chromatid fragmens.

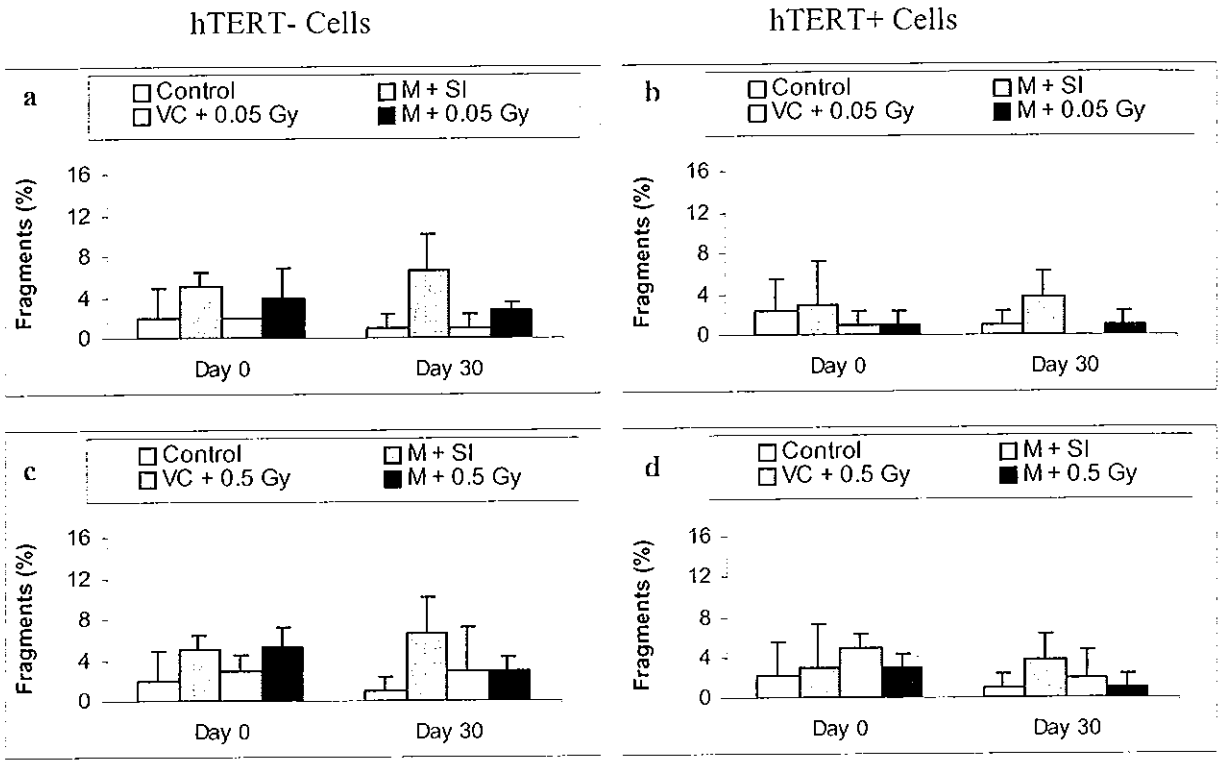

Figure 3.81. Chromatid Fragments. The figures show the percentage of chromatid fragments in hTERT– cells (Fig. 3.81a and 3.81c) and hTERT+ cells (Fig. 3.81b and 3.81d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS.

Combined Exposure of 0.05 Gy + M

Only metal treatment (SI + M) induced chromatid fragments, which reached statistical significance at *Day 30* ($p<0.05$) in hTERT- cells (Figure 3.82a and Figure 3.87). The hTERT+ cells had some fragments immediately after (*Day 0*) the metal treatment (SI + M) and the combined exposure (0.05 Gy + M), but they were not statistically significant (Figure 3.82b and Figure 3.87). At both survival times, after combined exposure (0.05 Gy + M), there was a slightly higher level of fragments in hTERT+ cells compared to hTERT- cells. However, all of this data was not significant (Figure 3.88). These results were expressed as a percentage of chromatid fragments.

Combined Exposure of 0.5 Gy + M

Only metal treatment (SI + M) induced chromatid fragments, which reached statistical significance at *Day 30* ($p<0.05$) in hTERT- cells (Figure 3.82c and Figure 3.87). The hTERT+ cells had some fragments immediately after (*Day 0*) the radiation exposure (0.5 Gy + VC), the metal treatment (SI + M) and the combined exposure (0.5 Gy + M), but they were not statistically significant (Figure 3.82d and Figure 3.87). At both survival times, after radiation exposure (0.5 Gy + VC) and combined exposure (0.5 Gy + M), there was a slightly higher level of fragments in hTERT+ cells compared to hTERT- cells. However, all of this data was not significant (Figure 3.88). These results were expressed as a percentage of chromatid fragmens.

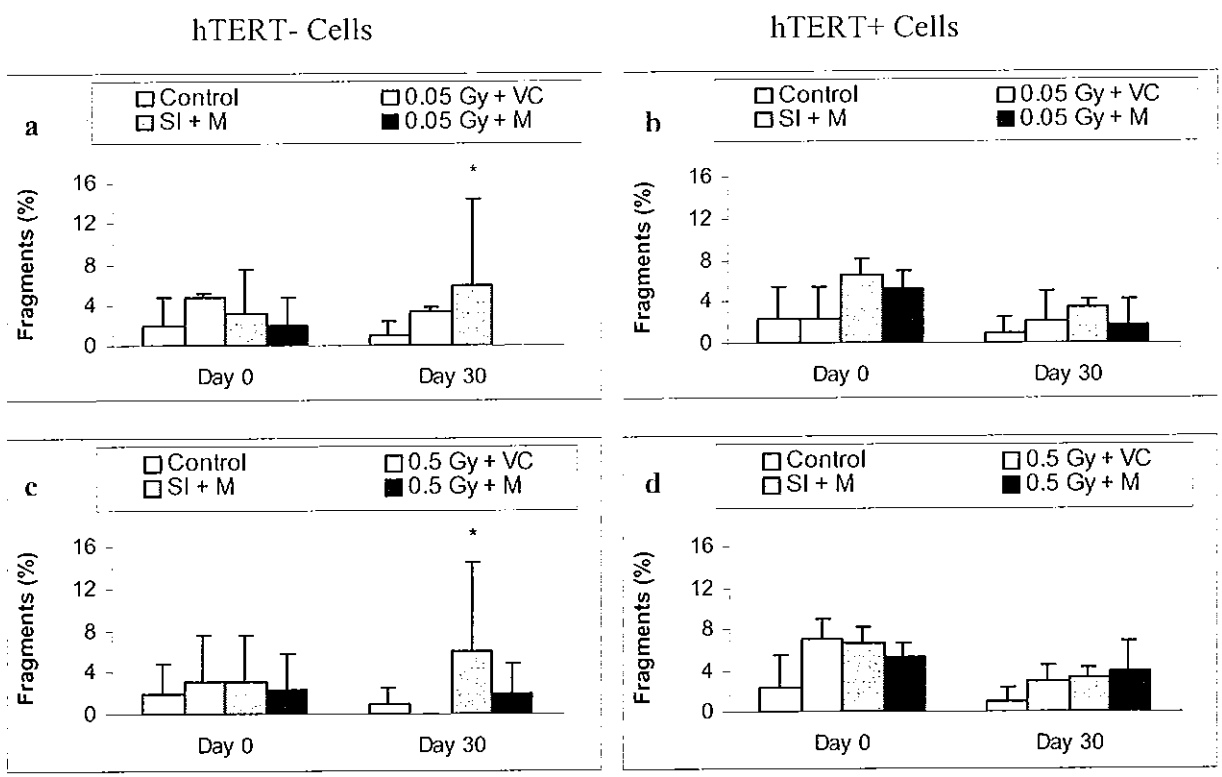

Figure 3.82. Chromatid Fragments. The figures show the percentage of chromatid fragments in hTERT– cells (Fig. 3.82a and 3.82c) and hTERT+ cells (Fig. 3.82b and 3.82d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS. * $p<0.05$ compared to the control (PBS).

3.3.2.6 Dicentric Chromosomes

Combined Exposure of M + 0.05 Gy

M + SI caused some dicentric chromosomes up to 30 days after metal exposure in hTERT- cells, but they were not statistically significant (Figure 3.83a and Figure 3.87). In contrast, there was very little induction of dicentrics in hTERT+ cells (Figure 3.83b and Figure 3.87). In all of the experiments, at both survival times, with or without either metal treatment (M + SI) or radiation exposure (VC + 0.05 Gy) or combined exposure (M + 0.05 Gy), there was a lower level of dicentrics in the hTERT+ cells compared to the hTERT- cells. However, all of this data was not statistically significant (Figure 3.88). These results were expressed as a percentage of dicentric chromosomes.

Combined Exposure of M + 0.5 Gy

Metal treatment (M + SI), radiation exposure (VC + 0.5 Gy) and combined exposure (M + 0.5 Gy) induced dicentric chromosomes up to 30 days in hTERT- cells, although this data was not statistically significant (Figure 3.83c and Figure 3.87). In contrast, there was no induction of dicentrics in hTERT+ cells (Figure 3.83d and Figure 3.87). In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of dicentrics in the hTERT+ cells compared to the hTERT- cells (Figure 3.88). A direct comparison of hTERT- and hTERT+ cells revealed that the excess of dicentrics in hTERT- cells after combined exposure (M + 0.5 Gy) was statistically significant, at both survival times ($p<0.05$), compared to that in the hTERT+ cells (Figure 3.95 and Figure 3.96). Furthermore, the level of dicentrics after radiation exposure (VC + 0.5 Gy) was significantly higher ($p<0.05$) at *Day 30* in hTERT- cells compared to hTERT+ cells (Figure 3.88). These results were expressed as a percentage of dicentric chromosomes.

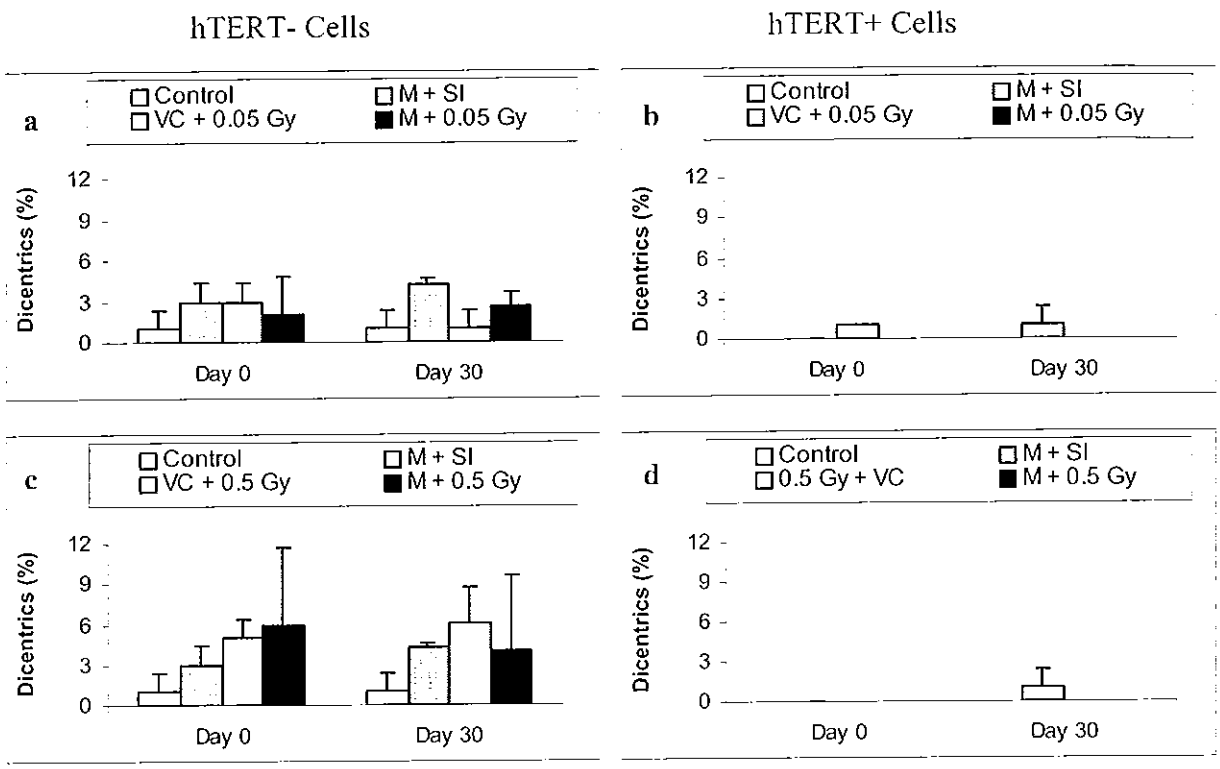

Figure 3.83. Dicentric Chromosomes. The figures show the percentage of dicentric chromosomes in hTERT– cells (Fig. 3.83a and 3.83c) and hTERT+ cells (Fig. 3.83b and 3.83d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS.

Combined Exposure of 0.05 Gy + M

Metal treatment (SI + M), at both survival times, and combined exposure (0.05 Gy + M), only at *Day 30*, induced some dicentric chromosomes, which were not statistically significant (Figure 3.84a and Figure 3.87). Generally, there were very few dicentrics in hTERT+ cells (Figure 3.84b and Figure 3.87). A direct comparison of hTERT- and hTERT+ cells showed that the excess of dicentrics in hTERT- cells after metal treatment (SI + M) was statistically significant, at *Day 30* ($p<0.05$), compared to that in the hTERT+ cells (Figure 3.88). These results were expressed as a percentage of dicentric chromosomes.

Combined Exposure of 0.5 Gy + M

Metal treatment (SI + M), at both survival times, and combined exposure (0.5 Gy + M), only at *Day 30*, caused a slight increase of dicentric chromosomes, but they were not significant (Figure 3.84c and Figure 3.87). In contrast, there were very few dicentric chromosomes in hTERT+ cells (Figure 3.84d and Figure 3.87). A direct comparison of hTERT- and hTERT+ cells revealed that the excess of dicentrics in hTERT- cells after metal treatment (SI + M) was statistically significant, at *Day 30* ($p<0.05$), compared to that in the hTERT+ cells (Figure 3.88). In all of the experiments, at both survival times, with or without either radiation exposure (0.5 Gy + VC) or combined exposure (0.5 Gy + M), there was a lower level of dicentrics in the hTERT+ cells compared to the hTERT- cells, but this data was not significant (Figure 3.88). These results were expressed as a percentage of dicentric chromosomes.

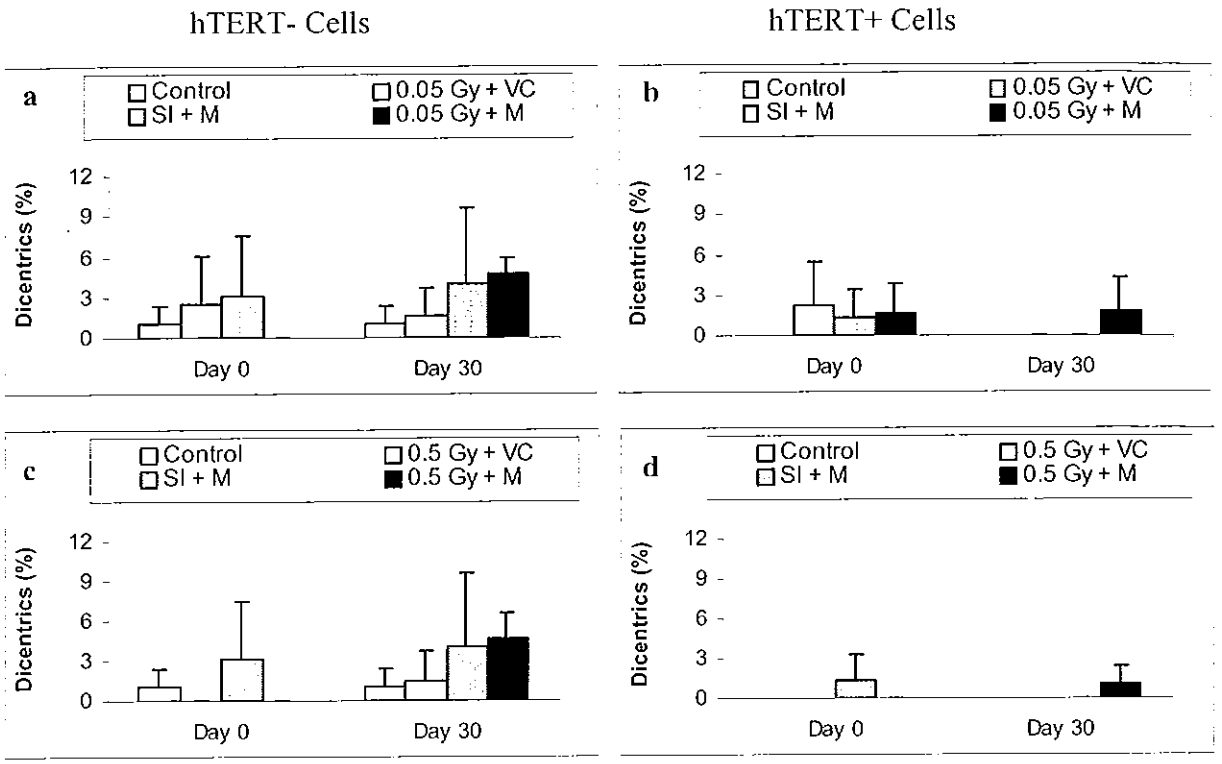

Figure 3.84. Dicentric Chromosomes. The figures show the percentage of dicentric chromosomes in hTERT– cells (Fig. 3.84a and 3.84c) and hTERT+ cells (Fig. 3.84b and 3.84d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS.

3.3.2.7 Tetraploidy

Combined Exposure of M + 0.05 Gy

M + SI, VC + 0.05 Gy and + M + 0.05 Gy did not cause a significant increase in tetraploidy in hTERT- cells (Figure 3.85a and Figure 3.87). The hTERT+ cells followed the same pattern (Figure 3.85b and Figure 3.87). However, a direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was more tetraploidy in the hTERT+ cells compared to hTERT- cells. However, the only significant data was seen immediately after (*Day 0*) the radiation exposure (VC + 0.05 Gy) ($p<0.05$) (Figure 3.88). These results were expressed as a percentage of tetraploid cells.

Combined Exposure of M + 0.5 Gy

M + SI, VC + 0.5 Gy and + M + 0.5 Gy did not induce a significant increase of tetraploidy in hTERT- cells (Figure 3.85c and Figure 3.87). The hTERT+ cells followed the same pattern, although the level of tetraploidy immediately after (*Day 0*) the combined exposure (M + 0.5 Gy) was higher than control (Figure 3.85d and Figure 3.87). In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a higher incidence of tetraploidy in hTERT+ cells compared to hTERT- cells. However, this data was not statistically significant (Figure 3.88). These results were expressed as a percentage of tetraploid cells.

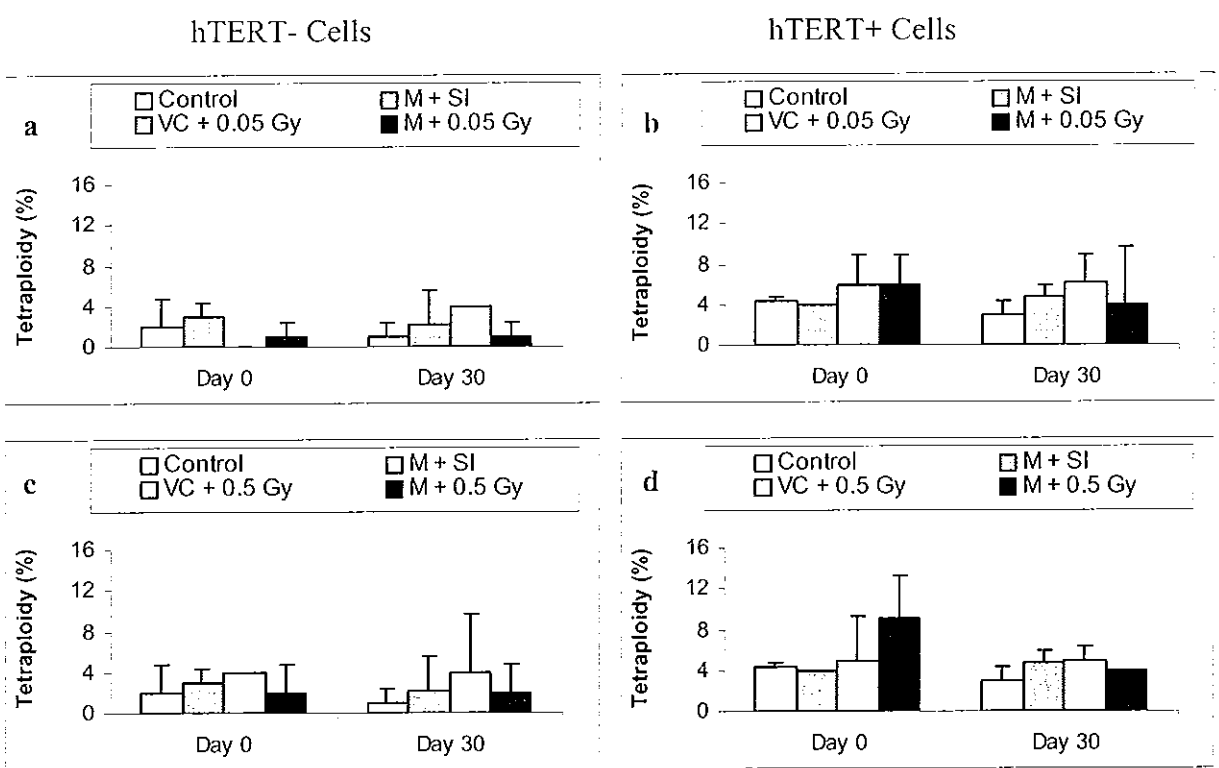

Figure 3.85. Tetraploidy. The figures show the percentage of tetraploidy in hTERT– cells (Fig. 3.85a and 3.85c) and hTERT+ cells (Fig. 3.85b and 3.85d) after a single exposure to Metal + Sham Irradiation (M + SI), either Vehicle Control + 0.05 Gy (VC + 0.05 Gy) or Vehicle Control + 0.5 Gy (VC + 0.5 Gy) and either a combined exposure to Metal + 0.05 Gy (M + 0.05 Gy) or Metal + 0.5 Gy (M + 0.5 Gy), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS.

Combined Exposure of 0.05 Gy + M

SI + M, 0.05 Gy + VC and 0.05 Gy + M did not determine a significant increase of tetraploidy in hTERT- cells (Figure 3.86a and Figure 3.87). The hTERT+ cells followed the same pattern, although the level of tetraploidy 30 days after the radiation exposure (0.05 Gy + VC) was higher than control (Figure 3.86b and Figure 3.87). In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a higher level of tetraploidy in hTERT+ cells compared to hTERT- cells. However, this data was not statistically significant (Figure 3.88). These results were expressed as a percentage of tetraploid cells.

Combined Exposure of 0.5 Gy + M

SI + M, 0.5 Gy + VC and 0.5 Gy + M did not result in a significant increase of tetraploidy in hTERT- cells (Figure 3.86c and Figure 3.87). The hTERT+ cells followed the same pattern, although the level of tetraploidy, after the combined exposure (0.5 Gy + M) was higher than the control, at both survival times (Figure 3.86d and Figure 3.87). In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a higher level of tetraploidy in hTERT+ cells compared to hTERT- cells. However, the only significant data was observed 30 days after combined exposure (0.5 Gy + M) ($p<0.01$) (Figure 3.88). These results were expressed as a percentage of tetraploid cells.

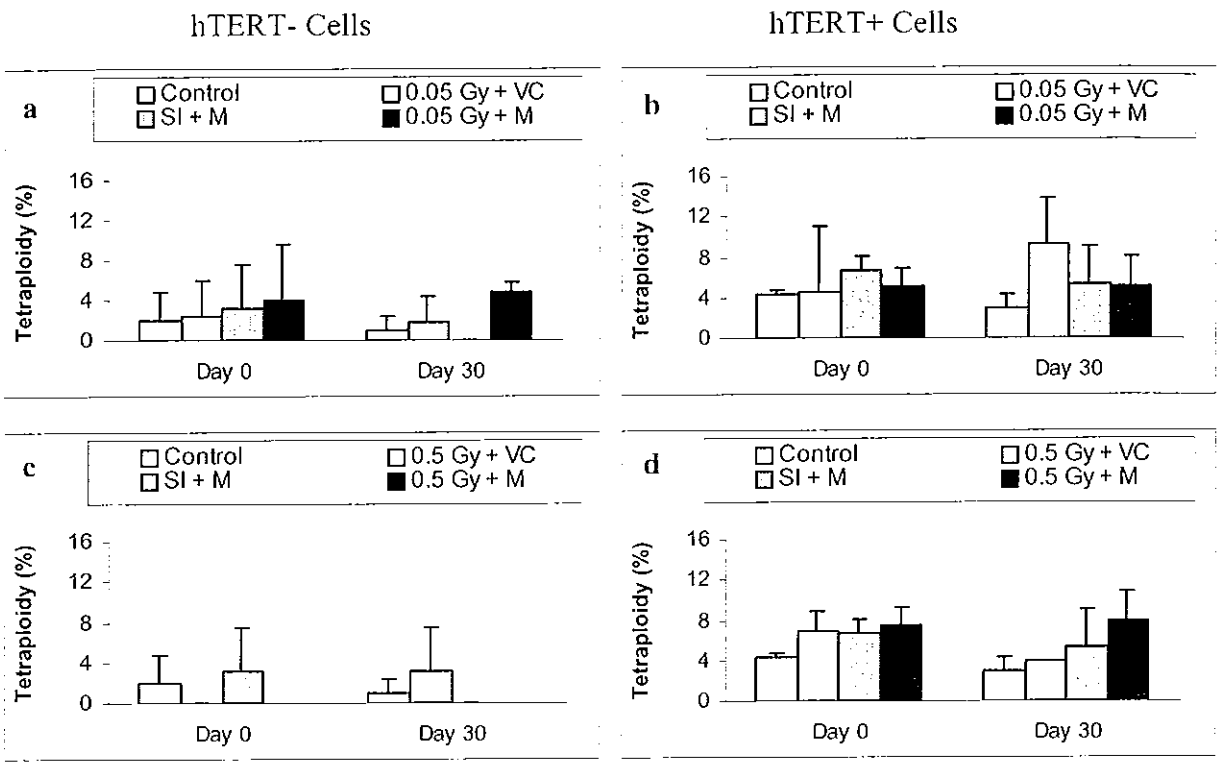

Figure 3.86. Tetraploidy. The figures show the percentage of tetraploidy in hTERT– cells (Fig. 3.86a and 3.86c) and hTERT+ cells (Fig. 3.86b and 3.86d) after a single exposure to either 0.05 Gy + Vehicle Control (0.05 Gy + VC) or 0.5 Gy + Vehicle Control (0.5 Gy + VC), Sham Irradiation + Metal (SI + M), and either a combined exposure to 0.05 Gy + Metal (0.05 Gy + M) or 0.5 Gy + Metal (0.5 Gy + M), and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is at room temperature and Vehicle Control (VC) is PBS.

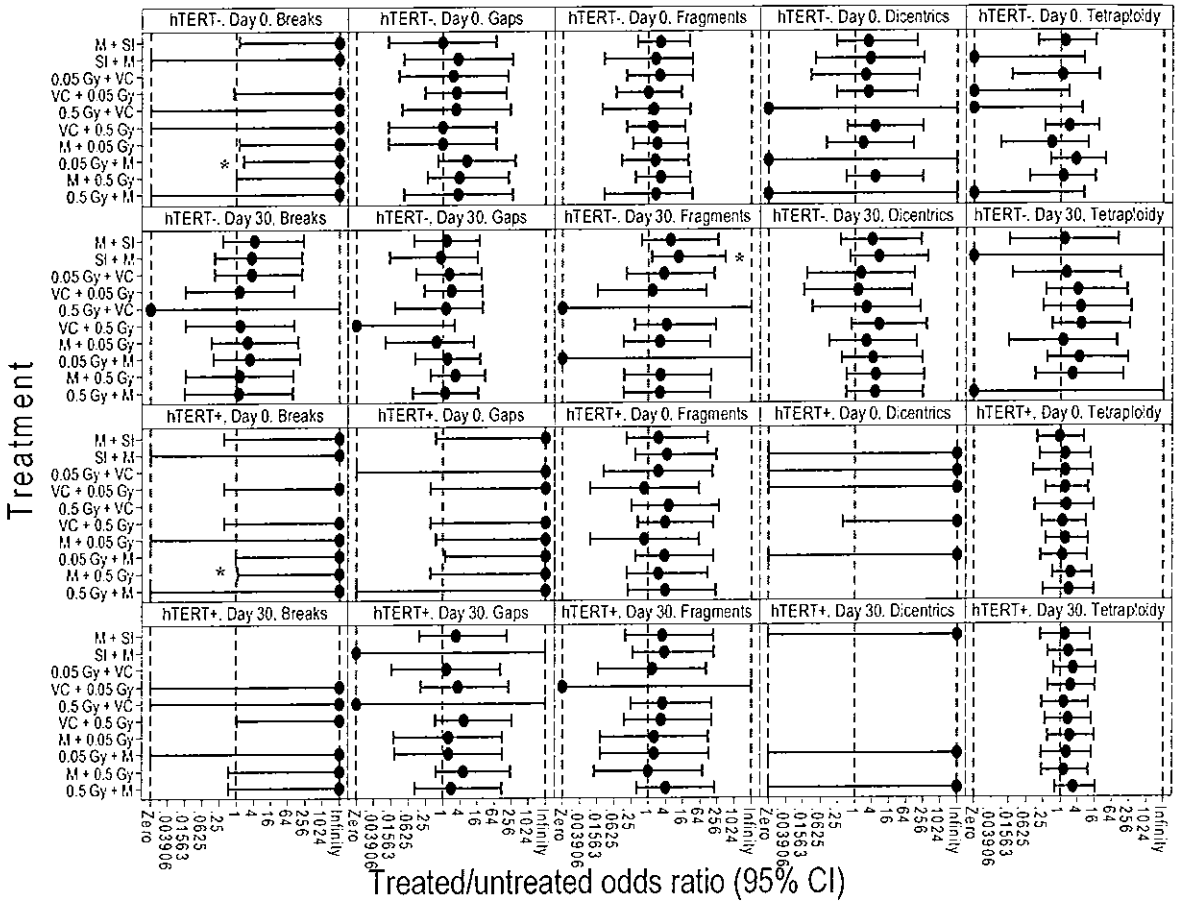

Figure 3.87. Breaks, Gaps, Fragments, Dicentrics and Tetraploidy. The figure shows the treated/control ratios of breaks, Gaps, Fragments, Dicentrics and Tetraploidy rates, with 95% confidence intervals, for all the combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure. * p<0.05 compared to the control (PBS).

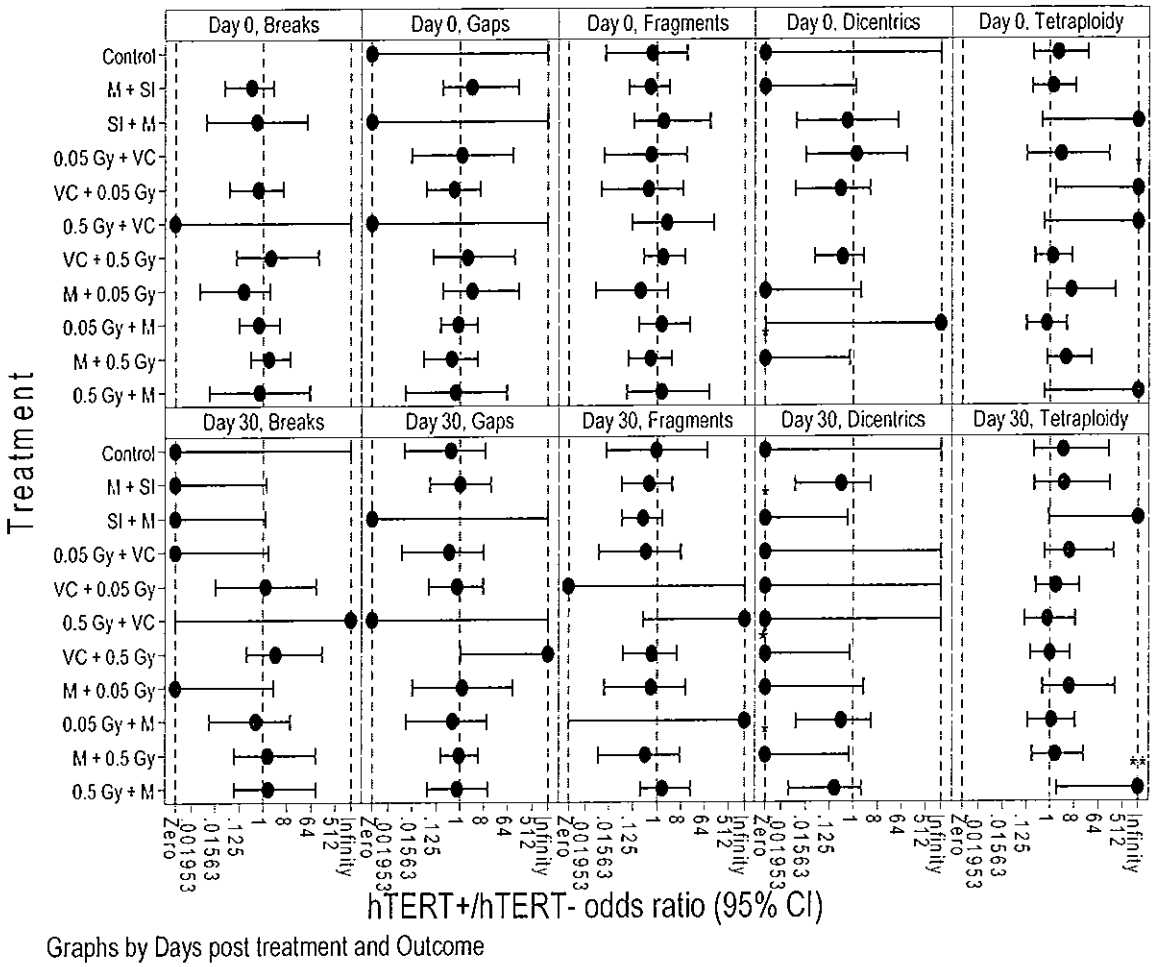

Figure 3.88. Breaks, Gaps, Fragments, Dicentrics and Tetraploidy. The figure illustrates the rate ratio of hTERT+/hTERT- for Breaks, Gaps, Fragments, Dicentrics and Tetraploidy, with 95% confidence intervals, showing combinations of exposure (metal only, radiation only and combined) and days (*Day 0* and *Day 30*) post exposure. * $p<0.05$, ** $p<0.01$ compared to hTERT+/hTERT- = 1.

CHAPTER 4

DISCUSSION

4.1 Metal Exposure

4.2 Radiation Exposure

4.3 Combined Exposure

4.1 Metal Exposure

4.1.1 General Comment

This work has provided direct evidence that Cr (VI) and V (V) are capable of inducing characteristics in progeny cells that are associated with metal ion-induced genomic instability. Previous work confirmed that a wide range of chemicals could reduce the reproductive integrity of progeny cells for several generations (Mothersill *et al* 1998, Coen *et al* 2001). In this work, evidence was provided not only of persistent reduction of cell survival (cell viability and clonogenic survival), but of increased frequency of a wide range of cell damage (cytogenetic abnormalities) such as chromatid breaks, micronuclei, dicentric chromosomes and nucleoplasmic bridges in progeny. In this present work, two other important features, telomerase activity and telomere length, were not found to be remarkably altered either by Cr (VI) or V (V).

4.1.1.1 Cell Survival

The results of cell viability showed that 4 μM Cr (VI) and 50 μM V (V) concentrations were toxic for both types of cells. However, this assay demonstrated that lower concentrations, for both chemicals, caused an increase of dead hTERT- cells in a dose dependent manner up to 30 days after exposure. The increase was most pronounced immediately after exposure (*Day 0*) and then decreased thereafter (*Day 5* and *Day 30*). There was a loss of cell viability in hTERT+ cells but this was significantly less than in hTERT- cells, especially with the highest dose of Cr (VI) (4 μM) and V (V) (50 μM). This difference was most pronounced at *Day 0* and diminished thereafter.

The hTERT+ cells grew faster than the hTERT- cells when cultured routinely at 70% confluency and there was a very slight reduction of growth in control hTERT- cells by *Day 30*.

Control hTERT- cells completed their doubling time (or cell cycle doubling time) in 24.42 hours at *Day 0* and in 28.60 hours at *Day 30*. Due to a much higher cell proliferation, control hTERT+ cells completed their cell cycle in 18.71 hours at *Day 0* and 19.29 hours at *Day 30*. The effect of the highest dose of Cr (VI) (4 μM) and V (V) (50 μM) did slow down the growth of both types of cells, but were remarkably less toxic in hTERT+ cells. The results of the clonogenic assay showed that 4 μM Cr (VI) and 50 μM V (V) concentrations were toxic for both types of cells and consequently there were few colonies detectable. However, this assay demonstrated that lower concentrations, for both chemicals, caused in hTERT- cells a decrease in colony formation from *Day 0* onwards. In contrast, hTERT+ cells showed no significant loss of clonogenic survival with either metal treatment and at any post exposure times studied. The results of the senescence partially explained the persistent loss of clonogenic survival in hTERT- cells. In fact, there was a progressive and dose dependent increase of β-galactosidase stained cells up to 30 days after metal treatment of hTERT- cells. No increase was observed in the hTERT+ cells. The apoptosis assay, as well as senescence, further explained the persistence of the reduction in clonogenicity in hTERT- cells. After metal exposure there was a persistent and dose dependent induction of apoptosis up to 30 days in hTERT- cells. No increase was seen in metal exposed hTERT+ cells. Surprisingly, the control level of apoptosis was higher in hTERT+ cells, compared to hTERT- cells.

4.1.1.2 Cell Damage

The micronucleus assay showed that hTERT- cells, for both chemicals, were more vulnerable to damage such as micronuclei and nucleoplasmic bridges, immediately after metal exposure. The initial damage of micronuclei (MNi) observed in hTERT- gradually increased with both metals up to 30 days after the treatment, whereas hTERT+ cells had very few MNi both initially and 30 days after metal treatment. The results of nucleoplasmic bridges (NPB) showed

that both metals increased the percentage of NPB in hTERT- cells immediately after both metal exposure, and this further increased up to 30 days, after metal exposure to both metals. hTERT+ cells behaved completely differently, since there were very few NPB at any post exposure times studied. The results of the chromosomal aberration analysis showed that hTERT- cells were more susceptible to genetic damage such as breaks, dicentrics and aneuploidy, compared to hTERT+ cells, which were more resistant.

Polyploidy (tetraploidy) followed a different pattern from the other aberrations described above. Tetraploid cells were observed, with both chemicals, immediately after metal exposure in hTERT- cells. This genetic damage persisted but did not increase 30 days after metal exposure. The behaviour of hTERT+ was different, since the initial damage after metal exposure was followed by a gradual increase in tetraploidy up to 30 days after metal treatment. This was reflected by an approximate five fold increase with the higher dose of Cr (VI) and a three fold increase with the higher dose of V (V) used in hTERT+ cells (compared to hTERT- cells), 30 days after metal exposure.

4.1.1.3 Cell Biology

The level of telomerase activity (total product generated), carried out with the TRAP assay, was much greater, as expected, in hTERT+ cells compared to the hTERT – cells. Interestingly, V (V) treatment caused a slight reduction of the telomerase activity in hTERT+ cells, which was particularly true immediately after metal exposure. Telomere length (telomere fluorescent intensity) in hTERT- cells was generally shorter than hTERT+ cells and this difference became more pronounced 30 days after metal exposure. The telomere lengths of untreated hTERT- cells and hTERT+ cells did not vary over the time course of this experiment. Telomere lengths were always slightly longer in control (untreated) hTERT+ cells compared to control (untreated) hTERT- cells. Furthermore, telomere lengths were generally slightly shorter after

metal treatment, either with Cr (VI) or V (V), compared to the control, but neither of these results were statistically significant. Despite this, the telomere lengths in metal treated hTERT+ cells were significantly longer (approximately two fold) compared to metal treated hTERT- cells, 30 days after the 24-hour exposure.

Analysis of the XpYp telomere, performed with STELA, confirmed that hTERT+ cells had longer telomeres compared to hTERT- cells. However, this was not due to an overall increase in all telomere lengths, but it was because the hTERT- cells had a neat bimodal allelic-like distribution of lengths, 0-3 Kb and 3-11 Kb, that was lacking in the hTERT+ cells, which have only the larger fraction 3-11 Kb. Telomere lengths on average were shorter, as expected, with increasing time. Both apoptosis and necrosis, were higher in hTERT- cells compared to hTERT+ cells, especially immediately after metal exposure. The results of the cell cycle distribution, showed that hTERT+ cells had a higher proportion of proliferating cells (S, G2/M) immediately after metal exposure compared to hTERT- cells. Interestingly there was a marked difference in the initial distribution of the cell cycle (*Day 0*) of V (V)-exposed hTERT- and hTERT+ cells. An initial increase of G0/G1 phase (83%) was observed in hTERT- cells, which did not persist till *Day 30*, whereas the hTERT+ cells had a much smaller percentage (63%) of cells in G0/G1 phase immediately after (*Day 0*) V (V) exposure.

The overall results clearly demonstrated that genomic instability was more pronounced in hTERT- cells. The only exception was the presence of tetraploidy, for which the hTERT+ cells were more susceptible. It was also interesting to observe that control hTERT+ cells had a higher percentage of apoptotic cells (in the colonies) compared to hTERT- cells. The overall results implied that insertion of the retrovirus carrying a cDNA encoding for the telomerase protein should have prevented damage in the hTERT+ cells. The much higher activity of telomerase enzyme in the hTERT+ cells was also reflected by the gradual increasing in

telomere length in these cells from *Day 0* onward. In contrast, hTERT- cells did not show any increase of telomere length from *Day 0* to *Day 30*.

4.1.2 Different Types of Genomic Instability

This study has shown that short term exposures of low doses of Cr (VI) and V (V) causes four types of long term genomic instability in human fibroblasts: 1) a persistent reduction in clonogenic survival occurred in hTERT- cells; 2) a persistent induction of chromosome end joining/fusion occurring in hTERT- cells; 3) a persistent incidence of chromosomal breakage occurring in hTERT- cells; 4) a higher percentage of polyploidy (tetraploidy) observed in hTERT+ cells.

4.1.2.1 Persistent Reduction of Clonogenic Survival

The first type of genomic instability was related to the persistent reduction of clonogenic survival, which occurred in hTERT- cells. The clonogenic assay demonstrated that both metals induced aberrations in surviving progeny that were lethal and therefore could not have been carried by the surviving cells. Non-clonality is a feature of this type of instability. The fact that this instability is non-clonal and lethal suggests that it is not due to a defect sustained by the cells at the time of the initial insult, as the defect should manifest in all clonal progenies of the affected cells, and not in any of the progeny of cells that did not sustain the damage (Mothersill and Seymour 1998). It is unlikely that residual chemical in the cells could account for the delayed loss of clonogenic survival, because in that case it would be predicted that the reduction in clonogenicity would reduce with time. In contrast, there was more clonogenic survival in the initially exposed hTERT- cells compared to 30 days after metal treatment. It has been observed that loss of epiregulin, an EGF growth factor, can lead to reduction of clonogenic survival (Lindvall *et al* 2003). Therefore, it may be possible that an upregulation of

this gene is responsible for the lack of reduction in colony formation in the metal exposed hTERT+ cells. Rubio *et al* (2004) have suggested that hTERT is only protective, for clonogenic survival, in cells with short telomeres, presumably near-dysfunctional. This is in line with the present study, since BJ human fibroblasts had been infected (with a retrovirus carrying a cDNA encoding hTERT) when they were quite old (PD approximately 50), and telomeres were already short. It may be possible that dysfunctional telomeres, in treated hTERT- cells, engaged cellular tumor suppressor mechanisms, which caused cells to permanently arrest growth and so became senescent, or died, via apoptosis (Kim *et al* 2002). In addition, it is known that telomeres (in hTERT- cells) can act as sensors of certain types of DNA damage, triggering senescence or apoptosis when the damage exceeds a particular threshold (Von Zglinicki 2002, Stewart and Weinberg 2002). According to other authors, telomerase enzyme can diminish the sensitivity to DNA damage, and thus reduce the senescence or apoptotic responses in hTERT+ cells (Lu *et al* 2001, Holt *et al* 1999, Gorbunova *et al* 2002). There is also general agreement that telomerase does not protect normal human cells with relatively long telomeres from ionizing radiation-induced senescence (Rubio *et al* 2002).

The reduction of clonogenic survival up to *Day 30*, after the metal treatment in hTERT- cells, can be partly explained by a delayed induction of senescence, observed only at *Day 30* after metal treatment within the colonies, and a persistent induction of apoptosis, observed from *Day 0* onwards. The increased background apoptosis in the colonies of untreated hTERT+ cells, compared to hTERT- cells, was also anomalous, but it was reminiscent of the high levels of apoptosis in the premalignant skin tumour Bowen's disease, which is also known to be hTERT positive (Park *et al* 2004) as well as present in many solid tumours. The lack of increased apoptosis (due to either metals) and reduced loss of cell viability in the hTERT+ cells after metal treatment, emphasises the link between cell death and telomerase state, which has been

noted in tumours during the development of drug resistance to chemotherapy including metal (cisplatin) (Mese *et al* 2001).

Interestingly, the results of this work showed that the growth of metal treated cells differed depending on whether they were plated out as colonies (as it was done in the clonogenic assay, senescence assay and apoptosis assay) or grown routinely at subconfluent levels (as it was done for all the other experiments). As already mentioned, the cells which were plated to form colonies, gave rise to a permanent reduction in clonogenic survival in hTERT- treated cells, which was partially explained by the metal-induced senescence and metal-induced apoptosis. In contrast, the lack of changes in doubling time after low dose of metal treatment (0.04 μM and 0.4 μM Cr (VI) or 0.5 μM and 5 μM V (V)) in cells grown at subconfluent levels, can also be related by only a small and temporary induction of apoptosis (determined by flow cytometry), and by only temporary changes in the proportion of these cells in different phases of their cell cycle, including G0/G1. These results demonstrated that there were different but consistent growth behaviour, in hTERT- cells, if they were plated (2000 cells) to form colonies in $25cm^2$ flasks or routinely subcultured (every three days) and kept subconfluent (approximately 70% confluence) in the $75cm^2$ flasks. It might be possible that some apoptotic cells were lost during the harvesting after the metal exposure. However, this would also have included cells, which were prepared for the clonogenic assay, as they too were grown at subconfluent levels with regular harvesting up to 30 days, before being plated (2000 cells) for colony formation in the clonogenic assay. The cause for the difference in growth between the cells in the colonies and the regularly split subconfluent cells, after metal treatment is not known. There might be two mechanisms by which this phenomenon could be explained.

a) In the first mechanism, it is possible that the level of genomic instability in the metal treated hTERT- cells was higher in the colonies as a result of the microenvironment (Bindra and Glazer 2005), which would lead to difference in growth of the colonies and the subconfluent

cells after metal treatment. It has been suggested that cell stresses, which are induced by the microenvironment, maybe the cause of this genetic instability (Reynolds *et al* 1996). Particularly, hypoxia has been proposed to be a key microenvironmental factor involved in the development of genetic instability, as it is linked with increased DNA damage, enhanced mutagenesis and efficient impairments in DNA repair pathways. All these phenomena include a considerable basis of genetic instability induced by hypoxia. Therefore, they could potentially accelerate the multi-step process of tumor progression. In contrast to hypoxia, there have been fewer studies that specifically concentrated on the impact of other microenvironmental factors on the induction of DNA damage and mutagenesis in tumors, such as low pH. In fact, decreases in pH, have been demonstrated to alter both the structure and function of proteins involved in DNA replication such as helicases and polymerases (Eckert and Kundel 1993).

b) In the second mechanism, it is possible that the difference in growth of the colonies and the subconfluent cells after metal treatment occurred as a result of an increased concentration of bystander effects, for example including those mediated by gap junctions. Little (2003) demonstrated that radiation-induced bystander effects occurred in cells that received no radiation exposure, as a consequence of damage signals transmitted from neighbouring irradiated cells. This transmission might have been provoked either by direct intercellular communication through gap junctions, as already mentioned, or by factors released in the nearby medium. The biological effect of this phenomenon seems to be related with an upregulation of oxidative metabolism (Little 2003). If this was true, hTERT might provide a genetic influence for the control of the bystander effect (Mothersill and Seymour 2004).

4.1.2.2 Chromosome End Joining/Fusion

The second type of genomic instability was related to chromosome end joining/fusion occurring in hTERT- cells, which was reflected by cytogenetic abnormalities such as

nucleoplasmic bridges and dicentric chromosomes. Nucleoplasmic bridges are thought to represent dicentric chromosomes and centric ring chromosomes (Thomas *et al* 2003). These authors showed, through the cytokinesis-block micronucleus (CBMN) assay, that inclusion of nucleoplasmic bridges in the CBMN assay provides a valuable measure of chromosome breakage/rearrangement. The CBMN assay allows nucleoplasmic bridge measurement to be achieved reliably because the inhibition of cytokinesis prevents the loss of nucleoplasmic bridges that would otherwise take place if cells were allowed to divide (Thomas *et al* 2003). These cytogenetic alterations (dicentrics and nucleoplasmic bridges) are referred to as clonal and the aberrations are not usually lethal (Morgan *et al* 1996, Mothersill and Seymour1998). These may arise as a result of complex rearrangements occurring at a high rate post-insult in surviving cells. This type of instability may involve a repair defect or other mutations sustained at the time of the insult. It is unlikely that residual chemical in the cells could account for the delayed effects, because in that case it would be predicted that the expression of the damage would decrease with time. In contrast, the percentage of nucleoplasmic bridges observed in hTERT- cells was much higher at *Day 30* compared to *Day 0* in all dose points, including the control. Dicentric chromosomes gradually increased from *Day 0* onward with the higher dose of Cr (VI), whereas they were persistent, but did not increase, with the lower dose of V (V). Furthermore, the washing and sub-culture protocols make it highly unlikely that any residual chemical remains in progeny cells 30 days after metal exposure. From these experiments it is clear that the genoxicity of both metals induced chromosome end joining/fusion. In theory, the persistent increase of dicentric chromosomes in the hTERT- cells could have been caused in three different ways.

1) In the first way, **two broken ends of chromosomes** maybe fuse and then possibly be processed by homologous recombination repair (HRR) or non-homologous end joining (NHEJ). Double strand breaks could be converted by HRR to either reciprocal translocations or

dicentrics and acentric fragments. Alternatively, double strand breaks may be converted by NHEJ to dicentric chromosomes and fragments (Obe *et al* 2002). HRR involves the members of the Rad52 gene group and strongly needs regions of extensive sequence homology, whereas NHEJ depends on the products of the genes XRCC4-7 and it does not necessarily require sequence homology (Pfeiffer *et al* 2000). The indispensable requirement of HRR for sequence homology is reflected by the fact that it occurs preferentially between sister chromatids, in cells undergoing mitotic cell cycles or between homologous chromosomes in cells undergoing meiotic cell cycles, where it could lead to gene conversion. HRR can also occur between homologous DNA sequences in different chromosomes (called ectopic HRR), which may lead to exchange type chromosomal aberrations such as dicentric chromosomes and translocations (already mentioned above). Normally, HRR is initiated by one single double strand break (DSB) in order to produce both correct intrachromosomal repair products and incorrect exchange type chromosomal aberrations. In this case, the two DNA ends of a single DSB interact with the two strands of a homologous unbroken DNA duplex. NHEJ rejoins the DSB in the absence of complete sequence homology and this means that it has a generally related pathway for the repair of DSB, which occurs within chromosome regions without sequence homology. In order to occur, NHEJ needs a single DSB to generate intrachromosomal repair products. In this case, the two DNA ends of a single DSB interact with each other. But to originate exchange type chromosomal aberrations, NHEJ requires at least two initial double strand breaks (DSBs). In this case, the four ends of two DSBs interact cross-wise with each other (Obe *et al* 2002).

2) In the second way, **telomeres could fuse with double strand breaks** in cells lacking telomerase and/or with eroded telomeres (Chan and Blackburn 2002). These authors reported that telomeres fuse rapidly (within hours) to the HO-generated double-stranded break in cells missing active telomerase. They noticed that the number of fusions increased when either

telomerase or Tel1p was deleted. The creation of telomere-DSB fusions occurred by the nonhomologous end-joining pathway, since they were depending on DNA ligase IV. Fusions also occurred in cells, in which telomerase could not elongate telomeres. The telomere-DSB fusion was observed carefully before critical telomere shortening caused any detectable cellular senescence, and it was even present in cultures where most of the telomeres were significantly elongated. This implies that significant loss of most of the telomeric DNA, and a resulting defect in chromosome end protection, could occur at a low incidence in cells lacking telomerase (Chan and Blackburn 2003). These authors also studied whether telomere-DSB fusion could occur in cells with long telomeres. Their results confirmed that it is not the telomere shortness itself, but rather the absence of telomerase and Tel1p, that caused rigorous telomere shortening and consequently telomere-DSB fusion. Furthermore, they demonstrated that telomerase is still necessary to protect long telomeres from high shortening. This showed that the telomere-protective function of telomerase is independent of its role in determining the telomere elongation (Chan and Blackburn 2003).

3) In the third way, **fusion between telomeres** could occur if a) they are dysfunctional, lacking TRF2 (Smorgorzewska *et al* 2002) and having DNA damage foci (Takai *et al* 2003); in this case telomeres are then processed as a NHEJ (Smogorzewska *et al* 2002), or b) if they were damaged by single strand breaks (Urushibara *et al* 2004).

a) Fusion between telomeres could arise if they were dysfunctional, lacking TRF2 (Smorgorzewska *et al* 2002) and having DNA damage foci (Takai *et al* 2003). Telomeres are required to prevent end-to-end chromosome fusions, but it was observed that end-to-end fusions of chromosomes occurred in cells with dysfunctional telomeres, due to reduced function of telomere-associated proteins, and in cells undergoing extensive erosion of telomeric DNA. Telomere fusions, resulting from inhibition of the telomere-protective factor TRF2, are produced by DNA ligase IV-dependent NHEJ. Therefore, NHEJ can then originate covalent

ligation of the C strand of one telomere to the G strand of another. The breakage of the resulting dicentric chromosomes results in nonreciprocal translocations. Telomere NHEJ takes place before and after DNA replication, and both sister telomeres contribute in the reaction. Telomere fusions are followed by active degradation of the 3′ telomeric overhangs. The main risk to dysfunctional telomeres is the degradation of the 3′ overhang and subsequent telomere end-joining by DNA ligase IV. The involvement of NHEJ in telomere fusions is paradoxical since the NHEJ factors Ku70/80 and DNA-PKcs are present at telomeres and protect chromosome ends from fusion. Several lines of evidence indicate that telomeres lacking TRF2 resemble the critically shortened telomeres of senescent cells (Smogorzewska and De Lange 2002, Karlseder *et al* 2002). Therefore, the conclusion that dysfunctional telomeres are treated like DNA breaks and repaired by NHEJ is also likely to be related to the shortened telomeres of primary human cells nearing replicative senescence or those of transformed cells undergoing telomere crisis (Smorgorzewska *et al* 2002).

b) Fusion between telomeres could occur if they were damaged by single strand breaks (Urushibara *et al* 2004). These authors demonstrated that a major type of delayed chromosome aberrations by ionizing radiation consists of dicentrics that might be triggered by end-to-end fusions. Interestingly, they found that the induction of delayed formation of dicentric chromosomes that retained telomere sequences at the fused position (tel+ end-fusion) increased in response to radiation dose, suggesting that ionizing radiation induces telomere dysfunction that might promote the tel+ end-fusions. These results suggest that telomeres are one of the critical targets for induction of delayed chromosomal instability by ionizing radiation. It has been observed that exposure to ionizing radiation induces enhanced and constant generation of reactive oxygen species (ROS) in irradiated progeny cells (Clutton *et al* 1996 and Limoli *et al* 2003). This persistent generation of ROS in irradiated cells partially explains why delayed effects of radiation appeared over many cell divisions post-irradiation. Single-strand breaks or

single-stranded regions induced by ROS are usually found in telomeric DNA, even in normal oxygen cell culture condition (Von Zglinicki *et al* 1995) (Petersen *et al* 1998). This shows that telomeres are vulnerable to oxidative damage. Thus, ionizing radiation has a potential to induce persistent instability of telomeres mediated by ROS. It would be interesting to see how the oxidative damage in telomeres induces telomeric fusions. A recent study on telomere integrity post-replication indicated that mammalian telomeres require strand-specific post-replicative processing (Bailey *et al* 2001). A defect in TRF2 or DNA-PKcs gives rise to damage processing of leading strands of the DNA, resulting in the formation of end-to-end fusions. Thus, it might be possible that elevated oxidative stress post-irradiation may decrease the integrity of post-replicative processing of telomeres and this could be provoked by the gathering of single-strand breaks. In addition to an indirect effect of radiation on telomere integrity, it was also demonstrated that a defect in DNA-PKcs support the end-to-end fusions characterised by telomeric instability such as the tel+ end-fusions. This sustains the evidence that DNA-PKcs plays a protective role in telomeric end-capping, in addition to repairing DNA double-strand breaks, as shown by other authors (Goytisolo *et al* 2001, Bailey *et al* 1999).

The results of this work suggest that the **second** and particularly the **third** mechanism might have been important for the following reasons. Ectopic hTERT expression protected against the formation of dicentric chromosomes, nucleoplasmic bridges and aneuploidy. Fusion of damaged telomeres is also considered to cause an increase in dicentric chromosomes after radiation. If this had created a cycle of chromosome fusion, bridging and breakage (McClintock 1941), this may have caused the persistence of the aneuploidy at the time of the increase in nucleoplasmic bridges, which was 30 days after metal exposure of hTERT- cells. Rubio *et al* (2004) have suggested that hTERT is only protective for clonogenc survival in cells with short telomeres. Certainly during ageing, chromosome fusion is restricted to the chromosomes with the shortest telomeres (Der Sarkissian *et al* 2004). This study is compatible

with this interesting theory. However, in the pre-senescent period of ageing in this study, there were no dicentric chromosomes in control (exposed only to PBS) cells, either in hTERT- or in hTERT+ cells, although there was an increasing population of some (XpYp) chromosomes, but not other (17p) chromosomes with short telomeres (1-3 Kb) with time. Besides, in the metal exposed hTERT- cells, which had an induction of dicentric chromosomes, there was no significant change in the average or the distribution of telomere lengths by metals. The hTERT- cells whether treated or not, showed essentially normal telomere dynamics. This could mean that metals may have caused structural or conformational changes in some telomeres, and/or their associated proteins, that were short and consequently susceptible to damage. Since in this work the hTERT+ cells had almost neither dicentrics nor nucleoplasmic bridges it is very interesting that hTERT+ cells are known to show an upregulation of the genes involved in HRR and NHEJ and/or changes in chromosome aberrations and tetraploidy. It might be possible that an upregulation of one of these genes could be responsible for the lack of chromosome/end joining fusion or the induction of tetraploidy in the metal exposed hTERT+ cells, which will be described later. This second type of instability (chromosome end joining/fusion) with the third type of instability described below (chromosomal breakage) could involve a cycle of dicentric formation/end joining and breakage, as originally proposed by McClintock (1941).

4.1.2.3 Chromosomal Breakage

The third type of genomic instability was related to chromosomal breakage occurring in hTERT- cells, which was reflected by cytogenetic abnormalities such as micronuclei (Fenech 2000) and chromatid breaks. These cytogenetic alterations are also referred as clonal and the aberrations are not usually lethal (Morgan *et al* 1996, Mothersill and Seymour 1998). Similarly to chromosomal end joining, these cytogenetic alterations may arise as a result of complex

rearrangements occurring at a high rate post-insult in surviving cells. This type of instability may involve a repair defect or other mutations sustained at the time of the insult. As already mentioned before, it is unlikely that residual chemical in the cells could account for the delayed effects, because in that case it would be predicted that the expression of the damage would decrease with time. In contrast, the expression of cytogenetic damage such as micronulei was less evident in the initially exposed hTERT- cells and gradually increased up to 30 days after metal treatment. The acute cytogenetic effects of the metals were different as expected from their toxicities. Cr (VI) at these concentrations induced chromatid breaks without aneuploidy, whereas V (V) caused aneuploidy without chromatid breaks. However, the instability produced by the two metals was similar. This suggests that genomic instability might have been provoked by a common mechanism such as single strand breaks (SSBs) (Mothersill *et al* 1998). The production of SSBs by Cr (VI) reduction, either directly as a consequence of Cr-DNA interactions or as a result of oxygen/carbon radical generation, represents one of the most commonly reported lesions arising from Cr (VI) treatment. Therefore, it is possible that the chromatid breaks and micronuclei observed in this work have been induced by DSBs, although conclusive experimental data linking Cr (VI) reduction to the development of DNA DSBs, as observed after ionising radiation or radiomimetic drug exposure, is still lacking (Bagchi *et al* 2002, Wise *et al* 2002, Hirose *et al* 2002). Alternatively, other types of mechanisms, such as hypermethylation (Sciandrello *et al* 2004) or adduct formation, could be responsible for the production of genomic instability in hTERT- cells.

It has been proven that a lack of SOD2 gene leads to increased DSBs, chromosomal translocations and loss of proliferative capacity (Samper *et al* 2003). Therefore, it might be possible that an upregulation of this gene is responsible for the lack of micronuclei (with both chemicals) and chromosomal breaks (with the higher dose of Cr (VI)) in metal exposed hTERT+ cells.

This third type of instability (chromosomal breakage) with the second type of instability described above (chromosome end joining/fusion) could involve a cycle of dicentric formation/end joining and breakage (McClintock 1941).

4.1.2.4 Polyploidy

The fourth type of genomic instability was related to the higher percentage of polyploidy (tetraploidy) observed in hTERT+ cells. The phenomenon of tetraploidy is known to be developed by several mechanisms: by cell fusion, by uncoupling of DNA replication from cell division (endoreduplication), by an abortive cell cycle, by stress and by ageing (Storchova and Pellman 2004). However, in this work the lack of cell cycle arrest at G0/G1 with 5 μM V (V) in the hTERT+ cells, and at G2/M with 0.4 μM Cr (VI) and 5 μM V (V) in the hTERT+ cells, but not in the hTERT- cells, imply that cell cycle checkpoints may play a role. Margolis *et al* (2003) originally proposed that there was a specific p53 dependent tetraploidy checkpoint at G0/G1 that prevents cell cycle progression of cells with abnormal chromosome failure. This has been challenged by Uetake and Sluder (2004), and Wong and Stearns (2005). In keeping with this, the tetraploidy in the metal exposed hTERT+ cells was not dependent on a G0/G1 arrest. However, it was interesting that V (V), and to a less extent Cr (VI), caused a temporary increase in G0/G1 in the hTERT- cells without tetraploidy, whereas hTERT+ cells had no temporary increase in G0/G1 and underwent tetraploidy (83% G0/G1 in hTERT- cells, 63% G0/G1 in hTERT+ cells). V (V), unlike Cr (VI), is known to inhibit microtubule assembly and induce tubulin depolymerization (Ramirez *et al* 1997) and Lanni and Jacks (1998) showed that adaption to another microtubule destabilising drug (nocodazole) caused a p53 dependent G0/G1 arrest. Therefore, it might be possible that abnormal p53 signalling in hTERT+ cells was partially accountable for the tetraploidy caused by V (V). The integrity of cell cycle checkpoints is a controversial aspect of hTERT biology. Some authors have noted that hTERT

+ cells express a normal cell cycle arrest and checkpoint protein response to specific challenges, including DNA damaging agents such as Cr (VI) (Gorbunova *et al* 2002, Pritchard *et al* 2001, Wood *et al* 2001, Jiang *et al* 1999, Morales *et al* 1999). In contrast, other authors have reported that there is a loss of p16INK4a and hyperphosphorylation of pRb in hTERT + cells (Piboonniyom *et al* 2003, Tsutsui *et al* 2002) and in one cell line a mutation in p53 and an abnormal G0/G1 checkpoint was observed (Noble *et al* 2004).

The results of the present work suggest that hTERT+ cells, at least in some circumstances, have a latent defect in checkpoint control. This may allow damaging agents like metals, in this work especially V (V), to cause tetraploidy, which would not be present in hTERT- cells. This mechanism could be important in carcinogenesis, in which the induction of telomerase positivity and tetraploidy are early steps in malignancy (Rajagopalan and Lengauer 2004, Dutrillaux *et al* 1991, Reid *et al* 1987). It is interesting that an example of transformation to a near tetraploid state has been described in tissue culture of Barretts epithelium, which was transfected with hTERT (Palanca-Wessels *et al* 2003). This transformation, joined with repeated exposure to DNA damaging agents, may drive a malignant process to complex aneuploidy with a consequent poor prognosis (Risques *et al* 2003).

Margolis *et al* (2003) have demonstrated that the mechanism of tetraploidy mainly depends on two steps: an **aberrant mitotic exit** and an **absence of G0/G1 surveillance** that would prevent cell cycle progression of cells with abnormal chromosome failure. These two phenomena could explain the observation that hTERT- cells, exposed to V (V), which is known to interfere with microtubule assembly (Ramirez *et al* 1997), showed a temporary induction of tetraploidy with G0/G1 arrest, which then reverted to normal cycling and a small increase of aneuploidy (Mailhes *et al* 2003). In contrast, hTERT+ cells had a perment increase in tetraploidy up to 30 days after the metal exposure, with no aneuploidy and no signs of G0/G1 arrest.

4.1.3 Future Directions

Telomeres are thought to be present in two forms, an open accessible form and a closed protected form (Blackburn 2001). Short telomeres can undergo a conformational change, as a result of a binding alteration of telomere-associated protein TRF2 (Karlseder *et al* 2002). It would be interesting to test whether there are differences in the binding of TRF2 between hTERT- and hTERT+ cells after metal exposure of either Cr (VI) or V (V), particularly because TRF2 is upregulated in cells exposed to carcinogens and in cancer cells exposed to arsenic but is not changed by oncogenic agents or hTERT alone (Nijjar *et al* 2005, Zhang *et al* 2005). A differential change in TRF2, for example, could provide a good contribution to both types of instability (loss of cell survival and cytogenetic damage in hTERT- cells and tetraploidy in hTERT+ cells). For example, it could help to explain (in conjunction with the short telomeres) the difference in the induction of dicentric chromosomes and senescence (Van Steensel *et al* 1998, Karlseder *et al* 2002). It could also give an explanation about the lack of response in the cell cycle and apoptosis to chromatid breaks in hTERT+ cells, as TRF2 prevents the induction of p53 and upregulation of p53 targets after radiation (Karlseder *et al* 2002).

4.2 Radiation

4.2.1 General Comment

This work has provided evidence that exposure to doses of radiation such as 0.5 Gy, and to a lesser extent 0.05 Gy, can induce characteristics in progeny cells that are associated with radiation-induced genomic instability in human fibroblasts. Furthermore, the higher dose of radiation (0.5 Gy) caused cell damage such as micronuclei (MNi) in human fibroblasts. Previous work confirmed that radiation causes damage to the cell and can reduce the reproductive integrity of progeny cells for several generations (Seymour and Mothersill 1986, Kadhim *et al* 1992, Morgan *et al* 1996).

4.2.1.1 Cell Survival

The clonogenic assay showed that only hTERT+ cells had a significant reduction ($p<0.01$) (10%) in clonogenic survival 30 days after the radiation exposure with the higher dose (0.5 Gy). However, there was an unexpected increase ($p<0.01$) in clonogenic survival 30 days after the radiation exposure with the lower dose (0.05 Gy). Moreover, it was also observed that 0.5 Gy caused more loss ($p<0.001$) (approximately 14.5%) in clonogenic survival, and also 0.05 Gy resulted in a significant increase in clonogenic survival ($p<0.01$ in VC + 0.05 Gy and $p<0.05$ in 0.05 Gy + VC), 30 days after the radiation exposure in hTERT+ cells when radiation was used as a "radiation control" in the combined exposure experiment (see Chapter 3, Section 3.3.1.1). Furthermore, 0.5 Gy also caused a significant reduction ($p<0.05$) in clonogenic survival 30 days after the radiation exposure in hTERT- cells when used as a "radiation control" in the combined exposure experiment (See Chapter 3, Section 3.3.1.1). Therefore, genomic instability was observed in both types of cells, although the reduction in clonogenic survival was more significant in hTERT+ cells.

It is important to clarify that in the "*Radiation Only Experiments*" the 0.5 Gy exposure was carried out 24 hours before the harvesting of the cells (that corresponds to the VC + 0.5 Gy in the combined exposure experiment), whereas in the "*Combined Exposure Experiment*" (when 0.5 Gy was used as a "radiation control") the 0.5 Gy exposure was carried out 48 hours before the harvesting of the cells (that is the 0.5 Gy + VC in the combined exposure experiment) (See Chapter 3, Section 3.3.1.1). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with radiation exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells.

4.2.1.2 Cell Damage

The micronucleus assay showed that hTERT+ cells were more vulnerable to damage such as micronuclei (MNi), immediately after radiation exposure of 0.5 Gy ($p<0.05$) compared to hTERT- cells (which also showed damage, but it was not significant). However, in all of the experiments, at both survival times, with or without radiation exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was particularly true 30 days after exposure to radiation, especially with the 0.5 Gy dose of radiation ($p<0.05$).

Neither hTERT- cells nor hTERT+ cells showed a significant increase in nucleoplasmic bridges (NPB), due to radiation. However, in all of the experiments, at both survival times, with or without radiation exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was particularly evident 30 days after radiation exposure, where both doses of radiation (0.05 Gy and 0.5 Gy) reached high level of significance ($p<0.01$).

Cytogenetic abnormalities such as chromatid breaks, chromatid gaps, chromatid fragments, dicentric chromosomes and tetraploidy, when observed, were not found to be statistically significant, in either types of cells. However, in all of the experiments, at both survival times,

with or without radiation exposure, there was a lower level of dicentric chromosomes in the hTERT+ cells compared to the hTERT- cells. This was statistically significant 30 days after radiation exposure to the 0.5 Gy dose ($p<0.05$). Furthermore, in all the experiments, at both survival times, with or without radiation exposure, there was a higher level of tetraploidy in the hTERT+ cells compared to the hTERT- cells. This was statistically significant immediately after the radiation exposure (*Day 0*) of 0.05 Gy dose ($p<0.05$).

4.2.2 Radiation Induces Genomic Instability

Radiation induced genomic instability in hTERT+ cells, and to a lesser extent, in hTERT- cells. Therefore, the telomerase activity in hTERT+ cells did not provide protection against genomic instability. The clonogenic assay demonstrated that radiation (mainly 0.5 Gy) induced aberrations in surviving progeny that were lethal and therefore could not have been carried by the surviving cells. Non-clonality is a feature of this type of instability. The fact that this instability is non-clonal and lethal suggests that it is not due to a defect sustained by the cells at the time of the initial insult, as the defect should manifest in all clonal progenies of the affected cells, and not in any of the progeny of cells that did not sustain the damage (Mothersill and Seymour 1998).

4.2.3 Radiation Induces Cell Damage

This study has shown that radiation of 0.5 Gy caused an incidence of MNi (which are supposed to be chromosomal breakage) (Fenech 2000), in hTERT+ cells immediately after (*Day 0*) the radiation exposure. This initial induction of MNi had no persistence up to 30 days after radiation exposure. Mothersill and Seymour have shown that cytogenetic alterations, such as micronuclei (or chromosomal breakage), are clonal and the aberrations are not usually lethal (Mothersill and Seymour 1998, Morgan *et al* 1996). These cytogenetic alterations may arise as

a result of complex rearrangements occurring at a high rate post-insult in surviving cells. This type of instability may involve a repair defect or other mutations sustained at the time of the insult (Mothersill and Seymour 1998, Morgan *et al* 1996). However, in this work exposure to either 0.05 Gy or 0.5 Gy doses of radiation did not cause genomic instability.

It has been suggested that DNA is the main target for the biological effects of radiation (Zirkle and Bloom 1953). In fact, if cells are irradiated with γ rays, they show susceptibility to several breaks in either of the single strands in the DNA molecule. It may be possible that this temporary induction of MNi, observed in hTERT+ cells, has been provoked by a mechanism such as single strand breaks (SSB) (Mothersill *et al* 1998). But it has been shown that the most lethal damage caused by ionizing radiation is the double strand break (DSB), where there is a break in both DNA strands, at the same position. Single strand breaks (SSBs) are less harmful to the cells, as the remaining DNA strand can be used as a template for repair (Zirkle and Bloom 1953, Munro 1970). If two single breaks may be opposite in the DNA, it means that a DSB has occurred, and therefore it can lead to a mutation or even cell death. It has been shown that the number of double strand breaks (DSBs) following irradiation of cells is approximately 0.04 times that of SSBs (Munro 1970). It has been demonstrated that all the DNA content of a cell is likely to be vulnerable to strand breaks and a DSB is essential in order to incur a lethal event in the cell (Kellerer and Rossi 1972). This has suggested that a low dose of radiation normally causes SSBs, while higher doses are likely to induce DSBs. Therefore, it may be possible that the higher dose of radiation used in this work (0.5 Gy) induced DSBs in the hTERT+ cells. It has been reported that the position of the cell in the cell cycle determines its potential of repairing post-irradiation damage (Sinclair 1973).

Damage to the nucleus after radiation exposure is believed to be the main cause of radiogenic cell death (Zirkle and Bloom 1953). According to these authors, nucleus and nucleolar lesions, increases in nuclear diameter, micronucleation, marginated chromatin and nuclear

fragmentation are the main endpoint damage resulting from exposure to radiation, which normally lead to the death of the cell (McClain *et al* 1990, Cornforth and Goodwin 1991). Moreover, ionizing radiation can induce damage not only to the nucleus and DNA, but also to the membrane and protein structure. Several biological effects are provoked by ionizing radiation through direct interaction with nucleic acid and the production of free radical species, or dysfunction of cellular organelles (Kantak *et al* 1993, Kasper *et al* 1993, Somosy *et al* 1995, Singh and Vadasz 1983).

The damage to the DNA caused by oxidative stress is detected in the bases and sugar-phospate in the structure of DNA, as well as SSBs and DSBs. In fact, SSBs and DSBs can be induced by direct ionizing radiation. However, indirect damage can be caused by radicals generated from radiation and lead to base damage. The majority of these indirect effects occur by free radicals in water, since this comprises 70-80% of the mammalian cells. The free radicals react with other molecules to form reactive oxygen species (ROS). The most important ROS are the superoxide radical, the hydroxyl radical and the hydrogen peroxide (Horsman and Overgaard 1997). There are more than twenty different types of base damage, which are induced by oxidative stress. The most frequent oxidative damage to purines is 7,8-dihydro-8-oxoguanine (8-oxodGuo), where the conformation is capable to mispair with adenine determining a transversion of G to T (Martinez *et al* 2003) The most common oxidative damage to the pyrimidines is the formation of thymine glycol (Tg) (Slupphaug *et al* 2003). Therefore, it would be interesting to investigate if the temporary induction of MNi (caused by SSB or DSB), observed in hTERT+ cells, could be due to radicals generated from radiation, which lead to base damage.

4.2.4 Why Telomerase Did Not Protect Against Radiation

In this work it was observed that ectopic expression of hTERT did not protect against radiation. In fact, radiation caused the following phenomena:

1. Increase of clonogenic survival at *Day 30* with 0.05 Gy and loss of clonogenic survival at *Day 30* with 0.5 Gy in hTERT+ cells, but not in hTERT- cells.

2. Incidence of MNi at *Day 0* with 0.5 Gy in hTERT+ cells, but not in hTERT- cells.

In contrast to the present work, some authors have shown that telomerase expression can prevent damage induced by radiation. Pirzio *et al* (2004) observed that human fibroblasts expressing hTERT showed remarkable karyotype stability after exposure to ionizing radiation. This long-term study illustrated that human fibroblasts immortalized by telomerase showed an unusual stability for chromosomes, both with and without exposure to ionizing radiation. Their work confirmed a role for telomerase in genome stabilisation by a telomere-independent mechanism (Pirzio *et al* 2004). Furthermore, Nakamura *et al* (2005) showed that cancer cells lacking hTERT had a significantly increased sensitivity, compared with control hTERT+ cancer cells, to ionizing radiation or chemotherapeutic agents that induce DNA double-strand breaks. Akiyama *et al* (2002) showed that hTERT expression, in hTERT-transfected K562 cells, protected against double-stranded DNA break inducing agents such as ionizing irradiation. These findings suggest that overexpression of telomerase by transfecting hTERT confers telomere-elongation and resistance to double-stranded DNA break inducing agents.

In contrast to this, Porter *et al* (2006) showed that telomerase-immortalized human fibroblasts retain UV-induced mutagenesis and p53-mediated DNA damage responses using the clonogenic assay. This is in line with the present work, since telomerase expression in hTERT+ cells did not protect against radiation exposure, in term of clonogenic survival. Therefore, hTERT+ cells showed genomic instability up to 30 days after the radiation exposure ($p<0.01$ with both 0.05 Gy and 0.5 Gy). It is unknown why the loss of clonogenic survival, and

therefore genomic instability, was statistically significant only in hTERT+ cells, and not in hTERT- cells, as well as the incidence of MNi immediately after the radiation exposure. It might be possible that the hTERT- cells, and to a lesser extent hTERT+ cells, used for this work, have become older (or alternatively have been damaged) since the first time they were used. In fact, the cell proliferation was observed to be lower in these *Old hTERT- Cells* compared to the *Young hTERT- Cells* (especially at *Day 30*), whereas the reduction in cell proliferation between the *Old hTERT+ Cells* (used for this experiment) and the *Young hTERT+ Cells* was minimal. Zhu *et al* (2006) have shown that a reduction of cell proliferation is likely to stop the cells at the G2/M phase of the cell cycle. This can induce apoptosis, and therefore it may reduce the damage that cells normally gain. This could be the reason why these *Old hTERT- Cells*, used for the present experiment, showed less damage with radiation, compared to the *Old hTERT+ Cells*.

4.3 Combined Exposure

4.3.1 General Comment

This work has provided direct evidence that either Cr (VI) followed by radiation or radiation followed by Cr (VI) can induce characteristics in progeny cells that are associated with combined exposure induced genomic instability. Both types of cells in this study (hTERT- and hTERT+ cells) showed genomic instability. Previous work confirmed that a wide range of chemicals could reduce the reproductive integrity of progeny cells for several generations (Mothersill *et al* 1998, Coen *et al* 2001). However, there has been no evidence yet that combination of metal and radiation could demonstrate genomic instability in human fibroblasts. In this work, evidence was provided of persistent loss of clonogenic survival and cell damage such as micronuclei (MNi) in human fibroblasts.

4.3.1.1 Cell Survival

Metal Followed by Radiation [(M + 0.05 Gy) and (M + 0.5 Gy)]

Metal treatment followed by sham irradiation (M + SI), used as a control treatment, caused a reduction in clonogenic survival in hTERT- cells, but not in hTERT+ cells at *Day 0*. M + 0.05 Gy caused a decrease in clonogenic survival in hTERT+ cells, 30 days after the combined exposure ($p<0.01$). Furthermore, the hTERT+ cells showed an unexpected increase in clonogenic survival 30 days after radiation exposure only (VC + 0.05 Gy) ($p<0.01$). M + 0.5 Gy caused a loss of clonogenic survival in both types of cells 30 days after the combined exposure, but it was more significant in hTERT+ cells ($p<0.001$) compared to hTERT- cells ($p<0.01$). Furthermore, the hTERT+ cells showed a reduction in clonogenic survival 30 days after radiation exposure only (VC + 0.5 Gy) ($p<0.01$). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with either metal treatment or radiation

exposure or combined exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells (with the only exception of M + 0.05 Gy at *Day 0*).

Radiation Followed by Metal [(0.05 Gy + M) and (0.5 Gy + M)]

Sham irradiation followed by metal treatment (SI + M), used as a control treatment, caused a reduction of clonogenic survival in hTERT- cells, which persisted up to 30 days ($p<0.01$). 0.05 Gy + M resulted in a decrease ($p<0.001$) in clonogenic survival in hTERT- cells 30 days after the combined exposure. The hTERT+ cells followed the same pattern, but the decrease 30 days after the combined exposure was less significant ($p<0.01$). Furthermore, in hTERT+ cells there was a loss of clonogenic survival with only radiation exposure at *Day 0* ($p<0.01$), and an unexpected increase in clonogenic survival at *Day 30* ($p<0.05$). 0.5 Gy + M caused a decrease of clonogenic survival in hTERT- cells at *Day 0* ($p<0.05$), which increased up to 30 days after the combined exposure ($p<0.001$). Furthermore, only radiation caused a decrease of clonogenic survival 30 days after the radiation exposure ($p<0.05$). In hTERT+ cells only the radiation exposure (0.5 Gy + VC), but not the combined exposure (0.5 Gy + M), resulted in a decrease in clonogenic survival, which persisted up to 30 days after the radiation exposure ($p<0.001$). A direct comparison of hTERT- and hTERT+ cells showed that at both survival times, with either metal treatment or combined exposure, there were more colonies in the hTERT+ cells compared to hTERT- cells.

4.3.1.2 Cell Damage

Metal Followed by Radiation [(M + 0.05 Gy) and (M + 0.5 Gy)]

The combined exposure of metal followed by the higher dose of radiation (M + 0.5 Gy) induced micronuclei (MNi) in both types of cells, whereas the combined exposure of metal followed by the lower dose of radiation (0.05 Gy) had no effect in either types of cells. M + 0.5 Gy caused a statistically significant increase in MNi immediately after the combined exposure

(*Day 0*) in hTERT- cells, which did not persist up to *Day 30*. The hTERT+ cells followed a similar pattern, but with M + 0.5 Gy there was also a significant persistence of MNi at *Day 30*, demonstrating that combined exposure had caused genomic instability in hTERT+ cells. Furthermore, the radiation only exposure (VC + 0.5 Gy) caused a significant increase in MNi at *Day 0* in hTERT+ cells only. In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was particularly true at *Day 30*, where most of the data was statistically significant.

Neither the combined exposure of metal followed by the higher dose of radiation (M + 0.5 Gy) nor the combined exposure of metal followed by the lower dose of radiation (M + 0.05 Gy) induced statistically significant nucleoplasmic bridges (NPB) in any type of cells. However, in all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was particularly true at *Day 30*, where all the data was statistically significant.

The combined exposure of metal followed by the higher dose of radiation (M + 0.5 Gy) induced chromatid breaks in hTERT+ cells only, whereas the combined exposure of metal followed by the lower dose of radiation (0.05 Gy) had no effect in either type of cells. M + 0.5 Gy caused a statistically significant increase in breaks immediately after the combined exposure (*Day 0*) in hTERT+ cells, which did not persist up to *Day 30*. There were no significant results of chromatid gaps and chromatid fragments showing differences in the experiments of metal treatment followed by radiation exposure in either type of cells. A direct comparison of hTERT- and hTERT+ cells revealed that the excess of dicentric chromosomes in hTERT- cells after combined exposure (M + 0.5 Gy) was statistically significant, at both survival times ($p<0.05$), compared to that in the hTERT+ cells. Furthermore, the level of

dicentrics after radiation exposure (VC + 0.5 Gy) was significantly higher ($p<0.05$) at *Day 30* in hTERT- cells compared to hTERT+ cells.

Neither the combined exposure of metal followed by the higher dose of radiation (M + 0.5 Gy) nor the combined exposure of metal followed by the lower dose of radiation (M + 0.05 Gy) induced statistically significant tetraploidy in any type of cells. A direct comparison of hTERT- and hTERT+ cells showed that in all the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was more tetraploidy in the hTERT+ cells compared to hTERT- cells. However, the only significant data was observed immediately after (*Day 0*) the radiation exposure (VC + 0.05 Gy) ($p<0.05$).

Radiation Followed by Metal [(0.05 Gy + M) and (0.5 Gy + M)]

The combined exposure of the higher dose of radiation followed by metal (0.5 Gy + M), as well as the higher dose of radiation itself (0.5 Gy + VC), induced MNi at *Day 0* in hTERT- cells only, whereas the combined exposure of lower dose of radiation followed by metal (0.05 Gy + M) had no effect in either type of cells. In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of MNi in the hTERT+ cells compared to the hTERT- cells. This was particularly true at *Day 30*, where most of the data was statistically significant.

Neither the combined exposure of the higher dose of radiation followed by metal (0.5 Gy + M) nor the combined exposure of the lower dose of radiation followed by metal (0.05 Gy + M) induced statistically significant NPB in any type of cells. However, in all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of NPB in the hTERT+ cells compared to the hTERT- cells. This was particularly true at *Day 30*, where all the data was statistically significant.

The combined exposure of the lower dose of radiation followed by metal (0.05 Gy + M) induced chromatid breaks in hTERT- cells only, whereas the combined exposure of the higher dose of radiation followed by metal (0.5 Gy + M) had no effect in either type of cells. 0.05 Gy + M caused a statistically significant increase in breaks immediately after the combined exposure (*Day 0*) in hTERT- cells, which did not persist up to *Day 30*. There were no significant results of chromatid gaps, chromatid fragments and dicentric chromosomes showing differences in experiments of radiation followed by metal in either type of cells.

Neither the combined exposure of the higher dose of radiation followed by metal (0.5 Gy + M) nor the combined exposure of the lower dose of radiation followed by metal (0.05 Gy + M) induced statistically significant tetraploidy in any type of cells. A direct comparison of hTERT- and hTERT+ cells showed that in all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was more tetraploidy in the hTERT+ cells compared to hTERT- cells. However, the only significant data was observed 30 days after combined exposure of the higher dose of radiation followed by metal (0.5 Gy + M) ($p<0.01$).

4.3.2 Combined Exposure Causes Genomic Instability (Loss of Clonogenic Survival) in Both Types of Cells

The first type of genomic instability was related to the persistent reduction of clonogenic survival, which occurred in hTERT- cells. The clonogenic assay demonstrated that both metals induced aberrations in surviving progeny that were lethal and therefore could not have been carried by the surviving cells. Non-clonality is a feature of this type of instability. The fact that this instability is non-clonal and lethal suggests that it is not due to a defect sustained by the cells at the time of the initial insult, as the defect should manifests in all clonal progenies of the

affected cells, and not in any of the progeny of cells that did not sustain the damage (Mothersill and Seymour 1998).

It is unknown why there was an increase in clonogenic survival 30 days after radiation exposure (VC + 0.05 Gy and 0.05 + VC) in hTERT+ cells, and why this phenomenon did not occur in hTERT- cells.

4.3.2.1 Combined Exposure of *Metal Followed by Radiation* Causes More Genomic Instability in hTERT+ Cells, while Combined Exposure of *Radiation Followed by Metal* Causes More Genomic Instability in hTERT- Cells

It was interesting to observe that *Metal Followed by Radiation* and *Radiation Followed by Metal* did not result in the same amount of genomic instability in both types of cells.

Metal Followed by Radiation with the lower dose of radiation (M + 0.05 Gy) caused a decrease in clonogenic survival immediately after (*Day 0*) the combined exposure in both types of cells ($p<0.05$), but it persisted and increased up to *Day 30* only in hTERT+ cells ($p<0.01$). *Metal Followed by Radiation* with the higher dose of radiation (M + 0.5 Gy) caused a loss of clonogenic survival in both types of cells 30 days after the combined exposure, but it was more significant in hTERT+ cells ($p<0.001$) compared to hTERT- cells ($p<0.01$).

Radiation Followed by Metal with the lower dose of radiation (0.05 Gy + M) resulted in a decrease ($p<0.001$) of clonogenic survival in hTERT- cells 30 days after the combined exposure. The hTERT+ cells followed the same pattern, but the decrease 30 days after the combined exposure was less significant ($p<0.01$). *Radiation Followed by Metal* with the higher dose of radiation (0.5 Gy + M) caused a decrease of clonogenic survival in hTERT- cells at *Day 0* ($p<0.05$), which increased up to 30 days after the combined exposure ($p<0.001$). The response of the hTERT+ cells was different, since there was no loss of clonogenic survival.

4.3.3 Combined Exposure Causes Genomic Instability (Incidence of Micronuclei) in hTERT+ Cells Only

This study has shown that short term combined exposures of *Metal Followed by Radiation* (M + 0.5 Gy) caused a long term genomic instability due to a persistent incidence of cytogenetic abnormalities such as MNi (chromosomal breakage) (Fenech 2000) occurring in hTERT+ cells, but not in hTERT- cells.

Cytogenetic abnormalities such as MNi (chromosomal breakage) are referred as clonal and the aberrations are not usually lethal (Morgan *et al* 1996, Mothersill and Seymour 1998). These cytogenetic alterations may arise as a result of complex rearrangements occurring at a high rate post-insult in surviving cells. This type of instability may involve a repair defect or other mutations sustained at the time of the insult. It is unlikely that residual Cr (VI) in the cells could account for the delayed effects, because the washing and sub-culture protocols make it highly unlikely that any residual chemical remains in progeny cells 30 days after combined exposure.

In order to understand the type of damage cause by the combined exposure of *Metal Followed by Radiation* (M + 0.5 Gy), it is important to remember the type of damage that metal only [Cr (VI)] and radiation only (0.5 Gy) normally induces. It is known that genomic instability, after metal exposure might be provoked by a mechanism such as single strand break (SSB) (Mothersill *et al* 1998). The production of single strand breaks (SSBs) by Cr (VI) reduction, either directly as a consequence of Cr-DNA interactions or as a result of oxygen/carbon radical generation or by the replication past/repair of DNA lesions, represents one of the most commonly reported lesions arising from chromium (VI) treatment. It has been suggested that DNA is the main target for the biological effects of radiation (Zirkle and Bloom 1953), since cells irradiated with γ rays, show susceptibility to several breaks in either of the single strands in the DNA molecule. It may be possible that the increase in MNi observed in hTERT+ cells,

after combined exposure of *Metal Followed by Radiation* (M + 0.5 Gy) have been provoked by a mechanism such as SSBs (Mothersill *et al* 1998). But it has been shown that the most lethal damage caused by ionizing radiation is double strand break (DSB), where there is a break in both DNA strands, at the same position. SSBs are less harmful to the cells, as the remaining DNA strand can be used as a template for repair (Zirkle and Bloom 1953, Munro 1970). If two single breaks are opposite in the DNA, it means that a DSB has occurred, and therefore it can lead to a mutation or even cell death. It has been shown that the number of double strand breaks (DSBs) following irradiation of cells is approximately 0.04 times that of SSBs (Munro 1970). It has been demonstrated that all the DNA content of a cell is likely to be vulnerable to strand breaks, and a DSB is essential in order to incur a lethal event in the cell (Kellerer and Rossi 1972). This has suggested that a low dose of radiation normally causes SSBs, while higher doses are likely to induce DSBs. Furthermore, it has been reported that the position of the cell in the cell cycle determines its potential of repairing post-irradiation damage (Sinclair 1973). Therefore, it is possible that the cell damage (MNi) observed in this work, after combined exposure of *Metal Followed by Radiation* (M + 0.5 Gy), was induced by DSBs, since conclusive experimental data linking Cr (VI) reduction to the development of DNA DSBs, as observed after ionising radiation (like the 0.5 Gy radiation used in this project) or radiomimetic drug exposure, is still lacking (Bagchi *et al* 2002, Wise *et al* 2002, Hirose *et al* 2002). Alternatively, other types of mechanisms, such as hypermethylation (Sciandrello *et al* 2004) or adduct formation, could be responsible for the production of genomic instability in hTERT+ cells.

Damage to the DNA caused by oxidative stress can be detected in the bases and sugar-phospate backbone in the structure of DNA, as well as SSBs and DSBs. In fact, SSBs and DSBs can be induced from direct ionizing radiation. However, indirect damage can be caused by radicals generated from radiation and lead to base damage. The majority of these indirect effects occur

by free radicals in water, since this comprises 70-80% of mammalian cells. The free radicals react with other molecules to form reactive oxygen species (ROS). The most important ROS are the superoxide radical, the hydroxyl radical and hydrogen peroxide (Horsman and Overgaard 1997). There are more than twenty different types of base damage, which are induced by oxidative stress. The most frequent oxidative damage to purines is 7,8-dihydro-8-oxoguanine (8-oxodGuo), where the conformation is capable to mispair with adenine determining a transversion of G to T (Martinez *et al* 2003) The most common oxidative damage to the pyrimidines is the formation of thymine glycol (Tg) (Slupphaug *et al* 2003). Therefore, combined exposure of *Metal Followed by Radiation* (M + 0.5 Gy) in this work, may have had the same effects, through direct interaction with nucleic acid and production of free radical species, or dysfunction of cellular organelles, in the hTERT+ cells, leading to genomic instability.

In this work, it was interesting to observe that ectopic hTERT expression had no effect in preventing the formation of MNi (chromosome breakage) after combined exposure of *Metal Followed by Radiation* (M + 0.5 Gy), since this combination caused significant MNi, which persisted up to *Day 30* in immortalised hTERT+ cells, but not in normal hTERT- cells. This was different to what was detected in the experiments of metal only treated [Cr (VI)] cells, where hTERT- cells showed significant cell damage (such as MNi and breaks) and this damage was less compared to hTERT+ cells.

4.3.3.1 Combined Exposure of *Metal Followed by Radiation* Causes Damage in hTERT+ Cells, while Combined Exposure of *Radiation Followed by Metal* Causes Damage in hTERT- Cells

It was interesting to observe that *Metal Followed by Radiation* and *Radiation Followed by Metal* did not induce the same damage in both types of cells.

Metal Followed by Radiation (M + 0.5 Gy) caused an increase in MNi immediately after (*Day 0*) the combined exposure in hTERT+ cells, which persisted up to *Day 30*, thereby demonstrating genomic instability. The hTERT- cells followed the same pattern only immediately after (*Day 0*) the combined exposure (M + 0.5 Gy) and there was no persistence of MNi at *Day 30*. The results of chromatid breaks, obtained with the chromosomal aberration analysis, followed the same pattern. As already noticed for the MNi, *Metal Followed by Radiation* (M + 0.5 Gy) caused an increase in breaks immediately after (*Day 0*) the combined exposure in hTERT+ cells. The response of the hTERT- cells was different, since there were no significant breaks.

Radiation Followed by Metal (0.5 Gy + M) caused an increase of MNi immediately after (*Day 0*) the combined exposure in hTERT- cells. The reaction of hTERT+ cells was different, since there was no induction of MNi. The results of chromatid breaks, obtained with the chromosomal aberration analysis, followed the same pattern. *Radiation Followed by Metal* (0.05 Gy + M) caused an incidence of breaks immediately after (*Day 0*) the combined exposure in hTERT- cells. The response of the hTERT+ cells was different, since there were no significant breaks

4.3.4 Why *Metal* + *Radiation* had effects on hTERT+ cells and *Radiation* + *Metal* had effect on hTERT- cells

The results obtained in the combined exposure experiment suggested that telomerase (in hTERT+ cells) could have conferred a protection to these cells when *Radiation was followed by Metal*, but not when *Metal was followed by Radiation*. In term of results, this was reflected by the normal clonogenic survival and low incidence of MNi, when hTERT+ cells were exposed to the combination of *Radiation* + *Metal*. It is unknown why this protection did not occur when hTERT+ cells were exposed to the combination of *Metal* + *Radiation*, and to date

there is no literature which offers any suggestions about this phenomenon. It may be possible that *Metal + Radiation* resulted in severe DNA damage (such as SSBs and DSBs), which could not be prevented by the telomerase enzyme of the hTERT+ cells.

Besides, it was interesting to observe that combined exposure in hTERT- cells induced loss of clonogenic survival, as well as increase in MNi, when *Radiation was followed by Metal*, but not when *Metal was followed by Radiation*. There is no literature either which can offer a suggestion about this phenomenon, but it may be possible that *Radiation + Metal* resulted in severe DNA damage (such as SSBs and DSBs), which could not be repaired by the DNA repair machinery of the hTERT- cells. Moreover, the combined exposure of *Metal + Radiation*, which was not as toxic as *Radiation + Metal*, may not have induced such severe damage or this damage may have been repaired by the DNA repair machinery of the hTERT- cells.

4.3.5 Synergistic Effects Caused by Combined Exposure of Metal and Radiation

The results of the combined exposure suggest that some of the biological effects provoked by combined exposure of metal and radiation have led to a synergistic action, compared to metal treatment only or radiation exposure only. In fact, in most of the significant results, the damage caused by the combination of metal and radiation was higher than the damage induced by either metal itself or radiation itself. However, this evaluation is very rudimentary, since there is no statistical data demonstrating that combined exposure of metal and radiation can lead to synergistic effects. Therefore, from now onwards in this section, it is only suggested that these results could have led to a synergistic outcome.

In this work, it was interesting to have observed the following characteristics of the combined exposure:

1. Synergistic effects were observed in both types of cells.

In hTERT- cells M + 0.5 Gy, and to a lesser extent 0.05 Gy + M and 0.5 Gy + M, caused a loss of clonogenic survival 30 days after the combined exposure. In hTERT- cells, MNi were induced by M + 0.5 Gy immediately after the combined exposure (*Day 0*) and chromatid breaks were increased by 0.05 Gy + M immediately after the combined exposure (*Day 0*).

In hTERT+ cells M + 0.05 Gy and 0.05 Gy + M caused a loss of clonogenic survival immediately after the combined exposure (*Day 0*), and M + 0.5 Gy resulted in a loss of clonogenic survival 30 days after the combined exposure (*Day 30*). In hTERT+ cells, MNi were induced by M + 0.5 Gy thirty days after the combined exposure (*Day 30*) and chromatid breaks were increased by M + 0.5 Gy immediately after the combined exposure (*Day 0*).

2. Synergistic effects were observed in both types of combinations.

The combined exposure of *Metal Followed by Radiation* showed synergism in the clonogenic assay, since there was loss of clonognic survival with M + 0.5 Gy at *Day 30* in hTERT- cells, M + 0.05 Gy at *Day 0* in hTERT+ cells and M + 0.5 Gy at *Day 30* in hTERT+ cells. In terms of MNi, this occurred in hTERT- cells immediately after the combined exposure (*Day 0*) and in hTERT+ cells 30 days after the combined exposure (*Day 30*), whereas in terms of chromatid breaks it only occurred in hTERT+ cells immediately after the combined exposure (*Day 0*).

The combined exposure of *Radiation Followed by Metal* showed synergism in the clonogenic assay, since there was loss of clonogenic survival with 0.05 Gy + M and 0.5 Gy + M in hTERT- cells 30 days after the combined exposure (*Day 30*), and 0.05 Gy + M in hTERT+ cells 30 days after the combined exposure (*Day 30*). In terms of chromatid breaks the synergism was shown only with 0.05 Gy + M in hTERT- cells immediately after the combined exposure (*Day 0*).

3. Synergistic effects may be related to genomic instability.

The evidence that synergism was present as delayed damage, and therefore likely to be linked with genomic instability, was provided by the loss of clonogenic survival with M + 0.5 Gy,

0.05 Gy + M and 0.5 Gy + M in hTERT- cells 30 days after the combined exposure, and with M + 0.5 Gy and 0.05 Gy + M in hTERT+ cells 30 days after the combined exposure. The evidence that synergism was also present as delayed damage was provided by the increase of MNi in hTERT+ cells 30 days after the combined exposure (*Day 30*) of *Metal Followed by Radiation* (M + 0.5 Gy). This demonstrated that genomic instability (delayed loss of clonogenic survival and delayed damage) occurred, in most of the cases, when metal treatment was in combination with radiation exposure.

Similar to the present work, Vitvitskii *et al* (1996) demonstrated that combined exposure of gamma rays and Cr (VI) can have enhancing effects on the outcome, compared to the single agent (either gamma rays or metal alone). In their studies, the effects of chromium ions (VI) on the mutagenic activity of gamma rays were assessed by a micronucleus test in mouse bone marrow polychromatocytes. They observed that chromium ions (VI) enhanced mutagenic effects of gamma rays in both acute and chronic experiments.

Other authors have shown the effects of synergism due to combined exposure of metal and radiation. Zaichkina *et al* (2001) demonstrated the induction of cytogenetic damages by combined action of heavy metal (lead and cadmium) salts and chronic/acute gamma irradiation in bone marrow cells of mice and rats. Anan'eva *et al* (2000) showed that combined exposure of rats to low-dose irradiation and heavy metal (Cu^{2+}) ions caused significant accumulation of the free-radical products proportional to exhausting antioxidant and oxidizing-reduction potential in various organs and tissues, compared to either radiation or metal only. Therefore, the synergistic effects observed after combined exposure of Cr (VI) + radiation (and *viceversa*) could be due to the accumulation of free-radical products. It was observed that neither exposure of mice to lead (in the drinking water) nor chronic gamma-irradiation of animals induces single-stranded DNA breaks in thymocytes (Chernikov *et al* 1998). Interestingly, combined exposure of acute gamma-irradiation (1 and 4 Gy) of mice, previously treated with

lead, resulted in an inhibition of repair of radiation-degraded DNA in thymocytes, and an increase of the level of DNA lesions detected in erythroblasts of bone marrow, detected by the micronuclear test method. This work reinforces the findings that combinations of metals and radiation are likely to induce DNA damage, due to their synergistic combined effects (Chernikov *et al* 1998).

4.3.6 Antagonistic Effects Caused by Combined Exposure of Metal and Radiation

Interestingly, there was also some data suggesting that the biological effects provoked by combined exposure of metal and radiation has led to an antagonistic action, compared to metal treatment only or radiation exposure only. These antagonistic effects were observed only in hTERT+ cells, since only the radiation exposure (0.5 Gy + VC), but not the combined exposure (0.5 Gy + M), resulted in a decrease (approximately 14.5%) in clonogenic survival immediately after the radiation exposure (*Day 0*) ($p<0.001$), which persisted up to 30 days after the radiation exposure ($p<0.001$) (Section 3.3.1.1, Figure 3.67d). However, this evaluation is very rudimentary, since there is no statistical data demonstrating that combined exposure of metal and radiation can lead to synergistic effects. Therefore, this data only suggests that these results could have led to an antagonistic outcome.

CHAPTER 5

CONCLUSION

5.1 Metal Exposure

5.2 Radiation Exposure

5.3 Combined Exposure

5.4 Discrepancies Among Different Experiments

5.5 Final Conclusion

5.1 Metal Exposure

• Both Cr (VI) and V (V) induced genomic instability in hTERT- cells.

• This phenomenon was reflected in several endpoints such as a persistent reduction in clonogenic survival (partially explained by senescence and apoptosis), a persistent induction of chromosome end joining/fusion (due to dicentric chromosomes and nucleoplasmic bridges) and a persistent incidence of chromosomal breakage (detected by chromatid breaks, chromosome fragments, chromatid gaps and micronuclei).

• Cr (VI) caused immediate chromosomal breakage, which was reduced with time possibly as a result of the death of severely damaged cells, leaving less damaged cells that might be vulnerable to chromosomal instability.

• Cr (VI) caused chromatid breaks without aneuploidy, whilst V (V) caused aneuploidy without chromatid breaks.

• Ectopic hTERT expression had a dramatic effect in preventing chromosome end joining/fusion with or without metal exposure.

• hTERT expression (using low doses of metals) protected against loss of metal-induced clonogenic survival, metal-induced senescence and metal-induced apoptosis.

• The increased apoptosis in the colonies of non metal treated hTERT+ cells compared to hTERT- cells was anomalous but reminiscent of the high levels of apoptosis in the premalignant skin tumour Bowen's disease, which is also known to be hTERT positive (Park *et al* 2004) as well as in many solid tumours.

• hTERT expression protected from an initial decrease in cell viability with the highest doses of metal used, 4 μM Cr (VI) and 50 μM V(V). However, there was a loss of cell viability in hTERT+ cells but this was significantly less compared to hTERT- cells.

• hTERT expression also protected from an initial increase in apoptosis and necrosis caused by both chemicals, in the cells regularly split and kept 70% confluent.

• Similar to other authors (Wise *et al* 2004), in the present work the hTERT+ cells showed an initial damage caused by metals as evidenced by increased chromosomal breaks. But there was no significant persistent increase in micronuclei formation or chromosomal breaks.

• However, hTERT+ cells did show a persistent increase in tetraploidy after metal exposure, which might be due to a dysfunctional G0/G1 checkpoint (Margolis *et al* 2003).

• Both Cr (VI) and V (V) caused a temporary (*Day 0*) high proportion of the hTERT- cells, but not hTERT+ cells, in the G0/G1 phase of the cell cycle, especially with V (V), and consequently a reduced proportion of the hTERT- cells in the S and G2/M phases of the cell cycle.

• Telomerase activity in hTERT+ cells was much higher than hTERT- cells and neither metals interfered significantly in the production of the telomerase activity, which was, as expected, much higher in hTERT+ cells compared to hTERT- cells.

• Neither Cr (VI) or V (V) caused a significant change in telomere lengths.

• Telomere length in hTERT- cells was generally shorter than hTERT+ cells and this difference became more pronounced 30 days after metal exposure. As others have shown (Sharma 2003), this small difference in telomere length, can be explained, by the fact that both cell lines were approximately examined at the same population doubling (after PD 50) and hTERT insertion would just have been expected to have become functional in hTERT+ cells.

• Doubling time in control hTERT- cells was 24.42 hours and in control hTERT+ cells was 18.71 hours. The effect of the highest dose of Cr (VI) (4 μM) and V (V) (50 μM) reduced the growth in hTERT- cells, and to a lesser extent in hTERT+ cells.

• The growth of metal treated cells differed depending on whether they were plated out as colonies (as it was done in the clonogenic assay, senescence assay and apoptosis assay) or

grown routinely at subconfluent levels (as it was done for all the other experiments). Cells plated to form colonies, gave rise to a permanent reduction in clonogenic survival in hTERT-treated cells, whereas the lack of changes in doubling time after metal treatment in cells grown at subconfluent levels, can also be related to only a small and temporary induction of apoptosis (determined by flow cytometer), and by only temporary changes in the proportion of these cells in different phases of the cell cycle, including G0/G1.

• The cause of this difference in cell growth is not known, but there might be two mechanisms by which this phenomenum could be explained.

a) It is possible that the level of genomic instability in the metal treated hTERT- cells was higher in the colonies as a result of the microenvironment (Bindra and Glazer 2005), which would lead to difference in growth of the colonies and the subconfluent cells after metal treatment.

b) It is possible that the difference in growth of the colonies and the subconfluent cells after metal treatment occurred as a result of increased bystander effects, for example including those mediated by gap junctions (Little 2003). Therefore, hTERT could provide a genetic influence for the control of the bystander effect (Mothersill and Seymour 2004).

5.2 Radiation Exposure

• The clonogenic assay demonstrated that radiation induced genomic instability in hTERT+ cells, but not in hTERT- cells. The higher dose of radiation (0.5 Gy) caused more genomic instability compared to the lower dose of radiation (0.05 Gy).

• This data showed that the telomerase activity in hTERT+ cells did not provide protection against genomic instability caused by the radiation insult.

• Neither 0.05 Gy nor 0.5 Gy induced chromosomal instability in either types of cells used (hTERT- and hTERT+ cells). However, the higher dose of radiation (0.5 Gy) caused cytogenetic damage in hTERT+ cells (but no significant damage in hTERT- cells), which did not persist up to 30 days after radiation exposure.

• hTERT+ cells were more vulnerable to damage such as micronuclei (MNi), immediately after radiation exposure of 0.5 Gy ($p<0.05$) compared to hTERT- cells.

• Neither hTERT- cells nor hTERT+ cells showed a significant increase in nucleoplasmic bridges (NPB), due to radiation.

• Cytogenetic abnormalities such as chromatid breaks, chromatid gaps, chromatid fragments, dicentric chromosomes and tetraploidy, when observed, were not found to be statistically significant, in either types of cells.

• At both survival times, with or without radiation exposure, there was a lower level of dicentric chromosomes in the hTERT+ cells compared to the hTERT- cells. This was statistically significant 30 days after radiation exposure to the 0.5 Gy dose ($p<0.05$).

• In all of the experiments, at both survival times, with or without radiation exposure, there was a higher level of tetraploidy in the hTERT+ cells compared to the hTERT- cells. This was statistically significant immediately after the radiation exposure (*Day 0*) of 0.05 Gy dose ($p<0.05$).

• Ectopic hTERT expression had no effect in preventing the formation of MNi (chromosome breakage) after radiation exposure, since 0.5 Gy caused significant MNi ($p<0.05$) in immortalised hTERT+ cells at *Day 0*.

• This was different to what it was seen in the experiments of metal only treated [Cr (VI)] cells, where hTERT- cells showed significant cell damage (such as MNi and breaks) and this damage was less compared to hTERT+ cells.

5.3 Combined Exposure

• Combined exposure caused genomic instability (loss of clonogenic survival) in both types of cells.

• Combined exposure of *Metal Followed by Radiation* caused more genomic instability (loss of clonogenic survival) in hTERT+ cells (91.06 in M + 0.05 Gy and 88.46 in M + 0.5 Gy), while combined exposure of *Radiation Followed by Metal* caused more genomic instability (loss of clonogenic survival) in hTERT- cells (84.35 in 0.05 Gy + M and 81.36 in 0.5 Gy + M).

• The results of the micronucleus assay showed that Cr (VI), followed by the higher dose of radiation (M + 0.5 Gy) can induce genomic instability.

• Combined exposure of *Metal Followed by Radiation* caused cytogenetic damage (MNi and breaks) in hTERT+ cells, while combined exposure of *Radiation Followed by Metal* caused cytogenetic damage (MNi and breaks) in hTERT- cells

• This genomic instability was shown by persistent cell damage such as micronuclei (MNi) in human fibroblasts. However, statistical significance of increased frequency of MNi, 30 days after combined exposure (M + 0.5 Gy), was only detected in hTERT+ cells, but not in hTERT- cells.

• In all of the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was a lower level of MNi and nucleoplasmic bridges (NPB) in the hTERT+ cells compared to the hTERT- cells. This was particularly true at *Day 30.*

• A direct comparison of hTERT- and hTERT+ cells revealed that the excess of dicentric chromosomes in hTERT- cells after combined exposure (M + 0.5 Gy) was statistically significant, at both survival times ($p<0.05$), compared to that in the hTERT+ cells.

• A direct comparison of hTERT- and hTERT+ cells showed that in all the experiments, at both survival times, with or without either metal treatment or radiation exposure or combined exposure, there was more tetraploidy in the hTERT+ cells compared to hTERT- cells.

• It is likely that some of the biological effects provoked by combined exposure of metal and radiation have led to a synergistic action, compared to metal treatment only or radiation exposure only. In fact, in most of the significant results, the damage caused by the combination of metal and radiation was higher than the damage induced by either metal itself or radiation itself.

• Synergistic effects due to combined exposure were observed in both types of cells.

• Synergistic effects due to combined exposure were observed in both types of combinations (*Metal Followed by Radiation* and *Radiation Followed by Metal*).

• Interestingly, there was also some data suggesting that the biological effects provoked by combined exposure of metal and radiation has led to an antagonistic action, compared to metal treatment only or radiation exposure only. These antagonistic effects were observed only in hTERT+ cells, since only the radiation exposure (0.5 Gy + VC), but not the combined exposure (0.5 Gy + M), resulted in a decrease in clonogenic survival at *Day 0*, which persisted up to 30 days after the radiation exposure.

• As well as in the experiments of radiation only exposed cells, it was interesting to observe that ectopic hTERT expression had no effect in preventing the loss of clonogenic survival after combined exposure of *Metal Followed by Radiation* (M + 0.05 Gy and M + 0.5 Gy), and *Radiation Followed by Metal* (0.05 Gy + M). In fact, these combinations (especially the *Metal Followed by Radiation*) caused a significant loss of clonogenic survival after combined exposure, which persisted up to *Day 30* in immortalised hTERT+ cells.

• As well as in the experiments of radiation only exposed cells, it was interesting to observe that ectopic hTERT expression had no effect in preventing the formation of MNi (chromosome

breakage) after combined exposure of *Metal Followed by Radiation* (M + 0.5 Gy), since this combination caused significant MNi, which persisted up to *Day 30* in immortalised hTERT+ cells, but not in normal hTERT- cells.

• This was different to what it was detected in the experiments of metal only treated [Cr (VI)] cells, where hTERT- cells showed significant cell damage (such as MNi and breaks), which was less compared to the damage observed in hTERT+ cells.

5.4 Discrepancies Among Different Experiments

The results obtained with Cr (VI) in the metal only treatment experiments were somewhat different compared to the results obtained with Cr (VI) - used as a control treatment - in the combined exposure experiments. Some examples have been listed below.

• Cr (VI) caused a significant incidence of chromatid breaks, dicentric chromosomes and nucleoplasmic bridges in hTERT- cells in the metal only treatment experiments, but not when used as a control treatment in the combined exposure experiments.

• Cr (VI) caused a significant increase in tetraploidy in hTERT+ cells in the metal only treatment experiments, but not when used as a control treatment in the combined exposure experiments.

• Cr (VI) caused a significant loss of clonogenic survival in the metal only treatment experiments ($p<0.001$ at *Day 30*), a slightly less significant loss of clonogenic survival when used as a control treatment (*Sham Irradiation Followed by Metal*, SI + M) in the combined exposure experiments ($p<0.01$ at *Day 30*), and no significant loss of clonogenic survival when used as a control treatment (*Metal Followed by Sham Irradiation*, M + SI) in the combined exposure experiments. However, Cr (VI) caused no significant loss of clonogenic survival in the metal only treatment experiments at *Day 0*, whereas *Metal Followed by Sham Irradiation* (M + SI) and *Sham Irradiation Followed by Metal* (SI + M), when used as a control treatment in the combined exposure experiments, caused a loss of clonogenic survival at *Day 0* ($p<0.01$ and $p<0.05$ respectively).

It is important to understand why there were discrepancies in the results with Cr (VI), depending if it was used either as a *Treatment* (in the metal only experiments) or as a *Control* treatment (in the combined exposure experiments).

It might be possible that these discrepancies of results are because both types of human fibroblasts used for this work (hTERT- cells and hTERT+ cells) had become older (or alternatively had been damaged) since the first time they were used. In fact, the metal only treatment experiments were carried out in the first year of this project, whereas the Cr (VI) was used as a control treatment in the combined exposure experiments in the third year of this project. Therefore, it was useful to determine the Mean Counts (MC) of the *Control* cells at both stages (in the period when the single treatment experiments were performed and in the period when the combined exposure experiments were performed). These results showed that the MC, and therefore the cell proliferation, was lower in the *Old hTERT- Cells* compared to the *Young hTERT- Cells*, especially at *Day 30* (Figure 5.1a). However, the MC of the *Old hTERT+ Cells* was slightly higher than the MC of the *Young hTERT+ Cells* at *Day 0* (Figure 5.1b). The MC of the *Old hTERT+ Cells* was slightly lower than the MC of the *Young hTERT+ Cells* at *Day 30* (Figure 5.1b). This shows that the reduction of the cell proliferation, between *Old Cells* and *Young Cells*, was more evident in hTERT- cells compared to the hTERT+ cells, especially at *Day 30*.

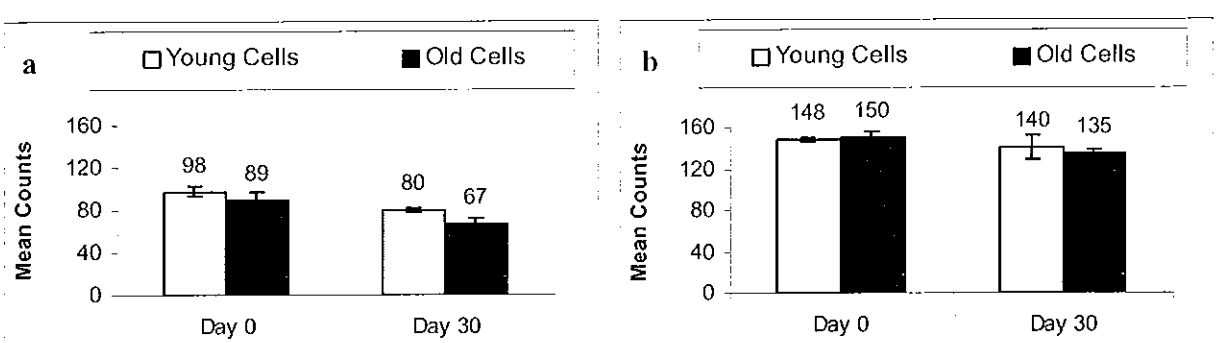

Figure 5.1. Mean Counts (MC). The figures show the MC in hTERT– cells (Fig. 5.1a) and hTERT+ cells (Fig. 5.1b) of the *Control* values and at different times. The MC values are displayed above the columns of the diagrams.

Zhu *et al* (2006) have shown that a reduction in cell proliferation is likely to stop the cells at the G2/M phase of the cell cycle. This can induce apoptosis, and therefore it may reduce the damage that cells normally gain. This could be the reason why the *Young hTERT- Cells*, used for the metal only experiments [where Cr (VI) was the *Treatment*] showed more damage with Cr (VI), compared to the *Old hTERT- Cells*, used for the combined exposure experiments [where Cr (VI) was the *Control* treatment]. Besides, the reduced proliferation in *Old hTERT+ Cells*, may have partially stopped the cell cycle in these cells, which therefore showed a lower incidence of tetraploidy caused by Cr (VI) in the combined exposure experiments [where Cr (VI) was the *Control* treatment].

Furthermore, it is important to specify that in the metal only treatment experiments the protocols for Clonogenic Assay, Micronucleus Assay, and Chromosomal Aberration Analysis were carried out in two days, whereas in the combined exposure experiments - when Cr (VI) was used as a control treatment - the same protocols were carried out in three days. This was necessary because the combined exposure experiments required two different 24-hour exposures (metal followed by radiation and *viceversa*). It may be possible that a longer time in culture of the hTERT- cells could have conferred more resistance to the Cr (VI) insult, and therefore reduced the cytogenetic damage which was detected when Cr (VI) was used as a *Treatment* in the metal only treatment experiments. Besides, a longer time in culture of the hTERT+ cells could have conferred more resistance to the Cr (VI) insult, and therefore reduced the percentage of tetraploidy which was observed when Cr (VI) was used as a *Treatment* in the metal only treatment.

Moreover, there are two important differences in the protocols (between the metal only treatment experiments and the combined exposure experiments), which need to be mentioned:

1. In the combined exposure experiments - when Cr (VI) was used as a *Control* treatment – Cr (VI) was added, to the cells in culture, 48 hours before harvesting [(in the *Metal followed by*

Sham Irradiation (M + SI) experiments], and obviously washed (eliminated) 24 hours before harvesting. Therefore, it might be possible that during the last 24 hours, when there was no Cr (VI) exposure, the cells could have partially recovered from the Cr (VI) insult, resulting in a reduction of damage caused by the metal.

2. In the combined exposure experiments - when Cr (VI) was used as a *Control* treatment – Cr (VI) was added, to the cells in culture, 24 hours before harvesting [(in the *Sham Irradiation followed by Metal* (SI + M) experiments], and obviously the number of cells in the flasks at that time, was higher since the cells had been in culture 48 hours (instead of 24 hours) when Cr (VI) was added to the flasks. Therefore, this higher number of cells in the flasks at the moment of treatment might have reduced the Cr (VI) toxicity, as it may have decreased the ratio of Cr (VI) ions per cell.

5.5 Final Conclusion

In conclusion, these have been the main achievements of this project.

1. Metals induced genomic instability in hTERT- cells (loss of clonogenic survival and cytogenetic damage) and hTERT+ cells (tetraploidy), radiation induced genomic instability only in hTERT+ cells (loss of clonogenic survival), and combined exposure caused genomic instability hTERT- cells (loss of clonogenic survival) and hTERT+ cells (loss of clonogenic survival).

2. Ectopic hTERT expression had a dramatic effect in preventing genomic instability (especially chromosome end joining/fusion) with metal exposure, whereas telomerase activity in hTERT+ cells did not provide protection against genomic instability (loss of clonogenic survival) caused by the radiation insult.

3. Combined exposure of *Metal Followed by Radiation* caused more genomic instability (loss of clonogenic survival) in hTERT+ cells, while combined exposure of *Radiation Followed by Metal* caused more genomic instability (loss of clonogenic survival) in hTERT- cells. Therefore, it might be possible that ectopic hTERT expression could prevent genomic instability in hTERT+ cells only in the combined exposure of *Radiation Followed by Metal.*

4. It is likely that the biological effects provoked by combined exposure of metal and radiation has led to a synergistic action, compared to metal treatment only or radiation exposure only. In fact, synergistic effects due to combined exposure were observed in both types of cells and in both types of combinations (*Metal Followed by Radiation* and *Radiation Followed by Metal*).

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APPENDICES

APPENDIX 1: Tables (Metal Exposure)

APPENDIX II: Tables (Radiation Exposure)

APPENDIX III: Tables (Combined Exposure)

APPENDIX I

Tables (Metal Exposure)

Table 1a. Dead Cells (%), Mean Counts (№ of Cells) and Doubling Time (hours) in hTERT-Cells after a 24 hours exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 0	Control	3.54 ± 0.55	97.67 ± 4.51	24.42 ± 0.49
	0.04 μM Cr (VI)	4.54 ± 0.55	92.67 ± 3.06	25.40 ± 0.54
	0.4 μM Cr (VI)	9.68 ± 2.60	95 ± 3.61	24.92 ± 0.51
	4 μM Cr (VI)	29.39 ± 2.33	58.67 ± 3.51	39.00 ± 1.53
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 5	Control	1.88 ± 0.94	94.33 ± 2.52	25.05 ± 0.52
	0.04 μM Cr (VI)	4.46 ± 1.48	95 ± 3.00	24.92 ± 0.51
	0.4 μM Cr (VI)	4.14 ± 1.13	92.33 ± 4.04	25.47 ± 0.55
	4 μM Cr (VI)	17.27 ± 5.66	72.67 ± 2.52	31.18 ± 0.90
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 30	Control	3.56 ± 0.59	80.00 ± 2.00	28.6 ± 0.73
	0.04 μM Cr (VI)	4.57 ± 1.48	79.33 ± 0.58	28.81 ± 0.74
	0.4 μM Cr (VI)	5.53 ± 1.47	77.67 ± 3.51	29.35 ± 0.78
	4 μM Cr (VI)	9.98 ± 1.35	63.67 ± 2.08	35.59 ± 1.24

hTERT- Cells

Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 0	Control	3.54 ± 0.55	97.67 ± 4.51	24.42 ± 0.49
	0.5 μM V (V)	7.67 ± 1.22	92.33 ± 3.79	25.47 ± 0.55
	5 μM V (V)	11.01 ± 4.52	82.33 ± 4.51	27.91 ± 0.69
	50 μM V (V)	43.44 ± 5.71	39.33 ± 1.53	73.41 ± 6.02
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 5	Control	1.88 ± 0.94	94.33 ± 2.52	25.05 ± 0.52
	0.5 μM V (V)	4.54 ± 0.58	92.67 ± 1.53	25.4 ± 0.54
	5 μM V (V)	3.75 ± 0.95	84.33 ± 3.51	27.36 ± 0.65
	50 μM V (V)	34.11 ± 5.75	43.33 ± 3.06	60.49 ± 4.04
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 30	Control	3.56 ± 0.59	80.00 ± 2.00	28.6 ± 0.73
	0.5 μM V (V)	5.70 ± 1.00	81.33 ± 5.51	28.2 ± 0.70
	5 μM V (V)	10.59 ± 1.77	73.33 ± 3.51	30.92 ± 0.89
	50 μM V (V)	9.27 ± 0.79	58.33 ± 2.52	39.27 ± 1.56

Table 1b. Dead Cells (%), Mean Counts (№ of Cells) and Doubling Time (hours) in hTERT+ Cells after a 24 hours exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 0	Control	2.61 ± 0.55	148.00 ± 2.00	18.71 ± 0.24
	0.04 µM Cr (VI)	2.25 ± 0.54	148.67 ± 2.52	18.66 ± 0.23
	0.4 µM Cr (VI)	4.48 ± 0.64	141.00 ± 7.55	19.23 ± 0.26
	4 µM Cr (VI)	18.36 ± 3.31	93.67 ± 4.04	25.19 ± 0.53
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 5	Control	2.21 ± 0.52	152.00 ± 2.00	18.43 ± 0.23
	0.04 µM Cr (VI)	1.30 ± 0.55	149.00 ± 1.73	18.64 ± 0.23
	0.4 µM Cr (VI)	1.90 ± 0.93	152.00 ± 3.00	18.43 ± 0.23
	4 µM Cr (VI)	11.84 ± 1.54	130.00 ± 2.00	20.18 ± 0.29
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 30	Control	2.23 ± 0.53	140.33 ± 11.15	19.29 ± 0.26
	0.04 µM Cr (VI)	3.81 ± 0.94	137.33 ± 7.02	19.53 ± 0.27
	0.4 µM Cr (VI)	6.45 ± 0.53	136.67 ± 10.02	19.59 ± 0.27
	4 µM Cr (VI)	6.18 ± 2.16	104.67 ± 7.02	23.24 ± 0.43

hTERT+ Cells

Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 0	Control	2.61 ± 0.55	148.00 ± 2.00	18.71 ± 0.24
	0.5 µM V (V)	5.40 ± 0.63	150.67 ± 3.21	18.52 ± 0.23
	5 µM V (V)	5.42 ± 1.88	130.67 ± 5.13	20.12 ± 0.29
	50 µM V (V)	22.50 ± 6.05	86.33 ± 5.13	26.85 ± 0.62
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 5	Control	2.21 ± 0.52	152.00 ± 2.00	18.43 ± 0.23
	0.5 µM V (V)	2.59 ± 0.52	142.33 ± 4.51	19.13 ± 0.25
	5 µM V (V)	3.88 ± 1.04	145.33 ± 2.52	18.9 ± 0.24
	50 µM V (V)	20.30 ± 4.81	110.33 ± 11.93	22.41 ± 0.39
Day Post Treatment	Treatment	Dead Cells	Mean Counts	Doubling Time (h)
Day 30	Control	2.23 ± 0.53	140.33 ± 11.15	19.29 ± 0.26
	0.5 µM V (V)	5.21 ± 1.22	127.67 ± 9.87	20.4 ± 0.30
	5 µM V (V)	4.79 ± 0.80	130.67 ± 11.59	20.12 ± 0.29
	50 µM V (V)	3.77 ± 0.97	126.33 ± 5.86	20.54 ± 0.31

Table 2a. Clonogenic Survival [these values were expressed as a percentage of control (PBS) and control was set to 100%], Senescence (%), Apoptosis (%) and Necrosis (%) in hTERT-Cells after a 24 hours exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 0	Control	100 ± 2.81	0.00 ± 0.00	15.33 ± 3.06	0.00 ± 0.00
	0.04 μM Cr (VI)	95.47 ± 6.31	0.00 ± 0.00	16.67 ± 6.11	0.00 ± 0.00
	0.4 μM Cr (VI)	75.89 ± 3.71	0.92 ± 1.60	27.00 ± 6.24	0.00 ± 0.00
	4 μM Cr (VI)	34.47 ± 4.54	0.00 ± 0.00	41.12 ± 4.39	0.67 ± 1.15
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 5	Control	100 ± 2.56	0.00 ± 0.00	14.17 ± 6.18	0.00 ± 0.00
	0.04 μM Cr (VI)	84.80 ± 3.55	0.00 ± 0.00	16.67 ± 4.16	0.00 ± 0.00
	0.4 μM Cr (VI)	66.50 ± 5.80	5.82 ± 1.84	28.00 ± 4.58	0.00 ± 0.00
	4 μM Cr (VI)	43.30 ± 5.35	5.81 ± 5.04	40.89 ± 5.54	0.33 ± 0.58
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 30	Control	100 ± 3.87	1.19 ± 1.05	23.00 ± 4.36	0.67 ± 1.15
	0.04 μM Cr (VI)	87.06 ± 4.28	0.81 ± 0.71	28.00 ± 4.00	0.67 ± 1.15
	0.4 μM Cr (VI)	63.65 ± 4.43	12.15 ± 9.46	37.33 ± 9.02	1.00 ± 1.73
	4 μM Cr (VI)	50.18 ± 5.05	16.61 ± 8.75	44.06 ± 12.52	1.29 ± 1.12

hTERT- Cells

Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 0	Control	100 ± 2.81	0.00 ± 0.00	15.33 ± 3.06	0.00 ± 0.00
	0.5 μM V (V)	93.85 ± 3.23	0.00 ± 0.00	23.33 ± 5.51	0.00 ± 0.00
	5 μM V (V)	68.28 ± 4.86	1.81 ± 1.59	26.33 ± 4.93	0.00 ± 0.00
	50 μM V (V)	2.27 ± 0.79	1.88 ± 1.79	30.69 ± 10.87	1.85 ± 3.20
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 5	Control	100 ± 2.56	0.00 ± 0.00	14.17 ± 6.18	0.00 ± 0.00
	0.5 μM V (V)	82.52 ± 5.66	0.00 ± 0.00	22.33 ± 3.21	0.33 ± 0.58
	5 μM V (V)	54.41 ± 7.28	8.14 ± 7.44	26.00 ± 7.21	0.00 ± 0.00
	50 μM V (V)	10.95 ± 1.44	4.41 ± 3.97	27.77 ± 5.99	0.79 ± 1.37
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 30	Control	100 ± 3.87	1.19 ± 1.05	23.00 ± 4.36	0.67 ± 1.15
	0.5 μM V (V)	71.28 ± 3.81	2.48 ± 2.17	31.08 ± 3.00	0.67 ± 1.15
	5 μM V (V)	67.02 ± 7.55	15.71 ± 8.10	40.34 ± 12.82	1.00 ± 1.73
	50 μM V (V)	33.16 ± 6.15	20.55 ± 4.20	37.80 ± 8.41	3.76 ± 0.29

Table 2b. Clonogenic Survival [these values were expressed as a percentage of control (PBS) and control was set to 100%], Senescence (%), Apoptosis (%) and Necrosis (%) in hTERT+ Cells after a 24 hours exposure to three different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 0	Control	100 ± 3.83	0.00 ± 0.00	32.33 ± 6.03	0.00 ± 0.00
	0.04 μM Cr (VI)	97.28 ± 3.66	0.00 ± 0.00	29.33 ± 4.51	0.00 ± 0.00
	0.4 μM Cr (VI)	98.53 ± 2.82	0.00 ± 0.00	35.33 ± 3.21	0.00 ± 0.00
	4 μM Cr (VI)	56.74 ± 2.46	0.00 ± 0.00	38.10 ± 9.03	0.33 ± 0.58
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 5	Control	100 ± 2.55	0.00 ± 0.00	31.00 ± 3.00	0.00 ± 0.00
	0.04 μM Cr (VI)	99.53 ± 2.01	0.00 ± 0.00	30.33 ± 4.93	0.00 ± 0.00
	0.4 μM Cr (VI)	94.64 ± 2.64	0.00 ± 0.00	36.00 ± 5.29	0.00 ± 0.00
	4 μM Cr (VI)	60.77 ± 3.80	0.40 ± 0.70	34.42 ± 2.67	0.00 ± 0.00
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 30	Control	100 ± 2.73	0.00 ± 0.00	28.67 ± 5.86	0.00 ± 0.00
	0.04 μM Cr (VI)	102.80 ± 3.44	0.00 ± 0.00	28.33 ± 4.16	0.67 ± 1.15
	0.4 μM Cr (VI)	96.23 ± 3.34	0.00 ± 0.00	30.00 ± 6.00	0.67 ± 1.15
	4 μM Cr (VI)	74.70 ± 7.32	1.95 ± 1.89	37.67 ± 7.77	2.67 ± 1.53

hTERT+ Cells

Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 0	Control	100 ± 3.83	0.00 ± 0.00	32.33 ± 6.03	0.00 ± 0.00
	0.5 μM V (V)	96.71 ± 2.30	0.00 ± 0.00	30.67 ± 6.11	0.33 ± 0.58
	5 μM V (V)	88.22 ± 1.69	0.00 ± 0.00	34.94 ± 3.10	0.00 ± 0.00
	50 μM V (V)	18.69 ± 2.64	0.00 ± 0.00	41.22 ± 3.75	0.33 ± 0.58
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 5	Control	100 ± 2.55	0.00 ± 0.00	31.00 ± 3.00	0.00 ± 0.00
	0.5 μM V (V)	97.79 ± 2.54	0.00 ± 0.00	29.33 ± 4.51	0.00 ± 0.00
	5 μM V (V)	91.85 ± 1.31	0.00 ± 0.00	34.65 ± 7.03	0.00 ± 0.00
	50 μM V (V)	35.04 ± 3.43	0.38 ± 0.66	43.38 ± 7.28	0.67 ± 1.15
Day Post Treatment	Treatment	Colonies	Senescence	Apoptosis	Necrosis
Day 30	Control	100 ± 2.73	0.00 ± 0.00	28.67 ± 5.86	0.00 ± 0.00
	0.5 μM V (V)	96.96 ± 1.49	0.00 ± 0.00	30.00 ± 2.00	0.33 ± 0.58
	5 μM V (V)	99.51 ± 2.38	0.00 ± 0.00	31.50 ± 6.87	0.67 ± 1.15
	50 μM V (V)	62.77 ± 4.40	2.80 ± 1.93	36.04 ± 13.20	1.39 ± 2.40

Table 3a. Micronuclei (MNi) (%), Nucleoplasmic Bridges (NPB) (%), № of Binucleated Cells (BNC), Nuclear Division Index (NDI) and Nuclear Division Cytotoxicity Index (NDCI) in hTERT- Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 0	Control	1.50 ± 0.30	1.50 ± 0.87	127.67 ± 28.73	1.255 ± 0.057	1.247 ± 0.059
	0.04 μM Cr (VI)	2.50 ± 0.36	2.23 ± 0.51	132.00 ± 3.61	1.261 ± 0.002	1.254 ± 0.011
	0.4 μM Cr (VI)	4.13 ± 1.70	2.83 ± 1.25	131.00 ± 14.93	1.262 ± 0.030	1.243 ± 0.31
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 5	Control	2.57 ± 0.21	2.17 ± 1.12	82.33 ± 13.58	1.165 ± 0.027	1.159 ± 0.028
	0.04 μM Cr (VI)	3.23 ± 0.21	3.07 ± 0.80	73.00 ± 25.53	1.146 ± 0.051	1.141 ± 0.052
	0.4 μM Cr (VI)	4.47 ± 1.27	2.60 ± 1.44	79.00 ± 35.00	1.158 ± 0.070	1.14 ± 0.064
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 30	Control	2.57 ± 0.68	3.23 ± 1.21	82.33 ± 36.56	1.165 ± 0.073	1.155 ± 0.075
	0.04 μM Cr (VI)	4.57 ± 1.07	7.17 ± 0.75	104.67 ± 33.86	1.209 ± 0.068	1.193 ± 0.073
	0.4 μM Cr (VI)	6.00 ± 1.75	6.47 ± 1.77	105.67 ± 31.77	1.211 ± 0.064	1.195 ± 0.066

hTERT- Cells

Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 0	Control	1.50 ± 0.30	1.50 ± 0.87	127.67 ± 28.73	1.255 ± 0.057	1.247 ± 0.059
	0.05 μM V (V)	3.10 ± 0.40	2.90 ± 0.89	126.67 ± 6.03	1.247 ± 0.005	1.245 ± 0.009
	5 μM V (V)	2.57 ± 0.25	2.73 ± 1.27	118.67 ± 6.03	1.237 ± 0.012	1.205 ± 0.020
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 5	Control	2.57 ± 0.21	2.17 ± 1.12	82.33 ± 13.58	1.165 ± 0.027	1.159 ± 0.028
	0.05 μM V (V)	3.43 ± 0.75	2.73 ± 0.50	76.33 ± 24.79	1.153 ± 0.050	1.146 ± 0.047
	5 μM V (V)	5.20 ± 0.89	2.83 ± 0.93	71.67 ± 20.82	1.143 ± 0.042	1.115 ± 0.039
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 30	Control	2.57 ± 0.68	3.23 ± 1.21	82.33 ± 36.56	1.165 ± 0.073	1.155 ± 0.075
	0.05 μM V (V)	4.23 ± 1.00	6.67 ± 0.32	105.67 ± 41.02	1.211 ± 0.082	1.199 ± 0.086
	5 μM V (V)	5.07 ± 1.89	7.97 ± 2.50	106.00 ± 37.64	1.212 ± 0.075	1.191 ± 0.077

Table 3b. Micronuclei (MNi) (%), Nucleoplasmic Bridges (NPB) (%), № of Binucleated Cells (BNC), Nuclear Division Index (NDI) and Nuclear Division Cytotoxicity Index (NDCI) in hTERT+ Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 0	Control	0.63 ± 0.32	0.47 ± 0.72	188.00 ± 27.73	1.376 ± 0.055	1.374 ± 0.056
	0.04 µM Cr (VI)	0.87 ± 0.29	0.40 ± 0.46	194.00 ± 6.56	1.388 ± 0.013	1.385 ± 0.015
	0.4 µM Cr (VI)	1.13 ± 0.06	0.27 ± 0.06	189.00 ± 16.70	1.378 ± 0.033	1.371 ± 0.034
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 5	Control	1.17 ± 0.29	0.63 ± 0.78	172.33 ± 33.71	1.345 ± 0.067	1.341 ± 0.067
	0.04 µM Cr (VI)	0.97 ± 0.49	0.57 ± 0.57	152.33 ± 10.69	1.305 ± 0.021	1.300 ± 0.021
	0.4 µM Cr (VI)	2.67 ± 1.03	0.93 ± 0.61	147.33 ± 14.15	1.295 ± 0.028	1.284 ± 0.030
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 30	Control	0.83 ± 0.23	0.27 ± 0.46	193.00 ± 33.51	1.386 ± 0.067	1.381 ± 0.068
	0.04 µM Cr (VI)	0.90 ± 0.44	0.33 ± 0.42	186.00 ± 58.64	1.372 ± 0.117	1.367 ± 0.115
	0.4 µM Cr (VI)	0.80 ± 0.20	0.43 ± 0.58	204.00 ± 42.93	1.408 ± 0.086	1.404 ± 0.088

hTERT+ Cells

Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 0	Control	0.63 ± 0.32	0.47 ± 0.72	188.00 ± 27.73	1.376 ± 0.055	1.374 ± 0.056
	0.05 µM V (V)	0.97 ± 0.42	0.53 ± 0.51	198.33 ± 39.93	1.397 ± 0.080	1.396 ± 0.081
	5 µM V (V)	1.22 ± 0.31	0.43 ± 0.51	190.33 ± 40.25	1.381 ± 0.081	1.37 ± 0.077
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 5	Control	1.17 ± 0.29	0.63 ± 0.78	172.33 ± 33.71	1.345 ± 0.067	1.341 ± 0.067
	0.05 µM V (V)	1.20 ± 0.56	0.40 ± 0.26	127.67 ± 11.59	1.255 ± 0.023	1.248 ± 0.024
	5 µM V (V)	2.33 ± 1.21	0.83 ± 0.93	117.33 ± 3.21	1.235 ± 0.006	1.224 ± 0.007
Day Post Treatment	Treatment	MNi	NPB	BNC	NDI	NDCI
Day 30	Control	0.83 ± 0.23	0.27 ± 0.46	193.00 ± 33.51	1.386 ± 0.067	1.381 ± 0.068
	0.05 µM V (V)	1.07 ± 0.42	0.47 ± 0.46	170.00 ± 61.94	1.340 ± 0.124	1.337 ± 0.123
	5 µM V (V)	0.90 ± 0.17	0.50 ± 0.36	156.33 ± 57.55	1.313 ± 0.115	1.307 ± 0.116

Table 4a. Chromatid Breaks (%), Chromatid Fragments (%), Chromatid Gaps (%) and Chromosome Rings (%) in hTERT- Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 0	Control	0.67 ± 0.58	0.67 ± 1.15	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	2.00 ± 2.00	0.67 ± 1.15	2.33 ± 1.53	0.67 ± 1.15
	0.4 µM Cr (VI)	10.33 ± 4.73	3.00 ± 1.73	0.33 ± 0.58	0.00 ± 0.00
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 5	Control	0.67 ± 0.58	1.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	0.67 ± 1.15	0.67 ± 1.15	1.00 ± 1.73	1.00 ± 1.73
	0.4 µM Cr (VI)	4.00 ± 1.00	1.33 ± 1.53	0.00 ± 0.00	0.00 ± 0.00
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 30	Control	0.33 ± 0.58	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	0.67 ± 1.15	0.33 ± 0.58	0.67 ± 1.15	0.67 ± 1.15
	0.4 µM Cr (VI)	2.33 ± 2.08	0.67 ± 1.15	0.00 ± 0.00	0.00 ± 0.00

hTERT- Cells

Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 0	Control	0.67 ± 0.58	0.67 ± 1.15	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	2.67 ± 2.52	0.33 ± 0.58	1.00 ± 1.73	0.33 ± 0.58
	5 µM V (V)	2.00 ± 1.73	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 5	Control	0.67 ± 0.58	1.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	3.00 ± 3.00	2.00 ± 2.00	0.67 ± 1.15	0.67 ± 1.15
	5 µM V (V)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.58
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 30	Control	0.33 ± 0.58	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	2.33 ± 0.58	0.33 ± 0.58	0.67 ± 1.15	0.33 ± 0.58
	5 µM V (V)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 4b. Chromatid Breaks (%), Chromatid Fragments (%), Chromatid Gaps (%) and Chromosome Rings (%) in hTERT+ Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 0	Control	1.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	2.33 ± 2.31	0.33 ± 0.58	0.67 ± 0.58	0.33 ± 0.58
	0.4 µM Cr (VI)	5.67 ± 3.21	1.00 ± 1.00	0.33 ± 0.58	0.00 ± 0.00
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 5	Control	0.67 ± 1.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	1.00 ± 0.00	0.67 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
	0.4 µM Cr (VI)	0.67 ± 1.15	1.67 ± 1.53	0.00 ± 0.00	0.00 ± 0.00
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 30	Control	1.33 ± 2.31	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	1.33 ± 0.58	0.67 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
	0.4 µM Cr (VI)	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

hTERT+ Cells

Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 0	Control	1.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	1.33 ± 1.15	0.00 ± 0.00	1.67 ± 2.08	0.00 ± 0.00
	5 µM V (V)	3.67 ± 2.89	0.33 ± 0.58	0.67 ± 1.15	0.00 ± 0.00
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 5	Control	0.67 ± 1.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	1.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	5 µM V (V)	2.67 ± 3.06	1.00 ± 1.00	0.00 ± 0.00	0.33 ± 0.58
Day Post Treatment	Treatment	Breaks	Fragments	Gaps	Rings
Day 30	Control	1.33 ± 2.31	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	1.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	5 µM V (V)	3.33 ± 2.08	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.58

Table 4c. Dicentric Chromosomes (%), Telomeric Associations (%), Tetraploidy (%) and Mitotic Index (%) in hTERT- Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 1.15	4.43 ± 0.21
	0.04 μM Cr (VI)	1.00 ± 1.73	1.33 ± 1.53	1.33 ± 0.58	2.67 ± 0.42
	0.4 μM Cr (VI)	1.67 ± 2.89	0.33 ± 0.58	3.67 ± 2.08	1.97 ± 0.25
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 5	Control	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 1.00	4.53 ± 0.40
	0.04 μM Cr (VI)	3.33 ± 3.06	0.67 ± 1.15	2.00 ± 1.00	3.57 ± 0.35
	0.4 μM Cr (VI)	4.33 ± 0.58	0.00 ± 0.00	1.00 ± 1.00	2.97 ± 0.25
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	2.67 ± 2.31	3.73 ± 0.35
	0.04 μM Cr (VI)	1.00 ± 1.73	0.67 ± 1.15	2.33 ± 0.58	3.57 ± 0.35
	0.4 μM Cr (VI)	6.33 ± 4.04	0.67 ± 1.15	2.67 ± 2.52	2.50 ± 0.53

hTERT- Cells

Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 1.15	4.43 ± 0.21
	0.5 μM V (V)	7.67 ± 3.21	2.67 ± 2.52	3.33 ± 1.53	3.10 ± 0.30
	5 μM V (V)	1.67 ± 1.53	0.00 ± 0.00	5.67 ± 2.52	0.87 ± 0.21
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 5	Control	0.00 ± 0.00	0.00 ± 0.00	3.00 ± 1.00	4.53 ± 0.40
	0.5 μM V (V)	3.00 ± 2.65	2.67 ± 1.15	4.33 ± 1.53	3.27 ± 0.35
	5 μM V (V)	2.33 ± 0.58	0.00 ± 0.00	5.00 ± 1.00	1.57 ± 0.15
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	2.67 ± 2.31	3.73 ± 0.35
	0.5 μM V (V)	3.67 ± 1.15	1.00 ± 1.73	5.00 ± 1.00	2.47 ± 0.40
	5 μM V (V)	0.33 ± 0.58	0.00 ± 0.00	4.33 ± 1.53	0.57 ± 0.15

Table 4d. Dicentric Chromosomes (%), Telomeric Associations (%), Tetraploidy (%) and Mitotic Index (%) in hTERT+ Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.53	8.27 ± 0.29
	0.04 µM Cr (VI)	0.00 ± 0.00	0.00 ± 0.00	2.67 ± 1.15	8.03 ± 0.42
	0.4 µM Cr (VI)	0.33 ± 0.58	0.00 ± 0.00	5.33 ± 5.77	7.03 ± 0.25
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 5	Control	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 4.36	8.60 ± 0.53
	0.04 µM Cr (VI)	0.67 ± 0.58	0.00 ± 0.00	4.33 ± 0.58	8.27 ± 0.42
	0.4 µM Cr (VI)	0.33 ± 0.58	0.00 ± 0.00	8.67 ± 4.16	6.73 ± 0.55
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	6.00 ± 4.58	7.07 ± 0.64
	0.04 µM Cr (VI)	0.00 ± 0.00	0.00 ± 0.00	4.67 ± 1.15	6.60 ± 0.70
	0.4 µM Cr (VI)	0.00 ± 0.00	0.00 ± 0.00	11.33 ± 6.11	7.13 ± 0.40

hTERT+ Cells

Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	1.67 ± 1.53	8.27 ± 0.29
	0.5 µM V (V)	0.67 ± 0.58	0.00 ± 0.00	3.33 ± 0.58	7.77 ± 0.76
	5 µM V (V)	0.00 ± 0.00	0.33 ± 0.58	3.67 ± 1.53	4.93 ± 0.31
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 5	Control	0.00 ± 0.00	0.00 ± 0.00	4.00 ± 4.36	8.60 ± 0.53
	0.5 µM V (V)	0.00 ± 0.00	0.00 ± 0.00	5.33 ± 1.53	6.53 ± 0.78
	5 µM V (V)	0.67 ± 1.15	0.00 ± 0.00	10.33 ± 4.04	5.57 ± 0.80
Day Post Treatment	Treatment	Dicentrics	Telom. Assoc.	Tetraploidy	Mitotic Index
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	6.00 ± 4.58	7.07 ± 0.64
	0.5 µM V (V)	0.00 ± 0.00	0.00 ± 0.00	8.33 ± 6.51	6.53 ± 0.25
	5 µM V (V)	1.00 ± 1.00	0.67 ± 1.15	14.00 ± 10.82	5.87 ± 0.42

Table 4e. Total Aneuploidy (%), Aneuploidy Gain (%), Aneuploidy (%), Hypodiploidy (%) and Hyperdiploidy (%) in hTERT- Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	3.33 ± 3.51	1.00 ± 1.00	0.33 ± 0.58	2.33 ± 2.52	0.67 ± 1.15
	0.4 µM Cr (VI)	1.67 ± 0.58	1.33 ± 1.15	1.33 ± 1.15	0.33 ± 0.58	0.00 ± 0.00
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 5	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	7.33 ± 2.31	0.67 ± 1.15	0.33 ± 0.58	6.67 ± 1.15	0.33 ± 0.58
	0.4 µM Cr (VI)	0.33 ± 0.58	0.33 ± 0.58	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	5.67 ± 3.79	1.33 ± 1.53	0.67 ± 0.58	4.33 ± 2.52	0.67 ± 1.15
	0.4 µM Cr (VI)	4.67 ± 0.58	2.00 ± 2.65	1.00 ± 1.73	2.67 ± 2.52	1.00 ± 1.00

hTERT- Cells

Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	5.33 ± 5.51	2.67 ± 3.06	1.67 ± 1.53	2.67 ± 2.52	1.00 ± 1.73
	5 µM V (V)	9.33 ± 6.66	5.67 ± 4.04	3.00 ± 1.00	3.67 ± 3.21	2.67 ± 3.79
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 5	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	4.67 ± 5.69	2.33 ± 3.21	1.00 ± 1.00	2.33 ± 2.52	1.33 ± 2.31
	5 µM V (V)	3.33 ± 1.15	3.33 ± 1.15	2.33 ± 0.58	0.00 ± 0.00	1.00 ± 1.00
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	8.67 ± 5.13	3.67 ± 3.06	0.67 ± 1.15	5.00 ± 4.58	3.00 ± 2.00
	5 µM V (V)	4.67 ± 3.06	3.00 ± 1.73	2.33 ± 1.53	1.67 ± 2.08	0.67 ± 1.15

Table 4f. Total Aneuploidy (%), Aneuploidy Gain (%), Aneuploidy (%), Hypodiploidy (%) and Hyperdiploidy (%) in hTERT+ Cells after a 24 hours exposure to two different doses of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 0	Control	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.58	0.00 ± 0.00
	0.04 µM Cr (VI)	0.33 ± 0.58	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.58
	0.4 µM Cr (VI)	1.33 ± 1.53	0.00 ± 0.00	0.00 ± 0.00	1.33 ± 1.53	0.00 ± 0.00
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 5	Control	0.67 ± 0.58	0.33 ± 0.58	0.00 ± 0.00	0.33 ± 0.58	0.33 ± 0.58
	0.04 µM Cr (VI)	2.33 ± 1.53	1.00 ± 1.73	0.33 ± 0.58	1.33 ± 0.58	0.67 ± 1.15
	0.4 µM Cr (VI)	1.33 ± 1.15	1.00 ± 1.00	0.33 ± 0.58	0.33 ± 0.58	0.67 ± 1.15
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.04 µM Cr (VI)	1.33 ± 2.31	1.33 ± 2.31	0.33 ± 0.58	0.00 ± 0.00	1.00 ± 1.73
	0.4 µM Cr (VI)	1.67 ± 1.53	1.33 ± 1.53	0.00 ± 0.00	0.33 ± 0.58	1.33 ± 1.53

hTERT+ Cells

Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 0	Control	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.58	0.00 ± 0.00
	0.5 µM V (V)	1.00 ± 1.73	1.00 ± 1.73	0.33 ± 0.58	0.00 ± 0.00	0.67 ± 1.15
	5 µM V (V)	2.00 ± 1.73	1.33 ± 1.15	1.00 ± 1.00	0.67 ± 0.58	0.33 ± 0.58
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 5	Control	0.67 ± 0.58	0.33 ± 0.58	0.00 ± 0.00	0.33 ± 0.58	0.33 ± 0.58
	0.5 µM V (V)	3.33 ± 1.15	2.33 ± 1.53	1.67 ± 2.08	1.00 ± 1.00	0.67 ± 0.58
	5 µM V (V)	1.00 ± 1.73	0.33 ± 0.58	0.33 ± 0.58	0.67 ± 1.15	0.00 ± 0.00
Day Post Treatment	Treatment	Total Aneuploidy	Aneuploidy Gain	Aneuploidy	Hypodiploidy	Hyperdiploidy
Day 30	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5 µM V (V)	1.67 ± 2.89	0.33 ± 0.58	0.33 ± 0.58	1.33 ± 2.31	0.00 ± 0.00
	5 µM V (V)	3.00 ± 3.61	0.33 ± 0.58	0.00 ± 0.00	2.67 ± 3.06	0.33 ± 0.58

Table 5a. Telomerase Activity expressed as a Total Product Generated (TPG) Units in hTERT- Cells after a 24 hours exposure to a single dose of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Telomerase Activity
Day 0	Control	0.007 ± 0.012
	0.4 µM Cr (VI)	0.038 ± 0.057
Day Post Treatment	Treatment	Telomerase Activity
Day 5	Control	0.012 ± 0.014
	0.4 µM Cr (VI)	0.020 ± 0.016
Day Post Treatment	Treatment	Telomerase Activity
Day 30	Control	0.012 ± 0.000
	0.4 µM Cr (VI)	0.021 ± 0.024

hTERT- Cells

Day Post Treatment	Treatment	Telomerase Activity
Day 0	Control	0.007 ± 0.012
	5 V µM (V)	0.073 ± 0.105
Day Post Treatment	Treatment	Telomerase Activity
Day 5	Control	0.012 ± 0.014
	5 V µM (V)	0.140 ± 0.103
Day Post Treatment	Treatment	Telomerase Activity
Day 30	Control	0.012 ± 0.000
	5 V µM (V)	0.043 ± 0.035

Table 5b. Telomerase Activity expressed as a Total Product Generated (TPG) Units in hTERT+ Cells after a 24 hours exposure to a single dose of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Telomerase Activity
Day 0	Control	3.957 ± 0.240
	0.4 µM Cr (VI)	3.029 ± 0.510
Day Post Treatment	Treatment	Telomerase Activity
Day 5	Control	2.156 ± 0.677
	0.4 µM Cr (VI)	1.991 ± 0.547
Day Post Treatment	Treatment	Telomerase Activity
Day 30	Control	2.915 ± 0.738
	0.4 µM Cr (VI)	3.464 ± 0.467

hTERT+ Cells

Day Post Treatment	Treatment	Telomerase Activity
Day 0	Control	3.957 ± 0.240
	5 V µM (V)	2.282 ± 0.658
Day Post Treatment	Treatment	Telomerase Activity
Day 5	Control	2.156 ± 0.677
	5 V µM (V)	1.485 ± 0.589
Day Post Treatment	Treatment	Telomerase Activity
Day 30	Control	2.915 ± 0.738
	5 V µM (V)	2.280 ± 1.172

Table 6a. Telomere Length expressed as a Telomere Fluorescence Intensity (TFI), Single Telomere Length Amplification (STELA), Apoptosis (%) and Necrosis (%) in hTERT- Cells after a 24 hours exposure to a single dose of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 0	Control	0.93 ± 0.38	5.70 ± 3.00	3.73 ± 0.78	3.93 ± 0.57
	0.4 µM Cr (VI)	0.87 ± 0.35	5.70 ± 3.10	6.67 ± 1.07	4.87 ± 0.21
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 5	Control	0.97 ± 0.06	4.50 ± 2.50	3.10 ± 0.35	3.40 ± 1.31
	0.4 µM Cr (VI)	0.80 ± 0.10	4.20 ± 2.70	3.03 ± 0.59	2.70 ± 0.44
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 30	Control	0.90 ± 0.10	4.20 ± 2.90	2.83 ± 0.57	3.73 ± 0.32
	0.4 µM Cr (VI)	0.67 ± 0.06	3.80 ± 2.40	2.27 ± 0.95	2.57 ± 0.38

hTERT- Cells

Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 0	Control	0.93 ± 0.38	5.70 ± 3.00	3.73 ± 0.78	3.93 ± 0.57
	5 µM V (V)	0.83 ± 0.38	5.70 ± 3.10	6.10 ± 0.44	7.10 ± 2.69
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 5	Control	0.97 ± 0.06	4.50 ± 2.50	3.10 ± 0.35	3.40 ± 1.31
	5 µM V (V)	0.83 ± 0.15	3.90 ± 2.50	3.47 ± 0.67	3.43 ± 0.31
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 30	Control	0.90 ± 0.10	4.20 ± 2.90	2.83 ± 0.57	3.73 ± 0.32
	5 µM V (V)	0.70 ± 0.10	3.90 ± 2.7	2.83 ± 0.42	3.57 ± 0.99

Table 6b. Telomere Length expressed as a Telomere Fluorescence Intensity (TFI), Single Telomere Length Amplification (STELA), Apoptosis (%) and Necrosis (%) in hTERT+ Cells after a 24 hours exposure to a single dose of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 0	Control	1.20 ± 0.61	8.00 ± 1.60	1.73 ± 0.45	1.80 ± 0.26
	0.4 µM Cr (VI)	1.30 ± 0.36	8.20 ± 1.70	1.33 ± 0.06	1.50 ± 0.20
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 5	Control	1.50 ± 0.17	6.80 ± 2.20	1.83 ± 0.35	1.20 ± 0.46
	0.4 µM Cr (VI)	1.23 ± 0.12	7.30 ± 1.50	2.07 ± 0.06	1.07 ± 0.45
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 30	Control	1.40 ± 0.00	6.50 ± 1.80	1.93 ± 0.29	1.47 ± 0.29
	0.4 µM Cr (VI)	1.27 ± 0.12	6.80 ± 2.00	2.53 ± 0.50	1.30 ± 0.36

hTERT+ Cells

Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 0	Control	1.20 ± 0.61	8.00 ± 1.60	1.73 ± 0.45	1.80 ± 0.26
	5 µM V (V)	1.10 ± 0.44	7.40 ± 2.30	2.00 ± 0.26	1.00 ± 0.10
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 5	Control	1.50 ± 0.17	6.80 ± 2.20	1.83 ± 0.35	1.20 ± 0.46
	5 µM V (V)	1.20 ± 0.10	6.70 ± 1.90	1.63 ± 0.06	1.63 ± 0.06
Day Post Treatment	Treatment	Telomere Length	STELA	Apoptosis	Necrosis
Day 30	Control	1.40 ± 0.00	6.50 ± 1.80	1.93 ± 0.29	1.47 ± 0.29
	5 µM V (V)	1.37 ± 0.31	6.90 ± 1.60	2.37 ± 0.40	0.90 ± 0.10

Table 6c. Cell Cycle Phase G0/G1 (%), Cell Cycle Phase S (%) and Cell Cycle Phase G2/M (%) in hTERT- Cells after a 24 hours exposure to a single dose of Cr (VI) and V (V) and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 0	Control	70.73 ± 0.95	13.80 ± 0.17	15.47 ± 0.81
	0.4 µM Cr (VI)	71.77 ± 0.65	13.33 ± 0.25	14.90 ± 0.53
Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 5	Control	78.17 ± 1.21	9.77 ± 0.91	12.07 ± 0.40
	0.4 µM Cr (VI)	77.27 ± 1.32	10.77 ± 1.11	11.97 ± 0.23
Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 30	Control	70.87 ± 2.61	13.07 ± 1.27	16.07 ± 1.37
	0.4 µM Cr (VI)	69.97 ± 2.41	15.10 ± 1.14	14.93 ± 1.37

hTERT- Cells

Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 0	Control	70.73 ± 0.95	13.80 ± 0.17	15.47 ± 0.81
	5 µM V (V)	81.33 ± 2.39	11.17 ± 1.19	7.50 ± 1.21
Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 5	Control	78.17 ± 1.21	9.77 ± 0.91	12.07 ± 0.40
	5 µM V (V)	78.77 ± 0.32	11.23 ± 0.55	10.00 ± 0.35
Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 30	Control	70.87 ± 2.61	13.07 ± 1.27	16.07 ± 1.37
	5 µM V (V)	68.80 ± 6.06	17.17 ± 3.93	14.03 ± 2.16

Table 6d. Cell Cycle Phase G0/G1 (%), Cell Cycle Phase S (%) and Cell Cycle Phase G2/M (%) in hTERT+ Cells after a 24 hours exposure to a single dose of Cr (VI) and V (V) and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 0	Control	63.50 ± 0.89	18.23 ± 0.23	18.27 ± 1.12
	0.4 µM Cr (VI)	62.23 ± 1.24	19.07 ± 0.84	18.70 ± 1.01
Day 5	Control	72.90 ± 3.89	13.17 ± 3.57	13.93 ± 0.35
	0.4 µM Cr (VI)	73.87 ± 4.13	13.80 ± 2.82	12.33 ± 1.66
Day 30	Control	67.40 ± 0.78	16.93 ± 2.05	15.67 ± 1.31
	0.4 µM Cr (VI)	62.83 ± 0.38	20.47 ± 1.67	16.70 ± 1.39

hTERT+ Cells

Day Post Treatment	Treatment	G0/G1 Phase	S Phase	G2/M Phase
Day 0	Control	63.50 ± 0.89	18.23 ± 0.23	18.27 ± 1.12
	5 µM V (V)	62.20 ± 1.04	17.63 ± 0.35	20.17 ± 0.74
Day 5	Control	72.90 ± 3.89	13.17 ± 3.57	13.93 ± 0.35
	5 µM V (V)	69.27 ± 1.36	16.93 ± 2.11	13.80 ± 0.90
Day 30	Control	67.40 ± 0.78	16.93 ± 2.05	15.67 ± 1.31
	5 µM V (V)	64.40 ± 1.47	16.67 ± 1.10	18.93 ± 2.57

APPENDIX II

Tables (Radiation Exposure)

Table 7a. Clonogenic Survival [these values were expressed as a percentage of control (PBS) and control was set to 100%] in hTERT- Cells after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Colonies
Day 0	Control	100 ± 0.00
	0.05 Gy	93.56 ± 6.94
	0.5 Gy	95.46 ± 0.41
Day Post Treatment	Treatment	Colonies
Day 30	Control	100 ± 0.00
	0.05 Gy	107.4 ± 8.54
	0.5 Gy	103.62 ± 2.56

Table 7b. Clonogenic Survival [these values were expressed as a percentage of control (PBS) and control was set to 100%] in hTERT+ Cells after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Colonies
Day 0	Control	100 ± 0.00
	0.05 Gy	99.28 ± 9.29
	0.5 Gy	98.43 ± 11.63
Day Post Treatment	Treatment	Colonies
Day 30	Control	100 ± 0.00
	0.05 Gy	109.91 ± 5.27
	0.5 Gy	90.00 ± 3.86

Table 8a. Micronuclei (MNi) (%) and Nucleoplasmic Bridges (NPB) (%) in hTERT- Cells after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 0	Control	1.60 ± 0.57	2.60 ± 0.57
	0.05 Gy	1.80 ± 0.28	3.10 ± 0.14
	0.5 Gy	3.90 ± 1.56	4.30 ± 1.84
Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 30	Control	2.80 ± 1.70	5.30 ± 3.25
	0.05 Gy	2.60 ± 1.41	6.40 ± 0.00
	0.5 Gy	2.80 ± 3.39	7.00 ± 1.41

Table 8b. Micronuclei (MNi) (%) and Nucleoplasmic Bridges (NPB) (%) in hTERT+ Cells after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 0	Control	0.90 ± 0.14	0.80 ± 1.13
	0.05 Gy	1.10 ± 0.14	1.80 ± 0.28
	0.5 Gy	3.30 ± 0.42	2.50 ± 0.71
Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 30	Control	0.20 ± 0.28	0.20 ± 0.28
	0.05 Gy	1.00 ± 0.85	1.00 ± 0.85
	0.5 Gy	0.60 ± 0.85	0.60 ± 0.85

Table 9a. Chromatid Breaks (%), Chromatid Gaps (%), Chromatid Fragments (%), Dicentric Chromosomes (%) and Tetraploidy (%) in hTERT- Cells after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure

hTERT- Cells

Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 0	Control	0.00 ± 0.00	1.00 ± 1.41	2.00 ± 2.83	1.00 ± 1.41	2.00 ± 2.83
	0.05 Gy	3.00 ± 1.41	3.00 ± 1.41	2.00 ± 0.00	3.00 ± 1.41	0.00 ± 0.00
	0.5 Gy	1.00 ± 1.41	100 ± 1.41	3.00 ± 1.41	5.00 ± 1.41	4.00 ± 0.00
Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 30	Control	1.00 ± 1.41	2.00 ± 0.00	1.00 ± 1.41	1.00 ± 1.41	1.00 ± 1.41
	0.05 Gy	2.00 ± 2.83	4.00 ± 0.00	1.00 ± 1.41	1.00 ± 1.41	4.00 ± 0.00
	0.5 Gy	1.00 ± 1.41	0.00 ± 0.00	3.00 ± 4.24	6.08 ± 2.72	4.00 ± 5.66

Table 9b. Chromatid Breaks (%), Chromatid Gaps (%), Chromatid Fragments (%), Dicentric Chromosomes (%) and Tetraploidy (%) in hTERT+ Cells after a single radiation exposure of either 0.05 Gy or 0.5 Gy and at different times after the exposure

hTERT+ Cells

Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	2.27 ± 3.21	0.00 ± 0.00	4.27 ± 0.38
	0.05 Gy	2.00 ± 2.83	2.00 ± 2.83	1.00 ± 1.41	100 ± 1.41	6.00 ± 2.83
	0.5 Gy	2.00 ± 2.83	2.00 ± 2.83	5.00 ± 1.41	2.00 ± 2.83	5.00 ± 4.24
Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 30	Control	0.00 ± 0.00	1.00 ± 1.41	1.00 ± 1.41	0.00 ± 0.00	3.00 ± 1.41
	0.05 Gy	1.35 ± 1.91	3.52 ± 1.16	0.00 ± 0.00	0.00 ± 0.00	6.22 ± 2.66
	0.5 Gy	4.00 ± 0.00	6.00 ± 2.83	2.00 ± 2.83	0.00 ± 0.00	5.00 ± 1.41

APPENDIX III

Tables (Combined Exposure)

Table 10a. Clonogenic Survival [these values were expressed as a percentage of control (PBS) and control was set to 100%] in hTERT- Cells after either a ***Single Exposure*** to Metal + Sham Irradiation (M + SI), Sham Irradiation + Metal (SI + M), 0.05 Gy + Vehicle Control (0.05 Gy + VC), Vehicle Control + 0.05 Gy (VC + 0.05 Gy), 0.5 Gy + Vehicle Control (VC + 0.5 Gy) and Vehicle Control + 0.5 Gy (VC + 0.5 Gy) or ***Combined Exposure*** to Metal + 0.05 Gy (M + 0.05 Gy), 0.05 Gy + Metal (0.05 Gy + M), Metal + 0.5 Gy (M + 0.5 Gy) and 0.5 Gy + Metal (0.5 Gy + M) and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS

hTERT- Cells

Day Post Treatment	Treatment	Colonies
Day 0	Control	100 ± 0.00
	M + SI	90.06 ± 4.34
	SI + M	92.42 ± 5.33
	0.05 Gy + VC	90.92 ± 8.89
	VC + 0.05 Gy	93.56 ± 6.94
	0.5 Gy + VC	94.78 ± 6.66
	VC + 0.5 Gy	95.46 ± 0.41
	M + 0.05 Gy	92.52 ± 4.46
	0.05 Gy + M	91.81 ± 5.47
	M + 0.5 Gy	91.03 ± 3.69
	0.5 Gy + M	91.31 ± 5.09

Day Post Treatment	Treatment	Colonies
Day 30	Control	100 ± 0.00
	M + SI	94.41 ± 6.81
	SI + M	90.45 ± 7.29
	0.05 Gy + VC	95.35 ± 19.35
	VC + 0.05 Gy	107.4 ± 8.54
	0.5 Gy + VC	92.27 ± 19.15
	VC + 0.5 Gy	103.62 ± 2.56
	M + 0.05 Gy	94.99 ± 6.35
	0.05 Gy + M	84.35 ± 0.58
	M + 0.5 Gy	88.61 ± 11.72
	0.5 Gy + M	81.36 ± 2.69

Table 10b. Clonogenic Survival [these values were expressed as a percentage of control (PBS) and control was set to 100%] in hTERT+ Cells after either a ***Single Exposure*** to Metal + Sham Irradiation (M + SI), Sham Irradiation + Metal (SI + M), 0.05 Gy + Vehicle Control (0.05 Gy + VC), Vehicle Control + 0.05 Gy (VC + 0.05 Gy), 0.5 Gy + Vehicle Control (VC + 0.5 Gy) and Vehicle Control + 0.5 Gy (VC + 0.5 Gy) or ***Combined Exposure*** to Metal + 0.05 Gy (M + 0.05 Gy), 0.05 Gy + Metal (0.05 Gy + M), Metal + 0.5 Gy (M + 0.5 Gy) and 0.5 Gy + Metal (0.5 Gy + M) and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS

hTERT+ Cells

Day Post Treatment	Treatment	Colonies
Day 0	Control	100 ± 0.00
	M + SI	97.06 ± 10.18
	SI + M	94.93 ± 7.17
	0.05 Gy + VC	89.25 ± 13.14
	VC + 0.05 Gy	99.28 ± 9.29
	0.5 Gy + VC	85.27 ± 6.55
	VC + 0.5 Gy	98.43 ± 11.63
	M + 0.05 Gy	92.12 ± 8.51
	0.05 Gy + M	92.73 ± 15.17
	M + 0.5 Gy	96.66 ± 13.00
	0.5 Gy + M	93.00 ± 8.78

Day Post Treatment	Treatment	Colonies
Day 30	Control	100 ± 0.00
	M + SI	97.62 ± 1.78
	SI + M	99.40 ± 4.71
	0.05 Gy + VC	108.71 ± 3.97
	VC + 0.05 Gy	109.91 ± 5.27
	0.5 Gy + VC	86.17 ± 1.29
	VC + 0.5 Gy	90.00 ± 3.86
	M + 0.05 Gy	91.06 ± 3.78
	0.05 Gy + M	89.85 ± 1.64
	M + 0.5 Gy	88.46 ± 0.89
	0.5 Gy + M	98.56 ± 0.61

Table 11a. Micronuclei (MNi) (%) and Nucleoplasmic Bridges (NPB) (%) in hTERT- Cells after either a ***Single Exposure*** to Metal + Sham Irradiation (M + SI), Sham Irradiation + Metal (SI + M), 0.05 Gy + Vehicle Control (0.05 Gy + VC), Vehicle Control + 0.05 Gy (VC + 0.05 Gy), 0.5 Gy + Vehicle Control (VC + 0.5 Gy) and Vehicle Control + 0.5 Gy (VC + 0.5 Gy) or ***Combined Exposure*** to Metal + 0.05 Gy (M + 0.05 Gy), 0.05 Gy + Metal (0.05 Gy + M), Metal + 0.5 Gy (M + 0.5 Gy) and 0.5 Gy + Metal (0.5 Gy + M) and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS

hTERT- Cells

Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 0	Control	1.60 ± 0.57	2.60 ± 0.57
	M + SI	2.53 ± 0.18	3.47 ± 0.19
	SI + M	2.50 ± 0.71	3.20 ± 1.70
	0.05 Gy + VC	1.26 ± 0.08	1.26 ± 0.08
	VC + 0.05 Gy	1.80 ± 0.28	3.10 ± 0.14
	0.5 Gy + VC	4.20 ± 0.28	3.60 ± 2.26
	VC + 0.5 Gy	3.90 ± 1.56	4.30 ± 1.84
	M + 0.05 Gy	2.31 ± 0.70	4.73 ± 1.03
	0.05 Gy + M	2.25 ± 0.92	3.45 ± 0.21
	M + 0.5 Gy	4.60 ± 1.13	2.95 ± 0.35
	0.5 Gy + M	4.74 ± 1.04	3.57 ± 1.18

Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 30	Control	2.80 ± 1.70	5.30 ± 3.25
	M + SI	4.58 ± 0.25	8.98 ± 5.97
	SI + M	5.33 ± 1.88	9.72 ± 7.47
	0.05 Gy + VC	2.20 ± 0.85	7.00 ± 1.41
	VC + 0.05 Gy	2.60 ± 1.41	6.40 ± 0.00
	0.5 Gy + VC	3.28 ± 2.93	5.19 ± 2.28
	VC + 0.5 Gy	2.80 ± 3.39	7.00 ± 1.41
	M + 0.05 Gy	2.59 ± 1.39	8.58 ± 5.40
	0.05 Gy + M	4.50 ± 0.71	3.17 ± 0.23
	M + 0.5 Gy	4.00 ± 0.00	9.50 ± 0.71
	0.5 Gy + M	3.20 ± 1.13	5.70 ± 3.82

Table 11b. Micronuclei (MNi) (%) and Nucleoplasmic Bridges (NPB) (%) in hTERT+ Cells after either a ***Single Exposure*** to Metal + Sham Irradiation (M + SI), Sham Irradiation + Metal (SI + M), 0.05 Gy + Vehicle Control (0.05 Gy + VC), Vehicle Control + 0.05 Gy (VC + 0.05 Gy), 0.5 Gy + Vehicle Control (VC + 0.5 Gy) and Vehicle Control + 0.5 Gy (VC + 0.5 Gy) or ***Combined Exposure*** to Metal + 0.05 Gy (M + 0.05 Gy), 0.05 Gy + Metal (0.05 Gy + M), Metal + 0.5 Gy (M + 0.5 Gy) and 0.5 Gy + Metal (0.5 Gy + M) and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS

hTERT+ Cells

Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 0	Control	0.90 ± 0.14	0.80 ± 1.13
	M + SI	1.60 ± 0.57	2.20 ± 0.28
	SI + M	0.50 ± 0.71	1.60 ± 0.57
	0.05 Gy + VC	0.60 ± 0.28	1.00 ± 0.28
	VC + 0.05 Gy	1.10 ± 0.14	1.80 ± 0.28
	0.5 Gy + VC	1.20 ± 0.00	1.20 ± 0.00
	VC + 0.5 Gy	3.30 ± 0.42	2.50 ± 0.71
	M + 0.05 Gy	1.60 ± 0.57	2.00 ± 0.57
	0.05 Gy + M	0.90 ± 0.71	1.00 ± 0.28
	M + 0.5 Gy	3.55 ± 0.64	2.20 ± 0.28
	0.5 Gy + M	2.60 ± 1.98	2.00 ± 0.00

Day Post Treatment	Treatment	MNi (%)	NPB (%)
Day 30	Control	0.20 ± 0.28	0.20 ± 0.28
	M + SI	0.49 ± 0.13	1.48 ± 0.39
	SI + M	0.70 ± 0.99	0.70 ± 0.14
	0.05 Gy + VC	0.20 ± 0.28	1.20 ± 0.57
	VC + 0.05 Gy	1.00 ± 0.85	1.00 ± 0.85
	0.5 Gy + VC	1.60 ± 0.00	1.00 ± 0.85
	VC + 0.5 Gy	0.60 ± 0.85	0.60 ± 0.85
	M + 0.05 Gy	1.40 ± 1.41	1.00 ± 0.85
	0.05 Gy + M	1.85 ± 0.35	0.60 ± 0.28
	M + 0.5 Gy	2.83 ± 0.53	2.12 ± 0.40
	0.5 Gy + M	1.20 ± 0.00	1.20 ± 0.00

Table 12a. Chromatid Breaks (%), Chromatid Gaps (%), Chromatid Fragments (%), Dicentric Chromosomes (%) and Tetraploidy (%) in hTERT- Cells after either a ***Single Exposure*** to Metal + Sham Irradiation (M + SI), Sham Irradiation + Metal (SI + M), 0.05 Gy + Vehicle Control (0.05 Gy + VC), Vehicle Control + 0.05 Gy (VC + 0.05 Gy), 0.5 Gy + Vehicle Control (VC + 0.5 Gy) and Vehicle Control + 0.5 Gy (VC + 0.5 Gy) or ***Combined Exposure*** to Metal + 0.05 Gy (M + 0.05 Gy), 0.05 Gy + Metal (0.05 Gy + M), Metal + 0.5 Gy (M + 0.5 Gy) and 0.5 Gy + Metal (0.5 Gy + M) and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS

hTERT- Cells

Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 0	Control	0.00 ± 0.00	1.00 ± 1.41	2.00 ± 2.83	1.00 ± 1.41	2.00 ± 2.83
	M + SI	5.00 ± 1.41	1.00 ± 1.41	5.00 ± 1.41	3.00 ± 1.41	3.00 ± 1.41
	SI + M	3.13 ± 4.42	3.13 ± 4.42	3.13 ± 4.42	3.13 ± 4.42	3.13 ± 4.42
	0.05 Gy + VC	0.00 ± 0.00	2.50 ± 3.54	4.77 ± 0.33	2.50 ± 3.54	2.50 ± 3.54
	VC + 0.05 Gy	3.00 ± 1.41	3.00 ± 1.41	2.00 ± 0.00	3.00 ± 1.41	0.00 ± 0.00
	0.5 Gy + VC	3.13 ± 4.42	3.13 ± 4.42	3.13 ± 4.42	0.00 ± 0.00	0.00 ± 0.00
	VC + 0.5 Gy	1.00 ± 1.41	1.00 ± 1.41	3.00 ± 1.41	5.00 ± 1.41	4.00 ± 0.00
	M + 0.05 Gy	5.00 ± 1.41	1.00 ± 1.41	4.00 ± 2.83	2.00 ± 2.83	1.00 ± 2.41
	0.05 Gy + M	4.00 ± 5.66	8.00 ± 2.83	2.00 ± 2.83	0.00 ± 0.00	4.00 ± 5.66
	M + 0.5 Gy	4.33 ± 3.30	5.00 ± 7.07	5.33 ± 1.88	6.00 ± 5.66	2.00 ± 2.83
	0.5 Gy + M	2.38 ± 3.37	2.38 ± 3.37	2.38 ± 3.37	0.00 ± 0.00	0.00 ± 0.00

Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 30	Control	1.00 ± 1.41	2.00 ± 0.00	1.00 ± 1.41	1.00 ± 1.41	1.00 ± 1.41
	M + SI	4.27 ± 0.38	3.27 ± 1.80	6.55 ± 3.60	4.27 ± 0.38	2.27 ± 3.21
	SI + M	2.00 ± 2.83	1.00 ± 1.41	6.00 ± 8.49	4.00 ± 5.66	0.00 ± 0.00
	0.05 Gy + VC	3.35 ± 0.32	3.35 ± 0.32	3.35 ± 0.32	1.56 ± 2.21	1.79 ± 2.52
	VC + 0.05 Gy	2.00 ± 2.83	4.00 ± 0.00	1.00 ± 1.41	1.00 ± 1.41	4.00 ± 0.00
	0.5 Gy + VC	0.00 ± 0.00	1.56 ± 2.21	0.00 ± 0.00	1.56 ± 2.21	3.13 ± 4.42
	VC + 0.5 Gy	1.00 ± 1.41	0.00 ± 0.00	3.00 ± 4.24	6.08 ± 2.72	4.00 ± 5.66
	M + 0.05 Gy	2.67 ± 0.94	1.00 ± 1.41	2.67 ± 0.94	2.67 ± 0.94	1.00 ± 1.41
	0.05 Gy + M	3.78 ± 2.51	2.00 ± 2.83	0.00 ± 0.00	4.78 ± 1.10	4.78 ± 1.10
	M + 0.5 Gy	1.00 ± 1.41	4.92 ± 1.53	2.92 ± 1.30	4.00 ± 5.66	2.00 ± 2.83
	0.5 Gy + M	1.00 ± 1.41	2.67 ± 0.94	2.00 ± 2.83	4.67 ± 1.89	0.00 ± 0.00

Table 12b. Chromatid Breaks (%), Chromatid Gaps (%), Chromatid Fragments (%), Dicentric Chromosomes (%) and Tetraploidy (%) in hTERT+ Cells after either a ***Single Exposure*** to Metal + Sham Irradiation (M + SI), Sham Irradiation + Metal (SI + M), 0.05 Gy + Vehicle Control (0.05 Gy + VC), Vehicle Control + 0.05 Gy (VC + 0.05 Gy), 0.5 Gy + Vehicle Control (VC + 0.5 Gy) and Vehicle Control + 0.5 Gy (VC + 0.5 Gy) or ***Combined Exposure*** to Metal + 0.05 Gy (M + 0.05 Gy), 0.05 Gy + Metal (0.05 Gy + M), Metal + 0.5 Gy (M + 0.5 Gy) and 0.5 Gy + Metal (0.5 Gy + M) and at different times after the exposure. Metal (M) is Cr (VI) (0.4 μM), Sham Irradiation (SI) is room temperature and Vehicle Control (VC) is PBS

hTERT+ Cells

Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 0	Control	0.00 ± 0.00	0.00 ± 0.00	2.27 ± 3.21	0.00 ± 0.00	4.27 ± 0.38
	M + SI	2.00 ± 0.83	3.00 ± 1.41	3.00 ± 4.24	0.00 ± 0.00	4.00 ± 0.00
	SI + M	1.39 ± 1.96	0.00 ± 0.00	6.62 ± 1.51	1.39 ± 1.96	6.62 ± 1.51
	0.05 Gy + VC	0.00 ± 0.00	2.27 ± 3.21	2.27 ± 3.21	2.27 ± 3.21	4.55 ± 6.43
	VC + 0.05 Gy	2.00 ± 2.83	2.00 ± 2.83	1.00 ± 1.41	1.00 ± 1.41	6.00 ± 2.83
	0.5 Gy + VC	0.00 ± 0.00	0.00 ± 0.00	6.94 ± 1.97	0.00 ± 0.00	6.94 ± 1.97
	VC + 0.5 Gy	2.00 ± 2.83	2.00 ± 2.83	5.00 ± 1.41	2.00 ± 2.83	5.00 ± 4.24
	M + 0.05 Gy	1.00 ± 1.41	3.00 ± 1.41	1.00 ± 1.41	0.00 ± 0.00	6.00 ± 2.83
	0.05 Gy + M	4.00 ± 5.66	5.61 ± 3.38	5.23 ± 1.73	1.61 ± 2.28	5.23 ± 1.73
	M + 0.5 Gy	6.00 ± 5.66	2.00 ± 2.83	3.00 ± 1.41	0.00 ± 0.00	9.00 ± 4.24
	0.5 Gy + M	2.17 ± 3.07	3.13 ± 4.42	5.30 ± 1.35	0.00 ± 0.00	7.47 ± 1.73

Day Post Treatment	Treatment	Breaks	Gaps	Fragments	Dicentrics	Tetraploidy
Day 30	Control	0.00 ± 0.00	1.00 ± 1.41	1.00 ± 1.41	0.00 ± 0.00	3.00 ± 1.41
	M + SI	0.00 ± 0.00	3.78 ± 2.51	3.78 ± 2.51	1.00 ± 1.41	4.78 ± 1.10
	SI + M	0.00 ± 0.00	0.00 ± 0.00	3.39 ± 0.87	0.00 ± 0.00	5.39 ± 3.70
	0.05 Gy + VC	0.00 ± 0.00	2.08 ± 2.94	2.08 ± 2.94	0.00 ± 0.00	9.25 ± 4.60
	VC + 0.05 Gy	1.35 ± 1.91	3.52 ± 1.16	0.00 ± 0.00	0.00 ± 0.00	6.22 ± 2.66
	0.5 Gy + VC	1.00 ± 1.41	0.00 ± 0.00	3.00 ± 1.41	0.00 ± 0.00	4.00 ± 0.00
	VC + 0.5 Gy	4.00 ± 0.00	6.00 ± 2.83	2.00 ± 2.83	0.00 ± 0.00	5.00 ± 1.41
	M + 0.05 Gy	0.00 ± 0.00	1.00 ± 1.41	1.00 ± 1.41	0.00 ± 0.00	4.00 ± 5.66
	0.05 Gy + M	1.79 ± 2.52	1.79 ± 2.52	1.79 ± 2.52	1.79 ± 2.52	5.04 ± 2.97
	M + 0.5 Gy	2.00 ± 2.83	5.00 ± 4.24	1.00 ± 1.41	0.00 ± 0.00	4.00 ± 0.00
	0.5 Gy + M	2.00 ± 2.83	2.00 ± 2.83	4.00 ± 2.83	1.00 ± 1.41	8.00 ± 2.83

COMMUNICATIONS

Publications

Presentations

Publications

Glaviano A, Mothersill C, Campisi J, Rubio MA, Nayak V, Sood A and Case CP. (**2003**). Metal-Induced Genomic Instability: Long-Term Effects of Chromium and Vanadium. *Cell Biology 2003* (page 16), the Press Book for the 43rd Annual Meeting of the American Society for Cell Biology.

Glaviano A, Nayak V, Cabuy E, Yin Z, Newson R, Ladon D, Rubio MA, Slijepcevic P, Mothersill C, Baird DM and Case CP. (**2006**). Effects of hTERT on Metal Ion-Induced Genomic Instability. *Oncogene* 25 (24): 3424-3435.

Presentations

Poster presentation at the 43rd Annual Meeting of the American Society for Cell Biology in San Francisco from the 13th to the 17th of December **2003**.

Title: Genomic Instability Caused by Metal Ions. *Molecular Biology of the Cell* (Abstracts Book). 2003. Supplement, Vol. 14, page 370a.

Authors: A.Glaviano,[1,2] C. Mothersill,[2] J. Campisi,[3] M.A. Rubio,[3] V. Nayak,[1] A. Sood,[1] C.P. Case,[1]. [1] Bristol Implant Research Centre, University of Bristol, Bristol, United Kingdom. [2] School of Physics, Dublin Institute of Technology, Dublin, Ireland, [3] Lawrence Berkeley National Laboratory, University of Berkeley, Berkeley, CA.

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Title: hTERT Expression Partially Protects Cells from Genomic Instability Caused by Metal Ions. *The American Society for Cell Biology* (Late Abstracts Book). 2004. Number of Reference: L354.

Authors: A.Glaviano,[1,2] V. Nayak,[1] C. Mothersill,[2] M.A. Rubio,[3] Z. Yin,[1] E. Cabuy,[4] P. Slijepcevic,[4] C.P. Case,[1]. [1] Bristol Implant Research Centre, University of Bristol, Bristol, United Kingdom. [2] School of Physics, Dublin Institute of Technology, Dublin, Ireland, [3] Lawrence Berkeley National Laboratory, University of Berkeley, Berkeley, CA. [4] Brunel Institute for Cancer Genetics and Pharmacogenomics, Brunel University, London, United Kingdom.

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Poster presentation at the 46th Annual Meeting of the American Society for Cell Biology in San Diego from the 9th to the 13th of December **2006.**

Title: Effects of hTERT on Genomic Instability Caused by either Metal or Radiation or Combined Exposure. *The American Society for Cell Biology* (Late Abstracts Book). 2006. Number of Reference: L82.

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