GROWTH ENHANCEMENT AND TOXIC EFFECTS OF LITHIUM ON HL-60 PROMYELOCYTIC LEUKAEMIA CELLS: THE INVOLVEMENT OF INSULIN

By

LEAH SNOW TEFFO

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In the Faculty of Mathematics and Natural Sciences, Department of Biochemistry,
University of the North, Private Bag X1106, Sovenga, 0727,
South Africa.

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Supervisor: Prof. Rolf W. Becker

DECLARATION

I, Leah Snow Teffo, declare that the thesis hereby submitted to the University of the North for the degree of Master of Science has not previously been submitted by me for a degree at this or any other University, that is my own work in design and execution, and that all materials contained therein has been duly acknowledged.

Signed : _____

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Dedication

To my daughter Bohlale Carol, I wish you a very successful and bright future.

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LIST OF ABBREVIATIONS

AP-1	-activator protein-1
BrdU	-5-bromo-2'-deoxyuridine
cAMP	-cyclic adenosine monophosphate
CAT	-catalase
CuZnSOD	-copper/zinc-superoxide dismutase
DMSO	-dimethylsulphoxide
DNA	-Deoxyribonucleaic acid
Dnase	-deoxyribonuclease
ECL	-enhanced chemiluminescence
EDTA	-ethylenediaminotetraacetic acid
FBS	-fetal bovine serum
GLUT4	-glucose transporter4
GPX	-glutathione peroxidase
GSH	-glutathione superoxide hydroxylase
HRP	-horse-radish peroxidase
IGF-1	-insulin-like growth factor-1
IL	-interleukin
IP_3	-inositol-1,4,5-triphosphate
IR	-insulin receptor
IRS-1	-insulin receptor substrate-1
I/S	-insulin/selenium
I/T	-insulin/transferrin
I/T/S	-insulin/transferrin/selenium
Kb	-kilobase pairs
MAP kinase	-mitogen-activated protein kinase
MnSOD	-manganese-superoxide dismutase
M_r	-relative molecular mass
mRNA	-messenger RNA
NBT	-nitroblue tetrazolium

PAGE	-polyacrylamide gel electrophoresis
PBS	-phosphate-buffered saline
PCNA	-proliferating cell nuclear antigen
PHGPX	-phospholipid hydroperoxide glutathione peroxidase
PI3K	-phosphatidylinositide 3-kinase
PIP_2	-phosphatidylinositol-1,4-bisphosphate
PKC	-protein kinase C
PMSF	-phenylmethylsulphonyl fluoride
RNA	-ribonucleic acid
RA	-retinoic acid
Rnase	-ribonuclease
SDS	-sodium dodecyl sulphate
SEM	-standard error of the mean
SePXs	-selenoperoxidases
Ser	-serine
TBS	-tris-buffered saline
TBST	-tris-buffered saline with Tween-20
TfR	-transferrin receptor
TCA	-trichloroacetic acid
TNF	-tumor necrotic factor
TPA	-12-O-tertradecanoyl phorbol-13-acetate
TRE	-TPA response element
WBC	-white blood cells

ABSTRACT

It is well documented that lithium, a drug used widely for the treatment of manic depression, affects the growth of HL-60 promyelocytic leukaemia cells. At low concentrations (5 mM) lithium is known to enhance proliferation of HL-60 cells, whereas at concentration of 10 mM and above lithium has been shown to be cytotoxic to these cells. However, these experiments were performed in media containing 10% fetal bovine serum. It was the aim of this study to ascertain that the same observations could be obtained using a well defined culture medium consisting of insulin, transferrin and selenium in RPMI-1640. Either insulin/transferrin/selenium (I/T/S), insulin/selenium (I/S) or transferrin/selenium (T/S) at concentrations of 5 μg/ml were used as growth factors for HL-60 cells and were treated with 0, 5, 10 or 20 mM lithium. The rate of HL-60 cell growth in defined medium was low as compared to serum-rich containing media. The behaviour of HL-60 cells grown in T/S containing medium seemed to be similar to that of cells grown in a serum-rich environment either in the presence or absence of lithium. The present results showed induction of apoptosis, evidenced by appearance of DNA ladders in cells treated with lithium (0-20 mM) in media containing 5 μg/ml I/T/S or I/S. Insulin was thus found to have an influence in the induction of apoptosis. When 5 μg/ml T/S was used, apoptosis, was only observed in cells treated with 10 mM lithium and above. The control and 5 mM treated cells did not reveal DNA ladders after 24 h of treatment. Gene expression analysis showed the same level of Bcl-2 in the presence of 5 μg/ml I/T/S or I/S and Bax levels were found to be increased. Phosphorylated Bcl-2 was observed in 10 and 20 mM lithium treated cells grown in 5 μg/ml T/S supplemented media and the Bax levels were the same in all lithium concentrations tested (0-20 mM). It is concluded that insulin is needed for lithium to induce apoptosis via pathways utilising the Bcl-2/Bax mechanism of action.

Chapter 1

1. Introduction

1.1. The haemopoietic system

The normal total circulating blood volume is about 80% of the body weight and about 55% of this volume is the plasma. The cellular elements of the blood, i.e the white blood cells, red blood cells and platelets are suspended in the plasma (Ganindg, 1991). The bone marrow is the most crucial site of haemopoiesis from 6-7 months of foetal life and during adult life, the marrow is the only source of new blood cells. Normally, 75% of the cells in the marrow belong to the white blood cell-producing lymphoid and myeloid series, and only 25 % are mature red blood cells (Ganindg, 1991, Hoffbrand and Pettit, 1993). The bone marrow pluripotent stem cells give rise, after a number of cell divisions and differentiation steps, to a series of progenitor cells: these are (1) erythroid, (2) granulocytic and monocytic and (3) megagakaryocytic, as well as to a common lymphoid stem cell. The white blood cells are divided into two broad groups: the phagocytes and the immunocytes. The phagocytes comprise of the neutrophils, eosinophils and basophils. The normal bone marrow contains up to 4% of the myeloblasts. Through cell division, myeloblasts give rise to promyelocytes and promonocytes. The promyeloblast are larger cells and their cytoplasm has primary granules. The promyelocytes can undergo several divisions and give rise to either eosinophils, neutrophils or basophils depending on the inducing factor (Hoffbrand and Pettit, 1993).

1.2. Leukaemia

Leukaemia can be defined as an abnormal neoplastic proliferation derived from one of the leukocytic tissues, often associated with abnormal WBC counts and abnormal increases in leucocyte mass, leading to anaemia, thrombocytopenia and in the absence of treatment, death. Leukaemia constitutes about 31% of all human cancers. Two varieties, ie. acute and chronic leukaemia have been described. However, a better description for these conditions is 'blast cell leukaemias' because the predominant cell type in all forms is a poorly differentiated immature cell defined as blast. Uncontrolled proliferation of these cells in the blood and bone marrow, and their invasion into the rest of the body organs is chiefly responsible for the devastating effects of the disease. The clinical manifestation and the response to therapy may vary with the type of

blast cell involved in the leukaemic process (Hoffbrand and Pettit, 1993).

1.3. Epidemiology

A number of environmental agents have been implicated in the induction of leukaemia, chiefly ionizing radiation and chemical carcinogens notably the alkylating agents used in the treatment of malignant conditions (Miller, 1979):

Ionizing radiation - X-rays and other ionizing rays were first identifiable agents associated with the induction of leukaemia. This became apparent in the survivors of the atomic bomb explosions in Hiroshima and Nagasaki, and the latter has been the single most important source of information on radiation leukaemogenesis in man (Miller, 1979).

Chemicals - The two types of chemicals which have been involved or are strongly suspected of being leukaemogenic are: (1) Benzene and other petroleum derivatives, and (2) alkylating agents. Benzene has been known for many years to be aetiologically related to the high incidence of leukaemia and aplastic anaemia in people occupationally exposed to it, e.g. shoe-makers in Italy and Turkey (Miller, 1979).

Viruses - New findings have indicated that viruses are also responsible for leukaemias and lymphomas in many animal species and by some direct findings in human leukaemia (Hoffbrand and Pettit, 1993). C-type RNA viruses are known to be oncogenic in chickens, cows and sub-human primates. Their relevance to humans relates to two facts: (1) type C viruses were isolated from some species of primates (baboon, woolly monkey, etc.), one of them, the gibbon ape leukaemia virus is responsible for the spontaneous leukaemia in gibbons, the species closest to man, and (2) in some instances human leukaemic cells were found to contain genetic information related to the primate type C virus. Reverse transcriptase, an RNA-dependent DNA polymerase, an enzyme unique to RNA oncogenic viruses was also found in some leukaemic cells, this made it possible to understand the mechanism of transcription from viral into DNA products (Wu and Gallo, 1977). Epstain-Barr virus DNA has been found integrated into the genome of the tumour cells of patients developing lymphomas who were receiving immunosuppressive therapy after organ transplantation. Human T-cell leukaemia virus-1 has been demonstrated by electron microscopy and by cell culture in the cells of patients with leukaemia (Hoffbrand and Pettit, 1993).

1.4. Diagnosis and Treatment

The diagnosis of leukaemia is made by careful examination of well-prepared blood and bone marrow films stained with Romanowsky dyes. Rarely a bone marrow trephine biopsy may be required to demonstrate the leukaemic infiltration when aspirates are unsuccessful: this may occur as a result of dense cellular infiltration and/ or increased reticulin fibres (Hoffbrand and Lewis, 1981).

The development of effective therapies for some of the childhood leukaemias has been a major, and particularly gratifying advance in cancer treatment. Chemotherapy now leads to cures for up to 75% children with acute lymphoblastic leukaemia (Cooper, 1992). The following anticancer drugs usually in combination with one another are used to treat acute leukaemia: vincristine, predispone, cyclophosphamide, methotrexane and cytarabine. In chronic leukaemia, busalfan, melphalan and chlorambucil are used (Cooper, 1992; Sham *et al.*, 1995). The genetic material of the cancer cells are less stable than that of the normal cells. Cancer cells usually have abnormal numbers or arrangements of chromosomes, resulting from translocation or loss of one or all of one member of chromosomal pair. The development of drug resistance is a problem encountered in cancer chemotherapy. Many tumours respond initially to a given therapeutic agent but then become resistant to the drug during the course of treatment (Cooper, 1992).

1.4.1. Mechanism of action of anticancer drugs

The mechanism of action for an anticancer agent in tumour/host tissue determines several parameters of drug cell interactions that can affect the toxicity of the agent. The anticancer drug may need a receptor molecule on the surface of the cell membrane to bind and penetrate the cell. Inside the cell it must inhibit the intracellular target site. The intracellular concentration of a drug will determine the duration over which cytotoxic levels remain within the intracellular compartment as the extracellular drug concentration falls following the administration of the agent. The higher the intracellular concentration the longer the critical drug level will remain within the cell. Anticancer drugs damage the tumour cells by interfering with the replicative process point or/and with actual process of cell division. Most of the drugs interact specifically with DNA, by interfering with the synthesis of essential proteins, i.e. The impairment of DNA synthesis, reaction with DNA helix/damage to DNA repair mechanisms. The anticancer drugs

may also inhibit DNA polymerase, thereby altering the synthesis and function of RNA, and therefore protein synthesis and function of the patient's cells will be impaired (Cooper, 1992; Sham *et al.*, 1995).

1.5. HL-60 Promyelocytic Leukaemia cells

The HL-60 promyelocytic leukaemia cell line was initially established by Collins *et al.*, (1977) from a patient with acute promyelocytic leukaemia. Human leukaemia cell lines are important models for studying haematopoietic cell growth and differentiation and in the present study HL-60 cells were investigated. HL-60 cells represent a human promyelocytic leukaemia cell line which is a well studied model of leukaemic cell differentiation (Djulbegovic *et al.*, 1987). They have rounded, sometimes folded nuclei with little heterochromatin but large nucleolus. In the cytoplasm, there are moderate numbers of mitochondria, many polyribosomes and little endoplasmic reticulum. Abundant azurophilic granules of different sizes and density are also found (Collins, 1987; Sham *et al.*, 1995).

Some cell lines originally showed a malignant growth response to haematopoietins, such as colony-stimulating factor, when cultured in serum-free media. Other hormones and nutrient molecules required for cell growth such as insulin and transferrin are provided by serum. Thus, conventional, serum-supplemented cultures of human leukaemia cell lines provide limited models for studying growth factor action (Sinclair *et al.*, 1988; Barnes and Sato, 1980; Breitman *et al.*, 1980).

Breitman *et al.*, (1980) showed that the HL-60 promyelocytic leukaemia cell line can be indefinitely grown and induced to differentiate into mature granulocytes in serum-free media, provided the media is supplemented with insulin, transferrin and selenium, of which insulin has been found to be the most important growth factor for HL-60 cells (Garbarino *et al.*, 1985). Their culture medium was, however, well-fortified with a full complement of trace minerals. Despite limited work in this area, the essentiality of iron (Higuchi, 1979) and selenium (Guibert and Iscove, 1976; McKeehan *et al.*, 1976) for the proliferation of culture cells has been established. HL-60 cells seem to be no exception in this regard (Breitman *et al.*, 1980). Insulin and IGF-1 each bind with high affinity to a specific cell surface receptor, but they also bind with lower

affinity to each others receptors (Rosenfeld and Dollar, 1982). Some studies suggests common metabolic effects of insulin and IGF-1 are mediated by the insulin receptor, but growth stimulation is mediated by the IGF-1 receptor. However, in some cell types, it remains unclear which hormone or receptor mediates mitogenic effects (Conover *et al.*, 1985, King *et al.*, 1980).

Both insulin and IGF-1 stimulate growth of leukaemia cells, each acting through their receptors, but do not show hormone-induced mitogenic responses (Sinclair *et al.*, 1988). Insulin receptors are also present on a variety of normal and malignant human blood cells, but their role in cell growth remains largely undefined (Chen *et al.*,1983). For many cell lines serum-free media have been developed and insulin is an obligatory component of most serum-free cultures. Most studies used microgram quantities (5 μg/ml) of insulin, and at this concentration, insulin acts through its own receptor, the IGF-1 receptor or both (Sinclair *et al.*, 1988).

Sinclair *et al.*, (1988) have shown in their studies that prolonged culture with high doses of insulin results in decreased insulin and IGF-1 binding, and decreased sensitivity to growth stimulation. These high insulin concentrations may reduce ligand binding by receptor down regulation, altering ligand-receptor affinity or receptor synthesis, or inducing failure of receptor recycling. Competitive blocking experiment using HL-60 showed that, at concentrations stimulating cell growth (1-10 ng/ml) each hormone bound only to its own receptor. At these hormone concentrations, IR-3, a specific antagonist of the IGF-1 receptor, blocked growth stimulation by IGF-1 but not insulin, confirming that each ligand can stimulate growth through its own receptor (Sinclair *et al.*, 1988). Insulin and IGF-1 receptors are examples of tyrosine kinases that stimulate cell growth. They differ from other receptors with similar enzymatic properties in that their distribution shows little apparent tissue specificity and the insulin receptor has well documented functions other than growth promotion (Sinclair *et al.*, 1988).

1.5.1. Proliferation

For a typical rapidly proliferating cell, the total cell cycle time might correspond to about 20 h, with approximately 1 h for M, 8 h for G_1, 8 h for S and 3 h for G_2. Different kinds of cells vary in their rates of replication and almost all this variation occurs in the G_1 phase. The rate at which

cell proliferates is thus determined by the length of time it spend in the G_1. Slowly dividing cells, become arrested in G_1 and do not continue progressing through the cell cycle unless an external signal induces the cell to resume proliferation. Such arrested cells appear to have entered a quiescent state which is referred to as the G_0. Cells that progress through G_1 are committed to proceed through the rest of the cell cycle and undergo mitosis. For most cell types, therefore reproduction is regulated in the G_1 phase of each cycle by the decision to enter G_0 or to proceed through G_1 and the rest of the division process. The protein synthesis occurs in the G_1, S and G_2 phases (Cooper, 1992).

Cell proliferation can be controlled by growth factors, thus the density at which the normal cells become quiescent is proportional to the amount of growth factors available. Transformed cells that continuously proliferate in culture may have lower requirements for growth factors or serum than normal cells have. Nevertheless, they depend on them to sustain the proliferative state (Barnes and Sato, 1980). When normal cells are propagated in culture, they divide until they reach a finite cell density. They stop dividing and become quiescent and arrested in the G_0 stage. Transduction of the proliferative (growth) signals mediated by growth factor-receptor interactions is most likely similar if not identical in normal and transformed cells. Binding of growth factor to a cell receptor evokes a cascade of biochemical and molecular events (Goustine *et al.*, 1986), transducing the signal from the cell surface to the nucleus and leading to the synthesis of DNA 12-24 h later (Stiles , 1983). A number of second messengers have been implicated in the control of cell proliferation, including growth factor receptors and their substrates, the cations Ca^{2+} and H^+, kinases and lipases and their products, and the products of transcriptional regulating genes such as *c-myc*, *c-fos* and *c-jun* (Metcalfe *et al.*, 1986; Rothenberge *et al.*, 1983; Moolenaar *et al.*, 1986; Berridge and Irvin, 1984; Goustine *et al.*, 1986; Mitchell *et al.*, 1986). Changes in intracellular pH, Ca^{2+} fluxes, and redistribution have been proposed to play important roles in growth factor-stimulated cell proliferation (Metcalfe *et al.*, 1986; Rothenberge *et al.*, 1983; Moolenaar *et al.*, 1986; Macara, 1986).

Myeloid leukaemic cell lines require insulin and transferrin for continuous growth in culture (Breitman *et al.*, 1980). Raphaeli *et al.*, (1990) have observed that, omission of transferrin or insulin from the medium of HL-60 cells grown in serum-free medium retards their growth rate.

Readdition of these growth factors was followed by enhancement of cell growth, thymidine incorporation and Ca^{2+} influx. Enhancement of the Ca^{2+} influx rate was observed 24 and 48 h after exposure to transferrin. Ca^{2+} influx rate of cells deprived of transferrin was low and constant over a period of 5-72 h and similar to the influx rate exhibited by the cells after 5 h exposure to transferrin. Transferrin stimulated the low thymidine incorporation activity of cells grown in transferrin-depleted, serum-free media, and addition of retinoic acid to these cells resulted in a lower thymidine incorporation activity. Inhibition of proliferation in the presence of retinoic acid was accompanied by increased cell maturation as evidenced by the increased percentage of cells capable of NBT reduction (33% at 48 h). Insulin affected the cells in a similar way to that described above for transferrin. Insulin was added at a super-physiological concentration (5 μg/ml). At this concentration it is known to bind to IGF-1 receptor (Kojima *et al.*, 1988), therefore, it could as well affect the cell via the binding to its receptor.

Most of the tumour cells, on the other hand fail to display the density-dependent inhibition of proliferation that is characteristic of normal cells in culture. Instead tumour cells in culture continue growing until they die as a result of exhaustion of essential nutrients in the culture media or production of excess toxic waste products. Leukaemic cells fail to differentiate normally, instead they become arrested at an early stage of maturation (myeloblasts) at which they retain their proliferation potential and continue to divide (Cooper, 1992). Although many cancer cells have lower requirements of intracellular Ca^{2+} than normal cells, there is no evidence to indicate significant differences in calcium metabolism between the two cell types (Beaven *et al.*, 1984). Myeloid leukemic cell lines, by virtue of these proliferation or differentiation responses to a variety of molecules, serve as a convenient model for studying the biochemical changes associated with cell growth (Raphaeli *et al.*, 1990). During the process of myeloid differentiation, bone marrow precursors of polymorphonuclear leukocytes undergo both proliferation and differentiation maturation. Cells in the proliferating pool pass through the successive stages of the cell cycle (G_1, S, G_2 and M) in the process of growth, DNA synthesis, and mitosis. Those in the maturational pool remain out of the cycle with a G_1 DNA content, also termed G_0 (Cooper, 1992).

HL-60 cells are capable of continuously proliferating in suspension culture with a doubling time of 18-24 h, but when they are seeded in insulin-free media they are unable to grow, whereas other cell lines such as K562 and KG-1 show no dependence on insulin for growth (Garbarino *et al.*, 1985). Surface expression of insulin and transferrin receptors seems critical for the proliferative capacity of HL-60 cells, as they can grow in serum-free medium supplemented with transferrin and insulin with a 44 h doubling time (Breitman *et al.*, 1980). The transferrin receptors have been considered to be a proliferation-associated antigen in HL-60 cells. In most of the studies performed on HL-60 cells a correlation was found between the presence of the receptor and proliferation and the stimulation of proliferation was associated with the appearance of the receptor. Furthermore, cell cycle control has been implicated as an important regulator of TfR expression in HL-60 cells.

1.5.2. Differentiation

HL-60 cells can be induced to differentiate into a variety of cell types, depending on the inducing agent, and upon reaching maturity these cells undergo apoptosis typically characterized by internucleosomal DNA fragmentation (Martin *et al.*, 1990). This induced differentiation of the HL-60 promyelocytic leukaemia cell line provides a useful *in vitro* model of myeloid differentiation *in vivo*. During the first several days after induction of differentiation, proliferation ceases and as in the bone marrow, maturation continues in the absence of DNA synthesis (Collins, 1987; Breitman *et al.*, 1980). Accompanying this process are marked decreases in expression of the genes for the cellular oncogene, *c-myc,* and TfR.

Induction of differentiation of HL-60 cells by treatment with RA or DMSO induces maturation along the granulocytic pathway, while alkaline medium and butyric acid induces these cells to eosinophilic differentiation. Exposure to TPA induces differentiation into monocytes/macrophages in serum containing medium (Collins, 1987). Exposure of HL-60 cells to DMSO, arrests cells in the G_1 phase of the cell cycle, causes a decrease in cell surface TfR. In uninduced cells, more TfR is found on the cells in the M, G_2 and S phase of the cell cycle than those in G_1. This indicates that the expression of TfR correlates with cell cycle stage in HL-60 cells both before and after induction of myeloid differentiation (Barker and Newburger, 1990).

Lee and co-workers (1987) have demonstrated that calcitrol caused concentration-dependent maturation of HL-60 cells grown in 10% fetal calf serum medium and serum-free medium, as evidenced by its ability to decrease cell proliferation and to induce chemiluminescent responsiveness and lysozyme production. In addition, HL-60 cells acquired monocyte-specific cell surface antigens in a dose-dependent manner. These data clearly indicated that HL-60 cells grown in serum-free media were also able to differentiate towards the monocyte/macrophage pathway after exposure to calcitrol. Moreover, the above data also suggested that calcitrol directly mediates this differentiation process rather than via a factor(s) contained within serum. These results are consistent with the findings of Breitman *et al.*, (1980), who demonstrated that HL-60 cells cultured in serum-free medium supplemented with transferrin and insulin could differentiate to mature granulocytes in the presence of DMSO.

Sergeant and Johnson, (1995) have grown HL-60 cells in a defined medium and they were found to be fully capable of differentiating along the granulocytic pathway in response to RA. The extent of differentiation was, however, promoted by a heat-stable, nonalbumin component of serum. Interestingly, the apparent decreased sensitivity of defined medium cells to RA was lost at 1 μM of RA. At this RA concentration, cells grown in FBS exhibited fewer NBT-positive cells than the cells from defined medium. Stevens *et al.*, (1990) made similar observations. Based on experiments at this single RA concentration (1 μM), they concluded that serum contains a component which antagonizes the process of differentiation. Observations by Sergeant and Johnson, (1995) shows clearly that serum enhances RA induced differentiation compared to defined-media at RA concentrations less than 1 μM.

Trayner and Clemens, (1992) have investigated the effects of TPA on the growth and differentiation of HL-60 cells in a fully defined medium consisting RPMI-1640 supplemented with selenium dioxide, insulin, and either transferrin or ferric citrate. High concentration of TPA (>1 nM) caused the expected inhibition of proliferation and induction of macrophage-like differentiation. In contrast, cells deprived of insulin continue to grow at a slow rate. Lower concentrations of TPA was found to stimulate proliferation without inducing differentiation. Concentrations of TPA (1 nM and above) induce differentiation of HL-60 cells into macrophage-like cells as assayed by morphological, functional and antigenic criteria (Rovera *et al.*, 1982).

This differentiation was associated with an inhibition of proliferation and the arrest of the cells in the G_0/G_1 phase of the cell cycle (Rovera *et al.*, 1980). Low concentrations of TPA which are insufficient to induce differentiation sometimes stimulate the proliferation of HL-60 cells growing in FCS-supplemented medium. However, this effect was variable and barely apparent when cells were growing in 10% FCS or a fully defined medium supplemented with insulin plus either transferrin or ferric citrate (Trayner and Clemens, 1992). The stimulation of proliferation by low TPA became pronounced and reproducible when HL-60 cells maintained in defined medium were deprived of insulin. Under these conditions the cells remain healthy and continue to proliferate at a low rate and the addition of low TPA concentrations (0.03-0.3 nM) causes a doubling of the rate of cell proliferation. Insulin itself stimulates proliferation to a greater degree than does low TPA (Trayner and Clemens, 1992). Indeed, both the growth and differentiation responses of HL-60 cells grown in defined-medium were different from cells grown in the presence of serum. Until all functions of serum and growth-promoting substances in serum are identified, the behaviour of cells in serum-free conditions will probably not be identical to that in serum (Sergeant and Johnson, 1995).

1.6. Transferrin and the transferrin receptor

Transferrin, the iron-binding protein of plasma, is the only source of iron for the metabolic needs of most vertebrate cell types. These transferrin molecules consists of a single 80 kDa polypeptide chain disposed in two lobes of a highly homologous amino acid sequence. Each lobe is arranged in two domains surrounding a cleft bearing its iron-binding site (Anderson *et al.*, 1987). Ligands of each iron-binding site are identical: two phenolic oxygen atoms from tyrosyl residues, one histidyl nitrogen atom, a single aspartyl oxygen atom, and two oxygen atoms from a carbonate anion. Without carbonate, or another anion capable of replacing the protein loses its iron-binding activity (Anderson *et al.*, 1987).

The iron-donating interaction of transferrin with cells may be complete in as little as 1-3 min, during which time the protein is internalized by the cell to an acidified compartment, relieved of one or both of its iron atoms, and returned to the circulation for another cycle of iron transport. Throughout its sojourn within the cell, transferrin remains complexed to its receptor (Klausner *et al.*, 1983; Dautry-Varsat *et al.*, 1983), a molecule composed of two identical 95 kDa subunits

(McClelland *et al.*, 1984). The role of TfR is not fully understood although Bali and co-workers, (1991) have done some experiments to find out the role of TfR in the release of iron from transferrin. Their results have shown that at the pH of the cell surface, 7.4, the initial rate of release of iron from transferrin to 0.05 M pyrophosphate is nearly 10 times faster from free transferrin than from the complex of transferrin and receptor. Even at this unphysiological concentration of pyrophosphate, however, only 35% of iron initially bound to transferrin is released in 60 min. At pH 6.4 release is much faster than that at pH 7.4, as would be expected from the weakening of the iron-transferrin bond as pH is lowered.

1.6.1 The mechanisms underlying the effect of receptor on iron release from transferrin

The mechanism of iron release from transferrin to acceptor chelating molecules is in two conformational states, one "closed" and unable to release iron and the other "open" and facilitating in releasing iron (Cowart *et al.*, 1982). This view, supported by other kinetics studies (Kretchmar and Raymond, 1986), is consistent with comparative X-ray crystallographic studies of differic lactoferritin and apolactoferritin (Anderson *et al.*, 1990), a protein homologous in primary and three-dimensional structure to serum transferrin (Bailey *et al.*, 1988). Both sites of lactoferritin assumed a closed structure in the presence of iron whereas in the absence of metal, the N-terminal site changed to a wide-open configuration due to a jaw-like pivoting of the surrounding domains about their hinge with the C-terminal site remaining in a closed conformation. Bali *et al.*, (1991) therefore, suggested that at low pH, the transferrin receptor forces one or both lobes of transferrin into the open confirmation, thereby facilitating release of iron. At extracellular pH, where the receptor has low affinity for apotransferrin, it may lock iron-bearing transferrin into the closed state and may impede iron release as well as by restricting access to the iron binding cleft (Tsunoo and Sussman, 1983; Young *et al.*,1984). However, the transferrin receptor binds transferrin more strongly at pH 5.0 than at pH 7.4 (Bali *et al.*, 1991).

1.6.2. Functions of the transferrin receptor

A well established function of the TfR is to sequester iron-bearing transferrin for internalization by iron-requiring cells, while ignoring iron-free transferrin. Transferrin is thereby able to donate iron to cells even in the presence of a preponderance of apotransferrin in the circulation, as in iron deficiency (Bali *et al.*, 1991). Many membrane proteins continually cycle between the cell

surface and endosomal compartments. The TfR is the model receptor of choice for studies of constitutive endocytic internalization and recycling. An advantage of using the TfR is that the complete endocytic cycle can be monitored using the receptor's native ligand, transferrin. Although in no single study has the trafficking of GLUT4 and TfR been directly compared, two characteristics are believed to distinguish GLUT4 trafficking from the trafficking of the transferrin (Yeh *et al.*, 1995; Subtil *et al.*, 2000). Bali *et al.*, (1991) have found another biological function of the TfR in modulating iron release from transferrin. At the pH of the cell surface, 7.4, formation of a complex between transferrin and its receptor impedes release of iron from transferrin. Although spontaneous release of iron at this pH is negligible in terms of cellular needs for iron, even trace amounts of iron release promoted by phosphate groups of the cell membrane may be sufficient to cause peroxidation of membrane lipids (Gutteridge, 1987). Thus, TfR may serve to protect the cell membrane against locally released trace iron. Perhaps more importantly, the receptor facilitates freeing of iron from transferrin in the pH range achieved by endosomes to which transferrin is internalized by the cell (Bali *et al.*, 1991).

1.7. Insulin and the insulin receptor

Insulin is a hormone that mobilizes responses to a nutritional state of plenty and is released from the beta cells of the pancreas in reaction to elevated blood glucose and amino acids. Insulin is a molecule consisting of an α-chain and a β-chain linked by a 31-amino acid connecting peptide and two pairs of dibasic amino acids (Ido *et al.*, 1997). This polypeptide hormone stimulates anabolic processes, including glycogen, lipid and protein synthesis. Insulin stimulation of glucose uptake by fat and muscle mainly through the translocation of GLUT4 from an intracellular location to the cell surface serves as an important mechanism for regulating whole body glucose homeostasis (Czech, 1995). An impairment in insulin-stimulated glucose uptake is a major factor leading to the development of non insulin-dependent diabetes mellitus (Cushman and Wardzala, 1980).

Moreover insulin acts on certain cell types in culture as a growth factor to cause cell proliferation. Exposure of a variety of cell types to insulin leads to an increased phosphorylation of ceratin proteins. These proteins includes the IR, ribosomal protein S6, ATP citrate-lyase, acetyl-CoA carboxylase, and a number of other proteins of unknown function (Avruch *et al.*, 1985). These

effects are initiated by the binding of insulin to its receptor (Garbarino *et al.*, 1985).

The insulin receptor is a tetrameric transmembrane glycoprotein consisting of two 120 kDa α- and two 90 kDa β-subunits. The two α-subunits are disulphide-linked, extracellular and contain the insulin-binding sites. Each β-subunit is disulphide-linked to an α-subunit, spans the membrane, and contains a tyrosine kinase domain in its intracellular portion (Garbarino *et al.*, 1985). The association of the insulin with the receptor stimulates its tyrosine kinase activity. After insulin has bound to its specific cell surface receptors, it activates the receptor tyrosine kinase activity, which in turn phosphorylates the receptor β-subunit and its cellular target proteins such as IRS1. These events are coupled to a series of cytoplasmic protein serine threonine kinases such as MAP kinase, S6 kinase and phosphatidyl 3-kinase (Garbarino *et al.*, 1985).

Autophosphorylation activates the kinase activity of the receptor towards other substrate proteins. Amongst these substrates is a 130 kDa protein known as insulin IRS1. IRS1 was first identified with antibodies against phosphotyrosine [Tyr (P)] as a Tyr (P)-containing polypeptide that appeared within seconds after treatment of a hepatoma cell line with insulin. Subsequently IRS1 was detected in a number of insulin responsive tissues and cell lines (Garbarino *et al.*, 1985).

Insulin stimulation also alters transcription of a variety of specific genes, however, the mechanism of transmission of the signal to the nucleus is undefined. Csermely and Kahn, (1992) have found that insulin stimulates the phosphorylation of several nuclear proteins capable of binding to DNA including lamins, numatrin and nucleolin, on serine residues.

Insulin-like growth factor-1 which is an essential growth promoting peptide that shares structural and functional features with insulin, stimulates the growth of HL-60 cells acting through their receptors induces granulocytic differentiation of RA-treated HL-60 cells and rescues these cells from apoptosis by activating phosphatidyl 3'-kinase (Liu *et al.*, 1997). This finding suggests that insulin may mimic IGF-1 in inducing differentiation of RA-treated HL-60 cells. Dikic *et al.*, (1994) reported that insulin induces neuronal differentiation in PC12 cells overexpressing the insulin receptor.

1.7.1. The insulin receptor

Insulin binding to its cell surface receptor activates the intrinsic tyrosine kinase activity of the insulin receptor and stimulates tyrosine phosphorylation of insulin receptor substrate proteins. Tyrosine-phosphorylated IRS proteins in turn recruit Src homology 2 domain-containing signalling proteins. Two main pathways have been identified downstream of IRS proteins, the MAP kinase pathway and the PI3K pathway. The PI3K pathway, through protein kinase B, has been shown to be necessary for insulin-stimulated glucose transport through various experimental approaches. In particular, platelet-derived growth factor stimulates a PI3K activity to the same extent as insulin in adipocytes but has relatively little effect on glucose transport (Hill *et al.*, 2000; Holman *et al.*, 1994). Variability in insulin receptor expression has been related to cell differentiation. Alteration in insulin receptor expression is selective in the bipotent HL-60 cell line. Yamanouchi *et al.*, (1982) have reported that both monocytic and myelocytic differentiation of HL-60 cells results in an increase in binding. In contrast, Palumbo *et al.*, (1983) have reported that granulocytic differentiation of HL-60 cells with DMSO is accompanied by a decrease in insulin receptor levels. Chaplinski and Bennett, (1987) have reported that granulocytic differentiation of HL-60 cells results in a decrease in insulin receptors, but monocyte differentiation results in a significant increase in receptor expression. In addition, treatment of U-937 with retinoic acid, a granulocytic inducing agent, decreases insulin binding levels, while induction of differentiation with Vitamin D increased binding.

1.7.2. Insulin receptor substrate 1

IRS1 is a hydrophilic protein and is highly conserved. The full role played by the IRS1 in insulin signalling is unknown, but one function appears to be to couple the insulin receptor to activation of the enzyme, phosphatidyl inositol triphosphate-kinase (Sun *et al.*, 1993). A striking feature of IRS1 is the presence of 18 potential sites for tyrosine phosphorylation that are distributed throughout the protein. IRS1 is in fact phosphorylated on multiple tyrosine residues in response to insulin. These Tyr(P)-containing sequences serve as a binding sites for a group of proteins containing Src homology 2 (SH2) domains. IRS1 also contains a plectrin homology (PH) domain in its N-terminal portion and is involved in signal transduction. In addition to the PH domain, IRS1 has a potential ATP-binding site that is homologous to the ATP-binding site in the kinase-like domain of the tyrosine kinase JAK1. IRS1 is directly phosphorylated by the receptor *in vivo*

rather than by an intermediate kinase. However, the insulin receptor is not the only tyrosine kinase that phosphorylates IRS1 (Keller and Lienhard, 1994). Following insulin stimulation, there is a large increase in PI3K kinase activity in a high speed pellet fraction. This fraction also contains the major insulin regulatable IRS protein (IRS1 and IRS2) found in insulin-sensitive cells. Alternatively, the activation of glucose metabolism by insulin may require activation of the PI3K pathway as well as an additional, insulin-specific pathway (Hill *et al.*, 2000).

1.7.2.1 The role of IRS1 in downstream effects of insulin

Several studies have indicated that IRS1 is required for insulin and IGF-1 stimulated DNA synthesis and cell proliferation. First, expression of IRS1 in a myeloid progenitor cell line, a 32D, that lacks both IRS1 and the related protein known as 4PS enables these cells to respond mitogenically to insulin and also IL-4. Second, microinjection of rat fibroblast with antibodies against the N-terminal domain blocks insulin and IGF-1 elicited DNA synthesis. Third, CHO cells expressing antisense IRS1 RNA have reduced growth rates, which correlates with decreased insulin-dependent transcription of a reporter gene (luciferase) under control of serum-response element. Expression of IRS1 restores insulin-enhanced transcription through this element. Last, the progression of *Xenopus* oocytes from the G_2-M boundary into the first meiotic division in response to insulin or IGF-1 requires prior microinjection of IRS1 (Chuang *et al.*, 1993). The role(s) of IRS1 in the various metabolic effects of insulin have not yet been established. There is a correlative suggestion that IRS1 is required for the stimulation of glucose transport by insulin. Prolonged insulin treatment of 3T3-L1 adipocytes causes a marked downregulation of IRS1 by increasing its rate of degradation (Rice *et al.*, 1993; Rice and Garner, 1994; Keller and Lienhard, 1994).

1.8 Selenium and Selenoproteins

Aerobic cells rely on an intricate network of non-enzymatic and enzymatic antioxidants for cytoprotection from the damaging effects of reactive oxygen species such superoxide, hydrogen peroxides and hydroxyl radical (Halliwell and Gutteridge, 1989). Included among the enzymatic antioxidants are: CAT, CuZnSOD, MnSOD, and SePXs, GPX and PHGPX. Hydrogen peroxide is a common substrate for CAT and the SePXs in eukaryotic cells (Thomas and Girotti, 1989). This being the case, a reasonable adaptation of cells to conditions which limit their selenium

supply (and hence lower SePX levels) would be to overexpress CAT and/ or possibly overproduce GSH. However, prolonged selenium deprivation resulted in a selection of cells that irreversibly express enormous amounts (>100 x normal) of active CAT (Lin *et al.*, 1995).

Eukaryotic cells also express selenium-dependent peroxidases and selenium-independent GSH-glutathione transferases, which are also cytoprotective. These latter enzymes catalyse the reduction and detoxification of hydrogen peroxides and organoperoxides at the expense of GSH, thus preventing the peroxides from initiating potentially lethal free radical reactions such as lipid peroxidation (Lin *et al.*,1995). Two intracellular selenoperoxidases are known to exist: (a) GPX, located in the cytosol and mitochondrial matrix, and (b) PHGPX, located both in the cytosol and endoplasmic reticulum. GPX acts on polar peroxidase such as hydrogen peroxides and fully acid hydrogen peroxides. By contrast, PHGPX can act on a broad range of hydrogen peroxides in membranes and triacylglycerol hydroperoxides in lipoprotein (Girsini *et al.*, 1991). The involvement of GPX and PHGPX in antioxidant defence can be established by making cells selenium deficient (Thomas and Girotti, 1989; Geiger *et al.*, 1991). Lin *et al.*, (1995), have observed a gradual weaning of L1210 cells from 10% serum to 1% serum in medium without selenium supplementation resulted in a dramatic drop in GPX activity, with correspondingly greater sensitivity to oxidant stress. Selenium deficient cells accumulate lipid hydroperoxides and lose viability more rapidly than selenium supplemented controls when exposed to photochemically generated Singlet O_2, *t*-butyl hydroperoxide or cholesterol hydroperoxides. Similar findings were observed with the HL-60 cell line.

Cellular GSH-Px, also termed GSH-Px is the best characterized of a large mammalian enzyme family that also includes plasma phospholipid hydroperoxide, GI glutathione peroxidases. In turn, this family is part of a larger class of selenoprotein, a unique group of prokaryotic and eukaryotic polypeptides incorporating the unusual amino acid selenocysteine. HL-60 cells as well as other myeloid cell lines such as PLB-985, also have the capacity to differentiate along granulocytic and monocytic-macrophage pathway after treatment with phorbol esters or dimethyl formamide respectively. During differentiation of phagocytic cells *in vivo*, their abilities to ingest and destroy foreign particles matures as does their ability to degrade the toxic products produced during the respiratory burst (Chu *et al.*, 1993; Newberger *et al.*, 1984). Shen *et al.*, (1994) have shown that

during differentiation of HL-60 cells to granulocytes, their ability to generate O_2 free radicals also matures. As GSH-Px activity is an important component of the cellular antioxidant defence system, cellular GSH-Px activity might also be expected to change during phagocytic differentiation, expression and regulation of GSH-Px gene during their induced, *in vitro* maturation along granulocytic and monocytic differentiation of (PBL-985 and HL-60 cells) was examined. Levels of selenoenzyme increase in conjunction with increases in steady-state mRNA levels and transcription rates for the GSH-Px gene. Thus, differentiation is regulated at least in part at the transcriptional level, in contrast with its translational regulation by selenium.

1.9. Lithium

Lithium, a monovalent cation has been used for more than 50 years for the treatment of manic depression (Calil *et al.*, 1990). Becker and Tyobeka (1990), have found that lithium enhances proliferation of HL-60 cells at low concentrations (2.5-5 mM) and inhibit growth of these cells at 10 mM and above. Lithium also induces leukocytosis and inhibits DMSO-induced terminal differentiation of murine erythroleukaemic cells (Zaricznyj and Macara, 1987). This effective drug is widely used and has drawn intense research interests because the mechanism of action underlying its therapeutic efficacy are still not clearly understood.

1.9.1. Mitogenicity

By treating HL-60 cells at varying concentrations of 0-5 mM lithium, it was noted that lithium stimulated HL-60 cell proliferation within a very narrow concentration range. Enhancement of growth was optimal at 5 mM, whereas 10 mM and above inhibited cell growth (Becker and Tyobeka, 1990). Gauwerky and Golde (1982), have found that lithium stimulated HL-60 colony formation at concentrations between 0.05-3 mM, with maximum stimulation occurring at 0.5 mM. Masemola *et al.,* (1991) showed that two different HL-60 sublines (HL-60 M and JE) had varying growth rates, both of which were enhanced when the cells were incubated in the presence of 5 mM lithium. However Knight *et al.,* (1989) found no enhancement of HL-60 growth at the concentrations ranging from 0.1-10 mM, but a reduction in growth occurred at 10 mM lithium and above. Sokoloski and Sartorelli, (1991) and Sokoloski *et al.*, (1993) could also not detect any mitogenic response of HL-60 cells to lithium treatment, but did ascertain that concentrations above 10 mM reduced cell proliferation. Sokoloski *et al.,* (1993) reported that 5-10 mM lithium

exhibited a concentration-dependent increase in the extent of differentiation of WEHI-3BD$^+$ cells and expression of Mac-1.

1.9.2. Mechanism of action

The mechanism of action of lithium is not well understood. However, lithium has been found to inhibit the formation or action of the adenylate cyclase cAMP system in many tissues (Christeson *et al.*, 1985). The inositol phospholipid system is another signal transduction pathway which is affected by lithium. Lithium inhibits the enzymatic action of myo-inositol 1-phosphatase which converts myo-inositol phosphate to inositol. As the hydrolysis of the membrane receptor requiring calcium it may be that the effect of lithium on the PI pathway is important in the action of lithium in many systems (Lazarus, 1993). Although 5 mM lithium stimulates HL-60 cell proliferation, addition of myo-inositol did not show any influence on lithium enhanced HL-60 proliferation (Becker and Tyobeka, 1993; Sokoloski *et al.*, 1993). However, when HL-60 cells were cultured in the presence of beryllium, an agent known to inhibit inositol monophosphate 5- fold more potently than lithium (Creba *et al*, 1989) enhancement of HL-60 cells growth was not observed, indicating that it is unlikely that signalling through this pathway is responsible for lithium enhanced proliferation of HL-60 cells (Becker and Tyobeka, 1993).

1.10. Apoptosis

The discovery of apoptosis, a widespread and morphologically distinct form of physiological cell death, has had an extraordinary impact on cell biology. The growing appreciation of the significance of this process has stimulated intense investigation into the molecular mechanisms involved and into its fundamental implications for developmental biology, immunology and oncology. Cell death is an important element in the pathogenesis of many diseases. There are two mechanisms of cell death, namely necrosis and apoptosis (programmed cell death) which are distinguishable by morphological and molecular characteristics (Kerr *et al.*, 1972). However, apoptosis and necrosis share common steps in signal transduction pathway (fig.1.1), since both modes of cell death can be suppressed by the anti-apoptotic Bcl-2 protein and caspase inhibitors (Tsujimoto, 1997). Necrotic cell death is a pathological form of cell death resulting from cellular injury, followed by rapid cell swelling and lysis and induction of inflammation. At high stress levels, cells die by necrosis, largely because they have no time to respond to the stimulus and die

instantly (Thompson, 1995).

Apoptosis on the other hand, is a highly regulated process involving gene expression, protein synthesis and activation of specific enzymes including an endonuclease responsible for internucleosomal DNA fragmentation. Characteristics include, cell shrinkage, membrane blebbing and chromatin condensation. Phagocytosis occurs before cell lysis and no inflammation is induced (Williams *et al.*, 1992; Goldstein, 1987). Apoptosis can be triggered by various external stimuli including DNA damaging agents such as chemicals, chemotherapeutic drugs and irradiation, hormones, toxins, growth factor withdrawal, cell-cell interactions, and physical trauma (Giannikis *et al.*, 1991; Wylie *et al.*, 1980).

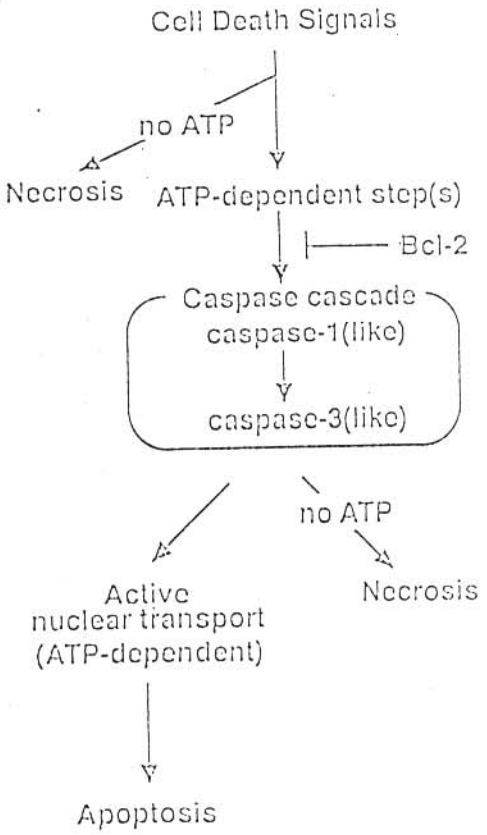


Fig.1.1. The above figure indicates the common signal transduction pathway shared by apoptosis and necrosis (Tsujimoto, 1997).

There are three known types of DNA fragmentation occurring during apoptosis: internucleosomal DNA cleavage, fragmentation into large (50-300 kbp lengths), and single-strand cleavage events (Bortner *et al.*, 1995).

1.10.1. Internucleosomal DNA cleavage

In almost all circumstances of morphologically well-characterized apoptosis, internucleosomal DNA cleavage has been the biochemical event used as the definitive apoptotic marker. This pattern of DNA degradation occurs by activation of an endogenous endonuclease that cleaves the DNA in the linker region between histones on the chromosomes. Since the DNA wrapped around the histones comprises ~180-200 bp, multiples of this interval are characteristically observed and are commonly referred to as 'apoptotic ladder'. This type of DNA degradation clearly occurs before cell death, once the cells have fragmented their DNA they are committed to die and cannot be rescued by removal of the apoptotic signal (Walker *et al.*, 1991).

1.10.2. Cleavage into large DNA fragments

Recently, several research groups have detected large DNA fragments of 50-300 kbp during apoptosis. Several investigations have proposed that large DNA fragmentation occurs before internucleosomal DNA cleavage and that these large DNA fragments serve as precursors for the smaller DNA fragments (Walker *et al.*, 1991).

1.10.3. Single-strand DNA cleavage

Recently, single-strand cleavage of DNA has also been suggested to occur during the apoptotic process by several researchers. In thymocytes induced to undergo apoptosis with steroid treatment, or stimulation of the T-cell receptor, numerous single-strand breaks in internuclesomal region and in the core-histone-associated DNA were observed (Peitsch *et al.*, 1993; Yoshida *et al.*, 1993).

1.10.4. ATP as an intracellular determinant of cell death by apoptosis versus necrosis

The level of ATP determines whether cells must die by apoptosis or necrosis. ATP depletion itself induces necrosis, indicating that necrosis does not require intracellular ATP. Eguchi and co-workers, (1997) have done some experiments to directly address the question whether

apoptosis is ATP-dependent. Depletion of intracellular ATP by incubating cells in glucose-free medium to halt glycolysis, in the presence of the mitochondrial F_0F_1-ATPase inhibitor oligomycin, completely blocked apoptosis induced by Fas stimulation, Vp16, dexamethasone or calcium ionophore. Oxidative phosphorylation restored the apoptotic cell death pathway, indicating that apoptosis is ATP-dependent (Eguchi *et al.*, 1997; Leist *et al.*, 1997). Shimizu *et al.*, (1995), measured intracellular ATP levels during cell death, and showed that intracellular levels remained unchanged until the very end of apoptotic process, such as disruption of the plasma membrane. Since it has been reported that loss of mitochondrial membrane potential, which halts mitochondrial ATP production, is an early step in apoptosis (Zamzami *et al.*, 1995; Shimizu *et al.*, 1996b), intracellular ATP required for the rest of apoptotic processes must be provided from glycolysis.

1.10.5. Ca^{2+} as a regulator of apoptosis

The cytosolic Ca^{2+} concentration can regulate apoptosis. In many cells, apoptosis is induced by treatment with thapsigargin or Ca^{2+} ionophores. Certain toxic chemicals may also promote apoptosis by stimulating PLC and disrupting intracellular Ca^{2+} homeostasis, leading to nonphysiological Ca^{2+} increase that promotes endonuclease activation and apoptotic cell death. Intracellular or extracellular Ca^{2+} chelators, Ca^{2+} channel blockers and calmodulin antagonists can all delay or abolish apoptosis in several model systems. Consistent with these effects, overexpression of the Ca^{2+} binding protein calbindin D28-K can block apoptotic cell death. Finally, anti-apoptosis onco-protein, Bcl-2 is involved in alterations in Ca^{2+} compartmentalization. Together these observations indicate that Ca^{2+} is the frequent trigger of apoptosis in diverse experimental systems. In addition, Ca^{2+} increases may promote apoptosis by altering the activity of particular transcription factors such a fos, jun or cAMP-response-element-binding protein, leading to changes in gene expression that may be required for the response (McConkey and Orrenius, 1994).

1.10.6. Oncogenes and proteins implicated in apoptosis

1.10.6.1 *p53*

The *p53* tumour suppressor gene is necessary for some but not all forms of apoptosis. This protein is known to be deleted in HL-60 cells (Dou *et al.*, 1995). A major cellular function of the

p53 is to suspend progression through the cell cycle in response to DNA damage so that it can be repaired before replication. Cell arrest occurs in the G_1 phase of the cell cycle, following DNA damage. This allows time to repair the damage before DNA synthesis occurs. If *p53* fails to respond to the extreme damage, apoptosis is triggered (fig. 1.2). An important pathway by which *p53* acts to arrest the cell cycle is through a protein variously called p21, waf-1, Cip1 or Sdi. Activated wild-type *p53* stimulates the transcription of Waf-1. Cells that lack *p53* can not induce Waf-1 in response to DNA damage although such cells can induce Waf-1 in response to growth factor stimulation. Waf-1 is an inhibitor of cell cycle kinases, whose activity is required for the cell cycle to keep going. Waf-1 specifically inhibits a range of cdk/cyclin complexes, including the G1 cyclins complexed to cdk2, which are most relevant to the G1/S-phase arrest mediated by *p53*. These kinases phosphorylates the Rb protein, which dissociates from E2F which in turn switches on the genes that are needed to go from G1 to the S phase. Overexpression of Waf-1 is sufficient to cause growth arrest, suggesting that it is a major control point in cell cycle progression. In other cell types, *p53* activates expression of Bax and suppresses that of Bcl-2, leading to cellular apoptosis. Most recently, studies using transgenic mice suggest that loss of retinoblastoma function is associated with induction of *p53*-dependent apoptosis (Dou *et al.*, 1995).

Recently, two members of the p53 family, p63 and p73 has been identified. The two proteins were found to have some similarities with p53. They share significant homologies in their DNA binding, transactivation and oligomerisation domains suggesting that all three function as transcription factors and may transactivate common target genes. There is already evidence that p73, for example, activates the promoters of some genes involved in the cell cycle and apoptosis that are also activated by p53, though quantitative and qualitative differences have been found (Levrero *et al.*, 1999). Moreover, in situations where p73 is overexpressed in p53-deficient cells, apoptosis is induced, suggesting that p73 at least may be able to substitute for p53 in the induction of cell death. Not p53, the guardian of genome but, the p53 family, the guardians of the genome. Not all forms of DNA damage activate p73, and while p53 mutations in human cancers are common, p63 and p73 mutations are extremely rare. The relative contribution of p53, p63 and p73, and the role they played in the induction of cell cycle arrest and apoptosis in response to different stressors remains to be clarified (Levrero *et al.*, 1999; Ikawa *et al.*, 1999). In situation where p53, but not p63 and p73, is mutated, the younger siblings fail in their role as

putative substitute guardians of genomes is still not understood (Arrowsmith, 1999).

1.10.6.2 Bcl-2/Bax

Bcl-2 is a pro-oncogene encoded by a gene of 230 kb in size. It is a protein of 24-26 kDa localized in the mitochondrial membrane. The mammalian gene *bcl-2* is homologous to the *ced-9* in the nematode worm *C. elegans*. The proteins are about 23% identical in sequence and human *bcl-2* can function in *C. elegans* to suppress apoptosis, indicating that the control of apoptosis has been highly conserved during evolution. Another member of the *bcl-2* gene family, *bcl-x* has been identified and it was found to share a significant homology with *bcl-2* in its BH1 and BH2 regions. By alternate splicing, *bcl-x* encodes for two important protein isoforms of which the longer Bcl-x_L inhibits apoptosis, whereas the shorter isoform Bcl-x_s facilitates apoptosis by acting as a dominant inhibitor of Bcl-2 and Bcl-x_L (Yang and Korsmeyer, 1996; Boise *et al.*, 1993). Bcl-x_L has a pattern of expression distinct from Bcl-2 and has been shown to block apoptosis where Bcl-2 is ineffectual (Gottschalk *et al.*, 1994). High levels of Bcl-2 or Bcl-x_L have been shown to inhibit apoptosis due to chemotherapeutic agents (Yang and Korsmeyer, 1996).

Bax, a 21 kDa protein, shows extensive amino acid sequence homology with Bcl-2 and forms homodimers and heterodimers with Bcl-2 *in vivo*. Bax itself does not cause cell death. Instead, overexpressed *bax* accelerates apoptotic cell death only if a death signal such as IL-3 deprivation is detected. Excess *bax* also converts the death repressor activity of *bcl-2*. When Bcl-2 is in excess, cells are protected from undergoing apoptosis. However, when Bax is in excess, and the Bax homodimer dominates, cells are susceptible to apoptosis. *bax* functions as a cell death effector gene that is neutralised by *bcl-2* (Dou *et al.*, 1995). Recently, Zha *et al.*, (1996) have demonstrated that Bax can homodimerize or heterodimerize with its partner through a novel BH3 domain.

Ibrado *et al.*, (1997), have demonstrated that treatment of HL-60 cells with paclitaxel, an antimicrobial drug that induces apoptosis results in a significant increase of free Bax and decline in Bax heterodimerized to Bcl-2, without affecting the total intracellular Bax, Bcl-2 or Bcl-x_L levels in transfected HL-60 cells (HL-60/neo cells). The precise mechanism by which paclitaxel causes a rise in free Bax levels in HL-60/neo cells is not clear. A previous report by Halda *et al.*, (1995) had indicated that exposure to palcitaxel caused serine phosphorylation and inactivated

of Bcl-2. Subsequently, they showed that paclitaxel-induced phosphorylation of Bcl-2 also inhibited its binding to Bax, thereby promoting apoptosis. In experiments performed by Ibrado *et al.*, (1997), it was reported that, HL-60/neo cells exposed to 500 nM of paclitaxel until 18 h results in Bcl-2 phosphorylation. This immediately precedes the rise in free Bax levels.

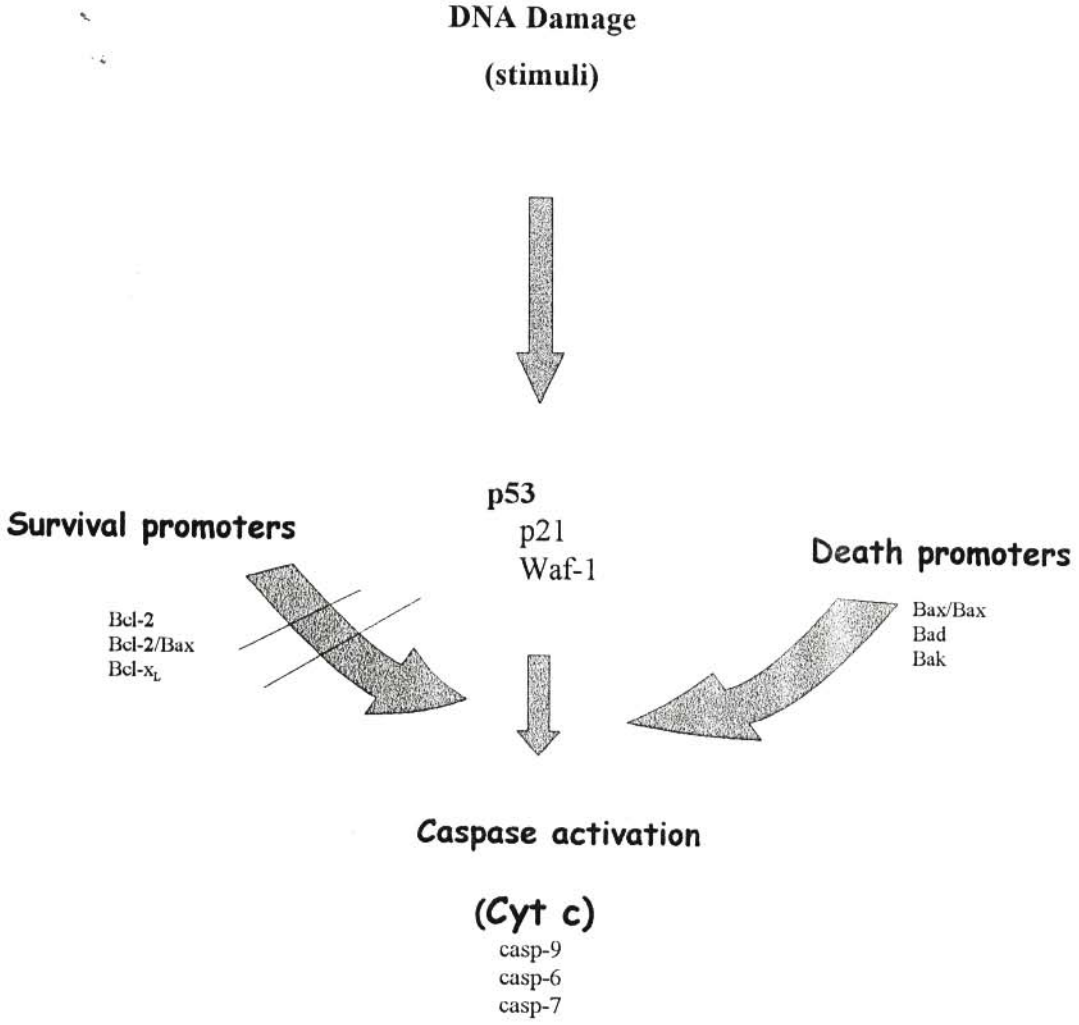


Fig. 1.2 The pathway involved in the induction of apoptosis following a stimulus and the genes involved.

Aims and Objectives

It is well documented that HL-60 cell growth and survival is dependent on the presence of insulin in the culture medium. Furthermore, lithium has been shown to require the presence of insulin in order to induce its proliferation enhancing and apoptotic effects on HL-60 cells. However, the exact biochemical mechanism by which these processes take place are not well described. Hence, this study was aimed at further elucidating these mechanisms of action. This was achieved by focussing on the gene expression systems which are found to have contributed in the induction of apoptosis. Specific objectives:

(a) To determine cell proliferation and viability of HL-60 cells in media containing either I/T/S, I/S or T/S as growth factors.

(b) DNA fragmentation and TUNEL to determine apoptosis of lithium treated HL-60 cells supplemented with either I/T/S, I/S or T/S.

© Determination of gene expression of *bcl-2* and *bax* by Western blotting of their protein products in lithium treated HL-60 cells supplemented with either I/T/S, I/S or T/S.

Chapter 2

2. Materials and Methods

2.1. Materials

Chemicals, assay kits and equipments were purchased from the following companies:

- HL-60 promyelocytic leukaemia cell line - **ATCC, Rockville, MD, USA**.
- RPMI-1640, Fetal bovine serum, Penicillin G - **Highveld Biologicals, RSA**.
- Streptomycin, Trypan blue, Insulin/Transferrin/Selenium, Insulin/Selenium, Transferrin/Selenium, Sodium selenite, EDTA, RNase, Proteinase K, Aprotinin, Sodium orthovandate, PMSF - **Sigma Chemicals Co. St. Louis, MO, USA**.
- Mycoplasma detection kit, Cell proliferation ELISA BrdU colorimetric assay kit, In Situ Cell Death Fluorescein (TUNEL) kit, BM purple AP substrate Anti-mouse Ig-AP Fab fragments, Sodium dodecyl sulphate - **Boehringer Mannheim, Germany**.
- Lithium chloride, Ethidium bromide, Phenol, Acrylamide, TEMED - **Fluka Chemi. AG., Buchs, Switzerland**.
- β-Mecaptoethanol, Tween-20, Ethanol, Methanol, Chloroform, Glycerol, 2-propanol - **E. Merk, Darmstadt, Germany**.
- 0.2 μm filter sterilizer - **Sartorius, Germany**.
- Agarose gel molecular biology grade - **Promega Corp. Madison, USA**.
- Tris- (Hydroxymethyl aminomethane), Bromophenol blue, Sodium chloride, Sodium dihydrogen orthophosphate, di-Sodium hydrogen orthophosphate - **Saarchem Holopro. Analytic, JHB, RSA**.
- Primary antibodies, anti-Bcl-2, anti-Bax - **Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA**.
- BCA* protein assay, HRP super signal Chemiluminescent substrate, Goat anti-mouse HRP - **Pierce, Rockford, USA**.
- ECL Western blotting detection system - **Amersham Int. Burkinghamshire, England**.
- Electrophoresis equipments - **Bio-Rad Laboratories, GmbH**.
- Co_2 Incubator (model Queue) - **Medical Distributors, JHB, RSA**.
- Coulter Counter model Zf, Centrifuges (model GS-6R and GS-15R) - **Beckman**

Coulter Electronics, Inc., Luton, UK.

- Computerized UVP-Scanner - **Vacutec, JHB, RSA.**
- Light microscope, Fluorescence microscope - **Zeiss, Germany.**
- Plasticware - **Sterilab Services cc., JHB, RSA; Costar Corp. Cambridge, MA, USA and Weil Organization, JHB, RSA.**

2.2. Methods

2.2.1. Cell culture

HL-60 promyelocytic leukaemia cells were maintained at 2-10 x 10^5 cells/ml in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated (56^{0}C for 30 min) fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin) at 37^{0}C in a humidified 95% air/5% CO_2 atmosphere. Cells were checked for mycoplasma contamination using a commercial mycoplasma detection kit.

2.2.2. Cell growth and viability

Prior to experiments, serum was removed from the cultures by centrifugation at 800 rpm and washed once with RPMI-1640. The cell pellet was resuspended in RPMI-1640, supplemented with either 5 µg/ml insulin/transferrin/selenium (I/T/S), 5 µg/ml insulin and 5 ng/ml selenium (I/S), 5 µg/ml transferrin and 5 ng/ml selenium (T/S), as growth factors. Selenium was considered as a detoxifying agent in all the experiments performed. HL-60 cells were treated with 0, 5, 10 or 20 mM lithium and seeded at 3.8 x 10^5 cells/ml and incubated for 3 days in the environment described above. The rate of cell growth before and after treatment with lithium was determined by counting the cells using a model Zf Coulter Counter and expressed as cells/ml.

The extent of cell proliferation was measured by using a commercial cell proliferation ELISA BrdU colorimetric assay kit following the manufacture's procedure. Briefly, treated and untreated cultures were transferred into a flat bottom 96-well MT plate to a final volume of 100 µl/well at a concentration of 5 x 10^5 cells/ml and incubated at 37^{0}C in a humidified atmosphere over 3 days. Samples were aliquoted after 24, 48, and 72 h. After 24 h of incubation, 10 µl/well BrdU was added and incubated further for 2 h at 37^{0}C. The labelling medium was removed by centrifugation at 300 x g by suction. Cell fixation and DNA denaturation was achieved by

addition of 200 µl/well FixDenant and incubated for 30 min at room temperature. FixDenat solution was thoroughly removed by flicking off and tapping and 100 µl/well anti-BrdU working solution was added and incubated for approximately 60 min at room temperature. The antibody was removed by washing with 300 µl/well washing solution three times. Substrate solution (100 µl/well) was added and incubated at room temperature until color development was sufficient (10 min) and the absorbence was measured at 405 nm using an ELISA reader with reference wavelength of 492 nm. The percentage of viable cells were detected by staining the cells with 0.1% trypan blue and examination under the light microscope. The cells which do not take up the dye were regarded as viable and scored as a percentage.

2.2.3. DNA fragmentation assay

For the determination of internucleosomal DNA fragmentation, HL-60 cells were treated with 0, 5, 10 or 20 mM lithium in the presence of either I/T/S, I/S or T/S in RPMI-1640. DNA was extracted at 24, 48 and 72 h, according to the method of Gunji *et al.*, (1992). Briefly, about 3 x 10^6 cells/ml pelleted by centrifugation and digested with lysis buffer (10 mM Tris-HCl pH 8.0; 100 mM EDTA; 0.5% (w/v) SDS and 0.5 µg/ml proteinase K) for 1 h on ice. DNA was extracted by phenol:chloroform (1:1), and further precipitated 100% ice-cold isopropanol containing 3M sodium acetate at pH 5.2 overnight at -20 ^{0}C. The DNA was recovered by centrifugation at 14 000 rpm for 15 min, washed with 70% ethanol and air dried for 30 min. The DNA was then resuspended in TE buffer and treated with 0.5 mg/ml RNase for 1 h at 37 ^{0}C. About 40 µg of DNA per lane was electrophoresed on a 1.5 % agarose gel for 2½ h at 56 volts. The gel was visualized for DNA laddering by staining with ethidium bromide and photographs taken using an image analyzer.

2.2.4. Cell death detection and quantification

HL-60 cells were treated with 0, 5, 10 or 20 mM lithium in the presence of either I/T/S, I/S or T/S in RPMI-1640 for 24 h. Detection and quantification of apoptosis at single cell level was determined using the In Situ Cell Death Detection kit, Fluorescein (TUNEL). The cells were washed twice with PBS at 4 ^{0}C and adjusted to 12-18 x 10 ^{6} cells/ml. The suspension was transferred into a V-bottom 96-well MT plate and fixed with 100 µl/well with paraformaldehyde solution (4% in PBS, pH 7.4) and incubated for 30 min at room temperature. The cell suspension was washed twice with PBS by centrifugation at 300 x g for 10 min and resuspended in 100

µl/well permeabilisation solution (0.1% TritonX-100 in 0.1% sodium citrate) for 2 min on ice. The suspension was once again washed as above and resuspended in 50 µl/well TUNEL reaction mixture and incubated for 60 min at 37 ^{0}C in a humidified atmosphere in the dark. For positive controls the cells were incubated with DNase I for 10 min at room temperature and for negative controls the fixed and permeabilized cells were resuspended in 50 µl/well label solution (without terminal transferase). The samples were washed once more and resuspended in 250 µl of PBS, cytospun onto a slide, examined under the fluorescence microscopy and micrographs taken. The percentage of individual apoptotic cells was determined.

2.2.5. Western blotting

2.2.5.1. Protein extraction and determination

HL-60 cells were treated as described previously (section 2.2.4) and they were incubated for a period of 24 h, washed by centrifugation with PBS and the cell pellet was resuspended in lysis buffer (1% NP-40, 20 mM Tris-Cl, 137 mM NaCl, 10% (w/v) Glycerol) with freshly prepared protease inhibitors (1 mM PMFS, 0.15 U/ml aprotinin, 1 mM sodium orthovandate). The cell suspension was centrifuged at 14 000 rpm and the supernatant used as total lysate. The amount of total protein in each extract was determined using the BCA protein assay. The absorbence was read at 562 nm and the protein concentration was calculated from the standard curve.

2.2.5.2. SDS-PAGE

About 50 µg/ml protein was suspended in a loading buffer (3% SDS, 15% 2-mercaptoethanol, 30% glycerol, 0.15% bromophenol blue, 19 mM Tris-HCl, pH 6.8). Samples were boiled for 90 seconds and loaded onto 12% SDS-polyacrylamide gel and electrophoresed at 30 mA for 2 h.

2.2.5.3. Electrotransfer of proteins

Following SDS-PAGE, separated proteins were transferred onto a nitrocellulose membrane according to the method of Towbin *et al.*, (1979). The transfer was carried out at 200 mA for 3 h in the transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, 0.5% (w/v) SDS, pH 8.3). The efficacy of transfer was assessed by staining the nitrocellulose membrane with 0.1% (v/v) Ponceau S in acetic acid and washing with Tris-buffered saline plus Tween-20 (TBST: 10 mM Tris-HCl, pH 7.5; 150 mM NaCl, 0.55 Tween-20) for 10 min.

2.2.5.4. Immunoblotting

For immunoblotting, the procedure recommended by the reagents suppliers was followed. Briefly, the non-specific binding sites were blocked with blocking buffer (3% fat free milk in TBST) and rocked for 30 min at room temperature, followed by washing with TBST buffer 2 times for 10 min. The primary antibodies, either anti-Bcl-2 or anti-Bax were diluted 1:200 with TBST buffer. The membranes were incubated with either one of the primary antibodies for overnight at room temperature with gentle rocking. The membranes were washed twice with TBST by gentle rocking for 10 min, followed by 1 h incubation with secondary antibodies, goat anti-mouse horse-raddish peroxidase for anti-Bcl-2 and anti-mouse IgG-alkaline phosphatase Fab fragments for anti-Bax detection, washed as above and once with TBS for 5 min. The membrane for anti-Bcl-2 detection was developed by HRP chemiluminescent substrate and exposed to X-ray film (Hyperfilm) for 5 min. The film was removed and immersed in the X-ray developing solution for 2 min, fixed with a fixing solution, stopped by rinsing with water and air-dried. For the detection of anti-Bax, BM purple alkaline phosphatase substrate was added onto the membrane and incubated for 45 min at room temperature in the dark. The reaction was stopped with running water, air-dried and photographed by using a UVP-scanner. For stripping and reprobing, the membranes were submerged in a stripping buffer (10 mM 2-mercaptoethanol, 2% SDS, 62.2 mM Tris-Hcl, pH 6.7) and washed with TBST for 15 min with gentle rocking and immunodetection performed as above.

2.2.6. Statistical Analysis

To evaluate significant differences, a statistical analysis was performed according to Student's paired t-test. Differences were considered significant at $p < 0.01$.

Chapter 3

3. Results

3.1. Growth Kinetics

3.1.1. Cell growth and viability in a media containing I/T/S

HL-60 cells are known to grow very well in serum-rich medium. Addition of lithium (10 mM and above) caused a lethal effect on these cells, whereas low lithium concentrations ($\leq$ 5 mM) were found to be growth enhancing (Becker and Tyobeka, 1990). In the present study, the effect of I/T/S on these cells in the presence and absence of lithium was investigated. HL-60 cells were treated with lithium concentrations of 0, 5, 10 and 20 mM, supplemented with 5 µg/ml I/T/S. The lithium treated and untreated (control) cultures were seeded at 4.5 x 10^5 cells/ml and incubated for 24, 48 and 72 h and maintained in an environment as described under materials and methods. Cell growth was determined by using a Coulter Counter and the findings indicated that there was a decrease in cell number with increasing lithium concentrations (fig. 3.1). The cell number in the control culture was 12.5 x 10^5 cells/ml after 48 h and this count was increased to 15.4 x 10^5 cells/ml after 72 h. In comparison to the control, the 5 mM lithium treated cultures were found to be at a significantly lower count ($p < 0.01$) with 9.8 x 10^5 cells/ ml after 48 h, which had increased to 11.8 x 10^5 cells/ml after 72 h, but was still significantly lower than control ($p < 0.01$). Similarly, higher concentrations of lithium also significantly decreased the cell growth.

In addition to evaluating cell counts, a cell proliferation study was performed to quantify cell growth by measuring BrdU incorporation during the synthesis of DNA (fig. 3.2). By following cell proliferation studies over 3 days with 24 h intervals it was discovered that 5 mM treated cells showed an initial non-significant enhanced rate of DNA synthesis compared to the control. However, significant inhibition of proliferation ($p<0.01$) was detected after 48 h and 72 h. At high concentrations of lithium (10 mM and above) the growth inhibitory effects of lithium on these cells were observed. After 48 h of 10 mM lithium treatment, BrdU incorporation was only 59% of control and this decreased to 41% of controls after 72 h ($p < 0.01$). The continuous presence of lithium decreased proliferation even further and after 72 h of treatment the BrdU incorporation was only 32% of control ($p < 0.01$). 20 mM lithium had an even more dramatic

effect, almost inhibiting BrdU incorporation completely.

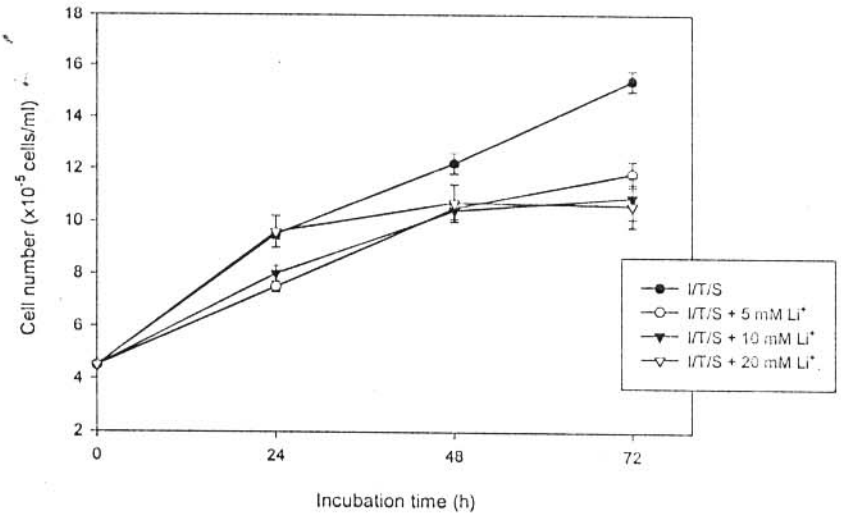


Fig 3.1. HL-60 cells were treated with lithium concentrations of 0-20 mM, supplemented with 5 µg/ml I/T/S. Aliquots were taken at 24, 48, and 72 h. The rate of cell growth was determined by counting the cells using Coulter Counter. The result represents the mean ± SEM of three independent experiments each performed in duplicate.

Viability studies were performed by staining the cells with 0.1% trypan blue and examining them under a light microscope. Cells with intact membranes exclude dye and are considered viable. Observations revealed 96% of viable cells in the presence of 5 mM lithium and the control was at 94% (fig. 3.3). However, this viability had fallen significantly to 58% after 48 h and to about 13% after 72 h ($p < 0.01$). Although the control cells also lost viability, it was not as rapid in the presence of lithium. At higher lithium concentrations (10-20 mM), a rapid

dose dependent increase ($p < 0.01$) in cell death was observed (fig. 3.3). The cytotoxic effects of lithium on HL-60 cells were shown to be mostly associated with inhibition of cell growth and DNA synthesis, as well as damage to the cell membrane which led to cell death. The mode of cell death observed in this study was further analysed by DNA fragmentation assays.

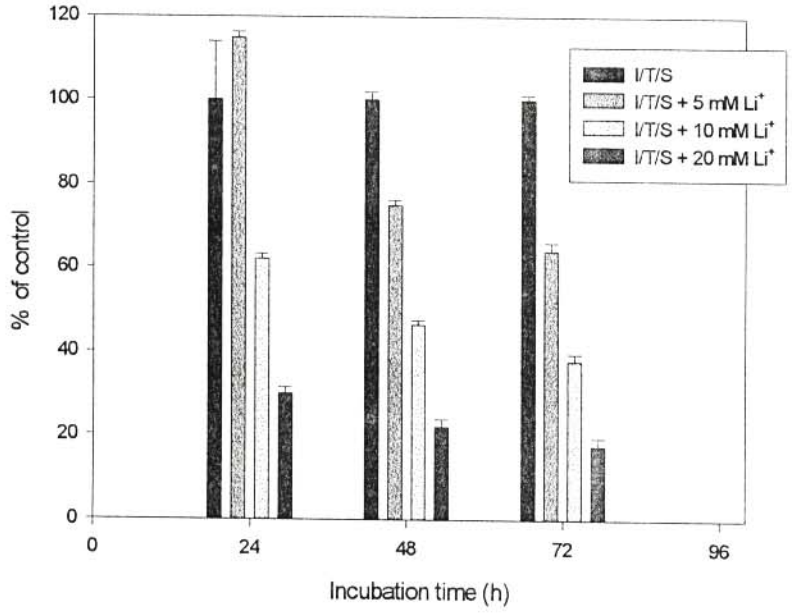


Fig. 3.2. HL-60 cells were treated with lithium concentrations of 0-20 mM, supplemented with 5 µg/ml I/T/S. Aliquots were taken at 24, 48 and 72 h. Cell proliferation studies were detected by using the Cell Proliferation kit and the procedure described under materials and methods was followed. The result represents the mean ± SEM of three independent experiments, each performed in duplicate.

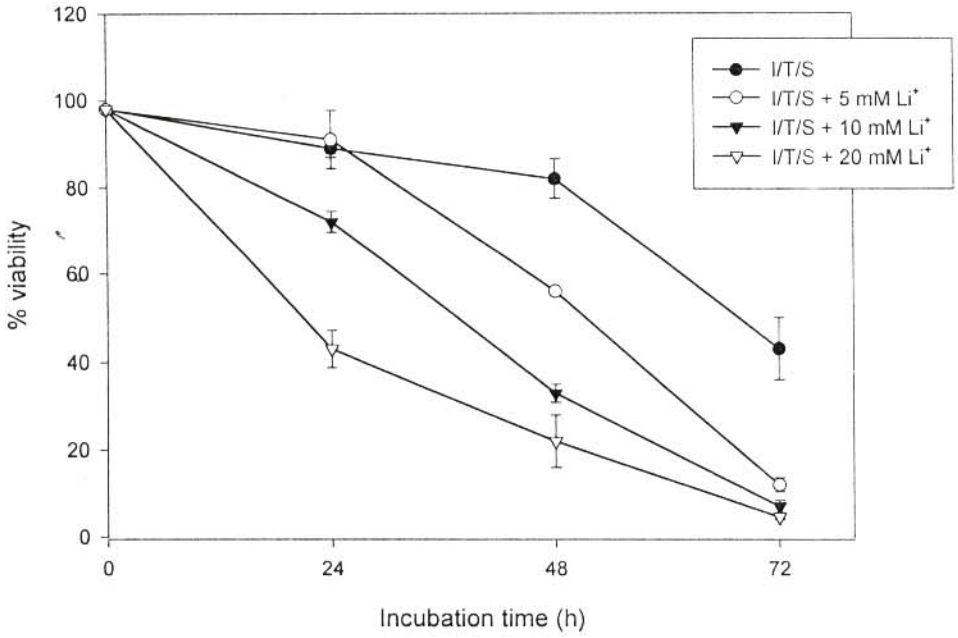


Fig. 3.3. HL-60 cells were treated with lithium concentrations of 0-20 mM, supplemented with 5 µg/ml I/T/S. Aliquots were taken at 24, 48, and 72 h. Percentage of viable cells was assayed by trypan blue exclusion method. The result represents the mean ± SEM of three independent experiments each performed in duplicate.

3.1.2. Cell growth and viability in a media enriched with I/S

In these experiments, transferrin was eliminated from the culture media and the effects of insulin and selenium on HL-60 cells were investigated. The HL-60 cells were treated with lithium (0-20 mM), supplemented with 5 µg/ml insulin and 5 ng/ml selenium, incubated for 24, 48 and 72 h and maintained in an environment described under materials and methods. The

cultures were initially seeded at a seeding density of 4.6 x 10^5 cells/ml. The observations revealed a decrease in the cell growth with increasing lithium concentrations (fig. 3.4). The cell number in the control cultures were increasing with an increase in incubation time. After 72 h, the cell count was above at 16.0 x 10^5 cells/ml. Similar to experiments done in I/T/S medium 5 mM lithium was found not to be stimulating growth (fig. 3.4). In fact, cell counts were significantly below those obtained with control cultures ($p<0.01$). In the presence of 10 mM and 20 mM lithium treated cells growth retardation was even more pronounced, reaching 10.5 x 10^5 cells/ml and 9.1 x 10^5 cells/ml respectively after 72 h ($p < 0.01$) for control compared to lithium treated. Hence the rate of cell growth was induced in a dose and time dependent manner.

Incorporation of BrdU into newly synthesised DNA was measured (fig. 3.5). Proliferation in the 5 mM lithium treated cells was not enhanced as was the case with I/T/S. DNA synthesis was further inhibited when 10 mM lithium was present. BrdU incorporation was 76% of control after 24 h and this percentage was further decreased to 48% after 72 h ($p < 0.01$). Moreover, cell proliferation was strongly inhibited when 20 mM was added to the cell cultures reaching only 18% of control after 72 h of incubation ($p < 0.01$). Hence, cell growth was significantly inhibited with increasing lithium concentration and prolonged exposure time.

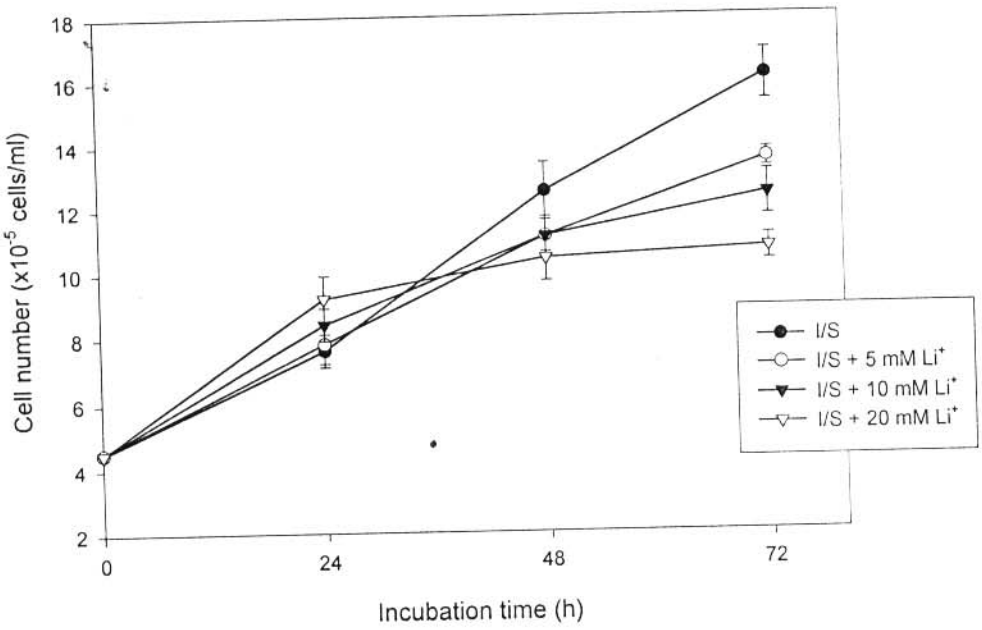


Fig. 3.4. HL-60 cells were treated with lithium concentrations of 0-20 mM, in media supplemented with 5 μg/ml insulin and 5 ng/ml selenium. The treated and untreated (control) cells were incubated for 24, 48, and 72 h. The rate of cell growth was determined by counting the cells using the Coulter Counter. The above data represents the mean ± SEM of three independent experiments each performed in duplicate.

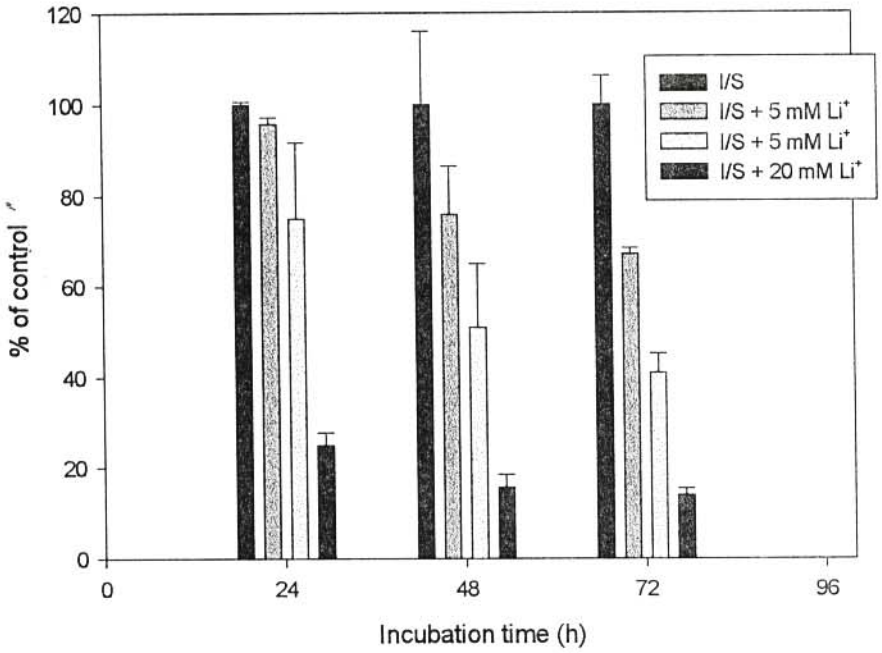


Fig. 3.5. HL-60 cells were treated with lithium concentrations (0-20 mM), supplemented with 5 µg/ml insulin and 5 ng/ml selenium. The treated and untreated (control) were incubated for 24, 48 and 72 h. The extent of cell growth was determined by using the Cell Proliferation kit following the manufacture's procedure. The above data represents the mean ± SEM of three independent experiments each performed in duplicate.

Viability studies showed 82% of viable cells in the controls after 24 h (fig. 3.6). Compared to the I/T/S medium, the presence of 5 mM lithium significantly affected the viability of these with only

48% of cells surviving after 72 h ($p < 0.01$). Cell death was induced to 82% of control in the presence of 10 and 20 mM lithium ($p < 0.01$). In these experiments, either in the presence or absence of lithium, growth and viability of HL-60 cells was inhibited. The viability studies demonstrated that there was cell death occuring characterised by a decrease in the number of viable cells. The mode of cell death was detected by DNA fragmentation studies.

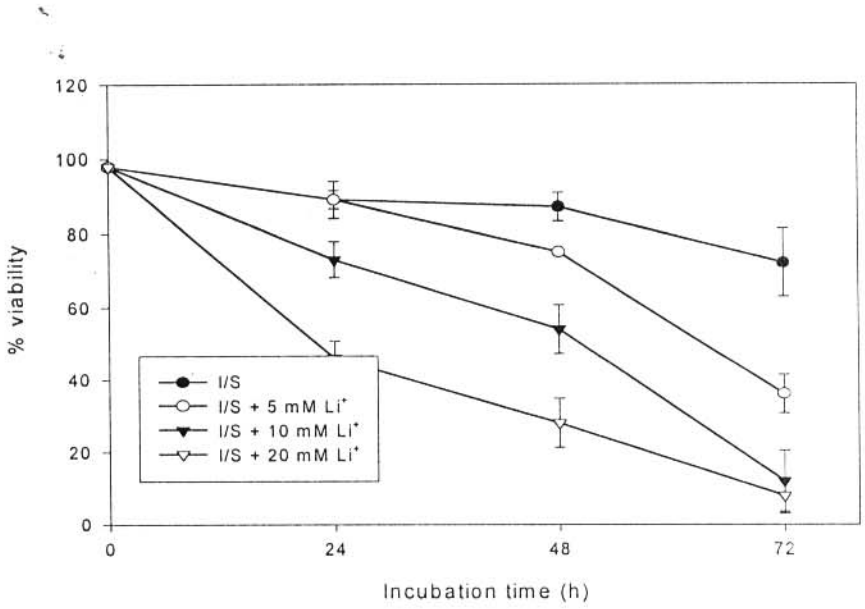


Fig. 3.6. HL-60 cells were treated with lithium concentrations (0-20 mM), supplemented with 5 µg/ml insulin and 5 ng/ml selenium. The treated and untreated (control) were incubated for 24, 48, and 72 h. The percentage of viable cells was examined by trypan blue exclusion method. The above data represents the mean ± SEM of three independent experiments each performed in duplicate.

3.1.3. Cell growth and viability in a media enriched with T/S

In these experiments, insulin was removed from the culture media and transferrin was used as the only growth factor. HL-60 cells were treated with 0-20 mM lithium, enriched with 5 μg/ml transferrin and 5 ng/ml selenium. The rate of cell growth was monitored over 3 days with 24 h intervals (fig. 3.7). The control showed an increased cell number from the original count of 4.5×10^5 cells/ml to 7.1×10^5 cells/ml at 24 h period, and a further increment to 13.8×10^5 cells/ml was observed after 72 h. Interestingly, the cultures treated with 10 mM lithium showed an initial rapid increase in cell number after 24 h. This effect was negated after 48 h.

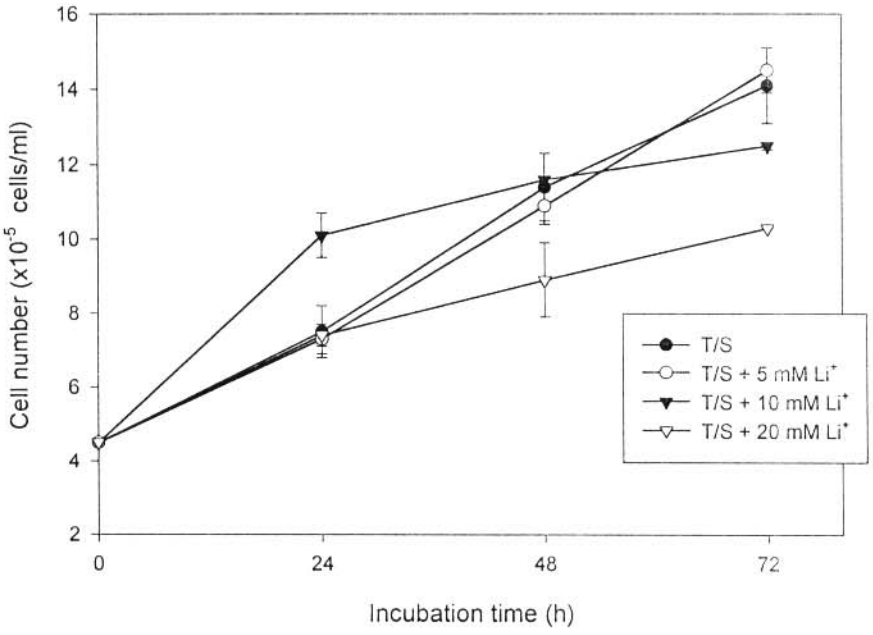


Fig. 3.7. HL-60 cells were treated with (0-20 mM) lithium, supplemented with 5 μg/ml transferrin and 5 ng/ml selenium. The treated and untreated (control) cells were incubated for 24, 48, and 72 h. The rate of cell growth was determined by using the Coulter Counter. The above data represents the mean ± SEM of three independent experiments, each performed in duplicate.

In order to ascertain the rate of DNA synthesis, the proliferation assay was performed. The observations made revealed growth enhancement when the HL-60 cells were treated with 5 mM lithium (fig. 3.8). DNA synthesised was 147% of the control after 24 h of incubation ($p < 0.01$) compared to the control. The synthesis of DNA reached 98% of control after 72 h with 5 mM lithium treatment. Transferrin boosted growth of HL-60 cells at low lithium concentrations (5 mM). Furthermore, lithium doses of 10 mM and above inhibited growth strongly. The results obtained from the transferrin-enriched media were in agreement with the findings reported by Becker and Tyobeka, (1990) for serum-rich cultures.

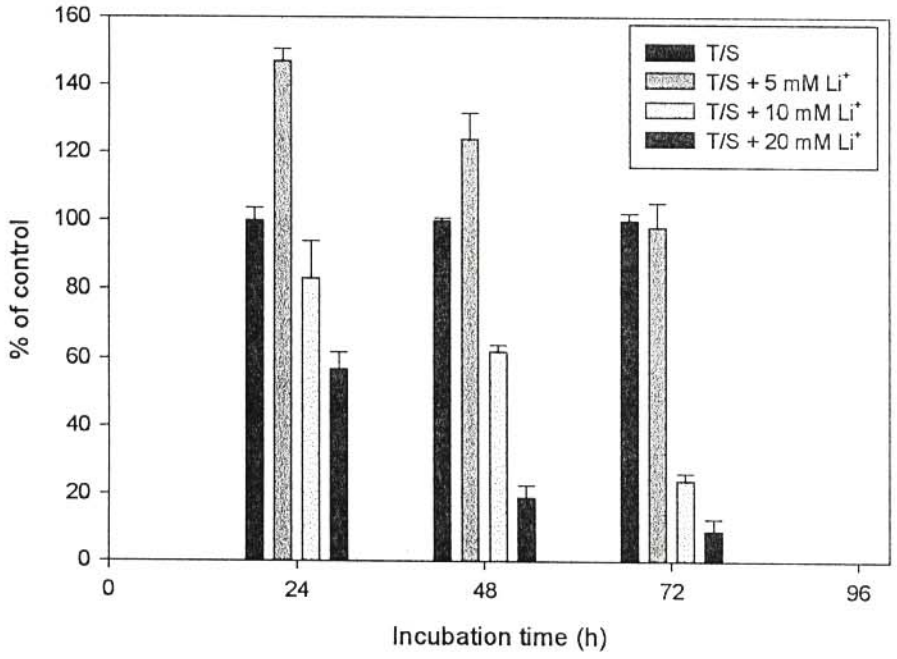


Fig. 3.8. HL-60 cells were treated with (0-20 mM) lithium, supplemented with 5 µg/ml transferrin and 5 ng/ml selenium. The treated and untreated (control) cells were incubated for 24, 48 and 72 h. The extent of cell proliferation was measured by using the Cell Proliferation kit following the manufacture's procedure. The above data represents the mean ± SEM of three independent experiments each performed in duplicate.

The cell membranes in the control cultures were still intact with survival of cells at 72 h (fig. 3.9). In the presence of 5 mM lithium the viability was at 96% at 24 h, and was increased to 98% after 72 h of incubation ($p< 0.01$). Upon addition of 10 mM lithium a time-dependent decrease in cell viability was observed. Above this lithium concentration (20 mM), cell viability was mostly affected, with 41% of viable cells observed after 24 h and dropping to 23 % after 72 h of treatment ($p < 0.01$ compared to control). Lithium at high concentration of 10 mM and above was found to be toxic to these cells which was characterised by a decrease in cell viability. Lithium at its low doses (5 mM) led cells to survive with viability being influenced by high lithium concentrations (10 mM and above).

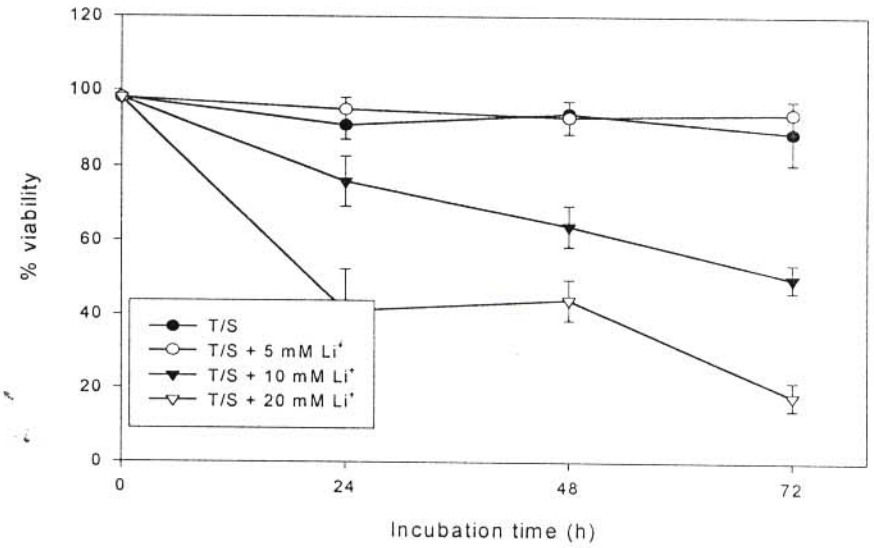


Fig. 3.9. HL-60 cells were treated with (0-20 mM) lithium, supplemented with 5 μg/ml transferrin and 5 ng/ml selenium. The treated and untreated (control) were incubated for 24, 48 and 72 h. The percentage of viable cells was determined by using trypan blue exclusion method. The above data represents the mean ± SEM of three independent experiments, each performed in duplicate.

3.1.4. Cell growth and viability in a media containing insulin

HL-60 cells were treated with 5 mM lithium, supplemented with increasing concentrations of 1, 2.5, 5, 10, 15 and 20 μg/ml insulin and 5 ng/ml selenium. HL-60 cells in RPMI-1640 without lithium or insulin was regarded as the controls. The cell growth experiments showed that insulin was needed by lithium to elicit its action on these cells. Growth was highly inhibited in the

cultures with insulin compared to those without insulin (fig.3.10).

In contrast to the experiments determining proliferation by cell number, cell growth as determined by BrdU incorporation was not enhanced by the presence of insulin. With an increase in incubation time, proliferation of HL-60 cells treated with 5 mM lithium in the presence of insulin (1-20 μg/ml) were found to be decreased with approximately 58-41% of DNA synthesised recorded after 72 h of treatment.

Increased insulin concentrations of 10-20 μg/ml maintained the lowest number of viable cells (15-9%) observed after 72 h of treatment (fig. 3.12). At the minimal concentrations of 1-5 μg/ml, insulin was affecting the viability of these cells and was diminished to approximately 49-38% after 72 h of exposure. Prolonged incubation time resulted in decreasing the number of viable cells (fig. 3.12). Insulin was inhibiting growth of HL-60 cells by inducing cell death provoked by lithium at low concentration. The above data implicates that a low lithium dose (5 mM) can provoke death of HL-60 cells and as such, insulin is needed by lithium to bring about its toxic effects on these cells.

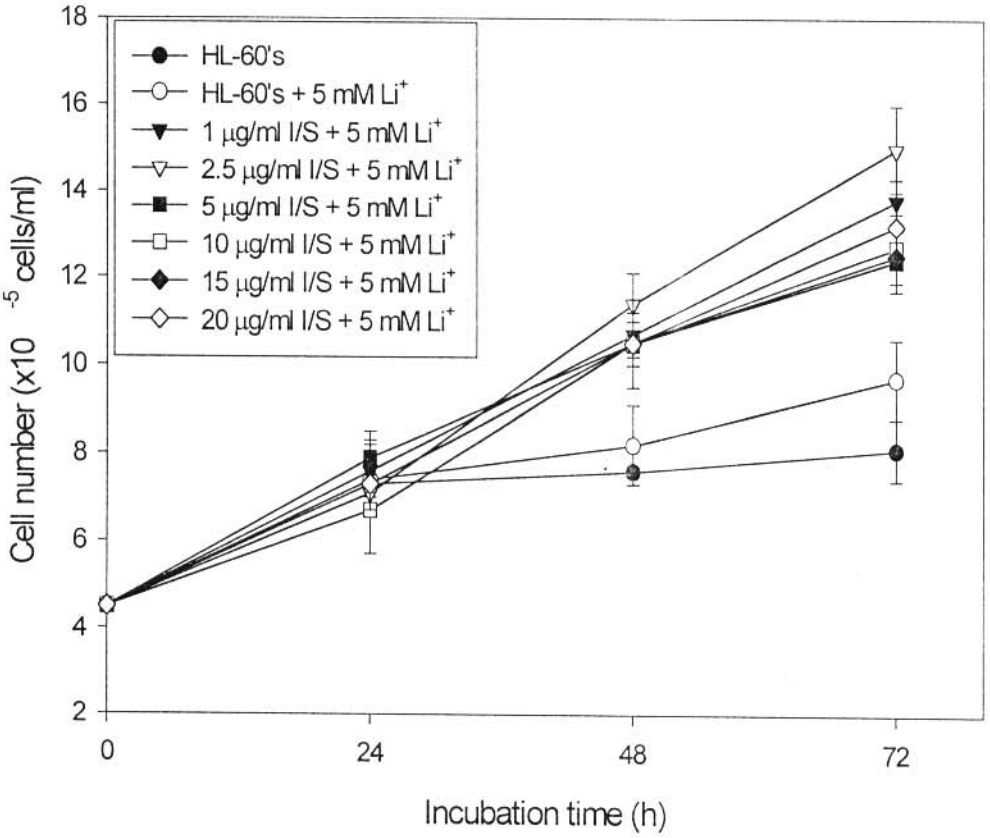


Fig. 3.10. HL-60 cells were treated with 5 mM lithium, supplemented with increasing concentrations of insulin (1, 2.5, 5, 10, 15 and 20 µg/ml with 5 ng/ml selenium or 5 mM lithium without insulin). The cultures were incubated for time interval 24, 48 and 72 h. The rate of cell growth was determined by using the Coulter Counter. The above results are representative of the mean ± SEM of three independent experiments each performed in duplicate.

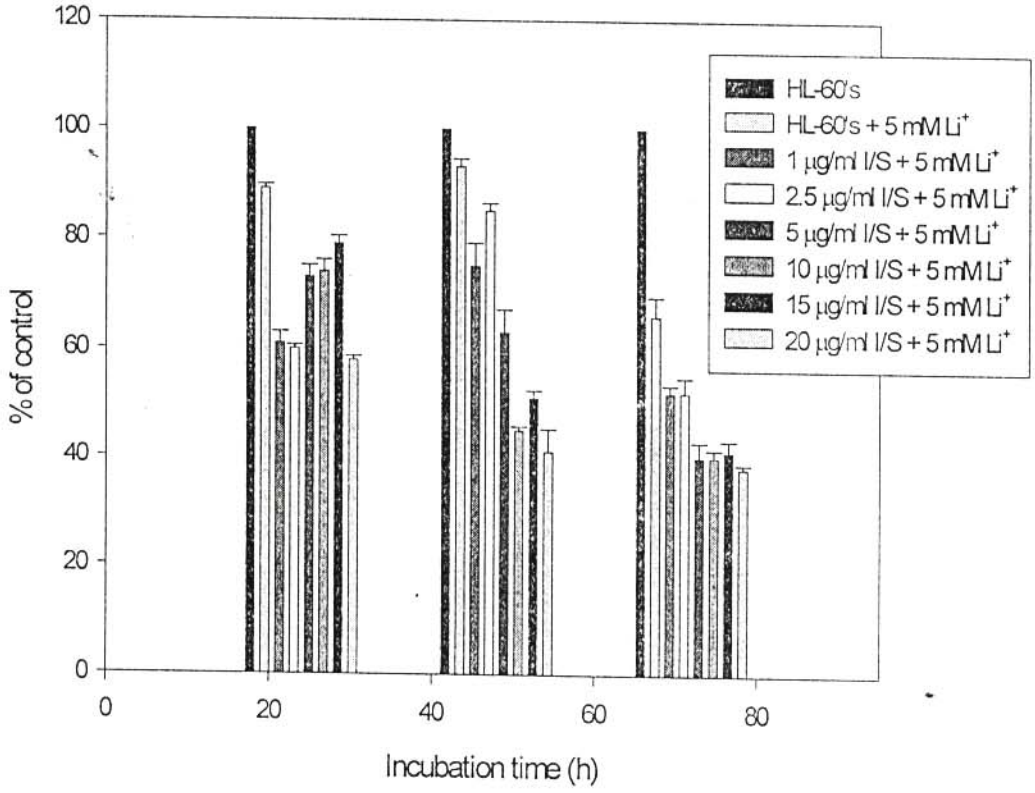


Fig.3.11. HL-60 cells were treated with 5 mM lithium, supplemented with increasing concentrations of insulin (1, 2.5, 5, 10, 15 and 20 µg/ml with 5 ng/ml selenium or 5 mM lithium without insulin). The cultures were incubated for time intervals 24, 48 and 72 h. The extent of cell proliferation was determined by using the cell proliferation kit following the manufacturer 's procedure. The above data are representative of the mean ± SEM of three independent experiments, each performed in duplicate.

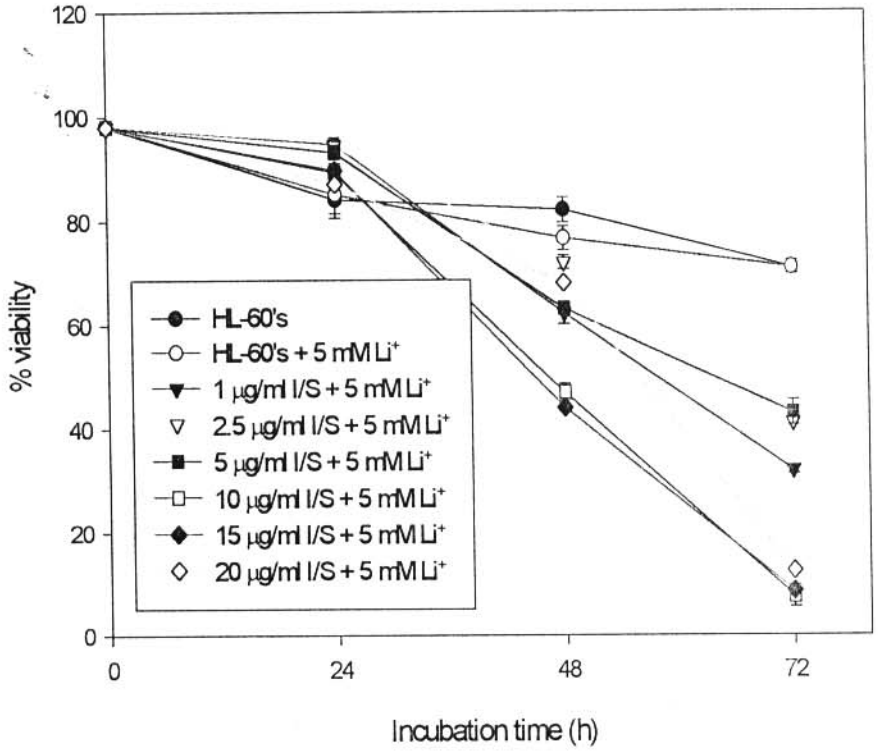


Fig.3.12. HL-60 cells were treated with 5 mM lithium, supplemented with increasing concentrations of insulin (1, 2.5, 5, 10, 15 and 20 µg/ml with 5 ng/ml selenium or 5 mM lithium without insulin). The cultures were incubated for time intervals 24, 48 and 72 h. The percentage of viable cells was determined by using the trypan blue exclusion method. The above data are representative of the mean ± SEM of three independent experiments, each performed in duplicate.

3.2. DNA fragmentation assay

3.2.1 Analysis of DNA by agarose gel electrophoresis in the presence of I/T/S

Madiehe *et al.*, (1995) have reported that lithium at high concentrations (10 mM and above) induces apoptosis by fragmentation of DNA and accumulation of cells in the G_2/M phase of the cell cycle. In this study, agarose gel electrophoresis was carried out to investigate the mode of cell death observed from the cell viability studies. These assays differentiate the two types of cell death, being the necrotic cell death and apoptotic cell death. HL-60 cells were treated with 0-20 mM lithium, enriched with 5 µg/ml I/T/S and incubated for 24, 48 and 72 h. DNA was isolated from the treated and untreated cells according to the method of Gunji *et al.*, (1992) with slight modifications. The extracted DNA was analysed on 1.5% agarose gel and visualized by ethidium bromide. The gels revealed the DNA ladders which were visible after 24 h of treatment with all lithium concentrations tested including the control (fig. 3.13 A). Similar trend of DNA laddering were observed after 48 h of treatment (fig. 3. 13 B) and furthermore at 72 h (data not shown).

Therefore cell death occurring in this study was following the apoptotic pathway evidenced by the appearance of DNA ladders following the agarose gel electrophoresis. The observations were in agreement with the findings reported by Madiehe *et al.*, (1995), that 20 mM lithium and above induces fragmentation of DNA of HL-60 cells after 24 h of treatment. The induction of apoptosis was found to be consistent with the cell growth and viability studies observed earlier in this study. Lithium at concentrations of 5 mM was found not to be protecting the cells from undergoing apoptosis in the presence of insulin. The viability and proliferation studies supported the above findings as there was cell death occurring evidenced by decrease in cell viability and inhibition of DNA synthesis. Apoptosis was further quantified by using the *in situ* cell death detection kit (section 3.3).

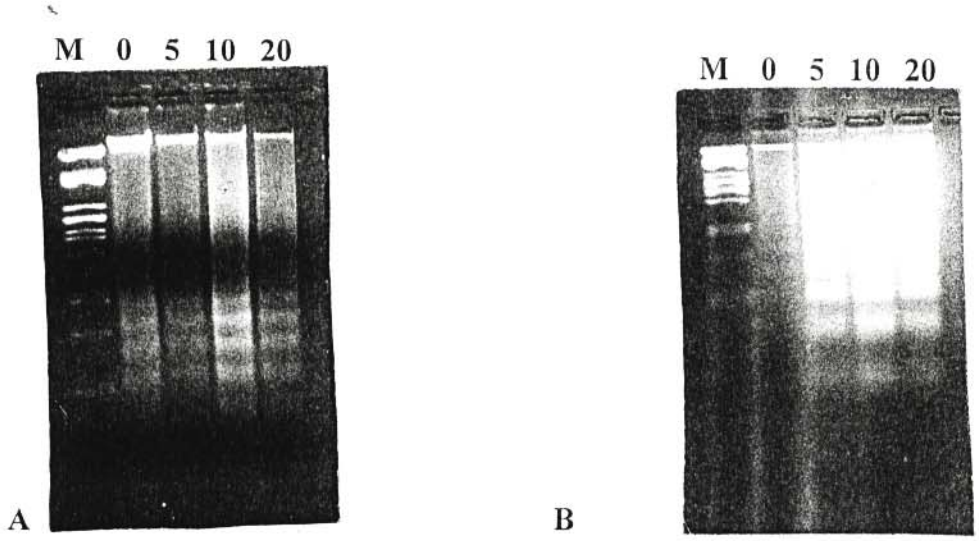


Fig. 3.13. Analysis of total DNA isolated from HL-60 cells treated with 0, 5, 10 and 20 mM lithium, supplemented with 5 µg/ml I/T/S. The cultures were incubated for time intervals 24 h (A) and 48 h (B). M- molecular weight marker, 0- control, 5- 5 mM lithium, 10- 10 mM lithium and 20- 20 mM lithium treated HL-60 cells.

3.2.2. Analysis of DNA by agarose gel electrophoresis in the presence of I/S

The growth kinetics studies demonstrated that there was cell death occurring and the mode of cell death was investigated by agarose gel electrophoresis. In this study, HL-60 cells were treated with lithium concentrations of 0, 5, 10 or 20 mM, supplemented with 5 μg/ml insulin and 5 ng/ml selenium. The cultures were incubated over 3 days and DNA extracted at 24, 48 and 72 h and analysed by agarose gel electrophoresis. The treated and untreated (control) cultures were lysed and DNA extracted according to the method described under materials and methods. Apoptotic cell death was attributed to the appearance of DNA ladders following agarose gel electrophoresis. After 24 h of treatment, agarose gel electrophoresis revealed DNA ladders of internucleosomal DNA fragmentation in all lithium concentrations as well as in the controls (fig. 3.14. A). A similar trend was observed after 48 h of treatment (fig 3.14. B), and after 72 h the same observations were made (data not shown). Lithium was found to induce apoptosis in HL-60 cells in the presence of insulin. The above results are in accord with the findings reported by Madiehe *et al.*, (1995), that lithium at 10 mM and above induced DNA fragmentation of HL-60 cells in a time and dose-dependent fashion. Quantification of apoptosis was attempted by the *in situ* cell death detection assay (section 3.3).

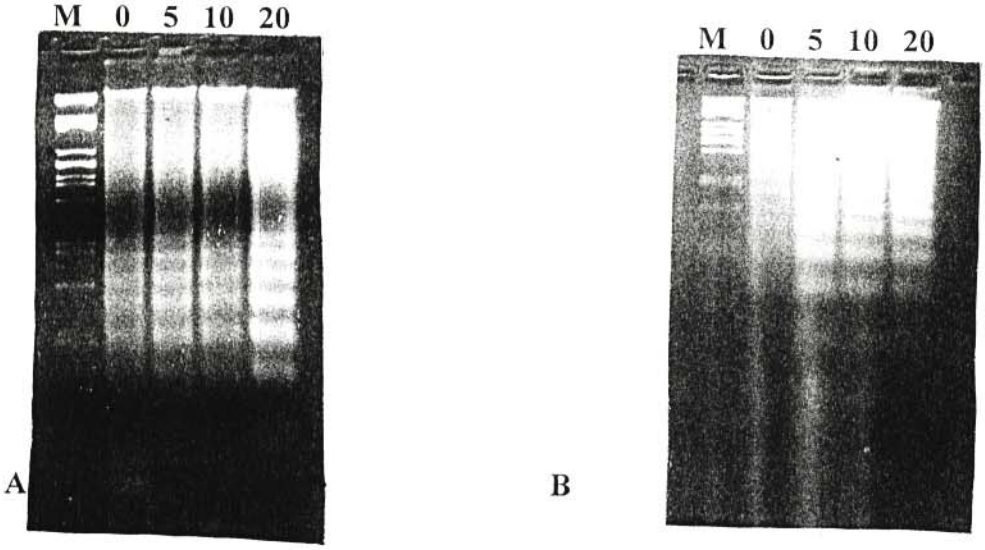
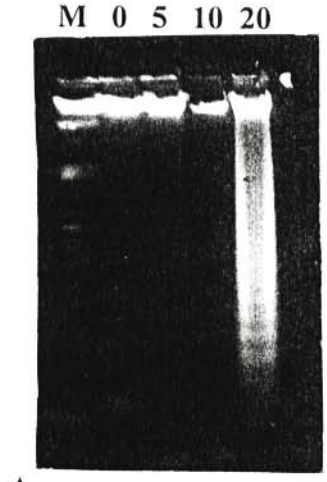


Fig. 3.14. Analysis of total DNA isolated from HL-60 cells treated with 0, 5, 10 and 20 mM lithium, supplemented with 5 µg/ml insulin and 5 ng/ml selenium. The cultures were incubated for time intervals 24 h (A) and 48 h (B). M- molecular weight marker, 0- control, 5- 5 mM lithium, 10- 10 mM lithium and 20- 20 mM lithium treated HL-60 cells.

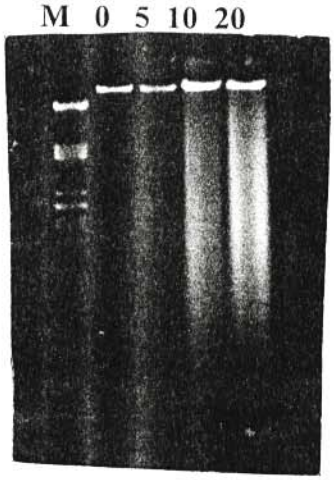
3.2.4 Analysis of DNA by agarose gel electrophoresis in the presence of T/S

The cell death observed from the growth kinetics was investigated and identified by the analysis of agarose gel electrophoresis. HL-60 cells were treated with lithium concentrations of 0-20 mM, supplemented with 5 μg/ml transferrin and 5 ng/ml selenium. Total DNA was extracted by harvesting the treated and untreated cells and the DNA isolated according to the method of Gunji *et al.*, (1992), with slight modification (section 2.2.3). Isolated DNA was analysed on 1.5 % agarose gel and visualized by ethidium bromide staining. After 24 h of treatment, the control and 5 mM lithium treated cells did not show any DNA ladders. Similar observations were observed after 48 h . After 72 h of lithium treatment with 10 mM and above, DNA appeared to be degraded.

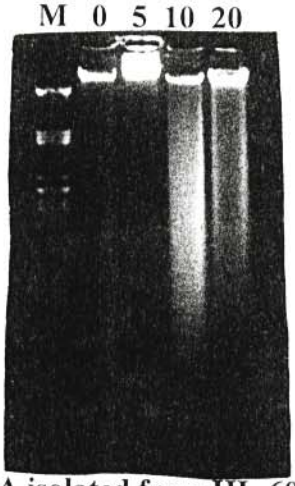
The pattern of cell death occurring in these studies was found to be apoptosis as confirmed by the appearance of DNA ladders following agarose gel electrophoresis and staining by ethidium bromide (fig. 3.15 A-C). The DNA in the control and 5 mM lithium was still intact and the fact that cells seemed to be surviving was in accordance with the high percentage of viability recorded (fig.3.9). However, at high concentrations of 10 mM and above, DNA laddering was observed. The obtained data strongly supported the findings reported by (Becker and Tyobeka, 1990 and Madiehe *et al.*, 1995) that lithium enhances growth at low concentrations of 5 mM and inhibited growth at high concentrations; and that lithium induces apoptosis at concentrations of 10 mM and above with accumulation of cells in the G_2/M phase of the cell cycle. The growth and viability studies (fig 3.8 and fig. 3.9) also supported the above results.



A



B



C

Fig. 3.15. Analysis of total DNA isolated from HL-60 cells treated with 0, 5, 10 and 20 mM lithium, supplemented with 5 μg/ml transferrin and 5 ng/ml selenium. The cultures were incubated for time intervals 24 h (A) and 48 h (B). M- molecular weight marker, 0- control, 5- 5 mM lithium, 10- 10 mM lithium and 20- 20 mM lithium treated HL-60 cells.

3.2.4. Analysis of DNA fragmentation by agarose gel electrophoresis (insulin)

Following cell growth, viability and proliferation experiments, HL-60 cells were treated with 5 mM lithium supplemented with increasing concentrations of insulin (1-20 μg/ml) and 5 ng/ml selenium. HL-60 cells without lithium or insulin were regarded as controls. Insulin concentrations were increased and its effect on HL-60 cells was further investigated by agarose gel electrophoresis to reveal DNA ladders, one of the biochemical markers of apoptosis. Total DNA isolated from the insulin and lithium treated and untreated cells was analysed by agarose gel electrophoresis and visualized by staining with ethidium bromide. The agarose gel electrophoresis displayed fragmentation of DNA after 48 h of treatment. After 24 h of treatment the cells were still surviving showing the intact DNA (fig.3.16. A). DNA fragmentation was visible after 48 h (fig 3.16. B) and became more prominent after 72 h of incubation (fig.3.16. C). These findings support the fact that insulin is required by lithium to induce apoptosis in these cells. The induction of apoptosis in insulin supplemented cells was concentration and time dependent. The longer the exposure time the more cell death was being induced. More importantly, lithium was able to induce apoptosis at a concentration known to be protective in the presence of normal levels of serum.

3.3. The TUNEL assay

The results obtained support the observations reported from the growth kinetics and the DNA fragmentation assays. The detection and quantification of apoptosis by the *in situ* cell death detection assay showed the percentage of individual apoptotic cells as labelled by the fluorescein dUTP at DNA strand breaks. In these experiments, HL-60 cells were treated with lithium (0-20 mM), supplemented with either 5 μg/ml I/T/S, I/S or T/S. The positive and the negative control are shown in fig. 3.18.A. The cells in the media containing I/T/S, individual apoptotic cells in the control was 48%, whereas for the 5 mM it was 57%. In the presence of 20 mM lithium a higher number of apoptotic cells (98%) was detected after 24 h with 10 mM lithium (61%). The above data is supported by photographs shown in fig. 3. 18 A-E.

When transferrin was removed and cultures enriched with I/S, lower percentages of 47% were obtained from the control, the 5 mM and 10 mM lithium treated was at 63% and 81% respectively. The toxic concentrations of 20 mM provided a higher count of 96% (fig. 3.17)

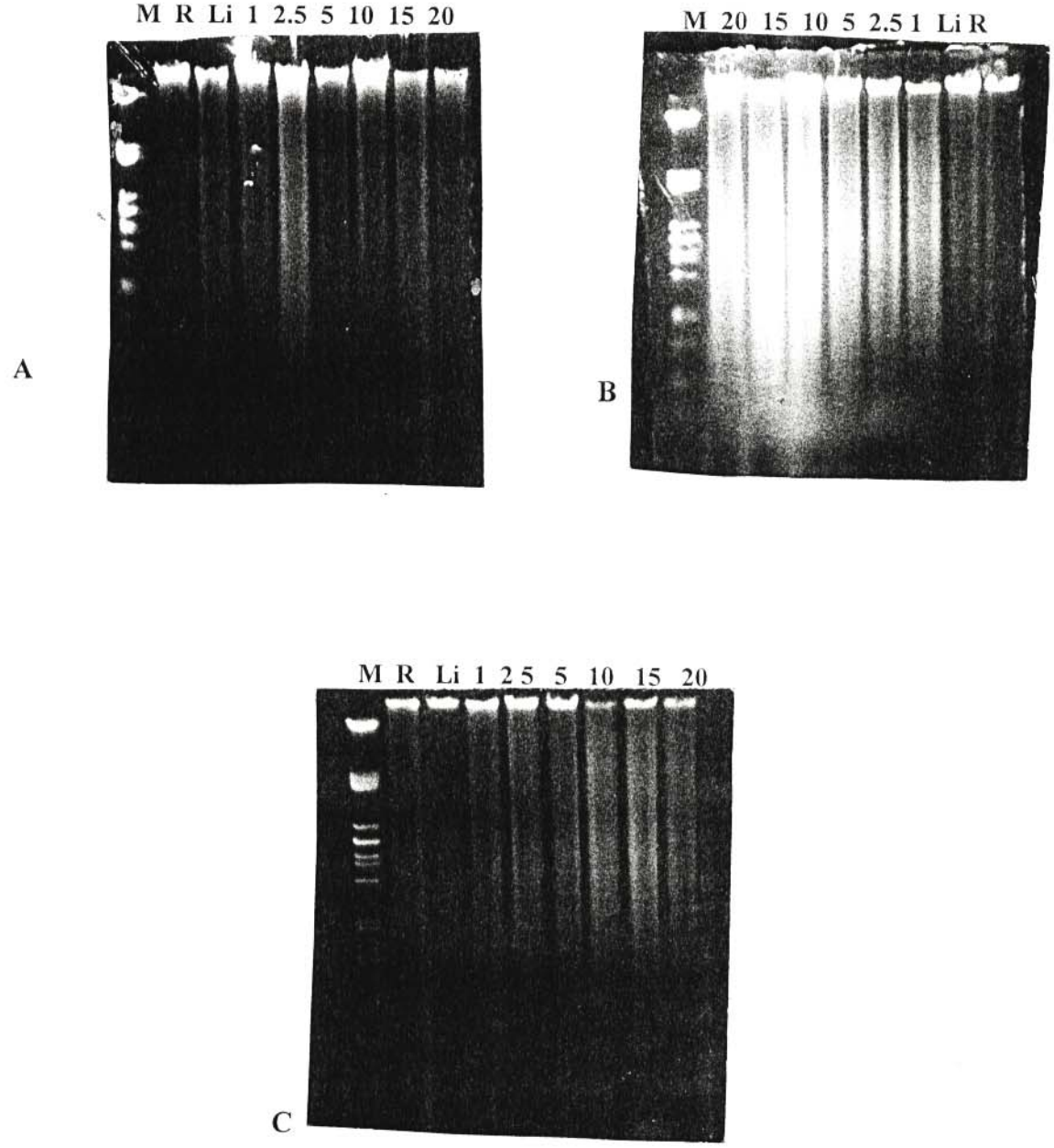


Fig 3. 16. The analysis of total DNA by agarose gel electrophoresis. The HL-60 cells were treated with 5 mM lithium with increasing concentrations of 0, 1, 2.5, 5, 10, 15 and 20 µg/ml and 5 ng/ml selenium. M- molecular weight marker, R- HL-60 cells in RPMI-1640, Li- HL-60 cells treated with 5 mM lithium, 1-20 - HL-60 cells enriched with insulin and treated with 5 mM lithium. The cultures were incubated for 24 h(A), 48 h(B) and 72 h (C).

which was close to the positive control (100%). In the lithium treated cultures containing T/S, the control and 5 mM cultures revealed a lower percentage of apoptotic cells (21% and 23%) respectively. The 10 and 20 mM lithium cultures were having approximately 59% and 80% (fig. 3.17). The data obtained from the media supplemented with I/T/S and I/S are more comparable with each other than the T/S supplemented cultures. The observations made in this study demonstrated that lithium seemed to be dependent on insulin and/ or transferrin to initiate its apoptotic effects on these cells. Insulin was found to have an influence in the induction of apoptosis supported by viability and fragmentation studies.

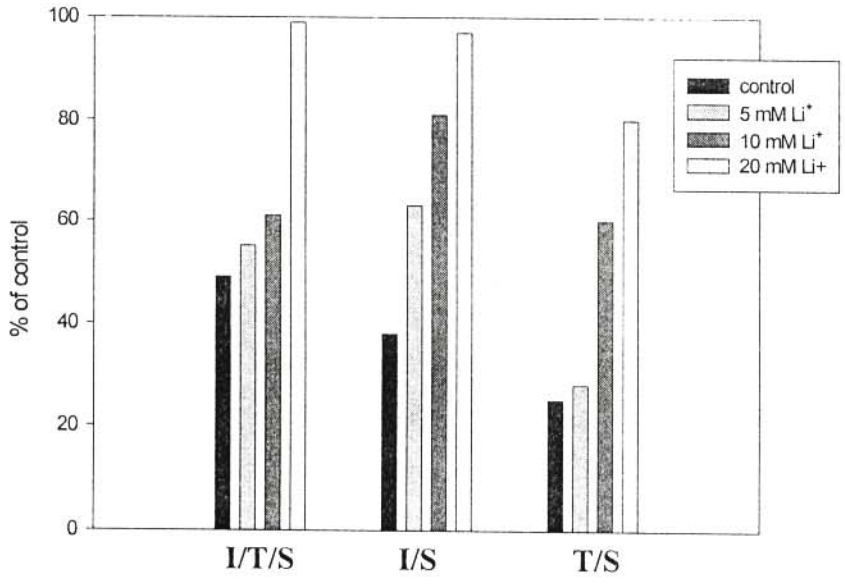
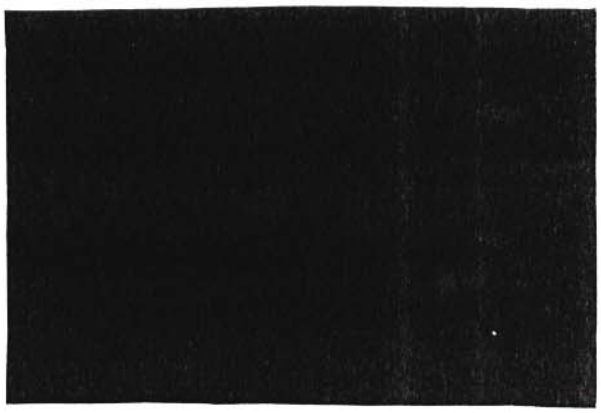


Fig. 3.17. HL-60 cells were treated with increasing lithium concentrations (0-20 mM) and supplemented with either 5 g/ml I/T/S, I/S or T/S. Apoptosis was quantified by using the In Situ Cell Death Detection kit following the supplier's protocol. Aliquots were taken after 24 h of incubation.

a



b

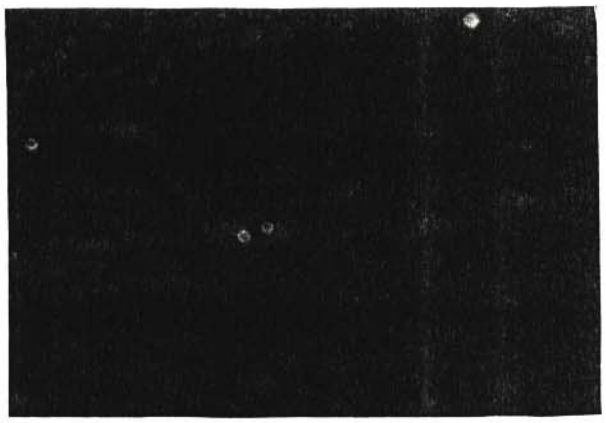


Fig. 3.18. (A). Photographs taken after HL-60 cells were stained for flourescein and apoptotic cell death detected. (a) negative control, (b) positive control treated with lithium (0-20 mM).

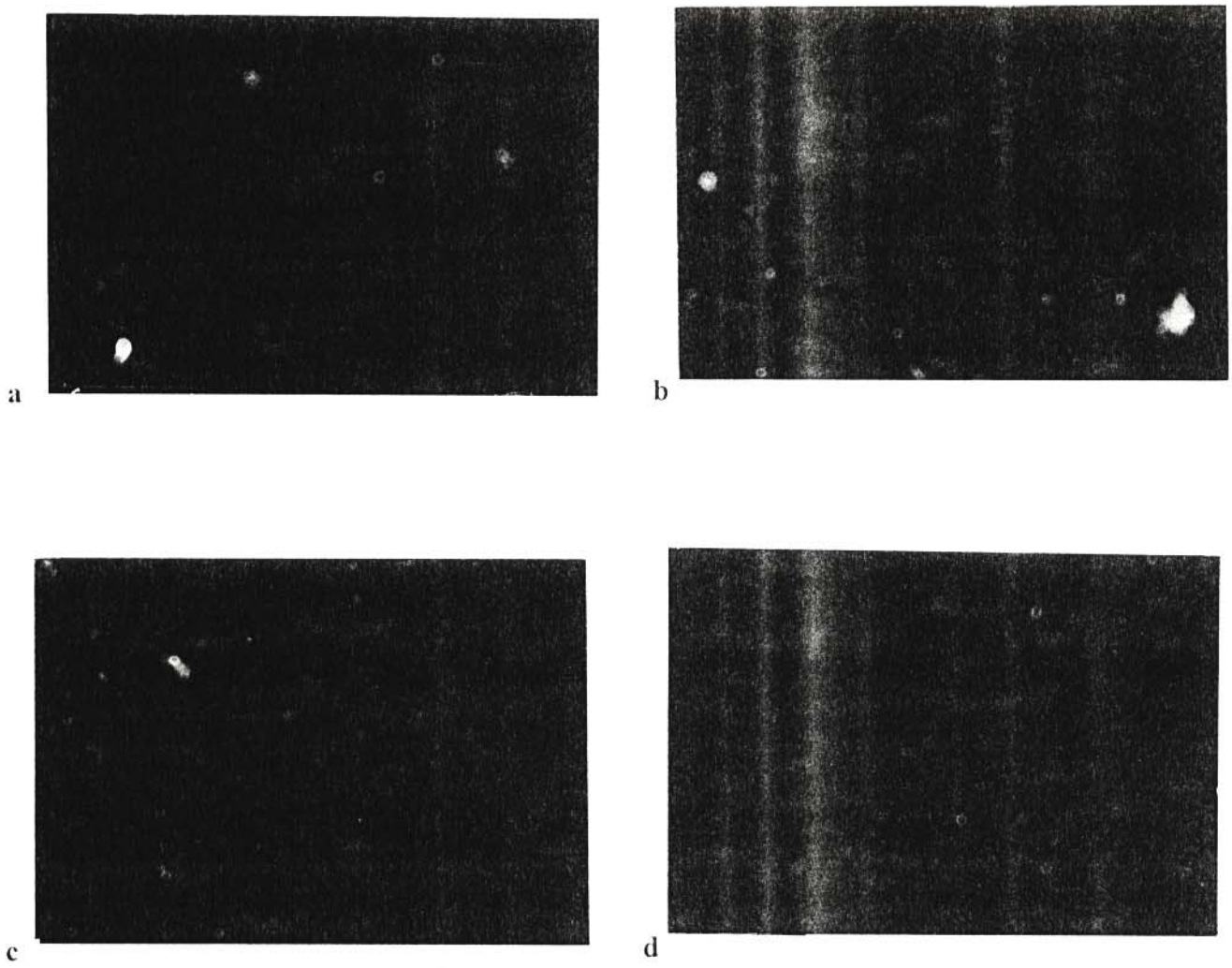


Fig.3.18. (B). Photographs taken after 24 h incubation of HL-60 cells treated with (0-20mM), supplemented with 5 µg/ml I/T/S and stained for fluorescein and apoptotic cell death was detected. a- HL-60 cells lithium untreated, b- 5 mM lithium treated, c- 10 mm lithium treated, d- 20 mM lithium treated, all supplemented with 5 µg/ml I/T/S.

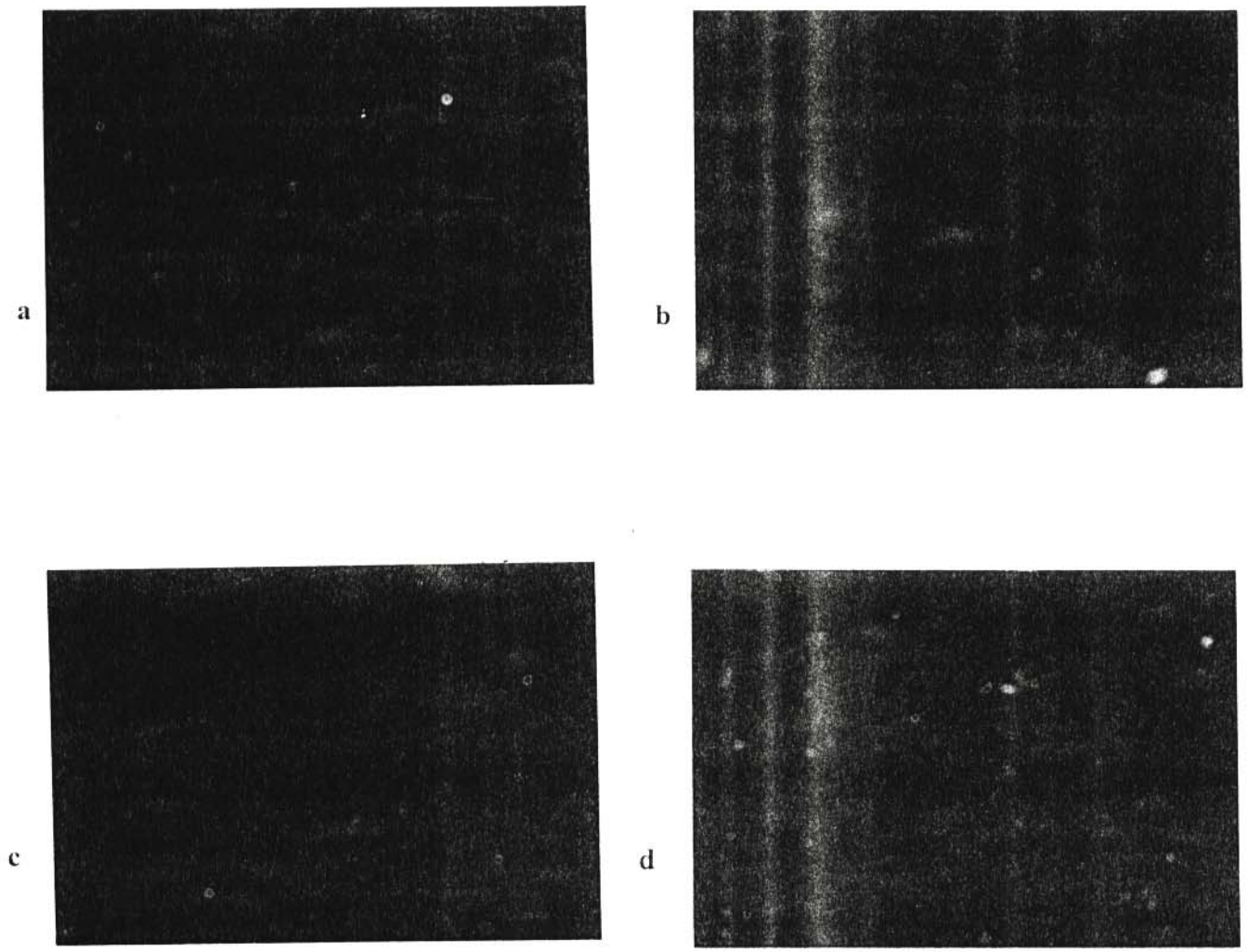


Fig.3.18. (C). Photographs taken after 24 h incubation of HL-60 cells treated with (0-20mM), supplemented with 5 µg/ml insulin and 5 ng/ml selenium and stained for fluorescein and apoptotic cell death was detected. a- HL-60 cells lithium untreated, b- 5 mM lithium treated, c- 10 mm lithium treated, d- 20 mM lithium treated, all supplemented with 5 µg/ml insulin and 5 ng/ml selenium.

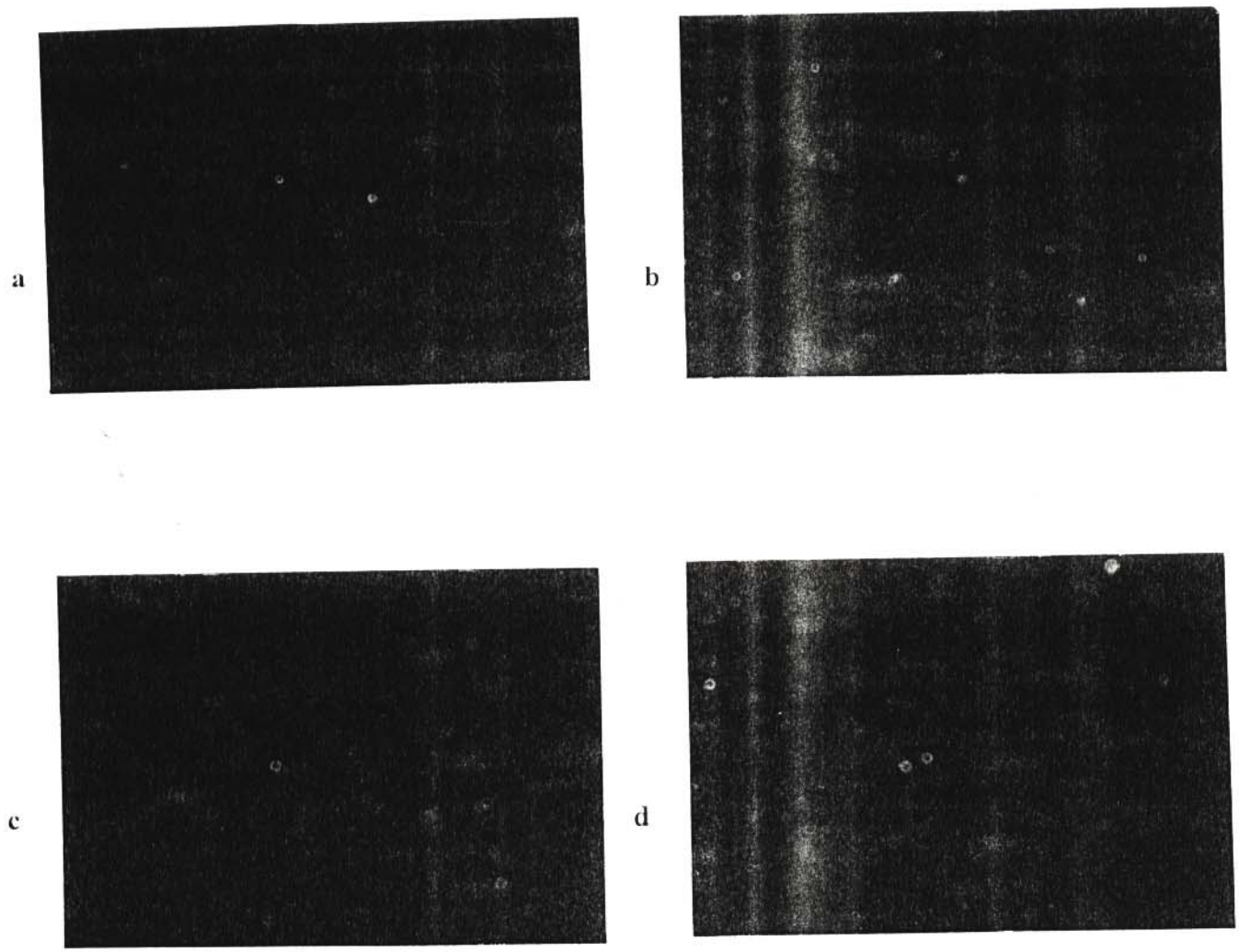


Fig.3.18. (D). Photographs taken after 24 h incubation of HL-60 cells treated with (0-20mM), supplemented with 5 µg/ml transferrin and 5 ng/ml selenium and stained for fluorescein and apoptotic cell death was detected. a- HL-60 cells lithium untreated, b- 5 mM lithium treated, c- 10 mm lithium treated, d- 20 mM lithium treated, all supplemented with 5 µg/ml transferrin and 5 ng/ml selenium.

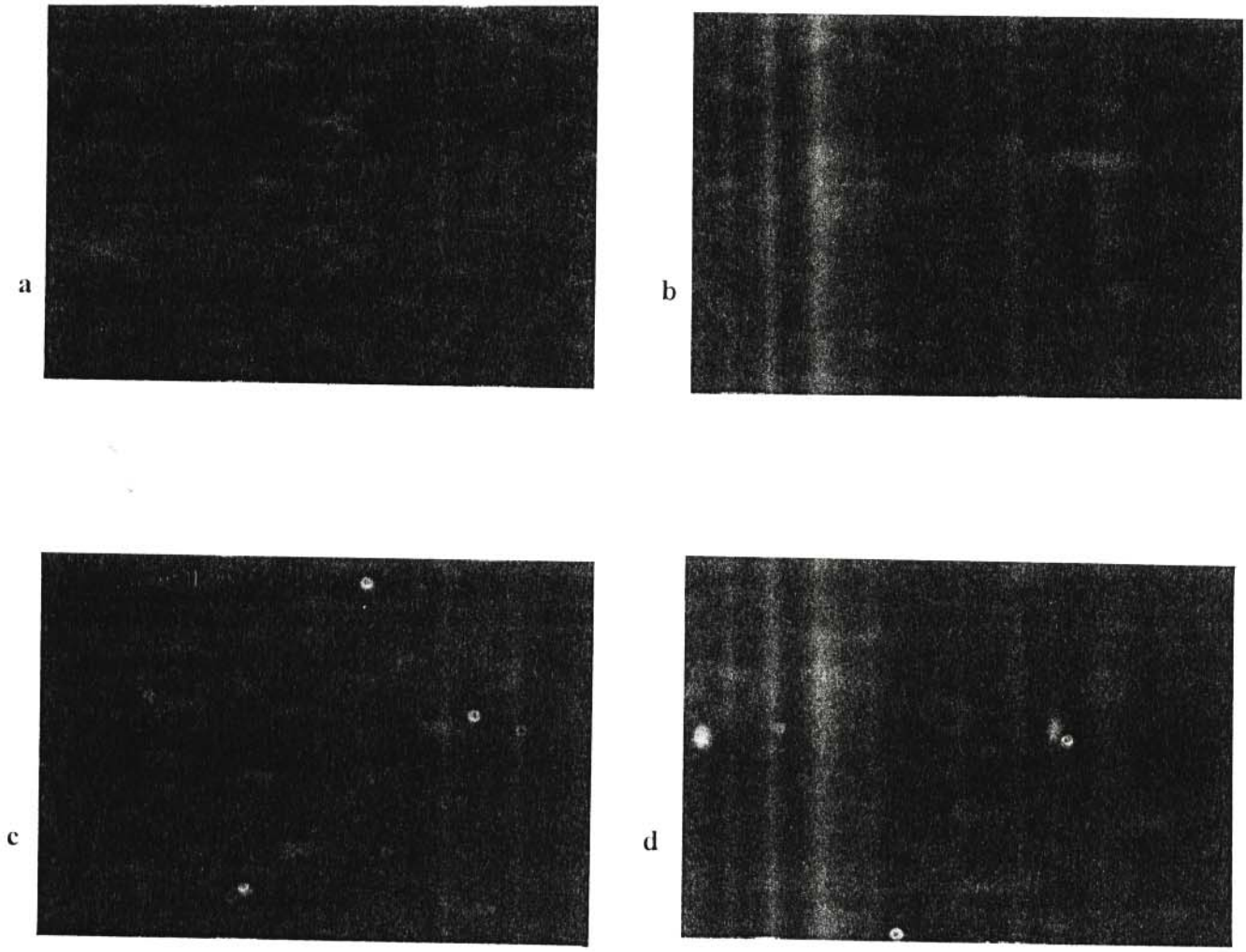


Fig.3.18. (E). Photographs taken after 24 h incubation of HL-60 cells treated with 5 mM, supplemented with 0-2.5 µg/ml insulin and 5 ng/ml selenium and stained for fluorescein and apoptotic cell death was detected. a- HL-60 cells in RPMI-1640, b- HL-60 cells treated with 5 mM lithium, c- HL-60 cells with 1 µg/ml I/S and treated with 5 mM lithium, d- HL-60 cells with 2.5 µg/ml I/S and treated with 5 mM lithium.

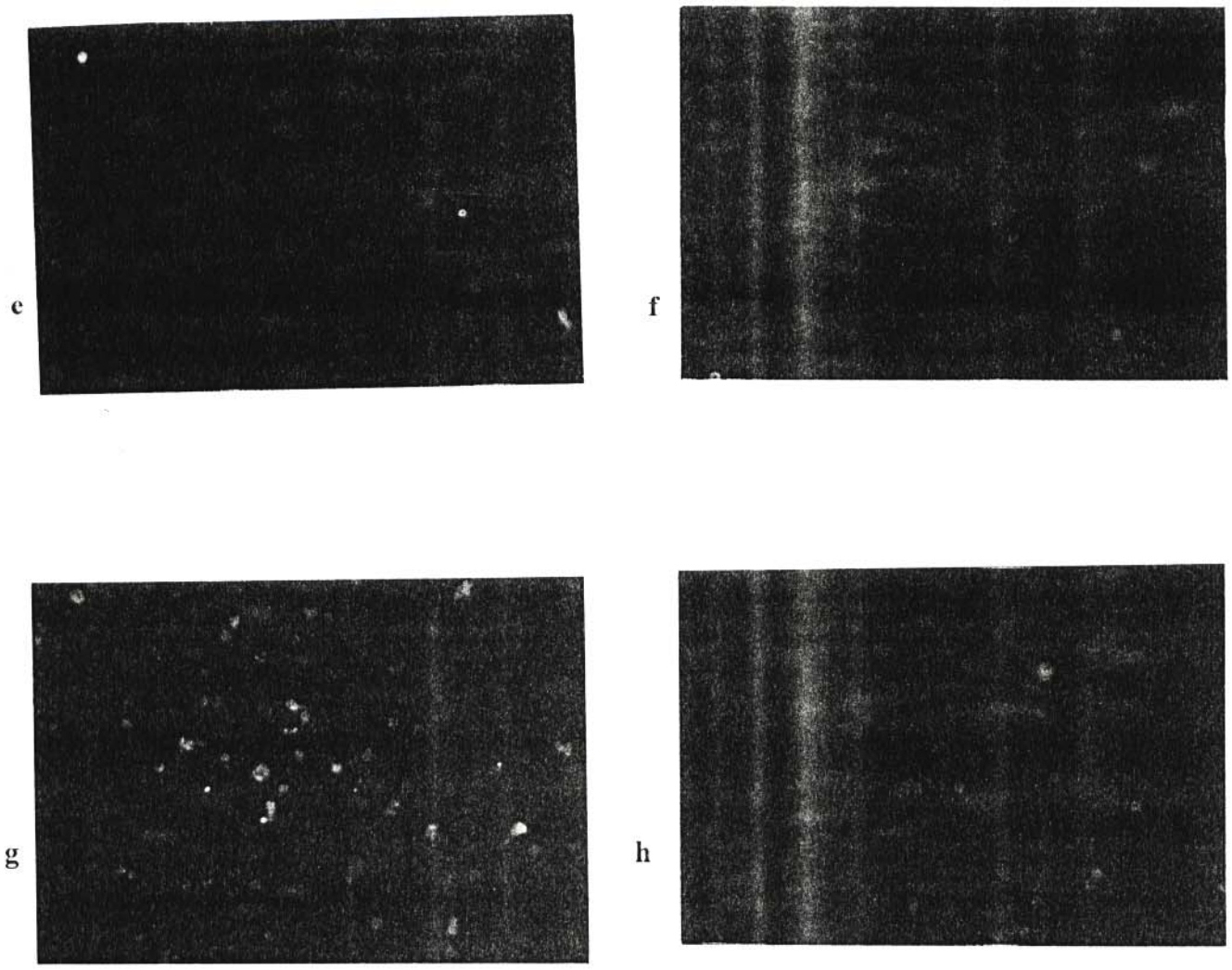


Fig.3.18. (E). Photographs taken after 24 h incubation of HL-60 cells treated with 5 mM, supplemented with 5-20 μg/ml insulin and 5 ng/ml selenium and stained for fluorescein and apoptotic cell death was detected. e- HL-60 cells with 5 μg/ml I/S and treated with 5 mM lithium f- HL-60 cells treated with 5 mM lithium, g- HL-60 cells with 10 μg/ml I/S and treated with 5 mM lithium, h- HL-60 cells with 20 μg/ml I/S and treated with 5 mM lithium.

3.4. SDS-PAGE and Western Blot analysis

3.4.1. Western blot analysis in the presence of I/T/S

To investigate the presence of genes that were involved during apoptosis, SDS-PAGE was performed followed by electrotransfer of proteins and immunodetection. HL-60 cells were treated with lithium (0-20 mM) and supplemented with 5 μg/ml I/T/S and incubated for 24 h. The patterns of expression the two genes, *bcl-2* and *bax*, were investigated in these experiments. It was found that, after 24 h of treatment with lithium, Bcl-2 and Bax were expressed differently. The level of expression exhibited are shown in fig.19. Bcl-2 was found to be expressed at all lithium concentrations tested. The level of expression showed a hint of phosphorylation with higher lithium concentrations.

On the other hand Bax exhibited an increased level in the lithium untreated and 10 mM lithium treated cultures, and its expression was slightly decreased in the 5 mM and 20 mM lithium treated cultures. Serum-rich cultures (10% FBS) expressed low levels of Bax compared to all cultures. Overexpression of Bax is indicative of the onset of apoptosis. Since the control and 10 mM lithium treated cells were showing overexpression of Bax compared to other lithium doses tested, it may therefore be suggested that lithium was inducing cell death via the apoptotic pathway to a larger extent in these cells. Equal loading was checked by staining the membranes with Ponceau S and scanning by UVP.

3.4.2. Western blot analysis in the presence of I/S

In this experiment, the level of expression of Bcl-2 and Bax were determined by performing the Western blotting after extraction of proteins. HL-60 cells were exposed to lithium concentrations of 0-20 mM and supplemented with 5 μg/ml insulin and 5 ng/ml selenium and incubated for 24 h. Bcl-2 was found to be equally expressed at all concentrations that were tested and showing a hint of phosphorylation. Bax was highly expressed in the control and 10 mM lithium treated cells, whereas in the 5 mM and 20 mM lithium its level of expression was slightly reduced (fig. 3.20). These observations are similar to the results obtained from the I/T/S media, and these findings suggests that apoptosis was occurring to the greatest extent in the control cells and 10 mM lithium treated cells. Insulin participated in the induction of apoptosis elicited by lithium. HL-60 cells growing in 10% FBS expressed low levels of Bax when there

was no lithium added in these cultures and therefore the cells were protected from apoptosis by Bcl-2.

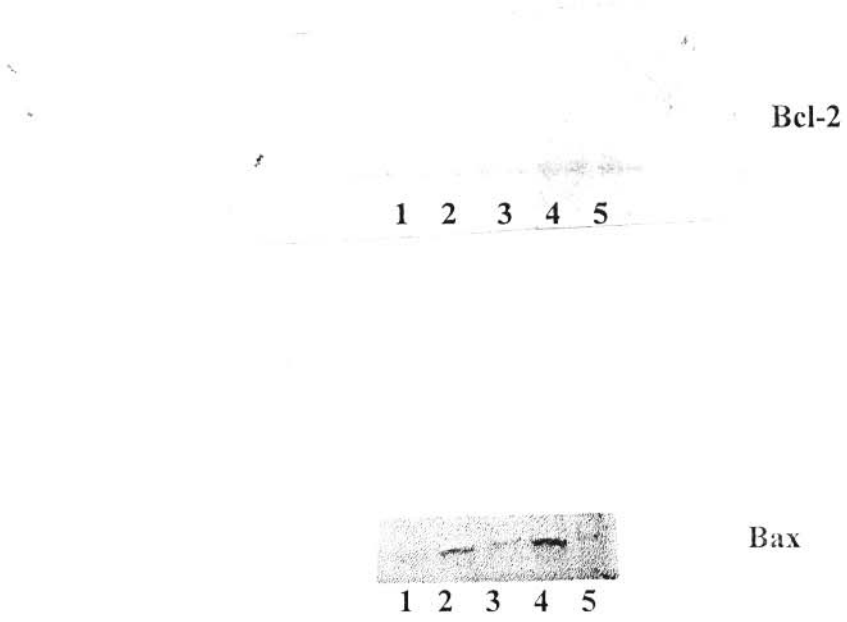
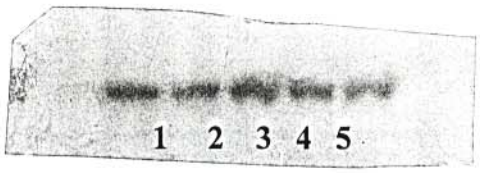
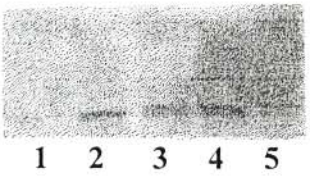


Fig 3.19. Western blot analysis of Bcl-2 and Bax expression in HL-60 cells treated with lithium (0-20 mM), supplemented with 5 μ g/ml I/T/S. 50 μ g/ml of proteins were analysed by western blotting after 24 h of incubation. Lane 1 from the left shows serum-rich HL-60 cells, lane 2- HL-60 cells (control), lane 3- HL-60 cells 5 mM lithium treated, lane 4- HL-60 cells 10 mM lithium treated, lane 5- HL-60 cells 20 mM lithium treated all in 5 μ g/ml I/T/S excluding lane 1.



Bcl-2



Bax

Fig 3.20. Western blot analysis of Bcl-2 and Bax expression in HL-60 cells treated with lithium (0-20 mM), supplemented with 5 μ g/ml I/S. 50 μ g/ml of proteins were analysed by western blotting after 24 h of incubation. Lane 1 from the left shows serum-rich HL-60 cells, lane 2- HL-60 cells (control), lane 3- HL-60 cells 5 mM lithium treated, lane 4- HL-60 cells 10 mM lithium treated, lane 5- HL-60 cells 20 mM lithium treated all in 5 μ g/ml I/S excluding lane 1.

3.4.3. Western blot analysis in the presence of T/S

HL-60 cells were treated with lithium (0-20 mM), supplemented with 5 μg/ml transferrin and 5 ng/ml selenium. After 24 h of treatment, expression of Bcl-2 in the control and 5 mM lithium was unphosphorylated and showing that these cells were protected from apoptosis and therefore surviving cell death. Addition of 10 mM and 20 mM lithium to the cells showed Bcl-2 which was phosphorylated which may be an indicative of Bax level accumulating leading to the onset of apoptosis. On the other hand, Bax showed an equal level of expression in all lithium concentrations tested.

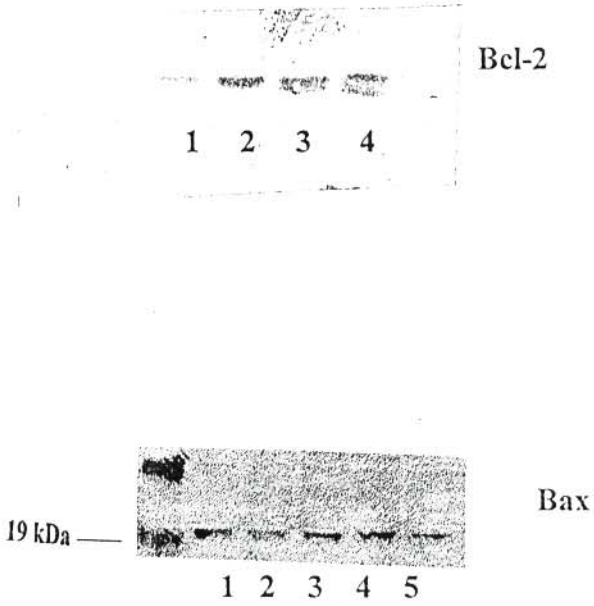


Fig 3.21. Western blot analysis of Bcl-2 and Bax expression in HL-60 cells treated with lithium (0-20 mM), supplemented with 5 μ g/ml T/S. 50 μ g/ml of proteins were analysed by western blotting after 24 h of incubation. Lane 1 from the left shows serum-rich HL-60 cells, lane 2- HL-60 cells (control), lane 3- HL-60 cells 5 mM lithium treated, lane 4- HL-60 cells 10 mM lithium treated, lane 5- HL-60 cells 20 mM lithium treated all in 5 μ g/ml I/T excluding lane 1.

Chapter 4

Discussion and conclusion

Lithium has been used to treat manic depressive disorders for 5 decades (Calil *et al.*, 1990). This effective drug is therefore widely used and has drawn intensive research interest because the mechanism of action underlying its therapeutic efficacy as well as its side effects are still not clearly understood (Calil *et al.*, 1990). Cancerous cells in general are known to grow and multiply at a faster rate than normal cells *in vivo* (Hoffbrand and Lewis, 1981). This condition can only be controlled by treatment with effective drugs to eliminate these unnecessary cells and it has been suggested that apoptosis, a programmed cell death, is the most convenient and safe way of cells to die. During the process of apoptosis the DNA is degraded and apoptotic bodies are formed and engulfed by phagocytes (Wylie *et al.*, 1980). The occurrence of apoptosis has been associated with numerous aspects of mammalian development and control of specific diseases, since the development and maintenance of tissues or cells depends on the balance between different processes, such as proliferation, differentiation and cell death. Failure of cells to undergo apoptosis have been demonstrated to perturb development and to contribute to diseases (Wylie *et al.*, 1980). At concentrations of 2.5-5 mM lithium, growth is enhanced and high concentrations of 10 mM and above lithium was found to be cytotoxic to these cells (Becker and Tyobeka, 1990). Madiehe *et al.*, (1995) have reported that lithium induces apoptosis of HL-60 cells propagated in serum-rich medium at concentrations of 10 mM and above.

Previous findings by Breitman *et al.*, (1980) have reported that HL-60 cells can grow and proliferate in serum-free medium supplemented with insulin and transferrrin. Sinclair *et al.*, (1988) have shown in their studies that insulin can bind to its own receptor or the IGF receptor or both to facilitate the grow of HL-60 cells. At cell growth stimulating concentrations (1-10 ng/ml) insulin can only bind to its own receptor and stimulates growth less than when bound to the IGF receptor and its own receptor. The amount of insulin receptors on the cell surface membrane is therefore a measure of cell growth. In the present study the effect of lithium on HL-60 cells in serum-free medium was investigated by following the growth of the cells before and after treatment. Cell proliferation studies indicate that in the HL-60 cells, both lithium

treated and untreated, containing either 5 μg/ml I/T/S or I/S as growth factors, cell growth was not enhanced. Due to the cells undergoing apoptosis, DNA was not synthesized and therefore the BrdU was unable to bind. In the cell cultures supplemented with T/S, growth of HL-60 cells was enhanced by 5 mM lithium as indicated by the increase in the number of cells. The cell growth experiments resulted in a decrease in cell number with increased incubation time. At 10 mM lithium and above proliferation was strongly inhibited. As the percentage of viable cells fell to approximately 51% in the control and to 18% in the 5-20 mM lithium treated, the cell growth was also decreasing according to the variation of lithium concentrations in the I/S or I/T/S supplemented experiments. In the presence of 10 mM lithium and above, there was no growth enhancement and the percentage obtained was below the control and decreasing with the increase in exposure time. The viability studies indicate that the percent of viable cells obtained from the control in I/T/S and I/S containing media, was decreased. Concentrations of 10 mM lithium and above caused cell death of HL-60 cells and this observation is in agreement with the findings reported by Madiehe *et al.*, (1995), that lithium (10 mM and above) causes a cytotoxic effect on HL-60 promyelocytes with induction of apoptosis and arrest of cells at the G_2/M phase of the cell cycle. The TUNEL assay studies confirm the above mentioned results as it was possible to quantitate individual apoptotic cells. The percentage of apoptotic cells obtained in the I/T/S or I/S supplemented cultures were comparable to the positive control. There was a slightly low percentage obtained in the control and 5 mM lithium, T/S supplemented cultures.

The agarose gel electrophoresis was used to investigate the mode of cell death in this study and to further quantitate cell death. Examination of cells undergoing apoptosis by agarose gel electrophoresis revealed DNA ladders of 200 bp fragments generated from the digestion of the chromatin between nucleosomes. This DNA fragmentation is rapid and common to all cells undergoing apoptosis as indicated by fig. 3.13-3.16. The endonucleases responsible for the DNA laddering have been suggested to be DNase I, II and other proteins related to cyclophilin A, causing accumulation of DNA fragments which appear as ladders following agarose gel electrophoresis (Goldstein *et al.*, 1987; Williams *et al.*, 1992). The occurrence of DNA fragmentation suggests that insulin is involved in the induction of apoptosis supported by appearance of DNA ladders in the untreated cells supplemented with either I/T/S or I/S. Our

results suggest that insulin took part in the induction of apoptosis and lithium was able to induce apoptosis at low concentrations of 5 mM. Transferrin supplemented cells revealed a striking similarity with previous results reported from cultures maintained in serum- rich medium, whereby 5 mM lithium enhances growth whereas 10 and 20 mM induced apoptosis (Becker and Tyobeka, 1990, Madiehe *et al.*, 1995). Lithium has been previously reported to induce DNA fragmentation of HL-60 promyelocytes maintained in serum-rich medium and its cytotoxic effects on these cells was of a concentration and time dependent manner (Madiehe *et al.*, 1995). However, in the current study, with T/S media, fragmentation of DNA was only occurring in the 10 and 20 mM lithium treated cells. The DNA in the control and 5 mM lithium treated cultures was still intact and DNA fragmentation was not found to be occurring. When HL-60 cells were treated with 5 mM lithium supplemented with insulin at increasing concentrations of 1-20 μg/ml, insulin affected both DNA synthesis and an increase in DNA fragmentation as insulin concentration and incubation time rose. The apoptotic effect induced by insulin became more apparent after 48 h of incubation, then increased in parallel with incubation time. Lithium effects seemed to be dependent on insulin and/or transferrin only to be present to elicit its metabolic effects in these cells.

Apoptosis was further quantitated by using the TUNEL assay which revealed supportive results with the agarose gel electrophoresis. In the TUNEL reaction, the terminal deoxynucleoidyl transferase (TdT) which catalyses polymerisation of nucleotides to free 3'-OH DNA ends in template-independent manner, is used to label the DNA strands breaks. The percentage of apoptotic cells indicated that there is apoptosis occurring in I/T/S or I/S supplemented HL-60 cells whether lithium treated or untreated. A higher percentage of apoptotic cells was found in the 10 and 20 mM lithium treated cells (60-90%); at these concentrations lithium was found to be toxic to HL-60 cells and apoptosis was found to be of a concentration dependent manner. The T/S supplemented HL-60 cells showed apoptotic cells with 10 and 20 mM lithium (62-81%), and the control and 5 mM lithium treated cells were both showing low percentages (26-28%) of apoptotic cells.

Insulin has been found to have an influence in the induction of apoptosis. When insulin has been removed from the culture media with supplementation of transferrin, a lower percentage of

apoptosis was observed in the control and 5 mM lithium treated cells. Our experiments have demonstrated that cell death was occurring in HL-60 cells, assessed both by growth kinetics and the ladder pattern of DNA fragmentation upon agarose gel electrophoresis. Moreover, that HL-60 cells have died via apoptosis was demonstrated by the TUNEL method which is a useful procedure for the *in situ* identification of programmed cell death.

Gene expression is one of the most important processes occurring during apoptosis. *Bcl-2* and *bcl-x* are known to be death suppressor genes whereas *bax* promotes apoptosis. It was our interest to analyse the apoptotic proteins Bax and Bcl-2 in this study and to investigate their parttern of expression since the occurrence of apoptosis may require changes in their expression. *bcl-2* is becoming a well understood gene in the cell death pathway and functions as a death repressor. Bcl-2 is known to form heterodimers with Bax and the ratio of Bcl-2:Bax will determine the survival or death of the cell following an apoptotic stimuli (Oltvai *et al.*, 1993). Bax induction does not appear to be a *de novo* response that follows a death signal and Bax in itself does not cause cell death. Instead overexpressed Bax accelerates apoptotic cell death only following a death signal. Excess Bax also counteracts the death represssor activity of Bcl-2. When Bcl-2 is in excess and Bax homodimers dominates, cells are susceptible to apoptosis. Therefore *bax* might function as a death effector gene that is neutralized by *bcl-2*. The two proteins of molecular weights, 24-26 kDa (Bcl-2) and 21 kDa (Bax) respectively, were found to be expressed as evidenced by the Western blot analysis. The Western blot studies performed, indicated that in HL-60 cells treated with 0-20 mM lithium, supplemented with I/T/S expressed equal levels of Bcl-2. The Bcl-2 in the 10% FBS containing medium was unphosphorylated. Bax was increased in the control and 10 mM and slightly decreased in the 5 and 20 mM lithium. Bax upregulation and overexpression may be responsible for the onset of apoptosis in the presence of lithium. The ratio of Bcl-2:Bax was decreased and could be explained as follows: during the onset of apoptosis Bax accumulated, thus binding Bcl-2 causing apoptosis. In the T/S containing cells, Bcl-2 was found to be phosphorylated only in the 10 and 20 mM lithium treated cells. Lithium induced phosphorylation of Bcl-2 and inhibited its binding to Bax and it decreased the amount of dimerized Bax. This suggests that lithium does not require overexpression of *bax* to induce apoptosis. However, overexpression of Bax with prolonged exposure time may occur.

In conclusion, treatment of cells with lithium in the presence of transferrin revealed a striking similarity to observations recorded with experiments done in a serum-rich environment. Transferrin can be used as a growth factor of HL-60 cells as compared to insulin. Furthermore, the observations made in this study demonstrated that lithium seems to be dependent on insulin to be present to elicit its metabolic effects on these cells. Insulin was found to have an influence in the induction of apoptosis and the mechanism of its action is still to be investigated. The exact pathways by which lithium induces cell proliferation when present in small amounts and apoptosis at high concentrations remains to be settled. Lithium need to be combined with other drugs to be more effective, and finally insulin, which is known to control diabetes mellitus, may also be of therapeutic importance in cancer therapy.

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