Apoptotic effects of lithium chloride on renal cells and potential alteration of renal antigens involved in systemic lupus erythematosus.

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DECLARATION

I, Kirsten Clare Lucas, declare that the thesis hereby submitted to the University of Limpopo for the degree of Doctor of Philosophy has not previously been submitted by me for a degree at this or any other University; that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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ADH	anti-diuretic hormone
anti-ds DNA	antibodies against double stranded DNA
anti-Sm	antibodies against Smith antigens
Apaf-1	apoptotic protease activating-factor 1
AQP2	aquaporin-2
AVP	arginine-vasopressin
Bad	Bcl-X_L-Bcl-2 associated death promoter
Bax	Bcl-2-associated x protein
BCA	bicinchoninic acid
Bcl	B-cell lymphoma
Bcl-X_L	Bcl-X long
Bid	BH3-interacting death domain agonist
BJP	Bens-Jones Protein
BP	bipolar
BSA	bovine serum albumin
CAD	caspase activated deoxyribonuclease
CARD	caspase recruitment domain
CD	cluster determinant
cdk	cyclin dependant kinase
CFU-GM	colony forming units-granulocyte-macrophage
CFU-Meg	colony forming units- megokaryocytes
cIAP	cellular inhibitors of apoptosis protein
C_{Li}	lithium clearance
CREB	cAMP responsive element binding protein
COX2	cycloogygenase 2

CXCR4	chemokine (cxc motif) receptor 4
DAB	3'3' diaminobenzidine
DAG	diacylglycerol
DED	death effector domain
Diablo	direct IAP-associated binding protein with low PI
DISC	death inducing signalling complex
dist. H_2O	distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulphoxide
FADD	Fas associated death domain
FAK	focal adhesion protein
FDA	food and drug administration
FE_{Li}	fractional lithium excretion in urine
GFR	glomerular filtration rate
GM-CFU	granulocyte-macrophage colony forming units
GN	glomerulonephritis
GSK	glycogen synthase kinase
HMDS	hexamethyldisilizate
HRP	horse-radish peroxidase
I-1,3,4-P_3	inositol 1,3,4 *tris*phosphate
I-1,4-P_2	inositol 1,4 *bis*phosphate
I-3-P	inositol 3 phosphate
I-4-P	inositol 4 phosphate
IAP	inhibitor of apoptosis
ICAD	inhibitor of caspase activated deoxyribonuclease
IgG	G-type immunoglobulins
IMPA	inositol monophosphatase
MAP	microtubule associated protein
MAPK	mitogen-activated protein kinase

MDM2	murine double minute 2
Mr	relative molecular mass
MW	molecular mass
NC	nitrocellulose
NDI	nephrogenic diabetes insipidus
NHS	normal human serum
NZB/W	New Zealand Black x New Zealand White mouse models of SLE
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PARP	poly (ADP) ribose polymerase
PBS	phosphate buffered saline
PCD	programmed cell death
PCNA	proliferating cell nuclear antigen
PGE2	prostaglandin E2
PI	phosphoinositol
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PP2	protein phosphatase 2
PTH	parathyroid hormone
®	registered trade mark
RB	retinoblastoma
RIP	receptor-interacting protein
RT	room temperature
SDF-1	stromal derived factor-1
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
sFas	soluble Fas

SLE	systemic lupus erythematosus
SMAC	second mitochondria-derived activator of caspase
snRNP	small nuclear rbonucleoproteins
SODD	silencer of death domains
SRP	signal recognition particle
TEM	transmission electron microscopy
TEMED	N,N,N',N', tetramethyl ethylene diamine
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRAF	TNFR associated factor

ABSTRACT

Lithium, a prophylactic drug for the treatment of bipolar disorder, is used with caution due to many toxicity implications. In particular, lithium dosage and renal toxicity has to be constantly monitored in these patients. Contrary to the above, lithium was found to prevent end-stage renal failure in a subset of female mouse models (NZB/W F_1) of systemic lupus erythematosus (SLE). The aim of this study was to investigate the direct effects of LiCl on two established porcine renal tubule cell lines, (LLC-PK_1 and PK(15)) to ascertain lithium toxicity, mode of cell death and potential alteration of renal antigens that may lead to the preservation of kidneys in lupus patients.

Lithium toxicity was determined using standard cell counts and viability studies. The cells were sensitive to lithium at concentrations of 10 mM and above. Light and electron microscopy, DNA fragmentation and TUNEL assays were performed and established the mode of cell death to potentially be apoptotic. The distal renal tubule PK(15) cells were more susceptible to lithium toxicity than the proximal renal tubule LLC-PK_1 cells. Proteins involved in apoptotic mechanisms were investigated for their involvement in lithium induced cell death of the renal cells. Immunocytochemistry (Bax, p53, proliferating cell nuclear antigen (PCNA), retinoblastoma protein (Rb)), Western blots (Bax, Bcl-2, p53) and enzyme activity assays (caspase 3, 8, 9) were used as assessment techniques. No significant difference in the expression of Bax, Bcl-2, p53 and PCNA proteins in LLC-PK_1 cells treated with concentrations of up to 20 mM LiCl for 24 h were observed. Conversely, a statistical significant upregulation ($p<0.05$) of the cell cycle protein, Rb, was noted in 5 and 10 mM LiCl treated LLC-PK_1 cells. Flow cytometry with propidium iodide stained cells, however, revealed no change in the cell cycle of the LLC-PK_1 cells after LiCl treatment for up to 48 h. Caspase enzyme activity, a hallmark of conventional apoptosis, was assessed using specific substrates. No changes were observed in the initiator caspase 8 or 9, nor in the executioner caspase 3 activities of lithium treated LLC-PK_1 cells. However, lithium chloride (20 mM) significantly increased Bax expression in PK(15) cells and the Bax:Bcl-2 ratio, an indicator of apoptosis, increased in a

LiCl concentration dependant manner ($p<0.05$). Furthermore, PCNA and Rb proteins were upregulated ($p<0.05$) at 10-20 mM and 5-10 mM LiCl respectively. Flow cytometry indicated that LiCl increased the G_2/M population of PK(15) cells. Although the results implicate apoptosis as a mechanism of PK(15) cell lithium induced cell death, they indicate that the cells are probably undergoing cell death via a caspase independent mechanism.

Well characterised SLE patient serum was used in Western blots to establish whether LiCl caused autoantigenic changes in PK(15) or LLC-PK_1 cells. Although initial experiments indicated some changes in autoantigens, particularly SSB-La antigens, further experimentation revealed no consistant changes. In addition, phosphorylation and glycosylation studies did not indicate any changes in autoantigenic structure within the cells.

To conclude, this study revealed that LiCl was toxic to established porcine renal tubular cells at concentrations (10 mM and above) that are much higher than those used therapeutically. Distal tubular PK(15) cells were more susceptible to lithium than proximal renal LLC-PK_1 cells and the toxicity seemed to be via a caspase independent mechanism of apoptosis. The study also revealed that lithium probably does not alter autoantigens to an extent that immunoprecipitation of antibodies can be prevented in SLE patients and thus the preservation of NZB/W F_1 female mice by lithium chloride may be due to another mechanism.

CHAPTER 1
INTRODUCTION

Literature based on health care and health care products is expanding yearly. Not only are new medical products being sought and produced for established diseases, but existing products are being used for newly diagnosed diseases. Millions of dollars are being spent yearly on health care research, development, treatments and preventions (http://www.nsf.gov /statistics/htm d.o.a.01/08/06). Ongoing research regarding the benefits and side effects of various drugs is taking place to ensure low-risk, but successful products being used by the population. One simple, yet effective drug that sparked off much interest nearly 60 years ago, lithium, is still being assessed today for its mechanism of action, its action on various aspects of disease and its toxicity. This literature review covers aspects of lithium from the description of the element to its benefits, risks, and more specifically, the scientific literature that attempts to elucidate lithium interaction at the cellular and molecular levels as well as progress made to resolve lithium's mode of action.

1.1 Lithium

Lithium ($^{3}Li_{6,941}$) is a monovalent cation found within group 1A, the alkali metals, on the periodic table. The name "lithium" originated from the Greek word "lithos", meaning stone. It is ubiquitous in the environment and naturally occurs in soils, rocks and water. As lithium is a trace element found in our foods and drinking water, we are exposed to a certain dosage every day. The amount of lithium absorbed by individuals varies in accordance to the geographic location of the individuals, some areas having higher lithium levels than others. The dietary amounts of lithium (1-2 mg/day), however, have been shown to be non-toxic (Weiner, 1993).

The most abundant isotope of lithium found is Li-7 (96.23%), although Li-6 (3.75%) is also found naturally (http:/www.webelements.com). Li^{7} has an ionic radius of 60 pm which is similar to the ionic radius of the divalent cation Mg^{2+} (Brady *et al*., 1980). This property of lithium established research into its

competitive nature for magnesium binding sites (Layden *et al*., 2003). Furthermore, the monovalent characteristic of lithium also has similarities to Na^+ and K^+, and this has led to lithium being used extensively as a tool for research on membrane transport processes (Birch and Padgham, 1993).

Medicinally, lithium has been used for over a century in dubious concentrations for various ailments (Summarised in Triffleman and Jefferson, 1990). John Cade, in his article entitled "Lithium salts in the treatment of psychotic excitement" published in the Australian Medical Journal, in 1949, is credited as the pioneer in the field of lithium and its uses in the treatment of bipolar disorder. This publication was met with great scepticism, but the use of lithium was later substantiated by thorough clinical trials (Müller-Oerlinghausen *et al*., 1991).

Although psychiatrists in Europe tested the efficacy of lithium in the mid 50's (Schou, 1999), the drug was only approved by the United States FDA in 1970 (Grof, 1999). Subsequently, lithium became the drug of choice for many psychiatrists, world-wide for the treatment of manic-depression (Manji and Lennox, 1998; Soars and Gershon, 1999) and although new drugs for bipolar disorder are being used, it is still preferred by many psychiatrists (Jamison, 2006). Bipolar disorder affects nearly 1-2% of the population worldwide and lithium has been found to be effective or partially effective in up to 60% of bipolar patients (Michelon *et al.,* 2006). It is usually prescribed in the form of lithium carbonate (Li_2CO_3) and the dosage is vigorously monitored to ensure blood serum levels of < 0.8 mM (Gitlin, 1992). This lithium dosage allows for a small margin of variation and toxic levels of lithium are deemed to be greater than 1.5 mM. Extensive historical evidence on the successes and failures of lithium use for psychiatric treatment can be found in Lithium: 50 years of Psychopharmachology, New Perspectives in Biomedical and Clinical Research (1999), edited by Birch, NJ, Gallicchio, VS and Becker, RW.

Lithium was also found to fulfil other roles in the medical field, for example, lithium causes haematopoeisis at low concentrations (5 mM or below). These mitogenic effects of lithium have been found in many cell lines including

human lymphocytes, human T-cells, murine pluripotent stem cells, myelocytic leukemia cells and in bone marrow cells (reviewed in Becker and Tyobeka, 1996). This property initiated many studies on lithium effects in the immune system (Hart, 1991; Beyaert and Fiers, 1992; Rapaport and Manji, 2001; Lieb, 2007). Much evidence points towards the favourable effects of lithium on the treatment of different viruses, in particular the herpes simplex virus (Rybakowski, 1999a) and the murine aquired-immunodefiecient virus (Gallicchio *et al*., 1993). Lithium uses in the HIV field potentially has enormous implications and may reduce the costs of AIDS treatment. Lithium is also useful as an indicator for disease such as renal clearance (Thomsen, 1990) and cardiac output (Jonas *et al*., 2001). Hence this small, seemingly insignificant, ion has an impact in many fields of medicine (Jefferson *et al*., 1999). The fact that no exact mechanism of action in any of the above mentioned treatment fields has been found, indicates that lithium may function in numerous ways at the molecular, cellular and physiological levels.

3.3. Mechanism of action of lithium as a psychomodulator

There are many studies still being undertaken by psychiatric research groups on the efficacy of lithium on bipolar disorder (Geddes *et al*., 2004; López-Muñoz *et al*., 2006). Through constant research, lithium doses have been regulated to 0.8 mM, which is lower than the 1.5 mM 12 hr blood serum levels previously advocated (Birch, 1999). Another aspect frequently investigated is the difference between patients that are good lithium responders and those that do not respond at all. This has led to increased investigations into heritable genes that may influence the response of the patient to lithium (Michelon *et al*., 2006). Further studies on the efficacy of lithium as a monotherapy or as a conjunctive agent with other bipolar drugs are constantly investigated and reviewed.

It has been discovered that lithium also has an effect on Alzheimers (Munoz-Montano *et al*., 1997) and Parkinson's disease (King *et al.*, 2001). Recently, the implications of lithium therapy to assist with proliferation and survival of neural progenitor cells in cell transplantation in spinal injuries has been

studied with some success and caution by Su *et al*. (2007). Although the molecular mechanisms of action were not investigated, these studies are encouraging for the use of lithium in this field. To date, much time and effort has been spent by clinical and laboratory researchers on the actual mode of lithium action at the cellular and molecular levels and varying hypotheses have emerged.

1.2.1 Lithium interference with cations

Lithium itself does not seem to have an important role in any metabolic process and thus it may replace, enhance or antagonise the actions of other cations. Since lithium is a cation, simple diffusion across polarised membranes has been ruled out and lithium uptake studies have been extensively researched, especially in erythrocyte membranes (Gallicchio, 1990).

The complexities involved in membrane transport of lithium have provided for a plethora of research papers which have led to the formulation of numerous hypotheses. Various transport routes were found to play a role in lithium influx or efflux from the cell (reviewed in, amongst others, Gallicchio, 1990; Birch and Phillips, 1991; Klemfuss and Greene, 1991; Birch, 1999).

a. Na-Li counter transport.

Lithium interferes with the Na_o-Na_i counter-transport system. This system was initially described by Morgan *et al*. (1989). Further work confirmed that lithium moves out of the cell against an electrochemical gradient, dependant on the external Na ion concentration, and that its binding affinity for lithium is 15-18 fold greater than that of sodium (Timmer and Sands, 1999). This transporter system has been shown to be important for lithium efflux from lymphoblastoma, red blood cells and neuroblastoma cells (Layden *et al*., 2003). An increase in the Na-Li counter-transport system in erythrocyte membranes has been found to correlate to hypertension for a subgroup of the general population

(Trevisan and Borrillo, 1992; Boero *et al*., 1993). Na-Li counter transport has also been implicated in kidney function, diabetes and IgA nephropathy. Boero *et al*. (1993) found that a genetically predetermined population of IgA nephropathy patients with high erythrocyte Na-Li counter-transport systems were predisposed towards arterial hypertension, lipid abnormalities and probably renal failure. Although this was a single study using a limited population group, it is indicative of using lithium action within cells as an indicator of prevailing disease conditions.

b. Na^+-K^+-ATPase pump.

Na^+-K^+-ATPase pump is normally responsible for pumping 3 Na^+ out of a cell and 2 K^+ into a cell, thus maintaining a membrane potential as well as maintaining interior K^+ levels. Studies have indicated that lithium can replace the K^+ and will be transported into the cell (reviewed in Birch, 1999). The order of magnitude for lithium binding is, however, less than that for potassium and is an unlikely event at physiological concentrations (Timmer and Sands, 1999). This pump was shown to be the main lithium influx pathway for lymphoblastoma cells but not for lipopolysaccharide stimulated splenocytes (Hart, 1982), erythrocytes and neuroblastoma cells (Layden *et al*., 2003).

c. $Na^+/K^+/2Cl^-$ co-transporter

With the use of furosemide, it has been shown the $Na^+/K^+/2Cl^-$ co-transporter is a mechanism via which lithium gains access into a cell.

This has been studied in erythrocytes (Birch, 1999), as well as in distal tubule renal cells (MDCK) cells where it appears to be the main transport system for lithium into this cell type (Suh *et al.*, 1992).

d. Na+ dependant cotransport systems

Sodium – glucose, dicarboxylic acid, amino acid and phosphate cotransport systems have been investigated as lithium transport systems into cells. Lithium substitutes poorly for sodium in some cell lines, such as renal cells, and well in others (Timmer and Sands,

1999). A good example is the Na_2CO_3 cotransporter in intestinal cells. Li^+, as opposed to Na^+, binds to bicarbonate resulting in a $(LiCO_2)^-$ complex. This allows for lithium to move into the cells (Birch, 1999).

e. Na/H (sodium – proton exchanger)

The Na/H exchanger is responsible for the major sodium reabsorption across the proximal tubules. It has been shown to transport lithium at a rate 50% slower than that of sodium, but plays an important role in lithium reabsorption (summarised in Timmer and Sands, 1999).

f. Leak diffusion

Lithium transport across the membrane utilises a Na^+-K^+ mechanism, this being the leak mechanism of the two cations down a concentration gradient via different proteins.

Although the above mechanisms have been found to influence the passage of lithium into and out of the cell, Birch (1999) cautions the scope of these mechanisms. Different cell lines utilise different mechanisms, and some, like the intestinal mucosal cells, use paracellular methods to transport lithium, though this has been ruled out as a route in renal cells (Timmer and Sands, 1999). This leads to variances of lithium concentrations within different cell types. Erythrocytes incorporate roughly 15% of the external lithium concentration, whereas hepatocyte and fibroblast cells incorporate approximately 5.7% and 7%, respectively (Birch and Padgham, 1993). Intestinal mucosal cells have low concentrations of lithium but renal cells can concentrate lithium to more than 100% (Suh *et al*., 1992), as is shown by Goldberg *et al*. (1988) in proximal renal cells.

One implication of lithium interfering in the above transport systems of Na^+ and K^+ is that it has led to the K^+ depletion hypothesis. A depletion of K^+ affects inositol phosphate metabolism as well as distal renal epithelial cell proliferation (Klemfuss and Greene, 1991; Suh *et al*., 1992).

Extensive work has been done on the influence of other cations on processes in which lithium has been implicated. Since lithium has a similar ionic size to Mg^{2+}, it has been hypothesised that lithium competes with Mg^{2+} in various enzymatic and transport reactions. Studies on magnesium binding enzymes have found pyruvate kinase and myo-inositol phosphatase to both be noncompetitively inhibited by lithium (reviewed in Geisler and Mørk, 1990). Studies by the group of Mota de Freitas and colleagues have shown Li^{+} to compete with Mg^{2+} binding sites on G-proteins, phosphate groups of ATP and erythrocyte membranes (Layden *et al*., 1999) and vasopressin stimulated adenylate cyclase activity (Goldberg *et al*., 1988). Layden *et al.* (2003) also showed that the extent of lithium immobilisation within a cell affects the Li^{+}/Mg^{2+} competition. This was found to vary within different cell lines.

Owing to the importance of Ca^{2+} in cellular signalling mechanisms, this is also an area of potential lithium interference. Many studies have been conducted on the effect of lithium on calcium levels, as well as its interference on Ca^{2+} binding sites. These studies, as well as the metabolic processes in which calcium is involved, have been reviewed in Warsh *et al.* (2004). Again, the inositol second messenger system appears to be influenced by lithium on Ca^{2+} signalling, and inositol monophosphatase inhibition in particular. The effects of lithium on intracellular calcium concentrations seems to be cell line specific and no conclusive evidence for the effect of lithium on bipolar disease can be established. Of particular interest are the studies by Dubovsky *et al*., as discussed in Warsh *et al*. (2004), which indicate that lithium treatment of bipolar patients normalised intracellular calcium levels.

Finally, lithium has also been found to alter DNA structure. Studies by Loprete and Hartman (1993) indicate that LiCl alters the conformation of DNA to a C-type conformation as opposed to the expected Z-type stabilised by Ca^{2+} and Mg^{2+}. Although lithium levels were high in these studies, it is an interesting observation.

Little new evidence for the action of lithium on the interference of cations has been forthcoming more recently. From the evidence presented above, it is

clear that lithium gains entry and exits the cell using K^+ and Na^+ pathways. It is also evident that lithium interferes with Mg^{2+} and Ca^{2+} pathways and proteins, though the exact mechanism of its bipolar altering activity has not been substantiated.

5.4.1 The inositol depletion hypothesis

The mechanism of action of lithium as a psychomodulator has not yet been completely elucidated, although evidence has pointed to the involvement of second messenger and signal transduction pathways. Specifically, lithium has been shown to inhibit adenylate cyclase after its stimulation by adrenaline (Ebstein *et al*., 1976), to interfere with the phosphoinositol pathway (Berridge, 1989), to compete with Mg^{2+} for ATP (Ramasamy and Mota de Freitas, 1989), to potentiate tumour necrosis factor (TNF)-mediated activities (Beyaert and Fiers, 1992), and more recently, to inhibit glycogen synthase kinase 3β, a key enzyme in signal transduction pathways (Klein and Melton, 1996).

The inositol depletion hypothesis depicting the involvement of lithium in the treatment of bipolar disorder was proposed by Berridge (1989) and has become one of the foremost theories describing the action of lithium in the brain. This hypothesis remains a prime topic of study (Belmaker and Bersudsky, 2007). Many critiques have been written concerning the inositol-depletion hypothesis and its merits or deficiencies. This has led to much evidence, speculation and thought provoking ideas addressing the mechanism of lithium action on bipolar disorder (Pacheco and Jope, 1996; van Calker, 1999; Harwood, 2005; Belmaker and Bersudsky, 2007).

Basically, the hypothesis suggests that a depletion of the brain inositol pool may reduce overactive brain neurotransmissions occurring in manic-depressive patients. Inositol has also been implicated in many other neurological and psychiatric disorders such as Alzheimers, epilepsy, Parkinsons, schizophrenia and depression, which has added substance to the inositol depletion hypothesis (reviewed in Pacheco and Jope, 1996).

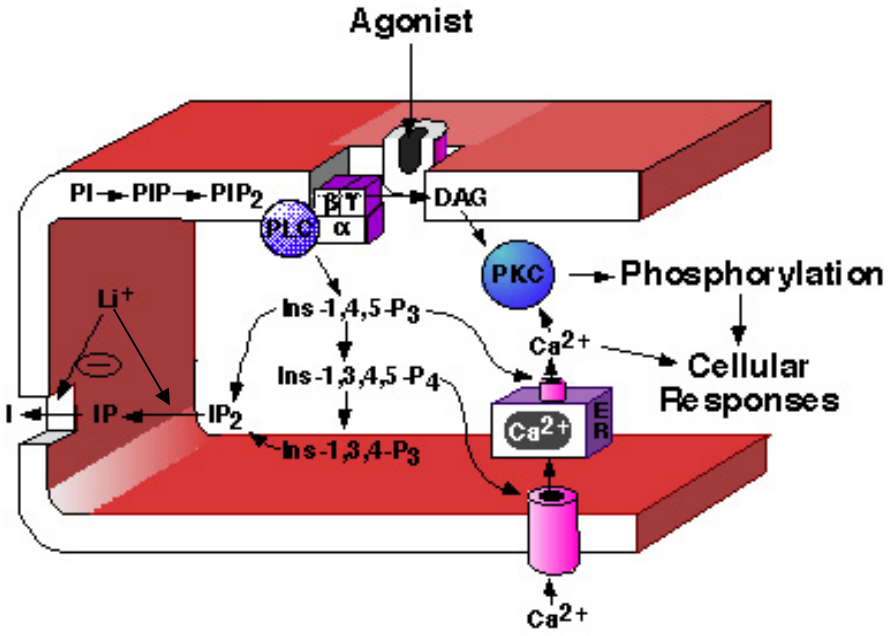

Figure 1.1: Phosphoinositide-linked signal transduction.
An agonist binds to its receptor and initiates the G-protein linked signal pathway. The effector enzyme is a phosphoinositide-specific phospholipase C which hydrolyzes membrane phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-trisphosphate (Ins-1,4,5-P3) and diacylglycerol (DAG). Ins-1,4,5-P3 then acts to release calcium from an endoplasmic reticulum - like store, and calcium can then activate a variety of calcium-dependent enzymes. DAG, which stays associated with the membrane, activates protein kinase C (PKC) causing phosphorylation of specific proteins leading to cellular responses. Ins-1,4,5-P3 can be phosphorylated to Ins-1,3,4 5-P4 which has been proposed to control influx of extracellular calcium. A series of dephosphorylation reactions control the levels of inositol phosphates. The inositol monophosphatase is inhibited by therapeutic concentrations of lithium and can disrupt the normal function of the phosphoinositide cycle. (Modified from Berridge, 1989 in Wilcox, R E. and Gonzales, R. A. (1995) Introduction to neurotransmitters, receptors, signal transduction, and second messengers. Textbook of Psychopharmacology, C.B, Nemeroff and A.F. Schatzberg, editors, American Psychiatric Press, 1st edition, pages 3-31).

In order to understand the effects of lithium on phosphatidylinositol signalling, a brief description of the pathway will be outlined in this review, adapted from review articles of the authors mentioned above, with reference to Figure 1.1. The phosphatidylinositol pathway is ultimately responsible for agonist induced second messenger responses by the cell, and forms a vital signalling system at the cell membrane. After an agonist, such as norepinephrine, has bound extracellularly to its receptor, a signal is sent via G-protein mediation to activate the internal extrinsic protein, phospholipase C. Activated phospholipase C hydrolyses the membrane phosphatidyl lipid, phosphatidylinositol-1,4,*bis*phosphate, to form inositol-1,4,5-*tris*phosphate (I-1,4,5-P_3) and diacylglycerol (DAG). Inositol-1,4,5-*tris*phosphate is an

important signal to stimulate calcium release from the endoplasmic reticulum and plasma membranes and thus elevates cytoplasmic calcium levels. Diacylglycerol is a second messenger that activates protein kinase C in a calcium-phosphatydylserine-dependent manner. Protein kinase C then phosphorylates other proteins in a signalling cascade. The I-1,4,5-P_3 undergoes one of two fates:

1. Further phosphorylation to form more inositol phosphates that are involved in downstream signalling (more than 20 soluble inositol phosphates in mammalian cells have been identified (reviewed in Majerus *et al*., 1999) or
2. It is dephosphorylated via protein phosphatases to form inositol-1,4-*bis*phosphate (I-1,4-P_2), inositol-4-phosphate (I-4-P) and eventually inositol (*myo*-inositol) which is recycled back to the membrane phosphatidylinositol phosphates via a synthase enzyme. The second fate described here is of importance to the inositol depletion hypothesis.

The inosoitol depletion hypothesis proposes that a downstream effect caused by the depletion of inositol, which is normally continuously replenished in the phosphoinositol pathway, causes the stabilising action that lithium has on bipolar disorder. Since it was initially thought that inositol could not traverse the blood-brain barrier, and that the inositol concentration in the brain could not be replenished by plasma inositol (Berridge, 1989), this was a plausible explanation.

Briefly, the magnesium dependant enzyme inositol monophosphatase which dephosphorylates I-4-P and inositol-3-phosphate (I-3-P) to form inositol in the phosphatidyl inositol pathway, is uncompetitively inhibited by lithium (Hallcher and Sherman, 1980). Lithium is also an uncompetitive inhibitor (K_i=0.8 mM) of the magnesium dependant inositol 1,4 *bis*phosphate-1-phosphatase which is important in the catalysis of I-1,4-P_2 to I-4-P (Inhorn and Majerus, 1987). This lithium mediated inhibition leads to the accumulation of intermediates such as I-1,3,4-P_3, I-1,4-P_2, I-3-P and I-4-P. Consequently, this causes a decrease in free inositol which minimises further cycling through the phosphatidyl inositol (PI) pathway and the ultimate depletion of various intermediates and thus of cell signalling (Figure 1.1). A further consequence

of the accumulation of inositol triphosphates, which are second messengers, is the release of Ca^{2+} from intracellular stores resulting in elevated intracellular Ca^{2+} levels (Schotte and Beyaert, 1999).

Lithium induced up-regulation of inositol monophosphatase (IMPA) transcription has been suggested as a mechanism used by the cell to compensate for IMPA inhibition and was shown by Nemanov *et al.* (1999) in lymphocytes of lithium treated bipolar patients. IMPA2 is another brain inositol monophosphatase enzyme initially thought to be an alternative to the inhibited IMPA1. Seelan *et al.* (2004) studied IMPA2 transcription in SK-N-AS neuronal cells and observed a lithium induced down-regulation of IMPA2 transcription. Ohnishi *et al*. (2007) recently discovered that IMPA2 has lower activity towards myo-inositol monophosphate and is inhibited by much higher concentrations of lithium, and suggest that IMPA2 may have a separate role to IMPA1 and, thus, a small effect in the inositol depletion hypothesis.

Further evidence for the involvement of lithium on inositol biosynthesis stems from studies by Williams *et al.* (2002) and Shaltiel *et al.* (2004). Both groups were investigating the potential of a common target for the various treatments of bipolar disorder viz. valproate, carbamazepine and lithium (Williams *et al.,* 2002; Cheng *et al.*, 2005; Daniel *et al*., 2006), and valproate and lithium (Shaltiel *et al*., 2004). Inositol depletion was implicated as a common action of the bipolar drugs. Furthermore, Williams *et al.* (2002) showed that prolyl oligopeptidase, a cytoplasmic protein that cleaves prolyl bonds in oligopeptides implicated in mood disorders, is involved in regulating inositol levels and that it's inhibition, by valproate and not lithium, abolished the drug induced effects on neuronal growth cones (Cheng *et al*., 2005). De Sarno *et al.* (2002), however, found that a common action of valproate and lithium on SH-SY5Y neuroblastoma cells was on the Akt/PI3K and GSK pathway as discussed below in section 1.1.3. Both bipolar drugs inhibited GSK via different mechanisms. These two results may indicate that both sites of action (inositol depletion and GSK) may be working together or that the mechanism of lithium action is cell specific.

The results of Williams *et al*. (2002) concurred with reports by O'Donnell *et al.* (2000) who used *ex–vivo* research on Sprague-Dawley rat brains. Nuclear magnetic resonance studies indicated that myo-inositol levels decreased and inositol monophosphate levels increased with chronic (15 day) lithium treatment (O'Donnell *et al*., 2000). Berry *et al*. (2004), however, disagreed with the inositol depletion hypothesis. In their experiments using murine brain inositol deficient mice (SMIT-/-), they showed that the phosphatydylinositol levels in the mice did not decrease when compared to control subjects. The authors did agree, however, that inositol may be important in brain homeostasis and suggested that alternate pathways may replenish the inositol in the neural cells. Although no lithium was used in the experiment, the authors concluded that phosphatidylinositol deficiency due to inositol depletion is not a mechanism of lithium action in bipolar disorder

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Much of the information on the inositol depletion hypothesis has been obtained using *in vitro* results or animal model studies. More recently, with the use of non invasive techniques such as proton magnetic resonance, *in vivo* results are coming to the fore. Moore *et al.* (1999) found that myo-inositol levels in the frontal lobe region of the brain decreased in lithium treated patients, though this did not correspond to an alteration in the patient's behaviour. Several further studies have been carried out using magnetic resonance spectroscopy on both lithium treated and untreated bipolar patients in various states of disease (Reviewed in Silverstone *et al.,* 2005). Although varying results were obtained in euthymic patients, evidence supporting the involvement of the PI pathway in lithium treatment has been shown.

The inositol effects appear to be neural cell specific. Becker and Tyobeka (1998) observed no changes in inositol concentrations in lithium treated HL-60 cells which led them to speculate that the phosphoinositide pathway does not play a role in lithium induced growth proliferation of HL-60 cells. Furthermore, inositol could not reduce lithium anti-proliferative effects in L6 myoblast cells (Laurenz and Smith, 1998).

The inositol depletion hypothesis has been supported for many years by various authors but perhaps, due to the complexities involved in cell signalling pathways, alternate pathway replenishing the inositol pools within a cell may be a plausible concept. Although other proteins have been implicated, *viz* glycogen synthase kinase 3β (GSK), no defining evidence to dispute or challenge the inositol depletion hypothesis has been forthcoming.

5.4.2 Glycogen synthase kinase-3β

GSK is a serine/threonine kinase, found ubiquitously in mammalian tissues. Named for its role in the phosphorylation of glycogen synthase, GSK plays an important role in many intracellular signalling pathways (Frame and Cohen, 2001; reviewed in Jope, 2004). *Inter alia* it participates in insulin signalling, the mitogen-activated protein kinase (MAPK) pathway, glycogen synthesis, PI-3 kinase dependant pathway, Wnt signalling, cell division (cyclin D1 and cyclin dependant kinases), modulation of transcription factors, such as c-jun and c-myc, and also in cell structure and architecture. It has recently been proposed that the functions of GSK in Wnt signalling and apoptosis are highly conserved, from hydra through to higher mammals, and has a significant role in embryogenesis (Klein and Melton, 1996; Rentzsch *et al*., 2005).

The influence of lithium on GSK was first reported by Klein and Melton in 1996. Their studies of lithium influence on the morphogenesis of Xenopus embryos showed that after complete inositol monophosphatase inhibition morphogenesis still occurred, and that lithium inhibited GSK activity with a K_i of 2.1 mM, a concentration slightly higher than that used in bipolar treatment. Although initially thought to be uncompetitive inhibition, lithium was found to compete with Mg^{2+} in this system (Ryves and Harwood, 2001). Later studies by Chen *et al.* (1998) supported Klein and Melton's hypothesis of an alternative mechanism of action by showing that the inhibition of inositol monophosphatase did not alter lithium induced glucose uptake in rat adipocytes. Since lithium (1 mM) was found to mimic insulin and increase glycogen synthesis by activating glycogen synthase (Cheng *et al.,* 1983) this

pathway was a plausible target of lithium action during embryogenesis. Lithium was shown to inhibit GSK which prevents the phosphorylation of glycogen synthase which subsequently remains active and promotes the formation of glycogen. De Sarno *et al*. (2002) later proved that lithium also inhibits GSK indirectly by increasing the level of phospho-Ser-9-GSK3β which leads to its inactivation. To substantiate this, it was shown that GSK affects protein phosphatase activity and in effect prevents its own dephosphorylation. A similar observation was made for phosphatase-1 by Zhang *et al.* (2003) who hypothesised that GSK autoregulated its own activity. Mora *et al*. (2002) showed that in the presence of lithium, ceramide stimulated neural cells had decreased dephosphorylation of PKB-P-Ser-473 (results not observed by De Sarno *et al*., 2002) and phospho-Ser-21-GSK, a GSK3α inhibition site. They hypothesised that lithium may inhibit protein phosphatase 2 (PP2) activities. Time intervals in these two studies varied which could account for the variability in the results. Interestingly, Wachira *et al.* (1998) were previously able to demonstrate that lithium and an inhibitor of PP2, calyculin-A, had similar, 24 h effects on the non-neural HL-60 cells. The observation that lithium can inhibit GSK indirectly generated great interest as it could explain the delayed onset of lithium therapy in bipolar disorder, as opposed to the direct inhibition which should provide more immediate effects (reviewed in Jope, 2004).

Rubinfeld *et al.* (1996) determined that GSK (zeste-white 3/shaggy in wnt signalling) forms a complex with adenomatous polyposis coli (a protein implicated in some colon cancers) and β-catenin (ARMADILLO in Wnt signalling). It phosphorylates β-catenin which marks it for degradation by the ubiquitin pathway. When GSK is inhibited, β-catenin is stabilised leading to altered gene transcription (Hedgepeth *et al.,* 1997). Beta-catenin translocates across the nuclear membrane and affects cell proliferation, the cell cycle and apoptosis by binding to transcription factors. The effect of lithium on GSK and c-jun activity was investigated by Hedgepeth *et al.* (1997). GSK phosphorylates c-jun and prevents DNA binding and thus transcription. Lithium was shown to increase c-jun activity in xenopus embryo lysates in a

dose dependant manner (Hedgepeth *et al*., 1997). Jun is a subunit of activating protein 1 (AP-1) which binds to DNA and it has been observed that lithium treatment enhances AP-1 activity in neural cell lines (reviewed in Williams and Harwood, 2000). No alteration in c-jun mRNA transcription was observed by Wachira *et al*. (1998) in HL-60 hematopoetic cells after treatment with 20 mM LiCl. This may, however, have been dependant on the time allowed for the lithium to act on the cells. Stabilisation of β-catenin was also implicated in fibroproliferative disease due to it increasing T cell factor-transcription and fibroblast proliferation (Cheon *et al*., 2002). Further studies have also indicated that β-catenin stabilisation due to lithium induced GSK inhibition have improved bone fracture healing in mice (Chen *et al*., 2007; Clément-Lacroix *et al*., 2005).

Glycogen synthase kinase inhibition by lithium may also play a role in Alzheimer disease. GSK phosphorylation of Ser/Thr-Pro sites of the tau protein results in hyperphosphorylated tau, a microtubule-associated protein (MAP), and causes microtubule destabilisation of the cytoskeleton. Lithium enhanced the dephosphorylation of the tau protein in primary cultures of rat pup cerebellar granule neurons, and has provided a hypothesis for the inclusion of lithium in Alzheimer treatment (Munoz-Montano *et al*., 1997). GSK has also been implicated in Parkinson's disease. Parkinson's disease is thought to occur due to excessive apoptosis. GSK has been found to be a pro-apoptotic protein and thus its inhibition should prevent apoptosis and thus protect neural cells from cell death (De Sarno *et al*., 2002). Lithium pre-treatment of neuronal cells triggered to undergo apoptosis via a protein thought to play a role in Parkinson's, reduced the effects (King *et al.*, 2001).

A distinct link between bipolar disorder, GSK abnormalities and the intervention of lithium has not yet been established. Indications of errors in Wnt signalling have been shown in mental disorders in some mice which may provide a link (reviewed in Williams and Harwood, 2000). Nishiguchi *et al.* (2006) investigated the association of GSK mapped to chromosome 3q13,3

and the potential locus for bipolar disorder on chromosome 3q in 280 patients but found no conclusive link.

From the above evidence it is clear that a conclusive mode of lithium action in bipolar disorder has not been determined. What has been established is that lithium does interfere in intracellular signalling mechanisms, be it via the inositol depletion hypothesis (Daniel *et al*., 2006), GSK direct and indirect inhibition (Jope, 2004), an integration between the two, or due to a molecule that has yet to be discovered.

3.4. Lithium toxicity and side effects

Lithium has a number of adverse effects such as gastrointestinal discomfort, nausea, vertigo, muscle weakness and a dazed feeling which frequently disappear after stabilization of therapy. The more common and persistent adverse reactions are: neurotoxicity (manifested as fine tremor of the hands, and, at times, fatigue); abnormal kidney function (manifested as thirst, polyuria, nephrogenic diabetes insipidus (NDI)); hypothyroidism, hypercalceamia, leucocytosis and aplastic anemia (reviewed in Lydiard and Gelenberg, 1982; Timmer and Sands, 1999; Chen and Petersen, 2000).

Much debate has ensued over the last 60 years or so on the beneficial versus the toxic effects of lithium. Most of the toxic related topics refer to the dosage required by patients to remain normalised. Presently it is easier for specialists to monitor the serum concentration of lithium using ion-electrodes as it eliminates waiting periods (Padgham *et al*., 1993). Although this negates many of the toxic concerns in patients, there are still questions regarding this issue. The organs most frequently researched are the nervous system, the thyroid and the kidney, whilst blood cells and the immune system are also often considered.

Lithium induced teratogenicity developments caused concern in the 70's and 80's (Schou, 1993). The 'lithium baby" scare was followed by extensive studies which led to the belief that the initial data was skewed and that the risk

of lithium induced teratogenicity was much lower than expected (Schou, 1993). Experiments assessing lithium induced teratogenicity in rats generated much criticism due to the excessively high lithium concentrations used and the intraperitoneal route of lithium administration. These reports did, however, indicate that lithium crosses the placenta and were confirmed by Hennequin *et al.* (1998) using neutron capture radiology studies. However, Hennequin *et al.* (1998) indicated that the lithium content in the embryo mimics that of the mother's, and lower levels of lithium, differentially distributed in the embryo, do not result in teratogen development. Cautious use of lithium during pregnancy, especially the first trimester, is prescribed by medical practitioners.

Lithium accumulates in the thyroid gland and is found there at concentrations two to five times that of the serum lithium levels. Lithium was found to cause hypothyroidism, hypothermia, and in a few contentious instances, involve the autoimmune system causing formation of antithyroid and antimicrosomal antibodies (Lydiard and Gelenberg, 1982; LeRoy *et al*., 1988; Rapaport *et al*., 1994). The higher incidence of non-toxic goitre occurs in lithium treated patients (Bauer *et al*., 2007) is thought to result from lower thyroid hormone production leading to increases in thyroid stimulating hormone (TSH). Studies on the metabolisms involved in the thyroid have found that lithium inhibits iodine uptake, iodination of tyrosine and the release of T3 and T4 (Rapaport *et al*., 1994). Since TSH binds to a receptor to stimulate cAMP production, lithium effects on cAMP production were investigated. Although studies have led researchers to believe the lithium effects to be post-cAMP production, contradictory reports have been described depending on the type of sample used (reviewed in Tasevski, 2000). Lithium did not play a role in TSH stimulated cAMP production in human-TSH receptor Chinese hamster ovary cells and a rat thyroid cell line (FRTL-5) (Gaberšček *et al*., 2003; Tasevski, 2000), though it did inhibit cAMP in primary cells (cited in Tasevski, 2000).

Lithium has also been associated with hyperparathyroidism and hypercalcaemia (reviewed in Timmer and Sands, 1999). Lithium induced hyperparathyroidism and hypercalcaemia have been observed in

osteoporosis, bone formation and bone turnover. A more positive side effect of lithium, hematopoesis, has led to the potential of its use as an agent to improve bone marrow transplantation (Gallicchio *et al.,* 1992). Recently, lithium was shown to enhance bone formation when administered during the healing process following a fracture (Chen *et al*., 2007). Furthermore, a clinical longitudinal study indicated that bipolar patients receiving lithium had a lower fracture risk than those not receiving it (Wilting *et al.,* 2007). In these studies, β-Catenin levels were shown to increase which implies that as discussed previously, GSK-3β is inhibited by lithium (See GSK) (reviewed in Krishnan *et al*., 2006).

Another lithium scare was the suggestion that lithium may cause diabetes mellitus. Extensive research has been performed on the diabetic effect of lithium on bipolar patients. Vestergaard and Schou (1987), however, diminished fears of lithium induced diabetes mellitus by not observing blood sugar changes in patients for up to six years. This was substantiated in the report by Markowitz *et al*. (2000) where 4.2% of renal biopsies from lithium treated patients indicated the presence of diabetes mellitus.

The above discussion advocates prudence when placing a patient onto lithium therapy. Furthermore, constant vigilance is necessary to prevent lithium induced side-effects. One area, not discussed above, is that of the renal system. The renal system, and kidney processing of lithium, is an area of concern that has stimulated much research and will be discussed in detail below.

3.5. Renal effects caused by lithium

Due to the development of common side effects, such as polydipsia (increased water intake), nocturia (increased nightly urinary output) and polyuria (increase in total urine output > 3 l/day), in lithium treated patients the effect of lithium on kidney function and renal disease has been extensively researched. The onset of lithium induced nephrogenic diabetes insipidus

(NDI) has also caused concern. The anatomy and physiology of the kidney will first be described to assist with the discussion on lithium toxicity.

5.2.1 Kidney anatomy and physiology

The kidney is the major secretory organ in the human body. Its function being to filter the blood, to selectively reabsorb fluid and solutes and to finally remove concentrated urine containing nitrogenous compounds out of the body via the ureter and the bladder. The kidney is a remarkable organ in its ability to adapt to various environmental and metabolic situations by altering the composition of the urine. Damage to the various components of the kidney leads to high levels of toxicity (White *et al.,* 1978).

The kidneys are two bean shaped organs connected to the ureter, a duct that carries urine from the kidney to the bladder (Johnson, 1983). Blood is supplied to the kidney via the renal artery and filtered blood leaves via the renal vein (Johnson, 1983). A longitudinal section through the kidney reveals three distinct areas. The renal cortex, the medulla and the renal pelvis. Urine leaves the kidney via distal ends of the collecting ducts and enters the bladder via the ureter. The kidney comprises of approximately 1.3 million nephrons, the basic functional units in which the blood is filtered and recirculated.

Nephrons are located in the cortex with some of them, the juxtamedullary nephrons, being located partly in the cortex extending down into the medulla. Nephrons contain various regions: the Bowmans capsule, the glomerulus, the proximal tubule, the thin limbs, distal tubule and the connecting segment (Figure 1.2). Each nephron has a loop of Henle composed of the thin limb segment and the straight portion of the distal tubule (Tisher, 1981).

The glomerulus filters the blood and removes plasma and red blood cells (Guyton, 1991). The permeability of the glomerular membrane is due largely to its unique composition and also to the high blood pressure in the glomerulus. Its permeability is 100 - 500 times greater than that of normal capillaries. Pressure in the glomerular capillaries forces the filtrate through

the glomerular membrane. This glomerular filtrate is an almost exact replica of blood plasma with a slightly higher composition of negative non-protein ions and a lower percentage of positive non-protein ions (Guyton, 1991). The glomerular filtration rate is autoregulated and remains relatively constant under normal circumstances.

The glomerular filtrate then enters into the proximal tubule. Reabsorption of water, glucose and amino acids takes place in the proximal tubule (Guyton, 1991). Many electrolytes such as Na^+ and Cl^- are reabsorbed by active processes involving Na^+-ATPase pumps (White *et al*., 1978). In the proximal tubules the composition of the glomerular filtrate changes rapidly (Burg, 1981). The chloride composition increases and the concentrations of other solutes, such as bicarbonate, glucose, amino acids and lactate decrease to low levels. Fluid is reabsorbed but the concentration of sodium remains isotonic to that of plasma. This iso-osmotic reabsorption of fluid is believed to be coupled to sodium transport. Sodium transport is the primary active process in proximal tubules and is coupled to the cotransport of bicarbonate, chloride, glucose, amino acids and lactate into the epithelial cell (Burg, 1981). Sodium is also transported by processes of facilitated diffusion out of the lumen of the tubules into the epithelial cells where it is actively pumped out into the interstitium. Subsequent movement of sodium out of the cell causes an osmotic pressure across the proximal tubule epithelial cells which allows for water to move from the lumen to the interstitial tissue. Hydrogen ions are secreted back into the lumen at this stage (Guyton, 1991). The filtrate then moves into the convoluted proximal tubule where there is not only a change in cellular anatomy but also in the absorption rate of various solutes (Burg, 1981). This part of the proximal tubule is able to maintain the concentration differences of the filtrate.

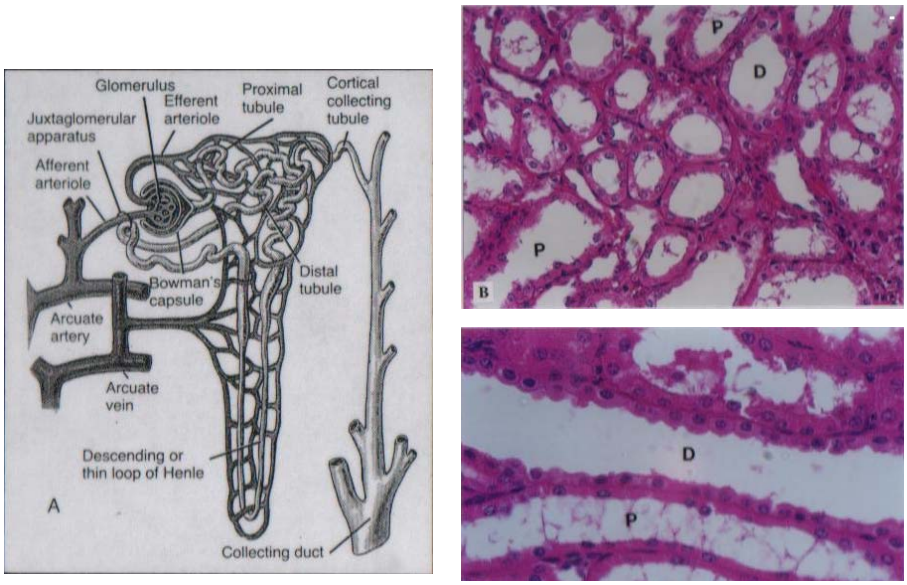


Figure 1.2: A nephron of the kidney.
Cross section depiction of the structural morphology of a nephron (A). A haemotoxylin-eosin stain of a cross section (B), and longitudinal section (C), of a porcine kidney cortex/medullary region depicting proximal (P) and distal (D) tubule epithelial cells. Figure 1.2(A) was extracted from Smith (1951) The Kidney: Structure and Functions in Health and Disease, Oxford University Press, New York, as referenced by Guyton, A.C. (1991) Textbook of Medical Physiology, 8th Ed, Wonsiewicz, M.J (Ed), W.B. Saunders, Philadelphia, pp287 and printed with the permission of Oxford University press.

The filtrate enters into the loop of Henle where no substantial active transport has been observed and the movement of ions and water out of the filtrate in this region occurs due to passive processes such as simple diffusion (Guyton, 1991). The thick loop of Henle is virtually impermeable to water though it actively transports sodium and chloride ions from the tubular lumen into the interstitial fluid (Burg, 1981), resulting in a dilute fluid with respect to that in the proximal tubule (Guyton, 1991). The same phenomenon is observed in the early distal tubules which contribute to the dilution of the filtrate. In the second half of the distal tubule the epithelial cells are similar in characteristics to those of the collecting ducts which give them a similar function. The tubule is impermeable to urea which allows all the urea to be excreted as urine. The cells are able to reabsorb sodium ions under the control of aldosterone, and secrete potassium ions back into the tubular lumen. Finally, the late distal tubule and the collecting duct are permeable to water under the influence of antidiuretic hormone (vasopressin), and impermeable in the absence of antidiuretic hormone. This system is very important in controlling the dilution of the urine (Guyton, 1991).

5.2.2 LLC-PK_1 proximal renal tubular cells

LLC-PK_1 cells were developed in the laboratory of Hull and colleagues in 1976. The cells were established from the kidneys of a juvenile Hampshire male pig. The cells grow easily in 5% FBS supplemented medium with a doubling time in the log phase of approx 9 h, although overall generation time was 30 h. The cells are epithelial-like cells, with large round nuclei sometimes containing up to three nucleoli. Sometimes domes of actively growing cells are observed. The karyotype of the cells was similar to that of a normal diploid domestic pig, i.e. majority of cells had 38 chromosomes, though it ranged from 36 – 40 chromosomes. The x and y chromosome were observed as normal in all passage numbers. The cells are stable through more than 300 serial passages and showed no evidence of transformation (Hull *et al*., 1976). The cells are non-tumourigenic, are susceptible to a number of viruses and characteristically produce high amounts of plasminogen activator, a protein catalytically the same as urokinase (Hull *et al*., 1976). They have been characterised to predominantly show proximal epithelial characteristics when confluent. The culture develops a fully polarised epithelium, forms tight junctions, transepithelial salt and water transport and brush border marker enzyme activity (Peng and Lever, 1995; Shikano *et al*., 2004). It also possesses an organic cation transporter which is characteristic of proximal epithelial cell renal organic cation secretion (Gründemann *et al*., 1997). Studies by Amsler (1994) indicate that induction of cell growth arrest as opposed to cell-cell contact density leads to induction of proximal epithelial-like features. It has a high expression of argenine vasopressin (Gorbulev *et al*., 1993) and has been used to study the vasopressin receptor adenylate cyclase system (Goldberg *et al*., 1988). Furthermore, the cell line possesses an active sugar transport system (Mullin *et al*., 1982), has been used as a model to assess sodium coupled glucose transport (Peng and Lever, 1995) and has high levels of alkaline phosphatase and γ-glutamyl transpeptidase (Rabito *et al*., 1984). This cell line has been used for many proximal renal tubular toxicology studies against toxins such as cadmium (Prozialeck *et al*., 1995; Zhang *et al*., 1995; Niewenhuis *et al*., 1997; Stinson *et al*., 2003), albumin (Erkan *et al*., 2001), bismuth (Leussink *et al*., 2002), Bence-Jones

monoclonal immunoglobulin free light chain proteins (Diemert *et al*., 1995), cellular stress by ATP reduction (van Why *et al*., 1999), gentamicin (Servais *et al*., 2004) and anti-cancer drugs (Pampillón *et al*., 2006).

5.2.3 PK(15) distal renal tubular cells

PK(15) cells are a porcine renal tubular cell line derived from a clone of the PK 2a cell line. The cells were established from an adult pig of undetermined strain or gender. The cells grow as a monolayer in 5 – 10% FBS supplemented medium under normal culture conditions (Aran and Plagemann, 1992a; Ward *et al*., 2000) and display an epithelial morphology. The population doubling time is approximately 28 h. The cells have a modal chromosome number of 37, one less than that of a normal diploid pig, and show characteristics of a single type of distal convoluted tubular cell (Aran and Plagemann, 1992 a and b). The cell line possesses an equilibrative nucleoside transport system with a high affinity for cytidine and uridine, but no Na^+-dependant concentrative nucleoside transport system, a transport system commonly seen in mammalian epithelial brush border membranes (Aran and Plagemann, 1992 a and b). Furthermore PK(15) cells express a cyclic AMP synthesis that is responsive to calcitonin, a hormone known to decrease the calcium and phosphate reabsorption by renal tubule cells, but not by parathyroid hormone or vasopressin (Aran and Plagemann, 1992a). The cells produce plasminogen activator and keratin (ATCC product information sheet). The cells display characteristic epithelial microtubule spreading and flattened cell morphology (Connolly *et al*., 1981). These cells have been found to be positive for porcine papovavirus, endogenous c-type retrovirus and porcine circovirus antigens. They are routinely used to screen for hog cholera virus and African swine virus, and are further used to study xenograft rejection between primate-porcine grafts such as human-pig or baboon-pig as they exhibit characteristic αGal-1-3-Gal disaccharides on their surface which have been characterised as the carbohydrate involved in porcine graft replacement therapy (Taniguchi *et al*., 1996; Neethling and Cooper, 1999).

5.2.4 Renal processing of lithium

Lithium urinary concentrations have been shown to be directly proportional to lithium concentrations within the plasma and in saliva (Thomsen and Schou, 1968; Kyroudis *et al*., 1987). Lithium is filtered in the glomerulus and 70-80% of it is reabsorbed primarily in the proximal tubule, at the same rate as that of water and sodium, with little reabsorption in the loop of Henle and none in the distal tubule (Thomsen and Schou, 1968; Walker and Fawcett, 1995; Walter *et al.,* 1996). The tubular handling of lithium may thus lead to high cellular lithium concentrations, and resultant toxicity (Walker and Fawcett, 1995). Most absorbed lithium is excreted in the urine, with a half-life of 90 – 120 min (Walker and Fawcett, 1995).

Lithium clearance C_{Li} usually remains constant but differences occur due to a change in glomerular filtration rate (GFR). This variation is usually proportional to give a constant fractional excretion rate of lithium (FE_{Li} = C_{Li}/GFR) (Thomsen, 1999). C_{Li} is altered under certain physiological and pathophysiological conditions, such as severe kidney damage. Presently C_{Li} is being used by many as a non-invasive estimate for the deliverance of tubular fluid from the proximal tubules (Reviewed in Thomsen, 1999).

5.2.5 Lithium induced renal toxicity

The literature reporting renal lithium toxicity needs to be reviewed critically due to isolated incidences being identified. A classic example can be found in Friedman *et al.* (1992) in which a single patient developed adult respiratory distress syndrome, NDI and other toxicities during lithium treatment. The patient's serum lithium concentration was, however, excessive (5 mmol/l dropping to 1.8 mmol/l) and once the lithium levels normalised the patient returned to normal. In a review article, Johnson (1998) discusses various long term studies (5-10 year) in which lithium treated patients do not show increased renal dysfunction that cannot be related to age. Bouts of lithium intoxication played a role in impaired renal function of some patients. Changes in urinary concentrating ability were observed in some long term

lithium treatment studies, but often in conjunction with the use of other anti-psychotic drugs (Johnson, 1998). Christensen and Schou (1990), in a review article, concluded that lithium does not lead to changes in glomerular filtration rate and that altered urine concentrating ability and volume output does not predict kidney failure. More recent reports of long-term lithium therapy reported that glomerular filtration rate was reduced when compared to age matched controls which could lead to lithium toxicity (Bendz *et al.*, 2001; Turan *et al*., 2002).

5.2.6 Lithium induced polyuria and nephrogenic diabetes insipidus

Many conflicting reports have been published relating to lithium induced polyuria and NDI. These have led to enhanced control of the prescription of lithium to bipolar patients and a strict monitoring regime. Early reports on lithium toxicity are cautious, in that the concentrating ability of the kidney is reportedly impaired leading to polyuria. Early studies insinuated that polyuria is a time dependant side-effect, i.e., the degree of polyuria increased as the length of lithium therapy increased. This was confirmed by various studies (See Table 1.1 below), and seemed not only to be related to time of therapy, but also the age and concomitant psychotropic medication of the patient (Bakris *et al*., 1981; reviewed in Lydiard and Gelenberg, 1982).

Lithium induced NDI is a disorder characterised by excessive urine excretion, lack of responsiveness to antidiuretic hormone (ADH, AVP or vasopressin), and defective kidney tubules. Vasopressin is normally released from the hypothalamus in response to various stimuli such as hypernatremia or hypercalcaemia (refer to section 1.4.1). One well established target of lithium is the inhibition of vasopressin sensitive adenylate cyclase which results in decreased cAMP levels (Hart, 1982). This inhibitory effect of lithium was further demonstrated by Goldberg *et al*. (1988) in LLC-PK_1 renal epithelial cells to be at the stimulatory multiunit G_s of the GTP binding protein which leads to downstream reduced cAMP levels. The adenyl cyclase inhibition, and the consequential reduced cAMP levels, was hypothesised to play a role

in lithium induced NDI (Lydiard and Glenenberg, 1982) due to reduced renal function of water reabsorption leading to polyuria.

Carney *et al*. (1996) and Li *et al.* (2006) in separate experimental models, dispute the influence of lithium on cAMP levels to influence NDI. Carney *et al*. (1996), using Wistar rats did not observe an inhibitory effect of lithium on vasopressin. These authors did note, however that released parathyroid hormone (PTH) acts as a partial agonist to the hydro-osmotic action of vasopressin (Carney *et al*., 1996), thus ultimately causing a vasopressin inhibitory effect. They thus hypothesised that the hyperparathyroidism caused by lithium, may play a role in NDI. Vasopressin binds to receptors V_2R, a G-protein type receptor, on the basolateral side of the collecting ducts which activates adenylate cyclase that subsequently increases the production of cAMP. PKA, activated by cAMP, phosphorylates both cAMP responsive element binding protein (CREB), a transcription factor responsible for aquaporin 2 (AQP2) transcription, and AQP2 itself (Reviewed in Bichet, 2006). Aquaporin 2 subsequently translocates to the apical membrane which allows water to be taken into the cells from the lumen and released to the interstitium via other water transporters, AQP3 and AQP4 (Devonald and Karet, 2004). Since lithium altered vasopressin-dependent cAMP levels (Christensen *et al*., 1985), the lithium induced changes on AQP2 expression and translocation to the apical membrane were investigated (Li *et al.,* 2006). Lithium treatment of mouse cortical collecting duct cells was found to decrease the expression of AQP2 proteins, but not its stability, in a cAMP independent manner (Li *et al*., 2006), thus reducing the amount of water reabsorbed by the kidneys leading to NDI.

Table 1.1: Percentage of patients showing polyuria after short, medium and long terms of lithium therapy.

Lithium dose mM	Short < 3 months	Medium 4-12 months	Long > 12 months	No polyuria Symptoms	Author
0,8-1,2 (C)	16%	21%	32%	11.5%	Bakris *et al*., 1981

0,25-1,32 (L)			22%		Walker *et al*., 1983
0,34-0,797 (L)			8%		Conte *et al*., 1988
Not described (L)			87%		Markowitz* *et al*., 2000

C= cross-sectional study, L=Longitudinal study

* Skewed report as renal biopsies used were from the renal Pathology Laboratory at Columbian Medical Centre

Further evidence of lithium involvement at a renal cellular level was afforded by Rao *et al.* (2005). Using cultured renal medullary interstitial cells, they showed that lithium increased renal cyclooxygenase 2 (COX2) expression *in vitro* and *in vivo* in kidneys of C57BL/6J mice, and related this observation to GSK inhibition (refer to section 1.2.3). Since COX2, a prostaglandin synthase which contributes to PGE2 production, has previously been shown to be elevated in lithium induced polyuria and NDI (Rao *et al.,* 2005), these results indicate that lithium induced GSK inhibition may be a causal effect of lithium induced renal toxicity.

Accumulating all the above evidence together, it seems as though lithium, collaboratively acting on various proteins in the kidneys, has the ability to stimulate lithium induced polyuria and NDI in patients.

5.2.7 Lithium induced renal cellular and tissue morphological changes

Hestbech *et al.* (1977) caused concern amongst psychiatrists with their report that patients on prolonged lithium therapy had chronic nephropathy. Many studies have since been initiated to determine short and long term effects of lithium on the kidney (amongst others: Tyrer *et al*., 1980; Walker *et al*., 1983 & 1986; Hetmar *et al.*, 1987 & 1989; Schou, 1988 (review); Conte *et al*., 1988 & 1989; Marcussen *et al*., 1989 & 1994; Nyengaard *et al*., 1994; Johnson, 1998 (review); Markowitz *et al*., 2000; Bendz *et al*., 2001; Turan *et al*. 2002). In these studies many aspects such as the number of patients, bipolar disorder controls, dosing strategy (whether once or twice per day), lithium

concentration, adjunct therapy, animal model, age of patients, and the water and diet regime used have been criticised as limitations of the experiments.

In short, despite the short comings in experimental design, the following has been established from lithium studies. Correlations between morphological abnormalities and renal function have been found in lithium treated patients (Hetmar *et al*., 1989), although the extent of the morphological damage was stated to be small in comparison to the beneficial effects of lithium. Lower lithium concentrations (0.34-0.797 mmol/l) seemed to prevent lithium renal effects on chronically treated lithium patients (Conte *et al*., 1988). Nevertheless these dosages are advocated with caution as some bipolar patients have experienced increased relapses (Gitlin, 1992).

Markowitz *et al*. (2000) cautions that the long term use of lithium could lead to lithium nephrotoxicity, particularly glomerular and tubulointerstitial nephropathy. Again, prudence must be applied to the findings of Markowitz *et al*. From 6514 renal biopsies in a renal clinic, they reviewed 24 biopsies - the total amount of biopsies taken from lithium treated patients in the time period of the study (0.37% of the total). They found tubular cysts in distal tubules and collecting ducts of 62% of the biopsies and tubular atrophy in all tubules of all the biopsies ranging from mild (4.2%) to severe (58.3%). They argue that many reports of long term lithium use and renal implications (Markowitz *et al*., 2000) have indicated chronic tubulointerstitial disease to be common amongst lithium treated patients.

Animal models of lithium induced morphological changes and altered renal handling have also been reported. Lesions of the distal convoluted tubules and the collecting ducts of rabbits were observed by Walker *et al*. (1983) after chronic (12 months) lithium treatment. Whilst Walker *et al*. (1983) observed glycogen granules in distal epithelial cells, this was not seen by Hetmar *et al.* (1987). Biopsy preparative techniques may be the cause for the disparity. Walker *et al.* (1986), using slightly higher than normal lithium concentration of 1.5 mM in rabbits, found distal tubule lesions as early as 1 month after treatment leading to the impaired renal function at 12 months of treatment,

previously observed. Wistar rats treated with lithium for up to 12 days showed irregularities in glomeruli size, interstitial nephropathy, dilation of distal convoluted tubules and collecting ducts, and glomerular proximal tubule formation abnormalities (Marcussen *et al*., 1989; Nyengaard *et al*., 1994). Christensen *et al.* (2006) observed that rats fed with lithium for 4 weeks had cellular composition changes in the inner medullary collecting duct and the cortical collecting duct with enhanced proliferation. Although the above lithium toxicity results are disturbing, the plasma lithium concentration of the rats was not stated and thus an objective conclusion concerning lithium toxicity on kidney cells could not be made from the aforementioned results.

Not only has lithium been shown to influence morphological changes in developed renal tissue, but it has also been implicated in the development of renal cells. Interestingly, Xenopus studies indicated that lithium induces a limited early stage kidney tubule differentiation and activates epithelial gene expression (Davies and Garrod, 1995). Since it was previously shown that kidney epithelial morphogenesis is induced by the Wnt signalling pathway (Herzlinger *et al.,* 1994; Stark *et al*., 1994), the results implied a link between lithium and the Wnt pathway inducing early stage kidney epithelial cell differentiation. Further studies have implicated Wnt signalling, β-catenin and lithium induced GSK inhibition in this early stage kidney tubule differentiation (Stark *et al*., 1994; Klein and Melton, 1996).

3.4 Lithium and the immune system

One well established side effect of lithium therapy is its haematopoetic effect on various blood cells, especially leukocytes. Many reviews have been written on the subject and much work investigating the potential benefiting effects of lithium therapy in immune challenging situations, such as herpes and human immunodeficiency viral (HIV) infections, has been done (Hart, 1990&1991; Gallicchio, 1991; Rybakowski, 1999a & 1999b).

Neutrophilia, or increased granulocyte (also known as polymorphonuclear leucocytes, PMN) concentration, the primary haematological response caused by lithium, has been used to attenuate myelosuppression during chemotherapy (Bray *et al*., 1981; Beyaert, 1999). Not only has lithium been reported to increase PMN concentrations but also to enhance degranulation, stimulate lysozyme but not elastase release, enhance the release of inflammatory mediators from psoriasis patients, and cause stimulator PMN responses (reviewed in Hart, 1990). The dosage of lithium used in the various *in vitro* and *in vivo* experiments played a critical role to the response of the PMN, with 1-5 mM LiCl doses giving a better response than higher lithium levels.

The influence of lithium to stimulate pluripotent stem cell production, colony forming unit-granulocyte-macrophages (CFU-GM) and megakaryocytes (CFU-Meg) was important to show that progenitor cells were influenced by lithium and not the differentiation (Gallicchio *et al*., 1992). Circulating neutrophil and platelet recovery of immunosuppressed mice receiving bone marrow transplants were much quicker with lithium treated transplants than untreated ones. These studies showed lithium to be a potential adjuvant in bone marrow transplant therapy (Gallicchio *et al*., 1992). It has been recently found that the amount of CXCR4, a chemokine receptor, is decreased in bone marrow neutrophils but not lymphocytes in response to lithium. CXCR4 and its ligand, stromal-derived factor 1 (SDF-1) are important to prevent haematopoetic cells from migrating out of the bone marrow. Thus, reduced CXCR4 chemokine expression, and a reduced SDF-1 response, may be the cause of neutrophilia in lithium treated patients (Kim *et al*., 2007).

Lymphocyte cells in the adaptive and innate immune system are also affected by lithium (Bray *et al*., 1981; Hart, 1991). Lithium induced proliferation in primed T cell was seen to occur in sup-optimum conditions (Hart, 1988) and in an IL-2 responsive manner (Kucharz *et al*., 1988). Hart (1991) summarised results found from *in vivo* and *in vitro* studies of lithium effects on lymphocytes and highlighted the discrepancies, species variations and anomalies involved.

Mast cells have not been reported to be susceptible to lithium induced changes. Macrophage/monocyte concentrations were not influenced by lithium, but reports of lithium induced changes in phagocytosis and their secretory activity have been made (reviewed in Hart, 1990). Natural killer cell concentrations were elevated in patients treated with lithium, especially those treated for more than two years (Rybakowski, 1999a).

Lithium influences cytokine production both positively and negatively. Studies by Beyaert *et al.* (reviewed in Beyaert, 1999) indicated that lithium potentiates tumour necrosis factor (TNF) effects in TNF sensitive cells. TNF is cytotoxic to many, but not all, tumour cells, the mode of death being either necrotic or apoptotic depending on the type of cells used (See discussion on apoptosis). Mice, with a human cervix carcinoma tumour, co-treated with TNF and low (0.5 mM) LiCl concentrations, survived much longer than those treated with TNF only. TNF is a pleiotropic cytokine and has a mitogenic effect on many immune cell types and lithium was found to intensify these TNF stimulated mitogenic effects (Beyaert, 1999). Furthermore, the expression of TNF and IL-1 was enhanced by lithium. Lithium also enhanced TNF induced expression of IL-6, a pro-inflammatory cytokine secreted by T-cells and macrophages and granulocyte macrophage colony stimulating factor (GM-CSF). In some cells lithium enhanced both the TNF and IL-1 stimulated expression of IL-3, a white blood cell mitogen, GM-CFU and IL-2 (Beyaert, 1999) implicating a common intracellular pathway between TNF and IL-1.

The cytokine responses, especially those of IL-2, seem to be stimulus related and conflicting results have been observed. Phytohaemoglobin stimulated T-cells incubated with lithium were shown to have increased levels of IL-2 but not of IL-2 receptors (Kucharz *et al.,* 1988). No change in IL-2 receptor levels was noted in *in vivo* studies using lithium treated control subjects (Rapaport *et al*., 1994), whilst blood cultures from normal patients treated with lithium caused an increase in IL-4 and IL-10 (Th2 cytokines) and a decrease in IL-2 and IFN-γ levels (Th1 cytokines) (Rapaport and Manji, 2001). Boufidou *et al.* (2004) observed a decrease in IL-2, IL-6, IL-10 and IFN-γ secreting cells in

lithium treated bipolar patients as compared to healthy controls. Interleukin-2 is a well characterised interleukin that plays an important role in CD4+ and CD8+ T-cell propagation, the distinction of self vs non-self antigens by regulatory T cell development in the thymus, and is involved in many intracellular pathways, the PI-3 kinase/Akt pathway being one of them (Nelson, 2004).

In addition, lithium opposed the eicosonoid, prostaglandin E2, action on the immune system. High amounts of prostaglandin E2 are known to activate microbial activity and suppress immune function (Lieb, 2007). The ability of lithium, and other antidepressants, to stimulate the immune system has been patented as an immunostimulatory drug for the treatment of disorders characterised by excessive prostaglandin E2 production such as infections and some autoimmune diseases (Lieb, 1984).

As mentioned earlier, lithium has been found to increase antithyroid antibodies, though conflicting evidence against the production of antithyroid antibodies was also reported (Rapaport *et al*., 1994). Furthermore, it has also been recorded that lithium exacerbates psoriasis and generate autoimmune antibodies (reviewed in Hart, 1991). Since lithium exacerbated the immune response in autoimmune disorders, investigations were carried out to further explain this mechanism of action as discussed below.

3.4.1 Systemic lupus erythematosus

SLE is an autoimmune disease affecting many different organs and systems. Patients with SLE may have more than one of the following affected: the skin and mucous membranes, joints, kidney, serous membranes, blood, heart, lungs and the nervous system. Diagnosis of SLE has previously been very difficult but, with a few definite criteria, is becoming more certain (Tan *et al*., 1982; Gladman *et al*., 2002). Diseases that are usually confused with SLE are amongst others, rheumatoid arthritis, scleroderma, drug-induced SLE-like symptoms and lupoid hepatitis (Davidson and Diamond, 2001).

Lupus affects nine times more women than men, and onset of the disease is usually diagnosed in the second to the fourth decade of life (Bonney, 2001). The disease varies between patients and ranges from mild and episodic to a severe and fatal disease. The range of symptoms includes fatigue, fever, inflammation, memory loss and severe organ damage. The most frequently affected organs are the heart (30% of cases), the lungs (40 - 50% of cases) and the kidney (around 50% of cases) (Bonney, 2001).

Autoimmune diseases are normally characterised by the type of autoantibody that they contain and therefore these autoantibodies are used as diagnostic tools for the disease. Autoantibodies characteristic for SLE are anti-ds DNA (double stranded DNA) and anti-Sm (Smith) antibodies. Anti-ds DNA antibodies target base-paired DNA and are capable of reacting with either native or single stranded DNA which has formed loops. Fifty-eight percent of SLE patients have anti-dsDNA antibodies which are strongly associated with renal, cardiovascular and respiratory facets of the disease (Dean *et al*., 2000). More than 30 autoantibodies have been identified in SLE patients, anti-snRNP (U1-U6 snRNAP), anti-SS-A/Ro, anti-SS-B/La and antiphospholipid antibodies being the most prevalent (Pollard *et al*., 1995). Many reports attempting to link disease prognosis to autoantibody profile have not proven to be successful and it is not easy for clinicians to predict disease targets and severity (Fritzler, 1997; Leslie *et al*., 2001; Mittleman, 2004).

The presence of immune complexes, and more especially anti-dsDNA immune complexes were observed early on in SLE patients with lupus glomerulonephritis (GN) (Koffler *et al*., 1967). These antibodies have been associated with renal tissue damage and enhanced anti-dsDNA antibodies have been observed in active stages of disease, and suppressed when patients were treated with corticosteroids (Putterman, 2004). Anti-nuclear antibodies, and other lupus associated antibodies, have proven to be an interesting phenomenon by their ability to actively penetrate and target intracellular antigens. They can thus bind extracellularly, in the cytoplasm or in the nucleus. This has led to many speculations about the way that cell penetrating autoantibodies may inflict injury. Previously, the entrapment of

immune complexes in the kidney or the binding of antibodies to an extracellular renal antigen was thought to trigger the immune system and cause inflammation and renal damage. This now seems to be more complex and the autoimmune antibodies have been implicated in cell proliferation, apoptosis, necrosis, immune stimulation, inflammation and cytokine expression. Although much research on anti-dsDNA and renal involvement has been carried out, no concrete mechanism of injury has been described (reviewed in Putterman, 2004; Rekvig *et al*., 2004).

The role of cytokine involvement in SLE has been well investigated and is a complex topic on its own. Cytokine production seems to differ depending on disease state of the patient, the stimulus involved and the type of study *in vitro* or *in vivo*. Cytokines observed to have reduced expression in SLE patients are IL-1 (involved in GN), IL-2 and IL-12 whilst those observed with enhanced expression are IL-2R, IL-6, IL-10, IL-12 and IL-16 (reviewed in Dean *et al*., 2000). The TNF: TNF-receptor ratio seems to decrease in lupus nephritis, as does transforming growth factor-β TGF-β (Dean *et al*., 2000; Gómez *et al*., 2004).

4.1.1 Accounts of lithium, lupus and the kidney.

Despite lithium's professed exacerbation of the immune system, its involvement in predisposing some bipolar patients to develop autoantibodies, and its haematopoetic ability on various cells, not many studies determining the effect of lithium on SLE patients have been carried out. Reports indicating that lithium is a safe drug to be used on SLE patients with BP disorder have been made (Terao *et al*., 1994) although the sample size was somewhat limited. More information has been gained using *in vitro* and animal models of lithium effects on lupus.

Although reports indicating that interleukin-2 and IL-2 soluble receptor expression seemed higher in patients with active SLE disease have been noted (reviewed in Huang *et al.,* 1988; Dean *et al*., 2000), many more have

described a decrease in T cell stimulated IL-2 release in active disease patients, though many of these studies were carried out *in vitro* (reviewed in Kucharz *et al*., 1993; Crispin and Alcocer-Varela, 1998). Interestingly, Kucharz *et al.* (1993) established that lithium increased the amount of IL-2 produced from T-cells of systemic lupus erythematosus (SLE) patients, as compared to reduced levels observed in control subjects. Although this result substantiates studies whereby lithium induced an increase of T-cell activated IL-2 (discussed above), Kucharz *et al*. in no way advocated the use of lithium as a modulator for SLE.

Tumour necrosis factor is another cytokine implicated in SLE disease, specifically lupus nephritis. Relatively low levels of TNF have been observed in patients with SLE, and a correlation between TNF levels and lupus nephritis has been observed. It was thus postulated that TNF has a protective effect on SLE patients (Gómez *et al*., 2004). A mouse model of SLE, the (New Zealand black x New Zealand white) NZB/W F1 mouse, known to have a diminished TNF expression, characteristically develop glomerulonephritis and perish due to end stage renal failure (Jacob *et al*., 1991). TNF expression was enhanced in a subset of these mice after they were treated with LiCl (Lenz and Hart, 1997). The authors had previously observed an increase in survival of a subset of NZB/W mice due to mice not entering into end-stage renal failure and postulated that lithium stimulated TNF expression may be the reason for it. This provides an interesting link between lithium which has been observed to increase TNF function and expression (Beyaert, 1999), lupus and glomerulonephritus.

1.6 Apoptosis

Apoptosis, a form of programmed cell death, originally described by John Kerr and colleagues in 1972 (Kerr, 2002), is an active process regulated by many molecular pathways that leads to cell death (Fig 1.3). It is a vital process in many cellular functions and in cell homeostasis and a plethora of articles relating apoptotic cell death due to various stimuli and mechanisms have been published. Amongst other functions, apoptosis:

2 plays a role in organism development and has been extensively studied in the developmental stages of many organisms from prokaryotes to humans (reviewed in Fabian *et al*., 2005).

3 eliminates self-recognising T-cells from the immune system (reviewed in Cline and Radic, 2004).

4 removes damaged cells from the general milieu (reviewed in Roos and Kaina, 2006).

5 prevents tumourigenesis and is vital in maintaining the health status of an organism (reviewed in Müllauer *et al*., 2001; Zörnig *et al*., 2001).

Basically, when there is an aberration in the apoptotic process a disease state occurs.

Apoptosis is typically characterised by cells that shrink, round up and lose contact with adjacent cells, while the plasma membrane integrity is maintained. Chromatin condensation, nuclear fragmentation and membrane budding accompany the process. Phagocytes then usually engulf apoptotic cells without eliciting an inflammatory response (reviewed in Renvoiz'e *et al*., 1998; Krieser and White, 2002; Böhm and Schild, 2003). These features distinguish apoptotic cell death from the alternate mechanism of cell death, necrosis. Necrotic cells die via pathological mechanisms, the integrity of the cell membrane is lost, the cell swells due to fluid intake, organelles are destroyed, the DNA is randomly sheared and inflammation occurs (Böhm and Schild, 2003). Further types of cell death have been described such as autophagy and paraptosis in which some of the above mentioned features of apoptosis and necrosis are shared (Sperandio *et al*., 2000; Lleo *et al*., 2007).

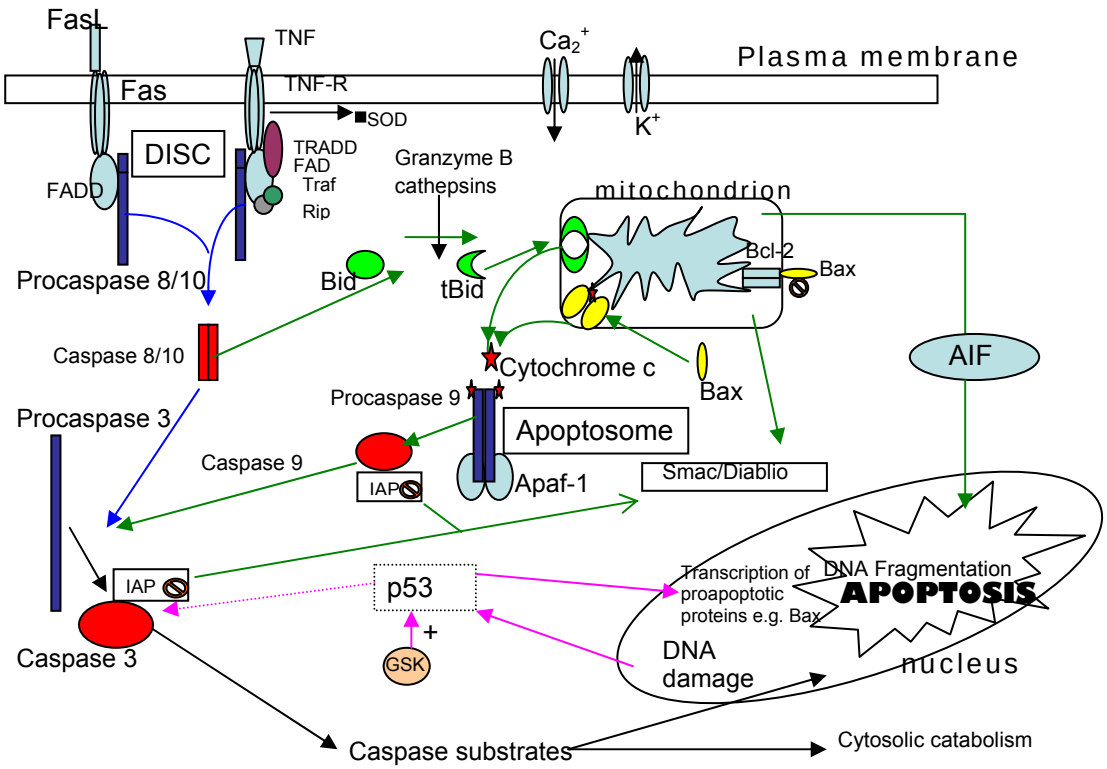


Figure 1.3: Diagram depicting different apoptotic pathways.
The blue pathway depicts the death receptor pathways. Once FasL or TNF binds, the receptors oligomerise in trimers, and recruit proteins FADD or TRADD via their death domains. Further proteins and pro-caspase 8 or 10 are recruited forming a DISC. The pro-caspase dimerises and via autolysis cleaves to form active caspase 8/10. This acts downstream to activate caspase 3 which cleaves various substrates leading to apoptosis. The green pathway depicts intrinsic mitochondrial mediated apoptotic responses. Once Caspase 8 or 10 is activated it cleaves Bid to form tBid which translocates to the mitochondrial membrane. The membrane potential is disrupted and cytochrome C is released via a pore in the membrane. Cytochrome C is also released via the translocation, homodimerisation and pore formation of proapoptotic proteins such as Bax or Bad. A heterodimerisation of proapoptotic proteins to antiapoptotic proteins such as Bcl-2 prevents the antiapoptotic function of these proteins. Cytochrome C forms an apoptosome complex with dimers of Apaf-1 and pro-caspase 9, which generates active caspase 9. A similar pathway as that of the death receptors is then followed by the activation of caspase 3 and its downstream effects. A change in the mitochondrial membrane also allows for the release of Smac/DIABLO protein from the mitochondrion into the cytoplasm where it binds IAPs which also lead to active caspase molecules. AIF, released from the mitochondrion mediates an apoptotic effect via a caspase-independent pathway. Lastly, p53 (pink pathway) plays a vital role in apoptosis. It is upregulated and stabilised by DNA damage and GSK activity. It then increases the transcription of many proapoptotic proteins such as Bax, Fas, TRAIL, and a link between caspase 3 activation and p53 has been observed. For more detail and references please refer to text. (Diagram was drawn using information and diagrams from; Israelis and Israelis, 1999; Kaufmann and Hengartner, 2001; Müllauer *et al*., 2001; Budd, 2002; Mak and Yeh, 2002; Wang *et al*., 2004; Buytaert *et al*., 2007; Mercer *et al*., 2007).

Apoptosis is a well regulated mechanism and broadly goes through three different stages; apoptosis activation, signalling through the cell followed by the effective death process. Two general modes of activation are known:

apoptosis via an extrinsic or death receptor mechanism and apoptosis via an intrinsic mechanism. Conventional extrinsic death receptor signalling is well described in Budd (2002). The two most widely known death receptor pathways are the Fas (CD95/Apo-1) pathway and the TNF-receptor pathway. Once a ligand (FasL and TNF respectively) has bound to the receptor a cascade of events is triggered leading to the activation of a set of zymogens, effector and executioner enzymes known as caspases, as well as other enzyme activation such as calpains, granzymes, serine proteases and proteosome-ubiquitin pathways, that ultimately leads to DNA fragmentation and apoptosis (Solary *et al*., 1998; Budd, 2002). Intrinsic signals such as irreparable DNA damage or increases in Ca^{2+} concentrations, triggers apoptosis once again through a network of signals until the effector enzymes are activated leading to cell death (Roos and Kaina, 2006). Figure 1.3 depicts the many apoptotic pathways occurring during apoptosis.

2.7.3. Death receptor pathways

Fas signalling has been reviewed recently by Wajant (2002). Briefly, binding of Fas-L, a homotrimer itself expressed under strict regulation, to three Fas molecules on the extracellular side of the cell membrane, causes the formation of an intracellular death inducing signalling complex (DISC). Adapter proteins known as Fas-associated death domain (FADD), cluster together with procaspase-8 and procaspase-10 to form a complex. Due to the oligermerisation of procaspase-8, it autocleaves and the activated caspase activates downstream caspases such as caspase 3 committing the cell to apoptosis (Ashkenazi and Dixit, 1998; Wajant, 2002). A review article by Lang *et al*. (2007) highlights the role played by Fas stimulated apoptosis on the plasma membrane K^+ and Ca^{2+} ion channels. Through the alteration of these channels and the loss of electrolytes such as Cl^-, HCO_3^-, K^+, and an increase of Ca^{2+}, the cell membrane potential is altered, cell shrinkage occurs and the cytosol is acidified.

Mutations in Fas have been implicated in the cause of lupus-like features in mice inheriting the *lpr* and *gld* mutations (Watanabe-Fukunaga *et al.*, 1992;

Takahashi *et al.,* 1994; Elkon and Marshak-Rothstein, 1996). Fas is also important in the immune system as it plays a role in the elimination of T cells in the thymus and autoreactive B cells, and it has been hypothesised that a defect in the Fas-FasL apoptotic system could lead to the presence of autoantibodies in autoimmune diseases (Jacobson *et al*., 1995). Fas has also been implicated in chronic glomerulonephritis. In particular, the levels of soluble Fas (sFas) are increased, which inhibits apoptosis from taking place in proliferative glomerulonephritis, thus causing an increase in damage in the kidneys (Sano *et al*., 1998). There seemed to be no correlation between sFas and autoimmune disease, although the study did not take into account patients with kidney disease (Goel *et al*., 1995). Similarly, no consistent defect or expression of Fas protein could be determined in patients with SLE (Mysler *et al*., 1994). Anti-Fas antibodies have, however, been implicated in causing apoptosis in mouse glomerular cells *in vivo* (Gonzalez-Cuadrado *et al.,* 1997).

TNF, or lymphotoxin-α, is a TNFR binding protein produced primarily as an inducer of inflammation, can also bind to TNFR on cell surfaces to initiate apoptosis. TNF binding to TNFR causes oligomerisation of the bound receptor and the formation of an intracellular DISC. A protein known as “silencer of death domains (SODD)” is removed from the intracellular portion of TNFR and then TNFR-associated death domain (TRADD) proteins oligomerise into a complex via their death domains together with other signaling proteins. These signaling proteins include TNFR associated factor (TRAF), receptor interacting protein (RIP) and FADD. TRAF and RIP are involved in recruiting antiapoptotic proteins whilst the binding of FADD leads to procaspase 8 recruitment and activation (Ashkenazi and Dixit, 1998; Chen and Goeddel, 2002). FADD and caspase 8 activation seem to be the position where Fas and TNF signaling converge in apoptosis.

The TNF-related apoptosis inducing ligand (TRAIL), another of the TNF family of proteins, has been implicated in neutropenia in SLE patients (Matsuyama

et al., 2004). Thus, both Fas and TNFR are important proteins linking apoptosis, the immune system and kidney disease with each other.

3.1.1 p53 and apoptosis

Apoptosis is not only mediated by the death receptor pathway but by others as well, the p53 pathway and mitochondrial pathways being two examples. p53, a 53 kDa protein, is known as a tumour suppressive protein or a pro-apoptotic protein. It is an important protein in the cell cycle and plays a role in preventing the cell cycle to continue through to the G2/M phase if there is DNA damage. The DNA is either repaired or the cell is moved into an apoptotic phase and destroyed if the DNA is irreparable. This is accomplished by the activation of *p53*, a transcription regulator gene (reviewed in Israelis and Israelis, 1999; Hofseth *et al*., 2004) and stabilisation and accumulation of p53 protein by the removal of Mdm2 (Zörnig *et al*., 2001). p53 promotes apoptosis via both transcriptional dependent and independent processes. It increases the transcription of proteins involved in the apoptotic process such as Bax, Bid, APAF-1, Fas, and a TRAIL Death receptor DR4,5 amongst others (Zörnig *et al*., 2001; Hofseth *et al.*, 2004). It has also been shown to increase caspase 3 activity in a death receptor, Bax independent fashion (Cummings and Schnellmann, 2002).

i. Intrinsic apoptotic pathway

Triggers from within the cell, such as at the endoplasmic reticulum, nucleus or lysosomes, and stress factors such as p53 mentioned above, trigger the intrinsic apoptotic pathway by their interactions at the mitochondrion (reviewed in Kranic *et al*., 2007). Permeabilisation of the mitochondrial membrane is enhanced and cytochrome c is released into the cytosol. Cytochrome c forms a complex with apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9. Procaspase-9 is activated which leads to the activation of caspase 3 and downstream events. Further mitochondrial proteins released that play a role in apoptosis such as Smac/DIABLO do not form an apoptosome complex but activate the executioner caspases directly.

Mitochondrial permeabilisation occurs via different proteins, the most well known being Bid, Bcl-2, Bax, Bad and AIF. Many proteins have been characterised as either having a pro-apoptotic function on cells or an anti-apoptotic function. Most of these proteins stem from the Bcl family of proteins. The Bcl-2 group of proteins are a family of proteins sharing at least one of four conserved regions (BH 1-4) that allow the proteins to dimerise in various combinations and thus play a vital role in the regulation of the proteins (Kirkin *et al*., 2004). The first protein of the Bcl-2 family was identified in a B-cell lymphoma from where the name was derived. Many members of the Bcl-2 protein family have been identified, though only a few will be mentioned. The members of the Bcl-2 family can be divided into anti-apoptotic proteins, also know as proto-oncogenes (Bcl-2 and Bcl-x_L)*, and pro-apoptotic proteins (Bax, Bad and Bid)*. * Please refer to "List of abbreviations for full Bcl family names

Bcl-2 acts as a suppressor of apoptosis and is localised in the mitochondrial membrane. Another anti-apoptotic protein is the cleavage product of Bcl-2, i.e., Bcl-x_L. The anti-apoptotic proteins can form heterogeneous associations with the pro-apoptotic Bcl family members such as Bax and Bad (Oltvai *et al*., 1993). Bax oligomerisation has been implicated in triggering cytochrome c release from the mitochondrial membrane, which forms a complex leading to the activation of caspase 9, which in turn can activate caspase 3 (Müllauer *et al*., 2001; Zörnig *et al*., 2001). Bax is normally found in the cytoplasm and translocates to the outer mitochondrial membrane due to an apoptotic stimulus, where it forms a heterodimer with Bcl-2 (Kaufmann and Hengartner, 2001). In some instances it seems that the heterodimerisation inhibits the pro-apoptotic activity of Bax, whereas in others Bax activity is promoted. It thus seems that the ratio of Bcl-2 to Bax is very important (Chao and Korsmeyer, 1998; Javelaud and Besancon, 2002), and that a higher Bax concentration can form homodimers and thus pore formation (Kaufmann and Hengartner, 2001; Böhm and Schild, 2003).

Another important Bcl-2 family protein that plays a role in mitochondrial-mediated apoptosis is Bid. Bid is activated via proteolysis, be it by Granzyme

B, cathepsins or caspases, in particular caspase 8, to release a truncated Bid (tBid) which then incorporates into the mitochondrial membrane mediating cytochrome C release. Proteolytic cleavage by cathepsins has also been seen to play a role in the inactivation of Bcl-2 and Bcl-X_L which could enhance tBid activity in the intrinsic pathway (Turk and Stoka, 2007).

AIF, apoptosis inducing factor, is also released form the mitochondrion and mediates apoptosis in a caspase independent manner. It seems to act directly to induce some of the nuclear morphology seen in apoptosis (Zörnig *et al.,* 2001).

ii. Caspases

Through the interaction of the activated death receptors, with cytoplasmic proteins containing various death domains, a set of proteins known as the caspases are activated. Caspases are zymogens that are activated by proteolytic cleavage. They are cysteine proteases and specifically require aspartic acid in position 1 of a tetrameric cleavage site, the P2-P4 amino acids being diverse (reviewed in Turk and Stoka, 2007). They are divided into cytokine processors (e.g., caspase 1), initiators of apoptosis (e.g., caspases -2, -8, -9 and -10) and executioners of apoptosis (e.g., caspases -3, -6 and -7). The executioner caspases are found downstream of the initiator caspases and are ultimately responsible for the morphological changes observed during apoptosis (Nicholson and Thornberry, 1997). Diverse apoptotic pathways trigger different caspases which show little redundancy in function (Zörnig *et al*., 2001) and much research is pursued to establish different mechanisms triggered by different stimuli and the caspases involved.

Procaspase 8, utilised in the death receptor pathways, is cleaved at the N-terminal. This prodomain contains the death effector domain (DED) that binds to DISC. The caspase is then cleaved into a large (17-20 kD) and a small (10-12 kD) subunit, which oligomerise with another caspase to form a tetramer of two large and two small subunits (Zörnig *et al*., 2001). Activated caspase 8 cleaves Bid, a Bcl-2 family protein, to release a 15 kD protein that

translocates in to the mitochondrial membrane. This leads to a cascade of mitochondrial events. The mitochondrial potential is altered, cytochrome c is released and together with apoptotic protease activating-factor (Apaf-1) and caspase 9 an apoptosome is formed which activates executioner caspases, viz, caspase 3, 6 and 7 (Böhm and Schild, 2003). The prodomain on caspase 9 is a caspase recruiting domain (CARD) which mediates a molecular interaction with Apaf-1, which has a similar domain.

Once initiator caspases are activated they cleave the executioner caspases which then act on multiple substrates. Caspase 3 acts on an inhibitor of caspase activated deoxyribonuclease (ICAD) to remove it from caspase activated deoxyribonuclease (CAD). This active CAD then translocates to the nucleus and cleaves DNA into its 200 bp fragments. Poly (ADP) ribose polymerase (PARP) is also cleaved by caspase 3, which prevents the repair of DNA double strand breaks. This cleavage is typically used as an indicator of caspase 3 activation. Acinus and AIF are two proteins, cleaved by caspase 3, that are involved in chromatin condensation (Böhm and Schild, 2003). Further substrates include proteins involved in cell structure such as gelsolin (caspase 3), lamins A and C (caspase 6), β-catenin (caspase 3) and focal adhesion kinase (FAK) (caspase 3 and 7). These proteins play a role in the morphological changes associated with apoptosis (Zörnig *et al*., 2001).

Caspases are regulated via different mechanisms: at the transcription level, anti-apoptotic proteins blocking proximity-induced activation of procaspases and via cellular inhibitors of apoptosis proteins (cIAPs). The cIAPs are separated from the caspases by SMAC/Diablo proteins which are released from the mitochondrial membrane in the intrinsic pathway (Kaufmann and Hengartner, 2001).

iii. GSK and apoptosis

GSK activity has been shown to have a pro-apoptotic function in many cells, particularly neuronal cells, an observation backed by many studies (Reviewed

in Frame and Cohen, 2001; Jope, 2004). In experiments using cultured rat primary neuronal cells, Chin *et al.* (2005) were able to prove that an increase in GSK-3β activity occurred in cells undergoing apoptosis due to low potassium concentrations. Watcharasit *et al.* (2003) showed that the lithium protective effect on neuronal cells stems from inhibiting the binding of active GSK to p53, an apoptotic protein. The binding of GSK to p53 stabilises p53 and allows it to cause its down stream effects and promote apoptosis. Beurel *et al.* (2004) concurred with the results above by establishing that lithium conferred resistance to cells induced to undergo apoptosis due to chemotherapy and related this to the disruption of the GSK3β-p53 complex, and repression of FAS expression. GSK also plays a role in destabilising the antiapoptotic protein MCL-1, a Bcl-2 family protein which functions in maintaining mitochondrial outer membrane permeabilisation, and thus promotes cytochrome c release in to the cytoplasm (Maurer *et al*., 2006).

1.6.6 Cell cycle and apoptosis

The cell cycle, like apoptosis, is a highly regulated entity and aberrations in the cell cycle also lead to anomalies in organisms. As the cell progresses from the G_1 (gap 1) through the S (synthesis), G_2 (Gap 2) and M (mitosis) stages back to G_1 it passes many check points (Figure 1.4). Both external and internal effectors, such as growth factor deprivation and DNA damage respectively, can mediate a cell cycle arrest at a position depending on the pathway triggered. The cells can then remain quiescent, mediate a repair process, or enter into apoptotic cell death where it is eliminated. Thus, the delicate workings of the proliferation and apoptosis pathways are interlinked and provide for cell homeostasis (reviewed in King and Cidlowski, 1998).

Cyclins and cyclin dependant kinases (cdk) are two families of proteins involved in mediating the checkpoints of the cell cycle. CDKs are activated by cyclins, which then phosphorylate proteins with a concomitant downstream effect on the cell cycle.

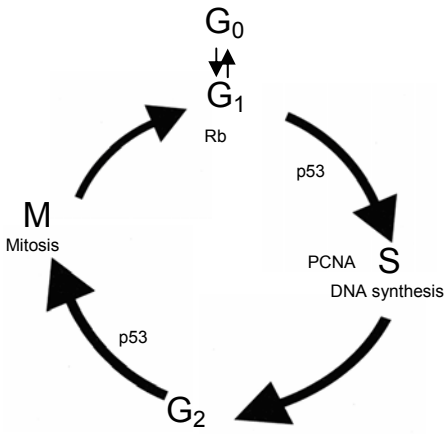


Figure 1.4: Cell cycle, highlighting the involvement of key proteins. (Adapted from Houtgraaf *et al*., 2006).

As mentioned in section 1.6.2 above, p53 is involved in maintaining the cell cycle. Depending on the extent of DNA damage within a cell, p53 up regulates p21 expression and mediates cell cycle arrest, DNA repair or apoptosis. It arrests the cells at the G_1/S interphase by accumulating in the cell due to an enhancement of its stability (reviewed in Maddika *et al*., 2007). MDM2 (murine double minute 2), is a transcription factor that down regulates *p53* expression. MDM2 also binds to the p53 protein which accelerates its degradation. Under stress conditions p53 is phosphorylated which prevents it binding to MDM2 and increases its half-life, and its activity (Israelis and Israelis, 1999). P21 is an inhibitor of some cdks. The inhibition of cdks prevents retinoblastoma protein hyperphosphorylation, which binds the transcription factor E2F, and prevents transcription of proteins to advance the cell into S phase. The *RB1* gene is a tumour suppressor gene and expresses a 105 kDa protein known as Rb. The loss of Rb protein leads to hyperproliferation of cells, especially with retinablastomas in the eye. In its active hypophosphorylated state, Rb holds the cell in the G_1 phase. Once it is phosphorylated it allows for movement through the cell cycle (Israelis and Israelis, 1999). The association between p53 and Rb seems to be the reason why many tumours show deficiencies in both the *p53* and *Rb* genes (Zörnig *et al*., 2001). Rb is also a suppressor of apoptosis and evidence has shown it to be cleaved by caspases during TNF mediated apoptosis. Loss of Rb may thus be required for apoptosis to be induced.

Proliferative Cell Nuclear Antigen (PCNA) is another protein important in the cell cycle. As its name implies PCNA is a protein that is found in the nucleus of actively dividing cells. It was initially described as an autoantigen in autoimmune diseases but has later been found to be involved in DNA processes (cited in Cox, 1997). It often acts as a marker for cell proliferation, tumour growth, and apoptosis inhibition (Kumaraguruparan *et al*., 2006; Mohan *et al*., 2006). In particular, PCNA seems to be actively involved in the S phase where it binds to replicating DNA and slides along providing an anchorage site for DNA polymerase δ and ε (Cox, 1997). It also assists in other essential DNA replication processes such as Okazaki fragment ligation, DNA repair and chromatin assembly (Reviewed Mohan *et al*., 2006). It has been established that not only does it play a role in the synthesis of DNA, it is also important in the degradation of the licensing system that ensures replication cannot be continued once the full genome has been replicated (Senga *et al*., 2006). Furthermore, PCNA seems to mediate its inhibition of the cell cycle through S phase by binding to the Bcl-2 antiapoptotic protein MCL-1 (Zörnig *et al.,* 2001).

1.6.7 Lithium and apoptosis

Lithium has been shown to have a protective effect on apoptotic stimulated neural cells. This protection seems to be due to the inhibition of GSK which plays a role in apoptosis (King *et al*., 2001; De Sarno *et al.,* 2002; reviewed in Jope and Bijur, 2002) which has led to increases of Bcl-2:Bax levels and decreases in p53. In addition Chin *et al*. (2005) have demonstrated that inhibition of GSK3β plays an important role in neuroprotection of cultured rat cerebellar granule neurons by survival factors such as insulin-like growth factor (IGF-1), cAMP analogues or elevating factors (forskolin 10 μM), 25 mM potassium and 10 mM lithium.

Lai *et al.* (2006) criticised most of the above work by arguing that the cell stress signals used and the concentrations of lithium in the studies were not

relevant to bipolar disorder related stresses or lithium concentrations. They have laid some doubt on the involvement of GSK-3β in lithium action of bipolar disorder by showing the neuroprotective effect to be cell and stress specific. Glial cells were not protected from mitochondrial or ER stresses, and neuroblastoma cells were only protected from mitochondrial induced stress (Lai *et al.,* 2006). Brewster *et al*. (2006) however found that primary cultures of rat cerebellar granule neurons were protected from ER stress apoptosis by increasing Ser-9- GSK phosphorylation, though, again, a high concentration (20 mM) lithium was used. Differences in the reports could stem from mature versus immature cells, as well as the use of serum in the cultures as noted by Marks *et al*. (2001).

Lithium has been shown to cause apoptosis in non-neural cell lines. Lithium, at concentrations higher than that used physiologically, caused apoptosis in HL-60 promyelocytic cells (Madiehe *et al*., 1995) and in K562 erythroleukemic cells (Becker *et al*., 1993; Tang *et al*., 2005). Van Gijn *et al*. (2001) noted that lithium did not induce apoptosis in all cell lines. Lithium, at low concentrations, induced apoptosis in African green monkey renal fibroblasts (COS-7) and human kidney epithelial cells (HEK 293), but not in rat adrenal cells (PC 12). Although lithium was not found to play a role in Fas induced apoptosis in a L929 cell line (reviewed in Schotte and Beyaert, 1999), it did potentiate TNF toxicity in some cell lines (Beyaert and Fiers, 1992 as discussed in section 1.5 above). Lithium was found to facilitate apoptosis of Fas stimulated Jurkat cells, differentiated to a neural form (Song *et al.,* 2004). Again, cell type as well as lithium concentration seem to be intricate players in whether the cell undergoes apoptosis or not.

1.7 Rationale for the study

Krause *et al.* (1992) investigated renal disease in murine (NZB/W) models of SLE. They found that treatment of female NZB/W mice with lithium salts led to prolonged survival of a subset of the mice. Prolonged survival was not accompanied by overt alteration in the murine autoantibody profile (ANA, anti-gp70, anti-DNA) (Lenz *et al*., 1997). Histological evaluation of kidney sections

from treated and untreated mice revealed that treated mice exhibited considerably less tubulointerstitial disease than did untreated, age-matched animals. Thus, it appeared that lithium treated mice had glomerulonephritis without progression to end-stage renal disease. Possible explanations for the NZB/W mouse findings are that:

1. There has been a change in the expression or posttranslational modification of a subset of possible tubule-specific autoantibodies, or other immune elements, or
2. The treatment induced an alteration in the expression or posttranslational modification of a subset of antigens in/on the tubule cells.

The possibility that the latter alternative is correct is supported by the finding that the lithium treatment regimen led to a rapid alteration in the expression of urokinase (Hart *et al*., 1998), an enzyme secreted from tubule cells. Verification of a possible direct effect of treatment on murine tubule cells was not possible due to the fact that such cells are not available. However, renal porcine tubule epithelial cells, PK(15) and LLC-PK_1, obtainable from the American type culture collection (ATCC), are well characterised.

The above mentioned experiments provide further interest due to the recorded renal toxicity effects of lithium in patients. Very few studies have been carried out determining the direct effects of LiCl on renal tubule cells, the amount of lithium that the cells can withstand, and the mode of cell death at toxic concentrations. Thus, the larger part of this study will focus on lithium toxicity in renal cells, whilst a second part will concentrate on lithium effects on renal antigens. The PK(15) and LLC-PK_1 cells have been used in preliminary experiments to ascertain that lithium treatment leads to an alteration in both gene expression for relevant molecules and a subset of autoantigens. These preliminary results confirmed the proof of concept. Therefore, part of this analysis will utilise sera from SLE patients with and without renal involvement, and investigate the impact of lithium treatment on expression of a number of biochemically undefined molecules of these cells.

1.8 Aim

The research program has provided data on two important levels. Firstly, the investigations lead to a new understanding of the influence of lithium on the biology of kidney tubule cells. Lithium is widely used therapeutically for the treatment of bipolar affective disorders. However, the mechanism of action in psychiatric disorders, as well as other organ systems, remains undefined. This study further enhanced our understanding of how lithium influences renal cell behaviour and outlines the biochemistry underlying these effects. Secondly, the study attempted to provide new approaches to detecting and characterizing novel autoantigens in kidney cells. Altering cell metabolism to induce changes in autoantigen expression is a relatively new approach to evaluating expression and function of autoantigens. Using this approach, the identification of unique autoantigens on or in renal cells may lead to new diagnostic and prognostic tools to assist in the management of patients with this disease.

The study addressed the following objectives:

1. Cell proliferation studies that determined the toxicity of lithium on porcine renal cells.
2. Determined whether lithium causes cells to undergo apoptosis or necrosis by using electron microscopy and DNA fragmentation methods.
3. Established whether lithium altered the expression of pro-apoptotic proteins (Bax, p53) or anti-apoptotic proteins (Bcl-2), as well as the activity of the different caspases which indicated the pathway affected by lithium.
4. Elucidated whether lithium alters the cell cycle of the porcine renal cells and determined which stage of the cycle was affected.
5. Lithium treated and untreated cell lysates were used as the antigen in Western blots. Autoimmune complexes were detected using serum from lupus patients with or without kidney damage.

6. Changes in immune complex formations due to lithium treatment were characterised using classical phosphorylation and glycosylation studies.

CHAPTER 2
MATERIALS AND METHODS

1. Introduction

All the general cell culture and apoptotic experimental methods used in this project are described within this chapter. Specific autoimmune experiments are described in Chapter 4. Materials used are listed initially in the materials section followed by descriptions of the methods themselves.

- Materials

Porcine kidney epithelial cells PK(15) (ATCC CCL-33), passage no.132 and LLC-PK_1 (ATCC CL-101) (Lily Laboratory Culture Porcine Kidney), passage no. 196 were purchased from American Type Culture Collection, Rockville, MD, USA.

Dulbecco's Modified Eagles Medium (DMEM) (with high glucose, pyruvate and L-glutamine) and foetal bovine serum (FBS) were supplied by Highveld Biologicals, PTY Ltd (JHB, South Africa). FBS was heat treated at 56°C for 45 min to inactivate the complement system in the serum.

Antibiotics (penicillin and streptomycin), the i*n situ* cell death detection kit and the cell proliferation ELISA, BrdU (colorimetric) immunoassay were obtained from Roche Diagnostics, JHB, South Africa.

Trypsin-EDTA (Gibco) and PSN (penicillin, streptomycin and neomycin) (Gibco) were sourced from Laboratory Specialists (South Africa).

The following reagents were purchased from Sigma (JHB, South Africa)
acrylamide, agarose, ammonium persulphate, aprotonin, N'N'N' bis-acrylamide, acetic acid, citric acid, chloroform, DMSO, EDTA, ethanol, ethidium bromide, glycerol, glycine, KH_2PO_4, KCl, LiCl (Fluka), mercapto-

ethanol, methanol, NaCl, Na_2HPO_4, Na orthovanadate, NP 40, phenol (ICN), PMSF, ponseau S, ribonuclease (RNase), Tris-base, Tween-20, TEMED.

Immobilon-nitrocellulose was obtained from Millipore, JHB, South Africa.

The BCA protein assay reagent kit (Pierce), 0.45 μm nitrocellulose membrane and chemiluminesence detection kit (Pierce) were purchased from Separations, JHB, South Africa.

Aclar embedding film, hexamethyldisilizate (HMDS) and LR-white resin were obtained from Agar Scientific Ltd, Essex, England.

Immunotech Universal staining kit and primary human antibodies (Bcl-2, Bax, p53, PCNA and Rb) were supplied from Beckman-Coulter, JHB, South Africa.

Antibodies for Western blots (Goat anti rabbit HRP, rabbit-antihuman Bcl-2, p53 and Bax) were obtained from AEC-Amersham, JHB, South Africa.

Whitehead Scientific (South Africa) procured the R & D system's caspase 3, 8 and 9 assays

Most plasticware was purchased from LASEC (South Africa) or from AEC-Amersham (South Africa). The plasticware was sterile.

All materials were of the highest grade and stored under optimal conditions.

- Protein concentration

The protein concentration of samples was analysed using the bicinchoninic (BCA) protein detection kit from Pierce. The standard used for each assay was bovine serum albumin (BSA) for which a uniform, concentration dependant, absorbance increase was obtained in the 0-1500 μg/ml range. This protein concentration determination method was used as the least

interference was obtained due to the presence of detergents such as NP-40, SDS and glycerol, in the cell lysates.

- Cell culture

The cell lines used in this study were both of porcine renal origin. The PK(15) cells take on characteristics of the distal tubule of the nephron whilst the LLC-PK cells take on proximal tubule characteristics. Both cell lines are epithelial cells that grow as adherent cultures and are polarised. PK(15) cells originated from an adult pig, sex unknown. PK(15) cells were derived from a clone of PK 2a cells originally established in 1955 (ATCC product information sheet). The LLC-PK_1 cells originate from a male Hampshire pig.

2.4.1 Medium preparation

Ten litres of DMEM were prepared by using 135.3 g of prepared DMEM powder supplemented with 100 U/ml penicillin and 0.1 g/l streptomycin, 40 x 10^{-6} M $NaHCO_3$ and 10 mM Hepes buffer. The pH was adjusted to 7.3. The medium was filter sterilised through a 0.45 μm filter and stored at 4°C in the dark.

Medium was also purchased in a ready-made form to which antibiotics (streptomycin and penicillin) were added.

2.4.2 Lithium chloride (LiCl)

LiCl solution (0.5 M) was prepared by dissolving 1 g of LiCl in 50 ml of cold DMEM. The LiCl was sterilised through a syringe filter (Millipore) of 0.2 μm. The solution remained stable at 4°C.

1. Cell culture maintenance

PK(15) and LLC-PK cells were maintained in DMEM and 10% or 5% (v/v) FBS respectively, in a humidified atmosphere containing 5 % CO_2 / 95% air at 37°C. Subconfluent cells were rinsed with trypsin and trypsinised with 0.25 %-0.03% (v/v) trypsin-EDTA and subcultivated at a 1:4 ratio twice weekly. Cells for experimentation purposes were grown to approximately 80 % confluency and treated with different LiCl concentrations for varying time intervals, as described in the results. Cells were maintained free of mycoplasma and were regularly checked using electron microscopy or the mycoplasma detection assay from Molecular probes, Oregon, USA. Cell passage numbers were restricted to less than 20 from that received, after which new cells were revived from stocks stored in DMEM containing 10% FBS and 5% DMSO as a cryo-protectant, at –145°C.

1. Cell number determination

Cells were initially plated into cell culture petri dishes at a concentration of 1 x 10^5 cells/ml, allowed to attach to the petri dishes for 24 h followed by treatment with varying concentrations of LiCl. To count, the cells were washed with PBS and trypsinised with 3 ml trypsin-EDTA for 8 - 10 min, until rounded. Cells were removed and separated by trituration. PBS was added to the dishes and any remaining cells were scraped off and added to the rest, followed by centrifugation for 10 min at 1000 rpm in a Beckman GS-6 benchtop centrifuge. The supernatant was carefully removed from the cell pellet and 10 ml of PBS was added. Cells were triturated again to obtain a single cell suspension, and counted on a Z1-Coulter counter.

1. Cell proliferation

Cell proliferation was determined using the Cell Proliferation ELISA, BrdU (colorimetric) immunoassay from Roche diagnostics. This assay determines the amount of viable cells in each reaction by measuring the DNA synthesis of

the cells. Five-bromo-2'-deoxyuridine (BrdU) is a pyrimidine analogue incorporated into the DNA by replacing thymidine.

Essentially, 100 μl of cell suspension at a concentration of 3 x 10^5 cells/ml was plated out into a 96 well microtitre plate. After 24 h the cells were 70-80% confluent. They were treated with 100 μl of various concentrations of LiCl in growth medium and were assayed at varying time intervals. Cells were labelled with the addition of 10 μl of BrdU for 4 h, this was removed and the cells were fixed and denatured for 30 min, anti-BrdPOD was added for 90 min, the cells were washed (3 x 10 min), substrate was added for 10 min and the reaction stopped with H_2SO_4. Absorbance was read in an ELISA plate reader at 450 nm. The average±SEM was calculated from three independent studies, each carried out in triplicate.

1. Microscopy

Scanning electron microscopy was employed to investigate the cell morphology of lithium treated and untreated cells, while light and transmission electron microscopy was used to study ultra-structural changes in the cells (All work in this section is referenced from Bozzola and Russell, 1991).

1. Light microscopy

PK(15) and LLC-PK_1 cells were grown in nunc cell culture Petri-dishes and treated with varying concentrations of LiCl for 30 h and 72 h respectively. To avoid trypsinising the cells, the cells were scraped off the Petri-dishes and washed three times in PBS. Samples were fixed in 2.5 % gluteraldehyde for 24 h at 4°C. After washing in PBS the cells were post-fixed with 1 % osmium tetroxide for 1 h, washed (3 x 10 min) with PBS and dehydrated through a gradient ethanol series. Samples were embedded in LR-White resin. Semi-thin sections were cut and stained with toluidine blue and viewed under a Zeiss light microscope. Photographs were taken and developed using standard techniques.

1. Scanning electron microscopy

Cells were grown to near-confluency on Aclar embedding film (AGAR) and treated with 0, 5, 10 and 20 mM LiCl for time intervals of 0, 30, 48 and 60 h. Samples were treated as for light microscopy to the ethanol dehydration step. Membranes were removed from the chambers, and either critical point dried or dried with HMDS overnight. The membranes were placed on a stub, gold coated and viewed in a Hitachi 450S scanning electron microscope.

1. Transmission electron microscopy

Cells were treated as for light microscopy and embedded in LR-White resin. Ultrathin sections were cut on a microtome and stained with Uranyl Acetate and Lead Citrate. Sections were viewed on a Phillips 301 transmission electron micrograph. Photographs were developed using standard photography techniques and micromarkers were calculated and placed on the original photograph.

1. DNA fragmentation

DNA fragmentation was studied as one of the methods used to establish whether lithium induced cell death occurs due to necrosis or apoptosis. The fragmentation of DNA was determined using the following two methods.

1. DNA laddering assay

Cells were treated with varying concentrations of LiCl for three days in petri dishes. The medium was removed and retained and the cells were washed with PBS and trypsinised, as described previously. Trypsinised cells were added to the cells from the spent medium and centrifuged at 500 rpm for 10 min in a Beckman GS-6 centrifuge. Medium was removed and cells were washed twice with PBS. The DNA was isolated using a method modified to

that reported by Di Jeso (1995). Cells were lysed in 400 μl of DNA lysis buffer (100 μg/ml proteinase K; 10 mM Tris-HCl, pH 8.0; 25 mM EDTA pH 8.0; 0.5% SDS) and left on ice for 30 min and incubated at 50°C overnight to allow for protein digestion. The DNA was extracted using one volume of phenol: chloroform (1:1 (v/v)). The DNA in the aqueous layer was precipitated with one volume of ice-cold isopropanol in the presence of 0.1 volume of 3 M sodium acetate (pH 5.2) and incubated at –80°C for 1 h. The precipitated DNA was recovered by centrifuging at 13 000 xg for 15 min. The pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 0.1% SDS) and treated with DNAse free RNAse (5 μl/ml) for 2 h at 37°C. Approximately 40 μg of DNA was run on 1.5 % agarose gel at 60 V for 3 h.

1. DNA fragmentation assays

A terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling method (TUNEL) for DNA fragmentation was carried out using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics). Cells were incubated in Nunc 4 chamber microscope slides at LiCl concentrations of 0, 5, 10 and 20 mM for 24 h and 48 h. Intact monolayer cells were air-dried and kit instructions followed. A positive control consisting of HL-60 cells treated with 20 mM LiCl for three days and a negative control using no terminal deoxynucleotidyl transferase were used. The cells were viewed with a Zeiss fluorescent microscope. Three random fields were photographed for each of three individual experiments. To eliminate bias, positive cells were counted using the spot blot application of a Syngene Gene Tools analyser.

1. Apoptotic protein expression and activity

Different methods were used to assess the expression or activity of particular pro- or anti- apoptotic proteins to LiCl treatment of LLC-PK_1 and PK(15) cells. The immunotechniques used included immunocytochemistry and Western blotting. Immunocytochemistry detects the prevalence of proteins in an *in situ* situation whilst Western blotting detects separated proteins from cell lysates.

A colourometric enzyme assay was used to determine the activity of caspase enzymes, the aspartic acid proteases involved in various apoptotic processes.

1. Immunocytochemistry

Cells were grown on four chamber Nunc microscope slides, and treated with LiCl in growth medium at varying concentrations for 24 h. The cells were washed twice, fixed in 100 % methanol for two min and air-dried. The slides were processed with the Immunotech Universal Immunostaining kit (Coulter), utilising anti-human primary antibodies (1:100 dilution) that targeted apoptotic (Bax) and cell cycle dependant (p53, PCNA and Rb) proteins. Blocking solution was used in place of the antibodies as a negative control, as preimmune serum was not available. A universal biotinylated secondary antibody and the streptavidin-peroxidase-DAB system were used for detection. Cells were counterstained with haemotoxylin and photographed using a Zeiss light microscope. One hundred cells in four random fields of each slide were assessed for positively stained cells. Each experiment was repeated three times. Since a positive control was not available, all results are presented as a percentage of the results obtained for the untreated experiments.

1. Western blot analysis

Lithium treated and untreated cells were harvested and washed using cold PBS. Cells were lysed using lysis buffer (20 mM Tris-HCl; 132 mM NaCl pH 8; 10 % glycerol; 1 mg/ml PMSF; 1 mg/ml leupeptin; 0.15 U/ml aprotonin; 1 mM sodium vanadate; 0.1 % NP40). Reduced, denatured lysates (40 μg/well) were run on 12% SDS-PAGE gels. The separated proteins were transferred to nitrocellulose membranes using conventional transfer methods. Standard Western blotting protocols were followed and blots were optimised for the most effective primary and secondary antibody concentrations. Negative controls were performed using no primary antibody and because a positive control for Bcl-2 was the only one available, molecular weights of positive bands were evaluated to ensure correct assessments. Primary antibodies

used were rabbit anti-p53 (1:100), anti-Bcl-2 (1:100) and anti-Bax (1:100). Goat anti-rabbit-HRP conjugated antibodies (1:1000) were used as a secondary antibody. Chemiluminescence was used to detect the presence of immune-complexes and the immunographs were developed using standard X-ray development techniques. Gels were documented using a Syngene Gel-Doc system and the results analysed using the Syngene Gene Tools analysis package.

1. Caspase enzyme analysis

Cells that were 80 % confluent, were treated with fresh medium containing the following concentration of LiCl (0, 1, 5, 10 and 20 mM) and incubated for 24 h. Caspase enzyme analysis, for caspase 3, 8 and 9, was carried out using a kit purchased from R & D systems. Briefly, adherent and non-adherent cells were harvested by trypsinisation and cell scraping. Due to variations in growth times, cells were counted on a Z1 Coulter counter and $2x10^6$ cells were used per experiment. Cells were washed in PBS and lysed in caspase assay lysis buffer (50 μl) on ice for 10 min, centrifuged at 10 000 xg for 1 min and the pellet discarded. Reaction buffer (50 μl), containing fresh DTT, was added to the supernatant. A caspase 3, 8 or 9 specific peptide, conjugated to a colour reporter molecule p-nitroanilide (5 μl), was added to the lysate, these being DEVD-pNA, IETD-pNA and LEHD-pNA, respectively, and incubated at 37°C for 90 min. The cleavage of the peptide by the caspase gave a colour that was measured spectrophotometrically on a microplate reader at 405 nm. A linear relationship exists between the amount of active caspase present and the colour of the assay. The average±SEM was calculated from three independent studies, each carried out in duplicate.

1. Cell cycle analysis

1. Cell synchronisation

For an accurate determination of the effects of LiCl on the cell cycle of PK(15) and LLC-PK_1 cells the cells need to be synchronised. The double thymidine

block method of Stein *et al*. (1998) was followed to do this. In this method, the majority of the cell populations incurred a metabolic block imposed at the the G_1/ S phase interface of the cell cycle, characterised by a similar DNA content (Stein *et al.,* 1998). Briefly, 2 mM thymidine was added to cells that are growing exponentially. The block was removed after 16 h and fresh medium was added for a further 8 h to allow the cells to progress through this stage. Thymidine (2 mM) was added once again for 16 h. Following this the cells were split into different cell culture dishes, fresh medium was added and the cells were left to attach for 24 h.

1. Flow cytometry

Various concentrations of LiCl were added to the cultures and incubated for different time intervals as indicated in the results. Cells were washed with PBS, trypsinised and stored in a citrate buffer (40 mM, pH 7.6) at –140°C until flow cytometry could be carried out. Cells were thawed and washed with 500 μl of PBS and resuspended in 500 μl phosphate-citrate buffer containing propidium iodide (33 μg/ml propidium iodide, 1 mg/ml RNase, 0.1% NP40 in 0.192 mM Na_2HPO_4, 4 mM citrate buffer, pH 7.8) and incubated at 37°C for 30 min. Due to clumping, the cells were separated by gentle trituration through an insulin syringe. DNA content was assessed using an ALTRA flow cytometer in the Department of Immunology, University of Pretoria, using an excitation wavelength of 488 nm. At least 10000 cells were counted in each experiment. The percentage of cells in each stage of the cell cycle was determined and normalised to control cells.

- Statistics

The paired t-test was carried out on three replicas of each experiment using the statistics package on SigmaPlot 4.0 (SPSS 1997).

CHAPTER 3
Lithium effects on cell proliferation, apoptosis and the cell cycle

1. Lithium effects on cell proliferation

3.1.1 Introduction

Lithium has a pleiotropic effect on cell proliferation and toxicity. Depending on the type of *in vitro* cell culture used, the effect of lithium could be proliferative, cytoprotective, cytostatic or cytotoxic.

Generally, lithium seems to have a cytoprotective effect on neural cells under various stress related conditions. This was confirmed recently, whereby lithium (1 mM) was found to have protective effects on SH-SY5Y human neuroblastoma cells after stress related incidences (Lai *et al*., 2006). These studies concur with many other studies of the effect of lithium on neural cells (cited in Lai *et al*., 2006). Although the cytoprotective effect of lithium on neural cells has been well reported, it was observed only when the cells were placed under stress conditions. On its own, lithium was shown to be cytotoxic (Lai *et al*., 2006). The toxic effect of lithium was noted by others on different types of neural cells (see Table 3.1).

Previous studies have shown lithium to have a proliferative effect on some established non-neural cell cultures (Becker and Tyobeka, 1993), it affects differentiation in others (Sokoloski *et al.,* 1993) and is cytotoxic to others (Gallicchio, 1985). The concentration of lithium and the type of cell line used in each instance was of particular importance. Table 3.1 summarises many of the lithium induced effects on established cell lines.

Table 3.1 Lithium inducing effects on various cell lines.

Cell Line	Lithium (mM)	Type of effect	Reference
Renal cells			
Mardin Darby Canine Kidney cells (MDCK)	1-20 20	Mitogenesis No toxicity or proliferation	Suh *et al.* (1992) Rybak & Stockdale (1981)
BS-C-1 African Green Monkey Kidney Cells	0.5-7.5 25	Mitogenesis of confluent cells DNA synthesis inhibited	Toback (1980)
CV1 African Green Monkey Kidney Cells	8 16	Cytostatic and morphology change Toxicity	Matthopoulos *et al.* (1995)
BUMPT Mouse proximal tubular cells	10	increased viability of growth factor deprived cells	Sinha *et al.* (2005)
Neuronal cells			
NT Neural precursor cell line	>5	Cytotoxic	Hasgekar *et al.* (1996)
PC12 Pheochromocytoma cells	5-10	Promotes cell survival Cytoprotective	Volonté & Rukenstein (1993) Hiroi *et al*. (2005)
SH-SY5Y human neuroblastoma cells	1	Cytoprotective	Lai *et al.* (2006) Layden *et al.* (2003)
Neuro-2a mouse neuroblastoma cells	10.5	Cytotoxic	Repetto *et al.* (2001)
SK-N-MC human neuroepithelioma	10	Inhibit cell proliferation	Smits *et al.* (1999)
Blood cells			
Human promyelocytic leukaemia cells (HL-60)	0.5-3 1-5 >10 >10 20	Colony stimulating Proliferative Toxic Toxic and effects on granulocytic differentiation Induces apoptosis	Gauwerky & Golde (1982) Becker & Tyobeka (1993) Masemola *et al.* (1991) Knight *et al.* (1989); Becker & Tyobeka (1993) Sokoloski & Sartorelli, (1991) Madiehe *et al.* (1995)
HL-60LiR (Li resistant cells)	12.5	Cytostatic	Matsebatlela *et al.* (2000)
Murine myelo-monocytic	0-20	Concentration dependant	Sokoloski *et al.* (1993)

cells WEHI-3B D+		differentiation to myeloid cells	
Human Myelocytes KG-1 KG (1a)	 0.5 0. 1	 Colony stimulating Colony stimulating	Gauwerky & Golde (1982)
Murune L1210 leukemia cells	3	Cell proliferation	Joyce (1991)
K562 human erythroleukemia cells	3 0.1-0.5 >10 30 Apoptotic	Growth enhancement Colony stimulating Toxic Apoptotic	Rosenstock *et al*. (1979) Gauwerky & Golde (1982) Becker *et al.* (1993) Tang et al. (2005) Madiehe *et al*. (1995)
Friend Murine erythroleukemia cells	0.1 0.5-50	Colony stimulating Toxic and inhibited DMSO differentiation	Gauwerky & Golde (1982) Gallicchio (1985) Zaricznyj and Macara (1987)
LP-BM5 MuLV bone marrow stromal cells	1-5 10-50	Proliferative cytotoxic	Mampuru *et al*. (1999)
Miscellaneous			
WI-38 embryonic lung cells	2	Cell growth in defined, serum-free medium.	Sorger and Cristafalo (1992)
A549 human lung adenocarcinoma	2	Cytotoxic	Allagui *et al*. (2007)
Balb/c/3T3 cells	10-25 0.1 20 1.5-20	Mitogenic in defined serum-free medium (insulin and EGF had an additive effect) As above but effect was synergistic with EFG and insulin. Mitogenic Synergestic with TNF	Rybak and Stockdale (1981) Rybak and Stockdale (1981) Beyaert & Fiers (1992) Beyaert & Fiers (1992)
Swiss 3T3-L1 Preadipocytes	2-20	Differentiation inhibited	Aratani *et al* (1987) Abotsi and Tyobeka (1993)
Brown preadipocytes	5-25	Differentiation inhibited	De la Concepción *et al.* (2005)
L929 Murine fibrosarcoma	1.5-10	Enhances cytotoxicity of TNF	Beyaert & Fiers (1992)
A14 mouse fibroblast	10	Decreased proliferation	Smits *et al*. (1999)
Rat-1 cells	10	No influence on proliferation	Smits *et al*. (1999)
Human HeLa cervix	12-16	Growth inhibition but no	Matthopoulos *et al*.

carcinoma		morphology changes	(1995)
B16 mouse melanoma		Cytotoxic and morphology change	Cited in Nordenberg *et al.* (1999)
HT-144 human melanoma		Cytotoxic and morphology change	Cited in Nordenberg *et al.* (1999)
Weri-Rb1 human retinoblastoma cells	40	Decreased viability	Tell *et al*. (2006)
Y79 human Rb cells	40	Decreased viability by 56%	Tell *et al.* (2006)
SJmRBL8 mouse Rb derived cells	40	Decreased viability by 71,6%	Tell *et al.* (2006)
P19 mouse embryonal carcinoma cells (differentiated and undifferentiated cells)	10	Cytotoxic Cell cycle arrest G2/M	Smits *et al.* (1999)
CZF mouse mammary tumour cells	10	Mitogenic	Ptashane *et al*. (1980)
Human MCF-7 Breast Cancer	5 <5 >5	Proliferative Additive proliferation with estradiol and EGF Toxic	Taylor *et al*. (1995)
FRTL-5 Rat Thyroid Cells (diploid)	2-12	Enhanced cell growth	Urabe *et al*. (1991) Tasevski *et al.* (1999)
UMR-106 osteoblast-like cell line	10	No effects observed	Tasevski (2000)
U_2OS human osteosarcoma	10	Inhibit cell proliferation	Smits *et al*. (1999)
L6 Myoblast cell	>5	Toxic	Laurenz & Smith (1998)
HepG2 hepatoblastoma cells	20	Resistance to chemical-induced apoptosis	Beurel *et al.* (2004)
Huh7 hepatocellular carcinoma Various Hepatocellular carcinoma cells		Growth arrest Growth arrest in 75% of cell lines tested	Erdal *et al.* (2005)
Bovine aortic endothelial cells BAEC	5	Decreased proliferation, viability unchanged	Mao *et al*. (2001)

According to the data in Table 3.1, the lithium induced effect on cells cannot be classified according to cell type. Generally, however, proliferation seems to

occur at low lithium concentrations (up to 10 mM), whereas cytotoxic effects are noted at higher lithium concentrations (above 5 mM). This is in line with general lithium toxicity studies where low levels of blood serum lithium must be maintained. Furthermore, lithium has not been shown to increase cancer risks in bipolar lithium treated patients, and thus the mitogenic effect observed in some cell lines have not caused overt concern.

Although trepidation regarding lithium induced kidney toxicity has been expressed, not many studies have been carried out on kidney cell culture lines. LLC-PK_1 cells were used in early studies to evaluate the effect of lithium on the vasopressin sensitive adenylate cyclase, although neither growth characteristics nor morphological changes were reported (Goldberg *et al*., 1988). The mitogenic effect of lithium has been observed in a number of kidney cell lines such as MDCK (Suh *et al*., 1992), BS-C1 African green monkey cells (Toback, 1980) and BUMPT mouse proximal cells (Sinha *et al*., 2005). Rybak and Stockdale (1981) did not observe an increase in DNA synthesis, hence mitogenesis, in lithium treated MDCK cells as was observed by Suh *et al.* (1992). They did, however, note an increase in protein synthesis but no morphological alterations. Similar conditions were used in both experiments. African Green Monkey kidney cells (CV1) showed a cytostatic response and accompanying changes in cell morphology. In this section the effect of lithium on the growth and proliferation of two porcine renal epithelial cell lines, PK(15) and LLC-PK_1 cells was investigated. PK(15) cells have characteristics of distal tubule cells, whilst LLC-PK_1 cells adopt characteristics of proximal tubular cells.

Results

To test the effects of LiCl on LLC-PK_1 and PK(15) on total cell number, cells were incubated in the presence and absence of varying concentrations of lithium up to 72 h. The results obtained for the total cell counts are presented in Figure 3.1a and b. These studies indicate that both the LLC-PK_1 and PK(15) cell growth is retarded by lithium in a time and concentration dependent manner. After 24 h LiCl treatment, there was little change with the

number of LLC-PK_1 cells in the various concentrations of LiCl used. Furthermore, the lithium effect on these cells was not too severe at relatively high concentrations and seemed to have a cytostatic rather than a cytotoxic effect on the cell numbers. After 72 h of 20 mM LiCl treatment the LLC-PK_1 cell numbers were 68% that of the control. An interesting observation made regarding the LLC-PK_1 cells is that they tended to plateau out after 48 h. These cells normally grow as a monolayer since growth is inhibited due to confluency and cell-cell contact inhibition. The plateau effect seems to indicate that LiCl does not affect the cells once they are confluent.

In contrast to the observations made with the LLC-PK_1 cells, the proliferation curves of the PK(15) cells do not plateau out quickly, possibly due to the slower growth profile observed (Figure 3.1 b). Although visual observations indicated the PK(15) cells to be confluent after 48 h the cells do not stop growing, but seem to grow little domes or clusters at growth initiation points (as observed in the microscopy studies in section 3.2). In comparison with the LLC-PK_1 cells, 60% of the cells as compared to controls remain after 20 mM LiCl treatment for 72 h. Furthermore, the lithium induced proliferation inhibition seemed to be statistically different for 10 mM and 20 mM treatments at all time intervals measured. Once again, the lithium effect seems to be cytostatic rather than cytotoxic as there is no decline in the cell numbers from the start of the experiment.

Further investigation into the mode of growth inhibition was investigated using the BrdU cell proliferation assay that assesses thymidine incorporation, and thus DNA synthesis. Using this method, results indicated that the cell proliferation of LiCl treated LLC-PK_1 and PK(15) cells remained unchanged from their untreated counterparts up to concentrations of 20 mM LiCl. BrdU-thymidine incorporation dropped to 65% after 50 mM LiCl treatment in both the LLC-PK_1 and PK(15) cells (Figure 3.2 a and b). This concentration of LiCl has proven to be toxic in many cell lines (reviewed in Becker and Tyobeka, 1996). LLC-PK_1 cells treated with 20mM LiCl maintained cell proliferation at about 80% whilst that of the PK(15) cells was above 80%. Unfortunately, due to the high variability within the experiments, statistical differences between

the results could only be shown within the 50 mM LiCl treatment in the PK(15) cells at all time intervals measured.

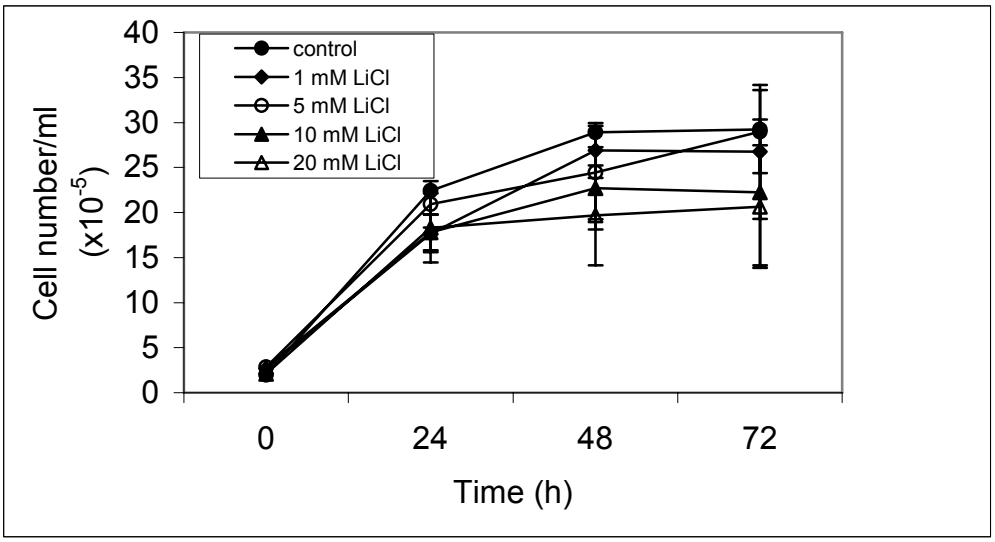


Figure 3.1a: Concentration and time dependent effects of LiCl on growth of LLC-PK_1 cells. Culture plates were seeded with $2.5x10^5$ cells and allowed to attach for 24 h. Plates were treated with various amounts of LiCl for upto 3 days. Data presented as mean ± SEM for three independent experiments, each performed in duplicate.

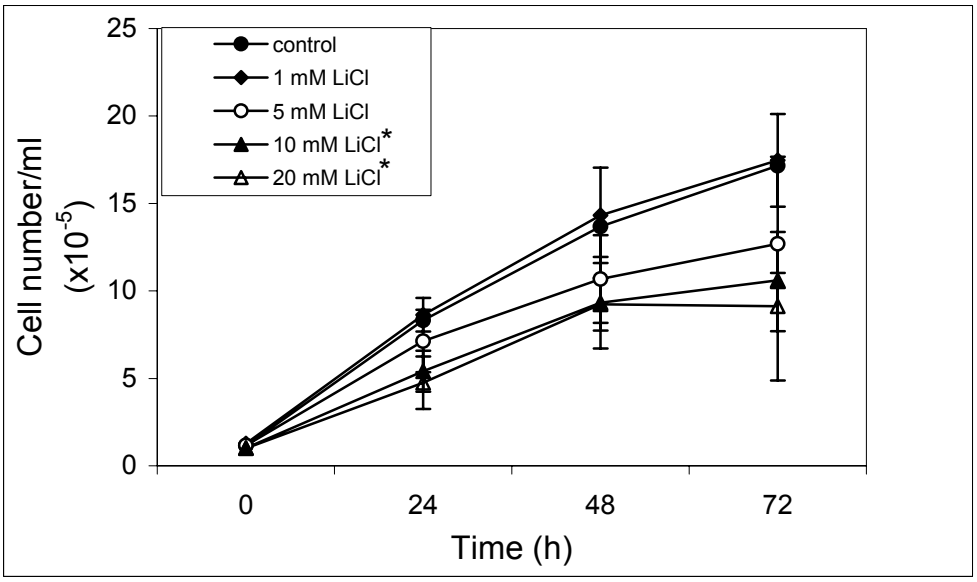


Figure 3.1b: Concentration and time dependent effects of LiCl on growth of PK(15) cells. Culture plates were seeded with $2,5x10^5$ cells and allowed to attach for 24 h. Plates were treated with various amounts of LiCl for upto 3 days. Data presented as mean ± SEM for three independent experiments, each performed in duplicate.*$p<0.05$ compared to control.

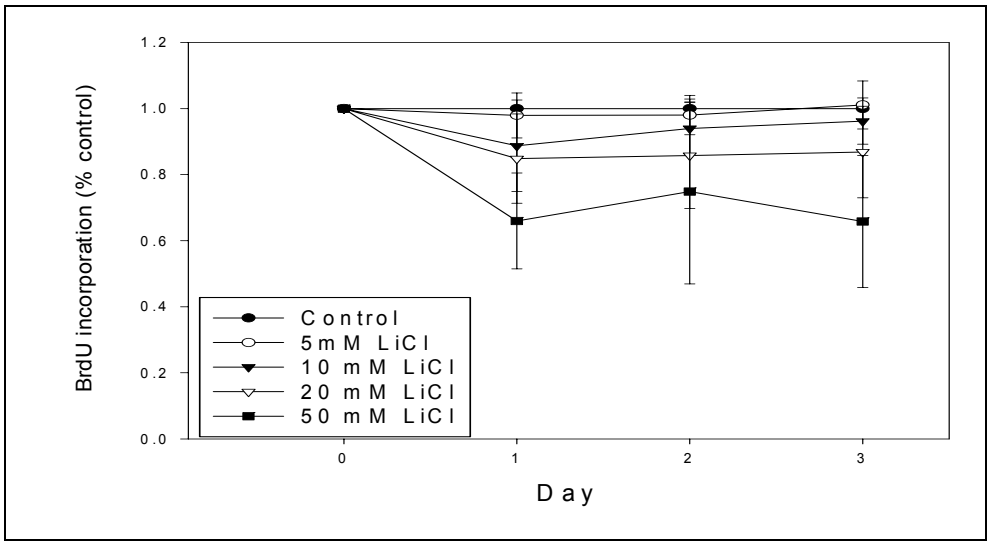


Figure 3.2a: Concentration and time dependant influence of LiCl on LLC-PK_1 cell proliferation.

Cells were grown to 80% confluency in a 96 well microtitre plate and treated with various concentrations of LiCl for up to three days. Data presented as mean ± SEM for three separate experiments, each performed in triplicate.

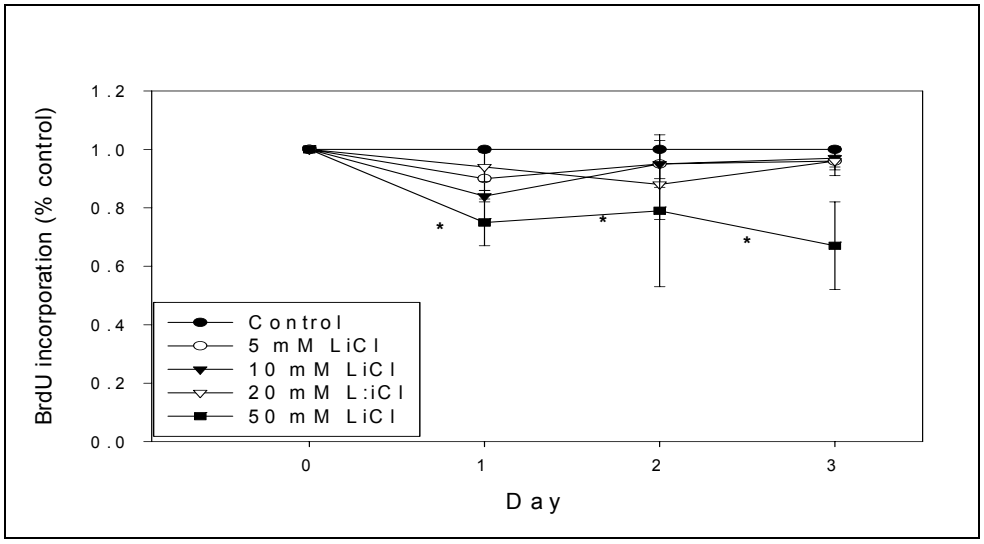


Figure 3.2b: Concentration and time dependant influence of LiCl on PK(15) cell proliferation.

Cells were grown to 80% confluency in a 96 well microtitre plate and treated with various concentrations of LiCl for up to three days. Data presented as mean ± SEM for three separate experiments, each performed in triplicate. *$p<0.05$ compared to control.

3.1.3 Discussion

Previous studies have indicated that lithium causes an enhanced proliferation on MDCK kidney cells at concentrations up to 20 mM (Suh *et al*., 1992), BS-C-1 African Green Monkey kidney cells at concentrations of 0.5-7.5 mM (Toback, 1980) and BUMPT cells (Sinha *et al*., 2005). These effects were, however, not observed for either the LLC-PK_1 cells or the PK(15) cells in the current study. The differences could be due to variations in experimentation between the different studies. In this study LLC-PK_1 and PK(15) cells were grown in the presence of serum, rather than in the absence, or limited quantities, as performed elsewhere. Sinha *et al*. (2005) reported that lithium promotes cell survival in growth factor deprived cells. It thus seems that many of these studies investigated cells under stress conditions in the presence of lithium. The cells in this study were not under stress conditions and the lithium induced effects were noted compared to control cells in the presence of the same batch of FBS.

Furthermore, previous studies on renal cell lines were performed on quiescent cells and not actively dividing cells. LLC-PK_1 cells only show tubular differentiation once confluent. In order to ascertain the effect of lithium on actively dividing cells, 80% sub-confluent cells were used to initiate the experiments. Many of the further experiments carried out in this report were conducted at 24 – 30 hr where the cells had become confluent. This was different to those conducted on the PK(15) cells as they did not become quiescent, as discussed below.

Lithium seemed to have a cytostatic effect on both LLC-PK_1 and PK(15) cells at similar concentrations, i.e., 10 mM and above (Fig 3.1a and b), seeing that the cell generation time slowed down in comparison to control studies. The BrdU incorporation assays, however, indicated little change throughout the different lithium treatments up to 20 mM. (Fig 3.2 a and b). This was most notable in the first 24 h of treatment and no decrease in cell number was noted once the cells were confluent (plateau effect). Again, this could be due to the presence of FBS, since Rybak and Stockdale (1981) noted a decrease

in MDCK DNA synthesis by LiCl in the presence of FBS leading them to believe that LiCl may antagonise events leading to DNA synthesis.

Cell proliferation studies (Figures 3.2 a & b) suggest that LiCl is more toxic to the LLC-PK_1 cells than it is to the PK(15) cells, though this was not observed in electron microscopy studies (detailed later). This may be an aberration of the BrdU incorporation method. PK(15) cells were observed to continue growing once they became confluent, and formed small masses of cells, hence the cell growth plateau effect was not as noticeable in this cell line as with the LLC-PK1 cells. The PK(15) cells may, thus, be masking the lithium effects during the assay due to the number of cells present. In addition, dying PK(15) cells tend to lift off the matrix more than LLC-PK_1 cells and in so doing the apoptotic cells may have been washed away during the experimental procedure and not detected. Both these cell lines adopt tubule epithelial characteristics once confluent and to ensure that the studies were carried out on non-quiescent epithelial cells, all experiments were initiated on 80% confluent cells. This did lead, however, to the PK(15) cells being quite overgrown, but not quiescent, by day three. This may have contributed to the aforementioned effects.

The cytotoxic effect of 10 mM LiCl compares favourably to the results obtained by Toback (1980) on BSC-1 African Green monkey kidney cells and Matthopoulos *et al.* (1995) on CV1 African Green Monkey kidney cells. The only reported studies of lithium treated LLC-PK_1 cells (Goldberg *et al.*, 1988) did not report on lithium cell proliferation effects but rather that 10 mM LiCl inhibited vasopressin-stimulated adenyl cyclase activity. Thus no comparison to this study can be made. It was of interest to note, however, that Goldberg *et al*. (1988) recorded intracellular lithium levels in LLC-PK_1 cells was 6 and 17 mM for extracellular levels of 5 and 10 mM. These concentrations are much higher than conventional therapeutic levels, indicating that the cells can withstand high levels of LiCl. These are the first reported lithium effects on the proliferation studies of PK(15) cells, also indicating that the cells can tolerate relatively high lithium concentrations.

Lithium effects on renal cell morphology.

Introduction

The cell proliferation studies indicated that lithium has a cytostatic effect on both the LLC-PK_1 and PK(15) cell lines at the higher LiCl concentrations used (10 mM and above). These studies did not, however, indicate the mode of cell death. Cells can die via different mechanisms: necrosis and programmed cell death (PCD) such as autophagy, paraptosis or apoptosis (reviewed in Bröker *et al*., 2005). Programmed cell death, involved in tissue homeostasis and cell development, has been morphologically characterised into three groups: type 1 (nuclear or apoptotic), type 2 (autophagic) and type 3 (cytoplasmic) (Fombonne *et al*., 2006). There are many markers of each mode of cell death, morphological and ultrastructural indicators being two of the more used characteristics.

According to Kerr (2002) a phenomenon called shrinkage necrosis was noticed by him on cell death studies in hepatocytes in 1969. The type of cell death did not resemble that of conventional necrosis and tissue inflammation was not observed. Electron microscopy studies on malignant cells and on developmental studies further proved the morphological differences between the shrinkage necrosis or apoptotic type of cell death compared to that of normal necrosis. After many studies, deliberations and collaborations, shrinkage necrosis emerged as the concept of apoptosis.

Necrosis typically occurs due to some kind of severe cell injury stimulus taking place from outside the cell (Bröker *et al*., 2005). Necrotic cells usually swell due to loss of membrane homeostasis, ion channels and ATP, and the ultimate hydration of the cell. Initially the nucleus looks preserved, but later, focal chromatin margination is observed (Falcieri *et al*., 1994). Finally the nucleus disintegrates. The cytoplasmic membrane ruptures and intracellular contents are released, which leads to cell digestion due to lysosomal enzyme release. Much debris is released into the extracellular environment, which leads to inflammation of the tissues (Wyllie *et al*., 1980; Bröker *et al*., 2005).

Apoptosis, also known as type I cell death, is a morphologically distinct type of cell death that results physiologically due to various conditions and stimuli (Falcieri *et al*., 1994). In contrast to the description above, apoptotic cells tend to shrink and the cytoplasm and nucleus condense. Externally, pseudopod formation is lost and microvilli on cells are blunted and recede (Wyllie *et al.,* 1980). Blebbing, cell membrane protrusions that do not have organelle structures in them, as well as budding occurs on the external membranous surface (Majno and Joris, 1995). It should be noted that necrotic cells could also have blebs extruding from the plasma membrane, which may add to the confusion of distinguishing apoptotic cells from necrotic ones.

In apoptosis, nuclear organisation is lost and the condensed chromatin usually forms dense areas at the nuclear membrane (Kerr, 2002). Membranous bodies, termed “apoptotic bodies” form from the nucleus and usually bud out of the cell. In *in vivo* studies the apoptotic bodies are engulfed by macrophages and other cells, and do not lead to the formation of inflammation. The organelle structure of an apoptotic cell is mostly conserved, especially the mitochondria as apoptosis is an ATP-dependent process (Wyllie *et al*., 1980). Adherent cells tend to round up and be released from the matrix support on which they are growing (Renvoizè *et al*., 1998) and epithelial cells tend to lose their polarity. An interesting feature of apoptosis is that the chromatin condensation appears differently in different cell lines and due to different stimuli. Cells may exhibit early chromatin clusters and dense margination; they can form a dense area around large portions of the nuclear envelope; extensive dense semi-circular areas may be observed; and crescents (protruding and non-protruding) and perinuclear cisternea may be found (Falcieri *et al*., 1994, Payne *et al.*, 1994; Dini *et al*., 1996).

Recently, the term “necroptosis” was coined by Degterev and colleagues (2005) to describe neuronal cells that share features of both apoptosis and necrosis. This type of cell death is blocked by a protein labelled necrostatin-1.

More research into this type of cell death, said to be influenced by p53, is required.

Another type of PCD is autophagy, or type II cell death (Bröker *et al*., 2005; Kim *et al*., 2006; Lleo *et al*., 2007). Autophagy is also involved in cell homeostasis, differentiation, tissue modelling, and plays an active role in regenerating amino acids from proteins during cell starvation (Lleo *et al*., 2007). Extensive double membranous structures, autophagosomes, are formed that fuse with endocytic compartments and lysosomes. Thus, extensive vacuolisation, with enlargement of the Golgi apparatus and the endoplasmic reticulum, is observed in autophagic cells (Kim *et al*., 2006).

Paraptosis is a relatively newly defined term in programmed cell death . The term parpaptosis was coined in 2000 by Bredesen's group to describe a form of cell death that does not conform to conventional apoptotic features such as DNA, nuclear and cellular fragmentation (Sperandio *et al*., 2000 & 2004; Castro-Obregon *et al.*, 2002). Paraptotic cells have extensive vacuolisation, swollen mitochondria and are resistant to agents that block apoptosis, such as caspase inhibitors. Paraptosis seems to exist in parallel to apoptotic mechanisms and may not be an exclusive mode of cell death due to various stimuli in different cell lines (Sperandio *et al*., 2000; Wyllie and Golstein, 2001). Although many authors have made reference to caspase-independent programmed cell death (Cummings and Schnellmann, 2002; Bröker *et al*., 2005; Buytaert *et al*., 2007), very few outside Bredesen's laboratory have used the term paraptosis (Wyllie and Golstein, 2001; Chose *et al*., 2002; Wang *et al*., 2004; Fombonne *et al.*, 2006), preferring to use apoptosis-like or caspase-independent apoptosis

One of the best methods to assess the type of cell death occurring due to specific stimuli are morphological and ultrastructural studies using microscopy (Falcieri *et al*., 1994; Renvoizé *et al*., 1998) The PK(15) and LLC-PK_1 epithelial cell morphology and ultrastructure were studied to establish the mechanism of lithium toxicity.

Results

Light micrographs of 72 h lithium treated LLC-PK_1 cells showed some morphological changes compared to their untreated counterparts. Untreated LLC-PK_1 cells clearly showed the polarity of the cells whereby the microvilli on the apical side were evident, which is distinguishable from the smoother basolateral side (Figure 3.3 (a)). The nuclei were large and typically had one or two nucleoli that tended to be apically situated. The junctions between the cells were tight. Although epithelial cells usually have a characteristically flat appearance when grown on a substratum, the cells depicted in Figure 3.3 no longer had this typically flat horizontal appearance, probably due to them being scraped off the matrix to do the study. LLC-PK_1 cells treated with 5 mM LiCl for 72 h (Figure 3.3(b)) did not show any changes in cell structure. Morphological changes started to appear after incubation of the cells with 10 mM LiCl (Figure 3.3 (c&d)). Although the cells still had a typical polar appearance, the microvilli on the apical surface were somewhat shorter. Furthermore, the cells appeared to be separating from each other. This is typical for epithelial cells undergoing apoptosis. Interestingly, however, the morphology of the nuclei had not changed much. The nuclei were still large and round and typically contained one or two nucleoli, though the apical distribution of the nucleus seemed to be lost. No typical features of cells undergoing necrosis such as pycnotic nuclei, swollen mitochondria, extensive vacuolisation, nor aberrations in the integrity of the cell and nuclear membrane were observed in these studies.

The consequences of LiCl treatment on the morphology of PK(15) cells were more profound. Untreated samples (Figure 3.4a&b) showed polarised cells with microvilli on the apical (luminal) surface. Nuclei were large and round containing one or two nucleoli. Again, it must be noted that the cells were scraped off the substratum on which they were growing and do not show the characteristic flat epithelial type cell in these studies. PK(15) cells treated with 10 mM LiCl for 30 h (Figure 3.4 (c&d)) indicated the presence of blebbing, a smoothing of the cell membrane, a distinct lack of microvilli, shrinking of the nucleus and the typical polar structure of the cells was lost. Of particular

interest was the production of apoptotic bodies. The apoptotic bodies typically extruded from the cytoplasmic membrane of the cells (Figure 3.4 (c)) and complete membrane bound structures (Figure 3.4 (c&d)) were released into the environment.

Since light microscopy results for LLC-PK_1 cells (Figure 3.3 (a-d)) did not show morphological changes at 10 mM LiCl treatment, cells were treated with higher lithium concentrations in order to determine whether alterations in cell morphology and ultrastructural composition could be detected when using scanning and transmission electron microscopy.

Scanning electron microscopy results indicated that there was little difference in the morphology of the LLC-PK_1 cells, even when treated with 20 mM lithium (Figure 3.5 (b-d)) compared to their untreated counterparts (Figure 3.5 (a)). Adherent cells had a flat appearance, cells did not seem to be pulling away from each other and the apical surface had many microvilli (Figure 3.5 (a-c)), though they seemed to be shorter than those in the control study (Figure 3.5 (c)). The basolateral surface, shown in Figure 3.5 (d), contained no microvilli but is one continuous structure. The 20 mM LiCl treated cells that had loosened from the substratum on which they were growing, had rounded up but still retained microvilli on the surface (Figure 3.5 (c)).

Scanning electron micrographs of untreated PK(15) cells indicated that they have a morphology similar to that of the LLC-PK_1 cells (Figure 3.6 (a)). The cells formed a flat layer and had numerous microvilli on their apical surface. A typical apical surface of the cells is seen more clearly in Figure 3.6 (b). Microvilli covered the entire upper surface with longer dendrite structures between the cells. As mentioned previously, the PK(15) cells did not stop growing once they were confluent. They started to develop regions of cell mass (clusters) that resembled bunches of grapes. This is clearly depicted in Figure 3.6 (c&d). Also evident from these illustrations is that the cells that grew above the monolayer tended to be round with clearly evident microvilli.

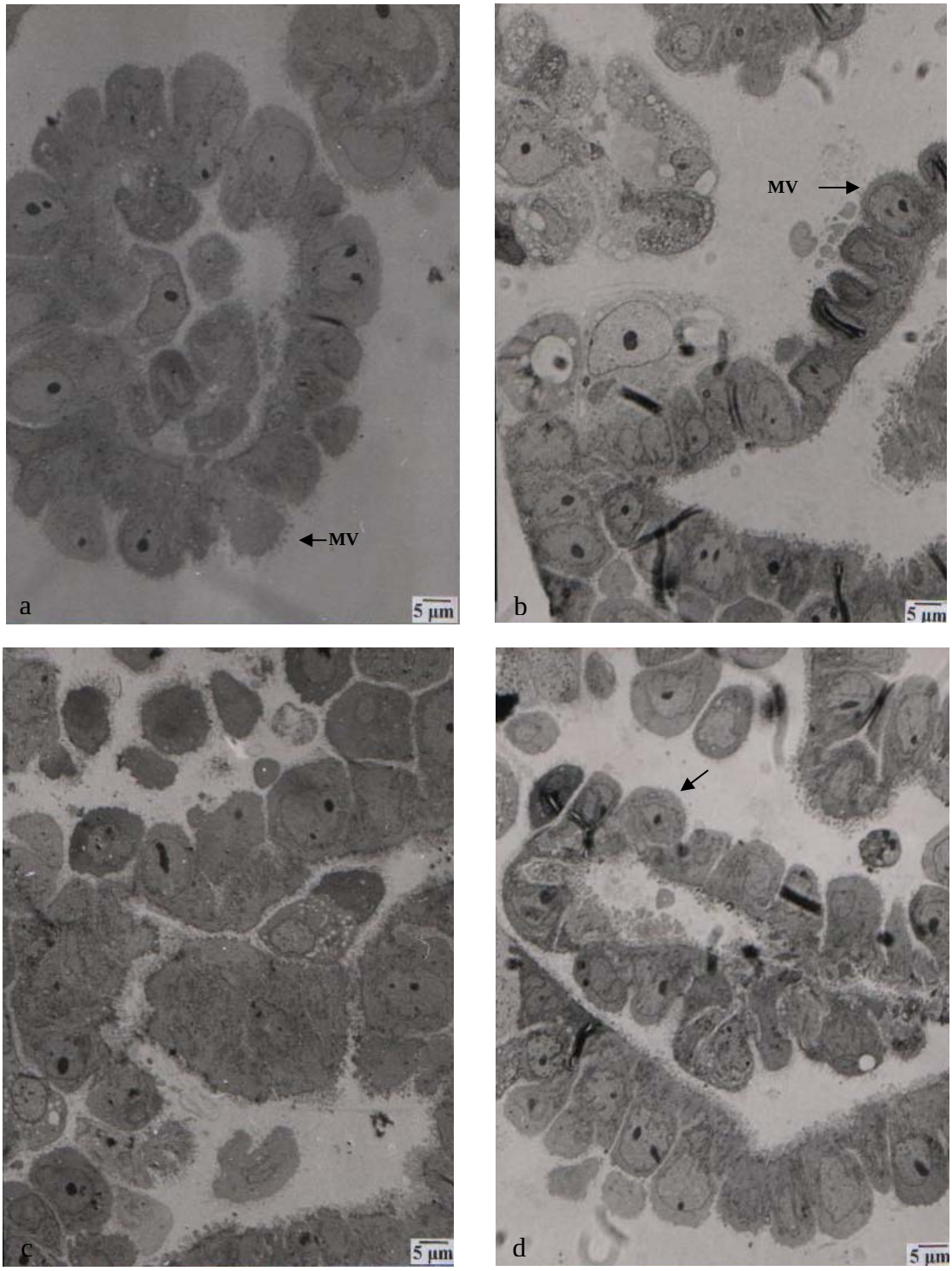


Figure 3.3: Light Microscopy study of the morphological changes of LLC-PK_1 cells treated for 72 h in the absence (a), and presence of 5 mM (b) and 10 mM (c,d) LiCl.
Microvilli (**MV**) were easily seen in the untreated and 5 mM LiCl treated cells but were shrunken or absent (**arrow**) on the luminal side of the epithelium after exposure to 10 mM LiCl.

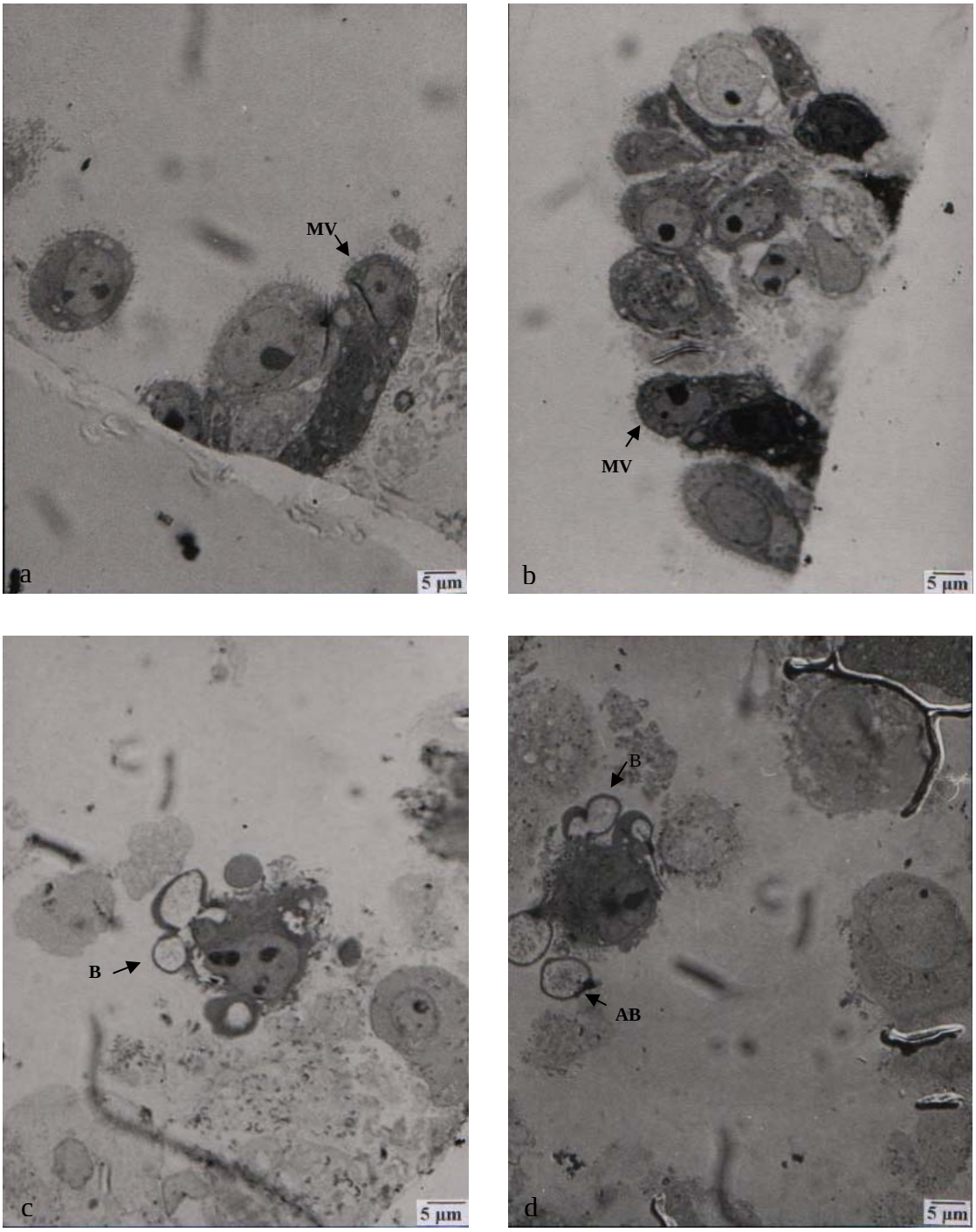


Figure 3.4: Light Microscopy study of the morphological changes of PK(15) cells treated for 30 h in the absence (a,b) and presence (c,d) of 10 mM LiCl.

Microvilli (**MV**) were clearly visible in the untreated samples (a,b), whilst blebbing (**B**) and apoptotic bodies (**AB**) were observed after 10 mM LiCl treatment.

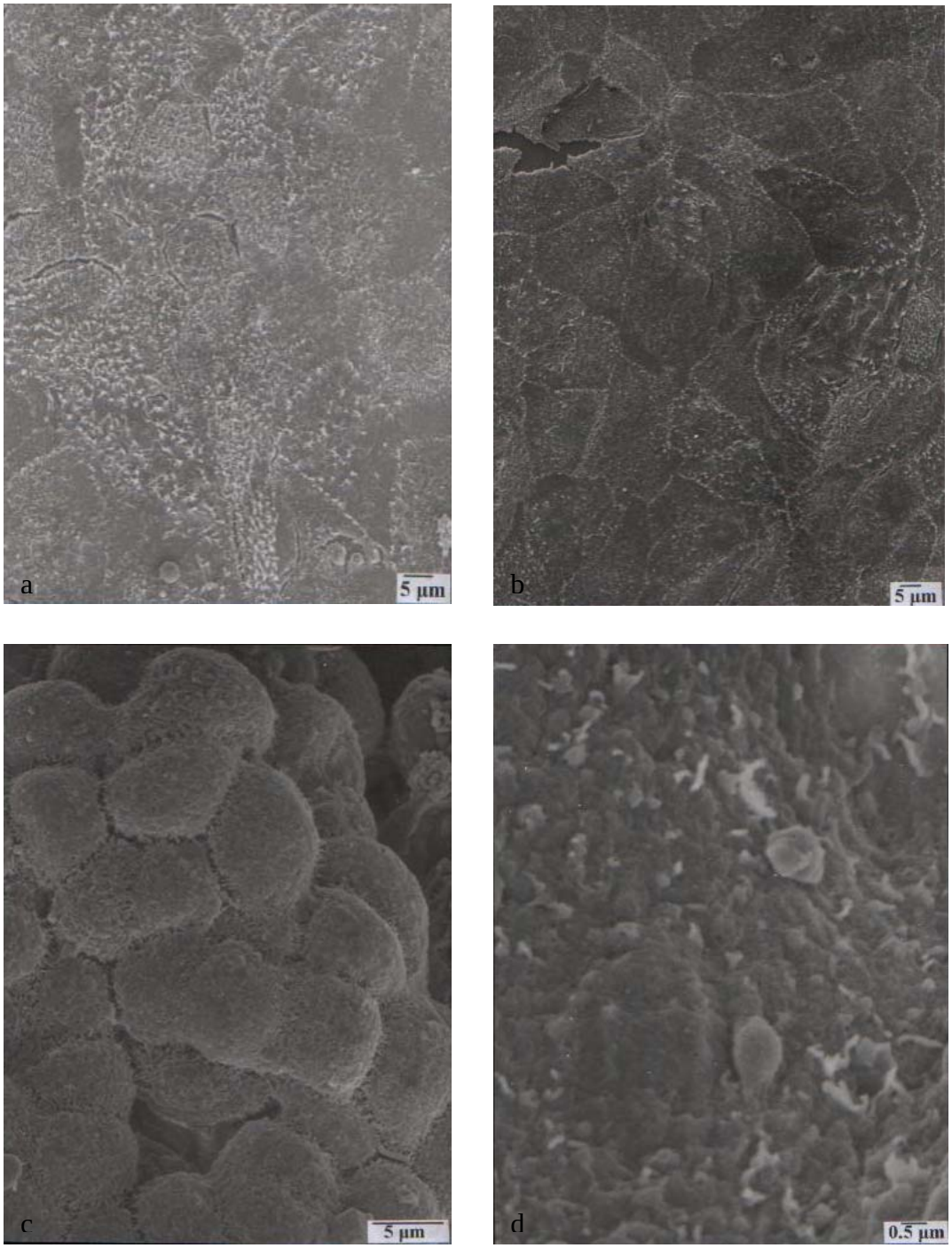


Figure 3.5: SEM studies of LLC-PK_1 cells untreated (a) or treated with 20 mM LiCl (b-d) for 72 h.

Cells were typically flat with microvilli on their apical surface (a-c) and a rough basolateral surface (d). Detached cells were slightly rounded up, but were still close in adherence to each other, and contained microvilli (c).

No blebbing was observed and the only difference between these cells and those growing on the substratum was that the cells were no longer flattened. The rounded cells were not necessarily dead cells as could be observed in Figure 3.6 (e), but it seemed as though these cells were released into the medium more readily than those on the support matrix.

Following 30 h of lithium treatment, PK(15) cells were easily released from the support matrix. This is clearly evident in Figure 3.6 (f) (10 mM LiCl) and in Figure 3.6 (g) (20 mM LiCl). A notable observation was the presence of distinct holes in positions where a cell cluster was present. This was presumed to be established due to cells being released from the cell mass, thereby leaving a smooth basal-membrane like layer behind (Figure 3.6 (f-h)).

Lithium (10 mM) seemed to blunt microvilli on the PK(15) cell surface (Figure 3.6 (i)), cause the cells to lose their uniform appearance and to create the formation of blebs on the cell surface as was seen in the light micrographs. A magnified version of the cell blebbing was observed in Figure 3.6 (j). Further blebbing and smoothing of the cell membrane was evident after 30 h of 20 mM LiCl treatment, which is clearly visible in Figure 3.6 (k). These structures are referred to as apoptotic bodies. Furthermore, the cell membranes of many cells treated with 20 mM LiCl that were released from the cell mass had a smooth appearance with little to no evidence of microvilli (Figure 3.6 l). The size of PK(15) cells treated with 20 mM LiCl appeared to be smaller in size than their untreated counterparts (as observed by comparing micrographs of treated vs untreated cells).

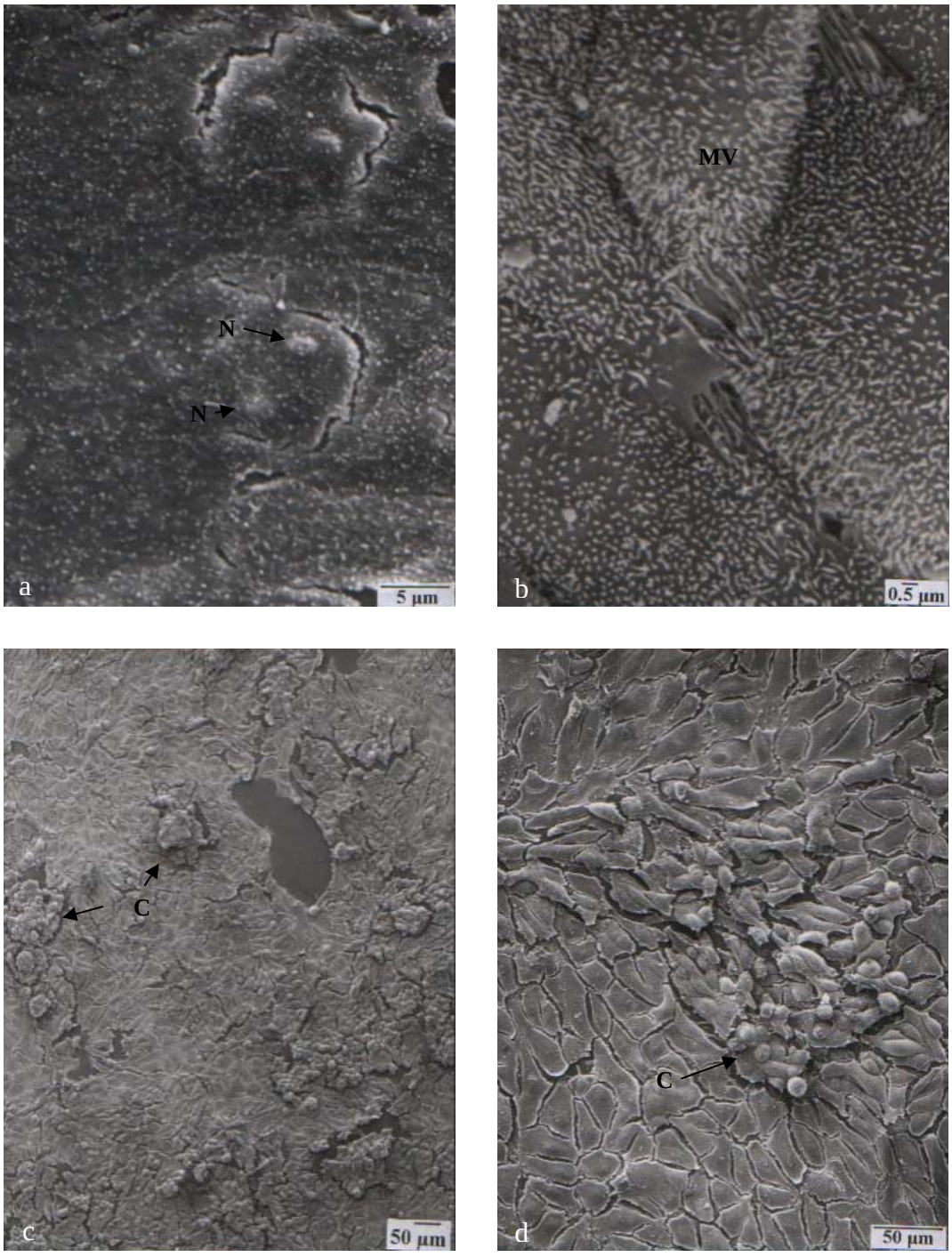


Figure 3.6: SEM studies of PK(15) cells untreated (a-e) or treated with 10 mM LiCl (f,i,j) and 20 mM LiCl (g,h,k,l) for 30 h.

(a) Cells were flat with a typical “fried egg” appearance of the nuclei (**N**). (b) Depicts the microvilli (**MV**) and the complexity of the cell layer. (c&d) illustrate the cell layer from far, indicating that the PK(15) cells did not stop growing once confluent but formed clusters (**C**). The cracked appearance of (d) was due to the drying process.

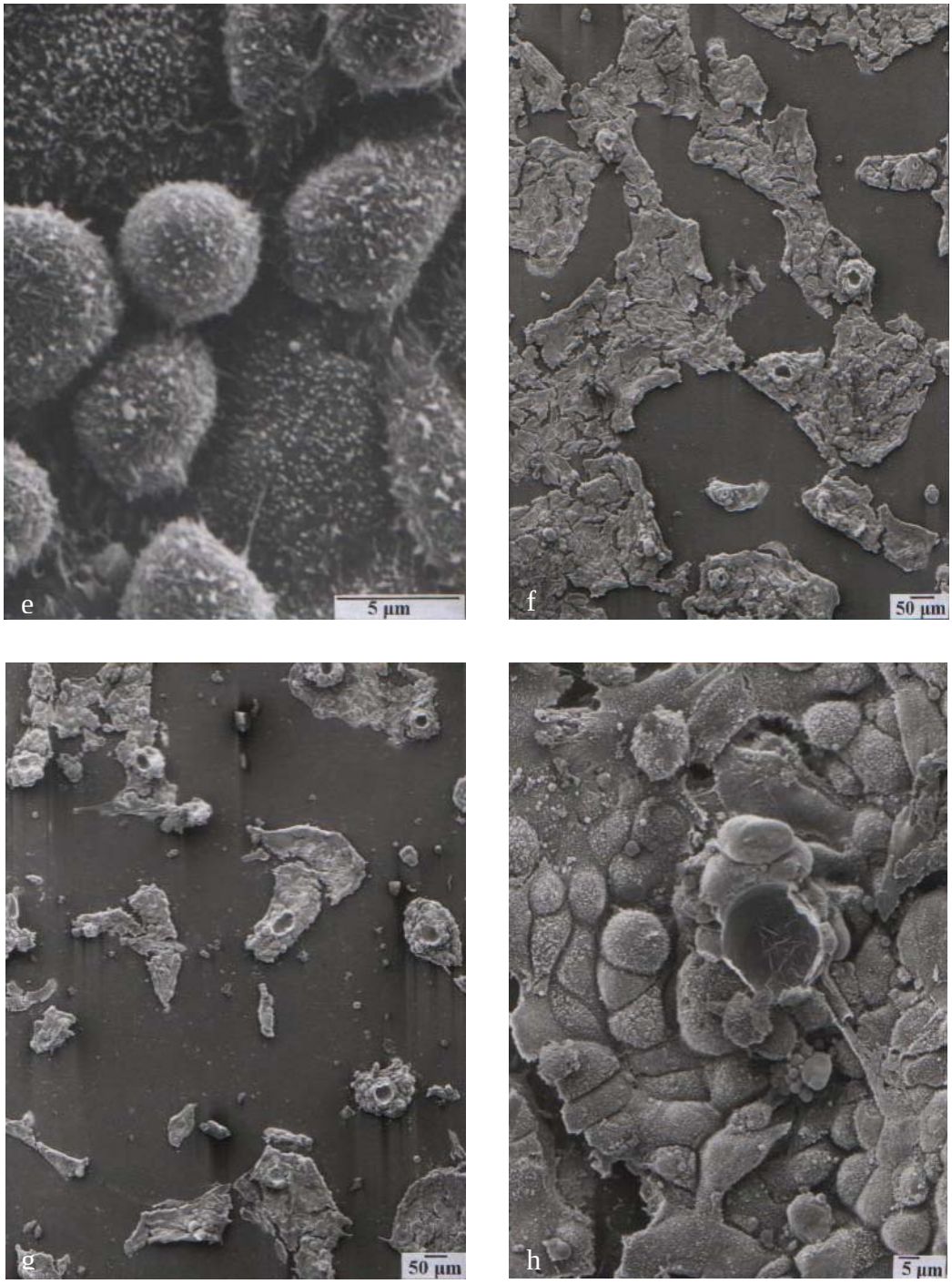


Figure 3.6 cont: SEM studies of control PK(15) cells untreated (a-e) or treated with 10 mM LiCl (f,l,j) and 20 mM LiCl (g,h,k,l) for 30 h.

(e) untreated cells that were not attached to the membrane, (f) cells treated with 10 mM LiCl, many cells have detached from the membrane, (g) 20 mM LiCl treated cells, more cells detached from the membrane and left crevasses on the clusters, (h) Crevasse left by cells released from the cluster, showing remnants of a basal membrane.

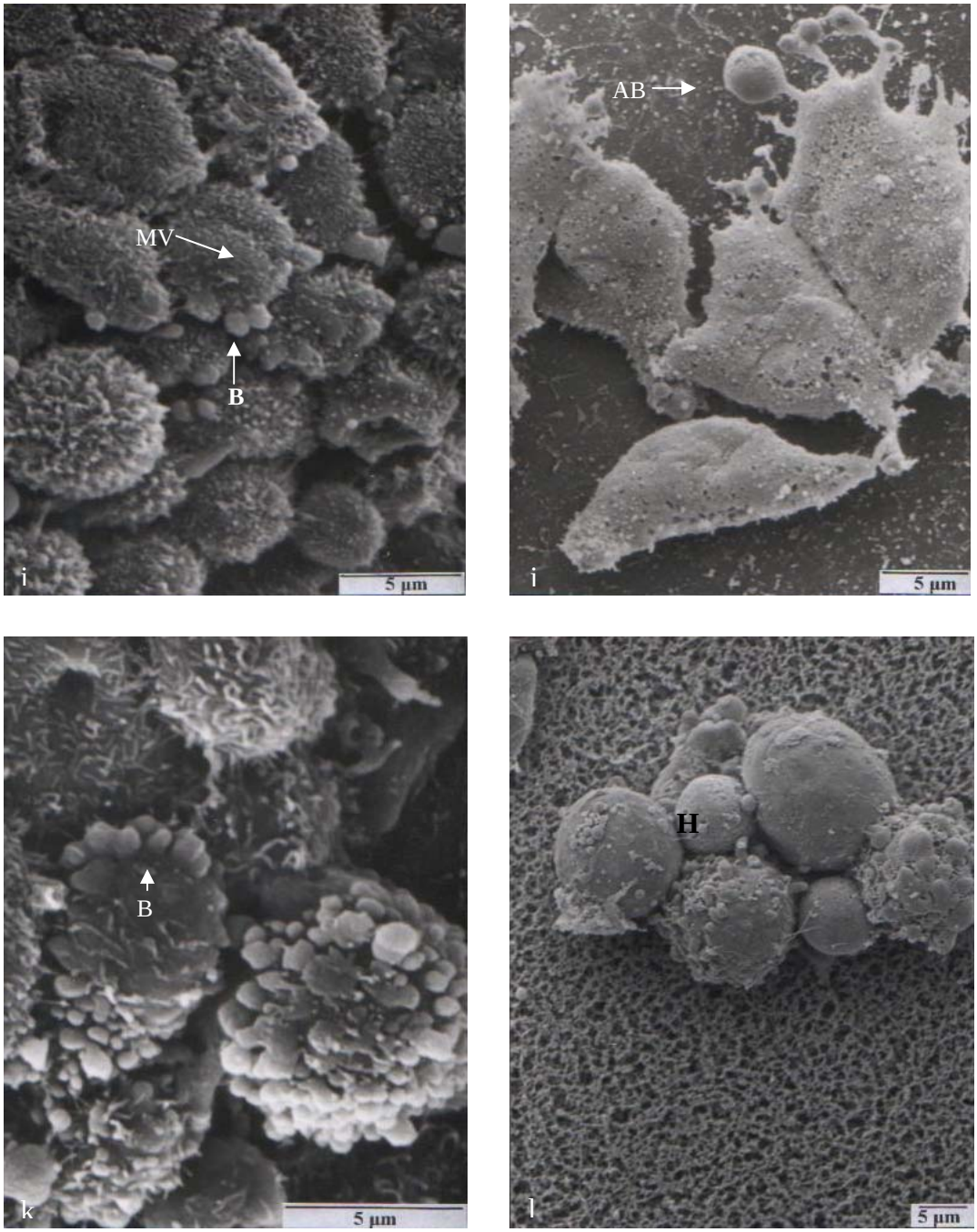


Figure 3.6 cont: SEM studies of control PK(15) cells untreated (a-e) or treated with 10 mM LiCl (f,i,j) and 20 mM LiCl (g,h,k,l) for 30 h.

(i) 10 mM LiCl treated detached cells with blunted microvilli (**MV**) and some blebbing (**B**), (j) apoptotic bodies (**AB**) on 20mM LiCl treated cells, (k) 20 mM LiCl treated detached cells with extensive budding, (l) 20 mM LiCl treated cells collected from the medium showed smoothing of the outer surface of the cell and extensive damage whilst remaining intact. Some of the cells seemed to be showing necrotic features by the presence of holes in the plasma membrane (**H**)

No ultrastructural changes were observed between untreated and 20 mM LiCl treated LLC-PK_1 cells (Figure 3.7 (a-d)). Euchromatin was evident in treated and untreated cells although their peripheral heterochromatin was also present, a normal feature in LLC-PK_1 cells. The nuclear membrane was convoluted in structure, the presence of many mitochondria was visible and apical microvilli were present. Tight gap junctions were evident as was the appearance of desmosome structures between the cells. The cells had a rounded appearance due to the microscopy that was done on cells scraped free from their supporting matrix. A cell with a morphologically distinct folded nuclear membrane can be seen in Figure 3.7(c) though this was not the norm, and could have been due to a cell undergoing normal homeostasis. The ultrastructural studies of the effects of LiCl on LLC-PK_1 thus concurred with the morphological results observed in the SEM studies.

Distinct ultrastructural differences were noted between untreated and LiCl treated PK(15) cells. Control cells that were growing on the membranous support appeared flat, microvilli were evident, and the nucleus had an oval appearance. Mitochondria were apparent and no unusual structures were observed (Figure 3.8 (a)). Figure 3.8 (b) illustrates control cells growing in a cell cluster. The nuclei were still oval in shape, one or two nucleoli were evident, and euchromatin, microvilli and mitochondria were present. The cells were more rounded than those observed in (a) due to their lack of adherence to a membranous support. No anomalies were observed in untreated PK(15) cells and the cells retained their polarized epithelial-like characteristics.

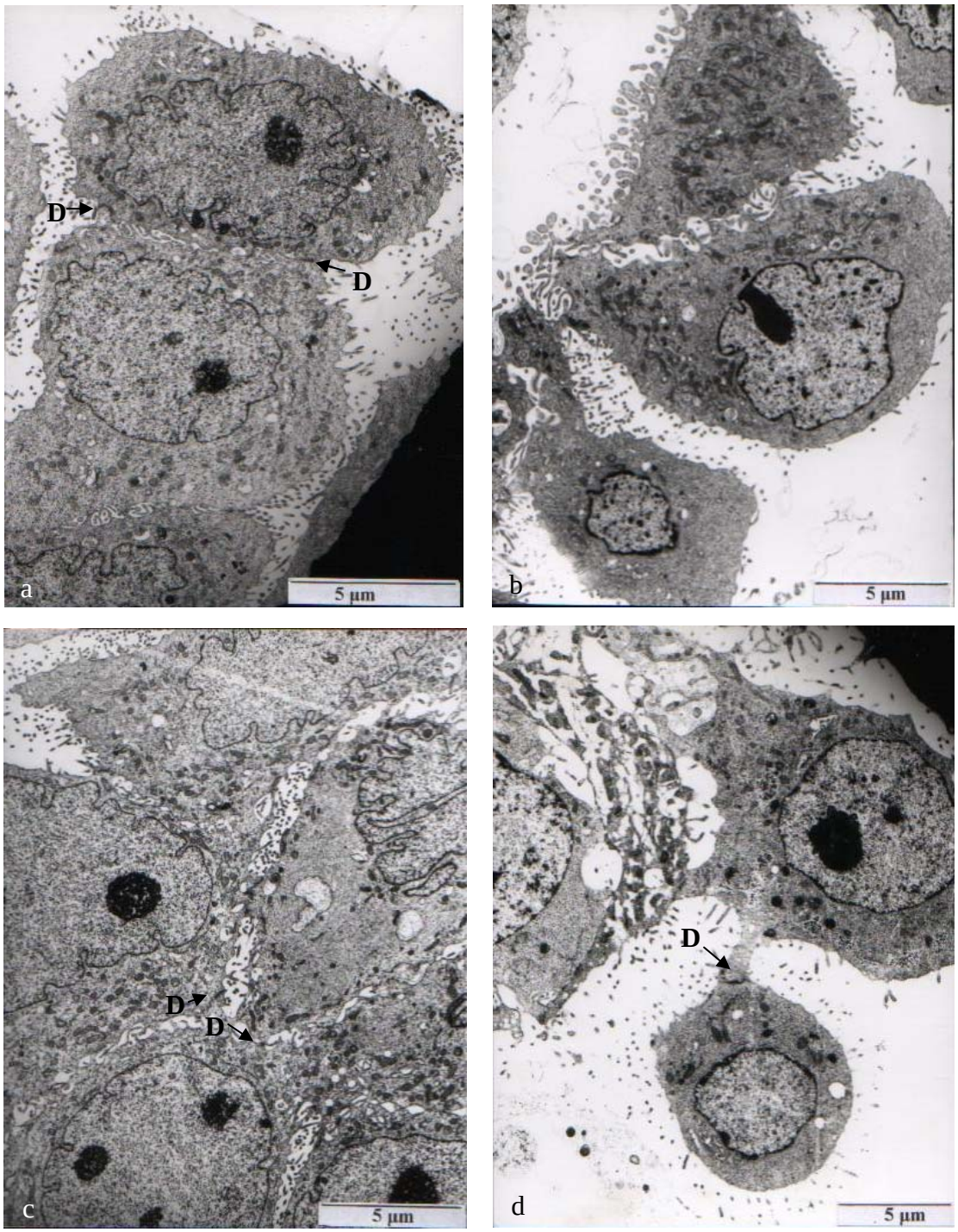


Figure 3.7: TEM studies of LLC-PK_1 cells untreated (a) or treated with 20 mM LiCl (b-d) for 72 h.

Very little change in the ultrastructure of the cells was seen. Desmosome structures (**D**) were still intact, nucleus morphology remained unchanged and microvilli structures were evident.

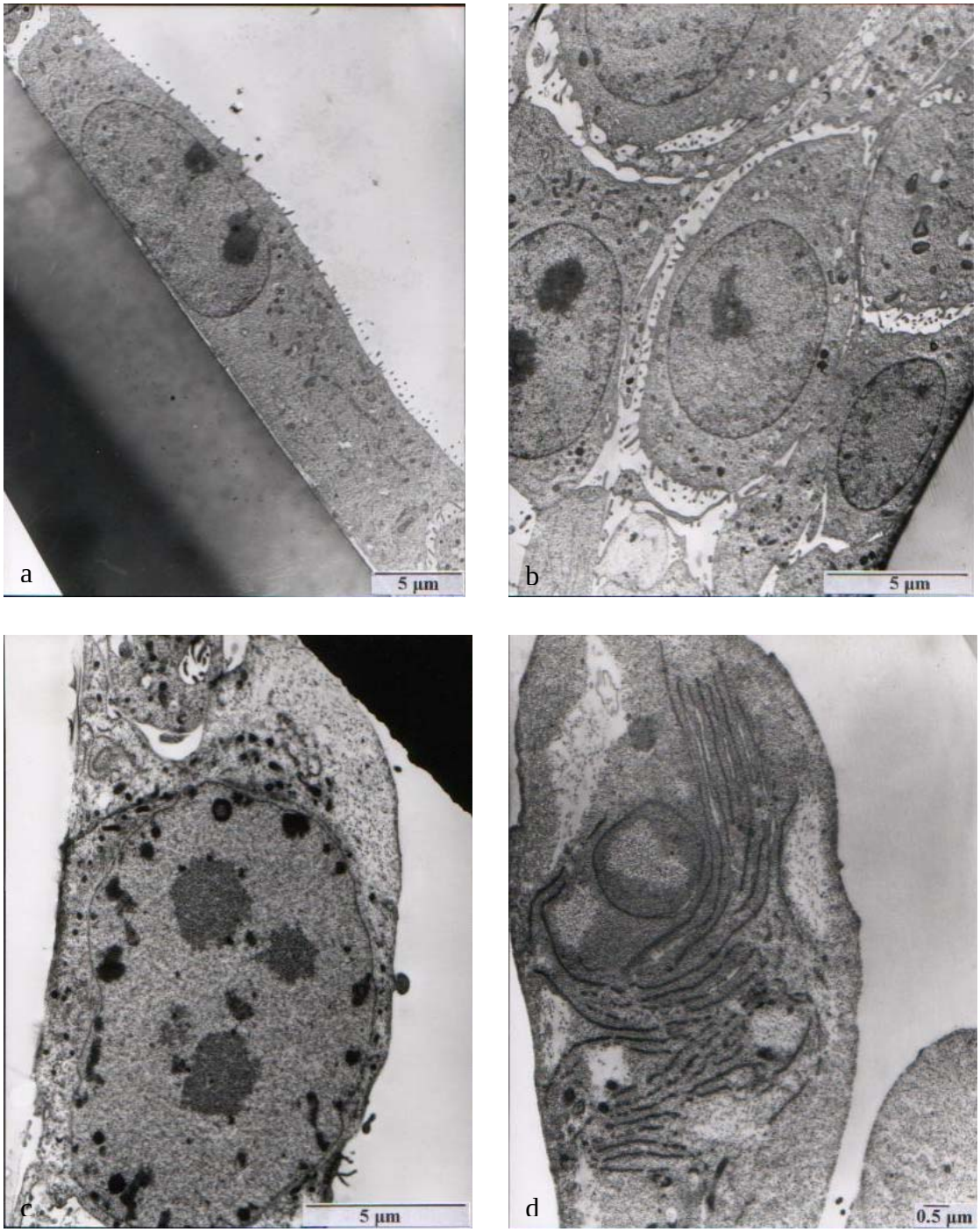


Figure 3.8: TEM studies of PK(15) cells untreated (a,b) or treated with 20 mM LiCl (c-f) for 30 h.

Control cells (a,b) had typical microvilli, the cell nucleus and cytoplasm was normal, and the cellular membrane was intact. Lithium (20 mM) (c,d) treated cells had smoothing of the cell membrane, no microvilli, chromatin condensation and abnormal ultrastructural features.

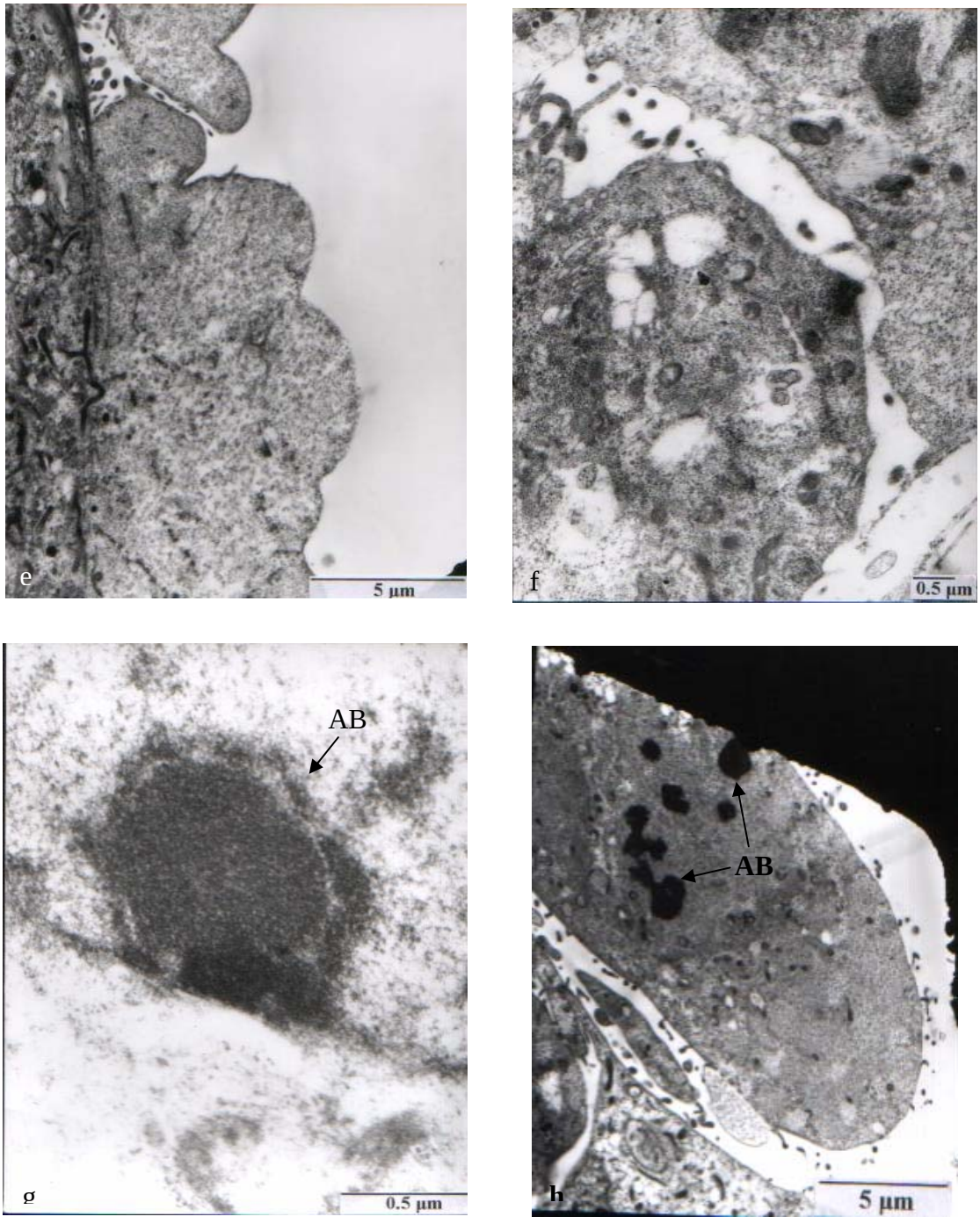


Figure 3.8 cont: TEM studies of PK(15) cells untreated (a,b) or treated with 20 mM LiCl (c-h) for 30 h.

Lobulated cell membranes (e), vacuolisation (f), and apoptotic bodies (**AB**) (g,h) were evident in 20 mM LiCl treated cells, although the cell membrane remained intact and mitochondria were evident.

Ultrastructural changes in PK(15) cells after treatment with 20 mM LiCl for 30 h were observed (Figure 3.8(c-h)). Distinct chromatin condensation was seen along with a ruffling of the nuclear membrane (Figure 3.8 (c)). There were no microvilli and distinct smoothing of the cytoplasmic membrane. These observations were evident throughout. Figure 3.8 (d) illustrates a distinct morphological change and condensation of the endoplasmic reticulum, a feature that was evident in many cells. The ER was fragmented and hyper-ribosylated compared to that in control cells where it was not clearly visible. Figure 3.8(e) illustrates the lobular appearance of many of the cells, a feature distinct from those of the control cells. Vacuolisation of the cytoplasm was apparent (Figure 3.8(f)), however, mitochondria and the integrity of the cell membrane were still evident. Finally, apoptotic bodies, a hallmark of apoptosis, were observed in many PK(15) cells treated with 20 mM LiCl (Figure 3.8 (g,h)).

3.2.3 Discussion

Confluent LLC-PK_1 cells are representative of renal tubular epithelial cells that display proximal tubular biochemistry (Shikano *et al.,* 2004). They have thus been used for toxicology studies and cell morphological changes have been extensively studied (Diemert *et al*., 1995; Hackett *et al*., 1995; Zhang *et al*., 1995; Niewenhuis *et al*., 1997; Zimmerhackl *et al.*, 1998; Filipovic *et al*., 1999; Wardelmann *et al*., 2000; Servais *et al*., 2004). In this study lithium, up to 20 mM did not seem to have any obvious effect on the cell growth and morphology of the LLC-PK_1 cells. The cells did not tend to release from the culture flask substratum, although the lithium treated cells did tend to trypsinise easier than their untreated counterparts (results not shown). These are similar to results reported by Matthopoulos *et al*. (1995) for lithium treated CV1 kidney cells. Furthermore, lithium treated cells were inclined to release easily from the Aclar embedding film used for the electron microscopy studies (results not shown).

Although some reduction of microvilli appearance could be seen on some cells, in essence, the cell morphology did not change in lithium treated cells.

No distinct apoptotic features were observed in these cells in the presence of lithium, compared to the distinctive apoptotic features observed when cells were treated with H_2O_2 (Filipovic *et al*., 1999) i.e. chromatin condensation, condensation and margination. Extensive non-apoptotic cell damage as seen previously by Hackett *et al*. (1995) due to oxalate toxicity, by Diemert *et al.* (1995) with kappa BJP- treatment, or by Wardelmann *et al*. (2000) after NH_4Cl treatment of LLC-PK_1 cells, was also not observed in these studies.

Few ultrastructural changes were noted in LLC-PK_1 cells after incubations with 20 mM LiCl. These results relate to those of the proliferation studies in which 20 mM LiCl had a low toxic effect on the LLC-PK_1 cells up to 72 h, a time interval that showed small differences in the cell proliferation studies (section 3.1), and where all cells are confluent and exhibit proximal tubular characteristics. A small number of anomalies such as blunted microvilli and extensive nuclear folding were observed in a few LiCl treated LLC-PK_1 cells, an observation reported by Niewenhuis *et al*. (1997) in cadmium-LLC-PK_1 toxicity studies. These cellular differences may indicate that the cells are entering into an early phase of apoptosis when external morphological changes are not obvious. It thus seems as though the LLC-PK_1 cells do not undergo extensive cellular damage due to high (20 mM) concentrations of LiCl treatment.

PK(15) cells have characteristics of distal convoluted tubular cells (Aran and Plagemann, 1992a). Although many toxicity related morphological studies have been conducted on MDCK, Mardin-Darby canine kidney cells, also a distal tubular epithelial cell type, no morphological studies on the PK(15) cells could be found. The microscopy studies of the PK(15) cells do, however, show that the cells conform to epithelial cell-like features, grow in a flat monolayer with clusters of growth forming at nucleation points, are polarized and display long microvilli at cell intersections.

Lithium treated cells, 10 mM and above, lifted easily off the culture flasks and Aclar membranes, but in contrast to the LLC-PK_1 cells, the remaining cells stayed firmly attached (results not shown). The loosening and rounding up of

the cells is a phenomenon noted in many other renal toxicity studies, albeit with different cell lines and stimuli (Rybak and Stockdale, 1981; Carranza-Rosales *et al*., 2005; Hackett *et al*., 1995). The damage caused by lithium observed in the light micrographs and the SEM studies seems to confirm an apoptotic – like cell death. Light micrographs clearly show cell blebbing and the production of membranous structures, the presence of condensed chromatin and fragmented nuclei are also visible. SEM micrographs indicate that lithium at concentrations above 10 mM causes extensive blebbing on the cell surface, the release of cells from the membrane support and the smoothing of the membrane. At high lithium concentrations (20 mM) some cells seem to be taking on a necrotic appearance, as manifested by the presence of holes in the plasma membrane. A similar effect was previously observed by Tyobeka and Becker (1990) when HL-60 cells were treated with 50 mM LiCl. This is perhaps an indication that initially cells undergo an apoptotic type death, but after treatment with high concentrations of the agent in question, necrotic type cell death takes over.

Ultrastructural studies confirmed that 20 mM LiCl caused the PK(15) cell plasma membrane to lose its microvilli, become smooth and undergo blebbing. Cell contacts were diminished and condensation of some of the ER was seen. Chromatin condensation, similar to that described by Dini *et al.* (1996) for early apoptosis in U937 cells, nuclear fragmentation, and apoptotic body formation are all seen in 20 mM LiCl treated cells. A similar effect was observed in distal tubular cells taken from renal biopsies of lithium treated patients (Markowitz *et al*., 2000). The PK(15) cells thus seem to be a mixed population of cells undergoing early and late apoptosis.

In comparison to the LLC-PK_1 cells, the PK(15) cells were more susceptible to LiCl treatment, a characteristic not observed in the cell proliferation studies in section 3.1. A notable difference between the two studies is that the LLC-PK1 cells were treated for 72 h without much in the way of overt effects, whilst the damage caused by lithium on the PK(15) cells occurred within 30 h. The PK(15) cells were also more susceptible to LiCl treatment than their MDCK counterparts, as reported by Rybak and Stockdale (1981) who did not

observe any morphological changes due to lithium treatment up to concentrations of 20 mM.

The microscopy studies indicated that LLC-PK_1 cells may be entering into an early apoptotic state after 72 h of LiCl treatment, whilst the PK(15) cells seem to apoptose at concentrations of 10 mM LiCl and above, after only 30 h of treatment. Since necrotic cells have also been observed to undergo some of the features mentioned above, such as blebbing, further characterisation to establish the mode of cell death was required.

3.3 Lithium effects on DNA fragmentation

3.3.1 Introduction

Initially, DNA fragmentation was considered to be one of the hallmark biochemical characteristics of cells undergoing apoptosis (Wyllie *et al*., 1980). The DNA of cells undergoing apoptosis is initially cleaved into large fragments of 500-3000 bp and later into fragments of 200 bp (Oberhammer *et al*., 1993; Walker *et al*., 1997). Some cell types undergoing apoptosis only cleave DNA into high molecular weight fragments whilst others cleave the DNA further into low molecular weight fragments, and some cells, having evidence of condensed chromatin, show no fragmentation at all (Oberhammer *et al*., 1993). A single strand binding nuclease initially cleaves the DNA at matrix attachment regions and later into small products by a nuclease activity on the inter-chromosomal linkages. Thus the cleavage products contain histone proteins as well as DNA (Walker *et al*., 1997). The low molecular weight fragments give a characteristic DNA ladder on agarose gel electrophoresis that is often used as a DNA marker (Wyllie *et al*., 1980). High molecular weight fragments do not form a DNA ladder and thus many cells show morphological characteristics of apoptosis but no DNA ladder.

Although DNA laddering is often used as a hallmark of classical apoptosis, certain anomalies occur. The DNA of cells undergoing necrosis is cleaved irregularly and usually runs as a streak on agarose gels, distinguishing this cell death type from that of apoptosis. Some reports have, however, suggested that some necrotic cells present a DNA ladder on agarose gel electrophoresis (Payne *et al*., 1994; Fujikawa *et al*., 2000). Paraptosis, another type of cell death, was initially described as apoptotic cells lacking in DNA fragmentation, although studies by Fombonne *et al*. (2006) have indicated that DNA fragmentation due to active DNAse II, may occur in this non-classical type of apoptosis. Autophagic cells, on the other hand, have not been shown to express low molecular weight DNA fragments though high molecular weight fragments have been observed (Kim *et al*., 2006).

Another test used to determine the extent of DNA fragmentation of cells is to label the 3'-hydroxyl ends of the DNA (Gavrieli *et al*., 1992). The more DNA strand breaks there are, the more labelling there will be which will make cells containing extensive DNA fragmentation easier to detect. The enzyme usually used to perform this function is terminal deoxynucleotidyl transferase (TdT) which adds labelled dUTP, *in situ*, to DNA fragments.

Although DNA fragmentation is not a conclusive test to determine the type of cell death a cell is undergoing, it is a good indicator to use. Together with other biochemical studies it provides evidence for an apoptotic type of cell death. The DNA fragmentation of both the LLC-PK_1 and PK(15) cell following lithium treatment was investigated.

Results

A DNA ladder was seen in PK(15) cells that were treated with 20 mM LiCl for three days, but was not observed after treatments with LiCl below this concentration. No DNA laddering was evident on the agarose gels for the LLC-PK_1 cells after LiCl treatment (Figure 3.9).

Since the DNA laddering technique is not conclusive for DNA fragmentation and apoptosis, a more sensitive technique was used to assess fragmentation of DNA after LiCl treatment. This was done to validate the results obtained in Figure 3.9 and to confirm that absence of DNA fragmentation in lithium treated LLC-PK_1 cells.

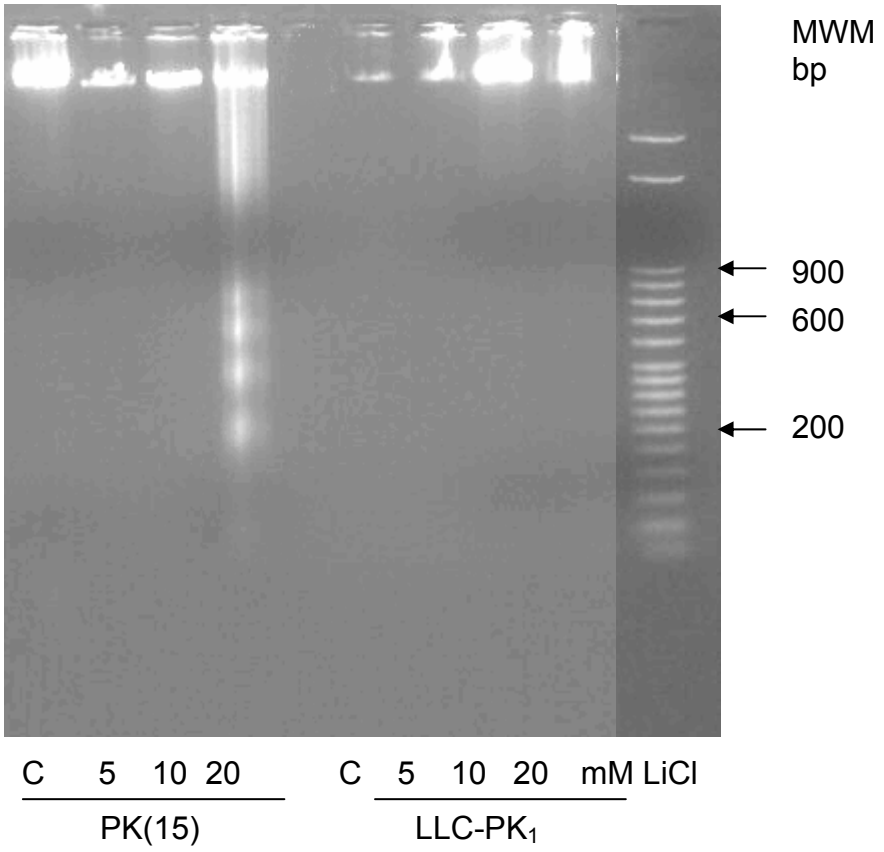


Figure 3.9: Analysis of DNA fragmentation of control and LiCl treated PK(15) and LLC-PK_1 cells. Cells were treated with varying concentrations of LiCl for 72 h.

A positive control of HL-60 cells treated with 20 mM LiCl for 72 h was run in parallel to these studies (Results not shown).

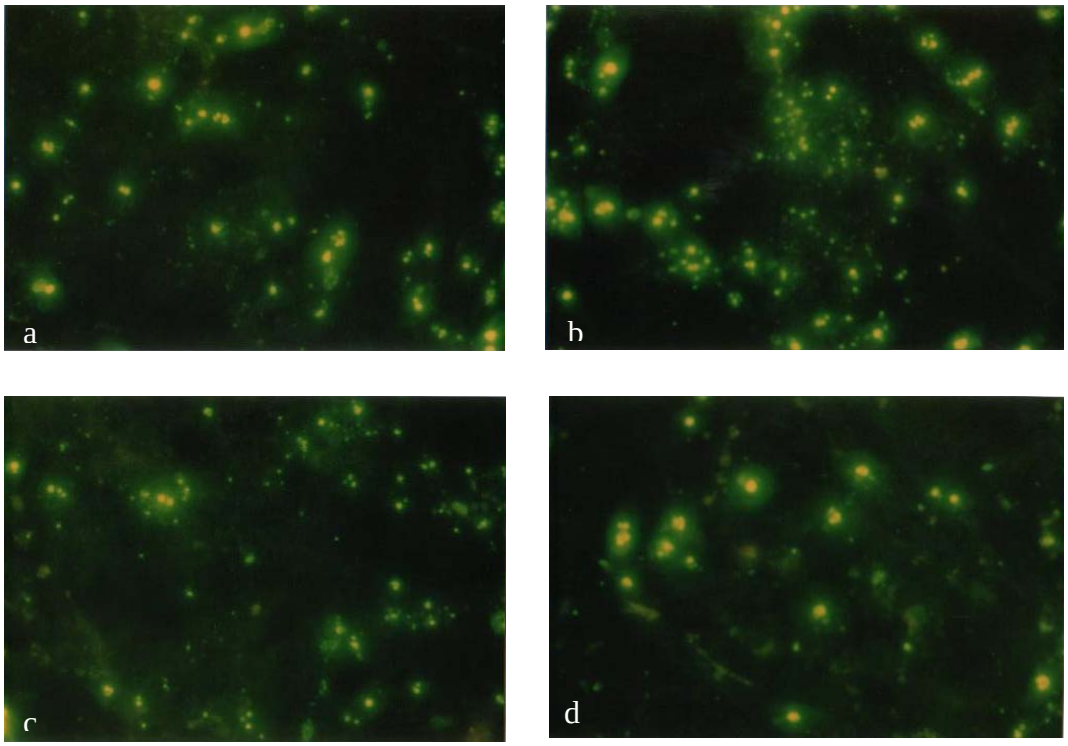


Figure 3.10: Representative micrographs of the TUNEL assay of LLC-PK_1 cells untreated(a) and treated for 72 h with 5 (b), 10 (c) and 20 (d) mM LiCl.
Original magnification 200x. Positive controls using 20 mM treated HL-60 cells and negative controls were conducted in parallel to these studies (not shown).

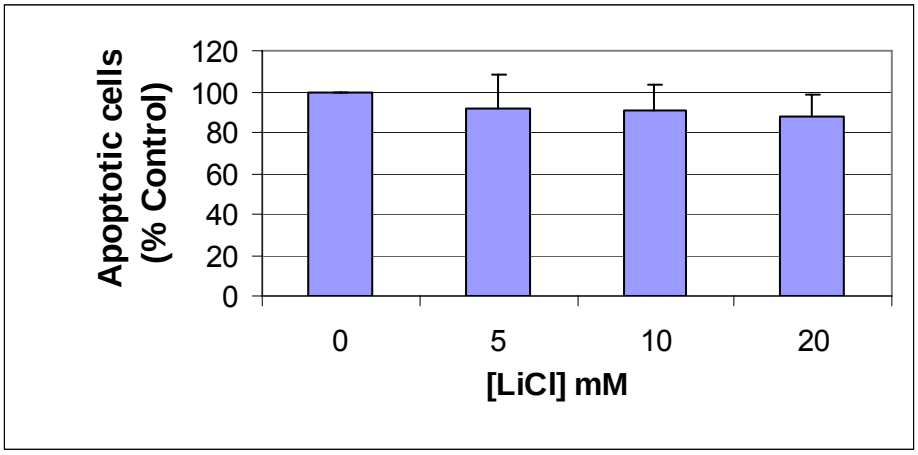


Figure 3.11: The influence of LiCl on the formation of apoptotic LLC-PK_1 cells.
Cells were treated for 72 h with varying concentrations of LiCl and assayed using the TUNEL assay from Roche diagnostics. The figure represents a quantification of the results observed in Figure 3.10 above. Positive cells were counted using the spot-blot application on a Syngene tools Analyser. Data presented as mean ± SEM for three separate experiments.

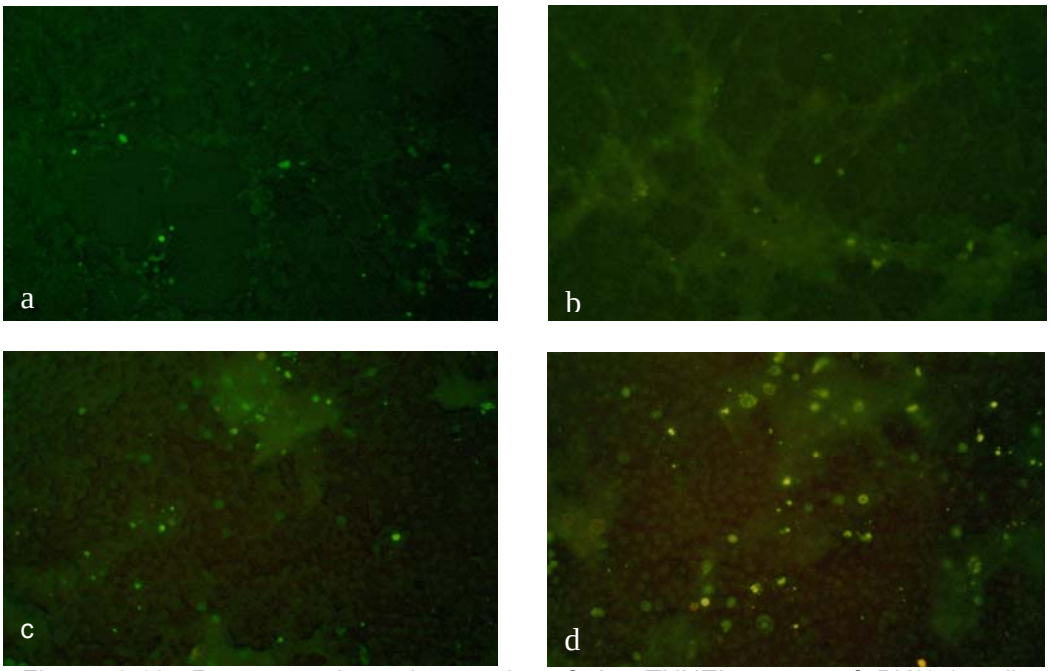


Figure 3.12: Representative micrographs of the TUNEL assay of PK(15) cells untreated (a) and treated for 72 h with 5 (b), 10 (c) and 20 (d) mM LiCl.
Original magnification 200x. Positive controls using 20 mM treated HL-60 cells and negative controls were conducted in parallel to these studies (not shown).

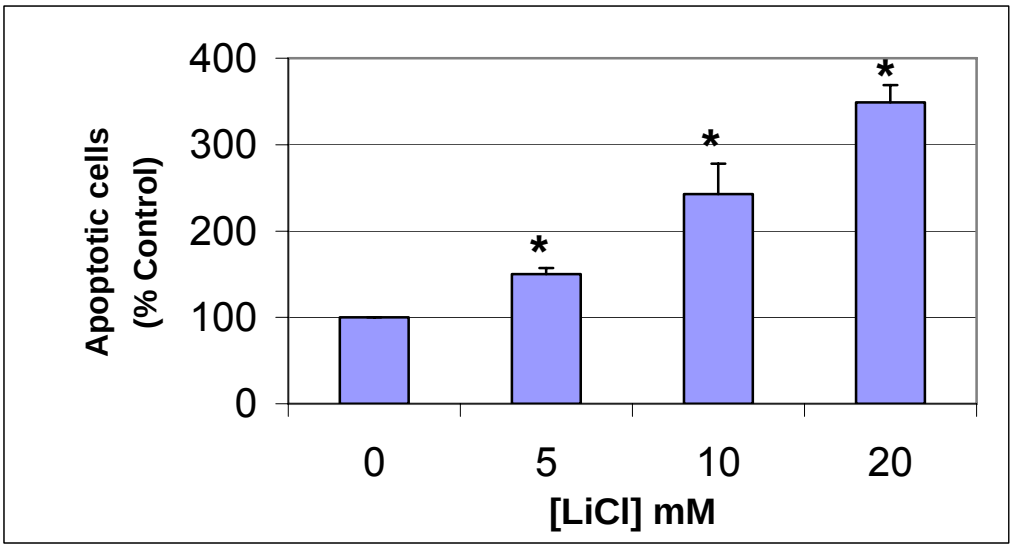


Figure 3.13: The influence of LiCl on the formation of apoptotic PK(15) cells.
Cells were treated for 30 h with varying concentrations of LiCl and assayed using the TUNEL assay from Roche diagnostics. The figure represents a quantification of the results observed in Figure 3.12 above. Positive cells were counted using the spot-blot application on a Syngene tools Analyser. Data presented as mean ± SEM for three separate experiments. (* $p<0.01$ compared to the control)

A terminal deoxinucleotidal transferase dUTP nick end labelling (TUNEL) assay (Roche Diagnostics) was carried out. Micrographs clearly indicate cells undergoing apoptosis (Figures 3.10 and 3.12), even in the control samples (Figures 3.10(a)). Interestingly, the LLC-PK_1 cells had a high amount of

fluorescence in the control as well as all the different lithium concentrations used, although no lithium induced changes were noted. This trend could, however, be a limitation of the method. The LLC-PK_1 cells are confluent at this time interval, and thus probably undergo apoptosis due to normal homeostasis. The procedure was carried out on cells that attached to and grew on a microscope slide. Slides were washed prior to staining and thus only remaining attached cells were analysed. Since dying cells tend to round up and are later released from the support matrix, the fluorescent, and hence DNA fragmentation, positive cells may not have been released from the substratum. Furthermore, the slight decrease in fluorescence due to LiCl treatment (Figure 3.11) may be explained by the later stage apoptotic cells being released, whilst those in early apoptosis are still bound to the substratum.

In contrast to the above mentioned results, PK(15) cells treated with LiCl had an increased amount of fluorescence, and thus DNA fragmentation, (Figure 3.12 (a-d)) in a lithium concentration dependant manner. Cells treated with 20 mM LiCl resulted in more than a three-fold increase that was significant ($p<0.01$) to that of the control (Figure 3.13).

Discussion

DNA laddering, a characteristic of apoptosis, was previously observed in LLC-PK_1 cells undergoing PCD because of cell injury caused by H_2O_2 (Filipovic *et al*., 1999) and albumin (Erkan *et al*., 2001). In addition, TUNEL assay positive LLC-PK_1 cells undergoing apoptosis caused by bismuth injury were observed by Leussink *et al.* (2002). It thus seemed reasonable that the lithium induced cytostatic effects observed from the cell proliferation studies (section 3.1) may be explained by the cells undergoing apoptosis.

The results observed from DNA laddering and TUNEL assays indicate conflicting results in the LiCl treated LLC-PK_1 cell studies. No DNA laddering was observed in agarose gels, but widespread fluorescence was noted throughout all the concentrations (0-20 mM) of lithium used. Similar studies,

performed on Wil-2 lymphoma cells, indicated that a low concentration of lithium caused cells to stain positively in the TUNEL assay, but did not show a DNA laddering effect on agarose gels (Mukhufi, 2000; Molepo, 2004). The Wil-2 cell DNA did, however, fragment into high molecular weight DNA fragments (Mukhufi, 2000), a phenomenon known to occur in apoptotic as well as autophagic cells (Kim *et al*., 2006). These results parallel those observed in the present study, although high fragmentation analysis was not conducted due to the absence of a lithium induced effect.

Doubt on the significance of the TUNEL assay has been laid by evidence indicating that DNA strand breaks are often observed after the formation of apoptotic bodies. Although DNA strand breaks have been detected in the pre-apoptotic phase of cells, discussions leading to the validity of TUNEL assay as an apoptosis assay have ensued (reviewed in Baima and Sticherling, 2002).

Although extensive DNA fragmentation was observed throughout the LLC-PK_1 studies using the TUNEL assay, there seemed to be no lithium induced differences between the control and 20 mM LiCl treatments and thus the fragmentation observed was not as a result of lithium treatment.

Lithium induced DNA fragmentation in PK(15) cells was observed with both the DNA fragmentation laddering method and the TUNEL assay. Similar DNA laddering was observed by Madiehe *et al*. (1995) when 20 mM LiCl was used to treat HL-60 cells and by D'Mello *et al*. (1994) on immature cerebellar granule cells, but not on mature granule cells.

Since low molecular weight DNA fragmentation and late *in situ* DNA end nick labelling (TUNEL) can occur in necrotic as well as apoptotic cells (Fujikawa *et al*., 2000), the above results only imply the presence of apoptosis but autophagy and necrosis cannot be ruled out. Sarkar *et al*. (2005) have previously shown lithium toxicity to be autophagic in African Green Monkey renal cells and lithium has been implicated in necrotic associated tubular nephrotoxicity in renal cells (Markowitz *et al*., 2000). Thus, the use of

biochemical markers to establish the mode of lithium induced cell death of the PK(15) and toxicity of LLC-PK_1 cells was deemed necessary.

Apoptotic and cell cycle proteins implicated in the apoptotic process

3.4.1 Introduction

Cell proliferation, survival and death are closely associated and various proteins can affect more than one pathway, depending on their state. After treatment with certain stimuli, proteins can be upregulated or downregulated, they can be activated by phosphorylation or dephosphorylation, or by cleavage from a zymogen to the active protein. Thus, many proteins directly involved in cell cycle inhibition or progression also have a role to play in cell death, be it necrosis or apoptosis.

In the previous section evidence presented strongly supported lithium to be a trigger of apoptosis at certain concentrations in PK(15) cells. Lithium may thus be the stimulus to an event that requires an entire network of proteins to be activated or deactivated to initiate the apoptotic response. Due to the complexity and the enormity of the protein complexes involved in apoptosis, it was not possible to evaluate the lithium induced effects on all of them. Only a few key proteins relevant in maintenance of the cell cycle and apoptosis were, thus, investigated in this study. Bax, Bcl-2, and caspases 3, 8 and 9 proteins implicated in the apoptotic processes, as well as p53, a multifaceted protein, were probed for their involvement in the cytotoxic effect caused by lithium on LLC-PK_1 and PK(15) porcine renal cells. Proliferating cell nuclear antigen and retinoblastoma protein (Rb) were used to establish their role in lithium induced cell cycle effects.

It has been previously reported that 1 mM LiCl upregulates Bcl-2 in stressed and unstressed human SH-SY5Y cells by 65 %. In the same cells chronic, but not acute, lithium treatment repressed cytochrome C release and caspase 3 activity (Lai *et al*., 2006). Furthermore, Yuan *et al.* (1998) and Chen and Chuang (1999) had previously observed that chronic lithium treatment of cerebellar granule cells led to decreased transcription of p53 and Bax mRNA with a parallel increase in the Bcl-2/Bax ratio. Lithium has thus been shown, using biochemical markers, to have a cytoprotective or anti-apoptotic effect in

stressed neural cells. Lithium has also been shown to be cytoprotective against etoposide treated HepG, hepatoblastoma cells. Biochemically, lithium inhibited Fas expression and clustering on the cell membrane, with concomitant inhibition of caspase 8 activity. Bax translocation to the mitochondrial membrane was also suppressed as was the release of mitochondrial cytochrome C (Beural *et al*., 2004). Together, the above mentioned results indicate that the lithium induced cytoprotection of these apoptotic stimulated cells, is manifested via the mitochondrial apoptotic route.

Lithium was found to enhance apoptosis in some non-neural cell lines (Madiehe *et al*., 1995; Beyaert, 1999). Specifically, lithium has increased TNF mediated apoptosis in rhabdomyosarcoma cells by increasing caspase 8 and caspase 3 activities (Schotte and Beyaert, 1999), and caspase 3 activities in Fas mediated apoptosis of neural differentiated Jurkat cells (Song *et al*., 2004). Mampuru *et al*. (1999) established that in bone marrow stromal cells, apoptosis occurred due to increased Bax/Bcl-2 levels, and Molepo (2004) established that Bax expression is increased in Wil-2 cells undergoing apoptotic death. Furthermore, Molepo (2004) found that Wil-2 cells undergoing apoptosis had elevated activity levels of caspase 3, 8 and 9.

Watcharasit *et al.* (2003) found that GSK3β binds to and promotes the actions of p53 after DNA damage. These important observations indicated that inhibition of GSK3β did not alter p53 binding but stabilised its association and prevented p53 mediated transcription of Bax and p21, and hence prevented p53 activation of caspase 3. This was also noted by Beural *et al*. (2004), and Mao *et al*. (2001) found that lithium stabilised p53 and inhibited cell cycle progression by arresting BAEC in the G_2/M stage. Evidence that lithium blocks cell cycle progression and arrests cells in the G_2/M stage was presented for promyelocytic cells (Madiehe *et al*., 1995; Wachira *et al.*, 1998), erythroleukemic cells (Tang *et al*., 2005), and for embryonal carcinoma, neuroepithelial and osteosarcoma cells (Smits *et al*., 1999).

Lithium thus seems to have a role in mitochondrial mediated cell protection as well as apoptosis, depending on the type of cells used. Furthermore, cell proliferation inhibition studies have clearly indicated that lithium prohibits cells from moving through the G_2/M stage of the cell cycle in some cell types. The following studies investigated the mode of lithium induced apoptosis and its effect on the cell cycle of LLC-PK_1 and PK(15) renal cells.

3.4.2 Results

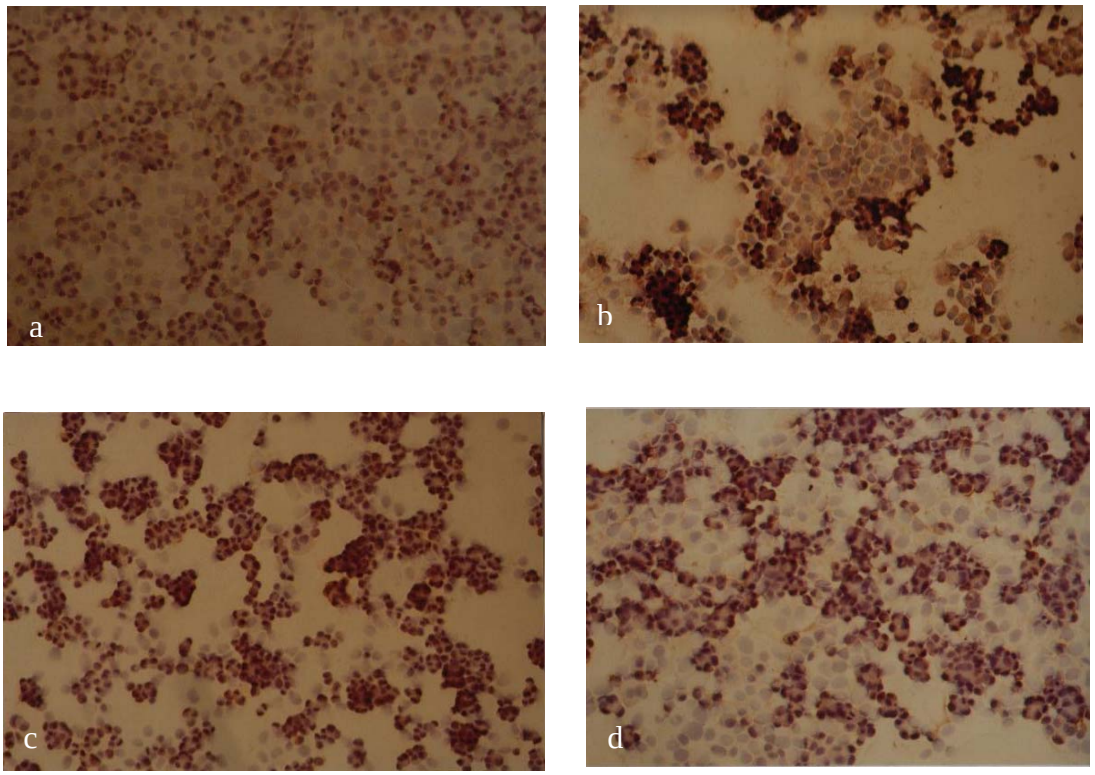
3.4.2.1 Apoptosis analysis

Immunocytochemistry revealed no lithium induced changes in Bax expression in LLC-PK_1 cells (Figure 3.14). In contrast, immunocytochemistry revealed that the percentage of PK(15) cells positive for a high expression of Bax protein increased in a LiCl concentration dependant manner, with a statistically significant two-fold difference after 20 mM LiCl treatment for 24 h (Figure 3.15). Western blot studies analysing the amount of Bax expression differed from immunocytochemical results (Figure 3.18). In these studies, Bax expression seemed to decrease with increasing lithium concentrations in LLC-PK_1 cells, whereas Bax levels increased in PK(15) cells after 5 mM LiCl treatment with a slight further increase in 10 and 20 mM LiCl treated cells, though the difference was not as large as that observed in the immunocytochemistry observations. Differences in these results may stem from the analysis of the results. In immunocytochemistry, the number of cells positive for the protein was assessed, whereas in the Western blot analysis, cell lysates were obtained and thus a change in total protein expression was observed. The results seem to indicate that Bax is regulated differently in the two cell lines following lithium exposure, with an increase in expression in the PK(15) lithium treated cells as compared to their untreated counterparts.

A uniform high percentage of cells positive for p53 was observed throughout the immunocytochemical studies of lithium treated LLC-PK_1 cells (Figure 3.16) and no change in total LLC-PK_1 cell p53 protein expression as revealed by western blot analysis (Figure 3.18). P53 was slightly, yet insignificantly, elevated, as shown by immunocytochemistry, after 24 h of 5 and 10 mM

treatment, returning to the control cell level at 20 mM lithium in the PK(15) cells (Figure 3.17). These results concur with those of the Western blot analysis of p53 protein in PK(15) lithium treated cells (Figure 3.18).

Western blot analysis of the anti-apoptotic protein, Bcl-2, revealed a lithium concentration dependant decrease in expression in both the LLC-PK_1 and PK(15) cells (Figure 3.18). Analysis of the Western blot results indicated that the Bax/Bcl-2 ratios in the two lithium treated cell lines differed substantially. No substantial difference in the Bax/Bcl-2 (Table 3.2) ratio was observed in the lithium treated LLC-PK_1 cells. This analysis agreed with the morphological and DNA fragmentation studies which indicated that lithium did not induce the cells to undergo apoptosis. The Bax/Bcl-2 ratio, however, increased notably for the lithium treated PK(15) cells in a dose dependant manner, a two-fold difference noted between the control and 20 mM LiCl treated cells. Again, these results supported the morphological and DNA fragmentation results of the previous section, whereby LiCl at 10 mM and above, seemed to induce the cells to undergo apoptosis. P53 analysis of the Western blots appeared to show little difference in LLC-PK_1 lithium treated cells, whereas the PK(15) cells had elevated levels at 5 mM LiCl followed by a decrease in p53 expression. Only two separate experiments were conducted using Western blot analysis, and thus statistical differences could not be calculated.



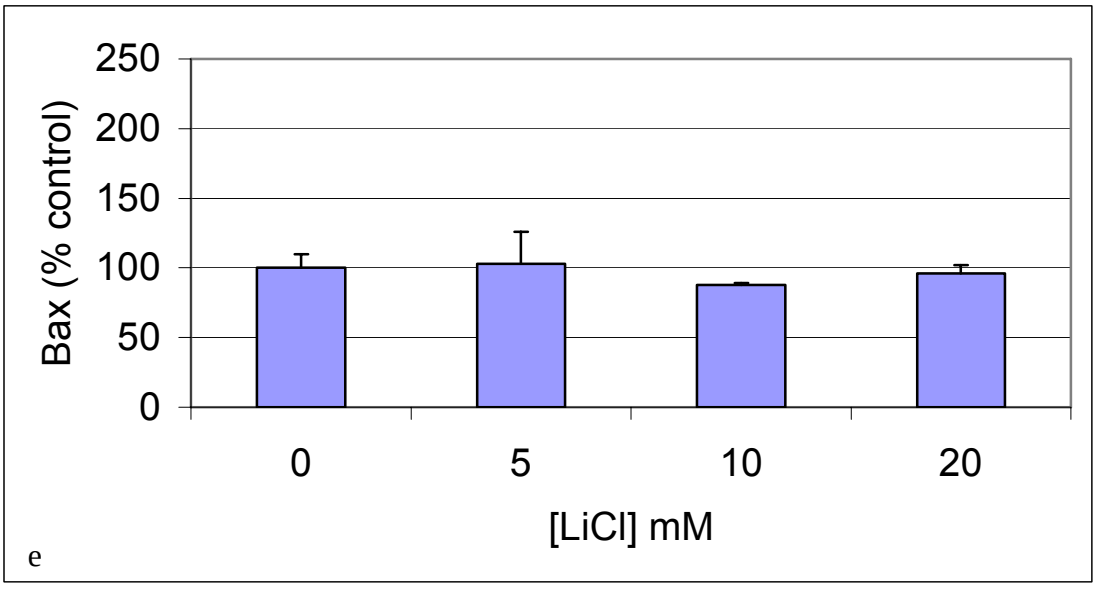


Figure 3.14: Immunocytochemistry of LLC-PK_1 cells treated with (a) 0, (b) 5, (c) 10, (d) 20 mM LiCl for 24 h to detect the expression of Bax protein.

Original magnification, 200x.

Protein expression was assessed (e) as described in chapter 2. No significant difference was found compared to control studies using Student's t-test, n=3.

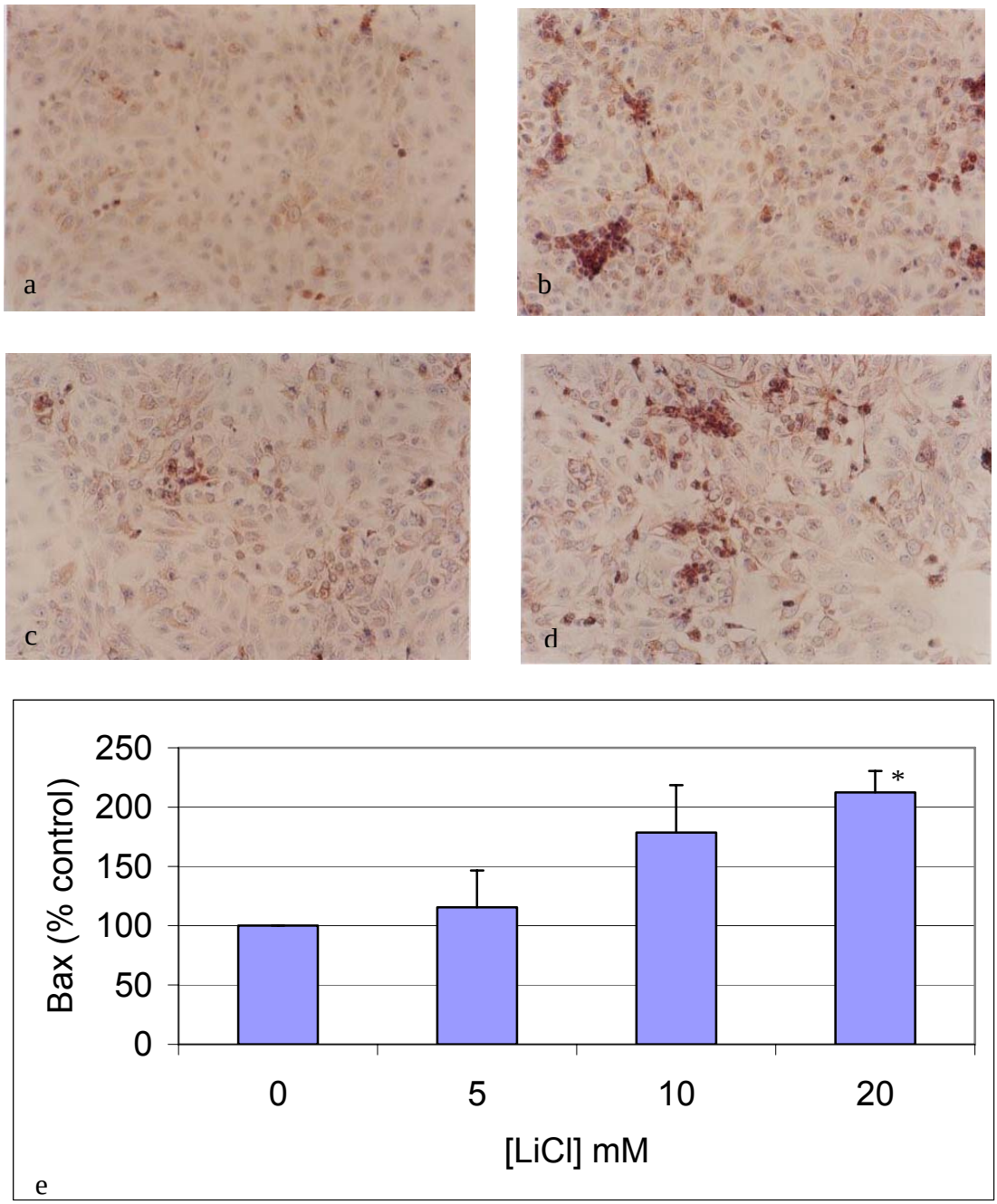
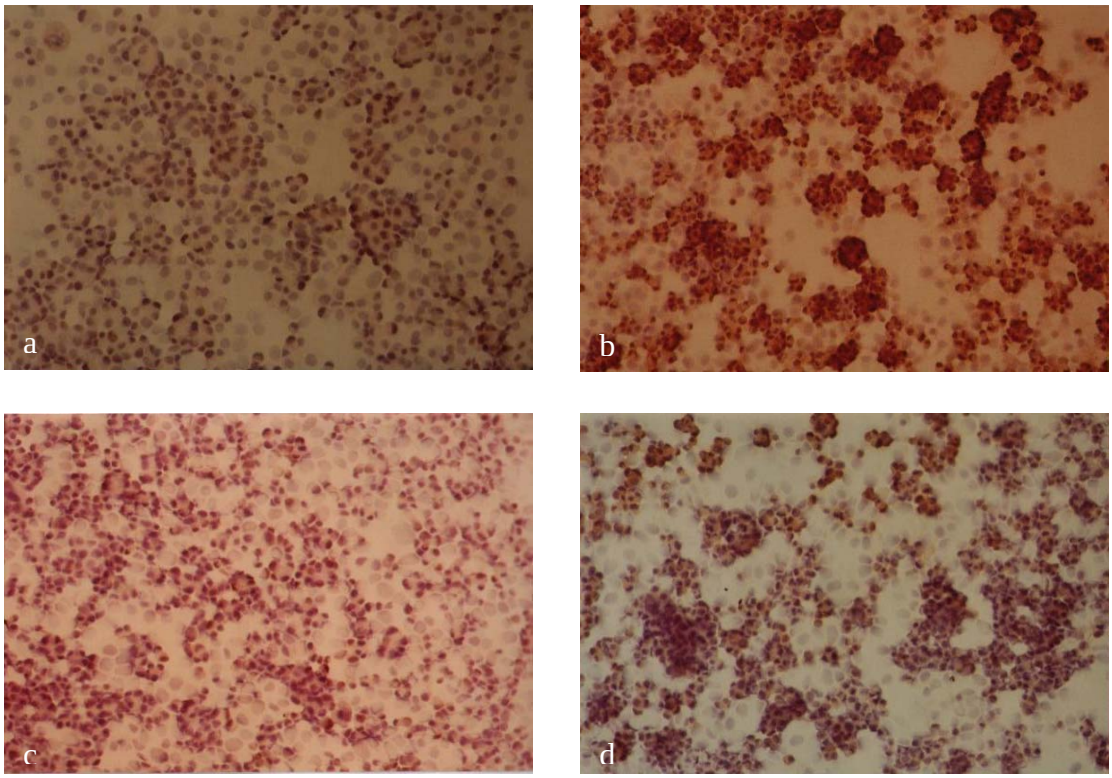


Figure 3.15: Immunocytochemistry of PK(15) cells treated with (a) 0, (b) 5, (c) 10, (d) 20 mM LiCl for 24 h to detect the expression of Bax protein.

Original magnification, 200x.

Protein expression was assessed (e) as described in chapter 2. Statistical difference was found compared to control studies using Student's t-test.*$p<0.05$, n=3.



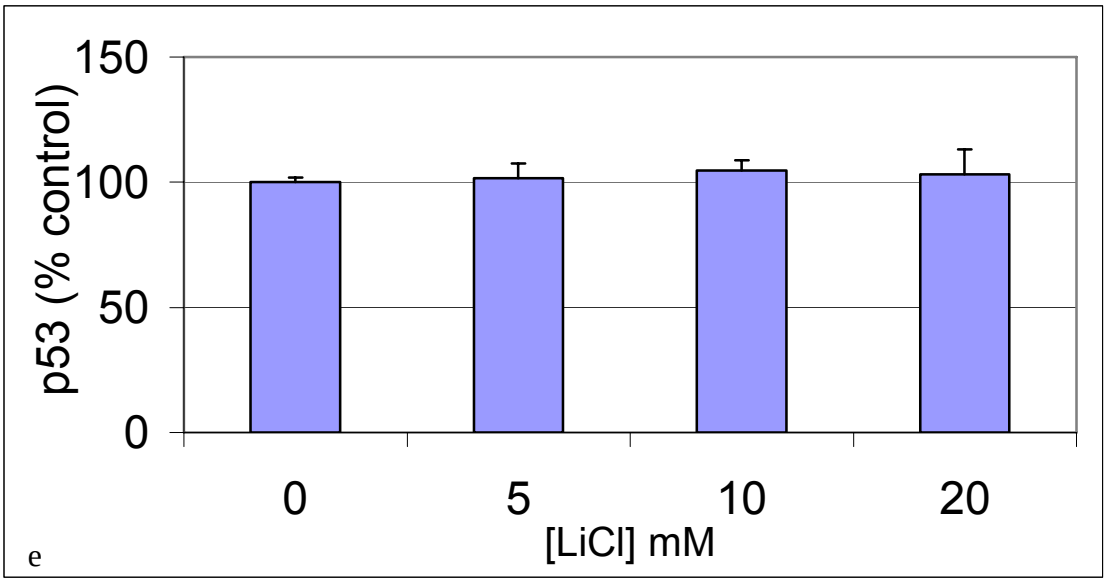


Figure 3.16: Immunocytochemistry of LLC-PK cells treated with (a)0, (b)5, (c)10, (d)20 mM LiCl for 24 h to detect the expression of p53 protein.

Original magnification, 200x.

Protein expression was assessed (e) as described in chapter 2. No significant difference was found compared to control studies using Student's t-test, n=3.

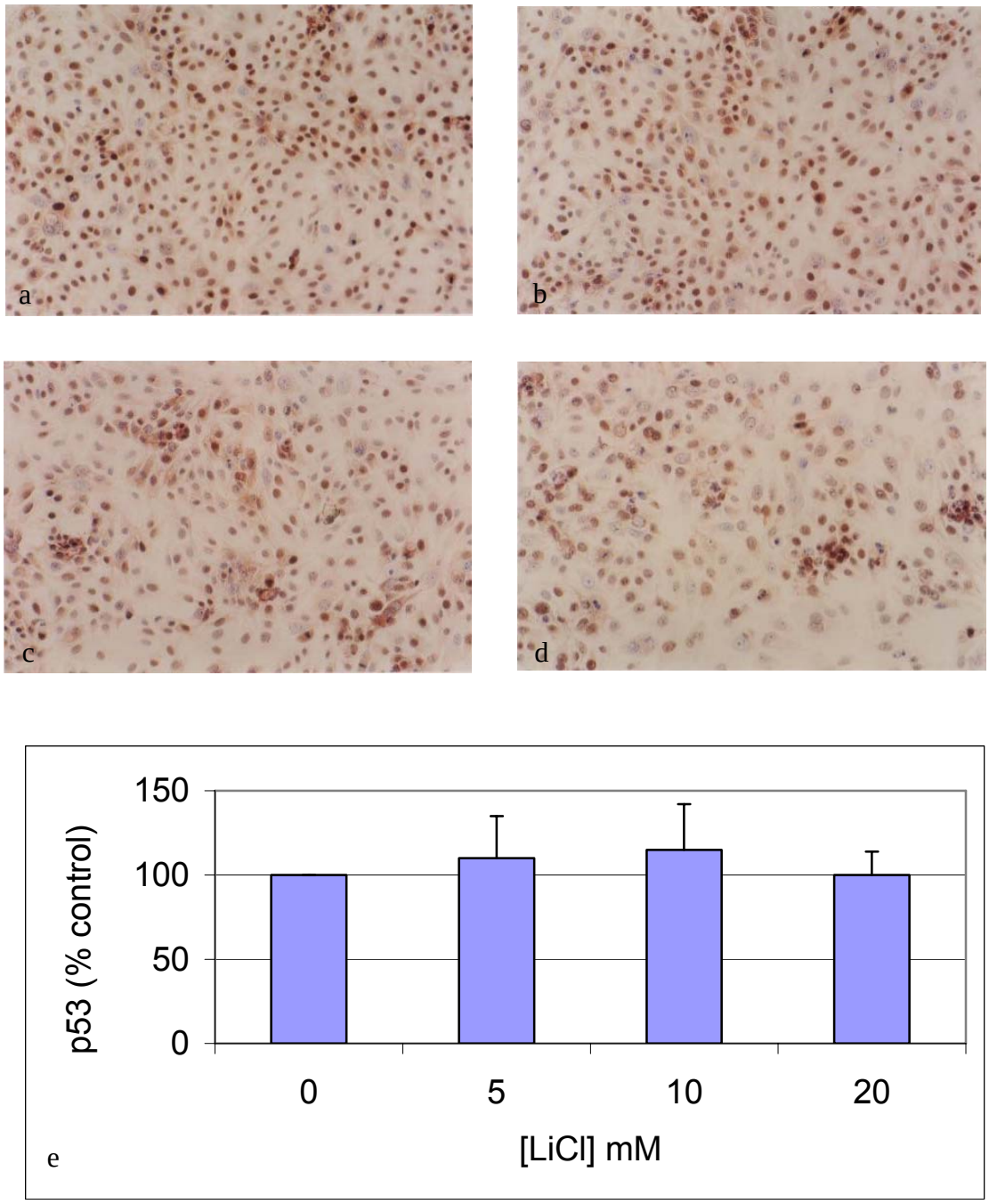


Figure 3.17: Immunocytochemistry of PK(15) cells treated with (a) 0, (b) 5, (c) 10, (d) 20 mM LiCl for 24 h to detect the expression of p53 protein.

Original magnification, 200x.

Protein expression was assessed (e) as described in chapter 2. No significant difference was found compared to control studies using the Student's t-test, n=3.

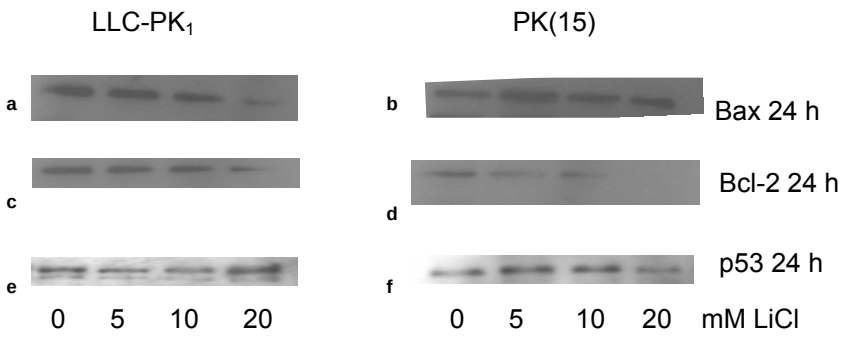


Fig 3.18: Western blot analysis of key apoptotic proteins, Bax (a,b), Bcl-2 (c,d) and p53 (e,f) of 24 h lithium treated and untreated cell lysates of LLC-PK_1 (a,c,e) and PK(15) (b,d,f) porcine renal cells.

Table 3.2: Analysis* of the Western blot results obtained for p53, Bax and Bcl-2 proteins from lysates of LLC-PK_1 and PK(15) cells treated or untreated with varying concentrations of lithium for 24 h.

Cell	Protein	mM LiCl for 24 hr			
		0	5	10	20
LLC-PK	p53	1.00	0.97	0.96	1.22
	Bcl-2	1.00	0.92	0.88	0.58
	Bax	1.00	0.86	0.78	0.73
	Bax/Bcl-2	1.00	0.94	0.89	1.26
PK(15)	p53	1.00	1.47	1.00	0.66
	Bcl-2	1.00	0.97	0.84	0.74
	Bax	1.00	1.54	1.59	1.64
	Bax/Bcl-2	1.00	1.59	1.88	2.21

* Analysis was conducted using a Syngene Tools analyser.

Caspases, proteins known to play important roles in apoptosis through the mediation of different apoptotic pathways, were assessed for lithium induced changes in activity. Control studies using no lysate, or alternatively no substrate, were run in parallel to the studies and thus all activities are related to the activity present in the control (absence of lithium) result. Caspase 8 (Figure 3.19) and caspase 9 (Figure 3.21) activities in the LLC-PK_1 cells increased only after cells were treated with 20 mM LiCl, with no change in the 1 mM, 5 mM nor 10 mM LiCl treatments. Due to the high amount of variance between repeated experiments, however, these changes cannot be

considered to be significant. Caspase 8 activity in LiCl treated PK(15) cells (Figure 3.20) was not altered by lithium treatment, whereas caspase 9 activity seemed to increase slightly in 10 mM LiCl treated PK(15) cells, returning to near control values after treatment with 20 mM LiCl (Figure 3.22). Once again, however, due to the high variance in these results no definite conclusions could be made in this regard. Very little difference in caspase 3 activity was observed for both LLC-PK_1 (Figure 3.23) and PK(15) cells (Figure 3.24) after LiCl treatment. These results imply that caspase 3 activity is not affected by LiCl treatment in these two cell lines.

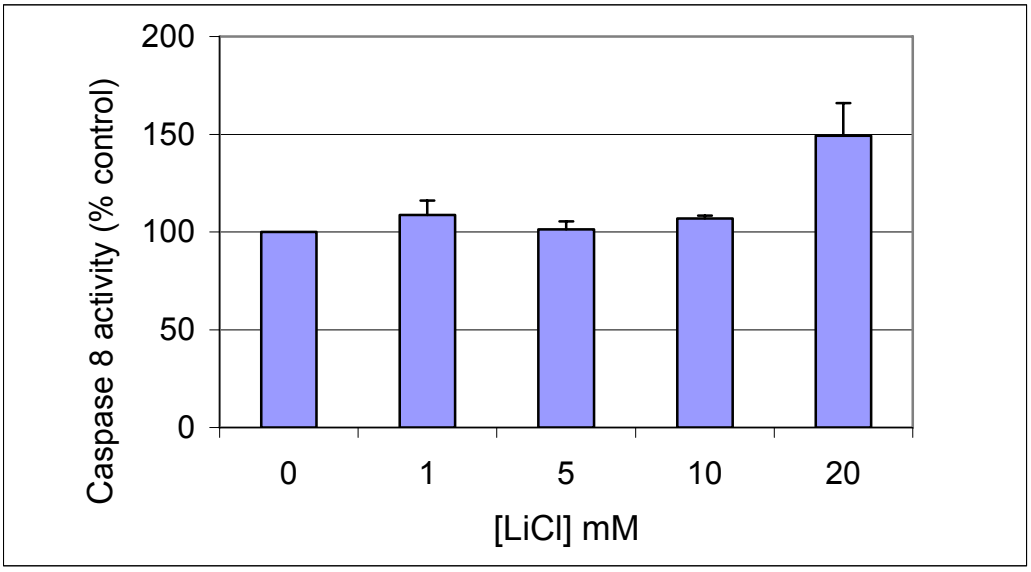


Figure 3.19: Caspase 8 assay of LLC-PK_1 cells treated with or without LiCl for 24 h. Results are presented as a percentage of control. SEM was determined from an average of 3 experimental results. No significant difference was found compared to control studies using Student's t-test.

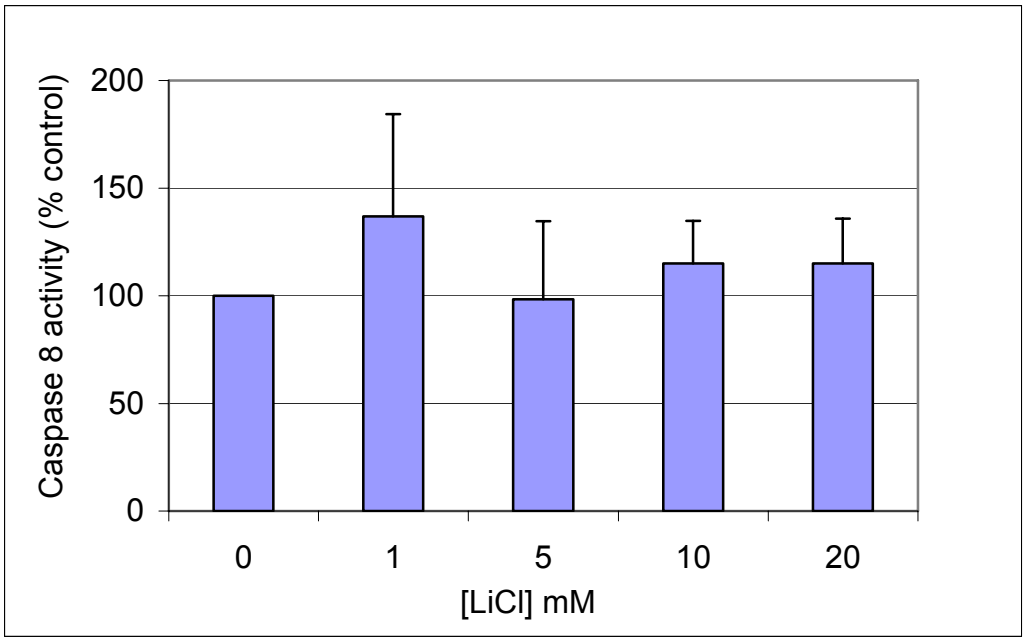


Figure 3.20: Caspase 8 assay of PK(15) cells treated with or without LiCl for 24 h. Results are presented as a percentage of control. SEM was determined from an average of 3 experimental results. No significant difference was found compared to control studies using Student's t-test.

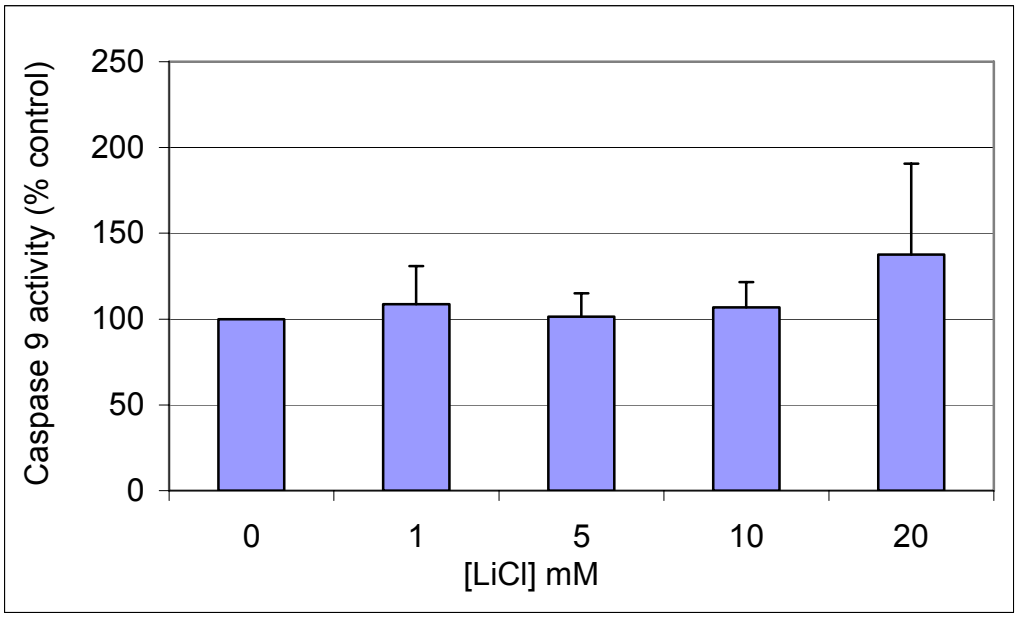


Figure 3.21: Caspase 9 assay of LLC-PK_1 cells treated with or without LiCl for 24 h. Results are presented as a percentage of control. SEM was determined from an average of 3 experimental results. No significant difference was found compared to control studies using Student's t-test.

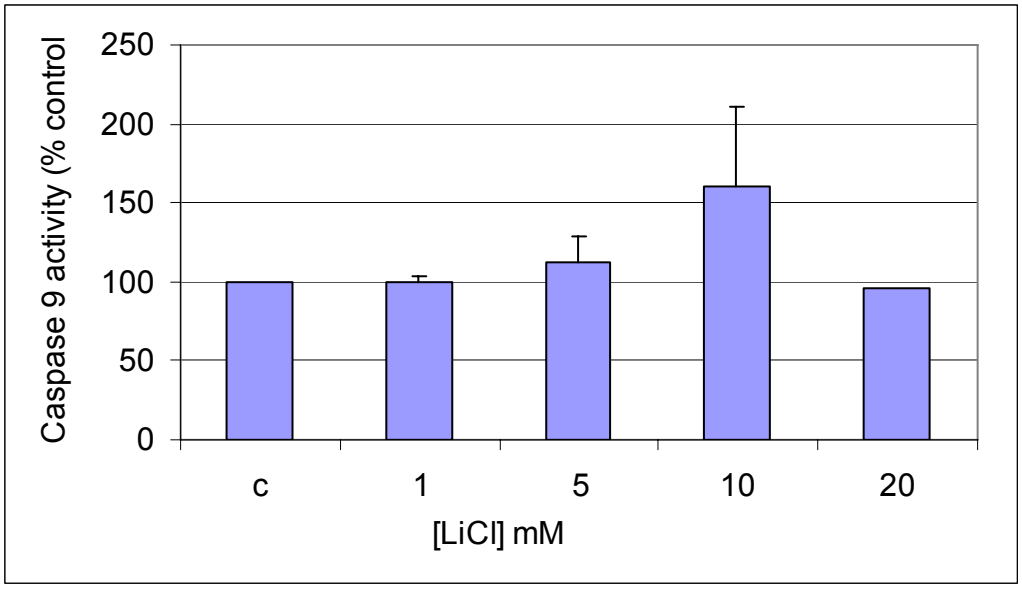


Figure 3.22: Caspase 9 assay of PK(15) cells treated with or without LiCl for 24 h. Results are presented as a percentage of control. SEM was determined from an average of 3 experimental results. No significant difference was found compared to control studies using Student's t-test.

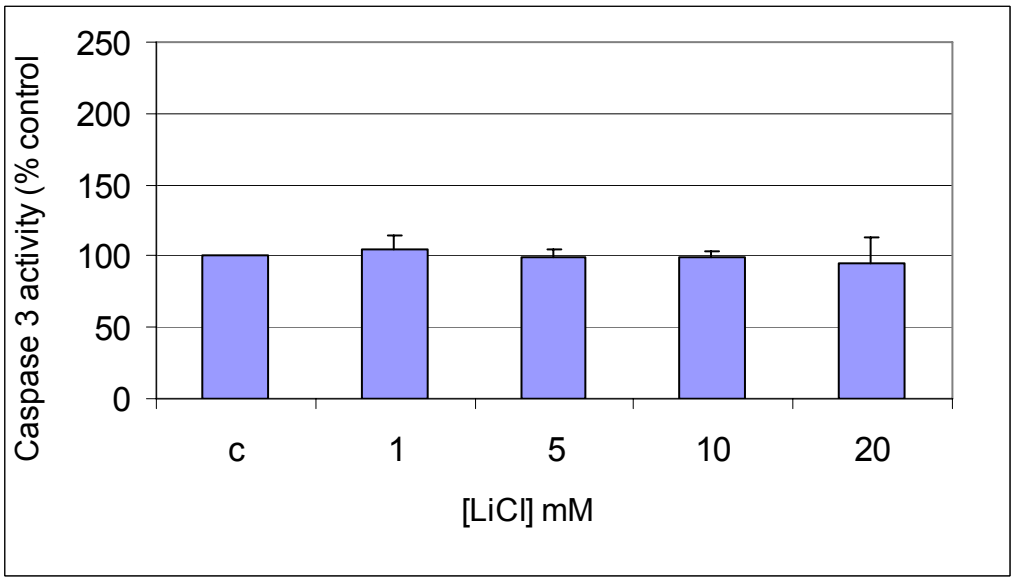


Figure 3.23: Caspase 3 assay of LLC-PK_1 cells treated with or without LiCl for 24 h. Results are presented as a percentage of control. SEM was determined from an average of 3 experimental results. No significant difference was found compared to control studies using Student's t-test.

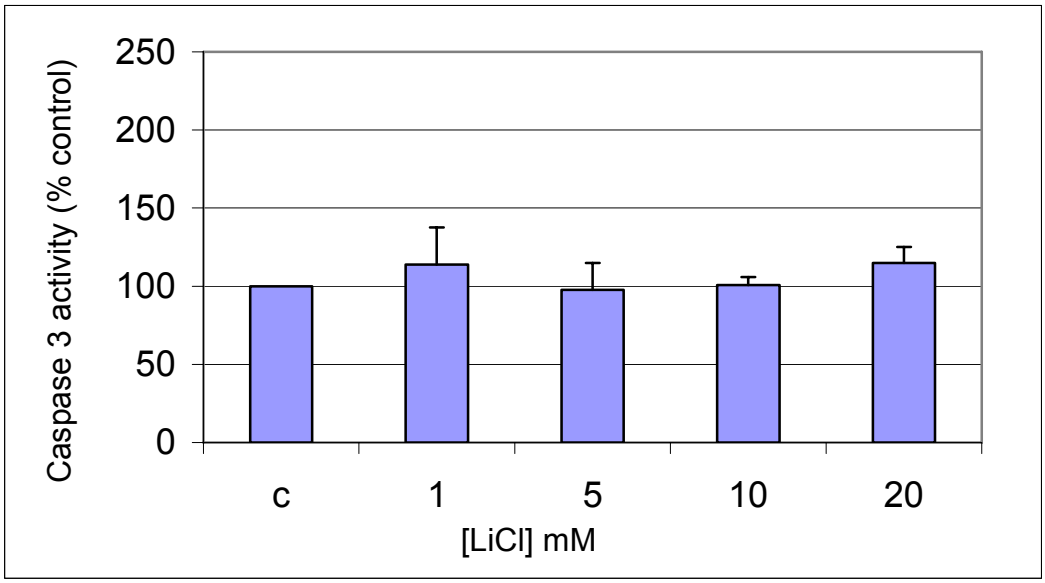
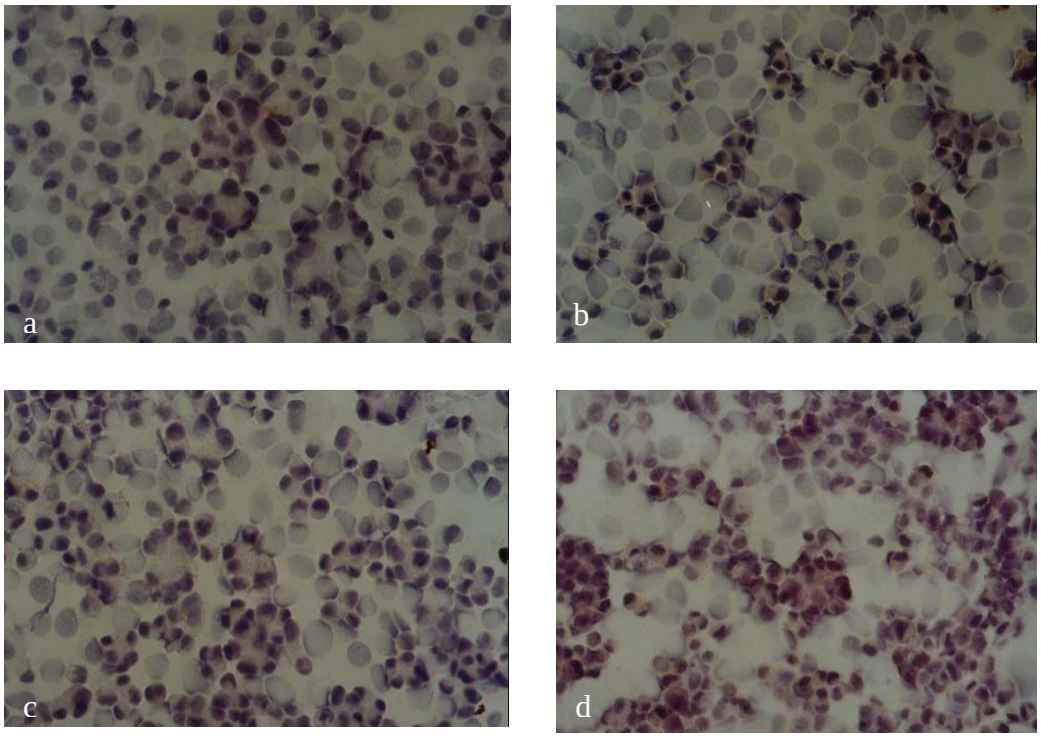


Figure 3.24: Caspase 3 assay of PK(15) cells treated with or without LiCl for 24 h. Results are presented as a percentage of control. SEM was determined from an average of 3 experimental results. No significant difference was found compared to control studies using Student's t-test.

3.4.2.2 Cell cycle analysis

Previous studies have determined that lithium inhibits cell cycle progression in some cell lines. The effect of lithium on the cell cycle and its key proteins, (PCNA, Rb, together with p53) was assessed. Immunocytochemistry assay results indicated that although a decrease in PCNA positive cells at 5 mM LiCl treatment was observed (Figure 3.25), there was no significant change in LLC-PK_1 PCNA protein expression in lithium treated samples compared to their untreated counterparts. In PK(15) cells, lithium treatment increased the amount of PCNA expressed in a concentration dependant manner, a significant difference being seen at 10 and 20 mM LiCl reaching as much as 2.5 times that of the control (Figure 3.26). LLC-PK_1 cells treated with increasing amounts of LiCl showed an increased content of Rb protein at 5 and 10 mM LiCl but returned to the same amount as the control at 20 mM (Figure 3.27). The amount of Rb protein at 5 and 10 mM LiCl were statistically different to those of the control study. Similar changes in the amount of Rb protein were observed in the LiCl treated PK(15) cells. Cells treated with 5 and 10 mM LiCl had an increase in the amount of Rb compared to their untreated and 20 mM LiCl treated counterparts.



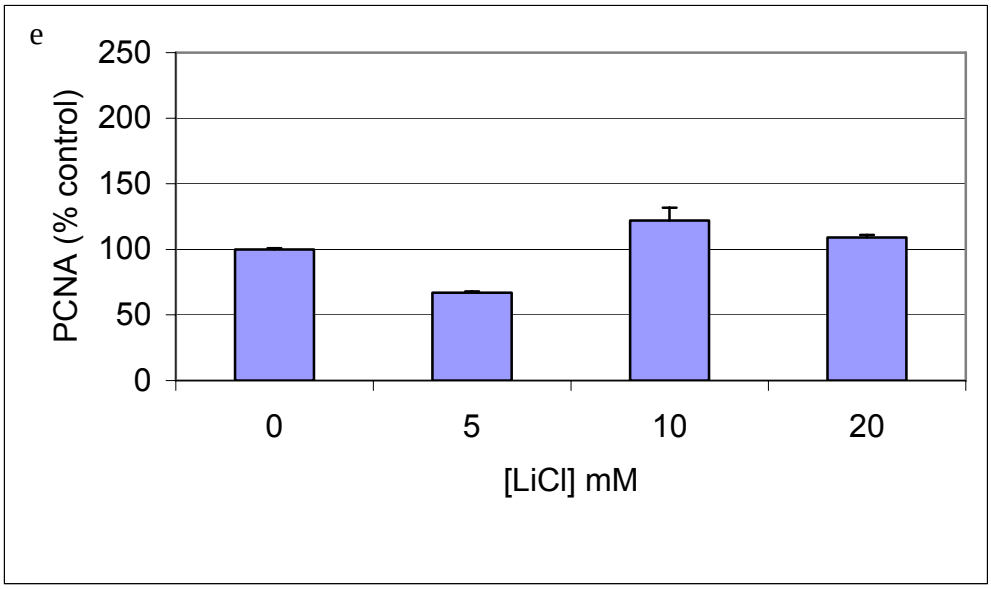


Figure 3.25: Immunocytochemistry of LLC-PK$_1$ cells treated with (a) 0, (b)5, (c) 10, (d) 20 mM LiCl for 24 h to detect the presence of PCNA. Original magnification, 400x.
Protein expression was assessed (e) as described in chapter 2. No significant difference was found compared to control studies using Student's t-test, n=3.

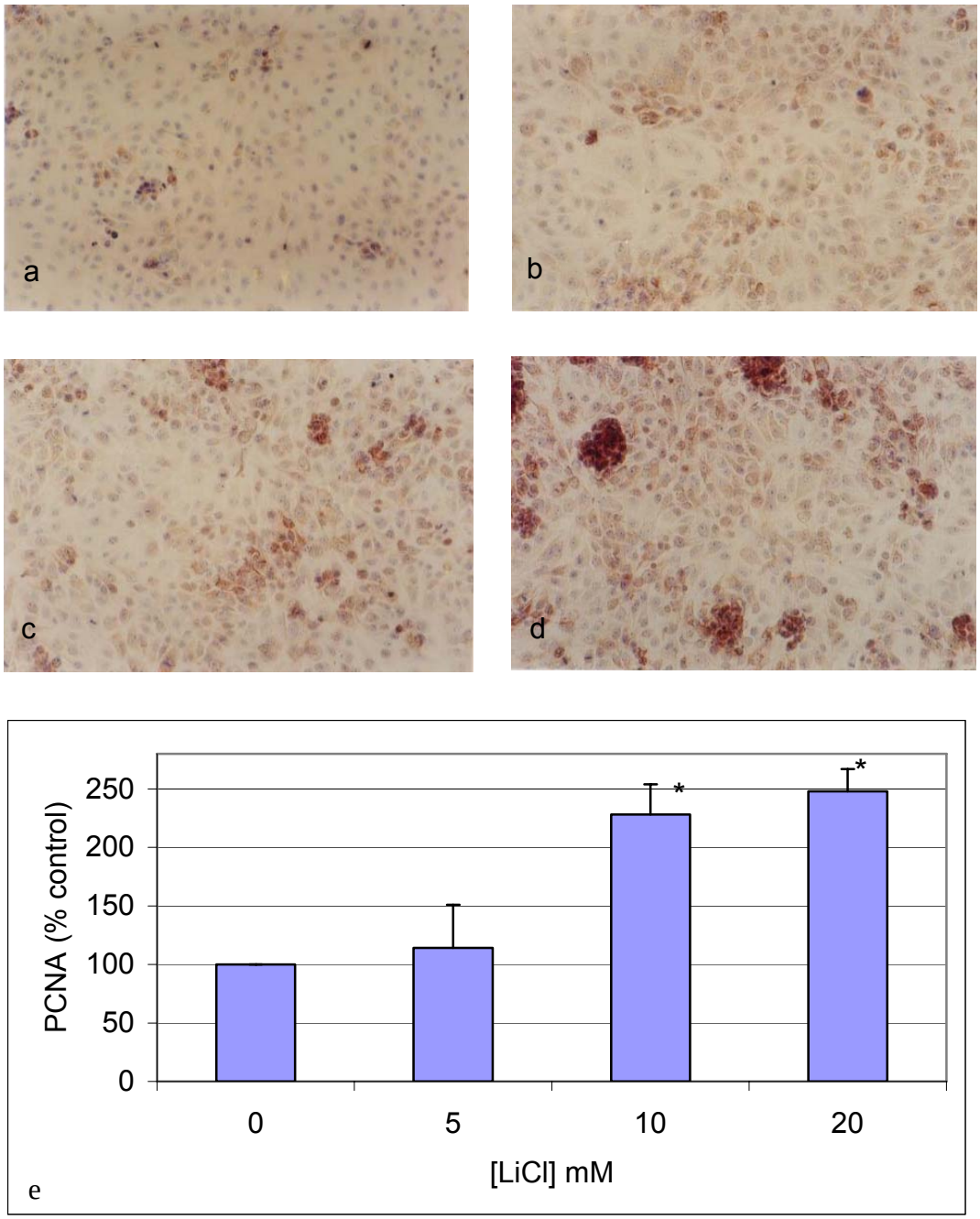


Figure 3.26: Immunocytochemistry of PK(15) cells treated with (a)0, (b)5, (c)10, (d) 20 mM LiCl for 24 h to detect the presence of PCNA. Original magnification, 200x. Protein expression was assessed (e) as described in chapter 2. *Significant difference was found compared to control studies using Student's t-test, $p<0.05$, $n=3$.

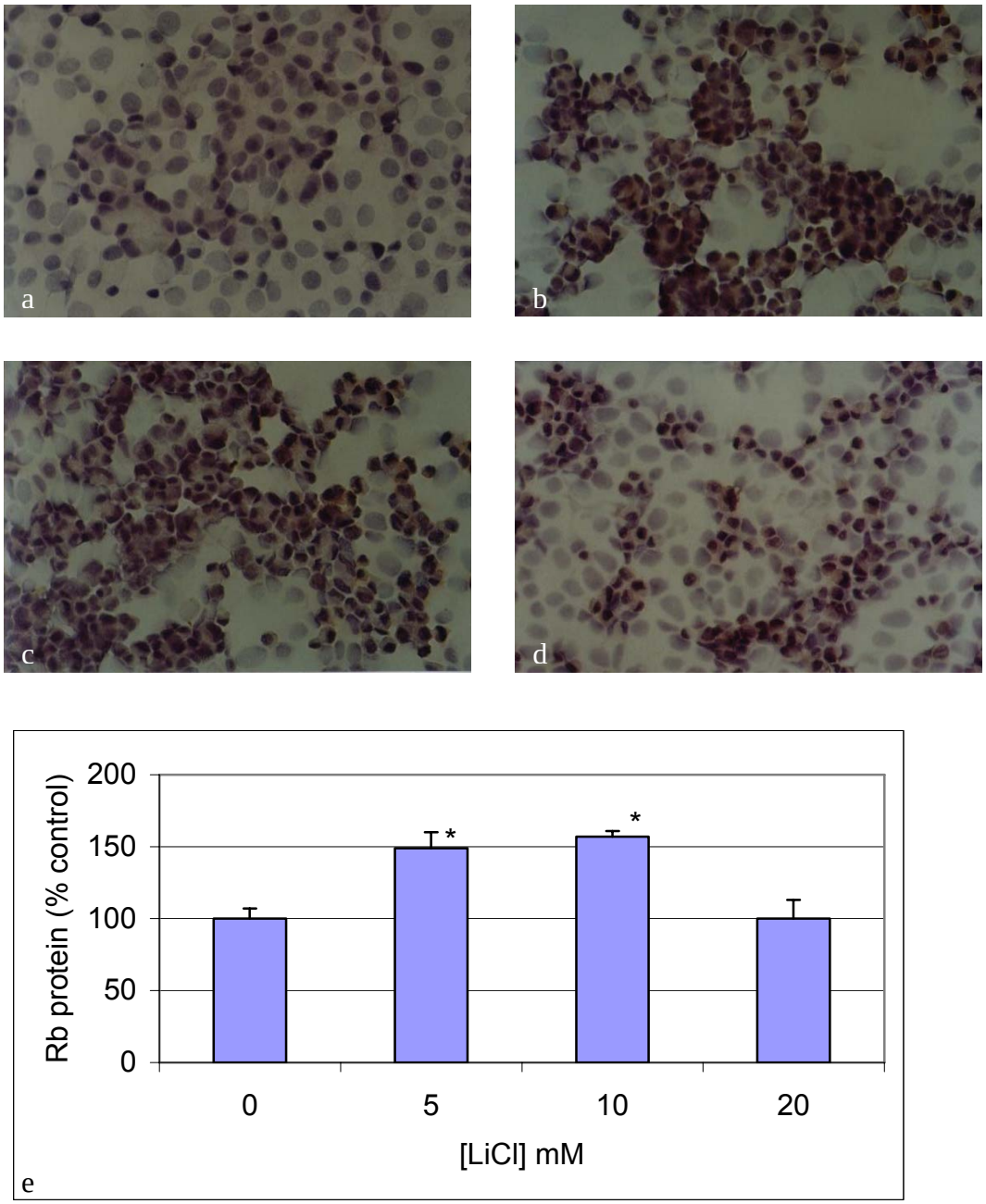


Figure 3.27: Immunocytochemistry of LLC-PK_1 cells treated with (a) 0, (b) 5, (c) 10, (d) 20 mM LiCl for 24 h to detect the presence of Rb protein.

Original magnification, 400x.

Protein expression was assessed (e) as described in chapter 2. *Significant difference was found compared to control studies using Student's t-test, $p<0.05$, $n=3$.

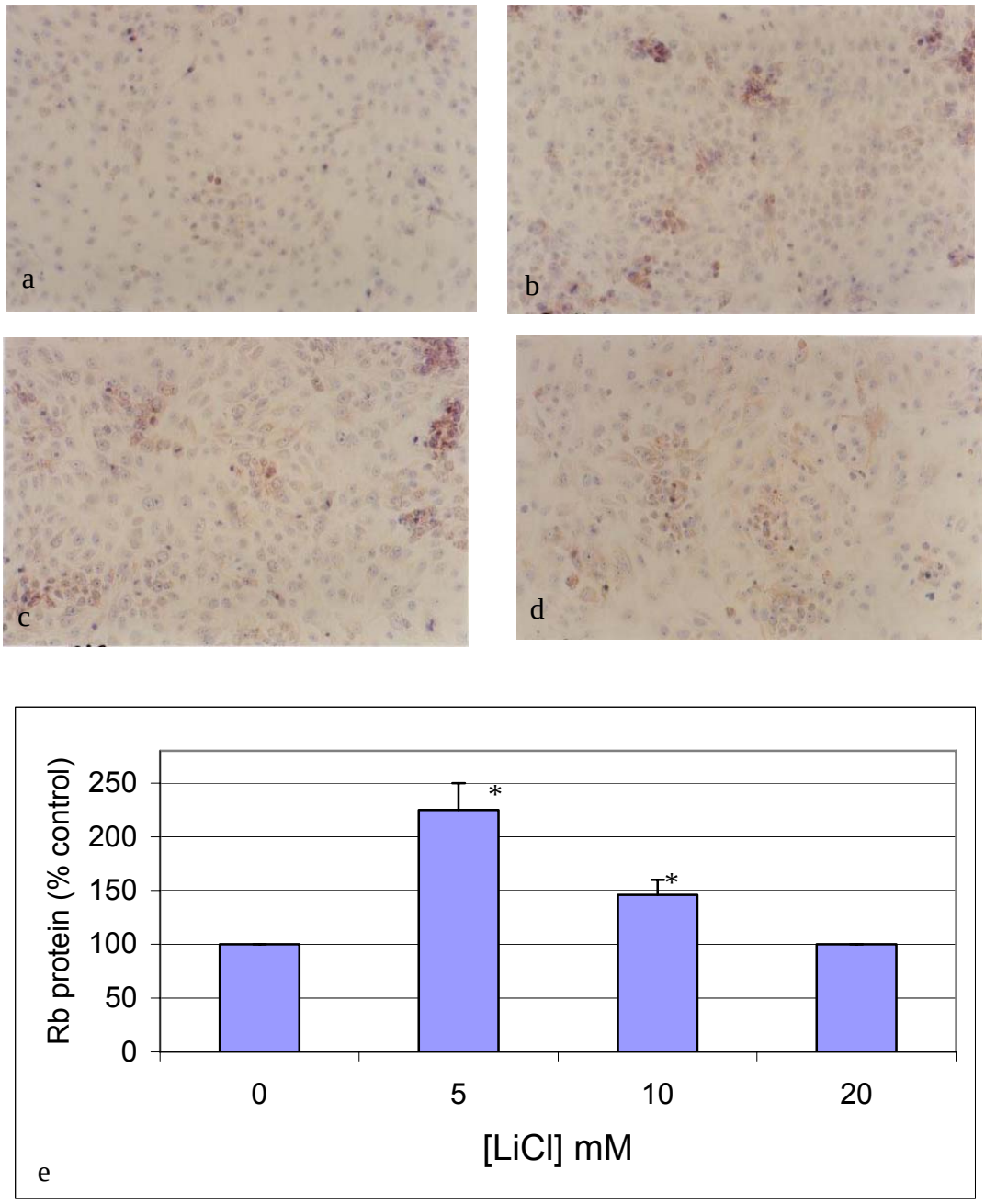


Figure 3.28: Immunocytochemistry of PK(15) cells treated with (a) 0, (b) 5, (c) 10, (d) 20 mM LiCl for 24 h to detect the presence of Rb protein.

Original magnification, 200x.

Protein expression was assessed (e) as described in chapter 2. *Significant difference compared to control studies was found using Student's t-test, $p<0.05$, $n=3$.

Flow cytometry was used to assess whether lithium prevented the cells from moving through the cell cycle. Scatter plots of the control and 20 mM LiCl treated samples of both the LLC-PK_1 (Figure 3.29) and PK(15) cells (Figure 3.30) indicated that the cell population used throughout the flow cytometry studies remained the same. Interestingly, the LLC-PK_1 cell population seemed to consist of two separate populations. Since the forward scatter, and hence the cell size, remained the same, the fact that the cells may not have been completely separated was eliminated. The side scatter was altered indicating a change within the cell composition.

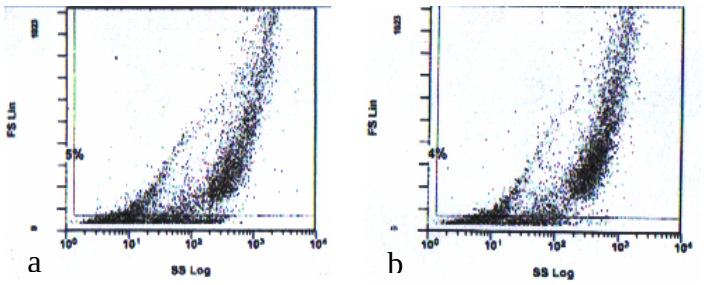


Figure 3.29: Scatter plots of the LLC-PK_1 cell population used in the flow cytometry analysis.
LLC-PK_1 cells were treated with (a) 0 mM and (b) 20 mM LiCl for 24 h.

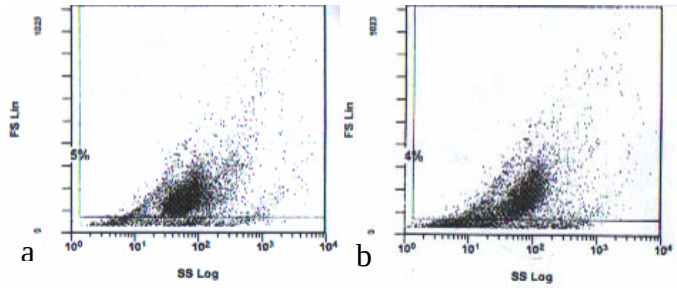


Figure 3.30: Scatter plots of the PK(15) cell population used in the flow cytometry analysis.
PK(15) cells were treated with (a) 0 mM and (b) 20 mM LiCl for 24 h.

The double thymidine block method was used to synchronise the cells into the G_0/G_1 phase of the cell cycle. Results from the flow cytometry pictograms show, however, that the cells were not completely synchronised (Figure 3.31 & 3.32). Nevertheless, since the largest portion of cells were synchronised, the cell cycle analysis was continued, with all lithium treated samples being

compared to the control. LLC-PK_1 cell progression remained unaltered after 24 h of lithium treatment with no significant accumulation of cells in any of the phases measured (Figure 3.33; 3.34 & 3.35). The PK(15) cells accumulated significantly in the G_2/M stage with 50 % more 20 mM LiCl treated cells being found in this phase than their untreated counterparts after 24 h (Figure 3.38). A simultaneous accumulation of 20 mM LiCl treated cells were found in the S phase (Figure 3.37) with a paralleled decrease in the G_0/G_1 phase (Figure 3.36).

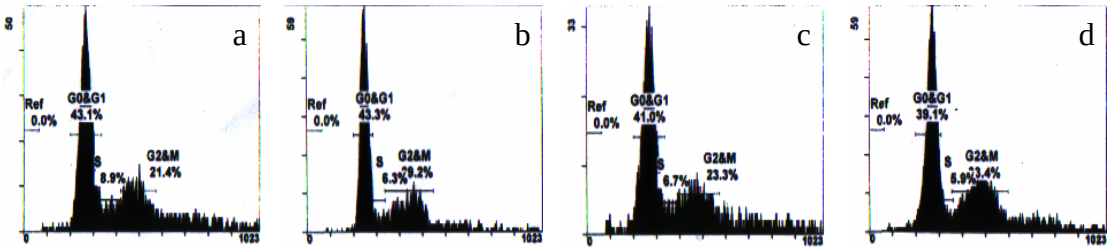


Figure 3.31: Effects of LiCl (a) 0, (b) 5, (c) 10 and (d) 20 mM on the cell cycle of LLC-PK_1 cells after 24 h of treatment.

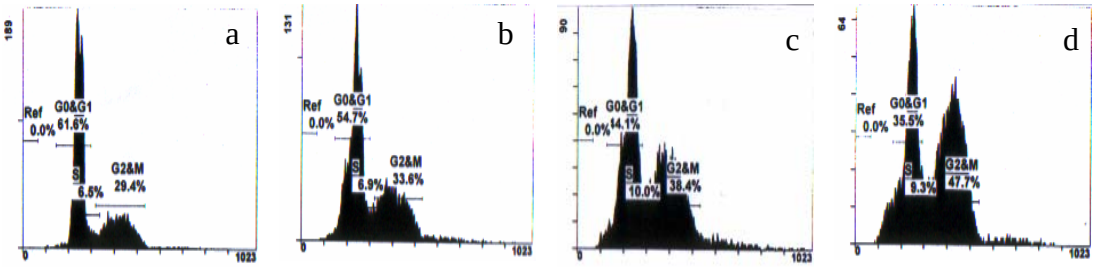


Figure 3.32: Effects of LiCl (a) 0, (b) 5, (c) 10 and (d) 20 mM on the cell cycle of PK(15) cells after 24 h of treatment.

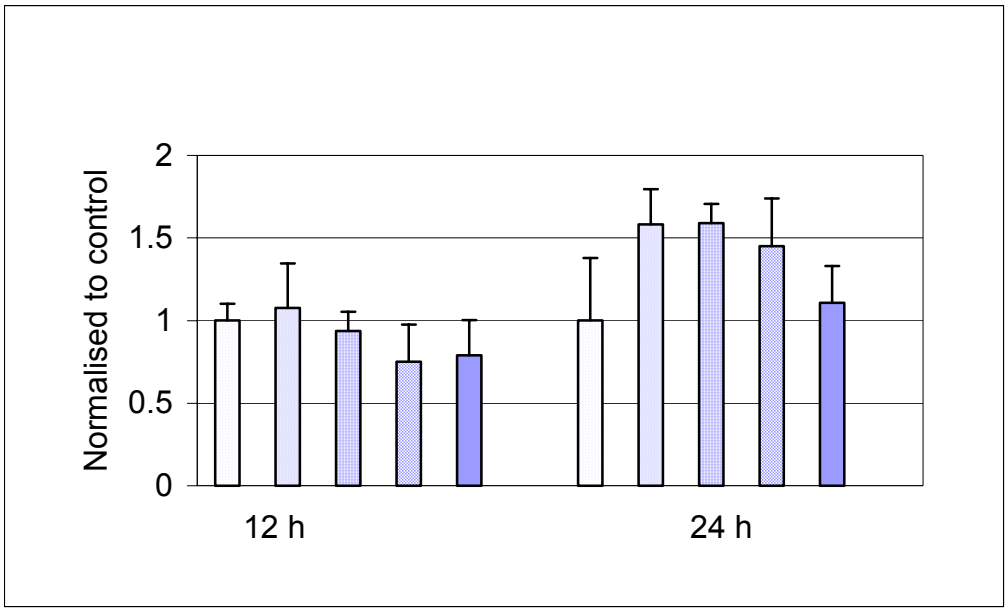


Figure 3.33: G_o/G_1 phase cell cycle analysis of lithium untreated and treated LLC-PK_1 cells. Control, 1 mM LiCl, 5 mM LiCl, 10 mM LiCl, 20 mM LiCl.
Results represent the fraction of cells found in each stage normalised to control cells. SEM calculated from three separate experiments, each performed in duplicate.

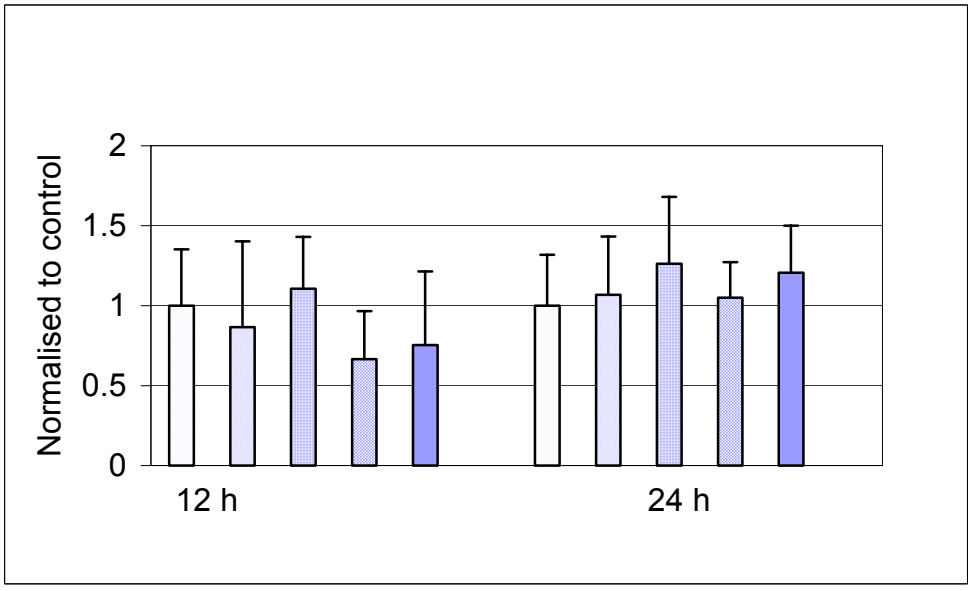


Figure 3.34: S phase cell cycle analysis of lithium untreated and treated LLC-PK_1 cells. Control, 1 mM LiCl, 5 mM LiCl, 10 mM LiCl, 20 mM LiCl.
Results represent the fraction of cells found in each stage normalised to control cells. SEM calculated from three separate experiments, each performed in duplicate.

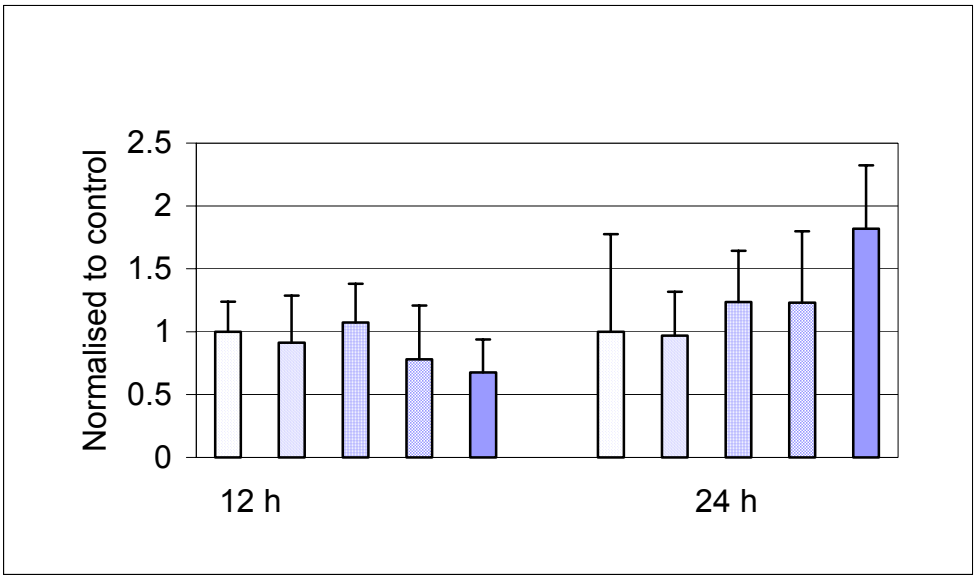


Figure 3.35: G_2/M phase cell cycle analysis of lithium untreated and treated LLC-PK_1 cells. Control, 1 mM LiCl, 5 mM LiCl, 10 mM LiCl, 20 mM LiCl.
Results represent the fraction of cells found in each stage normalised to control cells. SEM calculated from three separate experiments, each performed in duplicate.

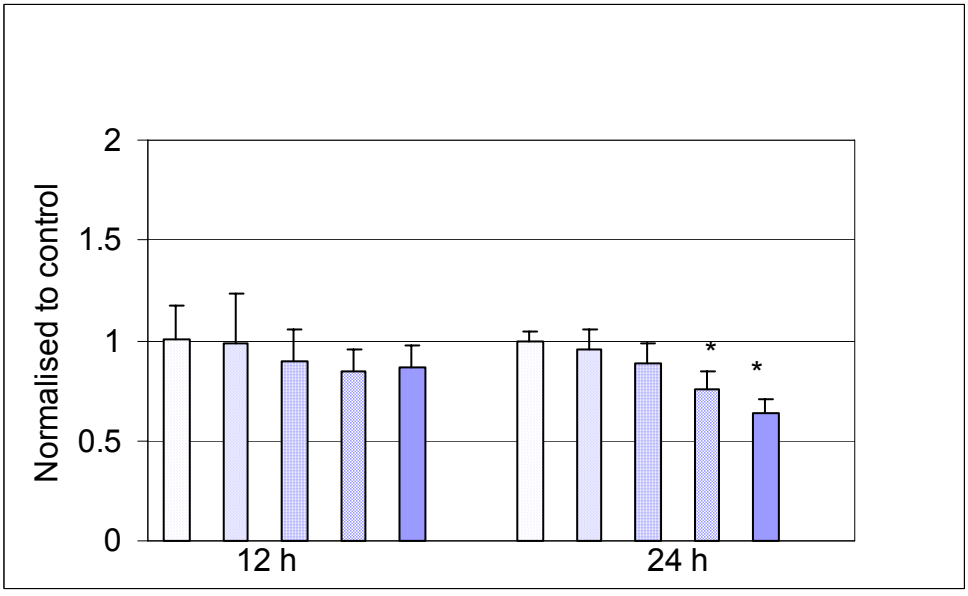


Figure 3.36: G_0/G_1 cell cycle analysis of lithium untreated and treated PK(15) cells. Control, 1 mM LiCl, 5 mM LiCl, 10 mM LiCl, 20 mM LiCl.
Results represent the fraction of cells found in each stage normalised to control cells. SEM calculated from three separate experiments, each performed in duplicate. *$p<0.05$

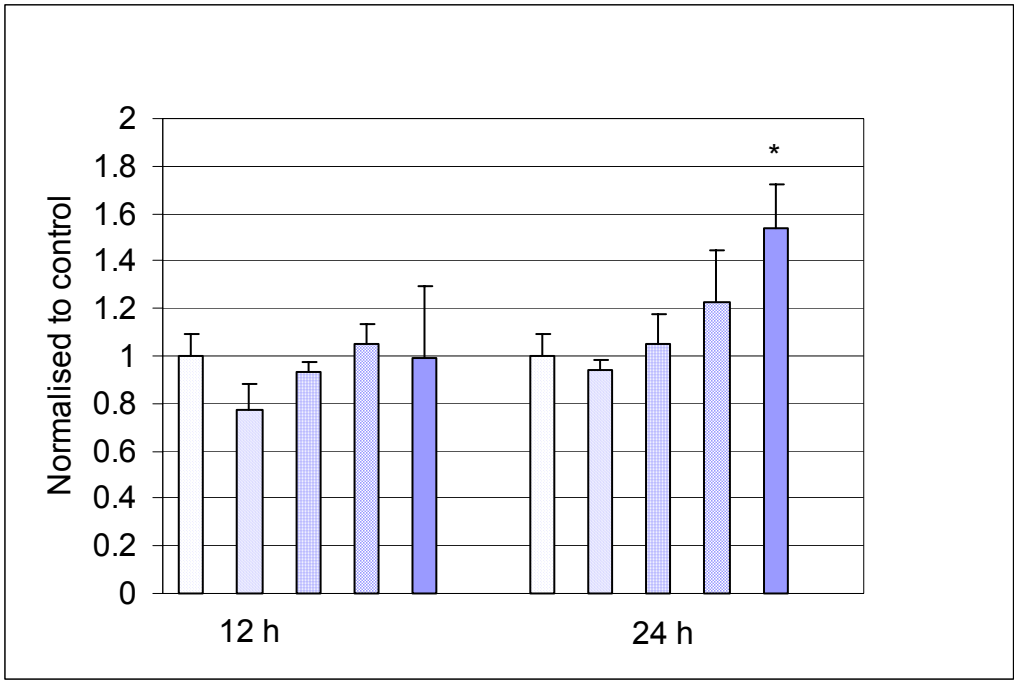


Figure 3.37: S phase cell cycle analysis of lithium untreated and treated PK(15) cells. Control, 1 mM LiCl, 5 mM LiCl, 10 mM LiCl, 20 mM LiCl. Results represent the fraction of cells found in each stage normalised to control cells. SEM calculated from three separate experiments, each performed in duplicate. *p<0.05

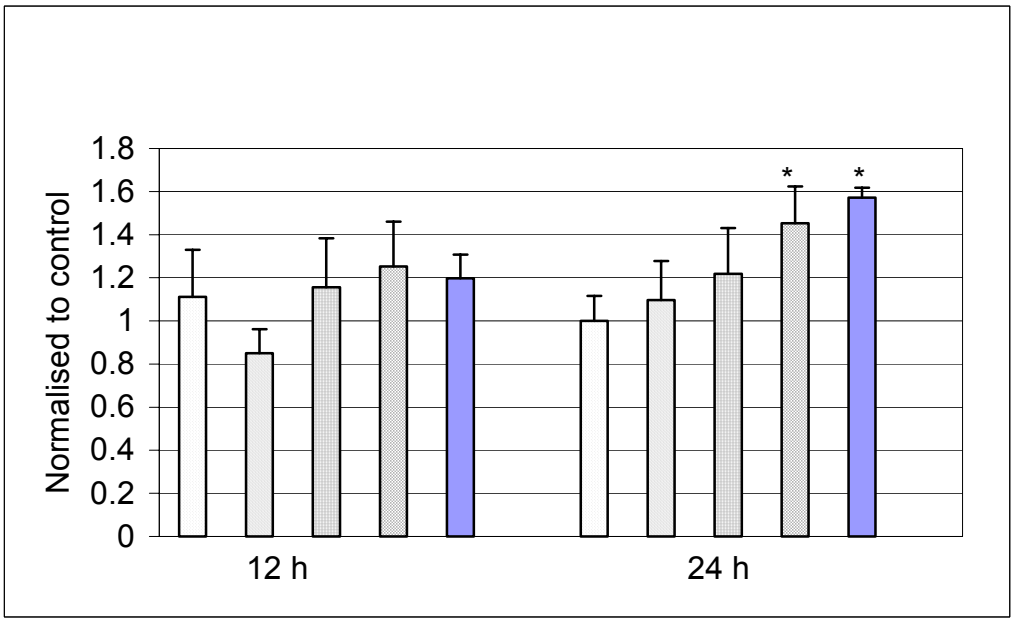


Figure 3.38: G_2/M phase cell cycle analysis of lithium untreated and treated PK(15) cells. Control, 1 mM LiCl, 5 mM LiCl, 10 mM LiCl, 20 mM LiCl. Results represent the fraction of cells found in each stage normalised to control cells. SEM calculated from three separate experiments, each performed in duplicate. *p<0.05

Discussion

Lithium renal toxicity has been shown to be reversible when lithium therapy has stopped and recurs after its re-introduction (Markowitz *et al*., 2000). It is thus tempting to postulate that lithium induces its toxicity in a non-pathological manner, such as through apoptosis rather than necrosis, keeping in mind alternatives such as autophagy, paraptosis and necroptosis.

Lithium toxicity on LLC-PK_1 cells has not been previously reported, though Goldberg *et al*. (1988) did report on lithium induced effects on adenylate cyclase activity in this cell line. Goldberg *et al*. (1988) also established that the intracellular lithium concentrations in these cells reached 6 and 17 mM with extracellular concentrations of 5 and 10 mM respectively. These seemingly high, physiologically toxic, concentrations of LiCl showed little to no toxicity or protein expression and activity alterations on this established cell line. This is in contrast to LLC-PK_1 toxicity results observed after using different insults on this cell line. LLC-PK_1 cells have previously been induced to undergo apoptosis (Filipovic *et al*., 1999; Erkan *et al*., 2001; Varlam *et al*., 2001; Servais *et al*., 2004; Erkan *et al*., 2005) and necrosis (Leussink *et al*., 2002). For example, cadmium was shown to prevent LLC-PK_1 cells from moving through the G_2/M phase of the cell cycle (Xie and Shaikh, 2006) and to cause cell toxicity. The mode of cell death was not well characterised with some authors professing apoptosis and others disputing it (Prozialeck *et al*., 1995; Zimmerhackl *et al*., 1998; Ishido *et al*., 1999; Stinson *et al*., 2003). Although LLC-PK_1 cells have been shown to be susceptible to low concentrations of some chemicals, the present studies indicate that these cells seem to be resistant to lithium induced insults. No overt changes in the expression or activity of the apoptotic or cell cycle dependant proteins investigated in this study were observed.

The antiapoptotic protein, Bcl-2, has been reported to prevent apoptosis in LLC-PK_1 cells by increasing expression within the cell (Ishido *et a*l., 1999). Furthermore, using synthetic peptides, Bcl-2 prevented apoptosis of LPS

stimulated LLC-PK_1 cells, whilst Bax and Bak stimulated the apoptotic effect. In the current study, although a small decrease in Bax protein occurred due to lithium treatment, the Bax/Bcl-2 ratio, a crucial apoptotic inducer, did not change. Bak was not measured in these experiments because it is not found within proximal epithelial cells (Peherstorfer *et al*., 2002).

The caspase enzyme activity did not show a notable change due to the lithium induced stimulus on LLC-PK_1 cells in these studies, a result expected as none of the present studies indicated that lithium induces apoptosis in these cells. A small but insignificant increase in caspase 9 activity in LiCl treated LLC-PK_1 cells was, however, observed. Caspase 9 acts downstream in the mitochondrial stimulated apoptotic pathway. As no change in the Bax/Bcl-2 ratio was observed in this study, no change in caspase 9 activity was expected. Furthermore, no indications of a lithium induced toxicity was seen in these LLC-PK1 studies, barring the observation that it was easier to release the 20 mM LiCl treated cells from the culture flask substratum than control cells, indicating a loss of cell contact integrin protein, a apoptotic marker (Wen *et al.*, 1997). The small increase in caspase 9 activity may, therefore, indicate that the cells have some LiCl-induced effects not detected by the previous assays performed in this study. Since caspase 9 is known to function upstream from caspase 3, it was expected that an increase in caspase 3 activity should follow an increase in caspase 9 activity. However, this was not observed. Caspase 3 activity did not change in lithium treated samples as compared to control lysates. Caspase 3 assays may not have shown results due to its activity being downstream to, and hence later than, caspase 9 activity. Timing of caspase activity studies seem crucial as seen in studies by Servais *et al.* (2004) where 16 h showed no differences in caspase 3 activity due to insult by gentamicin, followed by a time and dose dependant increase upto 48 h, and a sharp decrease of activity at 72 h. Similarly, Wong *et al*. (2001) indicated a dose dependant okodaic acid induced increase in caspase 3 activity in RPTE renal cells, with a decrease after 24 h.

Lithium was previously found to induce caspase 8 activity by enhancing TNF induced apoptosis in rhabdomyosarcoma cells (Beyaert, 1999), and caspase

3 and 8 activity in Wil-2 cells undergoing apoptosis (Molepo, 2004). Caspase involvement in LLC-PK_1 cells has also been observed, albeit not due to lithium as a stimulant (Erkan *et al.*, 2001; Servais *et al*., 2004). Caspase involvement in LLC-PK_1 cell apoptosis seems to vary depending on the insult used, and often the caspase cascade does not follow conventional studies (Ishido *et al*., 1999; discussed in Cummings and Schnellmann, 2002).

Cell cycle studies reflected a similar phenomenon with no significant differences being found in cell cycle protein studies of p53, PCNA or the flow cytometry results. The results showed a slight accumulation of cells in G_2/M phase which may have been due to a slowing down of the cell cycle and not necessary a halt in the cell cycle as the accumulation is small and insignificant. The results do, however, concur with the observations made by Mao *et al*. (2001) in their studies of the effects of lithium on the cell cycle of endothelial cells. The immunocytochemistry results of the Rb protein are, however pertinent. At 5 and 10 mM LiCl the number of cells positive for Rb increased by 50% compared to that of the control and 20 mM LiCl studies. Rb overexpression arrests cells in G_0/G_1 phase, where it acts by binding to the E2F family of transcription factors which are necessary for cell cycle progression to the S phase (Peeper *et al*., 1994; Hickman *et al*., 2002). Hyperphosphorylation of Rb by cdk, inhibits its growth-suppressive function, and results in apoptosis (Diederich *et al.*, 1998). The LLC-PK_1 cell studies indicated a similarity between Rb protein expression and the G_0/G_1 stage in cell cycle studies. A small, insignificant increase in Rb protein expressed was noted after 5 and 10 mM LiCl treatment for 24 h, with a concomitant accumulation in the G_0/G_1 phase of the cell cycle. The increase in the amount of Rb expression after 5 and 10 mM LiCl treatment may have been associated with cell repair mechanisms and p53. Since p53 and Rb have been associated together in apoptotic events (Hickman *et al*., 2002), and the fact that they both prevent cell progression to the S phase, could be a mechanism in the LLC-PK_1 cells to prevent and repair any DNA damage caused by LiCl, with the 20 mM LiCl insult probably being too high to overcome, setting some, as yet undetected, apoptotic signals into place.

The effects of lithium on the PK(15) distal renal tubule cells are more remarkable. Results of both the immunocytochemistry and the Western blot analysis indicate that the amount of Bax increases as the concentration of lithium increases. As previously noted, the Bax/Bcl-2 ratio is important in determining whether a cell will survive and whether it is triggered to undergo apoptosis (Maddika, *et al*., 2007). Bax binds to Bcl-2 at the mitochondrial membrane to form pores allowing for the release of cytochrome c which acts downstream from this event. The higher the proportion of Bax protein to that of Bcl-2 the more enhanced this effect (Oltvai *et al*., 1993). The increase in the Bax/Bcl-2 ratio seen in the lithium treated PK(15) cell study (Table 3.2) supports apoptosis as the mode of cell death, in particular, mitochondrial or the intrinsic pathways of apoptosis. Similar lithium induced effects have been reported by Mampuru *et al.* (1999), where bone marrow cells undergoing apoptosis had higher Bax/Bcl-2 ratios than those that were not. The above mentioned studies are contradictory to those of Chen and Chuang (1999), where Bax/Bcl-2 levels decreased after lithium treatment of cerebellar granule cells, although lithium was shown to have cytoprotective effects on these cells as opposed to the toxic effects observed in the afore mentioned studies.

An increase in caspase activity was expected in the lithium treated PK(15) cells as they seem to be undergoing classical intrinsic type apoptosis, and caspases 8, 9 and 3 have been implicated in previous studies of lithium induced apoptosis (Schotte *et al*., 2001; Molepo, 2004). Contrary to expectations, caspase activities showed no significant changes due to the presence of lithium in the PK(15) cells, though a non significant increase in caspase 9 activity was noted after 10 mM LiCl treatment. Caspase activity, especially -3 and -8 has been previously enhanced in PK(15) cell apoptosis induced by insults other than lithium (Fujino *et al*., 2000; Muneta *et al*., 2001; Liu *et al*., 2005; Klaric *et al*., 2007). Since caspases are involved in PK(15) cell death, and lithium stimulated apoptosis in other cell lines, it seemed as though the lithium treated PK(15) cells in this study were undergoing a caspase independent type of cell death. Apoptotic features have been observed in many examples of caspase independent cell death pathways (Donovan and Cotter, 2004). One cell line that previously showed pleiotropic

effects to different chemotherapeutic agents and stimuli are the MCF-7 breast cancer cells. Depending on the stimulus the cells undergo either classical apoptosis or autophagic cell death (reviewed in Kim *et al*., 2006). Caspase-independent cell death pathways have also been well described (Reviewed in Bröker *et al*., 2005). Specifically, growth factor withdrawal induced apoptosis of primary human erythroid progenitor cells occurred through increased Bax and GSK-3β expression, as shown via lithium inhibition studies, and not through caspase activation (Somervaille *et al*., 2001). A study by Cui *et al*. (2007) established that MCF-7 cells, after treatment with a plant anticancer compound, oridonin, underwent apoptosis due to upregulation of Bax and p53, a down regulation of Bcl-2, DNA fragmentation and most importantly through a caspase 9 dependant, caspase-3 independent manner. DNA fragmentation was shown to occur due to calpain cleavage. Similar results were observed in the present study, and perhaps the mode of cell death is an apoptotic-like mode where caspase activity is not involved, or involved in a non-canonical pathway. Further investigation is required to identify the mode of DNA degradation in lithium mediated cell death of PK(15) cells.

Lithium, at 5 mM, caused a significant increase in Rb expression in PK(15) cells, followed by a decrease after treatment with 10 and 20 mM LiCl. Since Rb is a tumour suppressive protein its upregulation implies a decrease in cell proliferation, as observed in cell proliferation studies (Figure 3.1b). Previously Rb protein was shown to be proapoptotic in radiation and TPA induced toxicity of prostrate cells (Bowden *et al*., 1998; Zhao and Day, 2001). The radiation induced apoptosis occurred in the absence of amongst others, caspase 8 and 3 expression (Bowen *et al.*, 1998), an observation also noted in the present study. The lithium induced decrease of Rb expression in PK(15) cells at higher concentrations may have occurred after the cells had already entered apoptosis at these concentrations. Tan and Wang (1998) hypothesised that upstream active caspases cleave Rb during apoptosis, but not sufficiently enough to kill cells. Interestingly, lithium has previously been found to accumulate some retinoblastoma deficient cell lines in the G_2/M stage at concentrations of 40 mM, with fewer cells being found in the G_1 phase. This

is in contrast to the PK(15) cells reported here, since the Rb protein was evident, even at 20 mm LiCl. The cells did, however, accumulate in the G_2/M stage of the cell cycle, and perhaps with an increase in the LiCl concentration, a decrease in Rb protein, below that of the control samples may have been observed. The studies of lithium effects on Rb deficient cells by Tell *et al*. (2006), were once again cell line specific, indicating the pleiotropic effect of lithium on different cells. It should be noted that in the studies presented here no distinction has been made between unphosphorylated, hypophosphorylated, hyperphosphorylated Rb and truncated Rb, and thus it is only the overall expression of the Rb protein that can be commented about in this report. The state of Rb is essential to its function, whether it is cell cycle suppressive, apoptotic, or involved in cell differentiation (Lundberg and Weinberg, 1999; Maddika *et al*., 2007).

Proliferating cell nuclear antigen, a cell cycle protein, has been found in different stages of the cell cycle, with a peak during S-phase (Reviewed in Cox, 1997; Green, 2006). The amount of PCNA protein positive cells was expected to decrease in non-proliferating or apoptotic cells and to mimic results obtained for the BrdU cell proliferation studies observed in section 3.1. Recently, PCNA immunohistochemistry has been used as a proliferative marker rather than the BrdU method, and it has also been adequately compared to the use of tritiated thymidine (Raucci *et al*., 2006). These results are clearly contradictory to this study. Furthermore, Allagui *et al*. (2007) found a down regulation of PCNA expression in lithium treated A549 cells. PCNA is, however, very prominent in DNA repair mechanisms which suggests a reason for its elevation due to lithium induced cell damage on the PK(15) cells after exposure to 10 and 20 mM LiCl for 24 h. The immunocytochemistry was only performed on attached cells, and thus represents viable cells or cells undergoing early stages of cell death. The down-regulation of PCNA seen by Allagui *et al.* (2007) may be due to the concentrations of lithium used being lethal to the cells and the protracted time interval (3 d) used for the study. If the immunocytochemistry results are combined with the cell cycle results, the increase in PCNA correlates to an increase in cell percentage in the S-phase.

The amount of PCNA is increased significantly during S phase due to DNA synthesis (Cox, 1997).

Normal cellular p53 levels are low due to the short half-life of the protein (Hickman *et al*., 2002). Once a signal causes its stabilisation and activation, however, the levels increase. This protein was previously found to control both G_2/M and G_1 cell cycle checkpoints, mediate growth arrest (Agarwal *et al*., 1995), and induce cells to undergo apoptosis, depending on the extent of DNA damage within the cell. Although a high amount of p53 seemed to be evident in the immunocytochemistry studies presented here, the concentration was similar in lithium treated PK(15) cells compared to untreated cells. This is an unusual observation due to the short-half life of the protein but cells that have lost their tumour suppressor functions frequently express high levels of p53 (Hickman *et al*., 2002). Since PK(15) cells continue growing into little tumour-like clumps after becoming confluent this may be one explanation for the observed high concentrations of p53 in all PK(15) samples. The Western blot analysis indicated that lithium induced changes in p53 expression in PK(15) cells. Expression after 5 mM lithium treatment was increased 1.5 times that of the control cells, whilst it decreased below control levels after 20 mM LiCl treatment. Stabilisation and accumulation of p53 by phosphorylation, promotes the transcription of p21 which ultimately prevents the progression of cells from the G_1 and the G_2/M phase of the cell cycle (Agarwal *et al*., 1995; Houtgraaf *et al.,* 2006). The flow cytometry results presented in this study suggest that lithium causes the cells to accumulate in both the G_2/M and the S phase, both phases showing a significant difference only at 20 mM LiCl. Although a higher percentage of cells were found in the G_2/M and S phase after 48 h (results not shown) they did not seem to accumulate more than those observed at 24 h leading to the suspicion that the cell doubling time may be altered, with slower progress through the G_2/M phase than through the rest of the cycle. The presence of high levels of p53 may influence the lower doubling time, manipulated by p53 interactions at cell cycle checkpoints. The relationship between p53 up-regulation, apoptosis and lithium in this study is contradictory to results obtained by Watcharasit *et al.* (2003). They

showed that p53 was stabilised by binding to active GSK-3β causing neural cells to undergo apoptosis. By using lithium, and inhibiting GSK-3β, p53 was no longer stable, suppressing apoptosis stimuli in neural cells. A study by Rössig *et al*. (2002), however, showed that lithium induced an increase in p21, the cell cycle protein transcribed by p53, and accumulated cells in the G_2/M phase of the cell cycle, closely mimicking the results obtained in the present study. Again the difference in lithium induced effects between neural and non-neural cells is evident from the above mentioned studies.

Clear apoptotic peaks were not observed in the cell cycle studies, though some evidence of a pre G_0/G_1 phase was noted in some of the 20 mM LiCl experiments. Surprisingly, this was observed more so with the LLC-PK_1 cells than with the PK(15) cells. A possible reason for this could be that cells undergoing apoptosis round up and are released from the petri dish, the PK(15) cells doing so more prevalently than the LLC-PK_1 cells (refer to micrographs in section 3.2). The remaining attached cells, used for cell cycle studies were, thus, mostly viable cells.

The results from the immunocytochemistry, the Western blot analysis, as well as the flow cytometry concur that LLC-PK_1 cells are resistant to LiCl toxicity, even at concentrations of 10 - 20 mM. In contrast, lithium treated PK(15) cells undergo cell death, the mode probably being Bcl-2 family related, and hence an intrinsic mitochondrial process. Little alteration in caspase 3, 8 or 9 activity was seen and thus the mode of cell death does not seem to be typically apoptotic. More investigations are necessary to establish which downstream proteins are involved, and thereby clarifying the type of cell death.

CHAPTER 4
DETERMINATION OF LICL ALTERED LUPUS ANTIGENS IN PORCINE KIDNEY CELLS.

Introduction

Various predictors of different autoimmune diseases have been determined, and are used for identification and treatments of diseases. Due to the systemic nature of SLE and the unpredictable involvement of many organs such as the heart, lungs, nervous system, joints and kidneys, biomarkers for disease prevalence and organ involvement have been sought after for many years. The prospects of elusive markers that can be used to pre-determine organ involvement in SLE so that monitoring and treatment can be carried out before irreparable organ damage occurs are still being investigated (Fritzler, 1997; Leslie *et al*., 2001; Mittleman, 2004 with reference to Illei *et al*., 2004). With new technologies emerging such as microarrays and proteomics, researchers are more hopeful that profiles will emerge (Hueber *et al*., 2002).

Studies in Hart's laboratory using mouse models of SLE with renal involvement indicated that a subset of lithium treated female mouse kidneys did not progress into end stage renal failure (Krause *et al*., 1992). Possible reasons for the enhanced survival of these mice were reported by Lenz (1995) and may be directly involved in the renal tissue or may influence the immune system itself. Lenz (1995) identified five specific areas that could be affected to enable lithium to induce its enhanced survival effect viz:

Inflammation due to cytokine changes or cellular involvement. Recently, lithium was found to play a role in the inflammatory response during wound healing (Cheon *et al*., 2002).

Extracellular matrix involvement due to regulatory enzyme involvement such as plasminogen activator inhibitors (PAI) or changes in collagen.

Autoimmune target expression.

Autoantibody changes either due to nephritogenic antibodies or non-pathological antibodies

T-cell function due to systemic immunosuppression or suppression of specific B cell clones.

Hart's group in Calgary has specifically investigated cytokine changes and inflammation, changes in the extracellular matrix proteins, and autoimmune profiles in the NZB/W mice models. Lenz (1995) found no statistical variation of kidney weight, total renal DNA and RNA, a small, insignificant decrease in total renal collagen, and a surprising increase, though insignificant, in ssDNA antibodies. Lenz (1995) further investigated urokinase, a fibrinolytic enzyme produced by tubular cells that is important in the maintenance of the renal extracellular matrix. Urokinase mRNA expression increased in lithium treated mice as opposed to their untreated counterparts. Interestingly these results were not observed in porcine renal cell studies, particularly LLC-PK_1 and PK(15) cells treated with lithium (Hart *et al*., 1998). A lithium concentration dependant decrease in urokinase mRNA expression was observed by these authors who hypothesised that the results may be species specific. Furthermore, no changes in PAI were observed in lithium treated LLC-PK_1 and PK(15) cells (Hart *et al*., 1998) Lenz (1995) also established that a subset of NZB/W mice had higher TNF-α expression levels. Since lithium is known to enhance TNF activity this may influence the effect of lithium on these mice. Hart *et al*. (2000) established that mRNA levels of some androgen regulated tubule proteins were not altered by lithium treatment of NZB/W F1 strains. They did however find that transcriptional proteins c-jun, c-fos and c-myc were influenced in lithium treated mice. The mRNA levels of c-jun increased in female mice only, c-fos levels decreased in lithium treated male mice and c-myc increased in lithium treated male and female mice. The above evidence indicates that lithium has the capacity to alter the expression of proteins that influence renal function in lupus-mice models as hypothesised by Lenz (1995). Lenz *et al*. (1997) also determined that antibody profiles from serum of NZB/W mice, untreated or treated with LiCl, binding to PK(15) and LLC-PK_1 cell antigens, remained unchanged, potentially eliminating the hypothesis that lithium alters the antibody profile in these mice.

Interest into the potential of lithium's ability to effect changes in auto-antigen structure has been influenced by many studies. Lithium was shown to cause cell surface changes on leukaemia cells. Rice *et al*. (2004) observed that lithium is able to induce changes in the expression of some cell surface markers of granulocytic and monocytic differentiation such as CD11b, CD15, CD14 and CD33 in leukemic cells. Apoptosis (Diederich *et al*., 1998) and apoptosis-like autophagy (Lleo *et al*., 2007) are known to effect changes in various proteins due to cleavage, and in particular in autoimmune diseases (Casciola-Rosen *et al*., 1995; Utz *et al*., 1998; Rosen and Casciola-Rosen, 1999; Utz *et al*., 2000). Furthermore, apoptotic bodies have been shown to have many autoantigens on their cell surface (Casciola-Rosen *et al*., 1994; Rosen *et al*., 1995; Utz *et al*., 2000; Cline and Radic, 2004) and some auto antigens are phosphorylated during apoptosis (Utz *et al*., 1998). Autoantigen proteins that were found to be altered by apoptosis are small nuclear ribonucleoproteins (snRNP), signal recognition particle (SRP), as well as Ro and La antigens. Many authors have alluded to the fact that poor apoptotic body clearance leads to apoptosis (Andrade *et al*., 2000; Bijl *et al*., 2001; reviewed in Gaipl *et al*., 2005), which has been particularly observed in kidney cells (Rosen and Casciola-Rosen, 1999; Cline and Radic, 2004). In addition, kidney disease was ameliorated by a caspase inhibitor in CBAxC57/BL10F_1 lupus mouse models (Seery *et al*., 2001).

Since previous results in this study, and those of others, have indicated that LiCl induces some cells to undergo apoptosis at concentrations of 10 mM and above, the question was asked whether lithium induces any antigen, in particular autoantigen, changes in the LLC-PK_1 and PK(15) cell lines, and whether these changes can be characterised due to protein cleavage, phosphorylation or glycosylation.

Materials and methods

Materials

Well characterised lupus serum was kindly provided by Dr M. Fritzler from the Department of Medicine, University of Calgary, Calgary, Canada. Serum from patients with renal disease, without renal disease, and from those who were in remission was kindly provided by Prof Mody and Dr Cassim from the Department of Medicine, University of KwaZulu-Natal, Durban, South Africa.

Cell culture

For methods of cell culturing techniques refer to the Materials and Methods section in Chapter 2.

Immunofluorescent studies

Cells were grown on polylysine treated microscope slides and treated with varying concentrations of lithium chloride for 24 and 48 h intervals as indicated in the results. I*n situ* immunofluorescent studies were carried out by J. Miller in the laboratory of M.J. Fritzler at the University of Calgary, Calgary, Canada using established methods (von Mühlen and Tan, 1995), and well characterised autoimmune lupus serum.

SDS-PAGE

Trypsinisation of cells was avoided to prevent any protein (antigenic) cleavage. Cells were thus scraped off the culture flask washed, lysed and prepared as before. Cell lysates were quantitated using the Pierce BCA protein determination. Reducing and non-reducing SDS-PAGE was carried out using 35 μg/well of protein on 10% gels on a Hoeffer mini-gel system. Some experiments were carried out in one continuous well equivalent to 10 wells (Figure 4.1a), alongside a molecular mass marker well, two separate

gels being used, one for the control sample and one for the LiCl treated sample on the same apparatus, under exactly the same conditions. In order to ensure absolute similarity between the control and their treated counterparts, the experiment was later changed to using two big wells, equivalent to 5 wells each per gel (Figure 4.1 b) so that the control and treated lysates were run on the same gel. Following electrophoresis the proteins were transferred to nitrocellulose membranes using conventional transfer methods.

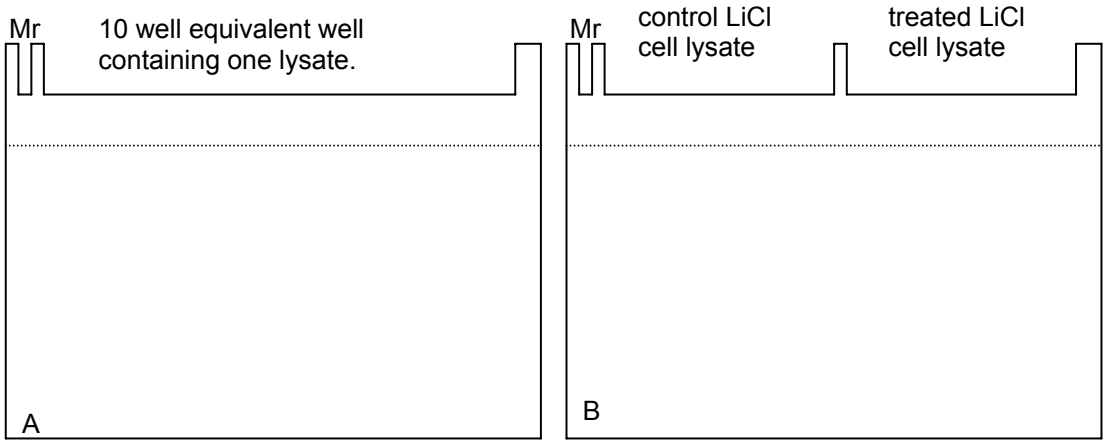


Figure 4.1: Diagram depicting the wells used in the SDS-PAGE autoimmune experiments.

Membranes were cut into strips and strips of control lysates and their untreated counterparts were incubated with different immune serum and developed using chemiluminescence alongside each other. The immune serum used was well characterised serum from lupus patients obtained in Calgary, Canada. The patients from which the serum was obtained had no record of renal disease. Autoimmune complexes were analysed to detect changes either in molecular weight differences or the presence or absence of bands due to lithium treatment.

To establish whether lithium changed antibodies in a specific manner depending on renal involvement or not, lupus patient serum was obtained, with permission from the ethical committees of the Universities of KwaZulu-Natal and Limpopo, and with the patient's consent. The serum was provided in a blind manner and stemmed from patients with renal disease, without renal

disease and from patients who had renal disease that had been controlled. Unfortunately age, sex and race matched controls were not available.

Lectin blots

Cell lysates were obtained and SDS-PAGE carried out as mentioned in section 4.2.4. After transfer to nitrocellulose membranes, strips were cut and treated with commercial biotin labelled lectins obtained from Sigma-Aldrich, Johannesburg. Concentrations were optimised and used as 1:500 or 1:1000 dilutions depending on the detection system used. Streptavidin labelled HRP was used and developed with either 3,3'diaminobenzidine or chemiluminescence depending on the lectin dilution used.

Results

Immunofluorescent studies were initially carried out to determine whether lithium induced any changes to fluorescent patterns created by human serum antibody binding to specific lupus characterised antigens. Good cross-reactivity was obtained between human serum and the porcine cell lines. Tables 4.1 and 4.2 depict results obtained for both the LLC-PK_1 and PK(15) cell lines treated with various concentrations of LiCl. Most notable changes were observed with the PK(15) cells, and the anti-golgi and anti-SSA antibodies. The PK(15) cell results were also the easier of the two to interpret.

Following the indications from the immunofluorescent studies that lithium may be altering the auto-antigenic targets in some way, Western blots were performed to determine, more specifically, whether lithium altered protein antigens in the renal cell lines. Normal human serum (NHS) did not form any complexes in any of the experiments performed and was thus used throughout the studies as a negative control. Using well characterised serum it seemed as though lithium altered some antigens. In particular, a 48 kDa SSB/La-like antigen from LLC-PK_1 cells, seemed to decrease in molecular weight (Figure 4.2).

Table 4.1: Immunofluorescence studies of lithium treated LLC-PK1 cells stained with antibodies against various SLE autoantigens.

	LLC-PK_1 24 h				LLC-PK_1 48 h			
	mM LiCl				mM LiCl			
Ab	0	1	5	10	0	1	5	10
Sm	0	0	+ cyto	+ cyto	0	0	0	0
Cell cycle specific anti-golgi GBM	0	-	-	-	0	0	0	0
Golgi	0	-	-	-	0	-	- -	- -
SSA	0	0	0	0	0	-	- -	- -
SSA/SSB	0	+	-	+	0	0	0	-
RNP	0	0	+ cyto	+ cyto	0	0	0	0
fib	0	+	+	+	0	0	0	0
NUMA	0	0	0	0	0	0	+	+
Scl 70	speckled	Change of pattern to nucleolar	nucl	nucl	0	0	0	0
NHS	0	0	0	0	0	0	+	+

(0) no change compared to control; (-) a decrease compared to control, (+)an increase compared to control.

Table 4.2: Immunofluorescence studies of lithium treated PK(15) cells stained with antibodies against various SLE autoantigens.

	PK(15) 24 h				PK(15) 48 h			
	mM LiCl				mM LiCl			
Ab	0	1	5	10	0	1	5	10
Sm	0	0	0	0	0	0	0	0
Cell cycle specific anti-golgi GBM	0	-	-	-	0	0	0	-
Golgi	0	-	0	0	0	0	0	-
SSA	0	-	+	+	0	0	0	-
SSA/SSB	0	-	-	-	0	0	0	-
RNP	0	-	-	-	0	0	0	-
fib	0	0	0	0	0	0	0	0
NUMA	0	-	-	-	0	0	-	-
Scl 70	0	0	-	-	0	0	0	0
NHS	0	0	+	0	0	0	0	-

(0) no change compared to control; (-) a decrease compared to control, (+)an increase compared to control.

Similar results were obtained using lithium treated or untreated PK(15) cell lines (Figure 4.3). Complex formation was also observed using serum from a patient characterised for Sm antibodies and it seems as though a 28 kDa antigen had a lower molecular weight in the lithium treated sample as compared to its untreated counterpart. This was observed in repeated studies. Furthermore, the PK(15) 48 kDa protein detected using patient serum characterised for SSA/SSB antibodies formed a complex and was presumed to be the SSB/La antigen due to its molecular weight. Lithium

caused the molecular weight of this protein to increase. This result was in contrast to the decrease in molecular weight observed with the LLC-PK_1 lithium treated cells (Figure 4.2). Serum against RNP (Figure 4.2 lane 6a and b) detected a 72 kDa protein after PK(15) cells were treated with LiCl, and absent in control sample. These results were, however, not consistent in all studies and are thus inconclusive. Although autoimmune complexes were seen using other patient sera, no differences were noted between lithium treated and untreated samples. The same results were observed in experiments using denatured reduced samples in the SDS-PAGE as well as denatured, non-reduced samples (results not presented).

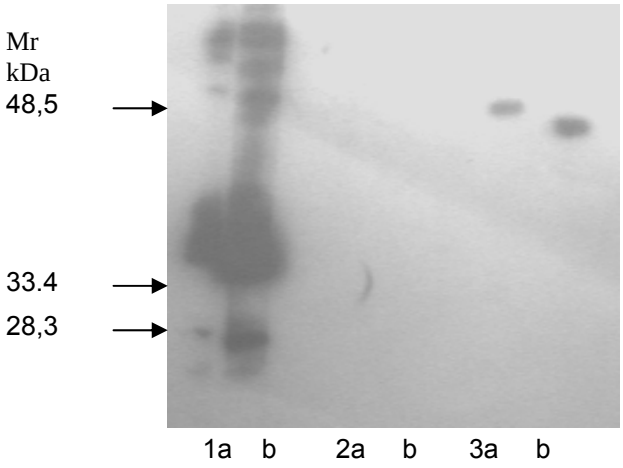


Figure 4.2: Immunoblot using autoimmune patient sera against 48 h, 0 (a) and 10 mM LiCl (b) treated LLC-PK_1 cells.
Sm ab (1); NHS (2); SSa/Rho Ab (3)

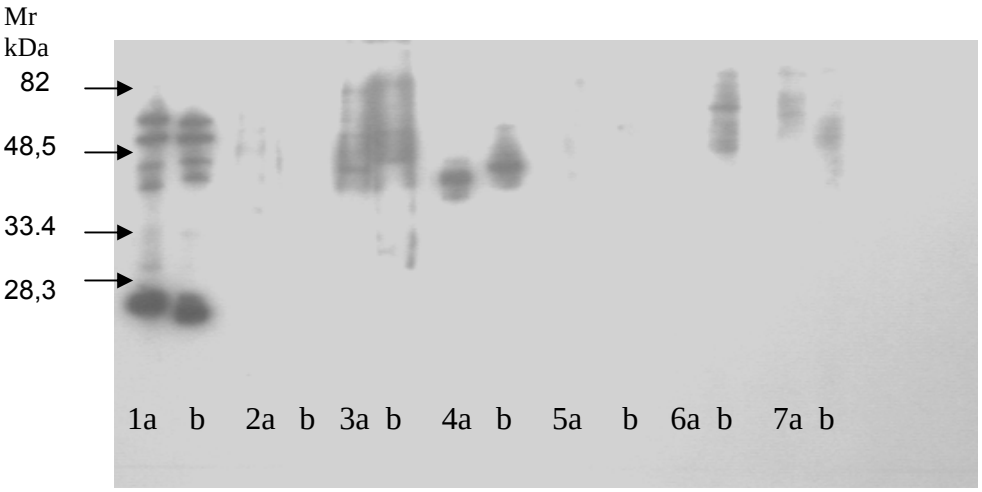


Figure 4.3: Immunoblot using autoimmune patient sera against 48 h, 0 (a) and 10 mM LiCl (b) treated PK(15) cells.
Sm Ab(1); Golgi Ab(2), 52 kDa Rho Ab(3); SSA/SSB Ab(4), NHS(5); RNP Ab (6), PCNA Ab (7).

The shift in molecular weight of some autoimmune complexes led to further investigation to establish possible causes. Phosphorylation of proteins was suspected and tyrosine phosphorylation in particular was characterised (Figure 4.4). No obvious alterations in tyrosine phosphorylation were noted between lithium treated and untreated PK(15) or LLC-PK_1 cells.

Protein glycosylation changes were also suspected in the alterations of molecular weights of autoantigens due to lithium treatment. With the use of lectins as glycosylation marker tools, lithium induced glycosylation changes were investigated. Lectin blots were performed using lectins obtained from various sources that were specific to some sugars. These being Concanavalin A that target glucose and mannose sugars, *Bandeirea simplificola* lectin that targets α-D-galactose, *Ulex europaeus* lectin binds to fucosyl-galactose, *Arachis hypogaea* lectin that binds to Galβ1-3GalNacα1-Ser/Tyr, and *Helix pomatia* lectin that binds to N-acetyl-D-galactosamine. Although the lectins bound to the porcine cell lysate glycoproteins, no particular differences between lithium treated or untreated glycoproteins were observed (Lucas, Moloto, Ngobeni, and Ncube, unpublished results).

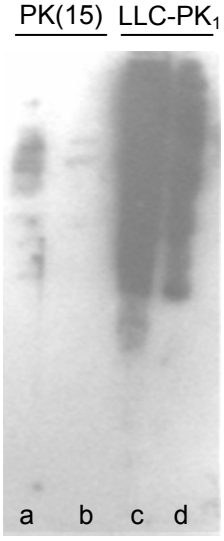


Figure 4.4: Immunoblot using antiphosphotyrosine against 0 (a,c) and 10 mM LiCl (b,d) treated PK(15) (a,b) and LLC-PK_1 (c,d) cells after 48 h

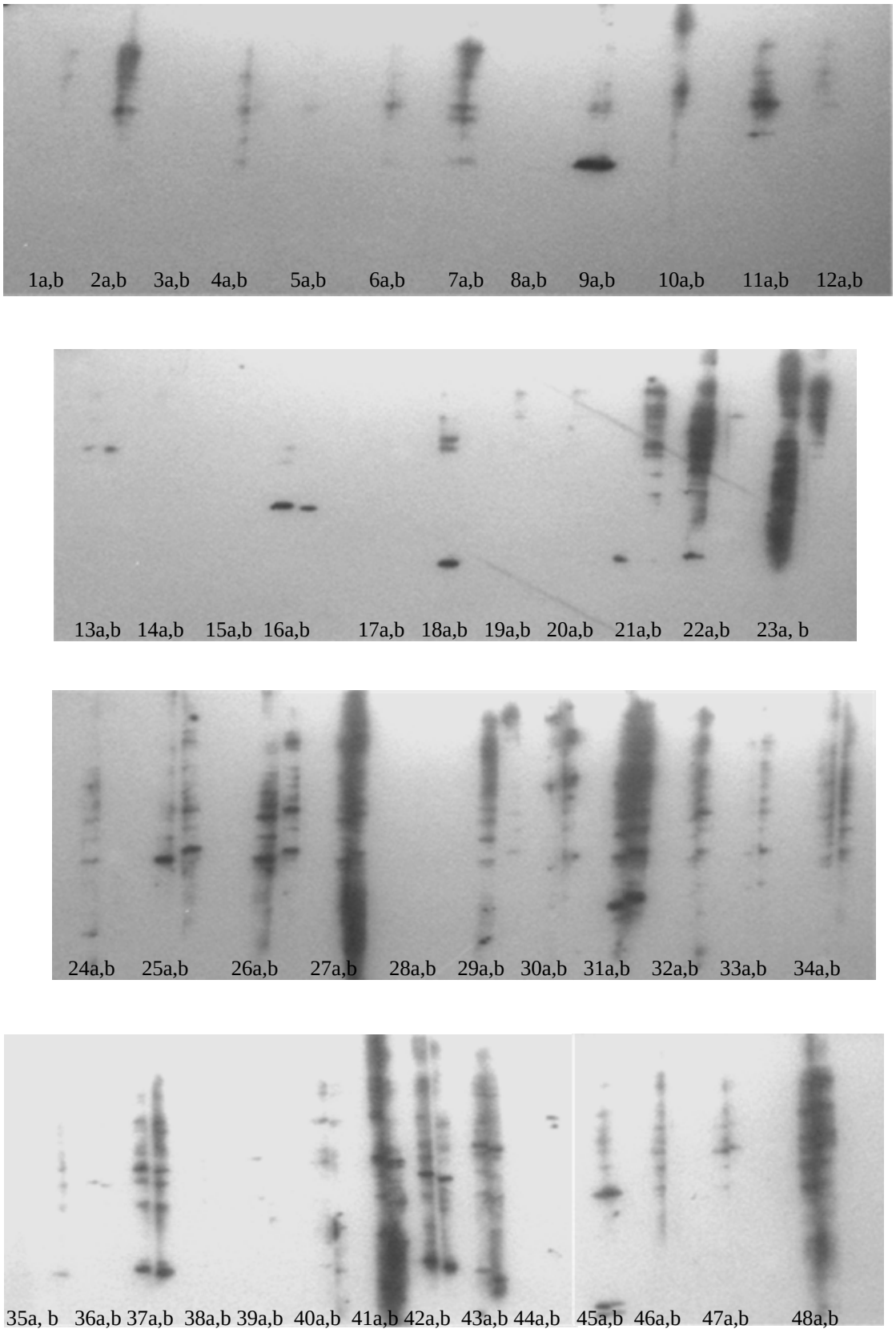
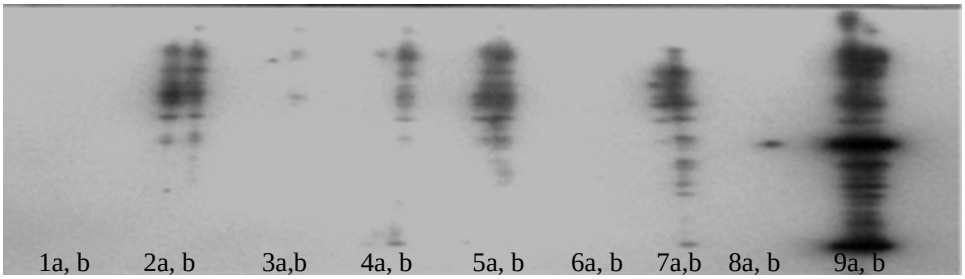
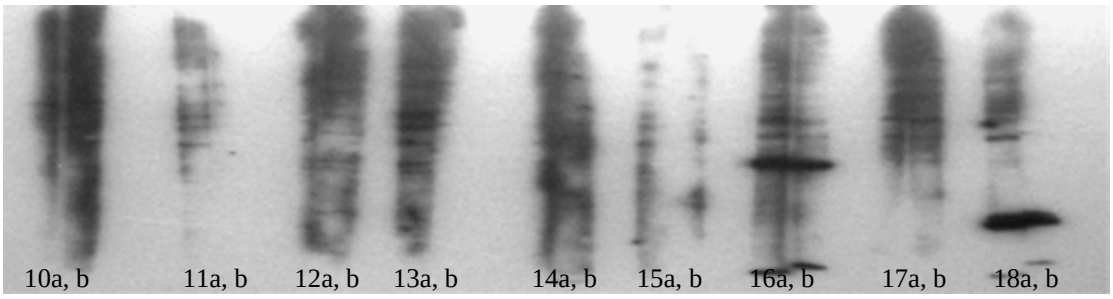
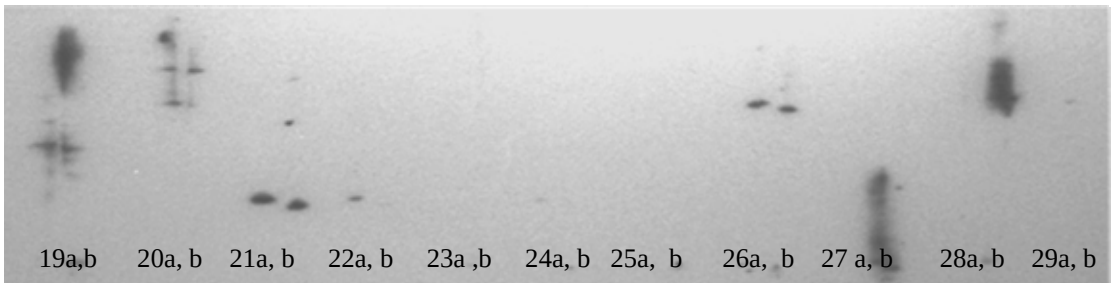
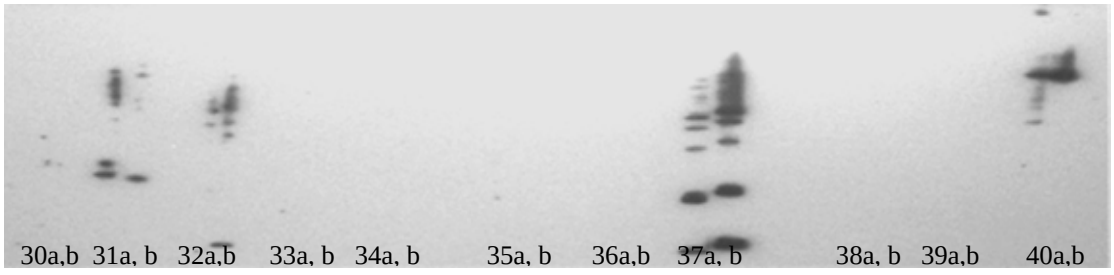


Figure 4.5: Western blot analysis of anti-autoimmune antibodies in LLC-PK_1 cells treated for 72 h with 0 (a) and 20 mM LiCl (b) using SLE patient sera.









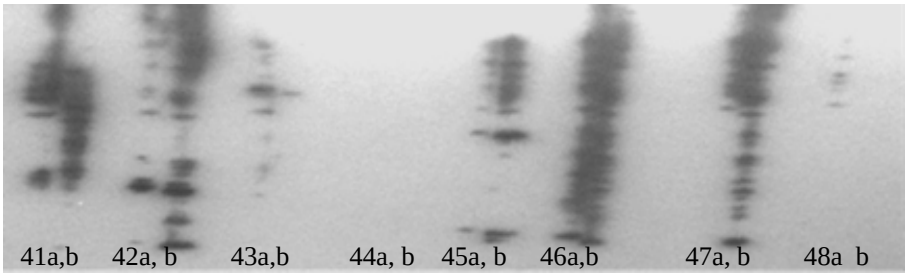


Figure 4.6: Western blot analysis of anti-autoimmune antibodies in PK(15) cells treated for 72 h with 0 (a) and 20 mM LiCl (b) using SLE patient sera.

To determine whether lithium induced changes in autoantigen formation could be used to differentiate between patients with or without renal disease, serum from 48 lupus patients was used in a blind manner to determine whether complex formation on lithium treated or untreated cells varied in a group dependant manner (Figures 4.5 and 4.6). Although complexes were observed in many of the patient sera used, no distinctions between lithium treated nor untreated complexes could be observed in either the LLC-PK_1 or the PK(15) cell lines.

Studies establishing whether glycosylation patterns of proteins within the sera of the 48 patients could be quantitated into groups were carried out using the previously mentioned lectins (Lucas, Madige, Dyantji and Ncube, unpublished results). Although the serum samples could be grouped into 7 different groups according to their concanavalin A binding pattern, the results cannot be validated as potential patient organ involvement markers without age, sex and race matched controls.

4.4 Discussion

Initial studies using well characterised human lupus serum in immunofluorescent (Tables 4.1 and 4.2) and Western blots (Figures 4.2 and 4.3) indicated that LiCl possibly altered autoimmune antigens in some way. Particularly, cell cycle specific golgi and SSA/SSB protein in the immunofluorescent studies and SSB in the Western blot analysis. The SSB autoantigen has been characterised as a calmodulin binding protein (Castro *et al*., 1996) that functions in dsRNA unwinding (Hühn *et al*., 1997), and is dephosphorylated and cleaved during anti-Fas induced apoptosis (Rutjes *et al.*, 1999) without affecting its subcellular position (Broekhuis *et al*., 2000). The results in this study, at concentrations of lithium known to initiate apoptosis in some cell lines (Madiehe *et al*., 1995), indicate no subcellular movement, but that the protein may be cleaved from a 50 to a 45 kDa fragment, as observed with the LLC-PK_1 cells. The results observed from lithium treated PK(15) cells were not the same, but instead a higher molecular weight protein was noted, possibly due to phosphorylation changes. Although

these results are in contrast to those reported by Broekhuis *et al*. (2000), they were persistent in all studies and indicate that lithium probably does effect some changes on this protein. Furthermore the Western blot studies indicated that a 28 kDa Sm autoantigen also seemed to be cleaved or dephosphorylated in both the LLC-PK_1 and PK(15) cells due to 10 mM LiCl treatment. The presence of Sm antibodies, that target B core proteins from snRNP splicesomal complex, although found in only 20-30% of lupus sera, are used as indicators for SLE (Kaufman *et al*., 2001). A further explanation for the changes observed in both the Sm and SSB/La proteins due to LiCl treatment is that there may be alterations in the splicing of the mRNA and hence protein formation as observed by previous authors (see Kaufman *et al*., 2001 for original references). Further studies need to be carried out to determine the relevance of this explanation.

Although alterations in molecular weight of the Sm and SSB/La proteins were observed in these studies, autoimmune complexes were still formed. This thus negates the implication that with a change in autoantigen structure, no immunoprecipitation would occur, and hence a lower amount of inflammation and damage to the organ. Furthermore, caution needs to be exercised as the sera used in the above mentioned studies were not from patients with renal disease, and thus the correlation to renal disease alteration by lithium cannot be made. Although it is known that a characteristic of autoantibodies is their cross-species specificity (Pollard *et al*., 1995), caution when using human serum across porcine antigens must be used. Normal human serum contains anti-gal antibodies that ordinarily target porcine antigens (Neethling and Cooper, 1999), however, the use of normal human serum as a control gave no complex formation which negates the above mentioned issue.

General antityrosine phosphorylation studies showed no changes in protein phosphorylation between control and lithium treated cells for both the PK(15) and LLC-PK_1 cell lines. These results mimic studies carried out previously in this laboratory using HL-60 cells and both Western blotting and ^{32}P labelling techniques (Lucas *et al*., 1995). Although these results seem to be in contrast to those of Rutjes *et al*. (1999) and Broekhuis *et al.* (2000), phosphorylation of

SSB/La antigens was specifically recorded at serine and not tyrosine residues, which is consistent with intracellular proteins. Few studies have reported lithium to alter tyrosine phosphorylation and those found refer to neuronal cells (Jope *et al*., 1991; Hashimoto *et al*., 2002) and in particular the effects on Src signalling. Further studies on lithium induced phosphorylation effects have indicated that serine-phosphorylation is altered in some proteins, in particular, GSK (reviewed in Jope, 2004).

As few lithium induced effects were noted between control and 10 mM LiCl treated cell autoantigens, additional experimentation was conducted with cell treatment of 20 mM LiCl. At this concentration the cells, especially the PK(15) cells, undergo apoptotic-like cell death and apoptosis-related antigen changes were expected. Using serum from SLE patients in remission, with, or without renal disease, immune complexes were investigated for any alterations. Figures 4.5 and 4.6 indicate that although complex formations did occur, there are no specific changes in the autoimmune complex formations occurring between human lupus serum and lithium untreated or treated proximal (LLC-PK_1) or distal (PK(15)) tubular-epithelial cells. Although no control samples were used in this study it is clear that lithium treatment of the cell lines used in this study cannot be used as an indicator of renal involvement in SLE.

Further limitations of this study were that Western blotting only determines changes in some autoantigens. Western blots do not detect changes in nuclear antigens, nor in phospholipid antigens. Since dsDNA is one of the prevalent autoantibodies within nephrogenic SLE, and antiphospholipid antibodies are prevalent in the disease status, further studies using alternate techniques such as immunofluorescence need to be conducted. Moreover, the cell lines used, and thus the antigens, were not derived from SLE or any other type of autoimmune patient. The results of these studies are thus only an indication of possible lithium induced effects and must be interpreted with caution.

Glycoproteins have been implicated in SLE disease (Li and Krilis, 2003) and as nephrogenic targets in NZB/W lupus mice (Lenz, 2005). Furthermore,

protein glycosylation of some proteins has been shown to be specifically changed during apoptosis (Brockhausen *et al*., 2002; Bilyy and Stoika, 2007). It was of further interest to determine the potential of glycosylation changes in these studies. Alterations in protein glycosylation states of both the SLE patient serum and the LiCl treated LLC-PK_1 and PK(15) cells were investigated to determine group changes in glycosylation profiles as well as lithium induced changes respectively. Using the relatively non-specific concanavalin A lectin profiles of patient serum, patients could be placed into 5-7 groups according to binding patterns. More specific glycoprotein binding lectins did not seem to show variations in binding profiles. These results show potential into variations of glycosylation due to disease status. Caution must be applied, however, as whole serum was used in this study. Thus no distinction between blood serum proteins including albumin, globulins, growth hormones, and complement proteins was made.

Studies using lectins to determine glycosylation pattern changes on lithium treated LLC-PK_1 and PK(15) cells, as opposed to their untreated counterparts, indicated few lithium induced changes. Allagui *et al*. (2007) using growth limiting concentrations of lithium, 5 and 10 mM, on lung alveolar cells, determined that lithium did not affect N-glycosylation in these cells. A recent report by Bilyy and Stoika (2007), published after completion of these studies, showed, using a variety of cell lines and apoptotic stimuli, that increased expression of α-D-mannose- and α-D-galactose-conjugated plasma membrane glycoproteins are a universal marker of apoptotic cells. The authors used many types of lectins including those used in these studies, but the lectins that could ultimately distinguish apoptotic markers were not used in this study. Further analysis using the responsive lectins described by Bilyy and Stoika (2007) should be performed in the future to determine whether the lithium induced apoptosis-like cell death of the PK(15) cells can be differentiated using this method.

In conclusion, this work suggests that lithium may not be altering renal antigens in these cells and therefore cannot be used as a tool to indicate

organ involvement within the disease. In addition, tyrosine phosphorylation did not seem to be involved with observed changes. Protein glycosylation of proteins within serum from lupus patients indicated some pattern differences whilst no glycosylation changes were observed due to the cell death status of the LLC-PK_1 or PK(15) cells due to treatment by high concentrations of LiCl, presumably due to the specificity of the lectins used.

CHAPTER 5
GENERAL DISCUSSION

1.6 Lithium toxicity on LLC-PK_1 and PK(15) cell lines

Choice of cell lines in any toxicity study is crucial to the results obtained. In this study the LLC-PK_1 and PK(15) cell lines were chosen as well characterized, established cell lines of renal tubular epithelial cells carrying markers for proximal and distal tubules respectively. Furthermore, both cell lines express high levels of urokinase, a protein expressed from tubular cell lines only. Since previous studies established that lithium prevented NZB/W mouse tubular-interstitial disease from progressing to end-stage renal failure (Krause *et al*., 1992), that urokinase transcription increased in these mice (Lenz, 1995), and that there were no established murine proximal and distal renal cell lines, the LLC-PK_1 and PK(15) cells were chosen. Furthermore, as the cell lines would later be used to assess human autoantibody binding studies, porcine cells were the alternative choice to using human renal cell lines, which were not available. A limitation identified using these cell lines was that lupus is a female prevalent disease and the LLC-PK_1 cells were established from a male Hampshire pig and the gender of the PK(15) cell line is unknown (ATCC information sheets). Thus any gender related autoimmune complex changes would not be relevant to this study.

Many toxicity studies of tubule cells have used LLC-PK_1 proximal cells and MDCK distal cells for comparisons. To my knowledge this is the first report comparing LLC-PK_1 to PK(15) with both cell lines established from the same animal species (porcine) and not from two separate ones i.e. porcine and canine. Since cell lines adapt characteristics of the tissues from which they are derived, but are somewhat different ones from intact cells that are part of those tissues (Shikano *et al*., 2004), it is important to select cell lines carefully. Evidence supporting this stems from studies by Bakker *et al.* (2002) who noted that human proximal epithelial cells were a more suitable model than LLC-PK_1 cells when studying the effects of cyclosporine A on apoptosis as the LLC-PK_1 cells were more susceptible to damage.

Previous studies that have compared LLC-PK_1 to MDCK cell toxicity have found the MDCK cells to be more resistant to cell injury producing agents than LLC-PK_1 cells. Agents that have caused this effect are oxaloacetate (Hackett *et al.*, 1995), amphotericin B (Varlam *et al*., 2001), and cadmium (Zhang *et al*., 1995; Zimmerhackl *et al*., 1998). Most of these findings have been explained as proximal cells absorbing higher amounts of the toxic agent than the distal cells. However, whether this was cell line specific, has not been explained. To date, very few lithium toxicity studies have been carried out on proximal and distal renal cells (see section 3.1.1). Only one previous study was found by this author on lithium treatment of LLC-PK_1 cells, though it was not a toxicity study (Goldberg *et al*., 1988), and to the best of my knowledge, no previous studies using lithium and PK(15) cells have been published. The lithium induced toxicity, or lack thereof, of these cells thus adds new information to lithium toxicity studies.

Interestingly, in this study the distal tubule cell line (PK(15)) was more susceptible to lithium treatment as opposed to the proximal cells (LLC-PK_1). The growth and viability studies indicated that both cell lines were able to withstand lithium chloride concentrations of up to 10 mM, the approximate urinary concentration in lithium treated patients (Goldberg *et al*., 1988; Godinich and Batlle, 1990), but light and electron microscopy studies clearly showed that the PK(15) cells round up and are released from the basal membranous support after 10 mM LiCl treated for 24 h. Of further interest was the observation that, although ordinarily easier to detach from their basal support than the PK(15) cells, LLC-PK_1 cells remained attached to the support after 72 h of 20 mM LiCl treatment showing no signs of cell death such as smoothing of the membrane and membrane blebbing, as did the PK(15) cells. Since 80% of filtered lithium is said to be reabsorbed in the proximal tubules with no appreciable absorption occurring in the distal tubule (Lydiard and Gelenberg, 1982) and the fact that Goldberg *et al*. (1988) found the intracellular LLC-PK_1 lithium concentration to be 17 mM as opposed to 10 mM extracellularly, more damage was expected in the proximal cells than in the distal cells. These results do, however, substantiate observations by Walker

et al. (1986) in which lithium treated rabbits showed signs of distal tubular damage. Perhaps the ion transport systems of the proximal renal tubular epithelial cells, adapted to moving lithium from the urinary filtrate to the blood, as opposed to distal tubules that are not predisposed to lithium of higher concentrations, may explain this finding. On the other hand, this explanation does not provide for the high concentrations of lithium tolerance observed in the MDCK cells (Rybak and Stockdale, 1981; Suh *et al*., 1992).

Cell culturing techniques are also pertinent in the interpretation of results. A possible shortcoming of this study is that no salt matched studies with NaCl were used in parallel studies. However, previous studies in this laboratory (Tyobeka and Becker, 1990; Madiehe *et al*., 1995) negated the effects of NaCl on the mitogenicity and toxicity of cultured cells. A further shortcoming may be the use of serum under experimental conditions. Both cell lines were grown in DMEM medium containing heat treated FBS, 5 or 10% in the LLC-PK_1 and PK(15) cells respectively. All experimentation was performed in the presence of FBS which adds to the dynamics of the results. Serum has often been omitted or reduced in the experimental phase of cell cultures in order to limit the unknown concentrations of serum proteins and their possible interference. Lithium enhances many cytokine and growth factor effects such as TNF (Beyaert and Fiers, 1992), insulin and epidermal growth factor (Rybak and Stockdale, 1981) and the same authors have noted differences in cell performance in the presence and absence of serum. However, the absence of serum in cell cultures has led to autophagic-type cell deaths due to amino acid deprivation, growth factor withdrawal, energy withdrawal and environmental stress (Lleo *et al*., 2007). In addition, Sinha *et al.* (2005) indicated that murine renal proximal cells underwent apoptosis during serum, and in particular, epidermal growth factor and insulin growth factor, deprivation. Lithium was able to limit apoptosis and increase the viability of cells in serum-deprived conditions, which could explain events observed by Suh *et al.* (1992) in MDCK cells. Since the aim of these experiments was to determine the toxic effects of lithium on renal cells, serum, with no batch variation, was kept constant in all experiments.

The results reported in the current study do, however, support the reported studies that lithium treatment, within the prescribed concentrations and well monitored, are not overly toxic to the renal tubular-interstitial cells.

Evidence for apoptotic cell death

Although the toxicity studies revealed that lithium chloride at low concentrations is not toxic to the cells, the mode of cell death at higher concentrations of lithium was investigated to determine cellular response to potentially toxic levels of lithium. No evidence for lithium induced apoptosis, necrosis, autophagy or paraptosis was observed using morphological or DNA fragmentation studies in LLC-PK1 cells. In addition, no participation of apoptotic proteins up to concentrations of 20 mM LiCl in the LLC-PK_1 cell was observed. The PK(15) cells were, however, susceptible to higher lithium concentrations (>10mM).

Morphological studies of lithium treated PK(15) cells indicated that the cells underwent an apoptotic cell death due to LiCl treatment. Furthermore, DNA laddering and terminal UDP-nick end-labeling studies further confirmed an apoptotic-like cell death. The immunocytochemistry and Western blot results indicated that the Bax:Bcl-2 ratio, found to play an important role in mitochondrial membrane permeabilisation during apoptosis, supported apoptosis as the mode of LiCl induced PK(15) cell death. P53, a role player in apoptosis after DNA damage, was not implicated in this role. Contrary to the above evidence caspase 8, 9 and 3 activity did not alter due to lithium treatment. Caspase 3 in particular, one of the executioner caspases towards which many apoptotic pathways converge, indicated no response. It thus appears as though lithium induces a caspase independent cell death in this cell line. In neuronal cells lithium attenuates caspase activity caused by apoptotic stimuli (King *et al*., 2001), and thus with no additional apoptotic stimulus in the present study, it is feasible that lithium may not activate caspase 3 activity. In addition, cisplatin, a toxic apoptotic stimulus of renal cells, was shown to function in a caspase 3- dependent or independent manner (Cummings and Schnellmann, 2002).

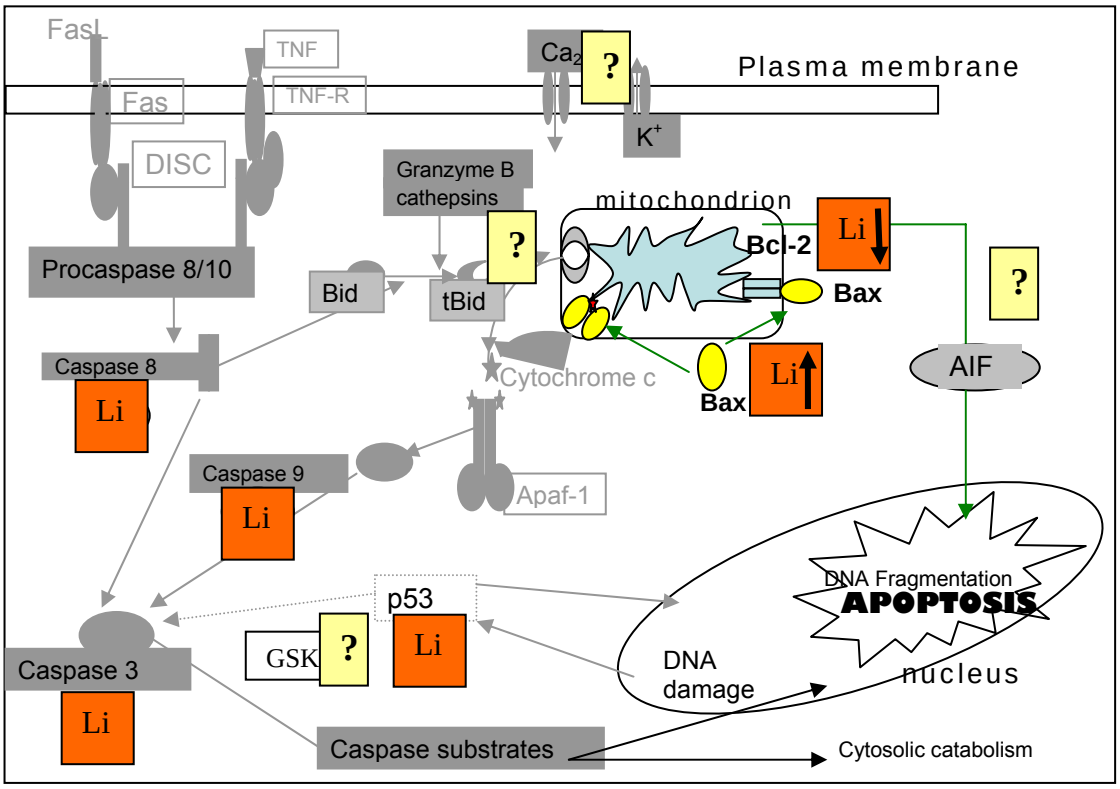


Figure 5.1: Proposed lithium induced apoptotic effects on PK(15) cells as determined in this study.

Li Indicate proteins investigated in the study, whilst accompanying arrows depict lithium induced expression of the proteins. Blocks with no arrows indicate that no response was observed between lithium treated and untreated ce ? Shows area still open for investigation into the mode of lithium induced cell death on PK(15) cells. (Diagram was drawn using information and diagrams from Israelis and Israelis, 1999; Budd, 2002; Mak and Yeh, 2002; Kaufmann and Hengartner, 2001; Müllauer *et al*., 2001; Wang *et al*., 2005; Buytaert *et al*., 2007; Mercer *et al*., 2007).

Figure 5.1 summarises the lithium effects on apoptotic proteins in the PK(15) cells elucidated in this study, and shows the possible pathways that may be involved. Further studies are required to assess whether proteases such as cathepsins or granzyme B are involved, or whether AIF, working downstream of Bax/Bcl-2 complexes, is the trigger for DNA fragmentation and cell death. Further possibilities to be investigated are the influence of lithium on potassium depletion (Toback, 1980) and apoptosis (Chin *et al*., 2005), as well

as lithium induced changes in intracellular calcium concentrations (Beyaert, 1999) and apoptosis (Bröker *et al*., 2005).

Lithium has been shown to cause an apoptotic type of cell death either directly (Madiehe *et al*., 1995; Tang *et al*., 2005), or indirectly, via TNFα (Beyaert and Fiers, 1992) in many cell lines. Furthermore, evidence of lithium induced apoptotic features have been seen in some renal biopsies of patients taking lithium for bipolar disorder (Markowitz *et al*., 2000). Contrary to an apoptotic mechanism, lithium has also been shown to cause an autophagic type cell death in African green monkey renal (COS-7) cells by inhibiting inositol monophosphatase and enhancing the clearance of autophagic proteins (Sarkar *et al*., 2005). Thus lithium may not cause the PK(15) cells to undergo conventional apoptosis as the mode of cell death, but further analysis using different techniques to assess caspase involvement, as well as alternate pathways should be performed.

5.3 Lithium effects on the autoantigens of renal porcine cell lines.

Although at first promising, no definite changes were observed in autoantigens in LLC-PK_1 and PK(15) cell lines. In particular, serum from SLE patients with renal disease, without disease, and in disease remission, did not show any group related changes between lithium treated and untreated cells.

Studies presented in this thesis relied on the ability of autoantibodies to cross-react and bind across species, possibly limiting the number of immune-complexes formed due to species variations. Subtle differences may easily have been missed. In addition, the concentrations of lithium used in this study were found to cause apoptosis-like cell death in the PK(15) cells. Apoptosis is known to create a host of differently expressed proteins, as well as cleavage and post-translated modified proteins. The flipping of the cell membrane to expose phosphatidylserine and other normally internalized proteins during apoptosis can also lead to the generation of more autoantigenic sites (Batisse *et al*., 2004). Apoptosis can therefore generate a variety of antigenic

determinants which could lead to possible anomalies in the results. in view of the fact that both apoptotic and non-apoptotic cell death has been largely implicated in the development of autoantigens in lupus patients, and the fact that SLE patients contain more circulating apoptotic debris than control subjects (Utz *et al*., 2000), the use of cells undergoing apoptosis may be useful in detecting new autoantigens, leading to more specific diagnosis and control.

The studies in this report do not substantiate those of Krause *et al*. (1992) that indicate less tubulointerstitial disease in lupus mice models possibly due to a change in autoimmune complex formations and reduced inflammation. Acute treatments of lithium were used in the present studies which contrast those of chronic lithium treatment of NZB/W mice performed by Krause *et al.* (1992) and Lenz (1995). Longer exposure to lithium may cause alternate changes to proteins as observed in neural cells by Chen and Chuang (1999) and in GSK inhibition studies (Jope and Bijur, 2002). To further support the implication of a chronically derived lithium induced pathway change, Lenz (1995) noted that the livers of lithium treated NZB/W mice were lower in weight than their untreated counterparts, but that there was no substantial change in their kidneys. Since lithium inhibits GSK, and hence glycogen formation which is ordinarily stored in the liver, this could be an indication that chronic LiCl treatment may effect changes not observed with acute treatments. In addition, the results presented in this report were performed under *in vitro* conditions. This is in contrast to the *in vivo* murine experiments performed by Krause *et al*. (1992). The porcine cell lines were thus investigated in the absence of a holistic system and in particular in the absence of an immune and inflammatory response. The results do, however, concur with similar observations made by Lenz *et al*. (1997) in their studies of NZB/W mice antibody binding profiles to both LLC-PK_1 and PK(15) cells.

Using these results, one cannot, however, speculate on the lithium induced changes on dsDNA autoantigens which are known to penetrate and bind directly to dsDNA within renal cells. The resulting damage is believed to be caused either by immunoprecipitation and inflammation, or have a direct toxic

effect on the cells (reviewed in Putterman, 2004). Furthermore, recycling and presentation of the antibody on the cell surface has suggested that the penetrating antibodies may themselves become immunogenic. Further studies using cells established from lupus patients or animal models should thus provide evidence as to whether lithium is able to alter autoantigens or not.

Conclusions

The following conclusions were made:

7. Cell proliferation studies indicated that growth and viability of both the LLC-PK_1 and PK(15) cell lines were not altered by lithium concentrations of 10 mM and lower. Cell proliferation decreased by approximately 15% and 20% after 20 and 50 mM LiCl treatment. These results indicate that therapeutic concentrations of lithium are not toxic to porcine renal proximal (LLC-PK_1) and distal (PK(15)) tubular epithelial cells in culture.
8. Electron micrographs showed clear apoptotic features of PK(15) cells treated with LiCl concentration of 10 mM and higher. No apoptotic-like characteristics were observed in the LLC-PK_1 cells. DNA fragmentation observed by both DNA laddering and TUNEL assay techniques substantiated that LiCl at 20 mM and above caused DNA fragmentation in PK(15) cells, but not in LLC-PK_1 cells.
9. Lithium did not alter the protein expression of the proapoptotic proteins Bax and p53, or that of the anti-apoptotic protein Bcl-2 in LLC-PK_1 cells. In addition, no change in caspase 3, 8 or 9 activity was observed in these cells, validating the results observed in 1 and 2 above. A two-fold increase in the Bax:Bcl-2 ratio, after 10 and 20 mM LiCl treatment of PK(15) cells was observed. This is indicative of mitochondrial involvement in the mode of cell death. No alteration in p53 protein expression, nor in caspase 3, 8 and 9 activity was observed due to lithium treatment of these cells indicating a caspase independent pathway of lithium induced PK(15) cell death.

10. Although the expression of retinoblastoma, a protein involved in both the cell cycle and apoptosis, was elevated after LLC-PK_1 cell treatment with 5 and 10 mM LiCl as opposed to the control, no change in PCNA protein expression, nor in the cell cycle was observed. Lithium did however elevate both Rb and PCNA proteins in PK(15) cells, and caused the cells to accumulate in the G_2/M phase of the cell cycle.
11. Initial studies, using well characterised lupus patient serum and Western blotting techniques, indicated that lithium altered the SDS-PAGE determined molecular weight of Sm and SSB autoantigens by decreasing and increasing them respectively. Again, the results were more prominent within the PK(15) cell line than the LLC-PK_1 cells. To further characterize whether lithium induced changes could be used as a marker for organ involvement in lupus patients, lupus serum from patients in remission, and with, or without kidney disease were assessed for lithium induced changes of the autoimmune complex formation. No changes in immune complex formations were observed due to lithium treatment of either the LLC-PK_1 or the PK(15) cells, and thus this technique cannot be used to determine organ involvement in SLE.
12. No changes in tyrosine phosphorylation of proteins treated with lithium in both the LLC-PK_1 and PK(15) cell lines were observed. In addition, studies investigating the glycosylation status of the lithium treated cell lysates, using a variety of lectins, did not show overt changes when compared to their untreated counterparts. Lectin binding studies did, however, show variation in lupus serum samples, providing interest that perhaps glycosylation studies could be used to assess disease progression in SLE patients.

In summary, lithium chloride did not adversely affect the porcine renal cells at concentrations used therapeutically, < 5mM, which substantiates reports that if monitored correctly, lithium will not damage renal tubule cells. At concentrations higher than 5 mM, lithium seemed to induce a type of cell death in the PK(15) distal cells that appeared to be apoptotic-like but caspase independent. The LLC-PK_1 proximal renal cells remained unaffected, even at

the higher lithium concentrations. In an effort to determine whether lithium altered SLE autoantigens in renal cells as a possible cause for lower kidney end stage renal disease previously observed in NZB/W mice, initial results indicated possibilities of this. Further studies to assess whether these changes could be used as a diagnostic tool for the indication of renal involvement in SLE were, however, inconclusive. However, tests that could determine the involvement of organs in SLE before damage occurs will provide an enormous benefit to patients and thus more work in this area may be pursued.

CHAPTER 6
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APPENDIX

This section contains a list of publications and presentations that have emanated from the work presented in this thesis.

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List of publications

Hart, D. A., Fritzler, M. J. and Lucas, K. C. (1998) Characterisation of Lithium Chloride mediated enhancement of survival in murine models of autoimmune disease: Evidence that lithium influences target organ resistance to immune insults. In The Biological and Clinical Actions of Lithium: New Perspectives, Becker, R. W., Lucas, K. C., and Gallicchio, V. S. (eds) Weidner Publishing, Cheshire, Connecticut, USA, pp 93-108.

Lucas, K.C., Moller, A., Becker, R.W. and Hart, D.A. (2000) Lithium chloride induces apoptosis in the porcine PK(15) renal tubular cell line, Journal of Trace and Microprobe techniques, **18**, 475-484.

Same article published, with permission, in Lithium - 50 years: Recent advances in Biology and Medicine (1999) Kirsten C. Lucas, Rolf W. Becker and Vincent S. Gallicchio (eds), Weidner Publishing group, Chesire, Connecticut, pp 189 - 200.

List of presentations

INTERNATIONAL

Lucas, K. C. And Tyobeka, E. M. (1996) The effects of lithium on the phosphorylation of proteins in HL-60 cells, Cell growth control symposium, Authers, Seat Hotel, Cape Town. (Poster)

Lucas, K. C., Becker, R. W., Moller, A. and Hart, D. A. (1999) The effects of lithium chloride on apoptosis in porcine renal tubular cells, Lithium-Kentucky conference, Lexington Kentucky USA, May 1999 (Poster)

Lucas, K.C., Möller, A., Becker, R. W. and Hart, D.A. (2001) Systemic Lupus Erythematosus, Lithium Chloride, and apoptosis: The relationship in different renal cell lines, IUBMB special meeting and SABMB meeting, Cape Town, November 2001. (Poster)

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Lucas, K. C. And Tyobeka, E. M. (1994) The effects of lithium on tyrosine phosphorylation patterns of HL-60 cells. S. A. Biochem. Congress, Stellenbosch. (Poster)

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Lucas, K. C., Fritzler, M. J. and Hart, D. A. (1998) A Potential role of Lithium in the Enhancement of Survival of Murine models of Systemic Lupus Erythematosus, 2nd Federation of African Society of Biochemistry and Molecular Biology, and, 15th SASBMB, Potchefstroom, South Africa. (Poster)

Moller, A., Hart, D. A. and Lucas, K. C. (1999) Lithium effects on the morphology of renal epithelial cells, Presented by A. Moller at the Microscopy Society of Southern Africa Meeting in Bloemfontein (Poster).

K.C. Lucas, A. Moller, R.W. Becker and D.A. Hart (2000) Lithium Chloride causes apoptosis in porcine kidney cells, Presented by RW Becker at the BIOY2K Combined Millinium Meeting, Grahamstown, 24-28 January 2000 (Poster).

A. Moller, D.A. Hart and K.C. Lucas (2000) The effects of lithium on distal renal epithelial cells, presented by A. Moller at the 39th annual MSSA conference, Grahamstown, 6th-8th December 2000.