Effects of psychoactive trace minerals on glial cell development *in vitro.*

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Abstract

Trace minerals are a group of nutritional elements appearing in low concentrations in body tissues. These minerals may have both regulatory and structural functions in the body, and therefore play an important role in human health and well being. Many of these elements have psychiatric actions, including lithium, copper and zinc. Lithium is the most commonly prescribed psychoactive trace mineral, as this is the current preferred treatment for bipolar disorder.

The research presented in this thesis has focused on the psychoactive nutritional trace minerals, copper and zinc and how they interact with lithium to affect glioblastoma cell proliferation, function and viability *in vitro*. Two different glioblastoma cell lines (U87MG and 1321N1) were cultured in media containing different concentrations of either zinc or copper above and below the physiological range (0.02 to 10 mg/L copper or 0.2 mg/L to 10 mg/L zinc), in the absence and presence of lithium (1 mg/L).

Cell viability was determined using tryptan blue staining. Proliferation rate was determined using both visual counting and an MTT (Methylthiazol tetrazolium) cell proliferation assay. Cell morphology was measured using a standard Harris haematoxylin/ eosin-y morphology stain and a computer software package. Cell function was determined by evaluation of cellular resting potential .

A comparison of two proliferation assays used in this study revealed the MTT assay to be the most accurate and precise. At trace mineral concentrations up to 7.5 mg/L, there were no dose dependent effects of either zinc or copper on cell proliferation, with or without lithium in the culture media. However, at a supraphysiological trace mineral concentration (10 mg/L), U87MG cells appeared to be tolerant to zinc, but sensitive to copper which resulted in marked reductions of cell number (2.91% of control), and cell

viability (24.36% of control). However, 1321N1 cells appeared sensitive to both supraphysiological zinc and copper concentrations with cell viability reduced to 73.63% and 68.63% of control, respectively.

Zinc uptake by 1321N1 cells appears to be highly regulated, and intracellular zinc levels detected using a fluorescent probe were not changed by extracellular medium concentrations of zinc up to 10 mg/L. This tight regulation was perturbed by treatment of cells with therapeutic (1 mg/L) levels of lithium. These results indicate an interaction between lithium and zinc in cell culture which may be of clinical interest if reproducible in other cell types.

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Glossary

AAS	Atomic Absorbance Spectrophotometer
ADHD	Attention Deficit Hyperactivity Disorder
AIDS	Autoimmune deficiency syndrome
ANOVA	Analysis of variance
B cells	B lymphocytes (formed in bone marrow)
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECT	Electroconvulsive therapy
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetra acetic acid
EPR	Electron parallel resonance
FBS	Foetal Bovine Serum
GABA	Gamma amino butyric acid
GST	Glutathione S-transferase
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP3	Insositol triphosphate
LD50	The dose of a drug required to kill 50% of a treatment test group
MDM2	Mouse double minute 2, human homolog of; p53-binding protein
MHz	Mega Hertz
MTT	Methylthiazol tetrazolium
NMDA	N-methyl-D-aspartate
PAM	Peptidylglycine alpha-amidating monooxygenase
PDGF	Platelet derived growth factor
PCRM	Physicians committee for responsible medicine
PTEN	phosphatase and tensin homolog gene
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
T cells	T lymphocytes (formed in thymus)
TRIS	(hydroxymethyl) aminomethane

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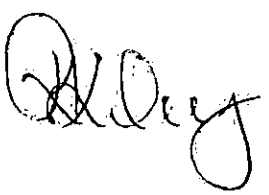
And, of course, heartfelt thanks go to my husband, Neil for his understanding, forbearance, and willingness to help and support me in any way possible!

DECLARATION

I declare that the work contained within this thesis is my own, and that appropriate credit has been given where reference has been made to the work of others.

The work published in this thesis has not been submitted for any other academic award and whilst registered for this degree I have not been a registered candidate for any other award by any other awarding body.



Ruth Kiley

CHAPTER 1

INTRODUCTION

1.1 Psychoactive trace minerals

The Austin Nutritional Research group, Texas define trace minerals as; "minerals that occur in tiny amounts or traces. They play a major role in health, since even minute portions of them can powerfully affect health. They are essential in the assimilation and utilization of vitamins and other nutrients."[1] These minerals have a wide range of biological activities, predominantly predicted by influences they have on hormonal and enzymatic activity within biological systems.

This group, the 'trace elements' include aluminium, arsenic, bromide, boron, caesium, calcium, chromium, cobalt, copper, iron, iodine, lead, lithium, magnesium, manganese, potassium, rubidium, scandium, selenium and zinc. The sale of forms of these as nutritional supplements to aid health, both mental and physical is a large and lucrative modern business and many of these do have significant health benefits, although many exceed the recommended uptake. Some elements have been more extensively researched than others, and found to have marked psychological or physiological effects. The best known of the trace minerals to be used in treatment of illness is lithium. The efficacy of lithium in treatment of bipolar disorder is well documented and it continues to be the preferred method of treatment[2].

Many of the other trace minerals have been demonstrated to have psychological effects and may interact with lithium. In schizophrenia, a disorder which can often have a similar presentation to bipolar disorder, levels of the trace elements cerium, indium, copper, boron, vanadium and zinc have been found to decrease, while the

concentrations of manganese, calcium, caesium and scandium increase[3]. Chromium has also been found to potentiate antidepressant treatment for dysthymic disorder in a limited number of patients observed in one trial[4].

Lithium carbonate treatment has been shown to specifically alter serum concentrations of some trace elements. Cobalt, potassium, iron, vanadium and rubidium have been found to decrease with increasing levels of lithium, but aluminium and bromide have been found to increase with increasing lithium[5]. The relationships between these elements within the periodic table and within enzymatic reactions within biological systems may provide some indication of modes of action of lithium prophyaxis.

Selenium and zinc have been found to have effects in a variety of disorders, including Alzheimer's disease, depression and hostile behaviour patterns. Patients with Alzheimer's' brains have been found to contain only 60% of the amount of selenium found in the brains of healthy subjects[6]. In addition, diminished selenium concentrations were associated with a depressed mood status and an increase in hostile behaviour in a group of young men living in a research compound[7]. Zinc concentrations in plasma have been shown to fall in Alzheimer's disease and in both unipolar depression and schizophrenia, both of which are often related to bipolar disorder[6].

Haepatic zinc levels have been shown to rise in lithium treatment[8]. Zinc is found in presynaptic vesicles, release from which is dependent on neural activity. Zinc has therefore been proposed as an indicator of neural injury in a variety of brain diseases, and is therefore likely to be particularly interesting to study *in vitro*.[9]

Plasma copper concentration has been found to be reduced in depressed patients, irrespective of treatment levels and observed effectiveness of the treatment, and has therefore been proposed as a 'trait marker' for depression[10]. Copper-dependent

enzymes play a vital role in cellular respiration and in the catecholamine biosynthetic pathway. Copper is therefore likely to influence neural cell development and activity[11].

Several trace mineral cations have been shown to have a regulatory effect on the action of the N-methyl-D-aspartate (NMDA) receptor in excitable cells. NMDA receptors are one of the 3 groups of the glutamate family of receptors. These receptors are permeable to calcium ions and are important to synapse plasticity[12], vital to learning and injury recovery processes within the brain[13]. These receptors can be blocked by either magnesium or zinc ions. The ions of calcium, magnesium, zinc, aluminium and lithium have all been shown to have effects on NMDA receptor activity and to have interactive effects[14]. Zinc, mercury, aluminium and lead have also been found to alter activity of voltage-activated calcium channels, but not sodium or potassium channels in excitable neural cells[15].

Some heavy metal complexes have also been found to have influences on the Gamma Amino Butyric Acid (GABA) receptor-channel complex. Mercuric chloride has been shown to augment GABA-induced current, while copper and zinc complexes suppress GABA-induced current[16]. GABA is a major inhibitory neurotransmitter in the central nervous system (CNS), inhibition of which can lead to seizures. Drugs with positive modulation of GABA receptor activity are very effective in seizure control and used in treatment of epilepsy.[17]

The above overview indicates that many trace elements have been shown to be psychoactive. The following review will focus on lithium, the most ubiquitous element in the treatment of neuropsychiatric disorders, and its interaction with two other trace elements of clinical importance, copper and zinc. In order to understand the efficacy and importance of lithium as a drug, it is first important to understand bipolar disorder, the illness for which it is most commonly prescribed.

1.2 Bipolar disorder

Bipolar disorder is a euthymic disorder featuring a cycling of mood state between a state of 'mania' and one of 'depression'. Onset is often in adolescence, with atypical features present in early, or adolescent bipolar disorder and the speed of cycle increasing with the number of previous cycles experienced.

The manic phase can last anywhere between 2 weeks and 4-5 months, the median duration being about 4 months. The manic phase may be prolonged by the existence of a prodrome (hypomania)[18]. The prodrome may last around 2-3 weeks, and usually features increased consumption of alcohol, disturbed sleep patterns, and milder forms of the symptoms of mania. Change between phases of the disorder and onset of a cycle may follow stressful circumstances, however, symptoms may vary between days with no external influences, and a diurnal variation in presentation is also common[19].

Common features of the manic phase include increases in thought speed, physical activity, sociability, talkativeness and sexual energy; along with elevated mood state, over-familiar behaviour, and a decreased perceived need for sleep. Other symptoms may appear to be similar to those of schizophrenia, and may cloud diagnosis, especially if the prodrome or hypomanic state has not been observed. These include hallucinations, delusions, ideas of persecution and irritable or boorish behaviour. A manic phase may disrupt all aspects of a patient's life, but can be pleasurable to the patient and lead to great creative achievements (for example Handel's Messiah and some of Virginia Woolf's writings)[20] This pleasurability can lead to non-compliance with medication which may initiate relapse into the manic-depressive cycling[21].

A depressive phase may last around 6 months, and shows many of the symptoms of unipolar depression. Common somatic symptoms of the depressive state include

anhedonia, emotional unreactivity, psychomotor retardation or agitation, loss of appetite and weight, reduced libido, and disturbed sleep patterns that lead to early waking (at least 2 hours early) and a greater degree of depression in the mornings. Other common features include a loss of self-esteem, a shortening of attention span, feelings of pessimism, and guilty thoughts that often lead to thoughts or actions of self-harm or suicide, frequently in the form of overdosing.

Adolescent patients may also show other symptomatic features of manic depression including anxiety, irritability, excessive alcohol consumption, phobias, obsessive behaviours and hypochondria. These symptoms may appear more prominently than those mentioned above[22].

There are many subclasses of the depressive state, which may be classified as being with or without somatic symptoms, (four or more somatic symptoms must be featured to be classified as "with"). Somatic symptoms may be mild, moderate or severe, and if severe, may be with or without psychosis.

Mild depression features; a depression of mood state, along with anhedonia and increased fatiguability, and must feature two others of the symptoms listed above.

Moderate depression should feature at least two symptoms of mild depression, plus three to four others. Symptoms are often marked if the range of them is limited, and an episode should last 2 or more weeks.

Severe depression almost always features somatic symptoms and loss of self-esteem. Feelings of guilt and attempts of self-harm or suicide are also common features of severe depression. Cycling of severe depression may also be referred to as recurrent depressive disorder.

Severe depression may also feature symptoms of psychosis, often including auditory or visual hallucinations commonly featuring accusatory or defamatory voices, or visions of rotting flesh or waste. A patient experiencing severe depression with psychotic symptoms may also believe that the loss of a loved one or family member, or world disasters in current affairs are caused by unrelated activities of their own, or that unless they perform certain ritualistic behaviours disasters will befall themselves or those close to them.[19]

Cycling between these two states of mania and depression may disrupt all aspects of patients' lives - work, home and social. Severe, unpredictable mood swings and distorted beliefs about self and others can result in loss of jobs, friends and family, leading to isolation and loss of self worth, compounding symptoms of the disorder. Rapid diagnosis and accurate treatment is therefore essential.

Some patients with bipolar disorder may show neuropsychological impairment, and it has been suggested that a higher level of impairment may indicate a poorer prognosis for the disorder[23]. Neuropsychological impairment symptoms often resemble those of schizophrenia, but are generally less severe. The symptoms shown vary between stages of bipolar disorder. Patients in a depressed state demonstrate impairment in frontal lobe function, while patients in a manic state show impairment in discriminatory and verbal learning skills[24]. The reason for a poor prognosis among neuropsychologically impaired patients is not clear.

1.3 Lithium.

Effective treatment of bipolar disorder using lithium carbonate (also called Carbolith or Lithane) was first recorded by John Cade in 1940. Initial treatment of symptoms is usually acute and vigorous in order to control symptoms, followed by a gradual reduction in additional treatments to provide a chronic stabilisation. Lithium carbonate,

the most ubiquitous form of therapy for bipolar disorder patients, is defined as a "mood stabiliser" and may be used in conjunction with other treatments such as reuptake inhibitors, eletroconvulsive therapy (ECT), carbamazepine (tegretol), divalproex, thyroxine (to counteract a major side effect of lithium prophylaxis; hypothyroidism), lamotrigine, gabapentin, and atypical antipsychotics. Response to each form of treatment may vary between patients and is dependent on many factors, primarily compliance with treatment. However, the stage of cycle, number of previous cycles and rate/symptoms of cycles have all been suggested as possible predictors of response. Level of response to treatment with lithium carbonate is often evident within approximately one week of commencement of treatment, but other treatments may have a longer period of latency. Carbamazepine, for example, may take up to 10 to 14 days.

There have been many theories as to how lithium actually acts to stabilise mood. One of the current theories is that lithium influences calcium levels in the intracellular space[25]. Inositol trisphosphate (IP3) is the second messenger molecule responsible for calcium release from storage within cellular organelles. The final stage of the recycling process of IP3 involves the enzyme inositol monophosphatase, which controls the levels of IP3 and controls mood swings. The initiation of action of inositol monophosphatase is dependent upon the action of 2 magnesium ions on receptors. Lithium acts by mimicking magnesium, with which it has a very similar charge: size ratio. Lithium thus blocks the Mg receptors on inositol monophosphatase and prevents the breakdown of IP3, interrupting the re-absorption of calcium.

Lithium may act to inhibit many phosphatases, as shown in figure 1, but its action is most potent on inositol monophosphatase. IP3 can also play a role in proliferation control and function of cells, and thus lithium's disruption of IP3 breakdown may be of interest in proliferation studies[26].

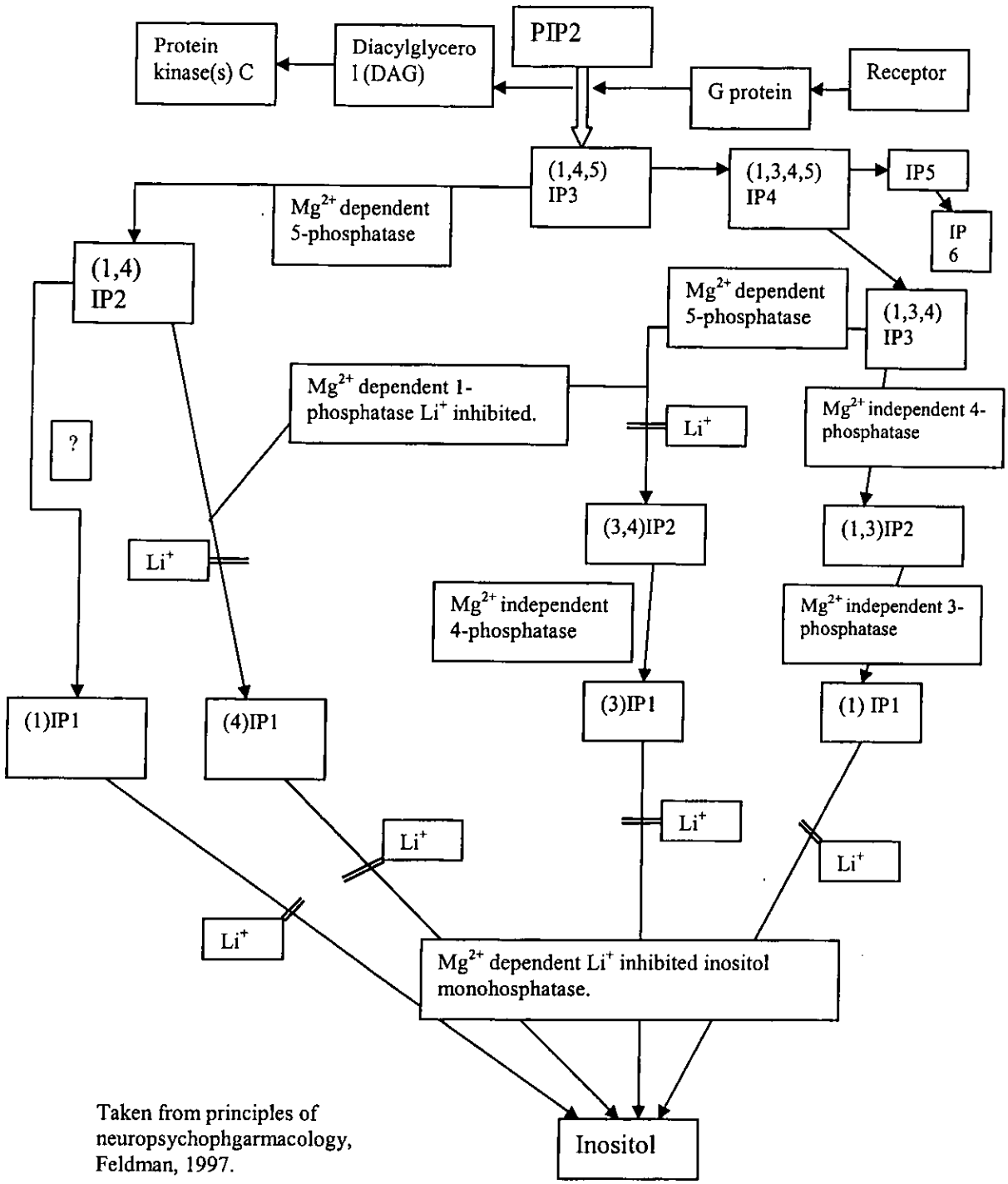


Figure 1. The phosphoionositide cycle, including the synthesis and metabolism of multiple second messengers.

Lithium affects many of the enzymes involved as shown in figure 1 above.

1.3.1 Variability in patient response to Lithium therapy.

Response to lithium treatment can be variable between bipolar disorder patients. One study has compared 3 techniques of assessment of lithium responsivity and efficacy. The 3 studies compared were:

- Discontinuation technique, in which patients treated with lithium have has their treatment discontinued, and the reoccurrence of symptoms recorded[27]. This method may, however, result in withdrawal effects.
- Before and after observations of lithium prophylaxis effects[28], this may be inaccurate owing to the cyclic nature if this disorder.
- Placebo studies[29]. This study appears the most reliable but is still prone to inaccuracies rising from assessment of a cyclic psychiatric disorder.

Each of these studies confirmed the existence of responders and non-responders to lithium prophylaxis. Cooper found a non-significant proportion of patients improved following lithium prophylaxis compared to placebo, but the only patients to display no symptoms were those receiving lithium treatment. This supports the hypothesis that some patients do respond to lithium prophylaxis and others do not, however for those who do not respond, there is no similarly effective alternative treatment at the present time[30].

Many studies have been carried out in order to find a method of predicting the responsivity of patients to lithium prophylaxis. Patients with a greater cognitive impairment appear to have a poorer prognosis than those of minimal or no impairment[23], but other indicators have also been proposed. Response to auditory evoked potentials has been suggested as a predictor of responsivity to treatment, as has

phosphocreatine response to photic stimulation. A reduced response to photic stimulation in treatment resistant patients has been found[31]

1.3.2 Side effects of lithium therapy.

Lithium prophylaxis may carry side effects, including electroencephalographic abnormalities associated with an increase in bromide and vanadium levels[32], alterations in levels of other trace elements, and hypothyroidism[33,34].

Hypothyroidism may confound results of lithium efficacy studies, as thyroid activity may affect mood. This means that lithium, unless used in conjunction with thyroxine, can help to contribute to the symptoms it is intended to treat[35]. Hypothyroidism has been observed to affect 2-5% of lithium patients, particularly ladies aged between 35 and 55 (14% women, 4.5% men). Thyroid screening and advice is therefore recommended for all potential lithium patients[21].

1.3.3 Interactions between lithium and other trace minerals.

The action of lithium illustrated in figure 1 is dependent upon the ability of lithium to interact with, and mimic other trace minerals. However, other interactions are also apparent.

Bromide has been proposed as a lithium activity potentiator, possibly enhancing effects of lithium in the human body. Bromide is therefore likely to have a role in the degree of responsivity a patient shows to lithium prophylaxis. However, Handorf *et al* have suggested that elevated serum bromide may actually be due to altered renal function shown to occur with high levels of lithium[36].

It has been proposed that rubidium may have a synergistic interaction with lithium as they both appear in the same group of the periodic table. Combined treatment with

lithium and lead has been shown to correlate with a decrease in serum arsenic levels, and red blood cell transport of vanadium, rubidium and caesium has also been shown to be altered with differing levels of lithium[37].

Many of the trace elements studied, including copper, iron, selenium, chromium, cobalt and zinc, can influence immune function[31]. It has been suggested that immune function may play a role in the responsivity to lithium prophylaxis[38]. Cytokines, such as interleukin-6, lymphocytes and killer cells, have all been shown to be altered in mental disorders such as unipolar depression, melancholia and schizophrenia[39], and in altered trace element status[40]. Lithium has also been shown to influence activity of the immune system. In humans, evidence has been found that at above normal therapeutic levels, lithium can increase the synthesis of immunoglobulins by B-lymphocytes, proliferation of T-lymphocytes and phagocytotic activity of macrophages[41]. Some work is being carried out as to uses of this function of lithium in the treatment of autoimmune deficiency syndrome (AIDS)[42]. It is, therefore, possible that it is immune system status, not trace element status that is affected in bipolar disorder, and it is here that differences should be sought in patients taking lithium. Trace mineral status, therefore, may play a key role in the response to lithium treatment.

1.4 Zinc

Zinc is a group II alkali metal forming tetrahedral compounds. It has a molecular weight of 65.37, and an atomic number of 30. Zinc is found in amino acids and nucleotides, and has a particular affinity for electron rich thiol, hydroxyl and nitrogen groups. Good dietary sources of zinc include red meat, shell fish, chicken, egg and whole grain[43]. Zinc is the second most prevalent trace mineral in the human body, following iron. There are approximately 3 grammes of zinc in the human body,

predominantly located within the bone, muscle, and liver. Most (90%) of the zinc in the brain is bound in metal-protein complexes[44].

Zinc is important in many biological systems, including

- transcription factor binding, regulation of tubulin polymerisation,
- activity of zinc-dependent enzymes (including alkaline phophatase, superoxide dismutase and lymphocyte 5'nucleotidase),
- biomembrane formation,
- stabilization of RNA, DNA, ribosomes and hormone receptors for a number of hormones (including somatomedin, prolactin, thyroid hormone, corticosterone, leuteinizing hormone, folicle stimulating hormone and hormone releasing hormone).

Clinical and severe zinc deficiency in humans may result in:

- general tissue dysfunction leading to dermatitis,
- decreasing spermatogenesis in males,
- abnormalities of bone growth,
- parturition difficulties and birth defects resulting from deficient cell division,
- degradation of insulin which leads to poor glucose tolerance and increased lipid oxidation,
- suboptimal cellar immune function (reduction in number of killer cells, lymphocytes and neutrophils) [11].

1.4.1 Psychoactive properties of zinc.

Animal studies have revealed that dietary zinc deficiency may result in behavioural changes. Feeding can become cyclic (possibly as an adaption to falling zinc levels) but

anorexia may also be seen. Noradrenaline levels increase, and the hypothalamus reduces in size and function. The loss of growth and brain function may partly be explained by the alteration of feeding habits, but exceeds levels fully explained by food reduction and must be specific to zinc depletion[11]. These effects can be seen very shortly after removal of zinc from the diet, as the functional zinc pool is small and may fall to less than 1/2 of the original pool in 12 hours or less.

In humans, zinc levels have also been shown to be altered in a variety of disorders. Zinc and albumin levels fall in unipolar depression, and lowered zinc in schizophrenia is proposed to have importance for neurotransmitter signalling[40]. Zinc appears to be involved in excitatory signalling, and in the modulation of inhibitory GABA. Zinc therefore, may contribute to seizures, and the reduction in zinc in schizoaffective disorders may be an adaptive response to excessive excitation of neurones in these illnesses[3,6].

Haepatic zinc levels have been shown to rise in treatment with lithium[5]. This lends credence to the theory that reduction in zinc in psychiatric disorders may be an adaptive response to excessive excitability. A recent double-blind placebo controlled study has also demonstrated a possible role for zinc as a supplementary medication in the treatment of Attention Deficit-Hyperactivity Disorder (ADHD) among 4-11 year old out-patients. This is proposed to be due to a regulation of the dopamine signal pathway, which is associated with pleasure and reward sensations[45]. This finding contradicts the theory that zinc is involved in neurone excitability, and thus seizures and mania, as increasing zinc reduces hyperactive behaviour amongst young people.

1.5 Copper

Copper has 3 ionic valencies: Cu^{+} which is very insoluble and complexed, Cu^{3+} which is very rare, and Cu^{2+} which is the most common and has 3 types in enzymes. Type I appears in oxidases, and is a deep blue colour. Type II is found in multicopper oxidases, is not coloured, but can be detected by electron parallel resonance (EPR). Type III is neither blue in colour or detectable by EPR.

The best food sources of copper are shellfish, nuts, seeds, legumes, bran, liver and organ meats[46]. According to the World Health Organisation , the average Western diet contains less than the reference nutrient intake[19]. However, clinical symptoms of deficiency are rarely seen. Total body copper levels are lower than those of other trace minerals such as zinc and iron (50-120 mg on average) and it is primarily stored in the liver, bound to metallothionine and other proteins.

Most copper absorbance occurs in the small intestine, with some in the stomach. Copper is carried by saturable active transport on ceruloplasmin carrier proteins or albumin to storage sites. There is also a small amount of passive diffusion. Most serum copper is bound to ceruloplamsin, while remaining copper is bound to albumin, metallothionein or other proteins[47]. Concentrations of copper stored in the liver is regulated by metallothionine. The main excretory route for copper is through the gastrointestinal tract, although other excretory routes may play a minor role. Copper may interact with other dietary constituents such as iron, zinc, molbydenum, ascorbic acid, carbohydrates, phyate, fibre and some drugs[11]. Copper occurs in many enzymes, including amine oxidases such as monoamine oxidase, diamine oxidase, lysys oxidase and PAM, ferroxidases I and II, cytochrome c oxidase, dopamine a hydroxylases, superoxide dismutase and tyrosinase.

There are several features of copper deficiency, and each can be explained by alterations in copper-dependent enzyme activity. For example:

- damage to connective tissue may be observed, due to reduction of cross linking of collagen and elastin and lysyl oxide, as lysyl oxidase is a copper-dependent enzyme.
- altered iron metabolism, leading to anaemia due to the altered activity of the copper-dependent enzymes ceruplasmin and ferroxide II oxidase decolouration of skin and hair following altered tyrosinase activity on melanin. Increased cholesterol levels are found in animal studies, although this effect is not found in humans.

Altered thermal regulation, glucose metabolism, blood clotting, pro and antioxidant function and immune function may also be associated with copper deficiency[11]. T cells appear to be more sensitive to copper depletion than B cells[48]. There appears to be a greater potential for cellular damage by calcium following impaired antioxidant activity and mitochondrial activity in copper deficiency.

1.5.1 Psychoactive properties of copper.

Copper deficiency also has some central nervous system effects. Cytochrome c oxidase is involved in the formation of myelin from phospholipids, thus the activity of this enzyme may influence the speed of neurotransmission. Monoamine oxidase inhibitors act as effective antidepressants, the copper dependency of monoamine oxidase may have implications for mood in copper deficiency. Copper is also a constituent of an enzyme involved in dopamine to noradrenaline conversion[11]. Copper has been found to be elevated in depressed patients, and antidepressant treatment has no effect on copper

levels. Copper has therefore been proposed as a trait marker for depression[10]. Plasma copper concentration has also been found to be depleted in schizophrenia[3] and increased in lithium treatment associated with diabetes, although it is unchanged in diabetes alone[5].

1.6 Glial cells

Much research has been done into the roles of neural cells in brain development and illness. However, little work has been done on the role of glial cells in mental illness. There are 50 times as many glial cells in the human brain as there are neurones; and they play important roles in the protection, support and nutrition of neurones.

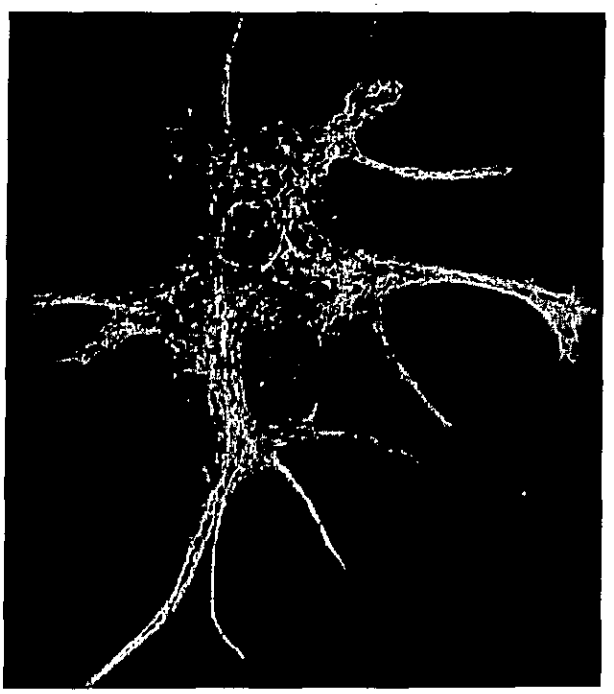


Figure 2. A typical astrocyte[49]

Current research is demonstrating that the astrocyte, a common brain glial cell (figure 2) has many more roles, and greater importance than had been previously thought. These cells appear to interact with neurones, affecting their ability to signal with each other. This suggests that they may influence the learning, thinking and development processes

of the brain.[50] Astrocytes are the cells in the central nervous system which can divide and reproduce, and it is because of this action that they may cause tumours[51]. Glial cells have been shown to play a role in the regulation of the neural medium by taking up neurotransmitters and ions[50]. These cells may, therefore have good control of trace minerals and remain unaffected in their proliferation and function by medium trace mineral status.

1.6.1 Cell culture as an animal model

The use of cell culture techniques has been proposed as a more accurate and less ethically contentious method of modelling human responses to medication than animal models alone. Cells suitable for culture lines tend to be immortal (either of tumour origin, or foetal origin) and thus do not stop dividing and growing with age or crowding. Cultured non-immortalised cells can provide a very accurate prediction of the action of normal cells.

For example, the Physicians Committee for Responsible Medicine (PCRM), in an article entitled 'new cell tests beat animal tests' cite results presented at the Conference of the Scandinavian Society for Cell Toxicology in which rat LD50 tests (the dose level of a chemical required to kill 50% of rats tested) were compared with human cell tests. In this test, the rat LD50 test was found to have a 59% accurate prediction of effects, while human cell test accuracy was found to be 77% with one cell line, or 80% when tested with 3 different cell lines and results combined.

Much work is being carried out, searching for methods of enhancement of longevity for cultured cells, in order to improve their range of uses. Immortalization may be carried out by the action of some viruses or by the use of cancerous cells. Either method may alter the features of cells to a greater or lesser extent compared to normal *in vivo* cells[52].

Drugs are now also being developed and tested without the use of animal models because of developments in cell culture technique. Several companies have ceased the use of animal experimentation in favour of cell culture use for non-prescription drugs (Gillette and Mary Kay UK), and others are seeking to develop prescription drugs without animal testing (Pharmagene Laboratories, with Glaxo Wellcome, SmithKline Beecham and Shire Pharmaceuticals, UK)[53].

The cell lines used in this study were 1321N1 and U87MG. These are glioblastoma multiforme/astrocytoma cells, taken from human tumour patients, subcultured and used in many cell biology studies within the Biomedical Research Unit at the University of Central Lancashire, and in many other research facilities.

1.6.2 How closely do glioblastoma multiforme cells mimic healthy glial cells?

Glial cells are defined as; "A type of cell that surrounds nerve cells and holds them in place. Glial cells also insulate nerve cells from each other"[54]. A glioblastoma multiforme is defined as; "A type of brain tumour that forms from glial (supportive) tissue of the brain. It grows very quickly and has cells that look very different from normal cells. It is also called grade IV astrocytoma." (National Cancer Institute, UK)[55]. There are 4 grades of glioma, each increasing in level of malignancy and morphological difference from healthy cells. A grade IV glioma is the most morphologically different to normal glial/astrocyte cells and, thus the most aggressive and difficult to treat form of glial tumour.[56] The differences occur for the genetic reasons outlined below.

1.6.3 The genetic alterations of glioblastoma multiforme.

Different grades and strains of Glioblastoma multiforme may have different genetic changes, the most common genetic alterations, however, include;

- p53, a tumour suppressor gene is frequently altered, inhibiting its activity and thus promoting excessive proliferation.
- Epidermal growth factor receptor (EGFR) gene, this gene controls cell proliferation and is amplified or over-expressed in over one third of glioblastoma.
- MDM2 is often over expressed in glioblastoma, this binds to p53, blocking its action, thus de-regulating cell proliferation.
- Platelet-derived growth factor (PDGF) gene acts as a mitogen for glial cells when bound to the PDGF receptor, this receptor is often amplified or over-expressed in glioblastoma.

PTEN encodes a tyrosine phosphatase. Phosphatases turn off signalling which is dependent on phosphorylation. Genetic alterations in PTEN, common in glioblastoma multiforme, can cause continuous signalling of these pathways, and thus cause excessive proliferation[57].

The 1321N1 cell line has been shown to have the stereotypical p53 protein mutation, as well as the presence of a c-myc oncogene, featuring t(8;14) chromosomal translocation. The genetic mutations of U87MG are not listed.[58]

All of these alterations are related to an inhibition of the down-regulation of cell division, and proliferation of this cell line compared to healthy glial cells. While glioblastoma cell lines can genetically differ from healthy glial cells in a number of ways, and should not, therefore, be used as a definitive image of healthy glial cell development, they may well give a useful indication of likely alterations with treatment.

1.7 Assessment of cell proliferation

Previous research investigating *in vitro* cell culture conditions on cell proliferation and viability have employed a number of different methods. This section provides a brief overview of these methods with a review of their strengths and weaknesses.

1.7.1 Trypan Blue staining

Trypan Blue is an established stain to study cell proliferation and viability. Cells are lifted from the base of their flask, homogenised, stained with trypan blue solution, and subsequently, they are counted manually by use of a haemocytometer. Viability is discernable from the level of staining in each cell. Viable cells do not allow entry of trypan blue and appear white under the microscope, but non-viable cells appear dark blue in colouration. This method is time-consuming and open to researcher bias in cell counting. Cells on the edge of the haemocytometer grid may be differentially counted and cells may clump together even following homogenisation, making accurate counting difficult. Several research companies are currently searching to produce more accurate, reproducible methods of obtaining the same data. Trypan Blue staining, however remains the technique to which new assays are compared.[59, 60, 61]

1.7.2 Visual counting

Visual counting is the simplest method of testing cell proliferation. Cells are grown, and then counted under a light microscope using a graticule. The key problems with this method are those associated with achieving a high degree of accuracy when cell populations are confluent, and cells tend to grow in homogenous clumps. Therefore, the selection of a section of cover-slip that is not representative of the population is major source of error. Efforts to minimise these sources of error must be considered if this technique is used.

1.7.3 Methylthiazol tetrazolium (MTT) counting

MTT is a water-soluble compound which is reduced to a non water soluble purple formazan by the action of dehydrogenases. Dehydrogenases are a large class of enzyme responsible for many of the reductive reactions within biology[12]. Different cells may contain different levels of dehydrogenase enzymes, but all can be expected to have some reductive enzyme. All cell types can, therefore, be expected to have a differently shaped calibration curve. The resulting formazan can then be solubilized using Sodium dodecyl sulphate (SDS), and concentration measured by optical absorbance at 550 nm wavelength.

Cells are grown in a 96-well plate for this method, and absorbance read using a plate-reader. This method claims accuracy up to approximately 10^6 cells per well, but can be confounded by the enzyme glutathione S-transferase (GST), which also reduces MTT. This method only counts viable cells, level of viability and presence of non-viable cells is not shown[62]. This assay is routinely used owing to its accuracy and ease of use. It can be used in conjunction with Trypan Blue counting to provide and accurate image of proliferation and viability.[61]

1.7.4 Protein assay.

This method is based on the principle that the protein content of a cell pellet is proportional to the number of cells in that pellet. Cells grown in medium can be lifted from the base of a flask and then centrifuged to form a pellet of cells. Protein content can be determined by a number of different methods.

The micro-Lowry protein assay kit was used in this study. The Lowry protein assay was first used to measure total protein content of samples in 1951[63], and remains one of the two more frequently used protein assays to date, the other being the Bradford

protein assay.[64] The Lowry assay is dependent upon the complexing of copper with protein in alkaline conditions, folin phenol reagent (phospho-molybdic-phosphotungstic reagent) then binds to the protein and is reduced, changing colour from yellow to blue. Optical absorbance is then measured at 650 nm. There is a variation in colour between protein types, a similar protein to those to be measured should be used for a calibration curve[65]. In the research presented in this thesis, the proteins assayed were common to cells, thus the use of a general protein standard was appropriate. The chemicals barbital, CAPS, caesium chloride, citrate, cyteine, diethanolamine, dithiothreitol, EDTA, EGTA, HEPES, mercaptoethanol, Nonidet P-40, phenol, polyvinyl pyrrolidone, sodium deoxycholate, sodium salicaylate, thimerosol, tricine, TRIS and Trition X-100 may all interfere with the Lowry assay results but there is no reason to believe that levels of these compounds will vary between treatment groups of the cells used in this study[65].

The Bradford protein assay was developed in 1972, and has fewer chemical interferences than the Lowry assay. It is dependent upon Coomassie Blue G-250 binding to proteins and darkening in colour[66]. This produces a linear relationship between protein content and absorbance at 595 nm, in the range 5-25 μg/mL[67]. A variance in colour production depending on protein type occurs in this assay, and calibration should be made with similar protein type to the protein to be measured.

1.8 Assessment of cell function

1.8.1 Morphology

Cell morphology can be quantified by measuring a number of parameters including process number and length, nuclear diameter and area and cell body diameter and area. Visualisation is aided by the use of a morphological stain, such as the Harris

hematoxylin stain, which differentially stains nuclear and cytoplasmic structures. Measurements of morphological parameters can then be made by use of a Spot 4.2.3 for windows software package (see section 2.8).

1.8.2 Ion transport.

Transport of zinc into cells can be monitored using zinc-specific fluorescent markers, of which several are available. These markers allow for imaging of cells and localisation of zinc ions within the cell as well as quantitative analysis of zinc influx[68],[69],[70]. In this study, zinc concentration and localisation were measured using Furazin-1, a highly zinc-specific fluorescent marker.

1.8.3 Electrical activity of cells

All cells which regulate ion levels in their cytoplasm have a 'resting potential'. This is the charge difference in resting cells between the interior of the cell, using ATP to create and maintain ion gradients and the exterior of the cell. This potential difference can be measured by impaling a cell with a fine glass electrode filled with an electrically conductive solution.

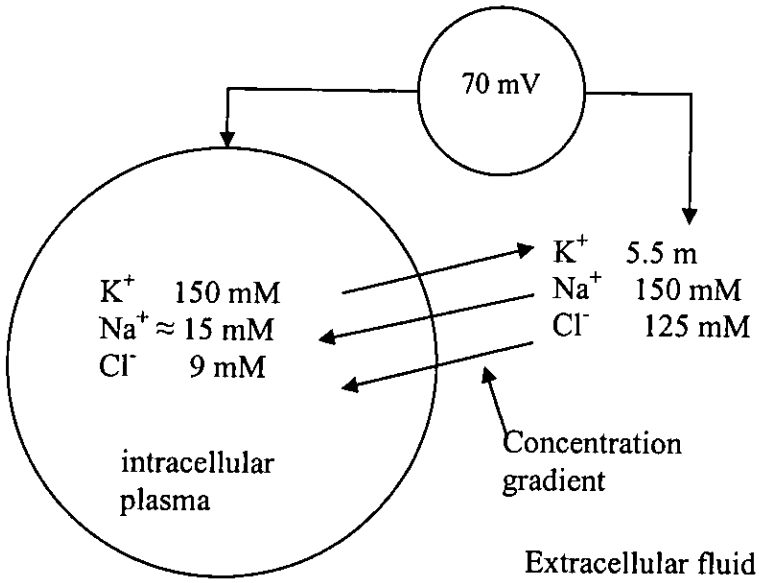


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Figure 3. The ion distribution of a typical excitable cell (cat motor neurone).

The resting membrane potential of a cell is the product of the ionic distribution across the plasma, and the selective permeability of the plasma to those ions[71]. The resting membrane potential, therefore, gives an indication of ion pump activity and membrane cohesion. In the cell illustrated in figure 3, the intracellular space has a negative charge compared to the extracellular fluid. This encourages positive ions (sodium and potassium) to enter the cell and negative ions chloride to leave the cell. The ionic concentration gradient also pushes sodium into the cell, but acts against the potential gradient for potassium and sodium. Electrical and concentration gradients in this case are not equal, and produce net forces on the ion. Active ion transport must occur to maintain this resting potential.

Expected resting potentials for excitable cells (neurones and muscle fibres) are in the -50 to -60 mV range, however, non-excitable cells (including glial cells) may have a

lower resting potential as sodium, potassium action potential formation are not part of their function. Excitable cells and *in vivo* preparations are more commonly tested for resting potential level than non-excitable cultured cells, however impalement of non-excitable cells is possible.

Potential differences may be affected by temperature changes of the medium in which cells are maintained, cell cluster size and correct impalement of the cells. An excessive hole diameter may rupture cells, thus removing any resting potential[72]. Some metal ions, such as copper, lead, silver, mercury, zinc and cadmium have been shown to influence conductance and permeability of excitable cell membranes, exceeding those expected by the alteration of charge associated with addition of ions. This may be due to metal ion receptors on the surface of cells, or competition for calcium binding sites within NMDA receptors and other cellular structures[73].

1.9 The project aims and hypothesis

The human diet is often associated in popular science with many disorders including those of mood, attention and learning. The sale of dietary supplements in the form of vitamins, mineral and trace elements is a common and lucrative business and there is scientific support to the idea that intake of certain elements may influence brain activity[74]. A number a trace minerals have been shown to influence psychiatric or physiological functions of human subjects, however, two have been selected for this study: zinc and copper. Previous research has indicated that these two minerals are likely to have roles in psychiatric illnesses through their effect on cellular respiration and proliferation. This study used cell lines of glial cells to observe the effects of

varying physiological concentrations of trace minerals, in the absence and presence of lithium to observe effects on growth, development and function.

Hypothesis

Interactions between lithium and other trace minerals alter cell proliferation and function within the brain. This in turn affects the prognosis for bipolar disorder patients undergoing treatment with lithium salts.

The aims of this study were therefore :

1. to study alterations in cell morphology, growth, division and signalling within trace mineral normal and enhanced cell culture medium
2. to observe whether these parameters are altered by either the presence or absence of therapeutic levels of lithium

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Media ingredients, trypsin, salt solution, trace mineral salts, trypan blue, plastics, micro-Lowry protein assay kit and poly-l-orinithine were obtained from Sigma-Aldrich Company Limited, Fancy Road, Poole, Dorset, BH12 4QH.

The MTT assay kit and zinc fluorescent probe were obtained from Molecular Probes Europe BV, Pootgebouw, Rijinsburgerweg 10 2333 AA Leiden, the Netherlands.

Glass used for the microelectrodes was obtained from Harvard Apparatus Ltd, Fircroft Way Edenbridge, Kent TN8 6HE.

Cell lines U87MG and 1321N1 were donated to the Biomedical Research Unit (BRU) at the University of Central Lancashire by Dr Jaleel Miyan, University of Manchester Institute of Science and Technology (UMIST).

2.2 Medium

Cells were grown in Dulbecco's Modified Eagles Medium (DMEM). The medium was made to contain 20 μm L-glutamine, 100 units penicillin, 0.1 mg streptomycin and 2.5 μg amphotericin-B per mL, diluted in DMEM (composition of DMEM as supplied is shown in appendix 1). 10% total solution volume of Foetal Bovine Serum (FBS) was then added. This mixture was sterile filtered (2 μm mesh bottle-top filter with vacuum pump) and stored in a sterile bottle in a refrigerator.

Media supplemented with trace mineral salts had a solution of these salts added in the filtration stage measured on a chemical balance and suspended in minimum volume deionised sterile water to ensure full transfer of salt. Concentration and volume of salt solution added was low enough to produce an unchanged osmolarity.

2.3 Trace mineral status

Concentrations of zinc and copper in the medium were assessed by Atomic Absorption Spectroscopy (Unicam 929 AA spectrometer). Media were then supplemented to test levels of either zinc or copper in either the presence or absence of 1 mg/L (0.07 mM) lithium (therapeutic serum level = 0.6 mg/L-1.2 mg/L (0.043 mM – 0.086 mM)). Medium not supplemented with additional zinc, copper or lithium was the control condition.

Test concentrations were:

For Zinc		For Copper	
Control= 0.227 mg/L	3.47μM	Control= 0.024 mg/L	0.38 μM
0.5 mg/L	7.65 μM	0.1 mg/L	1.57 μM
1.0 mg/L	15.29 μM	0.5 mg/L	7.87 μM
5.0 mg/L	76.45 μM	1.0 mg/L	15.73 μM
7.5 mg/L	114.68 μM	5.0 mg/L	78.68 μM
10.0mg/L	152.91 μM	10.0mg/L	157.37 μM

Lithium levels of control medium were negligible.

Salts used for the supplementation of test media were copper (II) sulphate ($CuSO_4$ RMM 159.61), zinc sulphate ($ZnSO_4 \bullet 7H_2O$ RMM 287.54) and lithium sulphate ($Li_2SO_4 \bullet H_2O$ RMM 127.96).

These salts were selected for use as sulphates are used in other copper and zinc cell culture studies[75,76]

2.4 Cell lines

U87MG and 1321N1 cells were stored in liquid nitrogen in a medium of 0.3 ml FBS, 0.6 ml DMEM, and 0.1 ml Dimethyl Sulphoxide (DMSO). Rate of freezing of cells was controlled using isopropyl alcohol, in order to prevent cell lysis due to water crystallisation in fast freezing or cell death due to cytotoxicity of DMSO.

When required, cells were warmed in sterile water in a water bath maintained at 36°C and placed into a sterile cell culture flask (base surface area 25 cm^2) with 7 ml DMEM overnight, to promote adhesion to the flask base. Medium was then changed in order to counteract the cytotoxic effects of DMSO.

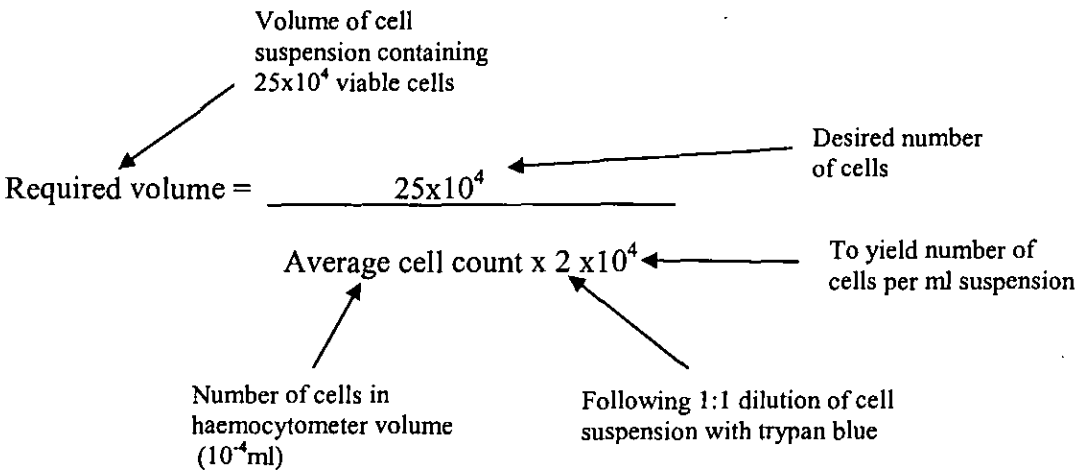
Cells were incubated in a 5 % CO_2, 95 % air, 37°C humidified incubator (Sanyo). All solutions coming into contact with the cells were warmed to 37°C in a water-bath prior to contact.

Medium was changed every 2 days or as needed to maintain glucose levels distinguishable by the colouration change of medium from pink to yellow as glucose is utilised by cells.

2.5 Sub-culturing

When cells became confluent in a flask, they were sub-cultured. Over confluence of cells can lead to cell death and confound results. 1321N1 cells needed sub-culturing within 7 days of seeding, but were found to be at a good confluence level for counting at 3 days. U87MG cells needed sub-culturing within 14 days of seeding but were found to be at a good confluence level for counting at 7 days.

Cells were washed twice in Hanks Balanced Salt Solution and then lifted from the bottom of the flask by incubation in 1 ml/25 cm^2 trypsin EDTA solution (0.25 %) for 5 min.. After this period, any un-detached cells were detached by tapping the flask or, if necessary, use of a sterile cell scraper. A volume of 2 ml/ 25 cm^2 medium was then added to counteract any toxic effects of trypsin. An aliquot of 100 µl of this solution was then mixed 1:1 with trypan blue solution. Cell number was then calculated using a 1 µl haemocytometer under a light microscope (Leica, 20 x magnification). Living cells did not take in the dye and thus appeared white under the microscope, dead cells appeared dark blue in colour. 4 counts were taken, and the mean of these counts used, and 25 x 10^4 living cells/ cm^3 were added to a new cell culture flask using the calculation:



Enough of the required medium was then added to each flask to cover the cells (7 ml approx).

2.6 Cell count methods

Three different methods of counting cell were used in this study, they included:

2.6.1 Trypan Blue Counting.

Cells were grown in a 25 cm^2 base area cell culture flask. Cells were lifted from the base of the flask by washing twice with HBSS, followed by 5-10 minute incubation at 37°C in 1 ml 0.25% trypsin. Any cells not lifted at this point were either lifted by tapping the flask or, removed by the use of a sterile scraper. A volume of 2 ml medium was than added to neutralise the toxic effects of trypsin, and the solution was homogenised using a small 100 µl Gilson pipette. A volume of 100 µl of this solution was mixed with 100 µl trypan blue, and the solution was placed into a 100 μm^2 haemocytometer and cells were counted. Dark blue cells were non-viable, bright ones viable. Viability and cells/ 100 μm^2 were calculated. This procedure was repeated 6 times for each condition and the means recorded.

2.6.2 Visual count method

Cover-slips were sterilised in a flame, then placed onto a drop of poly-l-orinithine in a Petri dish. A drop of poly-l-orinithine was placed on top and the cover-slips were left to dry. Poly-l-orinithine aids adhesion of cover-slips to the Petri-dish base and adhesion of the cells to the cover-slip. Cells were grown on these cover-slips for a number of days suitable to ensure exposure of the minerals without causing confluence, 3 days for 1321N1 and 7 days for U87MG. Cells were then counted using a Leica visual microscope with a graticule eyepiece. The number of cells visible within the graticule on a blindly-selected area of the cover-slip was recorded. This procedure was repeated 6 times for each condition and the means recorded.

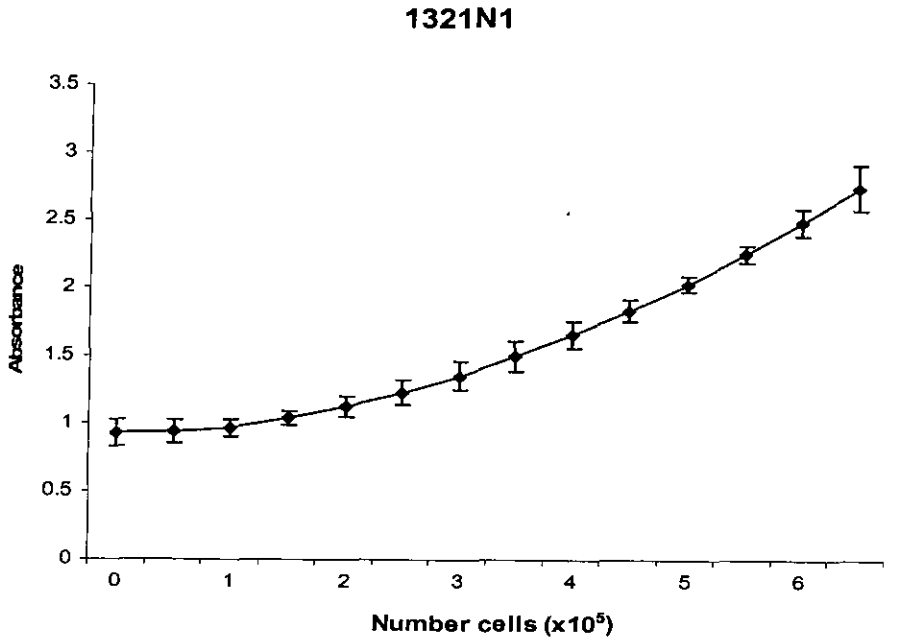
2.6.3 MTT assay method

Cells in 2 flasks were trypsinised and mixed; the cells were counted using trypan blue staining. A volume of 1 ml of the cell solution was placed into each of 6 micro

centrifuge tubes and centrifuged at 1500 rpm (250 g) in a 24 x 2 mL capacity bench-top micro-centrifuge for 3 min.. The number of cells in a pellet was calculated as the trypan blue count $x10^4$ which gave the number of cells in 1 ml. Pellets were then re-suspended in 200 µl DMEM, and 100 µl DMEM was added to each well of a 96 (12 x 8) well plate, and 100 µl of the re-suspended pellet added to each well of the top row (12 wells). This was then mixed and 100 µl added to the next row, which was then mixed and 100 µl added to the next until the plate was full. A volume of 20 µl of MTT was added to each well and the plate was incubated for 4 hours at 37°C. A volume of 100 µl of SDS was then added and the plates returned to the incubator overnight. Plates were then placed into an Athos htIII Plate Reader, shaken for 8 seconds, and then the absorbance was read at a wavelength of 540 nm. This was repeated at 3 different times with 3 different plates. A calibration graph was plotted for each cell line using the mean value obtained from the three calibration curves (figure 4). Error bars represent the standard error of the mean.

An equal number of cells were added to each well in a 96 well plate with 100 µl of the relevant medium. After a relevant number of days (3 for 1321N1 and 7 for U87MG), the MTT assay was carried out as described above. Mean absorbance for each condition was taken. Cell number was then read off the calibration curve and plotted on a graph.

A



B

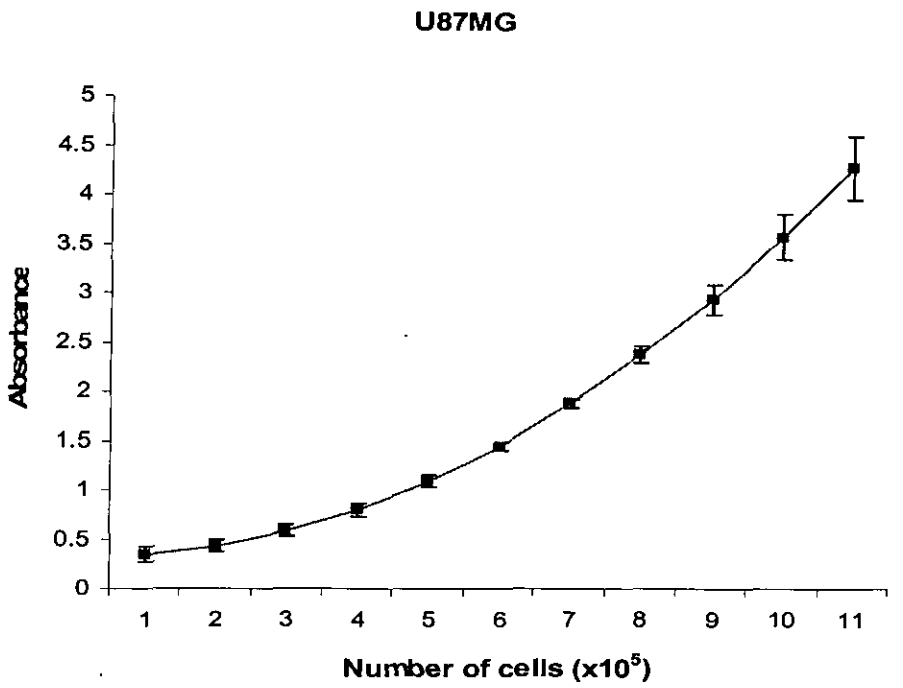


Figure 4. The calibration curves for the MTT cell proliferation assay. Points are mean ± SEM, n=3. Number of cells in each well was calculated using typan blue count, n=4.

2.6.4 Protein Assay

On day 7 (for 1321N1) or 14 (for U87MG) of treatment, cultured cells were trypsinised, re-suspended in medium and then centrifuged at 1500 RPM (250 g) for 3 min. The supernatant was discarded, and the pellet was then washed in pH 7.4 Phosphate Buffered Saline (PBS). This was centrifuged, then the pellet was washed again and centrifuged for a final time. The pellet was frozen and stored.

A protein assay was carried out using a micro-Lowry kit. Cells were suspended in 1 ml distilled H_2O and 1 ml Lowry's solution, and left for 20 min at room temperature. A volume of 500 µl Folin-Ciocalteu's solution was then added, and colour was allowed to develop for 30 min.. Absorbance was read at 650 nm using a Pharmacia LKB Novaspec II spectrophotometer. A calibration curve was plotted using protein standards supplied with the assay kit. Protein content of pellets was read from this graph. (Figure 5)

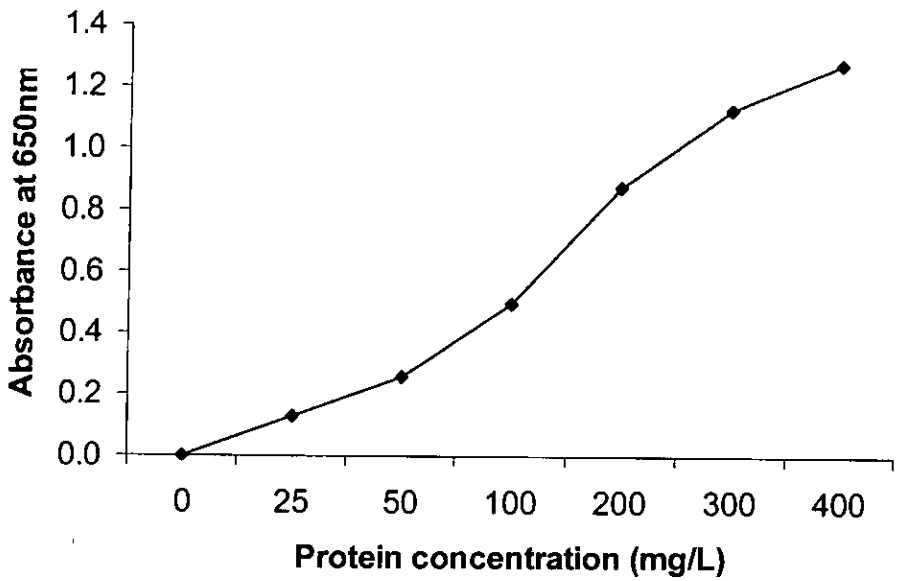


Figure 5. The calibration curve for the protein assay. Each point is the mean of 3 assays.

2.7 Cell staining for morphology

Cells were grown on flame-sterilised cover slips in each medium type for morphological staining. The cover-slips were covered in 50% methanol for 5 min. in order to fix the cells, and then washed in 75% ethanol for a further 5 min., followed by distilled water for 2 min. to remove any impurities. Cells were then stained with Harris Haematoxylin solution for 20 min. followed by 1% eosin y solution for 10 min.. Excess stain was washed away by submersion in increasing concentrations of ethanol, 80%, 90% and 100% for 5 min. each. Cover-slips were then rinsed in distilled water and mounted using DPX mountant and left to dry overnight.

2.8 Morphology

Cells were visualised using a Leica light microscope with camera attachment and the Spot 4.2.3 for Windows software package. Visible processes from cells were counted manually. Diameters of the cell body and nucleus were measured by "drawing" straight lines with the mouse, on the computer screen across the desired area which the software then measured. Areas of the cell body and nucleus were measured by outlining the structures as shown on the computer screen, using the mouse. The area was then calculated by the software. Processes were measured by tracing along the length of the process on screen and the software calculated the length of line drawn. 6 separate randomly selected cells were measured for each parameter and averages determined.

2.9 Electrophysiological parameters of cells.

Cells were grown on treated cover-slips in Petri dishes. After 3 days of treatment, cell clusters were impaled using a glass microelectrode filled with 3 M potassium acetate solution. Electrodes were pulled to a resistance of 20-25 MΩ using a CFP 1.5 Amp glass electrode puller (heat set to 86/100, solenoid to 32/50) with 1.2 mm outer diameter, 0.69 mm inner diameter filamented glass tube. Petri dishes were maintained at a constant temperature (37°C) by storing them on a plate connected to a hot water bath. When needed, dishes were placed on a Leica inverted microscope (20 x magnification) and cell clumps identified.

The circuit used to measure potential difference of cell clumps is illustrated in figure 6. The microelectrode was filled with 3 M potassium acetate solution, and placed into a microelectrode holder, also filled with 3 M Potassium acetate solution in order to ensure a perfect electrical connection. The electrode holder was plugged into an NL102G Digitimer connector, connected to the oscilloscope and ground wires. The electrode

was lowered into cell clumps and the change in direct current voltage from the electrode being in the medium to the electrode being in the cell cluster was recorded. Potential difference was visualised using a Gould 20 MHz digital storage OS1420 oscilloscope, connected to a neurolog system bridge balance and DC preamp set to 10x signal.

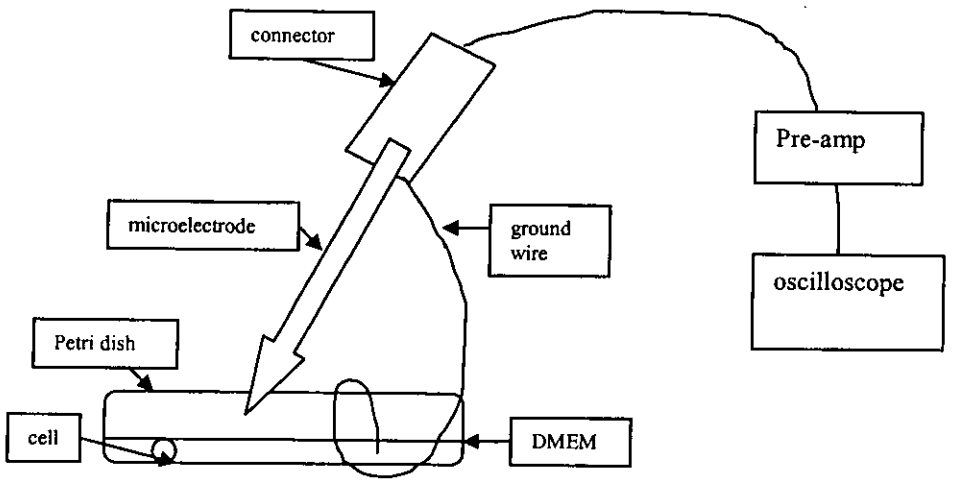


Figure 6. The circuit used for electrophysiological measurements.

Electrical "noise" from other electrical sources outside the circuit was eliminated by use of a Faraday cage to isolate apparatus from electrical signals within the lab and grounding of all equipment to the metal table top.

2.10 Zinc uptake determination.

The uptake of zinc for cells treated with different levels of exogenous zinc, and the influence of lithium treatment on this was tested. Cells grown in test media for 3 days were washed with HBSS and then incubated in control medium containing 5 μM Furazin-1, solubilised in DMSO, at room temperature for 1 hour. Cells were then washed in HBSS to reduce background fluorescence and trypsinised. A trypan blue

count was carried out, and number of cells per ml suspension determined. Fluorescence was read using the Perkin-Elmer LS50B Luminescence Spectrometer with the FLwinlab computer application (Perkin-Elmer). Excitation wavelength was set to 325 nm, and emission wavelength to 510 nm, gentle stirring, using a magnetic flea, was used to minimise separation of the cells from the medium. Cells were then centrifuged and the fluorescence of the supernatant determined to control for any background fluorescence not eliminated through washing.

Fluorescence minus background over cell number was determined for each medium type. Furazin-1 gives a linear relationship between zinc concentration and fluorescence.

Cells were grown on pre-treated cover-slips and incubated in fluorescence medium for an hour then washed and imaged using a Zeiss LSM 510 confocal microscope in order to observe localisation of fluorescent staining.

2.11 Experimental protocols

Four experiments were carried out using the above techniques.

2.11.1 Experiment 1: The effects of zinc and copper on cell proliferation, and their interactions with lithium.

Cells were grown in different test media for a period of time sufficient to ensure effects of trace minerals, without confluence of cells (3 days for 1321N1 and 7 days for U87MG) (section 2.5). Their proliferation was then measured by visual counting and by MTT assay.

Test media with zinc and copper concentrations close to the physiological range were used for this experiment (section 2.3). Each group was tested with and without 1.0 mg/L lithium supplementation. The visual counting protocol (section 2.6.2) and the

MTT assay (section 2.6.3) were used as an index of cell proliferation for each treatment group. Figure 7 illustrates the test sample arrangement for the MTT assay. Each 96-well plate was set up with an equal number of cells in each well and the test media as shown. The plate reader was used to measure absorbance values for each well. These absorbance values were read off the calibration curve (figure 4) to yield a cell number for each well. The mean number of cells for each medium type was then calculated.

	A	B	C	D	E	F	G	H	I	J	K	L
1	N c	1	2	3	4	5	6	7	8	9	10	11
2	N c	1	2	3	4	5	6	7	8	9	10	11
3	N c	1	2	3	4	5	6	7	8	9	10	11
4	N c	1	2	3	4	5	6	7	8	9	10	11
5	N c	1	2	3	4	5	6	7	8	9	10	11
6	N c	1	2	3	4	5	6	7	8	9	10	11
7	N c	1	2	3	4	5	6	7	8	9	10	11
8	N c	1	2	3	4	5	6	7	8	9	10	11

N c denotes no cells, and it was used to control for background staining. Numbers 1-11 denote different medium types

Figure 7. The set-up of a 96-well plate for MTT assay.

2.11.2 Experiment 2: The effect of trace minerals on morphological and functional parameters.

The influence of trace minerals with and without lithium supplementation on morphological parameters of glial cells.

Cells were grown in all test media used in experiment 1 for the previously determined period of time (see section 2.5). Cells were then stained using the Harris Haematoxylin method described in sections 2.7 & 2.8. Six cells from each treatment group were measured.

Resting potential; a functional parameter of glial cells. The resting potential functional parameter was measured for 1321N1 cells grown in lithium supplemented and control medium, using the technique described in section 2.9.

2.11.3 Experiment 3: The cytotoxicity of zinc and copper at supraphysiological levels and the influence of lithium on cytotoxicity.

Cells were grown in the control medium, and media containing either 10 mg/L zinc or 10 mg/L copper. The trypan blue method (see section 2.6.1) was used at regular intervals (every day for 5 days for 1321N1 or every other day for 11 days for U87MG) to record the proliferation of cells. Counts began on day 3 of exposure to test conditions. Cell viability and number counts were repeated 6 times. A protein content analysis for cell pellets collected at the end of each growth curve was performed using the Micro-Lowry protein assay kit described in section 2.6.4.

2.11.4 Experiment 4: The effect of the zinc and lithium concentration of the culture medium on cellular zinc uptake.

Cells were grown in the zinc test media (see section 2.3) each in either the absence or presence of lithium supplementation. Cells were stained using the protocol described in section 2.10, and fluorescence recorded. Cells were also imaged using the confocal microscope (section 2.10) to qualitatively assess the location of zinc within the cultured cells.

2.12 Statistical analyses.

Where possible, data are presented as mean ± standard error of the mean (SEM). Cell proliferation levels, cytotoxicity of supraphysiological levels of trace minerals, and fluorescence, were compared by 2-way ANOVA. Post-hoc tests used were Tukey and Bonferroni.

The effect of lithium on cytotoxicity of trace minerals was analysed for each trace mineral using a one-way ANOVA, with Tukey and Bonferroni post-hoc tests.

Cell morphology parameters (except process number) were compared using a 2-way ANOVA. Tukey and Bonferroni Post-hoc tests were used.

Process numbers for morphology were compared by Kruskal-Wallis non-parametric test with Tukey and Bonferroni Post-hoc tests.

Electrophysiological parameters were compared by an unpaired student's t-test (only 2 types of medium were used). Morphological parameters of the 2 cell lines in control medium were compared by unpaired student's t-test (equal variances not assumed).

In all statistical tests a value of $p<0.05$ was taken to be significant.

Where results are significantly different, characters appear on graphs. Bars not featuring the same character are significantly different.

CHAPTER 3

RESULTS

3.1 Experiment 1: The effects of zinc and copper on cell proliferation.

The effects of mineral concentrations above and below the physiological range on cell proliferation were examined using both the visual counting method (section 1.7.2) and the MTT assay (section 1.7.3). Counts were performed on day 3 for the 1321N1 cells and day 7 for the U87MG cells. The results of this experiment are summarised in Table 1 and graphs of proliferation are shown in figures 8-11. The graphs display mean values, ± SEM, n=6 for visual count methods and n=8 for MTT method. Values shown for MTT assay results are those determined from the calibration curves for the MTT apparatus, as shown in figure 4. Values shown for visual counting are the number of cells visible in a $1cm^3$ graticule area.

Many of the significant differences shown in Table 1 do not appear to be dose-related, as proliferation levels do not display a consistent pattern as the trace mineral concentration of the media increases. Lithium has some distinct significant effects in visual counting assay as can be clearly seen, in the graphs below. There is non-significant effect.

Table 1. Proliferation trends, as observed in the following cell number plots, comparing two different assay methods.

	Cell line	
Treatment	U87MG	1321N1
Zinc, by visual counting	Control significantly higher than all others ($p<0.05$) 7.5 mg/L significantly lower than 1 mg/L zinc ($p<0.05$), but not 0.5 mg/L or 5 mg/L ($p=0.059$). Lithium had no significant overall effect on proliferation ($p=0.444$)	0.5 mg/L & 5 mg/L zinc significantly lower than 1 mg/L & 7.5 mg/L zinc ($p<0.05$). Lithium had no significant overall effect on proliferation ($p=0.672$)
Zinc, by MTT assay	No significant differences between treatment groups $p=0.402$. Lithium had no significant overall effect.	0.5 mg/L zinc significantly higher than control,1 mg/L, or 7.5 mg/L zinc. ($p<0.05$) 7.5 mg/L zinc significantly lower than 5 mg/L zinc or 0.5 mg/L zinc.($p<0.05$) Lithium had no significant overall effect, cells grown in lithium were not significantly different to each other.
Copper, by visual counting	5 mg/L copper significantly higher than all other test groups ($p<0.05$). 1 mg/L and 0.5 mg/L copper were significantly lower than 5 mg/L copper, control, and 0.1 mg/L copper ($p<0.05$). Lithium had no significant overall effect on proliferation ($p=0.913$)	5 mg/L copper significantly higher than all other test groups ($p<0.05$). No other significant differences ($p=0.327$). Lithium had no significant overall effect on proliferation ($p=0.692$)
Copper, by MTT assay	0.1 mg/L copper significantly higher than control or 5 mg/L copper ($p<0.05$), not different to 0.5 mg/L copper or 1 mg/L copper ($p=0.052$). Lithium had no significant effect on proliferation ($p=0.217$)	Control & 0.5 mg/L copper significantly higher than 0.1 mg/L,1 mg/L & 5 mg/L copper. ($p<0.05$). Lithium had a significant overall inhibitory effect on proliferation ($p<0.05$). Cells grown in lithium were not significantly different to each other ($p=0.226$).

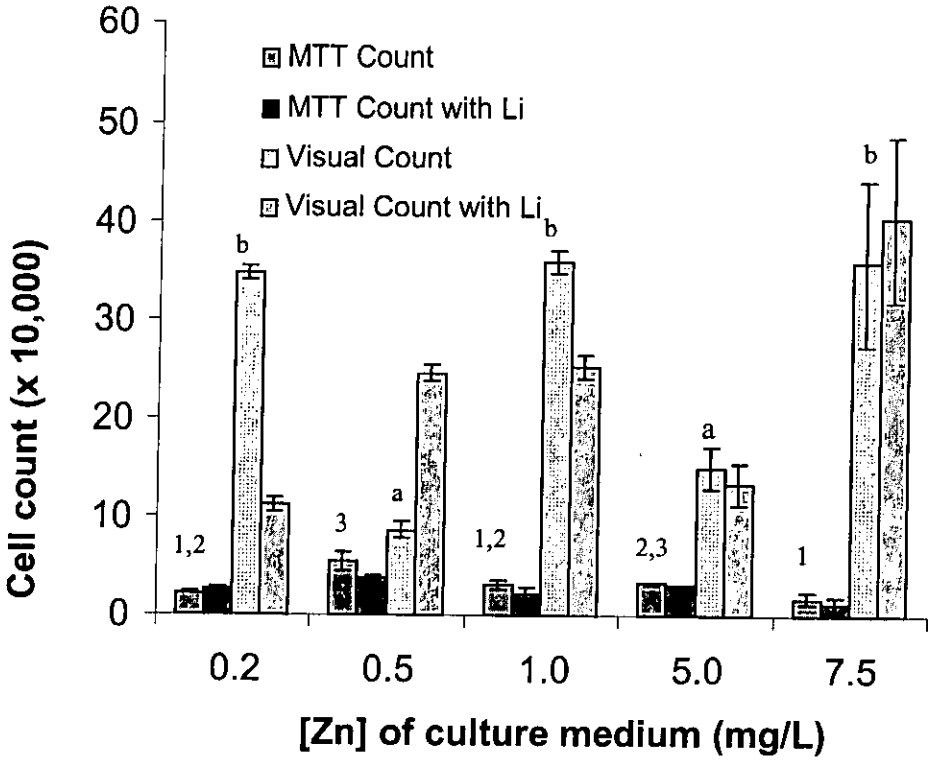


Figure 8. Bar chart showing the proliferation of 1321N1 cells cultured in zinc test media. Data are mean ±SEM. n=6 (visual count) n=8 (MTT)

[1,2] MTT counts with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[a,b] Visual counts with different superscripts are significantly different to each other Compared using 2 way ANOVA.

Neither the visual counting nor the MTT method revealed a dose dependent relationship between cell proliferation and the zinc concentration of the culture medium. The addition of Li to the culture media had no significant effect overall (2 way ANOVA).

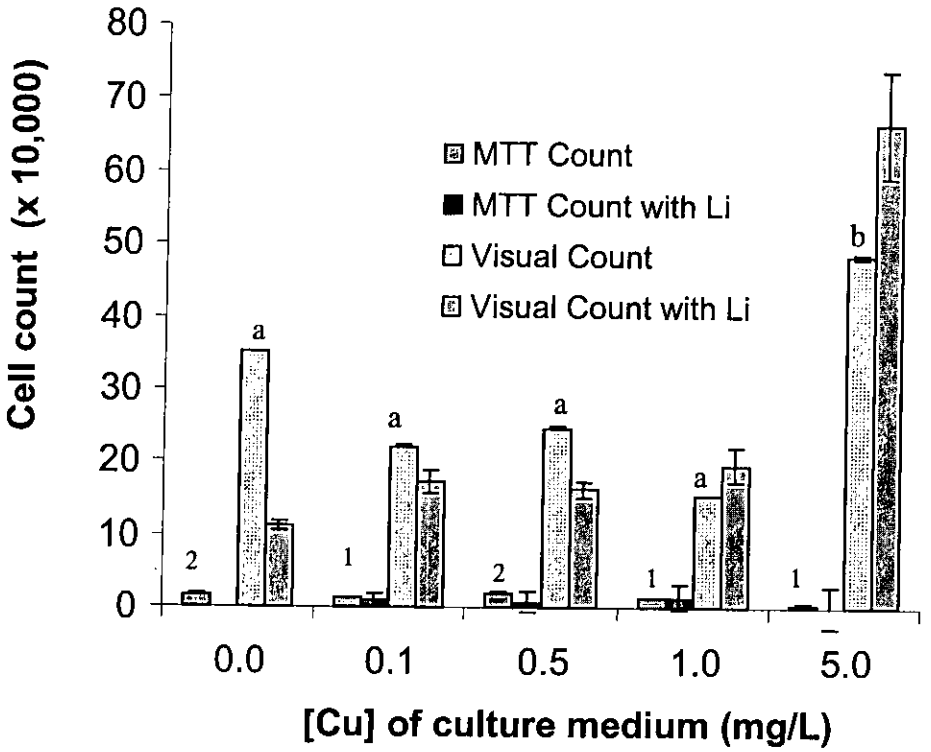


Figure 9. Bar chart showing the proliferation of 1321N1 cells cultured in copper test media. Data are mean ±SEM. n=6 (visual count) n=8 (MTT)

[1,2] MTT counts with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[a,b] Visual counts with different superscripts are significantly different to each other Compared using 2 way ANOVA.

Visual counting resulted in a significant peak at 5 mg/L, however there was no evidence for a dose dependent effect using either the visual counting or MTT assay. The addition of Lithium to the test media had no overall effect on cell proliferation.

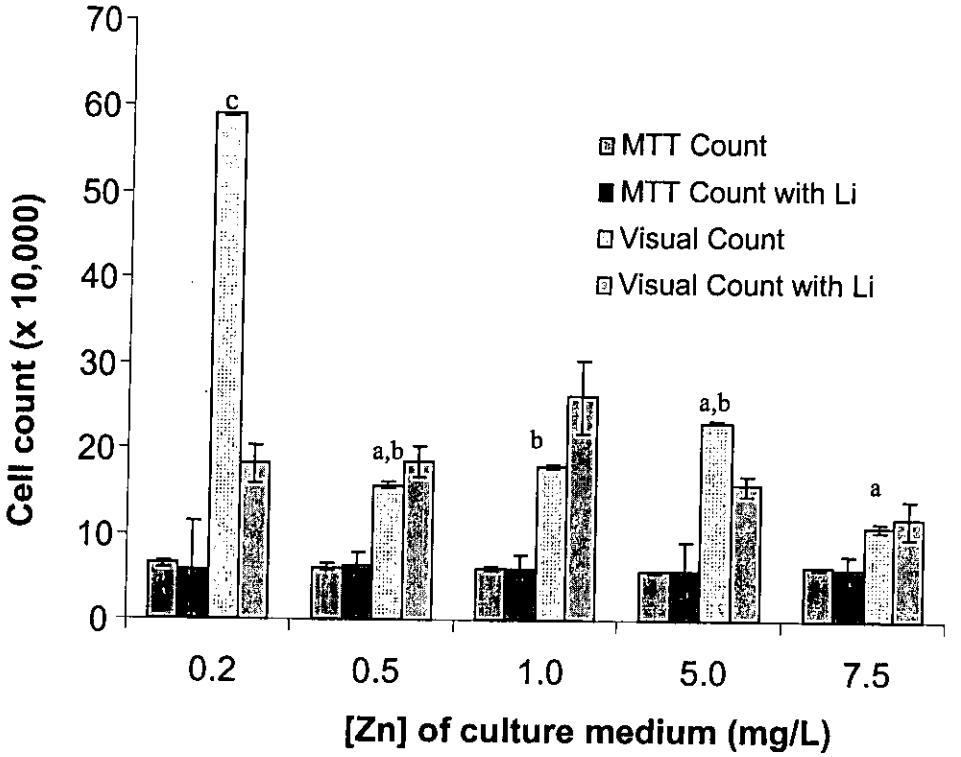


Figure 10. Bar chart showing the proliferation of U87MG cells cultured in zinc test media. Data are mean ±SEM. n=6 (visual count) n=8 (MTT)

[a,b] Visual counts with different superscripts are significantly different to each other Compared using 2 way ANOVA.

Using the visual counting method, addition of zinc to the test media resulted in an inhibition of cell proliferation. This was not observed using the MTT assay. The addition of Lithium to the culture media had no significant overall effect.

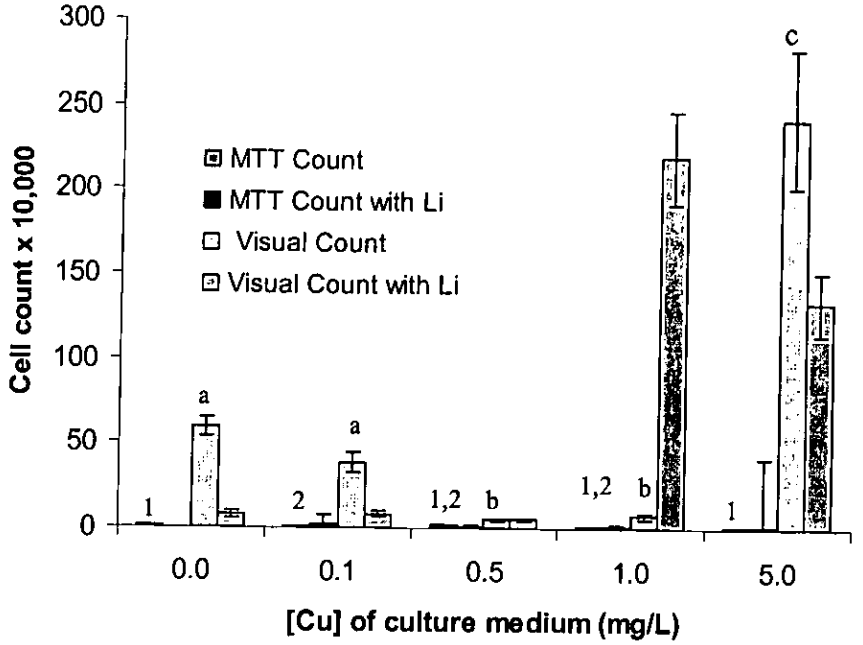


Figure 11. Bar chart showing the proliferation of U87MG cells cultured in copper test media. Data are mean ±SEM. n=6 (visual count) n=8 (MTT)

[1,2] MTT counts with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[a,b] Visual counts with different superscripts are significantly different to each other Compared using 2 way ANOVA.

A significant increase in cell proliferation at the highest copper concentrations was found using the visual assay technique. Several effects of lithium are apparent, however, whether proliferation rises or falls with lithium treatment is random, thus yielding no overall significant effect.

The MTT assay did not reveal any dose dependent responses to the copper content of the media.

There are some striking differences between the findings of the two assay techniques, for example, for 1321N1 cells cultured in the copper test media, the visual counting method indicates a significant peak at the highest dose used, while the MTT assay indicates a significant peak at 0.5 mg/L, one of the lowest doses. The MTT assay may be influenced by cellular functional parameters, as it is dependent on enzyme activity within the cell. Some functional parameters were measured to see if these were influenced by the treatments used. Functional parameters measured were morphology and resting potential.

3.2 Experiment 2: The effect of trace minerals on morphological and functional parameters.

3.2.1 The influence of zinc and copper, in the absence and presence of lithium supplementation on morphological parameters of glial cells.

The different cell lines had similar morphological characteristics;

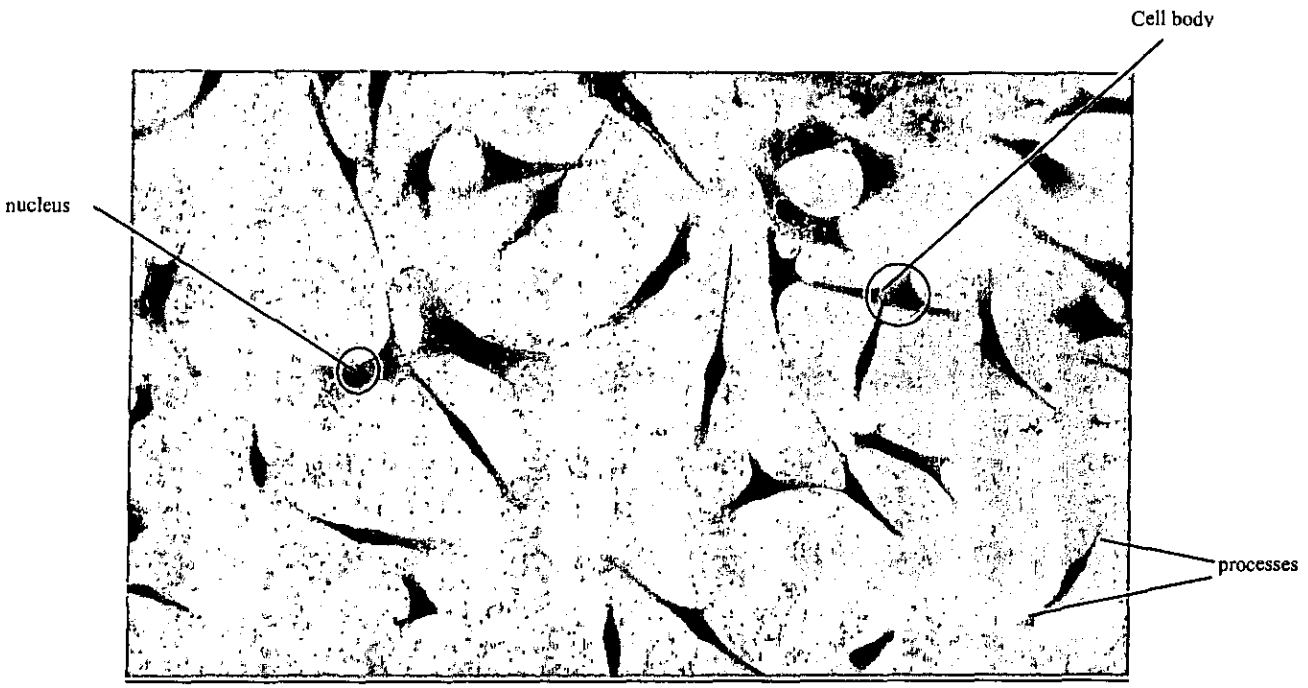


Figure 12. 1321N1 cells grown in control medium morphologically stained with Harris haematoxylin stain (x 20 magnification). This photograph is typical of 6 taken of 1321N1 cells grown in each medium condition.

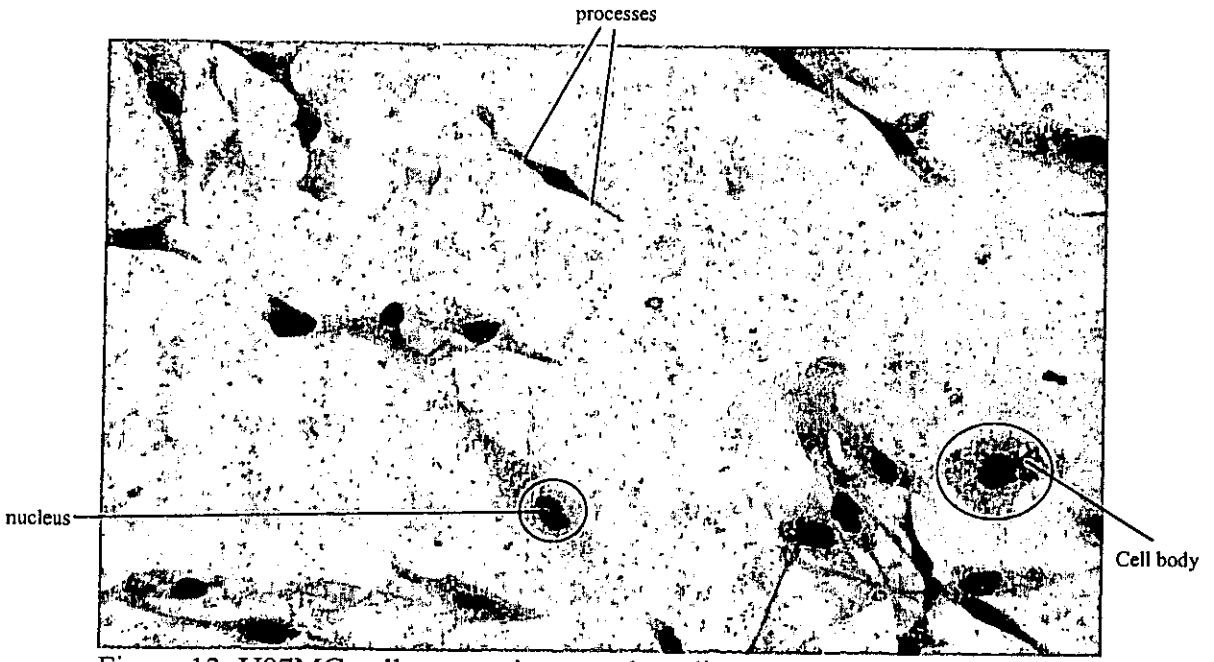


Figure 13. U87MG cells grown in control medium morphologically stained with Harris haematoxylin stain (x20 magnification). This photograph is typical of 6 taken of U87MG cells grown in each medium condition.

Comparison of the morphological features of the two cell lines using a students Student's t-test revealed that the 1321 cells had significantly larger nuclear area ($p=0.044$) and cell body area ($p=0.005$) when grown in control medium (3 cells from each of 5 different Petri dishes for U87MG and 3 cells from each of 6 different Petri dishes for 1321N1 were tested).

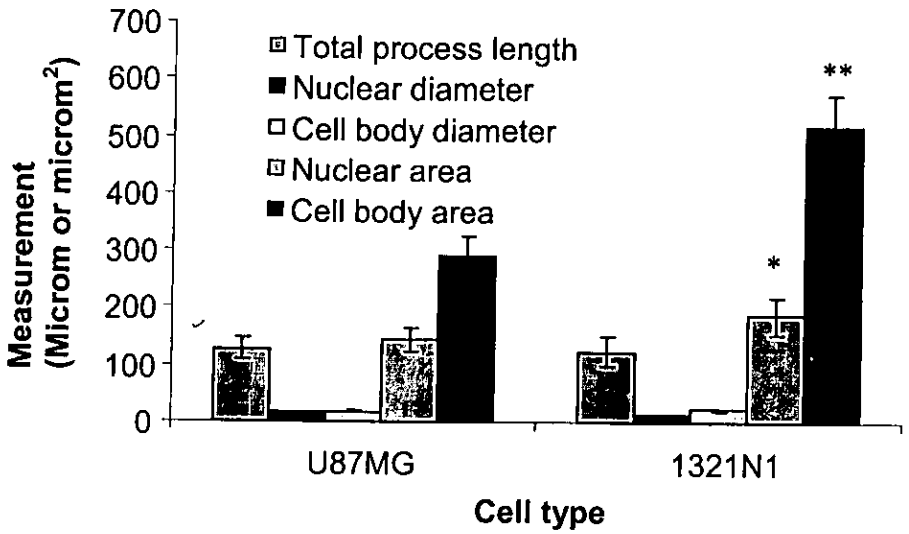


Figure 14. Bar chart showing the morphological parameters of each type of cell grown in control medium. Data are mean ±SEM, n=6.

*p= 0.044

**p=0.005

Some differences between treatment levels within cell lines were found. Lithium was found by ANOVA to have no significant effect on any treatment group. Numbers and lengths of processes were not altered in any treatment group in either cell line. Effects of zinc and copper on cell morphology are illustrated in tables 2 and 3, and in figures 15-22.

Table 2. Morphological trends for cells treated with zinc.

	1321N1 treated with zinc	U87MG treated with zinc
Number of processes	No significant difference between treatment groups ($p=0.312$).	No significant difference between treatment groups ($p=0.888$).
Total length of processes	No significant difference between treatment groups ($p=0.071$)	No significant difference between treatment groups ($p=0.355$)
Nuclear diameter	Cells grown in 7.5 mg/L zinc significantly larger than those grown in 0.5 mg/L zinc and 1 mg/L zinc ($p<0.05$), but not control, 5 mg/L zinc, or 10 mg/L zinc	Cells treated with 0.5 mg/L zinc significantly larger than those in 1 mg/L, 5 mg/L and 10 mg/L. ($p<0.05$), but those in control or 7.5 mg/L zinc were not significantly different to any other treatment group.
Nuclear area	Cells grown in 7.5 mg/L zinc significantly larger than those grown in control, 0.5 mg/L zinc, and 1 mg/L zinc, but not different to 5 mg/L zinc or 10 mg/L zinc.	Cells grown in 0.5 mg/L zinc significantly larger that those grown in 5 mg/L zinc or 10 mg/L zinc, not significantly different to control, 1 mg/L zinc or 7.5 mg/L zinc.
Cell body diameter	Cells grown in 7.5 mg/L zinc significantly larger than those in 0.5 mg/L zinc or 1 mg/L zinc. Not significantly different to control or 5 mg/L zinc	No significant differences between treatment groups. ($p=0.241$)
Cell body area	No significant difference between treatment groups ($p=0.306$)	Cells grown in 0.5 mg/L zinc larger than those grown in control medium, 1 mg/L zinc, 5 mg/L zinc and 10 mg/L zinc ($p<0.05$). Not significantly different to those grown in 7.5 mg/L zinc.

Patterns for different morphological parameters of cells when treated with zinc follow similar trends within cell lines, but are very different between cell lines. 1321N1 is enlarged in 0.5 mg/L zinc in several of the parameters listed above and U87MG is enlarged in 7.5 mg/L zinc. These trends are illustrated in figures 14 to 17, below, 6 cells from separate Petri dishes were measured for each condition, error bars indicate standard error.

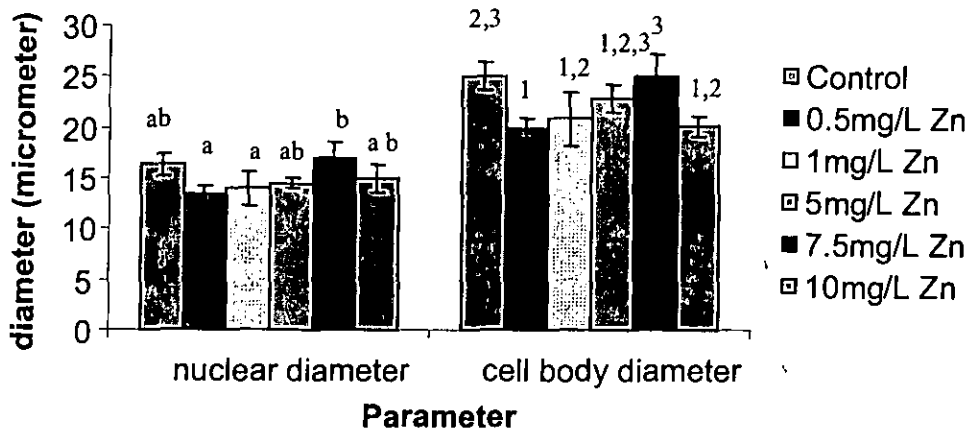


Figure 15. Bar chart showing the nuclear and cell body diameters of 1321N1 when treated with zinc. Data are mean ±SEM, n=6.

[a,b] Nuclear diameters with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[1,2] Cell body diameters with different superscripts are significantly different to each other Compared using 2 way ANOVA.

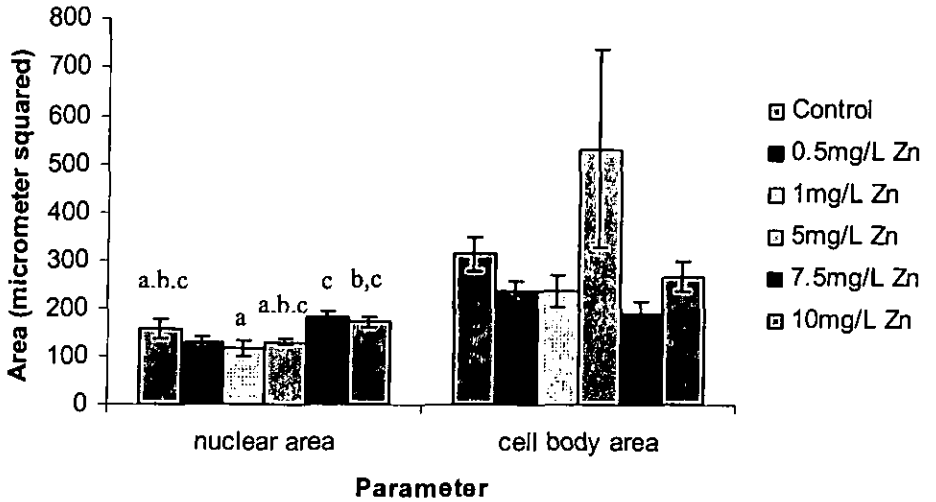


Figure 16. Bar chart showing the nuclear and cell body areas (um^2) of 1321N1 cells when treated with zinc. Data are mean ±SEM, n=6.

[a ,b] Nuclear areas with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[1, 2] Cell body areas with different superscripts are significantly different to each other Compared using 2 way ANOVA.

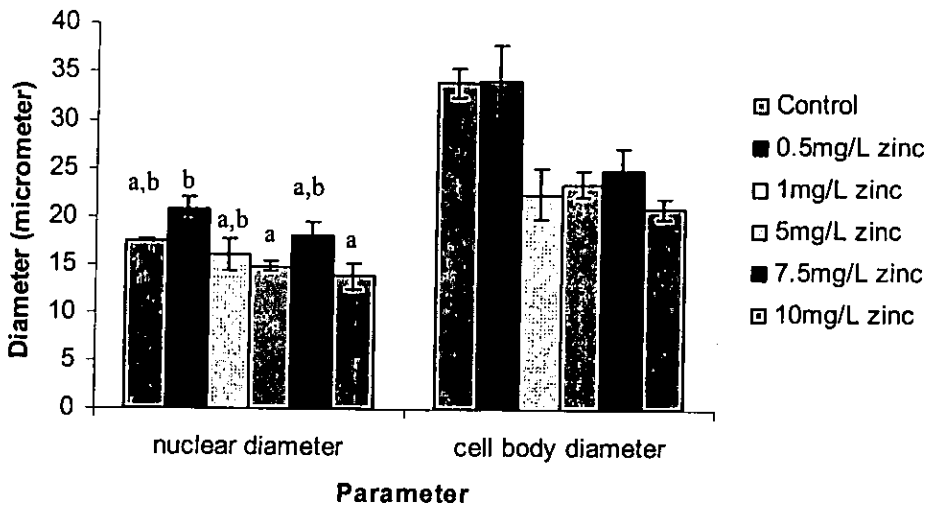


Figure 17. Bar chart showing nuclear and cell body diameter (um) of U87MG cells when treated with zinc. Data are mean ±SEM, n=6.

[a,b] Nuclear diameters with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[1, 2] Cell body diameters with different superscripts are significantly different to each other Compared using 2 way ANOVA.

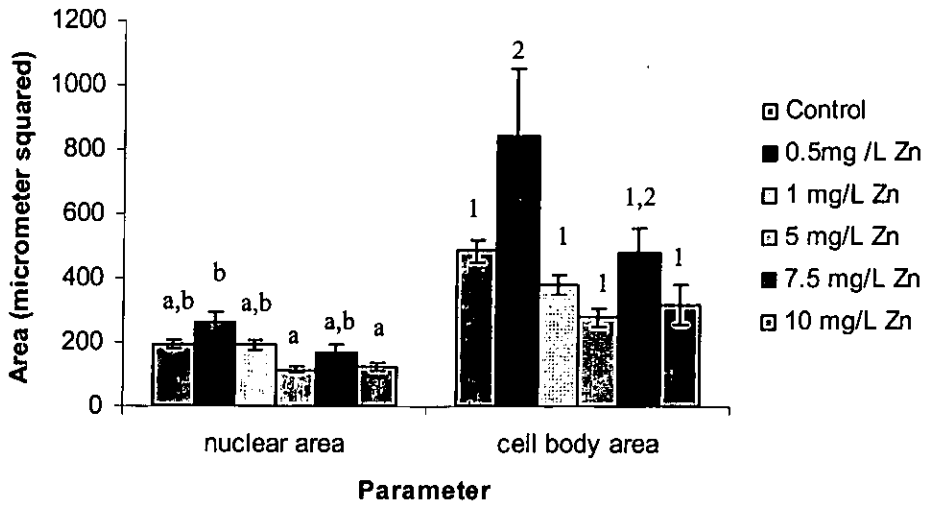


Figure 18. Bar chart showing nuclear and cell body areas (um^2) of U87MG cells when treated with zinc. Data are mean ±SEM, n=6.

[a, b] Nuclear areas with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[1, 2] Cell body areas with different superscripts are significantly different to each other Compared using 2 way ANOVA.

Table 3. Morphological trends for cells treated with copper.

	1321N1 treated with copper	U87MG treated with copper
Number of processes	No significant difference between treatment groups ($p=0.073$).	No significant difference between treatment groups ($p=0.401$)
Total length of processes	No significant difference between treatment groups ($p=0.397$)	No significant difference between treatment groups ($p=0.189$).
Nuclear diameter	Cells grown in 10 mg/L copper significantly larger that those in control, or 0.5 mg/L copper. Not different to 1 mg/L copper, 5 mg/L copper, or 0.1 mg/L copper.	Cells treated with 0.1 mg/L copper or 0.5 mg/L copper significantly larger than those grown in control medium ($p<0.05$). Cells grown in 1 mg/L copper or 5 mg/L copper not different to any other treatment group.
Nuclear area	Cells grown in 10 mg/L copper significantly larger than all other treatment groups ($p<0.05$).	Cells grown in 0.1 mg/L copper significantly larger than those grown in control or 5 mg/L copper, not significantly larger than those grown in 0.5 mg/L, or 1 mg/L copper.
Cell body diameter	Cells grown in 10 mg/L copper significantly larger than all other treatment groups ($p<0.05$), except 1 mg/L copper.	Cells grown in 0.1 mg/L copper significantly larger than those grown in control, 0.5 mg/L copper or 5 mg/L copper. Not significantly different to those grown in 1 mg/L copper.
Cell body area	Cells grown in 10 mg/L copper significantly larger than all other treatment groups ($p<0.05$).	Cells grown in 0.1 mg/L copper significantly larger than all other treatment groups ($p<0.05$).

Patterns for different morphological parameters of cells when treated with copper follow similar trends within cell lines, but are very different between cell lines. 1321N1 is enlarged in 10 mg/L copper in several of the parameters listed above, and U87MG is enlarged in 0.1 mg/L copper (U87MG proliferation was too low in 10 mg/L copper for morphological parameter to be measured). These trends are illustrated in figures 19 to 22, below.

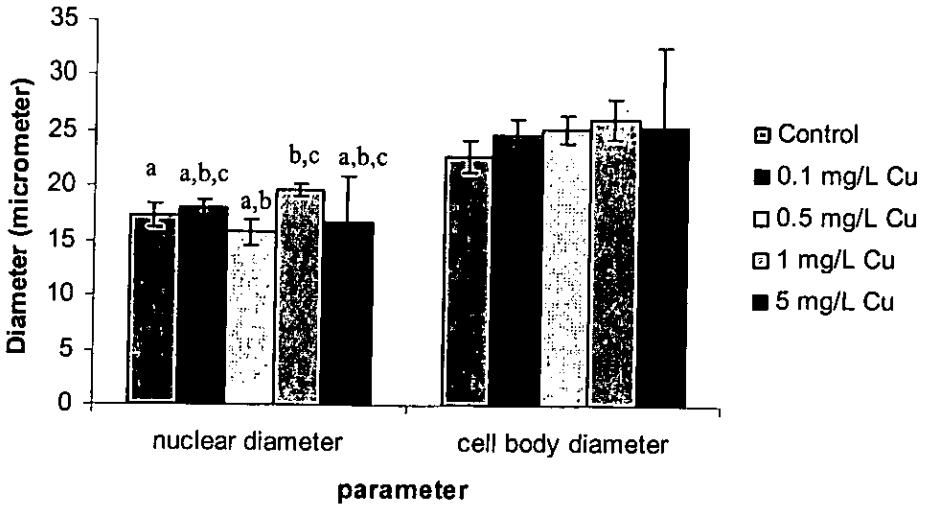


Figure 19. Bar chart showing the nuclear and cell body diameters (um) of 1321N1 cells when treated with copper. Data are mean ±SEM, n=6.

[a,b] Nuclear diameters with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

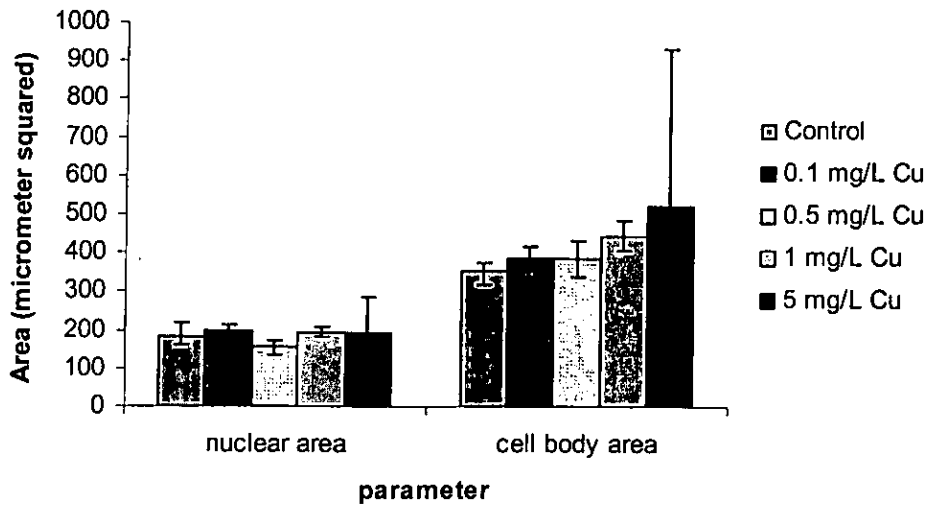


Figure 20. Bar chart showing the nuclear and cell body areas (um^2) of 1321N1 cells when treated with copper. Data are mean ±SEM, n=6.

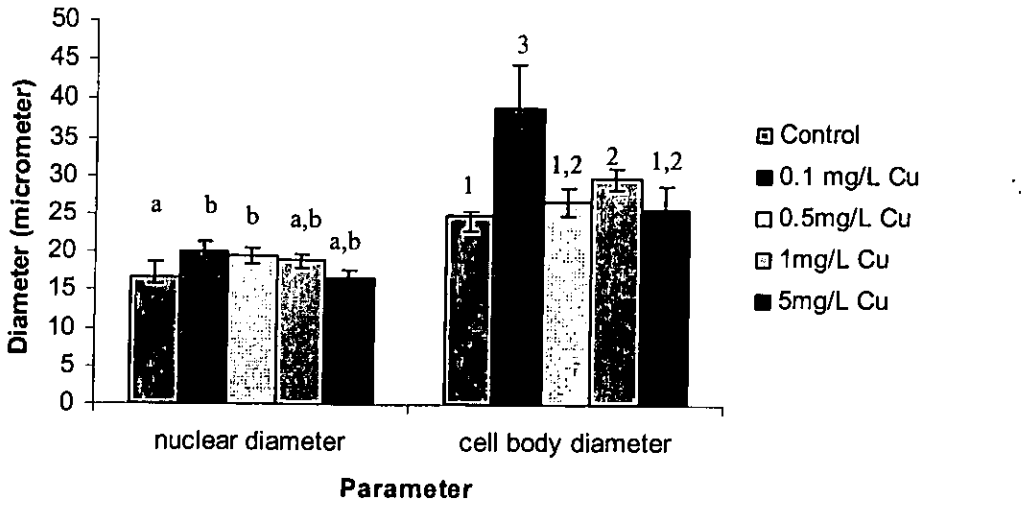


Figure 21. Bar chart showing the nuclear and cell body diameters (um) of U87MG cells when treated with copper. Data are mean ±SEM, n=6.

[a,b] Nuclear diameters with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[1, 2] Cell body diameters with different superscripts are significantly different to each other Compared using 2 way ANOVA.

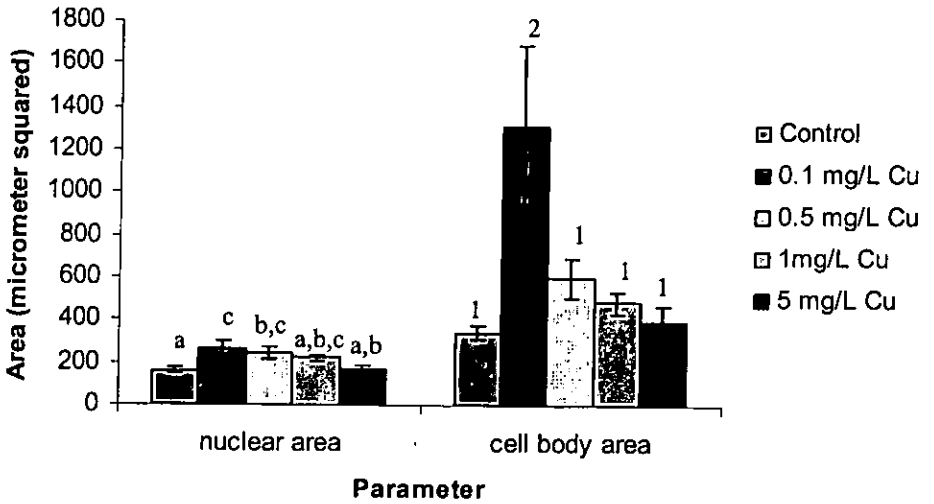


Figure 22. Bar chart showing the nuclear and cell body areas (um^2) of U87MG cells when treated with copper. Data are mean ±SEM, n=6.

[a ,b] Nuclear areas with different superscripts are significantly different to each other. Compared using 2 way ANOVA.

[1, 2] Cell body areas with different superscripts are significantly different to each other Compared using 2 way ANOVA.

3.2.2 Resting potential; a functional parameter of glial cells.

No difference was recorded in the potential differences observed when 132N1 cells were cultured in control either in the presence or absence of lithium.

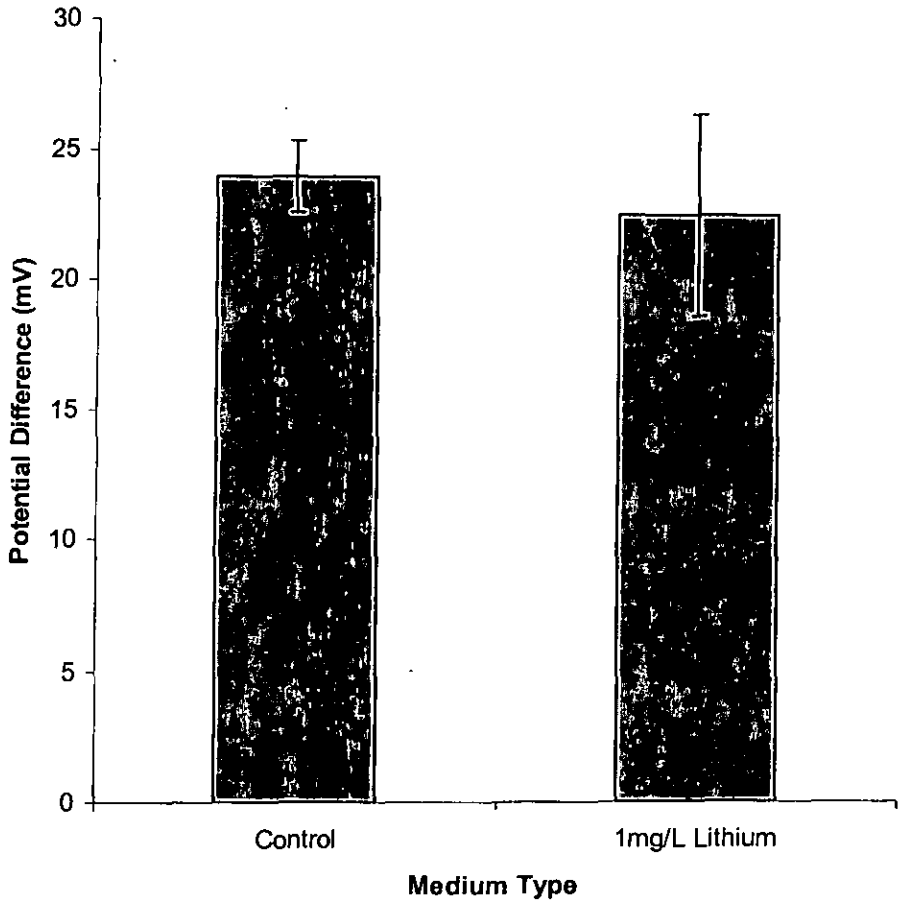


Figure 23. Histogram showing the resting potentials of 1321N1 cells with and without lithium supplementation. Data are mean ±SEM, n=6.

3.3 Experiment 3: The cytotoxicity of zinc and copper at supraphysiological levels and the influence of lithium on cytotoxicity.

On day 3 of exposure to trace minerals, no significant differences between cell proliferations were found between any of the medium types used. However, cell growth rates showed some significant effects ($p<0.05$) over a more prolonged time period. The data are as shown in figures 24-27. Protein content was measured to support growth rate data, the protein analysis data are shown in figures 28 & 29. Cell viability was also measured to determine whether cell division or cell death was affected in any changes found in the toxicity study, the viabilities are shown in figures 30- 33.

3.3.1 Proliferation rate of cells in supraphysiological levels of zinc or copper.

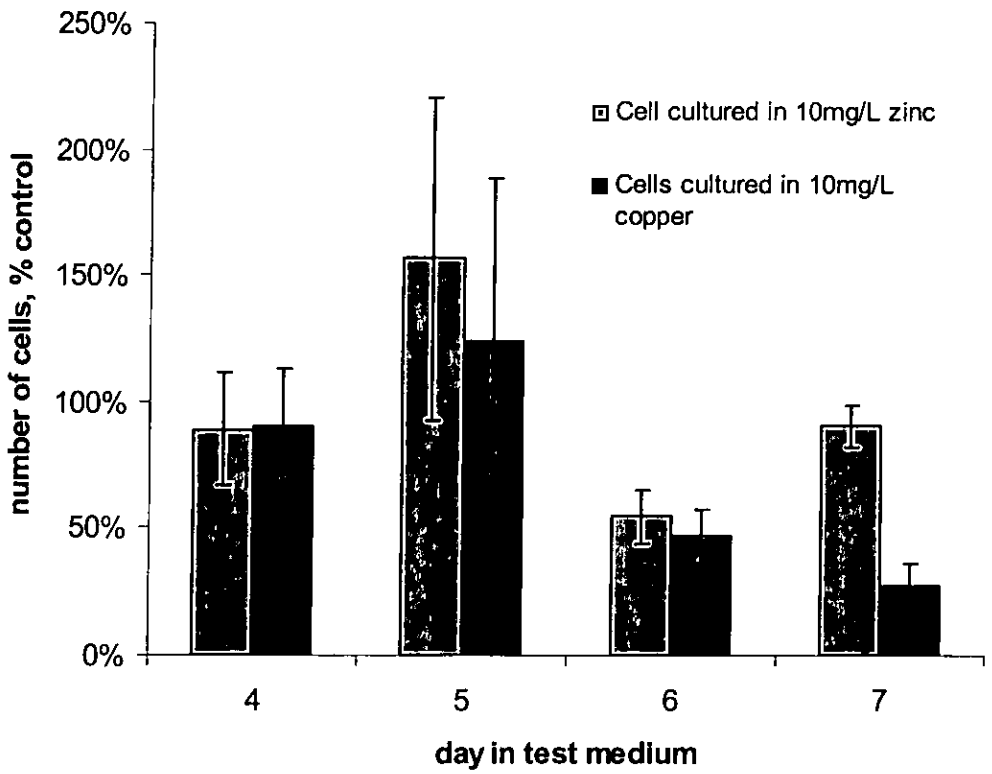


Figure 24. Bar chart showing the proliferation rate of 1321N1 cells cultured in supraphysiological levels of trace minerals. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell proliferation in control medium.

Analysis of the data presented in figures 24 and 25 by 2-way ANOVA and Tukey post hoc test revealed no significant differences between proliferation of cells in either Control medium and 10 mg/L zinc or control medium and 10 mg/L copper.

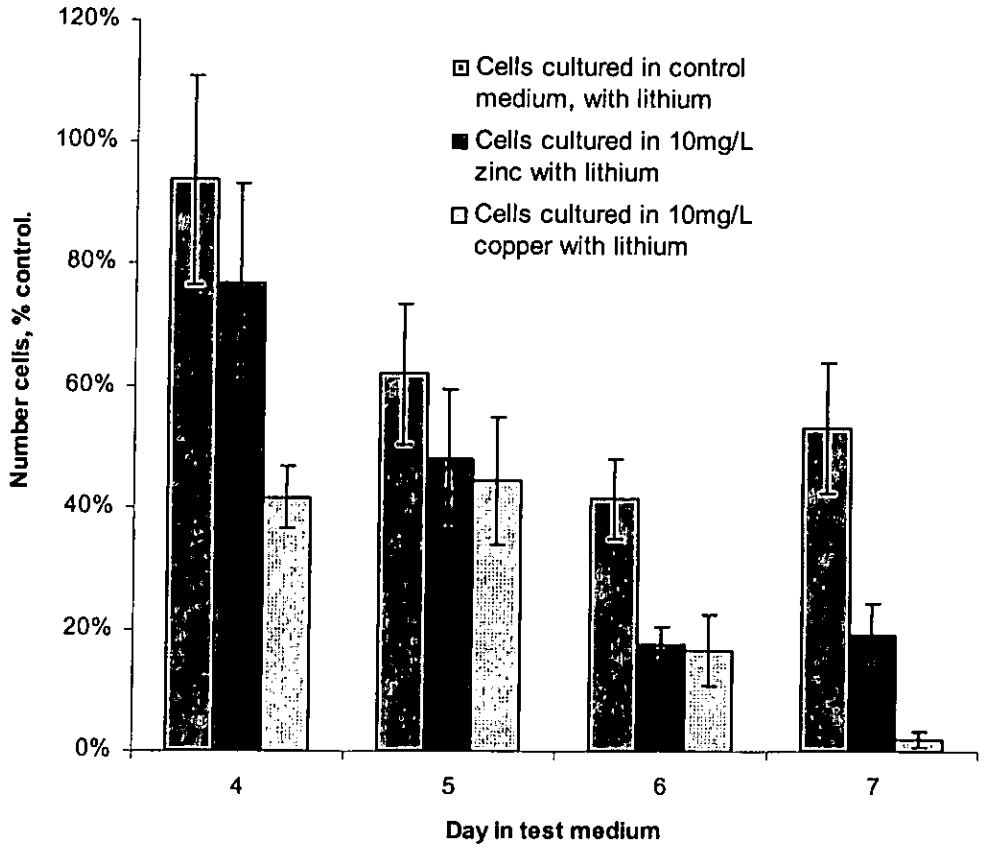


Figure 25 Bar chart showing the proliferation of 1321N1 cells cultured in supraphysiological levels of trace minerals with 1mg/L lithium. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell proliferation in control medium.

Lithium significantly reduced proliferation of cells grown in control medium ($p=0.045$) and zinc supplemented medium ($p=0.042$). The presence of lithium had no significant effect on cells grown in copper supplemented medium.

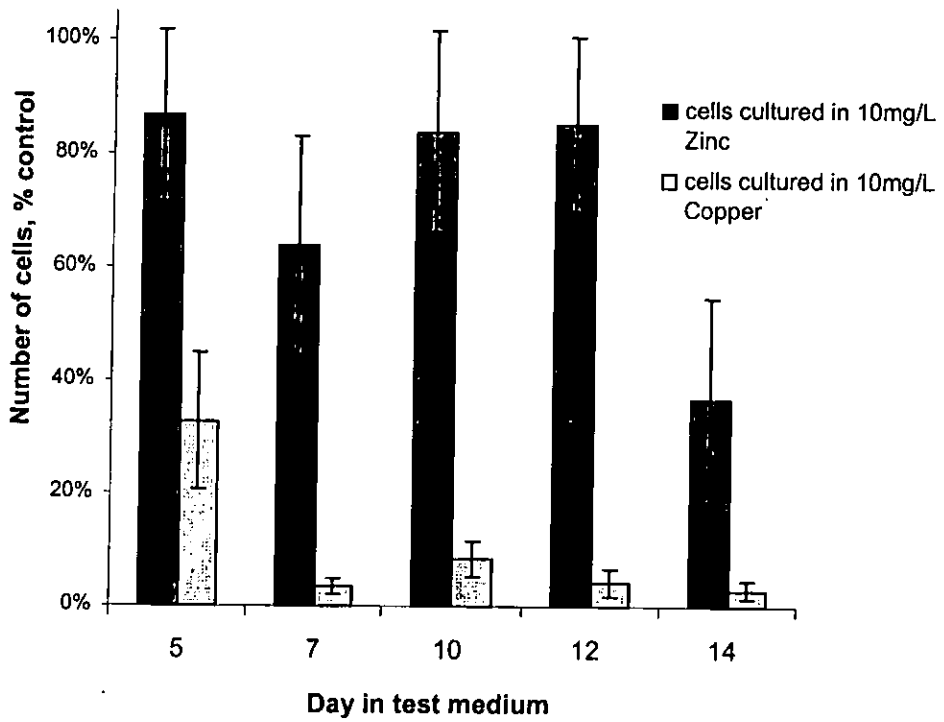


Figure 26 Bar chart showing the proliferation of U87MG cells cultured in supraphysiological levels of trace minerals. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell proliferation in control medium.

Analysis of the data presented in figure 26 by 2-way ANOVA and Tukey post hoc test revealed that the proliferation of cells grown in copper supplemented medium is significantly lower than that of cells grown in control medium, $p<0.05$.

Cells grown in zinc supplemented medium show no significant change in proliferation compared to control, except after 14 days, at which proliferation was reduced.

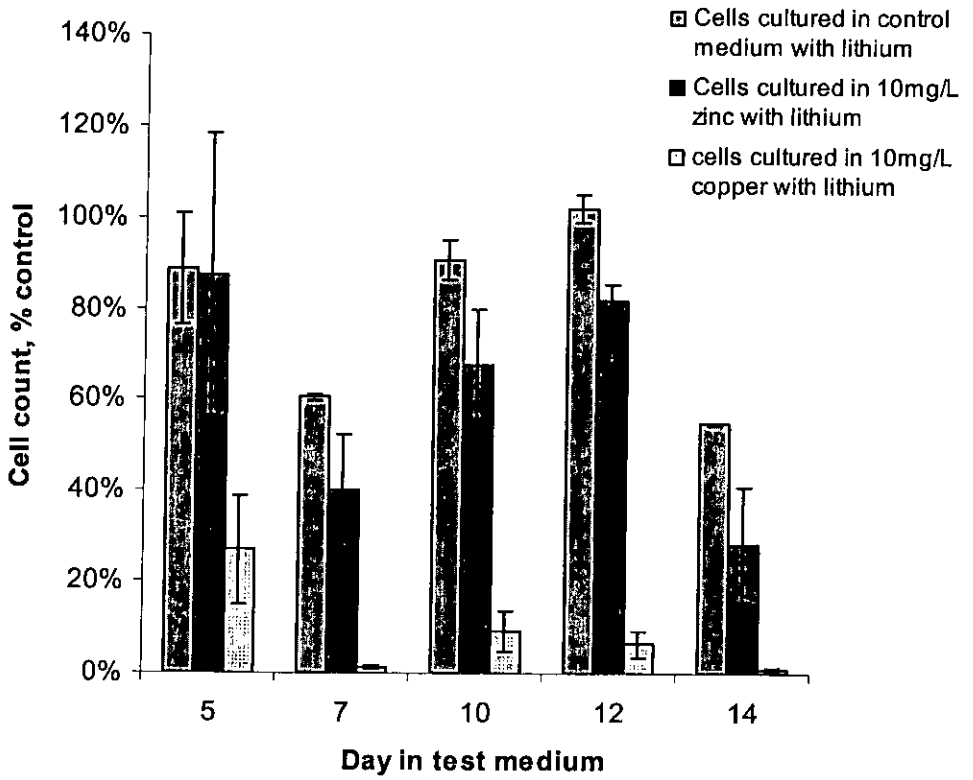


Figure 27. Bar chart showing the proliferation of U87MG cells cultured in supraphysiological levels of trace minerals, with 1mg/L lithium. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell proliferation in control medium.

The addition of lithium to the media significantly reduced the proliferation of cells grown in control medium ($p<0.05$), but not those grown in copper or zinc supplemented medium.

3.3.2 Protein content of cell pellets collected at completion of experiment 3.

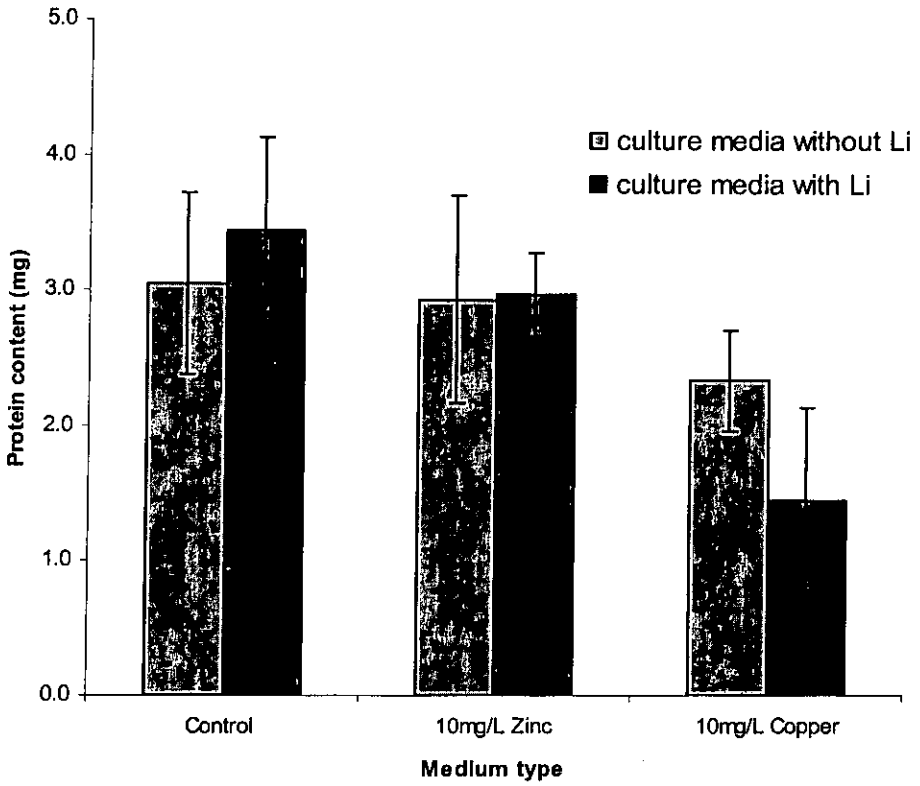


Figure 28. Bar chart showing the protein content of pellets collected from 1321N1 cell cultures. Data are mean ±SEM, n=6.

Note that there was no significant difference between protein content of cells grown in control medium, 10 mg/L zinc or 10 mg/L copper. Lithium had no effect on protein content of any group.

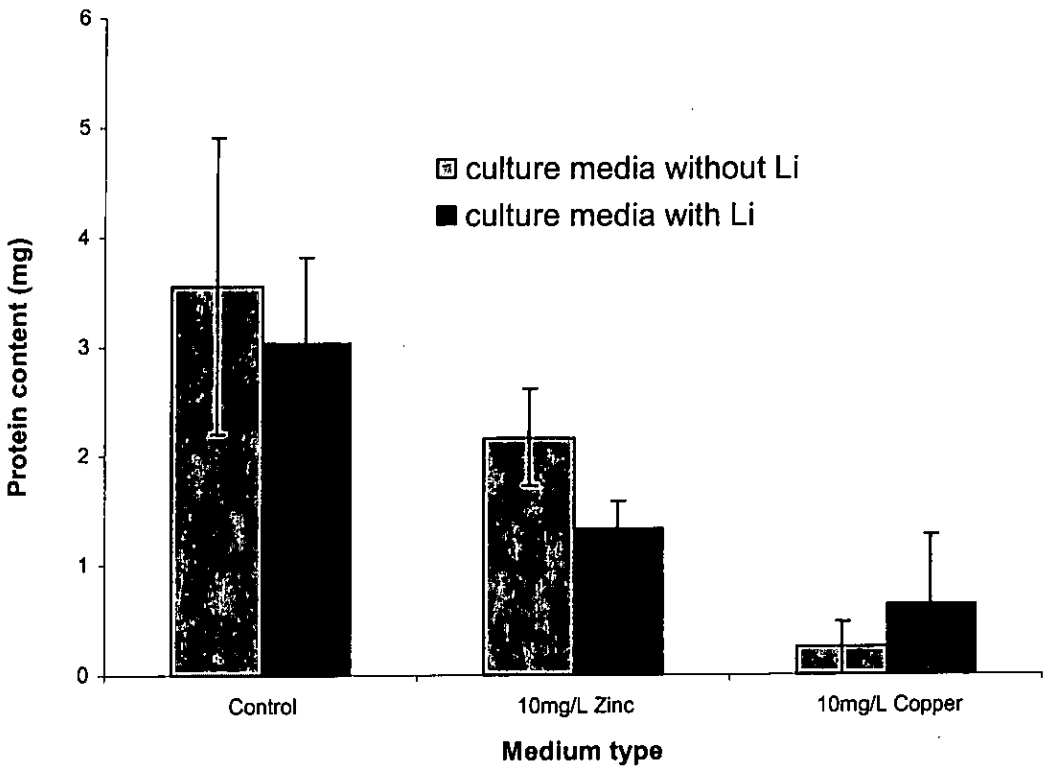


Figure 29. Histogram showing the protein content of pellets collected from U87MG cell cultures. Data are mean ±SEM, n=6.

Note that:

1) The protein content of cells grown in copper supplemented medium is significantly ($p<0.05$) lower than those grown in control medium.

2) There was no significant difference between protein contents in zinc and control groups' protein content.

3) Lithium had no significant effect on protein content of cells grown in any treatment group.

3.3.3 Viabilities of cells grown in supraphysiological levels of zinc or copper.

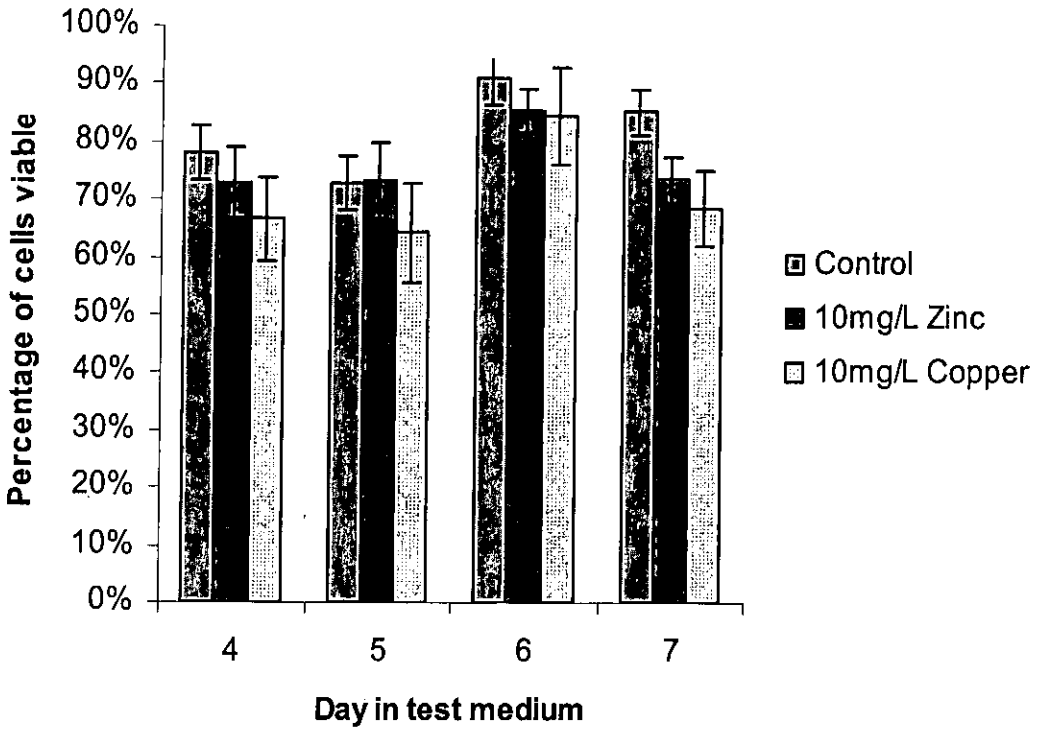


Figure 30. Histogram showing the viabilities of 1321N1 cultured in supraphysiological levels of trace minerals. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell viability.

Note that cells grown in control medium have significantly ($p<0.05$) higher viability to cells grown in either zinc supplemented medium or copper supplemented medium.

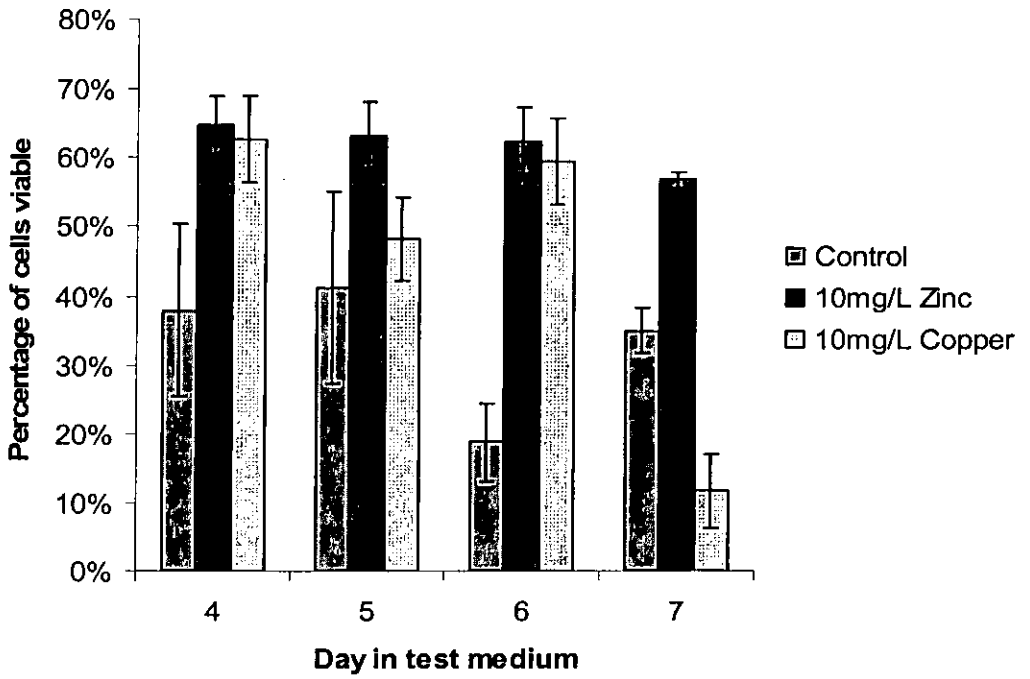


Figure 31. Histogram showing the viabilities of 1321N1 cells cultured in supraphysiological levels of trace minerals with 1mg/L lithium. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell viability.

Note that lithium significantly ($p<0.005$) reduced the viability of cells grown in each condition.

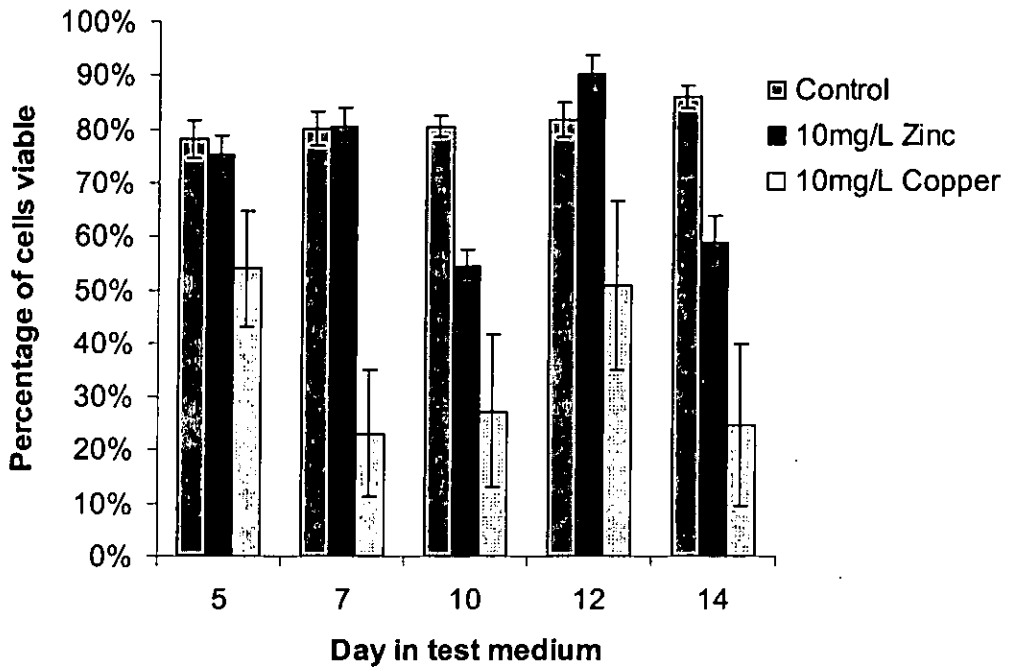


Figure 32. Histogram showing the viabilities of U87MG cells cultured in supraphysiological levels of trace minerals. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell viability.

Note that:

1) The viability of cells grown in copper supplemented medium was significantly lower ($p<0.05$) than those grown in control medium.

2) Cells grown in zinc supplemented medium showed no significant difference in viability to those grown in control medium, except after 14 days of exposure ($p<0.05$)

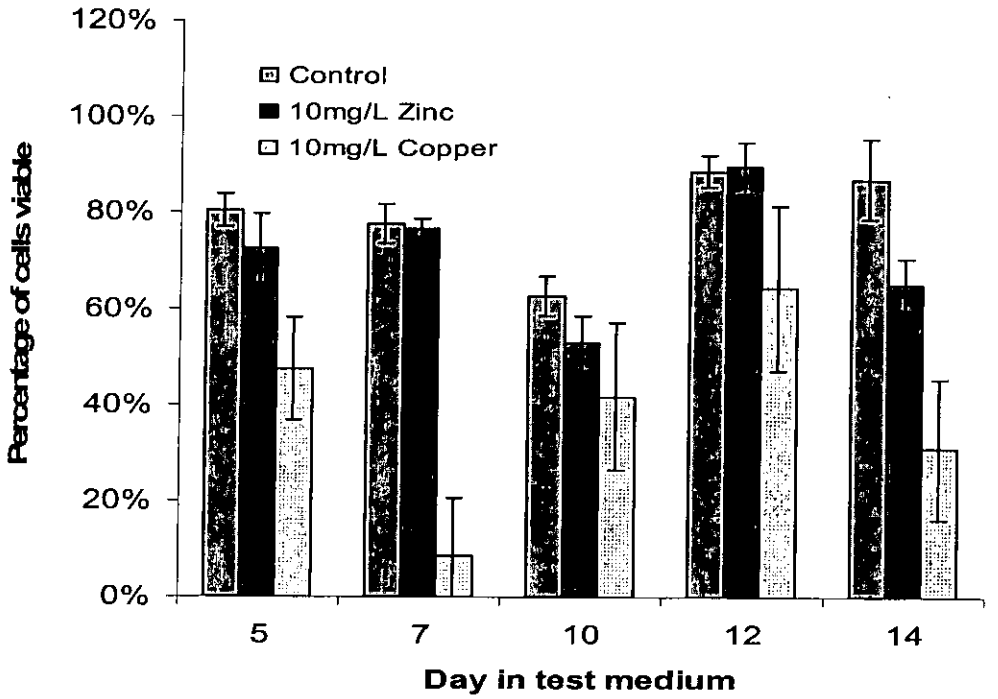


Figure 33. Bar chart showing the viabilities of U87MG cells cultured in supraphysiological levels of trace minerals with 1 mg/L lithium. Data are mean ±SEM, n=6 and they are expressed as a percentage of cell viability.

ANOVA revealed that lithium had no significant effect on viability of U87MG cells grown in either control, copper supplemented, or zinc supplemented medium, except for days 5, 7 and 14 of copper treatment, where proliferation was reduced $p<0.05$.

In summary (see figures 26-33), zinc supplementation at supraphysiological levels has no significant effect on cell proliferation in either cell line. Lithium affected the proliferation of 1321N1 cells grown in zinc supplemented medium, but not U87MG cells grown in zinc supplemented medium.

Copper supplementation affected U87MG cell proliferation negatively, but the same effect was not seen in 1321N1.

Viability of cells was not always affected in the same manner as live cell count.

3.4 Experiment 4: The effect of zinc concentration in medium on zinc uptake by the cell, and the influence of lithium on cellular zinc uptake.

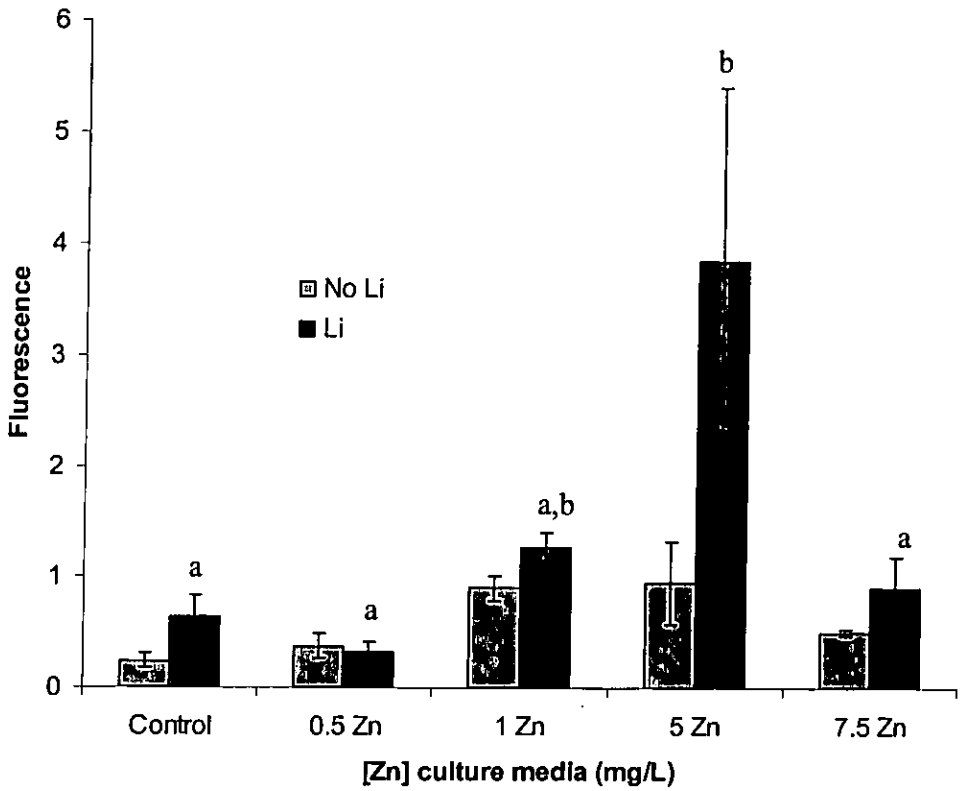


Figure 34. Histogram showing the zinc uptake of 1321N1 cells cultured in various concentrations of zinc, with and without lithium. Data are mean ±SEM, n=6.

There was no significant difference between fluorescence of cells grown in different concentrations of zinc, without lithium (p=0.057).

Cells cultured in lithium had an overall significantly higher fluorescence than those cultured without lithium (p=0.015). This effect is particularly marked in cells grown in 5 mg/L zinc. ANOVA analysis of the fluorescence of cells treated with lithium with Tukey post-hoc analysis revealed that cells grown in 5 mg/L zinc have a significantly higher fluorescence than those grown in control medium or 0.5 mg/L zinc, but cells

grown in 1 mg/L zinc and 7.5 mg/L zinc are not significantly different to any other group

Confocal microscopy imaging indicated that zinc is primarily located in the cell body and in particular the nuclei of glioblastoma cells. Less appears to be in the processes.

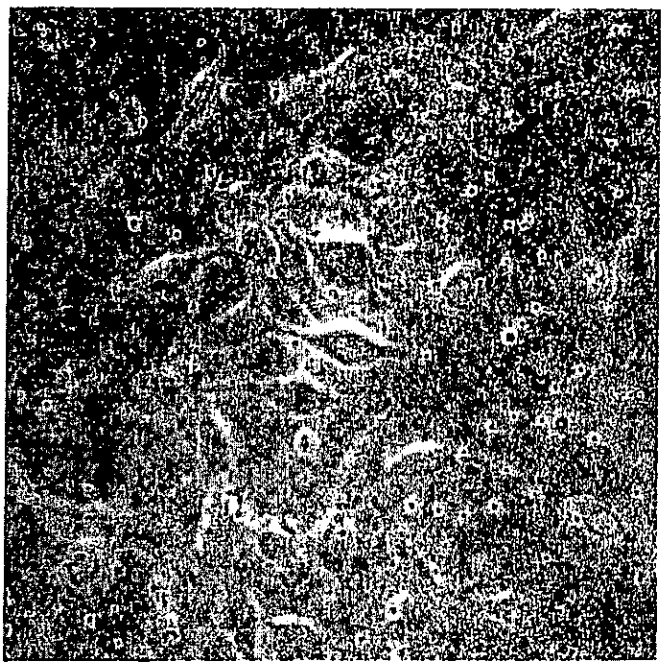


Figure 35. Visual image and fluorescent image using EYFP filter obtained using Confocal microscopy overlaid on day 3 of exposure to zinc. Photograph is typical of 3 such experiments.

CHAPTER 4

DISCUSSION

4.1 Experiment 1. The effect of trace mineral concentration on glial cell proliferation

Overall, neither the visual counting method, nor the MTT method revealed any consistent dose dependent effects of the addition of either zinc or copper to the culture medium on cell proliferation in either cell line. The addition of lithium to the culture media also had no significant overall effect on cell proliferation.

There were some inconsistencies in individual data produced by the two different assay techniques. Visual counting methods, while quick and easily completed have inherent accuracy difficulties. Cultured cells tend to grow in confluent patches, thus creating a wide variability between visualised areas. Firstly, a large number of sections must be taken to ensure an accurate representation of the overall slide. Second, randomisation of sections must be carefully controlled in this study. Third, the sections of slide to be counted must be selected prior to the observer looking down the microscope in order to minimise researcher bias effects. Fourth, cultured cells do not always grow in a perfect monolayer, and many layers superimposed on top of one another are frequently seen, and this may create difficulty in visually identifying separate cells. Finally, the processes of glial cells can also intertwine causing difficulties in visualisation of discrete cells.

In order to evaluate the reproducibility of the visual counting method, a simple inter-observer reliability test was conducted, two observers independently counted the number of cells visualised in a selected graticule area. This test indicated a reliability of

93.97% on the same sections, giving some confidence in the objectivity of the observers.

The MTT assay can only be as reliable as the count methods used for the seeding of the cells. The assay eliminates the subjectivity inherent in methods requiring researcher visual counting. The assay also has the advantage of rapid throughput enabling an increased number of sample replicates. The primary disadvantage of the MTT assay is that this technique relies upon spectrophotometric absorbance measurements which can be confounded by non-solubilisation of the formazan crystals in SDS. This effect can be reduced by shaking of the plates. The inter-assay and intra assay coefficient of variation of this assay was determined. The average intra-assay coefficient of variation was 26%, the average inter-assay coefficient of variation (calculated using the equation coefficient of variation = 100 (standard deviation/mean) of a set of data) was 11%. The calibration assays were each conducted 3-5 weeks apart, so such a low variation indicates a high reproducibility. Visual counting had an inter-assay coefficient of variation of 28%, higher than that of MTT. Visual counts were conducted on the same day. The high variability between trials in visual counts carried out on the same day, compared with MTT assays carried out weeks apart lends credence to the MTT assay over the visual count method, as do the objectivity of the MTT method and the closer agreement between the 2 cell lines with this method.

The lack of a significant effect of trace minerals at concentrations within the physiological range on cell proliferation in this study is surprising, given the clear and important roles of both zinc and copper in cell function and proliferation (section 1.4 and 1.5). Levels of zinc and copper in control medium are both below plasma levels in the human, therefore it was hypothesised that proliferation might be expected to increase as the concentration of Zn or Cu in the medium approached physiological

serum levels (approx 1 mg/L in serum)[77]. This suggests that there may be other factors limiting cell proliferation, and/or that control growth media contains sufficient mineral concentration for optimal growth of these cell lines.

4.2 Experiment 2: The morphological and functional parameters of cells grown in differing concentrations of trace minerals.

Harris haematoxylin is the most commonly used standard stain for cell morphology[78]. This study revealed that the nuclear and cell body diameters and areas of the 1321N1 cells were significantly ($p<0.05$) greater than controls when grown in 10 mg/L copper. This enlarged cell body and nucleus may indicate an osmotic effect of high copper concentrations. An inter-observer reliability test for measurement of the same cells by 2 independent researchers produced a reliability of 86%. The primary sources of error include; human bias in cell selection, for ease of measurement and a prior knowledge of required results. This was minimised by randomly selecting are area of the cover-slip without looking at the cells.

4.2.1 The resting potential of cells with or without lithium supplementation.

Previous research has shown that the resting potential of an excitable (neural or muscle fibre) cell is approximately -70 mV (cat motor-neurone) and greater than -70 mV (cancer pagarus muscle fibre cells)[71,72]. Glial cells are non-excitable, their resting potential was found to be around -20 to -25 mV, reflecting the lower resting potential of non-excitable cells. The addition of Li to the control medium did not significantly affect the resting potentials, indicating that there was no loss of membrane integrity, or alteration in ionic flow into and out of the cell following exposure to lithium.

4.3 Experiment 3: The cytotoxicity of zinc and copper at supraphysiological levels, and the influences of lithium on cytotoxicity.

There is much current research into the cellular mechanisms of zinc homeostasis[79]. Neurotoxicity can be preceded by zinc influx from presynaptic vesicles, but that this is often controlled by the capture of zinc ions by metallothionine, such that toxic levels of zinc do not always cause apoptosis[80]. Zinc-specific transporters also act in neurones to remove toxic levels of zinc from the intracellular space[81], reducing the probability of zinc-mediated death. These systems are either damaged or saturated in most cases of zinc-induced neurone death[80]. Neural cell ischemia can be induced by a rise in extracellular zinc, and this toxicity can then be reversed by introduction of a zinc chelating agent[82]. Whether similar controls exist for astrocytes and glial cells may be an interesting area for further research. Copper as a free ion is very toxic to cells owing to its high redox reactivity, and can be found to accumulate in astrocytes and glia, possibly to protect neurone cells from toxic effects[83]. Glia may not need to take up zinc as effectively as neurones have defined mechanisms for homeostatic control of zinc.

Trypan Blue stain counting was used to assess the effect of 10x physiological levels of zinc and copper on cell number and viability after incubation in the test media for either 7 days (1321N1) or 14 days (U87MG). Total protein content of the cell pellet was used as an additional index of total cell number.

For U87MG, the trypan blue counting method and protein analysis found no significant overall effect of 10 mg/L zinc on cell viability and number, however the 10 mg/L copper solution reduced viable cell count to 2.91% of control media on day 14 of measurement (figure 26) and this was accompanied by a reduction to 24.36% (figure 32) of control viability level and a fall to 6.92% pellet protein content (figure 29) compared to control. Lithium was found by Trypan Blue counting to reduce the

viability of U87MG cells grown in control medium to 87% of control, but there were no differences in total cell number assessed by visual counting or pellet protein content. No additional effect of lithium was observed in either of the trace mineral treated groups.

The viability of 1321N1 cells was reduced to 74% and 69% in cells treated with either 10 mg/L zinc, or 10 mg/L copper, respectively (figure 30). However, there did not appear to be a concurrent reduction in total cell number. Lithium was found to decrease cell number for those cells grown in control medium and zinc supplemented medium by Trypan Blue counting. The viability of cells in each treatment group was reduced by lithium treatment, control to 41%, zinc to 77% and copper to 17% of non-lithium treated cells (figures 30 and 31) . However, protein analysis found no effect of lithium in any treatment group (figures 28 and 29). Due to the high proliferation rate of 1321N1 cells, the proliferation study could not be continued to 14 days as U87MG was, it may be that given 14 days, similar effects to those seen in U87MG would emerge for 1321N1.

The high toxicity of copper for these cell lines is predictable by the high redox activity of copper, and the proposed activity of astroglia in protecting neural cells from these toxic effects.[83] The previously discussed tight regulation of intracellular zinc levels within neural cells[82,81,79], may also apply to astroglial cells thus protecting them from zinc toxicity. Evidence for the control of zinc uptake is shown in figure 34. The homeostatic regulation of zinc uptake appears to be perturbed by the presence of lithium.

4.3.1 Methodological issues

The trypan blue counting method is dependent on full trypsinisation of all cells. Trypsin is a cytotoxic solution when cells are exposed to it for a period of time and this

may confound viability studies. If cells do not lift from the base of a flask after the specified 5 min., they can be scraped using a sterile scraper in order to avoid toxic effects of trypsin. This is, however, also damaging to cells, and may also influence viability assays.

The variability between trials in this part of the study is very large, and proliferation levels appear to differ widely between repetitions of the experiment when up to 6 weeks apart. Flasks of cells subcultured on the same day for the purpose of a longitudinal study showed lower variability than those subcultured several weeks apart.

Four counts were taken for each flask used and the standard error between these counts was low. The large variation in cell count between trials may be a feature of the cell line, or an indication of imperceptible changes in the incubator in which cells were maintained between weeks, rather than a fault of the Trypan blue method of counting. Trypan blue is a commonly used cell proliferation assay technique, upon which many assay kits depend for their calibration.

Protein assays may be a good, objective calculation of proliferation, but could easily be confounded by cell size, cell loss during the PBS washing phase or accidental retention of some supernatant following washing, which with the small pellet size produced can affect protein concentration recorded to a large extent.

4.4 Experiment 4: The effect of zinc concentration in medium on zinc uptake by the cell, and the influence of lithium on cellular zinc uptake.

The results from experiments 1 and 3 indicated that zinc had no significant effect on cell proliferation in either the 1321N1 or U87MG cell lines, however, lithium significantly reduced the proliferation of 1321N1 cells grown in 10 mg/L zinc, but not in U87MG cells grown in 10 mg/L zinc. 1321N1 cell viability was affected by treatment with 10 mg/L zinc, but U87MG viability was not. This raises questions regarding the regulation of zinc uptake from the medium. Experiment 4 examined the uptake of a zinc-specific fluorescent probe into 1321N1 cells. The absence of a change in either zinc or lithium treatment indicates that glioblastoma cells regulate zinc transport into the cell. Several previous studies have demonstrated the existence of cellular control of zinc levels[79,80,81,82,] O'Halloran *et al*[84] have carried out much work searching for mechanisms of cellular control of zinc, and they propose that zinc is carried into the cell by a zinc carrier ZntR, and bound to an intracellular protein (Zur). Upon saturation of Zur proteins within a cell, no further zinc transport is allowed into the cell. This theory proposes a total absence of free intracellular zinc ions, all zinc is bound to intracellular proteins or excreted from the cell. This tight cellular control of zinc influx may explain the absence of an effect of zinc concentration on cell proliferation, morphology and viability.

When 1321N1 cells are cultured in media containing both lithium and zinc, zinc uptake of the cells is significantly enhanced. Zinc uptake is at its highest in 5 mg/L zinc with 1 mg/L lithium (significantly higher than in control medium and in 0.5 mg/L zinc, not significantly different to 1 mg/L zinc or 7.5 mg/L zinc). This study indicates that the cellular control of zinc influx is perturbed by lithium treatment at therapeutic levels in 1321N1 glioblastoma cells.

4.5 Conclusions

Copper showed low toxicity up to extracellular concentrations of 5 mg/L in this study, but higher doses (10 mg/L), had a profound effect on viability and proliferation of U87MG cells and a significant effect on 1321N1 cells. It is likely that the cells used in this study show control of copper ion influx, but that this is perturbed by high concentrations of copper, this hypothesis could be confirmed by use of a copper-specific fluorescent marker. Such a copper specific marker was not available for this study.

Zinc showed very low toxicity levels throughout this study, the fluorescence work, however, indicates that this is due to cellular control of zinc influx, which may be perturbed by simultaneous treatment with lithium, this may warrant further study.

Treatment with either trace mineral produced remarkably few changes in the functional parameters measured in this experiment. Cells treated with copper at toxic levels generally appeared enlarged, this may indicate that copper toxicity is initiated by an osmotic gradient caused by the influx of copper ions into the cell. Similar cellular enlargement did not occur in cells treated with toxic levels of zinc, indicating zinc may cause cell death by another means.

Glioblastoma cell lines *in vitro* are a limited but useful model of activity of glial cells *in vivo*. Glial cells may show different reactions to trace mineral treatment, but this study provides an indication as to likely glial cell responses to trace mineral exposure.

4.5.1 Methodology

The cell count methods have different reliability, despite the large error bars shown, trypan blue counting is an accurate and helpful method, although practical constraints make this inappropriate for a large number of conditions. The protein assay has many

conceivable flaws, but followed the trend of the trypan blue count method quite closely and supports the findings of this. The MTT assay has a good reproducibility and reliability for this application. Graticule-based visual counting is not a recommended proliferation assay technique.

4.5.2 Future research.

The investigation of ion uptake using a fluorescent probe should be repeated in the U87MG cell line, and other glioblastoma lines, to determine whether this effect is specific to the 1321N1 cell line, or whether it could be generalised to glioblastoma cells. If this effect is general, mechanisms for an inhibition of zinc influx control by lithium should be sought, as this may contribute further to the understanding of the method of action of lithium Carbonate prophylaxis. The current proposed action of lithium is primarily through blocking magnesium-dependent enzymes in the synthesis of inositol. The blocking is achieved by partially mimicking the magnesium ion on the enzyme receptor, thus blocking its action[25]. Lithium may similarly alter the action of zinc specific cell membrane channels, or zinc-dependent enzymes within the cell.

If the action of lithium on zinc uptake regulation is generalised in different cell populations, a clinical study of *in vivo* serum concentrations of zinc in bipolar disorder patients who respond to lithium prophylaxis and those who do not may be of interest in the search for predictors of responsivity to lithium prophylaxis.

A copper-specific fluorescent marker was not available to search for a similar effect in copper regulation, however the higher toxicity of copper found in experiment 3 implies that this may be less tightly controlled by the cell. Use of another, more general ion

fluorescent marker may help to shed light on control mechanisms for copper influx and any effect lithium may have on this.

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APPENDICES

Appendix 1

Composition of Dulbecco's Modified Eagle's Medium, as obtained from Sigma-Aldrich co.

Constiuent	Concentraion (g/L)	Concentration (mM)
INORGANIC SALTS		
$CaC_{l2}•2H_2O$	0.265	1203.397
$Fe(NO_3)3•9H_2O$	0.0001	0.247619
$MgSO_4$	0.09767	811.4081
KCl	0.4	5362.052
$NaHCO_3$	3.7	44047.09
NaCl	6.4	109514
NaH_2PO_4	0.109	908.6059
AMINO ACIDS		
L-Arginine•HCl	0.084	398.6711
L-Cystine•2HCl	0.0626	322.5142
Glycine	0.030	399.627
L-Histidine•HCl•H_2O	0.042	200.3817
L-Isoleucine	0.105	800.3049
L-Leucine	0.105	800.3049
L-Lysine•HCl	0.146	799.1242
L-Methionine	0.030	201.0724
L-Phenylalanine	0.066	399.5157
L-Serine	0.042	399.6194
L-Threonine	0.095	797.649
L-Tryptophan	0.016	78.35455
L-Tyrosine•2Na•$2H_2O$	0.10379	397.3583
L-Valine	0.094	802.7327
VITAMINS		
Choline Chloride	0.004	28.6533
Folic Acid	0.004	9.062075
myo-Inositol	0.0072	39.9556
Niacinamide	0.004	32.76003
D-Pantothenic Acid•½Ca	0.004	18.33181
Pyridoxine•HCl	0.004	19.45525
Riboflavin	0.0004	1.062699
Thiamine•HCl	0.004	11.85888
OTHER		
D-Glucose	1.0	5550.622
Phenol Red•Na	0.0159	42.24678
Pyruvic Acid•Na	0.11	1000