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Lithium Modulates Cancer Cell Growth, Apoptosis, Gene Expression and Cytokine Production in HL-60 Promyelocytic Leukaemia Cells and Their Drug-Resistant Sub-clones

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Abstract Lithium has been an FDA-approved and preferred drug for the treatment of mood disorders for many years, and cumulative evidence has pointed towards its potential use as an anti-cancer agent. Previous studies in our laboratory have demonstrated that lithium induces apoptotic cell death in HL-60 promyelocytes at concentrations of 10 mM and above. A lithium-tolerant HL-60 sub-clone, resistant to up to 15 mM lithium, was also generated and its growth profile reported. Treatment of cells with lithium resulted in a dose-dependent induction of p53, retinoblastoma (Rb) and bax expression which was accompanied by concomitant inhibition of bcl-2 expression as demonstrated using immunohistochemical microscopy. These results seem to suggest that lithium induced cell death in these cells by inhibiting expression of anti-apoptotic protein, bcl-2, while inducing higher expression of its pro-apoptotic counterparts which include bax. Expression of bax and bcl-2 is also linked to expression of inflammation-regulating cytokines. Using ELISA assays, lithium was demonstrated to induce production of pro-inflammatory cytokines, IL-6 and TNF-α, while inhibiting release of anti-inflammation-related IL-2 and IL-10 in a dose-dependent fashion. Our findings identify a critical function for lithium in modulating pro- versus anti-apoptotic gene expression and pro- versus anti-inflammatory cytokines in vitro and provide a rationale for suggesting a promising role of lithium in regulation of inflammation and cancer growth.

Keywords Lithium · Apoptosis · Cytokines · Lithium resistance · Cancer · Inflammation

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Introduction

Lithium is the gold standard treatment for bipolar disorder, a severe mental illness. Lithium, which has a well-established safety profile, has been FDA-approved and used to treat bipolar disorders for more than 60 years [1]. It is an effective treatment for mania and is also used as prophylactic therapy to prevent the recurrent manic and depressive episodes that characterise bipolar disorder [2]. A large body of evidence suggests that inflammation plays a role in the pathogenesis of bipolar disorder and that mood stabilisers exhibit anti-inflammatory properties. Pre-treatment with lithium 10 mM (but not 1 mM) significantly reduced LPS-induced secretion of TNF-α, IL-1β, prostaglandin-E2 and nitric oxide in rat primary glia cells. In addition, lithium significantly reduced the expression of cyclooxygenase-2 and inducible nitric oxide synthase, findings suggesting that lithium exhibits a potent anti-inflammatory effect [3]. Further evidence from our laboratory suggests potent anti-cancerous and apoptosis-modulating activities by lithium on HL-60 promyelocytic leukaemia cells and haematopoiesis [4–9]. This is compelling evidence that supports the notion that treatment with lithium may elicit strong anti-inflammatory effects in cancerous cells. Hence, a promising chemotherapeutic direction has emerged from an unexpected field of psychiatry and a monovalent cationic element, lithium.

Pioneering studies demonstrated that lithium directly inhibits glycogen synthase kinase-3 (GSK-3) [10, 11]. This

enzyme has been further established as a crucial target for lithium's cellular effects [12, 13]. GSK-3, consisting of α and β isoforms, is a serine/threonine kinase that regulates diverse cellular and neurophysiological processes. Lithium competes with magnesium to directly inhibit GSK-3 by binding to the active site of the enzyme and limiting its catalytic activity [14]. Lithium also indirectly inhibits GSK-3 activity by enhancing phosphorylation of GSK-3α at Ser21 and GSK-3β at Ser9 via activation of phosphatidylinositol 3-kinase (PI3-K)/Akt, protein kinase A and protein kinase C [12, 15]. In addition, lithium has been shown to increase the activities of two transcription factors, activator protein-1 (AP-1) and cyclic-AMP response element binding protein (CREB), both in vivo and in vitro [16]. Lithium also activates the mitogen-activated protein (MAP) kinase pathway [17]. These metabolic pathway intermediates play a crucial role in regulating apoptosis, cytokine production and differentiation in HL-60 cells [18].

Lithium is a potent inhibitor of GSK-3β and GSK-3β inhibition has been demonstrated to have anti-inflammatory effects, as shown by reduced TNF-α production via attenuated activation of NF-κB and JNK signaling cascades [18] and induction of the anti-inflammatory cytokine, IL-10 [19]. GSK-3β and NF-κB play a central role in cancer progression, and regulation of the factors by lithium may prove to be important in cancer treatment. In light of these findings, the effect of lithium on expression of apoptosis-related genes and inflammation-associated cytokines was studied.

Materials and Methods

Cell Cultures

Human HL-60 cells were obtained from ATCC and propagated routinely in RPMI-1640 supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in a humidified 95 % air/5 % CO_2 atmosphere. Parent cultures of normal HL-60 cells were maintained in continuous logarithmic growth between 2×10^5 and 1×10^6 cells/ml. Cell cultures were periodically checked for mycoplasma contamination using a detecting kit supplied by Boehringer Mannheim. Propagation of lithium-resistant HL60 sub-clones was achieved as previously described (Matsebatlela et al., 2000). Briefly, HL-60 cells were treated with 10 mM LiCl, and surviving cells were cultured and sub-cultured for about 52 weeks until gaining full resistance to 10 mM concentration. Lithium concentration was then increased in a stepwise manner until HL-60 cells proliferated normally and maintained viability of more than 95 % in the presence of 12.5 mM lithium. Cells were designated HL-60LiR to differentiate them from their non-resistant counterparts.

Treatment of Cells with Lithium

Lithium chloride was purchased from Fluka (Buchs, Switzerland) and stored in stock solutions of 500 mM in RPMI-1640 at 2–8°C. To initiate an experiment, HL-60 cells were seeded at 5×10^5 cells/ml and treated with 0 and 20 mM lithium, whereas HL-60 LiR cells were seeded at the same cell density and incubated in the presence of 12.5, 15, 20 and 30 mM LiCl, respectively.

Cell Growth and Viability

Cell growth was monitored daily for 4 days, and cell counts were electronically taken using a model Z1 Coulter Counter. Cells were considered viable if they could exclude 0.1 % trypan blue dye dissolved in physiological saline. At least 100 cells were scored to represent a data point. Cells in log-phase growth were incubated with the designated concentrations of lithium chloride and maintained as described above. Experimental incubations were terminated by centrifuging cells at 800 rpm for 10 min. Cell pellets were subsequently prepared for the immunocytochemical procedure described below.

Immunocytochemistry

After centrifugation and cytospinning, cells in microscope glass slides cells were assayed for Bcl-2, Bax, Rb and p53 protein expression. This analysis was done using the Streptavidin–peroxidase Universal Immunostaining ELISA kit obtained from Immunotech (Coulter, Miami, Florida, USA). Monoclonal antibodies purchased from Immunotech were used as primary antibodies and Streptavidin–peroxidase served as an indicator enzyme. As part of the substrate reaction, 3-diamino benzidine (DAB) addition resulted in the formation of a coloured precipitate which was visualised by counterstaining with haematoxylin. The prepared immunocytochemistry slides were immediately evaluated under a light microscope and relative amounts of stained cells and their expression patterns were determined by counting at least 100 cells per slide.

DNA Fragmentation Assays

DNA from harvested cells was extracted using a modified version of the method described previously [20]. Briefly, about 5–10×105 cells/ml were harvested by centrifugation at 200*g* for 10 min and then washed twice with 10 ml ice-cold sterile phosphate-buffered saline (PBS), pH 7.4. The cell pellets were suspended in 500 μl lysis buffer (10 mM Tris–HCl buffer, 0.5 % Triton X-100 and 1 mM EDTA) by gentle pipetting. The lysates were clarified by centrifugation at 13,000*g* for 10 min, and the supernatant was transferred

into a sterile microfuge tube. Cell pellets containing high molecular weight DNA and cellular debris were discarded. The DNA from the supernatant was extracted three times with an equal amount of phenol:chloroform (1:1) (500 μl) and precipitated in the presence of 55 μl 3 M sodium acetate, pH 5.2 and 500 μl of ice-cold propanol at −80°C for 15 min. The DNA was centrifuged, washed with 70 % ethanol and resuspended in 50 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). About 5–7 μl of 0.5 mg/ml RNase was added and the suspension incubated at 37°C for 1 h. The DNA was re-extracted with phenol–chloroform, precipitated with isopropanol and washed again with ethanol. The extracted DNA was air dried and resuspended in 50 μl TE buffer. For gel analysis, 20 μg DNA per lane was electrophoresed in a 1 % agarose gel for two and half hours at 56 V. The gels were visualised by illuminating ethidium bromide under ultraviolet light, and the gel photographs were taken using a polaroid camera.

Determination of Cytokine Production

Analysis of TNF-α and IL-2 was carried out using sandwich ELISA kits purchased from Sigma Immunochemicals, St. Louis, MO, USA. Sample supernatants from treated samples were harvested and applied on the 96-well microtitre plates as per kit procedure. An indirect immunoassay was developed for determining IL-6 and IL-10 levels in the cultured supernatants. The detection secondary antibodies were labeled with alkaline phosphatase and p-nitrophenyl phosphate was used as the substrate. After addition of 1 N HCl as a reaction stop, solution absorbances were read at 450 nm using a Bio-Rad model 550 microplate reader. All assays were repeated at least three times to ensure reproducibility, and error means were indicated on the result.

Results

Effect of Lithium on Proliferation of HL-60 and HL-60LiR Cells

The cytotoxic action of lithium was tested on the HL-60 promyelocytic leukaemia cells and their resistant sub-clones. Cells were treated with various concentrations of lithium for 24 h and cell proliferation was assessed. Lithium inhibited growth and viability of HL-60 and HL-60LiR in a dose- and time-dependent manner (see Fig. 1). Although 12.5 mM lithium was cytotoxic to HL-60 cells within 24 h of treatment, HL-60 LiR cells remained minimally affected by this concentration and maintained a high proliferation rate and high cell viability. Inhibition of HL-60 LiR cell proliferation and viability were observed only after treatment with 15 mM lithium. A highly pronounced inhibition of proliferation and viability of HL-60 LiR cells was only observed after treatment of cells with as high as 20 mM lithium. It is well documented that 10 mM lithium is highly cytotoxic to HL-60 cells, hence, our findings further show that HL-60 LiR sub-clones are resistant to cytotoxic concentrations of lithium.

Induction of Apoptosis by Lithium in HL-60 and HL-60 LiR Cells

A DNA fragmentation assay was carried out to investigate the mode of cell death used by lithium in inducing cytotoxicity in HL-60 leukaemic cells and their lithium-resistant sub-clones. Treatment of HL-60 cells with 12.5 mM LiCl resulted in DNA fragmentation as depicted through the laddering pattern of the DNA isolated from cells (Fig. 2, lane 3) compared to the untreated controls (Fig. 2, lane 2). Hence, apoptosis was evident when HL-60 cells were treated with 12.5 mM lithium. Increment of lithium concentration from 15 mM to 30 mM resulted in a much more pronounced laddering pattern of DNA isolated from HL-60 cells (Fig. 2, lanes 4–6). This shows that apoptosis was more noticeable when HL-60 cells were treated with lithium concentrations above 12.5 mM. On the other hand, treatment of HL-60 LiR cells with 12.5 mM lithium resulted in no apparent DNA laddering pattern (Fig. 3, lane 2). The laddering pattern became more evident when cells were treated with 15 mM, 20 mM and 30 mM LiCl. Thus, HL-60 LiR cells show acquisition of resistance to 12.5 mM lithium cytotoxicity, whereas their normal HL-60 counterparts succumb to this higher concentration of lithium. The results show evidence that HL-60 LiR cells have acquired resistance to apoptotic cell death induced by lithium at concentrations up to 12.5 mM.

Modulation of Apoptosis-Related Genes in HL-60 and HL-60 LiR Cells by Lithium

Since lithium seems to modulate apoptosis in HL-60 and HL-60 LiR cells, several apoptosis-related genes were analysed through immunohistochemical microscopy (Fig. 4). Cells were stained with labelled antibodies for bcl-2, bax, retinoblastoma (Rb) and p53 (Fig. 5). Positively staining cells were viewed under a light microscope, and the extent of gene expression was scored using the relative number of positively stained cells versus the unstained cell population. Bcl-2 expression was severely diminished in HL-60 cells treated with 20 mM lithium compared to the untreated to controls. More than 60 % of untreated HL-60 cells stained positive for bcl-2, whereas less than 1 % of cells stained for bcl-2 after treatment with 20 mM lithium. The converse was true for bax expression. More than 80 % of HL-60 cells treated with lithium stained positive for bcl-2 compared to

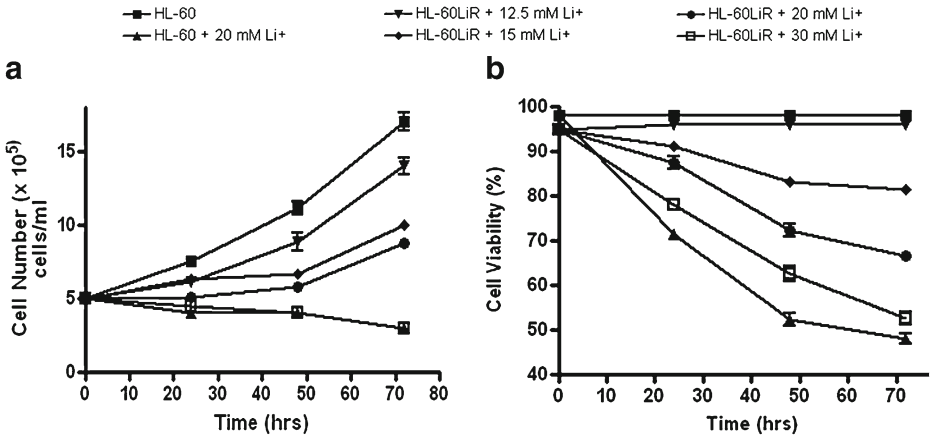

Fig. 1 Lithium-induced cytotoxicity in HL-60 and HL-60 LiR cells. HL-60 and HL-60 LiR cells were treated with 0, 12.5, 15, 20 and 30 mM lithium for 72 h and a Coulter Counter was used to assess cell count. The cell count was used as a measure of cell proliferation and a trypan blue dye exclusion assay was used to count the number of viable cells. The experiment was repeated three times in duplicates, and *error bars* represent the degree of variance

the less 5 % positive staining observed in untreated controls. Thus, treatment of HL-60 cells with lithium increased bax expression while diminishing bcl-2 expression. Therefore, lithium seems to favour elevation of the bax/bcl-2 ratio. In the HL-60 LiR cells, this ratio seems to be reversed, accounting for the acquired resistance towards lithium toxicity. HL-60 LiR cells were shown to express higher levels of bcl-2 and lower levels of bax. Hence, the bax/bcl-2 ratio is much lower in HL-60 LiR cells compared to their normal HL-60 cell counterparts. However, treatment of HL-60 LiR cells with lithium concentrations higher than 12.5 mM resulted in a dose-dependent increase of bax expression and decrease of bcl-2 expression. This observation corresponds to the pronounced DNA fragmentation seen when cells were treated with lithium concentrations higher than 12.5 mM. Lithium, therefore, seems to induce cell death in these lines by modulating the bax/bcl-2 ratio.

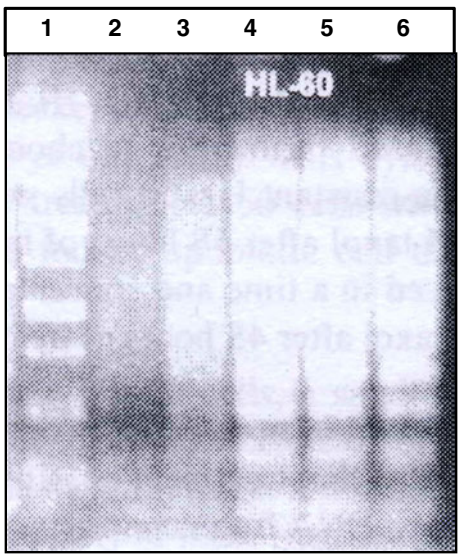

Fig. 2 Induction of DNA fragmentation in HL-60 cells by lithium. To measure the extent of apoptotic cell death, HL-60 cells were treated with 0, 12.5, 15, 20 and 30 mM for 24 h. Cells were harvested using centrifugation and DNA was extracted using phenol–chloroform method. The extracted DNA was ran on a 1 % agarose gel to assess the extent of DNA laddering as a measure of DNA fragmentation and apoptotic cell death. DNA molecular weight markers were included in *lane 1*

Regulation of Cytokine Production by Lithium in HL-60 and HL-60 LiR Cells

The effect of lithium on production of TNF-α, IL-6, IL-2 and IL-10 in HL-60 and HL-60 LiR cells were measured using a sandwich ELISA assay. After treatment of cells with lithium, culture supernatants were collected and assayed for cytokine content. Treatment of HL-60 and HL-60 LiR cells with lithium resulted in a general dose-dependent increase of TNF-α and IL-6 production. Production of TNF-α and IL-6 in HL-60 and HL-60 LiR cells was at its highest after treatment with 30 mM lithium (Fig. 6). Conversely, treatment of HL-60 and HL-60 LiR cells with lithium resulted in

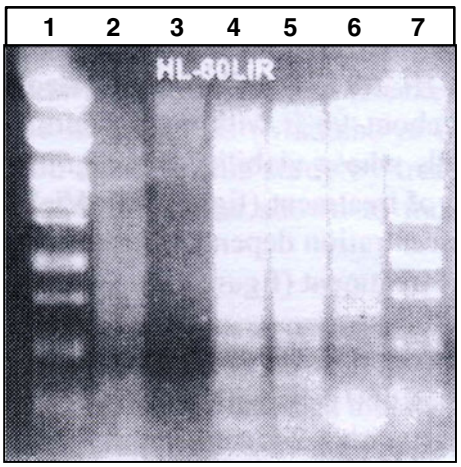

Fig. 3 Induction of DNA fragmentation in HL-60 LiR cells by lithium. To measure the extent of apoptotic cell death, HL-60 LiR cells were treated with 12.5, 15, 20 and 30 mM (*lanes 3–6*) for 24 h. HL-60 cells treated with 20 mM lithium was used as a positive control (*lane 2*). Cells were harvested using centrifugation and DNA extracted using phenol–chloroform method. The extracted DNA was ran on a 1 % agarose gel to assess the extent of DNA laddering as a measure of DNA fragmentation and apoptotic cell death. DNA molecular weight markers were included in *lanes 1* and *7*

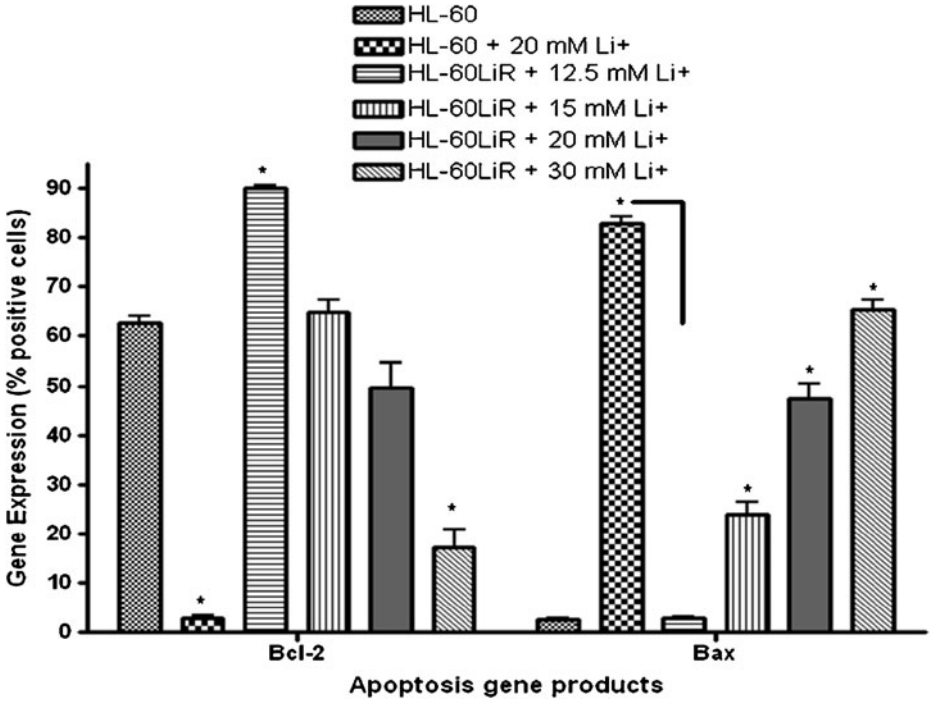

Fig. 4 Modulation of bcl-2 and bax expression in HL-60 and HL-60 LiR cells by lithium. HL-60 and HL-60 LiR cells were treated with 0, 12.5, 15, 20 and 30 mM lithium for 24 h and cells were attached onto microscope glass slides through cytospinning. Cells were stained with antibodies against bcl-2 and bax and visualised using a substrate solution after incubation with labelled secondary antibody as per kit instructions as outlined in "Materials and Methods". Positively staining cells were scored as a measure of extent of protein expression. Percentage gene expression was calculated as positively staining cells divided by the total number of cells. Experiments were repeated three times in duplicates, and $p<0.05$ represents significant differences

a dose-dependent decrease of IL-2 and IL-production. Generally, HL-60 cells expressed higher amounts of IL-10 than IL-2, and lithium decreases their production with maximal decrease observed after treatment of cells with 30 mM lithium (Fig. 7). These results show evidence to suggest that lithium increases TNF-α and IL-6 production while decreasing IL-2 and IL-10 production in HL-60 and HL-60 LiR cells.

Discussion

Although lithium was first identified for its use in the treatment of mania, during recent years attention has been focused on its anti-cancer potentials as well. It is now well accepted that lithium concentrations of 10 mM and above inhibit cell growth of HL-60 promyelocytic leukaemia cells

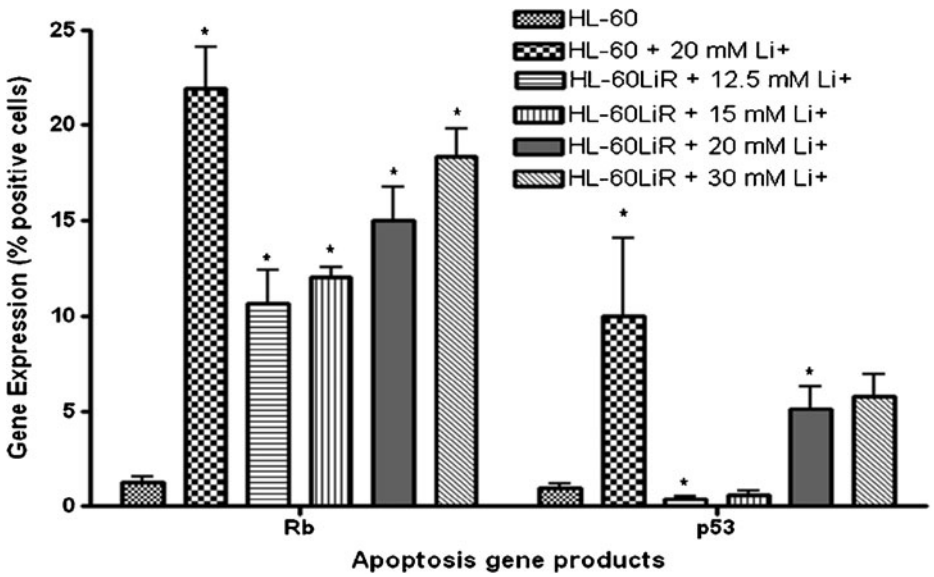

Fig. 5 Modulation of retinoblastoma (*Rb*) and p53 expression in HL-60 and HL-60 LiR cells by lithium. HL-60 and HL-60 LiR cells were treated with 0, 12.5, 15, 20 and 30 mM lithium for 24 h, and cells were attached onto microscope glass slides through cytospinning. Cells were stained with antibodies against Rb and p53 and visualised using a substrate solution after incubation with labelled secondary antibody as per kit instructions as outlined in "Materials and Methods". Positively staining cells were scored as a measure of extent of protein expression. Percentage gene expression was calculated as positively staining cells divided by the total number of cells. Experiments were repeated three times in duplicates, and $p<0.05$ represents significant differences

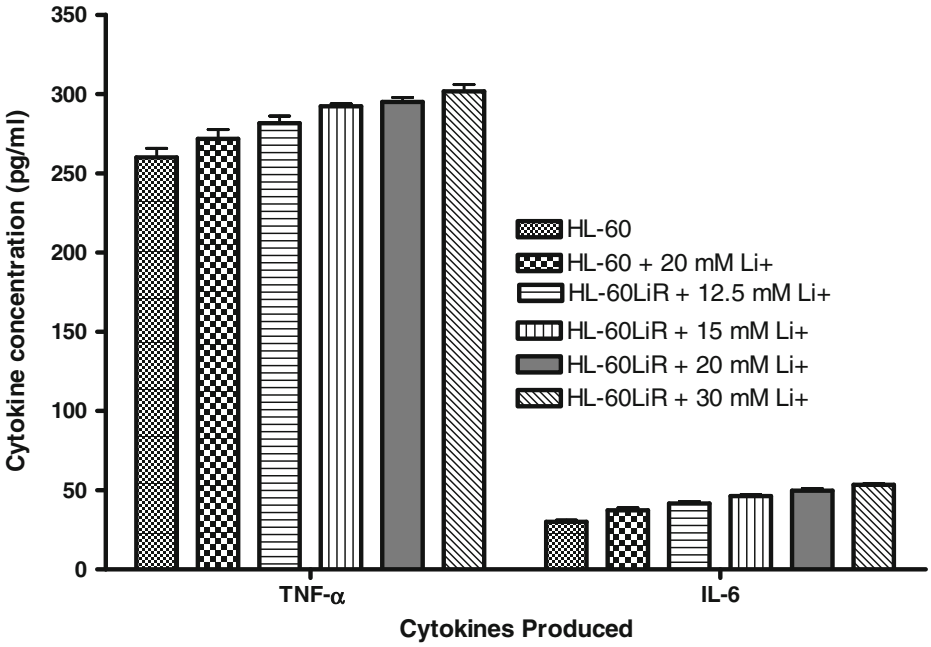

Fig. 6 Determination of TNF-α and IL-6 production in HL-60 and HL-60 LiR cells treated with lithium. HL-60 and HL-60 LiR cells were treated with 0, 12.5, 15, 20 and 30 mM LiCl for 24 h, and the culture supernatant was harvested for assessment of cytokine content. Cytokine content in harvested supernatant was assayed using a sandwich ELISA method using primary antibodies against TNF-α and IL-6. Alkaline phosphatase-labelled secondary antibodies were used as detection antibodies with p-nitrophenol used as the substrate. A standard curve was used to extrapolate the final cytokine concentrations. Experiments were repeated three times in triplicates to obtain the degree of variance as represented using the *error bars*

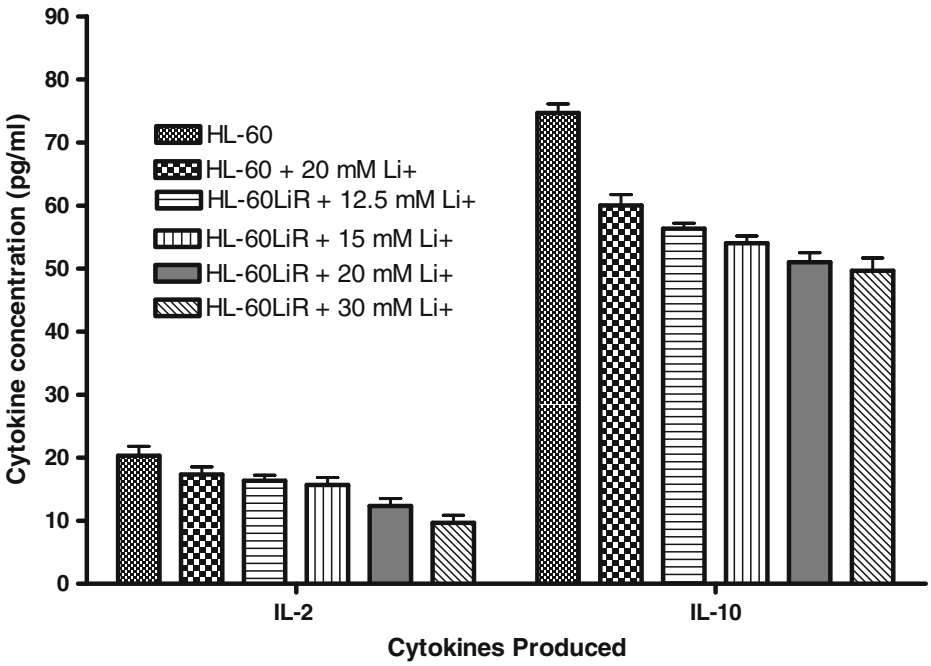

Fig. 7 Determination of IL-2 and IL-10 production in HL-60 and HL-60 LiR cells treated with lithium. HL-60 and HL-60 LiR cells were treated with 0, 12.5, 15, 20 and 30 mM LiCl for 24 h, and the culture supernatant was harvested for assessment of cytokine content. Cytokine content in harvested supernatant was assayed using a sandwich ELISA method using primary antibodies against IL-2 and IL-10. Alkaline phosphatase-labelled secondary antibodies were used as detection antibodies with p-nitrophenol used as the substrate. A standard curve was used to extrapolate the final cytokine concentrations. Experiments were repeated three times in triplicates to obtain the degree of variance as represented using the *error bars*

[4–9, 21, 22]. Although 10 mM LiCl induces apoptosis in HL-60 cells, a small fraction of the cell population remains viable, survives the cytotoxic effects of lithium and exhibit an altered gene expression pattern when sub-cultured for lengthy periods [4]. The resistance acquired by these HL-60 cells requires further evaluation as results from these studies will be of importance not only in psychiatry but in the proposed use of lithium as a chemotherapeutic agent. Results from our lab demonstrated that lithium seems to be selectively cytotoxic to cancerous cells while sparing non-cancerous cells, a phenomenon important in developing chemotherapeutic drugs [23].

Evidence from our laboratory has indicated that lithium resistance acquired by HL-60 cells and its anti-cancerous effects include a mechanism of action involving the induction of apoptosis accompanied by modulation of apoptosis-related genes [4, 9]. HL-60 cells express two important apoptosis-related genes: bax, a pro-apototic gene, and bcl-2, an anti-apoptotic gene. Lithium appears to induce apoptosis in HL-60 cells by increasing the bax/bcl-2 ratio, and when this ratio is reversed, HL-60 cells acquire resistance to lithium cytotoxicity. When lithium concentration was increased in the lithium-resistant HL-60 cells, bcl-2 expression decreased and cell death increased. In HL-60 cells, the increase in lithium concentration to 15 mM was accompanied by DNA fragmentation and a decrease in cell viability. Although treatment of HL-60 cells with 15 mM lithium resulted in DNA fragmentation, this phenomenon was not observed in their lithium-resistant sub-clones (HL-60LiR cells). Expression of other apoptosis-related gene products such as p53 and Rb increased proportionally with increased lithium dosage. The tumor suppressor protein, Rb, was significantly elevated especially after treatment of HL-60 cells with 20 mM lithium. Expression levels of Rb were generally lower in the HL-60LiR cells, an observation that could possibly be linked to the lithium resistance phenomenon acquired by these sub-clones. The p53 protein, like Rb, is a nuclear phosphoprotein that is postulated to arrest growth in the G1 phase of the cell cycle in response to a death signal or aberrant DNA activity [24]. When HL-60 cells were treated with higher doses of lithium, its expression levels increased albeit barely detectable in these cell lines, and as a result, very little could be deduced from these observations.

Since the successful growth and survival of leukaemic cells is partly due to their ability to respond to local paracrine growth factors and inhibitors, the levels of several growth-regulating or inflammation-related cytokines were carefully investigated. Tumor necrosis factor-alpha (TNF-α), IL-6, IL-2 and IL-10 are some of the cytokines which production by HL-60 and HL-60LiR cells were determined using ELISA assays after 24 h of lithium treatment. Both IL-6 and TNF-α have been shown to mediate apoptotic as well as necrotic forms of cell death in leukaemic cell lines. Furthermore, it was previously reported that lithium can considerably potentiate the antitumor activity of TNF-α and IL-6 both in vivo and in vitro. Lithium chloride has also been demonstrated to considerably increase the amount of TNF-induced IL-6 in murine skin [25]. Treatment of HL-60 and HL-60 LiR cells with lithium resulted in a dose-dependent increase of TNF-α and IL-6 production. The increase in TNF-α and IL-6 levels in both cells correlated with increase in lithium-dependent growth inhibition and cell death induction. Production of these cytokines was comparatively higher when cells were treated with 20 mM lithium, suggesting that induction of cell death in HL-60 and HL-60LiR cells might be achieved via modulation of TNF-α and IL-6 production. This report supports findings from previous studies which proposed that lithium and TNF-α work in synergy when inhibiting growth of HL-60 cells. Hence, the lithium-induced cell death may be a direct consequence or indirect stimulation of TNF-α.

Production of IL-2 and IL-10 in HL-60 cells is generally decreased by lithium in a dose-dependent fashion. Both IL-2 and IL-10 are reported as potent growth-promoting cytokines in HL-60 cell lines. Thus, IL-2 and IL-10 represent potent positive progression signals for the growth and differentiation of their cellular targets, especially, leukaemic subtypes [26]. Survival of cancer cells depends on the balance between growth-promoting cytokines and growth inhibitors; hence, when growth factors, like IL-2 and IL-10, are low and growth inhibitors, such as TNF-α and IL-6, are high, cells are destined to die. Surviving HL-60LiR cells may, therefore, be evading the process of cell death by decreasing levels of TNF-α and IL-6 while promoting IL-2 and IL-10 production. Thus, the survival patterns seen in HL-60 LiR cells might be a result of increased IL-2 and IL-10 levels with concomitant reduction in the production of TNF-α and IL-6. Induction of cell death by lithium in HL-60 cells may involve stimulation of IL-6 and TNF-α secretion and concomitant inhibition of IL-2 and IL-10 production. Taken altogether, these results suggest that lithium may be inducing cell death in HL-60 and HL-60LiR cells by modulating cytokine production and expression of apoptosis-related genes. The use of lithium in cancer management would improve treatment accessibility and reach a wider patient community, even those in developing countries, due to its affordability, stability and extensive distribution.

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