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Original Article

Lithium Affects Histogenesis of Embryonic Chick Retina

H. Ramchandran, Medha S. Rajadhyaksha

Department of Life Sciences, Sophia College for Women, Mumbai, Maharashtra, India

ABSTRACT

Background: Lithium, a drug used extensively for treatment of bipolar disorders, has also been shown to be neuroprotective *in vivo* and *in vitro.* While gross teratogenic effects of lithium at higher doses have been reported, in view of its potential wider use, it is necessary to investigate its effects on tissue formation at relatively low doses of lithium where no apparent teratogenic effects on morphology are observed. **Materials and Methods:** We have used retina of chick embryo to investigate its effects during neural histogenesis. Three major cellular events involved in retinal histogenesis have been monitored: Proliferation as measured by expression of proliferating cell nuclear antigen (PCNA); initiation of differentiation as observed by expression of p27/Kip1 expression; apoptosis as monitored by TdT-mediated dUTPX-nick end labeling. **Result:** We demonstrate that lithium at a dose of 60 mM has no effect on gross eye morphology; it disrupts histogenesis of chick retina by blocking proliferation, inducing apoptosis, and generating post mitotic cells prematurely.

Key words: Apoptosis, chick embryo, lithium, p27, retinal cell proliferation, retinal histogenesis

INTRODUCTION

Lithium is one of the most effective drugs for the treatment of bipolar disorder for last several decades.[1] It has also been found to be neuroprotective and is likely to find wider application in treatment of damage of the nervous system due to hypoxia, ischemia, radiation damage, and degenerative disease.[2,3] Therapeutically, lithium is effective in a narrow dose range. It causes renal toxicity at higher doses and is ineffective at lower doses.[4] Studies have shown that it dorsalize the vertebrate embryos,[5] but its teratogenicity in humans is equivocal.[6] Effects of lithium in early embryogenesis are well documented.[6] However, its effects on histogenesis at therapeutic doses are not adequately investigated. Lithium acts differently on mature and immature cells.[7] The protective effects of lithium on neural tissue and its potential clinical use for the same is of relevance to investigate its effect on developing neural systems that could be susceptible to lithium during tissue formation. We have, therefore, investigated effect of lithium on developing retina using embryonic chick as a model system.

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The chick embryonic retina by 7th day has three major layers: The outer layer containing the photoreceptors, the inner nuclear layer formed by the ganglionic cells, and the layer between the two formed of intermediate cells. Cellular differentiation starts on day 8 and continues till day 18, with major layers well established by day 12.[8] We investigated retinal architecture before and after histogenesis (Day 7 and 12). We further investigated the three major cellular processes involved in tissue formation early during histogenesis (day 7) by monitoring extent of proliferation by observing expression of proliferating cell nuclear antigen (PCNA), initiation of differentiation as observed by expression of p27/Kip1 expression and apoptosis as monitored by TdT- mediated dUTP-X- nick end labeling or

Address for correspondence: Dr. Medha Rajyadaksha, Department of Life Sciences, Sophia College for Women, B. Desai Road, Mumbai - 400 026, Maharashtra, India. E-mail: rajadhyakshamedha@gmail.com

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TUNEL. We report that lithium induces apoptosis, inhibits proliferation, and alters differentiation of retinal cells.

MATERIALS AND METHODS

Ex-vivo treatment of chick embryos

Fertilized eggs of *Gallus domesticus* were procured from local source and incubated at 37°C for 24 h. At Hamilton–Hamburger stage 6–8[9] eggs (n=4–6 per group) were inoculated with 60 mM lithium or distilled water (placebo) using insulin syringe by piercing the shell on the blunt end of egg (above the air space) using sterile 26 gauge needle. The eggs were further incubated for 6 days or 11 days. The embryos were weighed to confirm that lithium treatment was effective as reported earlier by the authors.[10]

Histology

Eyes of the chick embryos were excised and retinal sections of 10 μm thickness were cut using cold microtome. The slides (sections) were treated for 3 min with 95% and 75% absolute alcohol, washed in running water and stained with Haematoxylin for 4 min and with Eosin for 3 min. The sections were passed through grades of alcohol, cleared in xylene, mounted in DPX, observed, and photographed.

Localization of proliferating cell nuclear antigen and p27/Kip1

The retinal sections were fixed in chilled acetone and treated with 30% hydrogen peroxide to block endogenous peroxidase. After washing, sections were treated with 3% fetal calf serum (FCS) to block nonspecific staining and incubated with anti-PCNA or anti-p27/Kip1 antibody (Immuno Cruz) overnight at 4°C. Sections were incubated with HRP labeled secondary antibody and color was developed using diaminobenzidine (DAB) as a substrate. Localization of PCNA or p27/Kip1 was observed and photographed.

Detection of apoptosis

Cells undergoing apoptosis were observed by detecting DNA fragmentation *in situ* by TdT- mediated dUTP-X- nick end labeling carried out using the DIG-Oligonucleotoide Tailing Kit (Roche Diagnostics, Manheim, Germany). The procedure included the following steps.

1. Labeling with DIG: Slides with sections were air dried and fixed in 3% paraformaldehyde for 10 mins. After two washes in PBS, the slides were incubated with labeling mixture containing buffer $CoCl_2$ solution, DIG-dUTP, dATP, Terminal end transferase in water for 2 h at 37°C. The slides were transferred to ice and 1 μL of glycogen and chilled EDTA was added. This was followed by treatment with 3 μL sodium acetate and 100 μL chilled ethanol. The slides were treated with Ab. Alcohol for 10 min followed by overnight incubation in 70% alcohol at 4°C.
2. Blocking: Slides were incubated in Genius 1 (2% normal sheep serum+3% triton X 100) for half hour.
3. Conjugation with primary antibody: Slides were incubated overnight at 4°C with anti-DIG antibody was dilute 1:500 in Genius and washed with buffer.
4. Color development: Slides were incubated in dark at room temperature for 5–10 mins.

(With 4.5 μL NBT, 3.5 μL BCIP, and 50 μL levamisole in buffer). The slides were mounted in Canada balsam after washing. The sections were microphotographed.

RESULTS AND DISCUSSION

The vertebrate retina develops from morphologically identical neuroepithelial cells by a multistep process involving cell-cycle exit, migration, and dramatic change in cell morphology.[11] Developing and developed vertebrate retina offer an excellent model to study effect of lithium on neuronal cells. In our earlier studies, we have demonstrated dose-dependent toxicity of lithium.[10] Exposure of 24-h old chick embryo (Hamilton – Hamburger stage 6–8) to 60 mM lithium causes a significant reduction in body weight and brain/body ratio by 7th day and has therefore been used as a time point to observe retina.[10] The retina of the chick embryo between day 3 and 4 is mitotically active with cells not committed to specific fates. Between day 4 and 7 of chick embryonic development, the retina comprises of a mixture of proliferating neuroepithelium, cells that are undergoing terminal mitosis, and postmitotic cells in various stages of migration to their specific positions. Neural retina under low power appears as a compact multilayered structure at this stage [Figure 1]. The retinal ganglionic cells start differentiating by this time and by day 8 the progressively differentiated cells form multilayered structure and the retinal cells can be phenotypically identified.[11]

The chick embryos, in the present set of experiments, have received a treatment with lithium throughout the period its retina differentiated and the histology observed on the seventh day is a composite effect of lithium action on various processes of histogenesis. A loose arrangement of cells suggesting disrupted histogenesis is seen as a result of lithium treatment in developing retina. The pigmented retina is disorganized as clearly seen by 12th Day [Figure 1]. During eye development retinal pigmented epithelium (RPE) WNT/beta–catenin signaling has been demonstrated to be highly active and its spatio-temporal regulation is obligatory of normal RPE formation.[12] Lithium mimics WNT/beta catenin signals.[4] Lithium effectively results in ectopic regulation of WNT signaling and disruption in RPE as reported in mice.[13]

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One of the markers widely used to detect proliferating cells is proliferating cell nuclear antigen (PCNA/cyclin), 2800 dalton protein present in small amounts in normal non dividing cells.[14] It is an obligatory co-factor for DNA polymerase delta and is synthesized in a substantial amount in normal and transformed proliferating cells. It is synthesized in the S phase of the cell cycle, with a time course parallel to the DNA synthesis but slightly ahead of it in time. However, PCNA remains in the nuclei long after the S phase. Early histogenesis of the teleost fish retina has been reported using PCNA as an immunological marker and PCNA protein has been reported to be a conserved protein.[15] PCNA was, therefore, used as an immunological marker to investigate proliferating cells of retina of the chick embryo. The PCNA activity was detected in normal 7-day-old chick embryo neural retina suggesting ongoing or recent proliferation of cells in various layers of cells [Figure 2]. However, in lithium-treated embryonic retina proliferation appears to be restricted to innermost and outermost layers as indicated by the intensity of PCNA expression. This suggested reduction in proliferating cells could be responsible for distorted histology. It has been reported that in early phase of retinal development WNT signaling regulates cell-cycle progression.[16] Over expression of WNT signaling has been reported to cause accumulation of cells in S and G2/M phase of the cell cycle in transgenic fish, *Medaka*.[16] On the other hand, WNT was shown to attenuate proliferation of neural progenitors in *Xenopus*[17] suggesting contradictory effects of WNT on developmental processes. However, our earlier studies had demonstrated that lithium induced a block in G2/M phase of the cell cycle *in vitro* in mouse neuronal cells.[18] In the present study, a decrease in PCNA expression could be because of a modulation of cell-cycle progression by ectopic stimulation of WNT signal by lithium.

In retina of chick embryo p27/Kip1, a cyclin/CDK inhibitor, is expressed in cells that exit cell cycle, are postmitotic and initiate differentiation into various cell types.[19] In embryonic chick, retina expression of p27/Kip1 is seen on day 5 and increases as development proceeds.[19] In the present study, the p27/Kip1 expression is seen in neural retina. It is known that during embryonic day 7 and 8 more than 75% of ganglionic cells, photoreceptor, and amacrine cell precursors are postmitotic.[8] In retina of control embryos expression of p27/kip1 was relatively more clearly seen in innermost retinal layer suggesting cells exiting mitosis in these layers. The expression of p 27/Kip1 in retina of lithium-treated embryos is present throughout the retinal layers and is heightened as seen by visual quantitation [Figure 2] suggesting increased number of postmitotic cells that are possibly precursors initiated to differentiate into the various cell types. Lithium, thus appears to block cells in proliferation (as seen by PCNA expression), and possibly initiates increased differentiation prematurely as indicated by increase in p 27/Kip1 positive postmitotic cells. A similar cell-cycle block by lithium and enhanced differentiation has been indicated in our earlier study using Neuro 2a cells.[18]

Interestingly, in retina of chick embryo lithium appears to increase the number of cells positive for TUNEL labeling suggesting heightened apoptosis [Figure 2]

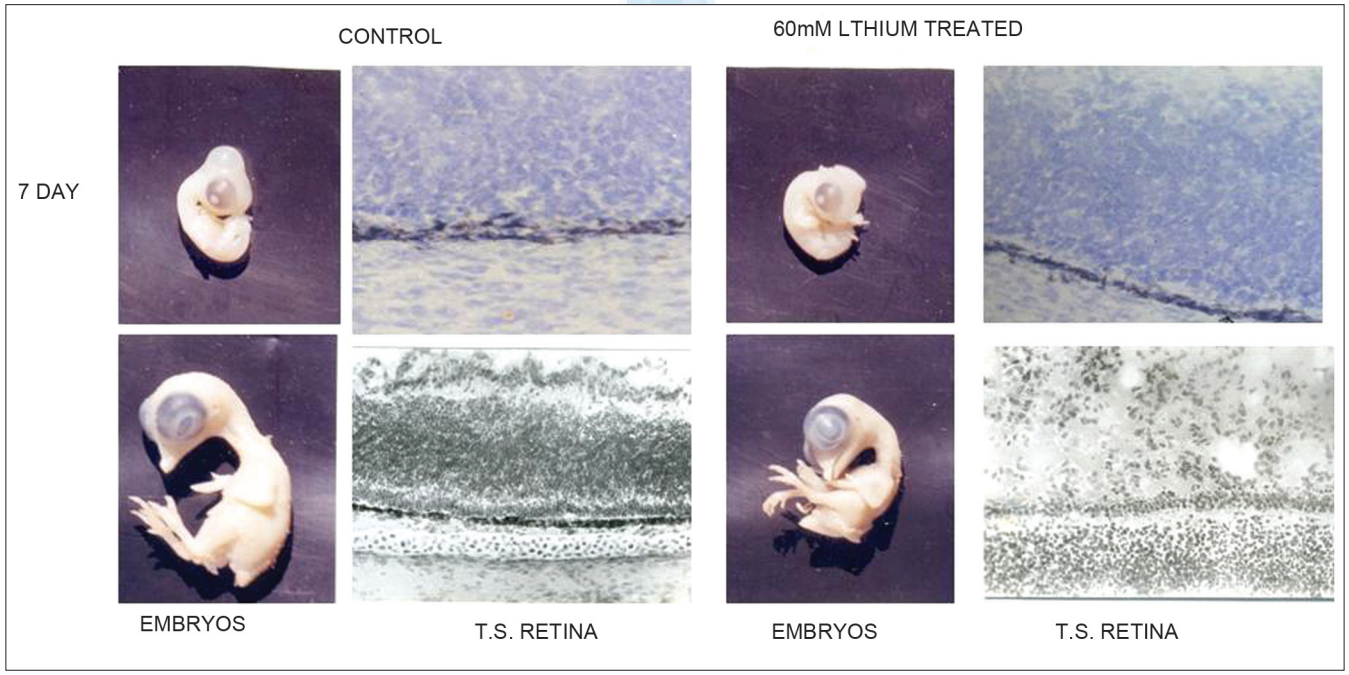


Figure 1: Morphology of the embryo and histology of embryonic retina with and without 60 mM lithium treatment on 7th day and 12th day of development. The embryos were apparently normal but significantly reduced in size and weight. Retinal histology on 7th day appears to be normal but completely disrupted by 12th day of embryonic development

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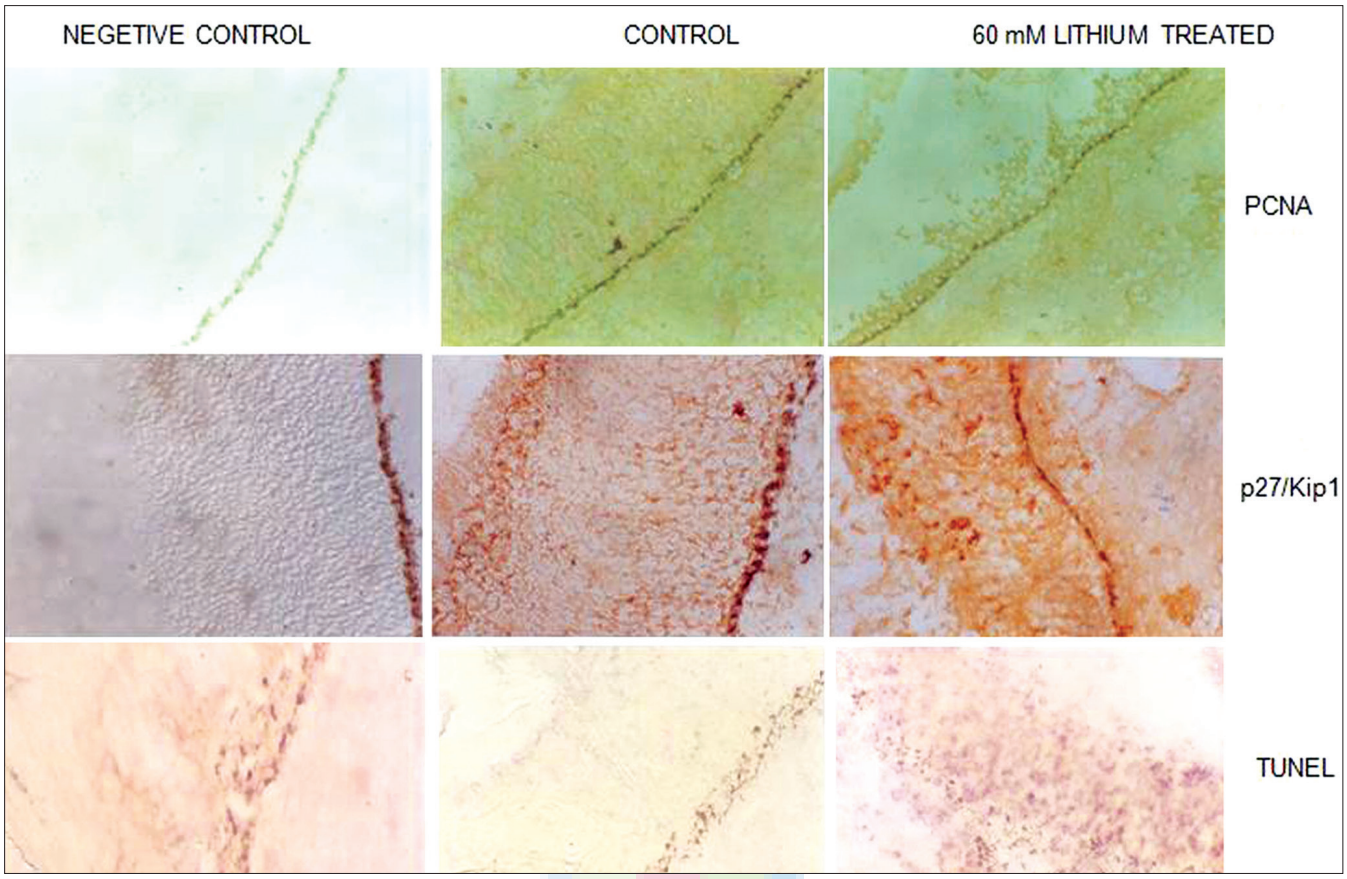


Figure 2: Histochemical expression of proliferating cell nuclear antigen (PCNA), p27/Kip1 and cells undergoing apoptosis (as monitored by TUNEL) in 7-day-old embryonic chick retina with or without lithium treatment. Negative controls expressed no or minimal activity. PCNA expression was reduced in most of the layers indicating decrease in proliferation. p27/Kip 1 expression was relatively intense in inner layer of retina in control but uniformly present in lithium treated cells. TUNEL positive cells are increased in lithium treated embryonic retinal cells as compared to controls

as seen visually. Caspase-dependent apoptosis has been demonstrated in normal developing chick retina, inhibition of caspase has been shown to result in enlargement of the ganglionoic cell layer.[20] Apoptosis normally occurring in retina at this stage appears to be enhanced due to lithium treatment. In adult mouse retina, however, lithium has been demonstrated to support retinal ganglionic cell survival by upregulating the antiapoptotic protein bcl2.[21] It is possible that lithium has differential action in mature and immature neuronal cells. Such differential action of lithium has been demonstrated in cerebellar granule neurons where lithium is protective to mature cells and causes apoptosis of immature cells.[7] Our studies demonstrated that in 7-day-old chick embryo lithium appears to enhance apoptosis in retina.

In conclusion, our studies demonstrate, for the first time, effect of lithium on three major processes involved in retinal histogenesis in 7-day-old chick embryo. Lithium, at a dose of 60 mM, though apparently has no effect on gross morphology of the eye, disrupts histogenesis by blocking proliferation and causing apoptosis. Further cells are pushed into post mitotic state prematurely. All these effects possibly lead to disruption of functional adult retina.

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