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Lithium carbonate teratogenic effects in chick cardiomyocyte micromass system and mouse embryonic stem cell derived cardiomyocyte – Possible protective role of myo-inositol

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ABSTRACT

The drug lithium carbonate (Li_2CO_3) use during pregnancy increases the possibility of cardiovascular anomalies. The earlier studies confirm its phosphatidylinositol cycle (PI) inhibition and Wnt pathways mimicking properties, which might contribute to its teratogenic effects. In this study the toxic effects of Li_2CO_3 in chick embryonic cardiomyocyte micromass system (MM) and embryonic stem cell derived cardiomyocyte (ESDC) were evaluated, with possible protective role of myo-inositol. In MM system the Li_2CO_3 did not alter the toxicity estimation endpoints, whereas in ESDC system the cardiomyocytes contractile activity stopped at 1500 μM and above with significant increase in total cellular protein contents. In ESDC system when myo-inositol was added along with Li_2CO_3 to continue PI cycle, the contractile activity was recovered with decreased protein content. The lithium toxic effects depend on the role of PI cycle at particular stage of cardiogenesis, while relation between myo-inositol and reduced cellular protein contents remains unknown.

1. Introduction

Lithium salts therapeutic profile ranges from treatment of acute manic depression [1,2] to management of alcoholic patients [3]. The serum therapeutic concentration of lithium in humans ranges from 0.6 to 1.2 mM [4], with a small therapeutic to toxic ratio (0.7), which on chronic exposure, even at therapeutic doses, produces malformations [5]. Since the discovery that lithium salts produce embryos with profound exogastrulation in sea urchins, it became evident that lithium may induce morphological deviations in primitive development [1]. In humans lithium carbonate (Li_2CO_3) crosses the placenta and perturbs normal development [6,7]. At serum therapeutic levels lithium chloride is shown to induce cardiovascular malformations specially the Ebstein's anomaly, disturbance in embryonic vascular development, yolk sac vessels vascular stasis and vascular dilation in the cranial region of the embryo [8]. In *Xenopus* embryos, lithium chloride treatment induces the formation of a second dorsal axis, but these teratogenic effects were reported to be preventable by a co-injection of myo-inositol [9].

Therapeutic and teratogenic mechanisms of lithium remain to be fully elucidated. However, its interference with the phosphatidylinositol (PI) cycle is thought to be the major factor in mood stabilising and in teratogenic properties [8,10,11]. More recently the toxic mechanism for lithium has been associated with inactivation of glycogen synthase kinase-3β (GSK3-β), which mimics the canonical Wnt signalling pathway [4,12,13].

In the PI cycle, phospholipase C (activated by G protein receptors) metabolises the plasma membrane PI into two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 regulates the release of Ca^{2+} from the non-mitochondrial pool (mostly ER). The Ca^{2+} activates the calcium and calmodulin dependent kinases and promotes extracellular Ca^{2+} influx, while DAG activates PKC [11,14,15]. The IP_3 is then dephosphorylated by a number of steps; finally inositol monophosphate is hydrolysed into free inositol by the enzyme inositol monophosphatase (IMPase). The free inositol is recycled back for the regeneration of phosphatidylinositol [10]. Lithium targets the enzymes IMPase in the PI cycle [11,16], thereby inhibiting the formation of free inositol. Inositol depletion consequently results in failure of PI to reconstitute at the cell membrane [8]. When exogenous inositol is not readily available, the cells do not respond to PI dependent

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extracellular signal transduction. IP_3 production is reduced and the regulation of Ca^{2+} is affected [8,16]. In the cardiovascular system IP_3 plays a key role in vascular smooth muscle mechanical coupling, thereby regulating peripheral resistance and blood pressure. In the developing heart IP_3 initiates the pacemaker activity, promotes cardiogenesis and regulates cardiomyocyte contraction by promoting calcium release and influx [17]. Inhibition of the PI cycle affects early developmental events, especially the development of the heart and vessel formation [8,17,18].

In another mechanism of teratogenicity, lithium mimics Wnt signalling and produces a hyperdorsalized embryo [7]. Wnt signalling has an important role in development, axis formation, cancer, stem cell biology and neural function. Wnt inactivates GSK3-β, which results in β-catenin cellular accumulation. In turn, β-catenin forms a transcriptional enhancer complex and up regulates expression of selected target genes [12,19]. In the absence of Wnt signals, GSK3-β phosphorylate β-catenin leading to its ubiquitination and proteosomal degradation [19]. Lithium mimics the Wnt pathway by inhibiting phosphorylation of GSK-3β resulting in β-catenin accumulation in the cell [13]. Wnt signalling inhibits early cardiac specification and the proper orchestration of cardiac development by acting via its major transducer β-catenin [13,20].

This study was designed to study the effects of Li_2CO_3 on primary culture of chick cardiomyocytes (micromass) and embryonic stem cells derived cardiomyocytes with possible protective role of myo-inositol. The micromass system (MM) involves the primary cell culture of different organs [21]. The basis of this test is the chemical's disruption of embryonic cell differentiation and re-establishment of the system [22]. The micromass culture system detects chemical interference in the normal process of cell differentiation, development, cell proliferation, cell–cell communication and cell matrix interactions [23]. The blastocyst derived pluripotent mouse embryonic stem cells (ES cells) [24] have the ability to differentiate into all three germ layers, including spontaneous differentiation into contracting cardiomyocytes. These cardiomyocytes exhibit the appropriate proteins, receptors and ion channels that closely resemble *in vivo* cardiogenesis [25]. Therefore ES cells can be used as a tool to predict tissue specific mutagenic, cytotoxic and embryotoxic effects of chemicals using *in vitro* systems [24,26]. The classical embryonic stem cell test (EST) for cytotoxicity testing by Spielmann et al. [27] became the basis of this test, with slight modification as described by Ahir et al. [28]. It is hoped that the results will clearly indicate the Li_2CO_3 toxic effects on cardiomyocytes, which might be counteracted with myo-inositol supplements.

2. Materials and methods

2.1. Chemicals and solution

All the chemicals were purchased from Sigma–Aldrich, UK unless otherwise stated. The lithium carbonate (Fischer Scientific, UK; Reference no. L/2100; CAS Number: 554-13-2) and myo-inositol (Sigma–Aldrich, UK; catalogue number I7508) stock solutions were prepared in culture medium [28]. The blank and control wells received culture medium only.

2.2. Micromass system

In chick cardiomyocyte micromass (MM) system the 5 day's old chick heart cells were cultured and treated with chemicals as described earlier by Memon et al. [29]. In brief, the chick hearts were trypsinized and 20 μl of single cell suspension (3 million cells ml^{-1}) in a defined culture medium (DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 50 U ml^{-1} penicillin/50 μg ml^{-1} streptomycin) was aliquoted at the centre of 24 well plates. The cells were allowed to attach for 2 h and flooded with more culture medium. The cardiomyocyte proliferated and established contractile foci within 18–24 h. The drug was added after 24 h of cell seeding and examined under microscope for contractile activity at 24 h (pre-exposure), 48 h and 144 h (post-exposure) time intervals [28].

Table 1
Morphological scoring system for cardiomyocytes contractile activity.

Morphological score	Contractile activity of cardiomyocytes
3	Entire well contracting
2	Numerous foci contracting
1	Few foci contracting
0	No contractile activity

2.3. Embryonic stem cell derived cardiomyocyte system

The embryonic stem cell derived cardiomyocyte (ESDC) system for toxicity testing was adopted from Ahir et al. [28]. In brief, the ES cells were cultured in undifferentiated state in a defined media (DMEM with 20% heat inactivated FBS, 2 mM L-glutamine, 50 U ml^{-1} penicillin/50 μg ml^{-1} streptomycin, 0.1 mM β-mercaptoethanol and 1% X100 non-essential amino acids) in the presence of leukemia inhibitory factor (LIF). For ES cells differentiation into cardiomyocytes the hanging drop method was utilised (in the absence of LIF), which results in the formation of embryoid bodies (EBs). When EB was allowed to attach it differentiated into contracting cardiomyocytes. The contractile activity was recorded on day 10th, 11th and 12th of experiment. For toxicity testing the process of differentiation was performed in the presence of drug.

2.4. End points

2.4.1. Cardiomyocytes contractile activity measurement

The cultured cardiomyocyte contractile activity was inspected morphologically using light microscope at given time intervals and recorded. A numerical value was assigned to the cardiomyocytes contractile activity scoring as described earlier by Ahir et al. [28] and Memon et al. [29]. The MM and ESDC cardiomyocytes forms contractile foci's. The scoring system depends on the number of foci's contracting in a well. If the entire well's foci are contracting a score 3 is given, if numerous foci's (not all) are contracting in a well it is given a score 2, which decreases to score 1 if few foci's (one or two) are contracting and became zero for no contraction (Table 1).

2.4.2. Resazurin assay

The resazurin cell viability assay was used as an indicator of the cellular toxicity of test chemicals compared to control. Resazurin is a non-toxic redox dye [30]. The assay involves the reduction of non-fluorescent resazurin (blue colour) to fluorescent resorufin (pink colour) by cellular oxidoreductases, which is commonly used as a tool to identify cell proliferation and cytotoxic potentials of the chemicals [31]. In brief, on the 6th day of MM and 12th day of ESDC systems, the medium was replaced with a 500 μl of pre warmed solution of 10 μg ml^{-1} resazurin in HBSS and incubated for 1 h at 37 °C, 5% CO_2. The living cells converted the blue non-fluorescent resazurin to pink fluorescent resorufin. Fluorescence was determined using a spectrofluorimeter (FLUORstar Galaxy BMG Lab Technologies, Buckinghamshire, UK) with an excitation filter of 530 ± 10 nm and emission filter of 590 ± 12.5 nm [29].

2.4.3. Kenacid blue assay

Measurement of cellular protein content may determine the extent of the process of cell proliferation [29]. The kenacid blue dye