Inhibition of neurite outgrowth and promotion of neuronal degeneration by the atropine in Neuro2a cells

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Abstract:
Myopia, a very common human eye disorder, affects up to 80% of young adults in some East Asian countries, including in Taiwan. Higher degree of myopia is associated with a number of visual terrorized complications, including retinal detachment, macular degeneration, cataract and glaucoma. The risk of these complications rises with increasing severity of myopia. Contrasting other blinding disorders such as aged-related macular degeneration, all these myopia-related complications tend to arise mainly in young adult. Atropine, a pan muscarinic cholinergic receptor antagonist, has been used for treated myopia since long time ago. However, the influence of innervation in the eyes was still not known. To address this question, the effect of atropine on nerve innervation and cell character, the pan muscarinic cholinergic receptor antagonist treated cultured cell were examined. The neuronal neurite lengths were assay with microscopy. To study the relationship between neuronal death and atropine-treated, statistical analysis was examined. Blocked the neuronal transmission caused the cells neurite lengths decreased compared with control. It also detected that the increase in the number of nuclear condensation cells after treated atropine with microscopy. In other words, neuroblastoma cells after atropine-treated, the cultured cell death ratio was increased compared with control. These results indicated that neuronal cells treated with atropine, a muscarinic receptor antagonist, reduced the neurite outgrowth and promoted neuronal degeneration.

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1. Introduction

Myopia, the most common ocular disorder in humans, affects up to 82% of young adults in Taiwan. Additionally, the decreased visual function from out of optical focus, higher degrees of myopia is associated with the risk of permanent blinding complications. These myopia-related complications, including retinal detachment, macular degeneration, glaucoma and cataract, tend to occur in young adults. Axial length of eyeball is the major determinant of refractive error [1] and has a well-known negative correlation with myopia. Restated, the longer the axial length, the more severe the myopia [2-3].

Atropine, a nonselective muscarinic cholinergic receptor antagonist, has a long history of use for treating myopia or for inhibiting axial growth of eyeball in human and animal models [4-8]. Atropine is a competitive inhibitor of the muscarinic acetylcholine receptor. It blocks the effect of acetylcholine and protects receptors from further stimulation. It has a minimal effect at nicotinic receptor sites [9-10]. The central nervous system effects observed in atropine that it is capable of crossing the blood-brain barrier.

However, the underlying mechanisms underlying the action of atropine in myopia treatment are ambiguous. At the outset, atropine was consideration to retard myopia by obstruction with accommodation through its cycloplegic effect. Another possible mechanism atropine regulates myopia is its interaction with the long-lasting increase in retinal dopamine release [11]. Otherwise, some studies also suggest that atropine-mediated growth signal may involve the retinal neural cells,

retinal pigmented epithelium, the choroids or the sclera, and effect on eyeball growth [6, 12-13].

Local reaction to atropine may derive from both toxic and allergic origins, which are not simply distinguishable by the clinical presentation alone [14]. Local toxic reaction to atropine includes conjunctival infection and periorbital dry, red skin. It evaluated the adverse reactions to atropine eye-drops among children, noted some reports of toxic reactions that resulted in eminent body temperature and dry, warm skin [15]. In some cases with red eyes and periorbital dermatitis, allergic reactions were suspected but skin allergy tests were negative. Other studies also demonstrated that atropine eye-drops toxic effects included local ocular reactions, mild systemic reactions, and severe reactions of asthmatic attack, convulsions and one of tachycardia [16].

After these years with use atropine for treating myopia or for inhibiting axial growth, studies indicated that incidence rate of myopia in Taiwan and some parts of world are not falling. Conversely, a high prevalence of myopia in young adults has been reported in Taiwan [17]. However, the influence of nerve innervation in eye balls was still not known. To address this question, the effect of atropine on nerve innervation and cell death, the pan muscarinic cholinergic receptor antagonist treated cultured cell were examined. The cell death ratio and neurite lengths were assay with microscopy. To study the relationship between neuronal death and atropine-treated, the cell morphology evaluation was examined.

2. Material and Methods

Retinoids

All-trans retinoic acid (atRA) was obtained from Spectrum Chemical Co. (New Brunswick, NJ, USA) and was deemed greater than 99% pure by reverse-phase HPLC.

Cell Culture

The murine neuroblastoma cell line (Neuro2a, American Cell Type Culture Collection), was used. Cells were cultured in Falcon dishes in DMEM supplemented with 10% fetal calf serum, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml potassium penicillin G, and 100 mg/ml streptomycin sulfate in 5% CO2, 95% air humidified incubator.

Neurite outgrowth

To induce neurite outgrowth, cells were plated at a 1.8 x 10^4/cm^2 cell density, and 48 h after plating the medium was replaced with 2% FCS-DMEM containing 20 mM retinoic acid, the incubation being continued for different times up to 48 h. Control experiment showed that incubation up to 48 h with 2% FCS-DMEM, in the absence of retinoic acid, caused only a very modest outgrowth of processes. The neurite outgrowth was used to measure average neurite length (μm per cell) as well as the number of neurites per cell. For each group, cells were examined in a minimum of 5 random non-overlapping images per replicate of each treatment condition.

Atropine treatment

All drug treatments were additions to DMEM (vehicle control). Cell viability of each treatment was determined morphologically after 24, 48, and 72 h.

Cell morphology evaluation

The cells were fixed in 4% paraformaldehyde for 15 minutes, washed 3 times in PBS, and covered with cold 100% methanol for 10 minutes. The cell nucleus was stained with hematoxlin (Sigma-Aldrich). After 5 more rinses in PBS, the cells were mounted and viewed on a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

The results are expressed as the mean ± SEM and were evaluated for significance. Statistical analysis was using a student's t-test. A *p* value of less than 0.05 was considered significant.

3. Results

3.1 Induction of neutrite outgrowth in neuroblastoma neuro2a cells treated with atRA

The time course of neurite outgrowth induction by atRA and changes in morphology produced by atRA were studied in neuroblastoma cell lines. An increase in the overall length of neurites was observed in Neuro2a cells exposed to atRA at both 24 and 48 h, with the effect more pronounced at the latter time point (data not shown). Thus, neuroblastoma cell lines showed the expected increase in neurite outgrowth after treatment with atRA.

3.2 Inhibition of neurite outgrowth after blocked muscarinic receptors

To assess the effects of muscarinic receptors expressed in neuronal cells, Neuro2a cultures were treated with muscarinic anagonists atropine. Neurite lengths were slightly decreased in the different atropine treatment groups (Fig. 1B-1D) compared with the control (Fig. 1A) after 1 day. After 2 days of atropine treatment, blocked muscarinic receptors substantially reduced the neurite outgrowth of atRA

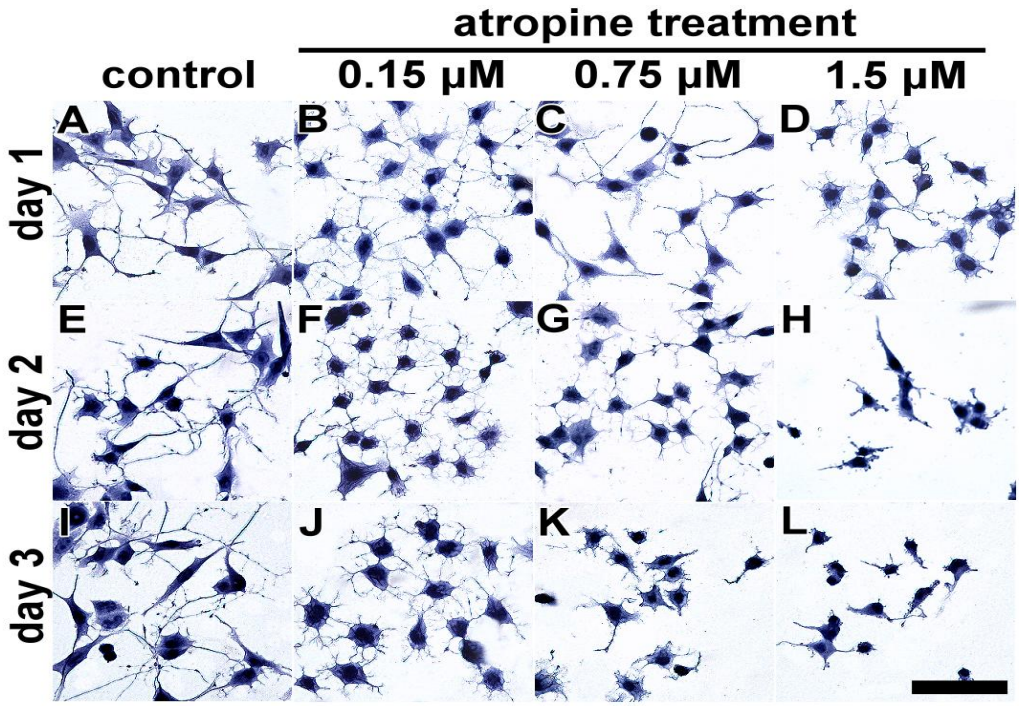

Fig. 1: Effects of atropine on the process length of Neuro2a cells
To enhance neurite outgrowth, Neuro2a cells were pretreated with 10 µM retinoic acid for 2 days. The cells were then either treated with atropine (0.15, 0.75 and 1.5 µM) or were not treated, and the neurite lengths of cells were assessed after 1-3 days. The neurite lengths were slightly decreased in the different atropine treatment groups (B, C, and D) compared with the control (A) after 1 day. However, after the cells were treated with atropine for 2 days, the neurite lengths of the cells were shorter (E-H). Moreover, the neurite lengths more significantly decreased after 3 days with atropine treatment compared with control cells (I-L). Scale bar = 50 µm.

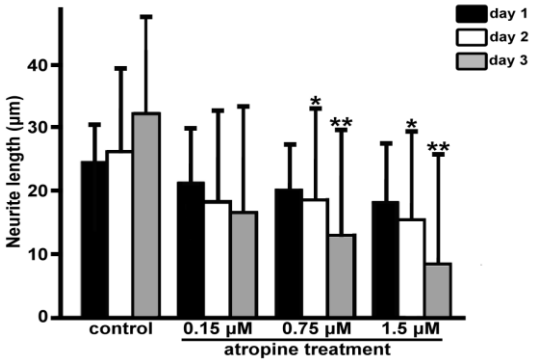

Table 1: Quantization of neurite length of Neuro2a cells after atropine treatment
The neurite length was not significantly different between any groups at 1 day after atropine treatment. Conversely, the neurite length of cells was significantly shorter on days 2 and 3 for both 0.75 and 1.5 µM atropine treatments than in the control. Quantification of neurits length showed a higher concentration of significantly shorter neurite after 2 days. ** indicates $p < 0.01$ and * indicates $p < 0.05$.

exposed Neuro2a , as indicated by cell morphology (Figs. 1E-1H). After 3 days, neurite lengths more significantly decreased in atropine treated cells (Figs. 1I-1L). It demonstrated that muscarinic receptors were involved in the regulation of axonal outgrowth as shown in the significant decrease in the axon length.

3.4 Notable differences between atropine treated and untreated cultured neuronal cells

Neuro2a cells were pretreated with 10 µM retinoic acid o enhance neurite outgrowth. The length of neurite from all the cells within an image was used to calculate the average neurite length. The neurite length did not significantly differ between any of the groups at 1 day after atropine treatment. The length decreased slightly after treatment with 0.0001% atropine for 2 and 3 days but did not significantly differ between the treatment groups. Conversely, the neurite length of cells was significantly shorter on days 2 and 3 for both 0.0005% and 0.001% atropine treatments than in the control. Quantification of neurite length showed a higher concentration of significantly shorter neurite after 2 days.

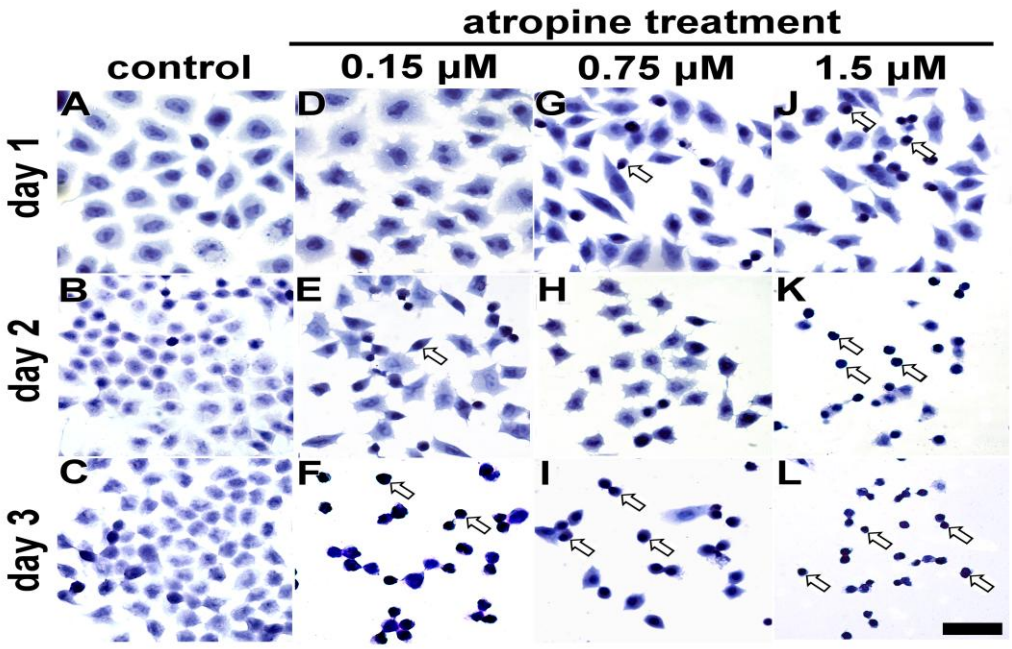

Fig. 2: Effects of atropine on the morphology of Neuro2a cells
Cellular morphology of untreated cells (A-C) and cells treated with 0.15 μM (D-F), 0.75 μM (G-I), or 1.5 μM atropine (J-L) for 1, 2, and 3 days. In the control cells (A-C), nuclei with normal chromatin structure were stained by hematoxylin. Atropine treatment of 1 day duration did not lead to significant differences in morphology among the different treatment groups (D, G, and J) compared with the control (A). However, after the cells were treated with 0.75 μM (H) or 1.5 μM atropine (K) for 2 days, cells showed nuclear chromatin condensation (arrows). At 3 days after treating the cells with different concentrations of atropine (F, I, and L), nuclear condensation (arrows) was evident in the cells. Moreover, the size of the cells decreased over time with atropine treatment. Scale bar = 50 μm.

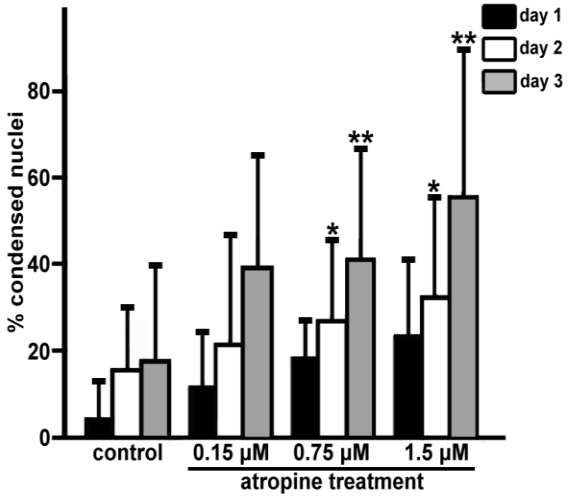

Table 2: Quantization of the percentage of nuclear condensation after atropine treatment
The percentage of nuclear condensation on days 2 and 3 of treatment in cells treated with 0.75 μM or 1.5 μM atropine was significantly higher than that in the control. ** indicates $p < 0.01$ and * indicates $p < 0.05$.

3.4 Promotion of nuclear condensation in Neuro2a cells

Cultured Neuro2a cells were either treated with atropine (0.15、0.75 and 1.5 μM) or were not subjected to treatment (control), and nuclear condensation was assessed after 1-3 days. The percentage of treated or untreated Neuro2a cells with condensed nuclei was assessed with microscopy. At 1 day after atropine treatment, the percentage of nuclear condensation was not significant different between all the groups. After the cells were treated with 0.15 μM atropine for 2 or 3 days, the percentage ratio of nuclear condensation slightly increased, but no statistically significant difference was observed between the untreated groups. Conversely, percentage rations of nuclear condensation after day 2 and 3 of Neuro2a cells treated with 0.75 μM or 1.5 μM atropine, were significantly higher than that of the control.

4. Discussion

The neural trophic factors have been shown to mediate various functions in the nervous system and in cell lines, including neuronal proliferation and

differentiation [18-19]. In vitro model systems, cells differentiate and acquire a sympathetic neuron-like phenotype in response to nerve growth factor (NGF) [20-21]. Our experiment data also indicated that blocked muscarinic receptors with the atropine, a nonselective muscarinic cholinergic receptor antagonist, substantially reduced the neurite outgrowth of atRA exposed Neuro2a , as indicated by cell morphology and statistical analysis(Figs. 1 and Table 1). The neurite length showed significantly shorter neurite after 2 days treated with 0.75 μM and 1.5μM atropine.

Cells death is the dependence of on trophic factors for cellular survival. The need for trophic support is not restricted to the stage of neuronal growth but has also been demonstrated in the adult [22]. The availability of trophic factors determines which neurons die and which survive. Neurons that fail to acquire sufficient trophic factor die [23-24]. Many reports have indicated neurotransmitters, which mediate synaptic communication, can also act as trophic factors in the nervous system [25-28]. The neurotransmitter acetylcholine is abundant in the nervous system. acetylcholine effect innervated tissues via two different receptor families, the nicotinic and the muscarinic receptors [29]. In our current study, this degeneration pattern after treatment with was demonstrated with cell morphology using microscopy. Rations of nuclear condensation after day 2 and day 3 in Neuro2a cells treated with relatively higher concentration of atropine, were significantly higher than that of the control.

Acetylcholine mediated neural functions are essential in central nervous systems and peripheral nervous systems. In particular, muscarinic type acetylcholine receptors are widely distributed in the full bodies, and play key roles in various aspects by normalizing the activity of many essential functions of the nervous system [30]. Many neurodegenerative conditions are associated with loss of acetylcholine function and increased cellular oxidative stress, for example Alzheimer's disease [31]. Stimulation of muscarinic receptors provides substantial protection from DNA damage, oxidative stress, and mitochondrial impairment. Action of muscarinic receptors prevents mitochondrial cytochrome c release, bcl-2 depletion, and bax accumulation [32]. Studies may be encountered by neurons of central nervous systems and peripheral nervous systems in growth, aging, or neurodegenerative diseases.

Atropine, a nonselective muscarinic cholinergic receptor antagonist, has a long history of use for treating myopia or for inhibiting axial growth of eyeball in human and animal models. After these years with use atropine for treating myopia or for inhibiting axial growth, studies indicated that incidence rate of myopia in Taiwan and some parts of world are not falling. However, influences of atropine on the nerve outgrowth and neuronal cell death were demonstrated in the study. These results suggest that the treated with nonselective muscarinic cholinergic receptor antagonist, atropine, may cause the degeneration of neurons with acetylcholine receptors.

5. Conclusion

The results indicated continued neuronal survival is dependent upon the environment of the cell, signals received from neighboring cells may provide the necessary drive to encourage defenses against death programs. Blocked muscarinic receptors with the atropine, a nonselective muscarinic cholinergic receptor antagonist, substantially reduced the neurite outgrowth and promoted neuronal degeneration. It is impossible to consider these degenerative changes are related unavoidably part of their mechanism of action or an avoidable toxic effect of use for treating myopia or for inhibiting axial growth of eyeball in human.

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