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PREVENTIVE EFFECT OF FOLIC ACID AND INOSITOL ON NEURAL TUBE DEFECTS INDUCED BY COTININE IN 2 DAY CHICK EMBRYO MODEL

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ABSTRACT

The present study was aimed to screen the preventive effect of folic acid and inositol on cotinine induced neural tube defects using a 2 day chick embryo model. The drugs (i.e., cotinine, folic acid and inositol) were injected into white leghorn, fertile, specific pathogen free eggs between 26-29 hours (8^{th} stage) of embryo development. After reaching the 12^{th} stage, the embryos were processed for morphological and histopathological studies. Cotinine treated groups were observed with abnormal development at thoracic region of neural tube and transverse sections of cotinine treated neural tube shown the degenerative cells (94±2.4; mean± S.E.M. (standard error of the mean). Folic acid (56±2.4; mean± S.E.M.) and inositol (66±2.4; mean± S.E.M.) were proved to be effective in decreasing the degeneration of cells. Combined treatment of folic acid and inositol has shown better efficacy (7±1.2; mean± S.E.M.) when compared with their individual treatments. Folic acid in combination with the inositol can be used to prevent the neural tube defects caused by cotinine.

KEYWORDS: Nicotine, Cotinine, Folic acid, Inositol, Neural tube defects

1. INTRODUCTION

Neural tube defects (NTDs) are the congenital deformities occurred by failure to closing of the neural tube. The occurrence of NTDs is highly differing from location to location and among the people.[1] The occurrence of NTDs greatly noticed worldwide mainly in Ireland and Wales *i.e.,* 6.38-10.92/1000 birth defects compared to 0.1-0.6/1000 in other European countries. High incidence of NTDs reported in Northern states of India like Punjab, Haryana, Delhi, Rajasthan, Uttar Pradesh and Bihar is about 3.9-9.01/1000 birth compared to 0.5-2.641/1000 in eastern, western and southern parts in India.[2]

A teratogen causes the toxic effects in pregnant women. Teratogen may present in the form of physical agents (cigarette smoking etc), drugs and its metabolites. [3]As well as genetic factors play a vital role in causing the neural tube defects.[4]

Now a day's many women are addicted to cigarette smoking results into 55% of cardiovascular disorders and 27% of cancer. Apart from that, secondhand smoke also plays a role in causing these disorders. Tobacco smoke contains 4 thousand chemicals from these 40 are vulnerable to cancer.[5] Nicotine is a non-ionized form; it penetrates into placenta and settled in fetal blood and amniotic fluid of maternal smokers. Nicotine metabolizes into cotinine, it has long half life. The urinary cotinine levels identified in breast-fed infants during smoking. It increases the risk of NTDs.[6] It also decreases the blood glucose levels, alters the glucose levels in brain, decreases the cholesterol levels and increases the acid phosphatase, alkaline phosphatase, aspartate transaminase levels in tissues of nicotine treated chick embryos. [7] Maternal smoking causes the impaired fetal growth during early pregnancy and it also shows raise in complications in neonates. [8] Nicotine is a neuroteratogen as it leads to neural tube deformities.[9] The low levels of folic acid were observed in smokers. [10] The low levels of folic acid also reduce the remethylation process of homocysteine to methionine consequently increases the homocysteine levels. Increased level of homocysteine leads to delay in the closure of neural tube due to the increased levels of S-adenosyl homocysteine by inhibition of transmethylation in chick embryos.[11]

In 1996, U.S preventive services task force (USPSTF) suggested that the supplementation of folic acid to a pregnant women or who are planning for conception, prevents the neural tube defects. [1] The effect of folic acid probably acts by donating a methyl group to metabolic and nervous system and it is essential for synthesis of DNA.[12]

Supplementation of inositol also reduces NTDs. Inositol showed a preventive effect on folic acid resistant neural tube defects.[13] Inositol is considered as a member of B-complex vitamin. It prevents the neural tube defects.[14] It provokes nuclear diacylglycerol (DAG) cycle results into activation of specific protein kinase C (PKC) isoenzymes. These play a role in closing the neural tube.[15]

Table 1: Drugs and their doses injected into eggs

Groups	Treatment	Dose
1	Control (Ethyl alcohol+ Physiological saline solution)	4 mg/dL [10]
2	Cotinine	100 pg [15]
3	Cotinine + folic acid (low dose)	100 pg + 100 pg [15]
4	Cotinine + folic acid (high dose)	100 pg + 200 pg
5	Cotinine + inositol (low dose)	100 pg + 100 pg
6	Cotinine + inositol (high dose)	100 pg + 200 pg
7	Cotinine + folic acid + inositol (low dose)	100 pg + 100 pg+ 100 pg
8	Cotinine + folic acid + inositol (high dose)	100 pg +200 pg + 200 pg

In the present study, we investigated the preventive effect of alone as well as combined effect of folic acid and inositol on neural tube defects. For this purpose we were selected cotinine for inducing the neural tube defects and chick embryo model to evaluate the preventive effect of both folic acid and inositol. Chick embryo model was widely utilized to learn the teratological and developmental studies. Additionally, the closing of neural tube occur within 2 days.[16]

2. MATERIALS AND METHOD

White leghorn chick eggs (Gallus gallus domesticus [17]) were chosen for the present study. These were procured from Tirumala hatcheries, Rampur, Warangal, India. The fertile and specific pathogen free eggs were selected. They were incubated at 37.5 °C and 75% Related Humidity (RH). Cotinine (98%) was purchased from Sigma Aldrich, Bangalore, India and Folic acid and Meso-inositol were purchased from S.D fine chem. Ltd, Mumbai, India.

2 day chick embryo

80 eggs were selected and incubated at 37.5 °C and 75% RH for 49 hours according to Hamburger and Hamilton stages.[18] During the eighth stage (26-29 hours) of embryo development, the eggs were divided into eight groups with 10 eggs in each group (Table 1).

A hole was made on blunt pole of the egg through which drugs were injected, sealed and incubated until the eggs were reached to 12th stage of embryo development. After incubation, the eggs were opened and the yolk was carefully transferred into petridish. [9] The embryo floated on the yolk was collected by placing a Whatman filter paper disc. Transfer the embryo adhered to Whatman filter paper into 10 % formalin solution.

All the embryos were collected and kept on individual slides and examined morphologically under photo-light microscope using hematoxylin-eosin dye. Embryos from each group were fixed with paraffin and cut into thin sections of 20 µ thickness. These sections were stained with hematoxylin-eosin dye and subjected to histopathological study.[9] The dose of drugs (100 ng and 200 ng) chosen from according to reference.[19] All the results were statistically analyzed by one way ANOVA using graph pad prism software version 5.0 followed by Dunnets comparison test.

3. RESULTS

During 12th stage of embryo development, all the eggs were subjected to morphological and histopathological studies. Among the 10 embryos from cotinine group, 50% embryos showed the neural tube defects rest of the 50% embryos shown normal development (Table 2).

Table 2: Normal development and neural tube defects among the groups

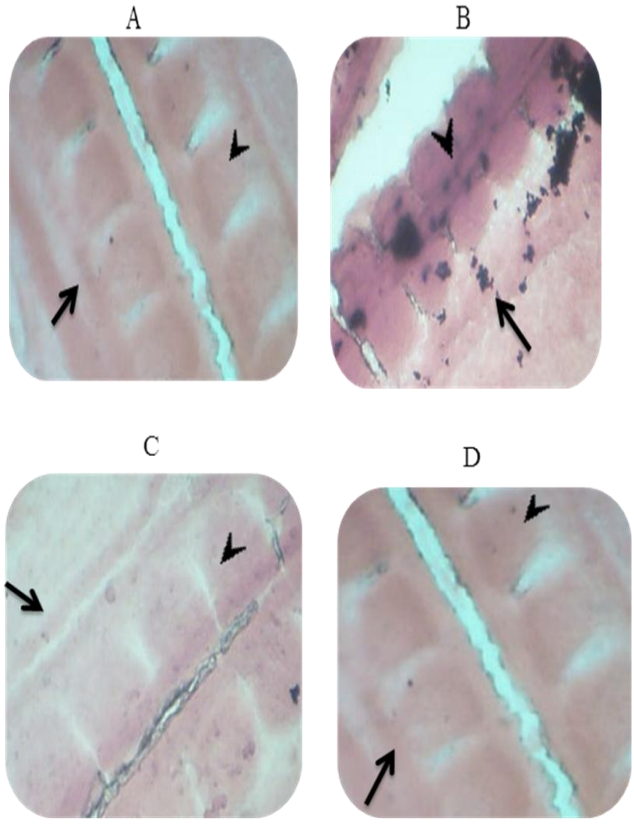
S. No.	Treatment groups	No. of eggs	Normal embryo development (%)	Neural tube defects (%)
1	Control	10	100	0
2	Cotinine	10	50	50
3	Cotinine + folic acid (Low dose)	10	70	30
4	Cotinine + folic acid (High dose)	10	80	20
5	Cotinine + inositol	10	70	30

	(Low dose)			
6	Cotinine + inositol (high dose)	10	80	20
7	Cotinine + folic acid + inositol (low dose)	10	80	20
8	Cotinine + folic acid + inositol (high dose)	10	90	10

3.1. Morphological examination

Neural tube was morphologically examined using hematoxylin-eosin dye under photo light microscope and the respective pictures were shown in (Figure 1). In control group, the embryos were observed with normal development at the caudal and rostral part of the neural tube. 16 pairs of somites and heart were noticed the normal development. Anterior neuropore was closed and side turning of head and amnion covers the total forebrain region (Figure 1A).

In cotinine treated group, 15 pairs of somites observed and abnormality was noticed at the thoracic region of caudal part and rostral part of neural tube (Figure 1B).
In folic acid treated groups, the high dose exhibited better preventive effect than low dose. In which we were observed the normal development at the caudal part of the thoracic region and rostral part of neural tube when compare to control group (Figure 1C and D). In inositol treated group, the high dose of inositol exhibited moderate preventive effect than low dose (Figure 1E and F). In combination of both folic acid and inositol, high dose of both folic acid and inositol exhibits significant effect (Figure 1G, H).



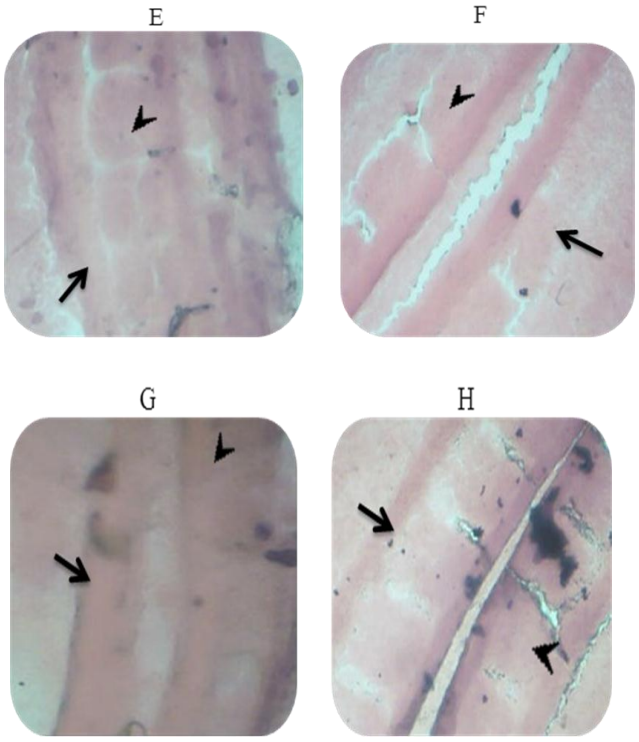
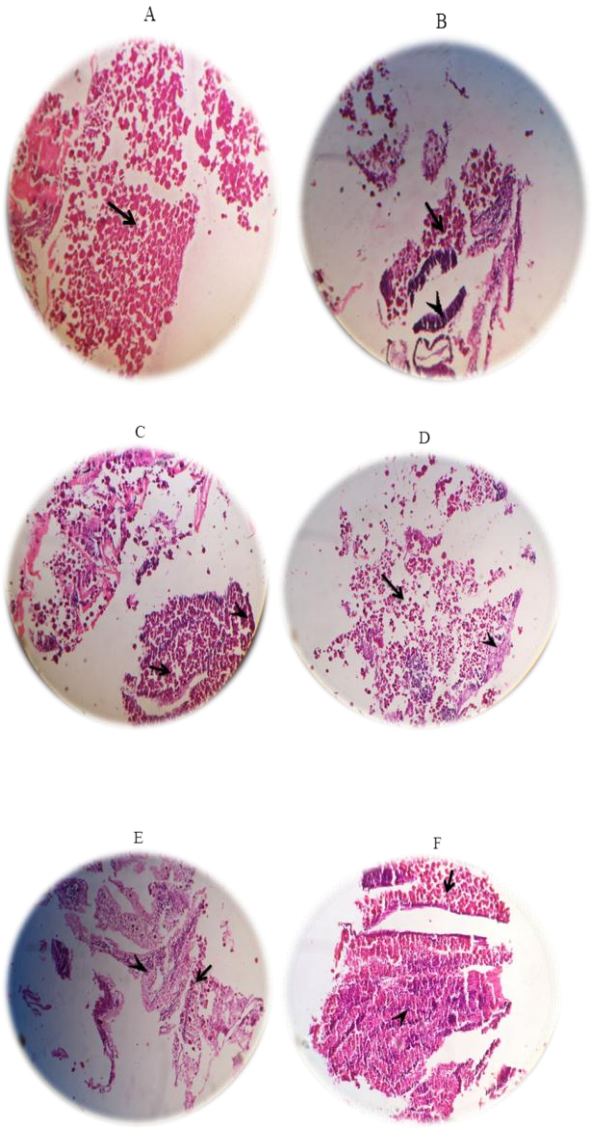


Figure 1: Morphological examination of neural tube. A. control B. cotinine C. cotinine + folic acid (low dose) D. cotinine + folic acid (high dose) E. cotinine + inositol (low dose) F. cotinine + inositol (high dose) G. cotinine + folic acid + inositol (low dose) H. cotinine + folic acid + inositol (high dose). ( )- Indicates somites and ( ) - Indicates neural tube (Hematoxylin-Eosin X60)

3.2. Histopathology

The transverse section of neural tube showed the normal and degenerative cells (Figure 2). In control group, transverse section of neural tube showed the normal cells and not yet finding any degeneration of cells. The open NTDs were not identified in control group (Figure 2A). The histopathological studies of cotinine treated group shown degenerative and normal cells (Figure 2B). The open neural tube defects were identified with presence of degenerative findings in chick embryo. In folic acid treated groups, high dose of folic acid reduces the degenerative cells effectively than the low dose (Figure 2C and D). In inositol treated group, high dose of inositol moderately reduced degenerative cells than the lower dose (Figure 2E and F). The high dose of both folic acid and inositol significantly reduced the degenerative cells than low dose (Figure 2G, H).



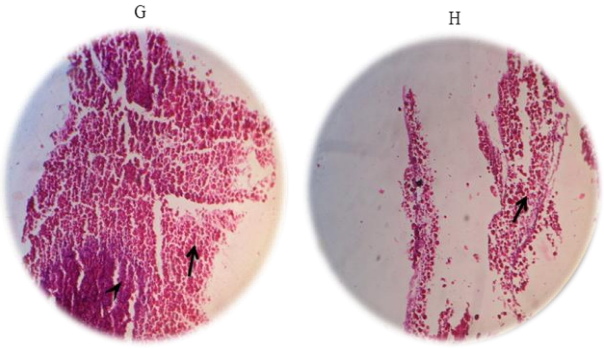


Figure 2: Histopathological transverse sections of neural tube. A. control B. cotinine C. cotinine + folic acid (low dose) D. cotinine + folic acid (high dose) E. cotinine + inositol (low dose) F. cotinine + inositol (high dose) G. cotinine + folic acid + inositol (low dose) H. cotinine + folic acid + inositol (high dose). (↑) - Indicates normal cells and (⋀)- Indicates degenerative cells (Hematoxylin-Eosin X60).

The transverse section of 2 day neural tube shown the highest degeneration in cotinine treated group (94±2.4; mean± S.E.M.) compared to control (0.0±0.0; mean± S.E.M.). The high dose of combination of folic acid and inositol (7±1.2; mean± S.E.M.) was shown significant preventive effect by reduced degeneration when compare to alone treated high dose of folic acid (56±2.4; mean± S.E.M.) and inositol (66±2.4; mean± S.E.M.) as shown in Table 3.

Table 3: Percentage of degeneration observed in transverse sections of normal and treated groups of 2 day embryo. (*)- indicates significant difference

S. No.	Treatment groups	Mean±S.E.M	No. of eggs
1	Control	0.0±0.0*	5
2	Cotinine	94±2.4*	5
3	Cotinine + folic acid (low dose)	80±5.4*	5
4	Cotinine + folic acid (high dose)	56±2.4*	5
5	Cotinine + inositol (low dose)	84±2.4*	5
6	Cotinine + inositol (high dose)	66±2.4*	5
7	Cotinine + folic acid + inositol (low dose)	15±2.2*	5
8	Cotinine + folic acid + inositol (high dose)	7±1.2	5

4. DISCUSSION

Results obtained from the present study proposed that the supplementation of folic acid and inositol shown preventive effect on cotinine induced neural tube defects using 2 day chick embryo model. There are some previous studies like Dalgic *et al*., exemplified that the cotinine induces the open neural tube defects from their results.[9] Helga and Sean *et al*., stated that the supplementation of folic acid reduces the occurrence and reoccurrence of neural tube defects from their results.[20] Patrica *et al*., depicted inositol prevents the folic acid resistant neural tube defects.[13] Vidia *et al*., proved that the supplementation of folic acid for 2 months before conception shown preventive effect of neural tube defects.[21]

We selected the chick embryo model which enlightens the development of vertebrate and guides the comparative, easily understanding the anatomy and evolution of vertebrate [22] and closing of neural tube is

fast and it happens at the 2 day of the embryo development.[16] It resembles with development of human fetus.[23] In this study, we investigated the effect of cotinine on 2 day chick embryo. We found that the cotinine causes the open neural tube defects and folic acid and inositol prevents the neural tube defects.

According to our results, in the morphological observation the control treated group exhibits normal development of neural tube like 16 pairs of somites, heart were noticed the normal development. Anterior neuropore was closed and side turning of head and amnion covers the total forebrain region (Figure 1A). In the cotinine treated group, we noticed the opening of anterior neuropore, abnormality in number and development of somites (Figure 1B). The combination of high dose of both folic acid and inositol exhibits significant development of neural tube compare to control treated group (Figure 1H).

The combined effect of folic acid and inositol at a dose of 200 pg shown significant preventive effect compared to alone treatment. Transverse section of neural tube shown degeneration in cotinine treated embryos. The high dose of folic acid and inositol significantly reduced the degeneration. Previously proved that the inositol shown preventive effect on folic acid resistant neural tube defects but in the present study addition of inositol to folic acid treated chick embryo showed significant effect compared to alone treatment.

5. CONCLUSION

The folic acid and inositol were shown preventive effect on cotinine induced neural tube defects in chick embryo model. Cotinine results into open neural tube defects. These were observed in 2 day chick embryo model. In morphological examination, the abnormality was observed at thoracic region of neural tube in cotinine treated 2 day chick embryo. The high dose folic acid and high dose of combination of folic acid and inositol were reduced the abnormality compared to cotinine treated group. The transverse section of 2 day neural tube showed degeneration. The reduced degeneration was observed with drug treated groups. So, the high dose of folic acid and high dose of combination of folic acid and inositol showed significant preventive effect. The addition of inositol to folic acid treated group showed good preventive effect compared to alone treatment of folic acid and inositol. In the present study, combination of inositol and folic acid prevents the open neural tube defects. Further studies needed to know about the preventive effect of inositol and combination of both folic acid and inositol.

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