Short Paper

Effect of arsenic on neural tube in mouse embryo and relation to reduced folate carrier (RFC-1)

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Summary

Arsenic is an important environmental toxicant which is usually found in drinking water in inorganic form. The hypothesis tested in this investigation is; arsenic exposure causes neural tube defects (NTDs) and these defects of the central nervous system are more likely related to folate deficiency during fetal life. In this study, sodium arsenate was administered via intraperitoneal route at a rate of 40 mg/kg body weight on the 6th day of gestation to every individual of 20 mated Albino mice. On the 8th (E8), 10th (E10), 16th (E16) and 19th (E19) days of the gestation, the pregnant mice in control and experimental groups were sacrificed by cervical dislocation. All embryos belonging to (E16) and (E19) were examined for external morphological neural tube defects. Histological staining techniques were haematoxylin and eosin and the immunofluorescence staining was also implemented. It was observed that, the intraperitoneal injection of sodium arsenate caused a number of morphological neural tube defects including; open fourth ventricle, exencephally, myelomeningocele and anencephaly. Difference in control and experimental specimens was significant (P<0.001) on the (E16) group. The histomorphologic changes of neural tube were significant in all of the experimental groups in comparison to the controls. Immunofluorescence study revealed reduced folate carrier (RFC-1) protein reduction in neural tissue, and these results demonstrate that the association between prenatal exposure to inorganic arsenic and NTDs is more likely related to a defect in reduced folate carrier protein.

Key words: Arsenic, Neural tube, Mouse embryo, RFC-1

Introduction

Arsenic is a naturally occurring metalloid released into the environment by natural events and human activities.

The main source of human exposure to arsenic is contaminated drinking water and some foods, such as rice, other grains, and fishes. Malformations found in embryos depend on the stage of gestational development, dose and the route of administration (Mead, 2005).

The mouse neural tube first fuses at the hindbrain/cervical boundary, at embryonic day 8.5 and finally closes embryonic day 10 (Finnell *et al*., 2003). Except for heart

defects, NTDs are the most common form of major congenital malformations with a birth prevalence of 1-5/1000 worldwide. Over the past decade, a considerable number of studies demonstrate the protective role of periconceptional folic acid supplementation in preventing neural tube malformations in humans and laboratory animals (Jason and Elizabeth, 2009).

The reduced folate carrier (RFC-1), a classical facilitative mechanism, delivers folates to neural and other tissues and plays a key role in mediating transport of antifolates. At day 10.5 RFC-1 protein is detectable in neural tube and dividing cells in the developing neural tube which is

particularly susceptible to folate deficiency (Dennis *et al*., 2003). Our hypothesis is that defective embryonic folate transport confers enhanced susceptibility to compounds that produce teratogenicity.

Materials and Methods

Male and female albino mice were obtained from the Animal House of Maulana Azad Medical College (MAMC) of Delhi University. Embryonic day 0 was designated as the day on which a vaginal plug was found. The pregnant mice were divided into two main study groups including the control and experimental, respectively, based on the administration of distilled water and aqueous solution of sodium arsenate. These groups were further divided into subgroups based on the day of sacrifice i.e., embryonic days (E8, E10, E16 and E19).

A single dose of arsenate was administered at the dose of 40 mg/kg body weight on the sixth gestational day. On embryonic day 8 (E8), E10, E16 and E19, the pregnant mice belonging respectively to the control and experimental subgroups were killed by cervical dislocation. All of the embryos were fixed in 4% paraformaldehyde (PFA) and examined for external morphological neural tube defects under stereomicroscope. For further histological analyses, the paraffin serial sections were cut at 5 μm and stained with haematoxylin and eosin. The immuno-fluorescence staining was carried out according to the protocols of Wang *et al*. (2001). Paired comparisons were conducted using an independent t-test and Fisher's test for statistical analyses.
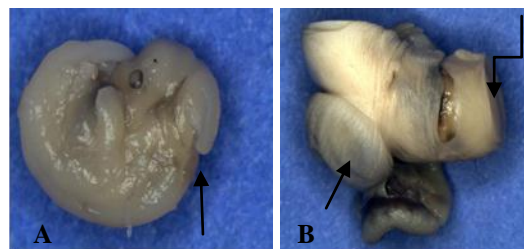
Results

Gross morphology

Maternal treatment with sodium arsenate on E16 and E19 resulted in the production of open fourth ventricle (Fig. 1A), exencephally, myelomeningocele (Fig. 1B) and anencephaly. This difference in control and experimental specimens was statistically significant on E16 (P<0.001).

Histomorphology

Transverse sections were taken at

Fig. 1: Photograph of mouse embryos exposed to sodium arsenate. (A) Open 4th ventricle (arrow) (E16). (B) meningomyelocele (arrow), anencephaly (elbow arrow) on E19

cervical, thoracic and sacral levels and stained with H&E. In the normal group on E8 neural folds were formed. Also, internal limiting membrane, dividing neuroepithelial cells, intermitotic neuroepithelial cells and external limiting membrane were clearly developed. The neural folds were closed at the level of the fourth and fifth somites. In the experimental animals gastrulation was established in a lesser number of fetuses (86.7%) and none of the embryos showed closure of neural tube in cervical region (Figs. 2A and B; Table 1). The results were significant with Fisher's test when compared with normal control animals (P<0.001).

Sections of normal embryos on E10 revealed that, the roof plates of neural tubes had several layers of neuroectodermal cells. The spinal cord was lined with the intact external and internal limiting membrane. (Figs. 2C and D). Sections of the treatment group showed open neural tube, disrupted internal membrane, and cell ejection. There was significant (P<0.05) neural tube defects in experimental animals (73.3%) as compared to the controls (26.7%).

On E16 in controls the appearance of the spinal cord was typical in transverse sections with white marginal and gray central mantle layers and an ependymal layer. The cartilaginous neural arches are clearly distinguished. In experimental animals, the uppermost edge of the neural tube did not show signs of fusion. The mantle and marginal layers were not well defined. There was significant (P<0.001) presence of neural tube defect in experimental animals (66.7%) in comparison the controls (0%).

Expression of reduced folate carrier (RFC-1)

Immunoreactivity against RFC-1 was

Table 1: Histomorphological study of neural tube defect between control and experimental on E8, E10, E16, E19 and expression of RFC-1 on E10, E16 and E19

| Group	Day of sacrifice	No. of fetuses	No. of NTDs (%)	Low RFC-1 (%)	P1-value	P2-value
Control	8	15	2 (13.3)	-	-	-
Experimental	8	15	13 (86.7)	-	<0.001[**]	-
Control	10	15	4 (26.7)	3.2	-	-
Experimental	10	15	11 (73.3)	68.3	<0.05[*]	<0.01[*]
Control	16	15	0 (0)	0	-	-
Experimental	16	15	10 (66.7)	52.1	<0.001[**]	<0.05[*]
Control	19	15	0 (0)	0	-	-
Experimental	19	15	7 (46.7)	48.5	<0.01[*]	<0.05[*]

[*]: Statistically significant (Fisher's test), and [**]: Statistically highly significant (Fisher's test)
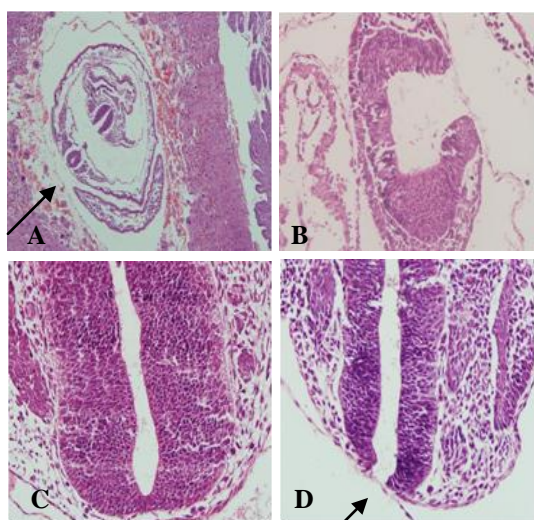
Fig. 2: Photomicroscopic of transverse sections of neural tube in control and experimental on E8 and E10 with H&E staining. (A) neural fold closed at the level of 4th somite (arrow) in control (×100). (B) Undifferentiated neural tube cells on experimental (×400). (C) Closed neural tube in control with different layers. (D) Open neural N.T. (arrow)

not detectable at early stages of development, but maturing neurons exhibited positive staining in control sections between days 10 and 19 of gestation. Immunofluorescence staining pointed out that, the RFC-1 protein is localized in the cells of the mantle layer on E10. In this stage, 68.3% of animals showed negative staining of RFC-1 in arsenic affected embryos, and it was found to be significant (P<0.01). On the E16 and E19, the RFC-1 was detected in white matter, and ependymal cells (Fig. 3A). Negative staining signal was noted in experimental groups in these stages (Fig. 3B). Also high expression of RFC-1 in cartilage model of intervertebral

disc was detected in controls as experimentals (Figs. 3C, D). Negative staining of experimental groups on E16 and E19 was 52.1% and 48.5%, respectively and with comparison to the normal control groups, it was significant (P<0.05, Table 1).
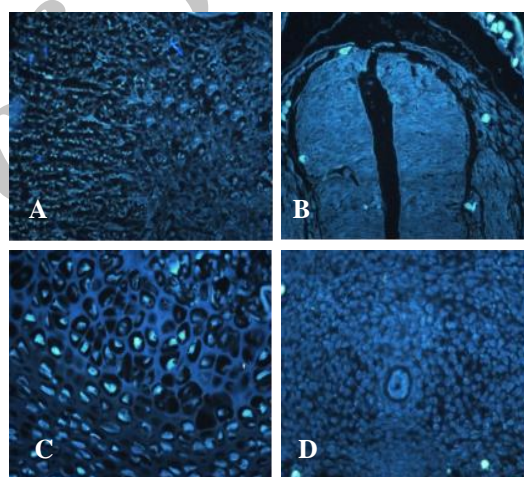
Fig. 3: Photomicroscopic of transverse sections of neural tube and intervertebral disc in control and experimental embryos on E19 with immunofluorescence staining (×400). (A) Expression of RFC-1 in white matter and neurons in control. (B) Low expression of RFC-1 with undifferentiated neurons in experimental N.T. (C) High expression of RFC-1 in intervertebral disc in control. (D) Low expression of RFC-1 in intervertebral disc in experimental

Discussion

The teratogenic effects of the inorganic arsenic has been reported in previous studies, but deep investigation on the mechanism of action of arsenic on the neural tube defects has not yet been reported. We observed failure of closure of neural tube in

both the morphological and histo-morphlogical studies. The gross features of experimental animals showed defects as, opening of hind brain, exencephally, myelomeningocele and anencephaly. The microscopic observations of neural tissue on E8 showed delay in development of neural tissue. Our results closely approximate those obtained by Willhite and Ferm (1984) who report that I.V. injection of 20 mg/kg sodium arsenate to hamsters results in delay in neural closure.

The most striking feature of the arsenic exposed embryos was the consistent lack of apposition of the neural folds on gestational day 8, 10 and 16, but on gestational day 19 it has been seen as a spina bifida, and lack of apposition of the vertebral arches.

According to one study, 24 h after administration of arsenic acid to pregnant mice the neuroepithelium has the highest concentration of radiolabelled arsenic of all embryonic tissue examined (Lindgren *et al*., 1984). This suggests that damage to the neural tube may be a direct effect of arsenate on the embryo during critical period of development. It is possible that necrosis of critical cells in the midline could occur to prevent closure (Wlodarczyk *et al*., 1996). Wlodarczyk *et al*. (1996) suggest that arsenic damage to DNA is responsible for inhibition of cell proliferation, thus delaying and preventing a normal neural tube closure.

Fisher examined the effect of sodium arsenate on DNA, RNA and protein levels in developing rat embryos. Arsenate resulted in a significant decrease in DNA, RNA and protein accumulation (Fisher, 1982). Electron microscopic study revealed that, cell necrosis occurred in the neuroectoderm of embryo firstly, and was then followed by vesiculation of the endoplasmic reticula. These authors considered that nucleolar atrophy results from the decrease in the synthesis of nucleolar RNA (Takeuchi, 1979).

With reference to the aforementioned reports, the reason for the reduction in the level of the RFC-1 prorien which is observed in our study could be due to the overall decrease in DNA and RNA levels as a result of arsenic neurotoxicity on the mice

embryo. We conclude that, due to the destructive effects of the arsenic on membranous carriers of the foliate which is present on the cell surfaces of the embryonic neural cells, the entrance of folate to the inside of these cells affects the developmental processes.

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