

Cyclophosphamide induced non-canalization of cerebral aqueduct resulting in hydrocephalus in mice

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ABSTRACT

This study aims to understand the mechanism of failure of canalization of cerebral aqueduct following intrauterine exposure to reference teratogen, cyclophosphamide in murine pups. Non-canalization of cerebral aqueduct was found to result in internal hydrocephalus. Cyclophosphamide was administered to pregnant mice on day 10, 11, or 12 of gestation in a single dose of 20 mg/kg body weight. Fetuses were dissected out on day 19 and studied for hydrocephalus and other cerebral or cranial malformations. Serial sections of brain in coronal and transverse planes exhibited incomplete development and failure of canalization of cerebral aqueduct. Pressure of cerebrospinal fluid (CSF) in non-canalized aqueduct resulted in its rupture leading to leakage and accumulation of CSF in brain substance causing a cavity full of CSF close to unopened aqueduct. The large pool of CSF in the brain substance in extreme cases communicated with the subarachnoid space pushing through the substance of brain causing external hydrocephalus. Internal hydrocephalus on the other hand was resulted from back pressure of CSF following blockage in its flow due to non-canalization of the cerebral aqueduct. In the extreme cases internal and external hydrocephalus were seen intercommunicating. Cyclophosphamide induced inhibition of mitosis and cell differentiation of ependymal cells and augmentation of apoptosis of brain cells were attributed as the major causes underlying the incomplete development of cerebral aqueduct. The study also suggested inductive role of CSF in the differentiation of ependymal cells lining the cerebral aqueduct. *Neuroanatomy; 2007; 6: 1–5.*

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Key words [apoptosis] [cerebral aqueduct] [cerebro spinal fluid] [cyclophosphamide] [ependymal cells] [hydrocephalus]

Introduction

Cyclophosphamide, a model teratogen, has been used to produce hydrocephalus experimentally by various researchers [1–4]. Although the precise mechanism of cyclophosphamide induced hydrocephalus remains elusive, hypertrophy of choroid plexus was considered as the primary cause [1,4]. Singh et al [4] observed agenesis or stenosis of the aqueduct as an associated reason. The present work has been undertaken to elucidate the mechanism involved in cyclophosphamide induced hydrocephalus in mice.

The present study reports a novel observation that intrauterine exposure of fetuses to cyclophosphamide leads to an incomplete canalization of cerebral aqueduct resulting in both internal and external hydrocephalus; i.e., CSF accumulation in both ventricles of brain and subarachnoid space. The report further discusses cyclophosphamide induced interference in cell differentiation and mitosis in ependymal cells as the possible mechanism of incomplete canalization of cerebral aqueduct. Furthermore, it provides evidence to show that CSF plays a pivotal role in the differentiation of ependymal cells lining the cerebral aqueduct.

Material and Methods

Approval of Animal Ethical committee of the Institute was obtained prior to the present study.

Sexually mature Swiss white female and male mice weighing 16–36 g obtained from Central Animal House of the Institute were used in this study.

Cyclophosphamide manufactured by Khandewal Labs Mumbai, India with trade name Endoxan-Asta was used in the experiment.

Female mice during their proestrous phase of estrous cycle were caged overnight with the males of the same stock (female:male=1:1). The vaginal smear was examined next morning at 8.00 a.m. Presence of spermatozoa in the smear or vaginal plug was taken as day 'zero' of pregnancy. A total of 40 pregnant female mice were sacrificed in the present study. They were divided into control group (n=10) and treatment group (n=30). The treatment group was divided further into three groups of 10 animals each, which received cyclophosphamide intraperitoneally on day 10, 11 or 12 of gestation in dose of 20 mg/kg body weight. In the control group only normal saline was injected on each of these respective days of gestation.

The pregnant mice were sacrificed with overdose of ether anesthesia on day 19 of pregnancy i.e. one day prior to full term. The uterine horns were exteriorized after opening the abdomen by midline incision. The fetuses were removed from the uterus and were dried by wiping on a blotting paper. Brains were dissected out from cranial cavities of fetuses. Bouin's solution was used for fixation and brains were prepared for light microscopic

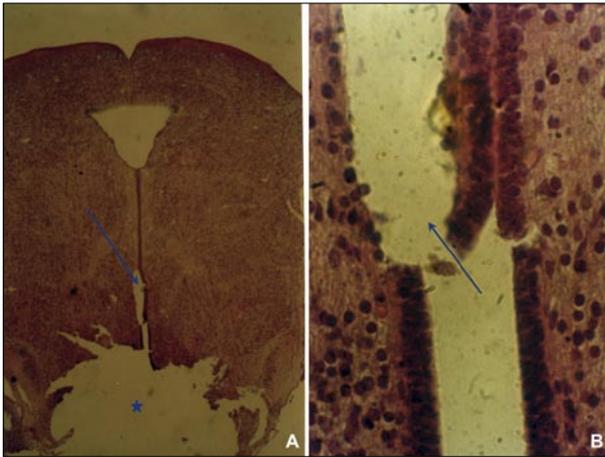


Figure 1. Photomicrographs of brain of fetus exposed to 20 mg of cyclophosphamide on day 12 and collected on day 19 of gestation. Color version of figure is available online. [A: Histological section of brain of treated fetus in coronal plane at low magnification showing incomplete canalization of cerebral aqueduct. Pressure of cerebrospinal fluid has created a separate passage parallel to unopened canal (arrow). Shape of the fourth ventricle is distorted due to CSF pressure (star). The wall lining the canalized part of aqueduct is deeply stained (H and E x42); B: Histological section of brain of treated fetus in coronal plane at higher magnification showing ependymal cells lining the opened and unopened parts of cerebral aqueduct and pressure of CSF creating a lateral cavity parallel to unopened canal (arrow). The ependymal cells lining the open part of the cerebral aqueduct showed deeply stained nuclei and differentiated cellular morphology. The cells surrounding the unopened part of the cerebral aqueduct showed weakly stained nuclei with less differentiated morphology. The wall of the unopened cerebral aqueduct facing the lateral cavity containing CSF showed differentiated cells as compared to the opposite wall not in direct contact with CSF (H and E x672)]

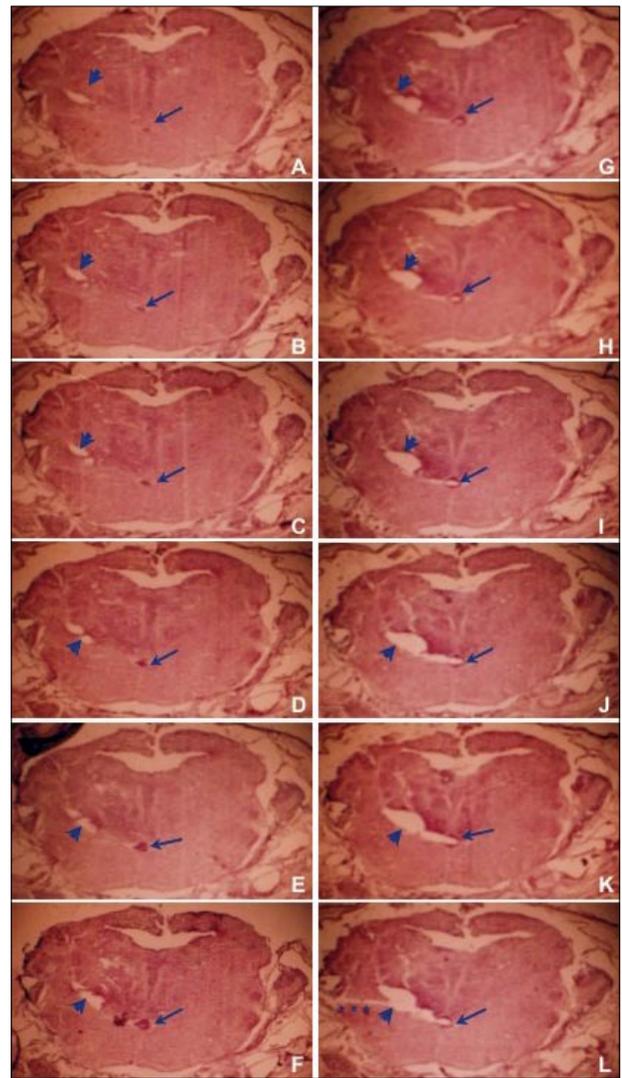
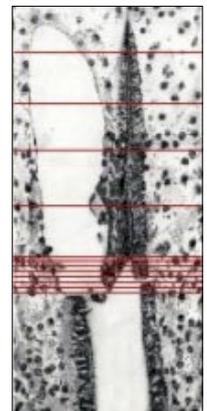


Figure 2. Photomicrographs of brain trans-sections of treated pup (20 mg on day 12) and collected on day 19 of gestation (H and E x16). Color version of figure is available online. (The twelve sections (A to L) show cerebral aqueduct having non-development and non-canalization in the proximal part (near to third ventricle) and gradual appearance of the lumen in the distal part towards fourth ventricle. The lumen marked by arrows showed gradual widening and rupture laterally due to CSF pressure from the fourth ventricle. A: arrow points out the site of aqueduct as a dark spot. Aqueduct shows complete agenesis (arrow), arrowhead points out at the cross section of lateral projection from the upper part of the duct (as shown in schematic drawing on the right top of figure) as a result of CSF pressure from the fourth ventricle into a blind aqueduct. B, C, D, E and F are serial sections of brain showing gradual widening of the aqueduct with appearance of lumen in F, (arrows). Arrowheads point out at the cross-section of lateral projection from the lower part of the aqueduct with well-developed lumen and ependyma. Sections show gradual sprouting of lateral extension from the aqueduct (lower part) with development of a zone (lake) in the substance of the brain accommodating CSF forced into the lower part of the aqueduct from the fourth ventricle. The last section (L) shows communication from the lateral extension to the subarachnoid space, resulting in hydrocephalus, (three asterisks). All the sections show communication in sagittal plane between the lateral ventricle and the subarachnoid space causing hydrocephalus.)

The schematic drawing shows level of trans-sections in coronal plane (right).



study by paraffin section. Brains were serially cut at 8 micron in coronal and transverse plane and stained with haematoxylin and eosin. Photomicrographs of histological findings were obtained at different magnification.

Quantitation of percent specific DNA fragmentation for evaluation of brain cells undergoing apoptosis in different groups

This method was carried out in two groups. First group included brains of control fetuses. In the second group, brains of fetuses obtained after cyclophosphamide treatment in dose of 20 mg on day 12 of gestation were studied. Percent DNA fragmentation was quantified following a method described by Sellins and Cohen [5] with slight modifications. Single cell suspension of brain cells was obtained. Brain cells (5×10^5 cells/ml) were

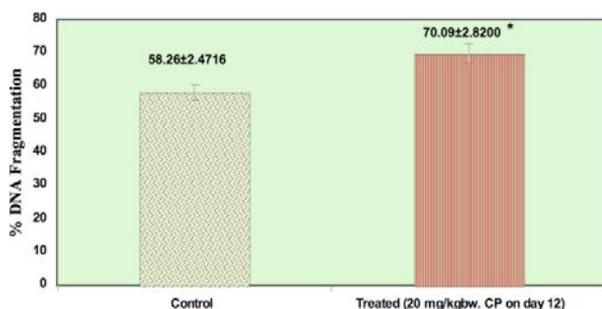


Figure 3. Percent fragmentation of DNA obtained from brain cells of control and cyclophosphamide treated groups was described in materials and methods. Values are mean \pm SD of percent fragmented DNA in total DNA isolated in each sample. * $P < 0.05$ vs. values for corresponding control. Color version of figure is available online.

suspended in 0.5 ml of lysis buffer [Tris-EDTA buffer, pH 7.4 containing 0.2% Triton \times 100 and were centrifuged for 15 minutes at 13000 \times gm at 4°C in a microfuge tube (labelled as B)]. Supernatant was transferred to another tube (labeled as T). 0.5 ml of 25% trichloroacetic acid was added to T and B tubes, followed by vigorous vortexing. Tubes were kept overnight at 4°C for precipitation. Supernatant was discarded after centrifugation at 13000 \times g for 10 minutes and then DNA in each pellet was hydrolyzed in 80 μ l of 5% trichloroacetic acid by heating on a water bath at 90°C for 15 minutes. 160 μ l of freshly prepared diphenylamine (150 mg diphenylamine in 10 ml glacial acetic acid, 150 μ l concentrated H₂SO₄ and 50 μ l of acetaldehyde solution) was added and the tubes were allowed to stand overnight at room temperature to develop color. 100 μ l of this colored solution was transferred to a 96 well flat bottom ELISA plate and absorbance was measured at 600 nm on an ELISA plate (Tarsons) reader (Lab systems, Finland). Percent fragmented DNA was calculated using the formula:

$$\% \text{ Fragmented DNA} = (T/T+B) \times 100$$

Where T = absorbance of fragmented DNA and T+B = absorbance of total DNA.

Statistical analysis

The results have been expressed as mean \pm SD. Statistics of the individual data were analyzed by using Fishers (F) test. Students (t) test was employed for all statistical comparisons. Any value of $p < 0.05$ was regarded as significant.

Results

Gross examination of the fetuses revealed significant growth retardation in the treatment groups (Table 1). There was a significant reduction in brain weight and distortion in shape of brain in all the treatment groups (Table 2). On microscopic examination in day 12 cyclophosphamide treated group, hydrocephalus was found to be a frequent finding as compared to the controls ($p < 0.001$). However, the difference between control and day 10 or 11 treated groups was only marginally significant ($p < 0.05$; Table 3).

The cerebral aqueduct was seen to be well formed in its distal part, i.e. near the fourth ventricle (Figure 1),

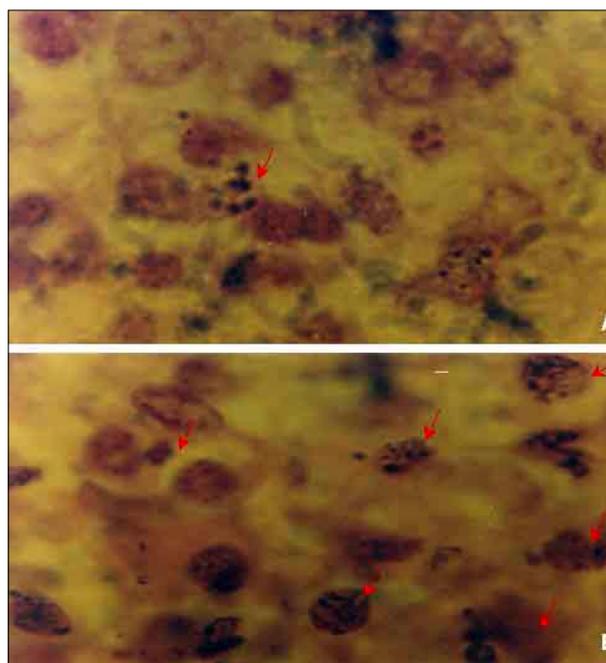


Figure 4. Photomicrographs of brain of pups collected on day 19 of gestation. A) Histological section of brain of control pup in coronal plane at very high magnification showing normal apoptotic process (arrow) (H&E \times 1024). B) Histological section of brain of treated pup (20mg on day 12) in coronal plane at very high magnification showing relatively higher incidence of apoptosis (arrows) as compared to control (A) group (H&E \times 1024). Color version of figure is available online.

Table 1. Incidence of growth retardation in pups of pregnant mice treated with cyclophosphamide (20 mg/kg body wt.) on days 10 or 11 or 12 of gestation.

Gestational day of treatment	Total fetuses studied (n)	AW of fetuses of different groups (g)	P*
10	43	0.771 \pm 0.019	<0.001
11	41	0.852 \pm 0.021	<0.001
12	38	0.921 \pm 0.022	<0.001
Control	50	1.471 \pm 0.051	

Values are Mean \pm SD of litters of 10 mothers. AW: Average weight. (* As compared with control \pm SD)

showing well-differentiated ependymal cells lining the duct having sufficient lumen (Figures 1, 2). At its proximal part, i.e. end near the third ventricle; the aqueduct had failed to open showing non-availability of lumen in this part. Consequently, the CSF, due to its pressure which in present cases was expected to be over produced due to hypertrophy of choroid plexus disrupted the continuity of the duct wall and CSF forced its way through the substance of the brain getting pooled by the side of the unopened aqueduct (Figure 1: long arrow; Figure 2: arrow heads). Continuous pressure of CSF further pushed its way through the substance of brain (Figure 2 L: three asterisks) resulting in communication between

Table 2. Findings of brain studies of pups collected from pregnant mice treated with cyclophosphamide (20 mg/kg body wt.) on days 10 or 11 or 12 of gestation.

Gestational day of treatment	Total fetuses studied (n)	AWB of different groups (mg)	P*
10	43	15.42 ± 0.41	<0.001
11	41	17.13 ± 0.52	<0.001
12	38	18.42 ± 0.58	<0.001
Control	50	29.41 ± 1.11	

Values are Mean ± SD of litters of 10 mothers. AWB: Average weight of brains. (* As compared with control ± SD)

the ruptured part of aqueduct and the subarachnoid space. It was conspicuous to observe (Figure 1) variation in the cellular morphology of ependymal cells lining the aqueduct. Well-differentiated cells with dark staining nuclei were observed in the portion of open part of cerebral aqueduct whereas relatively undifferentiated cells lined its unopened part. The nuclei of these undifferentiated cells showed weak staining. Thus, cells forming wall of the undifferentiated and non-canalized zone of the cerebral aqueduct showed weakly stained chromatin compared to the more differentiated cells lining the canalized part, suggesting inhibition of mitosis in the cells present around the non-canalized cerebral aqueduct. Cells showing proper differentiation were seen in direct contact of CSF whereas cells which did not differentiate completely were in the region of non-canalized part where cells were not in contact with CSF. Another interesting finding is seen in Figure 2 where all the sections showed additional communication between the lateral ventricles and the subarachnoid space in sagittal plane deep to scalp.

Significant reduction and distortion of brain following cyclophosphamide exposure can be ascribed to increase in rate of apoptosis caused by it, in addition to anti-mitotic effect of this alkylating agent. As seen in Figures 3 and 4, the rate of induction of apoptosis was significantly augmented by the cyclophosphamide administration resulting in small size brains. DNA fragmentation percentage quantifying apoptosis increased from 58.26 ± 2.4716 to 70.09 ± 2.82 after cyclophosphamide administration (Figure 3). Percent DNA fragmentation in the DNA of cyclophosphamide treated fetuses in dose of 20mg/kg body weight on day 12 of gestation was significantly higher than that of control group (Figure 3, p<0.05).

Discussion

In the present study, we provide evidence to demonstrate that hydrocephalus in murine fetuses exposed to cyclophosphamide during intrauterine life results in an incomplete development and non-canalization of cerebral aqueduct. Results of the investigations also indicate that such effects of cyclophosphamide could be attributed to:

- 1) The anti-mitotic action of the drug on the proliferation

Table 3. Incidence of hydrocephalus in mice brains treated with cyclophosphamide (20 mg/kg body wt.) on days 10 or 11 or 12 of gestation.

Gestational day of treatment	Total histological sections studied (n)	Incidence of Hydrocephalus (%)	P*
10	20	7.42 ± 0.02	<0.05
11	20	11.51 ± 0.04	<0.05
12	25	34.08 ± 0.07	<0.001
Control	43	0	

Values are Mean ± SD of litters of 10 mothers. (* As compared with control ± SD)

and migration of the ependymal cells which line the cerebral aqueduct; 2) The inhibitory action of the drug on the differentiation of ependymal cells; 3) Augmented induction of apoptosis in all cells of brain. Indeed, both proliferation and differentiation of the ependymal cells are essential prerequisite for the formation of cerebral aqueduct [6,7]. The effect of other factors, such as, the dose and time of the exposure to cyclophosphamide, and a zone-wise differential susceptibility of the ependymal cell to the inhibitory action of cyclophosphamide and augmented apoptosis are suggested as additional reasons for the non-development of cerebral aqueduct.

These suggestions are corroborated by a recent finding demonstrating that there are three distinct regions of cerebral aqueduct lined by different types of ependymal cells and each of these regions behave differentially in hydrocephalic mice, leading to obliteration of the proximal end of aqueduct [8]. Another interesting finding of the present study was the observation that only those ependymal cells showed proper differentiation and proliferation, which were in direct contact with CSF. It is, therefore, suggested that CSF may have an inducing role in the development and canalization of the cerebral aqueduct. Indeed, Miyan et al [9] demonstrated that CSF from lateral ventricle had a pivotal effect on the proliferation of neuronal progenitors. Cyclophosphamide could also have an interfering effect on the cell cycle of the ependymal cells that failed to receive mitosis-inducing signals from the CSF. Interference in the normal cycle and arrest of cells in S phase of cell cycle due to abnormal signals received from CSF have been attributed as one of the main causative factor in the defective development of neural tube [7,9,10]. The resulting diversion in the passage of CSF flow owing to the non-canalization of the cerebral aqueduct is suggested to manifest an early onset of hydrocephalus in cyclophosphamide exposed fetuses (Figures 1 and 2). However, it is difficult to assess if overproduction of CSF due to cyclophosphamide and rate of opening of aqueduct are unable to keep pace resulting in rupture of aqueduct or the aqueduct has altogether stopped opening. In any case, evidences from the study of Pourghasem et al. [11] who demonstrated that blockage of the connecting canal between the aqueduct and the fourth ventricle by apposition of its

walls led to dilation of lateral ventricles and showed the manifestations of hydrocephalus.

Cyclophosphamide is known to cause augmented apoptosis in a variety of cell types including those of central nervous system [12–15]. In the present study, when cyclophosphamide on one hand must have acted as an anti-mitotic and on the other stimulator of apoptosis, the combination of these two effects must have resulted in a substantial small number of brain cells. The resulting small size of brain in turn will not be able to put stretch on the aqueduct will cause the failure of its opening.

Conclusion

Taken together the observations of the present study provide novel evidences to demonstrate the mechanism

of cyclophosphamide-induced internal hydrocephalus in murine fetuses due to non-canalization and incomplete development of the cerebral aqueduct, complicated further by accelerated apoptosis, reducing the number of cells in whole of brain. The external hydrocephalus was secondary to internal hydrocephalus. Imbalance between rate of CSF secretion and opening of cerebral aqueduct is hypothesized as the primary cause of hydrocephalus.

Acknowledgement

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