

# Neuroimmune consequences of teratological insult induced by cyclophosphamide exposure during intrauterine life in mice

Published online 12 February, 2007 © <http://www.neuroanatomy.org>PRAKASH<sup>[1]</sup> ✦Gajendra SINGH<sup>[2]</sup>Sukhmahendra SINGH<sup>[3]</sup>

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## ABSTRACT

Present work is aimed to establish central nervous system and immune system changes in newly born murine pups produced after cyclophosphamide exposure during intrauterine life. Cyclophosphamide in single dose of 10 or 20 mg/kg body weight was administered to pregnant mice on day 11 of gestation. Fetuses were dissected out on day 19 and studied for gross malformations. Brains and thymuses were collected for further investigations. Cyclophosphamide treated brains and thymuses were smaller in size and distorted in shape as compared to control brains and thymuses. Protein estimation of brains revealed decline in both quantitative and qualitative aspect. Study of brain cells and thymocytes revealed 1) increase in apoptotic cell count and 2) increase in percent DNA fragmentation. In vitro cell proliferation assay exhibited decrease in both brain cells and thymocytes. Similar pattern of response shown by brain cells and thymocytes conclude interaction between central nervous system and immune system insults produced by cyclophosphamide. Loss of certain neuropeptides has culminated in thymic compromise besides direct actions. *Neuroanatomy; 2007; 6: 6–11.*

## RETRACTED ARTICLE

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## Introduction

A wide spectrum of the teratogenic effects of cyclophosphamide (CP) have been reported involving multiple tissues and organ systems of animals, but more population based studies may still be essential to conclusively demonstrate teratogenicity of CP in humans. However, a number of studies demonstrate that CP is a human teratogen due to its effects such as intrauterine growth retardation and multiple anomalies including microbrachycephaly, coronal craniosynostosis, hypotelorism, shallow orbits, proptosis, blepharophimosis, abnormal and small ears, unilateral preauricular pit, broad, flat nasal bridge, microstomia, high-arched palate, micrognathia, cranial anomalies, preaxial upper limb and post-axial lower limb defects consisting of hypoplastic thumbs, and oligodactyly like bilateral absence of 4<sup>th</sup> and 5<sup>th</sup> toes [1].

Tissue distribution of CP is wide. The drug can cross placenta, found in breast milk and ascitic fluid. Moreover, it is remarkable to note that CP and its metabolites can cross blood brain barrier. This may be particularly important in relation to the fact that CNS anomalies are the foremost among the malformations induced by the drug. Investigations provide evidences implicating CP induced cell death as a common event in the pathogenesis associated with tissues destined to be malformed [2]. Although the importance of this cell death is recognized, little information is available concerning the biochemistry of teratogen-induced cell death. Teratogen-induced cell

death is also selective, i.e. some cells within a tissue die while others survive. In addition, cells within some tissues die when exposed to CP while other cells are relatively resistant to the CP induced cell death [2].

The spectrum of teratogenic effects produced by cyclophosphamide in various species shows a striking similarity. Cyclophosphamide exposure leads to multiple central nervous systems (CNS) anomalies, the mode of neuronal damage need to be established and we have chosen murine system for our study. We also intend to investigate the teratological insult caused by cyclophosphamide on murine fetal thymuses. Since some preliminary studies have indicated the involvement of nervous system in cyclophosphamide induced immune disorders [3], neuroimmunological consequences of cyclophosphamide exposure need to be explored.

## Material and Methods

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

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 30 pregnant female mice were sacrificed in the present study. They were divided into control group (n=10) and treated group (n=20). The treated group were divided further into two groups of 10 animals each, which received intraperitoneally cyclophosphamide on day 11 of gestation in dose of 10 or 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. Thymuses were also dissected out. Bouin's solution was used for fixation and they were prepared for light microscopic study by paraffin section. Brains were serially sectioned at 8  $\mu$ m thickness in coronal and transverse plane while thymuses were cut at 4  $\mu$ m and both were stained with haematoxylin and eosin. Photomicrographs of histological findings were obtained at different magnification.

#### Qualitative estimation of proteins

It was done by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared by heating them to 100°C for 3 min in 1 x SDS gel loading buffer [0.5 M Tris Cl (pH=6.8), 100 mM  $\beta$ -mercaptoethanol, 20% SDS, 0.1% bromophenol blue and 10% glycerol]. 35  $\mu$ l of denatured samples (25  $\mu$ g of total protein) was resolved on a 10% SDS-polyacrylamide slab gel at 25 Ma in Tris-glycine electrophoresis buffer [25 Mm Tris Cl, 250 Mm glycine (pH=8.3) and 20% SDS] by a method as described by Orenstein and Davis [4,5]. The gel was stained by Coomassie blue R250 and analysed by gel documentation system software Quantity one (Biorad, Australia).

#### Quantitative estimation of proteins

Protein contents in different samples of cell lysates, prepared by repeated freeze thaw were determined by standard Folin's method. 200  $\mu$ l of reagent [alkaline copper solution: 25 ml of reagent A (2%  $\text{Na}_2\text{CO}_3$  in 0.1 N NaOH) + 0.5 ml of reagent B (0.5% of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 1% sodium potassium tartarate)] was mixed with 40  $\mu$ l of cell lysate followed by incubation at room temperature for 10 min. 20  $\mu$ l of Folin-Ciocalteu's Phenol reagent (freshly diluted with water in 1:1 ratio) was added to the above reaction mixture and allowed to stand at room temperature for 30 min. Absorbance was measured on an ELISA plate reader (Lab System, Finland) at 620 nm with water taken as blank.

#### Morphological evaluation of apoptotic cells

Thymocytes smeared on a slide were air dried, fixed in methanol, and stained with Wright staining solution, mounted in glycerine and analyzed under light microscope at 450 x magnifications. The percentage of apoptotic cells were determined by counting more than 300 cells in at least three separate fields.

Percent DNA fragmentation was quantified following a method described by Sellins and Cohen [6] with slight modifications. Brain cells or thymocytes ( $5 \times 10^5$  cells/ml) were suspended in 0.5 ml of lysis buffer (Tris-EDTA buffer, pH=7.4 containing 0.2% Triton x100 and were centrifuged for 15 min at 13000 x g at 4°C in a microfuge tube (labeled 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 x g for 10 min and then DNA in each pellet was hydrolyzed in 80  $\mu$ l of 5% trichloroacetic acid by heating on a water bath at 90°C for 15 min. 160  $\mu$ l of freshly prepared diphenylamine (150mg diphenylamine in 10ml glacial acetic, 150  $\mu$ l concentrated  $\text{H}_2\text{SO}_4$  and 50  $\mu$ l of acetaldehyde solution) was added and the tubes were allowed to stand overnight at room temperature to develop colour. 100  $\mu$ 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.

#### Cell proliferation assay of brain cells or thymocytes

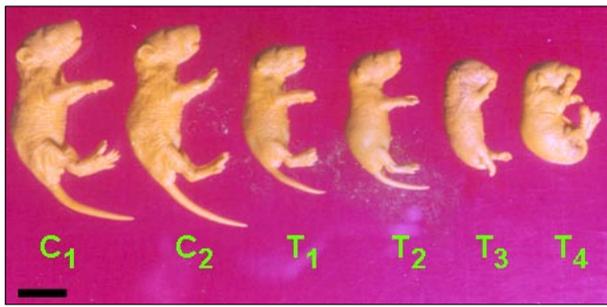
$1 \times 10^6$  cells were seeded in medium supplemented with 5% fetal calf serum in a 96 well tissue culture plate and incubated at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in air for 72 h in a  $\text{CO}_2$  incubator (Sheldon, USA). MTT (Tetrazolium) assay was carried out to estimate cell proliferation following a method described by Singh et al [7]. MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at a concentration of 5 mg/ml. 50  $\mu$ l of MTT solution was added to each well of culture plate containing 200  $\mu$ l medium and incubated at 37°C for 4 hours. Medium was then removed carefully without disturbing the dark blue formazan crystals. 50  $\mu$ l of DMSO was added to each well and mixed thoroughly to dissolve the crystals of formazan. The plates were then read on a microplate reader (Lab System, Finland) at a wavelength of 540 nm. Readings are presented as OD at 540 nm.

#### Determination of cell viability in brain

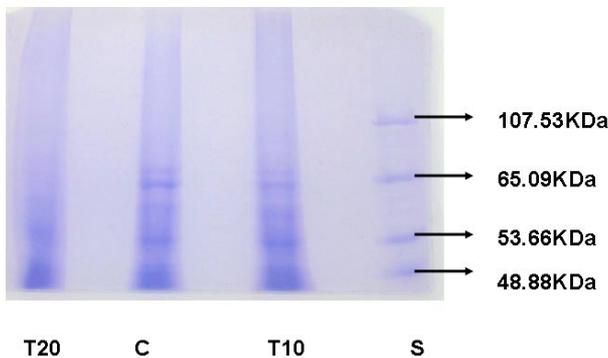
The percentage of viable brain cells was counted using Trypan blue dye exclusion test. 10  $\mu$ l sample of cell suspension was mixed with an equal volume of 0.4% trypan blue in PBS and the cells were counted using a haemocytometer. Cells that did not exclude the trypan blue dye were considered nonviable. Percent viable cells were calculated according to the formula:

$$\% \text{ viability} = [(TNC-TBSC)/TNC] \times 100$$

where TNC = total number of cells and TBSC = Trypan blue stained cells.



**Figure 1.** Photographs of pups collected on day 19 of gestation. The left side two pups  $C_1$  and  $C_2$  are controls and the other four pups on the right side were exposed to cyclophosphamide (CP) on day 11 of gestation with 10 ( $T_1$  and  $T_2$ ) or 20 ( $T_3$  and  $T_4$ ) mg/kg dose. The treated pups were smaller in size and showed gross malformations. The scale bar indicates 1 cm. Color version of figure is available online.



**Figure 2.** Gross photographs of qualitative assessment of brain protein profile collected from brain of pups collected on day 19 of gestation. Proteins in the Freeze thaw lysate of normal or cyclophosphamide (CP) treated brain were resolved by 10% SDS-PAGE under reducing condition at 25 mA. The gel was stained with Coomassie blue R250. Color version of figure is available online. (**T20**: 20 mg/kg CP treated brain protein showing absence of 65.09, and 53.66 KDa bands; **C**: Control brain protein showing presence of 65.09, and 53.66 KDa bands; **T10**: 10 mg/kg CP treated brain protein showing absence of 65.09 KDa band; **S**: Brain showing standard protein bands of 107.53, 65.09, 53.66, and 48.88 KDa)

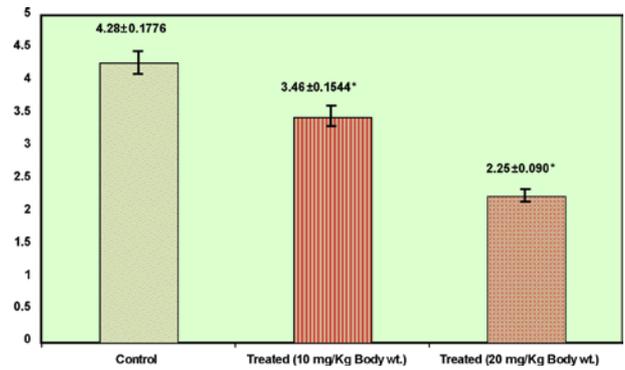
**Table 1.** Fetal effects following cyclophosphamide exposure in utero.

Groups	Body weight of fetuses (g)	Weight of fetal thymuses (mg)	Weight of fetal brains (mg)
Control (Normal Saline)	1.55 ± 0.121	8.76 ± 0.782	32.41 ± 2.181
Treated (10mg/kg)	1.02 ± 0.079*	5.14 ± 0.455*	21.68 ± 1.459*
Treated (20mg/kg)	0.68 ± 0.059*	3.23 ± 0.287*	14.52 ± 0.974*

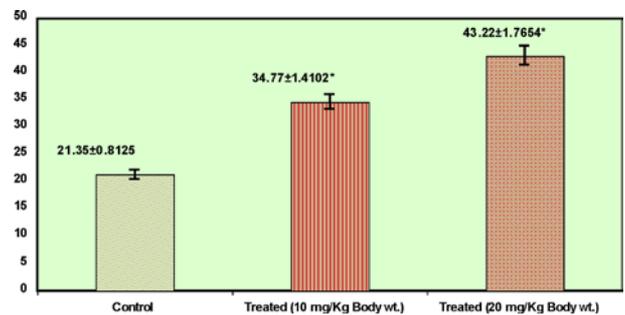
Values are mean ± SD of 3 independent experiments done in triplicate. (\* $p < 0.05$  verses values for normal or cyclophosphamide treated mice.)

### Statistical analysis

The results have been expressed as mean ± 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.



**Figure 3.** Effect of in-vivo administration of cyclophosphamide to pregnant mice on total protein content of fetal brain. Brain tissue obtained from normal or CP treated foetuses was lysed by freeze thaw technique and protein content in the lysate was measured by standard Follin's method. Values are mean ± SD of independent experiments done in triplicate. \* $p < 0.05$  verses values for corresponding control. Color version of figure is available online.



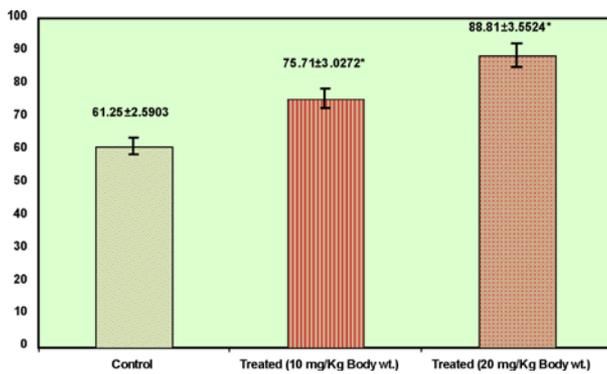
**Figure 4.** Effect of in-vivo administration of CP to pregnant female mice on the induction of apoptosis in fetal thymocytes. Percentage of apoptotic cells was determined on the basis of morphological features in Wright stained preparation of single cell suspension of thymocytes. Values are mean ± SD of 3 independent experiments done in triplicate. \* $p < 0.05$  verses values for corresponding control. Color version of figure is available online.

### Results

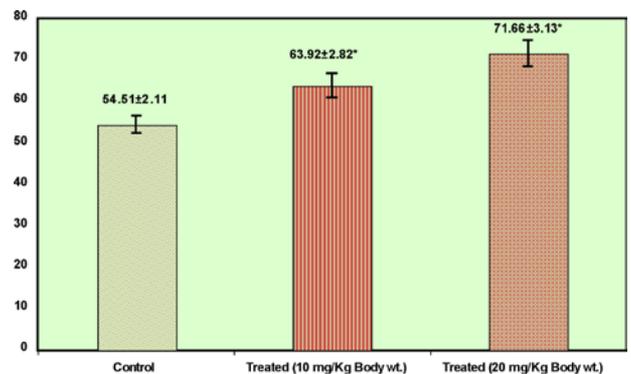
Gross examination of the fetuses exhibited significant growth retardation along with a significant reduction in weight of brain and thymuses (Fig. 1 and Table 1).

Important histological finding in brain was hydrocephalus and loss of lobular architecture was seen in thymus. Results of SDS-PAGE analysis is shown in (Fig. 2). Protein band corresponding to molecular weight of 65.09 KDa present in the control group was found disappeared in the brain of cyclophosphamide treated fetuses at both the dose tested. Whereas protein band corresponding to molecular weight of 53.66 KDa was found to decrease in intensity in 20 mg cyclophosphamide treated fetuses compared to that of control fetuses.

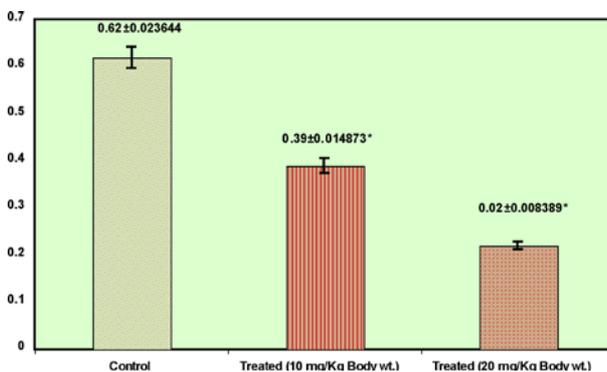
Results of quantitative protein estimation are shown in (Fig. 3,  $p < 0.05$ ). A dose dependent decline in protein content of brain tissue was observed following cyclophosphamide treatment to pregnant mice as compared to that of brain of fetuses obtained from normal. Protein contents were found to decrease in cyclophosphamide treated group.



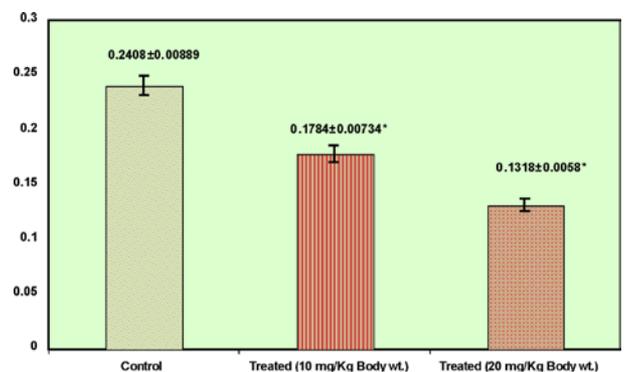
**Figure 5.** Effect of in-vivo administration of CP to pregnant female mice on % DNA fragmentation of brain cells of fetus. Percent fragmentation of DNA obtained from brain cells of control and CP treated groups was quantified described in materials and methods. Values are mean  $\pm$  SD from 3 independent experiments done in triplicate. \* $p < 0.05$  verses values for corresponding control. Color version of figure is available online.



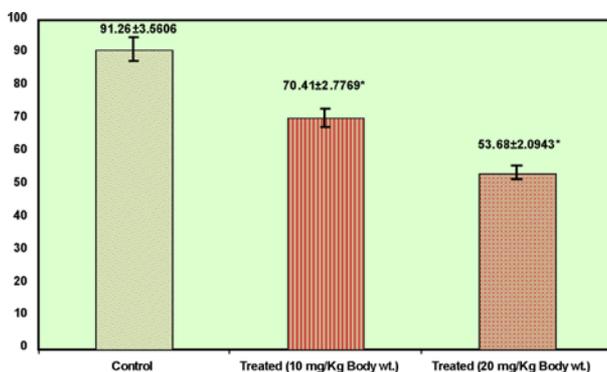
**Figure 6.** Effect of in-vivo administration of CP to pregnant female mice on % DNA fragmentation of thymocytes of fetus. Percent fragmentation of DNA obtained from thymocytes of control and CP treated groups was quantified as described in materials and methods. Values are mean  $\pm$  SD from 3 independent experiments done in triplicate. \* $p < 0.05$  verses values for corresponding control. Color version of figure is available online.



**Figure 7.** Effect of in-vivo administration of CP to pregnant female mice on proliferation of brain cells of fetus. Brain cells ( $1.5 \times 10^6$  cells) from control or CP treated groups were incubated in vitro for 72h in presence of phytohaemagglutinin (2  $\mu\text{g}/\text{ml}$ ). Cell proliferation was checked by MTT assay as described in materials and methods. Values are mean  $\pm$  SD of OD at 540 nm of 3 independent experiments done in triplicate. \* $p < 0.05$  verses values for corresponding control. Color version of figure is available online.



**Figure 8.** Effect of in-vivo administration of CP to pregnant female mice on proliferation of thymocytes of fetus. Thymocytes ( $1.5 \times 10^6$  cells) from control or CP treated groups were incubated in vitro for 72h in presence of phytohaemagglutinin (2  $\mu\text{g}/\text{ml}$ ). Cell proliferation was checked by MTT assay as described in materials and methods. Values are mean  $\pm$  SD of OD at 540 nm of 3 independent experiments done in triplicate. \* $p < 0.05$  verses values for corresponding control. Color version of figure is available online.



**Figure 9.** Effect of CP on the viability of brain cells of fetus exposed to CP in intrauterine life. The total count of viable brain cells was done in haemocytometer using the trypan blue dye exclusion test as described in materials and methods. Values are mean  $\pm$  SD for viable brain cells of 3 independent experiments done in triplicate. \* $p < 0.05$  verses values for corresponding control. Color version of figure is available online.

In order to check, the mode of cell death in thymocytes obtained from fetuses' cyclophosphamide treated mice; morphological analysis of such cells was performed. The occurrence of augmented apoptosis in the thymocytes of cyclophosphamide treated fetuses was further confirmed by counting of percentage of apoptotic cells by wright staining of thymocyte smears (Fig. 4,  $p < 0.05$ ). Cyclophosphamide administration resulted in an increase in the number of apoptotic thymocytes in a dose dependent manner.

This observation was later confirmed by quantification of percent specific DNA fragmentation. Percent DNA fragmentation in the DNA from both brain cells (Fig. 5,  $p < 0.05$ ) and thymocytes (Fig. 6,  $p < 0.05$ ) of cyclophosphamide treated fetuses showed a dose dependent increase as compared to that of control group. Effect of cyclophosphamide administration on cell proliferation assay of brain cells and thymocytes

obtained from fetuses of cyclophosphamide treated or control mice were assayed by standard MTT method as described in materials and methods. Cyclophosphamide administration resulted in a dose dependent massive inhibition in brain cell (Fig. 7,  $p < 0.05$ ) and thymocyte (Fig. 8,  $p < 0.05$ ) proliferation as compared to the level of proliferation in the brain cells or thymocytes of control fetuses.

The percentage of viable fetal brain cells decreased after cyclophosphamide administration in pregnant mice in a dose dependent manner (Fig. 9,  $p < 0.05$ ).

### Discussion

The precise reasons for the observed atrophy of brain are not clear however the possibilities can be considered. As cyclophosphamide has been reported to be a well known antimetabolic agent it is likely that the loss of brain weight might be contributed to by a gross loss of cell number in the growing fetal brain. Indeed we observed that the count of total cells in brain cell suspension of fetuses obtained from cyclophosphamide treated group was significantly lower than that of control group. The loss in the cellularity in the brain tissue could be attributed to two factors: 1) A decrease in proliferation of brain cells and 2) Induction of cell death in the brain cells of cyclophosphamide treated fetuses. The results of the present study corroborate both the possibilities. Brain cells obtained from cyclophosphamide treated fetuses upon incubation *in vitro* showed a decreased proliferative ability as compared to brain cells of untreated fetuses. Moreover, the brain cells of fetuses obtained from cyclophosphamide treated mice showed an increased population of cells with typical apoptotic morphology. Therefore, it is suggested that the brain atrophy in fetuses obtained from cyclophosphamide treated mice may not only be due to decreased proliferation of brain cells but also due to the 'switching on' of certain preexisting cell death inducing program. Our interpretations are also supported by reports of other workers who demonstrated that cyclophosphamide treatment can result in the induction of apoptosis in a variety of immature and mature cell types including neurons [8,9].

The overall suppression in the expression of proteins of cyclophosphamide treated brain cells may indicate a defect in gene expression in certain enzymes and other proteins responsible for the maintenance of cell viability. A loss of these proteins may culminate in the induction of apoptosis in cyclophosphamide treated cells [10–12]. Cyclophosphamide treatment has been shown to differentially alter expression of more than 100 genes associated with genes associated with drug detoxification, cell cycle control, and apoptosis [13,14].

The mechanism of cyclophosphamide induced DNA break in embryonic cephalic tissue in a time and concentration dependent manner. Thus this could be the additional mechanism by which cyclophosphamide could result in abnormal neurotransmission which in its turn may further end to the atrophy of the neuronal tissues. Indeed there are evidences indicating that cyclophosphamide administration results in inhibition in the production of

neurotransmitter acetylcholine esterase. The absence of which is associated with a massive neuronal damage [15,16]. Ogiso et al [17] showed that CP caused a considerable decrease in thymus weight and the number of thymus lymphocytes and caused a significant involution of the thymus. Mouse thymocytes were induced to apoptosis by cross linked recombinant soluble human Fas ligand both *in vitro* and *in vivo*, though human thymocytes were resistant to this mode of receptor ligation. Membrane-bound Fas ligand also induced apoptotic death in murine thymocytes but not in human thymocytes [18]. Furthermore, CP prevented specific proliferation of T cells and decreased cytotoxicity of alloantigen specific T cell lines by direct induction of apoptosis [19]. A number of investigations indicate that cyclophosphamide treatment of lymphocytes result in inhibition of antigen specific proliferation, decreased cytotoxicity, along with an increase in programmed cell death [20,21].

Several earlier reports have indicated conflict regarding the effect of CP on the proliferative and functional ability of T lymphocytes. While a majority of reports indicate the antimetabolic and apoptosis inducing effect of CP on thymocytes, sporadic observations also indicate that CP may result in an enhanced T cell proliferation. There could be several reasons for this discrepancy such as the age of host treated with cyclophosphamide may determine the extent of susceptibility of thymocytes to such treatment. While immature T lymphocytes have been considered to be more prone to exposure of alkylating agents like cyclophosphamide as compared to the more differentiated and mature lymphocytes, thymocytes and splenic T cells of young mice have been reported to be more susceptible to CP than those of old mice and were decreased in number after the treatment with even a low dose of cyclophosphamide [9,22,23]. The second reason could be the dose of cyclophosphamide used in the study demonstrating the augmentary effect of cyclophosphamide on T cell proliferation. Indeed it has been observed that treatment with low dose of cyclophosphamide is followed by an increased proliferation of thymocytes whereas a high dose of the drug induced suppression of the proliferative capacity [24]. Keeping in view above mentioned background our observation however was a dose dependent decline in proliferative ability of thymocytes obtained from fetal thymuses of same age.

The neuroimmunological aberration manifested by prolonged cyclophosphamide administration has been demonstrated by Farrell et al [25], who showed that cyclophosphamide administration caused abnormal infiltration of immunocompetent lymphocytes in choroid plexus of brain. This could be one of the reasons for the observed brain atrophy in our system as we observed several areas of brain showing development of lesions with massive loss of cells. Development of such lesions in brain have been attributed to immunological activation of glial cells and lymphocytes in the brain tissue and cyclophosphamide has been demonstrated to contribute to the development of such lesions in brain [3]. Neuroimmunological consequences of cyclophosphamide

administration have been demonstrated on the functions of T cells. Intraperitoneal injection of cyclophosphamide has been shown to result in inhibition of T cell function such as delayed type of hypersensitivity responses which was dependent upon a peptide of about 10,000 molecular weight of central nervous system. Our observations are also in the similar lines indicating that cyclophosphamide administration alters the profile of brain peptides, which

may have a direct impact on T cell differentiation and functions.

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