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Effect of cyclophosphamide administration during late gestation period on post natal survival and growth of rats

Anatomy

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ABSTRACT

The aim of this study was to compare the teratogenic effects cyclophosphamide among the pregnant female rats exposed with cyclophosphamide (CP) with the normal pregnant rats. 29 pregnant female rats were involved. 6 were control (injected normal saline) and rest were categorized as group 1(7 rats), group 2(7 rats) and group 3(9 rats), which received CP(10mg/kg body weight) in increasing order of single dose, double dose and triple dose respectively. The fetus or the newborns were analysed for litter size, mortality rate, weight, CR length, tail length and PC length. Litter size in control group was 100% while it decreased in dose dependent manner in the rats exposed to CP. The gestation period was also prolonged in these drug treated rats. We also found significant decrease in body weight, tail length, CR length and PC length in the offspring of the female rats treated with CP.

KEYWORDS:

Teratogen, Cyclophosphamide, Gestation

Introduction

Birth of malformed babies has taxed the ingenuity of men ever since the dawn of history. The causes of such anomalies dates back to Vedas in which Agonies asks the following question to Lord Punarvasu, "Could you kindly explain the reason why a woman gives birth to a defective offspring, like one having less or more organs or possessing any other type of defect in the sensory or motor organs. The discipline which deals with anomalies is known as "Teratology". The term teratology is derived from the Greek word Teras, which means monster. In turn the word monster is derived from Latin word monstrum which means something providing fore-knowledge of coming events (monstrase-to show monere-to warn). Developmental abnormalities in fetus can be induced by various chemical agents or drugs or pollutants or radiations or the disease that lead to production of free radicals and oxidative stress [1,2]. Study has revealed that almost 7 to 10 % of anatomic anomalies in human develop because of disruption drug action, viral injections or the environmental factors [3]. According to DeSannti's et al, drug therapy attributes to 1%m of congenital anomalies of known etiology [4].

Cyclophosphamide, a drug used in the treatment of neoplastic diseases [6] like lymphomas, leukemia, solid tumors and some brain cancer [7] and autoimmune disease [8], like SLF (Systemic lupus erythromatosus) , RA (rheumatoid arthritis), Granulomatosis and nephrotic syndrome [9], is a common teratogen inducing number of birth defects, when females during their pregnancy are being treated with this drugs [10]. Chemically cyclophosphamide is an alkylating agents [11] belonging to the oxazaphospharine class [12] and undergoes various complicated process for activation and inactivation [13,14]. The drugs remain inert in its parental form cyclo therefore in order to produce teratogenicity, it must undergo bioactivation process that require oxidase enzyme (cytochrome P450) converts cyclophosphamide to its active metaboloties phosphoramide, namely mustard and acroleun [15].This transformation mainly occurs in liver, however such metabolic activation of this drug can also occur in other tissues too.

Phosphamide inhibits synthesis of DNA nd forms inter and intra chain DNA crosslinks via the linkage of reactive alky groups [16] in the existing DNA strands resulting in the cell death [17]. On the other hand acrolein inducers the side effect of cyclophosphamide chemotherapy, most common one being cystitis [3] The exact underlying mechanism of CP induced teratogenicity is still debated, however some studies have demonstrated that reactive oxygen species (KOS) generated from the active metabolites of CP are responsible for the CP mediated anomalies. [15,18]. Previous studies conducted on rodents have revealed that cyclophosphamide when administered during gestation causes delayed fetal developmental or embryological fetal resorption, growth retardation followed by multiple anomalies such as exencephaly, limb and skeletal defects etc [5,19].

The exposure of fetus to ROS can be carefully managed by allowing exposure to occur only when the level of antioxidants are high thereby aiding in the shortening of ROS signal duration and facilitating the cells to repair the damaged DNA ([20]. However this condition is not feasible if the exposure of fetus to ROS occurs when their levels are excessive with the inadequate presence of antioxidants thereby causing neural tube defects death of fetus or skeletal malformations [5]. Study of Ashy et al demonstrated reduced embryotoxicity of CP drugs with the inhibition of P450enzyme system and induction of antioxidants (both enzymes and substances [21,22].

Therefore this research was initiated with aim of evaluating the effects of CP drug use at different doses, in the fetus during the gestational period, so that our result may be helpful in the furthers researchers and in the use of CP as potent chemotherapeutic agent with reduction of its side effects to the developing embryo.

Method and materials

This study was conducted in the department of anatomy, Banaras Hindu University, after taking the ethical clearance from the institute. We employed female rats of Charles Foster Strain for the study. The average age of rats was around 120 days with average weight of 200 grams. Initially theses rats were kept in separate cages in the air conditioned room whose temperature was maintained around 22^0C. Then the following procedures were carried out.

Mating

Vaginal smear of the rats was examined everyday to determine proestrous phase which was confirmed by the presence of large number of nucleated cells. The female rats in proestrous phase were caged overnight with the male rats of same strain. On the next day vaginal smear of the rats were examined to confirm the pregnancy.
It was confirmed by the presence of sperm which indicated impregnation thatmight have occurred during midnight and morning. This was considered as the "Day Zero" of the gestation. The pregnant rats were then kept in separate cages and fed with Hind Lever diet and water adlib. The weight of rats were taken daily till the time of delivery.

Drug Administration

The drug we used in this study was cyclophosphamide.
i. Preparation of drug solution

We used freshly prepared solution of CP as the solution remains potent for two hours only. The CP solution was prepared under sterile conditions by dissolving 100 mg of CP in 100 ml of normal saline.

ii. Intraperitoneal (IP) application of drugs
The pregnant rats were divided into four groups depending upon the dose of the drugs to be administered.

Group 1 (Single Dose Group)
Rats in this group were injected CP at the dose of10 mg/kg body weight IP on the 15th day of gestation.

Group 2 (Double Dose Group)
We injected CP (10 mg/kg body weight) on the 15th and 16th day of gestation in these rats.

Group 3 (Triple Dose Group)
The rats were given CP (10mg/kg body weight)on the 17th, 18th and 19th day of gestation.

Group 4 (Control Group)
The rats under control group were injected with vehicle (Saline without drugs) on the corresponding days.

Calculation of drug dose and mode of administration
The CP dose of 10mg/kg body weight of rats was calculated as:

$$\frac{weight}{1000} X\ Dose \frac{X100mg}{ml} = Divisions\ of\ the\ tuberulin\ syringe\ (1:100)$$

Drug was injected via tuberculin syringe in which 1ml is divided into 100 divisions. The CP drug solution was prepared that contained 10 mg CP/1ml of saline. Therefore, for the pregnant rats weighing 300 grams, required CP solution at the dose of 10 mg/kg will be:-

$$\frac{300}{1000} X\ 10\ X \frac{100}{10} = 30\ divisions\ of\ tuberculin\ syringe$$

The CP was injected intraperitoneally followed by recoding the weight of the animal everyday till delivery. The neonate rats born from the four different groups were weighed separately and the anthropometric measurements were taken from the day of delivery upto the post natal day 70. The measurements were taken with help of slide calipers.

1. Body Length (Crown Rump Length or CR length): It was measured from the tip of the nose to the root of the tail. The measurements were taken by applying gentle pressure over the backs of the neonates so as to straighten their vertebral column with least stress.

2. Tail Length: It was measured from the root of the tip of the tail.

3. Patellocalcaneal Length (PC length): It was measured from the lower border of patella to calcaneous in semiflexed condition.

Results

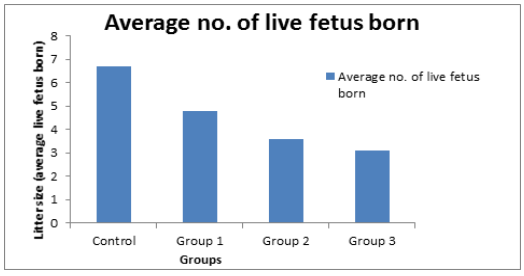
Our investigation showed that the drug cyclophosphamide reduces the number of live fetus born, increases the rate of mortality, increases the gestation period and produces the morphological changes in the offsprings.

Table 1. Litter size (number of life fetuses born)

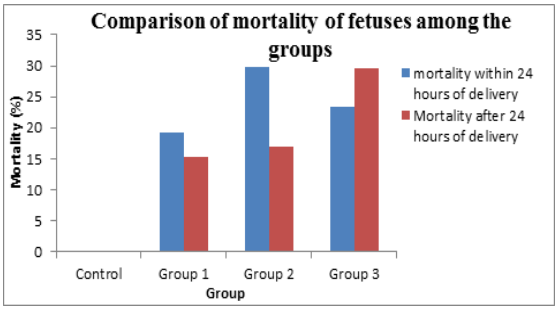
Groups	No. of Pregnant rats	No. of fetuses born	No. of live fetus born	Average No. of live fetus	% of live fetus
Control	6	40	40	6.7	100%
Group 1	7	52	34	4.8	65.38%
Group 2	7	47	25	3.6	53.19%
Group 3	9	64	30	3.1	46.87%

Table 2. Mortality of fetus due to effect of cyclophosphamide

Groups	Total no. of fetuses born	Mortality within 24 hrs of delivery	Mortality after 24 hrs of delivery
Control	40	0	0
Group 1	52	10 (19.23%)	8 (15.38%)
Group 2	47	14 (29.78%)	8(17.02%)
Group 3	64	15(23.43%)	19 (29.68%)



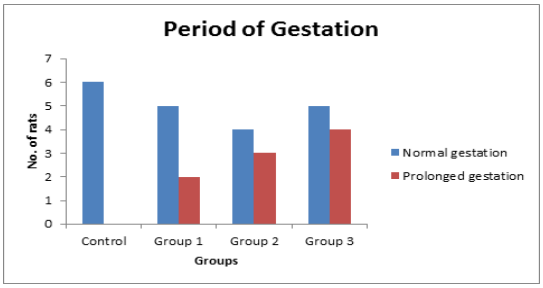
The average number of live fetus born greatly reduced in the drug treated group than in comparison to control. The mean litter size gradually declined as the gestation period was prolonged due to the effect of drug. The mean litter size was significantly reduced in all treated groups, most marked being on triple dose treated groups. Incase of controls, 100% of the fetuses survived while the survival rate significantly decreased depending upon the dose of CP. Only 46.87% of fetus survived at the time of birth in case of triple dose group.



We compared the mortality rate of the fetus within the 24 hours of delivery and after 24 hours of delivery till postnatal day of 70. We observed that the mortality of fetus within 24 hours of delivery was high in double dose group 29.78% whereas rate of mortality after 24 hours of delivery gradually increased depending upon the dose. Incase of control group all the fetuses survived while the number of fetuses that died within and after 24 hours of delivery were 10(19.23%) and 8(15.38%) for group 1, 14(29.78%) and 8 (17.02%) for group 2 and 15(23.43%) and 19(29.68%) for group 3 offsprings respectively.

Table 3. Effect of cyclophosphamide on Gestation period

Group	No. of pregnant rats	Normal gestation	No. of rats		Percentage of rats with prolonged gestation
			Normal gestation	Prolonged gestation	
Control	6	21 days	6	0	0
Group 1	7	21 days	5	2	28.5%
Group 2	7	21 days	4	3	42.8%
Group 3	9	21 days	5	4	44.4%



In control group 6 rats were included, all of which delivered in the expected day that 21st day of gestation and none of the fetuses were dead in this group. In case of rats in group 1 (those who received single dose of cyclophosphamade), out of 7 rats involved, 5 rats delivered on the expected day of gestation while in case of remaining 2 rats, one rat delivered on 22nd day of gestation and the other on 23rd day of gestation. In case of group 2 rats, we included 7 rats that were treated with double dose of cyclophosphamide. Out of 7 rats, 4 rats delivered on the normal day (21st day of gestation) and the rest 3 had delivered on 22nd day of gestation. They showed prolongation of gestation by one day. The triple dose group (group 3) contained 9 rats, out of which 5 rats delivered on the expected day and the rest 4 rats showed prolongation in gestation by one day (i.e. They delivered on 22nd day of gestation).

Morphological changes

The offsprings delivered from the treated rats in all three groups did not show any gross malformations and appeared morphologically similar to those of control groups. The post natal growth and the maturity were assessed by the measurement of body weight, CR length, tail length and PC length. The records were taken regularly upto the postnatal day of 70.

Body weight

The mean weight gain in the offspring of the control rats showed uniform increase till the post natal day of 70 where the maximum gain in weight was 142.41gm. The weight gain in offsprings of Group 1 rats was significantly less in comparison to controls and the gain in weight till postnatal day of 70. was 82.04 gm.

In case of the group 2 rats, the offsprings showed steady increase in weight but the gain in weight was significantly less than that of controls. The average weight gained by day 70 was 78.06. Similarly in case of triple dose group (Group 3 rats), there was maximum average weight gained was 76.46 gm. Therefore our study shows that, there is significant reduction in weight gain in the offsprings of the female rats treated with teratogenic drugs in comparison to control and the effect is dose dependent (table, 4 fig1)

CR length

We compared the gain in CR length among the offsprings of control rats and the offsprings of drug treated rats. We found that the gain in CR length was significantly less in the treated groups (group 1, 2 and 3) in comparison to controls and the decrease was dose dependent (table, fig). The maximum CR length gained was 17.47 cm in controls while they were 14.9, 14.71 and 14.15 cm in the treated groups 1, 2 and 3 respectively.

Tail length

The mean gain in tail length in the offsprings of treated groups was rapid and uniform but the increase was significantly lower in comparison to those of controls. The maximum CR length gained was 16.38 cm in controls while they were 13.56, 13.29 and 12.01 cm in the treated groups 1, 2 and 3 respectively till postnatal day 70.

PC length

Like other morphological features, PC length gain was also slow was steady in the offspribgs of treated groups but the gain was significantly less in comparison to the offsprings of the control group. Th maximum average PC length gain measured by the end of postnatal day 70 was 4.48 cm in control while the maximum average PC lengths were measured to be 3.76, 3.49 and 3.49 cm respectively in the newborns of group 1, 2 and 3 respectively.

Table 4: Comparison of weight gain, CR length, tail length and PC length among the groups

Parameter	Days	Control	Group 1	Group 2	Group 3	Parameter	Days	Control	Group 1	Group 2	Group 3
Weight gain (gm)	1	6.55	5.5	5.58	5.02	Tail length	1	2.13	1.58	1.48	1.52
	5	9.135	7.76	7.66	6.82	(cm)	5	2.78	2.17	2.45	2.13
	10	13.81	11.8	10.76	10.84		10	4	3.34	3.47	3.23
	15	20.34	16.23	15.06	14.81		15	5.8	4.92	5.11	4.29
	20	25.19	19.04	18.06	16.67		20	7.23	6.48	6.42	5.75
	25	34.1	22.24	19.87	19.32		25	8.89	7.55	7.24	6.81
	30	44.02	25.77	22.13	20.75		30	10.79	8.82	8.39	8.01
	35	57.02	34.05	27.19	26.45		35	12.51	9.75	9.32	9.06
	40	67.12	40.7	31.35	34.41		40	13.49	10.82	10.28	9.56
	45	80.92	47.01	36.71	39.47		45	14	11.44	10.75	9.92
	50	93.96	58.8	48.19	45.03		50	14.6	11.99	11.83	10.82
	55	103.18	68.51	61.94	60.6		55	15.02	12.14	12.41	11.07
	60	122.45	63.08	69.28	68.98		60	15.49	12.5	12.98	11.47
	65	134.98	76.3	72.35	70.03		65	15.74	12.76	13.22	11.83
	70	142.41	82.04	78.06	76.46		70	16.38	13.56	13.29	12.01
CR length (cm)	1	5.6	4.79	4.9	4.65	PC length (cm)	1	1.15	0.89	0.86	0.86
	5	6.07	5.75	5.58	5.4		5	1.36	1.16	1.19	1.14
	10	7.13	6.63	6.49	6.37		10	1.74	1.48	1.44	1.37
	15	8.42	7.46	7.38	7.22		15	2.09	1.87	1.76	1.62
	20	9.49	8.36	8.01	6.42		20	2.36	2.11	2	1.92
	25	10.74	9.23	9.04	8.84		25	2.69	2.33	2.22	2.11
	30	12.5	10.16	9.25	10.18		30	3.13	2.56	2.34	2.45
	35	13.92	11.26	10.09	10.96		35	3.36	2.88	2.56	2.61
	40	14.15	11.81	11.56	10.09		40	3.6	2.99	2.71	2.72
	45	14.81	12.62	12.34	11.43		45	3.83	3.11	2.91	2.86
	50	15.16	13.26	12.71	12.22		50	3.99	3.32	3.11	3.06
	55	15.73	13.68	13.1	12.95		55	4.13	3.44	3.22	3.15
	60	16.24	13.81	13.78	13.42		60	4.19	3.54	3.32	3.26
	65	16.95	14.16	13.86	14.35		65	4.38	3.62	3.38	3.4
	70	17.47	14.9	14.71	14.15		70	4.48	3.76	3.49	3.49

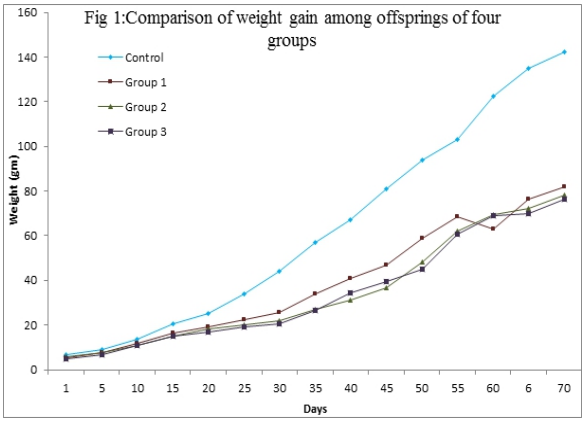


Fig 1:Comparison of weight gain among offsprings of four groups

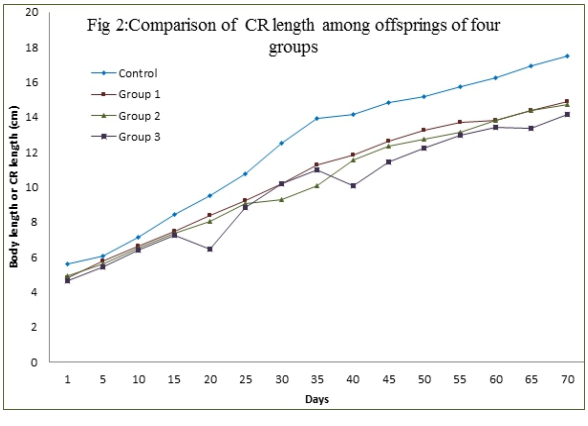
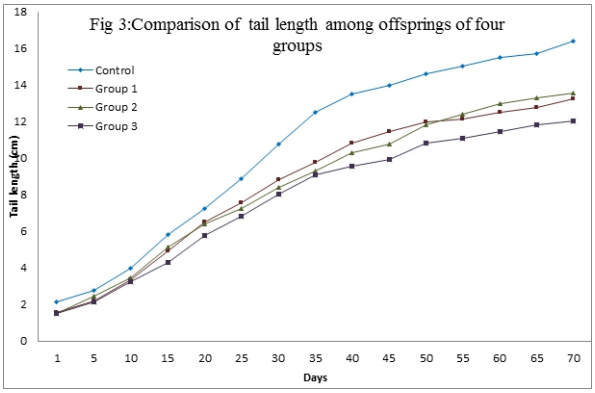
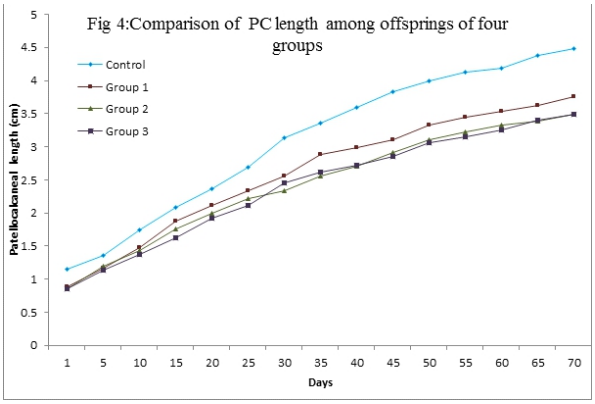


Fig 2:Comparison of CR length among offsprings of four groups





Discussion

In the present study, different doses of 10mg/kg of cyclophosphamide administration has revealed varying intensity of the effect of the drug on the fetuses depending on the day of insult. Our study showed the effects of cyclophosphamide on the average litter size, mortality of fetus, gestational period and post natal survival and growth of the fetuses.

There are many reports of the reduction in the litter size in mouse as well as rats following the maternal administration of teratogens during different days of gestation. Kreybig et al [23] reported reduced litter size on the maternal administration of cyclophosphamide. Similarly, Singh and Padmanabhan et al [24] showed significant reduction in litter size of the treated rats in comparison to controls when chlorpromazine was given to pregnant rats on the 14th day of gestation. The present study also served to prove that the litter size reduces significantly when the teratogens are administered to the pregnant rats and the effect was dose dependent.

In this investigation we also observed that cyclophosphamide besides being capable of causing teratogenic effects, also causes increase in fetal mortality. We classified fetal mortality into two categories namely fetal mortality within 24 hours of delivery and fetal mortality after 24 hours of delivery till post natal day of 70. Our study showed increased neonatal mortality within the 24 hours of delivery following the CP administration during the late gestation but it was not dose dependent. However, the mortality in the offsprings after 24 hours of delivery increased with increase in the dose of drug. In single dose group the moratality rate was 15.38% while in double and triple dose group the rates were 17.02% and 29.68% respectively. This shows that mortality rate of fetuses after 24 hours of delivery is dependent upon the dose of teratogen administered during the late gestation period. High mortality of fetuses of mice treated with cyclophosphamide was also reported by Gebhardt etal [25]. Lethal effects of cyclophosphamide were also reported by Gerlinger et al, who observed 60% mortality when the embryos were checked on 11th day of incubation and 77% were found dead by the 14th day.

Several teratogens also have shown to prolong gestation. Singh and Padmanabhan et al [24] reported prolongation of gestation in chlorpromazine treated rats. Similarly Hakenberger and Kreybig etal [23] showed maternal administration of cyclophosphamide in mice prolongs gestational period. Besides fetal and genetic control, the hormone level, uterine volume and placental aging are also the important factors initiating parturition. Alterations in any of these factors may prolong gestation. Therefore cyclophosphamide together with reduction in litter size and fetal size contributes to the reduction in the uterine volume. This may be the reason for the prolonged gestation. In our study the gestation was significantly prolonged in double dose group as comparison to single and triple dose group. This may be due to late treatment in the later part of gestation in case of triple dose group.

In the present study we also found that the postnatal survival of the offsprings decreased with the increase in the dose of cyclophosphamide which showed direct relationship between the dose of the teratogen and post natal viability of the offsprings.

Different teratogenic agents produce reduction in weight gain, tail length, CR length and PC length in the offsprings of the treated rats in comparison to the control. Study of Mahabady et al showed that CP when given intraperitoneally to the pregnant mouse on 10th day of gestation induced fetal anomalies [5]. Sloth and Hales et al experimentally observed that CP given at the dose of 10 and 15mg/kg to the rats on 13th day produced teratogenicity in 50% and 100% fetuses respectively [26]. Gibson and Becker et al demonstrated that CP induces teratogenicity in 67.3% of fetus when given at the dose of 20 mg/kg to the pregnant rats on 9-14th day of gestation [27]. They also showed reduced weight, CR lengths and tail lengths in the offsprings of the treated rats, which was similar to the findings of our study. The varying susceptibility of the different parameters may be attributed to the different growth potentials of various parts of body during different periods of development.

Some studies have stated that the teratogenicity induced by CP is due to increased susceptibility of tissue to undergo apoptosis and alterations in the expression of p53 gene, especially in neurons. The toxic metabolites of CP are eliminated from the body via phase II biotransformation reactions and via the mitochondrial antioxidants like glutathione and catalase systems [21,28]. Acrolein and phosphoramide, the products of CP, cause mitochondrial membrane lipid peroxidation and liberation of cytochrome c, hence activating caspases 3,8 and 9 along with p53 gene [29].

Goodman etal stated that CP induced teratogenicity involves the cellular death by limiting the synthesis of DNA. CP at high doses produces extensive cross linkages in DNA causing fragmentation. This capacity of CP to interfere normal mitosis and in cell proliferation serves as the basis of its therapeutic roles but also explains the toxic effects of this drug [30].

Number of teratogenic drugs such as anti neoplastics have been known to induce oxidative stress which is toxic to the growing fetus [31]. Oxidative stress results from the imbalance between reactive oxygen species (ROS) production and their clearance. CP by increasing the production of ROS causes both physiological and biochemical alterations [32].

Conclusion

Cyclophosphamide, a teratogen that causes fetal toxicity when given during pregnancy, can cause birth defect, retarded growth or miscarriages, due to its antimitotic and apoptotic effects . It can produce fetal abnormalities in number of species including mice rats, rabbits, hamsters and humans. The toxicity induced by CP is generally manifested as the deformities in CNS, skeletal system and facial anomalies. Therefore not only the drug should be avoided during the first trimester of pregnancy, also the women must be warned to avoid pregnancy while on treatment with CP.

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