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Research article

DEVELOPMENTAL TOXICITY OF IBUPROFEN TREATED MICE

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ABSTRACT

The effect of ibuprofen – an anti-inflammatory, analgesic drug was tested on pregnant females at different developmental phases. The fetus was collected on 12th, 15th and 18th gestation days (gd). Embryos on the day of parturition and 5 day old new born were considered for experimental study. Ovary and uterus of the mother was also taken into account for histopathological examination. The adverse effects on pregnancy outcome included a significant reduction in the number of implantation and significant increase in percentage of post implantation loss. The body weight as well as physical characteristics varied due to the treatment at different phases of gestation. The concentrations of brain DNA and RNA of the fetus and the pups were also lower compared to controls. A major change in the histological architecture was seen in the sections of ovary and uterus of the mother. These findings suggest the susceptibility of the drug to the mother as well as to the embryo.

Key Words: Ibuprofen, Fetus, Histopathology, DNA and RNA

INTRODUCTION

A number of reports suggest that non-steroidal anti-inflammatory drugs (NSAID) have been used primarily for the treatment of rheumatic disease, musculoskeletal disorders, dysmenorrhoea, inflammation and for post-operative pain[1-3]. Besides being anti-inflammatory these drugs are analgesic and anti-pyretic. More than 100 million NSAIDs are prescribed throughout the world[4]. The chemical class of 2-arylpropionate to which ibuprofen belongs is now being widely used as largest single group of NSAID. Nevertheless, ibuprofen has also been associated with gastric lesions and bleeding, which is due to the inhibition of COX, the enzyme which is responsible for synthesis of prostaglandin[5,6]. More recently another group of NSAID, a non-specific COX inhibitor, have gained attention as an effective therapy for tumor patient, against the incidence and mortality of colorectal cancer and reported to potentates the anti-cancer effects of cisplatin on human invasive bladder cancer[7]. Prior toxicological studies on ibuprofen were limited to rats

and dogs with repeated dose toxicity and the side effects of the drug thus showed gastrointestinal lesions. Drug developmental effects after triple daily doses administration of ibuprofen showed developmental anomalies in rats[8]. Though the drugs enter the fetal circulation, it does not show any teratogenic activity but influence to change in organ weight in rats. The present studies were conducted to examine the foetotoxic potential of ibuprofen in terms of the estimates of the dimension of each developmental period as well as measurements of the content of nucleic acid in the brain of mice. Microscopic examination of the reproductive tissues particularly the ovary and the uterus were used to characterize the effects of the drug on the reproductive parts.

MATERIALS AND METHODS

Animals

Sexually matured laboratory mice (*Mus musculus*) were housed in cages and received food and water *ad libitum.* The animal rooms were maintained at 22ºC to 25ºC and a relative humidity of 50 to 60 % with 12 h light and dark cycles. Female mice were allowed to mate to adult males of proven fertility overnight and were separated the following morning. Insemination was determined by the presence of sperm in the vaginal smears taken at the time of separation. The day on which sperm were found designated as day-0, and the female was placed in an individual cage at that time. The experiments have been conducted with due permission from Institutional Animal Ethics Committee (IAEC) formulated through CPCSEA.

Experimental designs

As per therapeutic doses pregnant females were fed 100 mg of ibuprofen (Boots India Ltd.)/ Kg of body weight, at different developmental phases separately according to the following schedule.

Phase	Gestation
Pre-implantation (PI)	1-4
Early Placental (EP)	5-10
Placental (PL)	11-19

The control set of 21 to 24 mice was similarly administered distilled water. The animals were sacrificed on 12^{th}, 15^{th} and 18^{th} day of gestation to collect fetus. The embryos on the day of parturition and 5 day old new born were also taken into consideration.

Maternal and fetal observation

Animals were observed daily for clinical sign of toxicity. On specific gd. mice were killed by cervical dislocation. Uterine contents (i.e. the number of implantation sites, resorptions, dead and live fetuses) were evaluated.

Assay procedure

Nucleic acid extraction was done following the method of Geel & Timirus[9]. DNA was assayed colorimetrically according to Burton Diphenyl procedure[10] using calf thymus DNA (Sigma) for the standard. RNA was assayed colorimetrically according to Ceriotti's orcinol procedure[11] using yeast RNA (Sigma) for the standard.

Histopathological examination

The ovary and uterus were fixed for histological examination. Tissues were trimmed, embedded in paraffin, sectioned and stained with Hematoxylene and Eosin. Representative cross sections were then examined for evidence of pathological changes.

Statistical evaluation

Statistical evaluation of quantitative data relative to fetus and pups (body weight, nucleic acid concentration) were performed using ANOVA and the means were compared using Student's 't' test. Probability test ($\propto = 0.05$) was used for pair-wise comparison between each ibuprofen treated group and the control group.

RESULTS

General reproductive performance

No maternal death or distinctive clinical sign were observed during the period under study. The results of the general reproductive performance are summarized in Table 1. Treatment related adverse effects on pregnancy outcome included a significant reduction in the number of implantation sites for litter both in PI and EP phase (Table 1).

Table 1 : Table shows developmental toxicity in mice following maternal exposure to ibuprofen

Control	Days				
	12	15	18	0	5
% pregnant	87.5	95.6	91.3	91.7	92.9
(No. preg. / No. mated)	21/24	22/23	21/23	22/24	24/26
Average No. of implantation sites/dam	13.20±0.10	14.40±0.50	13.77±0.40	13.83±0.40	14.01±1.10
%post-implantation loss/dam	0.30±0.02	0.32± 0.02	0.30±0.03	0.31±0.01	0.31±0.04
Average No. of life fetuses/dam	12.9±1.2	11.9±1.5	11.3±1.3	11.7±1.2	12.0±1.4
	Pre-implantation				
% pregnant (No.preg./ No. mated	88.2 15/17	94.7 18/19	90.0 18/20	94.4 17/18	95.0 19/20
Average No. of implantation sites/dam	5.47±0.02	5.87±0.02	5.58±0.02	5.85±0.02	5.89±0.02
Average No. of life fetuses/dam	NA	NA	NA	NA	NA
% post-implantation loss/dam	99.99	98.99	98.99	99.00	99.00

		Early placental			
% pregnant	89.47	95.0	94.4	90.0	95.4
(No.preg./No. mated	17/19	19/20	17/18	18/20	21/22
Average No. of implantation sites/dam	6.00±0.13	6.01±0.13	5.98±0.13	5.70±0.13	6.54±0.13
% post-implantation loss/dam	11.10±0.23	13.04±0.27	18.18±0.19	18.50±0.23	23.07±0.l2
Average No. of live fetus/dam	5.32±0.28	5.19±0.32	4.90±0.35	4.89±0.49	4.61±0.51
		Placental			
% pregnant				95.0	100
(No.preg./No. mated				19/20	20/20
Average No. of implantation sites/dam				6.30±0.07	7.01±0.03
% post-implantation loss/dam				7.69±0.01	7.80±0.03
Average No. of live fetus/dam				5.83±0.01	6.91±0.01

NA= Not available. Statistically significant as compared to control $p<0.05$

The implantation site did not show any further differentiation in PI phase whereas animals of EP phase treated group did show some pups lesser in number in comparison to controls suggesting implantation with further growth and differentiation. Similarly, the outcome of PL treated groups was more in terms of the implantation site and hence formation of pups than EP, but was lesser than control. No statistically significant differences between the groups EP and PL as to litter size and to ibuprofen feed were seen. Further adverse effect on pregnancy outcome included a significant increase in the percentage of post-implantation loss (resorptions and dead fetuses) for litter above control in EP phase and also the gradual increased percentage of loss with the advancement of age. Few abnormalities like shorten of one of the uterine horns (Fig.1) and stunted growth were noted. Negligible amount of post-implantation loss were also observed in PL phase. In PL phase the increased percentage of post implantation loss also evident during postnatal development. However this variation was not significant. A significant treatment related decrease in live litter size was seen in both EP and PL treated group of animals during different phases of development. The body weight (Table 2) of fetuses of the EP phase oriented groups of animals gradually increased in different gestation days except on 18^{th} gd. With the advancement of age of embryo the body weight of the postnatal embryo

increased compared to the controls. Then length of the embryo (Table 2) increased greatly between gd 12 to 5-day postnatal embryo resulting in significant change in length with time in control embryo. Likewise the width of the embryo (Table 2) increased reflecting the elongation of the embryo during embryogenesis in the control series.

Table 2 : Table shows physical characteristics and brain nucleic acid content of embryo of mice treated orally with ibuprofen.

Age of embryo	Embryonic length (cm)	Embryonic width (cm)	Body weight (gm)	DNA (mg/100mg)	RNA (μg/100mg)
12C	0.977±0.064	0.065±0.044	0.031±0.001	3.006±0.352	228.48±16.43
12EP	1.066±0.179	0.305±0.201	0.031±0.007	1.898±0.292	257.10±10.24
15C	1.377±0.213	0.716±0.030	0.048±0.005	2.624±0.301	205.94±10.57
15EP	1.700±0.105	0.416±0.024	0.032±0.002	0.163±0.013	173.86± 8.22
18C	3.200±0.207	0.800±0.024	1.163±0.109	2.103±0.264	174.45±22.89
18EP	3.788±0.139	0.538±0.024	1.075±0.034	0.170±0.008	166.29±11.31
0-day C	3.800±0.156	1.033±0.033	1.324±0.123	1.518±0.169	142.85± 5.46
0-day EP	4.555±0.122	0.899±0.038	1.905±0.094	0.163±0.021	111.59± 4.73
0-day PL	4.002±0.161	0.961±0.094	1.672±0.063	0.690±0.057	128.13± 7.29
5-day C	6.144±0.106	1.216±0.028	2.490±0.156	0.606±0.103	132.70±14.26
5-day EP	7.033±0.132	1.255±0.078	3.305±0.155	0.140±0.010	90.82± 4.14
5-day PL	6.700±0.159	1.000±0.035	2.710±0.105	0.492±0.035	106.38± 6.41

Values are expressed as mean ± SE, P value (C-T) significant at $p < 0.01$, C= Control, EP= Early Placental PL= Placental

A significant increase in the body weight for embryo and pups in both EP and PL treated animals was noted during embryogenesis. With the 6-fold increase in length between 12 gd and 5 postnatal embryos the width of the embryo increased 4 fold. Whereas, the length of the embryo showed an increase in body length compared to controls in EP treated animals, the width of the embryo did not increase, resulted an effect of the treatment. Similar results in increase of body length and decrease of body width were observed in embryo and pups of PL treated animals.

Brain nucleic acid concentration

Brain RNA content of the fetus and the pups of EP phase treatment animal showed both increase and decrease reflecting an infliction in the change in drug. However the PL phase treated animals showed a reduction in RNA content in their pups. When both doses were compared, significant reduction ($p< 0.01$) in RNA content was observed. Likewise brain DNA content of the

embryo and the pups developed out of EP and PL treated animals was reduced compared to the controls suggesting a drug effect. This drug effect of course made a change in DNA synthesis activity initially, which to some extent was repaired in next phase of development. Further EP phase treatment had more effect in fall of DNA level than PL phase.

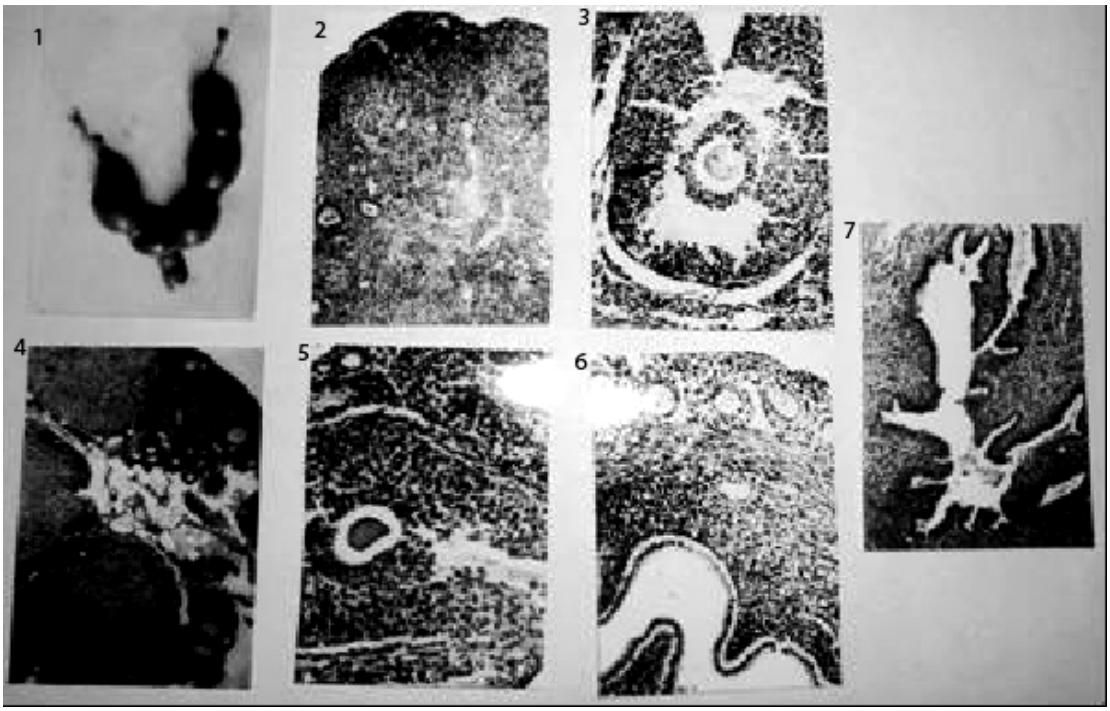

Fig. 1 : Uterus of early placental phase treated mouse showing shortening of one of the uterine horns, Fig. 2 : Magnified view of T.S.of ovary of treated mother of 12th gd of EP phase, showing atretic follicles and vacuolated stroma X 200, Fig. 3 : Higher magnified view of T.S. of ovary of treated mother of 18th gd of EP phase showing abnormal ovum. Note lyses of lutein cells. X 800, Fig. 4 : Higher magnified view of T.S. of ovary of treated mother on the 0 day PL Phase showing a number of corpus luteum and follicles of different developing stages X 400, Fig. 5 : Higher magnified view of T.S. of ovary of treated mother on 5th day after parturition of PL phase showing abnormal nucleus of the ovum X 800, Fig. 6 : Higher magnified of the T.S. of uterus of treated mother of 15th gd of EP phase showing necrosis of the epithelial layer of cavum X 400, Fig. 7 : Higher magnified view of T.S of uterus of treated mother of 18th gd of EP phase showing conspicuously tortuous cavum X 400.

Histopathology

Ovary

Oral administration of ibuprofen at therapeutic dose, on EP phase, there was histological evidence that a major change occurred in ovary of mice. On 12 gd, though there was a sequence of events of development and growth of ovarian follicles, the number of atretic follicles was more than the normal follicle. Matured follicles were with degenerated oocytes, distributed in vacuolated stroma (Fig.2). Besides the above changes corpus luteum was found to be more in number. Lutein cells of those were degenerated in the section of

15th gd ovary. Sometimes matured follicles were without ova. Likewise ovary of 18th gd had abnormal ovum and there was a tendency of lyses of follicular cells (Fig.3). Deshaped ovum in the developing follicles was distributed in loose stroma for the ovary of 0 day. The interesting features observed in the sections of the ovary of parturited mother, were crescent shaped ovum and the follicles were without any antrum. Lyses of ova were apparent in some follicles. Treatment of the drug at PL phase, the ovary of the mother on the day of parturition showed more number of corpus luteum than controls (Fig.4). Atretic follicles were less in number. But follicles with lytic ovum distributed throughout the stroma in the ovary of parturited mother. The deshaped ovum (Fig. 5) was also a prominent feature to note.

Uterus

Histopathologic changes associated with ibuprofen intoxication in 12 gd uterus at EP phase consisted of i) large number of fibroblast and connective tissue with few gland cells in the endometrium. ii) Villi like projections of the endometrium were altogether lost. Some variations were noted in the endometrial response in 15 gd by increased number of glands in the stroma and considerable folding of the endometrium make the cavum tortuous. The lining epithelium of the cavum showed degenerating character (Fig. 6). More conspicuous folding made the cavum commensurately tortuous (Fig. 7) in 18 gd. On the day of parturition, the uterus showed few gland cells and necrosis of epithelial layer of the cavum were very much prominent. Besides the normal feature of distribution of glands arrangement of layers, the cavum in the section of 5 day parturited mother looked slitting like. In PL phase treated animal, the uterus showed characteristic feature similar to control. But the endometrium of 0 day had few glands and the lumen was sometimes infiltrated with blood cells. The cells of endometrium were also degenerated. However much alterations were seen in the sections of uterus of 5 day parturited mother. Total architecture was lost. Serous layer was not conspicuous. Myometrium was much influenced in degeneration of cells by the drug. The endometrial wall of both sides made the uterine cavum squeezed.

DISCUSSION

Toxic effects on embryo are experimentally obtained by administering the agent on the mother. The drug is one such type of agent, which produces toxicity to the mother, and an effect would be expected in the intrauterine

environment surrounding the fetus. The administration of ibuprofen to mice during the period of organogenesis produced a variety of fetal alterations including reproductive performance, which may be attributed to induce toxicity. The mice treated at PI (1-4th gd) phase showed no sign of continuing of pregnancy, suggesting an interference and possibly ibuprofen may have a lethal effect in early pregnancy. Though there is no gross anomaly in the study group than in the control, in the reproduction studies, irregular phenomenon likes shortening of uterine horn, increased resorbtion rates, increased incidence of dead fetus was observed. In a recent study, a positive association between use of NSAID during pregnancy and miscarriages was reported[12] and both COX inhibitors were toxic to dams in the highest doses evaluated, which caused a significantly greater incidence of intrauterine growth retardation and developmental variations[8]. All NSAID produces an adverse effect during pregnancy, which are related to prostaglandin (PG) synthesis[13]. There is evidence of similar neonatal changes when maternal treatment was performed with indomethacin and diclofenac drug during pregnancy[14]. Fetal body weight significantly showed a greater value over the control except in 18 gd (NS) of EP phase group. The increased fetal weight is possibly a direct effect of the drug on fetal metabolism. Possibility of existence of membrane bound esterase for weight gain cannot be overruled. It is known that measurement of length, width reflect growth and development. The rate of growth of the embryo as estimated by change in length for both normal and experimental animals increases throughout the gestation period. Whether the increase of fetal length has any relationship with the drug is questionable, though there is evidence in change of head and crown rump length of embryos[15] in different developmental end points due to drugs. Drug may affect developing tissues or organs selectively due to their pharmacological activity and/or specific organ toxicity. However, significant effects on organogenesis were observed when rat embryos were exposed to ≥ 7.5 µg/ml of diclofenac[16]. The limitation of treatment in the gestational periods may disclose specific susceptivity of developmental phases of the fetus. Present study showed an increase of DNA content in brain during different gestation period in normal embryos, indicating synthesis of DNA due to proliferation of cells in brain, whereas, the content of fetal brain DNA

remarkably decreased in different gd for both EP and PL phase. The alterations observed in the synthesis of DNA in the treated animals might be due to non-availability of precursors, thus influencing normal cellular proliferation and differentiation in developing embryo.

Due to action of drug, neuronal division which is largely prenatal get stopped and consequently affects i) the proliferation of neuronal precursors reflecting decrease in brain DNA and ii) further neurogenesis. Fetal brain RNA content was also decreased except for 12 gd in EP followed by postnatal period. The synthetic activity thus has become hindered by the action of drug. Since prostaglandin synthesis is inhibited by NSAID by inhibiting the cycloxygenase (Cox) enzyme[5, 17, 18, 19]. The involvement of PG in the signal mechanism for the stimulation of RNA synthesis is also not possible. PG has been proposed as an intermediatory product in the ovulatory process. The present study shows an inhibition of ovulation manifested by degeneration of ova suggesting the essentiality of PG. The most obvious anatomical correlate of the drug related effect on ovary is lesser number of matured follicles and increased number of atretic follicles. The population of follicles in ovaries of mice treated with ibuprofen contains significantly more atretic follicles and increased number of corpus luteum than the controls. The increased number and altered morphology of ovarian follicles suggest follicular growth during drug treatment, but they may fail to survive for ovulation. The results of the present study indicate that the ovary of the ibuprofen treated mice interferes with the response of the uterine activity. However, the treatment causes certain deviations in the endometrial response in the form of lesser number of glands and absence of characteristic villi-like endometrial folding. It has been suggested that the endometrial changes may be due to the restricted synthesis of PG as PG inhibit the spontaneous motility of the myometrium. PG seems to exert an antifertility action possibly by indirect or direct effect on the implantation sites resulting a noteworthy effect of resorption by the drug ibuprofen. It is also known that ibuprofen suppresses estrogen-induced uterine growth[20]. Thus the present study reveals that the ibuprofen possibly blocks estrogen receptor-mediated activities causing intrauterine disturbances in the mice. The present study suggests that ibuprofen may have a destructive effect on the embryo of the mouse, when treated orally to the mother at different gestational phases.

The mother is also susceptible to the drug. It is that ibuprofen may have a potential in alteration of the normal architectural pattern of both ovary and uterus causing formations of more number of atretic follicles and having significant endometrial responses.

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