# Gross congenital abnormalities induced by leflunomide in mice embryos

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## SUMMARY

Leflunomide is an antirheumatic drug commonly used by females, as this disease is common in females and there are chances of pregnancy while taking this medication in initial months of pregnancy this commonly prescribed drug lacks studies related to its teratogenic potential. Present study was conducted to know about its teratogenicity in mice embryos. Pregnant mice were exposed to Leflunomide by oral route on gestational days 6 to 11 either as single dose in one of the gestational days or continuous doses. The embryos were collected on day 19 of gestation, were measured and examined for external anomalies. Findings suggested that Leflunomide was embryo lethal when given as continuous dose as there were 100% resorption of embryos. In the single dose group, maximum resorptions were found when was given in early pregnancy. Other anomalies included malrotated limbs, open eyes, kinking of tails, defect in anterior abdominal wall and visceroptosis and anencephaly. these anomalies were noted in embryos exposed to leflunomide only on gestational days 7 and 8.

The above findings suggest that leflunomide interferes with embryonic growth It also interferes with neural tube closure leading to anencephaly. Findings of open eyes and kinking tails suggest that the drug may affect epithelial and mesodermal growth. Leflunomide perhaps interferes with the lateral folding of the embryo leading to defect in the anterior abdominal wall and visceroptosis. The present study concludes that Leflunomide is teratogenic and embryolethal in mice and should be avoided in human pregnancy.

**Key words:** Leflunomide – Teratology – Gestation age – Malformation – Resorption

## INTRODUCTION

Leflunomide is a disease modifying antirheumatic drug that has been approved by food and drug administration for the treatment of rheumatoid arthritis, approval was based on data from a double-blind, multicentre trials in the United States in which leflunomide was superior to placebo and similar to methotrexate (Strand et al., 1999). Leflunomide is a novel isoxazole immunomodulatory agent that inhibits de novo pyrimidine synthesis and is also having anti proliferative activity. After oral administration, it is rapidly metabolized to an active metabolite (A77 1726), possibly in the gut wall, plasma and in the liver, which is presumed to be the active drug in vivo. No epidemiological studies have been completed with regard to the teratogenicity of lefluno-

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mide, since the drug was introduced in 1998. Of the few women who have been exposed during pregnancy, most have decided to interrupt their pregnancies. The pharmacokinetic level of the active metabolite of leflunomide reached clinically in humans is in the range of exposure that results in teratogenesis in the rat and rabbit. It is evident that leflunomide effects vary with species and it appears that the rat and rabbit are more sensitive to leflunomide inhibition of pyrimidine synthesis. But it is difficult to predict that metabolic difference into differences in teratogenic potential, because studies related to leflunomide's effect on the human embryo is lacking. Therefore, the conservative approach is to be considered and it is presumed that therapeutic blood levels of the active metabolite of leflunomide represent a teratogenic risk (Brent, 2001). Approximately 500 and 1500 patients in New Zealand have been prescribed leflunomide, up to the end of 2003 (Aventis Pharma Pvt Ltd, 2004). The adverse reactions reported in New Zealand for this medicine are similar to those seen internationally. Examples of the more serious local cases include:

- Elevated hepatic enzymes, along with neutropenia, thrombocytopenia and diarrhoea.
- Sepsis leading to multi-organ failure and death; concomitant medicines were methotrexate, ketoprofen and triamcinolone.
- Hypersensitivity pneumonitis, resulting in life-threatening respiratory compromise. The patient was taking leflunomide and methotrexate but did not relapse when methotrexate was re-introduced.
- Multiple bullous eruptions occurring within three weeks of starting leflunomide, and resolving upon discontinuation.
- In spite of the clear warning that the drug should not be prescribed for pregnant women, approximately 30% women have become pregnant while taking leflunomide as of December 1999 (Brent, 1999).
- In reproduction studies with pregnant rats during organogenesis, Leflunomide was reported to be teratogenic (anophthalmia or microphthalmia and internal hydrocephalus). This also resulted in an increase in embryo death and a decrease in maternal and surviving embryo body weight. However, the reports are only few. The present study aims to elucidate the different teratological outcome of leflunomide, its mechanism of teratogenesis in the developing organs exposed to Leflunomide in pregnant mice.

## MATERIAL AND METHODS

Forty-eight female albino mice of an average weight of 25-30 g and an average age of 80-100 days weeks were used in this study. Institutional Ethical committee approval was obtained before starting this study. Animals were housed in separate plastic cages in animal house on a light dark cycle of 12:12 hours. Mice were fed on diet pellets (Hindustan Liver Ltd, Mumbai, India) and tap water *ad libitum* and were treated with utmost aseptic care. Female mice were kept overnight with the males of same stock (female: male 3:1) at 16 hrs. In the next morning the vaginal smear was examined and if the presence of sperms was found in the smear it was considered as day "Zero" of pregnancy. The pregnant mice were weighed and kept individually in separate cages, date of pregnancy and weight of the pregnant mice was worked on the cage.

The leflunomide was administered orally with the help of sterile syringe either as single or continuous dose. The single dose was 50 mg/ kg body weight/day and continuous dose was 15 mg/ kg body weight/day from day 6-11 of gestation. Control mice were given equal volumes of normal saline. The pregnant mice were sacrificed with an overdose of ether anaesthesia on day 19 of pregnancy. The uterine horns were exteriorized after opening the abdomen by midline incision. The sacs were inspected for sites of resorption and viable embryos. The embryos were removed from the uterus and blotted on blotting paper. The weight and CRL of the embryo were recorded. These were examined for external abnormalities like cleft palate, limb and tail abnormalities. Embryo were fixed in 10% formalin solution and were preserved for histological examination. Controls were given equal volumes of normal saline. Anterior abdominal wall of the embryos was opened. The liver, lungs, kidney, heart and brain were dissected out and were examined for external mal-

formations. All the organs were washed and fixed in buffered formalin for tissue processing.

### Experimental groups

Each group were having 6 pregnant mice, total 48 pregnant albino mice were taken for study.

Group A: (Control) received equal volume of normal saline.

Group B: 50 mg/kg single dose on gestational day 6 (GD 6).

Group C:50 mg/kg single dose on gestational day 7 (GD 7).

Group D: 50 mg/kg single dose on gestational day 8 (GD 8).

Group E: 50 mg/kg single dose on gestational day 9 (GD 9).

Group F: 50 mg/kg single dose on gestational day 10 (GD 10).

Group G: 50 mg/kg single dose on gestational day 11 (GD 11).

Group H: 15 mg/kg/ once a day as continuous dose from GD-6 to GD-11.

### Statistical analyses

Data has been entered into an excel spread sheet. Mean, standard deviation and standard error were calculated.

## RESULTS

Various gross congenital abnormalities were reported in our study.

### Resorption of embryos

The rate of resorption in different treated groups are depicted in Table 1, Fig. 1. The resorption rate was around 67% when the pregnant mice were exposed to leflunomide on GD6. The resorption rate decreased to 50% on GD7 and 25% on GD8. However, there was no resorption when the mice were exposed to leflunomide on GD9, GD 10 and GD11 and in the control group. On the contrary, all the embryos were resorbed when the pregnant mice were exposed continuously from GD6 to GD11. Total implantation sites including resorptions (Table 1) were counted on pregnant mice in every group. Uterine implantations were ranging from minimum of 3 to maximum of 11 sites in different groups.

**Table 1.** Total implantation sites including rate of resorptions in the different groups.

| Group | Total number of implantations/ Number of implantations in each pregnant mice | Resorption/ Implantations (Resorption %) |
|---|---|---|
| **Group A – Control** | 41<br>7,6,7,5,7,9 | 0 % |
| **Group B – GD-6** | 24<br>3,5,4,4,3,5 | 16 (66.6%) |
| **Group C – GD-7** | 30<br>4,3,7,8,4,4 | 15(50%) |
| **Group D – GD-8** | 32<br>6,5,5,8,5,3 | 8(25) |
| **Group E – GD-9** | 35<br>7,4,3,8,9,4 | 35 (0%) |
| **Group F – GD-10** | 40<br>11,9,8,5,5,2 | 40 (0%) |
| **Group G – GD-11** | 40<br>10,7,8,5,6,4 | 40 (0%) |
| **Group H – GD-6-11** | 0 | 0 (100%) |

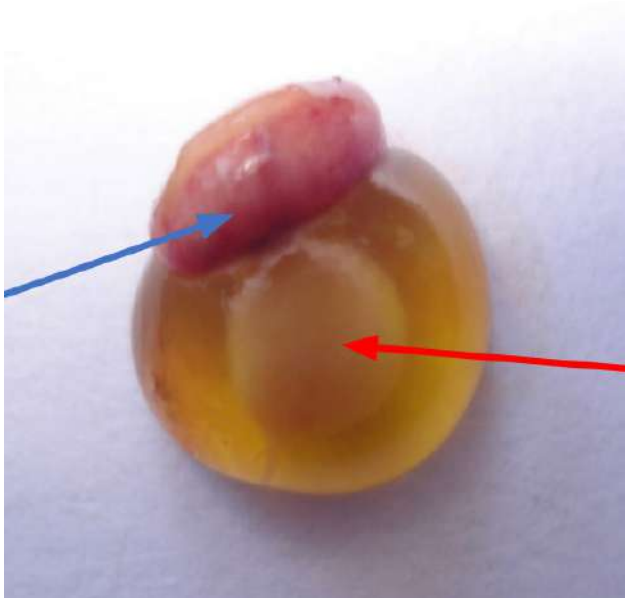

**Fig. 1.-** Fetal resorption Sac (red arrow - fetal resorption, blue arrow- placental site).

### Fetal parameters (length & weight)

The mean embryonal weight in various treated groups ranged between 1.33 to 1.69 g, while the mean weight in control group was 2.11 g. There was significant reduction in weight of the treated embryos (p value<0.001). The treated embryos were also markedly reduced in size, as compared to the control group (p<0.001). Mean CR length in control group was 1.02 cm. while it ranged between 0.63 to 0.86 cm in different treated groups.

### Gross malformations (Table 2)

- Large number of embryos showed haemorrhages either on forepaw or hind paw. About 52% embryos showed haemorrhages over forepaw while 58% showed haemorrhages over hind paw, when the pregnant mice were exposed to leflunomide on different gestational days. The forepaw haemorrhage rate ranged between 100% on GD6 to 40% on GD7. Similarly, the hind paw haemorrhage rates ranged from 75% on GD6 to 46.6% on GD7 (Table 2).
- Malrotated hindlimbs were found in 4.9 % embryos (Table 2). However, the findings were only confined to GD7 (20%) and GD8 (20.8%) groups.
- Open eye has been found in 3.7% embryos (Table 2) and confined to GD8 group.
- Kinking of tail was found in 2.4% embryos and only found in GD8 group (16.6%).
- Anterior abdominal wall defect and visceroptosis was found in 1.2% embryos and was limited to GD7 group (13.3%) (Table 2) (Fig. 1).
- Similarly, anencephaly was noted in 1.2% embryos and limited to GD7 group (13.3%). (Table 2, Fig. 1).

### Gross examination of viscera

Brain, lung, kidney and liver were markedly reduced in size as compared to that of control group.

## DISCUSSION

### Fetal parameters (weight & length)

Growth retardation has been widely accepted as an expression of maldevelopment in both human beings (Grovenwald, 1961) and experimental animals (Mc Laren and Michie, 1960; Jensh and Brent, 1967; Wilson, 1973).

We reported significant reduction in the weight of exposed embryos. Fukushima et al. (2007, 2009) reported reduction in maternal as well as embryonic weight in leflunomide exposed mice. The embryonic weights were especially low when exposed on gestational day 7 and 9. The authors (Fukushima et al., 2009) demonstrated that the single administration of leflunomide on GD7, GD8 or GD9 caused severe embryo toxicity and reduced intrauterine growth. In our study, there were significant body weight reductions which suggests that leflunomide has a potential to affect the embryonic growth.

### Skeletal malformations

Fukushima et al. (2009) reported 100% skeletal malformations in leflunomide treated embryos, and multiple malformations were reported such

**Table 2.** Various external malformations observed in leflunomide exposed mice fetuses.

| MALFORMATION | Group B GD-6 8 | Group C GD-7 15 | Group D GD-8 24 | Group E GD-9 35 | Group F GD-10 40 | Group G GD-11 40 | Total malformed fetuses / Total no. of fetuses examined |
|---|---|---|---|---|---|---|---|
| Haemorrhage on forepaw | 8(100%) | 6(40%) | 10(41.6%) | 18(51.4%) | 22(55%) | 20(50%) | 84/162(51.8) |
| Haemorrhage on hindpaw | 6(75%) | 7(46.6%) | 12(50%) | 19(54.2%) | 20(50%) | 30(75%) | 94/162(58%) |
| Malrotated hind limbs | | 3(20%) | 5(20.8%) | | | | 8(4.9%) |
| Kinking of tail | | | 4(16.6) | | | | 42.46%) |
| Anterior abdominal wall defect & Visceroptosis | | 2(13.3) | | | | | 2(1.23) |
| Anencephaly | | 2(13.3) | | | | | 2(1.23%) |
| Open eye | | | 6(25%) | | | | 6(3.70%) |

as absent palatine bone and deformities of the skull bone, cervical to lumbar vertebrae, forelimb, ischium/pubis, and hindlimb were seen at significantly higher frequencies than in the control group. However, in the present study, no skeletal malformations were noted observed after alizarin staining to colour bones and cartilages. Only 2 embryos showed anencephaly when exposed to leflunomide on GD7. Absence of significant skeletal anomalies is difficult to explain in our study. It could be due to different doses used or due to species difference. Cleft lip and cleft palate were not observed in the present study but it has been reported by Fukushima et al. (2009).

In the present study, all the embryos were resorbed when the pregnant mice were exposed to leflunomide continuously from GD 6 to GD11. Fukushima et al. (2007), observed that at 70 mg/kg, all embryos were resorbed, and at 30 mg/Kg, leflunomide reduced embryonic viability and increased the incidence of multiple external malformations. These observations suggests that leflunomide has potential to affect growth and in continuous dosing it can completely stop embryonal development resulting in resorptions.

**Visceral malformations**

In previous study by Fukushima et al. (2009) the incidence of embryos with visceral malformations in the groups treated with leflunomide was significantly higher than that in control group. The malformations included membranous ventricular septal defects, overriding of aorta and diaphragmatic hernia. Other malformations included hydrocephaly, absent rhinencephalon, mishappen retina, right sided aortic arch, persistent truncus arteriosus, small thymus, absent accessory lobe of the lung, hydronephrosis and retrocaval ureter. In our study, small sized kidney, Liver, Lung and brain were observed.

**External malformations**

In the present study, majority of embryos showed malformations, predominantly haemorrhages on forelimb and hindlimbs, followed by malrotated limbs, open eye, kinking of tail, anterior abdominal wall defect along with visceroptosis and anencephaly. However, the haemorrhage rate was much higher in our study as compared to those observed by Fukushima et al. (2009). Ob-

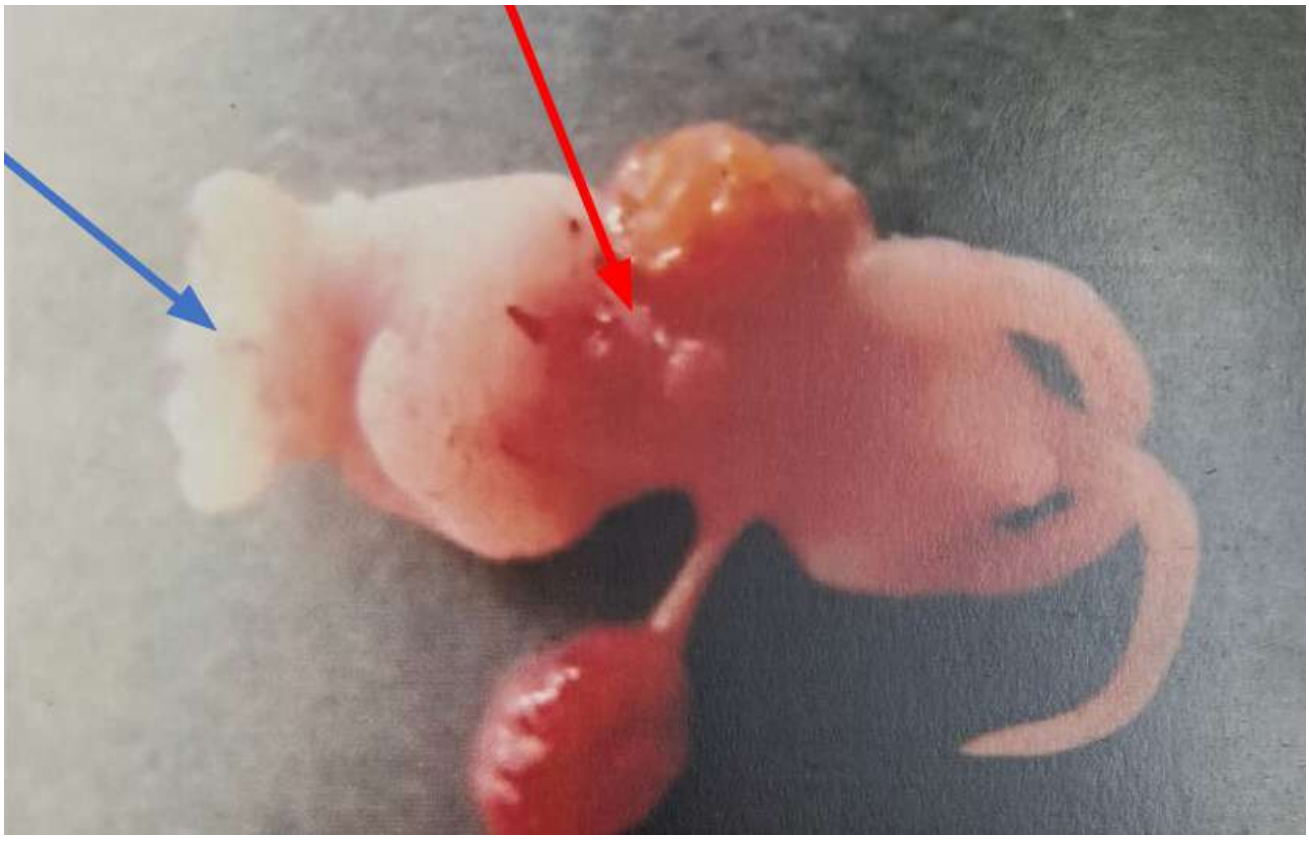

**Fig. 2.-** Anencephaly (blue arrow) with visceroptosis (red arrow).

served haemorrhages on forepaw and hindpaw in maximum number of mice embryos from GD6-11 (84 and 94 respectively). In previous study by Fukushima et al. (2009) hematoma was observed in 2/40 embryos on GD9,15/84 embryos on GD10 and 5/104 embryos on GD11. Since this drug inhibits de novo pyrimidine synthesis producing toxicities in organs where rapidly proliferating cells are located such as haematopoietic system, immune system, pancreas and developing embryos. Observed haemorrhages in embryos could be due to toxic effects on bone marrow.

Malrotated hind limbs were observed on GD7 & GD8 in 3 & 5 embryos respectively. In previous study by Fukushima et al. (2009) anomalies of hind limb were reported on GD6 & GD10 in 1 & 10 embryos respectively. These results show that this drug affects skeletal development also.

We observed open eye in 6 embryos on GD8 treated group. In previous study by Fukushima et al. (2009) it was observed in 7 embryos on GD11. This defect shows that leflunomide has a potential to affect developing epithelium.

Kinking of tail was observed in 4 embryos on GD8, in previous study by Fukushima et al. (2009) it was observed in 5, 48 and 63 embryos on GD9, GD10 & GD11 respectively. In another study by Fukushima et al. (2009) its overall incidence was 63.3±28.5(38/62). Kinking of tail may be due to defect in musculature of tail, which is mesodermal in origin so it is hypothesized that this drug has effect on development of mesoderm also.

Anencephaly was observed in 2 embryos on GD7 (Fig. 2) treated group. In previous study by Fukushima et al. (2009) incidence of exencephaly was (12.5±25.0) reported on GD7 (1 out of total 12 examined). These results show that this drug has effect on embryonic neural tube closure during early embryonic stages.

Anterior abdominal wall defect along with visceroptosis was observed in 2 cases on GD7 (Fig. 2) this has not been reported previously in literature. Probable mechanism of action of leflunomide producing this defect may be due to producing disturbance in the lateral folding of embryo resulting in defective closure of anterior abdominal wall. Fukushima et al. (2009) stated that leflunomide is a proven teratogen in rats, rabbits and mice, it inhibits de novo pyrimidine nucleotide synthesis by inhibiting enzymes tyrosine kinase and dihydroorotate dehydrogenase (DHODH) leading to its embryotoxic and teratogenic influences. Its immunosuppressive effect also occurs via inhibition of these enzymes. Inhibition of de novo pyrimidine synthesis can be attributed to teratogenicity via the anti-proliferative activity of Leflunomide on embryonic cells. It is evident that leflunomide effects vary with the species, and it appears that the rat and rabbit are more sensitive to leflunomide inhibition of pyrimidine synthesis (Brent, 2001). Women who become pregnant while taking leflunomide are at risk of reproductive effects based on studies on rats and rabbit, because pharmacokinetic level of the active metabolite (A77 1726) of leflunomide reach clinically in humans in the range of exposure that results in teratogenesis in rat and rabbit.

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