RESEARCH ARTICLE
A STUDY ON TOXIC EFFECTS OF NIMESULIDE IN PREGNANT SPRAGUE DAWLEY RATS

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ABSTRACT
The present study was aimed to evaluate the organ toxicity and teratogenic effects, if any, respectively in the dams treated with nimesulide in gestation period at different dose levels and in progeny. Thirty six female albino rats of Sprague dawley strain were equally divided into 3 groups and treated as follows. Group 1 served as control, group 2 received nimesulide @ 20 mg/kg body weight and group 3 received nimesulide @ 60 mg/kg body weight via intramuscular from 7th to 17th day of gestation. In each group, pregnant rats were subjected to caesarian section on 19th day of gestation for uterine weights with progeny, resorption sites, inborn progeny body weight, litter size, live and dead numbers, male: female progeny numbers, skeletal staining of progeny with Alizarin-Red S, Alcian blue-Alizarin Red S stains and soft tissue developmental anomalies. Thiobarbituric acid reacting substances (TBARS) and reduced glutathione (GSH) were estimated in kidney and liver on 19th day of gestation. Hepatic and renal biomarkers were estimated on 19th day of gestation. There was a significant difference in serum biochemical profiles of dams and was more evident with nimesulide treated @ 60 mg/kg body weight. Treatment with nimesulide at higher dose induced oxidative stress and tissue damage of liver and kidney as evident from altered biochemistry and histology. It is concluded that nimesulide at 60 mg/kg body weight in pregnant dams showed more significant damage to liver and kidney as compared to the dose of 20 mg/kg body weight and control, while no teratogenicity was reported in any of the tested doses.

INTRODUCTION
The Non Steroidal Anti-inflammatory Drugs (NSAIDs) are the most commonly prescribed drugs world-wide in the treatment of acute and chronic painful inflammatory musculoskeletal conditions and also in the management of pain and fever (Suleyman et al., 2007). The number of pregnant women and women of rheumatic diseases in child bearing age are receiving drugs during pregnancy to control maternal disease activity and to ensure a successful pregnancy outcome (Ostensen et al., 2006). Cyclooxygenase (COX) inhibitors are one of the most ingested drugs during pregnancy (Burdan, 2005). Treatment with NSAIDs is possible at some stages of pregnancy as well as during lactation (Ostensen, 2006). Sometimes such drugs cross the placental barrier, enter the fetal circulation and potentially alter fetal development, particularly the development of the kidneys. Increased incidences of maternal toxicity, intrauterine growth retardation and adverse renal effects have been reported. The fetus and the newborn infant may thus experience renal failure, varying from transient oligohydramnios to severe neonatal renal insufficiency leading to death. Such adverse effects may particularly occur when fetuses are exposed to NSAIDs (Balasubramaniam, 2000; Farid et al., 2006).

Although NSAIDs are effective, they are associated with adverse effects that predominantly affect the gastrointestinal tract (GIT), acute hepatitis and fulminant hepatic failure (Tan et al., 2007). In addition, they may decrease renal and platelet function (Saillant, 2009).

Nimesulide is a preferential COX-2 inhibitor with anti-inflammatory, analgesic and antipyretic effects (Thawani et al., 2003). Similar to other NSAIDs, prolonged use and more than therapeutic doses of nimesulide causes adverse effects (Goyal et al., 1998). Its adverse effects commonly involve the hepatobiliary, renal, cutaneous and gastrointestinal systems (Sbeity et al., 2001; Burke et al., 2005).

MATERIALS AND METHODS
Female albino rats of Sprague dawley strain weighing about 200-250 g were procured from NCLAS, National Institute of Nutrition, Hyderabad. The animals were housed in solid bottom polypropylene cages in Laboratory Animal House of Department of Pharmacology & Toxicology, College of Veterinary Science, Rajendranagar, Hyderabad, with 12 h – 12 h dark and light cycle and temperature of 22-24°C. Animals were placed on commercial standard mash feed for rat and provided water ad libitum. Before conducting the experiment, rats were acclimatized for 1 wk. Experiment was conducted according to the guidelines of Institutional Animal Ethics Committee with a single batch of 36 female rats. These rats
were mated and after confirming pregnancy, 36 female rats were divided into 3 groups consisting of 12 in each group. Group 1 was control maintained on basal diet, groups 2 and 3 were given nimesulide @ 20 and 60 mg/kg body weight, respectively via IM route daily from 7th to 17th day of gestation. Pregnant rats were subjected to caesarian section on 19th day of gestation. Litter was screened for soft tissue and skeletal developmental anomalies.

Blood samples were collected from the dams on 19th day of gestation in all three groups by retro-orbital puncture and sera samples were separated to estimate sero-biochemical profile (ALP, ALT, AST, BUN, creatinine, GGT and total protein). Dams of all groups at 19th day of gestation were anaesthetized for caesarian section and screened for gravid uterine weights, resorption sites, inborn progeny body weights, litter size, live and dead numbers, male: female sex ratio, skeletal developmental anomalies and soft tissue developmental anomalies. Tissues were collected and stored at -20°C for further estimation of GSH and TBARS in liver and kidney homogenates. Liver and kidney were immediately excised upon removal. They were fixed in 10 % neutral buffered formalin immediately upon removal. They were gradually dehydrated, embedded in paraffin, cut into 5 µm sections, stained with hematoxylin and eosin (H&E) for histological examination according to the standard procedure (Singh and Sulochana, 1997).

Skeletal staining in progeny: Alizarin red-S

All the embryos were eviscerated and cleared by the method of Dawson (1926) and the skeleton stained with alizarin red-S as given below.

1. Embryos were fixed in 95 per cent alcohol for 48-72 h.
2. Embryos were treated in acetone to remove any fat that appears as opaque white mass for 2-4 days.
3. The specimen was returned to 95 % alcohol and kept for 12-24 h.
4. Embryos were placed in 1 per cent KOH until bones were clearly visible through the muscle.
5. Finally they were kept in a solution of 0.1 % alizarin red S in 1 % KOH until bones was stained.
6. When clearing in the initial KOH solution has progressed to the proper stage, the bones took up stain. When the clearing was not complete, the muscle and other tissues took up stain almost as readily as the bone itself. In such cases the specimens were transferred to a fresh KOH solution.
7. Following the staining, embryos were transferred to jars containing
   a) KOH . . . 1 g
   b) Distilled water . . . 79 ml
   c) Glycerine . . . 20 ml
8. When the tissues were properly cleared they were passed through the increasing concentrations of glycerine and finally stored and maintained in pure glycerine.

Alcian Blue – Alizarin Red Skeletal Staining

All the embryos were eviscerated and cleared by the method of Humason (1967) and the skeleton stained with alcian blue-alizarin red-S as given below.

1. Neonatal progeny rats were dissected by removing the skin and organs completely. Fat was removed carefully without damaging the specimen and placed in 15 ml falcon tubes.
2. Specimens were fixed in 95 % ethanol for 12-48 h, slowly rocking at room temperature.
3. Specimens were again placed in 95 % ethanol with Alcian blue staining solution for 1-3 days slowly rocking at room temperature. For vertebral or appendicular skeleton 1-2 days is enough, whereas head staining requires 3 days.
4. Specimens, re-placed in Alcian blue solution with 95 % ethanol for 6 h slowly rocking at room temperature.
5. Specimens re-placed in 95 % ethanol with 2 % KOH solution for 12-24 hours slowly rocking at room temperature. Older animals require more time than younger ones. (Any remaining fat or skin can be removed at this point).
7. Cleared in the solution of 1 % KOH/20% glycerol, until the specimen is completely clear. Do not leave skeleton in the solution for too long because the skeleton still becomes extremely fragile.
8. Replaced clearing solution with 1: 1 glycerol: 95 % ethanol for 1 day.
9. To store skeletons for long periods of time, it was passed through glycerol/ethanol solutions.

Solutions

Alcian Blue (0.03 % Alcian Blue, 80 % ethanol and 20 % acetic acid)
Alizarin Red (0.03 % Alizarin Red, 1 % KOH and water)

The data were subjected to statistical analysis by applying one way ANOVA using statistical package for social sciences (SPSS) version 15. Differences between means were tested using Duncan’s multiple comparison test and significance was set at P<0.05.

RESULTS AND DISCUSSION

Results of sero-biochemistry are presented in Table 1. The activity of serum ALP (IU/L) in group 1 pregnant dams at 19th day was 239.36±10.75, which was significantly (P < 0.05) increased in group 2 (382.30±10.10). The group 3 showed a significant (P < 0.05) increase (515.58±43.86) as compared to groups 1 and 2. The activity of serum ALT (IU/L) in group 1 pregnant dams at 19th day was 22.27±0.73, which was significantly (P < 0.05) increased in group 2 (28.70±0.81). The group 3 showed a significant (P < 0.05) increase (43.07±1.14) as compared to group 1 and 2. The activity of serum AST (IU/L) in group 1 pregnant dams at 19th day was 223.50±7.64, which was significantly (P < 0.05) increased in group 2 (296.41±6.23). Group 3 showed a significant (P < 0.05) increase (408.41±4.75) as compared to groups 1 and 2. The activity
**Fig-1** Photomicrograph of liver showing mild hydropic degeneration of hepatocytes. H&E X 400 (Group 2)

**Fig-2** Photomicrograph of liver showing central vein congestion and bile duct hyperplasia H&E X 200 (Group 2)

**Fig-3** Photomicrograph of liver showing focal areas of lymphoid aggregates H&E X 200 (Group 3)

**Fig-4** Photomicrograph of liver showing moderate central vein congestion and bile duct hyperplasia H&E X 200 (Group 3)

**Fig-5** Photomicrograph of kidney showing mild intertubular congestion and hemorrhages. H&E X 400 (Group 2)

**Fig-6** Photomicrograph of kidney showing mild degenerative changes, intertubular hemorrhages and hyaline casts. H&E X 200 (Group 2)

**Fig-7** Photomicrograph of kidney showing moderate intertubular hemorrhages H&E X 100 (Group 3)

**Fig-8** Photomicrograph of kidney showing moderate degenerative changes in tubular epithelium and congestion. H&E X 200 (Group 3)
The concentration of blood urea nitrogen (mg/dl) in group 1 pregnant dams at 19th day was 20.87±0.79, which was significantly (P < 0.05) increased in group 2 (27.48±1.52). The group 3 showed a significant (P < 0.05) increase (42.36±1.48) as compared to groups 1 and 2. The concentration of serum creatinine (mg/dl) in group 1 pregnant dams at 19th day was 0.625±0.01, which was non-significantly increased in group 2 (0.710±0.01). Group 3 showed a significant (P < 0.05) increase (6.16±0.07) as compared to groups 1 and 2.

The hepatotoxicity of nimesulide has been linked to oxidative stress, generation of ROS caused damage to hepatocytes (Sarkar et al., 2005), and immunological and metabolic idiosyncratic reactions induced pathogenetic mechanism (Thawani et al., 2003), which was responsible for alteration in serum biochemical profile (Ramesh et al., 2001; Chatterjee et al., 2008 and Sozer et al., 2011). The mean concentration of GSH (uM/mg protein) in kidney and liver revealed a significant (P < 0.05) decrease in group 2 (21.34±2.54 and 25.39±3.25, respectively) as compared to group 1 (27.95±4.69 and 30.76±3.61, respectively) pregnant dams at 19th day. The group 3 showed a significant (P < 0.05) decrease in GSH as compared to groups 1 and 2. The mean concentration of TBARS (nM of MDA/mg protein) in kidney and liver revealed a significant (P < 0.05) increase in group 2 (1.96±0.06 and 0.41±0.01, respectively) as compared to group 1 (1.77±0.04 and 0.33±0.03, respectively) pregnant dams at 19th day. Group 3 showed a significant (P < 0.05) increase in TBARS as compared to groups 1 and 2 (Table 2).

These results suggest peroxidative stress and compromised antioxidant defense mechanisms. Sarkar et al. (2005) reported that nimesulide has the ability to provoke massive oxidative stress in vivo. Liver has the unique property of regeneration by itself after any drug and toxins injury. Nimesulide is exclusively metabolized and cleared by the liver (Davis et al., 1994). Hence, its injury can be induced by the exposure to various toxicants and by a number of drugs when taken very frequently or beyond therapeutic doses. These toxicants mainly damage liver by producing reactive oxygen species (Slater, 1995). These reactive oxygen species (ROS) have been
implicated in nimesulide-induced adverse effects including hepatotoxicity (Sozer et al., 2011). The mechanism of nimesulide-induced hepatic injury seems to involve generation of ROS, causing oxidative stress to hepatocytes as proposed by Sozer et al. (2011). Results of the present study indicated that nimesulide induced oxidative insult and suggested that the oxidant defense of the body has been suppressed effectively by nimesulide. The mean litter size (number) in group 1 pregnant dams at 19th day of gestation was 10.50±1.05, which was non-significantly decreased in group 2 (10.00±1.06). The group 3 showed a non-significant decrease in litter size number (8.83±0.60) as compared to groups 1 and 2. The mean live and dead number in group 1 pregnant dams at 19th day of gestation was 10.00±0.85 and 0.50±0.34, respectively, which was non-significantly decreased in group 2 (9.50±0.88 and 0.50±0.34, respectively). Group 3 showed a non-significant decrease in live and dead number (8.33±0.33 and 0.50±0.34, respectively) as compared to groups 1 and 2.

The mean male and female number in group 1 pregnant dams at 19th day of gestation was 4.83±0.70 and 5.16±0.70, respectively, which was non-significantly decreased in group 2 (4.66±0.49 and 5.33±1.25, respectively). Group 3 showed a non-significant decrease in male and female progeny number (4.66±0.95 and 5.00±1.25, respectively) as compared to groups 1 and 2. The mean gravid uterine body weight (g) in group 1 pregnant dams at 19th day of gestation was 42.30±1.66, which was non-significantly decreased in group 2 (41.70±2.33). Group 3 showed a non-significant decrease in uterine body weight with progeny (37.75±0.98) as compared to groups 1 and 2 (Table 3).
The histological sections of the liver from group 2 revealed mild hydropic degeneration of hepatocytes (Fig-1) with mild central vein congestion and bile duct hyperplasia (Fig-2) and group 3 revealed focal areas of lymphoid aggregates (Fig-3), marked central vein congestion and bile duct hyperplasia (Fig-4), whereas sections from group 1 did not reveal any lesions of pathological significance. The histological sections of the kidney from group 2 revealed mild inter tubular congestion and haemorrhages (Fig-5), hyaline casts and few tubules showing mild degenerative changes (Fig-6), and group 3 revealed moderate inter tubular haemorrhages (Fig-7) and moderatedegenerative changes in tubular epithelium (Fig-8), whereas sections from group 1 did not reveal any lesions of pathological significance. Gross photographs of progeny in

Table 1: Sero-biochemical parameters in different groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>ALP (IU/L)</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>GGT (IU/L)</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Total Protein (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>239.3±10.75</td>
<td>223.50±7.64</td>
<td>22.2±0.73</td>
<td>1.32±0.03</td>
<td>20.87±0.79</td>
<td>0.62±0.01</td>
<td>6.86±0.12</td>
</tr>
<tr>
<td>Nimesulide @ 20mg/kg body weight</td>
<td>382.30±10.10</td>
<td>296.4±6.23</td>
<td>28.7±0.81</td>
<td>1.77±0.39</td>
<td>27.48±1.52</td>
<td>0.71±0.01</td>
<td>6.67±0.09</td>
</tr>
<tr>
<td>Nimesulide @ 60mg/kg body weight</td>
<td>515.58±43.86</td>
<td>408.41±4.75</td>
<td>43.07±1.14</td>
<td>3.70±0.24</td>
<td>42.36±1.48</td>
<td>0.95±0.06</td>
<td>6.16±0.07</td>
</tr>
</tbody>
</table>

Values are Means±SE (n=12); One way ANOVA (SPSS); Means with different alphabets as superscripts differ significantly (P<0.05)

Table 2: Concentration of TBARS and GSH in liver and kidney in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDA/Mg protein</td>
<td>MDA/Mg protein</td>
</tr>
<tr>
<td>------------------------------</td>
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</tr>
<tr>
<td>Control</td>
<td>0.33±0.03</td>
<td>30.76±6.61</td>
</tr>
<tr>
<td>Nimesulide @ 20mg/kg body weight</td>
<td>0.41±0.01</td>
<td>25.39±3.25</td>
</tr>
<tr>
<td>Nimesulide @ 60mg/kg body weight</td>
<td>0.51±0.03</td>
<td>15.27±2.98</td>
</tr>
</tbody>
</table>

Values are Means±SE (n=12); One way ANOVA (SPSS); Means with different alphabets as superscripts differ significantly (P<0.05)

Table 3: Parameters pertaining to progeny of different groups of dams

<table>
<thead>
<tr>
<th>Group</th>
<th>Inborn progeny body weight (g)</th>
<th>Litter size number</th>
<th>Live and dead number</th>
<th>Male and female progeny number</th>
<th>Uterine weights with progeny (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.06±0.32</td>
<td>10.50±1.05</td>
<td>10.00±1.05</td>
<td>0.50±0.34</td>
<td>4.83±1.07</td>
</tr>
<tr>
<td>Nimesulide @ 20mg/kg body weight</td>
<td>2.82±0.33</td>
<td>10.00±1.06</td>
<td>9.50±0.88</td>
<td>0.50±0.34</td>
<td>4.66±0.49</td>
</tr>
<tr>
<td>Nimesulide @ 60mg/kg body weight</td>
<td>2.51±0.10</td>
<td>8.83±0.60</td>
<td>8.33±0.33</td>
<td>0.50±0.34</td>
<td>4.66±0.95</td>
</tr>
</tbody>
</table>

Values are Means±SE (n=12); One way ANOVA (SPSS); Means with different alphabets as superscripts differ significantly (P<0.05)

Fig-19 Photographs of rat uterus with ovary sacrificed on 19th day of gestation showing 10 number of fetus in group 1.
Fig-20 Photographs of rat uterus with ovary sacrificed on 19th day of gestation showing 9 number of fetus in group 2.
Fig-21 Photographs of rat uterus with ovary sacrificed on 19th day of gestation showing 10 number of fetus in group 3.

Table 1: Parameters pertaining to progeny of different groups of dams
The number of resorption sites was evaluated in sacrificed rats (Fig. 19, 20, 21). There were no resorption sites in groups 1, 2 and 3, which showed 10, 9 and 10 viable foeti, respectively.

**SUMMARY**

In conclusion, the study revealed that treatment with nimesulide @ 20 and 60 mg/kg body weight showed no teratogenicity. There was a significant difference in sero-biochemical profile of dams. Treatment with nimesulide at high dose induced oxidative stress and tissue damage of liver and kidney as evident from increased levels of MDA and decreased levels of GSH, histopathology of liver and kidney.

**References**


