A study of diclofenac-induced teratogenicity during organogenesis using a whole rat embryo culture model

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BACKGROUND: Diclofenac is a non-steroidal anti-inflammatory drug, commonly used by reproductive age women for the treatment of a variety of conditions. However, there is limited information regarding the teratogenic effects of this drug. METHODS: The effect of diclofenac on the developing embryo during the critical period of organogenesis was investigated by using a whole rat embryo culture model. Embryos were exposed to various concentrations of diclofenac and scored for growth and differentiation at the end of the culture period. RESULTS: Total developmental score and score for caudal neural tube, flexion and hindlimb were significantly lower in embryos exposed to high concentrations of diclofenac (7.5 and 15.0 µg/ml), but no difference in these parameters was observed when embryos were exposed to low concentration of diclofenac (1.5, 2.5 and 5.0 µg/ml). No significant differences in yolk sac diameter, crown–rump length and number of somites was found between embryos in the experimental and the control group. CONCLUSIONS: Our study has demonstrated that diclofenac exerts direct teratogenic effects on rat embryos. Until more is known about the effects of diclofenac (especially in moderate to high doses) in women of reproductive age, we suggest its use should be treated with caution.

Key words: diclofenac/teratogenicity/whole rat embryo culture

Introduction

Diclofenac is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAID) by women of reproductive age. It is used for the treatment of arthritis, soft tissue injuries (Todd and Sorkin, 1988), dysmenorrhoea and menorrhagia (Dawood, 1993). NSAID are effective analgesic and anti-inflammatory agents and their therapeutic effects are mediated through inhibition of prostaglandin synthesis via a cyclooxygenase enzyme (Van den Veyver and Moise, 1993). Since more than half of all pregnancies are unplanned (Bitto et al., 1997), women may incidentally become pregnant while receiving NSAID therapy. Counselling of these women is difficult because of the lack of information regarding the teratogenic effects of these drugs.

Aspirin and other NSAID act by inhibition of prostaglandin synthesis. Aspirin has been shown to induce a wide range of malformations when given in the first trimester to animals, including central nervous system abnormalities, spina bifida and hindlimb bud abnormalities (Kimmel et al., 1971; Klein et al., 1981). Teratogenicity of aspirin is gestation dependent. Studies on rat embryos showed that the incidence of abnormalities induced by aspirin treatment on gestation day 9 (the critical period of organogenesis) was higher than on gestation day 11 (Kimmel et al., 1971). Other malformations, such as cleft palate, have been reported when mice embryos were exposed to NSAID at gestational age of day 13.5 (Montenegro and Palomino, 1990).

Diclofenac has also been shown to inhibit implantation and embryonic development in rats when given on gestation day 5 (Carp et al., 1988). It was reported that diclofenac at a high concentration of 75 µg/ml was toxic to rat blastocysts. The same study also showed that diclofenac inhibited implantation and caused embryo growth retardation at a concentration of 40 µg/ml. In a recent study, a positive association between use of NSAID during pregnancy and miscarriages was reported (Nielsen et al., 2001). However, information regarding teratogenicity of NSAID during the critical period of organogenesis is lacking. Because aspirin and other NSAID share a similar mechanism of action, we postulated that NSAID might induce congenital abnormalities when given during the critical period of organogenesis.

The aim of the present study is to investigate the teratogenicity of diclofenac in explanted rat embryos undergoing organogenesis. The whole embryo culture model is a well recognized method of investigation in teratology (Webster et al., 1997). The advantage of this model is that the direct effect of the interested agent on the developing embryo can be studied (Webster et al., 1997). It is important to study the direct effect of diclofenac because in a previous experiment, we showed that diclofenac crosses the placenta readily in the first trimester of human pregnancy resulting in a fetal diclofenac...
concentration which is the same as the maternal serum concentration (Siu et al., 2000).

Materials and methods

Animals
Timed-gestation pregnant Sprague–Dawley rats were supplied by the animal house of the Chinese University of Hong Kong as the embryo donors in this study. The day on which spermatozoa were found in the vaginal smear was defined as day zero of pregnancy. The Animal Ethics Committee of the Institute approved this study.

Whole embryo culture
The whole embryo culture system was based on the model previously described (New, 1978). Animals were killed by diethyl ether overdose at gestational day 9.5 between 0900 and 1000 h in the morning and embryos were explanted. To minimize variation, only embryos with crown–rump length of 1.5 ± 0.3 mm were used for the experiment. Embryos were explanted from four pregnant rats at one time. They were then mixed together and three to five embryos were assigned to a culture bottle belonging to one of the experimental groups. The investigator who assigned the embryos was unaware of which experimental group the embryos were assigned to. Embryos were then cultured for 48 h using a rotating-bottle culture unit, rotating at a constant rate of 60 revolutions per min.

Each culture bottle contained 1 ml of culture medium per embryo. Each ml of culture medium contained: (i) equal volumes of Sprague–Dawley rat serum and Dulbecco’s modified eagle medium (DMEM) (Gibco BRL, USA); (ii) penicillin G (Sigma, UK) and streptomycin sulphate (Sigma) at a final concentration of 60 and 100 µg/ml respectively; and (iii) diclofenac sodium solution (Voltaren, Switzerland) at a final concentration depending on the study group.

During the period of culture, the system was continuously aerated with initially a gas mixture of 5% CO₂, 5% O₂ and 90% N₂ for 24 h, followed by 5% CO₂, 20% O₂ in 75% N₂ for the next 8 h and then 5% CO₂, 40% O₂ in 55% N₂ for the remaining 16 h. The switching of aerating gas was performed automatically by a timer-controlled system. The different types of gas mixtures were premixed and prepared commercially.

Experimental groups
During the first part of the experiment, embryos were randomly assigned to one of the following four study groups. Group 1 was the control group without diclofenac. Embryos in groups 2 to 4 were exposed to diclofenac at a concentration of 1.5, 7.5 and 15 µg/ml respectively. Based on the result of the first part of the experiment, the second part was performed to investigate the lowest teratogenic concentration of diclofenac, and the following diclofenac concentrations were used: 0 (control), 2.5 and 5.0 µg/ml. The concentrations of 1.5 and 2.5 µg/ml were the average peak plasma diclofenac concentrations after a single oral dose of 50 and 150 mg respectively of delayed-release (enteric-coated) diclofenac sodium tablets (American Society of Health System Pharmacist, 2000).

Morphological assessment
Embryos were examined after 48 h of culture at the equivalent of 11.5 days of gestation, by a researcher who was not aware of the study group assignment. Mean yolk sac diameter and crown–rump length were measured. Embryonic morphologies were studied according to a standard morphological scoring system (Van Maele-Fabry et al., 1990), which gives a numerical score (0–5) to 17 morphological features depending on their stages of development. To assess intra-observer error of the scoring process, 10 embryos were set aside and rescored later on the same day. The intraclass correlation coefficient was 0.92. Embryos with total morphological scores of less than two were the most likely embryos to be damaged due to explantation and were therefore excluded from the analysis.

Statistical evaluation
Between-group differences were analysed by the Kruskal–Wallis test; Dunn’s test was used as a posteriori test (Dunn., 1964), when a difference was found with the Kruskal–Wallis test. Analyses were performed by the Statistical Package for Social Sciences for Windows version 10.0 (SPSS Inc., Illinois, USA). A P value of <0.05 was considered statistically significant.

Results
For the first part of the experiment, rat embryos were exposed to a high concentration of diclofenac up to 15.0 µg/ml. Four embryos were excluded because of having morphological scores of <2 (three in the 15.0 µg/ml diclofenac group and one in the 7.5 µg/ml diclofenac group). There was no significant between-group difference in yolk sac diameter, crown–rump...
vivo animal models, this in-vitro model enables the direct

assessments of external factors on embryogenesis and is not

affected by any pharmacokinetic and pharmacodynamic differences

between human and animals. Webster et al. concluded that with this model ‘adverse embryonic outcomes (malformations or embryotoxicity) are directly related to the serum concentration of the compound being tested and can be compared to the serum concentration in the human. A similar

comparison is not possible after in vivo testing because for most compounds there are major pharmacokinetic differences between humans and experimental animals’ (Webster et al., 1997). Of course, fundamental biological differences exist between human and animals and therefore all results generated from animal studies should be interpreted with caution.

We have studied rat embryo exposure to diclofenac from gestational days 9.5 to 11.5. The overall embryonic growth, as measured by yolk sac diameter, crown–rump length and number of somites were not affected by diclofenac up to 15.0 µg/ml, which is equivalent to 10 times the plasma concentration after a single oral dose. However, significant effects on organogenesis were observed when rat embryos were exposed to ≥7.5 µg/ml of diclofenac. We also found that the caudal neural tube and hindlimb buds were particularly vulnerable. This pattern of abnormality is similar to those after exposure to aspirin treatment (McGarrity et al., 1981).

Our results showed that diclofenac demonstrates teratogenic effects in rat embryos at a relatively low concentration (7.5 µg/ml). Although this concentration is still higher than the mean peak plasma concentration achieved after a single oral dose of diclofenac, it is of clinical significance because there is a significant variation in mean peak plasma concentration

Discussion
In this study, we have chosen the whole embryo culture model to study the teratogenicity of diclofenac. With this model, rat embryos were cultured in vitro from gestational day 9.5 to 11.5, which is the critical period of organogenesis in the rat, equivalent to 3–6 weeks after fertilization in human embryos. This whole embryo culture model has been used extensively in studies in the field of teratogenesis and related mechanisms (Freinkel et al., 1984; Hewitt et al., 2000). Unlike other in-vivo animal models, this in-vitro model enables the direct

Table I. Developmental characteristics of rat embryos exposed to high diclofenac concentrations

<table>
<thead>
<tr>
<th>Diclofenac concentrations</th>
<th>Control (n = 29)</th>
<th>1.5 µg/ml (n = 34)</th>
<th>7.5 µg/ml (n = 31)</th>
<th>15.0 µg/ml (n = 32)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk sac diameter (mm)</td>
<td>4.1 (3.8–4.4)</td>
<td>4.2 (4.0–4.4)</td>
<td>4.0 (4.0–4.3)</td>
<td>4.0 (3.6–4.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Crown–rump length (mm)</td>
<td>3.3 (3.0–3.5)</td>
<td>3.5 (3.1–3.6)</td>
<td>3.3 (3.0–3.5)</td>
<td>3.1 (2.6–3.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Total morphological score</td>
<td>44.0 (39.5–49.5)</td>
<td>46.0 (42.8–48.3)</td>
<td>40.0 (38.0–45.0)</td>
<td>40.0 (31.0–44.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Number of somites</td>
<td>22.0 (20.5–23.0)</td>
<td>22.0 (20.0–23.0)</td>
<td>21.0 (19.0–22.0)</td>
<td>22.0 (17.5–22.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Caudal neural tube</td>
<td>4.0 (3.5–5.0)</td>
<td>4.0 (4.0–5.0)</td>
<td>3.0 (3.4–4.0)</td>
<td>3.0 (3.0–4.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hindlimb</td>
<td>1.0 (1.0–2.0)</td>
<td>2.0 (1.0–2.0)</td>
<td>1.0 (0.0–2.0)</td>
<td>1.0 (0.0–1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Flexion</td>
<td>4.0 (4.0–4.0)</td>
<td>4.0 (4.0–4.0)</td>
<td>4.0 (4.0–4.0)</td>
<td>4.0 (2.0–4.0)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

*aVersus control group, P < 0.05.
Data represent median (interquartile range).
NS = not significant.

Table II. Developmental characteristics of rat embryos exposed to low diclofenac concentrations

<table>
<thead>
<tr>
<th>Diclofenac concentrations</th>
<th>Control (n = 19)</th>
<th>2.5 µg/ml (n = 26)</th>
<th>5.0 µg/ml (n = 25)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yolk sac diameter (mm)</td>
<td>4.2 (4.0–4.9)</td>
<td>4.3 (4.0–4.8)</td>
<td>4.5 (4.0–5.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Crown–rump length (mm)</td>
<td>3.2 (2.9–3.6)</td>
<td>3.3 (3.1–3.7)</td>
<td>3.5 (3.2–3.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Total morphological score</td>
<td>47.0 (36.0–49.0)</td>
<td>48.0 (45.0–49.5)</td>
<td>48.0 (42.3–49.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Number of somites</td>
<td>22.0 (16.0–23.0)</td>
<td>22.0 (21.0–23.0)</td>
<td>22.0 (19.3–22.8)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data represent median (interquartile range).
NS = not significant.

length and number of somites. However, there was a significant
difference between the four groups in total morphological score (P < 0.001) (Table I; Figures 1 and 2). Post-hoc analysis showed that embryos in the high diclofenac concentration groups (7.5 and 15.0 µg/ml) had a significantly lower total morphological score compared with the control group (P < 0.05). Regarding individual morphological features, post-hoc analysis also revealed that embryos exposed to high concentrations of diclofenac had significantly lower scores for caudal neural tube, flexion and hindlimb compared with the control group.

During the second part of the experiment, rat embryos were exposed to low concentrations of diclofenac up to 5.0 µg/ml. Three embryos were excluded because of morphological scores of <2 (one in the control group and two in the 5.0 µg/ml diclofenac group). There was no significant between-group difference in all measured parameters for both growth and morphological development (Table II).

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between different subjects. It has been shown that after a single dose of 100 mg diclofenac sodium tablet (enteric-coated), the peak serum concentration ranged from 2.8–6.6 µg/ml (El-Sayed et al., 1988). Moreover, diclofenac has been shown to penetrate and accumulate in the synovial cavity; and synovial diclofenac concentrations are increased and sustained for periods up to 12 h following multiple doses, with a ratio of synovial fluid to plasma concentration of ~5 (Davies and Anderson, 1997). Our previous study also showed that fetal tissue concentration of diclofenac is higher than that in maternal plasma after two oral doses of diclofenac, indicating that the drug may also accumulate in fetal tissue with time (Siu et al., 2000). In a real clinical situation, diclofenac is usually given in multiple doses rather than a single dose. Therefore, it is possible that fetal tissue concentrations may well reach the teratogenic level in some patients who are taking diclofenac.

Although many mechanisms have been proposed, the exact pathway through which NSAID produce teratogenic effects is still uncertain (Montenegro and Palomino, 1990). It has been postulated that aspirin-induced malformations result from cellular death secondary to disturbed blood supply, which is a consequence of transient vasoconstriction due to the inhibition of synthesis of vasodilatory prostaglandins (Klein et al., 1980). Given the similar mechanism of pharmacological action and pattern of teratogenic abnormalities produced by aspirin and diclofenac, it is possible that diclofenac induces malformation through a similar pathway. Further studies are required to elucidate the mechanism of teratogenicity of NSAID.

In summary, our study has demonstrated that diclofenac exerts direct teratogenic effects on rat embryos. Since diclofenac can accumulate in fetuses, it is potentially teratogenic in humans. Although results from animal teratogenicity studies may not reflect the circumstances in humans, our findings suggest that adverse effects of diclofenac exposure during early pregnancy warrant further investigation and monitoring. Before more information in humans becomes available, the use of NSAID (especially regular and large therapeutic doses) in women of childbearing age should be treated with a degree of caution.

References


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Teratogenic effects of diclofenac