PHARMACIA

Pharmacia 71: 1–8
DOI 10.3897/pharmacia.71.e121947

Research Article

Teratogenic effect of 1.3 bis(*p*-Hydroxyphenyl) urea on Wistar rats (*Rattus norvegicus* L.)

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Received 29 February 2024 ♦ **Accepted** 26 March 2024 ♦ **Published** 9 April 2024

Citation: Satria D, Fauzi ZPA, Harahap U, Yuandani, Waruwu SB, Purnomo H (2024) Teratogenic effect of 1.3 bis(*p*-Hydroxyphenyl) urea on Wistar rats (*Rattus norvegicus* L.). Pharmacia 71: 1–8. https://doi.org/10.3897/pharmacia.71.e121947

Abstract

Pain is a physiological disorder that pregnant women often experience, so they take various medications to relieve the pain. Many pain relievers on the market have teratogenic effects on pregnant women. 1.3 bis(*p*-Hydroxyphenyl)urea is a modification of *p*-aminophenol and has analgesic and anti-inflammatory properties and fewer hepatotoxic side effects. However, its safety in pregnant women has not been studied. This research continues our previous research to determine the teratogenic effects on white rat fetuses and toxic effects on pregnant white rats after administering 1.3 bis(*p*-Hydroxyphenyl)urea. The teratogenic effect test was carried out on pregnant rats divided into five groups with doses of 50, 500 and 1000 mg/kg BW, CMC Sodium 0.5%, and Gabapentin 50 mg/kg BW as the control group. Rats marked as pregnant were given test preparations and observed for toxic symptoms, and then fetal weight, body length, internal malformations and bone malformations were observed surgically. The study showed that administration at 50 and 500 mg/kg BW doses did not have a teratogenic effect. However, at a dose of 1000 mg/kg BW, it causes teratogenic effects characterized by fetal bleeding and bone abnormalities.

Keywords

Pregnant, analgesic, anti-inflammatory, pain, *p*-aminopheno

Introduction

Pregnancy is a physiological process whereby, during pregnancy, a pregnant woman can experience physiological disorders such as vomiting, nausea, and back pain (Bryndal et al. 2020; Mascarenhas et al. 2022; Ertmann et al. 2023). The most frequently administered medications to pregnant women to relieve fever, discomfort, and inflammation are non-steroidal anti-inflammatory drugs (NSAIDs) (Black et al. 2019; Kwiatkowski et al. 2020; Saldanha et al. 2021). NSAIDs have the potential to cause many problems and adverse effects, including gastrointestinal bleeding, oedema, hypertension, and reduced kidney function (Moore et al. 2019). Several studies have provided evidence that NSAIDs can cross the placenta in humans, reach fetal circulation and provide side effects such as miscarriage, congenital heart disease, and vascular, brain, kidney and lung effects (Price et al. 2020; Zafeiri

et al. 2021; Elnashar et al. 2024). The two main isoforms of the cyclooxygenase enzymes, the principal NSAIDs' primary pharmacological mechanisms in the fetus, are reversibly inhibited to block the prostaglandin manufacturing pathway (cyclooxygenase-1 and cyclooxygenase-2). Because fetal urine is the primary source of amniotic fluid in the second and subsequent trimesters, prostaglandin blocking and decreased prostaglandin receptor activation by NSAIDs result in decreased renal perfusion (Campbell et al. 2017; Dathe et al. 2019; D'Ambrosio et al. 2023).

One of the modified p-aminophenol molecules, 1.3 bis(*p*-Hydroxyphenyl)urea, has an atomic charge of (-0.110), attaches to liver cells, and is expected to be more effective at relieving pain than paracetamol while having fewer hepatotoxic side effects (-0.107) (Purnomo et al. 2016; Purnomo et al. 2017). A previous study showed that the 1.3 bis(*p*-Hydroxyphenyl)urea inhibited carrageenan-induced inflammation in mouse paws while having fewer toxic side effects (Waruwu et al. 2022). 1.3 bis(*p*-Hydroxyphenyl)urea had a higher activity in binding COX-1 (1CQE) and TNF (2AZ5) than the control, dexamethasone and diclofenac, according to the in silico test for COX-1 and TNF-α (Harahap et al. 2021). The 1.3 bis(*p*-Hydroxyphenyl)urea can reduce neutrophil counts and COX-2, TNF, IL-1, and IL-6 expression in rat paw inflammatory tissue. Acute toxicity testing revealed that the substance 1.3 bis(*p*-Hydroxyphenyl)urea was essentially non-toxic because it did not produce toxic symptoms up to a level of 5000 mg/kg BW. It was determined through subchronic toxicity testing that this substance is safe at a level of 1000 mg/kg BW (Satria et al. 2022; Waruwu et al. 2022). This study is an extension of our earlier work, which attempts to demonstrate the teratogenic effect on white mouse fetuses by examining the mother's reproductive characteristics, external abnormalities, and skeletal deformities following administration of the 1.3 bis(*p*-Hydroxyphenyl)urea.

Materials and methods

Reagents and chemical

The materials used in this study were 0.9% NaCl, 0.5% CMC Sodium, 0.1% methylene blue powder, 1% KOH solution, 2% KOH solution, 4% KOH solution, clarifying solutions A, B and C, PA glycerin, Alizarin red powder, 95% ethanol, glacial acetic acid, picric acid crystals, 37% formalin, distilled water, Ketamine injection and Gabapentin. Surgical instruments and laboratory glassware were also used in this study.

Animals and study design

The procedures for using test animals have been reviewed by the Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, Department of Biology, Universitas Sumatera Utara, with approval number 0530/KEPH-FMIPA/2022. The animals used were Wistar strain rats (*Rattus norvegicus* L.) weighing 150–200 g and were about two months old. Five groups were formed from 25 rats consisting of 5 rats. Before testing, rats were acclimatized for 7–14 days. Rats were housed in a temperature-controlled environment with access to food and water. Experimental animals should be cared for in clean, well-ventilated cages two weeks before testing. Each group of animals is separated and treated individually during teratogenic testing. One rat was removed from each cage before the other rats. All procedures and animal care were performed at room temperature (20–22 °C), and additional precautions were taken to prevent environmental disturbances that could interfere with animal responses. Female rats declared fertile were mated with male rats in the afternoon for one night with a ratio of 3 males and two females in one cage (OECD 2018; Yuandani et al. 2021; BPOM RI 2022). The next day the female rats were separated from the male rats, and a vaginal swab was examined again. Thirty pregnant rats were utilized in the experiments, separated into five groups five. Following is a breakdown of the animal groups used in the teratogenic test:

- Group I: CMC sodium 0.5% as a negative control
- Group II: Gabapentin 50 mg/kg BW, a positive control.
- Group III: Treatment; 50 mg/kg BW of a test preparation was administered.
- Group IV: Treatment; 500 mg/kg BW of a test preparation was administered.
- Group V: Treatment; 1000 mg/kg BW of a test preparation was administered.

They administered suspension 1.3 bis(*p*-Hydroxyphenyl)urea from day 6 to day 15 of pregnancy. The mother rats were weighed every three days until the 19th day of pregnancy to determine the healthy development of the rats. The test preparation was given to pregnant rats during the organogenesis period and observed twice daily for 6 hours. Observations of the condition of pregnant rats were carried out every day during the test period for the presence of death and symptoms of toxicity such as piloerection, vaginal bleeding, diarrhoea, sedation and death. Food consumption of pregnant rats was weighed two times a week starting from the first day of pregnancy. The rats were put under anaesthesia on the 19th day, the fetuses were dissected, the fetuses were seen, the live fetuses and the resorption fetuses, their weight and body length were noted, and then the number was tallied (OECD 2018; Yuandani et al. 2021).

Fetal observation

The fetus is placed in a bouquet solution to preserve and color it so it is easier to observe. The fetus was also fixed simultaneously by soaking it in 95% alcohol for seven days. The fetal anatomy, including anencephaly, cleft palate, spina bifida, humpback body, limb abnormalities,

micromelia, liver, heart, and kidneys, were observed after three days of immersion. After seven days, the fixated fetus was removed and washed with distilled water. The fetus is then neatly peeled, dried with a tissue, and disposed of with the stomach contents and internal organs. Skinned and gutted fetuses are immersed in 1% KOH solution for 12 hours. The solution was changed to alizarin solution and allowed to soak for 24 hours after 12 hours in 1% KOH solution. The fetal skeleton was deemed red or purple if staining was successful. After being immersed in alizarin solution for 24 hours, fetuses were once more immersed in 2% KOH solution for 12 hours to remove any leftover alizarin dye. The purification was deemed effective if no trace of alizarin dye remained on the epidermal layer. To measure the growth in the number of bones, colored fetal skeletons were soaked in solutions A, B, and C for one hour each before being ready to be viewed under a binocular microscope at a magnification of 40 (Paumgartten 2010; Uche-Nwachi and McEwen 2010; Elgndy et al. 2019; BPOM RI 2022).

Statistical analysis

The results of this study are presented as the average ± Standard Error Mean. Statistical analysis used Statistical Product and Service Solution (SPSS) version 26 with ANOVA and Tukey methods.

Results and discussion

Body weight observation

After administration of the test preparation, body weight is measured on days 6 to 19 of pregnancy. There was no significant difference in changes in the body weight of pregnant rats ($p > 0.05$) in each group. Food intake in each test group also did not differ. The percentage of weight gain is calculated as in Fig. 1. However, based on the graph, it can be seen that the average weight gain was more significant in the CMC sodium control group. Meanwhile, the gabapentin group experienced lower weight gain than the other groups.

After receiving the test preparation (day 6–19 of pregnancy), the body weight of pregnant rats was measured to assess the toxicity of the test preparation. Ten days (days 6–15 of pregnancy) are the organogenesis phase in pregnant rats. The formation of organ systems must occur in the organogenesis phase. The same stage of fetal development during exposure determines the critical phase, a teratogenic reaction (Uche-Nwachi and McEwen 2010; Yuandani et al. 2021). 1.3 bis(*p*-Hydroxyphenyl) urea increased the body weight of rats based on acute and chronic toxicity tests (Waruwu et al. 2022). In this study, the body weight of pregnant rats in the 50, 500 and 1000 mg/kg BW treatment groups increased. However, there was no statistically significant difference between the gabapentin and CMC sodium control groups ($p > 0.05$). However, the gabapentin 50 mg/kg BW group experienced less weight gain than the test group and the CMC Sodium group. Changes in body weight and number of fetuses are dangerous in pregnant rats (Chen et al. 2020; Mozafari et al. 2020).

Toxic symptoms and mortality

Maternal fatalities or overt toxicity-related clinical indications are usually indicators of maternal toxicity (Hande and Veena 1993; Olayaki et al. 2009; Paumgartten 2010), such as decreased food or water intake, decreased body weight increase, and decreased body weight. Weight loss, feed consumption by pregnant rats, toxic symptoms, and death were all observed in this study as toxic effects of pregnant rats. None of the pregnant rats showed any toxic symptoms or death during pregnancy, including changes in their general behavior. Surgery was performed on the 19th day of gestation. No lesions were found on the organs of pregnant rats in all pregnant rats treated.

Table 1. The body weight of pregnant rats following treatment (Mean ± Standard Error Mean).

Days	Group				
	CMC Sodium 0.5%	Gabapentin 50 mg	1.3 bis(*p*-Hydroxyphenyl)urea		
			50 mg	500 mg	1000 mg
6	227.46 ± 9.28	230.2 ± 7.78	228.66 ± 4.53	230.78 ± 5.42	227.62 ± 6.03
7	228.98 ± 9.40	231.82 ± 9.44	231.66 ± 5.23	233.3 ± 4.24	229.94 ± 7.70
8	231.44 ± 9.33	234.2 ± 9.13	234.78 ± 4.80	238.24 ± 4.42	232.56 ± 8.92
9	236.16 ± 9.69	238.44 ± 10.70	236.7 ± 4.80	241.74 ± 3.72	239.36 ± 8.13
10	237.92 ± 8.69	242.08 ± 10.68	239.18 ± 5.49	244.52 ± 4.13	242.24 ± 8.56
11	240.88 ± 10.84	243.78 ± 10.12	240.24 ± 5.49	248.74 ± 5.73	245.52 ± 9.68
12	243.8 ± 9.14	245.78 ± 10.23	241.88 ± 6.82	253.08 ± 6.56	248.8 ± 10.64
13	245.76 ± 11.55	248.02 ± 11.12	243.58 ± 7.12	257.18 ± 6.38	253.1 ± 10.37
14	251.6 ± 9.66	250.92 ± 11.48	247.04 ± 8.10	260.52 ± 8.40	257.12 ± 10.00
15	257.52 ± 10.81	254.38 ± 10.24	250.56 ± 8.58	263.16 ± 9.11	260.42 ± 8.93
16	262.94 ± 10.83	259.12 ± 10.46	254.6 ± 10.11	268.3 ± 9.87	264.44 ± 8.93
17	274.48 ± 12.83	265.12 ± 9.70	260.12 ± 12.59	272.1 ± 10.03	269.62 ± 10.27
18	282.96 ± 14.82	271.36 ± 10.14	270.68 ± 14.96	279.6 ± 9.19	278.72 ± 10.19
19	290.2 ± 14.95	275.98 ± 9.45	278.68 ± 14.71	283.7 ± 8.40	283.86 ± 9.95

Number of fetuses

From the uterus, the entire fetus is removed and disinfected. All fetuses were still alive after administration of the 1.3 bis(*p*-Hydroxyphenyl)urea. Between the 1.3 bis(*p*-Hydroxyphenyl)urea group, the negative control group, and the Gabapentin group, there were no significant differences in the number of fetuses ($p > 0.05$) (Table 2). However, the CMC Sodium group showed the highest number of fetuses (50 fetuses) and the few in the Gabapentin group (41 fetuses). There was a significant difference in fetal body weight between the CMC Sodium 0.5% group and the group given 1.3 bis(*p*-Hydroxyphenyl)urea at a dose of 1000 mg/kg BW and gabapentin 50 mg/kg BW ($p > 0.05$). The fetus experienced weight loss at a dose of 1000 mg and Gabapentin 50 mg/kg BW. All groups did not experience changes in fetal body length ($p > 0.05$). Development and nutritional support during pregnancy can affect weight, body length and number of fetuses. Wistar rat mothers typically give birth to 1 to 13 offspring, with 10 being the most common number (Chahoud and Paumgartten 2009; Zouridis et al. 2021; Martínez-Oca et al. 2023).

Table 2. Number of fetuses.

Group	Number of rats	Number of fetuses		
		Life	Death	Mean ± SEM
CMC Sodium 0.5%	5	50	-	10.00 ± 0.54
Gabapentin 50 mg	5	41	-	9.60 ± 0.51
50 mg	5	48	-	8.20 ± 0.73
500 mg	5	48	-	9.60 ± 0.24
1000 mg	5	48	-	9.60 ± 0.24

Weight and length of fetal body

Body weight and length are the two main parameters in evaluating teratogenicity. Fig. 1 shows that the 1.3 bis(*p*-Hydroxyphenyl)urea at a dose of 1000 mg/kg BW has a significantly different decrease in the negative control group ($p < 0.05$). The effect of Gabapentin in reducing fetal weight was lower than in the 1000 mg/kg BW test group. Furthermore, the fetal body length in the compound group 1.3 bis(*p*-Hydroxyphenyl)urea at doses of 1000, 500 and 50 mg/kg BW did not have a significant difference compared to the positive control group ($p > 0.05$).

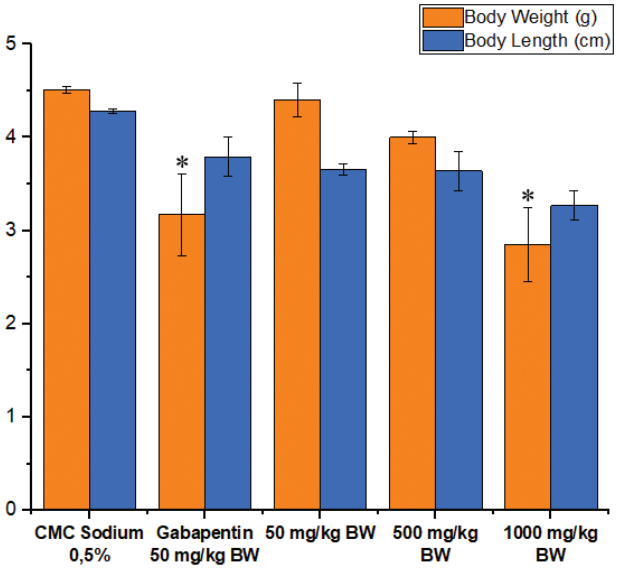

Figure 1. Fetal body weight and length after being treated (data: mean ± SEM, *$p < 0.05$ significant compared to CMC Sodium 0.5% group control).

The weight, length and number of fetuses can change depending on hormone levels. Growth hormone is essential for embryonic growth, influencing the metabolism of proteins, electrolytes, carbohydrates and fats (El Gendy et al. 2015; Caputo et al. 2021; Plante et al. 2022). The weight and body length of the fetus can increase or decrease due to a xenobiont, which interferes with the ability of the hypothalamus to excrete GHRH and GHIH (foreign substances in the body). Placental cells are the primary source of hormones required for embryonic growth and development (Braun 2020; Lewis et al. 2022; Chauhan et al. 2023). The placenta is vital for exchanging blood components between mother and fetus. During pregnancy, these hormones promote healthy fetal growth and development. The placenta connects fetal and maternal blood circulation and serves as a barrier to protect the fetus from xenobiotics in the mother's blood (Aplin et al. 2020; Tang et al. 2020; Gupta and Gupta 2022; Mohammed et al. 2022).

External malformation

Fetuses whose reproductive performance has been assessed are then divided into three parts. 2/3 of the fetuses from each mother were immersed in a boudin environment for three days to observe external malformations; the remaining 1/3 were immersed in 95% alcohol fixation solution for two weeks to prepare for observation of the fetal skeleton before being immersed in red alizarin solution. After immersing the fetus in Bouin's solution, external abnormalities were visible. Fig. 2 and Table 3 show that 1.3 bis(*p*-Hydroxyphenyl)urea at a dose of 1000 mg/kg BW causes external malformations in the form of hematomas in several parts of the fetal body. The 1.3 bis(*p*-Hydroxyphenyl)urea group at 50 and 500 mg/kg BW doses did not cause external malformations.

The 1.3 bis(*p*-Hydroxyphenyl)urea group at a dose of 1000 mg and the gabapentin group both showed hematomas in various places of the fetal body, consistent with

Table 3. Effect of administration 1.3 bis(*p*-Hydroxyphenyl)urea on external malformation appearance.

Parameter	Group				
	CMC Sodium 0.5%	Gabapentin 50 mg	1.3 bis(*p*-Hydroxyphenyl) urea		
			50 mg	500 mg	1000 mg
Number of fetuses examined	25	24	20	24	24
Humpback body	-	5	-	-	-
Dwarf	-	4	-	-	-
Hematoma	-	11	-	-	15

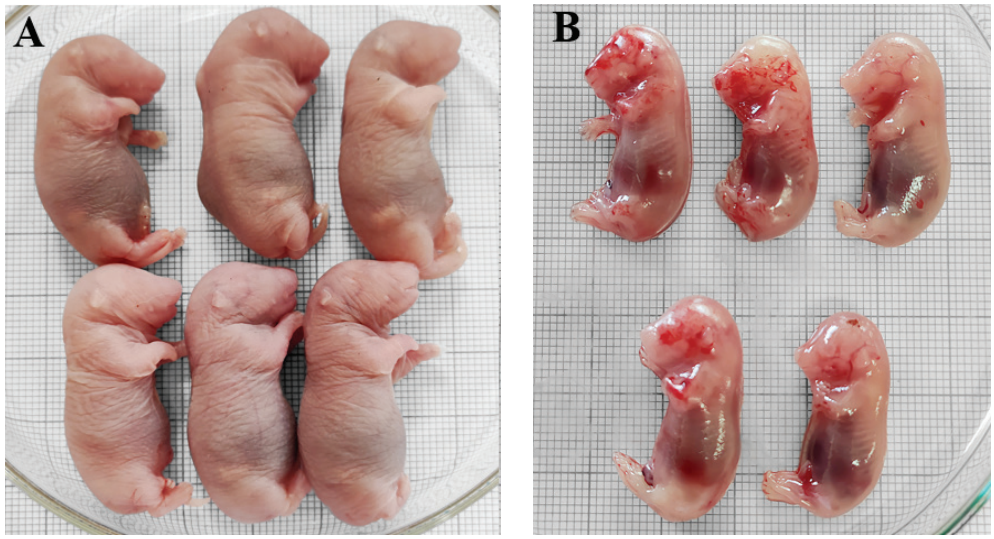

Figure 2. Effect of 1.3 bis(*p*-Hydroxyphenyl)urea on external malformations. **A.** Normal fetus; **B.** Fetus with hematoma.

external deformities. The fetus's head, neck, abdomen, front legs, and back all have hematomas, which are red or dark spots (Mokhtar et al. 2023). The presence of 1.3 bis(*p*-Hydroxyphenyl)urea can disturb osmotic balance, which can cross the placental barrier. Under normal circumstances, the embryo develops in the amnion, which is isotonic to body fluids. Impaired organ function, primarily metabolic and secretory organs, can cause the inability to maintain isotonic conditions between the amnion and the embryo's body fluids, resulting in chemicals in the embryo's blood circulation lasting longer and reaching higher levels (Menegola et al. 2001; Booth et al. 2022; Zin et al. 2022).

Skeletal malformations

The 1.3 bis(*p*-Hydroxyphenyl)urea at a dose of 1000 mg/kg BW caused skeletal malformations. Skeletal malformations that occur include malformations of the trunk and malformations of the front and hind paws (Table 4, Fig. 3). The gabapentin group experienced a humpback body, but this did not occur in the test group. Humpback body is a spinal structural disorder that causes the fetus to appear bent. Humpback body is caused by the death of the cells that make up the spine, resulting in the growth rate between the bones becoming unequal and the bones becoming bent (Muna et al. 1970; Yuandani et al. 2021). In skeletal observations, the 1.3 bis(*p*-Hydroxyphenyl) urea group at a dose of 1000 mg/kg BW caused skeletal deformities and abnormalities in the number of sternum bones, forelimbs and hind limbs. The fetal sternum measures six in typical conditions (Koehn et al. 2019). However, it was found that there was a decrease in the number of sternum, phalanges and metacarpals, which make up the front claws, as well as phalanges and metatarsals, which make up the back claws in the 1.3 bis(*p*-Hydroxyphenyl) urea group at a dose of 1000 mg/kg BW. Doses of 50 mg/kg BW and 500 mg/kg BW group 1.3 bis(*p*-Hydroxyphenyl)urea did not cause skeletal abnormalities.

Table 4. The impact of 1.3 bis(*p*-Hydroxyphenyl)urea treatment on the skeletal deformities.

Parameter	Group				
	CMC Sodium 0.5%	Gabapentin 50 mg	1.3 bis(*p*-Hydroxyphenyl)urea		
			50 mg	500 mg	1000 mg
Number of fetuses examined	**25**	**24**	**21**	**24**	**24**
Truncus malformation					
• Sternum	-	7	-	-	5
• Vertebrae caudalis	-	-	-	-	-
Front claw malformation					
• Metacarpals	-	3	-	-	3
• Phalanges	-	6	-	-	4
Hind claw malformation					
• Metatarsals	-	4	-	-	1
• Phalanges	-	4	-	-	3

It is currently unclear how 1.3 bis(*p*-Hydroxyphenyl) urea causes teratogenic consequences. However, many studies on p-aminophenol derivatives have found that paracetamol has a transfer through the placenta of around 40% to reach the fetus when given to pregnant women (Koehn et al. 2019). According to earlier research, paracetamol can cause congenital abnormalities and hurt the developing mouse embryo when taken in average amounts (Fernandes 2017). Because some of the skeletons in rat fetuses have not yet fully ossified at the end of the gestation period, more research needs to be done on the use of double staining of bone using Alizarin red and Alciant blue staining to identify the cartilage in the fetus.

Conclusion

According to this study, the compound 1.3 bis(*p*-Hydroxyphenyl)urea was given to pregnant rats during the organogenesis period at doses of 50 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW without having toxic effects

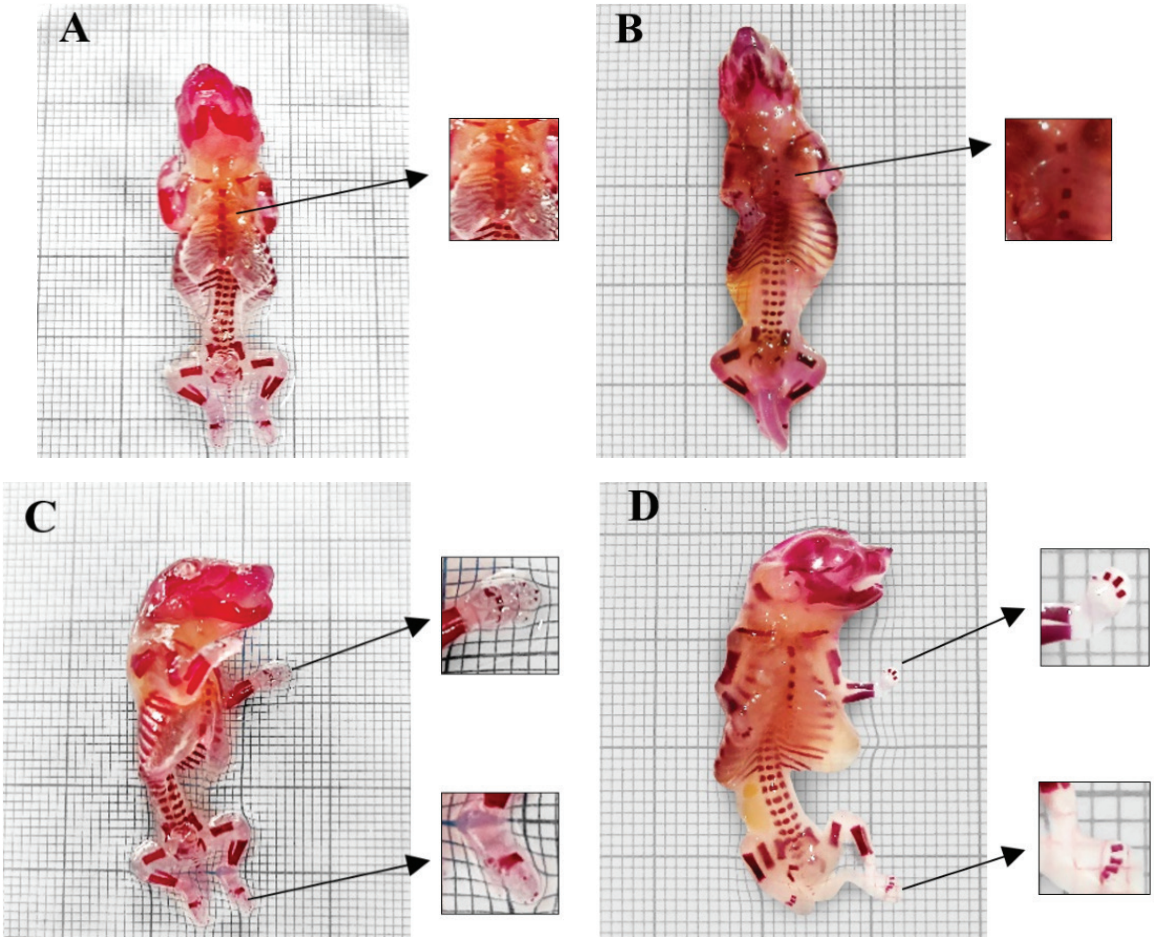

Figure 3. Effect of compound 1.3 bis(*p*-Hydroxyphenyl)urea on skeletal malformations. **A.** Normal sternum; **B.** Abnormal sternum; **C.** The front paws and hind claws are normal; **D.** Abnormal front paws and hind claws.

on the animals. However, the compound was found to be teratogenic in the fetus at the dose of 1000 mg/kg BW, where there are external malformations in the form of hematoma. The 1.3 bis(*p*-Hydroxyphenyl)urea should be used cautiously during pregnancy as it exhibits teratogenic effects at 1000 mg/kg BW.

Acknowledgements

Ministry of Education, Culture, Research and Technology Republic of Indonesia for providing financial support for the study through the "Hibah Penelitian Dasar" research grant 2022 (Contract No. 6/UN5.2.3.1/PPM/KP-DRTPM/L/2022).

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