Environmental Research 109 (2009) 922–929

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Dose–response relationships of rat fetal skeleton variations: Relevance for risk assessment

Ibrahim Chahoud *, Francisco J.R. Paumgartten [1]

Institute of Clinical Pharmacology and Toxicology, Charité University Medical School Berlin, Garystraße 5, 14195 Berlin, Germany

ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form
6 July 2009
Accepted 27 July 2009
Available online 13 August 2009

Keywords:
Hydroxyurea
5-Fluoro-2′-deoxyuridine
6-Mercaptopurine-riboside
No observed effect level
Skeleton malformations

ABSTRACT

In developmental toxicity studies, skeleton abnormalities found in fetuses at term are classified as variations or malformations. The relevance of skeleton variations for human risk assessment, however, is a controversial issue. This paper is a contribution to the discussion on the interpretation of fetal skeleton variations in the context of risk assessment. Dose–response relationships of skeleton variations and malformations induced by three antineoplastic drugs (FUDR: 5-fluoro-2′-deoxyuridine, HU: hydroxyurea and 6-MPr: 6-mercaptopurine-riboside) were evaluated. FUDR (0, 3, 14, 25, 35, 45, 55 and 65 mg/kg body wt sc) and HU (0, 250, 300, 350, 400, 450, 500 and 550 mg/kg body wt ip) were administered to rats on gestation day 11 (GD 11) while 6-MPr (0, 3, 7, 10 and 14 mg/kg body wt sc) was given on GD 11, or on GD 12. Caesarean sections were performed on GD 21 and all fetuses were cleared and stained with alizarin red S for skeleton examination. Drugs given on GD 11 increased the incidence of thoracic and lumbar vertebra (dumbbell-shaped and bipartite ossification center (o.c.) and sternum (misaligned sternebrae) variations in a dose-dependent manner. Occurrence of zygomatic bone fused with maxilla (a variation in our rats) was also increased by HU and 6-MPr (GD 11) but it was not altered by FUDR. Spontaneous occurrence of wavy ribs was reduced by all treatments. Malformations such as cleft palate, tympanic bone absent and tibia absent were also increased in a dose-dependent manner by the three compounds. No observed effect levels (NOEL) for variations, irrespective of the compound administered, were generally lower than NOELs for malformations. In the discussion, we supported the view that any dose-related increase in the incidence of variations should be taken into account for determination of NOELs in routine studies. Increased occurrences of skeleton variations in term fetuses are also to be considered in risk assessment, unless experimental evidence exists that a particular change has no detrimental effect on the animal survival or health after birth or that it does not occur in humans.

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1. Introduction

In developmental toxicity studies, skeleton observations in fetuses at term are classified as variations or malformations. The distinction between variation and malformation is based mainly on the transience/permanence and also on the potential adversity of the anatomical change for survival or health. According to current definitions, variations are structural changes that occur within the normal population and are unlikely to affect survival or health, whereas malformations are permanent changes that are likely to adversely affect survival, development or function (US EPA, 1991; Chahoud et al., 1999). Some skeleton observations may also be called "delayed ossification" if there is an apparent decrease in the amount of mineralized bone (e.g. "incomplete ossification" or "poorly ossified" bones) as compared with that expected for a given developmental age (i.e. term fetuses). Since in most cases cartilage anlagen is apparently intact, mineralization is likely to take place later and thus these observations are believed to be transient changes of minor impact on survival or health. Delayed ossifications are therefore included among the skeleton variations.

Whether or not a substance-induced increase in the incidence of fetal skeleton variations should be taken into account for human risk assessment is a long-standing controversial issue. It has been argued that chemical-produced increases in variations are not to be considered for risk assessment because they are "unlikely to adversely affect survival or health". The counter argument is that even not being overtly adverse and conveying no apparent selective disadvantage, a treatment-induced increase in the occurrence of variations means that the chemical agent has

* Corresponding author. Fax: +4930 8445 1761.
E-mail address: ibrahim.chahoud@charite.de (I. Chahoud).
[1] Present address: Laboratory of Environmental Toxicology, Department of Biological Sciences, National School of Public Health, Oswaldo Cruz Foundation, Rio de Janeiro, RJ 21045-900, Brazil.

0013-9351/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.envres.2009.07.013

the potential to perturb skeleton development. According to this view, under a different condition of exposure, or in another species, this perturbation of normal bone formation may give rise to a different and more severe outcome.

Another problematic area that contributes to this controversy is the interpretation whether a given fetal skeleton observation is a variation or a malformation. The difficulty in classifying skeletal anomalies in fetuses at term arises from the fact that little is generally known about their persistence into postnatal life and their consequences for survival, growth or health. Twenty-six years ago, Khera (1981) had already highlighted that sometimes distinction between variations (then called "aberrations") and malformations, based on observations of fetuses at term, may become extremely difficult due to our limited knowledge on the postnatal consequences of these anatomical changes. Today, classification of a number of rodent fetal observations still remains a difficult and sometimes questionable decision. This is illustrated by the outcome of a survey showing that a rather low agreement on the classification of a variety of skeleton observations ("grey-zone anomalies") was reached among experts (Solecki et al., 2001).

Recently, the interpretation of fetal skeleton variations in the context of human risk assessment has been brought again into discussion by a series of review articles focusing on different aspects of this topic (Augustine-Rauch, 2007; Carney and Kimmel, 2007; Daston and Seed, 2007; Tyl et al., 2007).

In this article we evaluated dose–response relationships for some skeleton variations and malformations induced by developmental toxicants, and in so doing we provided a set of data for discussing their relevance for quantitative risk assessment. We treated rats at mid-gestation (gestation day 11 or 12) with five to eight doses of an antineoplastic drug (hydroxyurea, 5-fluoro-2′-deoxyuridine and 6-mercatopurine-riboside) and examined the incidence of fetal skeleton abnormalities at term. It is of note that no more than 4 dose levels are usually tested in regulatory required developmental toxicity studies, a number of doses that may be at times insufficient to reveal a clear dose–response relationship. Restricting treatment to a single administration at mid-gestation, on the other hand, was an attempt to attenuating maternal toxicity and to avoiding marked embryo lethality.

2. Materials and methods

2.1. Animals

Nulliparous Wistar female rats (Bor:Wisw/spf, TNO), weighing 190–220 g, and supplied by Fa. Winkelmann, Borchen, Germany, were used in this study. Upon arrival at our animal quarters, rats were housed in standard plastic cages (Makrolon®) and kept under controlled temperature (21 ± 1 °C), relative humidity (50 ± 5%) and 12 h light/dark cycle (lights on at 9:00 a.m.). All animals were fed a standard pellet diet for laboratory rodents (Altromin®1324, Fa. Altromin, Lage, Germany) and received tap water ad libitum.

2.2. Mating

For mating, three females were placed into the cage of one male rat for 2 h (6:00–8:00 a.m.). The first 24 h period after this mating was designated as gestation day 0 (GD 0) if spermatozoa were found in the vaginal smears.

2.3. Treatment

Hydroxyurea (HU: 0, 250, 300, 350, 400, 450, 500 and 550 mg/kg body weight) and 5-fluoro-2′-deoxyuridine (FUDR: 0, 3, 14, 25, 35, 45, 55 and 65 mg/kg body weight) were administered once on GD 11 while 6-mercaptopurine-riboside (6MPr: 0, 3, 7, 10 and 14 mg/kg body weight) was given once either on GD 11 or on GD 12. Hydroxyurea was administered by intraperitoneal route, while other substances were injected subcutaneously at the dorsal side of the neck. The number of treated dams (*N*) per dose level was as follows: HU, 0 mg/kg = 53; 250 mg/kg = 18, 300 mg/kg = 17, 350 mg/kg = 21, 400 mg/kg = 34, 450 mg/kg = 17, 500 mg/kg = 15, 550 mg/kg = 13; FUDR, 0 mg/kg = 47; 3 mg/kg = 18, 14 mg/kg = 23, 25 mg/kg = 21, 35 mg/kg = 20, 45 mg/kg = 10, 55 mg/kg = 15, 65 mg/kg = 15; 6-MPr, 0 mg/kg = 41, 6-MPr GD 11, 3 mg/kg = 15, 7 mg/kg = 17, 10 mg/kg = 37, 14 mg/kg = 16, 6-MPr GD 12, 3 mg/kg = 11, 7 mg/kg = 13, 10 mg/kg = 25, 14 mg/kg = 21.

2.4. Caesarean section

Caesarean sections were performed on GD 21. Rats were killed by decapitation and gravid uteri were removed, weighed with their contents and opened. The uterine position and numbers of viable fetuses and resorptions were recorded. Implantation sites were determined as described by Salewski (1964). Living fetuses were weighed and inspected for external abnormalities. All fetuses were fixed in a buffered formaldehyde solution, cleared and stained with Alizarin red S for skeleton evaluation.

3. Results

3.1. Induction of skeleton variations by antineoplastic drugs: dose–response relationships

The exposure of dams to FUDR, HU or 6-MPr on GD 11 produced a variety of axial skeleton abnormalities in the exposed offspring. Tables 1–3 show some of these skeleton variations and malformations, the occurrence of which was altered in a dose-dependent manner by at least one of these antineoplastic agents.

As shown in Table 1, FUDR caused a dose-dependent increase in the incidences of dumbbell-shaped and bipartite vertebral ossification centra (Fig. 1) in lumbar as well as in thoracic vertebra. The frequency of misaligned sternebrae (Fig. 2) was higher in fetuses from dams treated with the two highest doses, while wavy ribs were increased only at the highest dose of FUDR (Table 1). The occurrence of zygomatic bone fused with maxilla (Fig. 3), however, was not altered by FUDR over the dose range tested (Table 1). Tympanic bone absent, clavicle bent and cleft palate, which are typical malformations, were increased only at higher doses of FUDR, i.e. doses equal to and higher than 35 and 55 mg/kg body weight, respectively (Table 1).

Similarly to FUDR, HU also caused a dose-dependent increase in the incidences of dumbbell-shaped and bipartite o.c. in thoracic and lumbar vertebra (Table 2). Nonetheless, HU produced a dose-related increase in the occurrence of zygomatic bone fused to maxilla as well (Table 2). The frequency of fetuses exhibiting misaligned sternebrae was also elevated at doses of HU equal to or higher than 350 mg/kg body weight (Table 2). HU seemed to have reduced wavy ribs (Fig. 4), the incidence of which ranged from 0% to 3% in the treated groups and was 6.1% and 8.8% in the historical and study controls, respectively (Table 2). The occurrences of tympanic bone absent, cleft palate, clavicle bent and ribs bent, on the other hand, were enhanced by HU at doses equal to or higher than 300, 400 and 450 mg/kg body weight, respectively (Table 2).

6-MPr administered on GD 11 also enhanced the occurrence of dumbbell-shaped and bipartite o.c. in thoracic and lumbar vertebrae in a dose-dependent manner (Table 3). Frequencies of zygomatic bone fused and misaligned sternebrae were elevated while wavy ribs were markedly reduced by administration of 6MPr to dams on GD 11 (Table 3). The two highest doses of 6-MPr given on GD 11 also caused cleft palate, tibia absent, clavicle bent and tympanic bone absent but, in all cases, the incidences were rather low (Table 3). Effects of exposure to 6-MPr on GD 12, however, were far less marked. 6-MPr administered on GD 12 still induced thoracic vertebra dumbbell-shaped and bipartite o.c. but incidences were lower than those noted after treatment on GD 11 and dose–response relationships were not clear. On GD 12, lumbar vertebrae dumbbell-shaped o.c. were slightly increased at the two highest doses of 6-MPr (10 and 14 mg/kg) while bipartite o.c. were not found at any dose

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