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## METHODS FOR TERATOGENIC SCREENING OF AIR FORCE CHEMICALS

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AEROSPACE MEDICAL RESEARCH LABORATORY  
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## TECHNICAL REVIEW AND APPROVAL


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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

**FOR THE COMMANDER**

  
ANTHONY A. THOMAS, MD  
Director  
Toxic Hazards Division  
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## PREFACE

The research presented in this report was performed by members of the Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory from August 1977 through October 1977. This research was performed in support of Project 6302, "Toxic Hazards of Propellants and Materials;" Task 630201, "Toxicology of Propellants and Materials;" Work Unit 63020104.

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## METHODS FOR TERATOGENIC SCREENING OF AIR FORCE CHEMICALS

### INTRODUCTION

With female personnel assuming positions formerly held only by males, the teratogenic screening of Air Force chemicals becomes necessary. Fisher 344 rats are commonly used in this laboratory, especially for oncogenicity studies, since they are a highly inbred strain and thus genetically stable and because they are routinely used in the Carcinogen Bioassay Program of the National Cancer Institute. To determine the suitability of the Fisher 344 strain of rat for teratogenicity studies and to gain experience in the procedures, pregnant Fisher 344 rats were exposed to two dose levels of hydroxyurea, a cancer chemotherapeutic drug and a known teratogen.

### PROCEDURES

Twenty-five, 200-gram female and five 250-gram male Fisher 344 rats were ordered. Twenty-six female and five male 100-gram rats were received. The animals were fed a commercial chow and water ad libitum and housed in a laboratory with a 14 hour-on, 10 hour-off light cycle. Toes were clipped for identification and after approximately five weeks delay for maturation, examination of vaginal smears for determination of state of estrus was begun. Female rats showing signs of proestrus (many nucleated vaginal epithelial cells with few leucocytes) were placed individually with males overnight. Vaginal washings from these rats were examined the next morning for presence of sperm. Animals with sperm present were considered pregnant, day 0. Male rats were not used for breeding more frequently than every other day.

Pregnant animals were weighed daily. On day nine of pregnancy, ten rats were injected intraperitoneally with 250 mg/kg body weight hydroxyurea. On the 11th day of pregnancy, ten additional rats were injected intraperitoneally with 750 mg/kg body weight hydroxyurea. Six rats were not injected and served as controls. Numbers were chosen at random to determine dosage group.

On day 20 of pregnancy, each female rat was anesthetized with chloroform, its abdominal cavity opened and diaphragm punctured. The uterus was examined for number of live and resorbed fetuses. Each fetus or resorption was identified by letter starting in the left horn at the site closest to the left ovary and extending to the site closest to the right ovary. Each fetus was examined under a 3X magnifying lens and weighed, and sex, weight and observed abnormalities were recorded. Fetuses were placed in wide-mouth jars containing Bouin's solution (25 ml/fetus minimum). Every third fetus was placed in absolute alcohol preparatory to clearing and staining the fetal skeleton.

Evaluation of fetuses was conducted in a manner similar to that performed at Children's Hospital Research Foundation, Cincinnati, Ohio (Wilson, 1973, Wilson and Warkany, 1965). Fetuses placed in Bouin's solution were fixed for a minimum of one week prior to sectioning. Before sectioning, each animal was again examined for external malformations. Legs and tail were cut off where they join the trunk. The fetus was then placed in a supine position on a flat block of paraffin and, using a 3X magnifying lens, a single-edge razor blade was placed in the oral cavity, and the upper portion of the head was removed above the ears. The palate was examined and the cut surface of the head was placed on a paraffin block where

1-mm thick transverse sections were cut beginning just anterior to the eyes and proceeding backward to the region of the ears. The trunk was again placed in a supine position on the block and transverse 1-mm thick sections were made beginning in the region of the shoulder joint. Sectioning was continued caudally, while sections through the heart region were cut as thin as possible in order to reveal as many of the septal and valvular structures as possible. The section immediately caudal to the heart was cut approximately 5-mm thick in order to obtain an intact diaphragm. Sectioning was continued posteriorly until section demonstrated both renal pelves. Sections were placed in individual compartments of a white porcelain spot-test dish and covered with 70% alcohol where they were examined in the order in which they were cut, using a 7X magnification dissecting microscope. Genito-urinary organs on the pelvic floor and along the posterior body wall were examined by removing the loosely attached intestines and looking directly into the pelvic cavity. If internal structures were not adequately demonstrated, the sections were dissected under the microscope with iridectomy scissors and fine-pointed forceps.

Fetuses preserved in absolute alcohol were eviscerated after two days, replaced in alcohol and after one week placed in 2% KOH for 48 hours and then placed in Alizirin Red S (50 mg/liter) 2% KOH solution for 24 hours. Fetal preparations were then cleared in increasing concentrations of glycerin (20% glycerin: 80% of 2% KOH solution for one day; 40% glycerin: 60% of 2% KOH solution for one day; 60% glycerin: 40% of 2% KOH solution for one day; 80% glycerin: 20% of 2% KOH solution for one day). The cleared embryos with stained skeletons were then stored in pure glycerin to which a small crystal of thymol had been added as a preservative. Skeletons were examined under 3X magnification for abnormalities.

#### RESULTS

Of the six rats designated as controls, three were successfully bred. No gross abnormalities were detected. Upon sectioning, cryptorchidism was detected in one fetus. The following data were recorded:

<u>MATERNAL RAT NUMBER</u>	<u>TOTAL NUMBER OF LIVE FETUSES</u>	<u>NUMBER OF RESORPTIONS</u>	<u>FETUSES</u>		<u>AVERAGE WEIGHT (g)</u>
			<u>FEMALE</u>	<u>MALE</u>	
3	6	0	4	2	3.4
6	9	0	4	5	3.2
12	9	0	4	5	3.1

Of the ten animals receiving 250 mg/kg hydroxyurea on day nine of pregnancy, seven were successfully bred and the following data recorded:

<u>MATERNAL RAT NUMBER</u>	<u>TOTAL NUMBER OF LIVE FETUSES</u>	<u>NUMBER OF RESORPTIONS</u>	<u>FETUSES</u>		<u>AVERAGE WEIGHT (g)</u>
			<u>FEMALE</u>	<u>MALE</u>	
1	8	5	4	4	2.4
5	7	2	4	3	2.3
7	8	2	1	7	2.6
8	5	1	1	4	2.5
20	0	10			
22	8	1	1	7	2.8
26	6	5	2	4	2.4

<u>Gross Abnormalities</u>	<u>Number of Fetuses with Abnormality</u>
Anophthalmia	36
Exencephaly	17
Missing, malformed or misplaced ears	11
Meningoencephalocoele	9
Micrognathia	9
Microophthalmia	7
Cleft palate	4
Cleft mandible	3
Cleft lip	3
Short or distorted limbs	2
Short, kinky tail	1
Agnathia	1
Hydrocephaly	1

<u>Skeletal or Microscopic Abnormalities</u>	<u>Number of Fetuses with Abnormality</u>
Hydrocephaly	14
Unfused centra	5
Ectopic testis	5
Wavy, fused ribs	4
Absent ovary	3
Small orbits	3
Absent uterus	2
Missing rib(s)	2
Herniated diaphragm	2
Distorted brain	1
Absent kidney	1
Ectopic kidney	1
Wavy ribs	1
Absent testis	1
Situs inversus of stomach, spleen, pancreas	1

Of the ten animals receiving 750 mg/kg hydroxyurea on day 11 of pregnancy, seven were successfully bred and the following data recorded:

<u>MATERNAL RAT NUMBER</u>	<u>TOTAL NUMBER OF LIVE FETUSES</u>	<u>NUMBER OF RESORPTIONS</u>	<u>FETUSES</u>		<u>AVERAGE WEIGHT (g)</u>
			<u>FEMALE</u>	<u>MALE</u>	
2	0	12			
4	0	11			
10	0	5			
11	6	3	3	3	1.9
13	7	4		7	2.2
14	0	12			
15	0	9			

<u>Gross Abnormalities</u>	<u>Number of Fetuses with Abnormality</u>
Short, kinky tail	12
Shortened or deformed limbs	10
Micrognathia	8

Cleft palate	5
Shortened body (dwarfism)	4
Microophthalmia	1

<u>Skeletal or Microscopic Abnormalities</u>	<u>Number of Fetuses with Abnormality</u>
Ectopic testis	3
Hydrocephaly	2
Absent testis	2
Missing ribs	2
Unfused centra	2
Missing sacral and coccygeal vertebrae	2

#### DISCUSSION

Wistar rats, a strain frequently used in teratologic studies, injected with 250 mg/kg hydroxyurea on day nine of pregnancy commonly demonstrated fetal abnormalities of the brain (exencephaly, hydrocephaly, meningoencephalocoele), anophthalmia, cleft palate, cleft lip, misplaced ears, clubbed hind limbs, fused vertebrae, fused ribs, split centra and scoliosis.\* In experimentation with hydroxyurea in Fisher 344 rats, at 250 mg/kg on day nine of pregnancy, our results showed anophthalmia, brain abnormalities, missing, malformed or misplaced ears, micrognathia, microophthalmia, cleft palate, cleft mandible, cleft lip, unfused or split centra and ectopic testis the most prevalent abnormalities detected.

Wistar rats injected with 750 mg/kg hydroxyurea on day 11 of pregnancy commonly presented fetal abnormalities including cleft palate, cleft mandible, kinky tail, diaphragmatic hernia, malformed limbs, micrognathia, split centra and digital abnormalities.\*

In Fisher 344 rats, injection of 750 mg/kg hydroxyurea on day 11 of pregnancy produced in order of prevalence, short, kinky tails, shortened or deformed limbs, micrognathia, cleft palate, dwarfism and ectopic testis.

The Fisher 344 strain did exhibit large numbers of fetal abnormalities when pregnant rats were injected with hydroxyurea, and there were definite differences in the prevalence of abnormalities seen depending upon the stage of gestation when subjected to hydroxyurea. In the 24 fetuses from rats not injected, only one fetal abnormality was detected.

One reason the Wistar strain of rat is commonly used in teratology studies is its high fertility rate. Personnel at the Children's Hospital Research Foundation in Cincinnati anticipate pregnancy in 80-90% of those rats demonstrating sperm in vaginal washings.\* With the Fisher 344 rat, only 65% of those rats demonstrating sperm in vaginal washings proved to be pregnant. Several reasons for this relatively low conception rate, however, can be given. Advised temperatures for housing

\* Personal Communications, Dr. J. G. Wilson, Children's Hospital Research Foundation, Cincinnati, Ohio 1977.



breeding rats is 72+5F (Hafez, 1970). Although the thermostat in the laboratory housing these animals was set at 70F, a recording thermometer demonstrated a baseline reading of 80F with wide fluctuation. Relative humidity of 50 to 60 percent is considered optimum (Hafez, 1970) while measurements showed fluctuation between 30 and 60 percent. Also, although animals were anesthetized in a separate laboratory, they were examined in the area where other animals were held and it has been reported that chloroform vapors in very small amounts are detrimental to the health of rodents and may be reflected by infertility (Hafez, 1970).

This experiment was initiated to determine the suitability of the Fisher 344 rat for teratogenic testing and to become familiar with the techniques required. The greatest problems arose in the preparation of stained skeletal specimens. Nine specimens were unable to be evaluated. Some fetuses were not developed enough to withstand clearing in 2% KOH. Others were inadequately fixed prior to attempted clearing and two were lost due to improper handling while changing solutions.

The Fisher 344 strain appears to be adequate for teratogenic testing of Air Force chemicals. Techniques for this evaluation have been developed and appear to be satisfactory. Fisher 344 rats will be used in future studies to evaluate teratogenicity of Air Force propellants.

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