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INFLUENCE OF FOOD RESTRICTION AND RESTRAINING ON PREIMPLANTATION, EMBRYONAL/FETAL AND POSTNATAL DEVELOPMENT OF RATS

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1 CONTROLLED BREEDING OF RATS

1.1 Sexual Cycle

After reaching sexual maturity rats having many mating periods of short duration each year (polyestrons). The length of the rodents' cycle is from 4 to 5 days (1, 2).

The sexual cycle is hormonally regulated and is also very much influenced by environmental conditions, such as temperature, time and intensity of light, season of the year and diet.

Constant light produces persistent estrus causing prolonged vaginal cornification in rats (3). Greatest sensitivity is to light in the orange-red region of the spectrum. Before mating, rats have to be maintained in a proper environment with a consistent day-night cycle to establish a carefully controlled mating routine.

The different periods of the cycle, especially the mating period, are reflected in the behavior of the rats as well as in the appearence of the external genitalia. The rat becomes irritable, aggressive and nervous; the reproductive instinct dominates its behavior.

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1.2 Vaginal Cycle

(1) Proestrus (stage I) - approx. 12 hours - (The vaginal smear contains epithelial cells which are oval in outline, contain large centrally placed nuclei and occur singly or in sheets. Leucocytes and mucus are rarely found.)

(2) Estrus (stage II) - approx. 14 hours - the mating period (The cells of the epithelium are cornified or horny, have lost their nuclei and, when desquamated, form a characteristic clumped plaque or aggregate. Leucocytes and mucus are absent.)

(3) Metestrus (stage III) - approx. 21 hours - occurs slightly after ovulation (During this phase the mating drive slowly declines. After passing the peak of the mating period, in addition to a few cornified cells, leucocytes and mucus reappear in the smear.).

(4) Diestrus (stage IV) - approx. 57 hours - the time interval between two mating periods (The vaginal mucosa is thin. An abundance of various epithelial cell types, mucus and especially leucocytes are present in the smear.)

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1.3 Vaginal Smears

A cotton swab (prepared by balling a small amount of cotton on the end of a small wooden stick, or purchased premade as Q-tips, etc.), which is predampened with water, is inserted into the vagina to obtain a sample of the vaginal mucosa.

The material obtained is spread onto the surface of a glass microscope slide and, if a more precise examination is required, stained with Giemsa stain or hematoxylin and eosin. The smear can be used to determine the stage of the vaginal cycle and the presence of sperms after mating. If sperms indicate a copulation the rat should be considered pregnant.

1.4 Control of Mating

Because of the dynamic state of tissues during the embryonic and fetal periods, the accuracy of information about the affect of drugs on a particular biological parameter depends upon whether the precise age, and therefore stage of development, of the tissue affected is known. To obtain such specific data, knowledge about the exact date and time of the initiation of pregnancy is necessary.

This is best achieved if females are brought to, and caged with males for a short, well defined period of time.

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1.4.1 2 methods for routine mating

(1) Estrous females are chosen with the aid of a vaginal smear or by visual observation, e. g. stroking the back of the rat which elicits a special response. Only selected females are caged with the males.

(2) Randomly selected, sexually mature females are caged with the males, and only checked after mating to determine if copulation occurred.

1.4.2 Some reasons implicate to use the 2nd method (see 1.4.1, (2))

(1) The vaginal smear is often difficult to interpret and is not always a sure indication of the stage of the vaginal cycle.

(2) The manipulation of the vagina required for obtaining the smear often results that the females are unreceptive to the males.

(3) Visual determination of the estrus phase is very subjective and therefore must be performed by an experienced handler. In addition, it is a time-consuming process and can only be used for a small number of animals.

(4) Even if only estrous females are mated the pregnancy rate does not reach 1oo percent.

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1.4.3 A reasonable schedule of mating

The dark period is routinely maintained between 18.oo and o6.oo (12 hours). During this period the females are caged with the males 3 : 1 for 3 hours - from oo.oo to o3.oo. According to this schedule oo.oo h will be the time of ovulation and the peak period of mating.

At o3.oo the rats are separated, vaginal smears are prepared and evaluated to determine if copulation occurred. The animals determined sperm-positive are considered pregnant and maintained separately until the gestational day on which they are required for experimental purposes. The 24-hour period following the time of identification of sperm in the vaginal smear is considered gestation day o (some investigators prefer to call this the 1st day of pregnancy). About 8o to 9o percent of sperm-positive rats are expected to become pregnant.

The length of the gestation period is from 2o to 23 days for rats. The exact duration is dependent upon the animal strain used and, to some extent, upon the age of the mother.

Litter size is also strain and species-dependent. A rat litter generally has between 9 and 1o pups (4, 5).

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2 GENERAL TECHNIQUES

2.1 Handling of pregnant rats

Rats can be handled daily during pregnancy, also too frequent or inappropriate handling of the animals can lead to abortion or intrauterine fetal deaths (6, 2).

2.1.1 Caesarean section

In teratological experiments dams are sacrificed before parturiton takes place, because mother animals will usually cannibalize malformed or not fully vital pups.

Day 2o of the gestation period is commonly used for caesarean section in rats.

Rats are killed with CO2 or by cervical dislocation. Immediately after killing the peritoneal cavity is opened. To prevent damage to the fetuses the uterus is opened with rounded forceps opposite the mesometrium.

2.1.2 Evaluation

Living and dead fetuses are distinguished immediately after the section. The criterion for a living fetus is the appearance of a moving reflex after touching the fetus in the unopened uterus with a pair of tweezers or some other instrument. The numbers of dead and living fetuses are recorded.

2.1.3 Resorption

The number of resorptions, abortions and the site of implantation in the right and left uterine horn are recorded (see protocol at the end).

Early resorptions and late resorptions are discerned as follows:
early resorptions: macroscopic discrimination between fetal residues and placental material not possible,
late resorptions: distinct macroscopic discrimination between fetal and placental possible

The dissected uteri, devoid of fetuses, plancental and resorptions are stained according to Salewski to get all implantation sites (1964) to reveal abortions or resorptions that have taken place shorthy after implantation.

2.1.4 Determination of implantation sites

Statistical analysis of the experimental data is often based on the total number of implantations. In cases where early fetal resorptions have occurred, visual inspection of the uterine horns and counting of the fetuses present, as well as resorption sites, may provide misleading information as to the exact number of implantations (7).

2.1.5 Fixation of the uterine horns (Method SALEWSKI, Arch. Pharmacol. exp. Pathol. 247, 367 (64))

The fresh unfixed uterine horns are placed in a 1o-percent solution of ammoniumsulfide for 1o minutes. After rinsing in running tap water, the uterine horns are then placed in a solution, consisting of equal parts of 1 percent hydrochloric acid and 2o percent potassium ferrocyanide. Implantation sites are stained blue-black and can readily be counted.

2.1.6 Numbering of the fetuses

When taking out the fetuses and in all further examinations it is necessary to keep to the positional sequence of the fetuses in the uterus. Therefore the fetuses are numbered beginning with the upper end of the left uterine horn and ending at the upper end of the right one. Numbering of the fetuses is done with a felt-tip pen.

2.1.7 Malformations

In rats after ceasarean section all fetuses are killed with chloroform, weighed and examined macroscopically for outer malformations, and in case of uncertainties by means of a stereomicroscope or a magnifier (8, 9, 1o).

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2.1.8 Skeletal malformations

(1) Absence of bones, parts of bone or parts of the bony system,

(2) distinct bendings, shortenings or gaps or clefts in bones or bony systems,

(3) markedly asymmetric structure of normally symmetric bones or ossification centers,

(4) fusion of bones which are normally distinctly separate

(see Protocols at the end of Proposal -/3o2o)

3 STAINING OF FETAL SKELETONS

In teratogenicity studies rodent fetal bones are mostly stained with alizarin red S to examine the morphological features of the skeleton (11, 12).

3.1 Method and Materials

3.1.1 Removal of fetal internal organs after opening of the abdominal cavity

3.1.2 Fixation

The fetuses are fixed with 96 o/o ethanol for at last 48 hours. A prolonged fixation period has been found to be advantageous.

3.1.3 Clearing of the soft tissues

For the clearing of the soft tissues, a o.7 o/o potassium hydroxide solution is used. The fetuses remain in the KOH solution until they are distinctly transparent and the light-colored skeletal system is well visible. This usually requires rats 5 to 7 days old.

The KOH solution is changed daily and the fetuses are frequently shaken in the solution.

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Fetal abnormalities produced by intraperitoneal injections of chemicals as Cyclophosphamide (1, 2), Cytosine (11, 12), Arabinoide (4 to 6), Streptonigrin (6, 7), Methylhydrazine (8 to 1o) and Hydroxyurea into pregnant rats on days 1o to 12 of gestation are described (13).

The here proposed dosages of Cyclophosphamide (2.5, 5 and 1o mg/kg BW) result according to (1), in approx. .LT.3. 1o and 4o o/o lethal effects on the embryo and leads in approx. .LT.3, 1o and 5o o/o to skeletal abnormalities.

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