Induction a Rat Model of Premature Ovarian Insufficiency (POI) Using D Galactose Feeding During the Critical Periods of Development: an Experimental Protocol

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Study Protocol

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Abstract

Introduction: Premature ovarian insufficiency (POI) affects about 1% of women of reproductive age, with no curative treatment. Several efforts have been made to develop an animal model of POI that mimics all manifestations of this disease; however, these models are limited due to increased mortality or high toxicity. Overall, well-developed animal models may improve our knowledge of diseases and can be suitable for identifying the underlying mechanisms and detecting the optimal treatment methods. In the present study, we aimed to present a functional rat model of POI, mimicking the clinical manifestations of POI in women.

Materials and Methods: This experimental study was performed in two steps. In the first step (induction of the rat POI model), a total of 40 pregnant Wistar rats were randomly divided into four groups. Three experimental groups were fed galactose-enriched diets at days 3-15 of pregnancy (group 1); on the third day of pregnancy until the end of pregnancy (group 2); and on the third day of pregnancy until the end of the weaning period (group 3). Also, group 4 (control group) was fed a standard pellet during pregnancy and breastfeeding period. In the second step of the study, the ovarian morphology (microscopic and macroscopic), reproductive hormones, and immunological and metabolic characteristics of the female offspring were examined in all experimental groups and compared with the controls at three time points, that is, postnatal days (PNDs) 45, 105, and 180 of age.

Results: In this study, we presented an optimal model of POI that highly mimics the clinical manifestations of this disease in women.

Conclusion: A functional rat model of POI may be suitable for increasing our understanding of this disease and filling the knowledge gaps.

Introduction

Premature ovarian insufficiency (POI), characterized by infrequent or absent menstrual periods, elevated follicle-stimulating hormone (FSH) levels >40 mIU/mL, and decreased estrogen levels in women below 40 years of age, is a critical condition for women of reproductive age (1). The prevalence of POI is about 1-5.5% in the general population (2, 3). The non-protective effects of estrogens on the cardiovascular system and bones in affected women may lead to many threatening health problems, such as osteoporosis, cardiovascular diseases, and metabolic disorders, and decrease their quality of life (4-6). Genetic factors, X-linked recessive disorders, immunological and metabolic disorders, environmental factors, and iatrogenic factors (chemotherapy, radiotherapy, or surgery) are the known causes of POI, while the majority of remaining cases have unknown or idiopathic causes (7).

So far, no treatment has been introduced for POI, and management strategies only include symptomatic and maintenance therapies (8). Evidence shows that the early depletion of the ovarian follicular reserve results in POI (9). The early and spontaneous discharge of the ovarian follicle reserve may be explained by one of the following hypotheses or a combination of both: (i) Germ cell deficiency during
embryogenesis causes primary stem cell deficiency; and/or (ii) the high acceleration rate of follicle apoptosis can drain the ovarian reserve (10-13). According to these two hypotheses, several animal models have been designed (14-16) to understand the mechanisms of the disease; however, an optimal model has not been introduced yet.

The ovotoxic effect of galactose (17), as the main monosaccharide of milk, is documented as a chromosomal defect of galactose metabolism in humans, resulting in galactosemia (18). POI is one of the common conditions in women affected by galactosemia (18). Some studies have used galactose to induce POI in laboratory animals (10, 19). A systematic review in 2019 showed that using different doses of galactose during the prenatal or postnatal period produced different ovarian follicle deficiency outcomes in rodents (20). Exposure to galactose during the prenatal period decreases the count of small ovarian follicles (primordial and primary), whereas postnatal administration increases apoptosis and loss of large ovarian follicles (preantral and antral) (20).

Prenatal galactose exposure appears to be a more successful model of POI than postnatal interventions, especially due to the reversible ovotoxic effects of galactose during the postnatal period (21). According to previous studies, gestational exposure of pregnant rats to 35% galactose feeding from day three of conception reduced the number of small ovarian follicles in the female offspring. This finding reinforces the first hypothesis, that is, the number of follicles is low since birth (20). On the other hand, some other studies have shown the acceleration of apoptosis in follicles, which reinforces the second hypothesis and suggests the high acceleration of follicle apoptosis (19).

Regarding the time- and dose-dependent ovotoxicity of galactose, in the present study, we aimed to produce a rat model of POI in the female offspring following maternal exposure to a galactose-enriched diet during gestation and weaning. We also aimed to examine the offspring of all experimental groups in terms of ovarian macroscopic and microscopic morphologies, reproductive hormones, and immunological and metabolic characteristics at postnatal days (PNDs) 45, 105, and 180 of age and to compare them with the controls.

**Materials And Methods**

**Methods and Design:**

This study, consisting of four groups (three experimental groups and one control group), was conducted since August 2018 at the Reproductive Endocrinology Research Center (RERC) of the Research Institute for Endocrine Sciences (RIES), affiliated to Shahid Beheshti University of Medical Sciences, Tehran, Iran. This experimental study was conducted according to the animal experimentation guidelines by the Ethics Committee of Shahid Beheshti University of Medical Sciences. It was also approved by the Ethics Committee of RIES (IR.SBMU.ENDOCRINE.REC.1398.001).

**Animals:**
Forty adult female Wistar rats (170-190 g) were obtained from the Animal House of RIES and maintained under standard husbandry conditions in a standard 12:12 h light/dark cycle (with lights on at 06:00 daily) with controlled temperature (22±3°C) and relative humidity of 45–55%. In the first step of the study, a total of 40 female Wistar rats were randomly divided into four groups (ten rats per group). To minimize variations between the groups, random allocation was conducted, using a previously described block randomization method (22). Animals had *ad libitum* access to food and water. In the first step of the study, each female rat was randomly assigned to each group and mated with a young fertile male overnight in a separate polypropylene cage (43 cm×30 cm×15 cm). The first day of pregnancy was confirmed by observing a vaginal plaque or sperm existence in the vaginal lavage.

**Food supplementation:**

The standard food pellets were purchased from Pars Co. (Iran) and enriched with D galactose in the animal house for further use in the experimental groups. To prepare one kilogram of galactose-enriched food, 650 g of standard food powder was mixed with 350 g of galactose powder in distilled water. After converting to pellets and drying, the prepared food was stored in a refrigerator (2-4°C) for further use.

**Steps of the study:**

**Step 1: Induction of POI model**

In this phase, pregnant rats in the experimental groups received a galactose-enriched diet during pregnancy or until weaning of the offspring. The three experimental groups were as follows: group 1, including ten pregnant rats that were fed a galactose-enriched diet from the third day of conception until the 15th day of pregnancy, followed by a standard diet; group 2, including ten pregnant rats that were fed a galactose-enriched diet from the third day of pregnancy until the end of pregnancy; and group 3, including ten pregnant rats that were fed a galactose-enriched diet from the third day of pregnancy until the end of breastfeeding period. Finally, group 4 (control group) consisted of ten pregnant rats that were only fed standard pellets during both pregnancy and breastfeeding periods. An overview of the study protocol is presented in Figure 1.

**Step 2: Model assessment in the female offspring**

The number of female offspring was recorded in each group. The vaginal opening time, regularity of the estrous cycle, number of nipples, vaginal length, anogenital distance (AGD), and anovaginal distance (AVD) were recorded for the female offspring in all study groups.

**Blood collection and extraction of ovaries:** Blood and ovarian samples were collected during the estrous phase of the sexual cycle at three time points, that is, PND 45, 105, and 180 of age; the day of birth was considered as PND 1. Sampling was carried out at 8-9 AM after confirming the estrous phase by preparing a vaginal smear. The rats were then anesthetized using an intraperitoneal (i.p.) injection of 5 g of pentobarbital sodium (60 mg/kg body weight; P3761; Sigma, St. Louis, MO, USA). The blood samples
were directly collected from the abdominal aorta after centrifuging at 6000 g for five minutes at 4°C. The sera were then separated and stored at −80°C for subsequent measurements of follicle-stimulating hormone (FSH), estradiol (E2), and anti-Müllerian hormone (AMH) levels. Next, the ovaries were removed, trimmed of excess fat, and fixed in 4% paraformaldehyde at room temperature for three days. They were then processed, based on the standard tissue processing protocol, and embedded in paraffin.

The histological assessment of ovarian tissues was carried out after tissue preparation. This process included fixation, in which the right ovaries of rats were placed in an automatic tissue processor (ATP) machine for 24 hours (23). The ATP machine automatically makes the necessary changes to tissues during the following steps, shown in box A.

**Box A. The automatic process in the automatic tissue processor (ATP) machine**

1. Fixation with 10% formalin;
2. Dehydration using an ethanol series;
3. Achieving transparency using xylol;
4. Infiltration and impregnation of tissue with molten paraffin;
5. Fixation of tissue by passing through a 10% formalin container;
6. Irrigation by passing through seven ethanol containers with ascending concentrations of 50%, 70%, 80%, 90%, 96%, and 100%, respectively;
7. Achieving transparency by passing through two xylene vessels; and
8. Passing through two molten paraffin containers with a temperature of 56±2°C.

A paraffin block was individually prepared from each ovary. To quantify the histological parameters, distinct sections (10 μm) with 10-section intervals were mounted onto glass slides to be stained with hematoxylin and eosin (H&E) and labeled.

**Morphological classification of follicles:**

The follicles were categorized, based on a classification described by Myers et al. (2004) (24). Briefly, in this classification, a primordial follicle refers to a follicle with an oocyte, surrounded by a single layer of flattened squamous granulosa cells. Occasional follicles, as the boundary between primordial and primary follicles, have both cuboidal and squamous cells. These follicles will be considered as primary follicles if the number of cuboidal cells is predominant. A primary follicle represents a follicle with an oocyte, which is surrounded by a single layer of cuboidal granulosa cells. On the other hand, secondary follicles refer to follicles with more than one layer of cuboidal cells with no visible antrum. Finally, early antral follicles represent follicles with one or two small areas of follicular fluid (antrum), and antral follicles represent follicles with a large antrum.

**Determination of ovarian volume:**

In this study, the Cavalieri method of segmentation with the point-counting technique, as a systematic random sampling (SRS) method, was used to estimate the ovarian volume (25). For this purpose, a set of
Cavalieri sections was prepared (Figure 2). For each ovary, ten sections were sampled with a fractionator. A point probe was placed randomly onto each ovarian section. Next, the number of points hitting the region of interest was counted. The whole ovarian tissue section was cut and counted for measuring the sample size. Finally, the ovarian volume was calculated using the following formula:

\[ V = \sum p \times \frac{a}{p} \times t \]

where \( V \) represents volume; \( P \) represents the points counted on the grid; \( a/p \) denotes the area associated with a point (0.02 mm in this study); and \( t \) denotes the section evaluation interval.

**Measurement of AMH, FSH, and E2 levels in the blood serum:**

The enzyme-linked immunosorbent assay (ELISA) is a widely used tool for detecting and quantifying proteins and antigens from various samples. The AMH, E2, and FSH levels were measured using an ELISA assay, based on the manufacturer's protocol.

**Determination of anti-ovarian antibodies (AOAs):**

The group with the highest similarity to POI was considered as the optimal group (POI+). To determine AOA (27), the ovaries of rats were removed in the experimental and healthy control groups under deep anesthesia. They were then rinsed thoroughly with phosphate-buffered saline (PBS) as a cold medium and then divided into segments of 1×1 mm\(^2\). Next, the ovarian segments were homogenized in a tissue grinder. The grilled content was passed through a mesh nylon filter (40 µm) and centrifuged at 450×g for 20 minutes at 4ºC. After centrifugation, the supernatant was removed and passed through a filter (0.22 um) to keep the liquid sterile.

Afterward, the protein concentration was determined by a Bradford assay. After determining the protein concentration, it was used as an antigen to cover the 96-well ELISA plate bed. The serum of rats was considered as a possible source of AOAs and was examined by serial dilution. By creating serial dilutions of the studied sera, we increased the accuracy and sensitivity of the ELISA assay, which is important for determining sera with positive reactions to ovarian antigens and eliminating serum cross-reactivity.

In the next stages of ELISA assay, the ELISA plate bed was covered with 0.5-5 µg of antigen, which is the protein extracted from the ovaries of healthy rats. The coated plates were then incubated with antigens in a refrigerator overnight. After removing the antigens from the refrigerator in the following morning, the plates were allowed to reach room temperature and washed three times with PBS solution. Next, dilutions of 1.10 to 1.100 of each serum sample were added to the antigen, and incubation was performed at room temperature for two hours. The reacting sera were removed and washed again with PBS three times.

By adding a peroxidase-conjugated antibody against the rat antibody, the main reaction was induced. Incubation was performed for one hour at room temperature to identify antibodies bound to the ovarian antigen. The contents of the wells were re-harvested and washed with PBS three times. Following that,
the reaction substrate (tri-methylbenzene) was added and incubated for 30 minutes in the dark at room temperature. Then, normal sulfuric acid (1 N) was added to terminate the substrate enzyme reaction. Finally, readings were obtained, using an ELISA spectrophotometer over a range of wavelengths (58 wavelengths), and the optical density was recorded. Also, comparisons were made with the cut-off level of reacting serum in healthy rats.

**Measurement of body weight and puberty:**

The female offspring of each mother was weighed, and its weight was documented at PND 1 and every 15 days until the end of experiments. The vaginal opening day was considered as a sign of puberty.

**Evaluation of the estrous cycle:**

The estrous cycle was monitored by daily observation of vaginal smears in all female offspring at 70-80 days of age (between 08.00 AM and 12.00 PM) for ten consecutive days. To collect vaginal samples, the lips of the vulva were parted, and a cotton-tipped sterile swab was inserted into the vagina. The swab was rotated two or three times against the vaginal wall and then withdrawn and rolled on a clean glass slide. The smears were fixed with 70% ethanol, stained with Giemsa, and examined under a light microscope (×100 magnification). The proportions of leukocytes, epithelial cells, and cornified cells were determined in daily vaginal smears. Overall, these proportions change characteristically in different stages of the estrous cycle. The rat estrous cycle (proestrus, estrus, metestrus, and diestrus) usually lasts four days (28).

**Intraperitoneal glucose tolerance test (IPGTT) and blood sampling for the measurement of serum glucose, insulin, and lipid profile:**

At 105-115 days of age, IPGTTs were performed on galactose-exposed and control rats after 6-7 hours of fasting at 2-3 PM. The animals in all groups were anesthetized by an i.p. injection of pentobarbital sodium (45 mg/kg body weight). The baseline (fasting) and all other blood samplings were performed through a tail cut. A baseline blood sample (min 0) was collected to determine the glucose level, insulin level, and lipid profile. Glucose (2 g/kg body weight) was intraperitoneally injected, and blood samples were collected at 10, 20, 30, 60, and 120 minutes after glucose injection. All blood samples were collected in Eppendorf tubes (blood volume of 0.3 mL at each time point) and centrifuged at 6000 g for five minutes at 4°C. The sera were stored at -80°C for subsequent glucose and insulin measurements, as well as lipid profile analysis.

**Intraperitoneal insulin tolerance test (IPITT):**

One week after IPGTT, the rats were examined to determine insulin resistance. The IPITTs were performed on galactose-exposed and control rats after 6-7 hours of fasting at 2-3 PM. The animals in all groups were anesthetized by an i.p. injection of pentobarbital sodium (45 mg/kg body weight). Regular human insulin (0.75 U/kg body weight) was also administered intraperitoneally, and blood samples were collected through a tail cut before (min 0) and 10, 20, 30, and 60 minutes after insulin injection. The blood
samples (0.3 mL at each time point) were finally centrifuged at 6000 g for five minutes at 4°C. The sera were stored at -80°C for subsequent glucose measurements.

**Measurement of glucose, insulin, and lipid in the blood serum:**

The serum glucose and insulin levels were measured by the glucose oxidase method (Pars Azmoon Co., Tehran, Iran) and the ultrasensitive rat insulin ELISA assay (Mercodia, Sweden), respectively. The serum levels of triglyceride (29), total cholesterol (27), and high-density lipoprotein (HDL) were measured by enzymatic colorimetric (Pars Azmoon Co., Tehran, Iran) and enzymatic photometric methods (Randox, UK), according to the manufacturers’ protocols.

**Laboratory materials:**

The following materials were commercially available in this study: phenobarbital sodium (5 g; P3761; Sigma, St. Louis, MO, USA); Rat AMH ELISA Kit (Lot ZB-MI19822; Zellbio, Germany); Rat FSH ELISA Kit (Cat. No.: ZB-10182C-R9648; Zellbio, Germany), E2 ELISA Kit (Cat. No.: 4925-300A; Monobind, USA), pentobarbital sodium (5 g; P3761; Sigma, St. Louis, MO, USA), and D-galactose (Souvenir Chemicals, Mumbai, India).

**Selection of the optimal model:**

The histological features of the ovaries, as well as the serum levels of some reproductive hormones (FSH, AMH, and E2) of female offspring, were compared at three time points (PND 45, 105, and 180 of age) between the experimental and control groups. The optimal rat model of POI included rats with the lowest number of primordial and primary follicles, the lowest level of AMH, and the highest level of FSH in the blood serum, compared to the other experimental and control groups.

**Results**

This study was initiated in August 2018. The results are being currently acquired and will be published by the end of 2020.

**Discussion**

In this experimental study, we aimed to propose a rat model of POI using a galactose-enriched diet and to investigate changes in the ovarian follicles and some reproductive hormones. Since a galactose-enriched diet can be toxic to the reproductive system, especially the ovaries (19), it can disrupt the folliculogenesis process; therefore, it can be potentially used to create the closest model for one of the phenotypes of POI.

Considering the heterogeneity of POI, there are several phenotypes of POI, such as ovaries with low follicular reserves or ovaries that contain enough follicles, but quickly undergo apoptosis (the ovaries are depleted of follicular reserves before the average time) (30). There is also a phenotype of POI, known as resistant ovary syndrome (31), where there is no response to FSH, despite the presence of follicles in the
ovaries. There are two hypotheses for the latter phenotype: (i) The FSH bioactivity is impaired and cannot stimulate the follicles; and (ii) FSH receptors on the follicle are disturbed and cannot be activated by FSH (32). Therefore, animal models of POI should be designed considering the target phenotype.

Folliculogenesis is a process that begins in the fetal period; therefore, POI must be induced during the fetal life, especially in POI phenotypes with low ovarian reserves from birth. The earliest studies using oral galactose have shown that a suitable dose of this monosaccharide (35%) has a relatively low mortality in animals, despite its association with ovarian toxicity (30). According to previous assumptions, in this study, we evaluated the prenatal and/or early postnatal exposure to galactose and examined the ovarian characteristics of the female offspring to develop a POI model.

Other methods for developing animal models of POI have some limitations, especially in case of exposure to chemotherapy drugs. It is not possible to design an animal model of the fetal period, given the high toxicity of some chemical agents (33). Also, other methods, such as radiotherapy, have similar limitations. In methods, such as ovarian removal (oophorectomy), significant information about the ovarian processes is lost. Therefore, it is more favorable to use a method associated with minimum mortality to mimic POI during critical periods of development.

Galactose is a monosaccharide that is naturally produced in the body and enters the body through nutrition (dairy products) (34). On the other hand, almost all breastfed newborns are exposed to this monosaccharide from birth and even earlier in the mother's body. However, only high levels of this monosaccharide can have the desired toxic effects on the ovaries, as its metabolism is beyond the animals’ tolerance (35). People with galactosemia, who are genetically deficient in galactose-metabolizing enzymes, are exposed to the toxicity of galactose aggregation or its metabolites. One of the target tissues of this toxicity is the ovarian tissue, and women with galactosemia will suffer from POI if they survive (36); the use of galactose to induce premature ovarian failure was based on this background knowledge. According to previous studies, exposure to a galactose-enriched diet (35%) is suitable for inducing a rat model of POI (30). However, there are very limited studies on the proper time of POI initiation (20), and no study has yet examined the long-term changes in the ovaries.

In this study, we investigated both short- and long-term effects of prenatal and early postnatal exposure of pregnant rats to enriched galactose feeding to propose a suitable model for one of the common POI phenotypes (i.e., gradual reduction of ovarian follicles) in the female rat offspring. We also studied the microscopic and macroscopic morphological changes in the ovaries and investigated hormonal, metabolic, and immunological changes in the experimental groups versus the controls.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Research Institute for Endocrine Sciences (RIES) (IR.SBMU.ENDOCRINE.REC.1398.001).
Consent for publication

Hereby declare that all authors participated in the study and in the development of manuscript titled “Induction a rat model of premature ovarian insufficiency (POI) using D galactose feeding during the critical periods of development: An experimental protocol”. We have read the final version and give our consent for the article to be published in Pilot ND Feasibility Studies Journal.

Availability of data and materials

Not applicable—protocol paper.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MRD designed, performed experiments, analyzed data, and writing the manuscript, MN performed experiments, NM supervised and performed immunologic tests, AP supervised histological tests, AZV analyzed data, MAA supervised and performed ovarian volume determination, FRT designed, supervised experiments, writing the manuscript.

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Abbreviations

POI Premature ovarian insufficiency

PNDs postnatal days

AGD anogenital distance

AVD anovaginal distance

FSH follicle-stimulating hormone

E2 estradiol

AMH anti-Müllerian hormone
ATP automatic tissue processor

H&E hematoxylin and eosin

SRS systematic random sampling

ELISA enzyme-linked immunosorbent assay

AOAs anti-ovarian antibodies

PBS phosphate-buffered saline

IPGTT Intraperitoneal glucose tolerance test

IPITT Intraperitoneal insulin tolerance test

**References**

The Reference section was not provided with this version.