CHAPTER 4

RESEARCH METHODS

4.1. Research Field

This research is in the field of Molecular Genetics.

4.2. Setting / Location and Research Periods

The research was performed at Department of Clinical Genetics and Endocrinology Department Erasmus Medical Center, Erasmus University Rotterdam, The Netherlands for 1 year.

4.3. Research Design

This is a cross sectional study, using mice as a model.

4.4. Population and Samples

4.4.1. Population

The population of this research were homozygous female premutation mice with Bl/6 and mixed FVB with Bl/6 background.

4.4.2. Samples

The samples of this research were ovaries from P6, P25, ~20wk and ~40wk from wt and premutation mice that already known its CGG repeat numbers, (obtained from Animal experimental facility, Erasmus Medical Center, Rotterdam, The Netherlands). P25 mice were used (n=5 for premutation mice and n=7 for wt mice) to measure the *Fmr1* mRNA levels. Several ages of mice were used (n=11 for premutation P6 and n=11 for wt P6, n=6 for premutation P25, n=7 for wt P25, n=10 for premutation ~20wk, n=6 for wt ~20wk, n=7 for premutation ~40wk and n=9 for wt ~40wk) to count primordial follicle number and especially to determine presense of recent corpora lutea, ~20wk and ~40wk were used.

Inclusion Criterion :

1. CGG repeat number 100-199
2. Healthy.

4.5. Research Variables

4.5.1. Independent Variables

Type of mice :

- Wt mice
- premutation mice

Scale : Nominal

4.5.2. Dependent Variables

- Primordial follicle number

  Scale : Numeric

- Presence of recent corpus luteum

  Scale : Nominal

- Fold change of *Fmr1* mRNA levels

  Scale : Numeric

4.6. Operational Definitions

1. Type of mice : Mice were distinguished into two group based on CGG repeat number in 5' untranslated region of *Fmr1* gene were determined by means of PCR. Approximate repeat lengths were determined using a standard curve, based on DNA samples of which repeat lengths were determined using sequencing with an ABI-based Fragile X size polymorphism assay in the past. There are two group, first wt mice and second premutation mice.
2. Primordial follicle number : Primordial follicle were recognized by the mean diameter of the follicle (< 20 μm) and morphological criteria (primordial follicles are in rest and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells). Primordial follicles were counted in every second section for P6 group, and every fifth section for P25, ~20wk, and ~40wk groups in whole of ovaries. Total number of primordial follicles in both ovaries were obtained from multiplication of counting results by 2,5 for P6 group and 5 for P25, ~20wk, and ~40wk groups, as described before by Durlinger et al. (65)
3. Presence of recent corpus luteum : Recent corpora lutea were determined by its appearance as a large rounded or irregularly shaped cells organization

stained homogeneously without oocyte in the middle. The samples were distinguished into two groups, first group is mice showed recent corpora lutea and old corpora lutea. Second group is mice showed only old corpora lutea or without corpora lutea.

4. Fold change of *Fmr1* mRNA levels: levels of *Fmr1* mRNA from ovaries were measured by means of RT and Q PCR . Efficiencies to be comparable. Ct (cycle of reaction to reach threshold of number cDNA) values of the reference gene was subtracted from the Ct value of the target gene, which gives the ΔCt. ΔCt of wild type mice then subtracted from ΔCt premutation mice, which is designated as the ΔΔCt. $2^{-\Delta\Delta Ct}$ then gives fold change.

4.7. Research Protocols

4.7.1. Mice Breeding

The samples were collected from Department of Animal experimental facility, Erasmus Medical Center, Erasmus University Rotterdam, The Netherlands.

Special breeding schedules were planned to obtain female homozygous premutation mice and female wt mice. The females were housed individually in cages placed near vicinity of a cage containing a wt male to stimulate regular estrous cycling.

4.7.2. Primordial follicles counting and presence of recent corpora lutea determination

Primordial follicle of ovaries were analyzed using Hemotoxyline-Eosin staining in four critical stages during reproductive life: 1) P6 mice because the ovaries predominantly contain primordial follicles, and no growing follicles; 2) P25 mice because they are immature and allow the study of the first wave of growing follicles; 3) ~20wk mice because they are at peak fertility; and 4) ~40wk irregular estrous cycling mice**,** and presence of recent corpus luteum were observed in ~20wk and ~40wk mice.

After mice were sacrificed, ovaries were dissected, and fixed overnight in Bouin's Fixative. Tissues were embedded in paraffin according to standard protocols. Whole tissues were cut into 8 μm, then all of them were spread on object glass and subsequently incubated at 37^{o}C overnight. Sections of both ovaries were stained using haematoxylin and eosine in Slide Stainer HMS 70 (Microm International) and mounted in Entellan.

Primordial follicles were counted under light microscope with 400 x magnification. Primordial follicles were recognized by the mean diameter of the follicle (< 20 μm) and morphological criteria (primordial follicles are in rest and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells). Primordial follicles were counted in every second section for P6 group, and every fifth section for P25, ~20wk, and ~40wk groups in whole of ovaries. Total number of primordial follicles in both ovaries were obtained from

multiplication of counting results by 2,5 for P6 group and 5 for P25, ~20wk, and ~40wk groups, as described before by Durlinger et al. (65)

Recent corpus luteum is corpus luteum with age range 48 hours until 5 days post ovulation. Presence of recent corpora lutea were determined by its appearance as a large rounded shaped structure stained homogeneously more blue than old corpus luteum without oocyte in the middle. Determination were done in whole ovaries.. The samples were distinguished into two groups, first group is mice showed recent or fresh corpora lutea and old corpora lutea. Second group is mice showed only old corpora lutea or without corpora lutea.

4.7.3. Measurement of Fmr1 mRNA levels

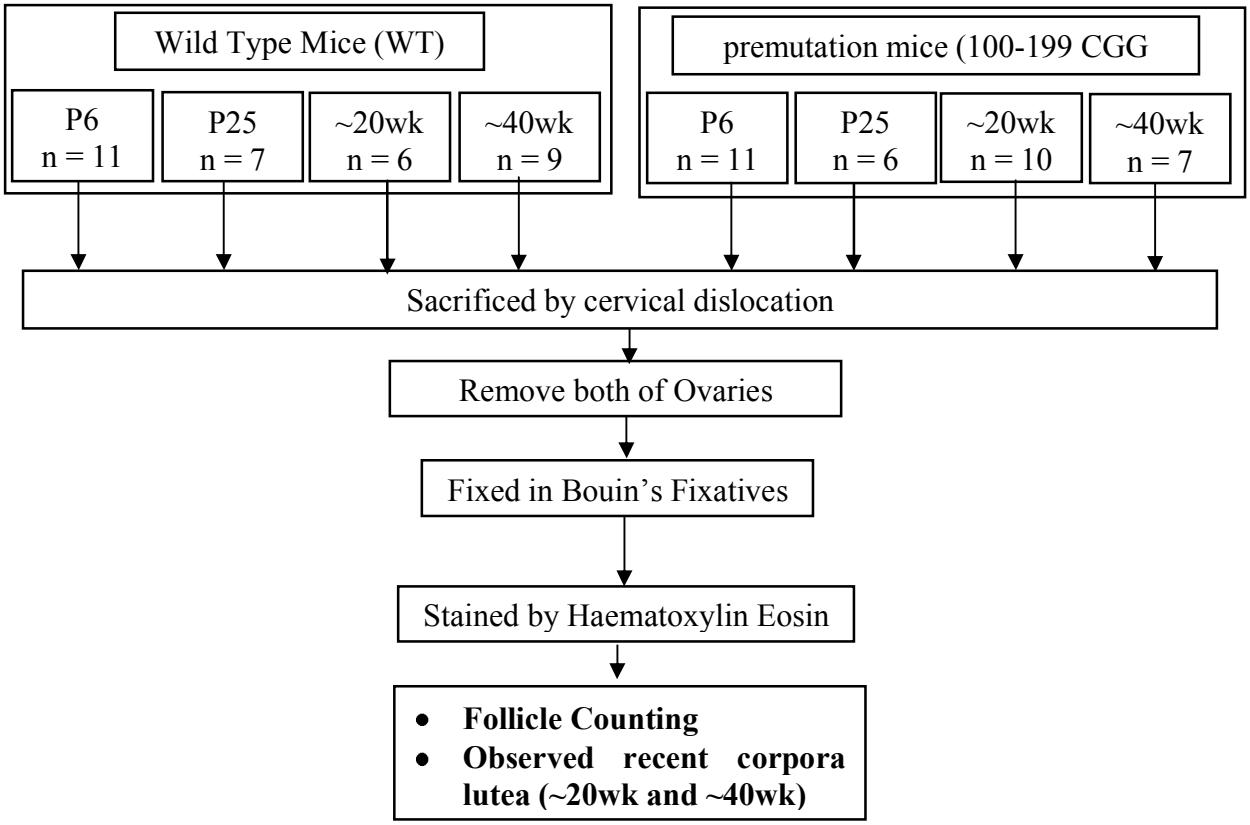
Fmr1 mRNA levels were measured using reverse transcriptase (RT) and quantitative (Q) PCR in ovaries of P25 mice because from previous study showed that Fmrp expression is located in granulose cells and P25 ovaries have highest number of granulose cells without corpora lutea. Ovaries were homogenized in 800 µl RNAbee using a plastic potter. Two hundred microliters of chloroform was added, then the mixture was spun at 4°C for phase separation. One volume of isopropanol was added to the aqueous phase to precipitate the RNA followed by centrifugation at 4^{o}C. The pellet was washed with icecold 80% ethanol and dissolved in DEPC-treated milliQ-H2O. RNA concentration and purity was determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies), and the quality of RNA were determined using electrophoresis in 1% agarose gel.

RT-PCR was performed on 1 μg RNA to synthesize cDNA using iScript cDNA Synthesis Kit (BioRad). PCR mix were made from 5x iScript reaction mix, iScript reverse Transcriptase, nuclease-free water, and RNA. PCR program were performed according to manufacturer's instructions.

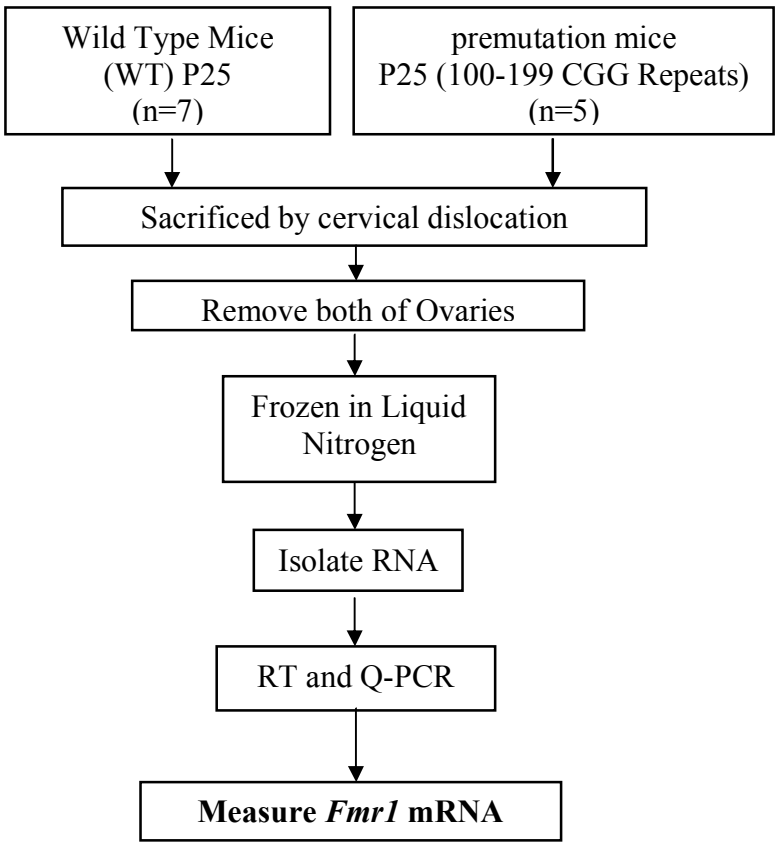
Quantitative PCR (Q-PCR) was performed to measure level of cDNA product of RT PCR. For Q-PCR one primer sets for *Fmr1* transition exon 5/6 (5′-AGATCAAGCTGGAGGTGCCA-3′ as forward primer and 5′-CAGAGAAGGCACCAACTGCC-3′ as reverse) and one primer sets for internal controls (Gapdh: forward: 5′-CCTGGAGAAACCTGCCAAGTAT-3′ and reverse: 5′-CCCTCAGATGCCTGCTTCA-3′ were used. Efficiencies were comparable. Ct values (cycle time to reach threshold of number cDNA) of the reference gene is subtracted from the Ct value of the target gene, which gives the ΔCt. ΔCt of wild type mice then subtracted from ΔCt premutation mice, which is designated as the ΔΔCt. $2^{-\Delta\Delta Ct}$ results in fold change.

4.8. Framework of Research Protocols

4.8.1. Follicle counting dan corpus luteum determination



4.8.2. Measurement of Fmr1 mRNA



4.9. Data Analysis

Data collected were analysed using SPSS 17 for Windows. T-Test was used to determine significant difference at $\alpha = 0,05$ for P5, P25 and ~40wk groups. Mann-Whitney Test was used to determine significant difference for ~20wk because the distribution of data is not normal. Data for proportion of presence of recent corpora lutea determination from ~20wk and ~40wk premutation mice and wt were analyzed using Fischer exact test.

4.10. Ethical Clearance

All experiments were carried out with permission from the local animal experimentation committee Erasmus Medical Center Erasmus University Rotterdam, The Netherlands (Ethical Clearance is attached)