ORIGINAL ARTICLE

Maternal Low-Protein Diet Affects Folliculogenesis and Mitochondrial Distribution Within Ovarian Follicles in Adult Offspring of Mice

Rahmadsyah[1], Junaedy Yunus[2], Mohammad Galih Pratama[2], Agung Dewanto[3], Shofwal Widad[3], Mochammad Anwar[3]

[1] Reproductive Endocrinology and Infertility Fellowship Program, Department of Obstetrics and Gynaecology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Jl. Farmako Sekip Utara, Yogyakarta 55281, Indonesia
[2] Department of Anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Jl. Farmako Sekip Utara, Yogyakarta 55281, Indonesia
[3] Department of Obstetrics and Gynaecology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Jl. Farmako Sekip Utara, Yogyakarta 55281, Indonesia

ABSTRACT

Introduction: Maternal nutrition intake affects the growth and development of foetal health. Poor nutritional intake contributes to adverse metabolic outcomes in the offspring at an advanced age, including its reproductive function. Maternal low-protein diet has been indicated to impair mitochondrial genetic expression in the offspring. This study aims to evaluate the impact of maternal low-protein diet on the folliculogenesis and mitochondrial distribution within ovarian follicles in female adult offspring of mice. **Methods:** Swiss Webster mice were nourished either normal protein diet containing 20% casein or low-protein diet (10% casein) during pregnancy and breastfeeding periods. Female offspring were weaned onto the control diet until 8 weeks of age. Female adult offspring were injected with 20 IU of FSH intraperitoneally to induce folliculogenesis. Folliculogenesis was assessed by morphological observation under a light microscope using haematoxylin-eosin staining, while mitochondrial distribution within ovarian follicles was identified by immunohistochemical staining of mitochondrial marker (ATP5A1). Immunostaining data were presented as fraction area (%) and quantified using ImageJ software. **Results:** The results from the low-protein diet group revealed a non-significant lower number of follicles at various stages compared to the normal protein diet group ($p>0.05$). The immunopositive expression of the mitochondrial marker in the low-protein diet group was notably lower than the control group (25.66% ± 4.90 vs 51.05% ± 1.51; $p<0.05$). **Conclusion:** Our study indicates that maternal low-protein diet during pregnancy and breastfeeding impairs folliculogenesis and mitochondrial distribution within the ovarian follicles in mice offspring later in life.

Keywords: ATP5A1, Folliculogenesis, Maternal low-protein diet, Mitochondrial distribution, Ovarian follicles

Corresponding Author:
Junaedy Yunus, PhD
Email: junaedy_yunus@ugm.ac.id
Tel.: +62-852-9203-5070

INTRODUCTION

Maternal undernutrition during preconception and pregnancy periods could influence foetal growth and development. This condition predisposes to adverse health outcomes in adult offspring (1). Indonesia Demographic and Health Survey estimates more than 50% of the pregnant woman both in urban and rural areas has energy intake less than 70% recommended dietary allowance. Furthermore, the proportions of the pregnant woman had inadequate protein intake (less than 80% recommended protein allowance) are estimated 49.6% in the urban region and 55.7% in the rural region (2). Nutrition at the molecular genomic level is essential for DNA synthesis and depends on macronutrients such as protein, fat, and carbohydrates to induce oocyte maturation and ovulation (3). Proteins themselves are involved in various biological processes, including reproduction, immunity, antioxidant system, embryogenesis, and other physiological functions (4).

The effect of inadequate protein intake on the preconception period is impairment of foetal development due to impaired gene expression in foetal tissue, reduced weight and size of the placenta, and reduced pregnancy rate (1). Visible abnormality caused by inadequate nutritional intake in mice can be observed in the development of follicles due to changes in glucose metabolic hormone levels such as insulin, leptin, and IGF-1 which resulting a decrease in anabolic hormones (insulin, IGF-1, thyroxin) and increase catabolic

hormones (cortisol and growth hormone) in reproductive hormones (5,6). A previous study concerning the effect of inadequate protein intake during the preconception period in rat embryos has also been shown to impact fertilization and embryo development (1).

Embryos at the preimplantation stage are vulnerable to environmental changes, including nutrient supply (7). The relationship between the development and quality of embryos with mitochondrial parameters, such as number, distribution, and shape, has been studied in several species (8). Previous study has shown the effects of pig mitochondrial function inhibition on poor foetal outcomes. The study results demonstrate that optimal oocyte and embryo metabolism influence the development and quality of the embryo after implantation, by increasing the production and storage of energy substrates in the mitochondria (9). Another previous study has examined the mitochondrial DNA copy number in sheep oocytes using real-time PCR. The study results show that there is a progressive increase in the mitochondrial DNA copy number from primordial follicular oocytes to mature meiosis II oocytes, but there is no difference in mitochondrial activity during meiosis (10). A previous study with immunohistochemical staining methods in mice shows the mitochondrial distribution patterns during the embryo implantation process and its association with hatching and implantation failures. There is a homogeneous mitochondrial distribution pattern in embryo successful implantation, whereas a heterogeneous pattern in failed implantation (11).

The potential for embryonic development and in vitro fertilization (IVF) results are related to the ATP content of human oocyte (12). Assisted reproduction technology (ART) procedures impact mitochondrial activity and function, while mitochondrial nutrition can improve mitochondrial functions on oocytes. Mitochondria influence all elements of mammalian reproduction and are crucial for optimum oocyte maturation, fertilization, and early development (13). Effective cytoplasmic maturation is determined by the oocyte mitochondrial distribution pattern in metaphase II with a homogenous pattern in successful implantation and a heterogeneous pattern in failed implantation (14). Mitochondrial distribution patterns are important for oocyte quality and correlate with a fertility problem, fertilization, implantation, and embryonic development (13).

This study aimed to examine the outcome of maternal low-protein diet on folliculogenesis and mitochondrial distribution within oocytes in female adult offspring of mice. The results of this study were expected to provide basic information regarding the impact of a maternal low-protein diet on ovarian anatomy (folliculogenesis) and improve the quality of oocytes through the mitochondrial distribution patterns, as well as to contribute to clinicians in regulating and making recommendations on the maternal nutritional intake to obtain optimal folliculogenesis and enhanced fertility.

MATERIALS AND METHODS

Animals

This study was a quasi-experimental research with a posttest-only control group design. Six female offspring Swiss Webster, aged 8 weeks old, were used per group. Female offspring were born from mother that was given the diet treatments. There were two diet treatment groups. The control group was given a normal protein diet (AIN-93G, 20% casein) while the treatment group was given a low protein diet (modified AIN-93G, 10% casein). The diet was produced by Department of Biochemistry, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada. The mice were nourished with either a normal protein diet or a low-protein diet during pregnancy and breastfeeding periods. The female offspring were weaned at 3 weeks of age onto the normal protein diet until 8 weeks of age. All mice were caged in a group in a 12-hour light/12-hour dark cycle at room temperature and supplied with food pellets and tap water ad lib. All research procedures using live animals were granted permission from the Medical Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada No. KE/0084/01/2020.

Assessment of folliculogenesis

The female adult offspring (6 mice per group), aged 8 weeks old, were injected with 20 IU of FSH (GONAL-f, Merck Serono) intraperitoneally to induce folliculogenesis. After 48 hours, the mice were profoundly anesthetized and transcardially infused with 0.9% sodium chloride solution for 5 minutes at ambient temperature. Bilateral oophorectomy was performed and the ovaries were transferred into the transport media (10% neutral buffered formalin). Then, the ovaries were desiccated in a stratified ethanol series, cleaned in xylene, and fixed in paraffin blocks. Paraffin-embedded tissue blocks were sectioned with a thickness of 4µm in the central part of the ovaries using a sliding microtome, installed on adhesive-coated glass slides, and a standard HE stain was performed (15). All types of ovarian follicles in both ovaries were observed by 3 blinded observers under a light microscope and counted according to a previous study with 4 follicle classifications: primordial follicle, primary follicle, secondary follicle, and antral follicle (16). The data were presented as a mean total number of ovarian follicles from all HE stained slides in each group for each type of ovarian follicle.

Mitochondrial immunohistochemistry and area fraction

The sections adjacent to the HE stained slices were immunohistostained according to a previous study (17) with anti-ATP5A1 (rabbit monoclonal, Abclonal Catalog No. A11217; 1:100 dilution). Mitochondrial area fraction was determined as a mitochondrial-immunopositive area in the ovarian follicle per total area in one field

of view. The area fraction was counted using ImageJ software (NIH) for 15 fields of view in bilateral ovary slides of each mouse and presented in percent unit as mean number of area fraction.

Statistical analysis

The data of folliculogenesis assessment were presented as a total number of ovarian follicles, whereas mitochondrial area fraction was displayed as mean ± standard deviation (SD) and analysed using SPSS Statistics 23 software. The normal distribution of data was determined using Saphiro-Wilk test. If the data were normal distribution data, an independent t-test was applied, otherwise, Mann Whitney U test was used for statistical evaluation.

RESULTS

Assessment of folliculogenesis

Macroscopically, there were no significant differences in the physical characteristics of the offspring between the two groups (data not shown). In addition, microscopic analysis through the central part of the ovaries (Fig. 1) showed no distinct difference in the morphology of ovarian follicles between the two groups. However, the mean total number of ovarian follicles in the maternal low-protein diet group was lower than the normal protein diet group (Table I). Detailed analysis on mean total numbers of each type of ovarian follicle in the maternal low-protein diet group were also lower contrasted to the normal protein diet group, despite not statistically significant, as shown in Table I. The low-protein diet group showed a higher proportion (64.84%) at the primordial follicle stage compared to the normal protein diet group (62.93%).

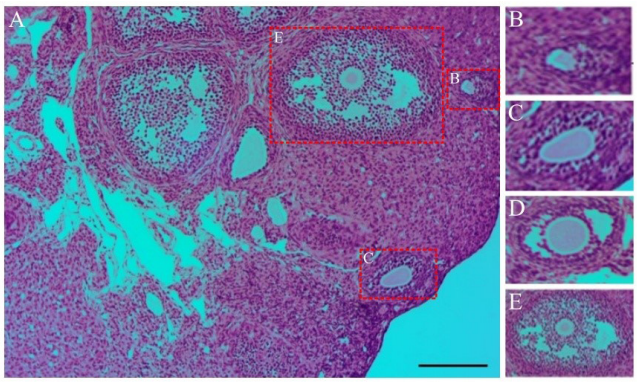

Figure 1: HE stained light micrographs of ovarian follicles at various stages. A: Low-power photograph of the ovary at 100x magnification; B: Primordial follicle; C: Primary follicle; D: Secondary follicle; E: Antral follicle. Scale bar = 10 µm

Mitochondrial area fraction

Mitochondrial immunohistochemistry demonstrated positive expression in the ovarian follicles, including granulosa cells and theca layers (Fig. 2). A homogenous pattern of the immunopositive area was observed in the normal protein diet group, while a heterogenous pattern was recognized in the low-protein diet group. The quantification results using ImageJ software showed a significantly lower mean area fraction of the low-protein diet group contrasted to the normal protein diet group (25.66% ± 4.90 vs 51.05% ± 1.51; $p<0.05$) (Table II).

DISCUSSION

The ovaries of the adult offspring in the low-protein diet group morphologically appear similar to those in the normal protein diet group. However, the total number of ovarian follicles in adult offspring of the maternal low-protein diet group is less than the normal protein diet group, despite not being statistically significant. These results show that maternal low-protein diet during pregnancy and breastfeeding periods could affect folliculogenesis at different stages in the female

Table I: Mean total number of ovarian follicles at various stages in normal protein and low-protein diet groups.

Follicle Stages	NPD Group (n = 6)		LPD Group (n = 6)	
	Mean Total (n)	Proportion (%)	Mean Total (n)	Proportion (%)
Primordial	460	62.93	402	64.84
Primary	220	30.10	181	29.19
Secondary	30	4.10	21	3.39
Antral	21	2.87	16	2.58
Total	731	100	620	100

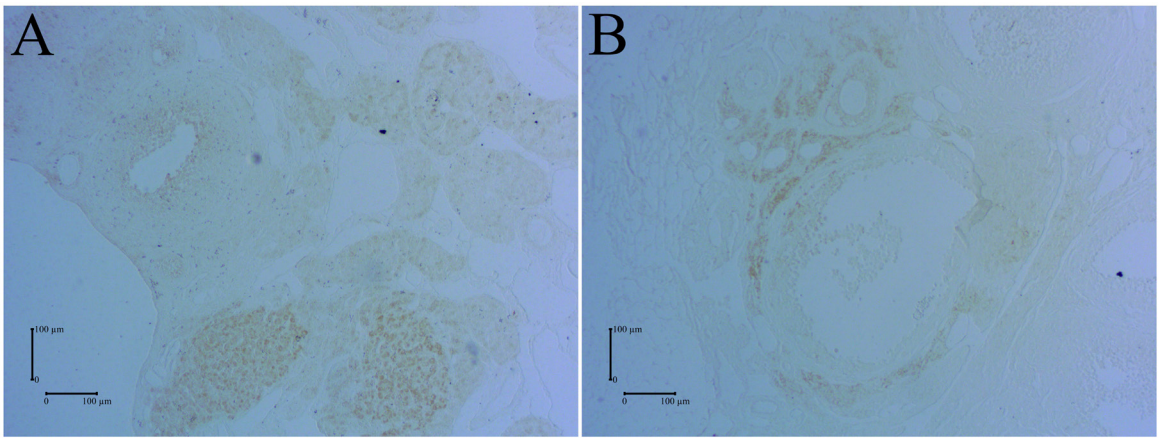

Figure 2: ATP5A1 immunohistochemical stains in ovarian follicles. Light micrographs at 100x magnification of ATP5A1-immunopositive expression in the normal protein (A) and low-protein diet groups (B).

Table II: Quantitative analysis on ATP5A1-immunopositive area fraction in the normal and low-protein diet groups

Groups	Mitochondrial Area Fraction (%, Mean ± SD)	P-Value
Normal protein diet (n = 6)	51.05 ± 1.51	0.000*
Low-protein diet (n = 6)	25.66 ± 4.90	

*= $p<0.05$

adult offspring. These study results are in accordance with the previous study that demonstrated the impact of poor nutrition in general on oocyte quality and follicular maturation (5). Low-protein diet, in particular, reduces primordial follicular reserve in adults. However, a low-protein diet does not directly involve follicular development, since protein malnutrition condition leads to wide-ranging changes in physiological function. The previous study has shown that a low nutrition diet decreases several hormones, including cortisol, ACTH, and insulin (18). This condition leads to disruption of the HPA (Hypothalamic-Pituitary-Adrenal) axis and therefore contributes to diverse abnormalities caused by hormonal imbalance, including disorganization of tissue structure and function. Another previous study has also demonstrated that maternal nutrition affects blood levels of insulin, IGF, thyroxine, glucocorticoids, and growth hormone. These maternal hormones will have an impact on the availability of hormones and their receptors in their offspring (19,20).

Our study result shows that mitochondrial protein expression in ovarian follicles of the female adult offspring in the maternal low-protein diet group is notably less than the normal protein diet group. Immunohistochemical staining shows the difference of mitochondrial distribution pattern in ovarian follicles between normal and low-protein diet groups. The heterogeneous pattern of mitochondrial distribution in ovarian follicles of the low-protein diet group indicates the disruption of energy (ATP) distribution into the nucleus, thus leading to a lack of necessary energy to carry out its physiological functions, such as proliferation and differentiation. In addition, the heterogeneous or clustered pattern of mitochondrial distribution may indicate that the cells are undergoing apoptosis due to mitochondrial dysfunction. Cells undergoing apoptosis have low energy (ATP), thus affecting cell growth and survival (18).

The development of preimplantation embryos is a critical window period in humans undergoing assisted reproductive technology procedures. The previous study in humans has shown that the mean mitochondrial DNA copy number in patients undergoing IVF is 256,000 - 213,000 (19). While another previous study in humans has revealed a correlation between the abnormal form of mitochondrial mural granulosa cells and age-related changes in which mitochondria are more elongated in the elderly patient, whereas the shapes are round or oval in young age (21).

The main target of nutritional programming is gene expression changes in foetal tissue cells, steroidogenesis and folliculogenesis in the ovary, foetal development, and placenta during pregnancy. Folliculogenesis requires ATP supply which is produced by mitochondria and distributed in the cell cytoplasm. The more ATP needed, the more mitochondria are distributed around the cell nucleus (22–24). The location of mitochondrial distribution could determine the condition of cell development. Mitochondria are normally distributed around the nucleus and thus ATP can be directly utilized by the nucleus and other organelles to carry out their functional activities (22,25,26).

CONCLUSION

Our study shows that maternal low-protein diet during pregnancy and lactation periods impairs folliculogenesis in female adult offspring indicated by the lower total number of ovarian follicles as well as lower area fraction of ATP5A1-immunopositive mitochondria. However, further study is needed to evaluate the effect of a low-protein diet on reproductive hormones involved in fertility and sexuality.

ACKNOWLEDGEMENTS

We are grateful to Mrs. Wiwit Ananda and Mr. Mulyana for providing technical assistance.

REFERENCES

1. Mitchell M, Schulz SL, Armstrong DT, Lane M. Metabolic and mitochondrial dysfunction in early mouse embryos following maternal dietary protein intervention. Biol Reprod. 2009;80(4):622–30.
2. ICF. Indonesia Demographic and Health Survey 2017. Jakarta, Indonesia : BKKBN, BPS, Kemenkes, and ICF ; 2018.
3. Silvestris E, Lovero D, Palmirotta R. Nutrition and Female Fertility: An Interdependent Correlation. Front Endocrinol (Lausanne). 2019;10:346.
4. Hosseini B, Eslamian G. Association of Dietary Factors With Male and Female Infertility: Review of Current Evidence. Thrita. 2014;3(3).
5. Ashworth CJ, Toma LM, Hunter MG. Nutritional effects on oocyte and embryo development in mammals: implications for reproductive efficiency and environmental sustainability. Philos Trans R Soc Lond B Biol Sci. 2009;364(1534):3351–61.
6. Diskin MG, Mackey DR, Roche JF, Sreenan JM. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. Anim Reprod Sci. 2003;78(3–4):345–70.
7. Eckert JJ, Fleming TP. The effect of nutrition and environment on the preimplantation embryo. Obstet Gynaecol. 2011;13(1):43–8.

8. Chappel S. The role of mitochondria from mature oocyte to viable blastocyst. Obstet Gynecol Int. 2013;2013:183024.
9. El Shourbagy SH, Spikings EC, Freitas M, St John JC. Mitochondria directly influence fertilisation outcome in the pig. Reproduction. 2006;131(2):233–45.
10. Cotterill M, Harris SE, Collado Fernandez E, Lu J, Huntriss JD, Campbell BK, et al. The activity and copy number of mitochondrial DNA in ovine oocytes throughout oogenesis in vivo and during oocyte maturation in vitro. Mol Hum Reprod. 2013;19(7):444–50.
11. Helmita R, Djuwita I, Purwantara B. Pola distribusi mitokondria sel-sel trofoblas blastosis mencit (Mus muculus albinus) dan pengaruhnya terhadap kegagalan hatching dan implantasi. Sainstek J Sains dan Teknol. 2016;7(1):16.
12. Schatten H, Sun Q-Y, Prather R. The impact of mitochondrial function/dysfunction on IVF and new treatment possibilities for infertility. Reprod Biol Endocrinol. 2014;12:111.
13. Van Blerkom J. Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. Reproduction. 2004;128(3):269–80.
14. de Cбssia S Figueira R, de Almeida Ferreira Braga DP, Semiro-Francisco L, Madaschi C, Iaconelli A, Borges E. Metaphase II human oocyte morphology: contributing factors and effects on fertilization potential and embryo developmental ability in ICSI cycles. Fertil Steril. 2010;94(3):1115–7.
15. Yunus J, Setsu T, Kikkawa S, Sakisaka T, Terashima T. Cytoarchitecture of the olfactory bulb in the laggard mutant mouse. Neuroscience. 2014;275:259–71.
16. Nie X, Dai Y, Zheng Y, Bao D, Chen Q, Yin Y, et al. Establishment of a Mouse Model of Premature Ovarian Failure Using Consecutive Superovulation. Cell Physiol Biochem. 2018;51(5):2341–58.
17. Yunus J, Salman M, Lintin GBR, Muchtar M, Sari DCR, Arfian N, et al. Chlorogenic acid attenuates kidney fibrosis via antifibrotic action of BMP-7 and HGF. Med J Malaysia. 2020;75(Suppl 1):5–9.
18. Tsuda Y, Iwasawa K, Yamaguchi M. Low-protein diet decreased the adrenal function and spontaneous activity of mice during chronic heat stress. Heliyon. 2019;5(4):e01463.
19. Fowden AL, Ralph MM, Silver M. Nutritional regulation of uteroplacental prostaglandin production and metabolism in pregnant ewes and mares during late gestation. Exp Clin Endocrinol. 1994;102(3):212–21.
20. Whorwood CB, Firth KM, Budge H, Symonds ME. Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11beta-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin ii receptor in neonatal sheep. Endocrinology. 2001;142(7):2854–64.
21. Liu Y, Han M, Li X, Wang H, Ma M, Zhang S, et al. Age-related changes in the mitochondria of human mural granulosa cells. Hum Reprod. 2017;32(12):2465–73.
22. Nagai S, Mabuchi T, Hirata S, Shoda T, Kasai T, Yokota S, et al. Correlation of abnormal mitochondrial distribution in mouse oocytes with reduced developmental competence. Tohoku J Exp Med. 2006;210(2):137–44.
23. Nadra K, Anghel SI, Joye E, Tan NS, Basu-Modak S, Trono D, et al. Differentiation of trophoblast giant cells and their metabolic functions are dependent on peroxisome proliferator-activated receptor beta/delta. Mol Cell Biol. 2006;26(8):3266–81.
24. Straszewski-Chavez SL, Abrahams VM, Mor G. The role of apoptosis in the regulation of trophoblast survival and differentiation during pregnancy. Endocr Rev. 2005;26(7):877–97.
25. Thouas GA, Trounson AO, Jones GM. Effect of female age on mouse oocyte developmental competence following mitochondrial injury. Biol Reprod. 2005;73(2):366–73.
26. Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis - the p53 network. J Cell Sci. 2003;116(Pt 20):4077–85.