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Unraveling the Epigenetic Impact of High-Fat Diet: Dnmt3b and Dnmt3l Expressions in Mouse Ovary

Yüksek Yağlı Diyetin Epigenetik Etkisinin Araştırılması: Fare Ovaryumunda Dnmt3b ve Dnmt3l Ekspresyonu

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ABSTRACT

Objective: Obesity is characterized by an increase in adipose tissue volume. It has emerged as a risk factor for infertility. Recent research has unveiled a strong correlation between obesity and epigenetics. Epigenetic mechanisms, primarily DNA methylation which is governed by DNA methyltransferases (Dnmts), regulates gene expression. In the present study, we aimed to evaluate Dnmt3b and Dnmt3l expression in ovary of HFD-induced group compared to control. **Materials and Methods:** For this purpose, C57BL6/J female mice (4 weeks old) (n=8) were fed either a normal diet consisting of 20% protein, 10% fat and 70% carbohydrate or an HFD consisting of 20% protein, 60% fat and 20% carbohydrate for 12 weeks. At the end of 12 weeks, animals were euthanized, and ovaries were transferred into buffered solution. In accordance with our research objective, we conducted immunohistochemistry to examine the presence of Dnmt3b and Dnmt3l proteins and their changes in the ovaries of HFD-induced mice. **Results:** Our findings revealed significant alterations in the protein levels in the ovary of the HFD-induced obese mice. Specifically, the total expressions of both Dnmt3b and Dnmt3l were remarkably increased in the ovary of the HFD group ($p<0.05$). Furthermore, we observed increased expression of Dnmt3b in each stage of folliculogenesis, while Dnmt3b and Dnmt3l expressions were elevated in granulosa cells of the HFD-induced group ($p<0.05$). **Conclusion:** These results indicate that HFD has a significant impact on the expression of Dnmt3b and Dnmt3l proteins in the ovaries, particularly affecting granulosa cells, which could be potentially associated with the observed changes in global DNA methylation levels.

Keywords: Dnmt3b; Dnmt3l; obesity; oogenesis; ovary

ÖZET

Amaç: Obezite, yağ dokusu hacmindeki artış ile karakterizedir. İnfertilite için önemli bir risk faktörü olarak görülmektedir. Son araştırmalar, obezite ve epigenetik arasında güçlü bir ilişki olduğunu göstermektedir. Başta DNA metiltransferazlar tarafından katalizlenen DNA metilasyonu olmak üzere epigenetik mekanizmalar gen ekspresyonunu düzenlerler. Bu çalışmada, YYD ile indüklenen grubun ovaryumlarında Dnmt3b ve Dnmt3l ekspresyonunu kontrol ile karşılaştırmalı olarak değerlendirmeyi amaçladık. **Gereç ve Yöntemler:** Bu amaçla, C57BL6/J dişi fareler (4 haftalık) (n=8) %20 protein, %10 yağ ve %70 karbonhidrattan oluşan normal bir diyetle veya %20 protein, %20 karbonhidrat ve %60 yağdan oluşan bir YYD ile 12 hafta boyunca beslendi. 12 haftanın sonunda hayvanlara ötenazi yapıldı ve ovaryumlar tamponlu solüsyona aktarıldı. Araştırma hedefimize uygun olarak, YYD ile indüklenen farelerin ovaryumlarındaki Dnmt3b ve Dnmt3l proteinlerinin varlığını ve değişikliklerini incelemek için immünohistokimya tekniğini kullandık. **Bulgular:** Bulgularımız, YYD'in neden olduğu obez farelerin ovaryumlarındaki protein seviyelerinde önemli değişiklikler olduğunu ortaya koydu. Spesifik olarak, hem Dnmt3b hem de Dnmt3l'nin toplam ekspresyonları, YYD grubunun ovaryumlarında önemli ölçüde arttı ($p<0.05$). Ayrıca YYD ile indüklenen grupta, folikülogenezin her aşamasında Dnmt3b ekspresyonunda artış gözlenirken, granüloza hücrelerinde Dnmt3b ve Dnmt3l ekspresyonlarında artış gözlendi ($p<0.05$). **Sonuç:** Bu sonuçlar YYD'in özellikle granüloza hücrelerinde önemli bir etkiye sahip olduğunu göstermiş olup, ovaryumlardaki Dnmt3b ve Dnmt3l ekspresyon seviyelerindeki artışın daha önceki çalışmamızda gösterdiğimiz artan global DNA metilasyonu ile potansiyel olarak ilişkilendirilebilir olması bakımından önemlidir.

Anahtar Kelimeler: Dnmt3b; Dnmt3l; obezite; oogenez; ovaryum

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Obesity, a prevalent and complex health condition, is characterized by the excessive accumulation of body fat. This condition poses significant risks to overall health and substantially increases the likelihood of developing various chronic diseases.[1] As obesity causes various pathological conditions and therefore leads to diseases irrespective of people age, genders, and socioeconomic statuses, it has been regarded as a global epidemic.[2] Obesity has a negative impact on the female reproductive system, affecting menstrual period and fertility.[3] It is known that, in obese women with polycystic ovarian syndrome (PCOS), elevated levels of the hormone leptin, which is released by adipose tissue, have been linked to hormonal imbalances and decreased fertility.[4] Obesity and PCOS share several similarities in their characteristics. Both conditions are associated with elevated testosterone and fasting insulin levels, along with reduced levels of sex hormone-binding globulin. These shared hormonal profiles may contribute to the overlapping phenotype observed in patients with obesity and those diagnosed with PCOS.[5] Oocyte quality is also essential for effective fertilization and embryo development, and obesity has been associated to a decrease in oocyte quality. Numerous studies have shown that obese women undergoing in vitro fertilization (IVF) tend to produce oocytes with lower developmental potential, lower fertilization rates, and diminished implantation rates when compared to their non-obese counterparts, suggesting that changes in metabolic and endocrine factors connected to obesity may have an negative impact on oocyte quality.[6,7] Obesity is also linked to alterations in the granulosa cells of the ovary and the follicular fluid surrounding the oocyte.[8] Differences in follicular fluid insulin, triglycerides, free fatty acids, proinflammatory cytokines, oxidized low density lipoprotein, and fatty acid composition have been identified in obese women, implying that oocyte development abnormalities are caused by a variety of processes.[9]

DNA methylation is an essential epigenetic alteration that is responsible for controlling gene expression and cellular growth and DNA methyltransferases (DNMTs), a category of enzymes, catalyze this activity.[10] DNMTs are involved in de novo DNA methylation, creating new methylation marks throughout embryonic development and cellular differentiation, as well as preserving the methylation patterns during DNA replication.[11] One of the important DNMTs is Dnmt1, which mostly performs maintenance function of methyltransferase. Dnmt3a and Dnmt3b, on the other hand, operate as de novo methyltransferases and help generate DNA methylation patterns.[11] Dnmt3l lacks the catalytic domain required for DNA methylation, in contrast to Dnmt3a and Dnmt3b. As a crucial cofactor, it interacts with Dnmt3a and Dnmt3b to control their enzymatic activity and target selectivity.[12] Although, it is well recognized that obesity alters the epigenome and we already showed that high-fat diet induces the levels of Dnmt1 and Dnmt3a proteins while decreases the global DNA methylation in ovaries.[13] The effect of obesity on Dnmt3b and 3l has not been elucidated yet. Therefore, in this study, we aimed to investigate the impact of high-fat diet-induced obesity on Dnmt3b and Dnmt3l protein expressions in various ovarian components of mice. Specifically, we assessed the effects on ovarian primordial, primary, and secondary follicles, as well as the corpus luteum. Additionally, we examined the oocytes within primary and secondary follicles, granulosa cells, and the ovarian stroma. By evaluating changes in Dnmt3b and Dnmt3l protein expressions in these different ovarian elements, we hope to shed light on the influence of obesity on the epigenetic mechanisms involved in folliculogenesis and overall ovarian function. This research will contribute to a better understanding of the molecular pathways affected by obesity in the ovaries and may provide valuable insights into the mechanisms underlying infertility associated with obesity.

MATERIALS AND METHODS

ANIMALS

This study was conducted in accordance with the principles of the Declaration of Helsinki. The experimental protocol was approved by the Animal Care and Usage Committee of Ankara University (protocol no. 2020-17-145 and date: 14/10/2020). C57BL6/J mice (4 weeks old) were housed in cages under a 12-h/12-h light/dark cycle with an ambient temperature of 22-25°C. Establishment of an HFD-induced obese mouse

model was revealed in our previous article.[13] After the females (n=8) were randomly allocated to the appropriate cages, they were fed either a normal diet (Research Diets, D12450J) consisting of 20% protein, 10% fat and 70% carbohydrate or an HFD (Research Diets, D12492) consisting of 20% protein, 60% fat and 20% carbohydrate for 12 weeks. Body weights were measured weekly. At the end of 12 weeks, animals were euthanized, following ablation of the ovaries, they were transferred to buffered solutions.

IMMUNOHISTOCHEMISTRY

The expression levels of the Dnmt3b and Dnmt3l in the ovary were evaluated by using immunohistochemistry. The paraffin sections of the tissues at 5 µm thickness were deparaffinized in fresh xylene and then rehydrated in a series of decreasing ethanol concentrations. After deparaffinization and rehydration processes, citrate buffer (pH 6.0) was used for antigen retrieval using microwave (7 min at 750 W). The endogenous peroxidase activity in the sections was blocked by 3% hydrogen peroxide for 25 min at room temperature (RT). Following three times washes with 1× phosphate-buffered saline (PBS, Sigma-Aldrich, P44417), sections were blocked with blocking buffer (Thermo Scientific, UK) at RT for 7 min to prevent non-specific binding. Then, the sections were incubated with the following primary antibodies specific for Dnmt3b (1:400; catalog no. ab2851, Abcam) and Dnmt3l (1:300; catalog no. ab194094, Abcam) at+4˚C overnight. For negative control, slides were incubated in PBS without primary antibody for each group. After that, sections were washed three times in PBS for 5 min each and subsequently incubated with anti-rabbit IgG secondary antibody (1:200; catalog no. BA-1000, Vector) at RT for 1 h. Finally, sections were incubated with streptavidin–horseradish peroxidase (HRP) complex (catalogue no. TS-125, Thermo Scientifc) for 20 min at RT. The immune reactions were visualized using 3,3′-diaminobenzidine (DAB) chromogen (catalogue no. D4168, Sigma-Aldrich) under a light microscope. Sections were washed in tap water and counterstained with Mayer's haematoxylin for 1 min. Slides were examined and images were obtained with a light microscope (ZEISS Scope.A1, Zeiss, Germany) at 20×(Zeiss, A-Plan 20×/0.45 Working Distance (W.D.)=0.46 M27) and 40× (Zeiss, N-Achroplan 40×/0.65 W.D.=0.60 M27) magnifications. Images were analyzed and captured with AxioCam camera (Zeiss, AxioCam MRc5) and AxioVision image software (Zeiss AxioVision v. 4.8). Their dimensions were at 1292×968 (width×height) pixels, and each of them has a depth of 24 bits. Also, all micrographs were evaluated for the related expression using the software and presented a resolution of 300 dpi in the panels. We applied H-score analysis for specific regions, cell type evaluations, total protein expressions. H-score analysis were performed by two independent observers. Primordial follicles, primary follicles, secondary follicles, corpus luteum, oocytes in primary and secondary follicles, granulosa cells and ovarian stroma were evaluated by H-score. Briefly, sections were evaluated using a numerical score based on the number of positive cells in the section (ten different fields in each section). H-score was calculated using the following equation: H-score = $\sum$Pi (i); where i is the intensity of staining with a value of 0, 1, 2, 3 (no signal, weak signal, moderate signal, high signal, respectively), Pi is the percentage of stained related cells for each intensity varying from 0% to 100%.

STATISTICAL ANALYSIS

Signal intensities of immunohistochemistry images were digitalized with ImageJ software. Data were transferred to SigmaStat software (version 3.5, Jandel Scientifc Corp) for statistical analysis. The Shapiro–Wilk test for normality was used to test whether the data were normally distributed. Logarithmic transformation was applied to non-normally distributed data, when applicable. Normally distributed data were presented as mean±standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) to examine the differences between the groups, and then a post hoc Tukey's test was applied. The significance level was set at $p<0.05$.

RESULTS

CHANGES IN THE OVERALL EXPRESSION OF DNMT3B AND DNMT3L IN THE OVARY INDUCED BY THE HIGH-FAT DIET (HFD)

Relative expressions of Dnmt3b and Dnmt3l in ovary sections from both HFD and control group were eval-

uated (Figure 1A, p<0.05). Expressions of Dnmt3b and Dnmt3l were observed at both cytoplasm and nucleus of the ovarian cells. Additionally, both Dnmt3b and Dnmt3l expressions significantly increased in total ovary sections of the HFD group compared to control (Figure 1B, p < 0.05).

EFFECT OF HFD-INDUCED OBESITY ON DNMT3B AND DNMT3L DURING FOLLICLE DEVELOPMENT

Relative expressions of Dnmt3b and Dnmt3l in primordial follicle, primary follicle, secondary follicle, and corpus luteum were evaluated in control and HFD group (Figure 2A, Figure 2B, p<0.05). In the HFD group, Dnmt3b expression showed a significant increase in primordial follicles, primary follicles, secondary follicles, and the corpus luteum compared to the control group (Figure 2C, p<0.05). On the other hand, Dnmt3l expression was significantly higher in primordial follicles of the HFD group (p<0.05), but it remained comparable in primary and secondary follicles. Notably, Dnmt3l was found to be decreased in the corpus luteum of the HFD group compared to the control group (Figure 2D, p<0.05).

DNMT3B AND DNMT3L LEVELS IN CELLULAR LEVEL: OOCYTE, GRANULOSA AND STROMAL CELLS

Dnmt3b and Dnmt3l expressions in oocyte, granulosa cells and ovarian stroma were individually evaluated in both the control and HFD group (Figure 3A). The expression of Dnmt3b and Dnmt3l in oocytes was relatively low compared to the other components of the ovaries. In the HFD group, oocytes in both primary and secondary follicles exhibited a significant increase in Dnmt3b expression compared to the control group (Figure 3B, p<0.05). Conversely, Dnmt3l expression was markedly decreased in oocytes of both primary and secondary follicles in the HFD group compared to the control group (Figure 3C, p<0.05). In granulosa cells, expression of both Dnmt3b and Dnmt3l remarkably induced in HFD group compared to control (Figure 3B,

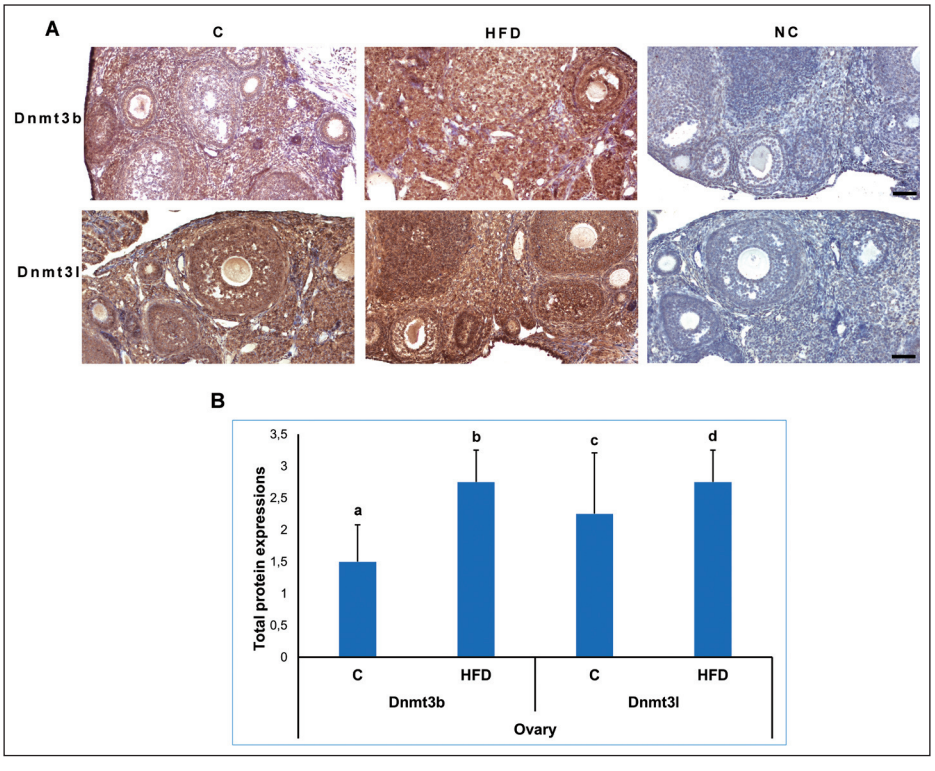

FIGURE 1: Changes in the overall expression of Dnmt3b and Dnmt3l in the ovary induced by the high-fat diet (HFD). **(A)** Demonstrative micrographs obtained from Dnmt3b and Dnmt3l stained sections **(B)** Total Dnmt3b and Dnmt3l expression levels in immunohistochemistry (IHC) analysis. The statistical significance was determined by using one-way ANOVA followed by post hoc Tukey's test. The p<0.05 was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. Scale bar: 20 µm.

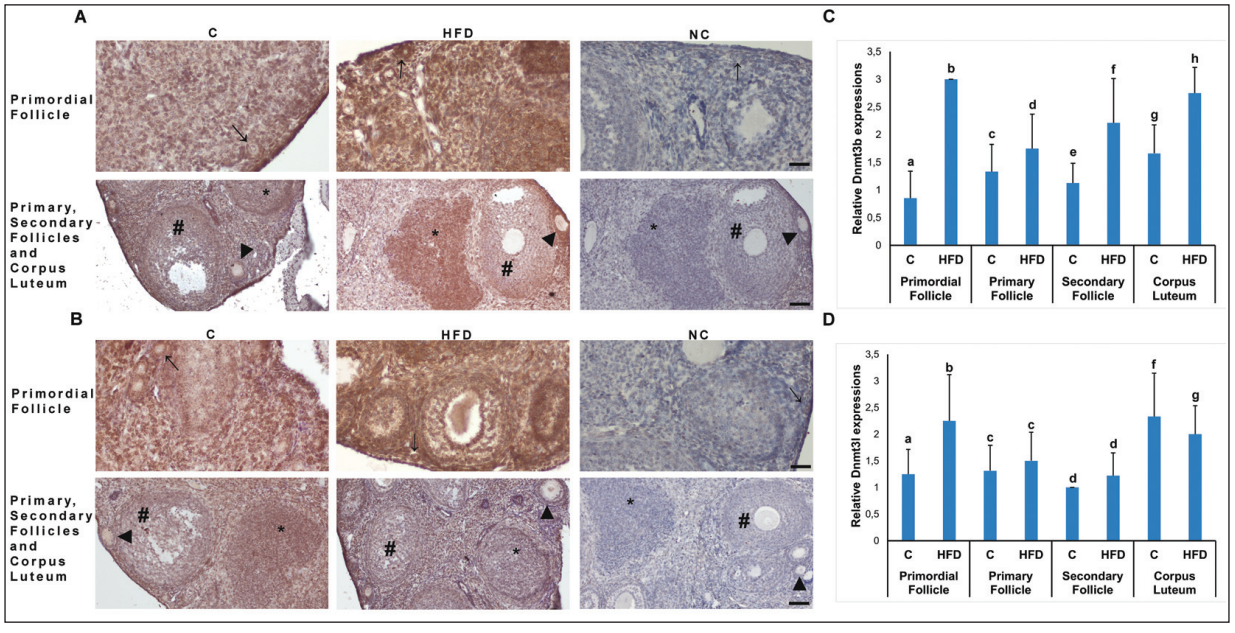

FIGURE 2: Effect of HFD-induced obesity on Dnmt3b and Dnmt3l during follicle development. (**A and B**) Demonstrative micrographs obtained from Dnmt3b and Dnmt3l stained sections. (**C and D**) Dnmt3b and Dnmt3l expression level in immunohistochemistry (IHC) analysis. The statistical significance was determined by using one-way ANOVA followed by post hoc Tukey's test. The $p<0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. Scale bar: 10 µm for primordial follicles. Scale bar: 20 µm for primary and secondary follicles, and corpus luteum. Indicator for primordial follicles is arrow; primary follicle is head of arrow; secondary follicle is hash; corpus luteum is asterisk.

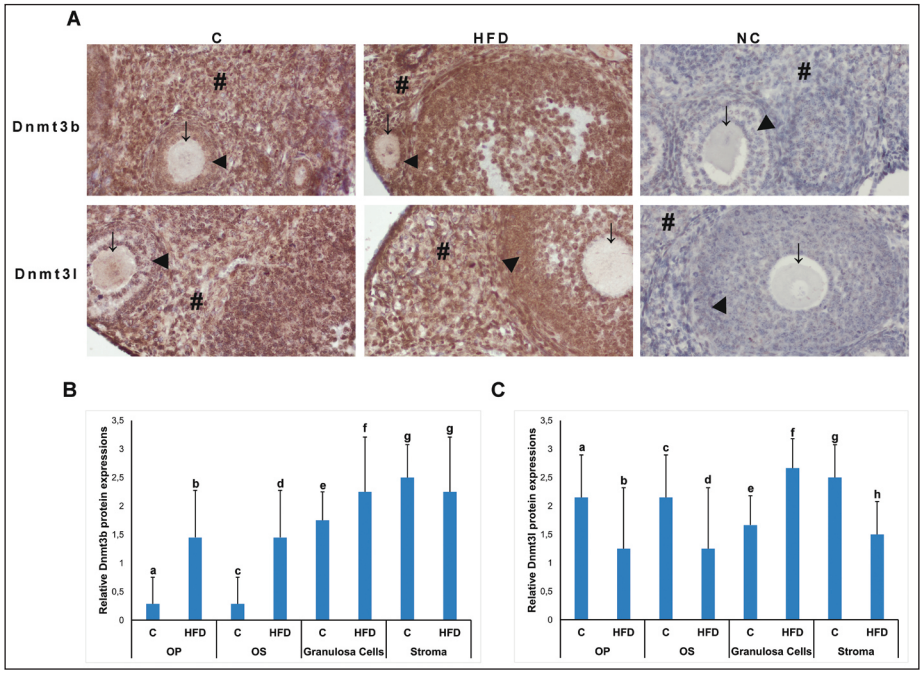

FIGURE 3: Dnmt3b and Dnmt3l levels in cellular level: oocyte, granulosa and stromal cells. **(A)** Demonstrative micrographs obtained from Dnmt3b and Dnmt3l stained sections. (**B and C**) Dnmt3b and Dnmt3l expression level in immunohistochemistry (IHC) analysis. The statistical significance was determined by using one-way ANOVA followed by post hoc Tukey's test. The $p<0.05$ was considered statistically significant, shown different letters on the columns. Bars in graphs are represented as mean ± SD. Scale bar: 10 µm for primordial follicles. Scale bar: 20 µm for primary and secondary follicles, and corpus luteum. Indicator for oocytes is arrow; granulosa cells is head of arrow; ovarian stroma is hash. OP; oocyte in primary follicle, OS; oocyte in secondary follicle.

Figure 3C, $p<0.05$). In stromal cells, Dnmt3b expression remained similar between the groups, while Dnmt3l expression was decreased in the HFD group.

DISCUSSION

Obesity has been found to have significant effects on epigenetic processes such as DNA methylation, histone modifications, and microRNA expression in various organs including reproductive system.[14,15] In this study, we found that both Dnmt3b and Dnmt3l expression dramatically increased in ovaries of HFD group. The findings are in line with our previous study, where we observed a significant increase in global DNA methylation levels, as well as elevated expressions of Dnmt1 and Dnmt3a.[13] Similarly, it was shown that obesity causes increased Dnmt1 and Dnmt3b levels in human blood cells.[16] Moreover, here, we found that overall Dnmt3b expression increased remarkably in all stages of folliculogenesis of HFD group compared to control while Dnmt3l increased in only primordial follicle and decreased in corpus luteum but did not change in primary and secondary follicles. These result suggest that Dnmt3b may play a significant role in the increased global DNA methylation during follicle development. Additionally, our results revealed that Dnmt3b expression increased in oocytes of both primary and secondary follicles, as well as in granulosa cells. Conversely, Dnmt3l levels in oocytes significantly decreased in the HFD group, while showing an increase in granulosa cells. These findings further highlight the distinct roles of Dnmt3b and Dnmt3l in the context of obesity-induced changes in the epigenetic landscape during ovarian development. Granulosa cells support the folliculogenesis and their differential expression patterns are critical for the development of follicles and maturation of oocytes.[17] Additionally, it was reported that granulosa cells of women with PCOS demonstrated altered DNA methylation pattern which resulted in impairment of steroidogenesis in granulosa cells.[18] Therefore, there are several studies showing the altered DNA methylation in promoters of specific genes involved in PCOS, however its direct relationship with obesity and epigenetics remains elusive.[19] In our previous study, we demonstrated that high-fat diet-induced obesity significantly increased expression of both Dnmt1 and Dnmt3a, and the level of global DNA methylation in mouse ovary. In this study, for the first time, alteration of Dnmt3b and Dnmt3l expression levels in HFD-induced obese mice ovary was demonstrated.

CONCLUSION

In conclusion, our results revealed that Dnmt3b and Dnmt3l protein expression were significantly altered in ovaries of HFD-induced obese mice. Our findings hold significant importance in illustrating the effects of obesity on ovarian follicle development. They offer a fresh perspective on potential therapies for obesity-induced female infertility. Moreover, our study underscores the need for further research focusing on the alterations of DNMTs preference for DNA methylation on the promoters of obesity-induced genes related to PCOS.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

***Idea/Concept:** Özgür Çınar, Fatma Uysal; **Design:** Özgür Çınar, Fatma Uysal, Nazlıcan Bozdemir, Tuba Kablan, Gözde Şükür, Meltem Sönmezer; **Control/Supervision:** Özgür Çınar, Fatma Uysal; **Data Collection and/or Processing:** Özgür Çınar, Fatma Uysal, Nazlıcan Bozdemir, Tuba Kablan, Gözde Şükür, Meltem Sönmezer; **Analysis and/or Interpretation:** Özgür Çınar, Fatma Uysal, Nazlıcan Bozdemir, Tuba Kablan, Gözde Şükür, Meltem Sönmezer; **Literature Review:** Özgür Çınar, Fatma Uysal, Nazlıcan Bozdemir, Tuba Kablan, Gözde Şükür; **Writing the Article:** Özgür Çınar, Fatma Uysal, Nazlıcan Bozdemir, Tuba Kablan, Gözde Şükür, Meltem Sönmezer; **Critical Review:** Özgür Çınar, Fatma Uysal; **References and Fundings:** Özgür Çınar, Fatma Uysal; **Materials:** Özgür Çınar, Fatma Uysal, Nazlıcan Bozdemir, Tuba Kablan, Gözde Şükür.*

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