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ORIGINAL PAPER

Altered DNA methylation and Dnmt expression in obese uterus may cause implantation failure

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Abstract

Obesity is defined by increased adipose tissue volume and has become a major risk factor for reproduction. Recent studies have revealed a substantial link between obesity and epigenetics. The epigenome is dynamically regulated mainly by DNA methylation. DNA methylation, which is controlled by DNA methyltransferases (Dnmts), has been widely studied because it is essential for imprinting and regulation of gene expression. In our previous study, we showed that the levels of Dnmt1, Dnmt3a and global DNA methylation was dramatically altered in the testis and ovary of high-fat diet (HFD)-induced obese mice. However, the effect of HFD on Dnmts and global DNA methylation in mouse uterus has not yet been demonstrated. Therefore, in the present study, we aimed to evaluate the effect of HFD on the level of Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and global DNA methylation in uterus. Our results showed that HFD significantly altered the levels of Dnmts and global DNA methylation in the uterus. The total expression of Dnmt1, Dnmt3a and Dnmt3b was significantly upregulated, while level of Dnmt3l and global DNA methylation were dramatically decreased ($p < 0.05$). Furthermore, we observed that the expression of Dnmt3b and Dnmt3l was significantly increased in endometrium including gland and epithelium ($p < 0.05$). Although Dnmt3b was the only protein whose expression significantly increased, the level of global DNA methylation and Dnmt3l significantly decreased in stroma and myometrium ($p < 0.05$). In conclusion, our results show for the first time that obesity dramatically alters global DNA methylation and expression of Dnmts, and decreased DNA methylation and Dnmt expression may cause abnormal gene expression, especially in the endometrium.

Keywords DNA methylation · Dnmt · High-fat diet · Obesity · Uterus

Introduction

Obesity, one of the most important health problems in developed and developing countries in recent years, is a metabolic syndrome that causes excessive fat accumulation in the body (Samblas et al. 2019; Wang et al. 2019), and it is known that about half of pregnant women are obese or overweight overweight (Kim et al. 2010; Yeh and Shelton 2005). Obesity can affect reproductive function by affecting both the ovaries and the endometrium. Some of these effects can be summarized as menstrual irregularity, infertility, and miscarriage (Broughton and Moley 2017). Evidence from experimental models suggests that maternal obesity, regardless of birth weight, may cause an increased predisposition to developmental abnormalities, insulin resistance, and metabolic disorders in the offspring (Shankar et al. 2011).

There is a strong association between a high prevalence of obesity and impaired reproductive function in women (Catalano and Shankar 2017). In addition, a few studies have examined that maternal obesity results in increased mitochondrial potential and oxidative stress in mouse zygotes and decreased blastocyst formation (Igosheva et al. 2010), smaller fetuses (Jungheim et al. 2010), altered gene

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expression in placenta (Mao et al. 2010), increased oxidative stress during fetal development (Sen and Simmons 2010).

Most importantly, there is almost no information on the effect of pre-pregnancy maternal obesity on the uterus of mice in estrus phase.

Embryo implantation is a critical step in embryo development and pregnancy outcome, and succesful implantation is highly depend on uterine receptivity. During implantation, the trophectoderm cells of the blastocyst interact with the luminal epithelium of the uterus in a series of defined events (Dey et al. 2004; Wang and Dey 2006). The purpose of implantation is to attach the developing embryo to the endometrial stroma, which is regulated by ovarian hormones and locally produced signaling molecules, including cytokines and growth factors (Dey et al. 2004). However, in the early stages, especially during implantation, the uterine environment is likely to have a major impact on the development of the offspring (Shankar et al. 2011). Recent studies have suggested that DNA methylation may be involved in endometrial changes during the preimplantation stages (Gao et al. 2012).

DNA methylation is one of the most well-studied epigenetic mechanisms leading for silencing gene expression in mammals and is required for normal development and for the cascade essential biological processes, including X-xhromosome inactivation, genomic imprinting, repetitive element siliencing, and carcinogenesis (Bird 2002; Li 2002). In mammals, DNA methylation mediates intergenerational gene silencing and is required for the generation and maintenance of cell type-specific expression during the progression of varios biological processes, not only during development but also in adult tissues (Futscher et al. 2002; Ko et al. 2010; Waterland et al. 2009). DNA methylation as a primary regulator of hereditary information is established by the addition of a methyl group to the fifth carbon atom of cytosine in CpG islands of DNA by DNA methyltransferases (Dnmts) (Law and Jacobsen 2010; Straussman et al. 2009). These CpG islands are typically located between the promoter regions of genes, and methylation of CpG islands results in transcriptional repression. This mechanism is known to be controlled in two ways: maintenance and *de novo* methylation. To date, six major Dnmtshave been characterized, including Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, Dnmt3c, and Dnmt3l. However, abnormal DNA methylation has been found to lead to abnormal embryonic development, birth malformations, and other diseases such as human carcinoma (Okano et al. 1999; You et al. 2017).

Dnmt1 is primarily involved in the methylation of the hemi-methylated DNA helix following its synthesis (Li et al. 1992). Dnmt3a and Dnmt3b are crucial for de novo methylation of unmethylated DNA strands (Chedin et al. 2002; Hata et al. 2002). Expression of Dnmt3a is observed in adult tissues, whereas expression of Dnmt3b is measured at low levels in most tissues (Okano et al. 1999). Dnmt3l lacks Dnmt activity, so it cooperates with Dnmt3a or Dnmt3b for de novo functions. Dysfunction of any of these Dnmts can have serious consequences for embryonic development and subsequent pregnancy, including embryonic lethality. The epigenetic status of genes or their repeat sequences has a profound effect on cell physiology and can markedly alter embryonic and fetal development (Dion et al. 2008; Li et al. 1992; Okano et al. 1999). For instance, Dnmt1 knockout (Dnmt1$^{-/-}$) mouse embryos experience genome-wide demethylation, loss of imprinting, and embryonic lethality in the early stages of pregnancy (Hirasawa et al. 2008). Similarly, Dnmt3a$^{-/-}$ and Dnmt3b$^{-/-}$ mice fail to initiate de novo methylation after implantation and also exhibit embryonic developmental arrest (Li 2002; Li et al. 1992). However, it is unclear whether dysfunction of Dnmts causes early pregnancy loss in humans (Deplus et al. 2002; Margot et al. 2003).

Research has demonstrated that Dnmts are regulated during the endometrial cycle in mammals(Ghabreau et al. 2004; Vincent et al. 2011a), and this regulation is associated with the expression of ovarian steroid receptors (Ghabreau et al. 2004). Dnmt1, Dnmt3a, and Dnmt3b are expressed at higher levels during the proliferative phase of the human endometrium, but at lower levels during the second secretory phase. Throughout the reproductive cycle, human endometrial changes rely on dynamic processes (Vincent et al. 2011b; Yamagata et al. 2009). Dnmt1, Dnmt3a, and Dnmt3b mRNA levels vary according to menstrual cycle phases(Vincent et al. 2011b; Yamagata et al. 2009). A previous study demonstrated the localization of Dnmt1, Dnmt3a, and Dnmt3b in the uterus of pregnant mice (Ding et al. 2012).

In our previous study, we showed that a high-fat diet has differential effects on Dnmt1 and Dnmt3a expression and global DNA methylation levels in the ovaries and testes (Sukur et al. 2023). In this study, we aimed to evaluate the effects of high-fat diet-induced obesity on Dnmt1, Dnmt3a, Dnmt3b, and Dnmt3l expression, as well as global DNA methylation, in the uterus of mice in the estrus phase. Briefly, the results showed that the high-fat diet-induced obesity has a differential effect on Dnmts and global DNA methylation levels in the uterus.

Materials and methods

Animals

The experimental protocol was approved by the Animal Care and Usage Committee of Ankara University (protocol

no. 2020-17-145). 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. After the female ($n=8$) and male ($n=8$) mice 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 uterus, they were transferred to buffered solutions. Establishment of a HFD-induced obese mouse model was revealed in our previous article (Sukur et al. 2023).

Immunohistochemistry

The level of the Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and global DNA methylation in the uterus was 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, sections were incubated with the following primary antibodies specific for 5-methylcytosine (5mC) (1:100; catalog no. ab214727, Abcam), Dnmt1 (1:400; catalog no. ab87654, Abcam), Dnmt3a (1:100; catalog no. ab23565, Abcam), Dnmt3b (1:400; catalog no. 48,488 S, Cell Signalling), 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 and global DNA methylation level. H-score analysis were performed by two independent observers. Uterine glands, epithelium, stroma, and myometrium 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 = ∑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 Dnmts and level of global DNA methylation in the uterus induced by the high-fat diet (HFD)

Expressions of Dnmts and global DNA methylation were evaluated in uterus sections from both the HFD and control groups (Fig. 1, $p<0.05$). The expression of Dnmt1, Dnmt3a, and Dnmt3b significantly increased in the HFD group compared to the control group (Fig. 1b, $p<0.05$). Conversely, the expression of Dnmt3l and the level of global DNA methylation dramatically decreased in the HFD group compared to the control group (Fig. 1b, $p<0.05$).

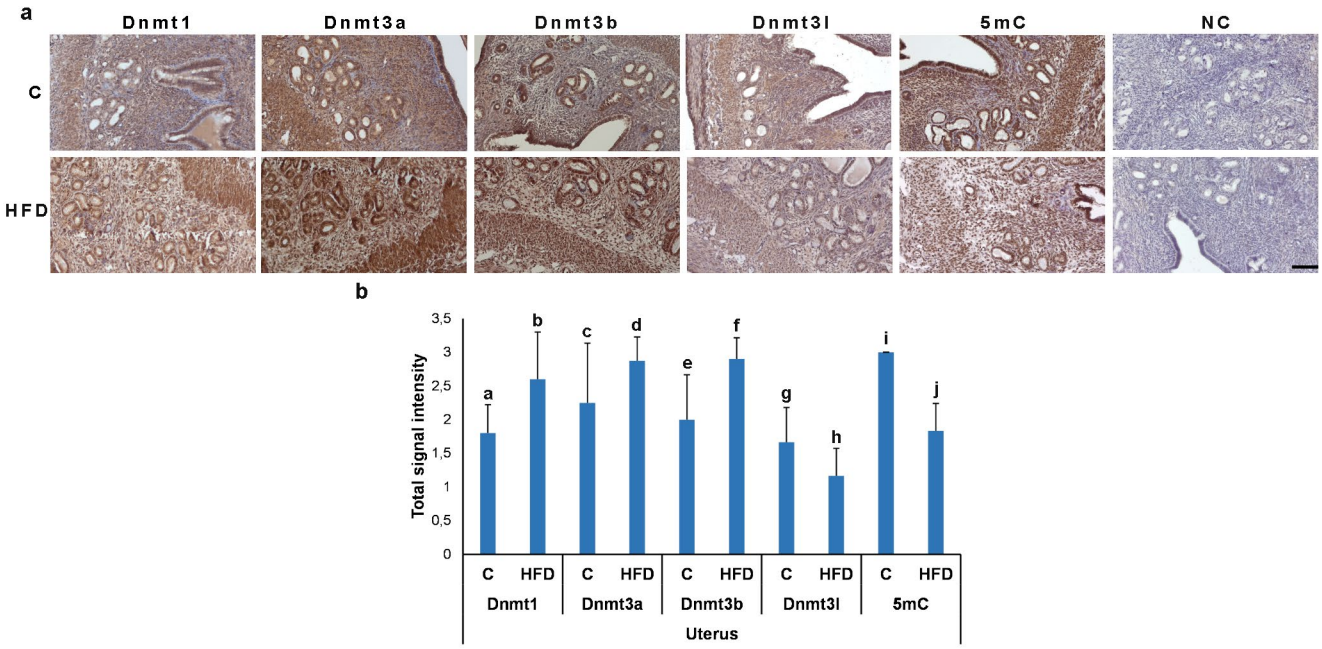

Fig. 1 Changes in the overall expression of Dnmts and level of global DNA methylation in the uterus induced by the high-fat diet (HFD). **(a)** Demonstrative micrographs obtained from Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and 5mC stained sections **(b)** Total Dnmts and 5mC levels in immunohistochemistry (IHC) 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: 40 µm

Relative level of Dnmts and global DNA methylation in uterine glands

The study evaluated the relative expressions of Dnmts and global DNA methylation in uterine glands of both the HFD and control groups (Fig. 2, $p < 0.05$). The expression of Dnmt3b and Dnmt3l was remarkably induced in the HFD group compared to the control group(Fig. 2b, $p < 0.05$). On the other hand, the expression of Dnmt1 and Dnmt3a, as well as the level of global DNA methylation, exhibited similar levels in both groups (Fig. 2b, $p < 0.05$). When considering the subcellular localizations of Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l, and 5mC in glandular cells, it was found that Dnmts were present in both the nucleus and cytoplasm in both the control and HFD groups. However, in the control group, 5mC staining was only observed in the nucleus, whereas in the HFD group, it was observed in both the nucleus and cytoplasm, but mainly in the nucleus.

Relative level of Dnmts and global DNA methylation in uterine stroma

The study evaluated the relative expressions of Dnmts and global DNA methylation in uterine glands from both the HFD and control groups (Fig. 3, $p < 0.05$). The expression of Dnmt1 and Dnmt3a remained comparable in the HFD group compared to the control group (Fig. 3b, $p < 0.05$). However, the expression of Dnmt3b significantly increased in the HFD group compared to the control group, while the level of Dnmt3l and global DNA methylation remarkably decreased in the HFD group compared to the control group (Fig. 3b, $p < 0.05$). Additionaly, the subcellular localizations of Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l, and 5mC were examined in stromal cells. Dnmts were present in both the nucleus and cytoplasm in both the control and HFD groups. On the other hand, 5mC staining was revealed in the nucleus of the control group, while it was observed in both the nucleus and cytoplasm, but predominantly in the nucleus, the in HFD group.

Relative level of Dnmts and global DNA methylation in uterine myometrium

The study evaluated the relative expressions of Dnmts and global DNA methylation in uterine glands of both the HFD and control groups (Fig. 4, $p < 0.05$). The expression of Dnmt3b significantly increased in the HFD group compared to the control group (Fig. 4b, $p < 0.05$). However, the levels of Dnmt1, Dnmt3a, Dnmt3l, and global DNA methylation dramatically decreased in the HFD group compared to the control group (Fig. 4b, $p < 0.05$). The subcellular localizations of Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l, and 5mC were investigated in myometrial cells. Dnmts were located in

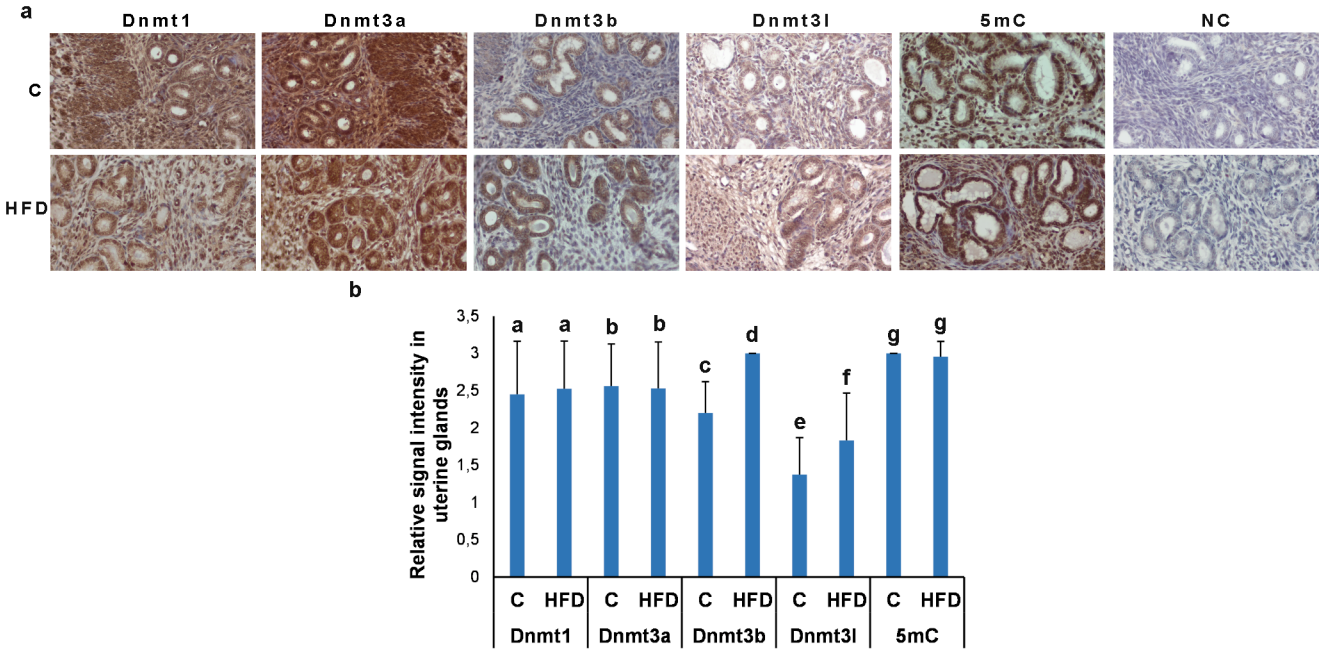

Fig. 2 Relative level of Dnmts and global DNA methylation in uterine glands. **(a)** Demonstrative micrographs obtained from Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and 5mC stained sections. **(b)** Dnmts and 5mC levels in immunohistochemistry (IHC) analysis. The statistical significance was determined by using 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

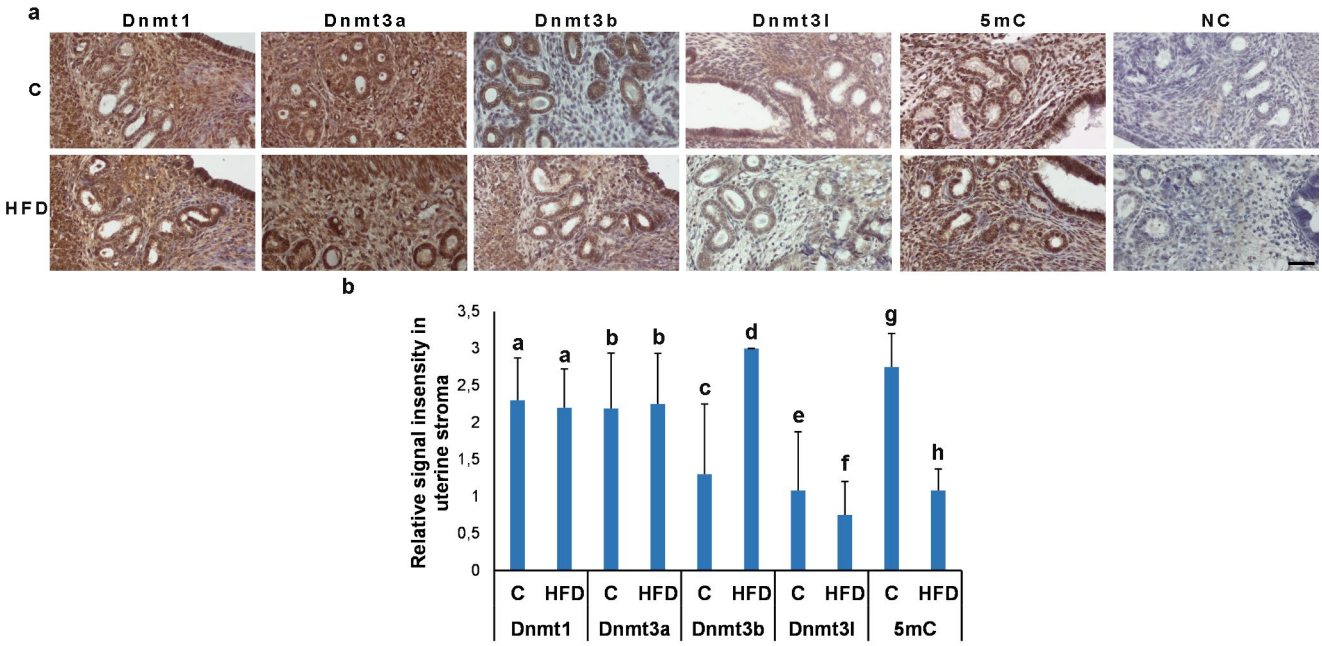

Fig. 3 Relative level of Dnmts and global DNA methylation in uterine stroma. **(a)** Demonstrative micrographs obtained from Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and 5mC stained sections. **(b)** Dnmts and 5mC levels in immunohistochemistry (IHC) analysis. The statistical significance was determined by using 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

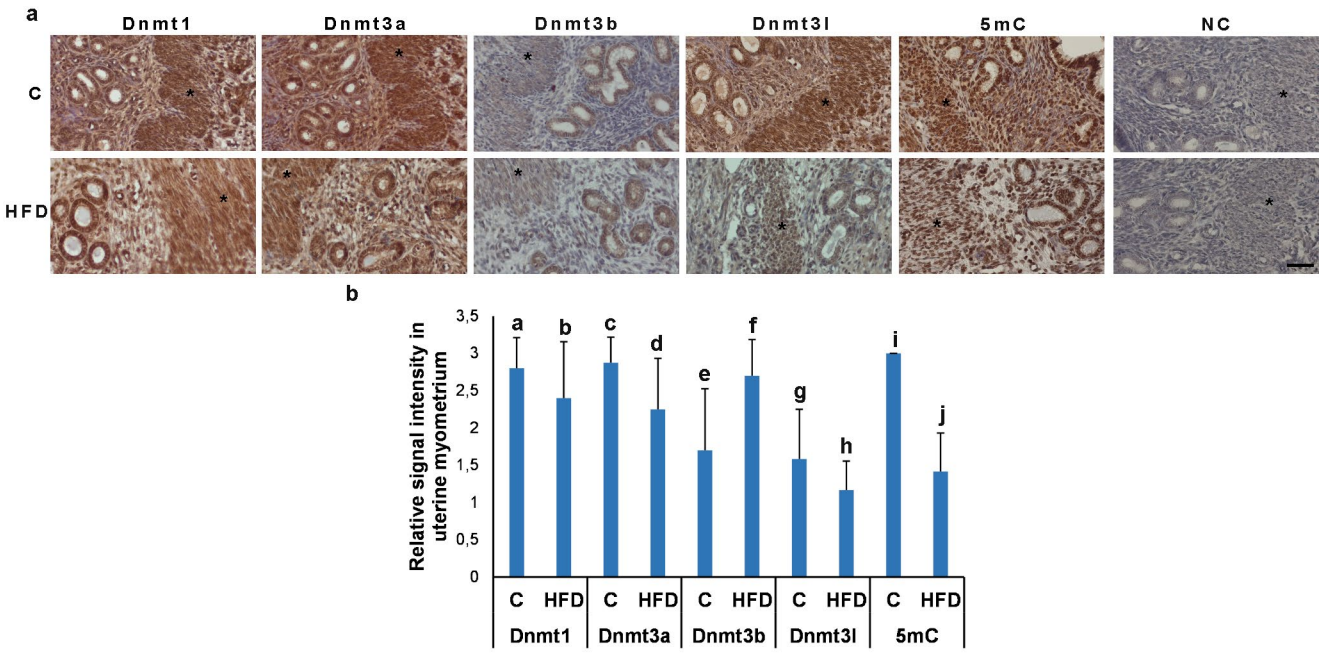

Fig. 4 Relative levels of Dnmts and global DNA methylation in uterine myometrium. **(a)** Demonstrative micrographs obtained from Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and 5mC stained sections. **(b)** Dnmts and 5mC levels in immunohistochemistry (IHC) analysis. The statistical significance was determined by 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

both the nucleus and cytoplasm in both the control and HFD groups. Notably, 5mC staining was observed in the nucleus of the control group, while it was observed in both the nucleus and cytoplasm, but mostly in in the nucleus, of the HFD group.

Relative level of Dnmts and global DNA methylation in uterine epithelium

The study evaluated the relative expressions of Dnmts and global DNA methylation in uterine glands of both the HFD and control groups (Fig. 5, $p < 0.05$). the expression of Dnmt3b and Dnmt3l significantly increased, while the expression of Dnmt3a dramatically decreased in the HFD group compared to the control group (Fig. 5b, $p < 0.05$). However, the expression of Dnmt1 and the level of global DNA methylation remained comparable in the HFD and control groups (Fig. 5b, $p < 0.05$). Subcellular localizations of Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l, and 5mC were evaluated in epithelial cells. Dnmts were observed in both the nucleus and cytoplasm in both the control and HFD groups. However, in the control group, 5mC staining was only observed in the nucleus, whereas in the HFD group, it was observed in both the nucleus and cytoplasm, with notable concentration in the nucleus.

Discussion

Obesity is a metabolic disease resulting from an increased body mass index. It can cause alterations in the uterus, resulting in impaired endometrial receptivity (Bellver et al. 2021). Recent studies suggest that obesity causes change in DNA methylation and histone modifications This is consistent with our previous study, which found significant changes in global DNA methylation and in the expressions of Dnmt1 and Dnmt3a in the ovaries and testes of mice with high-fat diet-induced obesity (Sukur et al. 2023).

Furthermore there is strong relationship between obesity and endometrial cancer (McDonald and Bender 2019). Moreover, a recent study has revealed that Hand2, a transcription factor expressed in the endometrial stroma, is strongly associated with endometrial cancer. The study demonstrated that *Hand2* was hypermethylated in the uterus of patients with endometrial cancer. Additionally, *Hand2* conditional knockout mice exhibited precancerous lesion development in the uterus and loss of Pten expression in the endometrial epithelium (Jones et al. 2013). However, the impact of obesity on Dnmts and global DNA methylation in the uterus has remained unclear. In this study, we revealed, for the firdt time, the alteration of Dnmts and global DNA methylation levels in the uterus of HFD-induced obese mice (Fig. 6).

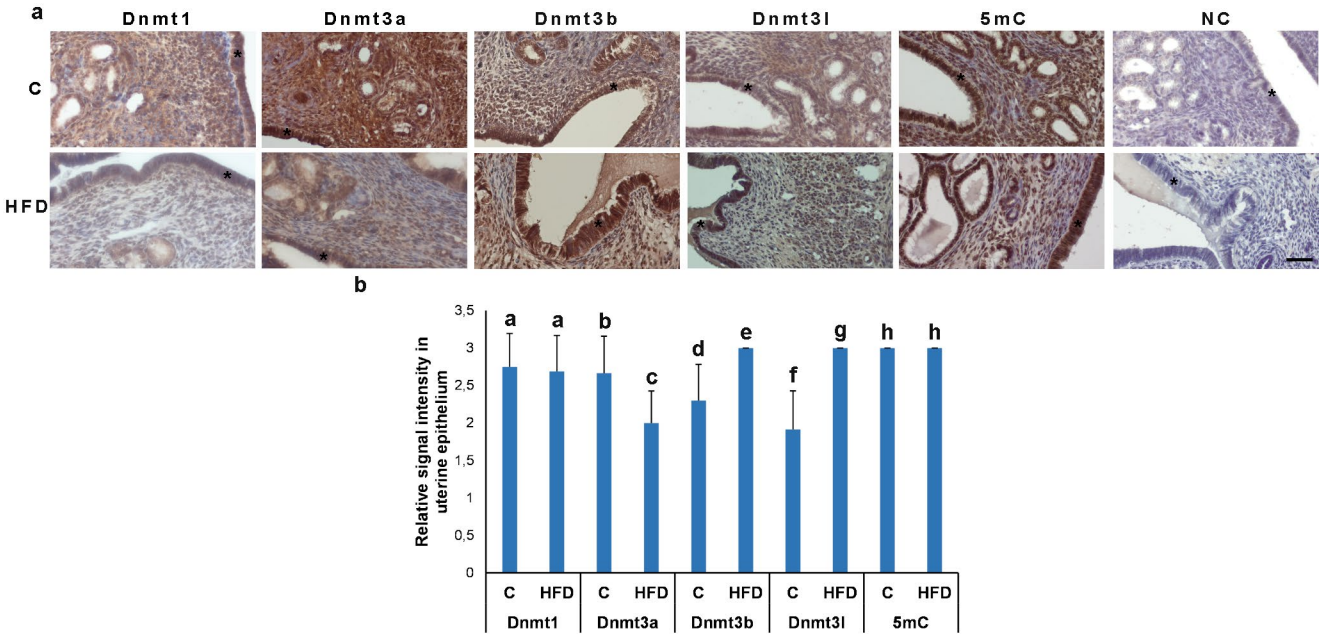

Fig. 5 Relative levels of Dnmts and global DNA methylation in uterine epithelium. **(a)** Demonstrative micrographs obtained from Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and 5mC stained sections. **(b)** Dnmts and 5mC levels in immunohistochemistry (IHC) analysis. The statistical significance was determined by 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

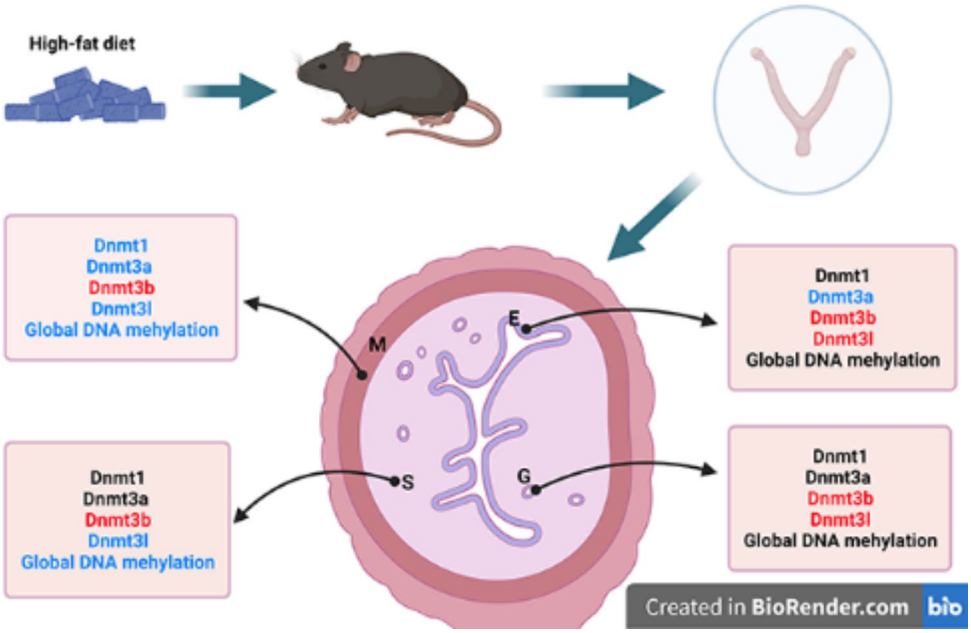

Fig. 6 Representation of HFD-induced alterations in levels of Dnmts and global DNA methylation in uterus. Demonstrative figure of HFD-induced Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l and global DNA methylation levels in uterine glands (G), epithelium (E), stroma (S) and myometrium (M). Red color indicates for significantly increased level, blue for significantly decreased level in HFD group compared to control and black for no significant difference between HFD and control group. Created with BioRender.com

The study found that in the uterus of the HFD-induced obese group, there was a significant increase in the expression of Dnmt1, Dnmt3a, and Dnmt3b, while global DNA methylation and Dnmt3l levels dramatically decreased. Additionally, although the global DNA methylation level was not altered, Dnmt3b expression was significantly induced in the uterine stroma of the HFD group. These findings suggest a potential effect of the increased level of Dnmt3b on the hypermethylation of *Hand2*. Furthermore, we observed a significant increase in Dnmt3b and Dnmt3l expression in the uterine epithelium of the HFD group. Reavey et al. (2021)demonstratedthat proper repairment of uterine epithelium after menstruation is crucial for preventing excessive bleeding. The HFD group exhibited impaired proliferation of uterine epithelium and subsequent repairment. Also in another study, overexpression of miR-124-3p

resulted in aberrant epithelial profile and reduced uterine receptivity by negatively affecting the proliferation and migration of endometrial cells through altering LIF, MUC1 and BCL2 expression in the mouse uterus and it has been shown that overexpression of miR-124-3p also caused significantly decreased global DNA methylation in blastocyst. Therefore, it is hypothesized that increased expression of Dnmt3b and Dnmt3l in epithelium may lead to repression of gene expression related to uterine receptivity. Furthermore, we found that the expression of Dnmt3b significantly increased, while the expression of the other enzymes and the global DNA methylation level decreased in the myometrium of the HFD group. Obese women are known to have dysfunctional myometrium, which can result in an increased rate of caesarean section. It has beensuggested that this dysfunctionality is caused by decreased in the expression of connexin 43 in the myometrium of obese women (Carvajal and Oporto 2021). The regulation of connexin 43 expression by nitric oxide is well-established (Roh et al. 2002). Therefore, further investigation is needed to determine the methylation status of *GJA1* and *NOS1*, which encode connexin 43 and nitric oxide synthase, respectively, in cases of obesity. Additionally, uterine glands secrete several components that are necessary for uterine receptivity, blastocyst implantation, and stromal cell decidualization (Kelleher et al. 2019). It was demonstrated that the expressions of Dnmt3b and Dnmt3l were significantly induced in the uterine glands of the HFD group.

In conclusion, this work demonstrates for the first time a significant alteration in the global DNA methylation and Dnmts in the uterus of high-fat diet-induced obese mice. Additionally, findings thoroughly describe the impact of obesity on the uterus through global DNA methylation and Dnmts. Further investigation is required to reveal differences in DNA methylation patterns and differentially expressed genes in the uterus of obese mice. Although this limitation may affect the study, our results are crucial for developing a potential therapeutic agent for infertile obese women and will pave the way future research.

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Author contributions All authors qualify for authorship by contributing substantially to this article. F.U. and N.B. developed the original concept of this study and wrote the manuscript. N.B., T.K., M.O.A. and G.S. collected data and F.U. and O.C. performed statistical analysis. F.U. and O.C. have contributed to critical discussion. All authors reviewed the final version of the article, and approved it for publication.

Declarations

Ethical approval The experimental protocol was approved by the Animal Care and Usage Committee of Ankara University (Protocol no: 2020-17-145).

Competing interests The authors declare no competing interests.

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