Superovulation alters DNA methyltransferase protein expression in mouse oocytes and early embryos

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Abstract

Purpose DNA methylation is an epigenetic mechanism that plays critical roles during mammalian oocyte and preimplantation embryo development. It is achieved by adding a methyl group to the fifth carbon atom of cytosine residues within *cytosine-phosphate-guanine* (CpG) and non-CpG dinucleotide sites using DNA methyltransferase (DNMT) enzymes for de novo and maintenance methylation processes. DNMT1, DNMT3A, and DNMT3B play important roles in establishing methylation of developmentally related genes in oocytes and early embryos. The purpose of this study is to identify the effect of superovulation on the expression and subcellular localizations of these three DNMT enzymes in the mouse oocytes and early embryos.

Methods Three groups composed of control, normal dose [5 IU pregnant mare serum gonadotropin (PMSG) and 5 IU human chorionic gonadotropin (hCG)], and high dose [7.5 IU PMSG and 7.5 IU hCG] were created from 4–5-week-old female BALB/c mice. The relative expression and subcellular localizations of the DNMT proteins in the control and experiment groups have been characterized by using immunofluorescence staining subsequently analyzed in detailed.

Results DNMT1, DNMT3A, and DNMT3B protein expression in the germinal vesicle and metaphase II oocytes and in one-cell and two-cell embryos differed significantly when some of the normal- and high-dose groups were compared with the control counterparts.

Conclusion This study has demonstrated for the first time that superovulation alters expression levels of the DNMT proteins, a finding that indicates that certain developmental defects in superovulated oocytes and early embryos may result from impaired DNA methylation processes.

Keywords DNMT · DNA methylation · Early embryo · Oocyte · Superovulation

Introduction

Assisted reproductive technology (ART) procedures are used to treat infertility, and 0.1–5% of newborn children are born this way in Europe and the USA [1, 2]. The ART procedure includes certain steps: superovulation/ovarian hyperstimulation, in vitro fertilization, in vitro culture, intracytoplasmic sperm injection, embryo transfer, and vitrification [3]. Recent studies showed that children born following ART application have increased epigenetic anomalies compared with those conceived spontaneously [3–7]. Children who were born by using ART techniques seem to have more possibility of birth defects [8], congenital or chromosomal anomalies [9], and imprinting disorders such as Beckwith-Wiedemann, Silver Russell, Angelman, and Prader-Willi syndromes compared with spontaneously conceived babies [6, 10].

DNA methylation is an epigenetic mechanism that plays critical roles in transcriptional repression/activation, X-chromosome inactivation, cell differentiation, and tumorigenesis [11]. DNA methylation is specifically carried out by certain enzymes, which are called DNA methyltransferases (DNMTs), with the capacity to accomplish maintenance and de novo methylation processes [12]. They add a methyl group to the fifth carbon atom of cytosine residues in *cytosine-phosphate-guanine* (CpG) and non-CpG

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dinucleotide sites by using s-adenosyl methionine (AdoMet) as a methyl donor [12].

To date, structurally and functionally, six different DNMTs have been identified in mammals: DNMT1, DNMT3A, DNMT3B, DNMT3C, DNMT3L, and DNMT2. DNMT1 fundamentally functions in maintenance methylation by adding methyl groups to the hemi-methylated DNA strands during DNA replication [13, 14]. In addition to being responsible for maintenance methylation, DNMT1 also contributes to de novo methylation events [15]. On the other hand, both DNMT3A and DNMT3B have a crucial role in the de novo methylation process, which occurs at CpG islands of unmethylated DNA strands [12]. DNMT3L does not have any catalytic domain; however, since it is capable of inducing DNMT3A and DNMT3B activity, DNMT3L participates in de novo methylation indirectly [16–18]. DNMT3C is recently identified de novo methyltransferase which protects male germ cells from retrotransposon activity and is essential for mouse fertility [14]. The DNMT2 protein methylates *cytosine 38* in the anticodon loop of *aspartic acid transfer RNA instead of methylating nuclear DNA* [19].

Superovulation is a protocol commonly used to obtain high numbers of oocytes and preimplantation embryos using pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), respectively, as substitutes for follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Recent research has shown that superovulation could negatively affect epigenetic mechanisms, oocyte quality, oocyte maturation, and expression levels of certain imprinted genes, and subsequently lead to abnormal blastocyst count, imprinting disorders, decreased implantation rate, fetal growth retardation, and delayed embryo development [20–24]. In accordance with those studies, mouse models also revealed that superovulation causes a decrease in global methylation level in zygotes when compared with control groups [3] and affects the imprinting of such genes as *H19* in blastocysts [25].

These findings revealed that superovulation may disturb oocyte maturation and early embryo development possibly due to impaired expression profiles of development-related genes. It is not fully understood whether the superovulation protocols used commonly in ART and experimental studies affects the spatial and temporal expression and subcellular localization of DNMT1, DNMT3A, and DNMT3B proteins. Recombinant FSH and hCG hormones are used in human superovulation protocols. Although the applied doses of these hormones differ between human and mouse, the underlying molecular mechanisms are similar. Determining the possible effects of PMSG and hCG hormones on the DNMT proteins in mouse oocytes and embryos may shed light on the potential effect of superovulation protocols on DNMT proteins in human oocytes and embryos, and this finding may be useful for clinical applications. The aim of the present study was to investigate the effect of superovulation with normal (5 IU) and

high (7.5 IU) doses of PMSG and hCG on the expression levels and subcellular localization of DNMT1, DNMT3A, and DNMT3B proteins in mouse germinal vesicle (GV), metaphase II (MII) oocytes, and one-cell and two-cell embryos.

Material and methods

Animals

The experimental protocol was approved by the Animal Care and Usage Committee of Akdeniz University (Protocol no: B.30.2.AKD.0.05.07.00/30) on 20th March, 2014. The BALB/C female mice at 4–6 weeks and male mice at 8–10 weeks of age were purchased from Research Animal Laboratory Unit of Akdeniz University Medical Faculty. All mice were hosted with free access to food and water and were kept in a 12-h light/dark cycle.

Collection of oocytes and early embryos

The GV and MII oocytes and one-cell and two-cell embryos were obtained from 4–6-week-old female BALB/C mice superovulated at normal dose (5 IU PMSG and 5 IU hCG) and high dose (7.5 IU PMSG and 7.5 IU hCG). Control GV oocytes and early embryos were collected from naturally ovulating female mice at the same ages. Importantly, control MII oocytes were obtained from 4–6-week-old female mice at the same time point as the superovulated groups, after mating with vasectomized male mice.

To obtain GV oocytes, 4–6-week-old female mice were intraperitoneally (i.p.) injected with 0.1 mL 5 IU or 7.5 IU PMSG (Intervet, Milton Keynes, UK). After 20 h, superovulated mice were killed and superovulated ovaries were punctured with a 23-gauge needle in human tubal fluid (HTF) medium (Vitrolife, Gotheburg, Sweden), and then we collected GV oocytes having a visible germinal vesicle by using a mouth-controlled pipette under a dissecting microscope (Zeiss, Oberkochen, Germany) as described previously [26]. In the control group, GV oocytes were recovered from normally cycling female mouse ovaries.

For collection of MII oocytes, mice were injected i.p. with PMSG (5 or 7.5 IU), and 48 h after the PMSG injection, hCG (Sigma-Aldrich) (5 or 7.5 IU) injection was given. Fourteen hours after hCG injection, cumulus-oocyte complexes (COCs) were obtained from the oviducts of the female mice. For the control group, female mice were mated with 8-week-old vasectomized male mice. Vaginal plug-positive female mice were sacrificed 14 h after their mating. The COCs obtained from either superovulated or control group were treated with HTF medium including 1 mg/mL hyaluronidase (Sigma-Aldrich) to remove cumulus cells from MII oocytes.

To obtain one-cell embryos from the normal- and high-dose superovulated groups, female mice were injected i.p. with PMSG (5 or 7.5 IU), and after 48 h, they were injected with hCG (5 or 7.5 IU). Then, each female mouse was mated overnight with one mature male mouse. The next morning, if the vaginal plug existed, the female mice were killed 20 h later and we collected one-cell embryos having two pronuclei from the oviducts of the female mice. The cumulus cells surrounding one-cell embryos were removed as described above. In the control group, vaginal plug-positive female mice were sacrificed 20 h after mating with fertile male to obtain one-cell embryos. Two-cell embryos were collected from the oviducts of superovulated female mice (see above) 42 h after hCG. In the control group, vaginal plug-positive female mice were sacrificed 42 h after mating to collect two-cell embryos.

Immunofluorescence staining of oocytes and early embryos

Immunofluorescence staining was performed to determine the subcellular localization and relative expression profiles of the DNMT1, DNMT3A, and DNMT3B proteins in the GV and MII oocytes and one-cell and two-cell embryos obtained from either superovulated or control groups ($n = 10$ per group). All oocytes and early embryos were fixed in 3% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and then permeabilized with 1% Tween-20 (Sigma-Aldrich) prepared in 1XPBS at room temperature. The fixed oocytes and early embryos were blocked with blocking solution including 20% normal goat serum (Vector Laboratory). Then, the oocytes and embryos were incubated overnight at 4 °C with primary antibodies specific for DNMT1 (Abcam, ab87654, reactive with the oocyte [DNMT1o] and somatic [DNMT1s] isoforms), DNMT3A (Abcam, ab23565), or DNMT3B (Abcam, ab2851). After washing three times for 10 min with 1X PBS including 2% bovine serum albumin (BSA) (Sigma-Aldrich), the oocytes and early embryos were incubated with anti-rabbit Alexa 488 secondary antibody (Invitrogen) for 1 h at room temperature in the dark. After incubating with secondary antibody, the oocytes and early embryos were washed three times for 10 min with PBS-BSA. After being counterstained with DAPI (Sigma-Aldrich, D8417) for 2 min at room temperature in dark, the oocytes and early embryos were washed with PBS-BSA. All staining steps were performed using mini well trays (VWR-Thermo Scientific) in a humidified chamber. The oocytes and early embryos were mounted onto slides using mounting solution (Vector Laboratory). All immunostaining experiments for oocytes and early embryos were performed under the same conditions. Staining of each material from the experimental or control group was performed at the same time. Fluorescence signals were detected with a motorized fluorescence microscope at $\times 400$ magnification (Olympus BX61, Tokyo, Japan). Then, the images were

evaluated with Image J software (National Institutes of Health, Bethesda, Maryland, USA), and the relative expression levels of DNMT proteins were identified. Negative controls were also analyzed, but no signal was detected. We carried out all experiments at least three times.

In addition to examining the total expression patterns of DNMT1, DNMT3A, and DNMT3B proteins in the control and superovulated oocytes and embryos, the relative expression levels of DNMT proteins in the nuclear region of each sample were analyzed. Fluorescence images were converted into gray value images and analyzed by ImageJ software for determining the line-scans of DNMT1, DNMT3A, and DNMT3B proteins at the central plain of each nucleus. The mean intensity level of each group was obtained by calculating the average of the line-scans which were produced for each oocyte and early embryo.

Statistical analysis

All results were analyzed with one-way analysis of variance (one-way ANOVA) test followed by Fisher's LSD and Dunn's post hoc tests. The statistical calculations were performed by SigmaStat for Windows, version 3.5 (Jandel Scientific Corp.). For all the tests, $P < 0.05$ was considered to be statistically significant.

Results

DNMT1 expression in control and superovulated oocytes and early embryos

The relative expression level and subcellular localization of DNMT1 protein in superovulated GV oocytes, MII oocytes, one-cell embryos, and two-cell embryos were analyzed and compared with their control counterparts.

In GV oocytes, DNMT1 protein expression was significantly decreased in the normal-dose group when compared with the control group ($P < 0.01$) (Fig. 1a, e). Although there was no significant difference between the control and high-dose groups, the high-dose group had remarkably higher DNMT1 protein expression than the normal-dose group had ($P < 0.01$). In MII oocytes, although there were expressional fluctuations for DNMT1 protein among the superovulated and control groups, it was not statistically significant (Fig. 1b, f). In the one-cell embryos, DNMT1 protein expression was found to be significantly decreased in the normal- and high-dose groups when compared with control counterparts ($P < 0.05$) (Fig. 1c, g). However, there was no significant difference between the normal- and high-dose groups. DNMT1 expression in two-cell embryos was higher in the normal- and high-dose groups than in the control group ($P < 0.01$), but no

Fig. 1 Expression of DNMT1 protein in mouse oocytes and early embryos obtained from control mice (C) and mice superovulated with normal (5 IU; ND) or high (7.5 IU; HD) doses of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The immunostaining of DNMT1 protein in germinal vesicle (GV) oocytes (a), MII oocytes (b), one-cell embryos (c), and two-cell embryos (d) is presented for the control and superovulated groups. A negative control (NC) including no primary antibody was used to identify whether there is any non-specific staining. In the micrographs, green, FITC staining for DNMT1; blue, DAPI (4',6-diamidino-2-phenylindole) staining for the nuclei; merge, DAPI-stained nuclei combined with green fluorescence signal thresholds. To determine relative expression levels of the DNMT1 protein in the three groups, we used ImageJ software. The relative expression levels (mean $\pm$ standard deviation) of DNMT1 protein in GV oocytes (e), MII oocytes (f), one-cell embryos (g), and two-cell embryos (h) are given. The statistical significance between the groups was analyzed by using one-way ANOVA test. $P < 0.05$ is considered significant. $P < 0.01$ for C versus ND and $P < 0.01$ for ND versus HD (e). $P < 0.05$ for C versus ND and $P < 0.05$ for C versus HD (g). $P < 0.01$ for C versus ND and $P < 0.01$ for C versus HD (h). The different letters in the columns depict statistical significance (for a and b, $P < 0.05$). ($n = 10$ per group)

difference was determined between the normal- and high-dose groups, as in the one-cell embryos (Fig. 1d, h).

In addition to analyzing the relative expression levels of DNMT1 protein, we further examined its subcellular localization and relative intensity in superovulated oocytes and early embryos. In all the groups, DNMT1 protein was strongly localized in the cytoplasm, but there was weak expression in the nuclear region of GV and MII oocytes and one-cell and two-cell embryos (Fig. 1a–d). It is important to note that DNMT1 was more intense in the cortical regions of all analyzed oocytes and early embryos, as was found in the previous studies (Hirasawa et al. 2008; Mertineit et al. 1998).

DNMT3A expression in control and superovulated oocytes and early embryos

The relative expression profile and subcellular localization of DNMT3A protein in the superovulated oocytes and early embryos was analyzed. DNMT3A protein expression in GV oocytes was not statistically significantly different between the groups (Fig. 2a, e).

In MII oocytes, DNMT3A expression was observed at a significantly lower level in the high-dose group in comparison to the control group ($P < 0.01$) (Fig. 2b, f). The low-dose group DNMT3A expression did not differ significantly from the control and the high-dose groups.

The expression level of the DNMT3A protein was significantly increased in one-cell embryos with a normal dose when compared with controls ($P < 0.001$), as was the high-dose group ($P < 0.05$) (Fig. 2c, g). Additionally, the high-dose group had significantly lower DNMT3A expression than the normal-dose group ($P < 0.05$). In two-cell embryos, DNMT3A expression was found to significantly decrease in the high-dose group compared with the normal-dose group

($P < 0.01$; Fig. 2d, h). However, the normal-dose and high-dose groups did not differ significantly when compared with the control group.

In all the groups, DNMT3A strongly localized at the nuclear regions of oocytes and early embryos. Additionally, there was weak DNMT3A expression in cytoplasm (Fig. 2a–d), but we detected no difference in the intracellular intensity of DNMT3A protein between superovulated and control oocytes and early embryos.

DNMT3B expression in control and superovulated oocytes and early embryos

The expression level and subcellular localization of DNMT3B protein in superovulated and control oocytes and early embryos was analyzed. In GV oocytes, there was no statistically significant change observed for DNMT3B protein expression in the normal- and high-dose groups when compared with controls (Fig. 3a, e).

In MII oocytes, the high-dose group had significantly lower DNMT3B expression than the control group ($P < 0.05$) (Fig. 3b, f). Although it was not statistically significant, the control group possessed higher DNMT3B expression than the normal- and high-dose groups.

Although there were differences between the superovulated and control groups, none were statistically significant in one-cell embryos (Fig. 3c, g). In two-cell embryos, both the normal- and high-dose groups had significantly higher DNMT3B expression in comparison to the control group ($P < 0.01$) (Fig. 3d, h). However, there was no difference between the normal- and high-dose groups.

In the detailed analysis of subcellular localization of DNMT3B protein, we observed no remarkable changes in the intracellular location or intensity of DNMT3B protein among the three groups (Fig. 3a–d). In all the groups, DNMT3B was intensively localized to the nuclear region of oocytes and early embryos, and there was weak expression in the cytoplasm.

Nuclear region quantification of the DNMT1, DNMT3A, and DNMT3B in the control and superovulated oocytes and early embryos

Nuclear DNMT1 protein expression was significantly decreased in GV oocytes and one-cell embryos in the ND (normal-dose) group compared with the control, but two-cell embryos from the ND group showed increased expression in comparison to those from the control group ($P < 0.05$) (Fig. 4a). In the HD (high-dose) group, we observed a decreased DNMT1 nuclear expression in the one-cell embryos; however, an increased expression in the two-cell embryo when compared with the control group ($P < 0.05$) (Fig. 4a).

Fig. 2 Expression of DNMT3A protein in mouse oocytes and early embryos obtained from control mice (C) and mice superovulated with normal (5 IU; ND) or high (7.5 IU; HD) doses of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The immunostaining of DNMT3A in germinal vesicle (GV) oocytes (a), MII oocytes (b), one-cell embryos (c), and two-cell embryos (d) is presented for the control and superovulated groups. A negative control (NC) including no primary antibody was used to identify whether there is any non-specific staining. In the micrographs, green, FITC staining for DNMT3A; blue, DAPI (4',6-diamidino-2-phenylindole) staining for the nuclei; merge, DAPI-stained nuclei combined with green fluorescence signal thresholds. To characterize relative expression levels (mean $\pm$ standard deviation) of the DNMT3A protein in the three groups, we used ImageJ software. The relative DNMT3A protein levels in these groups in GV oocytes (e), MII oocytes (f), one-cell embryos (g), and two-cell embryos (h) are presented. The statistical significance between the groups was analyzed by using one-way ANOVA. $P < 0.05$ is considered significant. $P < 0.01$ for C versus HD (f). $P < 0.001$ for C versus ND, $P < 0.05$ for C versus HD, and $P < 0.05$ for ND versus HD (g). $P < 0.01$ for ND versus HD (h). The different letters in the columns depict statistical significance (for a, b, and c, $P < 0.05$). ($n = 10$ per group)

In the nuclear region, DNMT1 protein expression in MII oocytes was similar in the three groups.

For the DNMT3A analysis, one-cell embryos obtained from the ND group possessed significantly higher nuclear expression than those from the control group ($P < 0.05$) (Fig. 4b). In the HD group, we found that MII oocytes had significantly decreased expression in comparison to those in the control group. On the other hand, one-cell embryos from the HD group exhibited increased expression when compared with control counterpart ($P < 0.05$) (Fig. 4b). In one-cell and two-cell embryos, DNMT3A expression was significantly lower in the HD group compared with the ND group ($P < 0.05$).

Finally, we have also analyzed the nuclear quantification of the DNMT3B protein in the superovulated and control oocytes and embryos. The two-cell embryos from the ND group had significantly increased expression than those of the control group. On the other hand, MII oocytes from the HD group exhibited a significant decrease, and two-cell embryos from the HD group possessed increased DNMT3B nuclear expression when compared with the corresponding control groups ($P < 0.05$) (Fig. 4c). Note that we did not detect any difference among other groups.

Discussion

In the present study, we demonstrated for the first time that superovulation alters to a large extent the expression levels of DNMT1, DNMT3A, and DNMT3B proteins in superovulated oocytes and early-stage embryos. DNMT1 protein expression in GV oocytes, one-cell embryos, and two-cell embryos exhibited alterations in the normal- and high-dose groups compared with control counterparts. Similarly, DNMT3A

exhibited expressional changes in superovulated MII oocytes and one-cell and two-cell embryos, but not in GV oocytes. Additionally, we found significant alterations of DNMT3B protein expression in MII oocytes and two-cell embryos obtained from the high-dose group in comparison to controls. Moreover, we have detected significantly different nuclear expression levels for the DNMT proteins in the certain superovulated and control oocytes or early embryos. Taken together, DNMT protein expression levels showed particular changes in the superovulated oocytes and early-preimplantation embryos. The alterations may have originated from the FSH and LH used in the superovulation protocols binding their receptors in the granulosa cells of growing-stage follicles, oocytes, and early embryos. This may affect intracellular signaling mechanism(s) leading to spatially and temporally altered DNMT protein expression. Notably, FSH and LH receptors have been identified in mouse oocytes and early-stage embryos as well as in the granulosa cells of growing follicles [27].

Epigenetic mechanisms play important roles in regulation of development-related genes of oocytes and early embryos. Recent studies demonstrated increased epigenetic anomalies in children born after ART when compared with children who were born through spontaneous conception [28]. The possible reasons for the development of epigenetic anomalies in ART children have not yet been characterized. Superovulation or ovarian stimulation is one of the ART applications, and it is frequently used in infertility treatment and experimental studies to obtain more oocytes. The effects of superovulation on many processes including genomic imprinting, fetal growth, oocyte maturation, oocyte quality, epigenetic mechanisms, and gene expression have been revealed in several studies [25, 29–35]. The latest clinical studies have also found an increased risk of imprinting disorders in children who were born using ART [32]. Furthermore, they found that DNMT1 deficiency causes genome-wide DNA methylation abnormalities, placental imprinting defects, and morphological anomalies [32].

PMSG is a member of the glycoprotein hormone family and serves as a follicle-stimulating hormone (FSH) analog. FSH plays an important role during folliculogenesis via binding its receptor on late-developmental stage follicles [36–38], as well as in GV oocytes from primary to antral follicles in human and pig ovaries [39]. Alternatively, hCG is being used as a luteinizing hormone (LH) analog, and its receptor is detected in granulosa, theca, and luteal cells [27]. Both FSH and LH receptors at the mRNA level have been identified in mouse and human oocytes and preimplantation embryos [40, 41]. These gonadotropins and their receptors have potential to regulate intracellular signaling mechanisms. In connection with these roles, their analogs may have similar direct or indirect effects on oocytes and early-stage embryos. Consistent with this, we demonstrated that PMSG and hCG

Fig. 3 Expression of DNMT3B protein in mouse oocytes and early embryos obtained from control mice (C) and mice superovulated with normal (5 IU; ND) or high (7.5 IU; HD) doses of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The immunostaining of DNMT3B in germinal vesicle (GV) oocytes (a), MII oocytes (b), one-cell embryos (c), and two-cell embryos (d) is presented for the control and superovulated groups. A negative control (NC) including no primary antibody was used to identify any non-specific staining. In the micrographs, green, FITC staining for DNMT3B; blue, DAPI (4',6-diamidino-2-phenylindole) staining for the nuclei; merge, DAPI-stained nuclei combined with green fluorescence signal thresholds. To determine the relative expression levels (mean $\pm$ standard deviation) of the DNMT3B protein in the three groups, we used ImageJ software. We compared the relative expression levels of the DNMT3B protein in GV oocytes (e), MII oocytes (f), one-cell embryos (g), and two-cell embryos (h). The statistical significance between the groups was analyzed using one-way ANOVA. $P < 0.05$ is considered significant. $P < 0.05$ for C versus HD (f). $P < 0.01$ for C versus ND and $P < 0.01$ for C versus HD (h). The different letters in the columns depict statistical significance (for a and b, $P < 0.05$). ($n = 10$ per group)

had different impacts on the expression and subcellular localization of the DNMT1, DNMT3A, and DNMT3B proteins in oocytes and embryos.

Fig. 4 Nuclear quantification of the DNMT1, DNMT3A, and DNMT3B proteins in the mouse oocytes and early embryos obtained from control (C) and superovulated mice with normal (5 IU; ND) and high (7.5 IU; HD) doses of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The nuclear quantification of the DNMT1 (a), DNMT3A (b), and DNMT3B (c) proteins was given for germinal vesicle (GV) oocytes, MII oocytes, one-cell embryos, and two-cell embryos. A sample of GV oocyte (ND, DNMT3A analysis) showing the gray values of nuclear line plot profile is represented (insert). Fluorescence images were converted into gray value images and

Fauque et al. (2007) found abnormal methylation of the *H19* gene in superovulated mouse blastocysts obtained through superovulation with 8 IU PMSG and 5 IU hCG [25]. The *H19* gene is an imprinted gene that represses *IGF2* gene expression. The paternal allele of *H19* is methylated to provide expression of the maternal *IGF2* allele [42]. In various studies, it was documented that superovulation leads to impairment in the methylation status of maternal and paternal imprinted genes in mouse blastocysts [36, 43, 44]. Additionally, superovulation adversely affects the methylation status of oocytes and expression levels of DNMTs, and this deleterious impact is maintained in early embryos from zygote to blastocyst [3]. In mouse zygotes, global DNA methylation was significantly decreased in the superovulated group with 5 IU equine chorionic gonadotropin (eCG) and 5 IU hCG. This abnormal decrease caused aberrant expression of *Dnmt1*, *Dnmt3a*, *Dnmt3b*, and *Dnmt3L* genes in the blastocysts [3].

In the current study, we found that DNMT1 expression was not significantly altered in MII oocytes between the control and superovulated groups. In a previous study, Liang et al.

analyzed by ImageJ software for determining the line-scans of DNMT1, DNMT3A, and DNMT3B proteins at the central plain of each nucleus. The mean intensity level of each group was obtained by calculating the average of the line-scans which were produced for each oocyte and early embryo. The statistical significance between the groups has been analyzed by using one-way ANOVA test. The $P < 0.05$ is considered as significant. The Yaxes indicate mean values and the bars indicate standard deviation. The different letters on the columns depict statistical significance in each group (for letters a, b, and c, $P < 0.05$). ($n = 10$ per group)

(2013) observed that *DNMT1* mRNA expression was not significantly changed in superovulated MII oocytes when compared with controls [45]. Although they analyzed mRNA expression of the *DNMT1* gene, their finding is comparable with our result. Although we detected a significant decrease in DNMT1 protein expression in one-cell-stage embryos for both the normal- and high-dose groups, Liang et al. (2013) found no remarkable change in *DNMT1* mRNA expression in superovulated one-cell embryos [45]. This difference between the two studies may derive from having examined protein versus mRNA expression of the *DNMT1* gene, from analyzing distinct types of *DNMT1* isoform or from using distinct gonadotropin doses because they treated with 6 IU PMSG/hCG for normal dose and 10 IU PMSG/hCG for high dose. Importantly, Huffman et al. (2015) revealed that there was decreased global methylation in superovulated one-cell embryos compared with control counterparts [3]. The decreased DNMT1 expression in the one-cell embryos identified in our study may lead to reduced maintenance methylation resulting in decreased global DNA methylation.

Although we detected a significant decrease of DNMT3A protein expression in MII oocytes from the high-dose group and an increase of DNMT3A in one-cell embryos from both the normal- and high-dose groups, Liang et al. (2013) observed unchanged *DNMT3A* expression in superovulated MII oocytes and one-cell-stage embryos [45]. This difference may originate from the gene product examined (in our study, DNMT3A protein expression and, in the later one, *DNMT3A* mRNA expression were analyzed) and from using different gonadotropin doses. Regarding DNMT3B expression, although the high-dose group had decreased expression in MII oocytes in comparison to controls in the present study, there was no change in *DNMT3B* mRNA expression in superovulated MII oocytes in the previous study [45]. However, changes in DNMT3B gene expression in one-cell embryos were similar in our study and the previous investigation.

In conclusion, this study is the first to analyze the impact of superovulation dosage on DNMT1, DNMT3A, and DNMT3B protein expression in superovulated oocytes and early-stage embryos. We found that superovulation differently and significantly altered DNMT expression in oocytes and embryos at distinct developmental stages. It was found in our and previous studies that superovulation alters the expression levels of DNMTs at mRNA and protein levels. Previous studies especially focusing on identifying methylation dynamics in oocytes and embryos obtained with superovulation should be evaluated in the light of our findings because superovulation protocol using PMSG and hCG hormones potentially affects DNMTs. Spatial and temporal expression of DNMTs is critical to establishing DNA methylation in oocytes and early embryos, which regulates transcriptional repression and activation of development-related genes. As is well

known, DNMT knockout studies have shown that lack of DNMT genes leads to imprinting disorders, epigenetic anomalies, embryonic lethality, or impairment of oocyte maturation. We suppose that more detailed molecular studies related to global methylation and DNMT-regulated gene expression analyses are required to determine the significance of expression changes of DNMT genes following superovulation in the developmental potential of oocytes and embryos.

Author's contribution F. Uysal performed the experiments. F. Uysal, S. Ozturk, and G. Akkoyunlu analyzed the data. F. Uysal, S. Ozturk, and G. Akkoyunlu wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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