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# The effect of the ovarian varicose vein on the DNA methylation in the rat's oocyte

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**ABSTRACT**

***Objective(s)***: We intended to determine whether the ovarian varicose which is one of the common etiologies of the pelvic congestion syndrome, has the ability to interfere with the DNA methylation reprogramming in the oocyte and thereby affect the oocyte quality or not.

***Materials and Methods:*** Varicose model was induced according to the Turner's method in the rats. Briefly, a 20-gauge needle was placed on the left renal vein and a thread was tied over both the needle and the renal vein medial to the insertion of the ovarian vein, and then the needle was removed. Evaluation of prooxidant-antioxidant balance (PAB) was assessed using specific kits and the expression level of the DNA methyltransferase genes Dnmt1, Dnmt3a and Dnmt3L was assessed by Real-time PCR. Immunofluorescent staining for 5-methylcytosine in the oocytes evaluated the global DNA methylation.

***Results:*** A significant PAB increase in the ovaries from varicose group was seen. Real-time PCR demonstrated a remarkable decrease in the expression of the Dnmt3a and Dnmt3L which are responsible for *de novo* DNA methylation in the oocytes. Immunofluorescent staining for 5-mC showed a reduction in the fluorescence intensity in the oocytes collected from the varicose group.

***Conclusion:*** Our findings from Real-time PCR and immunocytochemistry suggest that the epigenetic parameters in the oocyte could be affected by varicose induction and these epigenetic alteration has the potential to affect the oocyte quality. We suggest that the epigenetic changes could happen in the oocytes after the induction of ovarian varicose and lead to the oocyte quality reduction or even infertility.

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## Introduction

Chronic pelvic pain is experienced occasionally by about 40 percent of women (1-3). Although the pelvic pain could have various causes, in some cases the pain has something to do with the pelvic congestion syndrome (PCS) (1). In nearly 50 percent of the women with chronic pelvic pain, some evidence of varicose veins is seen (4). The most frequent cause of PCS is believed to be incompetent of ovarian veins, which is characterized by blood reflux and dilation of the ovarian veins (5, 6). Ovarian vein deficiency could happen as a result of either mechanical or endocrine factors (7).

The exact etiology of varicose veins is not clear yet. However, oxidative stress (OS) as a result of the excessive generation of reactive oxygen species (ROS) have the potential to harm the vascular endothelium, leading to the generation of varicose veins (8). An excessive level of ROS is reported to be found in tissues around varicose veins (9, 10).

A wide range of DNA defects such as deletions, strand breakage, chromosomal rearrangement and base modifications could occur following oxidative stress (11, 12). Such lesions in the DNA strands have the ability to interfere with the interaction between DNA molecules and DNA methyltransferase (DNMTs) enzymes, resulting in epigenetic alterations (13, 14). DNA methylation is associated with the mechanisms like gene silencing, chromatin condensation and histone deacetylation (15). DNA methylation has a significant effect on expression of the genes and interaction between transcriptional regulators and DNA sequences in the oocyte and since the pattern of DNA methylation could be transferred to the embryo and affect the gene expression of the embryo, establishment of the DNA methylation in the oocyte needs special attention (16).

Dnmt1 is believed to be the enzyme responsible for copying the pattern of methylation to the daughter strands during DNA replication and keeping the pattern

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of methylation over countless replications (17). The enzyme responsible for de-novo methylation in the oocyte is Dnmt3a (18). Dnmt3b seems to not have a significant role In the oocyte (19), while Dnmt3L is believed to be the major enzyme assisting Dnmt3a in establishing methylation pattern (20, 21).

DNA methylation plays a vitally important effect on the oocyte and the embryo's vitality (18, 20, 21). The aim of present study was to evaluate the effect of ovarian varicose veins on the expression of the genes responsible for establishing the DNA methylation pattern and also to assess the changes in the Global DNA methylation level.

## Materials and Methods

Thirty one-month-old female Wistar rats were divided into three groups. Each group contained ten Wistar rats. The first group was the varicose group, in which the ovarian varicose vein model was induced based on the Turner's method (22). The animals were anesthetized with the intra-peritoneal (IP) injection of ketamine (100 mg/kg) and xylazine (1 mg/kg). According to the Turner's method, left renal and left ovarian veins were approached by making a laparotomy incision on the left upper quarter of the abdomen. A 20-gauge needle was placed on top of the left renal vein and then a 4.0 thread was tied carefully over both the needle and the renal vein, medial to the insertion of the ovarian vein, and then the needle was removed gently. Based on this surgical method, about 50 percent reduction in the diameter of the renal vein could be achieved. The similar procedure was done in the second group with the exception of the ligation part, to serve as the sham group. The third group served as the control group. All three groups were kept for two months and then sacrificed.

### *Prooxidant-antioxidant-balance (PAB) assay*

Two months after the surgeries, animals were sacrificed and their ovaries were removed. After removing right and left ovaries, 40 mg of the tissue samples are frozen in liquid nitrogen and then stored at -80 °C. A tissue homogenizer in 1 mM cold 0.1 M phosphate buffer containing 1 mM EDTA is used to homogenize the tissue fragments. After being centrifuged at 10000 g for 15 min, total antioxidant substances and hydroperoxide are evaluated by removing the supernatant (23). By using one single assay, the antioxidant capacity and prooxidant burden are measured. By using 3, 3',5, 5'-tetramethyl-benzidine (TMB) and two different type of reactions, the PAB could be measured. In the first reaction, the chromogen TMB is oxidized to a color cation by peroxides and in the second reaction, the TMB cation is reduced to a colorless compound by antioxidants, giving a redox stress index. The photometric absorbency is compared with the absorbencies of a series of standard solutions (24).

### *Oocyte recovery*

Two months after the surgeries, the animals were super-ovulated by IP injection of 40 IU pregnant mare's serum gonadotropin (PMSG) followed by 40 IU of human chorionic gonadotropin (hCG) 48 hr later. In each group, 100 Mature MII-stage oocytes were collected from the oviducts 24 hr after the hCG injection and then treated with 0.1% hyaluronidase in the M2 medium in order to disperse the cumulus cells. After being washed three times in M2 medium, the oocytes were denuded of cumulus cells by pipetting multiple times with a narrow pipette. After washing, MII-stage oocytes with revealed first polar bodies were collected.

### *Immunofluorescent staining for 5-mC*

For Immunofluorescent staining, 50 MII-stage oocytes are used in each group. Cumulus cells and zona pellucida are removed from oocytes with 1 mg/ml hyaluronidase and acid Tyrode's solution at room temperature respectively. Zona-free oocytes are then fixed for 40 min in 2% formaldehyde and permeabilized in 10 mM PBS + 0.1% Triton X-100 for an additional 40 min (25). After fixation and permeabilization, samples are blocked for 1 hr in 10 mM PBS + 0.3% bovine serum albumin (BSA) + 1% fetal calf serum prior to incubation in humidified chambers with primary (Abnova, 5-methylcytosine monoclonal antibody, clone 5MC-CD) and secondary antibodies (SantaCruz, goat anti-mouse IgM-CFL 488), overnight at 4 °C and for 1 hr at room temperature, respectively. Samples are counter-stained for DNA using TOTO-3 at 10 µg/ml and RNase to eliminate RNA staining. Images are obtained using an Olympus fluorescent microscope, with laser lines at 488 nm wavelengths (Tehran University of Medical Sciences, Department of Embryology) and then processed using Adobe Photoshop 7.0. Negative controls are run in the absence of primary antibodies.

### *Real-time PCR*

The microarray results for selected genes were validated by real-time on an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) (30). Fifty MII-stage oocytes were put in each group for Real-time PCR assay. mRNA from each oocyte pool equivalent to 50 oocytes was DNase-treated (DNA-free, Ambion, Austin, TX, USA), annealed with random hexamer and reverse-transcribed into cDNA with ThermoScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR was used to quantify the mRNA transcripts levels of Dnmt1, Dnmt3a, and Dnmt3L with GAPDH mRNA transcript as endogenous references. Primers were designed using the Beacon Designer version 2.0 software (Bio-Rad Laboratories). The PCR thermal cycling conditions were 95 °C for 3 min for polymerase activation and the initial denaturation step, followed by 40 cycles with denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. A melting curve analysis was recorded at

**Table 1.** The primers used in real-time PCR

| Gene | Sequence | Base |
|---|---|---|
| GAPDH | Forward | 20 |
| | CTCTCTGCTCCTCCCTGTTC | 20 |
| | Reverse | |
| | CACCGACCTTCACCATCTTG | |
| Dnmt1 | Forward | 20 |
| | ACCCTATCGGATTGGTCGGA | 20 |
| | Reverse | |
| | TCAAGCAGGTCCTCTCCGTA | |
| Dnmt3a | Forward | 20 |
| | TTGCACGCGAGTCTGGATAA | 20 |
| | Reverse | |
| | AACATCAACCTCCACCTGGC | |
| Dnmt3L | Forward | 20 |
| | GCTGGGCTTTGGGATTCTCT | 20 |
| | Reverse | |
| | CCCATCGGGATCTTGTCCAG | |

the end of the amplification to evaluate the absence of contaminants or primer dimers.

### *Statistical analysis*

All data were analyzed by prism software (Version 5.0). Statistical analysis was performed using the one-way ANOVA and Tukey's tests are used for *post hoc* multiple comparisons and $P<0.05$ was considered statistically significant.

## Results

### *PAB assay*

Two months after varicose model induction, a remarkable increase in the PAB level in the left ovaries of the varicose group is seen which is considered statistically significant ($P<0.05$) (Figure 2). A slight increase in the PAB level on the right ovaries of the animals from the varicose group is also seen compared to the control group, it is not considered statistically significant ($P>0.05$).

### *Expression of the genes by real-time PCR*

A remarkable decrease in expression of the genes Dnmt3a and Dnmt3L in the oocytes collected from the left ovaries of the animals from the varicose group is seen and the difference is statistically significant compared to the control group ($P<0.05$) (Figure 3, 4). Although in the oocytes obtained from the right ovaries of the animals from the varicose group, a slight reduction in the expression of Dnmt3a and Dnmt3L is seen, but the reduction in the expression of these genes is not significant ($P>0.05$). The expression of the Dnmt1 in the left and right varicose group shows an insignificant decrease compared to the control groups ($P>0.05$) (Figure 5).

### *Immunofluorescent staining assay*

Global DNA methylation was measured in the oocytes obtained from each group by immunocytochemistry method. The result of Immunofluorescent staining for 5-mC on oocytes obtained from the varicose group shows a remarkable decrease in the global DNA methylation especially on oocytes obtained from the left ovaries. A slight decrease in the global DNA methylation of the oocytes from the sham group is also seen (Figure 6).

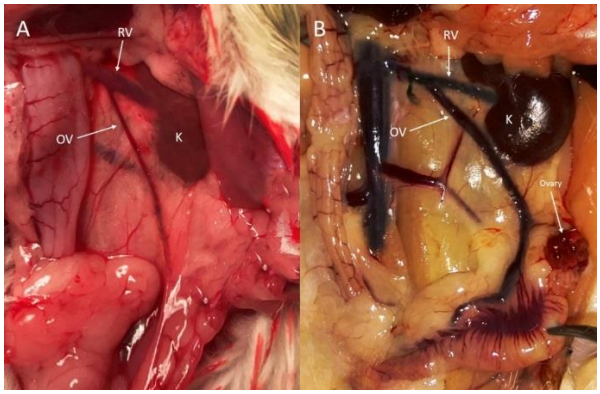


**Figure 1.** Ovarian vein before (A) and after (B) varicose induction. (OV: Ovarian vein, RV: Renal Vein, K: Kidney)

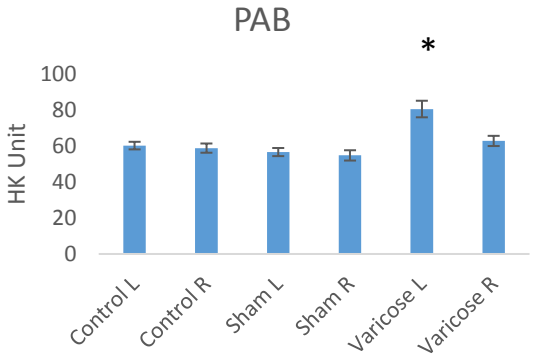


**Figure 2.** Analysis of the PAB levels in the ovaries varicose, sham and control groups. Values are expressed as mean±SD and ($P<0.05$) is considered to be statistically significant. The PAB level on the left varicose group has significantly increased compared to the control group

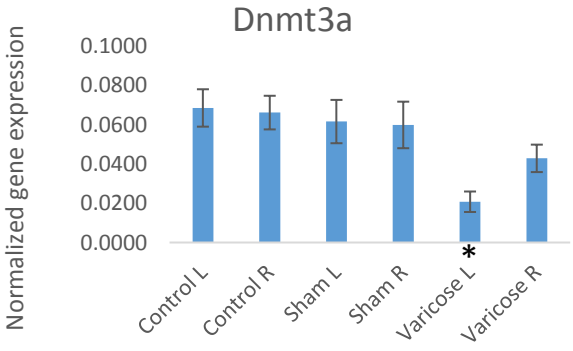


**Figure 3.** Normalized gene expression for Dnmt3a in the oocytes collected from varicose, sham and control groups. The expression of Dnmt3a has decreased in left and right varicose group but only on the left side, the reduction is statistically significant compared to the control group ($P<0.05$)

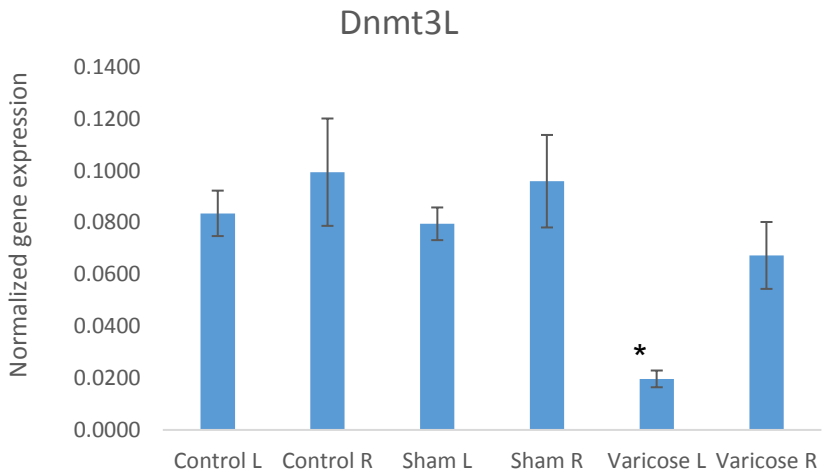


**Figure 4.** Normalized gene expression for Dnmt3L in the oocytes collected from varicose, sham and control groups. The expression of Dnmt3L has decreased in left and right varicose group but only on the left side, the reduction is statistically significant compared to the control group ($P$<0.05)

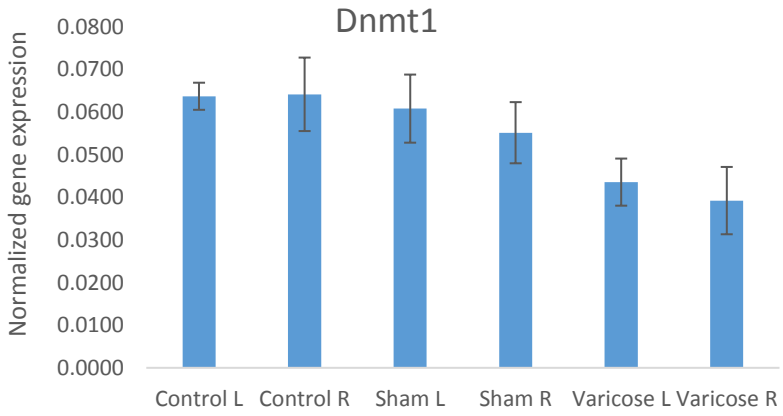


**Figure 5.** Normalized gene expression for Dnmt1 in the oocytes collected from varicose, sham and control groups. The expression of Dnmt1 has decreased in left and right varicose group but the differences are not statistically significant compared to the control group

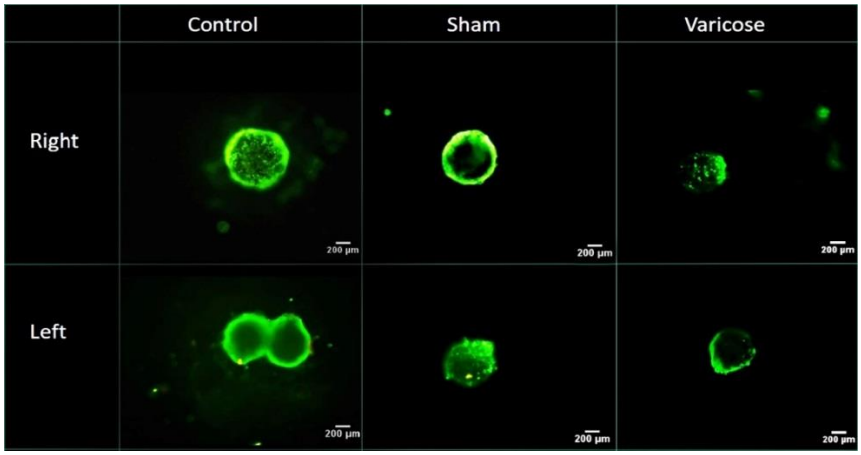


**Figure 6.** Immunofluorescent staining for 5-mC in rat oocytes. Fluorescence intensity was significantly reduced in the oocytes collected from the animals of the varicose group especially on the left side. Fluorescence intensity was assessed using ImageJ software

## Discussion

The purpose of the present study was to determine whether induced varicose veins around the ovaries and uterus, have the ability to interfere with the DNA methylation in the oocytes and thereby affect the oocyte quality or not. We assessed the expression level of the genes involved in establishing DNA methylation pattern during the oocyte maturation before and after varicose induction. We also performed immunofluorescent staining for 5-Methylcytosine (5-mC) to evaluate the effect of the ovarian varicose vein on the global DNA methylation level in the oocytes. We intended to determine whether induced ovarian varicose vein could disturb the normal epigenetic reprogramming in the oocyte by disrupting the DNA methylation pattern in the cell or not.

The pathophysiology of the varicocele and its role in reducing fertility have received a lot of attention over the past years. However, the exact explanation for how varicocele in males leads to infertility remains unclear.

For the first time, in 1857 Richet described the features of the ovarian varicosities (26). In 1991, Galkin *et al.* assumed that long-lasting ovarian varicose veins may lead to hypo-function of the ovaries and cause infertility just like the testicular varicocele (27).

There are several studies suggesting a relationship between the exceeded ROS level and infertility. In 2003 Flore *et al.* claimed that the level of ROS could get increased in tissues near a varicose vein (9). In 1996 Bell reported that increased ROS could be found in the seminal fluid in patients with varicocele (28). Increased ROS level is believed to be one of the major causes to reduce fertility in patients with varicocele (29). Hendin *et al.* also declared the fact that varicocele in males may cause infertility via high production of ROS (30).

In 2015 researchers have measured the level of malondialdehyde (MDA) and nitric oxide (NO) in the ovaries taken from the rats with induced ovarian varicose veins and demonstrated increased MDA and NO levels in the ovaries following the varicose induction (31, 32).

In this study, we evaluated the PAB in the ovaries collected from the varicose, sham and control groups and the results demonstrated a significantly increased level of PAB in the left side ovaries taken from the varicose group compared to the other groups. So our results seem to have no contradiction with the previous studies in this matter and like those studies confirm that ovarian vein varices can lead to a disturbance in the PAB level resulting in the OS in the female reproductive system.

DNA methylation has a significant role in reading the DNA sequence by transcriptional factors and because the pattern of DNA methylation could be copied at the time of replication, the accuracy of DNA methylation in the oocyte is crucial. DNA methylation could affect the expression of the genes in oocyte and also influence the embryo's gene transcription. The establishment of the DNA methylation is controlled dynamically by transcriptional events during oocyte growth and could be disrupted by any adverse conditions that have the potential to change the normal transcription program of the cell (33). OS can cause a wide spectrum of DNA defects which could consequently disrupt the interaction between DNA methyltransferase enzymes and the DNA sequences, resulting in epigenetic changes (13, 14).

Embryo's viability is dependent on the precise establishment of the DNA methylation pattern in the oocytes. Studies have shown that embryos conceived from Dnmt3a- or Dnmt3L-deficient oocytes will die by the 10$^{th}$ embryonic day (18, 20, 21).

Our findings showed a significant reduction in the expression level of the genes Dnmt3a and Dnmt3L in the oocytes collected from the varicose group, which could be as a result of exceeded ROS level in the cell. Decreased expression of the Dnmt3a and Dnmt3L would lead to hypomethylation in the cell and by doing this, alter the epigenetic pattern of the oocyte's genome and reduce the oocyte quality and the chance for a successful pregnancy.

Our results from immunofluorescent staining for 5-mC in the oocytes also confirmed that induced ovarian varicose vein could have a negative impact on the global DNA methylation in the oocyte and cause hypomethylation.

## Conclusion

According to the findings from the present study, specially the results from the expression assay of the genes Dnmt3a and Dnmt3L which we evaluated by real-time PCR and also the global DNA hypomethylation in the oocytes which was seen by the immunocytochemistry, we suggest that the epigenetic changes could take place in the growing oocytes after inducing the ovarian varicose veins and thereby lead to the oocyte quality reduction or even infertility. So the ovarian varicose vein could be suspected as one of the unknown etiologies causing infertility, but further studies on this particular topic is essential.

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