

## The effects of unilateral varicose ovarian vein on antioxidant capacity and oocyte quality in rat ovary

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### ABSTRACT

**Objective(s):** Several researchers have reported the relationship between infertility in male and varicocele for so many years but the implication of varicocele in female patients is remains elusive. Here, we aim to examine the effects of unilateral varicose ovarian vein on antioxidant capacity and oocyte quality of rat ovary after the experimental creation of varicocele in female rats.

**Materials and Methods:** In this study, thirty adult female albino rats were divided into three equal groups: Group 1 as the control group has 10 rats, Group 2 as the sham group has 10 rats and they underwent a sham operation and finally Group 3 has the varicocele group has 10 rats. Antioxidant assays for superoxide dismutase, glutathione peroxidase and catalase were performed using specific assay kits and gene expression for Bax, Bmp-15, Hsp-27 and Gdf-9 was done via real time PCR.

**Results:** The adverse effects of the experimentally induced varicocele were reported and recorded on the left ovary compared to the right sided ovary (no varicocele induction) in the varicocele group. Real time PCR data shows that the expression of Gdf-9, Hsp-27 and Bmp-15 genes were all significantly reduced at  $p \leq 0.05$ .

**Conclusion:** The results of this study show that reduced gene expression of Bmp-15, Gdf-9 and Hsp-27, increased gene expression of bax and an imbalance between pro-oxidant/ antioxidant ratio are few of the several mechanisms by which varicocele may lead to infertility in female.

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### Introduction

Generally, an estimated 84% and 92% of couples conceive after 1 year and 2 years of intercourse respectively (1). However the prevalence of female infertility depends on age of these couples and it ranges from 7% to 28%. A varicose vein is an anatomical and physiological disorder characterized by markedly dilated and tortuous veins (2). Previous clinical studies have demonstrated that the prevalence of varicose veins is at a doubled rate in women when compared to men (3).

There have so many factors identified as the cause of varicosity and they include Increase in hydrostatic venous pressure and dilated vein (2), weakened venous wall as a result of abnormal composition of extracellular matrix and connective tissues (3), abnormal vein wall composition such as intimal hyperplasia and variation of venous tone (4). Female pelvic varicocele

(FPV) is also known as a pelvic venous insufficiency (5), however when FPV is accompanied with chronic pelvic pain, it can then be called pelvic congestion syndrome (PCS).

Tinelli and co-worker, reported that the percentage frequency of PCS is almost as similar to the occurring frequency of ovarian varices (5). The anatomical implication of FPV has to its high occurrence rate at the left ovarian vein, is that it empties directly into the left renal vein whereas the right ovarian vein directly empties into the inferior vena cava (5).

According to Dubin and colleagues, the absence of venous valves in the superior aspect of the ovarian vein is visible up to 15% and 6% of cases on the left side and right side respectively (6). Several antioxidant systems that includes catalase (CAT), vitamin E, glutathione and carotenoids are responsible for regulating and controlling the activities of reactive oxygen species

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(ROS) at a normal physiological levels within the ovary (7).

The decomposition of superoxide into hydrogen peroxide and oxygen is catalyzed by an antioxidant known as superoxide dismutase 1 (SOD1) and it has been identified by Sugino and colleagues in the theca cells of an antral follicles as such there are suggestions that theca cells may be responsible for protecting the oocyte from surplus ROS during its maturation (8).

Oxidative stress (OS) arises from an imbalance pro-oxidant molecules/ protective antioxidants biological ratio. OS may lead to the destruction of lipid membranes, proteins and other molecules in the endothelium, stimulate the release of pro-inflammatory cytokines from damaged endothelial cells and affects the whole reproductive lifecycle of a woman. The follicular fluid milieu has been identified as a major source of ROS because it consists of white blood cells, antigen presenting cells like macrophages and cytokines. It should be noted that ROS might be important as a signaling molecules in some biological processes; however at surplus and unrestrained levels they may facilitate pathological processes especially in the female reproductive tract. There have been several clinical evidences to demonstrate the relationship between OS and infertility in female (9). ROS within the follicular fluid plays a role in modulating oocyte maturation, folliculogenesis, ovarian steroidogenesis, and luteolysis (10). Du and co-worker reported that during the final oocyte maturation stage, changes like fluctuations in cytokines, prostaglandins, proteolytic enzymes and nitric oxide leads to increase in ROS level thereby inducing ovarian blood flow and eventually aiding in follicular rupture (11).

However excess follicular ROS may disrupt the milieu antioxidant defense thereby leading to oocyte damage (7). Uncontrolled levels of ROS may also facilitate some pathological processes especially in the female reproductive tract thereby leading to luteal regression and reduced pregnancy maintenance hormones (7, 8).

Small stress proteins (Hsps) are molecular chaperones that modulate the ability of cells to respond to oxidative stress by rising of glutathione (GSH) levels in cells (12). Some studies declared that expression of Hsp-27 gene causes a rise in GSH levels and oxidative stress reduction in different cells (13).

It has been cleared that nitric oxide (NO) is produced in the rat ovary during follicular development and ovulation (14). Various studies have demonstrated the positive correlation between follicular NO<sub>x</sub> (nitrite plus nitrate) concentrations and follicular sizes (15). They demonstrated that the inhibition of Fas/FaL system induced apoptosis in rat granulosa cells is by NO via the inhibition of caspases activation, which are primary targets for NO (16). Regarding function of Bmp-15 and Gdf-9 genes in cumulus-oocyte complex development, there are several studies in literature.

Bmp-15 and Gdf-9 genes involve in control of apoptosis in granulosa cells and by this way, they stimulate growth of oocyte. Researches propose that BMPs and GDF9 may be responsible for regulating ovarian atresia (17-19).

It should be noted that the relationship between varicocele and male infertility has been extensively researched into for the past years but the implication of varicocele in female patients is yet to be unraveled. After intensive and extensive in-depth investigation into past and present literatures to find out any related study with our work, no study was found that reported the effects of experimentally induced female ovarian varicocele on oocyte quality and antioxidant capacity, as such we have decided to evaluate the effects of unilateral varicose ovarian vein on expression of quality related oocyte genes (Bmp-15 and Gdf-9) and apoptotic related (Bax) genes in rat ovary.

## Materials and Methods

### *Animals and experimental induction of varicocele*

Tehran University of Medical Sciences International Campus Ethics Committee approved all experimental procedures. Thirty adults female Wistar rats aged between 10 to 12 weeks were used in the course of this study. All rats were kept healthy in accordance to the laws and ethics of the International Council for Laboratory Animal Science. The rats were divided into three groups. Group 1: Control, Group 2: Sham and Group 3: Unilateral Varicose Vein.

### *Induction of experimental varicocele*

The induction of the varicocele was done at 37 °C body temperatures of the animals as measured and monitored by a rectal thermometer. For general anesthesia, ketamine (100 mg/kg) and xylazine (1 mg/kg) was administered intraperitoneally through a midline laparotomy incision, the upper left abdominal quadrant was approached. Left ovarian vein was dissected carefully medial to the insertion of the ovarian vein, and a 4-0 silk suture was tied around the ovarian vein over a 20-gauge needle.

Then the needle was carefully removed and approximately 50% reduction in the diameter of the left ovarian vein was achieved.

The midline incisions were sutured up with 3-0 silk sutures in all animals. In the sham group B, the rats underwent same surgical procedure as that of group A, but without ligation of ovarian vein and group C were the control group. The incidence of varicocele was checked for after eight weeks of induction. At the end of eight weeks, dilated left ovarian veins were seen in 10 of 30 rats that underwent laparotomy. All the animals used in this study were scarified in pro-estrus phase of the estrous cycle and the ovaries were removed for more laboratory examinations. Vaginal smears sample were taken from the animals in other to monitor the estrous cycles between the hours of 8:00 and 10:00 am.

The various cell population found within the cycle was quantified and recorded (20).

The ovarian tissue samples obtained were put in washed gently in phosphate buffered solution and were stored in -80 °C environment.

#### Measurement of antioxidant enzyme activities

Homogenization was carried out with a Teflon end homogenizator (Elvenjem Potter, Du Pont Instruments, and Newton, Conn.) and the homogenates were centrifuged at 1,500 rpm for 10 min at 4 °C. The plasma was divided and stored at 20 °C for a few days before the analyses were performed.

As demonstrated by Durak and co-worker, they measured the SOD1 level spectrophotometrically at 560 nm (21). glutathione peroxidase (GSH-Px) activity was measured as described by Paglia and colleagues by decreasing the absorbing rate of the reaction mixture at 340 nm, while NADPH is hydrolyzed to NADP (22). The decomposition of hydrogen peroxide, which is a measure of CAT activity, can be followed by measuring the decrease in absorbance at 240 nm. The decrease in absorbance was recorded every 15 sec, and, for measurement, absorbance differences with 1 min intervals were calculated (22). CAT activity in tissue homogenates was measured spectrophotometrically as described by Aebi at 240 nm by computing the rate of H<sub>2</sub>O<sub>2</sub> breakdown as a product of the CAT enzyme (23).

NO was determined by Griess method which involves converting nitrate to nitrite utilizing nitrate reductase and using of Griess reagents to convert nitrite to a deep purple azo compound (24).

#### Gene expiration by Real time-PCR

RNA extraction: total RNA was extracted from small ovarian tissue with use of an RNeasy kit (74104, QIAGEN, Germany). For cDNA synthesis, samples were. real-time PCR was performed in 96-well fast plates using the Taqman Fast Universal PCR Master Mix and the ABI 7900HT thermocycler (Applied Biosystems, Foster City, CA, USA). Additional reactions were performed on known dilutions of rat cDNA as PCR template to construct a standard curve relating threshold cycle to template copy number. Amplification efficiencies were validated and normalized against the housekeeping gene. Expression of  $\beta$ -actin as a housekeeping gene was evaluated as an internal control. Specific primer pairs of Gdf9, Bmp15, Hsp-27, Bax and  $\beta$ -actin (Table 1) were used for PCR reaction.

#### 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 were used for *post hoc*

**Table 1.** Sequence and amplicon size of primer pairs

Primer pair	Sequence	Product length (bp)	T <sub>m</sub>
Gdf9	F: CTTCCACGGGAGAATCCCAG R: GCCGGACGGTATTGTAGAGG	267	59
Hsp27	F: CGTGGTGGAGATCACTGGCAAGC R: CGGGCCTCGAAAGTGACCGG	153	65
Bmp15	F: GTGTCAGCAGCAAAAAGGCA R: CCGGACACTCCGCATAAGAA	184	59
Bax	F: GGCGAATTGGCGATGAACTG R: ATGGTTCTGATCAGTCGGG	217	60
$\beta$ -actin	F: TGCTCCTCCTGAGCGCAAGT R: ACGCAGCTCAGTAACAGTCCCG	161	63

multiple comparisons,  $P < 0.05$  was considered statistically significant.

## Results

#### Glutathione assay

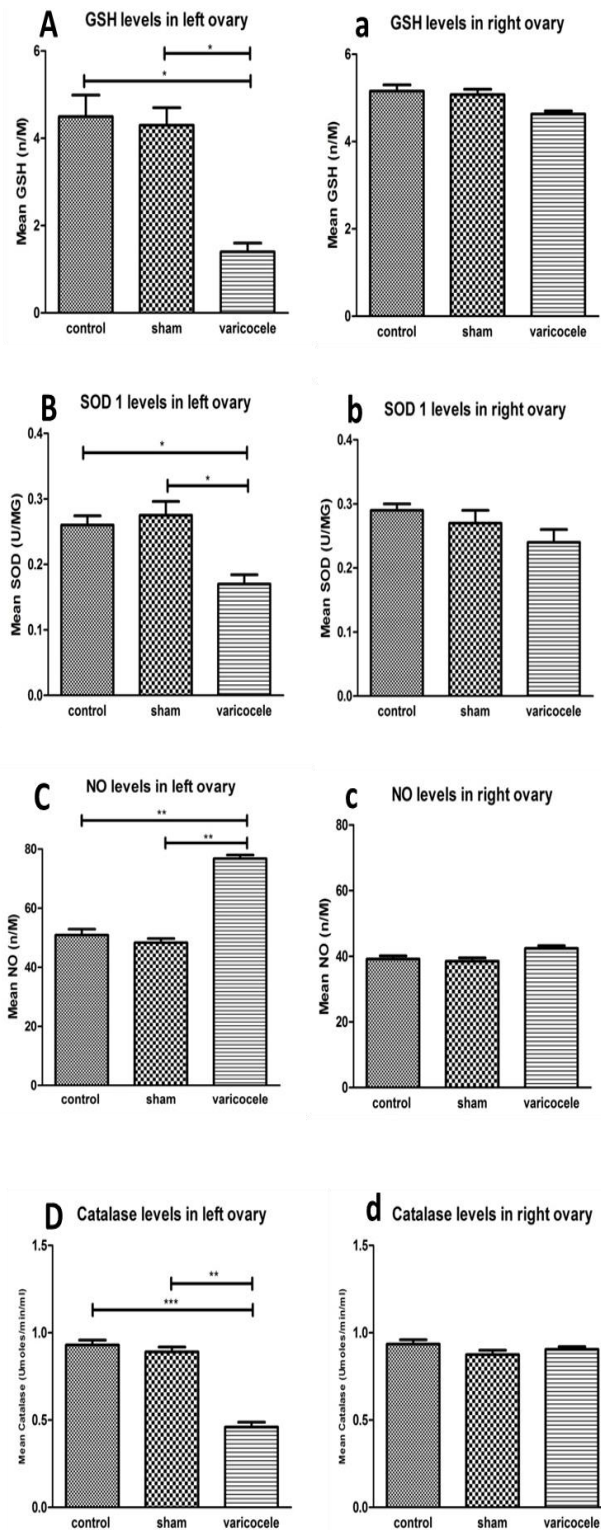
There was significant decrease in GSH-Px enzyme activities in group 3 animals on the left side when compared to group 1 and group 2,  $P \leq 0.05$  on the left side (Figure 1A). No significant difference was recorded between the control and sham group animals when compared statistically on the left side. Group 1 and group 3 animals showed the highest and lowest level of GSH-Px activities recorded on the left side respectively (Figure 1A). No significant differences recorded in the activities of GSH-Px enzyme in all the groups on the right side when compared statistically (Figure 1a).

#### Superoxide dismutase assay

There were no significant differences in the activities of SOD1 enzyme in all the groups on the right side when compared statistically. Respectively group 1 and group 3 animals showed the highest and lowest level of superoxide dismutase activities recorded on the right side respectively (Figure 1b). In group 3, there is a significant decrease in the activities of sodium dismutase enzymes which indicated a decrease in superoxide anion catalyze when compared to group 1 and group 2,  $P \leq 0.05$  on the left side (Figure 1B).

#### Nitric oxide assay

In group 3, there is a significant increase in the concentration of nitric oxide (NO) on the left side, when compared to group 1 and group 2,  $P \leq 0.05$  (Figure 1C). No significant difference recorded in the concentration of NO in all the groups on the right side,  $P \leq 0.05$  (Figure 1c).



**Figure 1.** Shows the graphical representation of biochemical analysis of the ovarian GSH-Px (A, a), SOD1 (B, b) levels, NO concentration (C, c) and catalase enzyme (D, d) levels in the three groups for the left ovaries (Left column) and right ovaries (right column). Values are expressed as mean ± SD. \* means statistically significant  $P \leq 0.05$ , \*\*,  $P \leq 0.01$ , \*\*\*,  $P \leq 0.001$  (N = 5 in each group)

**CAT assay**

In group 3, there was significant decrease in the activities of CAT enzymes on the left side which indicated an increase in the concentration of hydrogen peroxide when compared to group 1 and group 2,  $P \leq 0.05$  (Figure 1D). No significant differences recorded in the activities of CAT enzymes among the 3 groups on the right side (Figure 1d). However, group 1 and group 2 showed the highest and lowest level of CAT enzyme activities recorded respectively (Figure 1d).

**Bmp-15 gene expression**

In group 3, there was significant reduction in gene expression of Bmp-15 on the left side when compared to group 1 and group 2,  $P \leq 0.05$  (Figure 2A). However group 3 and group 1 showed the lowest and highest level of Bmp-15 gene expression recorded respectively (Figure 2A). No significant difference recorded in gene expression of Bmp-15 on the right side,  $P \leq 0.05$  (Figure 2a).

**Bax gene expression**

In group 3, there was significant increase in gene expression of Bax on the left side when compared to group 1 and group 2,  $P \leq 0.05$  (Figure 2B). No significant difference recorded in the gene expression of Bax among the groups on the right side (Figure 2b).

**Hsp-27 gene expression**

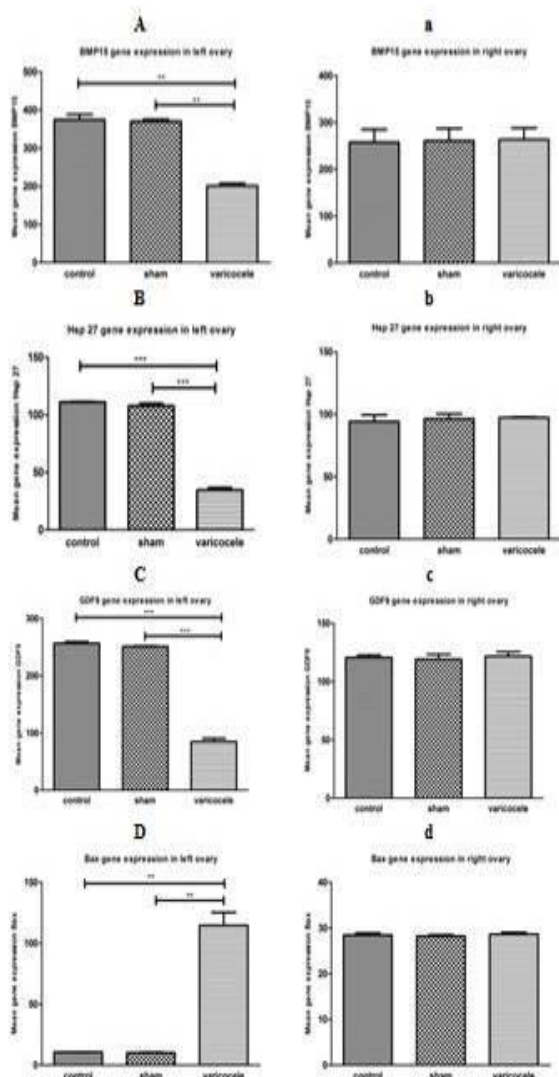
In group 3, there was significant decrease in gene expression of Hsp-27 on the left side when compared to group 1 and group 2,  $P \leq 0.05$  (Figure 2C). No significant difference recorded in the gene expression of Hsp-27 among the groups on the right side (Figure 2c).

**Gdf-9 gene expression**

In group 3, there was significant decrease in gene expression of Gdf-9 on the left side when compared to group 1 and group 2,  $P \leq 0.05$  (Figure 2D). No significant differences recorded in the gene expression of Gdf-9 in all the groups on the right side,  $P \leq 0.05$ . However group 3 showed the highest level of Gdf-9 gene expression (Figure 2d).

**Discussion**

In this study, it was investigated if increasing ROS and decreasing antioxidant, changes in genes expression of Bmp-15, Gdf-9, Hsp-27 and Bax have a resultant effect on ovarian tissue as a result of varicocele induction. In addition, biochemical examination was performed in unilateral varicocele induction, sham and control groups. Recently the pathophysiology of the varicocele has received extensive and in-depth studies, however because of the invasive nature of varicocele induction, mechanical and systemic data is almost impossible to obtain from



**Figure 2.** Shows the graphical representation of gene expression of Bmp-15 (A, a), Hsp 27 (B, b), Gdf-9 (C, c) and Bax (D, d) in the three groups for the left ovaries (Left column) and right ovaries (Right column). Values are expressed as mean±SD. \* means statistically significant  $P \leq 0.05$ , \*\*  $P \leq 0.01$ , \*\*\*  $P \leq 0.001$  (N = 5 in each group)

human models and in addition, factors like patient age, fertility or other health-related issues are variable among the subject population.

Acquisition of tissue is limited, surgical invasion for experimental purpose is forbidden and availability of appropriate numbers of control patient and varicocele patients of desired ages as such, animal models have been a good substitute for the advancement of recent researches and understanding varicocele and because of these limitations, animal models of varicocele have been developed in several species, the most common being the rat.

Several studies suggested a relationship between increased level of ROS and infertility (25). Sharma and co-workers reported there was high production of oxygen free radicals in patients with varicocele(25), it

should be noted that Hendin and colleagues also corroborated the fact that varicocele causes infertility via high production of oxygen free radicals (26). For the first time, ovarian varicosities were demonstrated by Richet in 1857 (27). Galkin *et al* showed that longlasting ovarian varicocele might influence hypofunction of the ovaries and similar to testicular varicocele, could be a cause of infertility (28), but there are few or no documentations on the prevalence of varicocele in female patients.

Although pathophysiology of the varicocele still remains unclear, Ikeda *et al* (29) reported that varicocele has shown to increase reactive oxygen species (ROS) and to suppress the activity of antioxidant enzyme activities, which is in accordance to our findings that varicocele caused a significant reduction in the activities of the GSH-Px, SOD1 and CAT enzymes. Our results support the potential role of ROS and antioxidants in the pathophysiology of varicocele induced apoptosis. In another study it was reported that in a treated left varicocele model, a dose dependent increase in the level of GSH-Px, SOD1 and CAT prevented the expression of pro-apoptotic Bax (30). This study corroborate our findings that varicocele when untreated causes a significant reduction in the activities of GSH-Px, CAT, SOD1 enzymes and an up-regulation/increased expression of pro-apoptotic Bax.

Preville *et al* and Arrigo proposed that Hsp-27 elevation causes increase in the activation of glutathione reductase enzyme (13, 31), which is in accordance with our findings that varicocele causes a significant reduction in gene expression of Hsp-27 and also a concomitant decrease in the activities of GSH-Px enzyme.

ROS and free radicals (NO, superoxides and H<sub>2</sub>O<sub>2</sub>) were reported to have a major role in the mediation of apoptosis (32). NO as a free radical inhibits apoptosis by acting through up-regulation of survival kinases, like Akt (33, 34). However, when NO combines with superoxides it forms nitrogen peroxides, which are efficient initiators of the apoptotic response, which is similar to our findings that varicocele increases the concentration of NO in the ovary that may lead to apoptosis in the ovary.

Micro-injected of Bax protein into isolated oocytes is, in itself, fully capable of inducing apoptosis (32), indicating that elevating cytoplasmic Bax levels is sufficient to drive apoptosis program in female germ cells, in addition several studies of the developing mouse ovary have demonstrated the apoptosis of fetal germ cells is related to high levels of Bax buildup without any significant changes in the levels of anti-apoptotic gene Bcl-2 (35), also studies of rat ovary (36-40), shows increased Bax expression has been consistently associated with granulosa cell death and follicular atresia (apoptosis).

All these studies are in support with our findings that varicocele is closely related with apoptosis by significantly increasing the gene expression of the pro-

apoptotic *Bax* gene at mRNA level. In our present study, the activities of the antioxidant enzymes were compared separately from each other (left from the right side ovaries), It should be noted that the left side is the induced varicose vein, the left side shown a significant reduction in the activities of all the antioxidant enzymes and a high concentration of NO free radical when compared to the right side.

No significant difference was observed statistically on the right side that is as a result of absences of varicosity and ROS. Hussein *et al* (18) reported that pro-apoptotic *Bax* expression was inhibited by Bmp-15 and expression of cumulus cell anti-apoptotic Bcl-2 was stimulated by Bmp-15, this study also corroborates our finding that varicocele causes a significant reduction in the gene expression of Bmp-15 which in turn causes an up-regulation of the pro-apoptotic *Bax* gene in the ovary. In addition, this is well-established that concentration of Bmp-15 and Gdf-9 in follicular fluid is directly related to oocyte and embryo quality and therefore, increasing in ROS content of follicular fluid will reduce concentration of Bmp-15 and Gdf-9 in follicular fluid and affect women fertility (41, 42). Our data show that induction of varicocele in female rat ovary induce ROS production and reduce Bmp-15 and Gdf-9 gene expression and therefore might reduce female rat fertility.

Finally according to a previous study performed in the field of classification for ovarian varicose veins (43), the varicocele induced in our study has aggravated to grade II (Figure 1) since all the changes are limited to the left side after two months.

Collectively, the evidence presented in this study demonstrates the effects of varicose ovarian vein on oocytes, like oocyte-secreted factors Gdf-9 and Bmp-15 gene expression is significantly reduced, the pro-oxidant/antioxidant ratio is rendered imbalanced thereby leading to suspicion in increase in oxidative stress, increase in free radicals as antioxidant enzymes activities are significantly reduced and increase in *Bax* expression, a pro-apoptotic gene suggesting that the varicose ovarian vein has a correlation with apoptosis.

## Conclusion

According to the results of this study we therefore concluded that the effect of varicocele is:

1. Decrease in gene expression of Gdf-9, Bmp-15 and Hsp-27
2. Increase in gene expression of *Bax* gene
3. Down regulation of the antioxidant enzymes like SOD1, GSH-Px and CAT
4. Increase in nitric oxide (NO) concentration
5. Conclusively, our data suggested an increase in oxidative stress with varicocele induction and suggests that damages by varicocele induction may lead to infertility in female rats.

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