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Effects of Ovarian Varicose Vein on Mitochondrial Structure, Malondialdehyde and Prooxidants - Antioxidants Balance in Rat Ovaries

Efectos de las Venas Ováricas Varicosas sobre la Estructura Mitocondrial, Niveles de Malondialdehído y Balance Prooxidantes-antioxidantes en Ovarios de Ratas

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SUMMARY: Oxidative stress is increased in varicose veins. Many studies have implicated oxidative stress in the pathogenesis of infertility causing diseases of the female reproductive tract. The aim of this study was to determine whether varicocele can cause raised levels of reactive oxygen species and denaturation of mitochondrial structure in ovaries of female rats or not. In each experimental study, 15 weaning-age female rats were divided equally in 3 groups: Unilateral Varicose Vein (A), Sham (B) and Control (C) groups. Mitochondrial structure and malondialdehyde levels as a product of lipid peroxidation and Prooxidants-Antioxidants Balance were evaluated 60 days after intervention in proestrus stage. Comparisons between groups were made by the measured test. After 2 months, our results showed that mitochondrial structure ultra-structurally was denatured with histologic examination, malondialdehyde and prooxidants-antioxidants balance levels of left ovaries increased significantly in varicocele group compared to control and sham groups ($P \leq 0.05$). In the right side, malondialdehyde increased significantly, but in prooxidants-antioxidants balance levels, there is no significant differences between groups. The data of control and sham groups were the same. These findings may support the concept that increased levels of malondialdehyde and PAB in varicocele may cause negative effects on fertility, so using antioxidants maybe useful.

KEY WORDS: Female varicocele; Proestrus phase; Mitochondrial structure; Malondialdehyde; Prooxidants-Antioxidants Balance.

INTRODUCTION

By definition a free radical is any atom with at least one unpaired electron in the outermost shell, which is capable of independent existence (Da Silva *et al*., 2010). They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any close molecule causing a cascade of chain reactions resulting in cellular damage and disease (Agarwal *et al*., 2005).

Reactive oxygen species (ROS) are formed endogenously during aerobic metabolism and as a result of various metabolic pathways of oocytes and embryos or as part of the body's defense mechanisms (Sekhon *et al*., 2010). Oxidative stress occurs as a consequence of exceeding formation of ROS and impaired antioxidant defense systems (Sugino, 2005). ROS can affect a variety of physiological functions in the reproductive tract, and excessive levels may result in precipitous pathologies affecting female reproduction (Sekhon *et al*., 2010). Some amount of ROS is needed in the ovarian follicle and also for normal sperm-oocyte interaction and sperm capacitation. Although, raised levels of ROS have a harmful effect on cell membranes, cellular DNA, mitochondria and eventually accelerates cell death either by apoptosis or necrosis (Gupta *et al*., 2006). Several theories have been reported to clarify the correlation between varicocele and infertility (Öztürk *et al*., 2012). A variety of designations has been used to describe the presence of ovarian and pelvic varices, also known as pelvic varicocele (Gandini *et al*., 2008).

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ROS cause oxidation of lipid membranes (Guzik *et al*., 2011). The lipid peroxidation product, malondialdehyde (MDA) is commonly used as a measure of the oxidative stress in cells (Pandey *et al*., 2012). The amount of MDA, which is formed from the breakdown of polyunsaturated fatty acids, perhaps taken as an indicator of peroxidation reaction (Motta *et al*., 2001). Along increased MDA, the balance of oxidants and antioxidants disturb so prooxidant-antioxidant balance (PAB) levels is expected to increase. The aim of this study was to evaluate the effects of varicocele induction on ovarian MDA and PAB levels.

MATERIAL AND METHOD

Adult wistar female rats (mean weight, 300±55 g) were maintained under standard laboratory conditions. The rats were kept throughout the study on a 12h light - dark cycle (lights on at 07:00, 4OW), room temperature of 22±2 ºC. Two-month-old rats were randomly divided into 3 groups: Unilateral Varicose Vein (A), sham (B) and Control (C). Rats were weighted and anaesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (1 mg/kg). The experimental left varicocele was induced in group A according to the method of Turner (2001). In brief, 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. In group B, rats underwent a similar procedure but without ligation of ovarian vein, and group C served as control. In 4 months, the animals were killed in proestrus phase. Ovariectomy was performed for examination.

The Stages of Estrus. Estrous cycles were monitored by daily vaginal smears, taken between 0800 and 1000 hr. Images are provided of unstained "wet" sample. The different cell population across the cycle were quantified and ratios determined to show trend between the predominant and other types in each stage of the estrus cycle (Hubscher *et al*., 2005) (Fig. 2). The rats were selected by proestrus stage of estrus cycle.

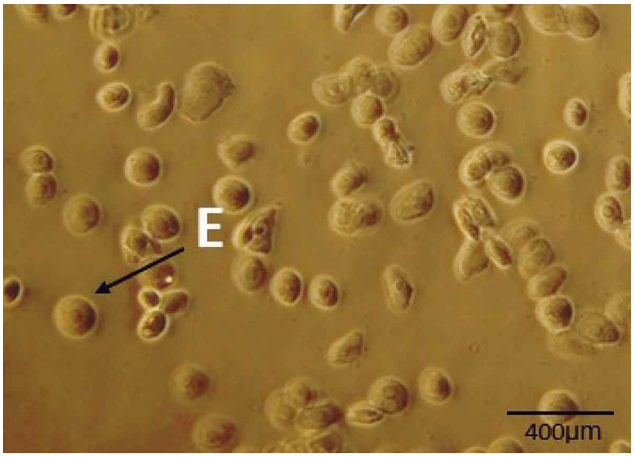

Fig 2. Vaginal smear in proestrus phase. Arrow refers to epithelial cell (E) as proestrus phase marker (Round or oval cells with a nucleus in the center).

Tissue

Electron microscope study: All rats were subjected to perfusion fixation under ether anesthesia on 60th day after the varicocele induction. Perfusion fixation was performed by immersion in 2.5% gluteraldehyde (Sigma, America) in 0.1 M phosphate buffer (Sigma, America) (pH 7.4) at room temperature for 4 hr and post-fixed in 1% phosphate-buffered osmium tetroxide (Sigma, America) for 2 h. Samples were dehydrated by being passed through the graded ethanol series and embedded in araldite–epoxy resin (TAAB, England).

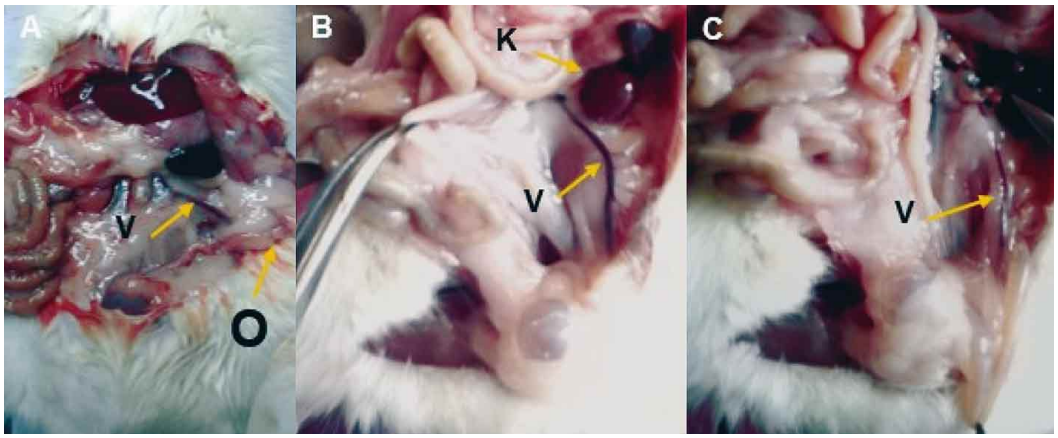

Fig 1. A, B: Distended ovarian vein in varicocele. C: Normal Ovarian Vein (V= Ovarian Vein, O= Ovary, K: Kidney).

Semi-thin sections were stained with toluidine blue (Sigma, America). Ultrathin sections were contrast stained with uranyl acetate (Sigma, America) and lead citrate (Sigma, America) and examined using a transmission electron microscope (Leo 906 E) (Cheville & Stasko, 2014).

Malondialdehyde (MDA) Measurement Method: The ovaries were eliminated quickly. Each ovary was weighed and tissue samples of 50 mg were frozen immediately in liquid nitrogen and stored at -80°C. The tissue sample was mixed with 2 volumes of cold 10% trichloroacetic acid (TCA) (mark, Germany) to precipitate protein. The precipitate is pelleted by centrifugation (3,000 g for 10 min), and 500 µl of the supernatant is reacted with an equal volume of 0.67% thiobarbituric acid (TBA) (Sigma, America) in a boiling water bath for 10 min. After cooling the absorbance is read at 532 nm (Esterbauer & Cheeseman, 1990). MDA assay was performed with a spectrophotometric assay. Data were expressed in nmol of MDA per ml of that tissue (Esterbauer & Cheeseman).

Determination-Prooxidants-Antioxidants Balance (PAB) Systems: Homogenization. The ovaries were eliminated quickly. Each ovary was weighed and tissue samples of 40 mg were frozen immediately in liquid nitrogen and stored at -80 °C. The ovary fragments were homogenized using tissue homogenizer in 1.25 mL cold 0.1 M phosphate buffer, pH 7.4, including 1 mM ethylenediaminetetraacetic acid (EDTA) (Riedel-deHaen, America). The homogenate was centrifuged at 10,000 g for 15 min. Then the supernatant fraction was removed for the definition of hydroperoxide, and total antioxidant substances (Chuffa *et al*., 2011).

Prooxidants-Antioxidants-Balance (PAB) Method: Tetramethylbenzidine 3, 3`, 5.5` (TMB) (Sigma, America) and TMB cations were used as oxidation-decrease index, due to their optical and electrochemical features. With This method, it is possible to measure the oxidant-antioxidant balance simultaneously in one experiment by two different reaction types: one enzymatic reaction where the chromogen TMB is oxidized to a color cation by peroxides and one chemical reaction where the TMB cation is reduced to a colorless compound by antioxidants. After that the photometric absorbency was compared with the given absorbencies of a series of standard solutions that are mixtures of uric acid and various proportions (0% to 100%) of hydrogen peroxide. They measured in an ELISA reader at 450 nm. PAB was arbitrarily expressed in HK units (Alamdari *et al*., 2009).

Statistical Analysis . All data are expressed as Means±SD. Statistical analysis was performed by analysis Kruskal-Wallis test and the significance between two means was determined by Mann-Whitney post-hoc test. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

After 60 days of varicocele induction, the mitochondrial structure of the ovarian tissue during proestrus is shown in Figure 3. Mitochondrial structure was denatured just in the left ovarian tissue.

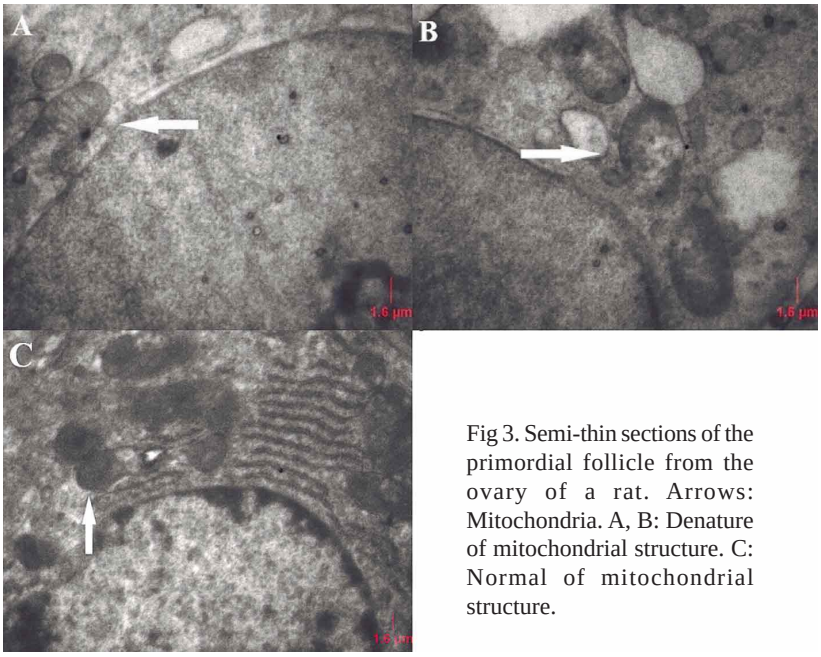

Fig 3. Semi-thin sections of the primordial follicle from the ovary of a rat. Arrows: Mitochondria. A, B: Denature of mitochondrial structure. C: Normal of mitochondrial structure.

The oxidative status of the ovarian tissue during proestrus is exhibited in Table I. The levels of the lipid peroxidation (MDA) content was significantly higher in A group ($P \leq 0.05$) than C group (Fig. 4A) and the group A showed a significant increase in PAB levels comparison with the C group ($P \leq 0.05$) (Fig. 5A). The results for Sham (B) and Control (C) groups did not differ significantly ($P > 0.05$). Data from the concentration of PAB at right ovarian tissue are shown in Figure 5B. There was no significant difference between the groups A and C in PAB levels ($P > 0.05$). However, the concentration of MDA significantly increased in the varicocele induction group ($P \leq 0.05$) (Fig. 4B).

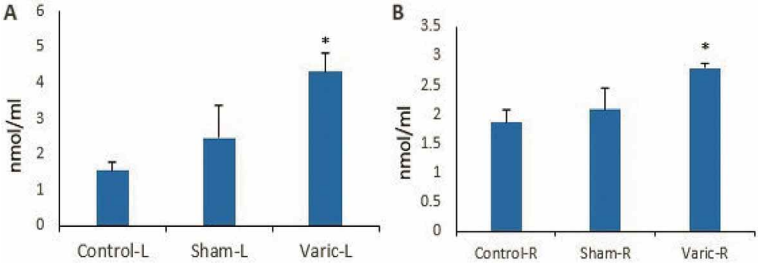

Fig. 4. Biochemical analysis of the ovarian MDA levels (A: left side, B: right side). Values are expressed as Mean±SD. Statistically significant difference between the groups (P≤0.05). Statistically significant difference was found between the groups for MDA activity in Varicose Vein group (n= 5 in each group).

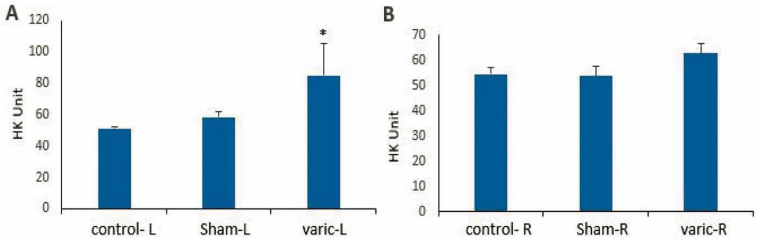

Fig. 5. Biochemical analysis of the ovarian PAB levels (A: left side, B: right side). Values are expressed as Mean±SD. Statistically significant difference between the groups (P≤0.05). Statistically significant difference was found between the groups for PAB activity in left side (n= 5 in each group).

Table I. Malondialdehyde (MDA) and Prooxidants-Antioxidants Balance (PAB) parameters of female rats after 60 days of varicocele induction.

Groups	MDA (ng/ml)	PAB (UK unit)
Left- varices	4.32±0.87*	85.11±34.80**
Left- Control	1.55±0.4	51.148±2.21
Left-- Sham	2.47±1.58	58.44333±6.05
Right- varices	2.79±0.15*	62.63333±6.93
Right- Control	1.86±0.4	54.48±4.46
Right- Sham	2.08±0.62	53.74333±6.46

*shows significant increase in MDA levels compared with control and sham groups (P≤0.05).
**shows significant increase in PAB levels compared with control and sham groups (P≤0.05).

DISCUSSION

In this study, it was investigated whether denaturation of mitochondrial structure and increasing ROS occurs in ovarian tissue by varicocele induction or not. In addition, biochemical examination was performed in unilateral varicocele induction, sham and control groups. Mitochondrial structure and amounts of MDA and PAB were investigated in ovarian tissue of all the rats.

The present results demonstrated a significant increase in PAB levels at 2 months after varicocele induction in left ovarian tissue. MDA also increase within this period, which was significant and mitochondrial structure was damaged. In right ovarian tissue, there was a significant increase in MDA levels but the increase in PAB levels was not significant in comparison to the other groups. This could be due to antioxidant increases; the prooxidant-antioxidant balance in the ovarian tissue is not affected significantly. Conversely, the above parameters of ovarian tissue at the right side, there were no significant alterations compared with the control group.

ROS induce lipid peroxidation with related influences in mitochondrial dysfunction (Da Silva *et al*.) so we evaluated mitochondrial structure in follicle's cells and observed denature of mitochondrial structure in the left ovarian tissue.

We have applied a previously described animal model of male varicocele to female rats (Gokdeniz, 1999). Physiologically, ROS are increased in ovary after the preovulatory gonadotrophin surge (Yener *et al*., 2013) so, in order to the same conditions of ovaries; In the present study, we selected proestrus phase.

Ovarian varicosities were first described by Richet in 1857 (Venbrux *et al*., 2012). In 1991, Galkin *et al*., hypothesized that long-lasting ovarian varicocele might cause hypo function of the ovaries and, similar to testicular varicocele, could be a cause of infertility (Galkin *et al*., 1991) but the significance of varicocele in female patients is not yet documented.

It has been experimentally shown that the severity of clinical changes is dependent on the efficiency of ROS scavenging (Krzysciak & Kózka, 2011), which additionally proves the participation of free radicals in the pathogenesis of varicocele induction, also increased ROS levels have been implicated in reduced fertility in patients with varicocele (Smith *et al*., 2006). We feel that there is no evidence in the literatures to date to state that oxidants play an important role as cause for infertility in female varicocele. Our study showed an increase in MDA and PAB

after induction of varicocele, therefore infertility in varicocele patient may be caused by increased of these markers.

About male varicocele, reported that semen ROS levels correlated positively with varicocele grade. In addition, some researchers reported that men with varicocele grade II or III had significantly higher semen ROS levels than men with varicocele grade I (Cocuzza *et al*., 2008). According to a study performed in the field of classification for ovarian varices vein (Hiromura *et al*., 2004), since all the changes of our study are limited to the left side, subsequently after two months, varicocele had probably progressed to grade II.

Our data suggesting an enhanced oxidative stress in varicocele induction and suggests that damages by varicocele induction may have negative effects on the process of fertility in female rat so in addition to the removal of varicose veins, using antioxidants may reduce the effects of varicose veins that which require further investigation.

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RESUMEN: El estrés oxidativo aumenta en las venas varicosas. Diversas investigaciones han implicado al estrés oxidativo en la patogénesis de las enfermedades que causan la infertilidad del tracto reproductivo femenino. El objetivo de este estudio fue determinar si el varicocele puede provocar niveles de especies reactivas del oxígeno y la desnaturalización de la estructura mitocondriales en los ovarios de ratas. En cada estudio experimental, cinco ratas hembras en edad de destete se dividieron por igual en 3 grupos: Várices unilateral (A), simulado (B) y control (C). La estructura mitocondrial y los niveles de malondialdehído como un producto de la peroxidación lipídica y el balance pro-oxidantes-antioxidantes (BPA) se evaluaron 60 días después de la intervención en la etapa proestro. Las comparaciones entre grupos se realizaron mediante la prueba de medición. Después de 2 meses, observamos que la estructura mitocondrial se desnaturalizó ultraestructuralmente, los niveles malondialdehído y el balance prooxidantes-antioxidantes de los ovarios izquierdos aumentaron significativamente en el grupo A en comparación con los grupos B y C ($P \leq 0.05$). En el lado derecho, los niveles de malondialdehído aumentaron significativamente, pero el BPA, no mostró diferencias significativas entre los grupos. Los datos de los grupos B y C eran los mismos. Estos hallazgos pueden apoyar el concepto de que el aumento de niveles de malondialdehído y BPA en presencia de varicocele, puede causar efectos negativos sobre la fertilidad, por tanto el uso de antioxidantes puede resultar útil.

PALABRAS CLAVE: Varicocele femenino; Fase proestro; Estructura mitocondriales; Malondialdehído; Balance prooxidantes-antioxidantes.

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