

## Do Pilea Microphylla Improve Sperm DNA Fragmentation and Sperm Parameters in Varicoceled Rats?

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**Abstract-** Varicocele is one of the most common causes of primary male infertility. Pilea microphylla (PM) is being used as folk medicine. This study was aimed to investigate the effects of PM in a rat model of varicocele. A total of 30 male Wistar rats were divided into control, sham, varicocele, accessory varicocele and PM-treated groups. After 10 weeks of varicocele induction, sperm parameters and chromatin (Aniline blue, acridine orange and toluidine blue) were evaluated, except for the treated and accessory groups that received 50 mg/kg PM orally daily for 10 weeks and then were sacrificed. Sperm parameters significantly decreased in varicocele groups ( $P < 0.01$ ). Moreover, there was a negative correlation between the DNA fragmentation and sperm parameters in varicoceled rats. Administration of PM led to significantly increased sperm parameters and AO staining ( $P < 0.05$ ). These findings suggest that PM improves sperm parameters and DNA fragmentation in varicoceled rats. PM can reduce the damage to sperm DNA but not chromatin condensation.

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### Introduction

Approximately 8% of men in reproductive age seek medical assistance for fertility-related problems. Among them, 1–10% carry a condition that compromise their fertility and varicocele alone accounts for 35% of these cases (1). Varicocele has an incidence of 4.4–22.6% in the general population, 21–41% of men with primary infertility, and 75–81% of those with secondary infertility have this condition (2). The varicocele is characterized by abnormal tortuosity and dilation of the gonadal veins that drain the testis (3-5). Although the physiopathology of varicocele and its association with male infertility has been discussed for the last 50 years, the exact mechanisms that would ultimately lead to an infertile/subfertile state are still controversial (6,7).

Varicocele leads to increased reactive oxygen species (ROS) generation, peroxidation of sperm plasma membrane, nuclear DNA damage and apoptosis rates,

both in the testes and in semen (8). An association between infertility and generation of ROS has been established and extensively studied (9). The incidence of sperm cells with fragmented DNA is higher in the ejaculate of participants with varicocele as compared to that infertile donors (10). Also, infertility in varicocele patients may be attributed to the production of spermatozoa with poorly condensed chromatin (11).

Seminal oxidative stress and testicular apoptosis are well-documented causes of increased sperm nuclear DNA fragmentation (12,13). It is well documented that the high level of ROS production correlates negatively with sperm parameters in the original semen (14). According to earlier studies, increased ROS formation has been correlated with a reduction of sperm motility (15,16). MacLeod in 1965 reported decreased sperm count, decreased motility and higher prevalence of abnormal forms in semen specimens collected from infertile men with varicocele (17).

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Clinical evidence shows that sperm chromatin defects have been correlated with the reduced ability of spermatozoa to fertilize oocytes in the context of assisted reproduction techniques and normal fertility (18,19). Any form of sperm chromatin abnormalities or DNA damage may result in male infertility (20,21). Toluidine blue (TB) test, acridine orange (AO), and aniline blue (AB) staining are used to assess sperm chromatin/DNA (22,23). To evaluate sperm chromatin quality and DNA integrity, after fixation of sperm smears, AB, TB and AO staining were applied in three groups.

Some researchers have suggested that the administration of antioxidants reduces the damage of DNA and improves sperm parameters (24-28) therefore, antioxidant supplementation may be used to treat infertile patients with varicocele (21). *Pilea microphylla* (PM) (family: Urticaceae) is being used as folk medicine to treat several allergies/wounds in and around Malaysia peninsular especially Penang island. It is reported to have antioxidant activity [9:8] and total phenolic content (20). PM is also used for infertility (29). However, the influence of PM on varicocele has not been reported yet. The aim of this study was to determine (1) the time required for complete infertility resulting from unilateral varicocele in an experimental varicocele induction model in the rat, (2) PM-induced improvement in the sperm quality in varicocele rat, and (3) the effect of PM administration for 10 weeks on the infertility of varicocele rats. Therefore, sperm parameters, DNA fragmentation, and mating outcomes were evaluated before and after PM administration.

## Materials and Methods

### Isolation of PM extract

*Pilea microphylla* were collected in Penang Island. The plant materials were washed, dried and cut into small pieces. The dried materials were then transferred into the Soxhlet extractor with methanol as a solvent for 72 hours as described by Modarresi *et al.*, (20).

### Animals

A total of 30 adult Wistar male rats (mean weight,  $300 \pm 55$  g) were maintained under standard laboratory conditions. The rats were acclimatized for 1 week under a 12 hour: 12 hour light: dark cycle at room temperature of  $22 \pm 2^\circ$  C, and subsequently the male animals were mated with adult female rats and their fertility was evaluated (mating action was checked by vaginal plaque). Male rats were randomly divided into five

groups: control, sham, varicocele, varicocele + PM-treated and accessory varicocele groups. Rats in group varicocele + PM-treated were orally administered 50 mg/kg PM, daily for 10 weeks after varicocele induction. Accessory varicocele rats were used for the evaluation of mating outcome. The ethical committee of the Tehran University of Medical Sciences approved all the experiments and animal handling procedures.

### Surgical Procedure

The rats in varicocele, accessory varicocele and varicocele + PM-treated groups were weighed and then administered a general anesthesia with an intraperitoneal (ip) injection of ketamine (100 mg/kg, Sigma/Aldrich Chemical Co.) and xylazine (1 mg/kg, Sigma/Aldrich Chemical Co.) (30). Left varicocele was experimentally induced according to the method of Turner 31. In brief, through a midline laparotomy incision, the upper left abdominal quadrant was approached. The left renal vein was carefully dissected medial to the insertion of the spermatic vein, and a 4.0-silk suture was tied around the renal vein over a 20-gauge needle. Then, the needle was carefully removed and approximately a 50% reduction in the diameter of the left renal vein was achieved. In the sham group, the rats underwent a similar procedure, but the ligation of the renal vein was not performed.

### Treatment

Ten weeks after surgery, the operated animals in varicocele + PM-treated group received 50mg of PM for 10 weeks. The drug was given to rats orally.

### Fertility rate assay

All rats were mated with fertile female rats after 10 weeks of varicocele induction for evaluation of mating outcome. Ten weeks later (i.e., 20 weeks after the operation), the fertility test was repeated for varicocele + PM-treated and accessory varicocele groups again. Sperm chromatin structure and semen parameters of the control, sham, and varicocele groups were evaluated 10 weeks after surgery and that of the accessory varicocele and varicocele + PM-treated groups were evaluated 20 weeks after inducing varicocele.

### Sperm collection

By laparotomy, the left caudal part of the epididymis was carefully separated from the testes, minced in 5 ml of Hanks' medium, and incubated for 15 min (8). The diluted sperm suspension (10 ml) was transferred to the hemocytometer, and the settled sperm were counted

with a light microscope at 400× magnification (million/ml) (32).

### Sperm motility

The motility assay was conducted by observing the sperm suspension on a glass slide at 37°C. The percentage of motile spermatozoa was determined by counting more than 200 spermatozoa randomly in 10 selected fields under a light microscope (Olympus BX51, Germany), and the mean number of motile sperm  $\times$  100/total number of sperms 33 was calculated.

### Sperm morphology

The sperm morphology was determined by eosin/nigrosin staining. Ten microliters of eosin Y (1%) and nigrosin was added to 50  $\mu$ l of sperm suspension. The prepared smear was used after incubation for 45–60 min at room temperature. In each field, 200 sperm were counted under a light microscope (1000×). The sperms were classified on the basis of the following abnormalities: double head, flattened, bent neck, bent tail, and multiple abnormalities (34).

### Rate of the sperm vitality

To determine sperm vitality, 40  $\mu$ l of freshly liquefied semen was thoroughly mixed with 10  $\mu$ l of eosin Y (1%), and 1 drop of this mixture was transferred to a clean slide. At least 200 sperms were counted at a magnification of 1000× under oil immersion. Sperms that were stained pink or red were considered dead, and the unstained sperms were considered viable (35,36).

### Chromatin structure of sperm in the caudal epididymis

To assess the sperm DNA integrity, three different cytochemical staining methods including acridine orange (AO), aniline blue (AB), and toluidine blue (TB) assays were applied. For evaluation of the excessive presence of histones, we used AB staining. For assessing sperm chromatin structure and packaging, TB and AO were used, respectively.

### Assessment of chromatin

#### AB staining

After sperm preparation, 5 mL of the prepared spermatozoa were spread onto glass slides, and the slides were allowed to dry. The smears were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 minutes. Then, the slides were stained with 5% aqueous AB mixed with 4% acetic acid (pH 3.5) for

5 minutes; 200 sperm cells per slide were examined, and the percentage of stained sperm heads was calculated. Sperm cell heads with good chromatin integrity were nearly colorless while those with diminished integrity were blue 37.

#### TB staining

Dried smears were fixed in 96% ethanol and acetic acid (1:1) for 30 minutes, and then hydrolyzed with 0.1 N, HCl for 5 minutes in 4°C. The slides were washed with distilled water 3 times for 2 minutes and stained with 0.05% TB for 5 minutes. Sperms with light blue heads were normal with good DNA integrity while those with dark heads (purple) were abnormal with poor DNA integrity (22,23).

#### AO staining

A small aliquot (20 mL) was smeared on pre-cleaned glass slides. The smeared slides were air-dried and later fixed overnight in freshly prepared Carnoy's solution (methanol/acetic acid, 3:1). After fixation, the slides were air-dried and stained for 5 minutes with freshly prepared 0.19 mg/mL AO stain as follows: Ten milliliters of 1% AO in distilled water was added to a mixture of 40 mL of 0.1 mol/L citric acid and 2.5 mL of 0.3 mol/L Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O. The AO solution was stored in dark at 4°C for four weeks. After staining, the slides were washed with distilled water, covered with glass cover slips, and immediately examined using a fluorescent microscope (Leitz, Germany; at the excitation wavelength of 450-490 nm). An average of 200 sperm cells were counted on each slide by the same examiner, and the duration of evaluation were not more than 40 seconds per field. Spermatozoa those show green fluorescence had normal DNA, whereas sperms show a spectrum of yellow-orange to red fluorescence had damaged DNA (38-40).

#### Statistical analyses

All values are presented as mean  $\pm$  standard deviation (SD). One-way analyzes of variance (ANOVA) and post-hoc Duncan test were performed to determine the differences in the staining characteristics of all groups using the SPSS/PC computer program (version 13.OSS). A probability of  $P < .05$  was considered to be statistically significant.

## Results

### Fertility assessment

The members of all 5 groups were still fertile when

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they mated with female rats 10 weeks after the operation. The pups were delivered within 25–30 days. Ten weeks later (20 weeks after varicocele induction), PM-treatment group and varicocele-induced group were mated with fertile females, all the rats in the varicocele-induced group turned out infertile, but significant number of the rats in the PM-treatment group were fertile and hence able to impregnate female rats.

### Evaluation of varicocele

All rats with varicocele showed conspicuous dilatation of the left spermatic vein with blood engorgement. In rats of control and sham groups, no dilatation of the spermatic vein was observed.

### Assessment of epididymal sperm parameters

The results of the comparison of sperm parameters in the five groups namely, control, sham, varicocele, accessory varicocele and treated groups are shown in Table 1.

The sperm count, vitality, motility, and morphology of left testis of varicocele group decreased significantly in comparison with control and sham groups ( $P<0.01$ ). Sperm parameters of left testis in the PM-treated group increased significantly in comparison with varicocele group ( $P<0.05$ ). However, no significant changes were recorded in comparison to the control and sham groups, also between varicocele and accessory varicocele groups.

**Table 1. Left epididymal sperm count, motility, vitality and morphology in the experimental groups**

Parameters	Sperm count (*10 <sup>6</sup> )	Normal morphology (%)	Motile sperm (%)	Sperm vitality (%)
<b>Groups</b>				
<b>Control</b>	12.84(0.45)	83.5(2.12)	(1.41)81	82(1.41)
<b>Sham</b>	12.45(0.35)	79(1.41)	81(4.24)	79(1.41)
<b>Varicocele &amp; Accessory V.</b>	6.5(1.35)*	45.5(0.71)*	39.5(0.7)*	41(1.41)*
<b>Treatment PM</b>	10.17(0.1)**	73.5(0.71)**	58.5(4.95)**	60(0)**
<b>Control</b>	12.84(0.45)	83.5(2.12)	(1.41)81	82(1.41)

Note: Results are presented as mean (SD)

\* $P<0.01$  compared to sham and control

\*\* $P<0.05$  compared with varicocele-induced group

### Chromatin staining assessment

The results of the comparison of sperm nuclear integrity (AB, AO, and TB staining) in the five groups are shown in Table 2. No significant differences were observed in the results of the control and sham groups, but the varicocele group showed significant differences

in all tests in comparison to the control group ( $P<0.05$ ). Sperm nuclear integrity improved significantly in the PM-treated rats compared to the varicocele groups in AO test ( $P<0.05$ ). However, no significant changes were recorded in comparison to the varicocele and accessory varicocele.

**Table 2. Characteristics of nuclear DNA integrity of spermatozoa in the 5 groups**

Parameters	AO	AB	TB
<b>Groups</b>			
<b>Control</b>	4.5(0.71)	11(1.41)	15(1.41)
<b>Sham</b>	5.5(0.7)	10(1.41)	14(1.41)
<b>Varicocele &amp; Accessory V.</b>	22(2.83)*	23.5(0.71)*	30(0)*
<b>Treatment PM</b>	18(0)	19(1.41)	20(1.41)**

Results are given as means (SD)

\* $P<0.05$  compared with control group

\*\* $P<0.05$  compared with varicocele group

## Discussion

The impact of varicocele on male fertility was not

suspected until the end of the 19th century when occlusion of dilated veins from the pampiniform plexus was shown to improve semen quality (2,3). There are a

lot of studies on semen parameters in adult varicocele (41-43). Some of them have proved a significant correlation with all parameters, and others just to a variety of different parameters, between varicocele and sperm quality (44). In the current experiment, we have observed that rats with varicocele exhibited a significant reduction in sperm count, motility, morphology and vitality. There were statistically significant differences between varicocele and control groups. These results are in agreement with our previous studies (25,28,45). These changes in sperm parameters after varicocele may be mainly due to the direct effects of increased ROS production and lower testosterone levels in dilated veins (45-47). Regarding the varicocele influence on androgen status, a recent report by Tanrikut *et al.*, evaluated men with clinical varicocele in a case-control study and reported that testosterone levels were significantly lower compared to men without varicocele (47). Jarow *et al.*, reported that men with varicocele were also shown to have lower total sperm count and testosterone levels, as well as reduced testicular size on the same side of varicose vessels compared with those without varicocele (48). Reduction in the number of sperm in varicocelized rat's epididymis may be related to cellular population reduction in seminiferous tubules and cell proliferation. Excessive ROS can induce germ cell apoptosis, decrease daily sperm production, which may account for the male fertility declining (49). Increased apoptotic germ cells were found in seminiferous tubules, mainly consisting of round spermatid and elongated spermatid (50).

Sperm chromatin abnormalities, such as enhanced ROS production and a high number of sperm cells with fragmented DNA were observed in rat models of varicocele (51). In the present experiment, varicocele rats exhibited a significant increase in chromatin abnormalities in the three tests (AO, TB and AB;  $P < 0.05$ ). Recent studies have shown that varicocele patients have a significantly higher proportion of sperm cells with fragmented DNA than fertile men (10,51,52). Smith *et al.*, found that high levels of sperm DNA damage were associated with varicocele even when semen analysis had been normal (53). Therefore, varicocele results in the production of spermatozoa with less condensed chromatin, and this is one of the possible causes of infertility due to varicocele.

According to one of the most important hypotheses, the molecular mechanism of sperm DNA damage involves ROS (54,55). Also Talebi *et al.*, showed that varicocele patients have a higher proportion of spermatozoa with abnormal DNA and immature chromatin than fertile men as well as infertile men

without varicocele (11). A number of studies have suggested that the presence of spermatozoa with damaged DNA may be the result of impaired chromatin packing or indicative of apoptosis (56,57). A study by Saleh *et al.* using the sperm chromatin structure assay (SCSA) (10) indicated that infertile patients with varicocele possessed a significantly higher DNA fragmentation index (DFI) than healthy fertile controls.

One of the factors responsible for sperm chromatin disorders in varicocele patients is apoptosis, which is more frequent in the spermatozoa of these patients (50). Varicocele may contribute to an increased sperm programmed cell death, necrosis, degeneration or physiological degeneration as a result of abnormal testicular temperatures (58). One study showed that apoptotic sperm cell count in patients with varicocele is approximately 100 times higher than those without varicocele (59).

In the present study, there was a negative correlation between sperm parameters and the percentage of sperm with high DNA fragmentation. This result is consistent with our previous results (27).

In this experiment, we also evaluate mating outcome after varicocele induction, the rats remained fertile 10 weeks after inducing varicocele. However, they became infertile after 20 weeks. These results confirm that unilateral varicocele affect fertility only 10 weeks after varicocele induction and complete infertility occurs only after a minimum of 5 months after varicocele induction. Thus, current results confirm the fact that in varicocelized animals the extent of damage caused by ROS on sperm parameters depends not only on the nature and amount of ROS involved but also on the duration of ROS exposure.

Varicocele is commonly treated with varicocelectomy, but because of the surgical risk and chance of the persistence of infertility, patients are also offered the choice of non-surgical treatment with certain drugs. Nonsurgical treatment modalities for varicocele-related infertility are poorly studied, and there is a need for well-designed trials. PM has been shown to have antioxidant properties; these properties have not been investigated in rats in which varicocele was experimentally induced. In this study, a statistically significant increase was observed in sperm count, vitality, morphology and motility in the treated rats compared to varicocelized rats. Positive changes in the sperm quality including sperm parameters and sperm DNA integration in this study may be because PM directly scavenges hydroxyl radicals and thereby inhibits lipid peroxidation. It is possible that interference of PM

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with free radical generation could be one of the causes of the decline in varicocele repair. Administration of PM in this experiment could reduce excessive ROS formation in varicocele rats.

The results are consistent with the data of previous reports on other antioxidant agents. A recent study using dietary vitamin E supplementation showed significantly low level of superoxide and possibly decreased apoptosis in varicocele rats (60). When we injected Aminoguanidine intraperitoneally into varicocele rats, significant changes were observed in all the sperm parameters (45). Although in other studies L-carnitine as an antioxidant in combination with the nonsteroidal anti-inflammatory agent cinnocicam have been studied but failed to show improvement in seminal parameters in men with clinical varicocele (61).

Although we observed improvement in the result of AO test after PM administration, DNA maturation (AB, TB) did not show significant improvement in the treated groups. Current results indicated that in addition to ROS, there might be other factors that influence DNA condensation and maturation. Immaturity is a consequence of defective spermatogenesis that could also lead to differences in disulfide cross-linking and insusceptibility toward DNA fragmentation (51).

In this study, we evaluated the animal fertility. After injection of PM for 10 weeks, half of the rats in the treated group could impregnate the female rats. It seems that PM improved the rate of fertilization in varicocele rats which may be due to a reduction in ROS production. However, we suggest further studies to understand the exact mechanism of PM infertility.

When we administered the PM to the varicocele rats, semen parameters, DNA fragmentation and fertility were improved. In conclusion, *Pilea microphylla* could be suggested as a natural source of antioxidant. Therefore, it could be useful for the treatment of varicocele and possibly other clinical conditions involving excess free radical production.

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