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Effect of *Alhagi camelorum* on Varicocele-Associated Epididymal Sperms and *in Vitro* Fertilization Disorders in Mature Rats

Peyman Montazemi[1], Ali Shalizar-Jalali ✉[2], Gholamreza Najafi[2], Sima Jafari[1], Negar Mahdavi ✉[1]

[1] *PhD Candidate, Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.* [2] *Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.*

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ABSTRACT

Varicocele is known to be induced by abnormal dilation and tortuosity of the pampiniform plexus veins within the spermatic cord and is considered the most common surgically remediable etiology of male infertility. This study aimed to uncover the effects of *Alhagi camelorum* (AC) on epididymal sperms characteristics and *in vitro* fertilization (IVF) outcomes in a rat model of varicocele. In this experimental study, 40 male rats were randomly categorized into five groups (n = 8), including control, varicocele, varicocele with high-dose AC (400 mg/kg), varicocele with medium-dose AC (100 mg/kg), and varicocele with low-dose AC (25 mg/kg). After a 60-day treatment period, malondialdehyde, total anti-oxidant capacity (TAC), sperm parameters, and IVF outcomes were determined. The varicocele group showed significant reductions in TAC, and sperm count, motility, and viability, as well as the number of zygotes, two-cell embryos, blastocysts, and hatched blastocysts, compared to the control group. The AC administration improved sperm traits, fertilization rate, and *in vitro* embryo development. These findings propound that AC can palliate the deleterious effects of varicocele on fertility in rats, likely due to its pharmacological properties, particularly anti-oxidant and anti-inflammatory activities.

Introduction

Varicocele is present in approximately 15% of all men and in 19%–41% of men presenting primary infertility, making it the most common surgically remediable etiology of male infertility. In men with secondary infertility, varicoceles are recognized as the underlying cause in 45%–81% of cases.[1] Varicoceles are progressive and typically develop during puberty, with the left side being more commonly affected (90%). Research has revealed that in men, varicocele is associated with reduced sperm count, motility, and morphology.[2,3] Over-production of reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide, singlet oxygen, and peroxynitrite radicals, has been implicated in varicocele occurrence.[4] These highly reactive species can lead to pathological damages through inducing oxidative changes in cellular lipids and proteins, resulting in sperm dysfunction.[5,6] Hence, ROS levels are important parameters for estimating the treatment of varicocele-induced infertility. Although, varicocelectomy can improve semen traits (sperm count, motility, and morphology) and there is substantial evidence suggesting that varicocelectomy leads to ameliorated reproductive outcomes, the role of varicocele repair in treating sub-fertile men has been debated over the past few decades.[7] Reportedly, it has been demonstrated that varicocele repair does not necessarily improve a couple's chances of conception.[8] Thence, varicocele repair is not always recommended for all infertile men

✉ Corresponding authors. Email: a.shalizar@urmia.ac.ir, mnegar60@gmail.com

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with varicocele, and current guidelines render specific considerations for selecting candidates for surgical or radiological treatments.

Anti-oxidant compounds react with free radicals in one-electron reactions to prevent oxidative damage. Accordingly, natural anti-oxidants play a crucial role in both disease prevention and adjunctive treatment, while also derogating adverse reactions. Correspondingly, a study of 219 men with varicocele stated promoted sperm concentration, motility, and morphology after 4 months of treatment with L-carnitine and acetyl-L-carnitine.[9]

Alhagi camelorum (AC) is one of the plants in traditional medicine being used to treat metabolic, gastrointestinal, and liver diseases, rheumatic disorder, migraines, and warts. Experimental studies point out that this plant extract reduces body temperature and heart rate. The extract also hinders the action of acetylcholine to relax the muscles, and is beneficial in opening the urinary tract and disposal of kidney stones.[10] It has been found that this plant contains flavonoids, coumarins, alkaloids, and vitamins, all of them have been shown to have repro-protective activities.[11-17]

Within this framework, the present study aimed to unveil the effects of ethanolic extract of the aerial parts of AC on varicocele-linked epididymal sperms and *in vitro* fertilization (IVF) impairments in mature rats.

Materials and Methods

Preparation of the Plant Extract

The aerial parts of AC were collected, washed three times with tap water and two times with distilled water, dried (under a shield), and milled to the fine powder. The AC powder was then soaked in a hydro-alcoholic solvent (70:30 v/v). Afterwards, a rotary evaporator was used to evaporate the filtrate at reduced pressure.[18]

Experimental Protocol

Forty mature male Wistar rats, 4 months old and weighting 187.59 ± 9.83 g, were used in this study. The rats were supplied from the Animal Resources Center, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, and accustomed to an environmentally controlled room (temperature: 23 ± 1 °C, relative humidity: 50 ± 5, and 12 hr light/12 hr dark) for two weeks. Food and water were also provided *ad libitum*. All experimental procedures involving animals were carried out in accordance with the ethical guidelines established by the Ethical Committee for Research on Laboratory Animals, Urmia University, Urmia, Iran. Following two-week acclimatization period, rats were randomly categorized into five groups (n = 8) as follows: Control group: The animals received no medication and the abdominal cavity was opened without inducing varicocele, varicocele group: The abdominal cavity was opened and varicocele was induced; however, the animals received no medication, varicocele + AC $_{\text{high dose}}$ group: The animals received 400 mg/kg/day of AC orally for 60 days following varicocele induction, varicocele + AC $_{\text{medium dose}}$ group: The animals received 100 mg/kg/day of AC orally for 60 days following varicocele induction, and varicocele + AC $_{\text{low dose}}$ group: The rats received 25 mg/kg/day of AC orally for 60 days following varicocele induction.[18]

Surgical Procedures

Varicocele induction was executed under anesthesia induced by an intra-peritoneal injection of 60 mg/kg 10% ketamine hydrochloride and 10 mg/kg 2% xylazine hydrochloride. The renal vein diameter was reduced to 1 mm through left renal vein ligation at the point directly medial to the junction of the adrenal and spermatic veins. Thereafter, the anastomotic branch between the left testicular and left common iliac veins was ligated. A ligature was placed around the probe being then removed, allowing the vein to be expanded within the boundary of the ligature. These procedures resulted in a renal vein diameter reduction to approximately one-half. The abdominal wall and anterior abdominal muscles incisions were then separately repaired.[19]

Spermatology

Cauda epididymal sperms were collected *via* dissecting this region of the epididymis into small pieces, being then placed in 5 mL of modified rat 1-cell embryo culture medium (mR1ECM) supplemented with 4 mg/mL bovine serum albumin (BSA). After that, sperm concentration was determined using a hemocytometer following incubation for 30 minutes at 37 °C in a 5% CO_2 atmosphere.[20]

Sperm motility was analyzed using light microscopy. In this respect, a drop of sperm suspension was situated on a glass slide, being then covered with a coverslip. The number of sperms displaying rapid progressive forward, slow progressive forward, and circumferential movements was recorded in at least 10 microscopic fields.[17]

Epididymal sperms viability, DNA damage, and chromatin quality were also respectively assessed through eosin/nigrosin, acridine orange, and aniline blue staining techniques, as described previously.[17,20,21]

Testicular Oxidant/Anti-Oxidant Status

Testicular total anti-oxidant capacity (TTAC) and malondialdehyde (TMDA) levels in homogenized testicular samples were measured using Benzie and Strain, and Tappel and Zalkin methodologies, respectively.[22,23]

In Vitro Fertilization

Each sexually mature female rat was given an intra-peritoneal injection of 15 IU pregnant mare's serum gonadotropin 48 hours before receiving the 2nd intra-peritoneal injection of 15 IU human chorionic gonadotropin (hCG). Twelve hours after hCG administration, the oviductal ampullae were carefully removed and placed in a Petri dish containing 5 mL mR1ECM having 4 mg/ml BSA. Using a stereomicroscope, the ovulated oocytes were cautiously dissected out and transferred into fertilization droplets under mineral oil.[21]

Following capacitation, the cauda epididymal sperms (1.00×10^6 *per* 1 ml of mR1ECM) were added to the fertilization medium. Later, the fertilization rate was computed after 10 hours based on male and female pronuclei observation. Newly transferred zygotes were subsequently monitored for 120 hours to determine two-cell embryos, blastocysts, and hatched blastocysts percentages.[24]

Statistics

The data were analyzed by one-way analysis of variance followed by Tukey multiple range *post-hoc* analyses using SPSS Software version 22. The Shapiro-Wilk and Levene's tests were also used to examine the normality of data distribution and variances homogeneity, respectively. All values were expressed as the mean ± standard deviation and a probability < 5.00% was considered significant.

Results

Spermatological Findings

As depicted in Table 1, varicocele caused pronounced ($p < 0.05$) decreases in cauda epididymal sperms count, motility, and viability, as well as significant ($p < 0.05$) escalations in percentages of cauda epididymal sperms having damaged DNA and immature nuclear chromatin compared to the control group.

However, varicocele + AC $_{\text{medium dose}}$ and varicocele + AC $_{\text{high dose}}$ groups exhibited marked ($p < 0.05$) improvements in the epididymal sperms quantity and quality compared to the varicocele group.

Biochemical Findings

Experimental varicocele induction in rats resulted in elevated lipid peroxidation in the testicular tissue, as evidenced by a clear upsurge in TMDA levels compared to the controls ($p < 0.05$). While, varicocele treatment in rats with medium and high doses of AC led to statistically marked ($p < 0.05$) reductions in TMDA levels (Table 1).

A statistically evident ($p < 0.05$) decrease was also observed in the TTAC levels of the varicocele group in comparison with control group. Whereas, pronounced ($p < 0.05$) rises were found in the TTAC levels of the varicocele + AC $_{\text{medium dose}}$ and varicocele + AC $_{\text{high dose}}$ groups compared to the varicocele group (Table 1).

Embryological Findings

The IVF rate, as well as two-cell embryos, blastocysts, and hatched blastocysts percentages were decreased significantly ($p < 0.05$) in the varicocele group compared to the control group (Table 2). Meanwhile, conspicuous ($p < 0.05$) higher percentages of IVF success rate, along with two-cell embryos, blastocysts, and hatched blastocysts than varicocele group were observed in the varicocele + AC $_{\text{medium dose}}$ and varicocele + AC $_{\text{high dose}}$ groups (Table 2).

Discussion

Herein, it was revealed that experimental varicocele in a rat model resulted in epididymal sperms quantity and quality drops, leading to the lower IVF outcomes and poor early embryonic development. Accordingly, there is a growing number of reports detailing that varicocele through various mechanisms, including heat stress,[25] androgen deprivation,[26] hypoxia,[27] and increased oxidative stress[28] can cause spermatogenic dysregulation and eventually fertility disorders.[29]

Moreover, it was found that AC, particularly at medium and high doses (100 and 400 mg/kg, respectively), promisingly alleviated varicocele-associated spermatogenesis, as well as early embryonic

Table 1. Spermatological and biochemical findings in different experimental groups.

Groups	Sperm count (10^6/ml)	Sperm motility (%)	Sperm viability (%)	DNA-damaged sperms (%)	Immature sperms (%)	TMDA (µmol/gr tissue)	TTAC (µmol/gr tissue)
Control	53.24 ± 6.30^a	88.47 ± 12.46^a	89.11 ± 5.34^a	6.56 ± 0.67^a	10.76 ± 0.56^a	7.33 ± 0.86^a	8.11 ± 0.65^a
Varicocele	25.11 ± 5.45^b	51.76 ± 3.98^b	52.57 ± 4.87^b	18.05 ± 1.43^b	19.54 ± 1.68^b	14.26 ± 0.82^b	4.12 ± 0.68^b
Varicocele + AC $_{\text{low dose}}$	27.09 ± 2.10^b	54.48 ± 4.06^b	56.69 ± 6.76^b	17.34 ± 0.88^b	17.88 ± 0.83^b	12.62 ± 0.90^b	4.97 ± 0.38^b
Varicocele + AC $_{\text{medium dose}}$	45.65 ± 5.27^c	70.33 ± 7.67^c	76.86 ± 4.91^c	9.65 ± 0.55^c	9.74 ± 0.74^c	8.13 ± 0.52^c	6.23 ± 0.56^c
Varicocele + AC $_{\text{high dose}}$	48.38 ± 4.09^c	73.02 ± 6.03^c	78.90 ± 4.71^c	7.99 ± 0.58^c	7.45 ± 0.63^c	7.89 ± 0.74^c	7.04 ± 0.43^c

AC; *Alhagi camelorum*; TMDA: Testicular malondialdehyde; TTAC: Testicular total anti-oxidant capacity. ^{abc} Values with different superscripts within one column differ significantly at $p < 0.05$.

Table 2. Embryological findings in different experimental groups.

Groups	IVF rate (%)	Two-cell embryos (%)	Blastocysts (%)	Hatched blastocysts (%)
Control	82.54 ± 4.68[a]	76.04 ± 6.23[a]	56.74 ± 3.24[a]	42.18 ± 2.39[a]
Varicocele	51.07 ± 5.00[b]	34.11 ± 4.75[b]	21.36 ± 2.08[b]	19.89 ± 1.73[b]
Varicocele + AC $_{\text{low dose}}$	52.67 ± 3.85[b]	39.08 ± 5.33[b]	24.01 ± 1.52[b]	24.33 ± 2.74[b]
Varicocele + AC $_{\text{medium dose}}$	70.36 ± 7.81[c]	68.92 ± 6.07[c]	42.69 ± 3.17[c]	32.69 ± 4.05[c]
Varicocele + AC $_{\text{high dose}}$	74.91 ± 4.13[c]	70.22 ± 8.06[c]	47.39 ± 5.27[c]	35.74 ± 3.80[c]

AC; *Alhagi camelorum*; IVF: *In vitro* fertilization. [abc] Values with different superscripts within one column differ significantly at $p < 0.05$.

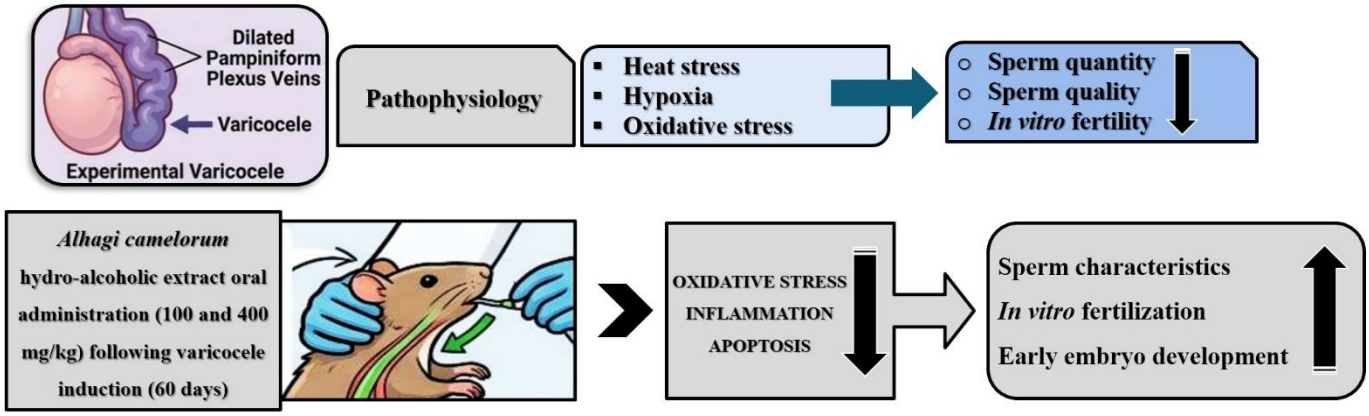

Figure 1. Effect of *Alhagi camelorum* hydro-alcoholic extract on varicocele-induced sperms and *in vitro* fertility disorders in mature rats.

abnormalities. In accordance with these results, recent report has indicted that AC polysaccharides have robust anti-oxidant effects through reducing lipid peroxidation, increasing superoxide dismutase and TAC levels, and lowering MDA levels, thereby regulating immune and anti-oxidant defense system.[30] Likewise, it has been stated that cyto-protective effects of hydro-alcoholic extract of AC are attributed to its high anti-oxidant activities, owing to the presence of flavonoids, phenols, tannins, and saponins.[18]

Similarly, varicocele treatment in rats with medium and high doses of AC (100 and 400 mg/kg, respectively) in this study led to statistically significant reductions in TMDA levels along with marked increases in the TTAC levels compared to the non-treated varicocele-induced ones. Hence, favorable repro-protective effects of hydro-alcoholic extract of AC being observed in the present study can be associated with testicular anti-oxidant defense machinery promotion due to the AC eminent anti-oxidant properties. In agreement with our findings, it has been reported that *Alhagi* extract protects male rats reproductive system against carbon tetrachloride-induced oxidative stress.[31]

Further, it has been well documented that *Alhagi* spp. exhibit notable anti-inflammatory and anti-apoptotic effects.[18,32] Considering the critical role of pro-inflammatory cytokines, as well as pro-apoptotic factors in the pathophysiology of varicocele-induced male reproductive dysfunction,[33] it can be assumed that AC anti-inflammatory and anti-apoptotic activities may play a role in the maintenance of regular spermatogenesis and IVF outcomes improvement in the current study. In alignment with that, several lines of evidence have emphasized that *Alhagi* ethanolic extract beneficial pharmacological activities are likely ascribed to its anti-oxidative, anti-inflammatory, and anti-apoptotic properties.[34,35]

These findings propound that AC hydro-alcoholic extract can palliate the deleterious effects of varicocele on fertility in rat, presumably due to its pharmacological properties, particularly including anti-oxidative, anti-inflammatory, and anti-apoptotic activities (Figure 1). Future research is imperative to further dissect the hormonal profile (*e.g.*, testosterone), additional oxidative stress markers, like xanthine oxidase, as a source of ROS generation,[36] and molecular pathways to fully provide a more comprehensive delineation of the AC repro-protective potential.

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Conflict of Interest

The authors declare no conflict of interest regarding this manuscript.

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