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ORIGINAL ARTICLE

Protective Effects of Ellagic Acid on Oxidative Stress and *in vitro* Fertilization in a Murine Model of Varicocele

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ABSTRACT

Varicocele is defined by abnormal tortuosity and pampiniform plexus veins dilation within the spermatic cord and is the most common surgically correctible cause of male infertility. This study aimed to evaluate the protective effect of ellagic acid (EL) on sperm parameters and *in vitro* fertilization (IVF) in a murine model of varicocele. In this experimental study, 50 mature male mice were randomly divided into five groups (n = 10), including control, varicocele, varicocele with a low dose of EL (25 mg/kg), varicocele with a medium dose of EL (50 mg/kg), and varicocele with a high dose of EL (100 mg/kg). After a 28-day treatment period, malondialdehyde, total anti-oxidant capacity (TAC), sperm parameters, and IVF were evaluated. In the varicocele group, TAC, and sperm count, motility, and viability, as well as zygotes, two-cell embryos, blastocysts, and hatched embryos were significantly reduced compared to the control group. Ellagic acid administration improved sperm parameters, fertilization rate, and embryo development. These findings suggest that EL owing to its anti-oxidant and free radicals scavenging abilities can inhibit the damaging effects of varicocele on fertility in mice.

Introduction

According to several clinical reports, varicocele is observed in 10-20 % of the general male population, 35-40% of men with primary infertility, and up to 80% of men with secondary infertility.[1] Varicoceles are progressive, often appear at puberty, and are more commonly (90%) found in the left side.[2] Despite numerous studies being focused on varicocele, the exact mechanisms by which varicocele induces testicular degeneration and dysfunction, and finally infertility have not completely understood. The suggested mechanisms include reflux of toxic metabolites from adrenal and/or renal origins, impairment of the hypothalamic-gonadal axis, and venous stasis, leading to testicular hypoxia and temperature elevation in testicles.[3] However, it has long been recognized that left-sided varicoceles can have bilateral effects.[4] The pathophysiological influence of the varicocele differs depending on time and the exact mechanism by which this deficiency affects the semen parameters. Often varicocele results in a generalized disruption of sperm production.[5] The relationship between infertility and generation of reactive oxygen species (ROS) has reportedly been established and widely studied.[6] According to several reports, following different toxicological, iatrogenic, and genetic reproductive disorders, mitochondria and plasma membrane of morphologically abnormal spermatozoa produce ROS.[7] In addition, oxidative stress has been shown to affect the integrity of sperm genome by causing high frequencies of single- and double-strand DNA breaks, being often detected in the ejaculates of infertile men.[8]

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Numerous plants, fruits, and berries contain high concentrations of ellagic acid (EL). Pomegranates, well-known sources of EL, have been found to have many biological activities, including anti-diabetic, anti-oxidant, and anti-inflammatory potentials.[9] Formerly, effect of EL on monosodium glutamate-induced testicular damage was evaluated and it was indicated that EL promoted reproductive performance.[10] In another study, EL showed a protective effect against oxidative damage of sperms caused by cigarette smoking and it efficiently reduced the number of abnormal sperms.[11]

The aim of the present study was to examine the effects of EL on oxidative stress, as well as sperms characteristics and *in vitro* fertilizing ability following experimental varicocele in mice.

Materials and Methods

Animals

Fifty mature male mice, 8 weeks olds and weighting 28 ± 5 g, were used in this study. The mice were provided from the Animal Resources Center of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, and acclimatized in an environmentally controlled room (temperature: 21-24 °C and 12 hr light/12 hr dark). Food and water were given *ad libitum*. In this study, all experiments conducted on animals were in accordance with the guidance of ethical committee for research on laboratory animals of Urmia University, Urmia, Iran. Following one-week acclimation, the animals were assigned into five groups (n = 10) as follows: Control group: Mice received no medication and the abdominal cavity was opened; however, there was no varicocele induction, varicocele group: Abdominal cavity was opened; animals underwent varicocele induction, and received no medication, varicocele + high dose of EL (Sigma-Aldrich, Calbiochem, USA) group: Abdominal cavity was opened; animals received 100 mg/kg of EL orally for 28 days, and were varicocele-induced, varicocele + medium dose of EL group: Abdominal cavity was opened; animals received 50 mg/kg of EL orally for 28 days, and were varicocele-induced, and varicocele + low dose of EL group: Abdominal cavity was opened; animals received 25 mg/kg of EL orally for 28 days, and were varicocele-induced.

Varicocele Induction

All surgical procedures were performed under anesthesia by intra-peritoneal injections of 60 mg/kg 10% ketamine hydrochloride and 10 mg/kg 2% xylazine hydrochloride. Diameter of renal vein was reduced to 1 mm; left renal vein ligation was performed at a direct medial to the junction of the adrenal and spermatic veins. Then, the anastomotic branch between the left testicular vein and left common iliac vein was ligated. The ligature was made around the probe, the probe was removed, and the vein allowed expanding within the boundary of the ligature. This procedure leads to a decrease in renal vein diameter to one-half (Figure 1). The incisions of the abdominal wall and anterior abdominal muscles were separately repaired.[12]

Sperm Count

Epididymal sperms were collected by slicing the caudal region of the epididymis into small pieces in 1 ml of human tubal fluid (HTF) + 4 mg/ml bovine serum albumin (BSA) and incubated for 30 min at 37 °C in 5% CO_2 to allow the sperms to swim out of the epididymal tubules. Sperm count was performed with a hemocytometer and results were expressed as millions of sperm/ml.[13]

Sperm Viability

To assess sperm viability, 10 µl of eosin/nigrosin was added to an equal volume of spermatozoa suspension. After 2 min of incubation at room temperature, slides were viewed at 400× magnification. Sperms with altered plasma membranes appeared pink and those with intact plasma membranes remained unstained. In each sample, 200 sperm cells were counted and the percentages of sperm viability were calculated.[14]

Sperm Motility

The percentage of sperm motility was evaluated visually by a light microscope (Olympus Co., Tokyo, Japan) at 400× magnification. For this process, one drop of sperm suspension was placed on a glass slide which was then covered with a lamella. The number of sperms that had rapid progressive forward movement, slow progressive forward movement, and circumferential motion in 10 microscopic fields of vision was recorded and the percentages of motile sperms were obtained. [13]

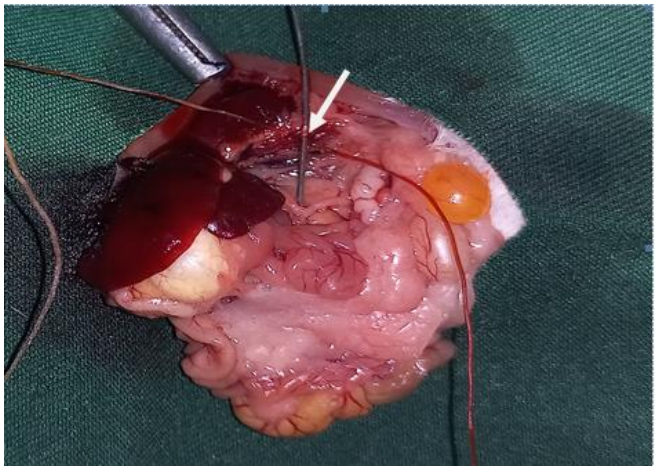

Figure 1. Experimental varicocele induction in a mature male mouse. The tunnel around the renal vein (white arrow) was dissected and partial ligation of left renal vein was performed.

Sperm DNA Denaturation Determination

The acridine orange (AO) staining is a simplified microscopic sperm chromatin structure assay, reflecting sperm chromatin denaturation. A drop of the sperm suspension was spread on the glass slides and allowed to air-dry. All smears were fixed in methanol-acetic acid (1:3 v/v) for 2 hr. The slides were then stained with 3 ml of 19% AO solution in phosphate citrate for 5 min and rinsed with deionized water. The sperms were evaluated by a fluorescence microscope (Zeiss Company, Germany) through two types of staining patterns, including green (double-stranded DNA) and yellow (single-stranded DNA).[15]

Sperm Chromatin Quality Assay

A drop of spermatozoa suspension was spread on glass slides and allowed to air-dry. All smears were fixed in 3% glutaraldehyde in phosphate-buffered saline. The slides were then stained with 5% aqueous aniline blue (AB) and mixed with 4% acetic acid (pH: 3.5) for 5 min. Sperm heads contained immature nuclear chromatin stained blue, whereas those with mature nuclei did not stain. The percentage of spermatozoa stained with AB was determined *via* counting 200 spermatozoa.[16]

Malondialdehyde (MDA) Level Measurement

Testis samples were minced and homogenized under ice-cold conditions. Then, 300 µl of 10% trichloroacetic acid was added to 150 µl of the homogenized sample and centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was transferred to a test tube with 300 µl of 67% thiobarbituric acid (TBA) and incubated at 100 °C for 25 min. After 5 min of cooling, a pink color appeared because of the MDA-TBA reaction. Absorbance was recorded using a spectrophotometer (Pharmacia, Novaspec II, Biochrom, England) at wavelength of 535 nm.[17]

Testicular Total Anti-oxidant Capacity (TAC) Assessment

The assessment is based on ferric reduction anti-oxidant power assay. Briefly, at low pH being provided using acetate buffer (300 mM; pH: 3.6), reduction of Fe^{III}-2,4,6-Tris(2-pyridyl)-s-triazine complex to the ferrous form produces an intensive blue color that could be measured at 593 nm. Aqueous solution of Fe^{II} ($FeSO_4.7H_2O$) and appropriate concentration of freshly prepared ascorbic acid were used as blank and standard solutions, respectively.[18]

Oocyte Pick-up

Each female mouse was received intra-peritoneal injection of 10 IU pregnant mare's serum gonadotropin (Boxmeer, Netherlands) 48 hr prior to an intra-peritoneal injection of 10 IU human chorionic gonadotropin (hCG; Folligon, Netherlands). The animals were euthanized 14 hr after hCG administration and their oviducts ampullae were removed and transferred to a Petri dish contained 1 ml HTF (Sigma, St. Louis, USA) medium plus 4 mg/ml BSA (Sigma, St. Louis, USA). Using a stereo microscope (Model TL2, Olympus Co., Tokyo, Japan), the oocytes were dissected out and moved to the fertilization droplets under mineral oil-containing HTF + BSA medium.

Sperm Preparation

Following male mice euthanasia, caudal epididymis was isolated and placed in a Petri dish containing 1 ml HTF medium combined with 4 mg/ml BSA which had reached equilibrium before. After making several cuts in the tail of the epididymis, sperm output was placed in an incubator at 5% CO_2 and 37 °C for 30 min. The capacitated sperms (1×10^6/1 ml HTF) were then added to the medium. Fertilization was determined about 4 to 6 hr after releasing of sperms through monitoring two pronuclei. After that, granulosa cells were denuded and washed, and the zygotes were transferred into the fresh pre-equilibrated medium and cultured for five more days. Two-cell embryos formation was recorded 24 hr after fertilization and the percentage of blastocyst-stage embryos was computed after 4 and 5 post-fertilization days.

Statistical Analyses

Statistical analyses were performed on all data using one-way ANOVA in SPSS Software version 21. All values were expressed as the mean ± standard deviation. The $p < 0.05$ was considered to be statistically significant.

Results

Sperm Count

Sperm count analysis showed that after varicocele induction the sperm count reduced significantly ($p < 0.05$) in comparison with control group. In the varicocele plus EL groups, increase in the number of sperms was seen, but this increase showed a significant difference only at the doses of 50 and 100 mg/kg EL compared to the varicocele group (Table 1).

Sperm Viability

The percentage of live sperms in the varicocele group decreased and had a significant difference compared to the control group. In the varicocele + EL groups, an increase in the number of live sperms was observed, but this increase showed a significant difference only at the doses of 50 and 100 mg/kg EL compared to the varicocele group ($p < 0.05$; Table 1).

Sperm Motility

In the varicocele group, the percentage of sperm motility decreased significantly ($p < 0.05$) compared to the control group. While, sperm motility in the groups received all doses of EL following varicocele induction showed a significant ($p < 0.05$) increase compared to the varicocele group. There was no significant difference among varicocele plus EL groups (Table 1).

Immature Sperms and Sperms with Damaged DNA

In animals with varicocele, the percentage of immature sperms and sperms with DNA damage showed a significant increase compared to the control group ($p < 0.05$). In the varicocele plus EL groups, a decrease in the percentage of immature sperms and sperms with damaged DNA was observed, but this decrease only at the doses of 50 and 100 mg/kg EL showed a significant difference compared to the varicocele group ($p < 0.05$; Table 1 and Figure 2).

Testicular MDA Level

Varicocele induced lipid peroxidation in the testicular tissue as evidenced by a significant rise in the MDA level in the varicocele group compared to the control group ($p < 0.05$). The MDA levels in the varicocele plus EL groups were lower than those in the varicocele group; however, this reduction was not statistically significant ($p < 0.05$; Table 1).

Testicular TAC Level

The TAC significantly ($p < 0.05$) decreased after varicocele induction in comparison with the control group. In varicocele + high dose of EL and varicocele + medium dose of EL groups, TAC significantly increased compared to the varicocele group ($p < 0.05$; Table 1).

Table 1. Effect of ellagic acid on sperm parameters in different experimental groups.

Groups	Sperm count (10^6/ml)	Sperm motility (%)	Sperm viability (%)	Sperms with DNA damage (%)	Immature sperms (%)	MDA (µmol/gr tissue)	TAC (µmol/gr tissue)
Control	62.24 ± 2.50[a]	89.26 ± 4.28[a]	91.36 ± 10.04[a]	11.83 ± 0.97[a]	6.26 ± 0.24[a]	5.64 ± 0.51[a]	7.04 ± 0.92[a]
Varicocele	31.55 ± 4.17[b]	49.33 ± 6.01[b]	58.03 ± 6.75[b]	21.67 ± 1.01[b]	16.37 ± 0.47[b]	10.34 ± 0.67[b]	3.57 ± 0.52[b]
Varicocele + low dose of ellagic acid	33.56 ± 3.69[b]	52.69 ± 4.93[b]	65.11 ± 8.82[c]	19.38 ± 0.55[b]	15.02 ± 0.66[b]	8.74 ± 0.74[b]	3.81 ± 0.75[b]
Varicocele + medium dose of ellagic acid	49.05 ± 6.33[c]	72.80 ± 5.02[c]	75.03 ± 9.06[c]	16.03 ± 0.96[c]	11.13 ± 0.97[c]	7.06 ± 0.81[b]	5.73 ± 0.30[c]
Varicocele + high dose of ellagic acid	55.27 ± 5.11[c]	87.23 ± 7.86[c]	87.92 ± 8.63[c]	10.59 ± 0.81[c]	6.17 ± 0.22[c]	7.02 ± 0.90[b]	6.13 ± 0.97[c]

[abc] Different superscript letters indicate significant differences ($p < 0.05$) between groups in the same column. MDA: Malondialdehyde; TAC: Total anti-oxidant capacity.

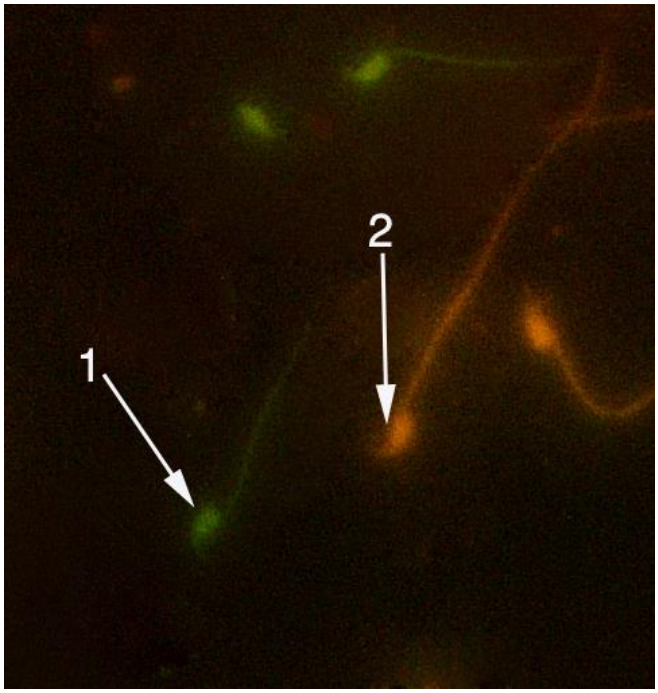

Figure 2. Sperms with normal DNA integrity (1) had green fluorescence and those with diminished DNA integrity (2) had orange-red staining (AO, 400×).

Fertilization and Early Embryonic Development

Varicocele caused a significant ($p < 0.05$) decrease in fertilization rate and percentages of two-cell embryos, blastocysts, and hatched embryos, and a significant increase in the percentage of arrested embryos. Ellagic acid at the doses of 50 and 100 mg/kg caused an increase in fertilization rate and percentages of two-cell embryos, blastocysts, and hatched embryos, and a decrease in percentage of the arrested embryos ($p < 0.05$; Table 2 and Figure 3).

Discussion

In the present study, it was found that sperm protamine-histone transition impairments, DNA missed integrity, plasma membrane peroxidation, and consequentially loss of motility increased by varicocele. Also, our results showed that the quality of sperm reduced by varicocele, resulting in lower *in vitro* fertilization (IVF) rate. Varicocele is an andrological disorder with a high incidence in the general population[19] and is commonly diagnosed among men with infertility.[20] Various factors

Table 2. Effect of ellagic acid on fertilization rate and early embryonic development in different experimental groups.

Groups	Fertilization rate (%)	Two-cell embryos (%)	Blastocysts (%)	Hatched embryos (%)	Arrested embryos (%)
Control	92.25 ± 9.74[a]	85.13 ± 10.06[a]	68.93 ± 8.69[a]	58.14 ± 7.05[a]	13.97 ± 1.37[a]
Varicocele	67.38 ± 4.78[b]	59.07 ± 7.13[b]	41.06 ± 5.11[b]	34.18 ± 4.73[b]	20.04 ± 0.95[b]
Varicocele + low dose of ellagic acid	71.25 ± 7.11[b]	63.92 ± 8.02[b]	46.85 ± 4.74[b]	37.01 ± 6.49[b]	18.33 ± 2.73[b]
Varicocele + medium dose of ellagic acid	84.09 ± 5.36[c]	68.04 ± 9.31[c]	58.07 ± 8.36[c]	53.08 ± 7.33[c]	13.52 ± 1.49[c]
Varicocele + high dose of ellagic acid	84.79 ± 7.04[c]	73.05 ± 6.48[c]	67.40 ± 3.08[c]	53.64 ± 5.37[c]	12.05 ± 1.37[c]

[abc] Different superscript letters indicate significant differences ($p < 0.05$) between groups in the same column.

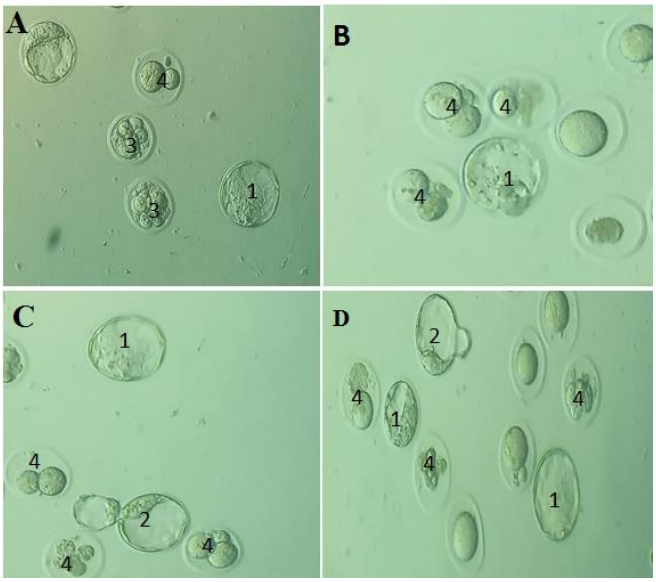

Figure 3. *In vitro* pre-implantation embryo development in control (A), varicocele (B), varicocele + medium dose of ellagic acid (C), and varicocele + high dose of ellagic acid (D) groups (200×). 1: Blastocysts; 2: Hatched embryos; 3: Morulae; 4: Arrested embryos.

associated with varicocele may cause several pathways leading to DNA damage, including heat stress,[21] androgen deprivation,[22] exposure to toxic agents,[23] testicular hypoxia,[24] and increased oxidative stress.[25] There are several independent reports indicating that following high scrotal temperature and/or elevated oxidative stress the cells in spermatogenesis series undergo apoptosis, thus remarkable cellular depletion occurs in seminiferous tubules after severe apoptosis.[26] Moreover, EL was found to be able to reduce testicular tissue destruction, as well as improve sperm *in vitro* fertilizing potential, leading to promoted early embryonic development. Accordingly, it has been reported that EL improves testicular histo-structure in streptozotocin-induced diabetic rats.[11] The EL has also been shown to protect testicles from adriamycin, monosodium glutamate, arsenic, acetic acid, and cisplatin.[27] In the current study, possible protective effects of EL on sperm traits, maturation, DNA integrity, and *in vitro* fertilizing ability following varicocele induction in mice were investigated. The significant loss of sperm count, viability, and motility, as well as fertilization rate, two-cell embryos, blastocysts, and hatched embryos in varicocele group in this study is consistent with several previous studies.[28] When spermatogenesis is damaged, the cytoplasmic extrusion mechanisms are not occurring in a normal condition. So, the released spermatozoa from the germinal epithelium carry surplus residual cytoplasm and considered as functionally defective spermatozoa. It is well-evidenced that the level of ROS generation correlates negatively with sperms quality.[29] In our study, mice in varicocele group showed high percentage of immature sperms. The immature sperms occurrence was found to be associated with imbalance in oxidative status,[30] being in conformity with our findings. Varicocele exerts its pathological effects in a higher degree by poor spermatogenesis and maturation arrest, and in a lower extent through anti-oxidant defense system debilitation.[31] While, EL treatment attenuated varicocele-induced spermatogenesis disruption, and boosted anti-oxidative defensive mechanisms. There are overwhelming reports indicating that any disorder resulting in a failure in epididymal sperm maturation, causes impaired sperm fertilizing ability.[32] Developments of abilities for forward motility, undergoing capacitation, and penetrating the zona pellucida of the oocyte are examples of the several important properties, which the spermatozoa acquire during epididymal sperm passage. Our observations revealed that in varicocele group the immature sperms increased remarkably and the IVF outcomes correlated reversely with this finding. On the other hand, it seems that the plasma membrane unsaturated fatty acids of notable numbers of sperms underwent a severe damage following varicocele induction. These unsaturated fatty acids are essential to give the plasma membrane the fluidity being needed to participate in the membrane fusion events being associated with fertilization. Considering that, the membrane fluidity reduction and the consequent loss of sperm function can lead to IVF success rate decrease and embryonic growth retardation. Additionally, the ability of embryo to survive appears to be negatively correlated with the DNA damage level in the germ line. Former reports showed that sperms having damaged DNA are impotent to fertilize the oocyte.[33] Correspondingly, in our study varicocele caused marked

DNA damage in sperms, leading to elevated embryo growth arrests. Interestingly, administration of EL, particularly at the doses of 50 and 100 mg/kg, could inhibit DNA damage formation in sperm cells, resulting in IVF along with *in vitro* pre-implantation embryo development furtherance. Similarly, it has been shown that EL has a repro-protective activity, being linked to free radicals over-generation inhibition and lipid peroxidation suppression.[34]

The cryo-protective and anti-oxidative properties of EL have also been formerly reported in several studies, confirming its promising effect against oxidative stress-related disorders, especially reproductive ones.[35,36] This study lays the groundwork for future studies to unearth the detailed EL-linked protective mechanisms in reproductive injuries, particularly varicocele.

In conclusion, the results indicated that EL may have beneficial effects against varicocele-induced oxidative stress, as well as sperm damage and early embryonic development failure in mice.

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Conflict of Interest

The authors declare that they have no competing interests.

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