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Protective Effects of Royal Jelly on Oxidative Stress and *In Vitro* Fertilization Outcomes in a Wistar Rat Model of Varicocele

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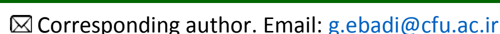
ABSTRACT

Varicocele, characterized by the abnormal tortuosity and dilation of the pampiniform plexus veins in the spermatic cord, is the most common surgically correctable cause of male infertility. This study aimed to evaluate the protective effects of royal jelly on sperm parameters and *in vitro* fertilization (IVF) outcomes in a rat model of varicocele. Thirty adult male rats were randomly divided into five groups (n = 6): control, varicocele, varicocele with low-dose royal jelly (50 mg/kg), varicocele with medium-dose royal jelly (100 mg/kg), and varicocele with high-dose royal jelly (200 mg/kg). After a 28-day treatment period, malondialdehyde (MDA), total antioxidant capacity (TAC), sperm parameters, and IVF outcomes were assessed. The varicocele group showed significant reductions in TAC, sperm count, motility, viability, and embryonic development outcomes, including zygote formation, two-cell embryos, blastocysts, and hatched embryos, compared to the control group. Royal jelly treatment improved sperm parameters, fertilization rates, and embryo development in a dose-dependent manner. These findings suggest that royal jelly mitigates the adverse effects of varicocele on fertility in rat, likely due to its antioxidant and free radical scavenging properties.

Introduction

Varicocele, a condition characterized by the abnormal dilation of the pampiniform venous plexus, is one of the most common causes of male infertility, affecting approximately 15% of the general male population and up to 40% of men with primary infertility.[1] The pathophysiological mechanisms underlying varicocele involve increased testicular temperature, hypoxia, and elevated oxidative stress (OS), which lead to impaired spermatogenesis and decreased sperm quality.[2] Oxidative stress, marked by an imbalance between reactive oxygen species (ROS) and antioxidants, induces lipid peroxidation, protein oxidation, and DNA damage, all of which compromise testicular function and fertility outcomes.[3]

Royal jelly (RJ), a secretion produced by worker honeybees, is a natural substance rich in bioactive compounds, including proteins, vitamins, lipids, and flavonoids. Its antioxidative and anti-inflammatory properties have been demonstrated in various experimental models, showing potential in reducing oxidative damage and promoting tissue repair.[4] Previous studies have indicated that RJ may enhance male reproductive parameters by improving sperm motility, count, and morphology, suggesting its potential application in managing infertility associated with oxidative stress.[5] In vitro fertilization (IVF) is an essential assisted reproductive technology for addressing infertility; however, its success depends heavily on the quality of gametes. Varicocele-induced



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oxidative damage can compromise sperm quality, negatively impacting fertilization rates and embryo development.[3] Interventions aimed at mitigating oxidative stress may thus improve IVF outcomes in varicocele-associated infertility.

This study aims to evaluate the protective effect of RJ against oxidative stress and reproductive dysfunction in a rat model of varicocele. Specifically, the research investigates its influence on testicular oxidative markers and IVF outcomes, providing insights into its potential as a therapeutic agent for enhancing fertility in varicocele-associated cases.

Materials and Methods

Animals

A total of 30 adult male Wistar rats (200–250 g) were obtained and maintained under standard laboratory conditions with a 12-hour light/dark cycle, controlled temperature (22 ± 2 °C), and ad libitum access to food and water. 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 = 6) as follows: Control group; rat 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 and high dose of RJ group: Abdominal cavity was opened; animals received 200 mg/kg of RJ orally for 28 days, and were varicocele-induced, varicocele and medium dose of RJ group: Abdominal cavity was opened; animals received 100 mg/kg of RJ orally for 28 days, and were varicocele-induced, and varicocele and low dose of RJ group: Abdominal cavity was opened; animals received 50 mg/kg of RJ orally for 28 days, and were varicocele-induced.

Varicocele Induction

Varicocele was surgically induced in the experimental groups following a previously described protocol.[6] Briefly, under anesthesia with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg), the left renal vein was partially ligated to simulate venous stasis. Sham-operated rats underwent the same procedure without vein ligation (Figure 1).

Administration of Royal Jelly

Royal jelly (provided by a certified supplier) was freshly prepared and administered to the RJ-treated group daily. The dosage was based on previous studies demonstrating its efficacy in oxidative stress models.[5]

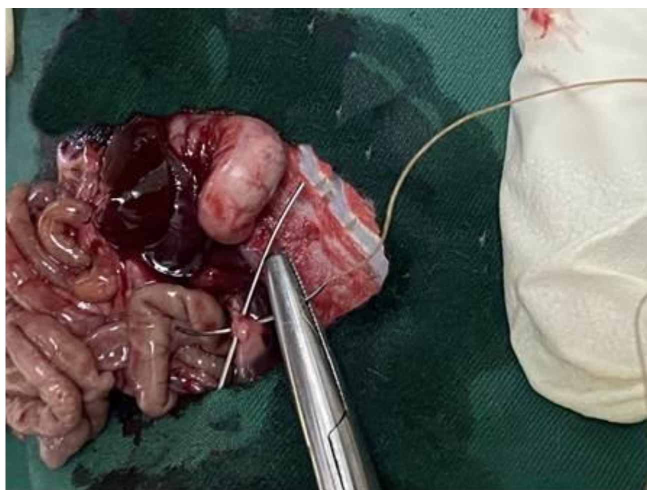

Figure 1. Experimental varicocele induction in a mature male rat. The tunnel around the renal vein was dissected and partial ligation of left renal vein was performed.

Sperm Count

Epididymal sperm were collected by slicing the caudal region of the epididymis into small pieces in 1 ml of human tubal fluid (HTF) supplemented with 4 mg/ml bovine serum albumin (BSA). The suspension was incubated for 30 minutes at 37 °C in an atmosphere of 5% CO_2 to allow the sperm to swim out of the epididymis tubules. Sperm count was determined using a hemocytometer. A 10 µl aliquot of the sperm suspension was loaded into the hemocytometer, and sperm were counted under a light microscope at ×200 magnification. Results were expressed as millions of sperm per milliliter (million/ml). The average of three independent counts was calculated for each sample to ensure accuracy.[7]

Sperm Viability, Motility, and DNA Integrity Assessment

To evaluate sperm viability, 10 µl of eosin/nigrosin stain was mixed with an equal volume of spermatozoa suspension. The mixture was incubated for 2 minutes at room temperature to allow staining. A smear was then prepared on a clean glass slide and observed under a light microscope at ×400 magnification. Sperm with altered plasma membranes appeared pink (stained), indicating non-viable sperm, while those with intact plasma membranes remained unstained, and indicating viable sperm. For each sample, 200 sperm cells were counted, and the percentage of viable sperm was calculated.[8]

Sperm motility was evaluated visually using a light microscope (Olympus Co., Tokyo, Japan) at ×400 magnification. A drop of the sperm suspension was placed on a clean glass slide and covered with a coverslip. The motility assessment was performed by observing 10 microscopic fields. Sperm were categorized based on their movement into the following groups: Rapid progressive forward movement, Slow progressive forward movement, Non-progressive or circumferential

motion. The number of motile sperm in each category was recorded, and the percentage of total motile sperm was calculated [7]

Acridine orange (AO) staining was used as a microscopic assay to evaluate sperm chromatin structure and detect DNA denaturation. A drop of the sperm suspension was spread evenly on a glass slide and allowed to air-dry. The slides were then fixed in a methanol-acetic acid solution (1:3 v/v) for 2 hours at room temperature. After fixation, each slide was stained with 3 ml of 19% AO solution in phosphate-citrate buffer for 5 minutes and subsequently rinsed gently with deionized water to remove excess stain. The stained sperm samples were examined under a fluorescence microscope (Zeiss Company, Germany). Sperm DNA integrity was determined based on fluorescence patterns: Green fluorescence indicated intact double-stranded DNA. Yellow fluorescence indicated denatured single-stranded DNA.

The proportions of sperm exhibiting each staining pattern were recorded to assess chromatin structure and DNA denaturation levels.[9]

Sperm Chromatin Quality Assay

A drop of spermatozoa suspension was evenly spread on a clean glass slide and allowed to air-dry. The slides were then fixed in 3% glutaraldehyde prepared in phosphate-buffered saline (PBS) for 30 minutes at room temperature. Following fixation, the slides were stained with 5% aqueous aniline blue (AB) solution mixed with 4% acetic acid (pH 3.5) for 5 minutes. After staining, the slides were gently rinsed with distilled water and air-dried. Sperm heads with immature nuclear chromatin stained blue, indicating incomplete chromatin condensation, while those with mature nuclei remained unstained. A total of 200 spermatozoa per slide were evaluated under a light microscope, and the percentage of AB-positive (blue-stained) spermatozoa was calculated.[10]

MDA and TAC Assay

In order to malondialdehyde (MDA) Level measurement, tissue samples were minced and homogenized in an ice-cold buffer to ensure the preservation of enzymatic activity and prevent oxidation. To 150 µl of the homogenized sample, 300 µl of 10% trichloroacetic acid (TCA) was added, and the mixture was centrifuged at 1,000 rpm for 10 minutes at 4°C. The resulting supernatant was carefully transferred to a test tube, mixed with 300 µl of 0.67% thiobarbituric acid (TBA), and incubated in a water bath at 100°C for 25 minutes to allow the formation of the MDA-TBA adduct. Following incubation, the tubes were cooled for 5 minutes at room temperature, during which a pink color developed as a result of the reaction. The absorbance of the solution was measured at a wavelength of 535 nm using a spectrophotometer (Pharmacia Novaspec II, Biochrom, England). MDA levels, indicative of lipid peroxidation, were expressed in nanomoles per milligram of protein, calculated using the extinction coefficient of the MDA-TBA complex.[11]

The total antioxidant capacity (TAC) of testicular tissue was assessed using the ferric reduction antioxidant power (FRAP) assay. Briefly, testicular homogenates were prepared and incubated in acetate buffer (300 mM, pH 3.6) to provide an acidic environment. In this medium, the reduction of Fe^{3+}–2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) complex to the ferrous form (Fe^{2+}) results in the formation of an intense blue color, which is directly proportional to the antioxidant capacity of the sample.

The reaction was measured spectrophotometrically at 593 nm. An aqueous solution of Fe^{2+} (prepared from $FeSO_4 \cdot 7H_2O$) was used as the standard, and freshly prepared ascorbic acid was used to generate a standard curve for quantification. The antioxidant capacity was determined by comparing the absorbance of the sample with the standard curve, and results were expressed as µmol ascorbic acid equivalents per milligram of protein.[12]

Ovulation Method

Each female mouse received an intraperitoneal injection of 10 IU pregnant mare's serum gonadotropin (PMSG; Boxmeer, Netherlands) 48 hours before an additional intraperitoneal injection of 10 IU human chorionic gonadotropin (hCG; Folligon, Netherlands). The animals were euthanized 14 hours after hCG administration. The oviducts were carefully removed, and the ampullae were transferred to a Petri dish containing 1 ml of human tubal fluid (HTF) medium (Sigma, St. Louis, USA) supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma, St. Louis, USA). Using a stereo microscope, oocytes were carefully dissected from the oviducts and transferred into fertilization droplets, which were placed under mineral oil in HTF+BSA medium.

Sperm Preparation

Following euthanasia of the male mice, the caudal epididymis was isolated and placed in a Petri dish containing 1 ml of HTF medium supplemented with 4 mg/ml BSA, which had been pre-equilibrated at 37°C. Several small cuts were made in the tail of the epididymis to release sperm. The Petri dish was then placed in an incubator at 5% CO_2 and 37 °C for 30 minutes to allow sperm capacitation. After capacitation, the sperm concentration was adjusted to 1×10^6 sperm/ml in HTF medium. The capacitated sperm were added to the fertilization droplets containing oocytes. Fertilization was assessed 4 to 6 hours later by monitoring for the presence of two pronuclei. After fertilization, granulosa cells were

removed by gentle pipetting, and the resulting zygotes were washed and transferred into fresh, pre-equilibrated medium for continued culture. The embryos were cultured for an additional five days. Formation of two-cell embryos was recorded 24 hours post-fertilization, and the percentage of blastocyst-stage embryos was calculated on days 4 and 5 post-fertilization.

Statistical Analyses

Statistical analyses were performed using one-way ANOVA with SPSS Software version 21. All data were expressed as the mean ± standard deviation (SD). A *p*-value of less than 0.05 was considered statistically significant.

Results

Sperm Count, Motility, and Viability

Sperm count analysis revealed a significant reduction ($p < 0.05$) in sperm count following varicocele induction compared to the control group. In the varicocele and RJ groups, an increase in sperm count was observed. However, this increase was statistically significant only at medium and high RJ doses when compared to the varicocele group (Table 1). In the varicocele group, the percentage of sperm motility decreased significantly ($p < 0.05$) compared to the control group. However, sperm motility significantly increased ($p < 0.05$) in all RJ-treated groups following varicocele induction compared to the varicocele group. No significant differences were observed among the varicocele + RJ groups (Table 1). The percentage of alive sperm significantly decreased in the varicocele group compared to the control group. In the varicocele + RJ groups, an increase in the percentage of alive sperm was observed. However, this increase was statistically significant only at medium and high RJ doses compared to the varicocele group ($p < 0.05$; Table 1).

Immature Sperms and Sperms with Damaged DNA

In animals with varicocele, the percentage of immature sperm and sperm with DNA damage significantly increased compared to the control group ($p < 0.05$). In the varicocele + RJ groups, a reduction in the percentage of immature sperm and sperm with DNA damage was observed. However, this reduction was statistically significant only at medium and high RJ doses compared to the varicocele group ($p < 0.05$; Table 1).

Testicular MDA and TAC Level

Varicocele-induced lipid peroxidation in testicular tissue was demonstrated by a significant increase in MDA levels in the varicocele group compared to the control group ($p < 0.05$). Although MDA levels were lower in the varicocele + RJ groups than in the varicocele group, the reduction was not statistically significant ($p > 0.05$; Table 1). The TAC levels were significantly decreased ($p < 0.05$) following varicocele induction compared to the control group. However, TAC levels significantly increased in the varicocele + high-dose RJ and varicocele + medium-dose RJ groups compared to the varicocele group ($p < 0.05$; Table 1).

Fertilization and Early Embryonic Development

Varicocele resulted in a significant decrease ($p < 0.05$) in the fertilization rate and the percentages of two-cell embryos, blastocysts, and hatched embryos, accompanied by a significant increase in the percentage of arrested embryos. Treatment with medium and high doses of royal jelly led to a significant increase ($p < 0.05$) in the fertilization rate and the percentages of two-cell embryos, blastocysts, and hatched embryos, along with a decrease in the percentage of arrested embryos (Table 2, Figure 2).

Discussion

Varicocele, a prevalent andrological disorder, is frequently diagnosed in infertile men.[13] This study demonstrated that sperm count, TAC, sperm motility, and viability were significantly reduced in the varicocele group compared to the control group. Treatment with RJ improved these parameters in all varicocele groups, with significant effects observed at medium and high doses. Similarly, Naughton *et al.* (2001) reported that varicocele induces DNA damage and apoptosis, leading to cellular depletion in seminiferous tubules.[2] Consistent with these findings, Asadi *et al.* (2019) reported that RJ improves sperm quality and IVF outcomes by mitigating testicular tissue damage.[14]

Table 1. Effect of royal jell 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	58.12 ± 1.2^a	90.22 ± 3.17^a	85.15 ± 9.41^a	8.96 ± 0.81^a	5.81 ± 0.33^a	4.91 ± 0.63^a	8.42 ± 0.78^a
VC	27.33 ± 2.52^b	50.03 ± 4.31^b	54.23 ± 4.7^b	18.62 ± 2.34^b	14.11 ± 0.23^b	12.13 ± 0.54^b	3.72 ± 0.18^b
VC + RJ(L)	28.41 ± 4.29^b	54.01 ± 3.41^b	63.02 ± 7.6^b	16.44 ± 0.63^b	13.31 ± 0.22^b	9.21 ± 0.63^b	4.11 ± 0.20^b
VC + RJ(M)	46.25 ± 5.61^c	69.23 ± 6.30^b	69.31 ± 4.62^c	13.75 ± 1.72^c	11.29 ± 1.03^c	8.15 ± 0.42^b	5.93 ± 0.30^c
VC + RJ(H)	52.32 ± 4.15^c	85.51 ± 4.64^b	73.81 ± 5.9^c	11.80 ± 0.63^c	8.73 ± 1.41^c	7.91 ± 0.19^b	7.12 ± 0.26^c

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

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

Groups	Fertilization rate (%)	Two-cell embryos (%)	Blastocysts (%)	Hatched embryos (%)	Arrested embryos (%)
Control	87.43 ± 8.21[a]	75.42 ± 6.71[a]	67.92 ± 4.71[a]	53.24 ± 9.64[a]	10.85 ± 6.04[a]
VC	59.64 ± 3.4[b]	48.55 ± 3.62[b]	39.28 ± 3.52[b]	29.64 ± 7.1[b]	22.13 ± 1.92[b]
VC + RJ(L)	63.18 ± 3.25[b]	56.84 ± 6.03[b]	43.23 ± 1.21[b]	31.21 ± 4.52[b]	20.46 ± 1.26[b]
VC + RJ(M)	71.02 ± 6.5[c]	69.82 ± 7.35[c]	50.16 ± 4.7[b]	41.73 ± 8.13[b]	18.24 ± 0.7[b]
VC + RJ(H)	74.91 ± 3.06[c]	70.32 ± 1.64[c]	55.40 ± 3.01[c]	45.68 ± 4.14[c]	15.65 ± 0.14[c]

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

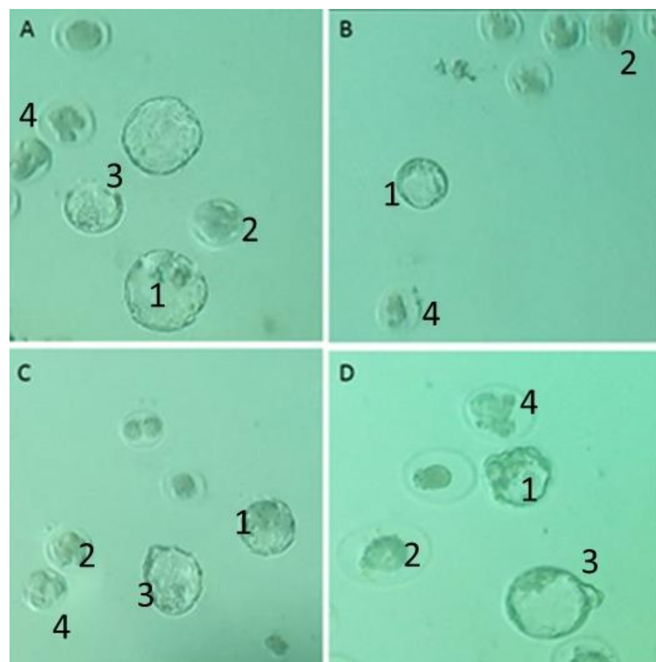

Figure 2. *In vitro* pre-implantation embryo development in control (A), varicocele (B), varicocele + medium dose of royall jell(C), and varicocele + high dose of royal jell (D) groups (×200). 1: Blastocysts; 2: Hatched embryos; 3: Morula; 4: Arrested embryos.

The study also showed that the varicocele group had significantly higher levels of sperm DNA damage, immature sperm, and malondialdehyde (MDA) compared to the control group. RJ treatment reduced these parameters, with significant improvements noted at medium and high doses. The underlying mechanisms of varicocele involve heat stress and increased oxidative stress, both of which contribute to DNA damage, apoptosis, and cellular depletion in seminiferous tubules.[15,16]

Moreover, the varicocele group exhibited significant reductions in fertilization rate, percentages of two-cell embryos, blastocysts, and hatching. RJ treatment significantly improved these outcomes, particularly at medium and high doses. Conversely, embryonic arrest increased in the varicocele group but decreased in the RJ-treated groups, with significant reductions observed at high doses. These findings align with Cho *et al*. (2015), who reported that varicocele reduces fertility by impairing sperm quantity and quality.[17]

RJ has demonstrated potential in reducing testicular tissue damage, improving sperm quality, and enhancing IVF outcomes by promoting early embryo development. Studies have shown that RJ improves testicular histology in animal models.[15] Additionally, there is a growing number of studies reporting that RJ enhances fertilization and zygote growth by reducing oxidative stress, corroborating the results of this study.[18-20]

This study underscores the protective effects of royal jelly (RJ) against varicocele-induced reproductive damage and oxidative stress, highlighting its potential as a therapeutic agent. Future research should delve into the detailed mechanisms by which RJ exerts its beneficial effects, particularly in mitigating reproductive injuries and enhancing fertility outcomes.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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