Resveratrol decreases apoptosis and NLRP3 complex expressions in experimental varicocele rat model

Elnaz Hajipour 1, Farideh Jalali Mashayekhi 2, Ghasem Mosayebi 3, Maryam Baazm 1*, Adib Zendedel 4, 5

1 Department of Anatomy, School of Medicine, Arak University of Medical Sciences, Arak, Iran
2 Department of Genetics and Biochemistry, School of Medicine, Arak University of Medical Sciences, Arak, Iran
3 Department of Immunology and Microbiology, School of Medicine, Arak University of Medical Sciences, Arak, Iran
4 Department of Anatomy, School of Medicine, Giulan University of Medical Sciences, Rasht, Iran
5 Institute of Neuroanatomy, Medical Clinic, RWTH Aachen University, 52074 Aachen, Germany

ABSTRACT

Objective(s): Varicocele is an abnormal dilatation in the testicular vein, which can cause hypoxia, reactive oxygen species accumulation, elevation in testicular temperature, and promote apoptosis and increase proinflammatory cytokine production. According to the varicocele pathophysiology, it is possible that a group of cytosolic receptors called nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes also involve in varicocele pathogenesis. Due to the important role of antioxidant in decreasing the testis tissue damage, in this study we investigated the protective effect of resveratrol (RES) on NLRP3 complex and apoptosis in experimental varicocele rats.

Materials and Methods: In this study, 40 male Wistar rats were randomly divided into 5 groups (8 rats in each group): Control, experimental left varicocele (ELV), ELV + ethanol, ELV + 20 mg/kg RES and ELV + 50 mg/kg RES. Varicocele was induced by partial ligation of the left renal vein. Three months after varicocele induction, RES was orally administered to rats for 1 month. The expression levels of NLRP3, ASC, NLRP1, NLRP2, NLRP3 and AIM), among different members of inflammasome (NLRP1, NLRP2, NLRP3 and AIM), the nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NALP3) inflammasome is involved in varicocele pathogenesis. The NLRP3 inflammasome is a complex of NLRP3, apoptosis associated speck-like protein (ASC) and caspase-1 (8). Once NLRP3 is activated, it cleaves procaspase-1 to caspase-1 and then caspase-1 converts the pro-interleukin-1β and 18 to their active forms (7, 9). Accumulation of these pro-inflammatory cytokines in the testis could disrupt testicular function, spermatogenesis and androgen production (10).

Conclusions: RES by reducing inflammatory factors and decreasing apoptosis might be used as adjuvant therapy to reduce varicocele complication.

Introduction

Varicocele is an abnormal dilatation in the testicular vein, which can cause hypoxia, reactive oxygen species accumulation, elevation in testicular temperature, and promote apoptosis and increase proinflammatory cytokine production. Considering the varicocele pathophysiology, it is possible that a group of cytosolic receptors called nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes also involve in varicocele pathogenesis. Due to the important role of antioxidant in decreasing the testis tissue damage, in this study we investigated the protective effect of resveratrol (RES) on NLRP3 complex and apoptosis in experimental varicocele rats.

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Conclusions: RES by reducing inflammatory factors and decreasing apoptosis might be used as adjuvant therapy to reduce varicocele complication.
Antioxidant therapy is one of the appropriate treatment modality for reducing the effects of varicocele on male fertility (11). Resveratrol (RES) (3,5,4’-tri hydroxy-trans-stilbene) that is found in some plants such as mulberries, peanuts and grapes has a widespread biological activities such as antioxidant, anti-inflammatory and anti-apoptosis (12). Previous studies demonstrated that RES can improve sperm parameters and histological changes occurred in the testis tissue of the experimental left varicocele rats (13, 14). In the current study, we investigated the protective effect of RES against inflammation and apoptosis induced by varicocele in rats.

Materials and Methods

Animals

Research and animal care were approved by the Ethics Committee of Arak University of Medical Sciences. In vivo experiments were performed on 14-weeks old adult male Wistar rats (250±20 g, Pasteur, Iran). Animals were housed at 24°C and controlled conditions with free access to water and food.

The animals were divided randomly into following groups prior to the operation procedure (8 rats in each group): Control, Experimental left varicocele induction, Experimental left varicocele induction + RES50 treatment (ELV + 20 mg/kg RES), Experimental left varicocele induction + RES100 treatment (ELV + 50 mg/kg RES), Experimental left varicocele induction + vehicle control (ELV + 10 % ethanol).

Surgical procedure

Rats were anaesthetized with intraperitoneal (IP) injection of 100 mg/kg ketamine and 10 mg/kg xyloazine (both from Alfasan, Iran). After shaving and cleaning the surgical area, a midline incision was performed and the left renal and spermatic veins were dissected from around tissue. A 0.85 mm wire was placed parallel to the left renal vein and a 4/0 silk suture was used for ligation around the wire and left renal vein proximal to the inferior vena cava (IVC). Then the wire was carefully removed and the abdominal wall was sutured (15).

Resveratrol administration

3 months after surgery, RES was given to the animals (20 and 50 mg/kg/daily by gavage) for one month. The animals were sacrificed under deep anesthesia and immediately transcardinally perfused with 150-200 ml phosphate-buffered saline (PBS; Sigma, Germany). Testis was removed and transferred to the liquid nitrogen and stored at -70°C until further use.

RNA isolation and cDNA synthesis

After sampling, the expression of NLRP3, ASC, caspase-1, Bax and Bcl2 genes in all groups was studied by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Total RNA was extracted using peqGold RNA TriFast (PeqLab, Germany) according to the manufacturer’s instructions. The RNA pellet was dissolved in diethylpyrocarbonate-treated water (DEPC treated water; Sigma, Iran) and quantified spectrophotometrically at 260 nm wavelength. The integrity of the extracted total RNA was assessed by agarose gel electrophoresis and verified by the presence of the 28S and 18S rRNA bands. Immediately after RNA preparation, 2 μg of total RNA was used for cDNA synthesis in a total volume of 20 μl by using RevertAid™ First Strand cDNA Synthesis Kit (Aryatous, Iran). The cDNA was stored at -80°C until use.

Quantitative RT-PCR

qRT-PCR was carried out using the Life Cycle Real time PCR (Roche, USA). qRT-PCR was performed in a total volume of 20 μl containing 2 μl of cDNA (5-fold diluted), 0.5 μl of 5 mmol/l solutions of each of the forward and reverse primers, and 10 μl of 2xSYBR green DNA PCR Master Mix. Each sample was loaded in duplicate.

The primer sequences used for amplifications are summarized in Table 1. Melt curve analysis was performed after each run to check for the presence of non-specific PCR products and primer dimers. All samples were normalized against Cyclo A (internal control) using the comparative CT method (ΔΔCT).

Statistical analysis

The results are expressed as means±standard deviation (SD). The statistical significance between the mean values was determined by one-way analysis of variance (ANOVA) followed by a Tukey’s post-hoc test with P<0.05 as the statistically significant criterion.

Table 1. Primer sets used for amplification

| Genes     | Primers sequences (5’ to 3’)               | Product length (bp) |
|-----------|-------------------------------------------|---------------------|
| NLRP3     | Forward: TCTGTTGATCGGCTGCAGAT             | 118                 |
|           | Reverse: GCCCTTTGAACTTGGCAGT              |                     |
| ASC       | Forward: GCTGAAGTTGACCCCATAG             | 174                 |
|           | Reverse: ACAGTGAGCTCCAAGCGA              |                     |
| Caspase-1 | Forward: CAAAGACCTTTTGATG                 | 154                 |
|           | Reverse: CTTGAGGAAACACTAGGT               |                     |
| Bax       | Forward: GCTAGGTTTCCATAGG                 | 212                 |
|           | Reverse: TCCAGATCGAACCAGG                 |                     |
| Bcl2      | Forward: AGCGTTAACACAGGAGG                | 118                 |
|           | Reverse: CCAAAAAGGATCCAGG                 |                     |
| Cyclo A   | Forward: GCGAAATGCTGGACCAAAAGC            | 196                 |
|           | Reverse: TCGAGTTTAACTTTTACAGG             |                     |

NLRP3: Nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3, ASC: Apoptosis associated speck-like protein.
**Results**

**Resveratrol down-regulates NLRP3 inflammasome components in varicocelezed rats**

To verify the anti-inflammatory effects of RES-treated animals, testis tissue was analyzed for gene expression of NLRP3 using real time PCR. As expected, experimental varicocele induced a significant up-regulation of NLRP3 gene expression 3 months after partial ligation of left renal vein ($P \leq 0.05$). The mRNA level of NLRP3 was significantly lower in RES$_{20}$ ($P \leq 0.05$) and RES$_{50}$ ($P \leq 0.01$) treated rats compared with the varicocelezed and ethanol-gavaged animals (Figure 1). In the line with this finding the gene expression of ASC and caspase-1 revealed (i) higher mRNA level of these genes in varicocele-induced and vehicle-treated animals and (ii) lower gene expression in RES-treated animals (Figures 2 and 3). Although western blot was not performed, RES possibly ameliorates varicocele-induced inflammation in this model.

**Resveratrol prevents cell apoptosis**

In varicocele, both death receptor and mitochondrialmediate apoptosis are responsible for testis tissue apoptosis (16). We evaluated whether different doses of RES could prevent apoptosis. For this purpose, the gene expression of Bax (a pro-apoptotic member of Bcl2 family) and Bcl2 (an anti-apoptotic gene) was analyzed in the testis tissue. The real time PCR analysis revealed that mRNA level of Bax was significantly lower in the RES$_{20}$ ($P \leq 0.001$) and RES$_{50}$ ($P \leq 0.001$) compared with the varicocelezed and ethanol-gavaged animals (Figure 4), and Bcl2 gene expression was meaningfully higher only in the RES$_{50}$ treated rats ($P \leq 0.01$) (Figure 5). These data strongly implicated that RES prevents apoptosis after varicocele in a dose dependent manner.
The expression level of Bcl2 is significantly potentiated in resveratrol 50 mg/kg (RES) compared to other treatment group. **: P<0.01

**Discussion**

The pathogenesis of varicocele, one of the common causes of male infertility, is not well understood and findings show that it is multifactorial (17). Due to the limitation in study design for investigation of human testis structure during varicocele, animal models of varicocele are valuable tools in this regard (15). In the present study, varicocele was induced by partial ligation of the left renal artery, a common model of varicocele which mimics the compression of the left renal vein between the aorta and the superior mesenteric artery (18).

Varicocele by producing stress oxidative and ROS accumulation in the testis tissue impairs spermatogenesis and male fertility (19). Therefore, antioxidant therapy has been considered as a treatment modality for varicocele (20). In this study, we used RES, a natural polyphenolic compound derived from grape (21) for reducing inflammation and apoptosis in the testis tissue during varicocele.

In the present study, we showed that three months after varicocele induction, the levels of NLRP3 inflammasome components including NLRP3, ASC and caspase-1 were up-regulated and RES administration for one month could decrease elevated levels of these genes. NLRP3 inflammasome is activated by pathological stress including stroke (22), spinal cord injury (23), and diabetic mellitus (24). Previous studies have shown that varicocele increases pro-inflammatory and inflammatory cytokines such as interleukin-1 (25), and 6 as well as tumor necrosis factor (26) and hypoxia induced factor (27, 28). In this study, we showed that RES by its anti-inflammatory properties decreases NLRP3 inflammasome activity in the testis tissue. RES by activation of sirtuin-1 (Sirt-1) signaling, can inhibit nuclear factor kappa beta (NF-kb) and then NLRP3 inflammasome activity (29). The dose dependent anti-apoptotic effect of RES on varicoceleized testis was another finding of this study. Our study revealed that the apoptosis rate decreases by using high RES concentrations. Low and high doses of RES was effective to decrease Bax gene expression. However, Bcl2 gene expression was higher at high dose compared to low dose. RES has been used in different doses in various studies. Liu et al. showed that 200 mg/kg administration of RES could decrease apoptosis induced by the spinal cord injury in rats (30), and Mendes et al. claimed that RES at the concentration of 300 mg/kg was able to improve sperm quality and decrease TUNEL positive cells in the experimental left varicocele rats; however, this dose could not decrease testicular levels of malondialdehyde (13). On the other hand, Oriquet al. observed that intraperitoneal injection of RES at the doses of 1 and 10 mg/kg improved sperm motility in the hyperthyroid rats (31). As mentioned previously, RES is a Sirt-1 activator and this activation could repress p53-dependent apoptosis. By this mechanism, RES can prevent cardiomyocytes from apoptosis induced by hypoxia (32). This polyphenol also exerts its anti-apoptotic properties by inhibiting caspase-7 activity in neuroblastoma cell line exposed to paclitaxel (33). The involvement of these pathways in varicocele needs more investigation.

**Conclusion**

In summary, our results suggest that RES might be an adjuvant therapeutic option in patients with varicocele by decreasing inflammatory events and apoptosis. Further studies are required to show if RES increase fertility outcomes in patients with varicocele.

**Acknowledgment**

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**Conflict of interest**

The authors declare no conflict of interest.

**References**

1. Pasqualotto F, Agarwal A. Varicocele and male infertility: an evidence based review. Arch Med Sci 2009; 5: S20-27.
2. Galan J, De Felici M, Buch B, Rivera M, Segura A, Royo J, et al. Association of genetic markers within the KIT and KITLG genes with human male infertility. Hum Reprod 2006; 21:3185-3192.
3. Krishna Reddy S. Varicocele and Male Infertility: Current Issues in Management-A Review. Med Surg Urol 2014; 3:137-142.
4. Sandlow J. Pathogenesis and treatment of varicoceles: controversy still surrounds surgical treatment. BJM 2004; 328:967-968.
5. Habibi B, Seifi B, Mougahi S-H, Ojaghi M, Sadeghipour H. Increases in interleukin-6 and interferon-gamma levels is progressive in immature rats with varicocele. Iran J Med Sci (1971-1) 2015; 18:4-53 1-537.
6. Zedler S, Faist E. The impact of endogenous triggers on trauma-associated inflammation. Curr Opin Crit Care 2006; 12:595-601.
7. Schroeder K, Tschopp J. The inflamasomes. Cell 2010; 140:821-832.
8. Fu Y, Wang Y, Du L, Xu C, Cao J, Fan T, et al. Resveratrol inhibits ionising irradiation-induced inflammation in MSCs by activating SIRT1 and limiting NLRP-3 inflammasome activation. Int J Mol Sci 2013; 14:14105-14118.
9. Latz E. The inflamasomes: mechanisms of activation and function. Curr Opin Immunol 2010; 22:28-33.
10. Hedger MP, Meinhardt A. Cytokines and the immune-testicular axis. J Reprod Immunol 2003; 58:1-26.
11. Kefer JC, Agarwal A, Sabanegh E. Role of antioxidants in the treatment of male infertility. Int J Urol 2009; 16:449-457.
12. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest 2001; 107:135-142.
13. Mendes TB, Paccola CC, de Oliveira Neves FM, Simas JN, da Costa Vaz A, Cabral REL, et al. Resveratrol improves reproductive parameters of adult rats varicoceled in peripuberty. Reproduction 2016; 152:23-35.
14. Abdel-Dayem M. Histological and immunohistochemical changes in the adult rat testes after left experimental varicocele and possible protective effects of resveratrol. Egypt J Histol 2009; 32:81-90.
15. Turner T. The study of varicocele through the use of animal models. Hum Reprod Update 2001; 7:78-84.
16. Ku JH, Shin HB, Kim SW, Paik JS. The role of apoptosis in the pathogenesis of varicocele. BJU Int 2005; 96:1092-1096.
17. Schoor RA, Elhanbly SM, Niederberger CS. The pathophysiology of varicocele-associated male infertility. Curr Urol Rep 2001; 2:432-436.
18. Paduch DA, Skoog SJ. Current management of adolescent varicocele. Rev Urol 2001; 3:120-133.
19. Romeo C, Santoro G. Free radicals in adolescent varicocele testis. Oxid Med Cell Longev 2014; 2014.
20. Semercioz A, Onur R, Ogras S, Orhan I. Effects of melatonin on testicular tissue nitric oxide level and antioxidant enzyme activities in experimentally induced left varicocele. Neur Endocrinol Lett 2003; 24:86-90.
21. Frémon L. Biological effects of resveratrol. Life Sci 2000; 66:663-673.
22. Fann DY-W, Lee S, Manzaneo S, Tang S-C, Gelderblom M, Chunduri P, et al. Intravenous immunoglobulin suppresses NLRP1 and NLRP3 inflammasome-mediated neuronal death in ischemic stroke. Cell Death Dis 2013; 4:e8790.
23. Zhang X, Ibrahim E, de Rivero Vaccari JP, Lotocki G, Aballa TC, Dietrich WD, et al. Involvement of the inflammasome in abnormal semen quality of men with spinal cord injury. Ferti Steril 2013; 99:118-124. e112.
24. Sulaiman M, Matta MJ, Sunderesan N, Gupta MP, Periasamy M, Gupta M. Resveratrol, an activator of SIRT1, upregulates sarcoplasmic calcium ATPase and improves cardiac function in diabetic cardiomyopathy. Am J Physiol Heart Circ Physiol 2010; 298:H833-H843.
25. Sahin Z, Celik-Özenci C, Akboyunlu G, Korgun ET, Acar N, Erdogru T, et al. Increased expression of interleukin-1α and interleukin-1β is associated with experimental varicocele. Ferti Steril 2006; 85:1265-1275.
26. Nallella KP, Allamaneni SS, Pasqualetto FF, Sharma RK, Thomas AJ, Agarwal A. Relationship of interleukin-6 with semen characteristics and oxidative stress in patients with varicocele. Urolrogy 2004; 64:1010-1013.
27. Kilinc F, Kayasaluk F, Aygun C, Guvel S, Egilmez T, Ozkardes H. Experimental varicocele induces hypoxia-inducible factor-1α, vascular endothelial growth factor expression and angiogenesis in the rat testis. J Urol 2004; 172:1108-1119.
28. Lee J-D, Jeng S-Y, Lee T-H. Increased expression of hypoxia-inducible factor-1α in the internal spermatic vein of patients with varicocele. J Urol 2006; 175:1045-1048.
29. Shao B-Z, Xu Z-Q, Han B-Z, Su D-F, Liu C. NLRP3 inflammasome and its inhibitors: a review. Front Pharmacol 2015; 6:262-270.
30. Liu C, Shi Z, Fan L, Zhang C, Wang K, Wang B. Resveratrol improves neuron protection and functional recovery in rat model of spinal cord injury. Brain Res 2011; 1374:100-109.
31. Ourique GM, Finamor IA, Sacco EM, Riffel AP, Pés TS, Gutierrez K, et al. Resveratrol improves sperm motility, prevents lipid peroxidation and enhances antioxidant defenses in the testes of hyperthyroid rats. Rep Toxicol 2013; 37:31-39.
32. Chen C-J, Yu W, Fu Y-C, Wang X, Li J-L, Wang W. Resveratrol protects cardiomyocytes from hypoxia-induced apoptosis through the SIRT1–FoxO1 pathway. Biochem Biophys Res Commun 2009; 378:389-393.
33. Nicolin G, Rigolo R, Miloso M, Bertelli AA, Tredici G. Anti-apoptotic effect of trans-resveratrol on paclitaxel-induced apoptosis in the human neuroblastoma SH-SYSY cell line. Neurosci Lett 2001; 302:41-44.