American Journal of Men's Health
March-April 1–9

DOI: 10.1177/15579883251321588
journals.sagepub.com/home/jmh
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Critical Roles of VEGFR1, VEGFR2, VEGFR3, BAX, and BCL-2 in the Pathogenesis of Varicose Veins: Unveiling Molecular Mechanisms

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Significance

This study offers new insights into the molecular mechanisms behind varicocele, a condition linked to male infertility. The discovery of elevated VEGFR3 expression in varicose veins points to its crucial role in the vascular changes tied to varicocele. Furthermore, the pro-apoptotic shift, reflected by the increased BAX/BCL-2 ratio, suggests that apoptosis may be a significant pathological mechanism. These results underscore VEGFR3 and apoptotic pathways as promising therapeutic targets, paving the way for better management and treatment of varicocele-related issues and infertility.

Abstract

Varicocele is characterized by the abnormal dilation of veins within the testicular pampiniform plexus, contributing to inflammation, pain, and infertility in males. The precise roles of vascular endothelial growth factor receptors (VEGFRs), B-cell lymphoma 2 (BCL-2), and BCL-2-associated X-protein (BAX) in the pathology of varicocele still need to be clarified. This study sought to investigate the protein expression levels of VEGFR1, VEGFR2, VEGFR3, BCL-2, and BAX in varicose and healthy vessels from patients diagnosed with varicocele. Tissue samples were collected from 20 varicose veins and 20 healthy vessels from patients diagnosed with varicocele. Western blotting was utilized to quantify VEGFR1, VEGFR2, VEGFR3, BCL-2, and BAX protein levels. Analysis revealed a statistically significant increase in VEGFR3 protein expression within varicose veins compared to healthy vessels (p = .0473), while no significant differences were observed in the levels of VEGFR1 and VEGFR2 between the two groups. Concerning apoptotic signaling proteins, no significant differences were noted in the individual expression levels of BAX and BCL-2; however, the BAX/BCL-2 ratio was approximately 1.29 in varicose vessels. This ratio, exceeding 1.0, may suggest a pro-apoptotic shift in varicose veins and indicates a potential involvement of apoptosis in the pathology of varicocele. These findings suggest that VEGFR3 may play a pivotal role in the pathogenesis of varicocele and could contribute to vascular alterations associated with this condition. Furthermore, the elevated BAX/BCL-2 ratio implies a pro-apoptotic environment within varicose veins, thereby implicating apoptosis as a possible mechanism in the development of varicocele. Further exploration of VEGFR3-related signaling pathways and apoptotic markers may yield valuable insights for identifying therapeutic targets in managing varicocele.

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Keywords
VEGF receptor, BAX, BCL-2, varicocele

Received December 12, 2024; revised January 28, 2025; accepted February 2, 2025

Introduction

Varicocele, or the abnormal expansion of the veins in the testicular pampiniform plexus, is considered one of the most well-known and significant factors related to infertility in men (Minas et al., 2023). This disorder exhibits a relatively high prevalence among men, with its occurrence in the general population of men of reproductive age reported to be approximately 15%–20% (Carto et al., 2022). Most men, approximately 85%, maintain normal fertility despite this disorder (GamalEl Din et al., 2023). The prevalence of varicocele in men experiencing fertility issues is approximately double that of the general population, estimated to be around 30% to 40% (GamalEl Din et al., 2023). Indeed, the increased prevalence of varicocele among infertile men is the most substantial evidence supporting the theory of a link between varicocele and male infertility (Bellastella et al., 2022). The surgical intervention for varicocele, when warranted by specific indications such as the palpable presence of varicocele, abnormalities in sperm parameters within semen analysis, and the normal fertility status of the spouse, may be regarded as the primary treatment option (Huang et al., 2023). Nonetheless, the impact of surgical intervention for varicocele on enhancing fertility, as well as the criteria for varicocele repair, remains a contentious subject (Syarief et al., 2023). A significant issue concerning varicocele lesions is whether their progressive or non-progressive forms may impact fertility over time (Deniz et al., 2023). Numerous urologists, drawing upon clinical evidence and research findings, assert that the detrimental effects of varicocele on spermatogenesis in adolescent males should be acknowledged (Belardin et al., 2016). Nonetheless, the ambiguity surrounding the progressive impacts of varicocele in adult men persists, as clinical evidence and studies yield contradictory findings. Furthermore, investigations have indicated that the prevalence of varicocele among patients experiencing secondary infertility is more significant than that observed in those with primary infertility, suggesting that varicocele may represent a progressive disorder over time (Lamy et al., 2023). It is recommended that men diagnosed with varicocele consider prophylactic treatment to prevent potential infertility in the future (Guarino et al., 2003). Currently, numerous experts neglect to acknowledge the indicators for varicocele repair in adults. However, the perpetuation of this viewpoint may incur substantial costs to the health care system, given the significant prevalence of varicocele within the general population. Consequently, incorporating additional indicators to validate the criteria for medication could assist physicians in making informed and precise decisions.

Numerous inflammatory and compensatory mechanisms are activated in the presence of varicose veins, resulting in an environment within the veins that is enriched with an accumulation of immune cells and inflammatory cytokines. This situation contributes to damage to the endothelial cells (J. D. Lee et al., 2012). It has been postulated that the factors inhibiting the programmed cell death of vascular smooth muscle cells (VSMCs), such as vascular endothelial growth factor (VEGF) and B-cell lymphoma 2 (BCL-2), in conjunction with the factor BCL-2 associated X-protein (BAX), which acts as an inducer of apoptosis, may contribute to the pathogenesis of varicocele (Nazari et al., 2020).

Previous studies indicated that VEGF facilitates its biological functions through interactions with its receptors: VEGF receptor 1 (VEGFR1), VEGFR2, and VEGFR3, inducing BAX and BCL-2 (Yang et al., 2023). However, VEGFR2 is the primary receptor that mediates the most recognized cellular responses to VEGF (Wichrowska et al., 2023). The roles of VEGFR1 and VEGFR3 remain fully elucidated. Previously, our findings showed that the mRNA levels of VEGFR2 exhibited a significant increase, whereas the BAX/BCL-2 ratio was reduced in varicose veins compared to normal veins in patients with varicocele (Nazari et al., 2020). However, it is necessary to confirm the results, as several factors can interfere with protein expression. This project was designed to investigate the protein levels of VEGFR1, VEGFR2, VEGFR3, BCL-2, and BAX in the endothelial cells of healthy vessels in adult patients diagnosed with varicocele compared to those with varicose veins.

Materials and Methods

Subjects

In this study, 20 male subjects diagnosed with varicocele participated in investigations concerning the

expression levels of VEGFR1, VEGFR2, VEGFR3, BCL-2, and BAX within the endothelial cells of healthy blood vessels compared to those in varicose veins. The participants were between 25 and 35 years old, as the prevalence of varicocele was notably higher during this period.

A urologist identified varicocele using physical examination and Color Doppler ultrasound, reporting that all patients presented with Grade 3 varicocele and required surgical intervention. Furthermore, fertile patients with varicocele, testicular injury, urogenital infections, inflammatory disorders, and those who had used anti-inflammatory medications in the last 6 months were excluded from the research. Moreover, patients with a history of smoking or alcoholism were not included in the study. Overall, multivitamin or antioxidant use was not significantly different among the participants. Therefore, patients with a history of epididymitis, orchitis, or any other inflammatory disease were excluded from the study. This exclusion criteria was implemented to reduce potential confounders such as scarring or inflammation-related vascular changes.

A healthy vein sample was obtained from the veins incised through the skin, often severed during surgical access to the spermatic vein. All the vein samples were collected from the same region for standardization. The control and varicose veins were harvested from the superficial femoral vein at the inguinal incision and from the inguinal part of the spermatic cord, respectively. Consequently, it was unnecessary to harm any portion of the healthy body to obtain the necessary access. Varicose veins are often excised via a surgical operation, with portions taken for further analytical examination. Consequently, the blood sample from the varicose vein is often obtained during the surgical procedure. The extracted tissues were stored in sterile containers filled with RIPA buffer and proteinase inhibitor at -80°C for future Western blot examination (Cocuzza et al., 2020).

Western Blotting

In order to investigate the protein levels of VEGFR1, VEGFR2, VEGFR3, BCL-2, and BAX, the Western blotting technique was employed, with beta-actin serving as the internal control for data normalization. Specifically, 50 mg of samples were homogenized in 500 mL of phosphate-buffered saline (PBS) at a pH of 7, supplemented with an anti-protease cocktail. The homogenized tissues were subsequently combined with a lysis buffer composed of Tris buffer at a pH of 8.6, 1% Triton X-100, 1% sodium dodecyl sulfate-polyacrylamide (SDS), 0.01% ethylenediaminetetraacetic acid (EDTA), 0.08% NaCl, and 0.028% sodium deoxycholate, followed by incubation for 30 minutes. The total protein concentration was quantified utilizing a BCA protein assay kit (KPG-TPBCA; Karmania Pars Gene Company, Iran). Consequently, the lysate (20 μg) was analyzed to evaluate the expression levels of VEGFR1, VEGFR2, VEGFR3, BCL-2, and BAX via Western blotting, as detailed in previous studies (Sun et al., 2023). In summary, tissue lysates were subjected to 10% SDS vertical gel electrophoresis and transferred onto a nitrocellulose membrane (Amersham Biosciences; Cytiva). The blocking of the membrane was conducted utilizing 5% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween-20 in tris-buffered saline (T-TBS), followed by an incubation period of 2 hours at room temperature. Upon completion of the washing process, the blocked membranes were incubated with diluted antibodies (Santa Cruz Company, USA) targeting VEGFR1, VEGFR2, VEGFR3, BCL-2, and BAX at a dilution of 1:2000. They were allowed to incubate overnight at 4°C. Afterward, washing was performed three times with T-TBS buffer for 10 minutes. Subsequently, the membranes were incubated with a 1:5,000 dilution of a secondary antibody (goat anti-human IgG-HRP [Sigma-Aldrich Company, USA]) for 1 hour at room temperature with gentle shaking (40 RPM). A chemiluminescent kit (Parstous Company, Mashhad, Iran) was utilized to visualize the bands on an X-ray film. Densitometric analysis was conducted using ImageJ software (ver. 1.46r; National Institutes of Health) to interpret the data.

Statistical Analysis

The raw data were analyzed utilizing SPSS software, version 18. A one-sample Kolmogorov–Smirnov test was conducted to assess the normal distribution of the raw data. Given the normal distribution of the data, the Student's *t*-test, a parametric statistical test, was employed to compare the data between individuals with varicose veins and those with healthy vessels. The data are presented as means $\pm$ standard deviation (*SD*), and a *p*-value of < 0.05 was deemed statistically significant.

Results

Subjects

The demographic and clinical characteristics of the varicocele patients are summarized in Table 1. The study included 20 patients diagnosed with varicocele, with a mean age of 30.54 $\pm$ 4.2 years. The average duration of infertility among these patients was 3.77 $\pm$ 2.1 years, reflecting prolonged reproductive challenges within this population.

Analysis of clinical parameters revealed a mean white blood cell (WBC) count of 6.21 ± 1.29 per high power field (HPF), suggesting a potential inflammatory response. In addition, sperm motility was observed to be significantly reduced, with an average motility rate of 30.3 ± 12.46%, which is below normal reference values and indicative of impaired sperm function in varicocele-affected patients. These findings highlight the demographic and clinical profile of individuals with varicocele, underscoring factors potentially associated with varicocele-related infertility.

Table 1. Demographic and Clinical Characteristics of Patients With Varicocele

Variable	Varicocele patients
n	20
Age (year)	30.54 ± 4.2
Duration of infertility (year)	3.77 ± 2.1
WBC (HPF)	6.21 ± 1.29
Sperm motility (%)	30.3 ± 12.46

Protein Expression

The protein expression levels of members of the VEGFR family were examined in tissues derived from control and varicocele vessel samples to explore potential differences in VEGF receptor levels. Quantitative analyses of VEGFR1 (Figure 1A) and VEGFR2 (Figure 1B) demonstrated no significant variations in protein expression between control and varicocele samples, indicating that these receptors are not appreciably upregulated in vessels affected by varicocele. Conversely, the expression of VEGFR3 (Figure 1C) was significantly elevated in varicocele vessels compared to controls ($p = .0473$), suggesting a specific

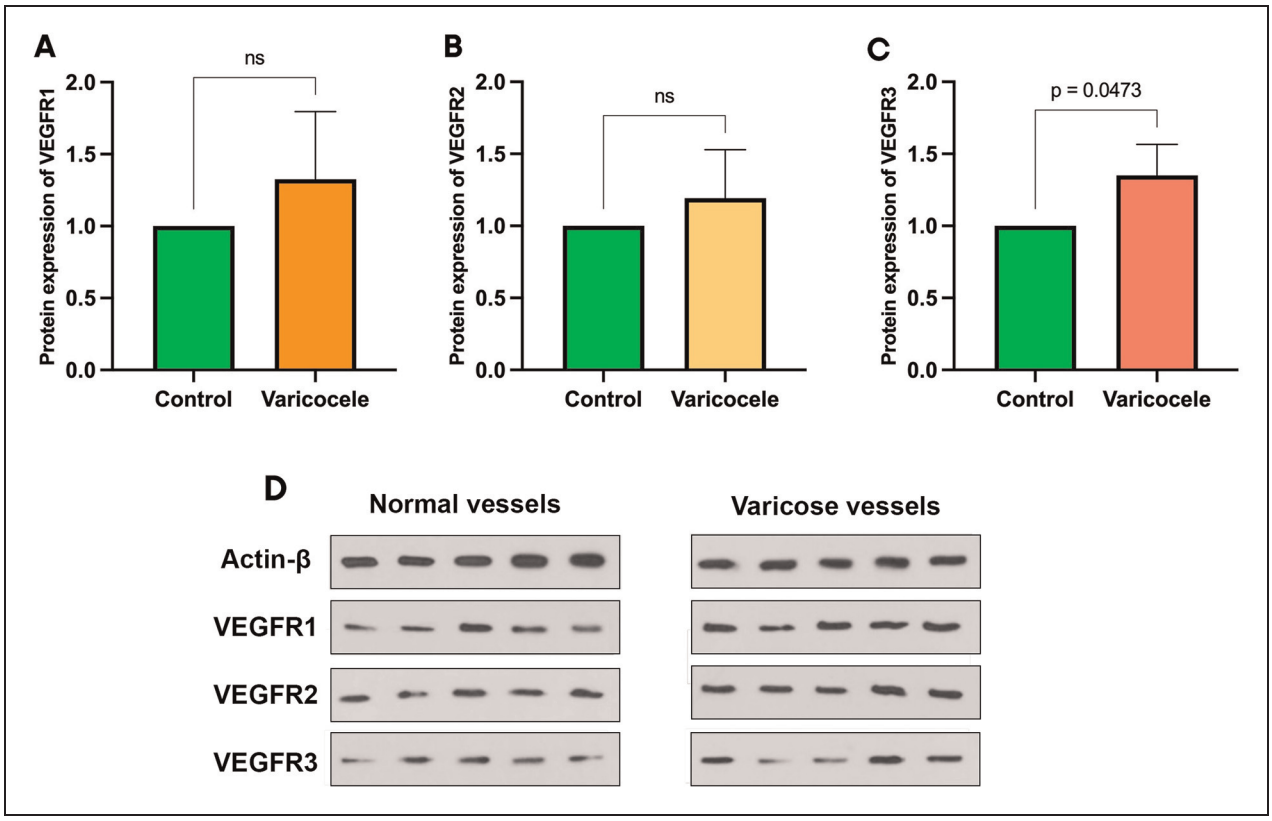

Figure 1. Protein Expression of VEGFR Family Members in Control and Varicocele Vessels. (A–C) Quantitative Analysis of VEGFR1, VEGFR2, and VEGFR3 Protein Expression Levels in Control and Varicocele Vessel Tissues. Expression Levels Were Normalized and Are Represented as Mean ± SD. (A) VEGFR1 Protein Expression Shows No Significant Difference Between Control (Green) and Varicocele (Orange) Vessels (ns). (B) Similarly, VEGFR2 Protein Expression Does Not Significantly Differ Between Control (Green) and Varicocele (Yellow) Vessels (ns). (C) VEGFR3 Protein Expression Is Markedly Higher in Varicocele (Red) Vessels Than in Control (Green) Vessels, With a p-value of .0473, Indicating Statistical Significance. (D) Representative Western Blot Images Show the Protein Levels of Actin-β (Loading Control), VEGFR1, VEGFR2, and VEGFR3 in Normal (Left Panel) and Varicose (Right Panel) Vessels, Consistent With the Quantitative Data Shown in Panels A–C

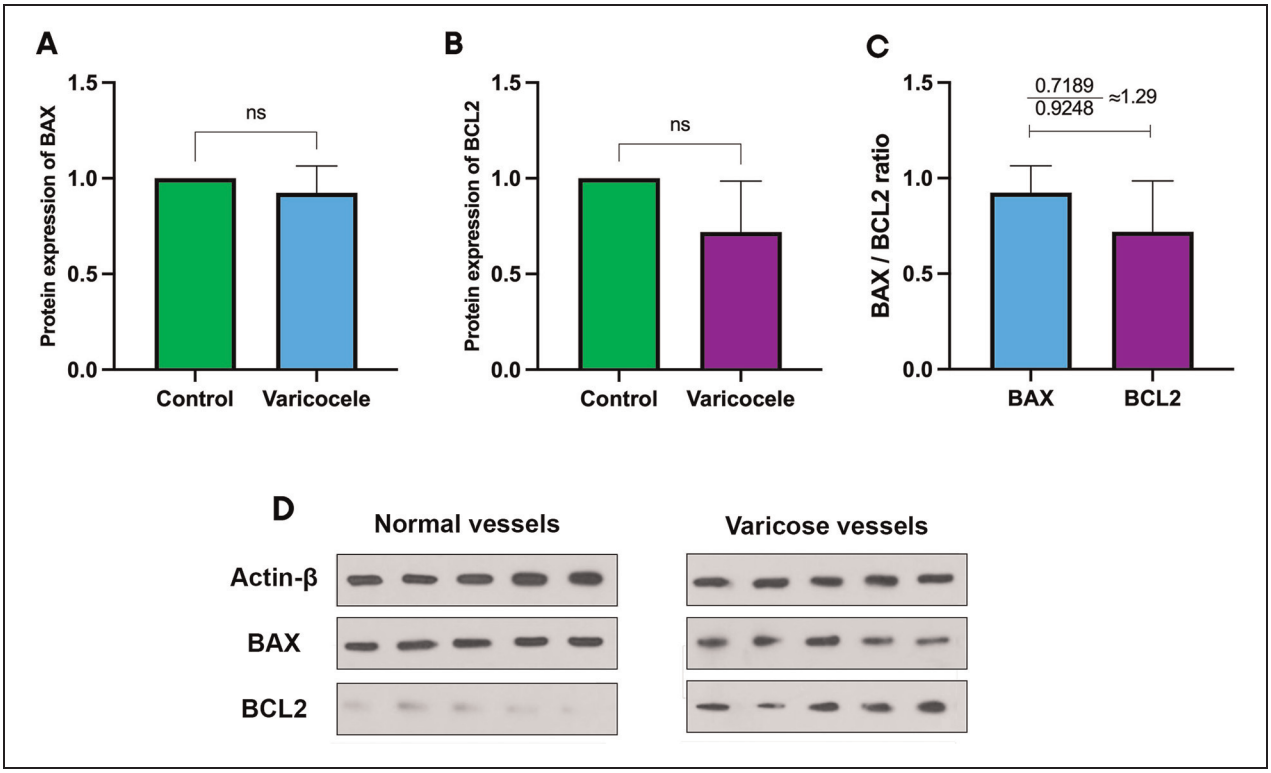

Figure 2. Protein Expression of Apoptosis-Related Markers BAX and BCL-2 in Control and Varicocele Vessels. (A–C) Quantitative Analysis of BAX and BCL-2 Protein Expression Levels and the BAX/BCL-2 Ratio in Control and Varicocele Vessel Tissues. Expression Levels Are Shown as Mean ± SD. (A) Protein Expression of BAX in Control (Green) and Varicocele (Blue) Vessels, With No Significant Difference Observed (ns). (B) Protein Expression of BCL-2 in Control (Green) and Varicocele (Purple) Vessels, Also Showing No Significant Difference (ns). (C) The BAX/BCL-2 Ratio Between Control and Varicocele Vessels, With No Significant Change, Indicating Balanced Pro-Apoptotic and Anti-Apoptotic Signals. (D) Representative Western Blot Images Display the Levels of Actin-β (Loading Control), BAX, and BCL-2 in Normal (Left Panel) and Varicose (Right Panel) Vessels, Confirming Similar Expression Patterns Across the Two Groups

upregulation of VEGFR3 in response to varicocele conditions. Representative Western blots (Figure 1D) corroborate these findings, displaying increased VEGFR3 bands in the varicocele group relative to controls, while VEGFR1 and VEGFR2 levels appear consistent across both conditions. Actin-β was utilized as a loading control to confirm uniform protein loading. These results imply that varicocele is associated with heightened VEGFR3 expression, which may contribute to the vascular pathology of varicocele.

We evaluated the protein expression levels of BAX (a pro-apoptotic marker) and BCL-2 (an anti-apoptotic marker), as well as the BAX/BCL-2 ratio in vessels affected by varicocele and control vessels to assess the implications of varicocele on apoptotic signaling. Quantitative analysis revealed no significant differences in the expression levels of BAX (Figure 2A) or BCL-2 (Figure 2B) between the control and varicocele tissues, indicating comparable expression of these markers in both groups. However, upon examination of the BAX/BCL-2 ratio (Figure 2C), it was observed that the ratio was approximately 1.29 in varicocele vessels. Although this value did not reach statistical significance, a BAX/BCL-2 ratio greater than 1 traditionally signals a shift toward pro-apoptotic signaling, as BAX induces apoptosis while BCL-2 inhibits it. This slight elevation in the BAX/BCL-2 ratio may imply a subtle pro-apoptotic tendency in varicocele vessels despite the change being insufficiently pronounced to attain statistical significance. Western blot analysis (Figure 2D) further supported these findings, revealing analogous band intensities for BAX and BCL-2 across both control and varicocele vessels. Actin-β was a loading control to ensure equal protein loading across the samples.

Collectively, these findings indicate that while varicocele does not significantly modify individual BAX or BCL-2 expression, a moderate increase in the BAX/BCL-2 ratio suggests a slight pro-apoptotic shift in the vascular environment linked to varicocele.

Discussion

It has been demonstrated that inflammation and heightened angiogenesis are characteristic of varicocele progression in adult males (Gökhan-Köse et al., 2014). Nevertheless, the functions of VEGFR1, VEGFR2, and VEGFR3 remain to be elucidated. Angiogenesis, characterized as the process by which new blood vessels are formed from pre-existing vasculature, is acknowledged as a vital phenomenon in various pathological conditions, including oncogenesis and age-related macular degeneration (Dudley & Griffioen, 2023). VEGFs have been demonstrated to promote angiogenesis and lymphangiogenesis by activating tyrosine kinases associated with the VEGFR in endothelial cells. Initially, VEGFR3 (FLT-4) was observed in all endothelial cells during developmental stages; however, its expression subsequently became restricted to the lymphatic endothelium in adult organisms (Shibuya & Claesson-Welsh, 2006). Noteworthy is the upregulation of VEGFR3 observed in both tumor and wound microvasculature (Paavonen et al., 2000). Research has established that VEGFR3 is markedly expressed in angiogenic sprouts. The genetic targeting or inhibition of VEGFR3 signaling, utilizing monoclonal antibodies, has decreased sprouting, vascular density, vessel branching, and endothelial cell proliferation in murine models (Tammela et al., 2008). Furthermore, the activation of VEGFR3 facilitated VEGF-induced angiogenesis and sustained angiogenesis, even in the presence of VEGFR2 inhibition (Zhao et al., 2015). The conjoint application of antibodies targeting VEGFR3 and VEGFR2 further inhibited angiogenesis and tumor growth (Guidolin et al., 2008). The interruption of Notch signaling was associated with the extensive expression of endothelial VEGFR3 and increased sprouting; these effects were alleviated by inhibiting VEGFR3 signaling (Tammela et al., 2008). The findings indicate that VEGFR3 serves as a regulator of vascular network formation, suggesting that the targeted inhibition of this receptor could potentially improve the effectiveness of anti-angiogenic therapies, especially in blood vessels exhibiting resistance to VEGF or VEGFR2 inhibitors.

The findings of our study indicated that the protein expression of VEGFR3, but not VEGFR1 and VEGFR2, exhibited upregulation in the varicose veins of patients with varicocele. Furthermore, our prior investigation involving infertile male patients diagnosed with Grade 3 varicocele revealed a significant upregulation of only VEGFR2 mRNA levels in varicose veins when compared with those in normal veins within this patient cohort (Nazari et al., 2020). Conversely, our recent data diverge from our previous findings, which indicated the upregulation of VEGFR3 at the protein level. It has been documented that VEGFR2 serves as the primary receptor for VEGF, thereby inducing the migration and proliferation of endothelial cells (Miettinen et al., 2012).

Furthermore, compared to healthy individuals, we have observed a notable increase in VEGF serum levels in the bloodstream of varicose veins, whether in peripheral blood or tissue (Nazari et al., 2020). Wang et al. reported that VEGF was significantly upregulated in the varicose veins of the varicocele animal model (H. Wang et al., 2021).

Varicose veins, identified as the most common manifestation of chronic venous disease (CVD), were observed as abnormally enlarged and tortuous superficial veins resulting from functional abnormalities in the venous circulation of the lower extremities, such as venous hypertension, venous valve incompetence, and venous reflux (Guidolin et al., 2008). Previous findings suggested that enhanced angiogenesis and inflammation contributed to the onset and progression of varicose veins (Pfisterer et al., 2014). Eighteen gene expression levels were analyzed in peripheral blood mononuclear cells (PBMC) using real-time polymerase chain reaction (PCR), and plasma levels of six proteins were measured using enzyme-linked immunosorbent assay (ELISA). Significantly higher levels of CCL5, platelet-derived growth factor A (PDGFA), VEGFC, transforming growth factor-alpha (TGF-α), TGF-β1, and VEGF-A, along with lower levels of VEGFB and VEGF-C, were observed in the varicose veins group compared to the controls without varicose veins. No association was found between the analyzed factors and the venous localization of varicosities (Guidolin et al., 2008). The study provided insight into dysregulated angiogenesis- and inflammation-related factors in varicose veins patients, suggesting potential biomarkers for this disease.

Another study reported that the upregulation of VEGF in the animal model of varicocele is induced by hypoxia-inducible factor-1α (HIF-1α; D. Wang et al., 2021). Several studies demonstrated that varicocele can result in tissue hypoxia and upregulation of HIF-1α and then an increase in expression of VEGF in the serum and endothelial cells of varicose veins, which leads to infertility in animal models (Ai et al., 2009; Goren et al., 2017; Kilinç et al., 2004; Minutoli et al., 2011; Shiraishi & Naito, 2008; Wang et al., 2017). Zhu and colleagues revealed that suppression of VEGF can inhibit the induction of varicocele in animal models (Zhu et al., 2019). Evidence revealed that this study

is the inaugural investigation assessing protein levels of VEGF receptors in patients with varicocele. Our findings indicate that VEGFR3 significantly contributes to the development of varicocele in Iranian patients with varicocele (Witmer et al., 2001). Accordingly, VEGFR3 inhibitors may be used as a molecular therapeutic approach for varicocele (Tammela et al., 2008).

Our prior investigation indicated that the mRNA BAX/BCL-2 ratio was decreased in varicose veins (Nazari et al., 2020). The findings from our analysis of BAX and BCL-2 protein levels offer valuable insights into the apoptotic signaling in varicocele-affected vessels. The lack of significant differences in the expression levels of BAX and BCL-2 between varicocele and control vessels suggests that varicocele alone may not dramatically alter the expression of these apoptotic markers. This result indicates that both pro-apoptotic (BAX) and anti-apoptotic (BCL-2) processes are comparably balanced in varicocele-affected and normal vessels, which might reflect a limited direct impact of varicocele on apoptotic regulation. However, the observed BAX/BCL-2 ratio of approximately 1.29 in varicocele vessels, while not statistically significant, hints at a potential shift toward pro-apoptotic signaling, as a ratio greater than one has historically been associated with increased apoptotic activity. This slight elevation may indicate a subtle tendency toward apoptosis within varicocele vessels. However, further research with larger sample sizes might be required to ascertain whether this tendency becomes more pronounced under different physiological conditions. In addition, Western blot analysis provided supportive evidence, as similar BAX and BCL-2 band intensities were observed across both control and varicocele samples. These findings suggest that while varicocele may not induce substantial apoptotic alterations, a marginal pro-apoptotic bias could exist, warranting further investigation into its clinical relevance and potential impact on vascular integrity in varicocele pathology.

An investigation evaluated seminal BAX and BCL-2 gene and protein expressions concerning fertility in men with varicocele (Mostafa et al., 2014). Findings showed that BAX levels were elevated and BCL-2 levels reduced in men with varicocele, especially in those with bilateral or severe varicocele. Elevated BAX correlated negatively with sperm concentration, motility, and morphology, indicating poorer sperm quality, while BCL-2 positively correlated with these parameters (Mostafa et al., 2014). The results suggest that BAX and BCL-2 expressions reflect apoptotic activity linked to varicocele severity and fertility outcomes, identifying them as potential biomarkers for assessing varicocele's impact on male fertility. Interestingly, a study investigated the expression of HIF-1α and BCL-2 in varicocele and varicose veins, characterized by thickened, dilated vein walls and hypoxia due to blood stasis and increased venous pressure. Protein analysis showed that both conditions had significantly higher HIF-1α and BCL-2 levels than controls, with the highest colocalization in the muscle layer and elevated BCL-2 expression in the endothelium under hypoxia (J. -D. Lee et al., 2012). These findings suggest that BCL-2 overexpression may protect vascular cells from apoptosis and contribute to vessel wall thickening, supporting a potential protective role of BCL-2 in hypoxic venous disease.

In our study, VEGFR1 and VEGFR2 also showed increased expression; however, this increase was not statistically significant, possibly due to various factors, including the small sample size. In addition, the semi-quantitative nature of the Western blot analysis may have influenced the results and statistical analysis. We dissected the entire vein without discriminating between the endothelial or muscular layers. This decision was made regarding the molecular expression of the whole vein system. Although it speculates that specific layers might behave differently during deoxygenation, this was not within the realm of the current method. Furthermore, an incision was made in the inguinal region, and no sublingual veins were used. This was done to ensure the same anatomical area was sampled in all the patients. Sublingual veins may have different features than others due to variations in oxygenation and gravitational factors. We have recognized this and suggest that this should be investigated further in future research. This study also faced limitations in the number of varicocele patients meeting the criteria for inclusion. Further studies with larger sample sizes could help better understand the role of angiogenic and apoptotic factors in the development of varicocele.

Acknowledgments

The authors wish to thank the staff of Islamic Azad University, Kerman Branch, for their warm cooperation.

Author Contributions

AN and HKH designed the study, edited the manuscript, and prepared for submission; SP wrote, revised, and edited the manuscript; MA revised the methodology and data collection; SV revised the methodology and technical lab comments.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Rafsanjan University of Medical Sciences supported this study (grant number: 97470).

Ethics Approval and Consent to Participate

All participants signed a written informed consent form. The Rafsanjan University of Medical Sciences Ethics Committee approved the study protocol (IR.RUMS .RIC.11399.082). This research was conducted in accordance with the Declaration of Helsinki.

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Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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