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Integrative Bioinformatics Analysis of The Cell Division Cycle and Ribosomal Pathways in The Rat Varicocele: Implications for Drug Discovery

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Abstract

Objective: Varicocele is a common cause of male infertility, affecting a substantial proportion of infertile men. Recent studies have employed transcriptomic analysis to identify candidate genes that may be implicated in the pathogenesis of this condition. Accordingly, this study sought to leverage rat gene expression profiling, along with protein-protein interaction networks, to identify key regulatory genes, related pathways, and potentially effective drugs for the treatment of varicocele.

Materials and Methods: In this in-silico study, differentially expressed genes (DEGs) from the testicular tissue of 3 rats were screened using the edgeR package in R software and the results were compared to 3 rats in the control group. Data was obtained from GSE139447. Setting a -1<LogFC>1 and P<0.05 as cutoff points for statistical significance, up and down-regulated genes were identified. Based on Cytoscape plugins, protein-protein interaction (PPI) networks were drawn, and hub genes were highlighted. ShinyGO was used for pathway enrichment. Finally, effective drugs were identified from the drug database.

Results: Among the 1277 DEGs in this study, 677 genes were up-regulated while 600 genes were down-regulated in rats with varicocele compared to the control group. Using protein-protein interaction networks, we identified the top five up-regulated genes and the top five down-regulated genes. Enrichment analysis showed that the up-regulated genes were associated with the cell division cycle pathway, while the down-regulated genes were linked to the ribosome pathway. Notably, our findings suggested that dexamethasone may be a promising therapeutic option for individuals with varicocele.

Conclusion: The current investigation indicates that in varicocele the cell division cycle pathway is up-regulated while the ribosome pathway is down-regulated compared to controls. Based on these findings, dexamethasone could be considered a future candidate drug for the treatment of individuals with varicocele.

Keywords: Cell Division Cycle, RNA-SEQ, Varicocele

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Introduction

Varicocele occurs in around 15 to 20% of all males and is reported in about 40% of infertile males. It is characterized by the abnormal enlargement of veins within the scrotum's pampiniform plexus which serve to drain oxygen-depleted blood from the testicles. Varicocele is commonly observed in the left testis and typically arises during or after puberty, likely owing to rapid testicular growth and the consequent need for increased blood supply (1). While varicocele typically does not severely impede blood flow, some individuals may experience venous backflow within the network of veins, leading to the development of varicocele and consequent male infertility. Interestingly, varicocele is also prevalent in fertile individuals, thus making the question of varicocelectomy a highly debated topic in the field of andrology, pertaining to when, why, and in whom surgical intervention should be undertaken (2).

In order to resolve these uncertainties, a comprehensive investigation into the factors that underlie varicocele is required. To this end, multiple studies have been conducted, with the majority of researchers suggesting that reactive oxygen species (ROS), resulting from increased testicular temperature, serve as the primary contributing factor. Given the role of ROS in the development of varicocele-induced male infertility, a growing number of researchers have explored the use of

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antioxidants as a potential therapeutic strategy to enhance fertility outcomes in affected males (3). However, recent studies suggest that it may not be "oxidative stress" but rather "reductive stress" that leads to increased ROS in the sperm of individuals with varicocele (4). To deepen our understanding of the etiology of varicocele, numerous research groups have taken advantage of an animal model of varicocele to evaluate the molecular and cellular mechanisms involved (5).

Members of our research team recently conducted a comprehensive review varicocele and oxidative stress with new insight to both human and animal studies in which we summarized the findings of numerous investigations into the mechanisms underlying varicocele-related infertility. Our review highlighted several cellular mechanisms that may contribute to the pathophysiology of varicocele, including ionic imbalance, hypoxia, hyperthermia, and impaired blood flow, all of which may ultimately result in severe, chronic oxidative and nitrosamine stress (5).

Despite extensive studies at the cellular and molecular levels, limited transcriptomic and proteomic analyses have been conducted in the field of varicocele. No single gene or single nucleotide polymorphism (SNP) directly associated with varicocele has been identified thus far to fully explain its common etiology. To gain a comprehensive understanding, researchers need to expand their focus to include transcriptomic and epigenetic analyses.

Our aim in this study was to find epigenetic regulatory mechanisms underlying varicocele by integrating the transcriptome and miRNA profiles of testicular tissue from rats with induced varicocele. Such identification of dysregulated gene expression patterns may contribute to the development of effective treatment strategies (6). Specifically, in this study, we analyzed the GSE139447 dataset, including control, varicocele, and treated varicocele groups. Our aim was to investigate differential gene expression in samples from varicocele and treated varicocele groups compared to controls. We constructed protein-protein interaction (PPI) networks to identify hub genes with significant interactions. Enrichment analysis using rat databases showed that cell division and ribosome pathways were prominently affected among the up-regulated and down-regulated genes respectively. Based on findings from these analyses, we utilized drug databases to identify candidate drugs for the treatment of varicocele.

Materials and Methods

Data collection and differential gene expression analysis

In this in silico analysis, we utilized RNA-Seq data obtained from an experimental study conducted at the Department of Human Anatomy and Histology and Embryology at Fujian Medical University in China. The dataset (GSE139447) consisted of nine rat testis tissues, including three samples from left varicocele testes, three normal samples and three varicocele treated with 300 mg/kg Morinda officinalis polysaccharide (MOP). As in the study by Zhang et al. (7), the rats used in this study were 67weekold Sprague Dawley rats, weighing 200 ± 10 g. Varicocele was induced by partial ligation of the left renal vein. The time post-analysis was 12 weeks, which is equivalent to chronic human varicocele commonly occurring on the left side.

RNA from the GSE139447 data set was extracted from the left testis using Trizol reagent and the quantity and quality of RNA was checked using nanodrop. The total RNA from each sample was used to prepare the RNA sequencing library. Random primed was used for first strand cDNA synthesis. This was followed by adaptor ligation and PCR amplification (7).

The GSE139447 normalized files, determined using Illumina HiSeq 4000 (Rattus norvegicus), were obtained directly from the Gene Expression Omnibus database. By utilizing the “edgeR” package in R software and by considering the counts per million (CPM) criterion (CPM less than 10 in 70% of samples), genes with low expression and close to zero were removed from the normalized data. A boxplot was used to measure the quality control of the normalized data. Differential expression analysis was then performed to compare differences between varicocele samples and normal samples. Genes with a $P<0.05$ and $|logFC|\geq1$ and $|logFC|<-1$ were defined as candidate genes. Volcano plots indicated differentially expressed genes (DEGs) using the "ggplot2" package in R software. A volcano plot is a type of scatterplot in which each point on the graph represents a gene. The log2-fold differences between the groups are plotted on the x-axis and the P value differences are plotted on the y-axis. The horizontal dashed line represents the significance threshold specified in the analysis.

Protein-protein interaction network and module analysis

One of the best tools for detecting PPI, is the Search Tool for the Retrieval of Interacting Genes (STRING), which is an online tool for recognizing and assessing PPI. The Cytoscape 3.9.1 application was used to establish a PPI network among the selected genes using the STRING plugin. Subsequently, the Molecular Complex Detection (MCODE) plugin was used to cluster the PPI network based on the following criteria: degree cutoff=2, node score cutoff=0.2, k-core=2, and max depth=100. Finally, we employed the CytoHubba plugin in Cytoscape to identify hub genes using 11 topological analysis methods for ranking nodes in the PPI network. In our study, hub genes were classified based on the maximal clique centrality (MCC) method. The MCC method was chosen because it captures essential proteins in both high-degree and low-degree regions, making it more effective than other methods.

Enrichment analysis

Pathway enrichment analysis helps researchers gain

mechanistic insight into gene lists generated from genome-scale (omics) experiments. ShinyGO, developed based on different R/Bioconductor packages, is a graphical web application that shows the functional acuteness of a set of genes. In this study, three enrichment databases including GO (molecular function, biological process, cellular component), KEGG, and Reactome were used for the identification of gene-related pathways via the ShinyGO (http://bioinformatics.sdstate.edu/go/) application. For the enrichment analysis, we set P<0.05 as the cutoff for statistical significance.

Drug discovery

Drug discovery can be described as the process of recognizing chemical entities that have the potential to become therapeutic agents. Several drug databases have been developed to introduce the drugs and explain the influence of the drugs on patients, and on the role of key genes in different diseases. To determine drugs that could target our selected genes, we made use of some of these drug databases including DTC, TdgClinicalTrial, GuideToPharmacology, ChemblInteractions and NCI. The GSE139447 dataset consisted of three treated samples with Morinda officinalis polysaccharide MOP and three untreated samples. We used this study to evaluate the influence of MOP on the expression levels of key candidate genes in rat varicocele samples (7). Thus, differential expression analysis was performed between three varicocele samples treated with MOP and three varicocele samples, as control, using the "edgeR" package. P<0.05 were considered sufficient to determine that the effectiveness of the drug on the genes was significant.

Statistics

All the analyses were performed using packages in R language 4.1.1 and Bioconductor packages in R software. GraphPadPrisme9 (Dotmatics, USA) was used for constructing the plots and performing the t test analyses. Cytoscape software (version 4.1, The Institute of Systems Biology, USA) was used to draw and categorize the data obtained. It is worth noting that the Limma package used the moderated t test.

Results

Indicating differentially expressed genes

Differential expression analysis showed a total of 1,277 different genes (P<0.05, |log2FC|≥ 1, |log2FC|< -1), of which expression of 677 different genes were up-regulated and expression of 600 different genes was down-regulated (Fig.1, Table S1, See Supplementary Online Information at www.celljournal.org).

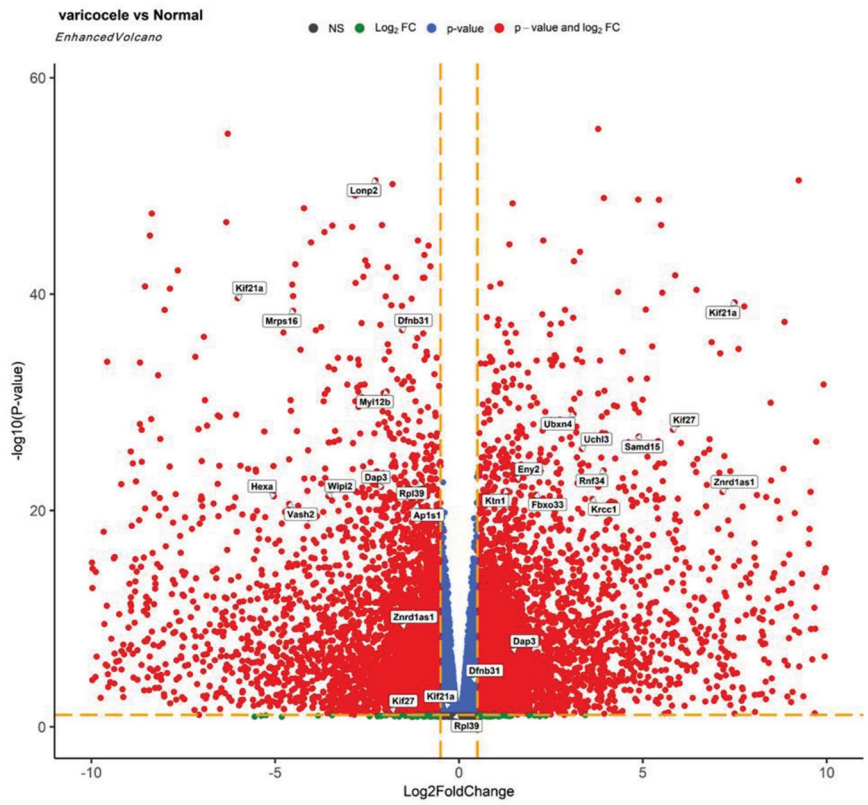

Fig.1: Differentially expressed genes in varicocele compared to normal samples. The expression level of differentially expressed genes (DEGs) was visualized using a volcano plot where the red dots represent the up-regulated genes and the down-regulated genes. Blue dots represent genes that not meet our criteria. The yellow line indicates P value and LogFC cutoffs. The LogFC>1, <-1 and P<0.05 was considered the cut point for statistical significance.

PPI network and generelated clustering module construction

To explore potential interconnections among the identified DEGs, we constructed PPI networks separately for up- and down-regulated genes based on the STRING database. Our analysis revealed that the up-regulated network consisted of 281 nodes and 710 edges, while the down-regulated network contained 181 nodes and 777 edges (Fig.2A, B). We selected the top modules for up- and down-regulated networks based on the highest MCODE score (Table S2, See Supplementary Online Information at www.celljournal.org). The top 5 genes among 30 cluster-related genes with the highest MCC score were designated as the top hub genes for the up-regulated network (*BUB1, SMC4, CENPE, KIF11,* and *ASPM*) and the down-regulated network (*RPL11, RPS15, RPS19, RPS55,* and *RPS2*) (Fig.3A, B, Table S3, See Supplementary Online Information at www.celljournal.org).

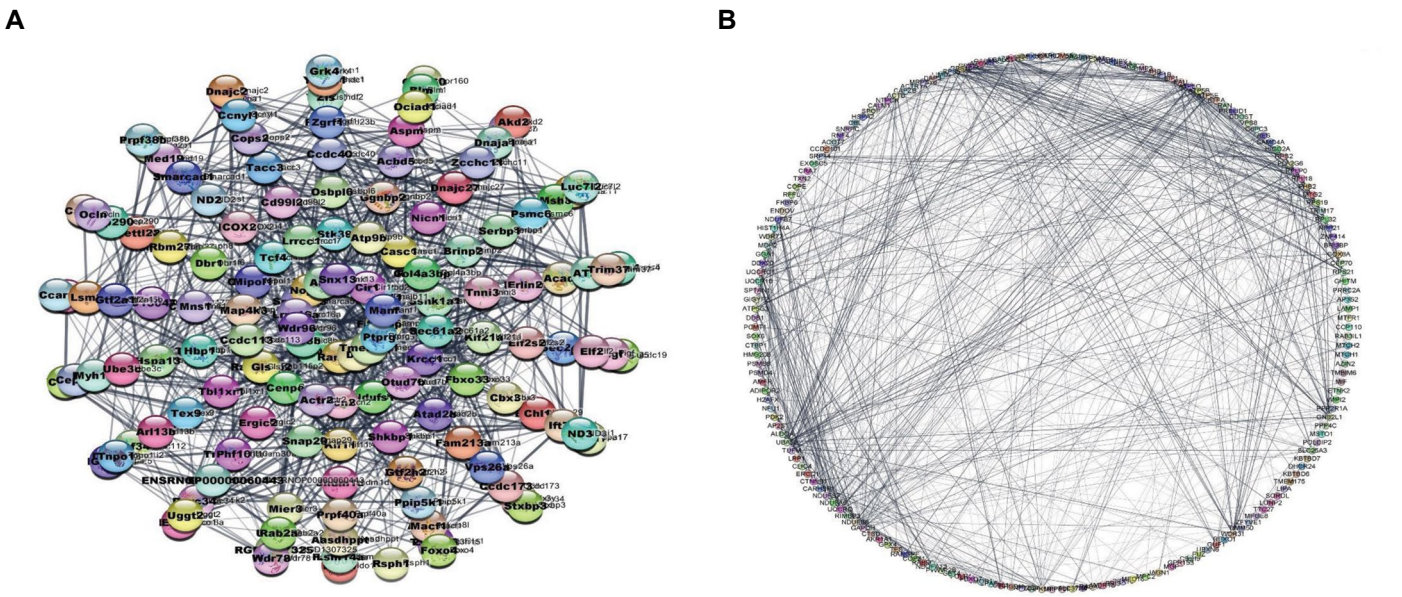

Fig.2: Protein-protein interaction networks. **A.** Up-regulated and **B.** Down-regulated genes.

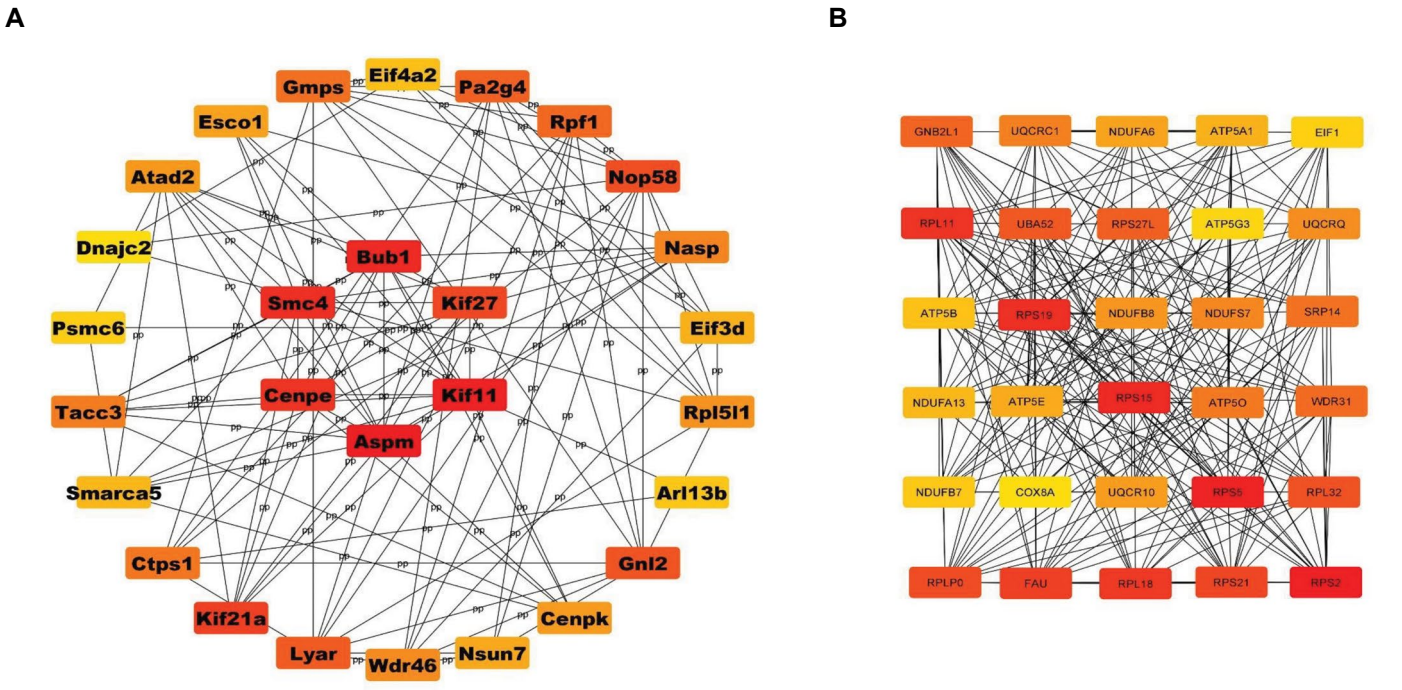

Fig.3: Identification of top hub genes using the Cytohubaa plugin in Cytoscape. **A.** Top hub up-regulated and **B.** Down-regulated genes. Darker colors represent higher Cytohubba scores.

Enrichment analysis for top hub genes

We utilized the ShinyGO online tool to perform functional annotation and pathway enrichment analysis of the top hub genes. To identify pathways related to selected genes with an adjusted P<0.05, KEGG, GO Biological Process, GO Molecular Function, GO Cellular Component, and Reactome datasets were used. The three top terms in each database for up- and down-regulated genes were highlighted based on the P value (Fig.4A-I). For up-regulated genes, the top KEGG pathways included the Cell cycle, oocyte meiosis, and progesterone-mediated oocyte maturation. The top GO Biological Process terms included nuclear division, organelle fission, and mitotic nuclear division. The top GO molecular function terms included microtubule motor activity, cytoskeletal motor activity, and ATP binding. The top GO Cellular Component terms included the chromosome, centromeric region, and mitotic spindle. Additionally, the top Reactome pathways were kinesins, COPI-dependent Golgi-to-ER retrograde traffic, and Golgi-to-ER retrograde transport. For down-regulated genes, the top pathways related to the GO Biological Process were translation, peptide biosynthesis, and peptide metabolism. The top GO Molecular Function terms included structural components of ribosomes, structural molecule activity proteins, and ubiquitin-protein transferase inhibitor activity. The top GO Cellular Component terms included ribosomes, small ribosomal subunits, and ribosomal subunits. The top Reactome pathways were l13a-mediated translational silencing of ceruloplasmin expression, SRP-dependent co-translational protein targeting to membranes, and eukaryotic translation initiation. Notably, the down-regulated genes were not significantly enriched in the KEGG pathway (Fig.4A-I). Therefore, our investigation showed that most varicocele-related pathways were involved in cell division and ribosomal pathways.

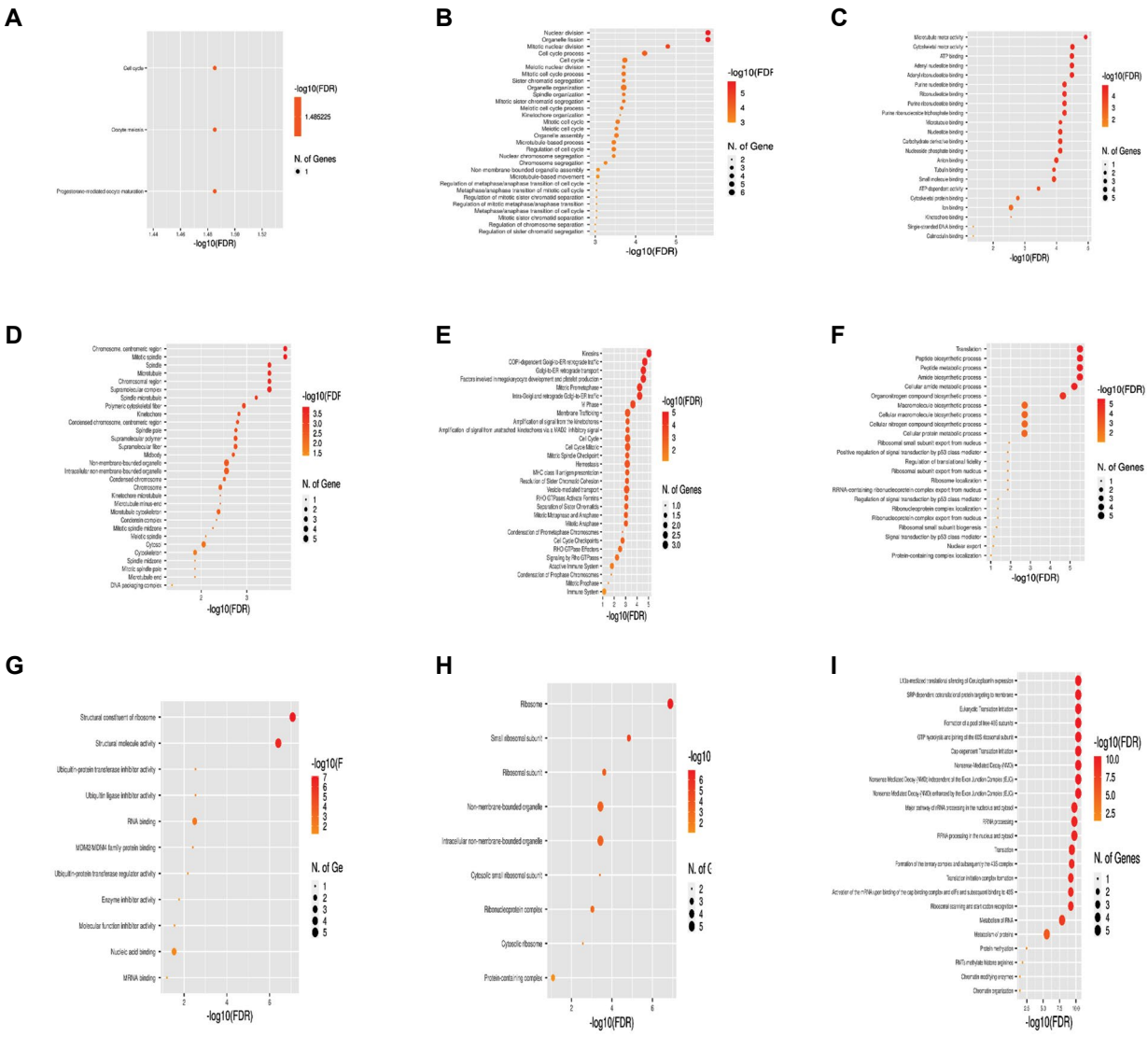

Fig.4: Enrichment analysis based on top hub up- and down-regulated genes. **A.** Dot plots represent top up-regulated genes in five selected pathways including KEGG, **B.** GEO biological process, **C.** Molecular function, **D.** Cellular component and **E.** Reactome datasets. **F.** Down-regulated genes include biological processes, **G.** Molecular function, **H.** Cellular component, and **I.** Reactome datasets. A P<0.05 was used to denote statistical significance.

Identification of suitable drugs

Recognizing drugs that could change the expression level of key genes with altered expression in varicocele could reduce the severity of this disease. Based on the Drug Gene Interaction Database (DGIdb) and the score obtained, drugs that could affect the expression level of candidate genes in varicocele patients were identified. The GSE139447 study was used to evaluate the effect of MOP on our candidate genes (Materials and Methods). Our results showed that among the up-regulated candidate genes, the expression levels of *BUB1, SMC4, CENP-E*, *ASPM*, were up-regulated by the effect of the MOP drug on varicocele samples, while the expression level of *KIF11* was not changed significantly. In addition, the expression of *RPS5* and *RPL11* were up-regulated significantly by the effect of MOP on varicocele samples, while the expression of *RPS19* was down-regulated significantly, and the expression of *RPS2* and *RPS15* were not significantly changed (Fig.5A-J). Our results indicated that the expression level of *KIF11* was lowered by some drugs like DIMETHYLENASTRON, CHEMBL392369, CHEMBL409102, ISPINESIB, AZD-4877, and LITRONESIB, some of which have been reported to have inhibitory roles. Furthermore, our results showed that the expression level of *RPS19* could be increased by DEXAMETHASONE (Table 1). Therefore, our outcomes suggested that MOP could have strong effects on the master genes in varicocele and could potentially impede its progression.

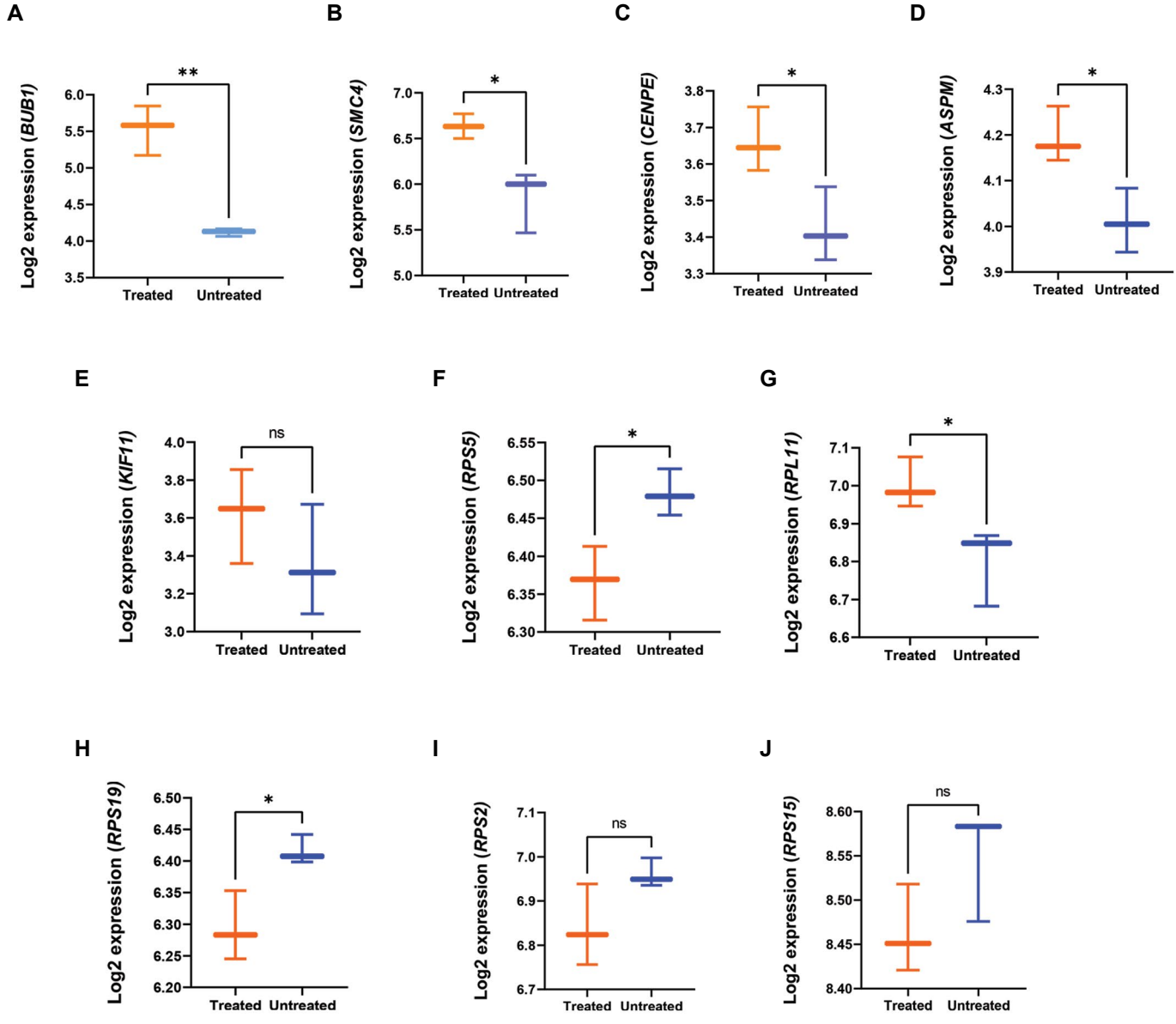

Fig.5: The effect of Morinda officinalis polysaccharide (MOP) on the expression levels of candidate genes. **A-E.** The effect of MOP drug on the expression levels of up-regulated and **F-J.** Down-regulated genes using the normalized count from the GSE139447 study. The Limma moderate t test was used to evaluate the degree of significance with the cutoff for statistical significance set at P<0.05, *; P<0.05, **; P≤0.01, and ns; P>0.05.

Table1: List of drugs effective on the candidate genes based on approved databases

Drug name	Score	Database	PMIDs	GENE	Specific action
DIMETHYLENASTRON	12.73	DTC	20138511	*KIF11*	Inhibitor
CHEMBL392369	12.73	DTC	19167222	*KIF11*	Inhibitor
CHEMBL409102	12.73	DTC	21344920	*KIF11*	Inhibitor
ISPINESIB	12.73	TdgClinicalTrial	NA	*KIF11*	Inhibitor
AZD-4877	12.73	GuideToPharmacology	NA	*KIF11*	Inhibitor
LITRONESIB	6.37	ChemblInteractions	NA	*KIF11*	Inhibitor
DEXAMETHASONE	5.89	NCI	15755903	*RPS19*	Stimulatory

NA; Not available.

Discussion

Varicocele is recognized as the primary and most remediable cause of male infertility. Nevertheless, some varicocele-affected men can procreate without intervention. This apparent contrast in fertility outcomes between varicocele-afflicted men who father at least one child and those who are infertile has remained a largely unresolved issue in the field of andrology (8). While obtaining semen and especially testicular biopsy from varicocele patients who have fathered a child can be challenging, animal models offer an alternative approach to deepen our understanding of the underlying causes of varicocele. The rat model has been widely utilized to explore the cellular and molecular mechanisms underlying varicocele and to advance our comprehension of this condition. Nevertheless, there is still potential for a more comprehensive approach (5).

In this study we utilized the transcriptomic profile of GSE139447 to investigate differentially expressed genes. Our analysis found 1,277 differentially expressed genes, including 677 up-regulated genes and 600 down-regulated genes. To identify key genes, we constructed PPI networks and obtained several modules. Using the cytohubba plugin, we identified the hub genes for the up- and down-regulated genes. The top five up-regulated hub genes were *BUB1, SMC4, CENPE, KIF11*, and *ASPM*, all of which are related to the cell division cycle. The top down-regulated genes were *RPL11, RPS15, RPS19, RPS5*, and *RPS2*, which are all involved in ribosomal pathways.

The findings of this study showed that all five of the top hub genes were intricately linked to the mechanism of cell division. It can be comprehended that even slight fluctuations in temperature can disrupt the organization of microtubules, which can ultimately lead to severe cellular consequences (9). These genes include:

BUB1 is a key protein mediating spindle-checkpoint activation and plays a key role in the inhibition of the anaphase-promoting complex/cyclosome (APC/C). Therefore, it is not surprising that a SNP in this gene predisposes humans to disease or affects drug response. In addition, BUB1 plays a central role in centromere cohesion and spindle assembly checkpoint (SAC). In human oocytes, the activity *BUB1* decreases with age while concomitantly the inter-kinetochore distances of bivalent chromosomes strongly increase with age (10). The consequence of this is aberrant sister chromatid separation in meiosis I, leading to aneuploidy (10, 11). Therefore, up-regulation of *BUB1* could be considered as a compensatory mechanism to overcome the heat-induced spindle-checkpoint aberrations in varicocele.

SMC4 one of the other up-regulated hub genes identified in this study, is another component of cohesion within multi-unit protein complexes (12). These complexes are involved in the maintenance of the chromosome family and are associated with "spermatoproteasomes" (13, 14). In this regard, the miRNAs related to *SMC4* have been shown to be differentially expressed between fertile and infertile males with abnormal semen parameters. Therefore, up-regulation of this gene is possibly another compensatory approach to reduce anomalies in mitotic and meiotic divisions by overcoming aberrant protein degradation.

The precise regulation of protein homeostasis is crucial for the proper functioning of specific protein groups during different stages of spermatogenesis. The inactivation or degradation of these proteins at specific stages is necessary for the step-wise progression of spermatogenesis. This process involves unique protein degradation events that are facilitated by testis-associated proteasome isoforms, known as “spermatoproteasomes”. Dysfunctions in these spermatoproteasomes have been linked to the pathogenesis of male infertility related to the ubiquitin-proteasome system, and may also be associated with chromatin defects that contribute to abnormal sperm morphology (12, 15, 16).

CENP-E similar to *BUB1*, regulates chromosome alignment and mediates kinetochore-microtubule attachment (17, 18). The specific inhibition of this protein significantly disrupts spermatogenesis and

can result in the arrest of the cell cycle, chromosome misalignment, spindle disorganization, and, ultimately, the formation of aneuploid cells. Moreover, the disruption of *CENP-E* function can lead to defects in spermatid formation, including impaired sperm head condensation and tail formation. For instance, Baccetti et al. (19) demonstrated that men with varicocele commonly exhibit mean frequencies of disomies and diploidy outside the normal range, indicating a severe disturbance in meiotic segregation.

KIF11 is involved in various kinds of spindle dynamics including chromosome positioning, centrosome separation, and establishing a bipolar spindle during cell mitosis (20). Thus, *KIF11* plays a crucial role in facilitating the proper progression of meiosis during spermatogenesis. Studies have shown that exposure to plasticizers can reduce its expression in male rat offspring. In addition, during spindle formation, its expression is altered in spermatogenesis-arrested mutant mice. Therefore, it serves as a pre-meiotic marker in germ cells and is essential for normal nuclear meiotic events (21).

Single-cell transcriptional analysis revealed that *KIF11* is notably overexpressed in spermatogonia and primary spermatocytes, as compared to other testicular cells. In these cells, the low expression of *KIF11,* as compared to cells with high expression of *KIF11*, cyclin binding, apoptotic processes, toxic substance binding, biological adhesion, DNA replication, identical protein binding, nuclear outer membrane of the endoplasmic reticulum membrane network, and the cell cycle are significantly enriched (20). Therefore, it is of note that *KIF11* is not only required for mitosis and meiosis but also is fundamental to a number of other biological functions, such as axonal transport in sperm motility.

ASPM is one of the genes involved in the regulation and evolution of brain size in primates and its mutation is associated with microcephaly and major defects in both male and female germlines (22). *ASPM* plays a central role in processes such as mitotic and meiotic spindle regulation, ciliary and flagellar function, and is essential for proper brain development. Given its critical role in these processes, it is not surprising that mutations in *ASPM* can lead to infertility. In the context of varicocele, the upregulation of *ASPM* may serve as a compensatory mechanism to improve the process of spermatogenesis

In summary, our analysis identified five up-regulated hub genes that are associated with cell division. Interestingly, the down-regulation of these genes is expected upon the reversal of varicocele, indicating that their up-regulation serves as a compensatory mechanism to rectify cell division-related disorders in the state of varicocele. However, in the long term, lack of recovery from varicocele, may possibly lead to a gradual decrease in the expression of these genes. The consequence of this depletion in these genes could result in reduced sperm count and motility, increased sperm abnormalities, and germline depletion in both human and rat models. It is important to note that the dataset used in this study was derived from rats with chronic varicocele, which is similar to the chronic condition observed in humans. In contrast, the only other available transcriptomic study in the literature utilized an acute rat model of varicocele. The observed differences between these two studies may be attributed to the distinction between the chronic and acute models. Although no transcriptomic analyses of human varicocele have been conducted yet, it would be intriguing to examine how future human studies align with the chronic or acute rat model, providing valuable insights into the condition in humans.

As stated above, the up-regulation of these genes is likely to be related to compensatory mechanisms, and to maintain spermatogenesis in surviving seminiferous tubules. Therefore, it is worth noting that MOP, which is also believed to have anti-inflammatory effects, increased the expression of these five up-regulated genes, of which 4 were significant. Observing that MOP in the state of varicocele increases the expression of these compensatory genes gives hope for remedying varicocele. Based on data presented in Table 1, it was interesting to note that 6 out of the 7 predicted drugs targeted *KIF11* via inhibitory action. Considering that *KIF11* is a pre-meiotic marker, its reduced expression may also be an alternative way to save pre-meiotic cells from entering a defective process of spermatogenesis; an hypothesis which needs future consideration.

Four of the top down regulated genes are associated with ribosomes, which are macromolecular machines involved in protein synthesis and composed of small and large 40S and 60S subunits, consisting of four RNA species and approximately eighty structurally distinct proteins. The "ribosome filter hypothesis" proposes that the specific functions of ribosomal proteins may make ribosomes selective for mRNA translation in a cell type- or tissue-specific manner, such as in the testis (23). By doing so, it minimizes the energy resources or cost of generating misfolded proteins at the earliest stages and occurs during ER-stress (24). This has important implications considering that seventy percent of cell energy is used for protein synthesis. In this analysis, the five down-regulated genes are:

RPL11 has been shown to be up-regulated in the normozoospermic group, as compared to the asthenozoospermic group. Moreover, in men with idiopathic infertility, antioxidant supplementation has been reported to increase the expression of this transcript, indicating its potential therapeutic value in the management of male infertility (25).

However, the deficiency of *RPL11* has been associated with reduced p53 responses and high C-MYC levels (RPL11-3). In the case of varicocele, the reduced expression of RPL11 may serve as a compensatory mechanism to transiently inhibit apoptosis induced by the condition. It is important to note that RPL11 is among the sperm-intact RNAs delivered to the zygote during

fertilization (RPL11-2), and its reduced level may have an impact on zygote quality (26).

RPS15 mutations rewire the translation program of primary CLL cells by reducing their translational efficiency. In addition, mutations in the C-terminal tail of human RPS15 have been shown to induce defective translation and impair the late pre-40S maturation step in the cytoplasm, the consequence of which could be reduced protein synthesis in varicocele (27).

In this regard, transcriptomic assessment of individuals with Klinefelter syndrome showed that RPS15 was among the hub genes in sperm, with higher expression observed in the control group. Interestingly, its expression showed a downward trend from SSC cells to sperm, indicating its potential role in spermatogenesis in normal individuals (28).

It was interesting to note that the expression of *RSP19*, a ribosomal protein, was reduced when sperm were exposed to the stress of both slow and rapid freezing (29, 30). It was also interesting to note that in an integrative network analysis of proteins, nine of the 10 hubs were cytoplasmic ribosomal proteins and *RPS19* was among these 9 hub genes (31). Gur and Breitbart (32) state that, despite spermatozoa being relatively transcriptionally and translationally silent, some studies have reported the existence of de novo protein synthesis under capacitation conditions. These proteins may be important for the replacement of some degraded proteins during capacitation events and their impairment may account for reduced motility, actin polymerization, acrosomal reaction, and *in vitro* fertilization. Therefore, low levels of ribosomal proteins, like *RPS19*, occurring in varicocele may account for poor-sperm quality.

RPS5 is another ribosomal protein shown to be down-regulated in asthenozoospermic and normozoospermic men compared to fertile or control groups (33). This statement indicates the critical role of ribosomes in producing healthy sperm and highlights the association between defects in their biogenesis and various diseases. It has been suggested that the copy number of ribosomal DNA is correlated with gene expression variation and mitochondrial DNA abundance in humans. As mitochondria have their own translational machinery and the down-regulation of ribosomal proteins could affect the assembly of ribosomes in mitochondria, it is possible that the consequence of reduced *RPS15* expression could lead to mitochondrial dysfunction in spermatozoa and, thereby, oxidative stress (34).

RPS2 has also been reported to be down-regulated during cryopreservation stress in human sperm (35). In addition, Talluri et al. (35) showed the expression of *RPS2* to be lower in low-fertile vs high-fertile bulls. They also concluded that this might be related to the role of the ribosomes in mitochondrial biogenesis which also accounts for oxidative stress.

Taken together, the analysis of both up-regulated and down-regulated hub genes, particularly those involved in mitotic and meiotic machinery for the up-regulated genes, and ribosomal machinery for the down-regulated genes, shows the significance of these biological processes in varicocele. However, a search in PubMed and Google suggests that these processes have not received much attention in the context of varicocele, despite their importance as highlighted in this network analysis. On the other hand, literature surveys have demonstrated that oxidative-reductive imbalance, endoplasmic stress, and altered metabolism are central factors in the biological and molecular pathways underlying varicocele pathophysiology (5). Differences observed through various biological approaches have contributed to the observed complexity of the pathophysiology of varicocele. However, these differences also provide an opportunity for further investigation to determine which biological or molecular pathways have a major impact on the infertility associated with varicocele. In this regard, a comparative study conducted by Xu et al. (36) evaluated the proteome profile of testicular tissues in rats with varicocele and after varicocelectomy. The study reported significant changes in the expression of several proteins, including 40S ribosomal protein S24, 60S ribosomal protein L38, and 60S ribosomal protein L32, which were ranked as the top first, fourth, and eighth proteins, respectively, in terms of the magnitude of the change in their expression in varicocele. This finding further confirms the effect of varicocele on protein machinery.

In addition, the study carried by Zhu et al. (37) which assessed key genes in varicocele rats via high-throughput sequencing and bioinformatics showed that oxidative stress-induced cell death and amyotrophic lateral sclerosis are the two major pathway involved in varicocele. These findings are consistent with the literature on the acute model of varicocele (8 weeks after surgery), while the data from this study pertains to the chronic model of varicocele (12 weeks), which resembles chronic varicocele in humans. Additionally, our study may shed light on the role of oxidative-reductive stress, ER stress, hypoxia, and related mechanisms that may be secondary to the altered regulation of these hub genes. Our study is the first bioinformatics study of chronic varicocele in a rat model. Differences between the two studies are likely to be related to difference between the acute vs the chronic model.

Considering the temperature sensitivity of mitotic and meiotic spindles, the up-regulation of genes related to the meiotic and mitotic machinery to improve the integrity of cell division is understandable. However, the altered or reduced expression of genes related to ribosomal biosynthesis is intriguing and novel, as it evidently affects protein biosynthesis. In this regard, it has been demonstrated that free ribosomal proteins regulate the Mdm2/p53 axis, leading to DNA damage, cell cycle arrest, apoptosis, and autophagy. Therefore, reduced expression of ribosomal proteins may inhibit the Mdm2/p53 axis, reducing the ability of the testis to remove DNA-damaged cells, and allowing processes like cell

cycle arrest, apoptosis, and autophagy to be executed.

Based on the Drug Gene Interaction Database analysis, Dexamethasone is the only predicted drug that targets ribosomes. However, it is interesting to note that MOP seems to increase the expression of *RPS5* and *RPS11*, while decreasing the expression of *RPS19*. In contrast, changes in the expression of *RPS15* and *RPS2* were insignificant (38).

It is noteworthy that dexamethasone has been demonstrated to decrease inflammation-induced oxidative and nitrosative stresses in a rat model of varicocele. In their study, Khosravanian et al. (39) showed that dexamethasone safeguarded testicular endocrine functions and facilitated the process of spermatogenesis. However, the effect of dexamethasone on ribosomal proteins in the context of varicocele remains to be investigated. Despite prior studies by Wang et al. (40) on teratozoospermia and by Zhang et al. (41) on asthenozoospermia, proposing a connection between ribosomal function and male infertility, the role and function of ribosomes in varicocele has not been explored. This is significant, given that varicocele is a well-known and treatable cause of male infertility. In this regard, unexpectedly, the second largest family of proteins, after the nucleoproteins, identified in the isolated human sperm nucleus corresponds to the ribosomal proteins (42). In addition, the background literature shows that among the ribosomal protein, *RPS6* regulates the viability of Sertoli cells in blood-testis barrier dynamics in rats, and deficiency of Rpl10lin in male mice and humans results in infertility (43-45). Collectively, these finding indicate that the disruption of ribosome biogenesis should be considered as one of the possible causes of male infertility. Considering the fact that ribosomal proteins constitute the second largest family of proteins in sperm, their reduced expression may be related to their abundance rather than their functions. This may also be true for other proteins, and, taken together, the functional importance of these findings requires validation.

Given the highly condensed and transcriptionally inert nature of the sperm nucleus, mitochondrial machinery is likely to be the sole player in translational processes within the sperm. As such, the altered transcription of ribosomal proteins may significantly impact mitochondrial function, which in turn may partially account for the oxidative-reductive stress observed in varicocele. The abundance of ribosomal protein in the sperm nucleus is not surprising, as it has been reported to be present in the cell nuclei of other eukaryotic cell types. The assembly of the 40S and 60S ribosomal subunits typically occurs in the nucleolar compartment of the nucleus, with later maturation events occurring in the nucleoplasm and cytoplasm. This suggests that altered ribosomal function in the context of varicocele could have implications beyond mitochondrial dysfunction, potentially impacting nucleolar and cytoplasmic processes as well (46, 47). This suggests that the sperm nucleus could potentially serve as a reservoir for ribosomal proteins that may play a role after fertilization, although further research is needed to explore this possibility. Alternatively, it is possible that these ribosomal proteins are simply remnants of spermatogenic differentiation.

Conclusion

Based on the results obtained in this study, it appears that cell division and ribosomal pathways are the most severely affected in chronic varicocele, and although the existing literature partly supports the outcome of this study, lab verification remains to be ascertained. The difference between this study and the only previous study on rat varicocele is likely to be related to the differences between the acute vs the chronic model of varicocele; an hypothesis which also needs verification. In addition, this type of study generates hypotheses for future lab studies and testing of candidate drugs, like dexamethasone, to remedy the damage induced by varicocele. It is important to note that this study was limited to rat models, and further research in humans is needed to validate these findings.

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Authors' Contributions

A.V.M.; Design, Conceptualization and Methodology of the study was undertaken. M.A.; Data mining, Formal analysis, and Investigation. A.N.-E., K.P.; Carried out the literature search and helped with interpretation and writing of the manuscript. M.H.N.-E.; Supervision, Validation, Visualization, Reviewed, Edited and Approved. All authors read and approved the final manuscript.

Reference

1. Paduch DA, Skoog SJ. Current management of adolescent varicocele. Rev Urol. 2001; 3(3): 120-133.
2. Cozzolino DJ, Lipshultz LI. Varicocele as a progressive lesion: positive effect of varicocele repair. Hum Reprod Update. 2001; 7(1): 55-58.
3. Arab D, Doustmohammadi H, Ardestani Zadeh A. Dietary supplements in the management of varicocele-induced infertility: a review of potential mechanisms. Andrologia. 2021; 53(1): e13879.
4. Swain N, Samanta L, Agarwal A, Kumar S, Dixit A, Gopalan B, et al. Aberrant upregulation of compensatory redox molecular machines may contribute to sperm dysfunction in infertile men with unilateral varicocele: a proteomic insight. Antioxid Redox Signal. 2020; 32(8): 504-521.
5. Razi M, Tavalaee M, Sarrafzadeh-Rezaei F, Moazamian A, Gharagozloo P, Drevet JR, et al. Varicocoele and oxidative stress: new perspectives from animal and human studies. Andrology. 2021; 9(2): 546-558.
6. Ni K, Steger K, Yang H, Wang H, Hu K, Chen B. Sperm protamine mRNA ratio and DNA fragmentation index represent reliable clinical biomarkers for men with varicocele after microsurgical varicocele ligation. J Urol. 2014; 192(1): 170-176.
7. Zhang L, Zhao X, Wang W. lncRNA and mRNA sequencing of the left testis in experimental varicocele rats treated with Morinda officinalis polysaccharide. Exp Ther Med. 2021; 22(4): 1136.
8. Jensen CFS, Østergren P, Dupree JM, Ohl DA, Sønksen J, Fode M. Varicocele and male infertility. Nat Rev Urol. 2017; 14(9): 523-533.

9. Nishio K, Yamazaki M, Taniguchi M, Besshi K, Morita F, Kunihara T, et al. Sensitivity of the meiotic stage to hyperthermia during in vitro maturation of porcine oocytes. Acta Vet Hung. 2017; 65(1): 115-123.
10. Lagirand-Cantaloube J, Ciabrini C, Charrasse S, Ferrieres A, Castro A, Anahory T, et al. Loss of centromere cohesion in aneuploid human oocytes correlates with decreased kinetochore localization of the sac proteins Bub1 and Bubr1. Sci Rep. 2017; 7: 44001.
11. Marchetti F, Venkatachalam S. The multiple roles of Bub1 in chromosome segregation during mitosis and meiosis. Cell Cycle. 2010; 9(1): 58-63.
12. Gómez L, Felipe-Medina N, Condezo YB, Garcia-Valiente R, Ramos I, Roig I, et al. Spermatoproteasome-deficient mice are proficient in meiotic DNA repair but defective in meiotic exit. bioRxiv. 2018; 384354. Available from: https://www.biorxiv.org/content/10.1101/384354v1.full.pdf (3 Aug 2018).
13. Siddiqui NU, Rusyniak S, Hasenkampf CA, Riggs CD. Disruption of the Arabidopsis SMC4 gene, AtCAP-C, compromises gametogenesis and embryogenesis. Planta. 2006; 223(5): 990-997.
14. Xiong Y, Yu C, Zhang Q. Ubiquitin-proteasome system-regulated protein degradation in spermatogenesis. Cells. 2022; 11(6): 1058.
15. Salas-Huetos A, Blanco J, Vidal F, Godo A, Grossmann M, Pons MC, et al. Spermatozoa from patients with seminal alterations exhibit a differential micro-ribonucleic acid profile. Fertil Steril. 2015; 104(3): 591-601.
16. Wen FP, Guo YS, Hu Y, Liu WX, Wang Q, Wang YT,et al. Distinct temporal requirements for autophagy and the proteasome in yeast meiosis. Autophagy. 2016; 12(4): 671-688.
17. Abrieu A, Kahana JA, Wood KW, Cleveland DW. CENP-E as an essential component of the mitotic checkpoint in vitro. Cell. 2000; 102(6): 817-826.
18. She ZY, Yu KW, Zhong N, Xiao Y, Wei YL, Lin Y, et al. Kinesin-7 CENP-E regulates chromosome alignment and genome stability of spermatogenic cells. Cell Death Discov. 2020; 6: 25.
19. Baccetti BM, Bruni E, Capitani S, Collodel G, Mancini S, Piomboni P, et al. Studies on varicocele III: ultrastructural sperm evaluation and 18, X and Y aneuploidies. J Androl. 2006; 27(1): 94-101.
20. Hara-Yokoyama M, Kurihara H, Ichinose S, Matsuda H, Ichinose S, Kurosawa M, et al. KIF11 as a potential marker of spermatogenesis within mouse seminiferous tubule cross-sections. J Histochem Cytochem. 2019; 67(11): 813-824.
21. Cannarella R, Condorelli RA, Mongioì LM, La Vignera S, Calogero AE. Molecular biology of spermatogenesis: novel targets of apparently idiopathic male infertility. Int J Mol Sci. 2020; 21(5): 1728.
22. Pulvers JN, Bryk J, Fish JL, Wilsch-Bräuninger M, Arai Y, Schreier D, et al. Mutations in mouse Aspm (abnormal spindle-like microcephaly associated) cause not only microcephaly but also major defects in the germline. Proc Natl Acad Sci USA. 2010; 107(38): 16595-16600.
23. Mauro VP, Edelman GM. The ribosome filter redux. Cell Cycle. 2007; 6(18): 2246-2251.
24. Sitron CS, Brandman O. Detection and degradation of stalled nascent chains via ribosome-associated quality control. Annu Rev Biochem. 2020; 89: 417-442.
25. Bansal SK, Gupta N, Sankhwar SN, Rajender S. Differential genes expression between fertile and infertile spermatozoa revealed by transcriptome analysis. PLoS One. 2015; 10(5): e0127007.
26. Sun YH, Wang A, Song C, Shankar G, Srivastava RK, Au KF, et al. Single-molecule long-read sequencing reveals a conserved intact long RNA profile in sperm. Nat Commun. 2021; 12(1): 1361.
27. Chan D, Delbès G, Landry M, Robaire B, Trasler JM. Epigenetic alterations in sperm DNA associated with testicular cancer treatment. Toxicol Sci. 2012; 125(2): 532-543.
28. He H, Huang T, Yu F, Chen K, Guo S, Zhang L, et al. KIF2C affects sperm cell differentiation in patients with Klinefelter syndrome, as revealed by RNA-Seq and scRNA-Seq data. FEBS Open Bio. 2022; 12(8): 1465-1474.
29. Corda PO, Silva JV, Pereira SC, Barros A, Alves MG, Fardilha M. Bioinformatic approach to unveil key differentially expressed proteins in human sperm after slow and rapid cryopreservation. Front Cell Dev Biol. 2022; 9: 759354.
30. Fu L, An Q, Zhang K, Liu Y, Tong Y, Xu J, et al. Quantitative proteomic characterization of human sperm cryopreservation: using data-independent acquisition mass spectrometry. BMC Urol. 2019; 19(1): 133.
31. Prakash MA, Kumaresan A, Sinha MK, Kamaraj E, Mohanty TK, Datta TK, et al. RNA-Seq analysis reveals functionally relevant coding and non-coding RNAs in crossbred bull spermatozoa. Anim Reprod Sci. 2020; 222: 106621.
32. Gur Y, Breitbart H. Protein synthesis in sperm: dialog between mitochondria and cytoplasm. Mol Cell Endocrinol. 2008; 282(1-2): 45-55.
33. Choucair F. Unraveling the sperm transcriptome by next generation sequencing and the global epigenetic landscape in infertile men. 2018. Avialable from: https://theses.hal.science/tel-01958881/preview/2018AZUR4058.pdf (6 Sep 2018).
34. Gibbons JG, Branco AT, Yu S, Lemos B. Ribosomal DNA copy number is coupled with gene expression variation and mitochondrial abundance in humans. Nat Commun. 2014; 5: 4850.
35. Talluri TR, Kumaresan A, Sinha MK, Paul N, Ebenezer Samuel King JP, Datta TK. Integrated multi-omics analyses reveals molecules governing sperm metabolism potentially influence bull fertility. Sci Rep. 2022; 12(1): 10692.
36. Xu F, Gao QQ, Zhu LL, Jiang HS, Chen H, Xu ZP, et al. Impact of varicocelectomy on the proteome profile of testicular tissues of rats with varicocele. Andrologia. 2018; 50(2).
37. Zhu J, Ou N, Song Y, Hu R, Zhang W, Liang Z, et al. Identification and verification of key genes in varicocele rats through high-throughput sequencing and bioinformatics analysis. Andrologia. 2020; 52(9): e13662.
38. Kang J, Brajanovski N, Chan KT, Xuan J, Pearson RB, Sanij E. Ribosomal proteins and human diseases: molecular mechanisms and targeted therapy. Signal Transduct Target Ther. 2021; 6(1): 323.
39. Khosravanian H, Razi M, Farokhi F, Khosravanian N. Simultaneous administration of dexamethasone and vitamin e reversed experimental varicocele-induced impact in testicular tissue in rats; correlation with Hsp70-2 chaperone expression. Int Braz J Urol. 2015; 41(4): 773-790.
40. Wang X, Ma S, Mao M, Li C, Shen X, Xu S, et al. RNA-sequencing and bioinformatics analysis of long noncoding RNAs and mRNAs in the prefrontal cortex of mice following repeated social defeat stress. Biomed Res Int. 2019; 2019: 7505260.
41. Zhang T, Wu J, Liao C, Ni Z, Zheng J, Yu F. System analysis of teratozoospermia mRNA profile based on integrated bioinformatics tools. Mol Med Rep. 2018; 18(2): 1297-1304.
42. de Mateo S, Castillo J, Estanyol JM, Ballescà JL, Oliva R. Proteomic characterization of the human sperm nucleus. Proteomics. 2011; 11(13): 2714-2726.
43. Jiang L, Li T, Zhang X, Zhang B, Yu C, Li Y, et al. RPL10L is required for male meiotic division by compensating for RPL10 during meiotic sex chromosome inactivation in mice. Curr Biol. 2017; 27(10): 1498-1505. e6.
44. Mok KW, Mruk DD, Cheng CY. rpS6 regulates blood-testis barrier dynamics through Akt-mediated effects on MMP-9. J Cell Sci. 2014; 127(Pt 22): 4870-4882.
45. Tu C, Meng L, Nie H, Yuan S, Wang W, Du J, et al. A homozygous RPL10L missense mutation associated with male factor infertility and severe oligozoospermia. Fertil Steril. 2020; 113(3): 561-568.
46. Antoine M, Reimers K, Wirz W, Gressner AM, Müller R, Kiefer P. Identification of an unconventional nuclear localization signal in human ribosomal protein S2. Biochem Biophys Res Commun. 2005; 335(1): 146-153.
47. Schmidt C, Lipsius E, Kruppa J. Nuclear and nucleolar targeting of human ribosomal protein S6. Mol Biol Cell. 1995; 6(12): 1875-1885.