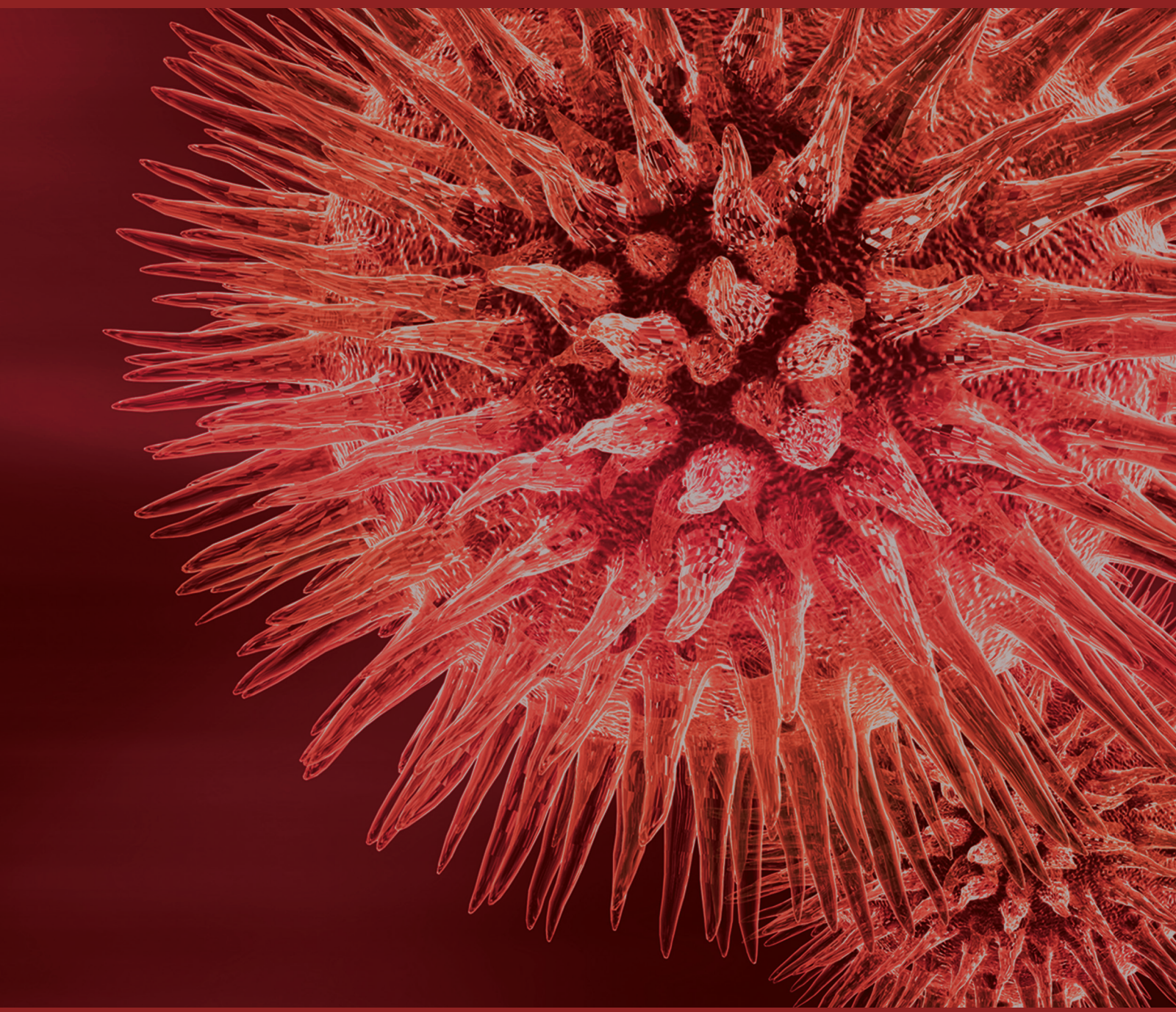
BioMed Research International

Male Infertility: Genetics, Mechanism, and Therapies

Guest Editors: Charles Coutton, Rafael A. Fissore, Gianpiero D. Palermo, Katrien Stouffs, and Aminata Touré



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Editorial

Male Infertility: Genetics, Mechanism, and Therapies

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The World Health Organization declaimed that infertility is a major global public health issue of the last few decades. Infertility is commonly defined as the failure to conceive after 1 year of unprotected intercourse and is estimated to concern 72.4 million people worldwide with 40.5 million currently seeking medical care. The overall burden of subfertility/infertility is significant, is likely underestimated, and has not displayed any decrease over the last 20 years. Male factors are estimated to be involved, at least partially, in half of the cases. While the diagnosis, medical treatment, and psychosocial management of infertility have rapidly evolved over the past 4 decades, some difficulties still persist. Little is known about the physiopathology of altered sperm production, its genetic causes, or the genetic and epigenetic consequences for the gamete and the forthcoming conceptus. The information generated by conventional semen analysis has historically classified patients into categories lacking knowledge of causality and leaving conventional therapy as somewhat empirical. One of the reasons for this lack of fundamental understanding is the heterogeneity of causal factors as male infertility is a typical multifactorial disorder with a strong genetic basis and additional factors such as urogenital infections, immunological or endocrine diseases, attack from reactive oxygen species (ROS), or perturbations from endocrine disruptors. Since assisted reproduction technology (ART) is widely used to achieve conception with gametes produced by compromised spermatogenesis, there is a clear need to detail the molecular pathogenesis of male infertility to improve long-term risk assessment on a case-by-case basis. In this context, research on the male partner will shed a much-needed light on the physiopathology of male reproduction, will enhance patient management, and constitutes a prerequisite for the development of new therapeutic solutions.

Acknowledgments

The editors thank the authors for their efforts and time spent for each manuscript. The lead editor would like to thank all editors for the time spent in reviewing, assigning reviewers, and commenting on submitted manuscripts. The editors hope that this special issue will prove useful to investigators, urologists, andrologists, and geneticists involved in the study of male infertility.

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Research Article

Sertoli Cell-Only Syndrome: Behind the Genetic Scenes

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Sertoli cell-only syndrome is defined by the complete absence of germ cells in testicular tissues and always results in male infertility. The aetiology often remains unknown. In this paper, we have investigated possible causes of Sertoli cell-only syndrome with a special focus on genetic causes. Our results show that, for a large part of the patients (>23% in an unselected group), the sex chromosomes are involved. The majority of patients had a Klinefelter syndrome, followed by patients with Yq microdeletions. Array comparative genomic hybridization in a selected group of "idiopathic patients" showed no known infertility related copy number variations.

1. Introduction

Spermatogenesis is a very complex process, involving thousands of genes, which can have an ubiquitous expression pattern or can have a specific function in reproductive tissues or spermatogenesis [1, 2]. Furthermore, many alternatively spliced spermatogenesis-specific transcripts are only detected in the testis, pointing at specialized functions of genes with an otherwise general expression pattern. The development of computation programs (fi the NCBI tool ORF finder: http://www.ncbi.nlm.nih.gov/gorf/gorf.html) has facilitated the prediction of presumable protein-coding genes. Moreover, massive parallel sequencing technologies have greatly facilitated the identification of transcripts and genes involved in spermatogenesis and other tissues. However, the characterization of the functionality and importance of newly identified genes remain a challenge.

In this study, we focus on a testicular phenotype in which all spermatogenic cells are missing: Sertoli cell-only syndrome. Patients with Sertoli cell-only syndrome are infertile due to nonobstructive azoospermia (NOA). Although, for most of these patients, artificial reproductive techniques such as intracytoplasmic sperm injection (ICSI) with the patients' own sperm cells will be impossible, it is important to gain insight into the origin of the fertility problems. For the small number of patients for whom still a few spermatozoa could be obtained, often after testicular sperm extractions (TESE), it is even more essential to gain insight into potential genetic origins of their problems. For these patients ICSI might be possible and, consequently, the fertility problems might be transmitted to the next generation.

In this study, we have examined patients with Sertoli cell-only syndrome. We first studied known (genetic) causes of Sertoli cell-only syndrome and then looked at the presence of copy number variations by array comparative genomic hybridization (array CGH) analysis in a selected group of patients with "idiopathic" Sertoli cell-only syndrome.

2. Material and Methods

2.1. Selection Criteria for Patients and Controls. The selection and elimination criteria for the patients are shown in Figure 1. A total of 171 azoospermic Caucasian patients with Sertoli cell-only syndrome were included in the present study. The diagnosis of azoospermia was based on at least two routine semen analyses, while a further classification as Sertoli cell-only syndrome was based on the histological examination of

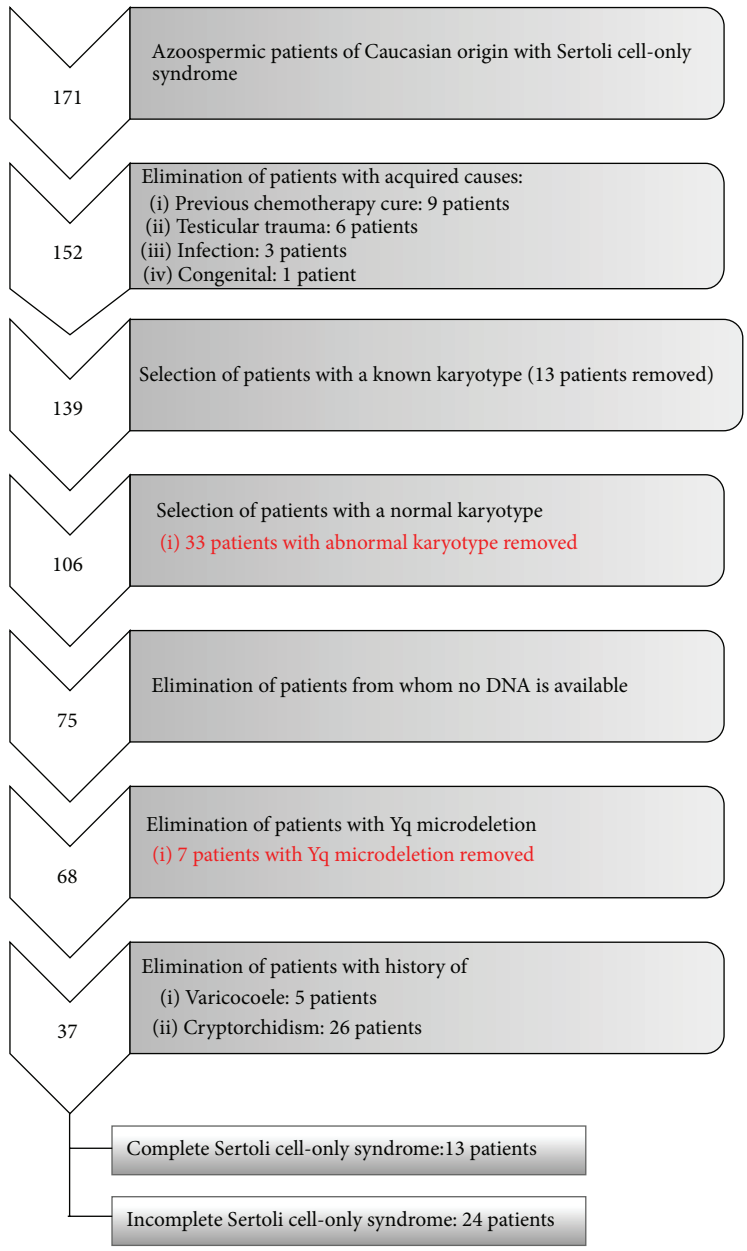

Figure 1: Overview of the patient selection.

a testicular biopsy within the frame of their fertility work-up [3].

For array CGH analysis, a selected group of patients was analyzed. The exclusion criteria included abnormal karyotype, presence of Yq microdeletion, presence of gr/gr deletion, previous varicocoele, or history of cryptorchidism. Furthermore, for all patients TESE was performed, and patients with residual spermatogenesis were excluded, defined as "incomplete Sertoli cell-only syndrome."

For the control group, DNA samples from men with normozoospermia, defined by routine sperm analysis, were used. These men were also from Caucasian origin from Belgium or The Netherlands. For array CGH analysis, 23 control samples were included, but these numbers are increased for the subanalyses as mentioned in the text.

2.2. DNA Preparation. Genomic DNA was isolated from peripheral blood using magnetic purification with the "Multiprobe II Plus EX + Gripper" liquid handling robot and "Chemagic Magnetic Separation Module I" (PerkinElmer, Belgium).

2.3. Yq Microdeletion Analysis and Karyotype Analysis. Karyotype analyses were performed using routine analyses. Yq microdeletion analysis was performed according to the guidelines proposed by Simoni et al. [4].

2.4. Array CGH. Array CGH analysis was performed using standard methods described [5]. In brief, 300 ng of genomic DNA was labeled with Cy3-dCTP or Cy5-dCTP (GE Healthcare, Belgium) using Bioprime array CGH genomic labeling system (Invitrogen, Belgium). For the labeling, we used the "triangle method": DNA samples from patients and controls were labeled and hybridized using a dye swap in trios consisting of at least one control per triangle. Samples were hybridized on 244K arrays (design ID 014693, Agilent, Belgium) for 40 h at 65°C. After washing, the samples were scanned at 5 μm resolution using a DNA microarray scanner G2505B (Agilent, Belgium). The scan images were analyzed using the feature extraction software 9.5.3.1 (Agilent) and further analyzed with "arrayCGHbase" [6]. Copy number variations were taken into consideration when two or more flanking probes were exceeding a value of the intensity ratios ± four times the standard deviation of $\log_2$ of all intensity ratios for that experiment. Always two experiments investigating the same sample with a dye swap were compared and only when an alteration is present in both experiments was the region included for further analysis. Inconsistencies were inspected manually.

2.5. qPCR. qPCR was performed on genomic DNA using predesigned Taqman Copy Number Assays (Applied Biosystems, Belgium) according to instructions of the manufacturer. Samples were run on the 7500 Real Time PCR system (Applied Biosystems, Belgium) and analyzed using Copy-Caller Software provided by Applied Biosystems (Belgium). The assays used are reported in Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/6191307. In each assay, we have analyzed the patient with the alteration detected by array CGH.

2.6. RNA Expression. The presence of RNA in testicular tissues was investigated using home-made RNA. Fresh testicular tissue was obtained from patients who came to the hospital for vasectomy repair and who signed an informed consent. The histology was determined on a second biopsy and showed normal spermatogenesis. RNA was extracted using the RNeasy Mini kit (Qiagen, Belgium) after which cDNA was prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Belgium). Primers for amplification of cDNA were designed according to the reference sequences and were overlapping at least one intron/exon boundary (primers available upon request). All amplified fragments from testicular tissues were sequenced to confirm specific amplification. Commercially available "Human Universal Reference Total RNA" (Westburg, Belgium) was investigated according to the above described method. The expression of RNA in multiple human tissues was analyzed using the Human MTC panels I and II including cDNA from 16 different tissues (Clontech, Westburg, Belgium). For MTHFD2L, two fragments were obtained after amplification with forward primer TTGTGCCTTGATCAGCATTC and reverse primer TGTCACTGGATCGTGGACAT, located in exon 4 and exon 7, respectively. The two obtained fragments were purified from an agarose gel after electrophoresis using the QIAEXII Gel Extraction kit (Qiagen) according to instructions of the manufacturer. The fragments were reamplified and sequenced using the above mentioned primers.

2.7. ncRNA Analysis. The analysis of "geneless" regions for the presence of noncoding (nc)RNA or presumed transcripts was performed using the following online programs: http://genome.ucsc.edu/, http://www.ensembl.org/index.html, http://diana.imis.athena-innovation.gr/DianaTools/index.php (miRGen2.0 was used) [7], and http://www.mirbase.org/search.shtml [8].

3. Results

3.1. Patient Selection. From 1994 to 2007, more than 600 patients with nonobstructive azoospermia have consulted the Center for Reproductive Medicine for a fertility treatment [9]. From this patient group, we have selected 171 Caucasian men that were categorized as having Sertoli cell-only syndrome. From this group, patients with idiopathic Sertoli cell-only syndrome were selected (Figure 1).

We were, in a first step, interested in known genetic causes of Sertoli cell-only syndrome. Therefore, we looked at the karyotype of the patients. For 139 patients, a karyotype was available (performed either in our lab or elsewhere). In this group 33 patients had an abnormal karyotype (24%). The majority of these individuals were Klinefelter patients (n = 29) or mosaics 46,XY/47,XXY (n = 3). The karyotype of the remaining patient was 46,X,der(X)t(Xp;Yp).

Next, we focused on Yq microdeletions. For the patients for whom DNA was available, we detected seven patients with a Yq microdeletion: four patients with an AZFc deletion, one patient with an AZFb deletion, and two patients with an AZFb + AZFc deletion.

Finally, we looked at potential risk factors for male infertility: a history of cryptorchidism or a (previous) varicocoele. These two conditions are known to potentially influence the fertility status of the patient, although a clear relationship remains controversial since, for some patients, sperm production is normal or only slightly subnormal.

For the remainder of the study, we ended up with 37 patients for whom the etiology of their fertility problems remains unknown. Thirteen of these patients had a complete Sertoli cell-only syndrome and for 24 patients some sperm cells could be retrieved during TESE. We focused on nine patients with a complete Sertoli cell-only syndrome. The four remaining patients were no longer available for further research.

3.2. Array CGH Analysis. In order to detect copy number variations, array CGH analysis was performed on the nine patient samples described above. Together with these patients, we have also analyzed 23 control samples.

In total, we have detected 800 CNVs, 213 in the patient group and 587 in the control group. The average number of CNVs was not different in the patient versus the control group: 23.7 versus 25.5. Next, we looked at the number of CNVs for each chromosome (Figure 2, red spots). From the 800 regions, 587 regions (163 in the patient group and 424 in the control group) could be selected out because they

Table 1: Overview of patient-specific copy number variations and the genes located in these regions, detected by array comparative genomic hybridization analysis.

	Patient	Deletion/ duplication	Region	Start position	Stop position		Genes involved
1	ESCO16	Del	1p21.1	104067184	104321250	13	RNPC3/AMY2B/AMY2A/AMY1A/AMY1C/AMY1B
2	**ESCO2**	**Dupl**	**1q23.1**	**156764562**	**156796736**	**7**	**PRCC/SH2D2A/NTRK1**
3	ESCO29	Dupl	2p22.1	38956776	38968369	3	GALM
4	ESCO2	Del	2p15	62258290	62277149	4	COMMD1
5	ESCO36	Del	2q13	110833640	110983703	20	MALL/NPHP1
6	**ESCO19**	**Del**	**4q13.3**	**75019400**	**75117648**	**10**	**MTHFD2L**
7	ESCO19	Dupl	4q21.1	76504370	76521476	2	CDKL2
8	ESCO34	Del	5p14.3	21943466	22002677	6	CDH12
9	ESCO25	Del	5p13.1	40779232	40785703	2	PRKAA1
10	ESCO29	Del	6p12.1	52624027	52682610	9	GSTA2/GSTA1
11	ESCO28	Del	6q26	162618199	162825032	23	PARK2
12	ESCO25	Dupl	7p22.2	3144476	3458017	28	SDK1
13	ESCO19	Del	7q11.22	66671745	66672634	2	TYW1
14	ESCO29	Del	7q35	143884029	143953472	4	FLJ43692/OR2A42/OR2A1
15	ESCO2	Dupl	8p23.1	6828426	6837339	2	DEFA1
16	ESCO34	Del	8p22	15403439	15409232	2	TUSC3
17	ESCO34	Dupl	11q24.2	124743538	124798029	11	ROBO3/ROBO4/HEPN1/HEPACAM
18	ESCO36	Del	12p12.2	21011077	21404166	37	SLCO1B3/LST-3TM12/SLCO1B1
19	ESCO25	Dupl	12q14.2	63947732	64116568	6	DPY19L2
20	ESCO19	Del	12q21.32	86695679	86703030	2	MGAT4C
21	ESCO29	Del	12q23.1	99994977	100005332	2	ANKS1B
22	**ESCO29**	**Dupl**	**13q12.11**	**20419333**	**20445320**	**5**	**ZMYM5**
23	ESCO36	Del	15q14	34671574	34841446	17	GOLGA8A/GOLGA8B + MIR1233-/DQ593032/DQ582939/KI110855
24	**ESCO18**	**Del**	**16p13.11**	**14968855**	**15115579**	**8**	**NOMO1/NPIP/PDXDC1**
25	ESCO28	Dup	16q12.2	55832511	55865159	5	CES1
26	**ESCO2**	**Del**	**18q21.2**	**52212057**	**52306572**	**10**	**C18orf26**
27	ESCO25	Del	18q21.31	55931229	55936547	2	NEDD4L
28	ESCO36	Dupl	22q11.21	18894835	19010508	19	DGCR6/PRODH
29	ESCO25	Del	22q13.33	50296855	50301369	2	ALG12
30	ESCO16	Dupl	X	134778328	134910134	12	CT45-1/CT45-2/CT45-4/CT45-3

were detected in patients as well as in controls. Figure 2 shows the average number of regions remaining after this selection procedure (green spots). In general, considering all chromosomes and after this selection, on average 5.6 CNVs were detected per patient, while this number was 7.1 in the control group. Finally, regions that were not containing any genes were eliminated. We ended up with 30 unique regions which were only altered in the patient group (Table 1). We also focused on "geneless regions" that are uniquely detected in the patient group and for which the reported CNV frequency is low (<5%). A total of 12 regions were analyzed through UCSC, Ensembl, the Diana database, and miRbase (Table 2).

3.3. Selection of Regions for qPCR Analysis to Look for CNVs in Patients and Controls. From the 30 unique gene-containing regions, the most promising CNVs were selected based on the reported population frequency and by searching the literature for the expression pattern, potential function of genes, and evolutionary conservation. Overall, 5 regions remained for further analysis (regions in bold in Table 1). These regions were three deletions located in chromosomal regions 4q13.3, 16p13.11, and 18q21.2 and two duplications located in 1q23.1 and 13q12.11. These regions varied in size from ~26 kb to ~147 kb. For all five regions, at least 87 extra normozoospermic controls were tested using Taqman copy number assays; together with the 23 normozoospermic controls tested through array CGH, a total of 110 controls were studied. In the analyses, a positive control, that is, the patient in which the CNV was detected, was always included. Consequently, all observed CNVs could be confirmed.

Multiple deletions have been detected for the region 16p13.11. We have tested a qPCR assay that was located in the PDXDC1 gene. This region was considered as probably nonpathogenic and therefore was not studied in more depth.

Table 2: Overview of patient-specific copy number variations not containing any genes.

Chromosome	Start	End	
12p12.1	21565934	21580503	—
16q23.3	83912597	83920609	—
2q14.3	122828556	122935716	DQ591124 (piRNA) and DQ583822 (piRNA)
3q13.32	118225639	118247407	EU250752
4p14	38458265	38467938	—
4p15.1	34033992	34053693	BC036345
5p14.3	20599622	20712049	AK093362
5p15.2	12559564	12572720	—
5q23.2	127077075	127082714	—
6p22.3	19774967	19791138	—
7q21.12	86941358	86947442	—
8q11.22	52042609	52074026	—
8q21.3	90423856	90458375	—
9p23	12163230	12357073	DB098556, HY017233, HY200407, and DB448686

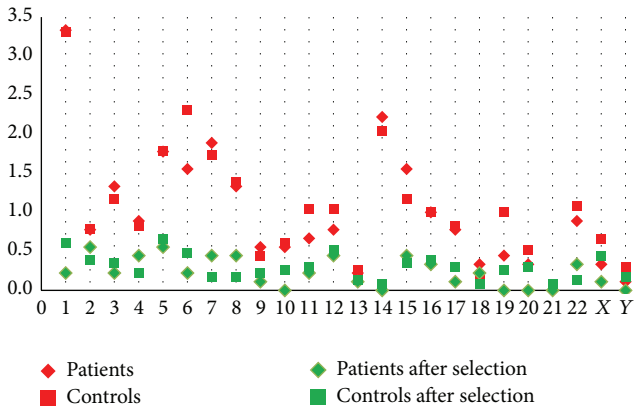

Figure 2: The average number of copy number variations per chromosome (CNVs) detected in the patient and control group before and after the removal of recurrent CNVs (i.e., CNVs that are detected in patients as well as in controls). The results do not differ between the two groups.

For all remaining regions 40 more controls were examined with qPCR. In these groups, no deletions/duplications were detected, except for the positive control.

3.4. Expression Analysis of Candidate Infertility Genes. We have tested the expression pattern of the following genes, located in the CNV regions of interest (Table 1) using multiple tissue cDNA panels or testis derived cDNA (see Section 2): C18ORF26, MTHFD2L, PDXDC1, and PRCC. The genes PDXDC1 and PRCC showed a ubiquitous expression pattern. No mRNA for C18ORF26 could be detected, which is in accordance with published data reporting that C18ORF26 is barely expressed in (normal) human tissues [10].

We could detect MTHFD2L when analyzing total human RNA. By amplifying a fragment compromising exon 4 to exon 7, two PCR fragments were detected. Excision of these fragments from a gel followed by reamplification and sequencing of each fragment individually showed two alternatively spliced fragments. The longest fragment showed an insertion of 98 bp, located in intron 6 bases c.806-20313 to c.806-20216 (minus strand) according to RefSeq NM_001144978.1. On the other hand, no transcripts were detected in the testis.

4. Discussion

In this study, we investigated the frequency of genetic causes of Sertoli cell-only syndrome and tried to identify new genetic causes. It is obvious from this study that karyotype abnormalities (24%), especially Klinefelter syndrome, are the most common abnormality seen in Caucasian azoospermic men with Sertoli cell-only syndrome. AZF deletions were detected in 9% of men with a normal karyotype and for whom DNA was available for analysis. Currently, AZF deletions are routinely examined in an infertile population with nonobstructive azoospermia or severe oligozoospermia. However, ~10 years ago, AZF deletions were not tested in every patient, explaining the difference in numbers of patients tested for karyotype analysis and Yq microdeletions.

In the Center for Reproductive Medicine, currently 3.10% of all ICSI cycles performed include male patients with NOA (after a successful TESE). Another 2.24% of patients have an obstructive azoospermia. Overall, the number of ICSI cycles performed with sperm cells from azoospermic male patients is very low (~5%).

In a recent study, Vloeberghs et al. [9] looked at the success rate of TESE ICSI cycles in patients with NOA. From the 714 patients included in the study, 464 were diagnosed as having "SCOS." For 38.4% of these patients, mature sperm cells could be detected. Consequently, for the majority of these patients, no spermatozoa could be retrieved. Since this study and our study are (partly) retrospective studies, we were not able to look for the presence of spermatogonia by testing

molecular markers. Consequently, the classification of SCOS patients in "complete" or "incomplete" SCOS is only based on the data obtained from (multiple) TESE attempts and a biopsy sample for diagnostics purposes.

For the final selection of patients for the present study, only Caucasians were included with an idiopathic Sertoli cell-only syndrome and for whom no sperm cells were found in multiple ejaculate samples, a diagnostic testis biopsy and, if available, a therapeutic TESE sample. Nine patients who fulfilled all criteria were included for further studies. The presence of a gr/gr deletion could be excluded, due to testing in previous studies [11].

These patients were analyzed through array CGH in order to detect CNVs that might be related to their fertility problems. Previous studies, performed in our center or abroad, have already shown that some CNVs might be related to male infertility. In the study of Krausz et al. X-linked CNVs were more abundant in the infertile patient group [12]. In our study, one X-linked CNV was detected that was absent in the control group. This CNV is removing multiple copies of the Cancer-Testis 45 gene family. In humans, at least six members of this family are present. The CT45 gene is expressed in testicular tissues, as well as in different cancer tissues. However, the role of this gene family in spermatogenesis remains unknown. We have not verified the presence of copy number variations in this gene since no Taqman copy number assays were available for this region. Furthermore, multiple copy number variations have been reported in the database of genomic variants (http://dgv.tcag.ca/dgv/app/home). When considering all X-linked CNVs detected in this study, three CNVs could be identified in the patient group and 15 in the control group, indicating that 0.3 and 0.7 X-linked CNVs were, respectively, detected per patient or per control. These X-linked patient CNVs were the one reported above involving the CT45 gene family and another CNV affecting TEX28 in two patients. In 5/23 controls, this region was also duplicated/deleted (and consequently the frequencies were similar). However, it should be noted that the average spacing of the probes on the X-chromosome specific array used in the study of Krausz et al. is much higher than the average resolution of the array of this study (4 kb versus 8.9 kb) [12].

We did detect, however, 30 patient-specific CNVs in which one or more genes were located. From these regions, five were selected out for further analysis. On the one hand, qPCR analysis was performed to confirm the presence of the CNV and to be able to test more control samples. On the other hand, the expression pattern of the genes located in these CNV regions was tested where appropriate. The knowledge of the expression pattern is useful in understanding the necessity of this gene in spermatogenesis. Genes that are not expressed in testicular tissues are not/less likely to be involved in spermatogenesis. Through qPCR analysis, we could conclude that the region 16p13.11 is very polymorphic, and consequently the PDXDC1 gene located in this region is presumably not crucial for spermatogenesis. Also the region containing C18ORF26 is most likely not involved in spermatogenesis since no transcripts of this gene were detected in testicular tissues. In a recent study, we have sequenced all (mRNA) transcripts from testicular tissues (unpublished data). Here again, we could show that C18ORF26 is not expressed in testicular tissues, while all other analyzed transcripts (including MTHFD2L) were present. We currently have no explanation for the discrepant results for the MTHFD2L gene. Massive parallel sequencing of RNA from testicular tissues from multiple patients, analyzed each individually, was able to show the presence of this gene, while PCR amplification with multiple primer sets from different commercially available testis libraries and in-house isolated testicular mRNA failed to amplify fragments of this gene.

The remaining two regions contained at least one gene that was shown or known to be expressed in testicular tissues: PRCC was tested in our center, while the expression pattern of ZMYM5 was described by Sohal et al. [13].

We also checked the presence of noncoding RNA sequences in regions where no known genes were located. Several testicular RNA transcripts were detected, but since their function and importance remain unknown, we decided not to focus on these regions for this paper.

Overall, we can conclude from this study that multiple CNVs could be (partially) causal for SCOS. However, it remains hard to determine the functional importance of genes located in these regions. In the present study ~24 CNVs were detected per individual (patient or control) analyzed. Part of the CNVs were unique for the control group. These neutral (or at least not-fertility causing) CNVs might be rare polymorphisms. Similarly, also the CNVs detected in the patient group might be rare CNVs, not related to the fertility problems of the patients. Consequently, array CGH might be useful in a research setting investigating male infertility, but it is still too early to implement as a routine test for idiopathic cases.

Altogether, these data show that especially the sex chromosomes are involved in the etiology of Sertoli cell-only syndrome, which was already suggested >20 years ago [14]. Moreover, Krausz et al. showed that CNVs on the X chromosome might be involved in male infertility [12]. Currently, the most frequent cause of nonobstructive azoospermia with Sertoli cell-only syndrome as the phenotypic background remains Klinefelter syndrome. Given the availability of next generation sequencing technologies, allowing genome sequencing as well as a better characterization of transcripts involved in spermatogenesis, it is expected that the knowledge of genetic factors involved in the etiology of Sertoli cell-only syndrome will increase over the next few years.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Variations in Antioxidant Genes and Male Infertility

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Oxidative stress and reactive oxygen species (ROS) are generated from both endogenous and environmental resources, which in turn may cause defective spermatogenesis and male infertility. Antioxidant genes, which include catalase (*CAT*), glutathione peroxidase (*GPX*), glutathione *S*-transferase (*GST*), nitric oxide synthase (*NOS*), nuclear factor erythroid 2-related factor 2 (*NRF2*), and superoxide dismutase (*SOD*), play important roles in spermatogenesis and normal sperm function. In this review, we discuss the association between variations in major antioxidant genes and male infertility. Numerous studies have suggested that genetic disruption or functional polymorphisms in these antioxidant genes are associated with a higher risk for male infertility, which include low sperm quality, oligoasthenoteratozoospermia, oligozoospermia, and subfertility. The synergistic effects of environmental ROS and functional polymorphisms on antioxidant genes that result in male infertility have also been reported. Therefore, variants in antioxidant genes, which independently or synergistically occur with environmental ROS, affect spermatogenesis and contribute to the occurrence of male infertility. Large cohort and multiple center-based population studies to identify new antioxidant genetic variants that increase susceptibility to male infertility as well as validate its potential as genetic markers for diagnosis and risk assessment for male infertility for precise clinical approaches are warranted.

1. Introduction

Reactive oxygen species (ROS), which are strongly linked with oxidative stress, are oxygen-derived free radicals that include superoxide anions, hydroxyl, peroxyl, alkoxyl radicals, and hydrogen peroxide [1]. ROS can be generated either from endogenous physical processes such as mitochondrial respiration and seminal leukocytes [2] or from various environmental factors, which include drugs, pollution, toxins, smoking, radiation, and diet [3]. In sperm, ROS can cause potential damage to plasma membrane and DNA integrity, motility, and overall semen quality [2, 4, 5]; therefore, scavenging excess ROS is mandatory for normal spermatogenesis and fertilization.

The nuclear factor erythroid 2-related factor 2/antioxidant response element (NRF2/ARE) signaling pathway and its regulated antioxidant enzymes have been shown to play crucial roles in cellular oxidative stress defense during spermatogenesis and fertilization [6, 7]. Antioxidant enzymes and molecules such as superoxide dismutases (SODs), glutathione (GSH), and catalases (CATs) are largely abundant in semen plasma or in sperm cells [8–10]. Most of these genes, including *NRF2*, *SOD*, *CAT*, glutathione *S*-transferase (*GST*), glutathione peroxidase (*GPX*), and nitric oxide synthase (*NOS*), harbor sequence variants in humans, which in turn may cause male infertility in different ways. As genetic variations are an important etiological factor in male infertility, these may significantly contribute to the incidence of male

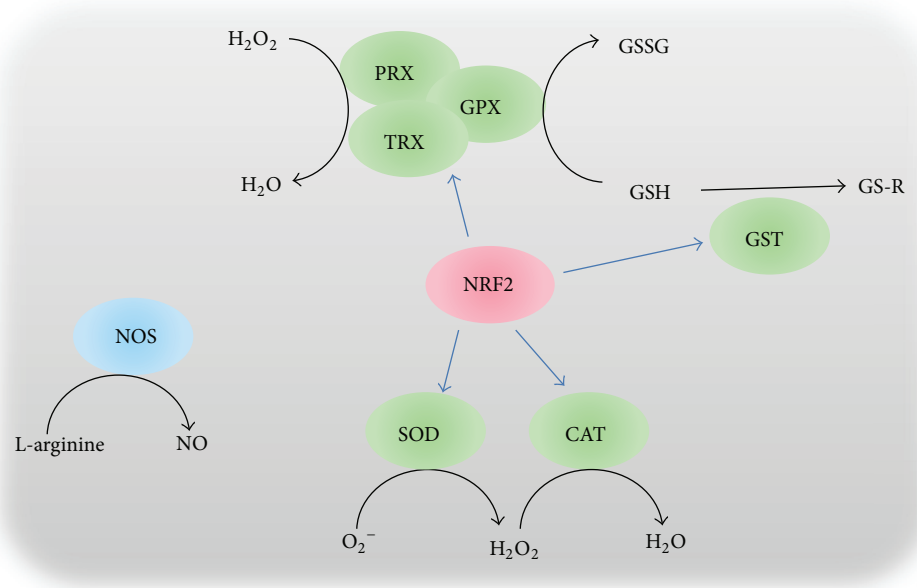

Figure 1: Major antioxidant gene products important for spermatogenesis. NRF2 regulates the expression of many antioxidant enzymes including peroxiredoxin (PRX), thioredoxin (TRX), glutathione peroxidase (GPX), glutathione S-transferase (GST), superoxide dismutases (SODs), and catalase (CAT). The principal form of ROS is anion superoxide (O_2^-), which can be converted into hydrogen peroxide (H_2O_2) by SODs. H_2O_2 can be catalyzed to H_2O by CAT, TPX, or PRX. GST catalyzes the conjugation of the reduced glutathione (GSH) to xenobiotic substrates. Nitric oxide synthases (NOSs) catalyze the production of nitric oxide (NO) from L-arginine. GS-R, GSH-xenobiotics adducts; GSSG, oxidized glutathione.

infertility, especially under environmental ROS stress [11]. To date, functional polymorphisms of antioxidant genes *NRF2*, *SOD*, *GST*, *NOS*, *CAT*, and *GPX* have been reported to be associated with male infertility in humans.

This review discusses the recent progress in the study of genetic variations in antioxidant genes that have associated with male infertility. The findings of these studies indicate that functional polymorphisms in the *NRF2*, *SOD*, *GST*, *NOS*, *CAT*, and *GPX* genes may potentially contribute to genetic causes of male infertility. As the incidence of male infertility continues to increase, the analysis of its association with sequence variants in antioxidant gene may not only help understand the roles of antioxidant signaling network in ROS-related male infertility but also facilitate validating its potential as genetic markers for the diagnosis and risk assessment for male infertility in the clinic.

2. Antioxidant Enzymes in Spermatogenesis

A number of antioxidant genes involved in spermatogenesis have been identified in mammals, which include *NRF2*, *SOD*, *CAT*, *GPX*, peroxiredoxin (*PRX*), glutaredoxin (*GRX*), thioredoxin (*TRX*), and *NOS* [6, 7, 55–57]. The enzymes encoded by these genes are widely involved in the cellular antioxidant response, GSH synthesis and reduction, and thiol redox cycles during spermatogenesis or involving sperm (Figure 1). Most of these genes also contain the ARE motif in its promoter regions, which facilitates the regulation of the oxidative stress-activated NRF2 transcription pathway [58].

NRF2 is the key gene in antioxidant defense, as it is the nuclear transcriptional factor that can induce antioxidant enzymes via ARE element [59]. In response to oxidative stress, NRF2 binds to AREs, mediating transcriptional activation of its responsive genes and modulating *in vivo* defense mechanisms against oxidative damage [60]. Kelch-like ECH-associated protein 1 (KEAP1) is the cytosolic regulatory protein of NRF2 and the sulfhydryl-rich sensor that responds to oxidants or electrophiles [61]. Under basal conditions, KEAP1 associates with NRF2 and targets it for degradation, and then modified KEAP1 by oxidative reagents will dissociate with NRF2 that could translocate into nucleus, bind to target gene ARE element, and promote many antioxidant enzyme gene expressions [62, 63].

Among the genes regulated by the NRF2-ARE signaling pathway, SODs and CATs are important enzymes that protect sperm from oxidative damage by superoxide and hydrogen peroxide (H_2O_2). SODs catalyze the dismutation of the superoxide radical into either ordinary molecular oxygen or hydrogen peroxide. Three families of SOD isoenzymes have been identified in humans: soluble SOD or CuZn SOD (SOD1), mitochondrial SOD or Mn SOD (SOD2), and extracellular SOD or EC SOD (SOD3) [13]. Among these, isoenzyme SOD2 is highly expressed in human semen [8, 13]. Seminal CAT catalyzes the degradation of H_2O_2 to oxygen

Table 1: Major antioxidant enzymes in spermatogenesis.

Enzyme	Name	Isoforms in human	Reference
NRF2	Nuclear factor erythroid 2-related factor 2	NRF2	[12]
SOD	Superoxide dismutase	SOD1, SOD2, SOD3	[13]
CAT	Catalase	CAT	[9]
NOS	Nitric oxide (NO) synthase	NOS-1, NOS-2, NOS-3	[14]
GST	Glutathione S-transferase	GSTA1–GSTA5, GSTZ1, GSTM1–GSTM5, GSTO1-GSTO2, GSTP1, GSTT1–GSTT4	[15]
PRX	Peroxiredoxin	PRX1–PRX6	[16, 17]
GPX	Glutathione peroxidase	GPX1–GPX8	[18]
TRX	Thioredoxin	TRX1, TXR2	[19]

and water [64], which are involved in the maintenance of normal levels of ROS and protection of spermatozoa against potentially toxic ROS [9].

NOSs are a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine [65], which is considered as an antioxidant that scavenges ROS at low concentrations [66–68]. The role of NO in sperm motility and its effect on fertility have been proven in penile erection, sperm motility and viability, metabolism, and acrosomal reaction [14]. Three NOS isoenzymes have been identified in mammals, which include neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2), and endothelial NOS (eNOS; NOS3) [69].

GSTs are abundant cytosolic proteins that catalyze the conjugation of GSH to electrophilic xenobiotic substrates, which usually form ROS *in vivo* [15]. The GST family consists of three superfamilies: the cytosolic, mitochondrial, and microsomal GSTs [15, 70]. In humans, GSTs include mitochondrial GSTK1, microsomal MGST1–MGST3, and cytosolic GSTA1–GSTA5, GSTZ1, GSTM1–GSTM5, GSTO1–GSTO2, GSTP1, and GSTT1–GSTT4 [71].

TPX, PRX, and GRX are enzymes involved in the redox of thiols in cells. TRXs and GRX collaboratively catalyze the reduction of protein mixed disulfides [72–74]. TRX isoenzyme TRX1 is located in the cytosol and the nucleus, and TRX2 is exclusively expressed in the mitochondria [75, 76]. PRX enzymes are a group of highly abundant peroxidases that eliminate organic hydroperoxidase and H_2O_2. The glutathione peroxidase (GPX) protein family catalyzes thiol redox with glutathione [18]. Among its isoenzymes, GPX4 is predominant in the testis and is currently considered vital for spermatogenesis [52]. GPX5 is solely expressed in the caput epididymis and possibly functions in maintaining sperm DNA integrity [54].

Studies employing animal models have further confirmed that mRNAs encoding several antioxidant genes can be detected at steady-state levels in the mouse testis [77]. For example, *SOD2* mRNA levels are developmentally regulated to reach maximal levels of expression in early post-meiotic germ cells, whereas the levels of *GPX* and *CAT* mRNAs are relatively constant [77]. *TPX* and *PRX* are extensively expressed in testis, and their roles in spermatogenesis have mainly been studied by gene disruption in mouse models [16, 17, 19]. In summary, antioxidant genes, including *NRF2*, *SOD*, *CAT*, *GPX*, *PRX*, *GRX*, *TRX*, and *NOS*, function at different stages of spermatogenesis, and defects in their expression may significantly contribute to the occurrence of male infertility (Table 1).

3. Genetic Variations in Antioxidant Genes Associated with Male Infertility

3.1. NRF2. *Nrf2* disruption has been demonstrated to affect spermatogenesis in an age-dependent manner in knockout mice model [7]. A mechanism study has shown that aged *Nrf2* knockout mice have elevated levels of lipid peroxidation in their testes and epididymis, as well as increased rates of testicular germ cell apoptosis and decreased levels of antioxidants compared to age-matched wild-type mice [7]. In humans, two SNPs (rs6721961 and rs35652124) have been associated with oligoasthenozoospermia, and individuals with 617 TT and 653 TT genotypes have a higher risk of oligoasthenozoospermia [20]. In addition, the *NRF2* rs6721961 TT genotype occurs at a higher frequency in heavy smokers with low semen quality than in those with high semen quality, and heavy smokers with this genotype have significantly lower sperm concentrations and counts compared to other genotypes [12]. At the mRNA level, *NRF2* expression was significantly lower in infertile patients than in controls [78], and a significant correlation was observed between the level of *NRF2* mRNA expression and specific sperm functional parameters such as concentration, progressive motility, immotility, and vitality [78]. Interestingly, the DJ-1 protein, which stabilizes *NRF2* by targeting 20S proteasomes in cells, has also been associated with male infertility [79–81]. The concentration of sperm DJ-1 was lower in moderate asthenozoospermia patients than in the controls [79]. Therefore, functional polymorphisms and expression level of *NRF2* as well as its regulators are associated with defective spermatogenesis in humans.

3.2. GST. Three types of *GST* SNPs, namely, *GSTT1*-null, *GSTM1*-null, and *GSTP1* Ile105Val, have been extensively demonstrated to be associated with male infertility in various ethnic populations [21–32, 34]. In a north Indian population, the *GSTT1*-null genotype was associated with nonobstructive azoospermia [21]. In Taiwanese patients with varicocele,

subjects with *GSTM1*-null genotype had significantly higher 8-OHdG levels in sperm DNA and lower protein thiols and ascorbic acid in seminal plasma than those with the *GSTM1*+ genotype [22]. In a Turkish population, increased oxidative damage of sperm was higher in patients with the *GSTM1*-null genotype than in controls [23], and similar results have also been reported in Egyptian, Iranian, and Brazilian infertile patients [24–26]. In a Chinese population, the null genotype of *GSTM1* and *GSTT1* is associated with an increased susceptibility to impaired spermatogenesis such as idiopathic azoospermia or oligospermia [27–30]. The association of polymorphisms in *GSTM1*, *GSTT1*, and *GSTP1* with idiopathic azoospermia or oligospermia was also observed in a southwest Chinese population [31]. Moreover, genetic polymorphisms in *GSTT1* may also affect the surgical outcome of varicocelectomies, and the *GSTT1* genotype can affect surgical outcomes of Japanese patients such as improvement of semen parameters after varicocelectomy [32].

Meta-analysis further confirmed that *GSTM1*-null and *GSTT1*-null polymorphisms are associated with male infertility risk [82–85]. A recent analysis encompassing 6934 subjects indicated that the *GSTM1*-null genotype was significantly associated with idiopathic oligozoospermia, while the null genotype of *GSTT1* was significantly associated with normozoospermia and azoospermia, and the association between *GSTM1* polymorphism and male infertility was observed in cohorts of both Asian and Caucasian groups [84].

GST enzymes are also important in protecting sperm from cryopreservation of semen, as this process can produce large amounts of ROS. In freeze-thawed bull semen, a C/G missense mutation in rs135955605 within the *GSTM1* gene is associated with cellular ATP content and total sperm motility [33]; therefore, genetic variations in GSTs may affect male fecundity, including sperm quality and the outcomes of semen cryopreservation.

3.3. SOD. It has long been known that seminal SOD activity is positively associated with sperm concentration and overall motility, whereas it is inversely associated with sperm DNA fragmentation [8, 38]. Genetic variations in SOD may also be related to reproductive outcomes. The Ala16Val polymorphism in the *SOD2* gene is associated with infertility and pregnancy rate in IVF cycles [39]. In a case-control study, the presence of the Ala-*MnSOD* allele (rs4880) was associated with a significant increase in the risk of infertility in male subjects [40]. Infertile men with *SOD2* rs4880 CC variants showed a low level of SOD activity [38]. In a Chinese population, the *SOD2* Val16Ala (rs4880) variant is associated with a significantly higher risk for male infertility, higher levels of sperm DNA fragmentation and 8-OHdG, and a low level of SOD activity [38, 41]. When multiple antioxidant gene variations were analyzed, the *PON1* Arg192Glu (rs662) and *SOD2* Val16Ala (rs4880) variants were associated with a significantly higher risk of male infertility and levels of sperm DNA fragmentation and 8-OHdG [41].

In rat models, it has been shown that SODs may play an important role in testicular development and spermatogenesis [86]. *SOD* mRNA transcripts were identified in rat testes and their highest level was detected in tubules just prior to spermiation [86]. In a Drosophila model, null mutants for *CuZn-Sod* (SOD1) are male sterile, and the transgene of a bovine *CuZn-Sod* can rescue its male infertile phenotype [42]. In addition, an accelerated impairment of spermatogenic cells was observed in *Sod1*-knockout mice under heat stress [43]. Therefore, genetic disruption or functional polymorphisms in both SOD1 and SOD2 can lead to defective spermatogenesis.

3.4. NOS. In the testis, eNOS is responsible for NO synthesis during spermatogenesis, and genetic variants of *eNOS* may be potential risk factors for impaired spermatogenesis [45]. Several *eNOS* alleles have been associated with sperm defects in various ethnic populations. In Egyptian infertile oligoasthenoteratozoospermic men, a significant relationship between *eNOS* polymorphisms T786C and G894T with decreased sperm parameters and increased seminal oxidative stress was observed [46]. In an Italian population, the *eNOS* 894G>T variant was associated with asthenozoospermia and sperm motility [48]. Similar results were reported in a Chinese cohort [49]. In Korean infertile men, sperm morphology was associated with the 4a4b eNOS polymorphism, a sequence variant with variable number of tandem 4a4b repeats in intron 4 [51]. In Iranian males, *eNOS* "-786C," "894T," and "a" alleles were associated with an increased risk for poor semen parameters [47]. In a Chinese case-control study, the *eNOS* rs1799983 polymorphism was positively associated with higher levels of sperm DNA fragmentation and an increased risk for male infertility [50]. Another study involving a Chinese population showed that four common polymorphism loci, namely, *eNOS* alleles -786C of T-786C and 4A of 4A4B, as well as genotype TC of T-786C and AB of 4A4B, were significantly associated with idiopathic male infertility [45]. Taken together, these studies demonstrate that genetic variations in *eNOS* are a risk factor for decreased sperm quality, including DNA fragmentation, sperm motility, and seminal ROS.

3.5. GPX. There are three isoforms of GPX, namely, cytosolic, mitochondrial, and nuclear GPX [87]. In a mouse model, cytosolic GPX4 was essential for embryonic development and spermatogenesis [53], and the deletion of mitochondrial GPX4 (mGPX4) also caused male infertility, which in turn led to impaired sperm quality and severe structural abnormalities, reduced sperm motility, and mitochondrial membrane potential [52, 88]. In bulls, subjects with the *ETFA* TT genotype presented the highest GPX activity in cryopreserved sperm [89]. In humans, GPX-defective spermatozoa were observed in 26% of infertile men diagnosed with oligoasthenozoospermia [90]. Another study has suggested that the expression of phospholipid hydroperoxide glutathione peroxidase (PHGPx) protein, a selenoprotein belonging to the family of glutathione peroxidases, may be associated with oligoasthenozoospermia; however, no *GPX* polymorphism has been associated with male infertility to date [91]. Further examination of *GPX4* polymorphisms as a potential cause of infertility is thus warranted.

3.6. CAT. Catalase enzyme activity (CAT) was demonstrated to be associated with low sperm quality [44, 92], and one

study reported that *CAT*-262T/T genotype was negatively associated with infertility in idiopathic infertile males [44].

4. Interaction of Antioxidant Genetic Variations and the Environment in relation to Male Infertility

Environment and genetic variation could synergistically affect male fertility. In a Danish twin study, both genetic background and environmental factors were associated with sperm quality, sex hormone levels, and sperm chromatin stability, in which heritability accounted for ≥20% of the observed variations in sperm density, hormone level, sperm morphology, and sperm chromatin parameters, whereas the rest of the variations in sperm quality were likely due to environmental factors [93].

Several studies have demonstrated that environment and antioxidant genes can affect male infertility. In terms of occupational exposure to PAHs, subjects harboring the *GSTM1*-null genotype showed significantly higher levels of PAH-DNA adducts in sperm [35]. In Russian men, the combination of *GSTM1*, *GSTT1*, and *GSTP1* gene polymorphisms and cigarette smoking was associated with a higher risk for idiopathic infertility [36]. Our study also demonstrated that heavy smokers with *NRF2* genetic variants had a higher risk of developing low semen quality compared to other genotypes [12].

Cytochrome P450 (CYP) families may contribute to the occurrence of endogenous oxidative stress *in vivo* because these are detoxification enzymes that interact with a wide range of environmental toxins and carcinogens that can form ROS. A previous study has shown a significant synergism between *GSTM1* and *CYP1A1* genotypes and infertility among human subjects [37]. A subject carrying the variants *CYP1A1* Val/Val or *CYP1A1* Ile/Val in association with *GSTM*-null genotype has a 6.90-fold higher risk for infertility than a subject carrying *CYP1A1* Ile/Ile in association with a *GSTM1* wild-type genotype [37]. Therefore, genetic polymorphisms of xenobiotic-metabolizing enzymes may also interact with antioxidant genes for environment-induced infertility [37].

5. GWAS Study in Male Infertility

With the development of new genetic analysis approaches, genome-wide association study (GWAS) has been utilized for male infertility recently, and new loci for male infertility have been identified using GWAS. In a large cohort of men of European descent, 172 candidate polymorphisms for association with oligozoospermia or azoospermia were evaluated and several SNPs were identified or confirmed to be significantly associated with oligozoospermia and/or azoospermia [94]. Another GWAS report identified candidate genes for male fertility traits and 9 SNPs found to be associated with reduced fertility [95]. In 2011 and 2014, two large scale GWAS in Chinese populations first discovered some new loci for the risk of nonobstructive azoospermia (NOA). A three-stage GWAS of 2,927 individuals with NOA and 5,734 controls identified significant associations between NOA risk and common variants near *PRMT6* (rs12097821), *PEX10* (rs2477686), and *SOX5* (rs10842262) [96]. A later extended three-stage validation study using 3,608 NOA cases and 5,909 controls further identified additional risk loci, including a new related gene *GEK* (Genghis Khan, orthologous to human CDC42BPA) which can cause severe male fertility in a Drosophila model [97].

A detailed summary of GWAS in infertile men has been described by Aston [98], which is not the focus of this review. However, except for the identification of new loci for male infertility, GWAS do confirm the association between previously identified SNPs in antioxidant genes and male infertility. For instance, in recent GWAS on genetic makers for sperm quality in bulls [99–101], the antioxidant genes *GSTT1*, *GSTM1*, and *NOS3* were identified as significant markers or suspected of being significantly associated with bull sperm concentration [100].

However, antioxidant signaling pathways involved in male infertility have not been analyzed at the genome-wide level to date. In addition, only a few diseases such as azoospermia or oligozoospermia have been studied at the genome-wide level. The most common male infertility disorders such as asthenozoospermia and oligoasthenozoospermia have not been extensively studied to date. Therefore, using advanced genetic analysis technologies to study antioxidant genetic variations in relation to male infertility at a genome-wide level is imperative.

6. Conclusions

As environmental pollution and lifestyle changes are prevalent in the current society, ROS from pollution, radiation, high-fat diets, and sedentary, physically inactive lifestyles will likely contribute to the increase in incidence of male infertility. The antioxidant enzyme system, which is largely regulated by the NRF2-ARE system, may be one of the key components that play a protective role against ROS damage during spermatogenesis and for sperm function (Figure 1). Therefore, it is expected that genetic variations in major antioxidant genes will alter the susceptibility of a male to infertility and defective spermatogenesis.

In the past two decades, numerous studies have demonstrated that functional polymorphisms or the genetic disruption of the *CAT*, *GPX*, *GST*, *NOS*, *NRF2*, and *SOD* genes was associated with male infertility (Table 2). In animal models, knocking out *Nrf2*, *Sod*, and *Gpx* all leads to mild or severe male infertility. Previous studies involving various ethnicities in different geographical regions and countries have described the association between SNPs in the *CAT*, *GPX*, *GST*, *NOS*, *NRF2*, and *SOD* genes and infertility. Several studies have also reported the synergistic effects of antioxidant gene polymorphisms and environmental ROS such as smoking and PAH exposure. Therefore, antioxidant-related genes may play a crucial role in spermatogenesis and sperm function, and their genetic variations may modify the antioxidant capability of the human reproductive system and increase the risk for male infertility.

However, most studies of the association between antioxidant gene variations and male infertility have been conducted

Table 2: Reported antioxidant genetic variations associated with male infertility.

Gene	Variation	Official description	Trait/effect	Species	Reference
NRF2	Deletion	—	Subfertility	Mice	[7]
	rs6721961 G>T	NC_000002.11:g.178130037 T>G	Oligoasthenozoospermia	Human	[20]
	rs35652124T>C	NC_000002.11:g.178130073 T>C	Oligoasthenozoospermia	Human	[20]
	rs6721961 G>T + smoking	NC_000002.11:g.178130037 T>G	Sperm concentration and count	Human	[12]
GST	GSTM1-null	Deletion	Male infertility, oligozoospermia, male infertility with varicocele	Human	[21–28]
	GSTT1-null	Deletion	Male infertility, male infertility with varicocele	Human	[24, 26–32]
	GSTM1 rs135955605 C/G	—	Sperm motility after cryopreservation	Bulls	[33]
	GSTP1 (Ile105Val)	NC_000011.10:g.67585218 A>G	Male infertility, oligospermia, oligoasthenoteratozoospermia, azoospermia	Human	[26, 31, 34]
	GSTP1 (Ala114Val)	NC_000011.10:g.67586108 C>T	Male infertility with varicocele, oligoasthenoteratozoospermia	Human	[27, 34]
	GSTM1-null + PAH exposure	Deletion	PAH-DNA adducts	Human	[35]
	GSTM1-null/GSTT1-null + smoking	Deletion	Idiopathic male infertility	Human	[36]
	GSTM1-null + CYP1A1	Deletion	Male infertility	Human	[37]
SOD	SOD2 rs4880 CC	NC_000006.11:g.160113872 A>G	Idiopathic infertility, male infertility, pregnancy rates in IVF, sperm concentration, sperm motility, and sperm DNA fragmentation	Human	[38–41]
	SOD1 knockout	—	Male infertility	Drosophila	[42]
	SOD1 knockout	—	Spermatogenic cell damage during heat stress	Mice	[43]
CAT	C-262T	NC_000011.10:g.34438684 C>T	Idiopathic male infertility	Human	[44]
NOS	eNOS T786C	NC_000007.13:g.150690079 C>T	Male infertility, oligoasthenoteratozoospermia, idiopathic male infertility	Human	[45–47]
	eNOS G894T	NC_000007.13:g.150696111 T>G, NP_001153582.1:p.Asp298Glu,	Oligoasthenoteratozoospermia, asthenozoospermia, idiopathic male infertility	Human	[46–50]
	eNOS 4a/b	NC_000007.14:g.150997188_150997214-AGGGGTGAGGAAGTCTAGACCTGCTGC(2)(3)	Idiopathic male infertility, sperm morphology	Human	[45, 47, 51]
GPX	GPX4 deletion	—	Male infertility, sperm chromatin condensation	Mice	[52, 53]
	GPX5 deletion	—	Sperm DNA integrity	Mice	[54]

in animal models or in a specific geographical population. In addition, systematic studies of the complete antioxidant signaling pathways in spermatogenesis and studies in multiple centers or large cohort studies are limited. Furthermore, epigenetic alterations in antioxidant genes, which may change their transcriptional activity *in vivo*, have not been examined to date. New technologies such as next-generation sequencing can yield large amounts of information at the genome-level. Therefore, the discovery and validation of antioxidant genetic variants as genetic markers for the diagnosis and risk estimation for male infertility may facilitate the improvement of clinical approaches for this particular disorder.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Varicocele Repair Improves Testicular Histology in Men with Nonobstructive Azoospermia

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Objective. To determine the histopathological differences after varicocele repair in testicular tissue in males with nonobstructive azoospermia. *Methods*. Between 2009 and 2014, 45 men with complete azoospermia and palpable varicocele, presenting with primary infertility of at least 1 year, undergoing varicocele repair at our institution were selected for the study. A standard systematic testicular 6-core Tru-Cut biopsy was performed during varicocele repair. Other biopsies were obtained from each testicle of all patients at the time of microscopic sperm extraction procedure. *Results*. Nineteen patients were selected for the study. Testicular biopsy specimens were classified as Sertoli cell only on preoperative histopathological analysis in 14 patients. After varicocele repair, focal spermatogenesis ($n = 3$) and late maturation arrest ($n = 2$) were found in these patients. Average Johnsen score was significantly increased after varicocelectomy ($P = 0.003$). Motile sperm was found in one patient on postoperative semen analyses and in 10 more patients in the microscopic sperm extraction procedure. Preoperative high serum follicle stimulating hormone level and venous reflux were significantly and negatively correlated with the increase in average Johnsen score ($P < 0.05$). *Conclusions*. Our findings suggest significant improvement in testicular histology after varicocele repair.

1. Introduction

There is no standard treatment for male infertility. Varicocele is the most common cause of male infertility and is generally correctable or at least improvable by various surgical and radiological techniques. It has been estimated that 5–10% of infertile males with azoospermia had a clinical diagnosis of varicocele [1, 2]. Urology guidelines recommend varicocele repair (VR) in infertile patients with semen abnormalities and palpable varicocele [3]. The benefits of VR for sperm concentration, motility, and morphology are well established in oligozoospermic males [4], but the efficacy of VR on testicular histology changes in cases of nonobstructive azoospermia (NOA) has not been examined yet.

Fathering a child for males with NOA is directly associated with obtaining spermatozoa by microscopic testicular sperm extraction (micro-TESE) and the success of intracytoplasmic sperm injection (ICSI). ICSI is the only way for males with NOA to have children. However, ICSI results in successful pregnancy for only a small percentage of males with NOA. Pregnancies and live births are eventually achieved in 30–50% of couples in which the male has NOA, when spermatozoa have been found on testicular biopsy [3]. Sperm recovery rates for ICSI treatment differ between 30 and 70% in various studies [5]. Consequently, auxiliary treatments are required to improve the recovery of testicular tissue, quality of spermatozoa, and probability of obtaining spermatozoa.

Tulloch first applied VR in one NOA patient with bilateral varicocele in 1952 and obtained spontaneous pregnancy [6]. After that, also other studies were published regarding VR in NO patients [1, 2, 7]. Subsequently, a number of studies were performed to determine the pathophysiology of varicocele and the role of VR in recovery of fertility. We examined the

histopathological differences associated with VR in testicular tissue. The parameters predictive of postoperative improvement in men with NOA were also determined in this study.

2. Materials and Methods

The study was carried out according to the Declaration of Helsinki and was approved by our institutional ethics committee with the number of IAEK 7/3-2009/54. Between 2009 and 2014, men with pellet (−) azoospermia and palpable unilateral or bilateral varicocele, presenting with primary infertility for at least 1 year, were selected for this study. All subjects underwent a standard basic diagnostic infertility evaluation. A detailed medical history was obtained, and a physical examination for complete infertility evaluation was performed. Varicoceles were identified on scrotal examination and classified as described previously [3]. Scrotal ultrasonography with real-time color Doppler imaging was used to confirm the presence of varicocele. At least three preoperative semen samples were obtained from all patients by masturbation after 4-5 days of abstinence. All analyses were performed in the same andrology laboratory (Kocaeli University Infertility Center).

All patients had the normal 46XY karyotype and did not have any Y-microdeletions. Patients with obstructive azoospermia, retrograde ejaculation, history of systemic disease and/or surgery that may affect testicular histology, and low serum testosterone levels were excluded. Patients' age, serum follicle stimulating hormone (FSH) values before surgery, grade and laterality of varicocele, time interval between varicocelectomy and micro-TESE, maximum diameter of varicose veins, and presence of retrograde flow (venous reflux) were obtained.

Informed consent was obtained from all participants. A total of 45 patients underwent microsurgical VR with subinguinal approach. After the VR a 5-mm midline dermal and subdermal scrotal incision was done, and a standard systematic testicular 6-core Tru-Cut biopsy (from each polar and midline on the right and left testicle) with a 20-mm 18-gauge needle was performed. Biopsies were laid separately on absorbent paper and placed into Eppendorf tubes filled with Bouin's solution and transferred to the pathology laboratory. Tissues were embedded in paraffin blocks after processing and cut into sections 5 μm thick, which were deparaffinized and stained with hematoxylin and eosin. All pathological analyses were performed by an expert uropathologist (KY) (preoperative histopathology). Any tissue obtained from the biopsy was not cryopreserved.

More than one semen sample was obtained from each patient beginning three mounts after the surgery. After complete gynecological evaluation of their partners, all patients presented to Kocaeli University Assisted Reproduction Treatment Center. Eighteen patients were not engaged in assisted reproductive treatments for financial reasons. During the evaluation of some biopsies of six patients, uropathologist could not find any testicular tissue, probably due to some technique issues. So these six and two patients with normal spermatogenesis in their preoperative biopsy were excluded. Micro-TESE and ICSI were performed for the remaining 19 subjects.

Micro-TESE procedure: Micro-TESE was performed under local anaesthesia by removing testicular tissue through a longitudinal incision of tunica albuginea. The testicular pulp was surged under operative microscope for dilated and enlarged tubules which are more likely to contain germ cells. Testicular tissues were obtained from different parts and levels.

Regardless of the success of the micro-TESE procedure, all extracted testicular tissues were sent to pathology laboratory for histopathological evaluation. Samples were put in tubes filled with Bouin's solution and were transferred to the pathology laboratory for histological evaluation (postoperative histopathology).

Histology was analyzed by scoring the seminiferous tubules at 400x magnification using the Johnsen score (JS) (10) according to the presence of germinal cells. All histopathological evaluation (Tru-Cut biopsies and micro-TESE material) was performed using JS. Each tubular section was given a score from 10 to 1 according to the presence or absence of the main cell types arranged in the order of maturity: scores 10, 9, or 8, presence of spermatozoa; 7 or 6, spermatids (and no further); 5 or 4, spermatocytes (and no further); 3, only spermatogonia; 2, only Sertoli cells; and 1, no cells. The germinal epithelium of at least 10, maximum 20, tubules was assessed for each testis, and the average Johnsen score was calculated for each patient. Testicular biopsy specimens were classified according to the histopathological criteria as follows: normal spermatogenesis (NS), hypospermatogenesis (HS), late maturation arrest (LMA), early maturation arrest (EMA), Sertoli cells only with focal spermatogenesis (SCOFS), Sertoli cell only (SCO), and hyalinization of tubules (HT).

2.1. Statistical Analysis. Preoperative high (above the reference value) FSH level, presence of venous reflux and bilateral varicocele, and presence of increased average JS in postoperative histopathology were modeled as dichotomous variables (yes/no). When bilateral varicocele was determined, the higher grade was used in the analysis. All data were analyzed using SPSS. The Wilcoxon matched-pair signed ranks test was used for nonparametric analyses. Univariate logistic regression (LR) analyses were performed and the presence of increased average JS was used as a dependent variable. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

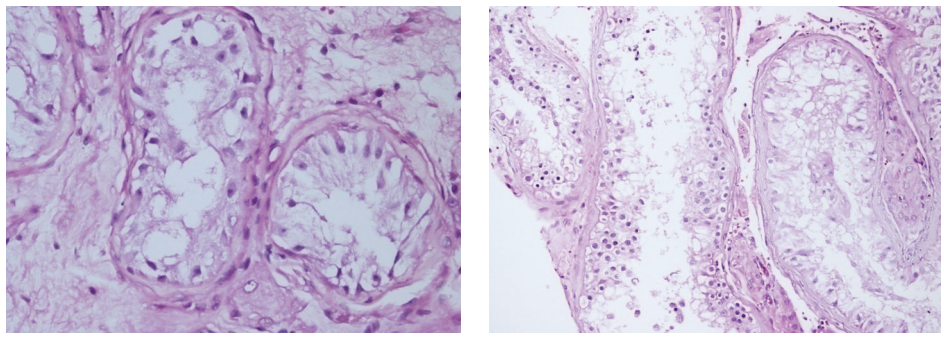
3. Results

Only patients having both preoperative and postoperative histopathological evaluation were included in analyses. Thus, we analyzed data on 19 patients. All patients had a diagnosis of the pellet (−) NOA and had palpable varicocele. The preoperative patient characteristics are shown in Table 1. Serum FSH level was high in 12 patients. Varicose veins of maximum diameter >3 mm were found in all patients.

Table 1: Patient characteristics.

Age (year), [median (IQR*)]	31.00 (29.00–35.00)
Serum FSH** level (mIU/mL), [median (IQR)]	26.50 (9.24–41.50)
The time interval between varicocelectomy and micro-TESE (month), [median (IQR)]	12.00 (8.00–20.00)
Maximum diameter of veins (mm), [median (IQR)]	3.20 (2.80–3.50)
Presence of venous reflux, % (*n*)	47.4 (9)
Varicocele grade, % (*n*)	
Grade I	21.1 (4)
Grade II	52.6 (10)
Grade III	26.3 (5)
Varicocele laterality, % (*n*)	
Unilateral	52.6 (10)
Bilateral	47.4 (9)

*IQR: interquartile range.
**FSH: follicle stimulating hormone.



(a) (b)

Figure 1: (a) Testicular histopathology indicating SCO. (b) Testicular histopathology showing SCO pattern with focal spermatogenesis in the same patient after VR.

Table 2: The percentages of histopathological findings according to preoperative and postoperative testicular biopsies.

Histopathology	Preoperative % (*n*)	Postoperative % (*n*)
HS	10.5 (2)	10.5 (2)
SCO-FS	10.5 (2)	26.3 (5)
LMA	0	10.5 (2)
EMA	5.3 (1)	5.3 (1)
SCO	73.7 (14)	47.4 (9)

HS: hypospermatogenesis, LMA: late maturation arrest, EMA: early maturation arrest, SCO-FS: Sertoli cells only with focal spermatogenesis, SCO: Sertoli cell only.

The median of average JS before and after VR was 2.00 (interquartile range (IQR) 2.00–2.40) and 3.30 (IQR 2.00–5.10), respectively. The average JS was significantly increased after VR ($P = 0.003$). The percentages of histopathological findings according to preoperative and postoperative testicular biopsies are shown in Table 2. Preoperative SCO was defined in 14 patients, focal spermatogenesis in two, hypospermatogenesis in two, and EMA in one. After VR, focal spermatogenesis was determined in three and LMA in two patients with SCO (Figure 1). Motile sperm were found in 1 patient on postoperative semen analyses and in a further 8 patients by the micro-TESE procedure. Average JS increased in 10 subjects and was unchanged in 9 (patients with SCO).

Univariate logistic regression analyses were performed to assess the factors correlated with the increased average JS (Table 3). Preoperative high serum FSH level and venous reflux were significantly correlated with failure to increase average JS ($P < 0.05$).

4. Discussion

The role of varicocelectomy in azoospermic patients was first studied in 1976 by Mehan [8]. He applied spermatic vein ligation to 10 NOA patients (2 of whom had varicocele) and confirmed two spontaneous pregnancies. Many subsequent studies have evaluated this issue [1, 2, 7, 9–12]. Weedin et al. performed a meta-analysis to evaluate studies of VR in patients with NOA performed during the previous 20 years [13]. They analyzed 11 publications and a total of 233 patients. Motile sperm were found on postoperative semen analyses in 91 of 233 (39.1%) patients, resulting in 14 (6%) spontaneous pregnancies [13]. Inci et al. and Haydardedeoglu et al. retrospectively evaluated NOA patients with or without

Table 3: The predictive factors for improvement of average JSs in univariate logistic regression analyses.

	OR (CI)	p
Age	0.93 (0.78–1.12)	0.466
Pre-op high serum FSH* level	0.08 (0.07–0.95)	**0.045**
The interval between surgery and micro-TESE	1.16 (0.99–1.35)	0.054
Varicocele grade		
Grade I	2.00 (0.11–35.80)	0.638
Grade II	0.44 (0.05–3.97)	0.468
Grade III	1.50 (0.18–11.92)	0.702
Presence of bilateral varicocele	0.21 (0.03–1.48)	0.119
Maximum diameter of varicose vein	0.30 (0.02–3.13)	0.317
Presence of venous reflux	0.07 (0.01–0.64)	**0.019**

*FSH: follicle stimulating hormone.

VR in two separate studies. They compared the presence or absence of motile sperm in the TESE procedure and found significantly higher rates of motile sperm in patients with varicocelectomy [14, 15].

Our five patients with a preoperative diagnosis of SCO had focal spermatogenesis (FS) and LMA on histopathological evaluation of micro-TESE specimens after VR. There have been several reports regarding motile sperm obtained in SCO patients. Pasqualotto et al. [16] found motile sperm in 4 of 10 SCO patients and Lee et al. [17] found motile sperm in 1 of 10 SCO patients, in their study. However, Kim et al. reported that three patients with the SCO pattern and three with EMA showed no improvement after varicocelectomy, while 5 of 10 (50%) with LMA and 10 of 18 (56%) with HS did show improvement. Similarly, Kadioğlu et al. and Çakan and Altuğ obtained no motile sperm after VR in SCO patients [7, 18]. Weedin et al. reported motile sperm in 5 of a total of 44 SCO patients, whereas motile sperm were detected in 30 of 55 HS and 24 of 57 maturation arrest patients in their meta-analysis [13].

There might be two possible explanations for detection of motile sperm in SCO patients. First, nonproductive testicular tissue would recover after VR and inactive germ cell precursors would be activated; the observation of focal spermatogenesis after VR in three preoperative complete SCO patients might support this hypothesis. Second, existing spermatogenesis may not have been detected during testicular biopsy and the improvement in histopathology might be a reason of more extensive tissue analysis at the time of micro-TESE procedure. It is well established that spermatogenesis can vary within a compromised testicle. Therefore, testicular biopsies may not always be representative of the most advanced histological pattern within the testis. However, we performed systematic testis biopsy in both testes with six samples in our prospective study design instead of obtaining only one biopsy from any testis. This was expected to lead to more representative findings than conventional testis biopsies. Nevertheless, although less likely, preoperative biopsy may be unable to detect existing spermatogenesis in the testicular tissue.

To our knowledge, this is the first report to compare the histopathological findings before and after VR. Although two thirds of our patients had preoperative SCO, we found a significant increase in the average JS. None of the patients showed a decrease in average JS. In conclusion, our findings represent clear evidence that VR positively affected the testicular histology. Histological changes were determined only in SCO group in our study. However, most of the patients in our study had this histology. We expect that larger study groups with different histologies might have represent improvement in all patients.

We evaluated the predictive factors according to histopathological improvement, unlike other studies, and our results were different from those reported previously. In the present study, we found that a preoperative high serum FSH level and the presence of venous reflux in color Doppler imaging negatively affected the testicular histopathological improvement. Similarly, Kadioğlu et al. showed that patients with normal FSH levels had higher rates of improvement in semen analyses [7]. However, several studies reported that a high FSH level was not associated with obtaining motile sperm after VR in NOA patients [1, 2, 18–20]. No previous study has evaluated whether the diameter of varicose veins and the presence of venous reflux affect the success of VR in NOA patients. However, in patients with varicocele and oligoasthenospermia, testicular vein diameter > 2.5 mm and the presence of venous reflux in preoperative color Doppler ultrasound were associated with significant improvements in sperm parameters after VR [21, 22].

This study has a number of limitations. There is not a control group in our study. But it is not easy to generate a real control group because of limited number of patients and much of the patients in our clinic with palpable varicocele and NOA did not accept to undergone testicular biopsy and VR before micro-TESE procedure. However some of these patients who had negative ICSI procedure were convinced of VR after the unsuccessful treatment. These patients might be used as a control group in another study and testicular histology can be compared before and after VR in the future. On the other hand, it is well established that spermatogenesis can vary within a compromised testicle. Although we performed systematic testicular biopsy, it would not be representative of the overall testicular tissue. In addition, the time interval between VR and micro-TESE was relatively long.

5. Conclusion

To our knowledge, this is the first report to compare the histopathological findings before and after VR in males with NOA. Our findings suggested significant improvement in testicular histology regarding average JS after VR. In addition, preoperative high FSH levels and the presence of venous reflux were negatively correlated with this improvement.

Conflict of Interests

None of the authors declare competing financial interests.

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Research Article

Candesartan Mediated Amelioration of Cisplatin-Induced Testicular Damage Is Associated with Alterations in Expression Patterns of Nephrin and Podocin

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Nephrin and podocin are known to be closely related to the pharmacological effects of angiotensin-II receptor blocker (ARB). The objectives of this study were to investigate the role of nephrin and podocin using cisplatin-induced testicular damage and to evaluate the effect of ARB. At first, we evaluated the effects of cisplatin either alone or in combination with ARB candesartan on changes in expression patterns of nephrin and podocin in the rat testes. We then conducted *in vitro* studies to investigate the effects of angiotensin using cultured Sertoli cells, line TM4. As a result, the expression of nephrin and podocin was shown to localize around the basal membrane of seminiferous tubules. Treatment with cisplatin resulted in a marked decrease in the expression of nephrin and podocin and induced a shift of both proteins from linear to granular expression patterns, accompanying the increased apoptotic index in the testes; these changes were partially restored by the additional administration of candesartan. *In vitro* studies with TM4 revealed the angiotensin-II mediated expression changes of nephrin and podocin. These findings suggest that candesartan can prevent cisplatin-induced testicular damage by regulating expression patterns of the nephrin-podocin complex in the testes.

1. Introduction

Nephrin and podocin are the well-known proteins, which are connected to each other and play an important role in maintaining the function of the slit diaphragm, a structure likely to be the filtration barrier of the glomerular capillary wall as a highly developed cell-cell junction [1]. Furthermore, several recent studies have reported that the development of proteinuria, not only in congenital nephrotic syndrome but also in acquired renal diseases, is regulated, at least in part, by renal slit diaphragm proteins, including nephrin and podocin [2].

To date, there have been a number of studies demonstrating the involvement of disorders of cell-cell junctions in the development of pathological conditions in the testes [3]. We previously reported the association between impaired spermatogenesis and disorganization of major components of tight junctions in Sertoli cells in both experimental and clinical studies [4, 5]. Collectively, these findings indicate that it might be interesting to investigate whether testicular function is regulated by nephrin and podocin, like some other molecules mediating pathophysiological roles in cell-cell junctions in the testes. In fact, the expression of nephrin and podocin was confirmed in murine testes, and Sertoli cells were suggested to be the testicular origin of these proteins [6, 7]. Furthermore, there have been several studies focusing on angiotensin-II mediated kidney damage through nephrin and podocin. For example, angiotensin-II receptor blocker (ARB) is reported to have antiproteinuric effects which act directly by protecting slit diaphragm molecules, including nephrin and podocin [8, 9], while cisplatin-induced kidney damage is considered to be mediated by angiotensin-II [10, 11]. In fact, the protective effects of ARBs on testis have been previously reported [12–14].

Taken together, in this study, we initially examined the effects of cisplatin-induced damage on expression patterns of

nephrin and podocin in rat testes, since platinum analogues like cisplatin are known to have toxic side effects on testicular function and frequently cause azoospermia through the impaired function of Sertoli cells and disruption of blood/testis barrier [15–18]. We then evaluated whether this damage could be restored by the angiotensin-II receptor blocker (ARB), candesartan.

2. Materials and Methods

2.1. Animals. A total of forty-two 9-week-old male Sprague-Dawley rats weighting 234 ± 23 g were purchased from Oriental Yeast Co. (Tokyo, Japan) and housed in a controlled environment at 22°C on a 12-hour light, 12-hour dark cycle. Animal experiments were approved by Committee of Animal Experiment at Kobe University School of Medicine and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [19].

2.2. Sertoli Cell Line. The mouse Sertoli cell line TM4 was purchased from the American Type Culture Collection (Manassas, Virginia). Cells were maintained in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Nacalai Tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum. This cell line TM4 is known to form tight junction and is considered suitable for this study [20].

2.3. Animal Studies. To examine the *in vivo* effects of treatment with cisplatin (Nichi-Iko Pharmaceutical Co., Toyama, Japan) and/or candesartan, kindly provided by Takeda Pharmaceutical Co. (Osaka, Japan), on expression patterns of testicular nephrin and podocin, a total of 42 rats mentioned above were randomly selected for the following six groups: Group 1 ($n = 7$), intraperitoneal (IP) injection of 2 mL isotonic saline; Group 2 ($n = 7$), IP injection of 2 mL isotonic saline and oral administration of candesartan 5 mg/kg/day; Group 3 ($n = 7$), IP injection of 4 mg/kg cisplatin; Group 4 ($n = 7$), IP injection of 4 mg/kg cisplatin and oral administration of candesartan 5 mg/kg/day; Group 5 ($n = 7$), IP injection of 7 mg/kg cisplatin; and Group 6 ($n = 7$), IP injection of 7 mg/kg cisplatin and oral administration of candesartan 5 mg/kg/day. After randomization, either candesartan at a dose of 5 mg/kg or water was orally administered once daily for 24 days, and on day 3, either cisplatin at a dose of 4 or 7 mg/kg or isotonic saline was given by IP injection. The dose and duration after treatment of cisplatin and candesartan were determined based on those reported in the previous study and our preliminary experiments [21, 22].

Three weeks after treatment, rats were sacrificed, and testes and epididymides were removed. Testicular weight measurement and epididymal semen analysis were performed on both sides, and mean values were used for the calculation. After semen analysis, testes were bisected, and one half was snap-frozen immediately and stored at −80°C until assessed, and the remainder was fixed in Bouin solution for histopathological examination.

2.4. Sperm Analysis. The epididymal sperm concentration was determined by using the modified method described by Okada et al. [23]. Briefly, the epididymis was finely minced by anatomical scissors within 1 mL of modified Whitten's medium (15 mM Hepes-sodium salt, 1.2 mM $MgCl_2$, 100 mM NaCl, 4.7 mM KCl, 1 mM pyruvic acid, and 4.8 mM lactic acid) in a dish and then allowed to incubate at room temperature for 1 hour to provide the migration of all spermatozoa from epididymis. Sperm analysis, including the number and motility, was performed using Makler counting chamber (Irvine Scientific, CA, USA). A total of 20 fields were analyzed for each sperm sample. In order to detect the spermatozoa with abnormal morphologies, the Diff-Quick kit (Medion Diagnostics International, FL, USA) was used to stain the smears of semen samples. Diff-Quick fixing and staining times were 5 minutes and the slides were washed in distilled water to eliminate excess staining, air-dried, and covered with a coverslip. At least 100 sperm per slide were counted to determine the percentage of spermatozoa with abnormal morphologies.

2.5. Treatment of TM4 Cells. To examine the changes in expression patterns of nephrin and podocin in the cultured TM4 cells, the cells were treated with 2×10^{-8} mL/L angiotensin-II (A9525, Sigma-Aldrich, Tokyo, Japan) for 1 hour in the presence or absence of 2.5×10^{-6} mol/L candesartan. Following treatment, cells were harvested and used for subsequent experiments. Angiotensin-II is considered suitable for our experiment, to investigate the involvement of angiotensin system through nephrin and podocin. That is, angiotensin receptor is located in Sertoli cell, while there is no production of angiotensin-II from Sertoli cell [24, 25].

2.6. Real-Time RT-PCR. Total ribonucleic acid (RNA) was extracted from the rat testes and cultured TM4 cells using TRIzol reagent (Life Technologies Japan, Tokyo, Japan). RT reactions were carried out using a GeneAmp RNA PCR Kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. To quantitatively determine the expression levels of nephrin, podocin, and GAPDH mRNAs in each sample, real-time PCR analysis using a standard curve method with SYBR Green I (Takara Bio, Tokyo, Japan) was then conducted as previously described [26]. The sequence-specific primers, synthesized by Operon Biotechnology Inc. (Tokyo, Japan) based on previous studies, are presented in Table 1 [27, 28]. Data were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) values and expressed as arbitrary units relative to samples from control, regarded as "1."

2.7. Western Blot Analysis. Samples containing equal amounts of protein (20 μg) from lysates of the rat testes or cultured TM4 cells were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was blocked in PBS containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 hour with antibodies against nephrin (1 : 200, sc-32532, Santa Cruz

Table 1: The species-specific primers of podocin, nephrin and GAPDH.

	Sense primer	Antisense primer
Rat		
Podocin	CCTGTGAGTGGCTTCTTGTCCTC	GGAGACGCTTCATAGTGGTTTGCA
Nephrin	CTGACTGGGCTGAAGCCTTCT	AAGAGCACAGGCAGCAGGGG
GAPDH	CTCTACCCACGCCAAGTTCAA	GGATGACCTTGCCCACAGC
Mouse		
Podocin	TGAGGATGGCGGCTGAGAT	GGTTTGGAGGAACTTGGGT
Nephrin	CCCAACACTGGAAGAGGTGT	CTGGTCGTAGATTCCCCTTG
GAPDH	CTCATGACCACAGTCCATGC	CACATTGGGGGTAGGAACAC

Biotechnology, Santa Cruz, California), podocin (1 : 200, P0372, Sigma-Aldrich, Tokyo, Japan), and β-actin (1 : 1000, A1978, Sigma-Aldrich). The filters were then incubated for 30 minutes with horseradish peroxide-conjugated secondary antibodies (1 : 2000, sc-2004, sc-2020 Santa Cruz Biotechnology), and specific proteins were detected using an enhanced chemiluminescence Western blot analysis system (RPN2106, GE Healthcare, Tokyo, Japan). The relative optical densities of the bands were quantified using Image J Program [29], and data were expressed as corresponding values of the ratio relative to the result from control group.

2.8. Immunohistochemical Studies. Immunofluorescence staining was used to investigate the expression of nephrin and podocin. For frozen sections, antibodies against nephrin and podocin as described above were added in dilution of 1 : 200 and 1 : 400, respectively, and then incubated for 2 hours. After staining with the secondary antibodies goat anti-rabbit IgG-TR (1 : 500, sc-2780, Santa Cruz Biotechnology) and donkey anti-goat IgG-FITC (1 : 500, sc-2024, Santa Cruz Biotechnology), the sections were mounted and the staining outcomes were evaluated by inverted fluorescence phase-contrast microscopy (Keyence BZ-9000, Osaka, Japan). For *in vitro* studies, cultured TM4 cells were seeded on coverslips and fixed in 4% paraformaldehyde and were stained with primary and secondary antibodies, the same as *in vivo* study, and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) was additionally performed.

2.9. TUNEL Staining. Apoptotic features in the rat testes were evaluated by Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End-labelling (TUNEL) assay using an *in situ* cell death detection kit (11684795910, Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Quantitative analysis was performed by counting the number of apoptotic cells and total germ cells in 100 randomly selected tubules in one slide per animal.

2.10. Immunofluorescence Staining of Carbonylated Protein. Carbonylated protein is known as a marker of oxidative stress, since protein carbonylation has been identified as a potential mechanism underlying mitochondrial dysfunction which is linked to oxidative stress [30, 31]. The expression of carbonylated protein in the rat testes was evaluated using a protein carbonyls immunohistochemical staining kit (Cosmo Bio, Tokyo, Japan). In accordance with the manufacturer's protocol, methacarn-fixed paraffin-embedded sections were incubated with anti-dinitrophenylhydrazine (DNP) antibody, followed by incubation with fluorescein isothiocyanate (FITC) conjugated secondary antibody for 1 hour at room temperature in the dark. After the labelling procedure was completed, the coverslips were mounted onto glass slides using a mounting medium containing 4′,6-diamidino-2-phenylindole.

2.11. Superoxide Dismutase (SOD) Assay. To quantify the production of superoxide in the rat testes, Superoxide Dismutase (SOD) activity was measured using an SOD Assay Kit-WST (DOJINDO, Kumamoto, Japan) based on the manufacturer's protocol. In this assay, homogenized tissue samples were centrifuged and the supernatant was processed for the measurement of SOD activity as previously described [32]. One unit of SOD activity was defined as the amount of enzyme that inhibits 50% of the WST-1 formazan per minute.

2.12. Immunoprecipitation. Immunoprecipitation analysis was performed as previously described procedure [33]. Homogenized samples were precleared by incubation with protein G-plus-agarose beads (sc-2003, Santa Cruz Biotechnology) at 4°C for 1 hour and then incubated with primary antibodies as described above. Agarose beads were washed with lysis buffer and suspended in SDS/PAGE sample buffer. After SDS-polyacrylamide gel electrophoresis, samples were analyzed by Western blotting with corresponding antibodies.

2.13. Statistical Analysis. The significance of differences was evaluated by the Mann-Whitney U test for unpaired observations. Values were expressed as the mean ± standard deviation, and P values < 0.05 were considered significant.

3. Results

The changes in testicular weight and sperm analysis following treatment with cisplatin and/or candesartan are summarized in Figure 1. Cisplatin caused the dose-dependent testicular damage; administration of high-dose (7 mg) cisplatin caused a significant decrease in testicular weights, while low-dose (4 mg) cisplatin did not affect the testicular weight. These

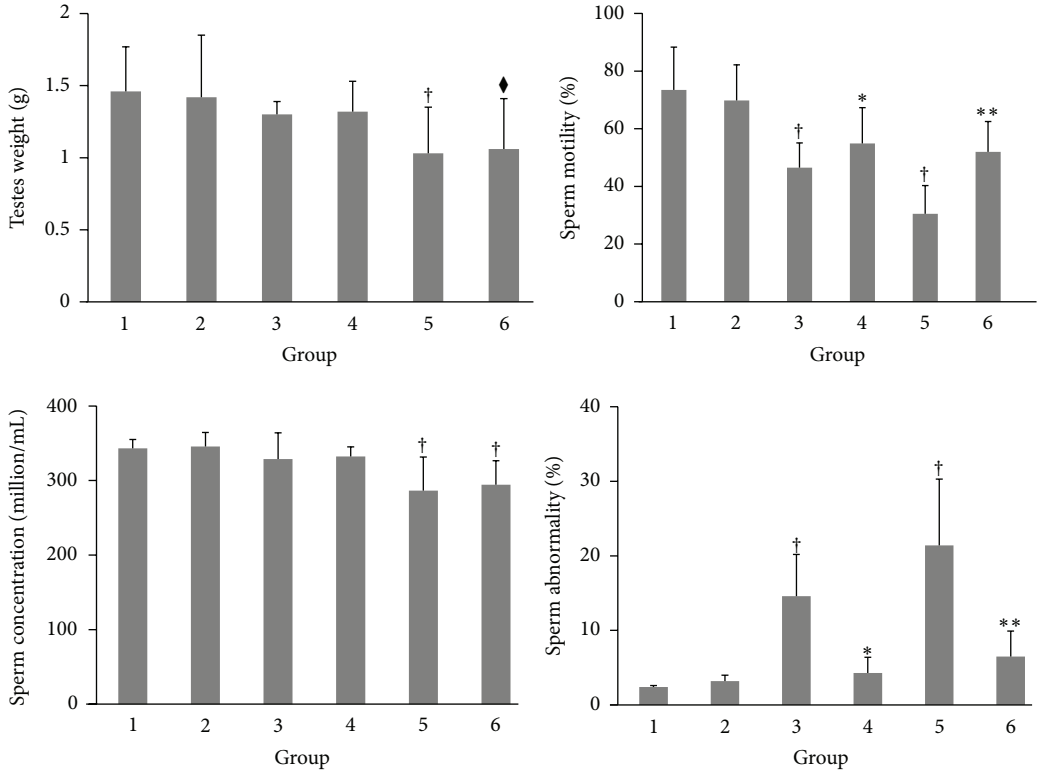


Figure 1: Weights of testes, epididymal sperm concentrations, and percentages of sperm motilities and abnormalities following treatment with cisplatin and/or candesartan. Group 1 ($n = 7$), intraperitoneal (IP) injection of 2 mL isotonic saline; Group 2 ($n = 7$), IP injection of 2 mL isotonic saline and oral administration of candesartan 5 mg/kg/day; Group 3 ($n = 7$), IP injection of 4 mg/kg cisplatin; Group 4 ($n = 7$), IP injection of 4 mg/kg cisplatin and oral administration of candesartan 5 mg/kg/day; Group 5 ($n = 7$), IP injection of 7 mg/kg cisplatin; and Group 6 ($n = 7$), IP injection of 7 mg/kg cisplatin and oral administration of candesartan 5 mg/kg/day. Each column represents the mean value with standard deviation. †, *, and ** differ from Groups 1, 3, and 5, respectively ($P < 0.05$).

changes were not affected by candesartan treatment. Sperm analysis revealed that sperm concentration and motility were significantly decreased by cisplatin administration, and increased proportion of abnormal sperm was observed after cisplatin administration. Coadministration of candesartan significantly recovered the motility rate and sperm abnormality rate. Sperm concentration was slightly recovered by candesartan, but the difference was not statistically significant.

We then assessed the changes in the expression levels of nephrin and podocin in the rat testes following treatment with cisplatin and/or candesartan. The expression of both nephrin and podocin mRNAs was detectable in the rat testes, and these levels were significantly decreased by treatment with cisplatin (Figures 2(a) and 2(b)); however, these changes were partially, but significantly, restored by combined administration with candesartan. Changes in the protein expression levels of both nephrin and podocin after treatment with cisplatin and/or candesartan were similar to those in the nephrin and podocin mRNAs (Figures 2(c)–2(e)).

Expression patterns of nephrin and podocin in the rat testes following treatment with cisplatin and/or candesartan are presented in Figure 3. Dual-labeling immunofluorescence staining revealed colocalization of nephrin and podocin near the basal membrane of seminiferous tubules. Moreover, the administration of cisplatin markedly reduced the staining intensities of both nephrin and podocin and resulted in a shift of both proteins from linear to granular expression patterns, accompanying the dissociation of these two molecules. These changes induced by cisplatin were partially restored by combined treatment with candesartan.

The effects of treatment with cisplatin and/or candesartan on apoptotic features in the rat testes were subsequently investigated. As shown in Figure 4, a marked increase in the number of TUNEL-positive cells was observed in the group treated with cisplatin in a dose-dependent manner, while the number of TUNEL-positive cells was significantly reduced in the group receiving candesartan in addition to cisplatin. Furthermore, the assessment of oxidative stress revealed that treatment with cisplatin induced a marked increase in the expression of carbonylated protein in the rat testes compared with the baseline expression in the control group without cisplatin treatment, and coadministration of candesartan partially inhibited the cisplatin-induced upregulation of this protein (Figures 5(a) and 5(b)). Similarly, a quantitative assessment of SOD activity in the rat testes showed a cisplatin-induced decrease in this activity and its partial restoration by candesartan (Figure 5(c)).

To confirm the findings of *in vivo* experiments, *in vitro* studies using a cultured mouse Sertoli cell line, TM4, were conducted. In TM4 cells as well, nephrin and podocin were

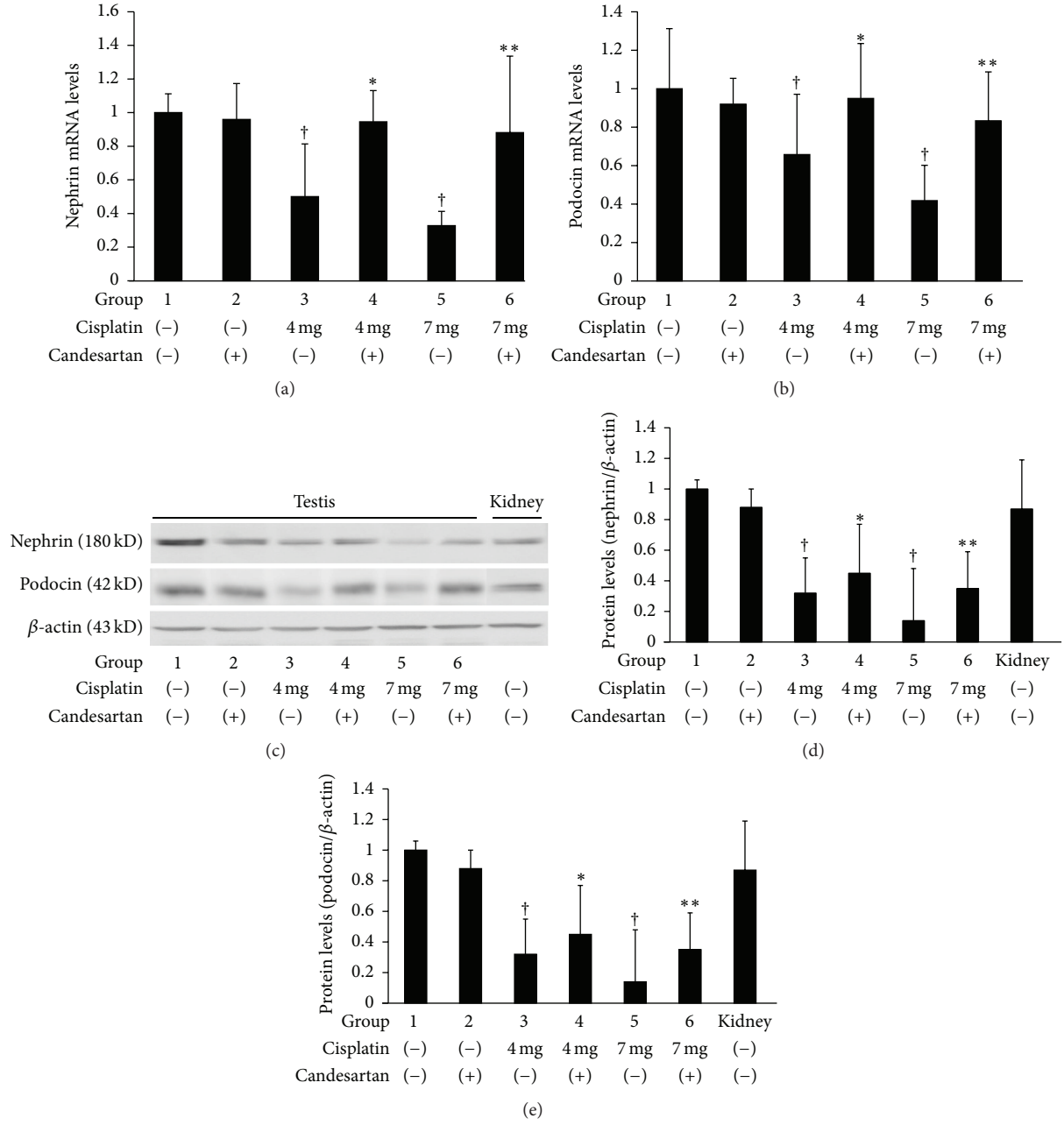


Figure 2: Changes in expression levels of nephrin and podocin in the testes of rats following treatment with cisplatin and/or candesartan. (a) Total RNA was extracted from the testes and analyzed for nephrin mRNA levels by real-time RT-PCR. Each column represents the mean value with standard deviation. †, *, and ** differ from Group 1, Group 3, and Group 5, respectively ($P < 0.05$). (b) Total RNA was extracted from the testes and analyzed for podocin mRNA levels by real-time RT-PCR. Each column represents the mean value with standard deviation. †, *, and ** are represented as in (a). (c) Protein was extracted from the testes, and expression levels of nephrin, podocin, and β-actin were analyzed by Western blotting. (d) Nephrin and β-actin signal intensity in (c) were quantified, and the ratio of nephrin over β-actin (d) and that of podocin over β-actin (e) were presented in histogram format. †, *, and ** are represented as in (a).

definitely expressed at both mRNA and protein levels, and treatment with angiotensin-II induced a reduction of their expression levels, which could be partially recovered by additional treatment with candesartan (Figure 6). As shown in Figure 7(a), immunofluorescence staining revealed that the expression of nephrin and podocin was mainly localized at junctions between TM4 cells cultured in standard medium; however, exposure of TM4 cells to angiotensin-II induced redistribution of these proteins, resulting in the emergence of diffused localization patterns. These changes in expression patterns of both proteins could be restored by combined treatment with candesartan.

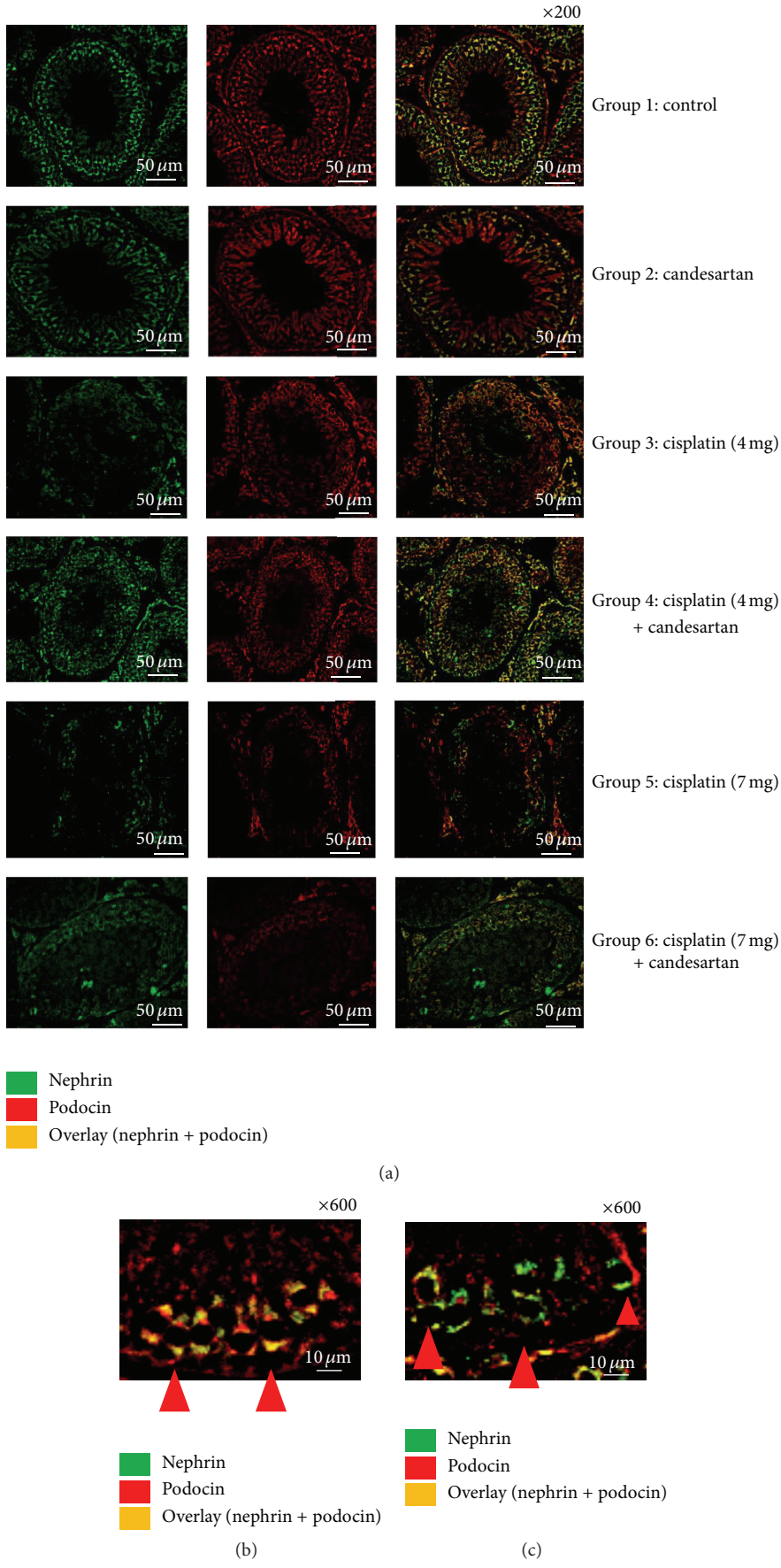
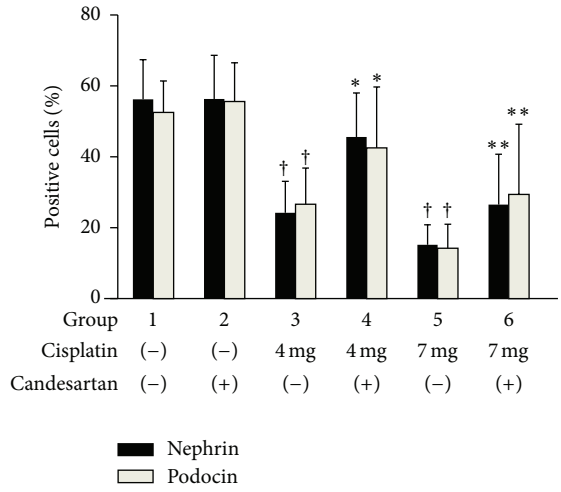
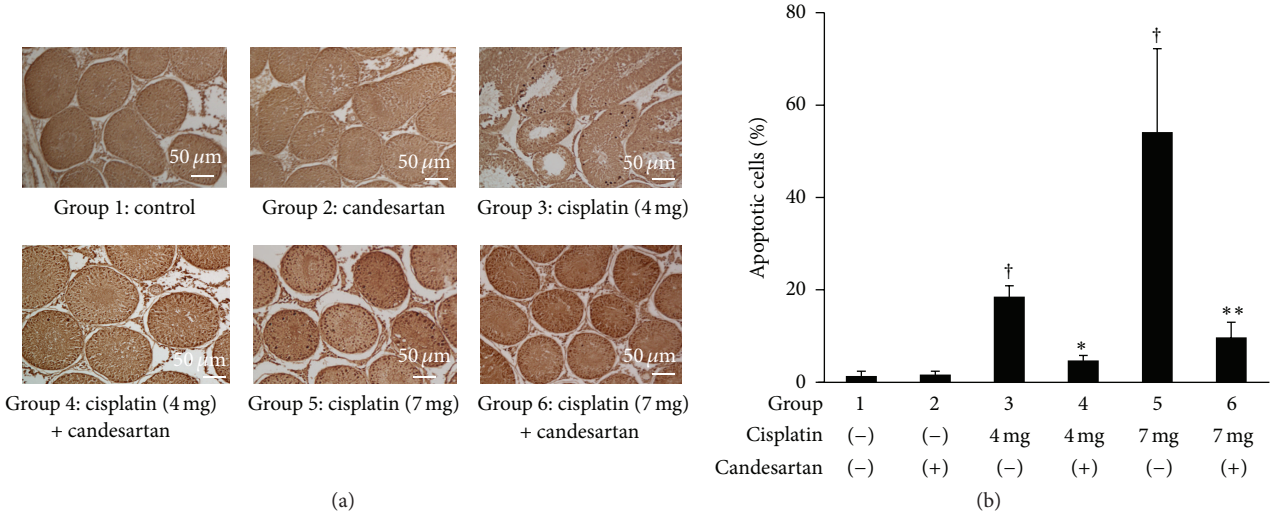


Figure 3: Continued.



(d)

Figure 3: Immunofluorescence double staining for nephrin and podocin in the testes of rats following treatment with cisplatin and/or candesartan. Nephrin was stained in green with FITC, podocin was stained in red with Texas Red, and the nuclei were stained in blue with DAPI. The overlaps of nephrin and podocin are indicated in orange. (a) Representative findings of immunofluorescence double staining and changes in each group. (b) High magnification images of control testis. Arrows indicate that nephrin and podocin are coexpressed in linear formation near basal membrane of seminiferous tubules. (c) High magnification images of testis following treatment with cisplatin. Arrows indicate that, after cisplatin (7 mg) administration, the expression patterns of nephrin and podocin were dissociated and in discrete granular structure. (d) Quantitative analysis of immunofluorescence positive cells for nephrin and podocin. †, *, and ** differ from Group 1, Group 3, and Group 5, respectively ($P < 0.05$).



(a) (b)

Figure 4: Apoptotic features in the testes of rats following treatment with cisplatin and/or candesartan. (a) Representative findings of TUNEL staining in rat testes. (b) The numbers of TUNEL-positive cells and total germ cells in 100 randomly selected tubules in one slide per animal were counted. Apoptotic index, defined as the percentage of TUNEL-positive cells, was then calculated for each slide. Each column represents the mean value with standard deviation. †, *, and ** differ from Group 1, Group 3, and Group 5, respectively ($P < 0.05$).

To examine whether nephrin specifically binds to podocin in TM4 cells, immunoprecipitation assay was performed. As shown in Figure 7(b), nephrin was coimmunoprecipitated with podocin, while podocin was also coimmunoprecipitated with nephrin.

4. Discussion

During the last two decades, several molecules have been identified to be associated with the structure of the slit diaphragm in the kidney podocyte, the primary filtration

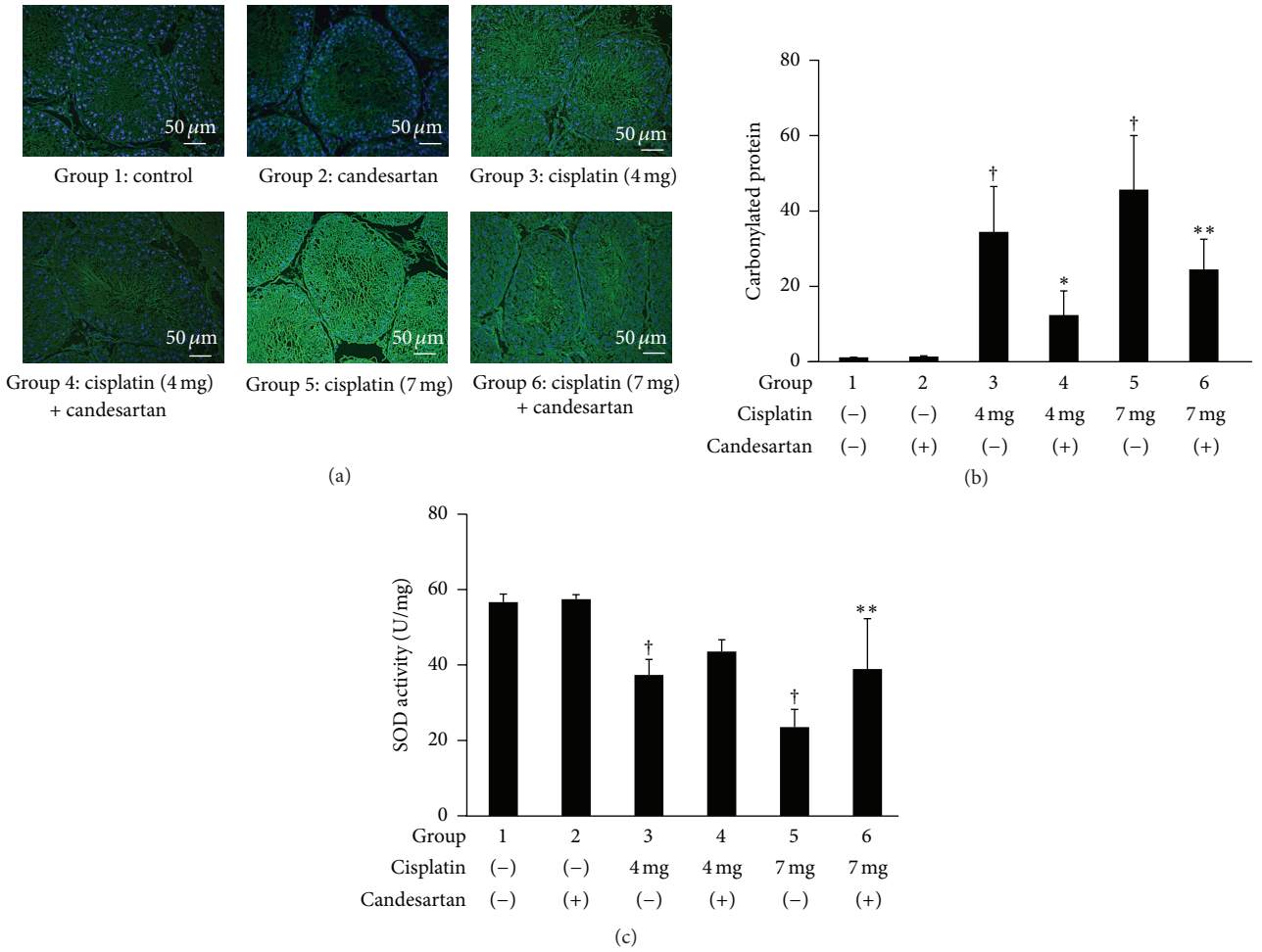


Figure 5: Oxidative stress status in the testes from rats following treatment with cisplatin and/or candesartan. (a) Representative findings on immunofluorescent staining of carbonylated protein in the rat testes. (b) Carbonylated protein signal intensities in (a) were quantified by Image J software and the ratio of each group over Group 1 was presented in histogram format. (c) Homogenized testicular specimens were prepared and analyzed for SOD activity. Each column represents the mean value with standard deviation. †, *, and ** differ from Group 1, Group 3, and Group 5, respectively ($P < 0.05$).

barrier responsible for macromolecule proteins. The initially reported molecule consisting of the slit diaphragm was ZO-1, originally identified as a component of the tight junction [34]. Nephrin and podocin, also well characterized components of the slit diaphragm, were shown to bind to each other, and several studies suggested that abnormal or insufficient signaling via the nephrin-podocin complex is likely to result in the development of proteinuria [8, 27, 35]. Recently, Relle et al. reported that the expression of podocin was detected in the testes and that confocal laser microscopy revealed colocalization of podocin with filamentous actin within Sertoli cells, suggesting a possible role of podocin in the blood-testis barrier [6]. Liu et al. reported the testicular expression of nephrin as well, which was colocalized with ZO-1 [7]. In this study, we revealed the colocalization of nephrin and podocin near the basal membrane of seminiferous tubules. These localization patterns were similar to those of tight junction protein, such as F-actin, β-catenin, and N-cadherin [6, 36]. Collectively, these findings indicate that nephrin and podocin may be involved in the function mediating the barrier system in cell-cell junction in the testis.

In this study, in order to investigate the relationship between testicular damage and expression patterns of nephrin and podocin in the testes, we treated rats with cisplatin, which has been shown to have a potential toxic effect on the testes through the impairment of functions associated with cell-cell adhesion [17, 37]. We initially confirmed a significant decrease in sperm concentration and motility as well as increased proportion of abnormal sperm in rats treated with cisplatin. Furthermore, cisplatin treatment induced marked inhibitory effects on the expression of testicular nephrin and podocin. Immunohistochemical analyses also showed that nephrin and podocin were colocalized near the basal membrane of seminiferous tubules without cisplatin treatment; however, the dissociated expression of these proteins, accompanying the changes in granular expression patterns, was observed following the administration of cisplatin. These findings suggest the possible role of nephrin and

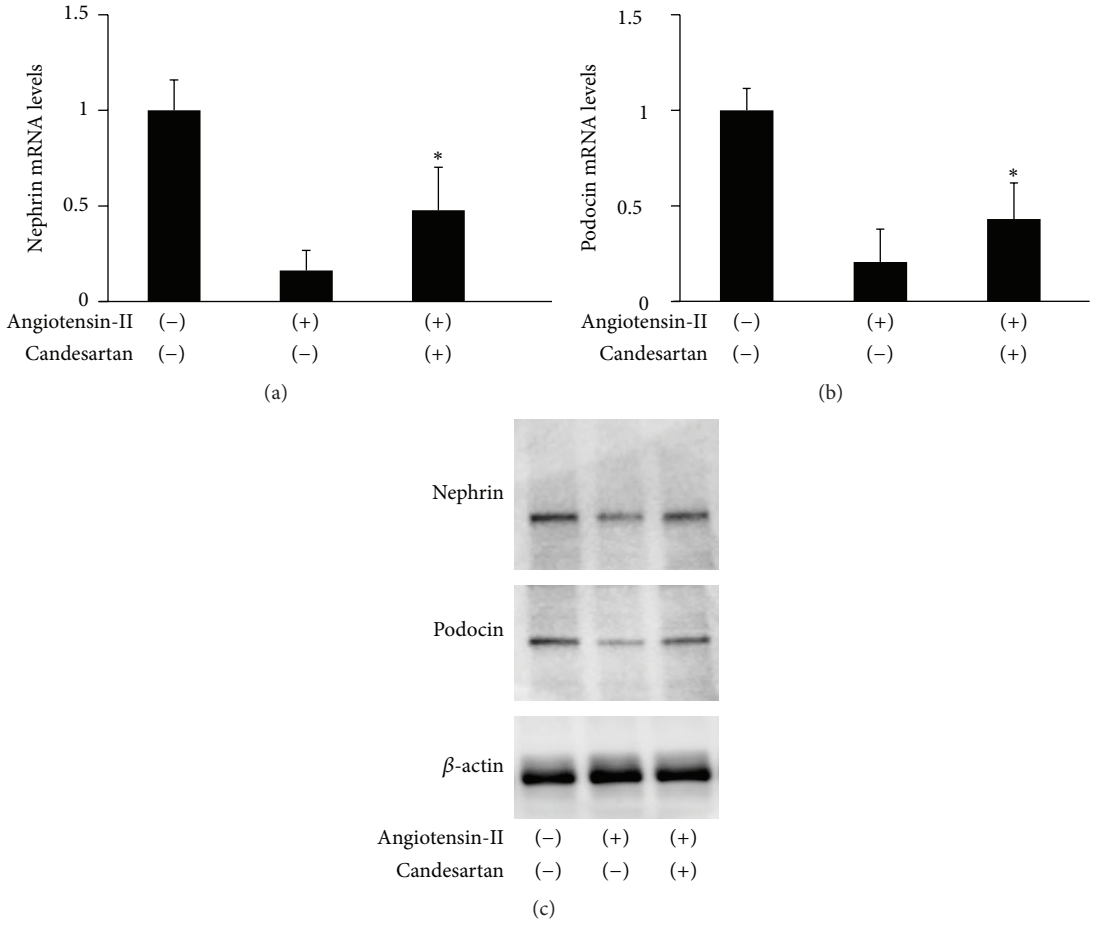


Figure 6: Changes in expression levels of nephrin and podocin in cultured mouse Sertoli cells, line TM4, following treatment with angiotensin-II and/or candesartan. (a) Total RNA was extracted from TM4 cells and analyzed for nephrin mRNA levels by real-time RT-PCR. Each column represents the mean value with standard deviation. * differs from TM4 cells treated with cisplatin alone ($P < 0.05$). (b) Total RNA was extracted from the testes and analyzed for podocin mRNA levels by real-time RT-PCR. Each column represents the mean value with standard deviation. * differs from TM4 cells treated with angiotensin-II alone ($P < 0.05$). (c) Protein was extracted from TM4 cells and expression levels of nephrin, podocin, and β-actin were analyzed by Western blotting.

podocin in cisplatin-induced damage through the disruption of the barrier function in the testes.

It is of interest to investigate whether cisplatin-induced testicular damage can be alleviated by an antiproteinuric agent. It has been well recognized that ARBs help reduce renal tissue damage and proteinuria in some renal diseases through specific blockade of angiotensin-II type 1 receptor (AT1R), independently of its function to lower blood pressure [32, 33]. Furthermore, several recent studies have demonstrated that AT1R-mediated action directly protects slit diaphragm molecules, including nephrin and podocin, from stimuli-inducing renal injury [8, 9, 38, 39]. To evaluate the regulatory mechanism of nephrin and podocin by angiotensin-II in addition to these findings, we assessed the effects of candesartan, one of the most prevalent ARBs, on the damage in rat testes following cisplatin treatment. Our results showed that the administration of candesartan resulted in the improvement of cisplatin-induced toxic events in the rat testis, including decreased sperm motility, and increased proportion of abnormal sperm. In addition, the reduced expression of nephrin and podocin and their dissociated expression patterns accompanied by apoptosis were partially restored by candesartan administration. Other than the present findings, there have been several studies revealing the involvement of an angiotensin-mediated system in the maintenance of testicular function [12–14, 40, 41]. For example, Shiraishi et al. reported that AT1R-dependent fibrosis in rat testes was observed after vasectomy and that impaired spermatogenesis caused by interstitial fibrosis was partially abrogated by ARB administration [14]. Similarly, Kushwaha and Jena reported that testicular toxicity induced by nicotine in streptozotocin-induced diabetic rat was attenuated by ARB treatment [13]. Taken together, these findings indicate that ARB may have protective effects from testicular damage induced by several types of toxic stimuli to the testes.

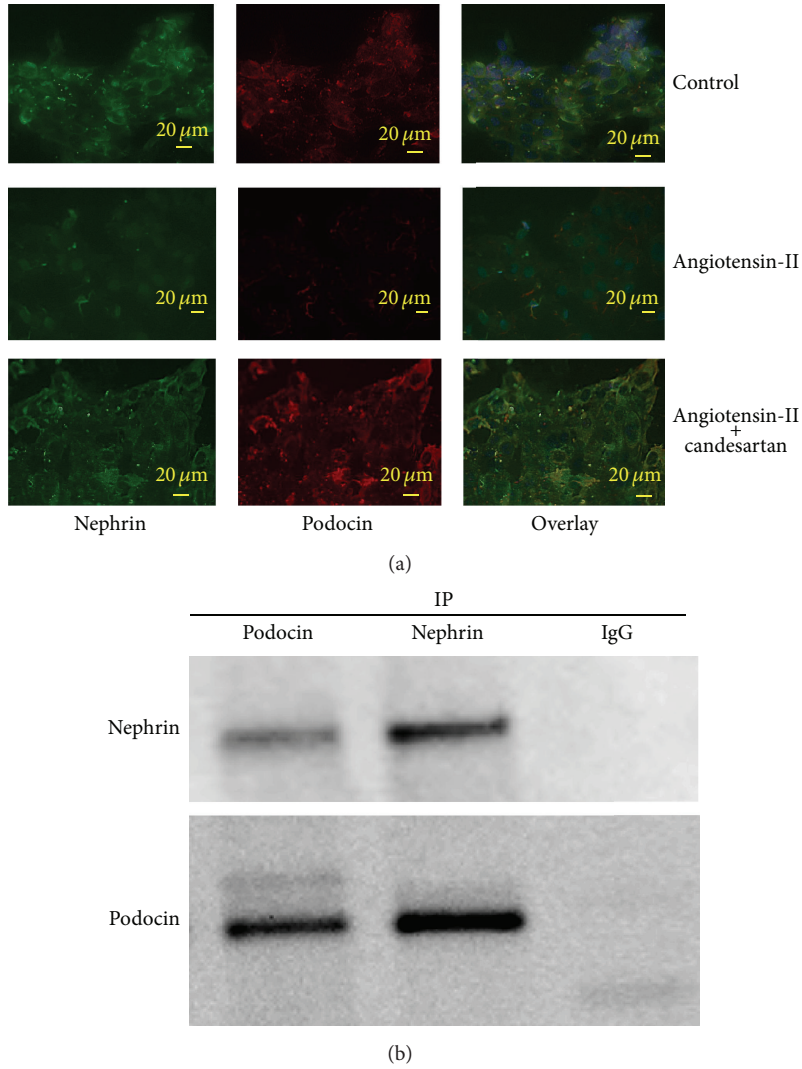


Figure 7: (a) Immunofluorescence double staining for nephrin and podocin in cultured mouse Sertoli cells, line TM4, following treatment with angiotensin-II and/or candesartan. Nephrin was stained in green with FITC, podocin was stained in red with Texas Red, and the nuclei were stained in blue with DAPI. The overlaps of nephrin and podocin are indicated in orange. Nephrin and podocin are mainly located at junctions between cells, and administration of angiotensin-II induced redistribution of nephrin and podocin. These changes were partially restored by additional treatment with candesartan. (b) Coimmunoprecipitation (IP) assays of nephrin and podocin in cultured mouse Sertoli cells, line TM4. Whole-cell lysates prepared from TM4 cells were immunoprecipitated with antipodocin or nephrin antibody and blotted with antinephrin or podocin antibody, respectively.

Another point of interest is the mechanism mediating the protective effects on cisplatin-induced testicular damage by candesartan. To date, a number of previous studies have reported the protective effects of ARB on testicular damage. Some of these reports clarified the antioxidative activity induced by ARB as one of the main mechanisms underlying its protective effect on testicular damage [13, 14]. In fact, there have been several studies showing the induction of reactive oxygen species (ROS) through AT1R as well as the antioxidant activity of ARB in various types of cells [42] (Figure 8). Griendling et al. reported that angiotensin-II specifically activates enzyme systems that promote superoxide generation in cultured vascular smooth muscle cells [43]. Similarly, Okui et al. showed that cisplatin increased serum levels of angiotensin-II and aldosterone, which synergistically prolonged the accumulation of cisplatin in renal tissues [10]. In the testes as well, ROS has been shown to produce detrimental effects on the maintenance of their physiological functions [44]. Furthermore, antioxidants have been regarded as having protective effects on testicular damage [45–47]. For example, Salem et al. also reported the attenuation of cisplatin-induced testicular toxicity by antioxidant supplementation with selenium and lycopene [48]. Similarly to these studies, we also confirmed the restoring effect of candesartan on increased oxidative stress induced by cisplatin in rat testes. Considering these findings, candesartan may alleviate cisplatin-induced testicular damage through the inhibition of oxidant activity. However, in addition to these

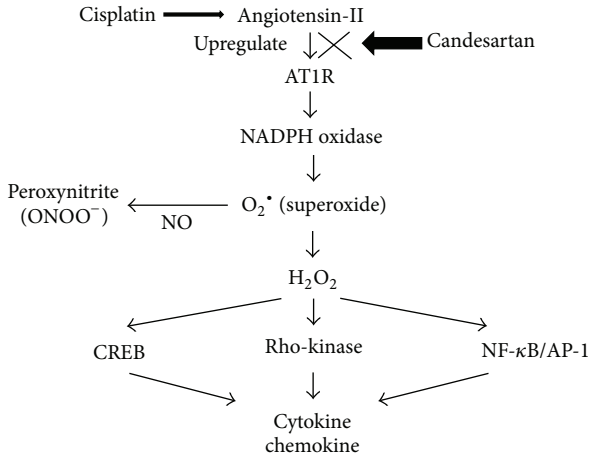


Figure 8: Signaling pathway of angiotensin-II type 1 leading to superoxide production. AT1R: angiotensin-II type 1 receptor, NADPH: nicotinamide adenine dinucleotide, CREB: cAMP response element binding protein, and NO: nitric oxide.

findings, we have to mention the toxic effects of cisplatin on cells in the adluminal compartment, which are normally protected by the blood/testis barrier, indicating that cisplatin and candesartan might modulate the functions of blood/testis barrier. In particular, ARB mediated protective effects on cell-cell junction in the testes, including nephrin and podocin. In this study, we confirmed that the dissociated nephrin-podocin complex was recovered by ARB administration, indicating that ARB could have protective effects among blood/testis barrier architecture.

In order to confirm and reinforce the findings obtained from *in vivo* studies, we also performed several *in vitro* experiments using a mouse Sertoli cell line, TM4. As for the changes in expression patterns of nephrin and podocin in TM4 cells following treatment with angiotensin-II and/or candesartan, we achieved findings that angiotensin-II directly acts on Sertoli cells and affects the expression of nephrin and podocin. Furthermore, the immunoprecipitation assay demonstrated binding between nephrin and podocin in TM4 cells. To our knowledge, this is the first study providing evidence concerning the binding of these proteins in cells other than those derived from the kidney. Although additional studies will be necessary to address the functional significance of the binding between testicular nephrin and podocin, these findings strongly suggest a direct interaction between these proteins in the testes like that in the renal slit diaphragm.

Here, we would like to emphasize several limitations of this study. Firstly, treatment of cisplatin was selected to induce damage in the testes; therefore, it is impossible to apply the findings of this study to all types of testicular injury. Secondly, we did not directly address whether testicular nephrin and podocin colocalized in tight junction in seminiferous tubules and regulate the physiological testicular functions or blood-testis barrier. Thus, it would be required to perform further study, such as that using immunoelectron microscopy and that assessing blood-testis barrier permeability. Thirdly, the significance of the use of candesartan together with chemotherapeutic agents from a clinical viewpoint should be evaluated. Finally, expression patterns of the nephrin-podocin complex in clinical testicular specimens from infertile men should be investigated to assess the role of these proteins in spermatogenesis.

5. Conclusion

Candesartan can prevent cisplatin-induced testicular damage by regulating expression patterns of the nephrin-podocin complex, which was shown to be localized near the basal membrane of seminiferous tubules in the testes. These findings suggest the possible involvement of angiotensin-II mediated action on these proteins in executing physiological functions as a component of cell-cell junction in the testes.

Abbreviations

ARB:	Angiotensin-II receptor blocker
AT1R:	Angiotensin type 1 receptor
DAPI:	4′,6-Diamidino-2-phenylindole
DMEM/F12:	Dulbecco's Modified Eagle Medium/F12
DNP:	Dinitrophenylhydrazine
FITC:	Fluorescein isothiocyanate
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
IP:	Intraperitoneal
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RT-PCR:	Reverse-transcriptase polymerase chain reaction
SDS:	Sodium dodecyl sulfate
SOD:	Superoxide Dismutase
TUNEL:	Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End-labelling.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Research Article

A Pilot Comparative Study of 26 Biochemical Markers in Seminal Plasma and Serum in Infertile Men

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Introduction. The relationships of the biochemical components in seminal plasma and serum, and their origins and physiological effects in male reproductive system have been poorly understood. *Methods*. Based on the calibration and quality control measures, 26 biochemical markers, in seminal plasma and serum samples from 36 male infertility patients with nonazoospermia were detected and compared. *Results*. Only PA was undetectable in all seminal plasma samples. There were significant differences of all other 24 biochemical markers in seminal plasma and serum ($P < 0.05$) except for UA ($P = 0.214$). There were rich proteins in seminal plasma, and globulin accounted for about 90%. There were also abundant enzymes in seminal plasma, and the activities of ALT, AST, AKP, GGT, LDH, CK, and αHBDH in seminal plasma were significantly higher than those in serum while ADA was inversely lower. There were relatively low levels of Glu, TG, TC, and hsCRP in seminal plasma, but Glu was undetectable in 8 of 36 cases. *Conclusions*. The differences of the levels of biochemical markers in seminal plasma and serum might be associated with the selective secretion of testis, epididymis and male accessory glands, and the specific environment needed for sperm metabolism and function maintenance.

1. Introduction

Currently, alpha-glucosidase, acid phosphatase, zinc, and fructose levels in seminal plasma have been determined in clinical andrology laboratories to evaluate the secretion function of male accessory glands [1–3]. Like serum, seminal plasma consists of rich biochemical components. It has been shown recently that seminal plasma proteins could serve as important biomarkers for male infertility [4]. In addition, functional proteomic analysis revealed that proteins are over- or underexpressed in the seminal plasma of men with poor semen quality [5]. However, the origins of these components and their correlations with those in serum are unclear. It is also unknown whether these components could be used to evaluate male fertility. Several studies have been done to compare the levels of biochemical markers in seminal plasma and serum, most of these studies focused on animal reproduction, and investigations on biochemical markers were limited. Moreover, the origins and potential physiological effects of biochemical components in seminal plasma have been still poorly understood; thus, we designed this study to detect the levels of 26 kinds of biochemical markers in seminal plasma and serum on the basis of quality control for each marker, and all the data were compared and analyzed.

2. Materials and Methods

2.1. Reagents. Kits for the determinations of total protein (TP), albumin (Alb), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AKP), and calcium (Ca) (Biosino Bio-Technology and Science Inc., Beijing, China), prealbumin (PA), high-sensitive C-reactive protein (hsCRP), gamma-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH), urea (Ur), creatinine (Cr), uric acid (UA), glucose (Glu), creatine kinase (CK), and adenosine deaminase (ADA) (Ningbo Medical System Biotechnology Co., Ltd., Ningbo, China), triglyceride (TG) and total

cholesterol (TC) (Shanghai Zhicheng Biotechnology Co., Ltd., Shanghai, China), alpha-hydroxybutyrate dehydrogenase (αHBDH) (Zhejiang Dongou Diagnostics Co., Ltd., Wenzhou, China), magnesium (Mg) and phosphorus (P) (Shanghai Fosun Long March Medical Science Co., Ltd., Shanghai, China), and potassium (K^+), sodium (Na^+), and chlorine (Cl^-) (Nanjing Panstar Electronics Instruments Co., Ltd., Nanjing, China) are commercially available from the abovementioned suppliers. Calibration and quality control products were provided by Randox Laboratories Ltd., Northern Ireland, United Kingdom.

2.2. Instruments. Olympus AU400 Automatic Biochemical Analyzer (Olympus Optical Co., Ltd., Tokyo, Japan), PSD-16a Electrolyte Analyzer (Nanjing Panstar Electronics Instruments Co., Ltd., Nanjing, China), and TGL-16B High Speed Centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China) were used to determine biochemical markers in this study.

2.3. Samples. All seminal plasma and serum samples were collected in the clinics of the Department of Reproduction and Genetics, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, China. Thirty six (36) infertility patients with nonazoospermia for over 1 year, aged from 26 to 33 years, who self-reported no other disorders, were included in this study. After abstinence for 2 to 7 days, semen samples were collected by masturbation, and for serum samples collection, fasting venous blood was drawn. Semen samples were placed at 37°C for liquefaction, followed by routine semen analysis, and the remaining semen samples were centrifuged at 12 000 ×g for 5 minutes. The upper layer seminal plasma was collected for the determinations of biochemical markers. Blood samples were centrifuged at 3 000 ×g for 5 minutes to isolate serum for the same analyses as for seminal plasma. All semen samples of nonliquefaction and volume less than 1.5 mL were excluded to avoid potential influence on the accuracy of sampling [6] and ensure all biochemical markers to be detected.

2.4. Biochemical Analysis. First, the calibration and the determination of quality control products for all biochemical markers were performed. After all results of quality control products were within the permitted ranges, the concentrations of K^+, Na^+, and Cl^- in seminal plasma and serum specimens were detected with a PSD-16a Electrolyte Analyzer and all other biochemical markers determined with Olympus AU400 Automatic Biochemical Analyzer. The samples outside of the linearity of the method and instrumentation were diluted to obtain effective results. All serum samples were directly determined without any dilution, while the measurements of LDH, CK, αHBDH, K^+, Ca, and Mg in seminal plasma were conducted after 1 : 5 dilution, and 1 : 25 dilution for GGT and P. The seminal plasma samples for the measurements of LDH, CK, αHBDH, Ca, Mg, GGT, and P were diluted with normal saline, and that for K^+ was diluted with deionized water.

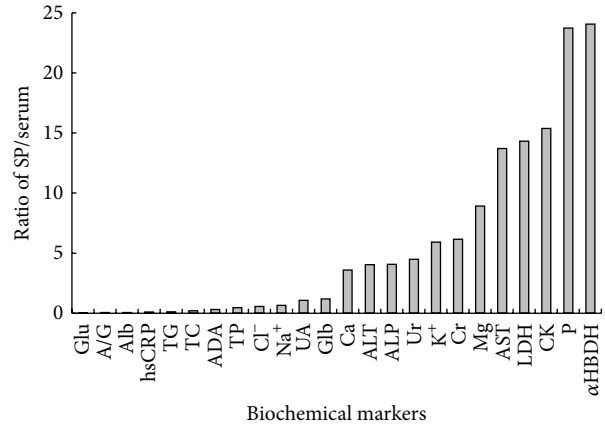

Figure 1: Ratios of 24 seminal plasma (SP) and serum biochemical markers except PA (undetectable) and GGT (the ratio of SP/serum up to 543.54).

2.5. Statistical Analysis. All data were saved in the Excel tables and analyzed with the SPSS 11.0 software (SPSS Inc., Chicago, USA). The values of biochemical markers in seminal plasma and serum were presented as mean ± standard deviation and compared by paired t-test, and $P < 0.05$ was considered as significant difference. The correlation of biochemical markers in seminal plasma and serum was analyzed with Pearson analysis, and $P < 0.05$ was considered as significant correlation.

3. Results

The comparison of the results of biochemical markers in seminal plasma and serum was shown in Table 1. Among all 26 kinds of biochemical markers, PA were undetectable, and there was no significant difference of UA in seminal plasma and serum ($P = 0.214$), but all the results of other 24 markers in seminal plasma and serum had significant difference ($P < 0.05$). The levels of Glb, ALT, AST, ALP, GGT, LDH, Ur, Cr, CK, αHBDH, K^+, Ca, Mg, and P in seminal plasma were significantly higher than those in serum, while the levels of TP, Alb, A/G, ADA, Glu, TG, TC, Na^+, Cl^-, and hsCRP in seminal plasma were significantly lower than those in serum (Figure 1). The level of seminal plasma Glu in 8 out of 36 cases was undetectable. The correlation analysis for 24 kinds of biochemical markers except PA (undetectable in seminal plasma) and A/G (with the same value) showed no obvious correlation of biochemical markers except significant positive correlations of UA ($r = 0.513$, $P < 0.01$) and hsCRP ($r = 0.861$, $P < 0.01$), significant negative correlation of Glu ($r = -0.356$, $P < 0.05$), and some negative correlation of TC ($r = -0.328$, $P = 0.051$) in seminal plasma and serum.

4. Discussion

Over the past seven decades, seminal plasma have been extensively studied. However, the comparison of biochemical markers in seminal plasma and serum has been poorly

Table 1: Comparison of biochemical parameters in seminal plasma and serum ($n = 36$).

Parameters	Seminal plasma (SP)	Serum	Ratio (SP/serum)	Correlation (r)
Total protein (TP) (g/L)	33.91 ± 6.99	74.67 ± 3.83*	0.46 ± 0.09	0.063
Albumin (Alb) (g/L)	2.87 ± 0.51	48.30 ± 1.93*	0.060 ± 0.011	0.112
Globulin (Glb) (g/L)	31.05 ± 6.86	26.37 ± 3.09*	1.19 ± 0.27	0.166
Albumin/globulin (A/G)	0.10 ± 0.00	1.86 ± 0.22*	0.055 ± 0.0065	—
Prealbumin (PA) (mg/L)	Undetected	344.63 ± 68.17*	—	—
Alanine aminotransferase (ALT) (U/L)	76.75 ± 24.29	28.39 ± 24.59*	4.04 ± 2.57	0.042
Aspartate aminotransferase (AST) (U/L)	439.31 ± 110.57	34.61 ± 12.31*	13.71 ± 4.55	−0.043
Alkaline phosphatase (AKP) (U/L)	311.33 ± 337.10	79.61 ± 19.65*	4.07 ± 4.24	0.107
Gamma-glutamyl transpeptidase (GGT) (U/L)	12133.33 ± 4278.56	36.31 ± 34.96*	543.54 ± 344.21	0.006
Lactate dehydrogenase (LDH) (U/L)	2427.08 ± 724.34	172.11 ± 26.17*	14.32 ± 4.54	0.115
Adenosine deaminase (ADA) (U/L)	2.92 ± 1.11	9.58 ± 2.31*	0.31 ± 0.12	0.109
Urea (Ur) (mmol/L)	24.00 ± 3.81	5.58 ± 1.20*	4.49 ± 1.23	0.185
Creatinine (Cr) (μmol/L)	505.61 ± 147.19	82.77 ± 6.97*	6.15 ± 1.86	−0.011
Uric acid (UA) (μmol/L)	383.14 ± 105.44	363.56 ± 74.61	1.07 ± 0.25	0.513$^{\triangle}$
Glucose (Glu) (mmol/L)	0.073 ± 0.095	5.30 ± 0.60*	0.0147 ± 0.20	−0.356$^{\#}$
Triglyceride (TG) (mmol/L)	0.12 ± 0.11	1.56 ± 0.98*	0.11 ± 0.13	−0.168
Total cholesterol (TC) (mmol/L)	0.90 ± 0.50	4.92 ± 1.00*	0.20 ± 0.13	−0.328
Creatine kinase (CK) (U/L)	1725.56 ± 1730.48	128.67 ± 41.22*	15.37 ± 19.79	−0.111
Alpha-hydroxybutyrate dehydrogenase (αHBDH) (U/L)	2898.61 ± 1506.08	122.06 ± 20.09*	24.06 ± 12.02	0.157
Potassium (K^+) (mmol/L)	24.06 ± 5.82	4.10 ± 0.29*	5.92 ± 1.58	−0.144
Sodium (Na^+) (mmol/L)	93.78 ± 9.66	142.73 ± 1.52*	0.66 ± 0.069	−0.112
Chlorine (Cl^-) (mmol/L)	56.54 ± 11.37	101.86 ± 1.44*	0.56 ± 0.11	−0.087
Calcium (Ca) (mmol/L)	8.47 ± 2.86	2.37 ± 0.10*	3.59 ± 1.24	−0.050
Magnesium (Mg) (mmol/L)	6.70 ± 2.62	0.76 ± 0.052*	8.92 ± 3.51	−0.105
Phosphorus (P) (mmol/L)	24.89 ± 7.16	1.08 ± 0.20*	23.73 ± 8.11	−0.065
High-sensitive C-reactive protein (hsCRP) (mg/L)	0.066 ± 0.064	2.05 ± 4.78*	0.097 ± 0.091	0.861$^{\triangle}$

The data were presented as mean ± standard deviation. $^{*}P < 0.05$ versus the corresponding parameter in seminal plasma. $^{\#}P < 0.05$ and $^{\triangle}P < 0.01$ for the correlation of the same parameter in seminal plasma and serum.

documented. So far, up to 19 biochemical markers have been reported, and the seminal plasma samples were obtained from 6 patients with spinal cord injury and 6 volunteers [7]. Rosecrans et al. [8] reported 16 biochemical markers, including Ca, Mg, K^+, Na^+, Cl^-, Zn, P, glycerylphosphorylcholine (GPC), carnitine, fructose, UA, acid phosphatase (ACP), AKP, AST, LDH, and ALT in seminal plasma and serum from 24 volunteers, and their results showed that the levels of all biochemical markers except UA between seminal plasma and serum were significantly different ($P < 0.05$). Our results presented in this study were similar to them. Rosecrans et al. [8] also reported that there were significant correlations of K^+ ($r = 0.51$), carnitine ($r = 0.54$), and AST ($r = 0.70$) in seminal plasma and serum, but we have not observed such phenomena in present study. Other comparative studies on biochemical markers in seminal plasma and serum were focused mostly on a single marker, such as the level of Mg in seminal plasma and serum samples obtained from normal fertile men and patients with premature ejaculation [9], and the levels of Ca and Mg in seminal plasma and serum from

113 men [10]. Moreover, the levels of protein, electrolytes, enzymes, and other components in seminal plasma samples in animals such as brown bears [11], rabbits [12], stallion [13], and bactrian camels [14] have been investigated, but the useful information was very limited in these studies.

In present study, we investigated the levels of the other 26 biochemical markers in seminal plasma except alpha-glucosidase, acid phosphatase, fructose, and zinc, which had been extensively evaluated for their standardized operation and quality control [1–3]. In order to avoid potential influence of particles in semen, such as sperm, lecithin body on the level, and the determination method of biochemical components in seminal plasma, seminal plasma was isolated from semen samples by centrifugation at 12 000 ×g for 5 minutes. The results of preliminary experiments for such seminal plasma samples showed good repeatability (CV < 5%). In previous studies [1–3], we have shown that there were still many spermatozoa in some seminal plasma samples obtained at 3 000 ×g centrifugation for 15 minutes. So, the inconsistency for some previously reported results may be due to the sperm residue in seminal plasma. Second, we obtained the results of all 26 markers from samples with the volume above 1.5 mL and ensured the accuracy of all results for the calibration and quality control measures made. All these efforts provided a guarantee for drawing meaningful conclusions.

Similar to serum, seminal plasma is composed of various components, and each of them has physiological significance. von Wolff et al. [15] reported that the injection of cryopreserved seminal plasma into the cervix and the posterior fornix of the vagina just after follicle aspiration in IVF or intracytoplasmic sperm injection (ICSI) treatment cycles has the potential to improve pregnancy rate. Moreover, seminal plasma was important for sperm metabolism, the maintenance of sperm function, and the survival and transport of sperm in the female reproductive tract. Our results showed that despite the fact that the UA levels in seminal plasma and serum were comparable, the other markers were significantly different in seminal plasma and serum, suggesting that the origins of the biochemical components in seminal plasma may be the secretion of epithelial cells in the reproductive tract, Sertoli cells, and spermatogenic cells in the male reproductive system for the existence of blood-testis, blood-epididymis barrier and other barriers.

Over 2 545 unique proteins in seminal plasma have been identified [16]. In our studies, TP level in seminal plasma was 33.91 ± 6.99 g/L, close to the level of 37.9 ± 5.606 g/L reported by Verdejo et al. [17]. Unlike serum, the dominant proteins in seminal plasma were globulins, which accounted for 90% of TP, while albumin accounted for about 10%. Previous studies [7, 18–21] described the possible role of albumin in seminal plasma. However, there were abundant globulins in seminal plasma, and their roles in male reproduction have been poorly understood. Moreover, in contrast to the previously reported results [22], our findings showed that PA was undetectable in seminal plasma.

A great number of enzymes in seminal plasma have been investigated. Guerin et al. [23] reported that the existence mode of enzymes in seminal plasma was similar to that in sperm, indicating that the enzymes in seminal plasma might play a role in ensuring the normal metabolism of sperm. Our results showed that the activity of ALT and AST was 76.75 ± 24.29 U/L and 439.31 ± 110.57 U/L, respectively; both of them were significantly higher than those in serum; in particular AST activity in seminal plasma was 13.71 times higher than that in serum, similar to the result reported by Hirsch et al. [7], that is, ALT: 62.3 ± 25.7 IU/L and GOT: 412 ± 191.1 IU/L. Mortimer and Bramley [24] also reported that the level of AST in seminal plasma was about 15 times higher than that in serum. Moreover, as reported by Dhami and Kodagali [25], the activity of ALT was positively correlated with that of AST ($r = 0.514$, $P = 0.001$). Whether ALT and AST in seminal plasma were derived from testis, epididymis, prostate, or seminal vesicle [7, 13, 26] was uncertain. In general, high activity of ALT and AST in seminal plasma might have some adverse effects on sperm function [25, 27–30], which may be used as a marker to evaluate sperm quality. However, further studies are needed to analyse the origins of the high levels of ALT and AST in seminal plasma, and we might speculate that some of damaged sperm or epithelial cells in the reproductive tract contribute to such elevated ALT and AST levels in seminal plasma.

GGT and AKP are two important enzymes that reflect hepatobiliary function. Our results showed that the levels of AKP and GGT in seminal plasma were 311.33 ± 337.10 U/L and 12133.33 ± 4278.56 U/L, respectively, and 4.07 and 543.54 times higher than that in serum. Chen et al. [31] reported that GGT in seminal plasma was mainly from prostate, which could be used to evaluate the secretion function of prostate instead of ACP, and that the accuracy of seminal plasma GGT detection was superior to that of ACP. However, studies on the detection of AKP in human seminal plasma were limited [7]. In boar [32] and stallion [13], the level of AKP in seminal plasma was significantly correlated with semen volume and sperm concentration. In bull [33], after semen was frozen and thawed, the level of AKP in seminal plasma increased significantly with sperm motility and fertilization rate decreasing, indicating that AKP in seminal plasma might come from the secretion of reproductive tract epithelial cells and the release of damaged sperm. However, the source of AKP in seminal plasma and its role in male reproduction need to be further investigated.

Our results also showed that LDH, CK, and αHBDH were rich in seminal plasma, up to 14.32, 15.37, and 24.06 times higher than those in serum, respectively. There were also some ADA in seminal plasma. The level of LDH in seminal plasma was similar to those in other published results [7, 17, 34]. It was well known that lactic acid could be converted to pyruvic acid catalyzed by LDH, which played a role in the production of ATP in sperm mitochondria [35]. Similarly, there was CK in sperm [36, 37], which was very important for sperm energy metabolism. Such high LDH and CK concentrations in seminal plasma might suggest that LDH and CK could be used as the marker to predict whether sperm energy metabolism is normal. Therefore, it is necessary to further analyse their origins and potential physiological functions. Recently, a PubMed search showed that the detection of αHBDH and ADA in seminal plasma has not been found in

MEDLINE database, and their possible physiological roles in male reproduction remain unclear.

The results in the determination of TG, TC, Glu, UA, hsCRP, Ur, and Cr in seminal plasma showed that the level of UA was similar to that in serum, while the levels of Ur and Cr were significantly (4.49 and 6.15 times) higher than those in serum, respectively. In contrast, the levels of TG and TC in seminal plasma were only about 1/5 of those in serum, and hsCRP was only about 1/10 of that in serum, and the level of Glu in seminal plasma was extremely low, even undetectable in 8 out of 36 cases, which may be due to the fact that fructose is the main energy source for sperm capitation.

It was shown that the level of UA in seminal plasma from normal fertile men was significantly higher than that from patients with azoospermia, infertility, or the vasectomized [38]. Moreover, the level of UA in seminal plasma was significantly correlated with sperm count [38] and the percentage of sperm with normal morphology [39]. All these reported findings indicated that the UA in seminal plasma might come from testis and/or epididymis and play an important role in protecting sperm from the damage of free radicals [40]; thus, UA could be used as an antioxidation marker in clinical diagnosis. In addition, investigations on Ur, Cr [7, 38], and hsCRP [41] in seminal plasma were very limited; thus meaningful information was not available. At least three questions remain unclear: (1) whether the hsCRP in seminal plasma was a kind of stress protein in male reproductive system, (2) whether the Ur and Cr in seminal plasma were the products of sperm metabolism, and (3) what their possible physiological function and clinical significance were.

There was rich cholesterol in human sperm cell membrane, which played an important role in maintaining the normal function of sperm. It was shown that there were exchanges of cholesterol and phospholipids between sperm and seminal plasma [42]. Therefore, it was possible that the level of cholesterol in seminal plasma would lead to the disorder of the cholesterol in sperm cell membrane. Although Meseguer et al. [43] reported no significant correlation of the concentration of cholesterol in seminal plasma with the level in sperm cell membrane, Cross [44] revealed that the medium with rich cholesterol could inhibit the acrosome reaction induced by progesterone. All these observations suggested that the extremely high or low concentration of cholesterol in human seminal plasma could affect the exchange of cholesterol between sperm and seminal plasma, and the detection of cholesterol in sperm cell membrane may be more meaningful than that in seminal plasma.

There were many reports about the detection of electrolytes in seminal plasma, but the results were not entirely consistent, which might be related to the different detection methods. Our results showed that the levels of Na^+ and Cl^- in seminal plasma were about 1/2 to 2/3 of them in serum, while the levels of K^+, Ca, Mg, and P in seminal plasma were significantly higher than them in serum, especially for P up to 23.73 times. Moreover, there were significantly positive correlations between the levels of Na^+ and Cl^- ($r = 0.654$, $P = 0.000$) and between Ca and Mg ($r = 0.930$, $P = 0.000$). In general, there were parallel concentrations of Na^+ and Cl^- in seminal plasma, and both were significantly higher than those of K^+. In contrast, the concentration of K^+ in sperm was significantly higher than that of Na^+ [45].

It was shown that the levels of Na^+, Ca, and Mg in seminal plasma were positively correlated with the percentage of motile sperm [46, 47], while that of K^+ was inversely correlated [47], indicating that K^+ in seminal plasma might inhibit sperm motility, but Na^+ promotes it. There were specific ion channels in sperm cell membrane for transferring K^+, Na^+, and Ca^{2+} into sperm or seminal plasma; accordingly sperm membrane potential changed and sperm motility promoted [48]. Therefore, the levels of electrolyte ions in seminal plasma should have an optimal range, and the increase and decrease of these ions will lead to the disorders of sperm motility [49]. However, whether these ions can be used to evaluate sperm motility needs to be confirmed.

There were very few reports about the detection of P in seminal plasma. Adamopoulos and Deliyiannis [50] found that there was higher level of P in seminal plasma in the patients with asthenospermia and lower level in patients with azoospermia compared with the males with normal sperm and that the level of P was positively correlated with that of fructose in patients with asthenospermia, oligospermia, or azoospermia, indicating that the origin of P in seminal plasma might be the secretion of seminal vesicle, and the detection of P in seminal plasma might reflect the secretion function of seminal vesicle.

In summary, among all the 26 biochemical markers we studied, only the level of UA was no significant difference in seminal plasma and serum. There were rich proteins in seminal plasma, but unlike serum, globulin was the dominant protein. There were also rich enzymes in seminal plasma, and the activities of ALT, AST, AKP, GGT, LDH, CK, and αHBDH except ADA in seminal plasma were significantly higher than those in serum. There were low levels of Glu, TG, TC, and hsCRP in seminal plasma, Glu was undetectable in 8 out of 36 cases, and PA was undetectable in all subjects. At present, the causes leading to the difference of these markers in seminal plasma and serum were unclear, due to the selective secretion of testis, epididymis and male accessory glands, and the specific environment required for sperm metabolism and function maintenance. The limitation of this pilot comparative study was relatively small sample size. Thus, further investigations should be focused on the identification of the origins and potential roles of these biochemical components in male reproduction, and comparison of the difference between fertile and infertile men based on bigger sample size.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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