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STUDY ON GENETIC VARIATIONS OF ANTIOXIDANT GENES IN MALE INFERTILITY

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INTRODUCTION

The necessity of research

The balance of oxidative stress or the final antioxidant factor may determine the success of future reproductive outcomes. With evidence of the correlation between oxidative stress components and the antioxidant reaction catalysts in sperm conditions, studies on the activity of these enzymes in semen samples have become a promising field in the context of research and addressing male infertility, which requires new and improved directions.

Knowledge regarding the polymorphic characteristics of genes encoding essential enzymes involved in antioxidant pathways in the Vietnamese population and their relationship with male infertility is still limited. Updating the genetic polymorphism data of the aforementioned gene group is a crucial supplementary foundation for previous basic studies on male infertility, aiming to elucidate comprehensive risks and mechanisms. Based on this, providing proposals, specific recommendations, as well as standardized procedures contribute to addressing infertility issues, including the aspect of treatment in Vietnam.

Research objective:

- Determine the level of oxidative stress in the semen of male diagnosed with primary infertility
- Determine the polymorphism rate in certain antioxidant genes influencing the spermatogenesis process in male diagnosed with primary infertility.
- Identify the correlation between genetic variations in antioxidant genes and male infertility.

Research approach:

The study subjects are men of reproductive age, including 107 male diagnosed with primary infertility, along with a control group of 85 healthy male with normal reproductive capability, each having at least one biological child under 2 years old. The infertility group will exclude causes of infertility due to chromosomal abnormalities and deletions on the Y chromosome, along with any reproductive organ-related diseases that may affect infertility status.

CHAPTER 1. OVERVIEW OF LITERATURE

1.1. Concept of male infertility

According to the World Health Organization (WHO), infertility is a condition in which a couple of reproductive age, with normal health, desires to have a child but is unable to conceive after 12 months of regular unprotected sexual intercourse. Depending on whether there has been a previous pregnancy or not, infertility is classified into two types: primary infertility and secondary infertility. Primary infertility, also known as infertility I, occurs when a couple has never conceived before. Secondary infertility, also known as infertility II, is when a couple has had at least one pregnancy but is unable to conceive again despite having regular unprotected sexual intercourse for a year and not using any contraceptive methods.

1.2. Overview of infertility and male infertility in Vietnam

In Vietnam, some research works on infertility indicate an increasing trend in infertility rates. The report by Nguyen Viet Tien at the International Workshop "Updates on Reproductive Support" (2009) in Hanoi, studying 14,396 couples of reproductive age (15 - 49 years old) across 8 provinces representing the 8 ecological regions of the country, revealed an overall infertility rate of 7.7%. Within this, male infertility accounted for 25-40%, female infertility for 40%, and the remaining cases were attributed to both partners or unknown causes.

1.3. Causes of male reproductive disorders and risk factors for male infertility

Factors that disrupt endocrine functions or affect processes such as spermatogenesis, erectile function, ejaculation, etc., not necessarily with a genetic cause, can contribute to infertility. In recent years, the advancement of gene sequencing technology has allowed the identification of numerous genetic variations as the causes of abnormalities in the male reproductive system, including fertilization, endocrine regulation of spermatogenesis, differentiation of stem cells, and sperm functions. Genetic mutations causing abnormalities in male reproduction include mutations affecting the structure and function of sperm, mutations affecting the vas deferens and testes, and abnormalities in the Y chromosome. Additionally, concerning

epigenetic aspects, the methylation process of many genes becoming inactive has been studied in abnormal semen samples from patients with oligozoospermia and deformed sperm. Among them, *MEST* and *H19* are the most extensively studied genes, with repeated data indicating that variations in the methylation levels of these two genes play a role as risk factors for male infertility. Moreover, unhealthy lifestyles leading to obesity, smoking habits, frequent use of electronic devices are also considered negative factors affecting the reproductive capacity of men.

1.4. Oxidative stress and male infertility

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the ability to eliminate ROS by available antioxidants. Sperm cells are vulnerable to ROS because they contain many polyunsaturated fatty acids in the membrane and cellular content. Additionally, sperm have limited antioxidant capacity and DNA repair systems. Several antioxidant genes play a role in the spermatogenesis process in mammals, including *NRF2, SOD, CAT, GST, GPX, PRX, GRX, TRX,* and *NOS*. The enzymes encoded by the mentioned gene group participate in antioxidant reactions, glutathione synthesis, and reduction, as well as the redox cycle during spermatogenesis. Genetic variations are a significant factor contributing to male infertility, and variants in genes encoding antioxidant enzymes may be responsible for male infertility, particularly in an environment exposed to oxidative stress. To date, genetic variations in genes responsible for antioxidant defense, such as *NRF2, SOD, GST, NOS, CAT*, and *GPX*, have been reported to be associated with male infertility..

In humans, two SNPs of *NRF2*, namely rs6721961 and rs3562124, are linked to weak sperm conditions (low count and poor motility). Individuals carrying the 617TT and 653TT genotypes have a higher risk of experiencing such sperm deficiencies. Three genetic variants of the *GST* gene, including the null alleles *GSTT1 (GSTT1* null*)*, *GSTM1* (*GSTM1* null), and *GSTP1* 6624A>G (p.105Ile>Val), have been demonstrated to be associated with male infertility in various human populations. In individuals lacking GPX, observed in 26% of diagnosed low sperm count and poor motility cases, genetic

variations in the *SOD* gene may also be partially related to reproductive capabilities. For example, the polymorphism c.47T>C (p.16Val>Ala) (rs4880) in the *SOD2* gene is associated with male infertility and successful pregnancy rates during in vitro fertilization (IVF). Certain alleles of the NOS gene have been reported to be linked to impaired sperm function in various human populations. Furthermore, the activity of the catalase (CAT) enzyme has been shown to be correlated with poor sperm quality.

1.5. Research on male infertility in Vietnam and worldwide

The genetic spectrum of male infertility patients is extremely complex due to the heterogeneity of semen and the histological structure of the testes. Until now, at least 2,000 genes have been identified that may influence the spermatogenesis process. Common genetic tests for male infertility in current clinical practice include karyotype analysis, screening for deletions on the Y chromosome, and testing for mutations in the *CFTR* gene.

Despite significant advances in the field of medical genetics, up to 80% of male infertility cases remain unexplained. With the rapid development of genetic analysis technologies, strategies for genomic analysis over the past 20 years include methods based on microarray platforms (SNP array, exome array, CGH array) and next-generation sequencing (NGS).

In Vietnam, the genetic cause of infertility due to *AZF* gene deletions in male patients without sperm has been investigated by PGS. Researchers at Military Central Hospital 108 and Hanoi University of Medicine have also initiated research to assess the impact of trace elements (such as zinc) or fructose in serum/semen on male infertility. Additionally, the correlation between oxidative stress and the genetic polymorphisms of *NAT2* and *GSTP1* in the primary male infertility group has been studied by Vu Thi Huyen and colleagues. To date, there has been no comprehensive study on the relationship between genetic variations in antioxidant system genes, such as *SOD1, SOD2, CAT, NOS3*, and male infertility in Vietnam.

CHAPTER 2. MATERIALS AND METHODS

2.1. Subjects and time for research

Research subjects included 107 patients (aged 21-50) diagnosed with primary infertility and a control group of 85 healthy men (aged 23-43), all within the reproductive age range. Data collection took place from January 2019 to December 2020, involving the enrollment of 107 patients and 85 control subjects.

The data collection form was established prior to the sampling phase. Detailed data information of the participants was securely stored at Hanoi Medical University. Administrative medical records included demographic information of the couples, along with forms for semen analysis and oxidative stress testing information.

2.2. Study design and sample size

- Study design: Cross-sectional with control group
- The sample size is calculated based on the frequency of occurrence of polymorphisms, following the formula as:

$$n = \frac{4C}{(\ln OR)^2 pq}$$

In which: n is the number of required collected samples; C is the constant related to type I and type II errors. Taking the values $\alpha = 0.05$ and $\beta = 0.20$, then C = 7.85; OR: Odds ratio; p: frequency of gene polymorphisms occurrence.

2.3. Instruments and chemicals

The machinery and equipment used in the sperm analysis are from the Center of Clinical Genetics and Genomics, Hanoi Medical University Hospital. Machines, equipment, and chemicals serving molecular biology experiments are from the Institute of Genome Research, Vietnam Academy of Science and Technology.

2.4. Methodology

2.4.1. Determining the oxidative stress level of semen samples

The Oxisperm kit (Halotech, Spain) was used to assess the level of oxidative stress based on the excess amount of superoxide anion

present in the semen. All semen samples were evaluated for oxidative stress immediately after liquefaction and 60 minutes after ejaculation to avoid false positive conditions.

2.4.2. Total DNA extraction and concentration

Total DNA was extracted from blood samples following the protocol of the Exgene Blood SV mini Kit (GeneAll, South Korea). The concentration of total DNA was assessed using the Qubit dsDNA HS Assay kit (Life Technologies, USA).

2.4.3. PCR amplification of specific gene segments containing the target variants

The sequences of specific primer pairs were designed using Primer 3 software (v.0.4.0) based on the reference gene sequence, which was synthetically produced by PHUSA Biochem Company (Can Tho, Vietnam). Using the designed specific primer pairs, the gene regions containing target variants *SOD1* 7958G>A, *SOD2* c.47T>C (p.16Val>Ala), *CAT*-262C>T, and *NOS3*-786C>T were amplified following standard procedures. All PCR products were purified using the Multiscreen PCR 96 Filter Plate according to the supplier's instructions.

2.4.4. Sanger sequencing

The purified PCR products were used as templates for the sequencing reaction. The DNA sequences were electrokinetically preserved and the fluorescent signals were read on the ABI 3500 Genetic Analyzer system. The signals were recorded automatically, analyzed, and stored on the computer.

2.5. Data analysis

The raw data was analyzed by using SeqScape 3.0 software (Applied Biosystems, Waltham, Massachusetts, USA). Statistical algorithms used in this study including Chi-square test ($\chi 2$), One-way ANOVA, Wilcoxon test, and logistic regression.

2.6. Ethics in research

This research was conducted after obtaining approval from the Ethics Committee of the Hanoi Medical University (76/HMU-IRB).

CHAPTER 3. RESULTS

3.1. Demographic and clinical characteristics

Table 3.1. Demographic characteristics and semen parameters

Characteristics	Patients (n=107)	Control (n=85)	P OR (95% CI)
Demographics			
Age (year/mean ± SD)	31.93 ± 6,3	31.96 ± 4,87	0.920[b]
BMI (Kg/m^2/TB± SD)	24.84 ± 2.31	23.53 ± 2.55	**0.00026[b]**
Smoke			0.550[a]
Yes (%)	65 (60.75)	48 (56.47)	
No (%)	42 (39.25)	37 (43.53)	
Drinking alcohol			
Yes (%)	103 (96.26)	75 (88.23)	**0.034[a]**

Note: BMI: Body Mass Index; SD: Standard deviation.

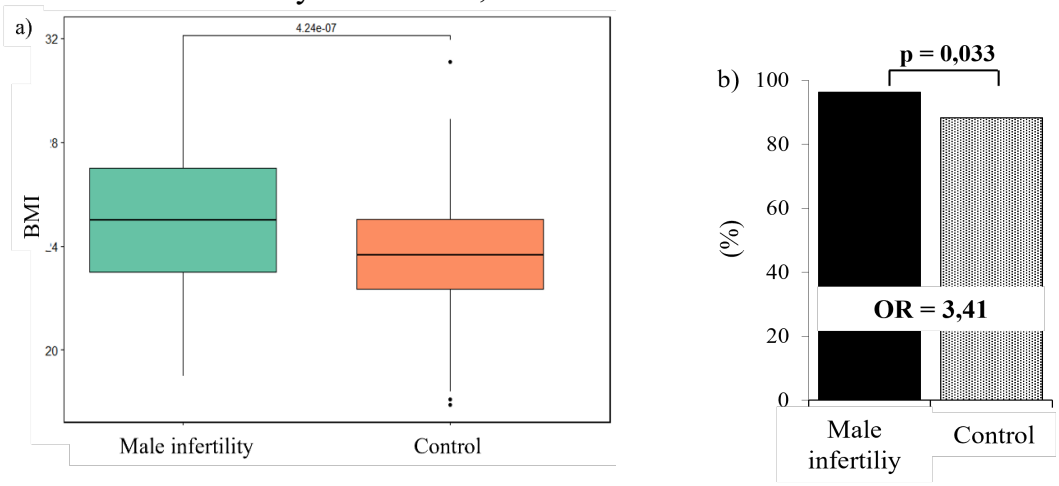

Figure 3.1. The impact of BMI and alcohol consumption on male infertility

a) The BMI in the male infertility group (24.84 ± 2.31) was significantly higher compared to the control group (23.53 ± 2.55), p = 0.00026, b) The rate of infertility patients with alcohol consumption was higher than the control group (OR = 3.41; p = 0.033).

3.2. Determining the level of oxidative stress in semen samples

Semen was collected from patients identified as idiopathic infertility. The level of oxidative stress was determined by measuring the concentration of superoxide anion using the Oxisperm kit in samples within 60 minutes after ejaculation. The oxidative stress levels obtained

from the Oxisperm kit results were categorized into 4 levels from 1 to 4. In this study, the oxidative stress levels of semen samples from patients were classified into two levels: high (levels 3 and 4) and low (levels 1 and 2). Due to the characteristics of the processing time of semen samples after collection not ensuring 100% compliance with the standards for excluding false positives (processing time exceeding 60 minutes after collection), only 90 out of 107 semen samples from patients had information on the level of oxidative stress.

Specifically, 21 out of 90 samples were determined to have high oxidative stress, accounting for 23.3%, and 86 out of 90 samples were determined to have low oxidative stress, accounting for 76.7% of the total samples.

3.3. Determining the polymorphisms of some antioxidant genes

3.3.1. Results of DNA extraction and amplification of gene segments containing the target variants.

The total DNA was extracted from peripheral blood, then was electrophoresed on a 0.8% agarose gel. The gel electrophoresis image showed bright, distinct bands, reflecting intact total DNA with high purity, ensuring quality for subsequent experimental steps.

In all study samples (107 patient and 85 control), we successfully amplified all four gene segments containing the variants 7958G>A (rs4998557) in the *SOD1* gene (298 bp), c.47 T>C (rs4880) in the *SOD2* gene (218 bp), -262C>T (rs1001179) in the *CAT* gene (282 bp), and -786C>T (rs2070744) in the *NOS3* gene (280 bp).

3.3.2. Analysis of gene variants

3.3.2.1. Variant 7958G>A (rs4998557) in the SOD1 gene

For the variant 7958 G>A (rs4998557) in the *SOD1* gene, in the male infertility group, there were 23/107 individuals with the homozygous GG genotype (21.5%), 66 individuals with the heterozygous GA genotype (61.68%), and 18 individuals with the homozygous mutated AA genotype (16.82%). In the control group, there were 26/85 individuals with the wild-type GG homozygous genotype (30.59%), 36 individuals with the heterozygous GA genotype (42.35%), and 23 individuals with the homozygous mutated AA genotype (27.06%). The allele frequency in the

patient group was 47.66% for allele G and 52.34% for allele A. In the control group, the allele frequency was 48.24% for allele G and 51.76% for allele A.

Table 3.3. Allele and genotype frequency of *SOD1* 7958 G>A (rs4998557) variant

Genotype/Allele	Patient n = 107 (%)	Control n = 85 (%)
GG	23 (21.5)	26 (30.59)
GA	66 (61.68)	36 (42.35)
AA	18 (16.82)	23 (27.06)
Allele A	112 (52.34)	88 (51.76)
Allele G	102 (47.66)	82 (48.24)

Note: n: number of samples

3.3.2.2. SOD2 c.47 T>C (rs4880) variant

For the *SOD2* c.47 T>C (rs4880) variant, in the male infertility group, there were 51 individuals with homozygous TT genotype (47.66%), 48 individuals with heterozygous TC genotype (44.86%), and 8 individuals with homozygous mutated CC genotype (7.48%). In the control group, there were 56 individuals with the wild-type TT homozygous genotype (65.88%), 27 individuals with the heterozygous TC genotype (31.76%), and 2 individuals with the homozygous mutated CC genotype (2.35%). The allele frequency in the patient group was 70.09% for allele T and 29.91% for allele C. In the control group, the allele frequency was 81.76% for allele T and 18.24% for allele C.

Table 3.4. Allele and genotype frequency of *SOD2* c.47 T>C (rs4880) variant

Genotype/Allele	Patient n = 107 %)	Control n = 85 (%)
TT	51 (47.66)	56 (65.88)
TC	48 (44.86)	27 (31.76)
CC	8 (7.48)	2 (2.35)
Allele T	150 (70.09)	139 (81.76)
Allele C	64 (29.91)	31 (18.24)

Note: n: number of samples

3.3.2.3. CAT -262 C>T (rs1001179) variant

Table 3.5. Allele and genotype frequency *CAT* -262 C>T (rs1001179) variant

Genotype/Allele	Patient n = 107 (%)	Control n = 85 (%)
CC	88 (82.24)	75
CT	18 (16.82)	10
TT	1 (0.93)	0 (0)
Allele C	194 (90.65)	160
Allele T	20 (9.35)	10

Note: n: number of samples

For the *CAT* -262 C>T (rs1001179) variant, in the male infertility group, there were 88 individuals with the homozygous CC genotype (82.24%), 18 individuals with the heterozygous CT genotype (16.82%), and only 1 individual with the homozygous mutated TT genotype (0.93%). In the control group, there were 75 individuals with the wild-type CC homozygous genotype (88.24%), 27 individuals with the heterozygous CT genotype (11.76%), and no individuals with the homozygous mutated TT genotype. The allele frequency in the patient group was 90.65% for allele C and 9.35% for allele T. In the control group, the allele frequency was 94.12% for allele C and 5.88% for allele T.

3.3.2.4. NOS3-786 C>T (rs2070744) variant

For the *NOS3*-786 C>T (rs2070744) variant, in the male infertility group, the homozygous mutated TT genotype had the highest frequency (57.94%), followed by the heterozygous CT genotype (40.19%), and the homozygous wild-type CC genotype had the lowest frequency (1.87%). Similarly, in the control group, the genotype frequencies for homozygous wild-type CC, heterozygous CT, and homozygous mutated TT were 1.18%, 17.65%, and 81.18%, respectively. The allele frequency in the patient group was 78.04% for allele T and 21.96% for allele C.

Table 3.6. Allele and genotype frequency *NOS3* -786 C>T (rs2070744) variant

Genotype/Allele	Patient n = 107 (%)	Control n = 85 (%)
CC	2 (1.87)	1 (1.18)
CT	43 (40.19)	15
TT	62 (57.94)	69
Allele T	167	153
Allele C	47 (21.96)	17 (10)

Note: n: number of samples

3.4. Surveying the relationship between genetic variants of antioxidant genes with infertility and oxidative status

3.4.1. Evaluation of the genetic characteristics of the male infertility group and the control group in correlation with the basic parameters of sperm.

The differences in genotype and allele frequencies of the variants continue to be investigated between the two groups: 107 patient and 85 control samples, to elucidate the relationship between genetic characteristics and male infertility status.

For the *SOD1* 7958G>A variant, the distribution of genotypes between the patient and control groups showed statistically significant differences ($p = 0.027$). Specifically, the heterozygous GA genotype in the patient group had a higher frequency than the control group ($p = 0.004$). Conversely, the homozygous mutated AA genotype in the control group had a higher frequency than the patient group ($p = 0.044$).

For the *SOD2* c.47T>C (rs4880) variant, it was observed that the homozygous wild-type TT genotype in the control group had a higher frequency than the patient group ($p = 0.006$). Conversely, the heterozygous TC genotype in the control group had a lower frequency than the patient group ($p = 0.033$). Additionally, the frequency of the wild-type T allele in the control group was also higher and statistically significant compared to the male infertility patient group ($p = 0.019$).

For the *CAT* -262C>T variant, the differences in the distribution of genotypes and alleles between the two study groups did not reach statistical significance.

For the *NOS3* -786C>T variant, the heterozygous genotype had a higher frequency in the patient group (p = 0). The frequency of the mutated C allele in the patient group was significantly higher than the control group (p = 0.02). Logistic regression model indicated that the odds of individuals carrying the wild-type allele in the patient group were higher than the control group (p = 0.001).

3.4.2. The correlation between genetic characteristics and clinical features of sperm in the male infertility group.

In addition to infertility characteristics, we continued to investigate the correlation between the genetic characteristics of the patient group and the basic parameters of sperm in these patients. These parameters included shape, motility characteristics, survival rate, density, and total number of sperm.

Specific results showed that, for *SOD2* c.47T>C, there were differences in the average values of progressive motility and survival rate among different genotypes. Specifically, the percentage of surviving sperm in patients with wild-type or heterozygous genotypes (TT or TC) was higher compared to the data obtained in patients with the homozygous mutated genotype (CC) (Figure 3.9). Investigation of the remaining variants including *SOD1* 7958G>A, *CAT* -262 C>T, *NOS3* -786 C>T, revealed no significant differences in sperm characteristics among different genotypes.

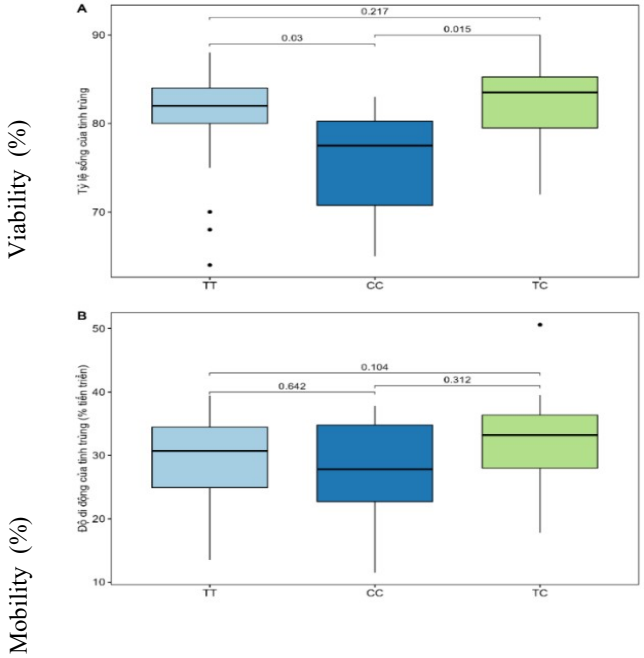

Figure 3.8. The correlation between the genetic polymorphism *SOD2* c.47T>C and the clinical parameters of sperm in the male infertility group.

A) The correlation between the *SOD2* genotypes and the sperm survival rate, B) The correlation between the *SOD2* genotypes and the sperm motility. Light blue boxes represent the wild-type homozygous genotype TT, dark blue boxes represent the homozygous mutated genotype CC, and green boxes represent the heterozygous genotype TC.

3.4.3. Evaluating the relationship between the oxidative stress level with the clinical and genetic characteristics of sperm in male infertility.

3.4.3.1. Oxidative stress levels of sperm in the male infertility group and their correlation with sperm parameters.

Only 90/107 sperm samples from male infertility patients met the criteria for assessing oxidative stress. The results showed that in samples with high levels of oxidative stress, the total sperm count was significantly lower compared to the samples with low oxidative stress, and this difference was statistically significant (p = 0.0334) (Figure 3.9a). Conversely, when assessing the relationship between oxidative stress and other sperm parameters such as semen volume, morphology, progressive motility, percentage of live sperm, and density, no statistically significant differences were observed between the two groups of sperm samples with high and low levels of oxidative stress.

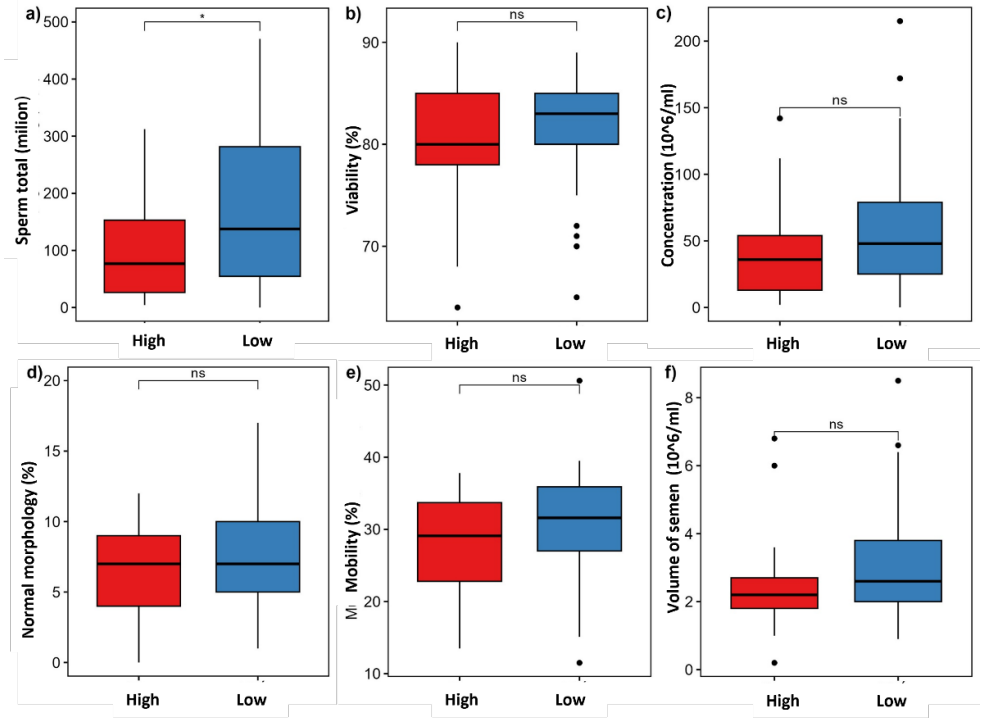

Figure 3.9. The relationship between sperm parameters in patient groups and the level of oxidative stress

Note: The red-colored box represented high levels of oxidative stress, and the green-colored box represented low levels of oxidative stress. *: $p<0.05$, ns: not statistically significant.

3.4.3.2. The oxidative stress level of sperm in the male infertility group and the correlation with the genetic characteristics of the antioxidant gene group.

There was no statistically significant difference in comparing the gene type frequency and allele frequency of *SOD1* (rs4998557), *SOD2* (rs4880), *CAT* (rs1001179), and *NOS3* (rs2070744) variants between the two male infertility groups with high and low sperm oxidative stress levels.

3.4.4. Assessing the relationship between certain gene combinations studied and the male infertility status and sperm oxidative stress level.

3.4.4.1. Investigating of SOD1, SOD2, and CAT gene combinations between the patient and the control group.

Statistical analysis data indicated that patients carrying both genotypes in the heterozygous state of *SOD1* 7958GA and *SOD2* c.47TC had a more than 2.5 times higher risk of infertility compared to those carrying the wild-type genotype [p = 0.006; OR = 4.343 (1.467 – 13.799)] (Figure 3.11). Patients with heterozygosity for both *SOD1* 7958GA and *CAT* -262CT genotypes had a higher risk of disease than the group with wild-type genes [p = 0.039; OR = 4.101 (1.103 – 20.93)]. Specifically, the risk of male infertility in individuals carrying the combined genotypes *SOD1* 7958GA/AA, *SOD2* c.47TC/CC, and *CAT* -262CT/TT increased sevenfold compared to those with wild-type genes [p = 0.007; OR = 7.614 (1.582 – 62.709)]. Conversely, two gene combinations showed a reducing risk of male infertility, including the wild-type gene combinations of *SOD1* 7958 GG and *SOD2* c.47TT, as well as the wild-type gene combinations of *SOD2* c.47TT and *CAT* -262 CC. Specific results presented in Figure 3.10.

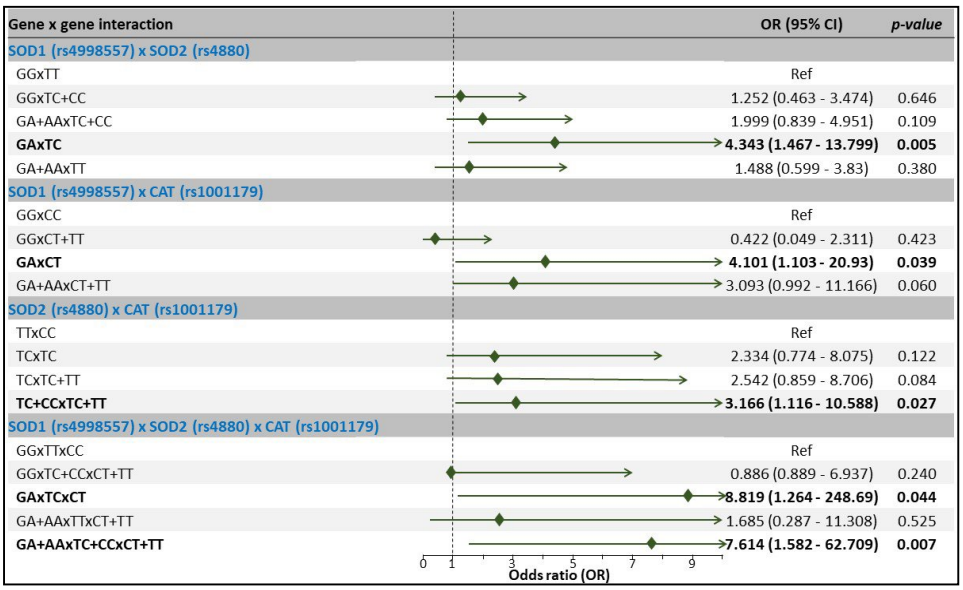

Figure 3.10. Forest plot is a statistical chart evaluating the correlation between certain gene combinations and male infertility.

Note: 95% CI: 95% confidence interval.

3.4.4.2. Correlation between SOD1, SOD2, CAT gene combinations and sperm oxidative stress level.

The correlation between gene combination variants of SOD1, SOD2, and CAT genes and sperm oxidative stress level in the male infertility patient group continues to be evaluated. Gene combinations investigated involve variants from pairs of genes *SOD1-SOD2*, *SOD1-CAT, SOD2-CAT*, or all three genes *SOD1-SOD2-CAT*.

Specifically, the combinations of *SOD1* (rs4998557) and *SOD2* (rs4880) were examined including the genotypes GGxTT, GGxTC+CC, GA+AAxTC+CC, GAxTC, and GA+AAxTT. In patients with high levels of sperm oxidative stress, the frequencies of these combinations ranged from 4.76% to 42.9%, while in the group with low sperm oxidative stress, the frequencies ranged from 10.1% to 39.13%..

Examining the combinations of *SOD1* (rs4998557) and *CAT* (rs1001179), four genotype combinations were investigated: GGxCC, GGxCT+TT, GAxCT, and GA+AAxCT+TT. In patients with high sperm oxidative stress, the frequencies of these genotypes ranged from 4.76% to 19%, whereas in the low sperm oxidative stress group, the frequencies ranged from 1.4% to 20.29%.

Considering the combinations of *SOD2* (rs4880) and *CAT* (rs1001179), four genotype combinations were examined: TTxCC,

TCxTC, TCxTC+TT, and TC+CCxCT+TT. The frequencies of these genotype combinations were quite common in the group with high sperm oxidative stress, ranging from 19% to 42.9%. In the low sperm oxidative stress group, the most prevalent combination was the homozygous mutant combination of the two genes (44.9%), while the lowest was TCxTC (7.2%).

Examining the combinations of *SOD1* (rs4998557), *SOD2* (rs4880), and *CAT* (rs1001179), four genotype combinations were investigated: GGxTTxCC, GGcTC+CCxCT+TT, GAxTCxCT, and GA+AAxTTxCT+TT. In the group with high sperm oxidative stress, the two genotypes with the lowest frequencies were GA+AAxTTxCT+TT and GGxTTxCC (4.76%), while the two genotypes with the highest frequencies were GGxTC+CCxCT+TT and GAxTCxCT (9.52%). In contrast, in the low sperm oxidative stress group, the genotype combination GA+AAxTTxCT+TT had the lowest frequency (4.35%), and the combination GGcTC+CCxCT+TT had the highest frequency (11.59%).

3.4.4.3. The correlation between several antioxidant gene combinations and the clinical characteristics of sperm in the patient group.

The gene combinations carrying variants of *SOD1, SOD2*, and *CAT* were investigated, including 4 combinations of *SOD1* (rs4998557)-*SOD2* (rs4880), 2 combinations of *SOD1* (rs4998557)-*CAT* (rs1001179), 2 combinations of *SOD2* (rs4880)-*CAT* (rs1001179), and 3 combinations of *SOD1* (rs4998557)-*SOD2* (rs4880)-*CAT* (rs1001179).

Examining gene combinations involving *SOD1* and *SOD2* compared to the reference GGxTT, only the sperm motility parameter in individuals with heterozygous in both *SOD1* and *SOD2* (GAxTC) showed a statistically significant difference ($p = 0.032$) (Figure 3.11).

Examining gene combinations involving *SOD2* and *CAT* compared to the reference TTxCC, the sperm viability rate in the patient group with the gene combination TCxCT (84.09%) was higher than the control group (80.81%) ($p = 0.036$) (Figure 3.12).

Examining gene combinations involving *SOD1, SOD2*, and *CAT*: the percentage of normal sperm morphology in the patient group with GA+AAxTTxCT+TT was higher than in the group with the wild-type ($p = 0.0057$) (Figure 3.14).

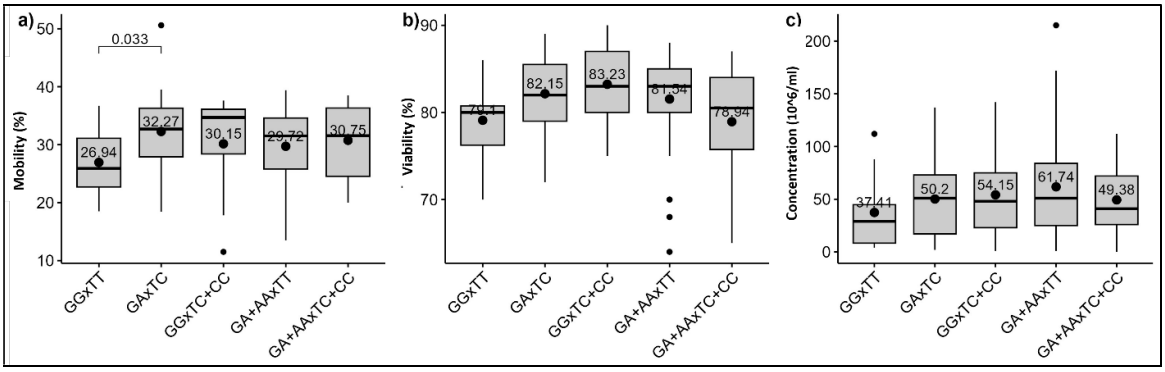

Figure 3.11. The correlation between *SOD1-SOD2* and sperm parameters

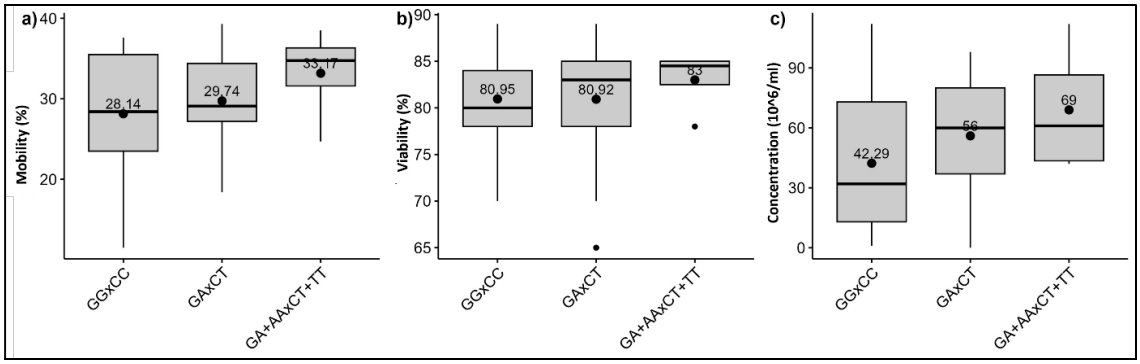

Figure 3.12. The correlation between *SOD2-CAT* and sperm parameters

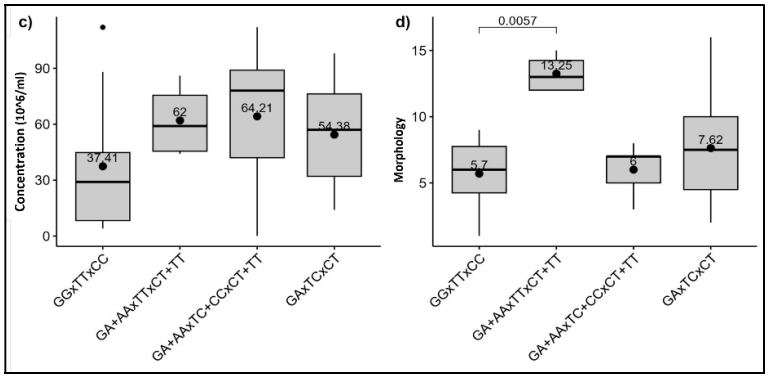

Figure 3.14. The correlation between *SOD1-SOD2-CAT* and sperm parameters.

3.5. The impact of genetic variations and BMI on male infertility

We continued to assess the interaction between the risk genotypes for infertility analyzed in the previous content and the Body Mass Index (BMI) with male infertility. Based on the BMI values of 18.5-24.9, considered normal for Vietnamese individuals, the male infertility patient group was divided into two categories: Low/Normal BMI (≤ 24.9) and High BMI (> 24.9).

The values of -2LL, R Square, BIC, and AIC for all four models indicated the suitability of logistic binary regression models. Specifically, the -2LL values in the empty regression models consistently decreased by introducing independent variables into the models.

The results of logistic binary regression analysis showed that the NOS3 -786CT genotype still had an impact on the risk of male infertility in both the NOS3 -786CT with BMI model ($p = 0.003$) and the model assessing all four gene variants along with BMI ($p = 0.007$). In the model evaluating the effect of the heterozygous SOD1 GA genotype and BMI on infertility, this genotype did not influence the risk of infertility ($p > 0.05$). However, in the comprehensive model considering all four gene variants along with BMI, the SOD1 GA genotype still had an impact on the risk of male infertility ($p = 0.049$).

Nevertheless, a high BMI continued to have an effect on the risk of male infertility in all four models assessing individual gene variants and the model evaluating the combined impact of all four studied gene variants (SOD1, SOD2, CAT, and NOS3) ($p < 0.05$).

CHAPTER 4. DISCUSSION

4.1. Role of genetic variations involved in the antioxidant pathway in male infertility risk

4.1.1. SOD1 gene polymorphism

Although there has been no study on the impact of *SOD1* gene polymorphism on infertility in humans, studies on mice have indicated a reduced reproductive capacity in sperm-deficient *SOD1* mice or a decrease in germ cell proliferation in mice exposed to high temperatures. Our disease/condition study identified the heterozygous genotype 7958 GA of the *SOD1* gene in infertile men, which showed a statistically significant association compared to the control group. This reflects that the heterozygous GA genotype may be a risk factor for male infertility. This is a highly valuable initial observation, as the frequency of rs4998557A is prevalent in geographical areas worldwide, especially high in Asia (52.9% in East Asia and 20% in West Asia). However, further investigations on a larger scale and across diverse human populations are needed to validate the influence of rs4998557 within the *SOD1* gene on idiopathic male infertility.

4.1.2. SOD2 gene polymorphism

The results of this study indicate that the frequency of the TT homozygous genotype and T allele in the male infertility group is significantly lower compared to the control group, demonstrating the protective role of the T allele. Conversely, individuals carrying the C allele and the heterozygous TC genotype have a higher risk of infertility, a observation consistent with previous studies. This trend could be explained by the partial reduction in the activity of the SOD2 enzyme in the mitochondria, creating conditions for the accumulation of reactive oxygen species (ROS), leading to oxidative imbalance and ultimately damaging the quality of sperm in individuals with the TC heterozygous genotype. As expected, the analysis of the dominant genetic model for all individuals carrying the C allele (TC+CC/TT) also showed a tendency for the dominant genetic model to contribute to male infertility.However, this study did not show a statistically significant difference in the frequency of the homozygous mutant CC genotype between the two groups. Although not reaching statistical significance, the proportion of individuals carrying the CC genotype in the patient group (7.48%) was still higher than in the control group (2.35%). Notably, data from this study indicate that the CC genotype is a risk factor associated with a reduced sperm viability rate in the male infertility patient group. Specifically, the sperm viability rate in patients with the CC genotype was significantly lower compared to patients with the TT or TC genotype. Thus, the male infertility risk associated with the c.47C allele may be linked to a decrease in sperm quality in men.

4.1.3. CAT gene polymorphism

Regarding the *CAT* -262C>T variant (rs1001179) is located in the promoter region of the *CAT* gene. Some analyses indicate a significant reduction in CAT enzyme activity in certain subgroups within the male infertility group compared to the control group. Considering the crucial role of semen in sperm function, the expression of the CAT enzyme in semen has been reported to be positively correlated with the forward motility of sperm. In the limited sample size of the study, consisting of 107 patients and 85 controls, statistical data did not reflect a relationship between the -262C>T variant of the *CAT* gene and infertility characteristics, sperm clinical parameters, or sperm oxidative stress levels. However, the heterozygous -262CT genotype tended to be higher

in the patient group, while the homozygous -262CC genotype tended to be higher in the control group. Among the 107 patient samples and 85 control samples, only one patient had the homozygous mutant -262TT genotype. As the frequency of the -262T allele of CAT in Asia and Africa is approximately 2-3%, the observed trend contradicts previous reports in Europeans and Western Asians (where the frequency of the -262T allele of CAT is > 20%) and needs further confirmation with a larger sample size. Moreover, the inconsistent data on the influence of the rs1001179 variant in the *CAT* gene on male infertility status and sperm quality warrants continued investigation across diverse human populations.

4.1.4. NOS3 gene polymorphism

The genetic variations of the *NOS3* gene may be associated with abnormal sperm morphology. Previous studies have indicated that the *NOS3* -786C allele is related to poor sperm quality in Iranian individuals or an increased risk of sperm DNA fragmentation and male infertility in Chinese men. Mechanistically, NOS3 is a crucial enzyme that catalyzes the synthesis of nitric oxide (NO), an important antioxidant that helps reduce the formation of superoxide anions. However, an excess of NO can also contribute to oxidative stress. Physiologically, NO at moderate levels protects tissues from damage caused by oxidative stress, while high concentrations of NO activate sperm DNA destruction and the apoptosis process of male germ cells. Based on this mechanism, we hypothesize that the -786C>T substitution in the promoter region of NOS3 reduces the expression of the NOS3 enzyme. Therefore, individuals carrying the -786C wild-type allele may accumulate higher NO levels compared to those with the homozygous -786TT genotype, leading to oxidative imbalance.

Observations revealed that the frequency of the heterozygous *NOS3* -786 CT genotype and the -786C allele in the patient group was higher than in the control group, indicating that the CT heterozygous genotype and C allele are risk factors for male infertility. The *NOS3* -786 C>T polymorphism did not show a statistically significant correlation with sperm parameters. In the future, it is essential to investigate which sperm parameters the *NOS3* -786C>T variant affects in cases of unexplained male infertility and whether there are differences between cases with varying levels of oxidative stress. Additionally, the

validation of the impact of the -786C>T variant should be conducted on sperm samples from individuals carrying this variant through the assessment of *NOS3* expression levels.

A limitation of this study is the relationship between genetic variations and an important sperm parameter, DNA fragmentation, was not investigated due to laboratory constraints. On the other hand, when comparing with data on oxidative stress levels in 90 male infertility patients, no relationship was found between genetic variations or combinations of gene variants and sperm oxidative stress. The impact of oxidative stress on sperm DNA fragmentation has not been explored in the participant group of this study. Therefore, this is a crucial aspect that needs to be addressed in future study designs to elucidate the direct correlation of genetic variations with sperm quality, which could be a factor influencing male reproductive health.

4.2. The interaction between antioxidant gene groups in relation to oxidative stress levels and male infertility

The rinteraction between polymorphisms of genes related to antioxidants may influence male infertility. Regarding the polymorphisms of the three genes *SOD1, SOD2*, and *CAT*, after analyzing single gene data, an analysis combining genotypic combinations with a tendency to cause male infertility also yielded certain results. We observed that patients carrying both the *SOD1* 7958 GA and *SOD2* c.47TC genotypes, patients with the *SOD1* 7958GA × *CAT* -262 CT genotype, and those with the *SOD1* 7958GA/AA × *CAT* -262CT/TT genotype all had an increased risk of male infertility. Specifically, the combination of the *SOD1* 7958GA/AA, *SOD2* c.47TC/CC, and *CAT* -262CT/TT genotypes had a nearly 8-fold higher risk of male infertility compared to individuals with the wild-type combination. Notably, the heterozygous combination of *SOD1, SOD2, CAT*, namely GAxTCxCT, had the highest risk of male infertility among the investigated genotypic combinations (OR = 8.819). When combining the evaluation of combinations of risk genotypes, the data obtained will bear the individual's signature and have a higher predictive significance than when assessing each genetic variant individually. Thus, these results provide important insights for genetic counseling related to antioxidant genes in cases of unexplained male infertility. When feasible, genetic factors related to idiopathic male infertility need to be

comprehensively assessed because the genetic risk factors in each patient may not be entirely similar to each other.

4.3. The importance of molecular signatures related to oxidative stress/male infertility and application in idiopathic male infertility treatment

In natural fertility, sperm quality is closely related to the success rate of pregnancy. Improving sperm function and vitality by supplementing antioxidants has shown positive effects on successful fertilization. A recent Cochrane review of high-quality studies concluded that supplementing antioxidants in infertile male patients increased the pregnancy rate from 12% to 14-26% (a 1.2-2.1 times increase). Assisted reproductive techniques (ART) are supportive treatment options for couples identified as infertile, and the availability of ART methods has been steadily increasing at rates ranging from 2.4-18.3% per year in Europe, Australia, and New Zealand during the period 2012-2016. Oxidative stress is a significant factor influencing the efficacy of ART methods. In the ART environment, both eggs and sperm are no longer in their natural milieu, becoming more sensitive to the presence of reactive oxygen species (ROS) as the antioxidant barrier is somewhat lost. Three meta-analyses have reported a connection between increased sperm fragmentation and reduced pregnancy rates after intrauterine insemination (IUI) and in vitro fertilization (IVF). In the laboratory setting for ART methods, factors contributing to elevated ROS production can arise from embryos or external factors such as oxygen levels in the air, temperature, humidity, equipment used, embryo culture environment quality, and frozen embryo storage tubes. Therefore, strategies aimed at reducing oxidative stress often focus on limiting ROS production or enhancing antioxidant capacity to help control the oxidative imbalance in ART.

However, evidence regarding the direct relationship between polymorphisms in antioxidant gene groups and the efficacy of ART, such as successful pregnancy rates and live birth rates, remains limited. Accumulated evidence from various research groups across different populations has shown associations between polymorphisms in antioxidant gene groups and male infertility, sperm quality, and oxidative stress. Supplementing diverse genetic information from these crucial gene groups along with the success rates of ART cases will

provide a significant scientific foundation for using molecular signatures as part of fertility support. In the future, large-scale and multi-population studies, combined with research from multiple centers, are needed to continue exploring the genetic variations in antioxidant gene groups as potential risk factors for male infertility.

4.4. Limitations in the screening of antioxidant gene polymorphisms related to idiopathic male infertility and overcoming trends

In the field of male infertility, there is currently no comprehensive study conducted to assess the impact of non-genetic factors on the expression of antioxidant gene groups. This would be a crucial approach in the future to deepen information and comprehensively exploit the dataset on the genetic system responsible for antioxidant defense mechanisms and mechanisms related to male infertility, as well as mechanisms causing various other diseases. Analyzing the entire encoded genetic system is predicted to be a feasible approach due to cost-effectiveness, high reliability with large datasets covering all coding regions, including new variants. Given the complexity of male infertility conditions and the diverse group of antioxidant genes, consideration should be given to this screening approach for the descendants of families with a history of infertility. The body's antioxidant system plays a complex coordinated role in performing functions; therefore, examining individual gene variants separately is not convincingly meaningful because a single gene alone does not decisively determine the phenotype. Conducting focused genome-wide association studies (GWAS) on genes within protective signaling pathways against oxidative stress is necessary.

CONCLUSION AND FUTURE PERSPECTIVE

Conclusion

1. The oxidative stress level was determined in sperm samples from 90 severe oligozoospermic men: 21/90 (23.3%) samples had high oxidative stress, and 86/90 (76.7%) samples had low oxidative stress. High oxidative stress was associated with a decrease in total sperm count in the infertility patient group, independent of other sperm structural and quality parameters.
2. The genotypes and allele frequencies of variants in the 4 genes *SOD1* 7958G>A, *SOD2* c.47T>C, *CAT* -262C>T, and *NOS3* -786C>T was

investigated in a group of 107 idiopathic male infertility patients and 85 control samples.

3. The relationship between polymorphisms in antioxidant genes and idiopathic male infertility was identified. Combinations of antioxidant genes were identified as a risk factor for male infertility.

Future perspective

1. Further research on a larger scale focusing on unexplored antioxidant gene groups in the Vietnamese population is needed. Additionally, the relationship between genetic factors and sperm fragmentation levels in infertility patients should be investigated.
2. The mechanism influencing the risk of male infertility associated with the *SOD1* 7958G>A variant may be related to alterations in SOD1 enzyme fucntion. Further functional models are essential in order to clarify its function.

LIST OF THE PUBLICATIONS
RELATED TO THE DISSERTATION

1. **Huy Anh Bach**, Phuong Nhung Vu, Thi Huyen Thuong Ma, Hai Ha Nguyen, Phan Tran Duc, Duc Bui Minh, Van Hai Nong, Dang Ton Nguyen (**2023**) Genetic variations of antioxidant genes and their association with male infertility in Vietnamese men. *Journal of Clinical Laboratory Analysis*, DOI: 10.1002/jcla.24829.
2. Lê Thị Quyên, **Bạch Huy Anh**, Trần Đức Phấn, Nguyễn Thị Trang (**2021**) Khảo sát đa hình gen *SOD2* C47T và *CAT* C262T ở nam giới vô sinh nguyên phát. *Tạp chí Y học Việt Nam*, 507(2):119-123.
3. **Bạch Huy Anh**, Trần Văn Khôi, Trần Đức Phấn, Lê Thị Minh Phương, Lê Thị Quyên, Vũ Thị Huyền, Nguyễn Thị Trang (**2020**) Nghiên cứu đa hình gen SOD1 ở nam giới vô sinh nguyên phát. Tạp chí Y học Việt Nam, tập 493, tháng 8, số 2.