Original Article

Down-Regulated Expression of Cystathionine β-Synthase and Cystathionine γ-Lyase in Varicocele, and Infertile Men: A Case-Control Study

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Abstract

Objective: Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are two important enzymes involved in One-Carbon metabolism. These enzymes play important roles in modulating oxidative stress and inflammation in male factor infertility through participating in the synthesis of glutathione (GSH) antioxidants in the trans-sulfuration pathway. Besides, the direct release of hydrogen sulfide (H_2S) has anti-inflammatory and antioxidant effects. Therefore, the expression of *CBS* and *CSE* genes at mRNA levels in infertile and varicocele men was evaluated and compared to the healthy counterparts to clarify their possible role in the pathology of male infertility.

Materials and Methods: In this case-control study, semen parameter assessment (concentration, morphology, and motility of sperms) was performed on 28 men with varicocele, 43 infertile men with abnormal sperm parameters, and 19 fertile men. RNA was extracted from sperm samples followed by cDNA synthesis and real-time polymerase chain reaction (PCR) using *CBS, CSE,* and *GAPDH* primers.

Results: Sperm concentration and motility in infertile and varicocele groups were significantly lower (P=0.001), while spermatoza normal morphology was higher than fertile group (P=0.05). The expression levels of both *CBS* and *CSE* genes in infertile (P=0.04 and P=0.037 respectively) and varicocele (P=0.01 and P=0.046 respectively) groups were significantly lower than fertile group. Additionally, *CBS* gene expression indicated a positive correlation with expression of *CSE* gene (r=0.296, P=0.025) and sperm parameters.

Conclusion: In light of our findings, there is a valid rationale to consider the primary role of *CBS* and *CSE* enzymes impairment in male factor infertility which specifically may point to a deficit in the release of essential antioxidants including the H_2S as a molecular basis of infertility and warrants further investigation.

Keywords: Cystathionine β-Synthase, Cystathionine γ-Lyase, Hydrogen Sulfide, Male Infertility, Oxidative Stress

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Introduction

According to the WHO, infertility is a global health issue affecting around 15% of couples. Available data suggests that about half of all infertility cases are caused by male factors including lifestyle, genetic, and environment (1, 2). Several lines of evidence have identified oxidative stress as a major contributor to abnormal semen parameters and subsequent male infertility (3). Oxidative stress is mainly occurred due to the imbalance of antioxidant capacity and reactive oxygen species (ROS) content. Increasing the level of ROS could result in the molecular and cellular damage of the reproductive system cells especially spermatozoa with a low amount of cytoplasm and limited repair mechanism potential. Therefore, the high level of ROS could lead to an extreme vulnerability to oxidative stress such as lipid peroxidation of sperm membrane, DNA damage, and apoptosis (1).

Spermatogenesis is a complex process controlled by a large number of genes and interacts with various cell signaling pathways (4). One-carbon metabolism has long been acknowledged to affect spermatogenesis and sperm quality by regulating the biosynthesis of nucleotides and methylations, maintaining genomic integrity, and protecting DNA from damage (5). This folate cofactor-mediated pathway transfers one-carbon (methyl) units for accomplishing the metabolic processes. Folate cycle, methionine cycle, and trans-sulfuration pathway are three main constituent interlinking pathways of one-carbon metabolism (6).

The canonical trans-sulfurations pathway for sulfur amino acid metabolism uses homocysteine as a substrate to generate cysteines for protein synthesis and biosynthesis of glutathione (GSH). GSHs are a family of the most abundant natural antioxidants in human tissues which

are well known as a powerful cellular antioxidant and constitute, together with NADH/NADPX, the main redox buffer of the cells (7, 8). Cystathionine-γ-lyase (CSE) and cystathionine β-synthase (CBS) are trans-sulfuration pathway enzymes known for an unspecific recognition of their substrate. These enzymes allow their substrates to act in a sort of reverse manner, an alternative pathway of CBS and CSE (9). In addiotion, these enzymes are involved in the synthesis of hydrogen sulphide (H_2S). This is a gaseous transmitter with antioxidant and anti-inflammatory properties. H_2S behaves as a powerful reducing substance as the standard two-electron redox potential of H_2S/S0 couple, at pH=7, versus the standard hydrogen electrode, is −0.23 V and is in the same range as that of GSH disulfide/GSH (E°′=−0.262 V) (10). Thus, the transsulfuration pathway is a master regulator of the redox homeostasis in many tissues, which also applies to the testis where the activity of the pathway is well documented (11, 12).

The role of H_2S as a homeostasis modulator is established and several reports proved that it has protective effects against ROS in vascular and neural systems and behaves as a signaling molecule in the regulation of vasodilation and blood pressure (13, 14). Besides its direct ability to neutralize ROS, H_2S also increases the expression of antioxidant enzymes through activation and translocation of the nuclear factor (erythroid-derived 2)-like 2 transcription factor (*NRF2*) (15, 16). Recent studies confirm the role of H_2S release as a homeostatic modulator also in the reproductive system of males and females. Indeed, the vasodilation induced by H_2S occurs at the time of penile erection. Therefore, the H_2S release has been proposed as a target for the treatment of erectile dysfunction (12, 17).

CBS acts at the cross-road of the one-carbon metabolism and is responsible for partitioning homocysteine to either trans-sulfuration, towards GSH and H_2S generation, or re-methylation, feeding the production of activated methyl groups as S-Adenosyl-Methionine (SAMe) (6). SAMe is the main activator of CBS, meaning that antioxidant effectors GSH and H_2S are released only if transmethylations are working (18). H_2S resulting from trans-sulfurations in turn an activator of Methionine Synthase Reductase (MTRR), a key regulator of the folate and methionine cycle (19). Thus, H_2S is the link between the activity of the endogenous antioxidant system and the efficiency of transmethylation reactions. Accordingly, impaired CBS activity is demonstrated to impair the cell methylation (6).

Both CBS and CSE are widely detected in the testes with CSE found mainly in Sertoli cells and immature germ cells and CBS abundant in Sertoli cells, Leydig cells, and germ cells (14), but their relative contribution to H_2S generation within the testes is unknown. However, CBS was identified as a major contributor to H_2S production as it may account for up to 70% of total endogenous H_2S in hypermetabolic cells like astrocytes (7). Moreover, stoichiometric calculations indicate that CSE can be a main source of H_2S in peripheral tissues only in conditions of very high homocysteine as seen in homocystinuria (20).

Several studies have proven that dysfunction of one-carbon metabolism, especially the imbalance of CBS and CSE enzymes in the trans-sulfuration pathway, plays a role in male infertility (14, 21-23) and a specific deficit in the output of H_2S is reported (23). The dysregulated content of CBS and CSE enzymes protein at human semen as well as their expression level at the RNA level in the testis of infertility mouse models were reported previously, however, to the best of our knowledge, there is no report about the specific mechanisms of dysregulation of the trans-sulfuration pathway in human seminal plasma.

This case-control study was undertaken to analyze the expression of *CBS* and *CSE* genes in sperms of infertile men in comparison to healthy fertile counterparts at the RNA level to clarify the connection between these enzymes and male infertility.

Materials and Methods

Design of experiment

This case-control study was conducted following the approval of the Institutional Review Board from the Royan Institute (IR.ACECR.ROYAN.REC.1398.244). Twenty-eight infertile men with varicocele (II or III grade) and 43 infertile men with abnormal sperm parameters (oligozoospermia, asthenozoospermia, teratozoospermia, austenotratozoospermia, oligostenotratozoospermia, and oligosthenospermia) referred to Isfahan Fertility and Infertility Clinic (IFIC) were recruited before receiving any treatments. Nineteen fertile men referring to the same center for family balancing served as fertile controls. Written informed consent was obtained from all subjects before participation in the study.

Semen collection and sperm parameters analysis

Semen samples were collected by masturbation following 3-4 days of sexual abstinence and delivered to the laboratory within 30 minutes after ejaculation. Sperm parameters were analyzed on one portion of the semen samples according to the WHO criteria (24). Briefly, sperm counting chamber (Sperm Processor, India), computer-assisted semen analysis (CASA) software, and Papanicolaou staining were used for assessing the sperm concentration, motility, and abnormal morphology respectively. White blood cells (WBCs) were assessed by peroxidase assay and all the samples showed WBCs below 0.5 million/ml.

RNA expression analysis

The remaining semen samples were washed twice with phosphate-buffered salin (PBS, Sama Tashkhis, Iran) and used for total RNA extraction using YTzol pure RNA (Yekta Tajhiz Azama, Iran) according to the manufacturer's protocol. cDNA was synthesized from extracted total RNA using Yekta Tajhiz cDNA Synthesis Kit (Yekta Tajhiz Azama, Iran). As shown in Table 1, primers

for *CBS*, *CSE,* genes, as well as *GAPDH* housekeeping gene, an internal control, were designed using Gene Runner (version 3.05; Hastings Software, Inc. Hastings, USA). mRNA expression analysis was accomplished via real-time polymerase chain reaction (PCR) by YTA SYBR Green qPCR MasterMix 2X (Yekta Tajhiz Azama, Iran) according to the manufacturer's instruction in StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA). The cycling condition consisted of an initial denaturation at 95˚C for 15 minutes, accompanied by 40 two-steps cycles of 95˚C for 15 seconds and 60˚C for 1 minute. eventually, the process was ended by a dissociation step for documenting the melt of the PCR product.

Statistical analysis

All data in the present study were analyzed by the statistical package for the social sciences for windows, version 26 (SPSS, Inc., Chicago, IL, USA). All parameters had a normal distribution and gene expression was calculated by the $2^{\Delta\Delta Ct}$ method. An independent sample t test was used to compare the mean expression of variables of each group along with the Pearson correlation coefficient test. Data were presented as mean ± standard error of the mean (SEM), and $P<0.05$ was assumed as significant.

Results

Sperm parameters

The conventional sperm parameters are presented as bar charts in Figure 1. The mean sperm concentration (10^6/mL) was significantly lower in varicocele (50.78 ± 6.7, P=0.002) and infertile (30.02 ± 3.8, P=0.000) groups compared to the fertile men (80.23 ± 5.725, Fig.1A). Similarly, motility showed a significant decline in men with varicocele (48.54 ± 3.8, P=0.006) and infertile group (34.52 ± 3.107, P<0.001) compared to the control (63.11 ± 2.816, Fig.1B). On the other hand, the mean percentage of sperm with total abnormal morphology (abnormal head, neck, and tail) was higher in varicocele (96.74 ± 0.3, P=0.018) and infertile (97.13 ± 0.2, P=0.019) groups in comparison with the fertile group (96.06 ± 0.4, Fig.1C).

Gene expression analysis

The comparison between the expression of messenger RNAs of *CBS* and *CSE* genes in healthy, varicocele, and infertile men in Figure 2. indicate a significant lowe expression of *CBS* in varicocele (0.11 ± 0.3, P=0.01) and infertile men (0.52 ± 0.1, P=0.04) in comparison with the fertile counterparts (2.6 ± 0.9, Fig.2A). *CSE* expression also revealed a significant decline in varicocele (0.6 ± 0.08, P=0.046) and infertile men (0.55 ± 0.08, P=0.037) compared to fertile male matched controls (1.6 ± 0.46, Fig.2B).

Table 1: Sequence of designed primers of real-time polymerase chain reaction assay

Gene	Oligonucleotide sequence (5´-3´)	GC (%)	Optimal temperature (˚C)	Size (bp)
CBS	F: GGGCGAAGTGGTCCATCTC	63.16	60	94
	R: GTTGGCAAAGTCATCTACAAGCA	43.48		
CSE	F: GACTCTACATGTCCGAATGG	50	58	149
	R: AACCTGTACACTGACGCTTCA	47.62		
GAPDH	F: CCACTCCTCCACCTTTGACG	60.00	60	107
	R: CCACCACCCTGTTGCTGTAG	60.00		

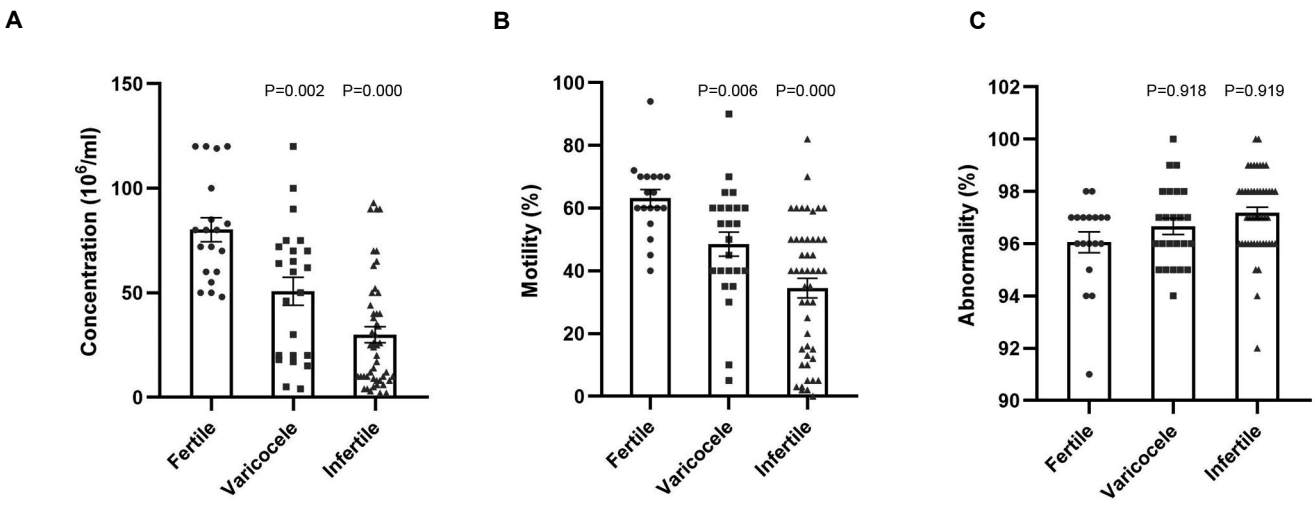

Fig.1: Sperm parameters of fertile, varicocele, and infertile groups. **A.** Sperm concentration (10^6/mL). **B.** Sperm motility (%). **C.** Sperms with the abnormal morphology (%). Data are expressed as means ± standard error of the mean (SEM), significant P values are reported in the figure.

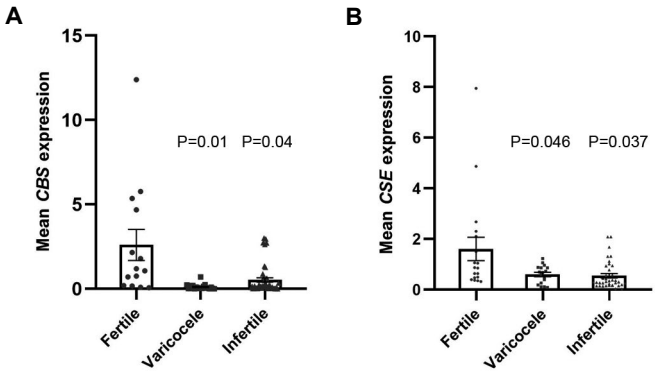

Fig.2: Comparison of mean expression of *CBS* and *CSE* between infertile men (n=43), infertile men with varicocele (n=28), and fertile individuals (n=19). Real-time polymerase chain reaction analysis of **A.***CBS* and **B.** *CSE* genes expression at RNA level relative to the *GAPDH* housekeeping gene (internal control for normalization) between study groups. Values are expressed as mean ± standard error of the mean (SEM), significant P values are reported in the figure.

Correlation of sperm parameters and gene expression

Pearson correlation analysis showed a significant positive correlation between the relative expression of *CBS* and *CSE* mRNAs (r=0.296, P=0.025). The correlation between sperm parameters (sperm concentration, motility, abnormality) and the relative expression of *CBS* and *CSE* genes were also analyzed. As shown in Table 2, a significant positive and negative correlation was observed between *CBS* and sperm concentration and sperm abnormality, respectively. However, no significant correlation between the relative expression of the *CBS* gene with sperm motility and the *CSE* gene with any sperm parameters was observed.

Table 2: Analysis of correlation between different sperm parameters with mRNA expression of *CBS* and *CSE* genes

Sperm parameters		*CBS* expression	*CBE* expression
Sperm concentration	r	0.291*	0.17
	P value	0.01	0.159
Sperm motility	r	0.159	0.155
	P value	0.164	0.205
Sperm abnormality	r	-0.351**	-0.086
	P value	0.002	0.484

*; P<0.05, **; P<0.01, and r; Correlation.

Discussion

In the present study, the decreased expression of *CBS* and *CSE* genes in varicocele and infertile men in comparison with healthy counterparts was documented. These two genes encode core enzymes of the trans-sulfuration pathway, which is a main effector of the endogenous antioxidant system through contributing to GSH generation and direct release of H_2S via CBS and CSE enzymes (7, 9, 11). Accordingly, reduction in the expression of *CBS* and *CSE* in infertile men points to a deficit of the endogenous antioxidant defenses and confirms the relation of male infertility with oxidative stress in addition to the involvement of the H_2S system in sperm DNA methylation.

GSH is produced in trans-sulfuration pathway by CBS and CSE enzymes and its depletion in male infertility are well documented. Naher et al. (25) found that infertile men, despite having the same level of erythrocyte GSH as their fertile controls, had far lower GSH in their seminal plasma indicating a testis-specific defect. Fafula et al. (26) found diminished levels of GSH and oxidized GSH ratio (GSH: GSSG) in the sperm of infertile men. This level decreasing was coupled with a reduction in the activity of GSH peroxidase (GPX) that was interpreted as a consequence of decreased substrate (i.e. GSH) after consumption to counteract primitive oxidative aggression, i.e. The fall of GSH could consider as the consequence, not the cause, of the oxidative damage. This interpretation is questioned by the findings of Wang et al. (23) reporting decreased amounts of CBS protein in infertile men for the first time and also by the findings of the present study at RNA level. According to the data in this study, the low GSH in infertile men is likely due to a decreased synthesis and might be a primary reason for oxidative imbalance and damage.

Wang al. (23) also reported a decreased amount of H_2S in seminal plasma of infertile men and its positive effect on motility from exposure of their sperms to H_2S. The decrease in seminal H_2S was related to the decrease of the CBS protein. However, it could not be established whether the lower amount of the enzyme was due to a primary suppression of the gene or any post-translational modification.

DNA methylation occurs at sperm more than any other cell which is fundamental for appropriate gene expression, DNA compaction, and proper development of the embryo. It is also well-known to contribute to male infertility (22, 27, 28). Hypo-methylation is a common phenomenon in infertile men with varicocele and oligozoospermia although the mechanisms through which hypo-methylation occurs remain unclear (29, 30). In a recent study, in spite of sperm hypomethylation, varicocele patients exert a paradox higher expression of DNA de-novo methylation enzymes DNMT3A and DNMT3B that are likely reverting their activity toward DNA de-methylation during oxidative stress (31). However, decreased generation of H_2S from CBS may also explain the link between male infertility, oxidative stress, and impairment of sperm DNA methylation. The activity of CBS is indeed strictly linked with the activation of transmethylations including DNA methylation, hence epigenetic programming.

The connection between trans-sulfuration and DNA methylation is also supported by animal and clinical models

(22). The administration of cocktails of micronutrients including methyl donors in infertile patients is reported to be effective in reducing both sperm DNA fragmentation, clear oxidative damage, as well as in improving chromatin packaging and protamination (29). Based on the data from Toohey et al., the release of H_2S from CBS (and CSE) is the link between the two pathways and explains why a resumption of the endogenous antioxidant pathways parallels an improved methylation activity (19). This qualifies H_2S as a primary regulator of sperm functions and explains our finding of correlation of *CBS* expression with sperm morphology that is expected to respond to epigenetic regulations, including DNA methylation.

Regarding the role of H_2S as a homeostasis modulator, Nuño-Ayala et al. (32), reported that CBS deficiency leads to pregnancy loss in female mice. The imperative role of H_2S in sustaining the germ cells in normal physiological conditions, especially in heat exposure, was demonstrated in a study by Li et al. (33) on rat testes as the down-regulation of CBS and CSE enzymes were observed following the heat shock injuries. They also showed that the exogenous H_2S exerts protecting effects on germ cells against heat exposure owing to its antioxidant properties, although the administration of a high dose of this gas could have toxic effects. Finally, Morales et al. (16), demonstrated that an H_2S prodrug acts as an antioxidant with recovery effects on spermatogenesis and sperm parameters including sperm count, motility, and morphology in men with oligoasthenozoospermia. Data in this study are showing that the dysregulation of H2S in male infertility is likely dependent on decreased expression of one of the producing enzymes, CBS.

In this study, the gene expression of both the enzymes is also studied and the results showed that *CBS* expression inversely correlated with sperm concentration and normal morphology whereas *CSE* expression did not. A possible explanation is that CSE down-regulation is just the consequence of reduced CBS activity resulting in lower amounts of cystathionine, the CSE substrate. This led our attention to a possible specific CBS defect of H_2S generation. In summary, a major role of CBS down-regulation in the oxidative imbalance of our patients supports the idea that a fall of H_2S generation is involved in male infertility.

A possible limitation of the present study is the expression of another H_2S generating enzyme, 3MST, which is not yet studied. 3MST is well expressed by all cells in the seminiferous tubules with a weaker expression in Leydig cells (33). However, 3MST expression is suppressed in conditions of oxidative stress and is unlikely to contribute to this setting (20). Another possible limitation of this study is not checking GSH and H_2S in the seminal plasma of patients. They could not directly link the lower expression of CBS and CSE to a decreased level of the resulting antioxidant effectors. However, a reduced release of GSH and H_2S in these patients has been well demonstrated by others and was likely to occur also in our model (23, 25, 26).

Conclusion

All things considered, the downregulation of GSH and H_2S releasing enzymes, CBS and CSE, at the RNA level was observed in the present study in men with varicocele or unexplained infertility in addition to the inverse correlation of *CBS* expression with semen parameters. The main role of *CBS* down-regulation points to a specific defect in the H_2S system as well as GSH, which justifies both the oxidative imbalance and the DNA methylation dysregulation in these patients. Although the CBS and CSE proteins were evaluated in human semen previously, this study is the first report of downregulation of *CBS* and *CSE* genes at the RNA level in human samples. Efforts aimed at supporting the activity of CBS and/or the release of H_2S may be of help in the treatment of male infertility.

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Authors' Contributions

F.A.; Patients management, preparation of samples and tests, collection, analysis of data, and manuscript writing. M.T.; Design, collection and/or assembly of data, data analysis, interpretation, and manuscript writing. M.D.; Conception, design, interpretation, and manuscript writing. M.H.N.-E.; Conception, design, data analysis, interpretation, manuscript writing and final approval of the manuscript. All authors read and approved the final manuscript.

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