

## Original Research

## Expression of Endothelial Nitric Oxide Synthase in Testicular Cells of Men with Obstructive Azoospermia; a Case-Control Study

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### Abstract:

**Introduction:** Obstructive azoospermia is one of the causes of post-testicular infertility in men and previous studies have reported inconsistent levels of endothelial nitric oxide synthase (eNOS) enzyme in these patients. Accordingly, the present study aimed to provide further evidence on the expression of eNOS enzyme in patients with azoospermia. **Materials and Methods:** In this case-control study, 10 patients, who were diagnosed with azoospermia and were referred to the infertility center for treatment or diagnosis, and 7 healthy fertile men were recruited. An informed written consent was obtained from included subjects and they underwent testicular biopsies. Samples were assessed via immunohistochemical methods to determine their levels of eNOS expression.

**Results:** Both leydig and sertoli cells were found to express eNOS, while this enzyme was not expressed in normal germinal cells. The only significant difference between the two groups was the level of eNOS expression in sertoli cells which was found to be higher in patients with obstructive azoospermia compared to the control group ( $P < 0.001$ ). **Conclusion:** According to the results of this study, sertoli cells and their interactions with germinal cells of seminiferous tubule might play an important role in sperm quality and a subsequent successful fertilization.

**Keywords:** Immunohistochemistry; endothelial Nitric Oxide Synthase; Obstructive azoospermia

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### Introduction:

Global statistics estimate infertility problems to affect 10 to 15% of couples (1), of which nearly half is attributed to male factor problems (1, 2). Azoospermia is one of the causes of male factor infertility (2), defined as absence of sperms in the centrifuged seminal vesicle fluid (3). Azoospermia is classified into two groups of obstructive and non-obstructive (4). In obstructive azoospermia spermatogenesis is normal, but there is obstruction along the pathway through which sperm travels from testes to be ejaculated. On the other hand, in non-obstructive azoospermia spermatogenesis is diminished or absent and severe testicular defects are

present. Obstructive azoospermia could be caused by pathologies within epididymis, vas deferens and ejaculatory duct. One of the main causes of vasa obstruction is vasectomy. Other etiologies of obstructive azoospermia include genitourinary infections, scrotal and inguinal injuries during surgical interventions, genetic factors and varicocele (5).

Recent studies have suggested a role for Nitric Oxide (NO) as a possible cause of obstructive azoospermia (6). This free radical gas is generated during conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) (7). This enzyme is present in three isoforms that are classified into two groups of calcium dependent constitutive (cNOS) and calcium independent inducible (iNOS or NOS2). The former group includes the two endothelial (eNOS) and neuronal (nNOS) isoforms that mediate vasodilatation and neurotransmission, respectively (8,

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9). Current literature suggests that NO plays an important role in various physiological processes within the male genitourinary system including erection, androgen release, sperm motility, capacitation and maturation, quality of sperms and attachment of sperm to the ovum (10). Immunohistochemical localization of eNOS enzyme in epididymis, prostate and seminal vesicle has pointed towards its role in the vascular balance, spermatogenesis and sperm maturation (8-11). Expression of eNOS enzyme has been identified in various cells within the normal human testes including endothelial cells, leydig and sertoli cells, and myofibroblasts around the seminiferous tubules (7, 10), but such evaluations have not been conducted in patients with obstructive azoospermia. In this regard, the present study aimed to assess the expression and role of eNOS enzyme in patients with obstructive azoospermia using immunohistochemical methods, and compare their findings to that of normal subjects.

#### **Methods:**

In this case-control study, 10 patients diagnosed with azoospermia and 7 healthy fertile men, referred to infertility center for treatment or diagnosis, were included and underwent immunohistochemical evaluations. After obtaining approval from the Ethics Committee of Shahid Beheshti University of Medical Sciences and written informed consent from the participants, testicular biopsy was performed. It should be mentioned that biopsies were performed during the procedures conducted to access sperms as a part of assisted reproductive technologies, and did not put patients at any additional risks.

#### **Immunohistochemistry:**

The methods applied in this study were in accordance with previous surveys (9). After confirming the diagnosis of obstructive azoospermia (presence of sperms in testicular biopsy specimens and their absence in the seminal vesicle fluid), the biopsied specimens were placed in Bovine solution and after 4 hours were transferred to 10% fixative formalin solution to be prepared for immunohistochemical analyses.

Paraffin-embedded tissues were sectioned at 5  $\mu$ m thickness, transferred onto poly-L-lysine-coated slides and deparaffinized. Endogenous peroxidases were neutralized using 3% hydrogen peroxidase and methanol (Merck, Germany) for 15 min in darkness. Goat serum (DAKO, Denmark) was used for blocking of unspecific antibodies. Endogenous Biotin was also blocked using Biotin blocking solution (Merck, Germany) for 15 min.

The slides were then incubated with 1:50 dilution of rabbit-derived primary monoclonal anti-human eNOS antibody (cat num# ab5589, USA) for one hour. The sections were then washed and incubated with 1:200 dilution of goat-derived anti-rabbit biotinylated secondary antibody (cat num#6721, USA) for one hour. After being washed again, the sections were incubated with 1:2000 dilution of Streptavidin-horseradish peroxidase (HRP) conjugate as the tertiary antibody for 30 min. The slides were then subjected to 3,3'-diaminobenzidine tetrahydrochloride (DAB) stain (DAKO, Denmark). Slides prepared from human placenta specimens obtained from mothers with full-term deliveries were considered as external positive controls, while sections with exposure to PBS instead of the primary antibody were considered as negative controls.

Eventually, the slides were reviewed by three independent pathologists blinded to the group of patients, and their staining intensities were scored on a scale of 0 to 4 (0: no immunoreaction, +: mild staining, ++: moderate staining and +++: severe staining) (9-12).

#### **Statistical analysis**

SPSS software for windows v.19 was used for data analysis. Immunohistochemical findings of the two groups were compared using the Kruskal Wallis non-parametric test. A p value less than 0.05 was considered as statistically significant in all analyses.

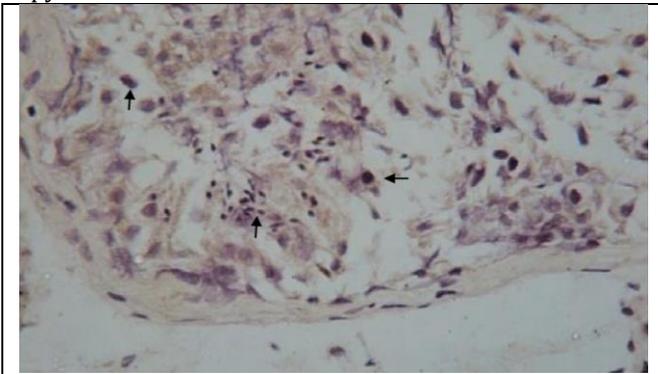
#### **Results:**

Figures 1 and 2 depict expression of eNOS in leydig cells, sertoli cells and immature spermatids that seem to be in the process of apoptosis. In both groups, no staining was observed in normal germinal cells of most tubules; however, a few of them showed mild staining. Leydig cells in both the obstructive azoospermia group and the control group had similar staining intensities (Table 1). The only significant difference between the two groups was the level of eNOS expression in sertoli cells which was found to be higher in patients with obstructive azoospermia compared to the control group (Table 1). Germinal cells with heterochromatic pyknotic nuclei that seem to be going through apoptosis also showed similar staining in the two groups (Figure 2). Other than the cells mentioned, the endothelial cells of testicular vasculature and peritubular myoid cells showed comparable levels of eNOS expression (Figures 1 and 2).

**Table 1:** Immunohistochemistry assessment of eNOS expression in testicular biopsies from the two control and obstructive azoospermia groups

Cells	Groups		P value
	Control	Obstructive Azoospermia	
Leydig cells	++ <sup>a</sup>	++	NS <sup>b</sup>
Sertoli cells	+	++	<0.001
Immature spermatid (cytoplasm)	+	+	NS
Normal germinal	0	0	NS
Abnormal germinal <sup>c</sup>	+	+	NS

<sup>a</sup> 0: no staining; +: mild staining; ++: moderate staining; +++: severe; staining; <sup>b</sup> P value>0.05; <sup>c</sup> Germinal cells with pyknotic nuclei.

**Figure 1:** Immunohistochemistry evaluation of eNOS expression in the obstructive azoospermia group (x40); sertoli cell (arrow on the right), leydig cell (arrow on the top left) and spermatozoa (arrow on the bottom left).**Figure 2:** Immunohistochemistry evaluation of eNOS expression in the control group (x40); sertoli cell (arrow) and endothelial cell (\*).**Conclusion:**

Although the NO present in the seminiferous tubules is generated by different NOS enzymes, it seems that the eNOS enzyme is the main regulator of spermatogenesis process. This hypothesis can provide the basis for treatment strategies for prevention of NO-dependent azoospermia.

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**Conflict of interest:**

All authors declare that there is no conflict of interest in this study.

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**Author's contributions:**

All the authors have contributed to drafting/revising the manuscript, study concept, or design, as well as data collection and interpretation.

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