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Research Article

Aberrant Wnt/β-Catenin Signaling Pathway in Testis of Azoospermic Men

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Abstract

Purpose: The Importance and key role of Wnt/β-catenin signaling pathway in spermatogenesis is known. Abnormalities of this pathway in Sertoli and germ cells leads to infertility. Leydig cells play an important role in spermatogenesis and male reproduction. As of now, exact position of the Wnt/β-catenin signaling pathway disorders in the tissue and possible involvement of Leydig cells has not been investigated.

Methods: Samples of our previous study were used for common Y chromosome microdeletions screening and common CFTR gene mutations.[1] β-catenin gene expression were evaluated and compared between testicular tissue obtained by testicular sperm extraction (TESE) in two groups of obstructive (n=10) and non-obstructive (n=10) azoospermic infertile men. Location of β-catenin accumulation was detected by immunofluorescence technic and quantitatively compared in the tissue followed by counterstaining with anti-vimentin antibody. It was used as specific marker of leydig cells to determine and confirm the cells in which this gathering was occurred.

Results: β-catenin gene expression does not have a significant difference between the obstructive azoospermia (0.998) and non-obstructive azoospermia group (0.891). β-catenin was abnormally aggregated in leydig cell of non-obstructive azoospermic men.

Conclusion: Gathering β-catenin in cytoplasm of leydig cells can disrupt spermatogenesis and cause infertility in men.

Introduction

Male sex hormone testosterone is made by leydig cells in the interstitial tissue of the testes form about 20 percent of the adult testes weight. Function of these cells is controlled by LH (Luteinizing hormone) and it is secreted from the pituitary gland in the brain. Leydig cells in childhood are almost absent and there is no secretion of testosterone from the testes. Many of these cells during the first three months after birth in boys and adult men are present, thus; the large amount of testosterone secretion happen by the testes during childhood. Testosterone inhibits the formation of the female sexual organs in contrast it increases the formation of the prostate gland, seminal bags, and male genital tract.

Azoospermia is the most important factor for male infertility. It can be divided into two categories; Obstructive and non-obstructive azoospermia. Spermatogenesis is normally done in obstructive azoospermia, however; produced spermatozoa are not found in seminal fluid for various reasons. The good example is, anatomical abnormalities or infections.[2] On the other hand, spermatogenesis is impaired in non-obstructive azoospermia and no spermatozoa is made in seminiferous tubules. Non-obstructive azoospermia happens mainly due to congenital genetic and metabolic reasons. Intercellular communications andintracellular signaling pathways play an important role in spermatogenesis. Their abnormalities can affect this process with various intensities from low (Oligospermia) to high (Azoospermia).

Wnt/β-catenin signaling pathway is one of the major cellular signaling pathways which plays a key role in the lives of humans and animals. This pathway involves in cell polarization, migration, early differentiation, neurogenesis and somitogenesis during embryonal development. On the other hand, in adulthood cell proliferation and differentiation is deeply under control of Wnt/β-catenin signaling pathway. In which impairments causes many pathological diseases most notably cancers. Wnt/β-catenin signaling pathway role in reproductive processes has been demonstrated.[3] In general, two routes are defined for Wnt/β-catenin signaling pathway: canonical and non-canonical. In canonical pathway Wnt molecules connect to the Frizzled (FZD) receptors caused inhibition in β-catenin phosphorylation by GSK3-β (a serine/threonine protein kinase with two isoforms of GSK3-α and GSK3-β), caused migration of b-catenin into the nucleus and expression of specific target genes. In contrast, in the absence of activating Wnt signal, β-catenin is phosphorylated by GSK3-β-Axin (a cell scaffold protein)-APC (Adenomatous polyposis coli) complex. It

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is ubiquinated and degraded by proteasome. This chain reaction causes repression of target gene.[4-7] Non-canonical pathway acts through alternative molecules other than β-catenin.[8]

Several types of Wnt proteins including 1, 3, 4, 5a and 7a in the testes of fetal and adult rodents and humans have been reported.[9-13] Some components of the Wnt/β-catenin signaling pathway such as Fz9, Nkd1 and β-catenin have been identified in the testis.[14] β-catenin has a high level expression in mice germ and sertoli cells.[15] Impaired β-catenin signaling in Sertoli cells of the mouse embryo results in the destruction of the testicular cord and Mullerian duct.[16] The aberrant activity of Wnt/β-catenin signaling pathway leads to abnormal differentiation of the PGCs.[17]

Wnt/β-catenin signaling pathway and androgen receptors have direct effect each other in many aspects. β-catenin binds to the ligand-receptor complex and serves as transcription co-activator. Canonical Wnt/β-catenin signaling pathway leads increase in expression of the androgen receptors.[18] GSK3-β phosphorylates the androgen receptors and regulates its ability to activate gene transcription.[19] Altogether, interaction between Wnt/β-catenin signaling pathway and androgen receptors is very complex, it depends on the conditions of the cell. During early development, estrogen injection in newborn rats increased expression of Wnt5a; the increase in expression could be an indirect response because promoter of the Wnt5a gene has no response factor for estrogen.[20,21]

The removal of β-catenin in the mouse spermatid increases apoptosis, acrosome defects, abnormal chromatin condensation, and degradation of Sertoli-germ cell junctions in which can lead to infertility.[22] Overexpression of β-catenin leads to uncontrolled proliferation, inhibition of differentiation in Sertoli cells and their function to support germ cells in mice.[23]

As a result, relationship of Wnt/β-catenin signaling dysfunction in sertoli, germ cell, and infertility has been confirmed. Leydig cells have not been studied in this regard. Therefore, this study was aimed to investigate the probability of disruption of Wnt/β-catenin signaling pathway in leydig cells of azoospermic men.

Materials and Methods

Human subjects

Human subjects, sample size, sampling technique, and screening were described completely in the previous article.[1] Briefly, the test group included 10 patients with non-obstructive azoospermia. Samples were prepared by testicular sperm extraction (TESE) aseptically by an urologist and local anesthesia. 10 men with obstructive azoospermia were considered as the control group (n=10). Non-obstructive azoospermia subjects were screened for Y chromosome microdeletions and obstructive azoospermia patients were checked for CFTR mutations before the study. Samples were transferred to the laboratory within an hour in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, U.S.A.).

Realtime-PCR

Expression of β-catenin was quantitatively evaluated using Realtime-PCR method. cDNA was made from extracted RNA by Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA), according to the manufacturer's instructions. Realtime-PCR were done using the Power SYBR green kit and Step one device manufactured by Applied biosystems (Applied biosystems, USA). 20 microliter reactions were made including 10 ml of Power SYBR green master mix, 500 micrograms per microliter of cDNA, 0.5 microliter of forward primer, 0.5 microliter of reverse primer, and 8 microliter of water (*β-catenin* forward: 3'-CAGCAGCAATTTGTGGAGGG-5', reverse: 3'-GCAGCTGCACAAACAATG GA-3' and *GAPDH* forward: 5'-ACAGTCAGCCGCATCTTCTT-3', reverse: 5'-ACGACC AAATCCGTTGACTC-3'). Results were analyzed by Rest 2009 software following normalization using LinRegPCR software.[24]

Immunofluorescence

Immunofluorescence analysis was performed to determine the location of β-catenin accumulation. The samples were first rinsed with phosphate buffered saline (PBS) and fixed with %10 formalin. In the second step they were dehydrated using ascending grades of ethanol and 5 micron sections were made using microtome (Thermo scientific, USA). Samples were placed on glass slides and hematoxylin-eosin staining was performed. Photomicrographs were taken by Nikon TS-100 microscope (Nikon, Japan). Some samples were placed on poly L-lysine coated glass slides and were deparaffinised with xylol and rehydrated with descending grades of ethanol. Following 3 times rinse with deionized water unspecific antigens were blocked with 10% goat serum. Incubation in 4°C with primary antibody (Mouse anti human β-catenin; SC-7963, Santa Cruz biotechnology, USA) was done overnight after 3 times with deionized water. Secondary antibody exposure was performed in 4°C and dark following 3 times rinse with deionized water. Slides were mounted by mounting fluid. Fluorescent photomicrographs were immediately taken with TS-100 microscope (Nikon, Japan). Leydig cells were labeled by specific primary antibody (Mouse anti human Vimentin; V6389, Sigma, USA) against vimentin in a similar manner. Finally, the taken photomicrographs were analyzed and quantitatively compared using Image J (version 1.40g) software.

Results

Realtime-PCR

Samples were quantitatively analyzed regarding the expression level of β-catenin gene. The gene expression of β-catenin did not have significant difference between the obstructive azoospermia (0.998) and non-obstructive azoospermia group (0.891). (Table 1 and Figure 1).

Table 1. Realtime-PCR results analysed by Rest software. β-catenin (NO: Non-obstructive azoospermia) sample group is not different to control group. P(H1)=0.139 β-catenin (O: Obstructive azoospermia) sample group is not different to control group. P(H1)=0.924P(H1) - Probability of alternate hypothesis that difference between sample and control groups is due only to chance. TRG– Target, REF– Reference.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P(H1)	Result
GAPDH (NO)	REF	1.0244	0.999	-	-	-	-
β-catenin (NO)	TRG	0.8538	0.891	0.712 - 1.131	0.618 - 1.353	0.139	-
β-catenin (O)	TRG	0.8288	0.998	0.848 - 1.166	0.715 - 1.220	0.969	-
GAPDH (O)	REF	1.0244	1.001	-	-	-	-

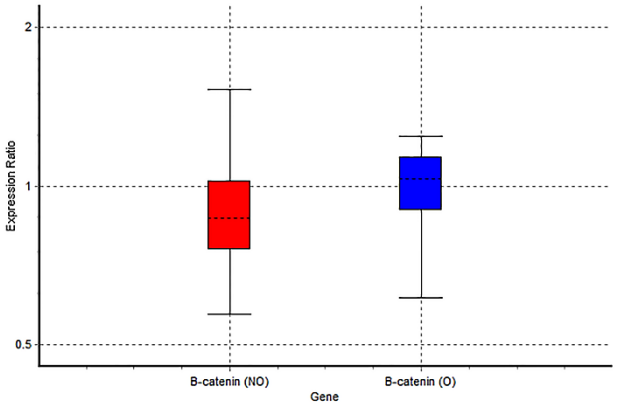

Figure 1. β-catenin gene expression has no significant difference between the obstructive azoospermia (O) (0.998) and non-obstructive azoospermia (NO) group (0.891).

Immunofluorescence

The Location of β-catenin accumulation in the tissue were detected using immunofluorescence technique. As it is shown in the images, β-catenin was aggregated in interstitial tissue. Counterstaining with vimentin antibody revealed that this accumulation was done specifically in leydig cells. Comparison of fluorescence intensity by Image J software displayed, accumulation of β-catenin was significantly more in non-obstructive azoospermia than obstructive azoospermia samples (Figure 2).

Discussion

In this experiment, the Wnt/β-catenin signaling pathway was examined in testicular tissue and compared between obstructive and non-obstructive azoospermic men. The result of realtime-PCR showed β-catenin gene expression in both groups were not significantly different. According to our previous study, expression of GSK3-β gene was down regulated in non-obstructive azoospermic men significantly. The data showed that over expression of β-catenin gene is not obligatory for its increasing and accumulation in cytoplasm, but down regulation of GSK3-β could lead to aberration gathering of β-catenin in cytoplasm. Tanwar et al. reported that overexpression of β-catenin leads to uncontrolled proliferation, inhibition of differentiation of Sertoli cells, and their function in protecting spermatogonia. It leads to cessation of spermatogenesis in mice. They used mutant mices which their β-catenin gene was manipulated in a way that GSK3-β could not phosphorylate and degrade it. In this situation AMH (Anti mullerian hormone) and GDNF (glial cell-derived neurotrophic factor) is only expressed in immature Sertoli cells which continue to express at a high level. Their results showed that the accumulation of β-catenin in Sertoli cells and spermatogonia increased apoptosis in the cells.[23] These researchers studied only Sertoli cells and the importance of other cells especially leydig cells were not considered. Leydig cells play a crucial role in spermatogenesis and fertility. Immunofluorescence test was performed to identify the accumulation of β-catenin. Results showed that β-catenin unusually accumulated in interstitial tissue of non-obstructive azoospermic samples. Counterstaining with anti vimentin antibody (a marker of leydig cells) revealed this aberrant accumulation of β-catenin was done in leydig cells. The results confirmed the similar study of Boyer et al used mutant mice whose sertoli and leydig cells Wnt/β-catenin signaling pathway was continuously active which confirmed the results. These mice lack germ cells with testicular atrophy and became infertile from fifth week.[25]

Conclusion

Overall, aberrant accumulation of β-catenin in leydig cell cytoplasm can cause impaired spermatogenesis and lead to non-obstructive azoospermia and infertility.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

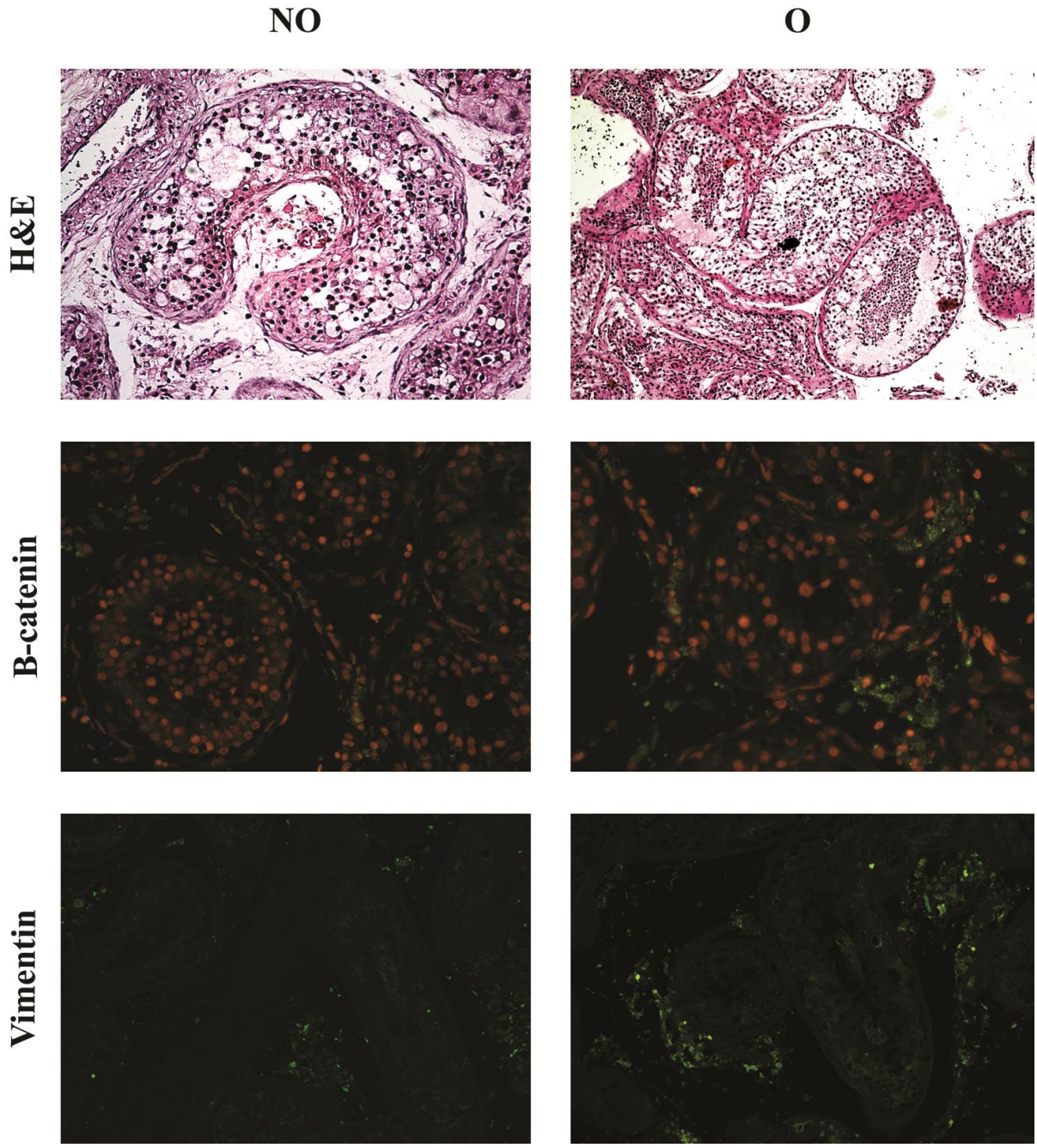

Figure 2. Histologic and Immunofluorescence microscopy. *Left column:* Non-obstructive azoospermic samples. *Right column:* Obstructive azoospermic samples. *First row:* 5µ sections and H&E staining. *Second row:* Detection of β-catenin accumulation site in the tissue by anti β-catenin antibody. Comparison of fluorescence intensity by Image J software revealed that β-catenin has significantly more accumulation in interstitial tissue of testis in non-obstructive azoospermic men. *Third row:* Immunofluorescence staining of leydig cells by specific anti-vimentin antibody. β-catenin was accumulated in leydig cells.

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