

Identification of VASA Gene Expression in *In-vitro* Culture From Non-Obstructive Azoospermia (NOA) Testicular Biopsy Cells: A Study to Unlock Knowledge of Male Infertility

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ABSTRACT

The expression product of VASA gene is widely conserved germ line marker and participates to regulate the development of reproductive system and spermatogenesis in healthy man. Azoospermia is a condition which the man is unable to produce any sperm cells for reproduction activity. This condition produced has bad impact to the man since the ability to produce their own progeny will be interrupted or blocked forever. Not only the family bloodline of the man would be ended, the psychological issue like shamefulness and low self-esteem occurs. In Islam, seeking knowledge is an obligatory to Muslims in order to solve problems and improve lives. Thus to unveil the problem of azoospermic man we aimed to determine the level of VASA gene expression in samples of testes cells of non-obstructive azoospermic (NOA) and compare it with sperms of healthy man. Samples were taken from three NOA patients by testicular sperm extraction (TESE) to obtain testicular biopsies. Testicular cells were isolated and cultured in supplemented knockout DMEM media. VASA gene expression was determined by reverse transcriptase polymerase chain reaction (RT-PCR). The VASA gene expression from sperm of healthy man was also determined for comparison purpose. It was demonstrated that the addition of growth factor significantly increased SSC-like cells colony formation in tissues obtained from NOA patients. No VASA expression was detected in spermatogonial-like stem cells culture on day 1, 7, 14 and 21 in each of the azoospermic samples. Our findings shown VASA gene was not expressed in spermatogenesis *in vitro* culture that might be associated with the abnormal differentiation of primordial germ cells that lead to male infertility. Islam also teaches us to not have feelings of despair and for problems that we are facing but to find the solution and consider the problem as a test from the Almighty. All the tests should be handled with strong believe since only Allah (SWT) knows what the best for us and each of the tests will have justified wisdom and benefits which we as human being are unable to realise thoroughly.

KEYWORDS: VASA, non-obstructive azoospermia, spermatogenesis, testicular cells, justified wisdom

INTRODUCTION

About 10 to 15% in general population, couples suffer due to infertility problems.⁵ Roughly, 50% of these cases can be attributable to issues in the male partner. Of these infertile men, 10 to 20% of all men suffer from azoospermia. There are two etiologies of azoospermia known as non-obstructive azoospermia

(NOA), a lack of spermatozoa production in the testes, and obstructive azoospermia (OA), an inability of produced spermatozoa to reach the emitted semen. From the total amount of azoospermic men, it is estimated that 49% to 93% caused by NOA.¹⁹ NOA is due to a failure of spermatogenesis process. This condition produces bad impact to the man since the ability to produce their own progeny will be interrupted or blocked forever. Not only the family bloodline of the man will cease the psychological issue like shamefulness and low self-esteem will be experienced by the sufferer.

In Islam, seeking knowledge is an obligatory to each Muslims in order to solve problems and live better. Problem should be solved by finding solutions and wise decision. But somehow, there

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are problems that sometime better left unsolved since no better solution could be achieved e.g. azoospermic in men. When a man has been diagnosed as azoospermic patient, there is no more possibility for him having his own biological offspring, and no solution is available. Such thought is against Islamic principles, which asks the humans to find the solution as what the Prophet (PBUH) said, "go and gain knowledge starting from birth till die. Therefore male azoospermic problem should be considered as unsolved problem that is waiting to be discovered.

Spermatogenesis is a cellular differentiation process that produces spermatozoa in which diploid spermatogonia differentiate to haploid spermatozoa through meiotic reduction.⁷ There is major interest in the field of andrology to understand the self-renewal and germ-associated networks of human adult spermatogonial stem cells (SSCs). The establishment and improvement of *in vitro* culture of SSCs has provided important information about germ cell biology and future fertility treatment for azoospermia patients.⁵

Current knowledge regarding the molecular markers of SSCs is still limited. This is due to the rarity of human testicular tissue, the relatively low number of adult stem cells in the testis, the lack of unique surface markers, and the absence of a robust proliferative *in vitro* culture system to support their self-renewal for efficient isolation and high purity propagation.⁵ It is estimated that more than 2000 genes are involved in the regulation of spermatogenesis.⁴ Lately, it has been proclaimed that the VASA gene has turned into hereditary determination germ-cell line marker derived from undifferentiated embryonic cells.²² VASA expression is limited to the testis and ovary, and is unnoticeable in somatic tissues.⁴ VASA protein is specifically limited to polar granules and then specifically exhibited during the whole of germ cell stages.¹² Earlier studies indicated that VASA were detected in spermatids, spermatocytes and spermatogonia. According to Castrillon *et al* (2000), no sign was noticed in testicular biopsies from a sterile patient with Sertoli cell only syndrome thus VASA is a possible gene marker for male infertility. Therefore this study is to determine the present of VASA gene expression in NOA patient using RT-PCR. This finding may help to discover the male fertility problems.

MATERIAL AND METHODS

Sample collection

Testicular biopsy tissues were obtained from the TESE procedure at International Islamic University Malaysia (IIUM) Fertility Centre, IIUM Malaysia. The use of human testicular biopsies and the experimental protocol were ethically approved by the IREC, IIUM (IIUM/305/20/4/10/1). For controls, semen samples were obtained by masturbation with signed informed consent and analysis and performed according to World Health Organization (WHO)

criteria.

Isolation of Human Spermatogonial Stem Cells and In vitro Culture

The testicular biopsy tissues were washed with sperm washing media (Irvine Scientific, USA). The testicular tissues were minced into small pieces and digested with 2 ml trypsin for 20 minutes in 37°C temperature. An amount of 2 ml of Basic human embryonic stem cell (bhESC) media was added to deactivate the function of trypsin and the cells were released into solution. Then, the dissociated cells were collected by centrifugation at 800 rpm for 5 min. Briefly, testes tissues were placed in bhESC media which consists of knockout Dulbecco's Modified Eagle's Medium EM medium (DMEM; Gibco, Paisley, UK), knockout serum replacement, L-glutamine + 2-mercaptoethanol, 100x non-essential amino acids, recombinant basic fibroblast growth factor (bFGF) (Sigma, St Louis, MO), and gentamycin (Gibco, Paisley, UK).

The collected cells were incubated in a humidified atmosphere at 37 °C and 5 % CO₂. The cells were cultured in uncoated 24-well culture plates (Cole-Parmer, Vernon Hills, Illinois) and the culture medium was replaced for every 3 days. In order to propagate the clusters and prevent SSCs from differentiating, cells were passaged every 5-7 days, with trypsin- EDTA (Invitrogen) and re-cultured. The culture was studied using an inverted microscope (Zeiss, Jena, Germany). The resulting cells were collected on different day of culture at 1, 7, 14, and 21 days for RT-PCR analysis.

Identification of VASA gene expression

Total RNA from the control sperm samples, and culture of testicular cells were extracted using PureLink® RNA Mini Kit (life technologies, USA). The quantification and the purity of RNA was checked by a 260/280 nm ratio measurement by using NanoDrop Spectrophotometers (NanoDrop Technologies, Inc., Wilmington, DE, USA). First-strand cDNA was performed by using oligodT primers and SuperScript® III First-Strand Synthesis System for RT-PCR Kit. All reverse transcription reagents were purchased from Invitrogen (USA). Samples were then reverse transcribed at 42°C for 15 minutes and denatured at 99°C for 10 minutes. After that procedure, the cDNA was stored at -80°C before use for amplification by PCR.

VASA primer sequence was taken from previous study by Medrano *et al*. (2010). The primers specific for VASA were designed using previously described human sequences (GenBank) (Table 1). PCR products were validated by sequencing in all cases. RT-PCR was performed using the prepared cDNA, the primers, and TopTaq DNA Polymerase (Qiagen). Firstly, dNTP mix and primer solutions were thawed at room temperature. The PCR master mix was prepared consist of; 10x TopTaq PCR Buffer, dNTP mix (10mM of each), 10x Coral Load concentrate,

primer forward, primer reverse, RNase-free water and cDNA. The tubes containing master mix were briefly centrifuged to assure all the mixtures were well mixed. The PCR tubes were placed in Techne TC-512 Thermal Cycler to run the samples. The RT-PCR protocols were started by denaturation step at

94°C for 30 seconds, followed by annealing step at 55.0°C for 30 seconds and final extension step at 72°C for 1 minute for 35 cycles. The bands were visualized by gel electrophoresis at 120V for voltage 40 minute by using EC 300 XL Power Supply.

Table 1.Primer Sequence used for measuring VASA expression.

Gene	Primer Sequences (5'-3')	Annealing temperature (°C)	Size (bp)	GenBank accession no.
VASA	Forward primer: 5'-ATGGATGATGGACCTTCTCG-3' Reverse primer: 5'-CCTCTGTTCCGTGTTGGATT-3'	54.1 and 55	228	NM_024415.2 (positions 228 and 456)

RESULTS

In this study, a small portion of adult human testicular biopsy cells obtained from (NOA) patients were cultured. This study demonstrated that the addition of growth factor significantly increased SSC-like cells colony formation in tissues obtained from NOA patients. It was also shown that these SSC-like cells clusters managed to grow and propagated for 3 weeks of culture (21 days). The addition of basic fibroblast growth factor (bFGF) in the supplemented knockout DMEM, increased the numbers of SSC-like cells clusters by self-renewal *in vitro* (Fig. 1).

This study had shown that VASA gene was detected in normozoospermic man as a control. However, there is no expression of VASA gene in NOA samples on day 1, day 7, day 14, and day 21 of cultures (Fig. 2).

DISCUSSION

This study demonstrated that the addition of growth factor significantly increased SSC-like cells colony formation in tissues obtained from NOA patients. It was also shown that these SSC-like cells clusters can be successfully cultured and propagated for 3 weeks (21 days). This is supported by Ebata *et al.* (2011) showed that cluster disassociation and serial passaging for prolong periods can increase the SSCs in number. In this study, the addition of basic fibroblast growth factor (bFGF) in supplemented knockout DMEM, increased the numbers of SSC-like cells clusters by self-renewal *in vitro* (Fig. 1).

This study has also shown that VASA gene was detected in normozoospermic man (as a control. This finding is consistent with Guo *et al.* (2007) that verified that VASA was expressed in human ejaculated sperm, and its expression was notably declined in the infertile patients, those with oligozoospermia. The statement above correlates with the finding of this research that there was no expression of VASA on day 1, day 7, day 14, and day 21 on NOA samples. These patients may have a complete depletion of the spermatogenic process, so the expression of VASA are reduced or terminated totally until it almost disappears. Normally, the

expression in the spermatogenesis enters the meiotic stage.

Growth factors in a culture medium may also play an important role in differentiating cells into other stages. In our study, the use of only one growth factor; bFGF in bHESC and in short term of culture was not enough to propagate SSCs. Most of other studies emphasized that combination of growth factor instead of one, have greater impact to induce differentiation to a precursor of spermatogenesis. Growth factors included in that modified medium, i.e., basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), glial cell line-derived neurotrophic factor (GDNF) and leukemia inhibitory factor (LIF), have the ability to induce spermatogonial stem cell differentiation and survival.²⁰ Ebata *et al.* (2011), which stated that the combination of fibroblast growth factor 2 (FGF2) and GDNF had propagated the number of dividing SSCs opposed to a no-growth-factor condition. It means that combination of growth factors may promote a differentiation pattern of SSCs that sustains the size of the stem cell pool while producing committed predecessor. Another study also supported that growth factor play a vital role in differentiation pattern. Ardekani *et al.* (2009) stated that they used a special formulated culture medium to support the SSCs development. Lim *et al.* (2010) stated that combination of growth factors such as GDNF, FGF and EGF in long-term culture has improved propagation of SSCs in NOA and OA patients. It has been unclear how growth factors contribute in controlling the patterns of SSCs division and fate decision. Since a SSCs vital function is to support the differentiation of spermatogenic and transmission of genes to the next generation, it is possible that growth factor may induce division of SSCs that cause the production of progenitors.

The least discovery of azoospermic condition in men, teach us how grand is the knowledge of the Almighty Allah (SWT). Islam also teaches us to not feel despair and give up when facing any problem, but find a solution and consider the problem as a

test from the Almighty. All the tests should be handled carefully with strong believe since only Allah (SWT) knows what the best for us and each of the tests will have justified wisdom and benefits which we as human being are unable to realise thoroughly. In order to helps men who are azoospermic, more research needs to be done and thus help such men in the population from alleviating their anxiety in order to have a child through sperm donation or some other way that are not forbidden in Islam but popular in the Western world.

CONCLUSION

Our finding shown that VASA gene was not expressed in spermatogenesis *in vitro* culture that might be associated with the abnormal differentiation of primordial germ cells that lead to male infertility. In order to helps the men with azoospermic condition, more profound needs to be done. Be aware that Islam also teaches us not to despair and give up, but try to solve problems and consider such problems as a test from the Almighty.

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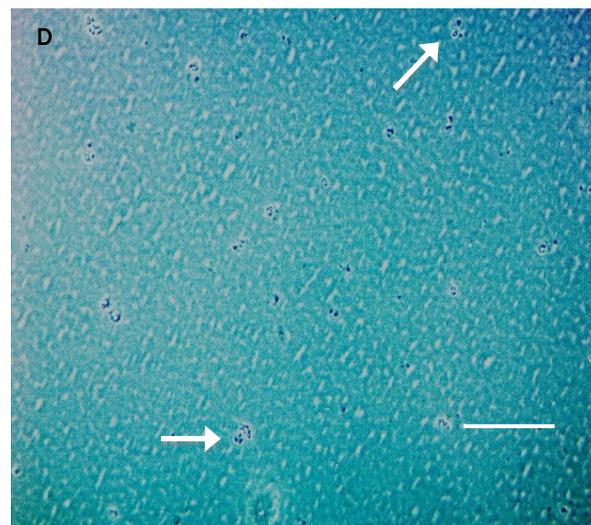
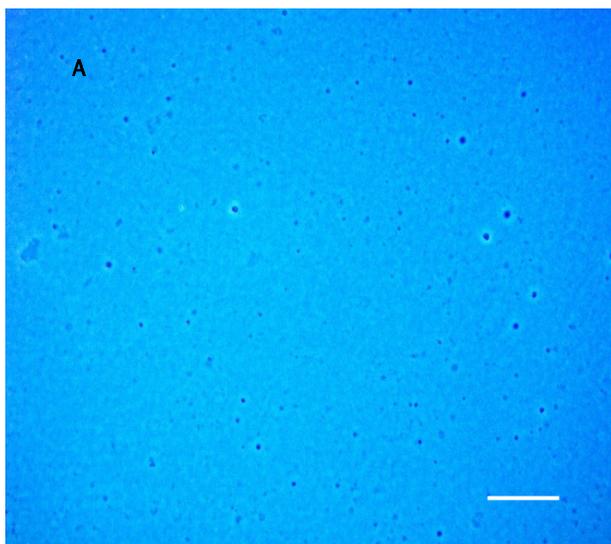
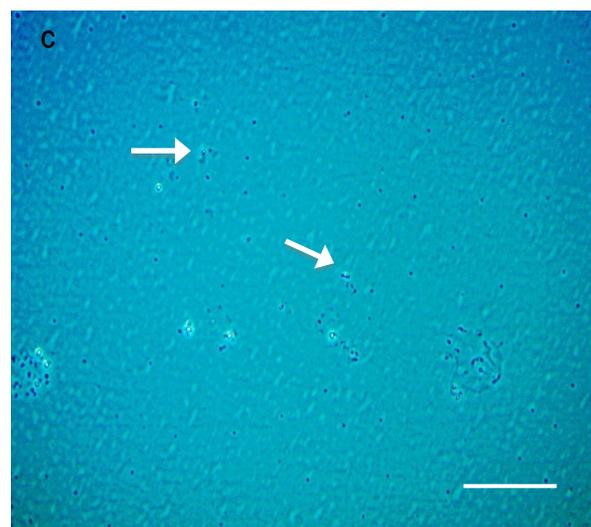
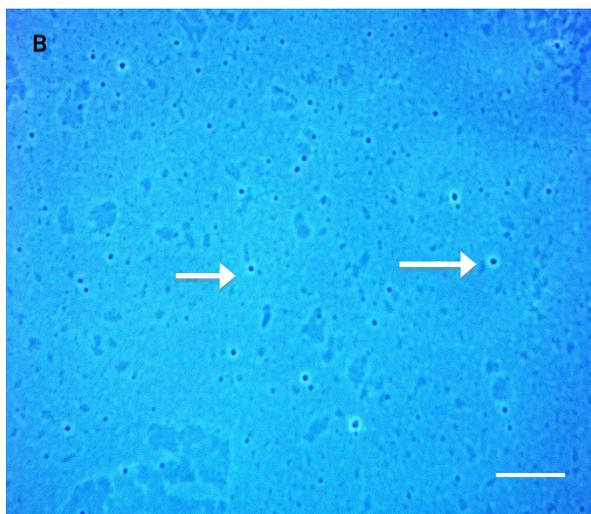


Figure. 1. Propagation activity of SSCs *in vitro* culture condition. A: 24 h culture, SSCs adhered to the culture plate; B: Day 7, SSCs were seen as round or oval shape (arrows); C: Day 14, SSC started to form colony (arrows); D: SSC colonies (arrow) after 21 days of culture. Scale bars = 324 μ m (A), 198 μ m (B), 590 μ m (C), 408 μ m (D) and 324 μ m (E). The cells were observed under inverted microscope (200X).

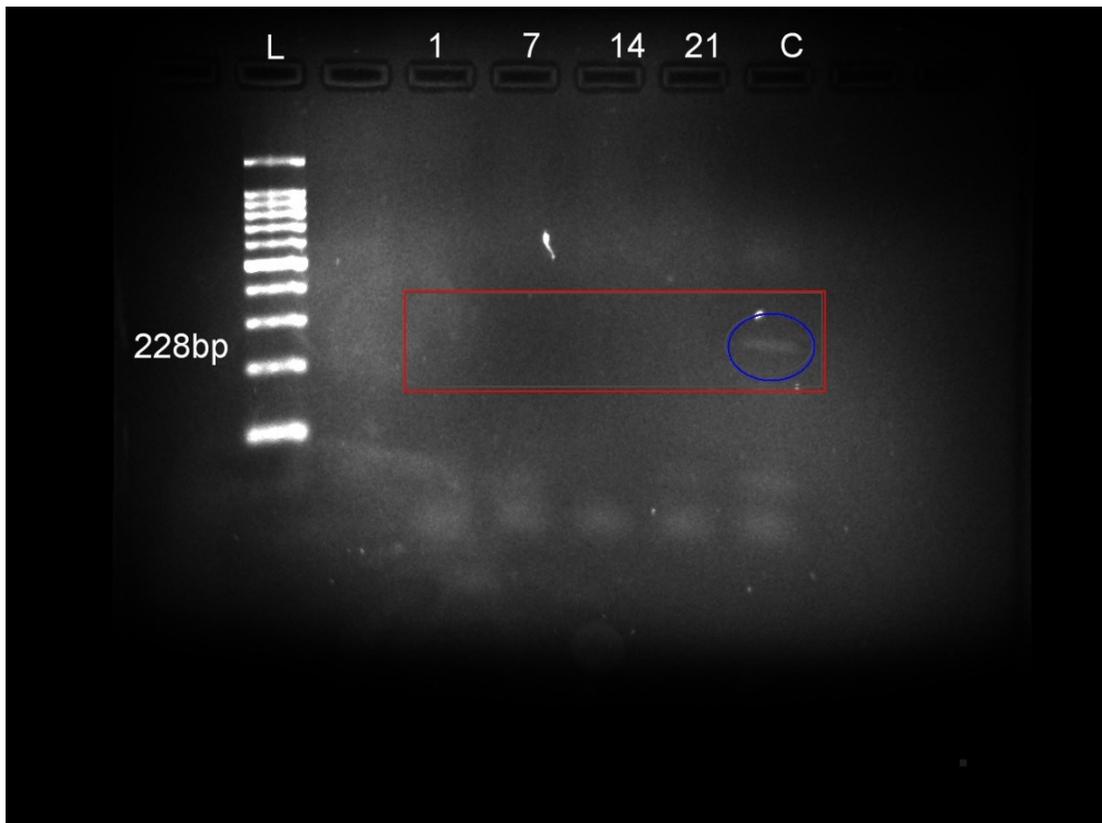


Figure. 2. Representative gels of RT-PCR amplification products of VASA (228 bp) for each sample of testis biopsy culture. L is indicator for 100 bp ladder marker which is on the left.

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