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Expression of *Catsper* Genecombination in Improves Sperm Parameters *in Vitro* of Normozoospermic Men with Anti Sperm Antibody Factor

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Abstract: Such a defect, any such one that causes infertility of the *CATSPER* cation channel, a particular calcium channel of the sperm tail, results in hyper-activation of sperm motility. In an attempt to enhance some of the sperm characteristics that are relevant by ART clinics, this research aims to investigate the relationship between *CATSPER* Channele and anti-sperm antibodies in Normozoospermic men before and after in vitro activation. From September 2024 to February 2025, 75 semen samples of men were received at Kamal Al-Samarrai Hospital for Fertility and Infertility's Andrology Laboratory, Bagdad, Iraq. 50 normal viable controls and 25 normozoospermic men with Antisperm anti body (ASA) were considered after seminal fluid analysis. In vitro sperm activation by stacking technique and FertiCultTM Flushing media was used in all the semen samples. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was applied to quantify *CATSPER* gene expression in semen samples. Statistical Package for Social Sciences (SPSS) software version 23.0 was applied for statistical analysis. Descriptive statistics such as mean, standard error, frequency, and range were employed, and Students t-test was applied for data comparison between treatment and control groups. Mean ± SEM was applied to represent treatment and control group data. All analyses indicated a highly significant ($p< 0.001$) increase in active grade A sperm motility, expression of down-regulated genes after the layering method, and highly ($p<0.001$) progressive motility % and significantly enhanced MNS compared to the control group, whose level of gene expression did not exhibit non-significant difference upon activation ($P>0.05$). In vitro activation method of layering should be applied to sperm activation and preparation in ART programs, according to the current study, since it has the ability to improve sperm quality in male infertility.

Keyword: Gene expression, in vitro activation, ASA, normozoospermia, *CATSPER*, and layering technique

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Introduction

Infertility is one of the most serious problems facing the countries. It is a disease of reproductive system defined by failure to achieve the clinical pregnancy after 12 months or more of regular unprotected sexual intercourse which is approximately 15% of couples(1). The World Health Organization (WHO) performs a pivotal position in standardizing health definitions in shaping the medical network's consensus on diverse health-related terms. According to the WHO, infertility is described as a disease of the reproductive gadget defined by

using the failure of girls age 35 years or much less to achieve scientific pregnancy after twelve months or greater (and girls more than 35 years inside 6 months) of everyday unprotected sexual sex. This specific expression not simplest highlights the period of unsuccessful attempts to conceive, however also assumes the presence of infertility inside the spectrum of medical situations, calling for the same gravity and compassion as another infection (2,3). Up to 12% of men have been estimated to be sub-fertile, according to more recent US estimates from a survey of 22,682 men and women between the ages of 15 and 44 years (4). Other recent research has been highly positive towards semen analysis as a marker of the general health of men and explained deteriorating semen parameters as being due to the rising disease and mortality rates among aging men compared to women (5).

Spermatogenic failure and subsequent infertility are outcomes of a mix of highly interconnected aetiologies such as genetic, medical, environmental, dietetic, and socioeconomic in nature (6,7).

Adult mammalian sperm initiate a series of necessary physiological events, including sperm hyperactivation, chemotaxis towards the egg, capacitation, and acrosome reaction, prior to the sperm entering the female reproductive tract and coming into contact with eggs for fertilization. The majority of studies propose that changes in calcium ion levels [Ca2+] control all the physiological events directly (8).

Fertilization is a highly complicated process involving the addition of numerous various chemicals. Capacitation and acrosome reaction (AR), (9) explain, are obligatory for successful fertilization. AR enables spermatozoa penetration through inside the egg. Intracellular Ca2+ increase by quite considerable amounts, initiates membrane-bound PLC (on the order of μM), which increases with capacitation (10). Ca2+ therefore controls sperm motility and fertilization. An efficient process of fertilization relies upon the distinct pattern of motility of the sperm during capacitation, known as hyper-activated motility (HAM). *CATSPER* is the principal Ca2+ channel responsible for initiating HAM and is localized in the sperm tail (11). Two primary stores of calcium ions in sperm are a calcium pump in the sperm head, the midpiece mitochondria that are loaded with excess calcium ions, and a duplicate nuclear envelope in the neck region—which also contains the inositol 1,4,5-trisphosphate (IP3) receptor—report (12). Studies have illustrated that the sperm flagellum has specific Ca2+ channel proteins known as *CATSPER* 1, which are associated with sperm hyperactivation and play a role in fertilizability, sperm motility, and penetration of the egg (8).

The Cation channel sperm-associated protein is the product of the 9,768 kb *CATSPER*1 gene, having 12 coding exons. It is situated at chromosome 11q 13. *CATSPER* protein family, i.e., these proteins, consists of four proteins (*CATSPER*1-4) that function together to form a tetrameric pore. The four members of the family bind auxiliary proteins *CATSPER*β, *CATSPER*γ, and *CATSPER*δ (13). The four members of the family are expressed at various stages of spermatogenesis (14). In contrast to all the other genes of this type, CATSPER 1 was discovered earlier. Its histidine-rich cytoplasmic domain, which detects intracellular shifts in pH, initiates the opening of the *CATSPER* channel. Dysfunction of the functional channel causes male

infertility and abnormal hyperactivation motility. But deletion in any one of the four *CATSPER* genes can make it dysfunctional. (15) *CATSPER*2 and *CATSPER*1 defects, however, are linked to human infertility. The results suggest that the *CATSPER* channel should be put at the forefront of male infertility research and male contraceptive drug development (16). Immunologic infertile men and idiopathic infertile men both prior to and following in vitro sperm production should be investigated for their *CATSPER* gene polymorphism and gene expression. (3,17).

In an effort to achieve such goals, the following strategies will be utilized:

1. Have the semen analyzer services to ascertain sperm motility, morphology, agglutination, and count after and prior to sperm activation in vitro.
2. Quantitative gene analysis of *CATSPER* was examined pre- and post-in vitro sperm activation usingqRT-PCR.

2. Persons, Equipment, and Procedures

Persons

75 fertile and infertile male persons aged between 18 and 60 years were utilized in the present prospective study. They were from the Kamal Al-Samarrai Hospital for Fertility and Infertility in Bagdad, Iraq, to be specific, the Andrology Laboratory. They were enlisted by 50 healthy fertile controls and 25 ASA normozoospermic men after seminal fluid analysis. A very interesting questionnaire was drawn up from the patient's thorough and comprehensive medical history. All male volunteers were explained the aim of the study in simple language.

The initial fluid analysis

Semen samples were also piped directly into the clean, dry, and sterile laboratory-provided container after three to five days of continence. They were incubated at 37°C to liquefy. The specimens were then microscopically examined and according to the WHO 2021 standards.

In vitro stacking technique sperm activation procedure Activation of FertiCult® Flushing Medium:

It can be utilized for both normozoospermic as well as asthenozoospermic semen. It was initially explained by United Kingdom's Bourn Hall Clinic in 1992. It is the disadvantage of this procedure that it is laborious. (18)

CATSPER 1 gene expression:

After the isolation of total RNA, the reverse transcription quantitative polymerase chain reaction (RTqPCR) method was used to measure *CATSPER* 1 gene expression. TransZol (TransGen, No. ET101), a commercial reagent, was used to isolate total RNA. (19)

RNA extraction:

From the semen samples by TransZol (TransGen, biotech. ET101). By using TransZol reagent and according to the manufacturer's instructions, all semen samples were extracted to obtain the RNA.(19)

Real Time Quantitative Polymerase Chain Reaction (RT qPCR)

This technique was used to amplify gene to detection of gene expression in all tested samples by using real time kits (GoTaq® qPCR and RT-qPCR Systems) and 7500 real time PCR system from applied biosystem company. The mixture was prepared with final volume 20 µl by mixing all contents.(20).

Culture Media

FertiCult flushing medium is an inter cytoplasmic sperm injection (ICSI) sperm injection cell culture medium, sperm activation, embryo transfer, and intrauterine insemination medium. The

supplements in this FertiCult flushing media, which was developed from HEPES-buffered medium. (21)

Direct Sper Mar test for test IgA Antibodies:

The reagents and specimens were allowed to attain room temperature.Ten microliters of Eprom MarTest Immunoglobulin A Latex particles and ten microliters of fresh semen should be applied on a microslide. After the cover glass slip was placed over the mixture, it was observed under a light microscope. They were read after three minutes. looked for latex particles bound to the sperm as they move. Count 100 of sperm cells to determine the proportion of responding sperm. Results were again read after ten minutes. Suspected immunological infertility if 10–39% of motile spermatozoa bind to latex particles; it is very probable when 40% or more of spermatozoa bind. (22)

Direct Sper Mar test for IgG Antibodies

We equilibrated the agents and specimens at room temperature in a microslide. There are ten microliters of raw semen.Ten microliters of Sperm Mar Test Immunoglobulin G antiserum and Sperm Mar Test Immunoglobulin G Latex particles. The Latex reagent and sample were mixed five times by utilizing the edge of a cover glass.(22)

Statistical analysis

Statistical Package for Social Sciences (SPSS) software version 23.0 was applied for statistical analysis. Descriptive statistics such as mean, standard error, frequency, and range were employed, and Students t-test was applied for data comparison between treatment and control groups. Mean ± SEM was applied to represent treatment and control group data.

Result and Discussion

Table(1): Comparison between patients and control groups in *Means Ct* of *CATSPER* before acivation

Groups	Means Ct of *CATSPER* e before	Means Ct of GAPDH	ΔCt (Means Ct of *CATSPER*)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Control (Normozoospermia without AsA)	22.88576	22.22653	0.659230	0.633215	0.633215/0.633215	1.000
Normozoospermia with AsA	22.5264	22.1844	0.342	0.788946	0.788946/0.633215	1.246

Table (2): Comparison between patients and control groups in *CATSPER 1* gene before activation.

Groups	Mean ±SD	P-VALUE
Control(Normozoospermia without AsA)	1.1466 ±0.7380b	0.001**
Normozoospermia with AsA	1.3498 ±0.5767b	

Mean ± SD has been used to present the data. Statistical testing for ANOVA and Post Hoc test was conducted. Two-tailed test of ANOVA significance. Bold was applied to denote significance. A and B: Denote letters to represent significant difference between the variables. If the two meanings never

use the same letter, then there can never be any risk of differentiation between them. SD: Common or Typical. Standard deviation has been shortened to SE. No modification has been observed, as per the NS mean error.

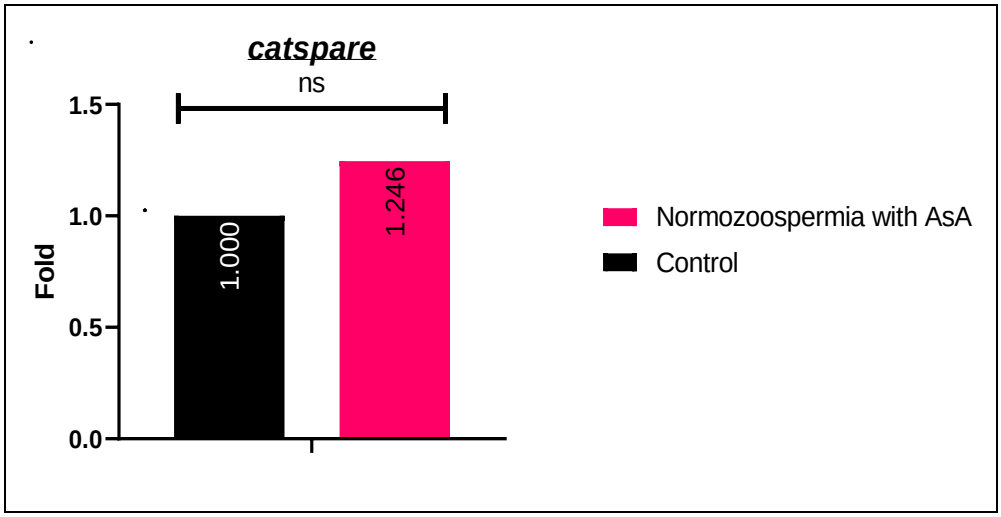

Figure (1): *CATSPER 1* expression levels in the patient and control group before activation.

Table (3): Comparison between patients and control groups in *Means Ct of CATSPER 1* after acivation

Groups	Means Ct of *CATSPER* after	Means Ct of GAPDH	ΔCt (Means Ct of *CATSPER*)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Control (Normozoospermia without AsA)	23.33231	24.32	-0.98769	1.98301	1.98301/1.98301	1.000
Normozoospermia with AsA	25.2492	24.228	1.0212	0.492706	0.492706/1.98301	0.248

Table(4): Comparison between patients and control groups in *CATSPER 1* gene after activation.

Groups	Mean ± SD	P-VALUE
Control (Normozoospermia without AsA)	1.01976± 0.5216b	0.001**
Normozoospermia with AsA	0.30571± 0.1421c	

Results were expressed as mean ± SD. ANOVA and Post Hoc test, or Duncan's test for multiple comparison, were used to statistics. ANOVA two-tail significance test. Significant is indicated by Bold. A and B: Different letters show there is a significant difference. It cannot be told which of two meanings is there when they have the same letter. SD: Standard. SE is short for standard deviation. The NS mean error suggests that nothing has changed.

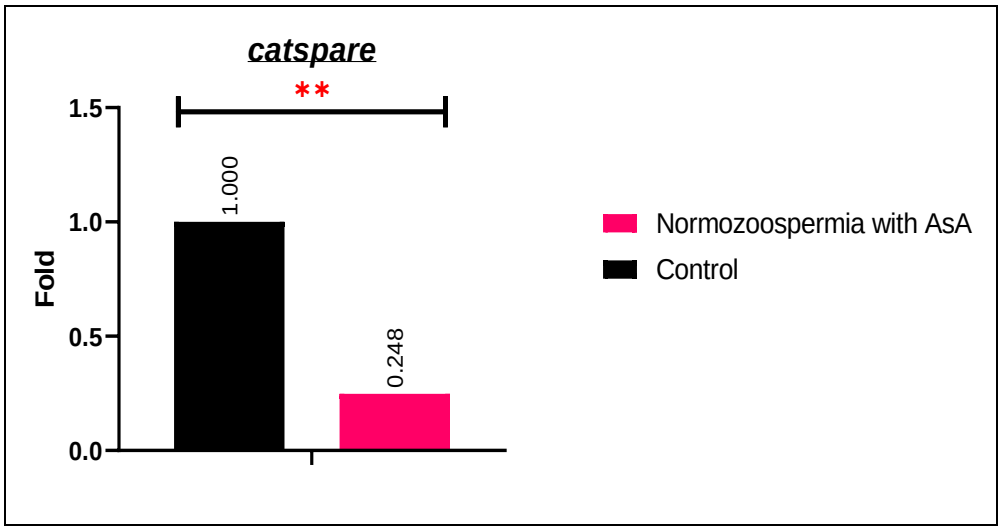

Figure (2): *CATSPER 1* expression levels in the patient and control group after activation.

Table (5): Impact of FertiCultTM Flushing medium on individual sperm function parameters in normozoospermic with ASA before in vitro activation by layering methods

Groups		Concentration /million (before)	Progressive % (before)	Non-progressive % (before)	Immotile% (before)	Normal morphology % (before)
Control	Mean	36.6538 a	19.0385 a	35.5769 a	44.6154 b	52.50
	Std. Deviation	5.76154	9.27569	7.52687	11.74079	19.300
Normozoospermia with AsA	Mean	34.2000 b	16.6000 b	36.8000 a	46.6000 b	45.28
	Std. Deviation	3.34166	6.24500	5.75181	8.38153	12.016
p-value		0.001**	0.03*	0.02*	0.001**	0.1 NS

Table (6) : Impact of FertiCultTM Flushing medium on some parameters of sperm function in normozoospermic with ASA following in vitro activation with layering methods

Groups		Concentration /million (after)	Progressive (a + b) % (after)	Non-progressive (after)	Immotile % (after)	Normal morphology % (after)
Control	Mean	27.0000 a	33.0769 a	40.5769	64.81	26.7308 b
	Std. Deviation	4.39090	12.65519	7.65858	18.893	13.03398
Normozoospermia with AsA	Mean	26.7200 a	34.8000 a	43.0000	68.80	24.2000 b
	Std. Deviation	4.12836	7.83688	7.77282	10.436	9.20598
p-value		0.001**	0.04*	0.2 ns	0.01**	0.4 NS

Male Infertility and the Role of *CATSPER* Gene

Male infertility is a difficult issue that many couples around the world deal with. (23) Sperm motility and morphology are two of the most important parameters influencing reproductive outcomes among the many reasons causing male infertility. Infertility can result from any interference with sperm motility, which is necessary for a healthy fertilization process. One important component of male infertility is sperm motility, which can be hampered by defects in the *CATSPER* gene or its channel. Regardless of size or shape, sperm cannot attain the hyperactivated motility necessary for fertilization when this channel's function is interfered with.

To improve male fertility results, especially with assisted reproductive

technologies (ART) such intra-cytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF), research on increasing sperm motility in situations of *CATSPER* failure is essential. (24).

Anti-Sperm Antibodies' Impact on Sperm Function (25,26) Male infertility is frequently caused by anti-sperm antibodies (ASA) in addition to *CATSPER* dysfunction. ASA immunoglobulins incorrectly link sperm cells to invasive intruders. The motility, shape, and general capacity of sperm to fertilize an egg can all be impacted by these antibodies. (27,28) The production of antibodies by the immune system against sperm antigens, such as the *CATSPER* channel, can further hinder sperm motility in males with ASA. Because ASA reduces the effectiveness of conventional sperm preparation methods, its prevalence poses a major danger to ART. Since ASA-mediated sperm failure is one of the primary causes of male infertility, it is imperative to investigate ways to mitigate its effects in ART settings. This study investigates the relationship between ASA and *CATSPER* gene expression in normozoospermic males to determine whether in vitro sperm activation utilizing a layering technique might improve sperm motility and other important sperm characteristics. (29)

In Vitro Activation and the Layering Method

Techniques for in vitro sperm activation have grown in significance in ART, especially for boosting sperm motility in cases when sperm malfunction is suspected or obvious. For sperm to fertilize an egg, the female reproductive system must naturally activate them. Replicating this mechanism is the aim of activation techniques. The layering approach, which uses a particular sperm activation medium, such FertiCultTM, has been demonstrated to increase sperm motility by increasing the sperm's capacity for hyperactivation.

FertiCultTM Flushing Medium is frequently used in ART facilities to boost sperm motility and enhance sperm quality for conception. The layering technique is used to add activation medium to sperm samples in a controlled laboratory setting. It has been demonstrated that this method encourages sperm hyperactivation, which improves sperm motility and, occasionally, morphology. (30) By increasing sperm activity, this method can increase the likelihood of successful fertilization in ART, particularly for those experiencing male infertility as a result of conditions like ASA or *CATSPER* gene malfunction. Using the layering approach, the current study investigated the effects of in vitro sperm activation on sperm motility, morphology, and *CATSPER* gene expression. Based on the findings, the method can be a useful addition to ART treatments and assist men with ASA in improving their sperm parameters. When compared to pre-activation measurements, the proportion of progressive motility, active sperm motility (Grade A), and morphologically normal sperm (MNS) all rose significantly ($p<0.001$) following activation; these improvements were particularly apparent in the group that tested positive for ASA. The results of the study also demonstrated that, following in vitro activation, the layering technique dramatically enhances sperm motility and morphology. (31)

The significance of the discovery is that layering technique can reverse ASA's negative impacts on motility and quality of sperm (32). The activating medium, by supposition, aids in propelling the calcium ion within the

sperm tail, enhancing hyperactivation and the motility of sperm. In addition to the motility, the percentage of normally formed sperm is enhanced, which is significant. It also implies that enhancing the functional properties of sperm would also improve or rectify the morphological defects resulting from ASA-induced damage. These results are consistent with previous research indicating that sperm activation techniques can improve motility in sperm under infertile conditions resulting from ASA. The protocol is of use as an adjuvant to ART in men with ASA-induced infertility because there is improved morphology and motility of sperm after stimulation. (33).

CATSPER Gene Expression Pre- and Post-Activation

The identification of whether in vitro activation of sperm has an influence on *CATSPER* gene expression was one of the most important objectives in this study since variation in *CATSPER* (34) gene expression is associated with motility of sperm directly. The significant and new contribution was when findings pointed towards the reduction of expression for the *CATSPER* gene following activation in the ASA-positive population.

To reverse the inhibitory action of ASA, activation has been shown to be able to modify the molecular equipment of the sperm, as evident from decreased *CATSPER* gene expression following activation. Increased motility of sperm is typically paralleled by increased *CATSPER* expression, and the decrease in gene expression recorded here could well signal the sperm to have crossed over from being non-hyperactivated to the hyperactivated condition. In reversals of ASA's adverse effect to restore movement, it would be a compulsory procedure. *CATSPER* expression was not altered before and after activation in the control group, i.e., fertile healthy men without ASA ($p>0.05$). This finding suggests that healthy sperm are already maximally functional and activation is not providing significant advantage, thereby justifying the argument that the procedure is more advantageous for application in dysfunctional function sperm (26).

Implications for ART Programs

This study has important clinical implications for ART programs, particularly for men who are infertile and ASA (32). The increase in sperm motility and morphology after in-vitro activation, and potential modulation of *CATSPER* gene expression, supports the advancement of the hypothesis that layering can be used as a sufficient adjunct therapy to sperm preparation for ART. By boosting the quality of the sperms, this technique has the ability to enhance fertilization and pregnancy possibilities in such cases where normal sperm preparation techniques have yielded nothing due to ASA-related malfunctioning of sperms (35).

One such possible way of enhancing ART results would be the application of in vitro sperm activation methods, especially when ASA is involved (26). The method could be especially valuable where ASA is involved and routine sperm processing methods, e.g., swim-up or density gradient centrifugation, fail to provide optimal sperm quality (36).

Limitations of the Study

While results of this study are promising, the focus must be placed on a variety of limitations. There are just 75 in the sample, 25 ASA-positive and 50 controls. Other large studies with more patients would be needed to confirm findings and establish their generalizability in other treatment settings.

In addition, other forms of sperm activation or media that can otherwise be beneficial to sperm quality were not examined with this study; rather, it examined advantages of the FertiCult Flashing Medium and stacking technique. Perhaps the best way that sperm motility and morphology can be maximized for ART is through comparison among different types of activation. Long-term effects of in vitro activation on sperm function and ART outcome like fertilization rate, embryo development, and pregnancy rate were not studied here. Long-term effect of sperm activation on the success of ART with fertilization would be evaluated by future studies.

Conclusion

This research proves that in vitro stimulation of sperm by the layering method increases significantly the sperm motility, morphology, and expression of the *CATSPER* gene in ASA-normozoospermic patients. The findings indicate that treatments of sperm stimulation, which augment the quality of sperm and promote the chances for fertilization, can be a productive supplement for ART protocols on ASA-induced male infertility. Additional studies on the molecular mechanism of sperm activation would likely be aimed at the post-activation switch in *CATSPER* gene expression that characterizes the molecular process resulting in enhanced sperm motility. With the development of technology in ART, sperm activation methods such as the layering strategy could be a critical parameter to attain enhanced reproductive success in ASA men with infertility.

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