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Glycogen Synthase Kinase 3 Enzyme Gene Expression in Asthenozoospermic Men Before and After Sperm Activation *in vitro*

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Abstract: The study was conducted to treat the asthenozoospermic samples *in vitro* by swim-up technique and determine the gene expression of Glycogen synthase kinase (GSK3A) in semen samples of Iraqi men before and after activation *in vitro*. The semen samples of 50 men were collected from Al-Najaf Fertility Center in Al-Sader Medical City, Ministry of Health in Najaf-Iraq from 2024 to 2025. Following seminal fluid analysis ,25 patients with asthenozoospermia and 25 healthy fertile controls were enrolled. *In vitro* sperm activation by FertiCult^{TM} Flushing medium using swim-up technique was performed for all semen samples. The expression levels of the GSK3A gene in semen samples were analyzed using reverse transcription quantitative polymerase chain reaction (RT-qPCR). The results of present work revealed a highly significant improvement in active sperm motility ($P<0.006$) and normal sperm morphology percentages ($P<0.001$) in both control and asthenozoospermic (AS) patients group compared to before activation. The expression levels of GSK3A gene after activation showed a significant ($P<0.01$) difference in asthenozoospermic patients group compared to before activation, while the gene expression of control group after activation was shown non-significant ($P>0.5$) difference compared to before activation.

Keyword: Gene expression, GSK3A, Asthenozoospermia, *In vitro* activation

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Introduction

Infertility is described as not getting pregnant after trying regularly without protection for at least one year (1). Multiple factors may impair male fertility, such as testicular dysfunction, abnormalities of the male reproductive system, immune-related conditions, varicocele, genetic disorders, hormonal imbalances, systemic illnesses, cancer, infections of the reproductive tract, exposure to environmental toxins, unhealthy lifestyle habits, and contact with substances harmful to the gonads(2). 48.5 million couples worldwide suffer from infertility. The failure of a male to get gestation in a fertile female is mention as male infertility which form about 40-50% of the incidence (3). Sperm cells rely on energy to carry out their functions, which is primarily generated in the form of adenosine triphosphate (ATP) through two main pathways: glycolysis and oxidative phosphorylation (OXPHOS) (4). As a result, enzymes that regulate ATP production are thought to be crucial in managing vital sperm functions, including movement and the acrosome reaction. Among

these enzymes is glycogen synthase kinase 3 (GSK3), a serine/threonine kinase that participates in various cellular activities, such as programmed cell death (apoptosis), cell division, and cell growth. Dysregulated activity of GSK3 has been associated with several health conditions, including cancer, Alzheimer's disease, and diabetes (5). GSK3 is widely expressed in the body and is encoded by two separate genes, producing two isoforms: GSK3α and GSK3β. These isoforms differ mainly at the N-terminal region; GSK3α contains a unique glycine-rich sequence at its N-terminus, which is well conserved across mammalian species-suggesting it may have distinct functional roles (6). In humans, inhibition of GSK3 by valproic acid has been shown to interfere with sperm development and motility, indicating that GSK3 is likely involved in male reproductive function (7). Although both isoforms are generally present throughout the body and often function redundantly, research has shown that the absence of GSK3α specifically can lead to male infertility (8). Interestingly, GSK3 activity decreases as sperm mature in the epididymis. This implies that GSK3 activity is necessary during the early stages of sperm development. Furthermore, sperm that lack GSK3α show reduced energy production and impaired motility, often displaying stiffness in the midpiece region (9). Based on these findings, the current study aims to examine the expression levels of the GSK3A gene in infertile men, both before and after *in vitro* sperm activation, in order to better understand the enzyme's role in male infertility.

Materials and methods

Semen collection

Fresh human semen samples were collected through masturbation from men and put it directly into a clean, dry and disposable sterile plastic petri dish in the especially allocated room for this purpose. For each subject with acquaintance in the abstinence period from 3-7 day (10).

Semen analysis

The semen sample was delivered for analysis within 30 to 60 minutes. Each sample was then permitted to liquefy according to the procedure outlined in (11). Once the semen had fully liquefied, it underwent both macroscopic and microscopic examination As defined by the World Health Organization (WHO) standards from 2010.

Swim-up procedure for sperms Activation by FertiCult^{TM} Flushing medium

Swim-up procedure (according to WHO,2021).

Expression of GSK3A gene

Following total RNA isolation, the expression of the GSK3A gene was measured by Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used. Total RNA was extracted using the ready-to-use *TransZol* reagent. (TransGen Biotech, ET101) according to the manufacturer's guidelines (TransGen, China).

RNA extraction

From the semen samples by *TransZol* (TransGen, biotech. ET101).

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

This technique was used to amplify the gene and detect its expression in all tested samples using real-time PCR kits (GoTaq® qPCR and RT-qPCR Systems) and 7500 real time PCR system from applied biosystem company. The reaction mixture was prepared to a final volume of 20 µl by combining all components listed in Table 1.

Table (1): The Components of RT-qPCR Mixture.

Item	Volume
GoTaq ® qPCR master mix 2X	10 µl
RT mix for 1-step RT-qPCR 50x	0.4 µl
Forward primer	1.5 µl
Reverse primer	1.5 µl
$Mgcl_2$	1.6 µl
CXR	0.3 µl
RNA	4 µl
Nuclease free water	~ 0.2 for complete volume to 20 µl

The cycling protocol was programmed using the following optimized cycles, as outlined in the thermal profile shown in Table 2.

Table (2): Thermal profile of GAPDH and GSK3A gene expression.

Stage		Condition	No. of cycle
Hold		95 °C/10 min	1
Cycling	Denaturation	95 °C/15 sec	45
	GAPDH Annealing	58 °C/1 min	
	GSK3A Annealing	57 °C/1 min	
	Extension	72 °C/30 sec	
Melting curve		95 °C/15 sec 60 °C/1 min 95 °C/30 sec	1

Equation Delta Ct equations to analyse RT-qPCR results

The levels of GAPDH and GSK3A gene expression were determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR), a sensitive technique for quantifying steady-state mRNA amounts (12). To validate the expression of the target gene, an RT-qPCR SYBR Green assay was employed. Primer sequences for GAPDH and GSK3A were designed specifically for this study, synthesized by OLIGO (Macrogen), and stored lyophilized at –23°C. The primer sequences are provided in Table 3. The mRNA of the endogenous control gene (GAPDH) was amplified and utilized to normalize GSK3A gene expression. GAPDH primer sequence is also shown in Table 3.

Table (3): Designed Primers used in the current study.

Primer	Sequence (5′→3′ direction)	Product size
GSK3A (primer-Gene Expression)		643 bp
Forward	TCCATTTCCCGACGTACAGC	
Reverse	GGGAACCCTAATCACCACCC	
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase		94 bp
Forward	TCTTTTGCGTCGCCAGCCGAG	
Reverse	TGACCAGGCGCCCAATACGAC	

Gene expression or gene fold or RQ (Relative quantification) value were calculated by (12): $RQ = 2^{-(\Delta\Delta CT)}$

Statistical analysis

To evaluate statistical differences among multiple groups, Analysis of variance (ANOVA) was applied, with data presented as mean ± standard deviation (SD). Correlation analysis was performed to investigate relationships between groups. All statistical analyses were conducted using SPSS for Windows (version 20; SPSS Inc., Chicago, IL, USA).

Result and discussion

In vitro activation of semen samples

Effect of *in vitro* activation by FertiCult^{TM} Flushing medium on certain function parameters of sperms for control group, that show in Table 4.

Table (4): Effect of *in vitro* activation by FertiCult^{TM} Flushing medium on certain function parameters of control group of semen samples.

Parameters function of sperm	Activation status of control group		p-value
	Before activation	After activation	
Sperm concentration (million/ml)	74.4 ± 33.26	49.5 ± 24.27	0.001
Progressive motility (%)	65.16 ± 14.51	73.24 ± 14.21	0.006
Non progressive motility (%)	13 ± 6.99	5.14 ± 3.87	0.00001
Immotile (%)	22 ±11.84	16.62 ±11.24	0.02
Normal morphology(%)	73.08 ±4.73	81.7± 3.03	0.001
Round cells (m/ml)	1.60±0.15	0	0.0001

And effect of *in vitro* activation by FertiCult^{TM} Flushing medium on certain function parameters of Asthenozoospermic (AS) patients group that show in Table 5.

Table (5): Effect of *in vitro* activation by FertiCult^{TM} Flushing medium on certain function parameters of Asthenozoospermic (AS) patients group.

Parameters function of sperm	Activation status of (AS)		p-value
	Before activation	After activation	
Sperm concentration (million/ml)	37.12±18.43	24.5±13.92	0.008
Progressive motility (%)	23.54± 5.33	45.15±9.72	0.000
Non progressive motility (%)	19.96±9.57	6.96±4.14	0.000
Immotile (%)	58.38±12.43	47.88±11.81	0.003
Normal morphology(%)	72.04±2.12	81.19±2.46	0.000
Round cells (m/ml)	2.86 ±0.29	0	0.0001

Seminal analysis is essential for assessing male fertility, yet it lacks the depth to reveal subcellular abnormalities in the spermatozoa of infertile patients. This limitation highlights the need for further molecular-level analyses to better elucidate these underlying alterations (13). The study results found a positive effect of FertiCult^{TM} Flushing Medium *in vitro* leading to enhance the certain sperms function parameters (motility, normal sperm morphology and round cell). However, the significant decrease in concentration of sperm after activation *in vitro* was notice, may be resulted from lack of ability of the immotile and low grade activity sperm to move up and swim from the bottom to the upper layer in the FertiCult^{TM} Flushing Medium. This results agree with the other studies by (14). There was an increase in the percentage of normal sperm morphology and progressive sperm motility were observed after activation. This detected may be due to the rapid normal sperm motility from the lower layer of the culture medium that which contain of calcium and energy supplements to increase of sperm motility in addition to the effect of some components present in the seminal plasma such as white blood cells and other decapitation factors which in turn lead to avoid of sperm from stress factors and producing

reactive oxygen species which lead to poor sperm motility and DNA damage (15). This leads to a considerable decrease in abnormal, non-progressive, and immotile spermatozoa, respectively. (16). The number of round cells significantly decreased after activation using FertiCult™ Flushing Medium compared to their levels before activation. This reduction is attributed to the survival of cells at the base of the tube, as these immotile cells are unable to move upward or downward due to the effect of gravity. This finding aligns with the observations reported by (17). The current study demonstrates that FertiCult™ Flushing Medium has a positive effect on certain sperm function parameters in asthenozoospermic men. Specifically, the assessment of sperm parameters after the swim-up procedure, such as total motile sperm count and motility, demonstrated notable improvement. According to (18), the swim-up technique enhances sperm count, motility, and pregnancy rates. Additionally, following sperm preparation, sperm washing combined with the swim-up method resulted in lower recovery rates in concentration but higher recovery rates in morphology(19). As reported by (20), there was a significant increase in sperm motility, progressive motility (grades A and B), total progressive motility (grades A and B), and normal sperm morphology from pre-activation to post-activation. The sperm concentration, active sperm motility, and sperm morphology are all indicative of the normal fertilization capacity in healthy men (21).

Quantitative Expression of GSK3A gene

In the semen samples from asthenozoospermic patients, total RNA concentrations varied between 82 and 197 ng/µl, with purity levels ranging from 1.91 to 2.11 ng/µl. The Ct values and melting curves obtained from the qPCR analysis provide crucial information about the gene expression levels and the specificity of the amplified products. Figure (1) displays the Ct values for the target gene GSK3A before and after activation, while Figure (2) displays the Ct values for GAPDH before and after activation. The results facilitate comparison of gene expression levels among various samples and help reveal significant differences. Additionally, the melting curve analysis provides insights into the specificity of the amplification, ensuring that the detected signals are indeed derived from the target genes rather than any non-specific amplification or background noise, as seen in Figure (3,4).

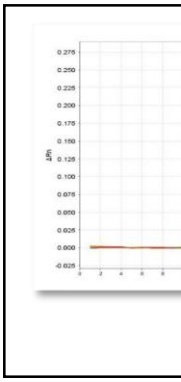

Figure (1): RT-PCR Amplification plots of GSK3A gene: (A) Before activation. (B) After activation.

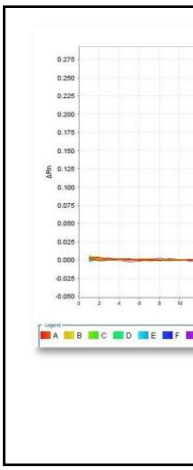

Figure (2): RT-PCR Amplification plots of GAPDH gene (A) Before activation. (B) After activation.
Figure (3): Melt curve of GSK3A gene: (A) Before activation. (B) After activation.

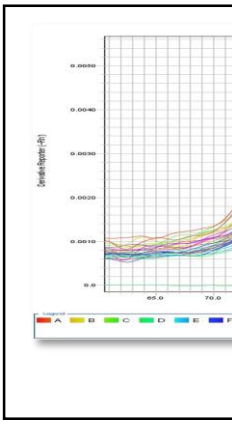

Figure (3): Melt curve of GSK3A gene: (A) Before activation. (B) After activation.

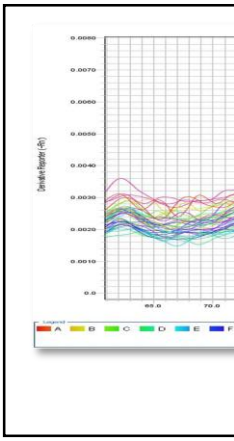

Figure (4): Melt curve of GAPDH gene: (A) Before activation. (B) After activation.

According to the study results, the semen samples of the studied groups compared together before and after activation, the mean of GSK3A gene expression in semen samples of asthenozoospermia (AS) patients before activation by FertiCult^{TM} Flushing medium was (2.03±0.61) and (1.2±0.41) after activation, While the mean of GSK3A gene expression in semen

samples of control group after activation by FertiCult™ Flushing medium was (1±0.32) and (0.87±0.51) before activation for control group. The GSK3A gene expression in semen samples of (AS) patients before activation showed a highly significantly (P<0.01) with the after activation of semen samples, while the control group before activation non-significant (P> 0.5) with the gene expression of the control group after activation. In general, the GSK3A gene expression after activation in control and (AS) patients group decreased significantly with the GSK3A gene expression before activation in Table 6. For a more detailed overview of the fold change values for each group before and after activation, refer to Table 7 and Table 8.

Table (6): Expression level of GSK3A in the semen of studied groups.

Men groups	Mean ± SD GSK3A gene expression Before activation	Mean ± SD GSK3A gene expression After activation	P-value
Control	0.87±0.51	1±0.32	0.5
AS	2.03±0.61	1.2±0.41	0.01

Table (7): Fold change in gene expression of GSK3A and GAPDH genes before activation of studied groups

Group	Mean Ct of GSK3A	Mean Ct of GAPDH	Mean ΔCt of GSK3A	ΔΔCt	Fold change
Control	25.440	29.930	-4.490	-2.440	1.000
AS	24.110	29.980	-5.870	-3.820	2.603

Table (8): Fold change in gene expression of GSK3A and GAPDH genes after activation of studied groups

Group	Mean Ct of GSK3A	Mean Ct of GAPDH	mean ΔCt of GSK3A	ΔΔCt	Fold change
Control	28.820	28.710	0.110	-0.160	1.000
AS	28.590	28.180	0.410	0.140	0.812

The results in the current research found the GSK3A gene expression after activation in control and (AS) patients group reduced significantly with the GSK3A gene expression before activation. However, The GSK3 enzyme plays a central role in the male reproductive system. Applying the swim-up technique with FertiCult™ Flushing Medium as a pharmacologically inhibit GSK3 activity results in a significant rise in the proportion of spermatozoa exhibiting rapid and medium speeds, along with enhancements in all sperm velocity metrics. Furthermore, pretreating porcine spermatozoa with a GSK3 inhibitor notably increased the percentage of capacitated sperm (22). However, there are conflicting reports regarding the phenotype resulting from disruption of the GSK3A gene (23). Initially, it was reported that homozygous null mice lacking GSK3A exhibited normal fertility (24). More recently, however, studies originally intended to create a brain region-specific disruption found that male mice lacking GSK3A were infertile (23).

The seminiferous tubules of mouse testes express GSK3A, which intensifies during spermatogenesis onset and reaches maximum levels in adult testes (9). Although disruption of GSK3A does not affect spermatogenesis itself, it results in mature sperm with decreased motility and impaired metabolism, leading to male infertility (8).While the role of GSK3 in male fertility is well established in bovine and mouse models, knowledge about its function in human sperm remains limited. Besides reduced progressive motility and velocity, Sperm deficient in

GSK3A show a significantly diminished flagellar beat. Both activated and hyperactivated motility, marked by vigorous flagellar movements, are crucial for egg penetration and fertilization (25).

Conclusion

Observations from the current research, it was concluded, there was a significant decrease in the mean of sperm concentration, non-progressive sperm motility and round cells with significant increase of the percentage of progressive motility and normal sperm morphology after activation by swim - up technique compared to before activation. In asthenozoospermic patients group, the expression level of GSK3A gene after activation was significantly different compared to before activation.

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Conflict of Interest

The authors state, this work has no associated conflicts of interest.

Ethical Clearance

The Ethical Approval Committee of the Institute granted approval for this study.

Source of funding

There is no financial disclosure.

Author contributions

Dr. Saad S. Al-Dujaily was responsible for conceiving and designing the study. Noor Hani Al-Naji conducted the research, provided the necessary materials, and managed data collection and organization. Dr. Haider Ali Al-Naji handled data analysis and interpretation. The initial draft of the article was prepared by Dr. Saad S. Al-Dujaily and Noor Hani Al-Naji. Logistic support was provided by Dr. Saad S. Al-Dujaily and Dr. Ismail Hussein Aziz. All authors have thoroughly reviewed and approved the final version of the manuscript and take responsibility for its content and similarity index.

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