In vitro effect of Granulocyte-macrophage colony-stimulating factor (GM-CSF) on the expression of related genes to sperm motility and energy metabolism, and ICSI outcomes in obstructive azoospermic patients

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Abstract

Background
Granulocyte-macrophage colony-stimulating factor (GM-CSF) expressed in the human reproductive system, holds a pivotal role in the reproductive processes. This study investigates the in vitro effect of GM-CSF on the testicular sperm of obstructive azoospermia (OA) patients and assesses the effectiveness of GM-CSF-supplemented sperm media in Intracytoplasmic sperm injection (ICSI) outcomes.

Methods and Results
Following testicular sperm extraction from 20 patients diagnosed with OA, each sample was divided into two parts: the experimental samples were incubated with the medium containing 2 ng/ml GM-CSF at 37°C for 60 min, and control samples were incubated with medium without GM-CSF. Subsequently, the oocytes retrieved from the partner were injected with sperms from treatment and the control groups. The sperm parameters (motility, viability), the expression level of sperm motility-related genes (PIK3R1, PIK3CA, and AKT1), and sperm energy metabolism-related genes (GLUT1, GLUT3, and GLUT14) were assessed. Furthermore, the fertilization and cleavage rates and embryo quality were evaluated. Supplemented testicular sperm with GM-CSF significantly increased motility parameters, the mRNA expression of PIK3R1, AKT1, and GLUT3 compared to the non-treated group (p < 0.05). However, no significant differences in mRNA expression of PIK3CA, GLUT1, or GLUT14 were identified. Based on ICSI outcomes, the GM-CSF treatment group exhibited significantly higher fertilization rates (p = 0.027), cleavage rates (p = 0.001), and the proportion of good-quality embryos (p = 0.002) compared to the control group.

Conclusions
GM-CSF increased gene expression related to motility and energy metabolism pathway and effectively had a positive effect on the motility of testis-extracted spermatozoa and, consequently yielding positive clinical outcomes.

Introduction
Azoospermia accounts for approximately 10 to 15 percent of male infertility, manifesting in two predominant forms: obstructive and non-obstructive. Obstructive azoospermia is due to obstruction in the male genital tract. Conversely, non-obstructive azoospermia, being the more prevalent form, arises from a failure of spermatogenesis within the testis [1]. Intracytoplasmic sperm injection (ICSI) following testicular sperm extraction (TESE) has become an effective treatment for men with azoospermia in assisted reproduction programs [2]. The absence of motile spermatozoa is a common observation in specimens obtained through epididymal sperm aspiration and testicular biopsy, adversely affecting ICSI outcome and fertilization rates [3, 4]. Based on studies, testicular spermatozoa should be incubated in a sperm medium before ICSI [5]. The incubation of testicular spermatozoa for a duration of 1 to 2 hours before ICSI has been observed to induce motility. However, it is noteworthy that, in certain instances, spermatozoa may remain immotile even following the incubation period [6]. The application of certain compounds appears to enhance sperm parameters in vitro
among individuals diagnosed with azoospermia. Subsequently, the ICSI success rate and fertilization rates could be increased [4]. Researchers have reported that Pentoxifylline (PTF) functions as a chemical motility enhancer, eliciting an increase in cyclic adenosine monophosphate (cAMP), glycolysis, and energy production within testicular sperm [7]. However, apprehensions exist regarding the safety profile of this chemical compound. PTF has toxic effects when incubated with sperm for a prolonged period [8]; it is imperative to substitute the natural compounds within the male reproductive system to ameliorate sperm quality in vitro.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a crucial member of the cytokine family, primarily recognized for its role in the immune system where it governs the maturation and proliferation of granulocytes and macrophages [9]. GM-CSF promotes embryonic growth and development and regulates the implantation process and maternal immune response [10]. Studies have shown that GM-CSF supplementation to embryo culture media can increase the implantation rate and the number of live births [11]. The location and distribution of GM-CSF and its receptor alongside the male reproductive tract (epididymis and testis), seminal plasma and spermatozoa cells were previously proved [12–14]. Its receptor was expressed on the mid-piece and tail of mature spermatozoa [12, 13]. According to studies, about 25% of the interstitial cells in the testis are macrophages [15]. Interestingly, macrophages in the testis produce more GM-CSF than macrophages in other body parts [16]. On the other hand, it was demonstrated that exogenous GM-CSF increases testosterone concentration in prostatic adenocarcinoma [17].

It has been shown that infertile men have lower levels of GM-CSF in seminal plasma than fertile one [18]. GM-CSF is known to act in seminiferous tubules and spermatozoa, but its role on sperm function is not well understood. However, GM-CSF seems to have a significant role in sperm physiology [13], for example, it has been shown that GM-CSF can be beneficial as a post-freeze additive and may improve sperm quality after post-thawing incubation [19].

GM-CSF activates different signaling pathways in different cell types; one of them is the phosphoinositide-3-kinase/protein kinase B (PI3K/AKT) signaling pathway, which is involved in intracellular metabolic and anti-apoptotic processes; AKT indirectly promotes cell survival by regulating glucose-related metabolic processes [20].

It has been shown that GM-CSF stimulates glucose uptake by translocating glucose transporter 1 (GLUT1) via the PI3K/AKT pathway [21]. The PI3K/AKT pathway is a main pathway in regulating the motility and hyperactivation of spermatozoa [22]. GLUTs family are found in various tissues, including testes and spermatozoa, and help transport mono hexoses [23, 24] in sperm; also, the energy is produced through metabolic pathways between sertoli cells and germ cells [25]. Through GLUTs, such as GLUT1, GLUT3, GLUT8, and GLUT9, glucose is transported from interstitial space into sertoli cells [26]. These transporters are located in the plasma membrane [27]. Studies showed that GM-CSF increased bovine and human sperm motility [12, 28]. GM-CSF supplement improves sperm parameters and increases glucose transporter expression via the PI3K/AKT pathway in Oligoasthenoteratospermia (OAT) men [28]. Its effect on testicular biopsy in patients with obstructive azoospermia has not been evaluated yet. The present study assessed the in-vitro effect of GM-CSF on the gene expression of GLUTs, PIK3CA, PIK3R1, AKT1, and the motility and viability of sperm from testicular biopsies. In addition, the clinical outcomes of these sperm injections were assessed.

**Materials and Methods**
A total of twenty testicular samples were obtained from men with obstructive azoospermia treated for infertility. All patients who participated in the study underwent testicular biopsy as part of their treatment and provided written informed consent. The experimental protocol was approved by the ethical committee of Zanjan University of Medical Sciences, Zanjan, Iran, (IR.ZUMS.REC.1398.357).

All patients were evaluated by semen analysis, hormone levels (serum FSH, LH and testosterone), and physical examination. In the present study, all male patients with infertility were diagnosed with azoospermia. Lack of spermatozoa in semen samples and the pellet after centrifugation (20 minutes at 3000 g), used as the diagnosis for azoospermia. Reconstructive surgical interventions were considered as a therapeutic modality to repair obstructive anomalies within the male reproductive tract. In instances where reconstructive procedures proved unfeasible, male patients proceeded to undergo diagnostic TESE as an alternative approach to perform intracytoplasmic sperm injection-embryo transfer. Male partner with non-obstructive azoospermia, including Y-chromosome deletions, spinal cord injuries, hypogonadotropic hypogonadism and Klinefelter syndrome were excluded from this study. Following TESE, individuals displaying a lack of spermatozoa in the biopsy specimens were subsequently excluded from the study cohort. Demographic information of the studied couples are shown in the Table 1.

Table 1
Demographic information of the studied couples

<table>
<thead>
<tr>
<th>Male partners</th>
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<tr>
<td>Age (year)</td>
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<tr>
<td>BMI</td>
<td>24.45 ± 3.9</td>
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<td>FSH (mIU/ml)</td>
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<table>
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<td>BMI (Kg/m²)</td>
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<td>AMH (ng/μl)</td>
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</table>

**TESE procedure, sperm extraction, and incubation**

The TESE procedure was carried out by skilled urologists. In brief, a transverse incision measuring 2 cm was meticulously made on the anterior scrotal skin and tunica albuginea, followed by the injection of lidocaine into the underlying tunica. Subsequently, small testicular parenchymal specimens were excised from the testis, placed in a Petri dish containing sperm wash medium, and meticulously separated using sterile needles. The
dissected tissue fragments were subjected to microscopic scrutiny using an inverted microscope to ascertain the presence of spermatozoa.

Following sperm observation, the homogenate underwent a wash procedure and centrifugation at 2000 rpm for 5 minutes. The resulting precipitated sample was then partitioned into control and treatment groups in cases where sperm retrieved through TESE exhibit poor motility. In the treatment group, the sample underwent incubation in an IVF medium (Cook, USA) enriched with 2 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) [28]. Conversely, the control group’s sample was incubated in the same medium without the inclusion of GM-CSF. Both groups were subjected to incubation for one hour at 37°C and 5% CO₂ (27).

**Ovarian stimulation and ICSI**

Ovarian stimulation was performed with long agonist protocol; stimulation began with the administration of Gonadotropin-releasing hormone (GnRH) agonist (triptorelin) at a dose of 0.1 mg on day 21 of the menstrual cycle. Subsequently, gonadotropin (Cinal-F or Gonal-F) was administered daily at a dose ranging from 150 to 225 IU, adjusted based on individualized female parameters, including anti-Müllerian hormone (AMH) levels, age, and antral follicle count (AFC). Continuous administration of both GnRH agonist and gonadotropin persisted until the visualization of at least two follicles measuring 16 to 18 mm in diameter on ultrasound. Upon achieving this criterion, the administration of gonadotropin and GnRH agonists was halted and human chorionic gonadotrophin (hCG) was administered. Follicular aspiration was conducted 36–38 hours subsequent to hCG injection [29].

Subsequent to the oocyte retrieval procedure, cumulus cells enveloping the oocytes were isolated through mechanical pipetting and the application of hyaluronidase enzyme. Post-isolation, the oocytes underwent meticulous examination to assess both morphological characteristics and maturation stage. The Metaphase II oocytes were subsequently randomly divided into two groups, wherein one group received sperms treated with GM-CSF, and the other group received untreated sperms. The oocytes, following the injection procedure, were cultured in a one-step medium (ORIGIO). Fertilization status was assessed 16–18 hours post-insemination, and on day 3, both cleavage rate and embryo quality were evaluated. The embryo quality was evaluated based on the grading criteria previously described [28].

**Sperm parameter assay**

Sperm parameters, including motility and viability, were evaluated after incubation time. The percentage of sperm with progressive and non-progressive motility was evaluated under a phase-contrast microscope (×40 magnification) (Olympus, Japan). The eosin-nigrosine staining technique was used to assess sperm viability; each slide was stained, then counted under the light microscope (×100 magnification) [30].

**Real-time quantitative PCR**

Total RNA from about 50 mg of testicular biopsy was extracted using RiboX reagent (Gene ALL) according to the manufacturer’s instructions. The concentration and quality of RNA were determined by using a spectrophotometer (Epoch Microplate, Biotech, USA); then, the extracted RNA was applied for the cDNA synthesis using the cDNA Reverse Transcription Kit (SMO-BIO) according to the instructions of the cDNA synthesis kit. Real-time PCR analysis was performed using the SYBR-Green master mix (AMPLIQON). Primers for the target genes PIK3R1, PIK3CA, GLUT1, GLUT3, GLUT14, AKT1, and the reference gene (GAPDH) were designed
according to Gene Runner and BLAST. The specific primer pair sequences for individual genes are presented in Table 2. The $2^{-\Delta \Delta CT}$ process was used to calculate the relative expression levels of the target genes. All assays were performed in duplicate for two original amounts of total RNA.

<table>
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<tr>
<th>Gene names</th>
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<th>Reverse sequence (3′→5′)</th>
<th>Annealing temperature (°C)</th>
<th>Size (bp)</th>
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<tr>
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<tr>
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<td>TAAGCAAAATCCTCCAGCGGTT</td>
<td>GAGCACAACGGAATGATGATG</td>
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<td>CATCAAGAAGGTGGTGGAAGCAG</td>
<td>GCGTCAAGGATGAGGAGTGA</td>
<td>60</td>
<td>120</td>
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</table>

### Statistical analysis

Statistical analyses were performed utilizing the Statistical Package for the Social Sciences (SPSS 24.0, SPSS Inc., USA). The comparison between the two groups was executed through the t-test, assuming normal distribution of the variables, and significance was defined at a P value less than 0.05. The results are presented as mean ± standard deviation.

### Results

#### Effect of GM-CSF treatment on sperm parameters

The impact of granulocyte-macrophage colony-stimulating factor (GM-CSF) on sperm motility and viability was investigated following a one-hour incubation period at 37°C with 5% CO₂. Comparative analysis revealed a statistically significant increase in progressive motility (12.81 ± 4.89 vs. 6/81 ± 2.63, respectively) and non-progressive motility (23.63 ± 9.26 vs. 14.27 ± 10.82, respectively) in the GM-CSF-supplemented group compared to the control group (p = 0.001 and p = 0.04, respectively). On the other hand, immotile sperm cells were significantly decreased in the GM-CSF-supplemented group compared to the control group (63.63 ± 11.89 vs. 79.54 ± 11.99). Conversely, no significant difference in sperm viability was determined between the GM-CSF-treated and untreated groups (p > 0.05) (Figure 1).

#### Testicular GLUTs, PIK3R1, PIK3CA, and AKT1 genes mRNA expression

In the present study, the GM-CSF treatment significantly increased the testicular mRNA expression of PIK3R1 and AKT1 compared to the control group. Also, among GLUTs, GM-CSF treatment significantly increased the
testicular expression of GLUT3 compared to the control group. Unlikely, no significant differences in testicular mRNA expression of PIK3CA, GLUT1, or GLUT14 were identified between the two groups (Fig. 2).

**Evaluation of ICSI outcomes in obstructive azoospermia patients**

The results of the clinical data are shown in Table 3. Significant differences were observed between the control group and GM-CSF treatment in ICSI clinical results (fertilization rate and embryo quality). The fertilization rate in the GM-CSF treatment group (84.26±11.14) showed a significant increase compared to the control group (75.52±13.94) (p = 0.02). The percentage of high-quality embryos (Grade 1) in the treatment group (50.28% ± 28.07) showed a significant increase compared to the control group (32.88%±24.41) (p = 0.002). The percentage of poor-quality embryos (Grade 3) in the GM-CSF treatment group (12.19%±15.32) was decreased significantly compared to the control group (26.03%±27.14) (Fig. 3, Table 3). The cleavage rate in the GM-CSF treatment group (80.50%±14.17) were significantly increased compared to the control group (66.47%±20.81) (P = 0.001).

<table>
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<th>Parameters</th>
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<th>GM-CSF treatment</th>
<th>P Value</th>
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</thead>
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<tr>
<td>Fertilization rate (%)</td>
<td>75.52 ± 13.94</td>
<td>84.26 ± 11.14</td>
<td>0.027*</td>
</tr>
<tr>
<td>Cleavage rate (%)</td>
<td>66.47 ± 20.81</td>
<td>80.50 ± 14.17</td>
<td>0.001*</td>
</tr>
<tr>
<td>Grade 1 (%)</td>
<td>32.88 ± 24.41</td>
<td>50.28 ± 28.07</td>
<td>0.002*</td>
</tr>
<tr>
<td>Grade 2 (%)</td>
<td>39.46 ± 21.42</td>
<td>34.95 ± 21.39</td>
<td>0.153</td>
</tr>
<tr>
<td>Grade 3 (%)</td>
<td>26.03 ± 27.14</td>
<td>12.19 ± 15.32</td>
<td>0.011*</td>
</tr>
</tbody>
</table>

* significance at P<0.05

**Discussion**

The results of this study reveal a multifaceted impact of GM-CSF on both sperm functionality and testicular gene expression, with consequential effects on in vitro fertilization outcomes. Sperm extracted from testicular tissue has poor motility in azoospermia patients undergoing ART Procedure [8]. Various investigations have been undertaken to enhance the in vitro quality of testicular spermatozoa, encompassing interventions such as pentoxifylline (7), 2-deoxyadenosine (30), L-carnitine, and L-acetyl-carnitine. such as pentoxifylline [7], 2-deoxyadenosine[31], L-carnitine, and L-acetyl-carnitine [8, 32]; these compounds could significantly improve sperm motility. However, Pentoxifylline and 2-deoxyadenosine can cause embryotoxic effects, as well as deplete the metabolic resources of sperm, which makes treated sperm permanently immotile [6].

To date, no studies have been conducted to assess the in vitro impact of GM-CSF on the quality of testicular spermatozoa. Our data elucidates that the supplementation of granulocyte-macrophage colony-stimulating factor (GM-CSF) in testicular sperm culture media results in a notable improvement in total motility. This observation aligns with the hypothesis that GM-CSF, as a cytokine secreted by the testis, plays an essential role in the process of sperm maturation [12]; In accordance with the investigation conducted by Vilanova et al., GM-
CSF demonstrated a stimulatory effect on sperm motility in bovines [12]. Remarkably, the in vitro supplementation of GM-CSF demonstrated a beneficial impact on sperm parameters in individuals with OAT [28]. In the present investigation, the inclusion of GM-CSF in the testicular sperm medium resulted in an elevation of the total motility rate. Conversely, the proportion of viable testicular sperm exhibited comparability between the treatment and control groups, indicating that GM-CSF did not exert cytotoxic effects. Rodriguez et al. demonstrated that GM-CSF serves as a protective agent against cryoinjuries, preserving motility in ram spermatozoa during the freezing/thawing process [14]. Hence, according to these findings, granulocyte-macrophage colony-stimulating factor (GM-CSF) has the potential to enhance sperm motility during a one-hour incubation period and exhibits no cytotoxic properties or adverse effects on testicular spermatozoa.

As per other research, GM-CSF is documented to instigate the activation of cellular metabolic signaling pathways. This activation, in turn, facilitates the uptake of glucose through the direct activation of GM-CSF receptors and interaction with glucose transporters [21]. Our previous investigation showed that GM-CSF supplement in culture medium improved sperm parameters via PI3K/AKT signaling pathway in OAT men and elevated GLUT1 and GLUT3 in spermatozoa [28]. This cytokine also increases the glucose and vitamin C uptake in the other cell types including neutrophils, monocytes, and HEK 293 cells [21]. In the sperm cells [12], mouse embryos [33], and granulosa cells [34], GM-CSF facilitates hexose transporters, thereby promoting the uptake of glucose. Human testes tissue and spermatozoa cell express different hexose transporters; therefore spermatozoa demonstrate the capacity to transport fructose, glucose, and vitamin C facilitated by the activity of these specific hexose transporters [35]. However, GLUT3 exhibited the highest affinity among the glucose transporters. According to immunocytochemistry studies, GLUT3 is expressed in the human testes' sperm and seminiferous tubule [36]. In the present investigation, GM-CSF upregulated the expression of GLUT3 mRNA in the testicular tissue, potentially influencing glucose uptake. The proximity of GLUT3 to the mitochondria was observed, leading to an elevation in mitochondrial membrane potential (MMP) and metabolic activity [28]. Recent investigations, such as that conducted by Wessendarp et al., have substantiated novel roles of granulocyte-macrophage colony-stimulating factor (GM-CSF) in regulating metabolism and mitochondrial functions. These roles encompass a substantial increase in glycolysis, activation of the pentose phosphate pathway, enhanced amino acid synthesis, modulation of the tricarboxylic acid cycle, stimulation of oxidative phosphorylation, and augmented ATP production [37]. In the present investigation, the GM-CSF treatment group exhibited a substantial increase in the expression levels of key genes associated with the PI3K/AKT pathway, specifically PIK3R1 and AKT1, implying the activation of this signaling pathway within testicular cells. Furthermore, our prior findings have already demonstrated the activation of the PI3K/AKT pathway in spermatozoa of individuals with OAT following GM-CSF treatment, thereby aligning with the outcomes observed in this study.

In addition to protein kinase B (or AKT), other types of protein kinases including protein kinase G (PKG) and PKA and mitogen-activated protein kinases (MAPKs) have also been found in sperm cells which regulate the cell biology of sperm motility [22, 38, 39]. During the incubation period of spermatozoa cells in a culture medium enriched with energy substrates, there is a progressive reduction in motility. However, it appears that the phosphorylation status of PI3K/Akt serves to rescue sperm cells from this phenomenon, as illustrated in Fig. 4. In other words, the disruption or inhibition of either PI3K or Akt leads to cellular descent into an apoptotic cascade marked by a substantial suppression of motility, the initiation of oxidative DNA damage, and ultimately, the activation of caspases [40, 41]. Some growth factors/cytokines, identified as prosurvival factors by previous
investigators, have been proposed to safeguard sperm cells from degenerative processes. Notably, prolactin, progesterone, and extrapancreatic insulin have been recognized for their involvement in the PI3K/Akt signaling pathway [22, 40, 42]. An in vitro study by Zhang et al. demonstrated that leucine supplementation also was able to activate PI3K/AKT pathway to enhance the motility of spermatozoa [39].

To date, investigations exploring the in vitro impact of GM-CSF on the quality of testicular sperm and subsequent ICSI outcomes are lacking. Nevertheless, in OAT patients, the addition of GM-CSF to sperm media demonstrated an enhancement in fertilization rates and improvements in embryo quality [28]. The present study confirms the previous findings and establishes that GM-CSF exerts a positive influence on ICSI outcomes in azoospermia patients. Aliabadi et al. similarly identified the beneficial impact of additional substances, such as L-carnitine and L-acetyl-carnitine, in enhancing testicular sperm motility [8]. Pentoxifylline can improve sperm motility; however, studies showed this compound has embryotoxic effects; based on research, Pentoxifylline significantly reduced fertilization rates and embryo development compared to controls [43, 44]. In contrast to Pentoxifylline and 2-deoxyadenosine, the current study demonstrates that granulocyte-macrophage colony-stimulating factor (GM-CSF) augments intracytoplasmic sperm injection (ICSI) outcomes, including improvements in embryo quality, cleavage rate, and fertilization rate. As an alternative to Pentoxifylline, GM-CSF exhibits the potential to enhance testicular sperm motility preceding ICSI.

Conclusion

GM-CSF upregulated gene expression associated with motility and the energy metabolism pathway, including the PI3K/AKT pathway. Additionally, GM-CSF enhanced glucose uptake through GLUTs, culminating in a positive impact on the motility of spermatozoa extracted from the testis and subsequently influencing clinical outcomes. The supplementation of GM-CSF in sperm culture media holds the potential to improve sperm parameters in individuals diagnosed with obstructive azoospermia.

Abbreviations

GM-CSF: Granulocyte-macrophage colony-stimulating factor
ICSI: Intracytoplasmic sperm injection
ART: assisted reproductive technology
TESE: testicular sperm extraction
PTF: Pentoxifylline
GLUTs: glucose transporters
GLUT1: glucose transporter 1
PI3K/AKT: phosphoinositide-3-kinase/protein kinase B
OAT: Oligoasthenoteratospermia
GnRH: Gonadotropin-releasing hormone

hCG: human chorionic gonadotrophin

**Declarations**

**Ethics approval and consent to participate**

The experimental protocol was approved by the ethical committee of Zanjan University of Medical Sciences, Zanjan, Iran, (IR.ZUMS.REC.1398.357). All participants signed informed consent to participate.

**Consent for publication**

Not applicable

**Availability of data and materials**

All data generated or analysed during this study are included in this published article

**Competing interests**

The authors declare that they have no conflict of interest.

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**Authors' contributions**

FTKS: Original draft preparation. EH., FSA., ZZ., and MM: Conceptualization, and Methodology. EH., and FSA: Supervision. MA., and FM: patient recruitment. FTKS., and RK: Performing laboratory works, collecting the data and analysis. All authors read and approved the final manuscript.

**Acknowledgements**

Not applicable

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Figures

Figure 1
A comparison of sperm parameters (a: motility and b: viability) in control and GM-CSF treatment groups in patients with obstructive azoospermia. Data are expressed as means ± standard deviation. ** P < 0.005 * P < 0.05

**Figure 2**

Assessment of gene expression of GLUT1, GLUT3, GLUT14, PIK3R1, PIK3CA, and AKT1 in control and GM-CSF treatment groups. Results were presented as mean ± standard deviation. * P<0.05, *** P< 0.0005
Figure 3

Final oocyte grade in two groups (Control and GM-CSF treatment)
Figure 4

Schematic representation of regulatory mechanisms involved in sperm motility. Created with Biorender.com