



# Expression of *MCM6* in the Sperm of Teratozoospermia Infertile Males

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## Abstract

**Objectives:** *MCM6* is an essential member of the MCM family and a key component of the DNA replication machinery that regulates cell cycle progression and genome stability. Previous studies suggested that genome integrity is an important factor in male infertility. Therefore, reducing the expression of this gene may be effective in male infertility, which has not been investigated yet. We aimed to evaluate the expression level of this gene in the sperm of infertile males with teratozoospermia and compare it with control individuals.

**Material and Methods:** In this study, we included a total of 20 fertile healthy males in the control group and 40 individuals with teratozoospermia in the case group. After cDNA synthesis and total RNA extraction, relative quantification was carried out. The normalizer gene utilized was GAPDH, and the fold change was computed using the  $2^{-\Delta\Delta Ct}$  formula. Considering a significance threshold of  $P < 0.05$ , a t-test was employed to compare the expression levels between the case and control groups.

**Results:** According to our findings, teratozoospermia males had significantly lower levels of *MCM6* ( $\log_{2}FC=0.065$ ) than controls. Moreover, the DNA fragmentation index of normal individuals was  $16.00 \pm 3.00$ , while this index was  $33.72 \pm 5.098$  for teratozoospermia patients.

**Conclusions:** Since *MCM6* downregulation is associated with teratozoospermia, it might be proposed as a possible candidate gene for the progression of this condition. Also, it could be considered as a potential diagnostic marker and have other therapeutic uses.

**Keywords:** Infertility, Teratozoospermia, DNA replication, *MCM6*, and DNA integrity

## Introduction

About one-fifth of couples attempting to conceive have infertility, which is a problem with the reproductive system that affects people worldwide (1). Male factors account for 50% of infertility cases, and 75% of these instances are caused by unidentified variables since the molecular processes underlying the problems are unclear (2). Male infertility is clinically analyzed through different semen factors, including quantity, count, motility, and other morphological evaluations (3). However, in recent years, researchers have focused on genetic variations, which can be another important factor causing infertility.

Several genetic and environmental variables, including old paternal age and psychological stresses as well as genetic factors like aberrant expression of DNA replication machinery, have been linked to teratozoospermia, which is the outcome of a failure in cell differentiation during spermatogenesis; however, its molecular origin remains unknown in most cases. Sperm DNA undergoes mutations as a result of higher cell division cycles, decreased DNA replication fidelity, ineffective DNA repair, and buildup of mutagens from both internal and external sources

(4,5). Thus, the importance of sperm DNA integrity has been highlighted in different studies on male infertility. Moreover, male infertility seems to be primarily caused by DNA damage, notably in the form of DNA fragmentation (6). In fact, spermatozoa with DNA fragmentation are more common in infertile men's semen samples than in fertile individuals (7). Therefore, different studies are investigating the onset and progression of infertility, with a focus on DNA replication.

As a replicative DNA helicase, the minichromosomal maintenance family (MCMs) plays a crucial part in DNA replication and provides a hexameric ring-shaped complex around DNA (8, 9). MCM proteins, which are crucial for DNA replication in all eukaryotic cells and play a significant role in regulating replication throughout each cell cycle, were initially identified in the yeast *Saccharomyces cerevisiae* (10). At least ten human homologues have been identified. Of these, the MCM2-7 complex shows helicase activity that recruits DNA polymerase and starts DNA replication and elongation (11). It also takes role in the creation of the pre-replication complex. As a crucial component of the MCM family, *MCM6* can interact

Received 2 June 2023, Accepted 24 August 2023, Available online 6 September 2023

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## Key Messages

- ▶ The expression of the *MCM6* gene decreased in individuals with teratozoospermia.
- ▶ The expression of the *MCM6* gene decreased with increasing sperm DNA fragmentation

with Chromatin licensing and DNA replication factor 1 (Cdt1) protein at the N-terminal domain (NTD) to maintain the coil structure, be phosphorylated by Dbf4 dependent kinase (*DDK*) at the NTD terminus to activate prereplication complex, and be engaged in translocation along single-stranded DNA in the MCM2-7 complex (12). Teratozoospermia is a common cause of male infertility, affecting sperm morphology and quality. Several genetic and environmental factors have been linked to teratozoospermia, but its molecular mechanism is not yet fully understood. Previous studies have suggested that DNA replication and cell cycle regulation in genome stability can affect teratozoospermia. *MCM6* is a key member of the DNA replication machinery that regulates genome stability. However, the role of *MCM6* in teratozoospermia is not still fully understood.

This study aimed to investigate the changes in *MCM6* expression in men with teratozoospermia and oligozoospermia so that the molecular mechanisms affecting teratozoospermia can be more clearly identified and provide a basis for future studies. Accordingly, we investigated the sperm expression level of *MCM6* from teratozoospermia in comparison to the healthy individuals to find a possible abnormal alteration in its expression level.

## Material and Methods

### Study Population

The population of this study included all males referred to the medical clinics in Tehran, Iran from January 2021 to January 2022. A short medical history was gathered using a combination of direct questioning and reviewing the clinical records or self-reported questionnaires. Samples of sperm were collected in accordance with the World Health Organization guidelines (2010). We collected the semen of 40 males with teratozoospermia (case group) and 20 healthy individuals (control group). Individual information was also reviewed using a questionnaire. The age range of participants was 29-44 years. We excluded all individuals younger or older than the mentioned age group, those with specific medication or radiotherapy use, and people with a history of diseases such as cancer, autoimmune disorders, diabetes, varicocele, and infection.

### Semen Collection

All participants provided a semen sample by masturbation after a period of abstinence for three to five days. The samples were classified as normal or teratozoospermia

based on standard sperm counts (sperm concentration, motility, morphology, and viability) in accordance with the WHO guidelines (2010). To assess the semen quality, we considered the appearance of all samples; the samples with a milky color were accepted and all bloody, yellowish, or brownish samples were rejected. The volume of the samples was between 2 and 6 ml, and sperm morphology was checked for defects in head, midpiece, and tail. In this study, sperms were classified according to the WHO's (2010) definition of sperm motility as progressive (grade A+B), non-progressive (grade C), and immotile (grade D).

### RNA Extraction, cDNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction

The samples were washed with phosphate-buffered saline (PBS) solution. Then, one to 5 million cells were collected by centrifugation at 300 rpm for 5 minutes. Next, RNA extraction was performed according to the kit protocol and total RNA extraction was conducted from sperm pellets using a RiboEXSL RNA extraction kit (Favorgen Biotechnology). Total RNA purity and quantity were measured by spectrophotometry using a Nanodrop 2000 (Thermo Scientific, USA). The RNA samples were frozen at 80 °C after extraction. RNA clean up and concentration was done (Norgen Biotek Corp kit). To remove DNA contamination from RNA samples, the kit of SinaClon company was used (SinaClon Company, Iran). Following the instructions provided by the manufacturer, we synthesized cDNA from 0.5 g of total RNA using the Reverse Transcription kit. Based on the manufacturer's protocol, real-time polymerase chain reaction (PCR) was conducted using the RealQ Plus Master Mix Green high ROX™ kit gene (Ampliqon, Denmark). All reactions were performed in accordance with the guidelines provided by the manufacturer using an ABI Applied Biosystems StepOne Plus Real-Time PCR instrument (Fisher Scientific, Germany). In a total volume of 25 l, we utilized 12.5 μL of SYBR Premix, 0.5 μL of each primer, and 20 ng of cDNA as a template. To thermally cycle the amplification processes, they were first denatured at 95 °C for 30 seconds, then annealed at 60 °C for 30 seconds, and then extended at 72 °C for 30 seconds. Target mRNA expression levels were normalized to those of human glyceraldehyde-3-phosphate dehydrogenase beta (*GAPDH*), and the fold change in expression was determined using the  $2^{-\Delta\Delta Ct}$  relative expression formula. To guarantee that RNA and not genomic DNA is amplified, the primer sets were constructed at exon-junction or between two neighboring exons separated by a large intron. The expression of *MCM6* and *GAPDH* was measured using the primers given in (Table 1).

### Sperm Morphology

The morphology of the sperm was assessed according to the WHO guidelines (2010) and using Diff-Quik dye

**Table 1.** The List of Primers Used in This Study

Gene name	Primer sequence (5' to 3')
MCM6 (Forward)	ACCTGCCTACCAGACACAAG
MCM6 (Reverse)	ACAGAAAAGTTCCGCTCACAAG
GAPDH (Forward)	CATCAAGAAGGTGGTGAAGCC
GAPDH (Reverse)	AAATGAGCTTGACAAAAGTGGTCG

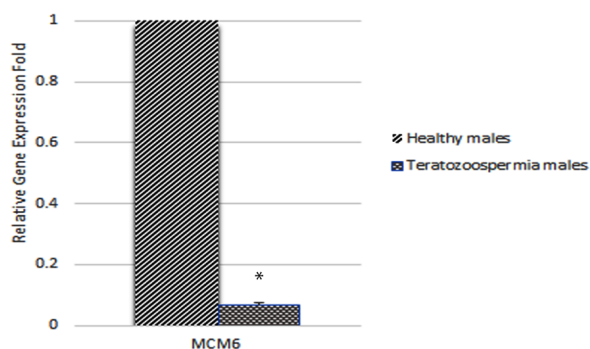
solutions. One drop of each of the samples was applied, dried, and stained. The dry slides were incubated for a total of 3 minutes: 75 seconds in fixation solution, 60 seconds in staining solution, and 35 seconds in detaining solution. After washing and drying with distilled water, a microscope was used to reveal their appearance.

### Sperm Chromatin Dispersion Method for DNA Fragmentation Detection

Sperm chromatin dispersion testing was done utilizing a halo sperm kit (Idevarzan-e-Farda Co., Tehran, Iran) to find DNA fragmentation (Figure 1). The preparation of the sperm samples followed the manufacturer's instructions. Before adding 50  $\mu$ L of the samples to the agarose, each sample was rinsed twice in PBS. After that, 30  $\mu$ L of the sample was put on a glass slide, covered with foil, and put in the freezer for 5 minutes. The slides underwent denaturation and were treated with slip solutions. Each sample was washed with distilled water for 5 minutes prior to being dehydrated with an increasing gradient of ethanol (70%, 90%, and 100%). The samples were dried after being washed. Using a light microscope, 200 sperm cells from each plate were examined for halo analysis (1000x magnification). Sperms without or with small halos were assumed to have fragmented DNA, while sperms with medium-sized or larger halos were assumed to have intact DNA.

### Statistical Analysis

The Statistical Package for the Social Sciences, version 16 (SPSS, Chicago, IL, USA) was used for all statistical analyses. The mean differences between the two groups were evaluated using a t-test. All data were expressed as mean  $\pm$  SD. *P* value < 0.05 was considered as statistically significant.

**Figure 1.** Diagram of MCM6 Gene Expression (\**P* value < 0.05)

## Results

### Demographic Data

A Shapiro-Wilks test showed that the data in the research were normal. The mean age of participants in both groups was 36.5 years, with a minimum age of 29 years and a maximum of 44 years (*P* < 0.05). Table 2 displays the findings of the semen analysis. Sperm morphology (*P* < 0.001) and motility (*P* < 0.001) significantly varied between the two groups (Table 2).

### MCM6 Expression in Teratozoospermia

Ejaculated sperm samples from teratozoospermia men and controls were analyzed for *MCM6* and *GAPDH* mRNA expression levels using quantitative real-time PCR. In the teratozoospermia male group, we observed that *MCM6* was downregulated by fold-change of  $0.065 \pm 0.01$  on average, compared to the control group (Figure 1).

### DNA Fragmentation Index in Teratozoospermia

Our halo sperm results revealed that teratozoospermia males possess higher index of DNA fragmentation rate; in this regard, our results revealed that the index of normal individuals was  $16.00 \pm 3.00$ ; this is while this index for teratozoospermia patients was  $33.72 \pm 6.40$  (Table 2).

## Discussion

The descriptive microscopic assessment of ejaculate sperm, including quantity, motility, and morphology, forms the backbone of regular and conventional semen analysis (13). Since regular semen analysis does not represent the integrity of the male genome, it is not the sole approach to properly discern between fertile and infertile men (14-16). Therefore, the most definitive information on sperm DNA may be obtained by DNA integrity evaluation, which has been proposed as an independent supplementary measure of fertility (17). It is possible that biochemical steps during embryogenesis, including DNA replication, transcription, and translation, are disrupted when a sperm with damaged DNA fertilizes an oocyte. Increased DNA fragmentation rate in sperm has been shown to have a deleterious impact on both spontaneous and aided conception (18-20).

**Table 2.** Spermogram Characteristics in Males With Normal and Teratozoospermia Sperms

Parameters	Control	Teratozoospermia
Age	37.22 $\pm$ 6.55	40.05 $\pm$ 2.94
Sperm count	44.44 $\pm$ 18.85*	29.85 $\pm$ 22.45*
Sperm motility	73.88 $\pm$ 7.96**	47.30 $\pm$ 23.18**
Grade A	10.55 $\pm$ 7.45*	5.90 $\pm$ 7.04*
Grade B	51.11 $\pm$ 7.96**	33.15 $\pm$ 17.7**5
Grade C	12.22 $\pm$ 4.91*	9.15 $\pm$ 5.13*
Non-motile	26.11 $\pm$ 7.96**	52.70 $\pm$ 23.18**
Sperm morphology	5.77 $\pm$ 0.17**	1.05 $\pm$ 0.65**
DNA fragmentation index	16.00 $\pm$ 3.00*	33.72 $\pm$ 6.40*

\**P* < 0.05; \*\**P* < 0.001.

Our results indicated that infertile males had higher rate of DNA fragmentation compared to the healthy individuals. Consequently, standard clinical investigations of infertile males should incorporate sperm DNA integrity as an important contributing factor in distinguishing fertile from infertile males. Numerous studies have shown that rising DNA fragmentation negatively impacts reproductive success (21, 22). Consequently, uncovering new genes associated with infertility may be done by studying dysregulation and aberrant expression of any components, such as those involved in DNA replication machinery and proteins that preserve DNA integrity. MCM complex family members coordinate the onset and elongation phases of DNA replication and the DNA damage response. Moreover, their role is clear in the maintenance of DNA integrity (23). In this regard, in human tumors, such as epithelial ovarian carcinoma, researchers found a negative correlation between RBM3 expression and that of the *MCM3* gene and protein, indicating the fact that MCM family members may at least partially play a crucial role in the DNA integrity and that of infertility pathogenesis (24,25). Since the MCM complex is responsible for DNA repair, DNA replication, and DNA integrity maintenance, we hypothesized that a change in *MCM6* gene expression may play a role in the development of male infertility.

Most studies have focused on the importance of different MCM family members in the progression of infertility, and studies on the *MCM6* are limited. Accordingly, male and female human gonads rely on *MCM8*, a component of the pre-replication complex, for proper development and function (26). Furthermore, deleterious missense variants in *MCM8* have been reported to have a role in both female and male infertility based on a whole-exome sequencing study (27). Two sisters suffering from premature ovarian insufficiency were found to have a homozygous frameshift mutation in the *MCM8* gene; functional investigation confirmed that this mutation is harmful (28). Reproductive system abnormalities and a loss of germ cells were seen in both male and female *MCM9*-mutant mice, leading researchers to hypothesize that *MCM8* and *MCM9* play a role in genome maintenance during meiotic replication-induced stress (29,30). However, other members of MCM family are poorly investigated. In the present study we showed that the expression level of *MCM6* is downregulated in teratozoospermia males in comparison to healthy individuals. Further in silico and in vitro research can help to reveal the specific mechanism of action associated with *MCM6* and its other family members in the progression of infertility among males.

#### Limitations of the study

The limitation of our study was the sample size, particularly due to our restricted access to normal samples in infertility centers.

## Conclusions

According to our results, reducing the expression of the *MCM6* gene was associated with DNA fragmentation and teratozoospermia in infertile men. Therefore, this gene may be used as a marker for teratozoospermia, and further research may suggest therapeutic therapies for male infertility. To the best of our knowledge, this is the first study to report an alteration in the expression of an MCM family element in teratozoospermia males; this result can shed light on more precise pathological mechanisms involved in male infertility.

#### Authors' Contribution

**Conceptualization:** Mina Moayeri and Mohammad Salehi.

**Data curation:** Mina Moayeri, Mohammad Salehi, and Marefat Ghaffari Novin.

**Formal analysis:** Mohammad Salehi.

**Funding acquisition:** Mina Moayeri.

**Investigation:** Mohammad Salehi and Shiva Irani.

**Methodology:** Shiva Irani and Iman Salahshourifar.

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**Supervision:** Shiva Irani, Marefat Ghaffari Novin and Mohammad Salehi

**Validation:** Mina Moayeri and Mohammad Salehi.

**Visualization:** Mina Moayeri.

**Writing—original draft:** Mina Moayeri.

**Writing—review & editing:** Mohammad Salehi.

#### Conflict of Interests

Authors declare that they have no conflict of interests.

#### Ethical Issues

All couples included in the study signed an informed consent, and the research was approved by the ethical board of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics No. IR.SBMU.RETECH.REC.1396.1296).

#### Financial Support

This study was supported by Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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