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Research Article

Phenotype Compensation in Reproductive ADAM Gene Family: A Case Study with ADAM27 Knockout Mouse

Jafar Khezri, Ehsan Hasheni, Mehdi Shams Ara, Aidin Rahim Tayefe, Farid Heidari*

Department of Animal Biotechnology, Faculty of Agriculture Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

**Corresponding author*: Farid Heidari, Department of Animal Biotechnology, Institute of Agricultural Biotechnology, National Institue of Genetic Engineering and Biotechnology(NIGEB),Tehran Iran. Tel: +98-44787314, Fax: +98-2144787399, E-mail: heidari@nigeb.ac.ir

Background: A disintegrin and metalloproteinase (ADAM) cell surface proteins are expressed in different cells and are involved in biological processes such as cell-cell interactions, cell differentiation, sperm attachment and fertilization. A significant number of ADAM isoforms are expressed in the reproductive tracts of male mice and other mammals, which shows the importance of this gene family in reproduction.

Objectives: The role of ADAM27 protein in reproduction was investigated.

Materials and Methods: ADAM27 knock-out mutant mice were generated using the blastocyst microinjection technique. The knock-out mice were analyzed genetically and phenotypically to discover any abnormalities.

Results: The results of this study revealed that the homozygote mutant male mice were fertile and showed no significant differences compared to wild-type male mice. A histological exam, sperm analysis and in-vitro fertilization experiments showed no statistical differences.

Conclusions: We can conclude that the role of deficient ADAM27 protein is probably compensated mainly by other ADAM isoforms which are expressed in the reproductive system.

Keywords: ADAM27, Fertility, Knockout mouse

1. Background

A disintegrin and metalloproteinase (ADAM) has been reported in a wide range of organisms. At least 40 members of the ADAM family have been identified in chromosomes in different cells. Most ADAMs have a preserved structure that comprises several domains. The disintegrin ADAM domain contains motifs that bind to integrin receptors in target cells (1). Integrin receptors are heterodimer cell membrane proteins that play a critical role in cell-cell and cell-matrix interactions (2). There are cysteine-rich and EGF-like domains in the ADAM protein structure that contribute to the recognition and binding of specific substrates (3). Some cysteine-rich ADAM domains contain virus-like fusion peptide sequences and probably are involved in cell fusion (4).

Some ADAM domains play critical and specific roles in protein localization (5), protease activity (6-7) as well as cell signaling (8-10) and regulation (11-13). Multiple functions are expected, especially in cellular process such as neuron differentiation (14), cell migration (15-16), cell determination (17), skin differentiation and hair cycling (18), cell-cell interactions (19) and membrane fusion (20-22). Several functional domains have been reported in ADAM.

ADAM genes are expressed in different tissues. A remarkable number of them are expressed in the male reproductive system and this expression bias increases the possible role of these proteins in reproduction (21). Several cell-cell and cell-matrix interactions participate in the spermatogenesis and fertilization processes. These interactions are essential for sperm maturation, sperm

migration in uterus and oviduct, binding to cumulus cells, attachment to oocyte extracellular matrix (zona pellucida), binding to oocyte cell membrane and sperm-oocyte fusion. ADAM proteins play an important role in all these interactions (23-25).

Several methods have been applied to investigate the role of ADAM family proteins and their integrin partners in the process of reproduction. One study showed that monoclonal antibodies that work against the disintegrin domain of ADAM can reduce fertilization rates (26). The disintegrin domain is responsible for integrin receptor binding of ADAM. Monoclonal antibodies that act against oocyte integrin receptors have been recommended for inhibition of fertilization (27).

In addition to oocyte integrin receptors, monoclonal antibodies that act against integrin-related proteins such as CD9 and CD151 can partially inhibit oocyte-sperm fusion (28). The saturation of the oocyte receptors with ADAM mimic peptides can hinder oocyte-sperm binding and fusion (29). Furthermore, the functional domains of ADAM expressed in hosts such as *E. coli* can prevent sperm-oocyte binding and fusion (30). These processes provide valuable information about the fertilization process. However, owing to the expression of several similar ADAM isoforms in sperm and the multi-domain structure of these proteins, it is difficult to restrict experiments to a specific ADAM isoform or to investigate all the functions of a protein using such processes.

The use of mutant knockout mouse can make it possible to examine the effects of a specific isoform without disadvantages. In some cases, the mutation could cause infertility, while others have shown no significant difference in wild-type mice. For example, homozygote mutant mice for the ADAM1a gene are infertile because the sperm cannot migrate from the vagina to the oviduct (31); however, the knockout mouse mutated with in ADAM1b gene are fertile and it seems that this gene is not essential to the reproductive process (32). The sperm of the ADAM3 mutant knockout mouse are infertile because they lack the ability to interact with zona pellucida. After the removal of zona pellucida, the sperm were able to fertilize the zona-free oocytes (33).

2. Objective

ADAM27 gene knockout mouse mutated for the transmembrane domain previously have been produced and evaluated (34). The aim of the current study was to assess the effect of ADAM27 on reproduction. Another mutated allele belonging to exons 1, 2 and 3 from the ADAM27 gene was used to generate the ADAM27 knockout mouse. The mutant male mice were studied genetically and phenotypically to spot any abnormalities.

3. Materials and Methods

3.1. Mice

NMRI and C57BL/6J mice strains were obtained from Pasture Institute (Tehran, Iran). The mice were kept under the conditions of 50% humidity in a 14:10 h light: dark cycle. They were fed a standard pellet diet and tap water supplied as desired. All animal handling was conducted based on European guidelines (EU Directive 2010/63/EU).

3.2. Knockout Generation

3.2.1. Cells

The R1 mouse embryonic stem cells (mESCs) were used for knockout mouse production. The ADAM27 allele in these cells was inactivated by a genetic construct containing two homologous arms flanking a neomycin resistant gene cassette. In the mutant cells, a segment of the ADAM27 gene that includes the Kozak sequence, initiation codon, exons 1, 2 and 3, introns 1 and 2, and part of intron 3 were replaced with the neomycin gene (**Fig. 1**).

The mESCs were co-cultured with mouse embryo fibroblast (MEF) cells in high glucose DMEM medium (Gibco; 884155) supplemented with 15% fetal bovine serum (FBS), 2 mm L-glutamine (Gibco; 073), 0.1 mm non-essential amino acids (Gibco; 11140), 0.1 mm 2-mercaptoethanol (Sigma; M7522), 50 $U.mL^{-1}$ penicillin-streptomycin (Sigma; M074) and 1000 $U.mL^{-1}$ leukemia inhibitory factor (Millipore; DAM1770435)) in a tri-gas incubator (5% CO_2, 5% O_2, 90% N_2) incubator at 37 °C and 95% humidity.

Additional essential supplements for the growth of the mESCs were provided by a monolayer of inactivated MEF feeder cells as described previously (35). Briefly, the MEFs were isolated from 12.5 dpc mouse embryos and grown in high glucose DMEM (Gibco; 884155) containing 10% FBS, 2 mm of L-glutamine (Gibco; 073), 0.1 mm of non-essential amino acids (Gibco; 11140) and 50 $U.mL^{-1}$ of penicillin-streptomycin

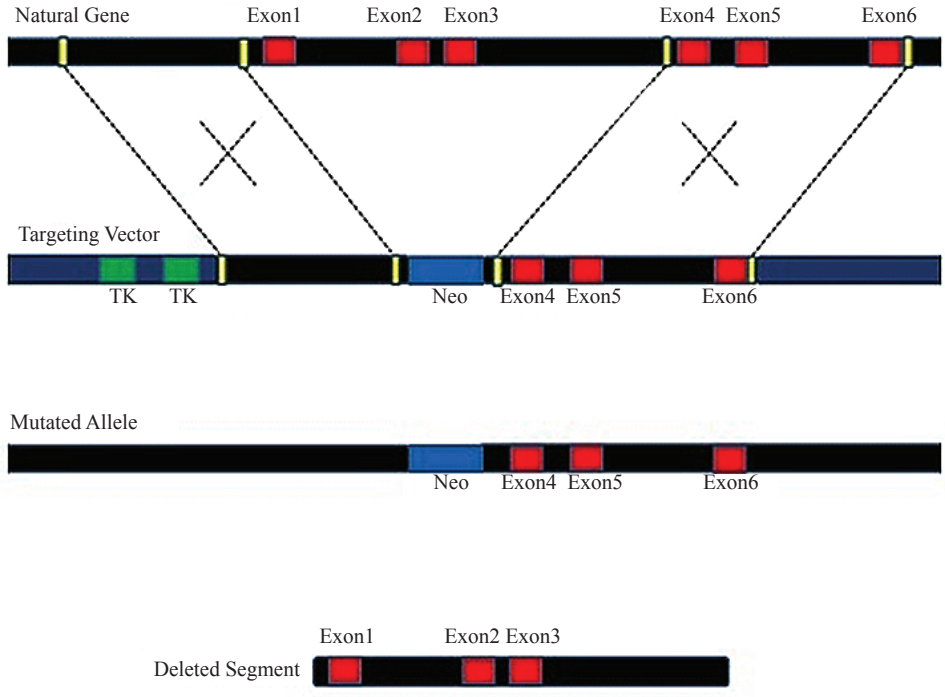

Figure 1. The mechanism of homologous recombination and the exons used in this study

(Sigma; M074)) in a tri-gas incubator (5% CO_2, 5% O_2, 90% N_2) incubator at 37 °C and 95% humidity. The MEFs were inactivated by mitomycin C (Sigma; M0503) treatment and were applied as the mESC feeder layer.

3.2.2. Blastocyst Injection

Chimeric mice were generated by blastocyst microinjection as described by Khezri *et al*. (36). In brief, ESC clones were turned into single cells by treatment with Trypsin-EDTA 0.05 and pipetting. The cells were washed with PBS and resuspended in M2 embryo culture media. They were kept at 10 °C until injection. Only 3.5 dpc, grade-A blastocysts were used. Eight to 16 single mutant cells were injected into the blastocoel cavity using a Narishige micromanipulator. The injected blastocysts then were cultured in M16 embryonic culture media in 37 °C, 5% CO_2, 5% N_2 and 90% air for 2-3 h.

3.2.3. Generation of ADAM27$^{-/-}$ Knockout Mouse

The injected blastocysts were transferred in groups of 7 to 12 to the uterus of 2.5 dpc recipient female mice (NMRI strain) by embryo transfer surgery as described by Hogan *et al*. (37). After birth, the chimeric pups could be identified by their eye and skin colors. Male agouti chimeric mice were mated with female C57BL/6J mice and the heterozygote pups were selected after polymerase chain reaction (PCR) testing for the ADAM27 mutated allele. The mature heterozygotes were mated for the production of knock-out homozygote ADAM27 mutant mice.

3.3. Knockout Confirmation

3.3.1. PCR Analysis

The pups resulting from ADAM27 heterozygote breeding were analyzed by PCR testing using ADAM27 wild and mutated allele primers. Primers for wild-type gene amplification (KOATGfp, AAGTGCAAGAAGCTCAGCCGA and KOATGrp, CCTGAGCTGGTAGTTCTGAAC) and for mutant gene amplification (KOATGfp, and NeoRI, AGGA GCAAGGTGAGATGACAG) were generated (Gen Fan Avaran, Iran) and optimized to a 50 °C annealing temperature. The cycling conditions comprised 4 min of heating at 95 °C and 35 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s.

3.3.2. Gene Sequencing

To confirm the deletion of the ADAM 27 gene, the mutant part of gene was sequenced using the knockout mouse gene.

3.4. Knockout Analysis

3.4.1. Breeding

Homozygote ADAM27 mutant male mice and wild-type control male mice were mated with C57BL/6J wild-type female mice to determine fertility. The mating rate, pregnancy rate and number of newborns were recorded and statistically analyzed.

3.4.2. In-vitro Fertilization

In-vitro fertilization was performed to evaluate the ability of the mutant sperm to fertilize the oocyte naturally as described by Sztein *et al*. (38). Briefly, the oocytes were isolated from the ampulla of superovulated NMRI female mice at 13 h after the hCG injection. Grade-A oocytes were selected and used for IVF. Mutant sperm ($ADAM27^{-/-}$) and wild-type sperm (as control) were used in the IVF procedure. The fertilization and blastocyst rates were recorded and analyzed.

3.4.3. Histological Examination

A mouse from each knockout and wild-type was sacrificed and necropsies were performed. Samples from the kidney, liver, heart, intestine, lung, brain and testis were taken for histopathological assessment. Also investigated were abnormalities in the process of maturation of the primary spermatocytes to spermatozoa in the testicular tissue to determine whether or not the mutation negatively affected spermatogenesis. The biopsy samples were preserved in 10% buffer formalin, then dehydrated and embedded in paraffin with a paraffin tissue processor (DS 2080/H; Did Sabz) and paraffin dispenser (DS 4LM; Did Sabz). The sections were prepared by cutting the paraffin blocks into 5 µm thicknesses (Rotary Microtome RM2145; Leica). They then were stained with hematoxylin-eosin and observed by light microscopy (E600; Nikon) (39).

3.4.4. Sperm Motility Analysis

Epididymal sperm from homozygote mutants and wild-type mice were evaluated for morphological and motility features using phase contrast microscopy (Nikon). The sperm motility was assessed by computer-assisted semen analysis (CASA) (39). The sperm samples were kept at 37 °C in HTF media for 1 h before CASA analysis. The following parameters were measured: total motility (%), progressive motility (%), distance average path (DAP; mm), distance curved line (DSL; mm), distance straight line (DSL; mm), velocity average path (VAP, mm/s), velocity curved line (VCL; mm/s), velocity straight line (VSL; mm/s), variation on straightness (STR), VSL/VAP (%), linearity (LIN), VSL/VCL (%), wobble (WOB), VAP/VCL (%), amplitude of lateral head displacement (ALH; mm), and beat cross frequency (BCF; Hz).

3.4.5. Histological Studies

The testicular tissues were biopsies from both $ADAM27^{-/-}$ and normal mice and fixed in 10% formalin. The paraffin-embedded tissues were subjected for microtome dissection and histopathological studies.

3.4.6. Statistical Analyses

All data were statistically analyzed using SAS version 9.1 (SAS; USA). The data was tested for univariate normality and the mean values were subjected to *t*-tests.

4. Results

4.1. Generation of $ADAM27^{-/-}$ Knockout Mouse

The blastocyst microinjection of the ADAM27-mutant mESC was resulted in chimeric mouse, which was distinguished from wild-type mouse by the agouti-white color skin (**Fig. 2**). Mutant homozygote mice were used to investigate the role of ADAM27 protein in fertilization. We obtained heterozygote mice ($ADAM27^{+/-}$) by mating high-percentage chimeric male mice with C57BL/6J wild-type female mice and then mating the heterozygotes to gain mutated homozygotes The number of new born pups in different groups were summarized in **Table 1**.

4.2. Knockout Confirmation

4.2.1. Genetically Confirmation

We confirmed the genomic DNA of the heterozygote mice ($ADAM27^{+/-}$) and homozygote mutant mice ($ADAM27^{-/-}$) by PCR. The offspring of male chimeras also were evaluated by PCR. The PCR results confirmed that a number of pups were heterozygote for the ADAM27 gene. ADAM27 mutated homozygotes

Figure 2. Agouti and white chimeric mouse (Right) compared with a wild mouse which is completely white (Left)

Table 1. The number of new born pups in different groups

	ADAM27$^{+/+}$×ADAM27$^{+/+}$		**ADAM27$^{+/-}$× ADAM27$^{+/+}$**		**ADAM27$^{-/-}$ × ADAM27$^{+/+}$**	
	♂	♀	♂	♀	♂	♀
Pups	**73**		**69**		**65**	
Male pups	**39**		**32**		**27**	
Female Pups	**34**		**37**		**38**	

were also identified by PCR in the offspring of male and female heterozygotes (**Fig. 3A**).

The results of gene sequencing show that the exons 1, 2, and 3 of the ADAM 27 gene were replaced by the neomycin resistant gene in the genome (**Fig. 3B**). This figure shows that a part of ADAM 27 gene was replaced by neomycin resistant gene.

4.3. Knockout Analysis

4.3.1. Breeding

The heterozygote male mice (ADAM27$^{+/-}$) were mated with wild-type female mice (ADAM27$^{+/+}$). They showed normal fertilization rates and there was no significant decrease in the number of newborn in the ADAM27$^{+/-}$ group compared with the wild-type group. The average number of newborns was 8.5 for the ADAM27$^{+/-}$ group and 9.1 for the wild-type group.

In order to investigate the fertility rate of the homozygote mutant mice (ADAM27$^{-/-}$), the male ADAM27$^{-/-}$ mice were mated with normal females (ADAM27$^{+/+}$) and the results were compared with the wild-type male group. The average number of newborns was statistically similar in both groups. The average number of newborns was 8.9 for the ADAM27$^{-/-}$ group and 9.1 for the wild-type group. The male and female ADAM27$^{-/-}$ mice showed normal behavior and were similar in weight, size and longevity compared to the wild-type male and female mice.

4.3.2. In Vitro Fertilization

In-vitro fertilization was carried out to compare the rates of fertilization by homozygote mutant sperm and wild-type sperm. A total of 327 oocytes were incubated with ADAM27$^{-/-}$ sperm and 172 embryos were obtained (52%). A total of 289 oocytes were incubated with

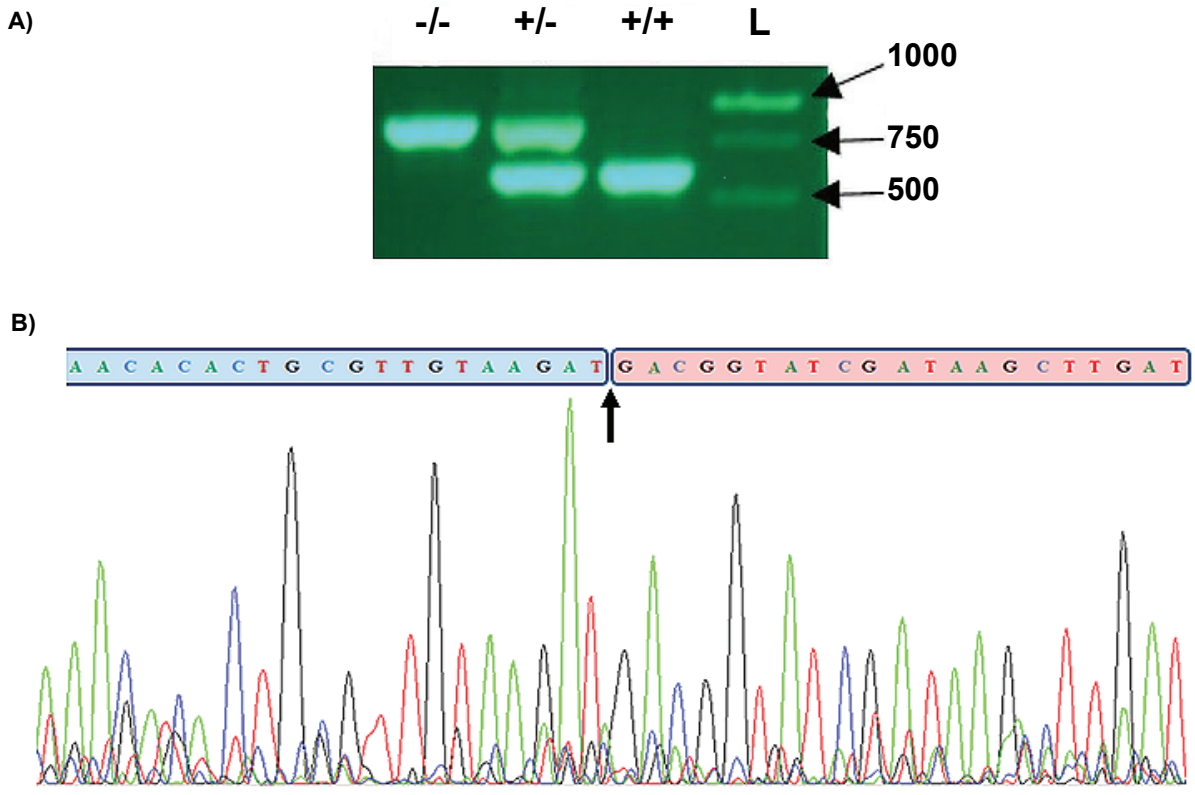

Figure 3. The pups genotype analysis. **A)** The KOATGfp and KOATGrp primers amplified the natural allele and KOATGfp and NeoRI primers amplified the mutated allele. **B)** The result of gene sequencing of mutant part. The blue nucleotides are the remaining part of ADAM27 gene and the red nucleotides are beginning part of neomycin resistant gene.

ADAM27$^{+/+}$ sperm and 156 embryos (54%) were obtained. There was no significant difference in the rate of fertilization between the ADAM27 homozygote mutant and wild-type groups **(Fig. 4**).

4.3.3. Pathology

4.3.3.1. Sperm Motility

The epididymis sperm of the ADAM27$^{-/-}$ mice were analyzed morphologically and compared with the sperm of ADAM27$^{+/+}$ mice. There was no remarkable difference in the shape, size and motility of the two types of sperm. Moreover, the ADAM27$^{-/-}$ mice sperm concentration showed no decrease compared to the wild-type mice (**Fig. 5**).

4.3.3.2. Histopathology

The histopathological sample from the examined organ was normal (**Fig. 6**).

5. Discussion

The ADAM27 gene is expressed exclusively in testis tissue. Although transcription of this gene begins from day 15 after birth, the ADAM27 protein is inactive in spermatogonial stem cells and even in testicular sperm. In the epididymis, ADAM27 is activated by detaching the prodomain (40). Testis-specific expression of ADAM27 raises questions about the role of this protein in testis development, spermatogenesis and fertilization. ADAM27$^{-/-}$ knock-out mice were produced to investigate the role of ADAM27 in reproduction. The male ADAM27$^{-/-}$ mice then were mated with female wild-type mice. These mutant mice showed normal reproductive behavior and the number of newborns was similar to that of wild-type mice. The testis histopathology demonstrated normal morphology and the CASA results for both the ADAM27$^{-/-}$ and wild-type sperms indicated similar behavior and features.

The ADAM27 gene shows the most similarity to the

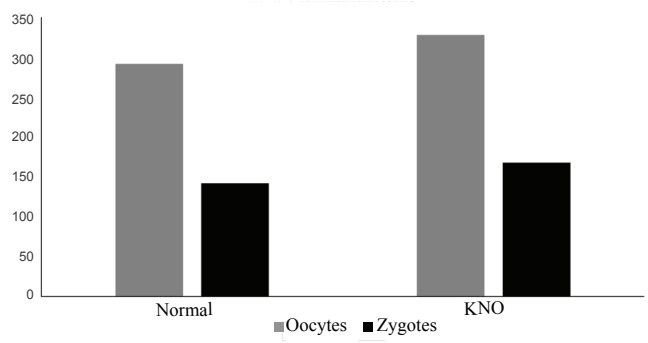

Figure 4. *In Vitro* Fertilization rate of sperm in two groups. Gray columns represent the number of oocytes used for IVF and black columns shows the successful zygotes.

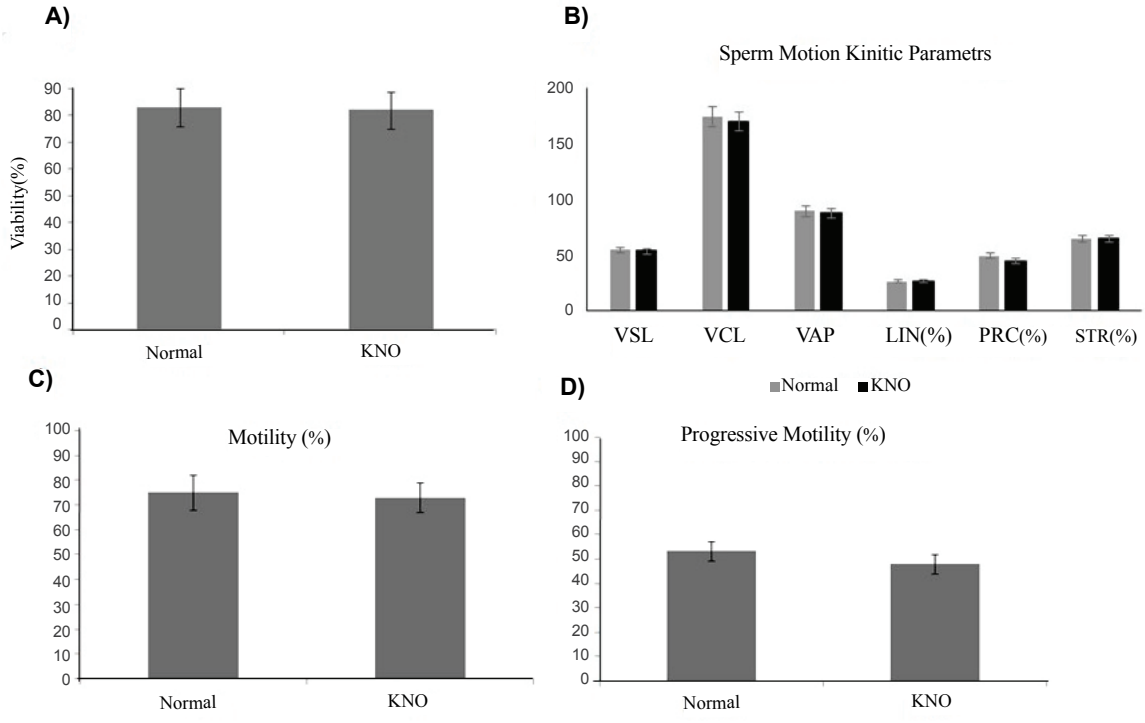

Figure 5. A) Viability, **B)** sperm kinetic, **C)** motility and **D)** progressive motility of knock-out (KNO) and normal sperms

ADAM3 gene (40); however, knock-out mice with the ADAM3 gene are infertile as their sperm are unable to bind to the zona pellucida of oocytes (33). Accordingly, it was assumed that the ADAM27 protein could play a role in binding to oocyte envelopes. Although the knock-out mice exhibited natural fertility, the mutant sperm appeared to require more time for binding and crossing the oocyte envelope than the wild-type sperm. Thus, IVF was conducted to investigate the ability of homozygote mutant sperm (ADAM27$^{-/-}$) to bind and pass through the oocyte envelope.

The fertilization rate in mutant and wild-type groups was similar and there was no significant difference between the two groups. This suggests that the deletion of this gene neither prevented fertilization nor significantly affected the fertilization rate.

Several ADAM genes (at least 18) are expressed in the reproductive tracts of male mice (41). Because the structure and function of ADAM genes are identical, it is possible that other ADAM genes compensated for the absence of the ADAM27 gene. The ADAM27 homozygote mutant sperm was able to maintain their

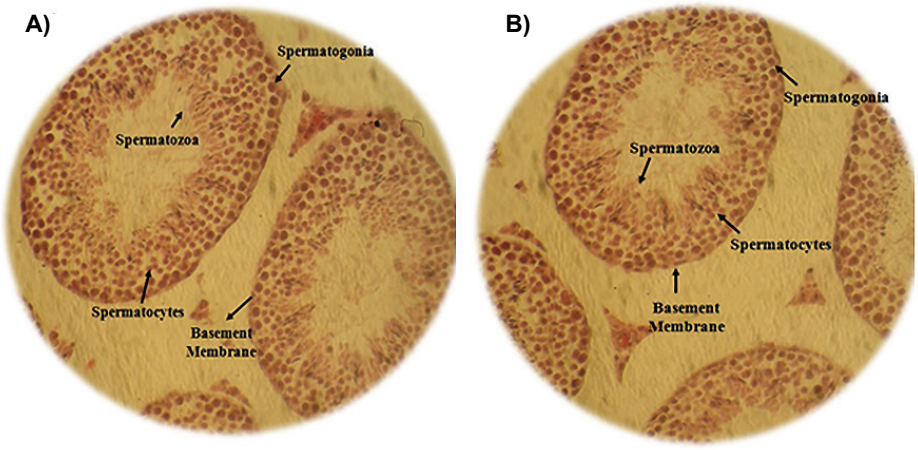

Figure 6. Histopathology results of testis of normal and ADAM27-/- mice. **A)** Image of normal mouse testis, **B)** testis of ADAM27-/-. Spermatogonia, spermatocytes and spermatozoa and the basement membrane were indicated with arrows.

fertilization ability and showed a normal phenotype.

Normal reproduction has been reported in mutant mice having different ADAM family members (32) or incapacitated receptors (42). These mutant mice had a normal fertilization phenotype (32, 43). These findings have been reported in other studies examining a member of this family expressed exclusively in the reproduction system. However, mutations in other members of the ADAM family have been shown to reduce the fertilization rate or even cause infertility (31, 33). These findings show that not all ADAM genes expressed in the mice reproduction system are essential for fertilization. Although some, such as ADAM2, ADAM7 and ADAM24, play a key role in normal reproductive system development and fertilization, the presence of others is not critical for normal fertilization (44).

These results pose the questions of whether or not the expression of some ADAM genes in the reproduction system is essential for fertilization and why do they remain in the genome and continue to be expressed in the male reproductive system. Studies have increased the number of testis-specific ADAM genes after several rounds of duplication and positive selection (45-46). Fertilization may be possible only with some isoforms of the ADAM genes, but sperm expressing more ADAMs tend to be more fertile than other sperm types, which provides them more opportunities to participate in the next generation's gene pool.

In addition to the need for interaction with different environments, envelopes and cells (critical ADAMs), sperm competition may provide a force through redundant reproduction of ADAM genes in the male rodent reproduction system to keep these genes in the genome (redundant ADAMs). Although some ADAM genes expressed in the testes do not play a critical role in fertilization and reproduction, they appear to increase the ability of the sperm to fertilize the oocyte when competing with sperm that lack these genes.

Although a mutation in one or some genes involved in the phenotype might not disrupt the fertilization process, mutant sperm fertilization ability could decrease compared with that of the wild-type sperm. If the decrease in fertilization ability is low, more effective methods are required to determine the precise effects. In some cases, the observation of a gene effect could require the organism to be exposed to a condition other than standard laboratory conditions.

6. Conclusion

The fertilization ability of sperm is a polygenic phenotype. Like several similar studies, we could not observe a significant effect on the normal reproductive ability of mutant mice in a reproductive ADAM gene. ADAM27 is not essential for reproduction in mice. Considering the expression of several other isoforms of the ADAM family in sperm, the role of this gene is probably covered and compensated by other isoforms.

ADAM genes play a role in some signaling pathways and some exhibit proteolytic activity, which could be considered in future investigations. Knock-out line generation from different ADAM genes could pave the way for planning more experiments on the process of fertilization. Planning and implementing such investigations would help us to better understand the fertilization process, as well as the proteins involved and their roles.

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