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Spermatozoa from male mice with infertility due to *Odf4* deficiency can fertilize oocytes by in vitro fertilization

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Abstract

Purpose: The purpose of this study is to confirm whether in vitro fertilization (IVF) with spermatozoa from *Odf4*-deficient infertile males ($Odf4^{-/-}$ spermatozoa) can lead to the development of zygotes, which was reported in a previous in vivo study.

Methods: In vitro capacitation and IVF were performed using $Odf4^{-/-}$ spermatozoa in a small drop of TYH medium with pyruvate and glucose, for 60 min or up to 4 days. A capacitation test was performed by immunoblotting using an anti-p-Tyr antibody. A sperm movement test was performed using a computer-assisted sperm motility analysis system (SMAS). An IVF fertilization test was also performed to evaluate zygote production. Videos were taken by a DMi8 stereomicroscope equipped with a high-speed camera.

Results: In in vitro condition, $Odf4^{-/-}$ spermatozoa with hairpin flagella harboring large cytoplasmic droplets (CDs) underwent capacitation, about 30% of large CDs were removed from spermatozoa, and the flagella became straight (capacitation test). The $Odf4^{-/-}$ spermatozoa with straight flagella swam forward (movement test) and fertilized $Odf4^{+/+}$ oocytes, which eventually developed into zygotes (fertilization test).

Conclusions: By conventional IVF, spermatozoa from *Odf4*-deficient male mice can fertilize oocytes that then develop into zygotes. These findings can be translated to human males with infertility caused by *ODF4* deficiency.

KEYWORDS
adenylate kinase, fertility and infertility, flagellar motility and shape, IVF, Odf4

1 | INTRODUCTION

Morphologically normal spermatozoa of mammals, including mice and humans, have straight flagella and swim forward, which are essential for normal fertilization in vivo. Proper shape and movement of sperm flagellum are supported by the fundamental structure and energy production-consumption system in flagella. For example, ATP is produced by oxidative respiration (pyruvate cycle: tricarboxylic acid (TCA) cycle) which takes place in the mitochondria of the midpiece of a sperm flagellum, and by glycolysis, which takes place in the fibrous sheath of the principal piece; the ATP that is produced is subsequently transported to energy-consuming sites such as the axoneme where it is used by dynein.[1] The cytoplasmic droplet (CD) is termed the cell remnant in sperm flagella; therefore, the size and localization of the CD affects flagellar shape and movement. However, it has long been unclear how the ATP that is produced is transported to dynein and the CD or how the CD is removed from the flagella. CD affects flagellar

shaping and causes various types of multiple morphological abnormalities in flagella (MMAF), such as bent and hairpin flagella, which are frequently caused by gene mutations.[2] MMAF is a main factor associated with male infertility, as reported by the World Health Organization.[3] Recently, we reported that *Odf4*-deficient male mice with *Odf4* gene deletion are infertile due to abnormally shaped spermatozoa with hairpin flagella (bent tails) harboring large CDs.[4] Human *ODF4* gene-deficient male infertility has not been reported until now.

Outer dense fiber of sperm tails 4 (ODF4) is a subtype of ODF that is a flagellar cytoskeletal protein integrated into the outer dense fibers (ODFs).[1] The ODFs emanate from the axoneme in the neck and run down in the center of the whole flagellum. The ODFs are covered by helically arranged mitochondria (mitochondrial sheaths) at the midpiece and by fibrous sheaths at the principal piece surrounding the microtubular axoneme containing dynein; thus, the ODFs support flagellar shape and movement. The ODFs are reported to be connected to the axoneme.[5]

Here, we show additional information about ODF4 in Figure S1 and Table S1 based on the previous findings.[4] First, in wild-type $Odf4^{+/+}$ spermatozoa, soluble ODF4, which is readily solubilized in mild detergent such as RIPA buffer for SDS–PAGE, is present in sperm cells and interacts with adenylate kinases (AKs) as ODF4-AK1- and AK2-complexes, where soluble ODF4 corrects the position of the AK. AK is a cellular nucleotide synthetic machinery known as myokinase (EC 2.7.4.3), a phosphotransferase ubiquitously distributed throughout the body that catalyzes the interconversion of adenine nucleotides and regulates adenine nucleotide ratios in different intracellular compartments. In other words, AK shuttles ATP to energy-consuming sites in sperm cells and controls the concentration equilibrium of adenine nucleotides to balance energy production and consumption.[6] AK1 is mainly localized in the cytosol[7] and in cytoskeletal ODFs throughout the flagellum.[8] AK2 mainly localizes to the mitochondria at the midpiece.[8] Second, the large CD frequently expands from the midpiece to around or beyond the annulus region or cortex ring in mature $Odf4^{-/-}$sperm flagellum (Figure S1 −/−). In contrast, the CD in mature $Odf4^{+/+}$ sperm flagellum is small or absent (Figure S1 +/+) because the CD is generally removed from flagella during epididymal transit or during ejaculation soon after copulation.[1,4] Third, AK1 and AK2 are produced normally in the testes of $Odf4^{-/-}$ mice, but they are freely present with reduced amounts in mature spermatozoa.[4] Fourth, $Odf4^{-/-}$ spermatozoa can actively produce ATP by oxidative respiration and glycolysis at high levels, similar to $Odf4^{+/+}$ spermatozoa, but the control of the concentration and balance of ATP and ADP is impaired in mature spermatozoa due to a reduction in AK.[4] Thus, $Odf4^{-/-}$ sperm abnormal tail shaping and reduced motility are not caused by failures of oxidative respiration and glycolysis, but by reduced amount of AK to shuttle ATP.

Capacitation is termed for the state of gaining fertilizing ability of spermatozoa, showing activated and vigorous tail movement change leading to hyperactivation and tyrosine phosphorylation of 65 kDa protein by western blotting (phosphorylation test).[9] Sperm movement is routinely analyzed by computer-assisted analysis (SMAS in this study), and can be further analyzed by high speed recording system as reported previously.[4] Immunostaining intensity obtained by tyrosine phosphorylation test is quantitatively analyzed by western blotting. One of the most basic information we want to know in this study is whether IVF condition can induce capacitation in $Odf4^{-/-}$ spermatozoa. However, since $Odf4^{-/-}$ sperm flagella are bent due to large CDs and show reduced motility as described above and reported previously,[4] $Odf4^{-/-}$ sperm capacitation is somewhat different from general definition for mammalian sperm capacitation in terms of the motility and swimming pattern. Therefore, in this study, we quantitatively analyzed not only the immunostaining intensity of the tyrosine phosphorylation test but also approximate number of highly activated $Odf4^{-/-}$ spermatozoa in IVF condition, including the individual sperm movement obtained by SMAS images, and these results were compared between $Odf4^{-/-}$ and $Odf4^{+/+}$ spermatozoa.

IVF is an assisted reproductive technique (ART) that is widely used in the field of human reproductive medicine worldwide, especially in the field of infertility treatment. IVF is usually performed using chemically defined medium in a small dish. IVF conditions are favorable for the formation of a zygote, e.g., an appropriate concentration of spermatozoa are used to increase the chances of the eggs being fertilized; therefore, spermatozoa can bypass chemical and physical barriers such as fluid viscosity and utero-tubal junctions, which are present in the female reproductive tract. TYH[10] medium is widely used in conventional animal reproduction research studies, including studies of mice. TYH medium contains enough sodium pyruvate for oxidative respiration and glucose for glycolysis, which is especially ideal for spermatozoa. Similarly, IVF for human infertility treatment involves the use of various types of conditioned media suitable for gametes, which are commercially available.

Based on these findings, two questions have arisen: can IVF with spermatozoa from infertile males with *Odf4* deficiency fertilize oocytes that then develop into zygotes, and how does a large CD in $Odf4^{-/-}$ sperm flagellum behave during in vitro capacitation and IVF? This study addresses these questions.

2 | MATERIALS AND METHODS

2.1 | Ethical approval and animals

All procedures were performed in accordance with the institutional guidelines for animal research and approved by the Animal Care and Use Committee of Chiba University (#A4-026 and A5-001) for *Odf4*-deficient ($Odf4^{-/-}$) mice, C57BL/6JJmsSlc (B6) and B6D2F1 (BDF1) mice (Japan SLC). $Odf4^{-/-}$ mice were generated using CRISPR/Cas9 system as previously reported.[4] These animals are maintained in the Animal facilities of Chiba University. The mice used in this study were 7–9-week old. $Odf4^{-/-}$ mouse (termed as $Odf4^{em1Ktos}$) is registered in the mouse genome information (MGI). This study was

carried out in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines as described in our previous report.[4] This article does not contain any studies with human subjects performed by any of the authors.

2.2 | Capacitation

Capacitation was performed in small IVF dishes supplemented with TYH medium containing pyruvate and glucose.[10] The dishes were incubated at 37°C for 60 min in a CO_2 incubator. The TYH medium contained the following components (mg/100 mL [mM]): NaCl 697.6 (119.37), KCl 35.6 (4.78), KH_2PO_4 16.2 (1.19), $MgSO_47H_2O$ 29.3 (1.19), $NaHCO_3$ 210.6 (25.07), sodium pyruvate 5.5 (0.5), glucose 100 (5.56), $CaCl_22H_2O$ 25.1 (1.71), penicillin G 7.5, and BSA 400 mg/100 mL (mM) at the final concentrations. The osmolarity of the TYH medium was adjusted to 297 mOsm/kg in this study, which corresponds to the osmolarity of the plasma; this TYH medium was similarly used in our previous report.[4]

2.3 | Capacitation test or tyrosine phosphorylation test, SDS–PAGE and western blotting

Tyrosine phosphorylation test was performed according to the original method reported previously,[11] and SDS-PAGE and western blotting were performed according to the previous study,[12] with slight modifications in this study as follows. The samples were collected from the spermatozoa used for the test (starting at 0 min and ending at 60 min) after the same number of sperm cells were added to a dish. The spermatozoa were subsequently lysed in sample buffer (Nacalai) and subjected to western blotting as described below. A positive judgment was made for the western blotting sample, in which an approximately 65 kDa band (p-Tyr) appeared after immune staining with the anti-p-Tyr antibody on the blot obtained from the sample at the end of the training session (60 min). The staining intensity of the band increased when compared with that of the sample at 0 min (start), as shown in Figure 1A, and then the staining intensity image was quantitatively analyzed by Image Lab Software installed in Molecular Imager ChemiDoc XRS Plus (Bio-Rad) which took the original image, as shown in Figures 1B and S2. Experiments were duplicated using different males.

2.4 | In vitro fertilization (IVF)

IVF with TYH medium was performed under conditions similar to those of the capacitation test, as described above and previously reported,[13] with slight modifications. In TYH drops covered with mineral oil, wild-type *Odf4*$^{+/+}$ oocytes were inseminated with *Odf4*$^{-/-}$ spermatozoa at a concentration of approximately 2×10^5/mL. After incubating for 9 h in a CO_2 incubator at 37°C, the oocytes were transferred to fresh TYH medium in different IVF dishes and then incubated for 2–4 days for observation of the status of the inseminated spermatozoa and oocytes.

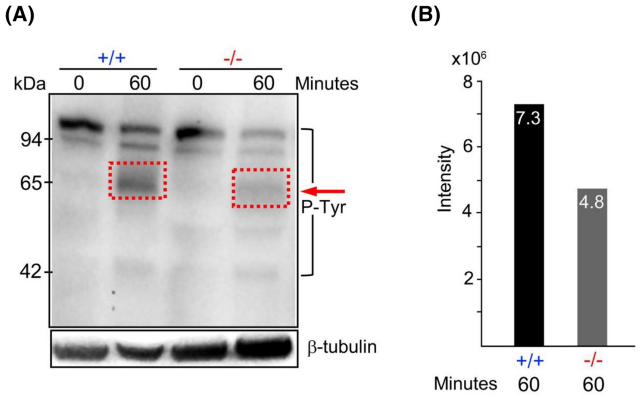

FIGURE 1 Capacitation test of *Odf4*$^{+/+}$ (control) and *Odf4*$^{-/-}$ spermatozoa by western blotting; tyrosine phosphorylation (p-Tyr) test. +/+: *Odf4*$^{+/+}$. −/−: *Odf4*$^{-/-}$. Samples were obtained at 0 min (start) and 60 min (end) of the capacitation test. The method for evaluating the result (positive or negative) is described in the Methods. (A) Tyrosine phosphorylation test: A partial image of the raw data. Full length images of the raw data for (A) and (B) obtained by ChemiDoc™ XRS+ imager (BioRad) are shown in Figure S2. This test is done to confirm the occurrence of capacitation in *Odf4*$^{-/-}$ spermatozoa in TYH medium. The intensity of 65 kDa-band (indicated by a red arrow) increased in both *Odf4*$^{+/+}$ and *Odf4*$^{-/-}$ spermatozoa after 60 min incubation, comparing with those of 0 min (start); this result indicates that both *Odf4*$^{+/+}$ and *Odf4*$^{-/-}$ spermatozoa are positively capacitated. However, the intensity was lower in *Odf4*$^{-/-}$ than in *Odf4*$^{+/+}$ spermatozoa. (B) Bar graph of quantitative data (Volume (Int)) at 60 min (end) show the value of −/− was lower than that of +/+; 7.3×10^6 (+/+) versus 4.8×10^6 (−/−). This point is briefly discussed in the Discussion. β-Tubulin, internal protein control.

2.5 | Morphology and motility assays

Spermatozoa were collected by squeezing the cauda epididymis of *Odf4*$^{+/+}$ (+/+) and *Odf4*$^{-/-}$ (−/−) males. The spermatozoa were processed for general light microscopic and stereoscopic examination, including motility tests by SMAS, as reported previously.[4]

2.6 | SMAS

SMAS was used for the movement test. Fresh spermatozoa were collected from the cauda epididymis. The samples were preincubated in a CO_2 incubator for 30 min to allow the cells to disperse so that they could be counted precisely. Sperm motility and the related characteristic parameters were quantitatively analyzed using an automated SMAS (DITECT Co. Ltd.) as reported previously[4]; with slight modifications in this study as follows. Automatically obtained raw data are presented as bar graphs in Figure 3A–C. In addition, we present the examples of original SMAS image

(Figure 3D) to show the approximate number of highly activated spermatozoa indicated by white dots, of which trajectory line length was longer than 100 μm (scale bar); we call these trajectory lines indicated by white dots as good trajectory lines in this study. Here, it should be noted that we excluded the following cases from the count, because it was technically difficult to precisely calculate; when the original line was shown by a black color and when more than two trajectory lines were complexly overlapped in the original image.

2.7 | Video and movies taken with a high-speed camera

Flagellar movement images were recorded at 30 fps for 15 s under a DMi8 stereomicroscope equipped with Neo-PLAN ×20 or ×40 objective lenses and an MC170 HD camera (Leica). The videos were used to analyze sperm movement using Premier Pro software (Adobe) and Adobe Photoshop CC 2019 (Adobe), depending on the purpose; these methods were described previously.[4] In addition, varieties of images of interest were taken from the original movies and used in this study (Figure 4). Since the status of the flagellar CD was not clearly shown in the movie images in this report, the results are summarized in Figure S1 and Table S1, based on a previous study.[4]

2.8 | Statistics

The data are shown as the mean ± SEM after statistical analysis ($p < 0.01$) by Student's *t* test for Figure 2 wild type (+/+) and Figure 3, and by Mann–Whitney's *U* test for Figure 2 homozygous type (−/−).

3 | RESULTS

3.1 | Capacitation test or tyrosine phosphorylation (p-Tyr) test

First, we examined whether *Odf4*$^{-/-}$ spermatozoa are capacitated by an in vitro capacitation. The method for evaluating the test image is described in the Methods section. As a result, the staining intensity of the 65 kDa band was greater in *Odf4*$^{+/+}$ and *Odf4*$^{-/-}$ sperm samples recovered after 60 min (end of incubation) than at 0 min (start of incubation; Figure 1A). Therefore, these samples were judged as positive, which indicated that both *Odf4*$^{+/+}$ and *Odf4*$^{-/-}$ spermatozoa were capacitated. In addition, the quantitative analysis at 60 min showed that the staining intensity was 4.8 in *Odf4*$^{-/-}$ sperm samples, and 7.3 in *Odf4*$^{+/+}$ sperm samples (Figure 1B); the raw data are shown in Figure S2. Such a trend with lower values in *Odf4*$^{-/-}$ spermatozoa is also found in the % sperm motility, straight line velocity and average velocity (Figure 3).

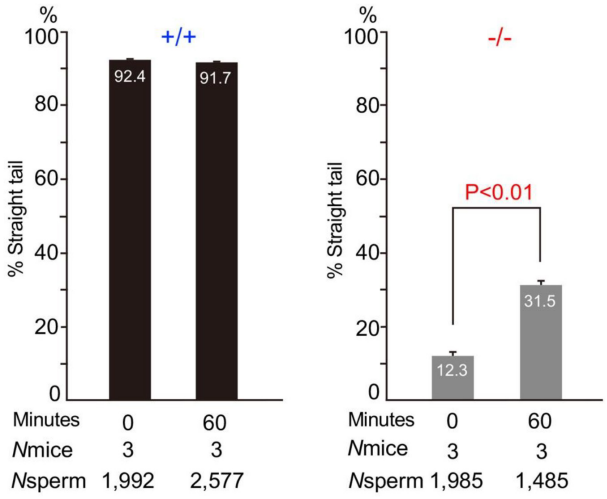

FIGURE 2 Comparison of percentage (%) straight tail after in vitro capacitation between *Odf4*$^{+/+}$ and *Odf4*$^{-/-}$ spermatozoa. +/+ (analysis by Student's *t* test) and −/− (analysis by Mann–Whitney's *U* test). The percentage (%) of *Odf4*$^{-/-}$ sperm straight tail significantly increased at 60 min after capacitation ($p < 0.01$), showing 31.5% at 60 min and 12.3% at 0 min, while that of *Odf4*$^{+/+}$ sperm straight tail did not increase, showing similar values 91.7% at 60 min and 92.4% at 0 min.

3.2 | Comparison of percentage (%) straight tail after in vitro capacitation between *Odf4*$^{+/+}$ and *Odf4*$^{-/-}$ spermatozoa

The % straight tail in *Odf4*$^{-/-}$ spermatozoa after 60 min incubation in TYH medium (31.5%) was higher than that at 0 min (start, 12.3%), even though the values were low; this increment was statistically significant ($p < 0.01$, Figure 2 right). In contrast, in *Odf4*$^{+/+}$ spermatozoa, the % straight tail after 60 min incubation in TYH medium (91.7%) was almost similar to that at 0 min (92.4%); thus, the % straight tail in *Odf4*$^{+/+}$ spermatozoa did not increase (Figure 2 left).

3.3 | *Odf4*$^{-/-}$ sperm flagellar movement significantly increases during in vitro capacitation (movement test)

Next, we compared *Odf4*$^{-/-}$ sperm flagellar movement between 0 min (start) and 60 min (end) of the test. For this, we used an SMAS instrument equipped with a live video recording system (see Methods for details). In addition, these results were compared with the videos taken of *Odf4*$^{+/+}$ sperm flagellar movement (Figure 3A–C). The examined parameters and the results are as follows. In *Odf4*$^{-/-}$ spermatozoa, the % sperm motility at 0 min was 28.5% (Figure 3A −/−, Figure 4A still image for Movie S1), containing 7 highly activated spermatozoa (Figure 3D −/−) with good trajectory lines, and the % sperm motility at 60 min significantly increased to 35.1% ($p < 0.01$, Figure 3A −/−, Figure 4B still image for Movie S2), containing 15 highly activated spermatozoa (Figure 3D −/−) with good trajectory

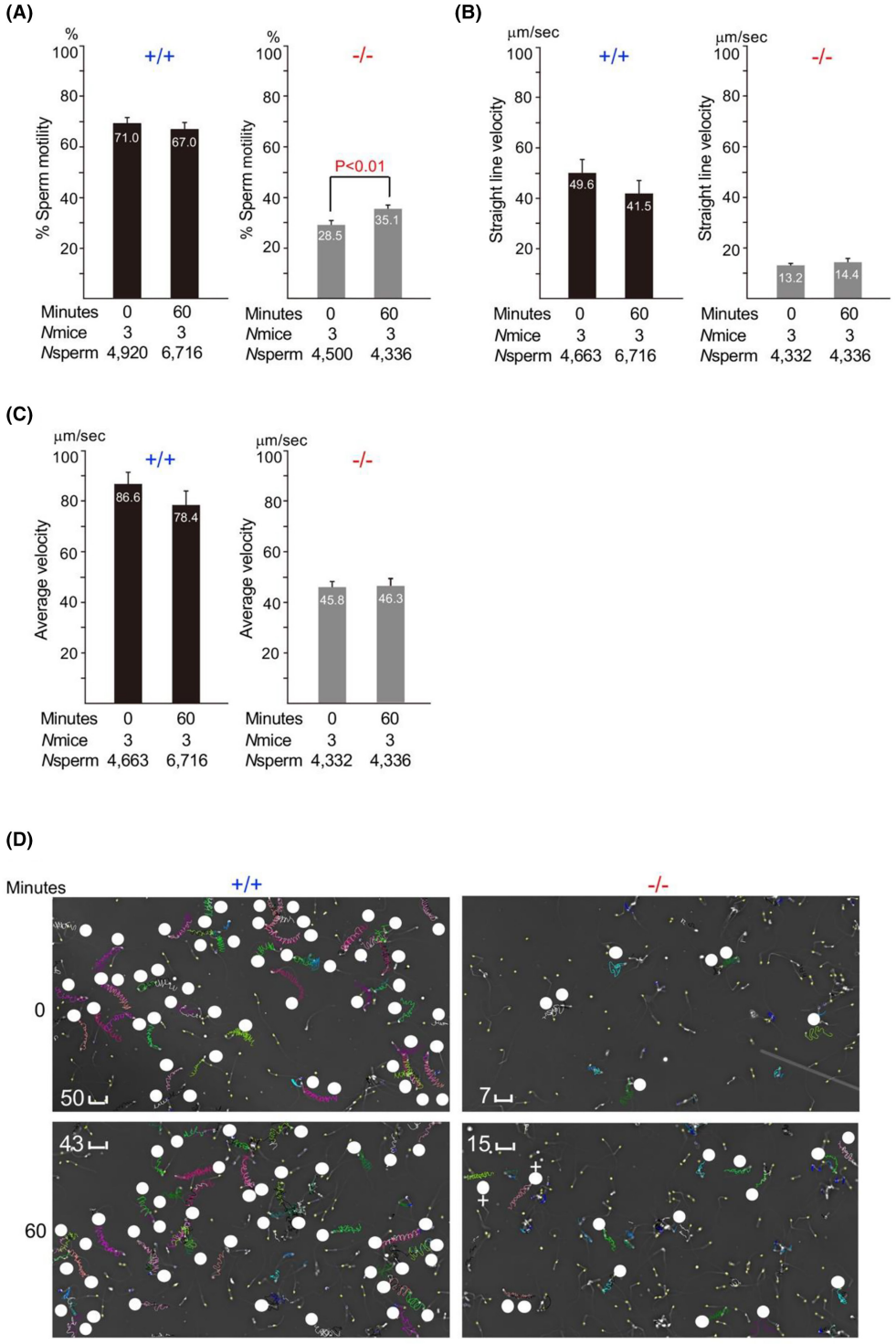

FIGURE 3 Comparison of sperm movement between *Odf4*$^{+/+}$ and *Odf4*$^{-/-}$ mice during in vitro capacitation (movement test). +/+: *Odf4*$^{+/+}$ (control). −/−: *Odf4*$^{-/-}$. (A–C) Bar graphs of the % sperm motility (A), straight line velocity (B) and average velocity (C). (A) The statistical significance of differences between the start (0 min) and end (60 min) of the capacitation test was examined by Student's *t* test. The % *Odf4*$^{-/-}$ sperm motility at 60 min (35.1%) was significantly higher ($p < 0.01$) than that at 0 min (28.5%). (B, C) The straight line velocity (B) and average velocity (C) of *Odf4*$^{+/+}$ spermatozoa were higher than those of *Odf4*$^{-/-}$ spermatozoa, but the values were slightly decreased at 60 min compared with those at 0 min; this trend with decreasing values is also observed in the raw data obtained by SMAS (D). (D) Examples of SMAS raw data at 0 and 60 min. Each color in the image indicates the trajectory of each spermatozoon. Sperm trajectory line larger than the scale (100 μm) indicated by the white dot is called good trajectory lines in this study (see the criteria in Materials and Methods). The number of spermatozoa with good trajectory lines was compared between the start and end. The increase was more pronounced in *Odf4*$^{-/-}$ spermatozoa than in *Odf4*$^{+/+}$ spermatozoa, as shown in (A, D); in detail, in *Odf4*$^{-/-}$ spermatozoa, 7 (0 min) versus 15 (60 min), and in *Odf4*$^{+/+}$ spermatozoa, 50 (0 min) versus 43 (60 min).

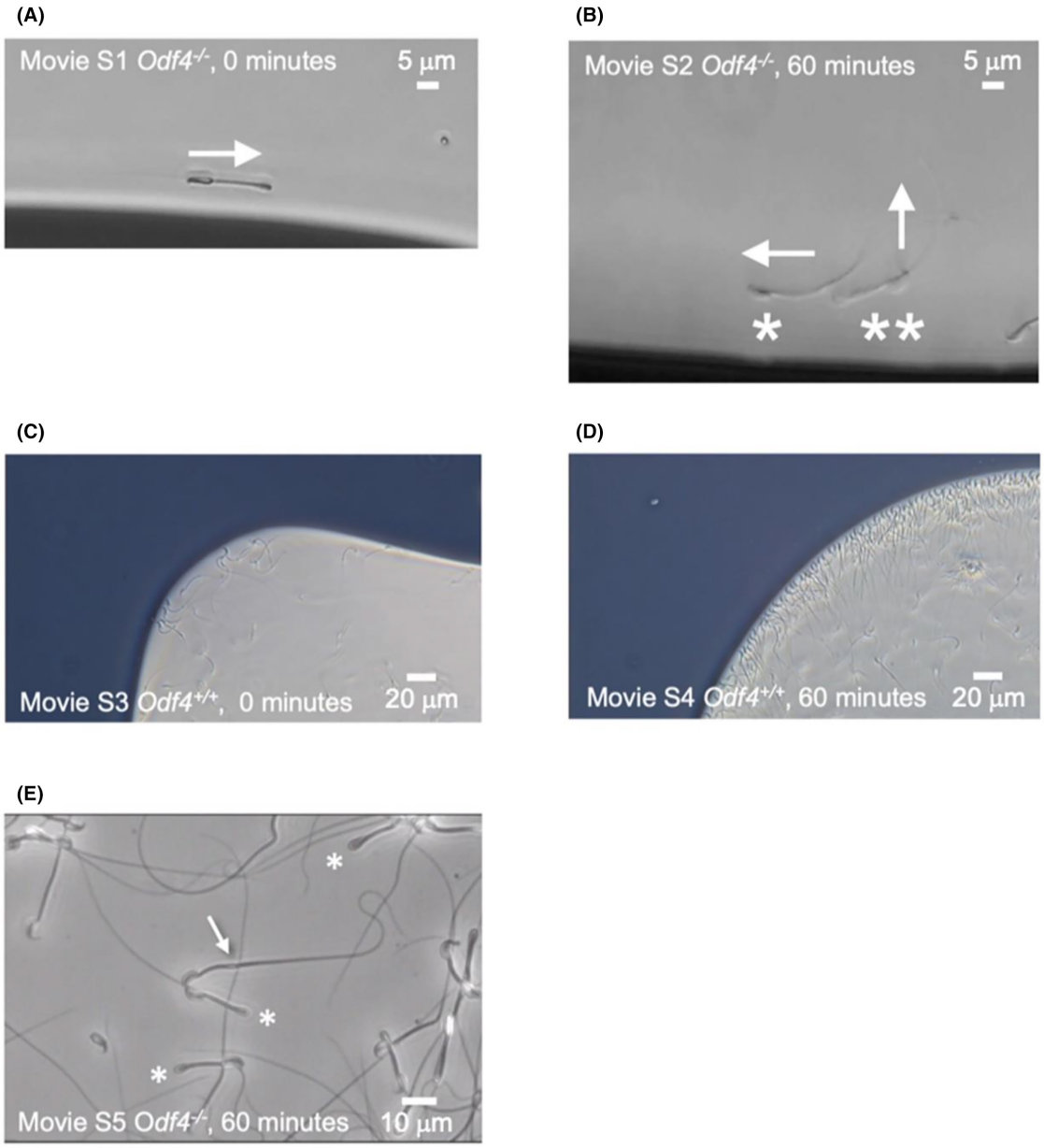

FIGURE 4 Still images for morphology and movement of sperm flagella during in vitro capacitation. −/−: *Odf4*$^{-/-}$ (high magnification). +/+: *Odf4*$^{+/+}$ (control, low magnification). Capacitation was performed in a dish containing TYH medium. Images were obtained from the movies taken using a DM8 video recording system and an MC170 HD camera (Leica) and Premier Pro software (Adobe) for Movies, as described in Method. (A–E) Capacitation test. (A) Movie S1 and (C) Movie S3: At 0 min. (B) Movie S2 and (D) Movie S4: At 60 min. (A, B) Arrow indicates the direction of spermatozoa. Comparisons were made between the start and end. In general, *Odf4*$^{-/-}$ spermatozoa swam backward at 0 min (A). However, at 60 min, there were some spermatozoa with straight flagella that swam forward (arrow with * shown in B), but other *Odf4*$^{-/-}$ spermatozoa with bent flagella swam backward or asymmetrically upward while rotating (arrow with ** shown in B); therefore, most *Odf4*$^{-/-}$ spermatozoa hardly reach the edge of the TYH medium drop. A similar image is shown from the high-speed movie (E), where one sperm flagellum has a quite straight flagellum with very small cytoplasmic droplet (arrow); however, many other sperm flagella are still bent with a large CD (*) and show reduced movement. In contrast, *Odf4*$^{+/+}$ spermatozoa swam forward at 0 min (C), and readily reached the edge of the incubation medium drop at 60 min (D).

lines. Among them, we found two highly activated *Odf4*$^{-/-}$ spermatozoa indicated by white dots with plus (+) in Figure 3D −/− 60 min, of which line images were quite similar to those of highly activated *Odf4*$^{+/+}$ spermatozoa in Figure 3D +/+ 60 min. Such a trend with increasing values in *Odf4*$^{-/-}$ spermatozoa was also shown for the other parameters, but the differences were not statistically significant: straight line velocity and average velocity, 13.2 versus 14.4 μm/s and 45.8 versus 46.3 μm/s at 0 and 60 min, respectively (Figure 3B,C). In contrast, 71.0% of the *Odf4*$^{+/+}$ spermatozoa were very active at 0 min (Figure 3A +/+, Figure 4C still image for Movie S3), containing

50 highly activated spermatozoa (Figure 3D +/+); however, 67.0% of the spermatozoa showed high motility at 60 min, which was slightly decreased (Figure 3A +/+, Figure 4D still image for Movie S4), containing 43 highly activated spermatozoa (Figure 3D +/+). Such a trend with decreasing values in *Odf4*$^{+/+}$ spermatozoa was also shown for the other parameters, as follows: straight line velocity and average velocity, 49.6 versus 41.5 μm/s and 86.6 versus 78.4 μm/s at 0 min and 60 min, respectively (Figure 3B,C). The difference in these findings is briefly discussed in Discussion section.

Next, we examined whether the large CDs disappeared from *Odf4*$^{-/-}$ sperm flagella during in vitro capacitation and whether *Odf4*$^{-/-}$ spermatozoa with bent flagella became straight. For this purpose, we used similar methods to those used for the in vitro capacitation test. The flagellar shape and movement were recorded by the live video recording system described above, and the obtained images were compared between 0 and 60 min. The images of *Odf4*$^{-/-}$ spermatozoa were compared with those of *Odf4*$^{+/+}$ spermatozoa. As a result, some *Odf4*$^{-/-}$ sperm flagella, which were originally bent with large CDs at 0 min (Figure 4A still image for Movie S1), had removed the large CD, and the flagella without CDs became rather straight at 60 min (Figure 4B still image for Movie S2). However, even at 60 min, there were still many spermatozoa with varying degrees of bent flagella (Figure 4E still image for Movie S5), suggesting that *Odf4*$^{-/-}$ spermatozoa are unevenly or heterogeneously capacitated and that some *Odf4*$^{-/-}$ spermatozoa are highly activated and others are not fully activated. In contrast, almost all *Odf4*$^{+/+}$ spermatozoa (control) at 0 min already had straight flagella without CDs (Figure 4C still image for Movie S3), suggesting that almost all *Odf4*$^{+/+}$ spermatozoa without CDs are evenly or homogeneously capacitated at 60 min (Figure 4D still image for Movie S4).

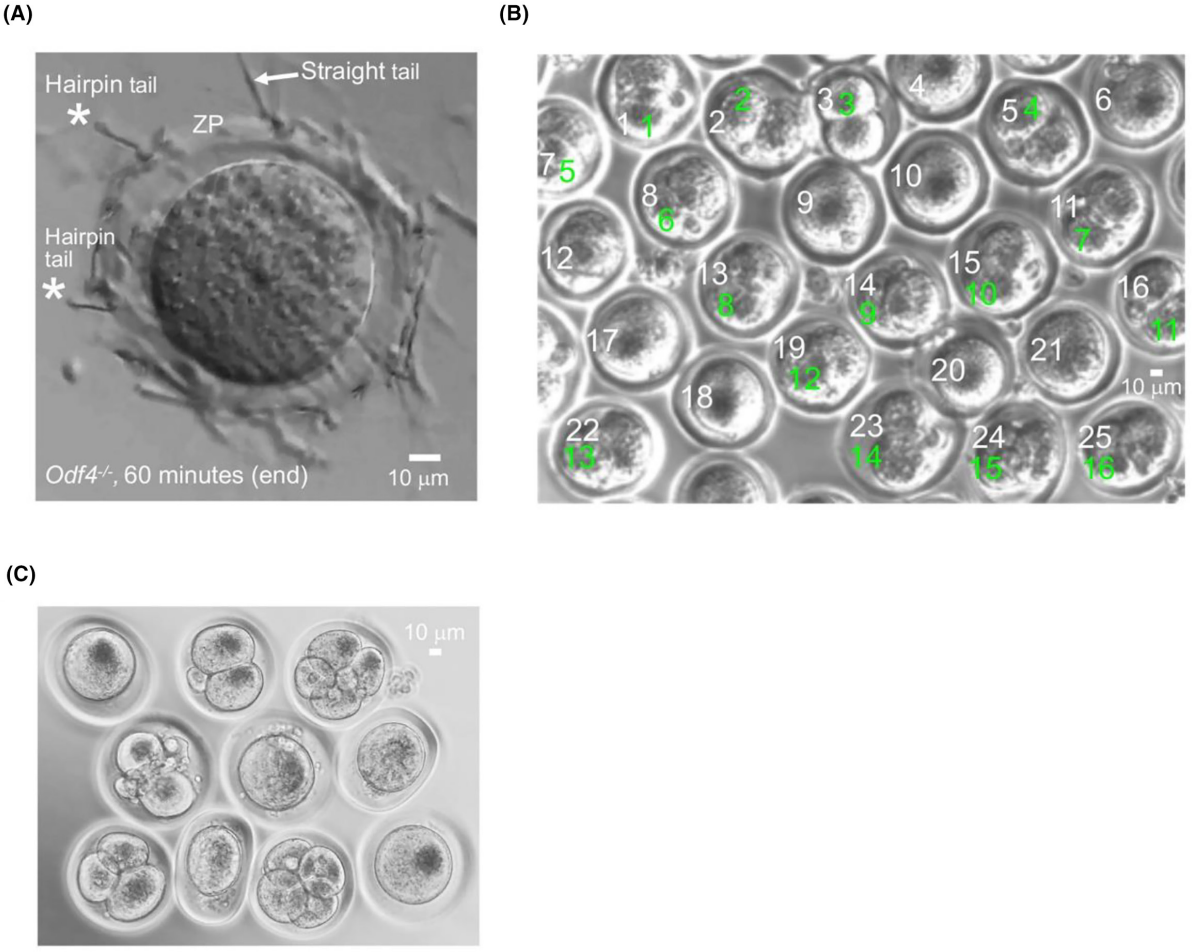

FIGURE 5 Stereoscopic image of fertilized eggs and zygotes during IVF using *Odf4*$^{-/-}$ spermatozoa. (A–C) Fertilized eggs during IVF. (A) A still image of an oocyte from Movie S6 at 60 min after insemination. Spermatozoa attach to the oocyte surface or zona pellucida (ZP), but they exhibit various states of capacitation. Several spermatozoa with straight tails (arrows) are highly capacitated, but other spermatozoa with hairpin or bent flagella bearing large CDs (asterisks) are remained at low state of capacitation. In this image, many spermatozoa are out of focus because the oocyte with attached spermatozoa was moving vigorously, as shown in Movie S6. (B) Examples of 2-cell images taken at 1 day (approximately 20 h) after the start of IVF; sixteen (*; green) out of twenty-five oocytes were found in total (64.0%). (C) Example of a zygote image 4 days after the start of IVF. Total number of zygotes (1–4 cells) is shown in Table 1; 47.8% (66/138) of zygotes had 2–4 cells. *Odf4*$^{-/-}$ spermatozoa from 4 different males were incubated with oocytes from 4 different *Odf4*$^{+/+}$ females.

3.4 | Capacitated *Odf4*$^{-/-}$ spermatozoa can result in the formation of a zygote during IVF (fertilization test)

Finally, we examined whether capacitated *Odf4*$^{-/-}$ spermatozoa without CDs can result in the formation of a zygote. To address this question, we inseminated *Odf4*$^{-/-}$ spermatozoa to wild-type *Odf4*$^{+/+}$ oocytes under IVF conditions, similar to what was done in the capacitation test. The fertilization events were recorded by a live video recording system equipped with the high-speed video camera system described above. Many *Odf4*$^{-/-}$ spermatozoa reached the oocyte at the end of 60 min and exhibited varying degrees of activated flagellar movement on the zona pellucida; among them, some spermatozoa had straight flagella without CD, but many other sperm flagella were still bent or hairpin shaped (Figure 5A and Movie S6), as was also observed during in vitro capacitation (Figure 4E and Movie S5). At 24 and 48–72 h after insemination started, we found 2-cell (Figure 5B) and 2-4-cell zygotes (Figure 5C and Table 1), respectively.

4 | DISCUSSION

This study revealed that the spermatozoa of infertile mice with *Odf4* deficiency can successfully result in the formation of a zygote by IVF with TYH medium containing pyruvate and glucose. We summarize the results in Figure 6 and discuss the mechanism of rescue using Figure 7. First, the evidence that *Odf4*$^{-/-}$ spermatozoa are capacitated in TYH medium (Figure 1) is supported by the previous finding that *Odf4*$^{-/-}$ spermatozoa can produce enough ATP at a high level, similar to wild-type *Odf4*$^{+/+}$ spermatozoa.[4]

In this study, an important information is that *Odf4*$^{-/-}$ spermatozoa can be capacitated in IVF condition (Figures 1 and 2 right) and fertilize oocytes (Figure 5). Image analysis of SMAS data indicates that the % sperm motility of *Odf4*$^{-/-}$ spermatozoa can significantly increase during capacitation, from 28.5% at 0 min (start) to 35.1% at 60 min (end) (Figure 3A −/−). Video image analysis indicates that some *Odf4*$^{-/-}$ spermatozoa become straight in tail, remove CDs, and gain active forward progression during capacitation, although many *Odf4*$^{-/-}$ spermatozoa were still bent in tail with large CDs and moved backward even at 60 min after incubation in TYH medium, suggesting that there were *Odf4*$^{-/-}$ spermatozoa in various states of capacitation (Figure 4B for Movie S2 and Figure 4E for Movie S5). The result in tyrosine phosphorylation test showing that the staining intensity of 65 kDa band protein in *Odf4*$^{-/-}$ spermatozoa (4.5) was positive, though the intensity was lower than that in *Odf4*$^{+/+}$ spermatozoa (7.3) (Figure 1). Considering all these lines of evidence, some *Odf4*$^{-/-}$ spermatozoa can be highly capacitated with forward swimming, which is important to succeed IVF (Figure 6), although total number of highly capacitated spermatozoa with straight tails was smaller in *Odf4*$^{-/-}$ spermatozoa (31.5%) than in *Odf4*$^{+/+}$ spermatozoa (91.7%), as judged by the % straight tail appearance at the end (60 min) of the capacitation test in TYH medium (Figure 2). Thus, IVF with an appropriate concentration of spermatozoa in a small drop of TYH medium is thought to be a better method for oocyte fertilization by *Odf4*$^{-/-}$ spermatozoa as opposed to in vivo fertilization, where many spermatozoa get stuck in the uterus.[4]

TABLE 1 Total number of zygotes (1–4 cells).

Cells (zygotes)	1	2	3	4	Total
Number of cells	72	29	15	22	138

Note: Percentage (%) of 2–4 cells: 66 (29 + 15 + 22) 138 = 0. 478 (47.8%).

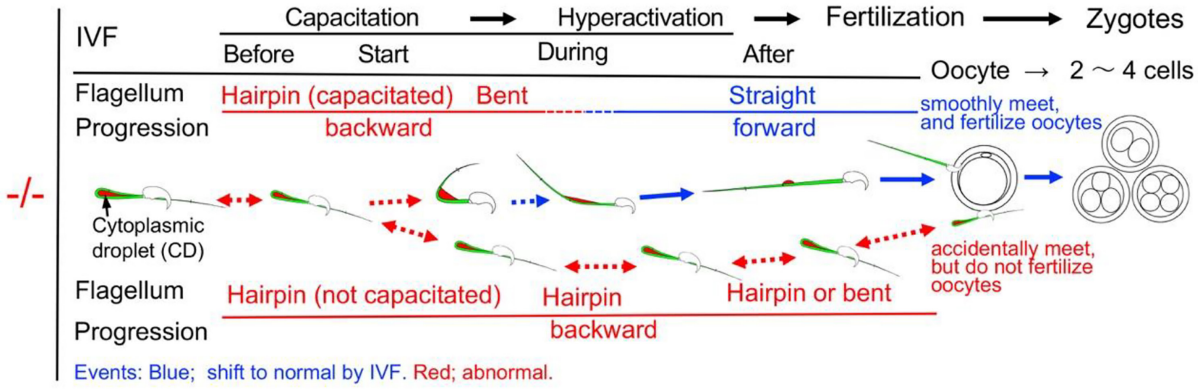

FIGURE 6 Summary of the present results. Before capacitation, the flagella of the *Odf4*$^{-/-}$ spermatozoa were hairpin or bent in the shape (indicated by a red color). In contrast, during IVF (indicated by blue) in TYH medium, *Odf4*$^{-/-}$ spermatozoa which could be capacitated (Figure 1) gradually reduced the volume of large cytoplasmic droplets (CDs) (Figure 4B,E). Among them, there were some *Odf4*$^{-/-}$ spermatozoa without CD that developed straight flagella to swim forward (Figure 4B); thus, several spermatozoa could attach to the zona pellucida (ZP), resulting in the formation of a zygote (blue arrows). On the other hand, there were many *Odf4*$^{-/-}$ spermatozoa with hairpin flagella harboring CD (Figure 4B), but they accidentally attached to the zona pellucida (red arrows); these spermatozoa failed to enter the oocyte due to the reduced motility and abnormal shape of the flagella.

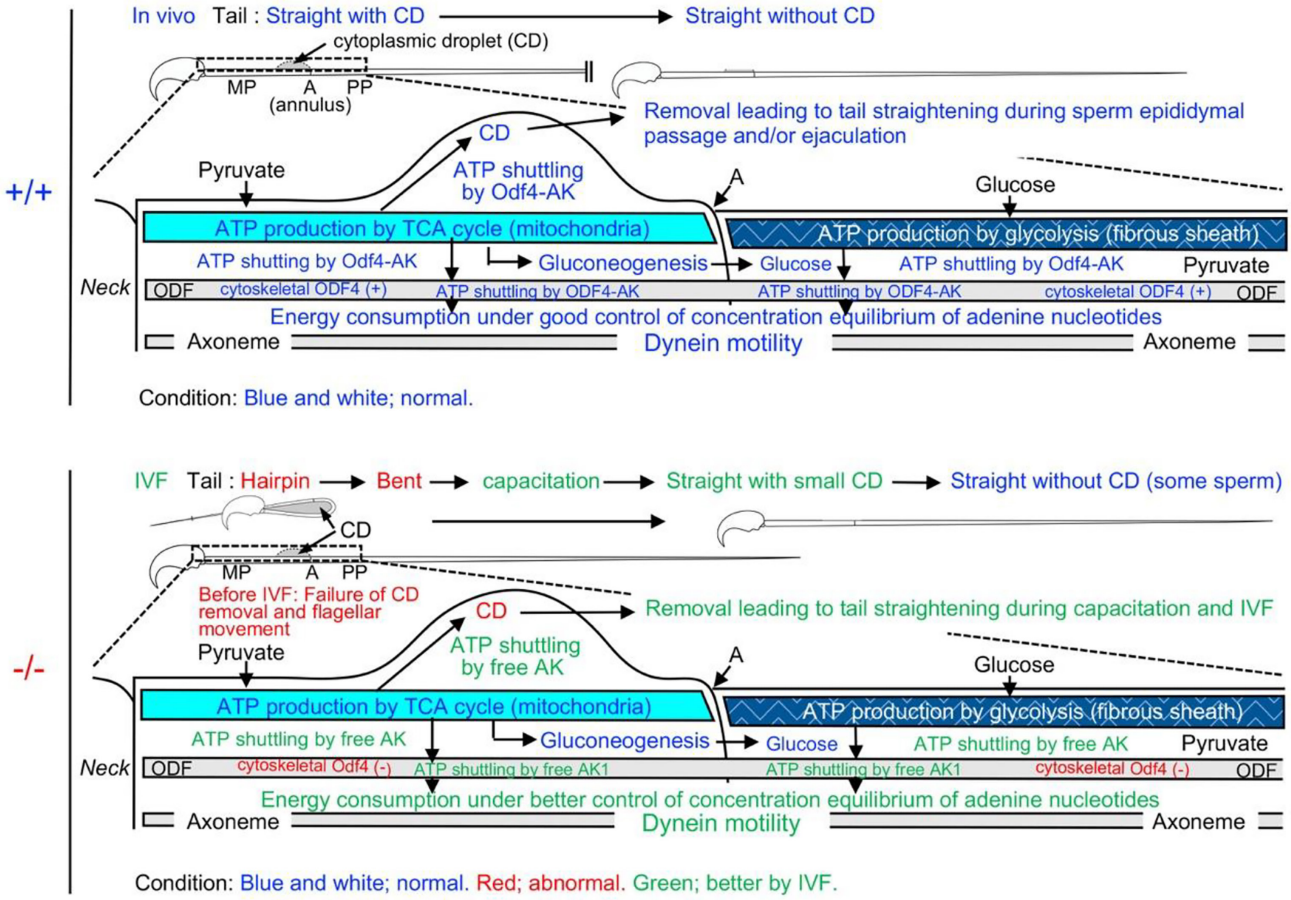

FIGURE 7 Mechanism of the reversal of *Odf4*$^{-/-}$ male infertility by IVF. (Top) +/+: *Odf4*$^{+/+}$ spermatozoa (control). In vivo. (Bottom) −/−: *Odf4*$^{-/-}$ spermatozoa. IVF. (Top) As reported previously,[4] in *Odf4*$^{+/+}$ flagellum, ATP is normally produced by TCA cycle and glycolysis, and shuttled to energy-consuming sites, dynein and cytoplasmic droplets (CDs), by adenylate kinase (AK), where AK controls the ATP/ADP balance, and soluble ODF4 controls the AK positioning. ATP is well consumed, and *Odf4*$^{+/+}$ spermatozoa develop straight flagella and fertilize oocytes in vivo. (Bottom) As reported previously,[4] in *Odf4*$^{-/-}$ flagellum, ATP is actively produced by TCA cycle and glycolysis, similar level to *Odf4*$^{+/+}$ flagellum, but the amount of AK is reduced, and the AK is freely present due to lack of interaction with soluble ODF4. Therefore, the produced ATP is not smoothly shuttled to energy-consuming sites, and the ATP consumption is worse in flagella, eventually leading to sperm stacking in the uterus and infertility in vivo. However, IVF for *Odf4*$^{-/-}$ spermatozoa in a small dish, which contains rich pyruvate and glucose in TYH medium, provides good conditions for flagella, where ATP is continually produced by TCA cycle and glycolysis. ATP can be shuttled to energy-consuming sites by free AK in capacitated spermatozoa, even in the condition of a reduced amount of AK (Figure 1A). In fact, some highly capacitated *Odf4*$^{-/-}$ spermatozoa with straight tail appear and fertilize oocytes, eventually leading to a zygote formation (Figures 5B,C and 6). *Odf4*$^{-/-}$ spermatozoa with bent tail move backward (Figure 4B,E and Movies S2 and S5), and accidentally reach oocyte surface, but cannot enter into oocytes.

A possible mechanism for the reversal of *Odf4*-deficient male infertility at the molecular level is shown in Figure 7, considering previous findings[4] and the discussion above. Basically, in *Odf4*$^{+/+}$ spermatozoa, AK in the ODF4-AK complex shuttles ATP to energy-consuming sites, in which soluble ODF4 functions to correct the positioning of AK[4] (Figure 7 +/+). AK controls the concentration equilibrium of adenine nucleotides to balance energy production and consumption.[6] In contrast, in *Odf4*$^{-/-}$ spermatozoa, ATP can be shuttled to energy-consuming sites by free AK1 and AK2, where the shuttling efficiency is presumably insufficient or uncontrolled in in vivo fertilization because the amount of AK1 and AK2 is reduced and because the positioning of AK1 and AK2 is poorly controlled due to the lack of soluble ODF4.[4] This also means that in in vivo fertilization the control of the ATP/ADP concentration and the balance between ATP production and consumption are worse in *Odf4*$^{-/-}$ spermatozoa than in *Odf4*$^{+/+}$ spermatozoa. However, even in such poor conditions, AK1 and AK2, even in small amounts, can shuttle ATP to energy-consuming sites in flagella to induce capacitation in in vitro fertilization; in fact, the total number of activated *Odf4*$^{-/-}$ spermatozoa was slightly increased at the end (60 min) of the capacitation test, as shown in Figure 3D (−/−: 7 spermatozoa at 0 min versus 15 spermatozoa at 60 min). Thus, in in vitro fertilization there were some activated *Odf4*$^{-/-}$ spermatozoa with straight tail after 60 min incubation (31.5%, Figure 2), in which the volume of large CDs was greatly reduced and the spermatozoa swam forward, and reached the

oocyte to form a zygote (Figure 6; progression indicated by blue arrows and characters). However, there were still many *Odf4*$^{-/-}$ spermatozoa that were low state of capacitation; in this case, the large CDs cannot be removed from the flagella, and the *Odf4*$^{-/-}$ spermatozoa with large CDs swim backward and do not fertilize the oocyte to form a zygote (Figure 6; progression indicated by red arrows and characters).

Concerning other molecules involved in the mechanism, we cannot completely deny the possibility that AK7 and/or AK8[14] are involved; however, there is currently no information about how AK7 or AK8 are related to ATP shuttling to energy-consuming sites.

Regarding the slight reduction in sperm motility shown in the bar graphs calculated by SMAS, where the straight line velocity and average velocity of *Odf4*$^{+/+}$ sperm motility at 60 min were lower than those at 0 min (Figure 3A–C), our explanation is as follows. Since spermatozoa were preincubated for 30 min in a CO_2 incubator before starting SMAS analysis as described in the Methods section, it is presumable that *Odf4*$^{+/+}$ spermatozoa had already been activated at the start of the examination (0 min), as shown in the Movie S3 and SMAS trajectory data (50 spermatozoa at 0 min as opposed to 43 spermatozoa at 60 min; Figure 3D). This means that many *Odf4*$^{+/+}$ spermatozoa quickly reach maximum motility, indicating that ATP is quickly consumed by sperm cells; thus, the motility in total, which is shown by the SMAS parameters in the bar graphs, was slightly reduced before the end of the experiment (Figure 3A–C).

The detailed molecular mechanism of how the CD is removed from sperm flagella is unclear at present, but CATSPER3, AQP3,7, and 8, and SLC22A14, which are related to solute carriers or water or ion exchangers, are not involved in this mechanism because these proteins are normally present in *Odf4*$^{-/-}$ spermatozoa, as shown by immunoblotting in our previous report.[4]

5 | CONCLUSION

ODF4-deficient male infertility has not been reported in humans. Although this study was conducted using mouse models, the findings reported here and previously can be translated to the case of humans,[4] i.e., infertility due to the *ODF4*-deficient spermatozoa getting stuck in the uterus, which is caused by the backward movement of spermatozoa due to morphologically abnormal hairpin flagella bearing large CDs. Therefore, the present results provide strong evidence for how to manage *ODF4*-deficient infertility in humans given that a sufficient number of zygotes (consisting of 2–4 cells) can be obtained by conventional IVF. These zygotes can then be transferred into the female reproductive tract to develop into fetuses. Thus, simple combinations of ARTs (IVF and embryo transfer into the female reproductive tract), which are now widely performed worldwide, constitute the first methods that can be used for infertile human males with *ODF4* deficiency to impregnate their partner. Such a combination treatment will also be applicable to similar cases of infertility in other mammalian *ODF4/Odf4* gene-deficient males.

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CONFLICT OF INTEREST STATEMENT

All the authors have no conflicts of interest to declare relevant to this study.

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SUPPORTING INFORMATION

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