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Testosterone-Induced Metabolic Changes in Seminal Vesicle Epithelial cells Alter Plasma Components to Enhance Sperm Fertility

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eLife Assessment

Seminal plasma is a crucial component of semen that can affect sperm capacitation. However, the role of seminal plasma components, including fatty acids, in sperm function and fertility is poorly understood. In this **important** study, the authors provide a **solid** evidence of the testosterone-induced metabolic shift in the epithelial cells of seminal vesicle to support an fatty acid synthesis and also describe the potential effect of oleic acid on sperm motility.

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Abstract

Male factors account for almost half of the causes of infertility. In rodents and humans, most of the components of semen are supplied by the seminal vesicles, and they support male reproductive ability, but there are many unknown details. In this study, the metabolic changes in the seminal vesicle epithelial cells were focused on, and the mechanisms by which testosterone affects the seminal plasma composition were investigated. A factor that improves the linear motility of sperm was secreted from the seminal vesicles, which were synthesized in an androgen-dependent manner. Bioassays, gene expression, and flux analysis studies demonstrated that testosterone promotes glucose uptake in seminal vesicle epithelial cells via GLUT4, resulting in oleic acid synthesis. Oleic acid was shown to be taken up by sperm and to promote linear motility, thereby improving fertilization rates both *in vitro* and *in vivo*. ACLY was a critical factor in this metabolic change, which produces oleic acid and enhances their fertilization ability *in vivo*. In conclusion, the critical role of testosterone-induced metabolic changes in the seminal vesicles is to ensure the synthesis of oleic acid, which is important for sperm fertilization *in vivo*. These findings provide new perspectives for the development of potential biomarkers of male fertility and advances in the treatment of male infertility.

One Sentence Summary

Testosterone induces ACLY expression in seminal vesicles, a key factor in forming seminal plasma to acquire *in vivo* fertilization ability of sperm.

Introduction

Infertility is defined by the World Health Organization (WHO) as the failure to conceive within 12 months despite contraceptive-free sexual intercourse. Its prevalence has increased over the decades and accounts for 17.5% of couples worldwide (1). The male partner is thought to be solely or in combination responsible for 50% of infertility cases, and the male factor in the etiology of infertility is highly recognized. Male fertility depends largely on the quality of semen composed of sperm and seminal plasma and decreases with aging(2–4).

Spermatogenesis occurs in the testes. While passing through the epididymis, sperm undergo a maturation process, but sperm do not fully acquire their fertilizing ability in the epididymis. (5–7). Therefore, sperm collected from the epididymis cannot fertilize oocytes unless they acquire the ability to fertilize in *in vitro* and cannot maintain sustained liner motility required for migration from the uterus to the oviduct (8, 9). Thus, when epididymal sperm are artificially inseminated to the cervix or uterus, fertilization rates are low (10–12). However, when epididymal sperm are mixed with seminal plasma at the time of ejaculation or when sperm are exposed to seminal plasma *in vitro*, even for only a brief period, they acquire *in vivo* fertilization potential and are capable of passing through the female reproductive tract and fertilizing ovulated oocyte *in vivo* (13). Thus, sperm formation occurs in the testis; they mature in the epididymis but only acquire the *in vivo* fertilization ability after exposure to seminal plasma.

Seminal plasma is mainly produced in the seminal vesicles (approximately 65-75%), with approximately 20-30% produced by the prostate, and small amounts secreted by the bulbourethral and urethral glands (14). The mixing of seminal fluid with accessory reproductive gland fluid during ejaculation causes semen to clot and liquefy in humans and causes vaginal plug formation in rodents. Vaginal plugs not only inhibit continuous mating but also play a role in maintaining sperm in the uterus and ensuring male fertility (13). TGFβ, a component of seminal plasma, increases antigen-specific Treg cells in the uterus of mice and humans, which induces immune tolerance, resulting in pregnancy (15, 16). Furthermore, SVS2, secreted from the seminal vesicle, is also known to be required for sperm to escape attack by the female immune system in the uterus (17). In addition, seminal plasma contains various biochemical components that support sperm metabolism, including lipids and organic acids. Differences in the relative size of the accessory reproductive glands reflect differences in the seminal plasma composition. Accordingly, there are considerable differences in the properties of seminal plasma between animal species. Specifically,

Rosecrans et al. (18) found significant differences in the levels of most biochemical components in seminal plasma compared to blood, with specific factors like fructose being rarely detected in blood but abundant in seminal plasma. Our previous studies (19–21) documented that seminal plasma also contains creatine and fatty acids taken up by sperm within minutes. These substances play crucial roles in regulating sperm metabolic pathways and enhancing sperm motility. These observations indicate that seminal plasma not only alters the female immune system to protect sperm but also directly influences sperm metabolic pathways, facilitating the transformation of sperm capable of *in vivo* fertilization.

Some specific factors that male accessory glands contribute to seminal plasma and impact male fertility have been elucidated. For example, surgical removal of seminal vesicles in male mice and rats was associated with infertility (17, 22, 23). The observation that fructose (24) and citric acid (25) concentrations in seminal plasma of control mice and rats are higher than in castrated animals indicates that specific functions of the accessory glands might be affected by testicular-derived testosterone that is known to activate the intracellular androgen receptor (AR; NR3C4), required for gene regulation of transcription. AR-deficient mice have been found to be infertile (26), and long-term administration of androgen receptor antagonists, like flutamide, abolished male fertility (27, 28). AR-deficient mice and transcriptome analyses have identified specific targets of AR (29, 30). In both mice and human, androgen levels decrease with aging, and their concentrations have been reported to cause a decrease in male fertility, especially ejaculated sperm motility. Therefore, the reduction of androgen concentrations may affect the metabolic mechanisms of the accessory reproductive glands and alter the composition of seminal plasma with aging. However, the metabolic mechanisms that impact sperm functions are governed by a complex of enzymatic reactions. Therefore, the intricate interplay between testosterone and seminal plasma synthesis in the accessory reproductive glands requires further investigation.

In this study, we first performed various bioassays to identify which accessory reproductive glands are essential for sperm fertility. Subsequently, comparative *in vivo* and *in vitro* analyses were performed using a hypoandrogenic model. In addition, biochemical and molecular techniques such as RNA-seq, flux analysis, mass spectrometry, and knockdown experiments were employed. These analyses revealed that testosterone promotes the synthesis of oleic acid in seminal vesicle epithelial cells and its secretion into seminal plasma, and the oleic acid ensures the linear motility and fertilization ability of sperm.

Results

The effect of androgen-dependent changes in mouse seminal vesicle secretions on the linear motility of sperm

A direct comparison was conducted to clarify the effects of secretions from the prostate and seminal vesicles on the motility parameters of epididymal sperm. Since it is difficult to collect the ejaculated semen of mice, a pseudo-seminal plasma was prepared by preparing fluids from the seminal vesicle secretion and homogenized prostate tissue (**Fig.1A**, **Supplemental Fig.1A**). When seminal vesicle secretions were added to the HTF medium, the sperm motility, progressive motile, the straight line velocity (VSL), and the percentage of linearity (LIN) values increased significantly compared to when only prostate extract was used. However, the curvi-linear velocity (VCL) did not change (**Fig.1B**-F). Meanwhile, the secretions from the prostate and seminal vesicles did not affect the viscosity of the medium (**Fig.1G**). These results indicate that sperm exposure to seminal plasma factors significantly improves sperm linear motility (high VSL and LIN).

Androgens regulate the functions of seminal vesicles. Therefore, to elucidate whether the seminal vesicle factors, which enhance sperm linear motility, are synthesized in an androgen- dependent manner, bioassays were performed using seminal vesicle secretions collected from control mice or mice treated daily for 7 consecutive days with flutamide, an androgen receptor antagonist (**Fig. 1H**, **Supplemental Fig.1B**). The results showed that Motile, Progressive motile, LIN, and VSL were significantly reduced in the sperm cultured in seminal vesicle secretions from flutamide-treated (Flutamide) compared to the sperm cultured in seminal vesicle secretions from controls (Ctrl) (**Fig.1I**-L). The reduction of VSL, Motile, and Progressive motile were also significantly lower in the sperm treated with seminal vesicle secretions from over 12 months old mice that showed low circular testosterone levels as compared with the sperm treated with seminal vesicle

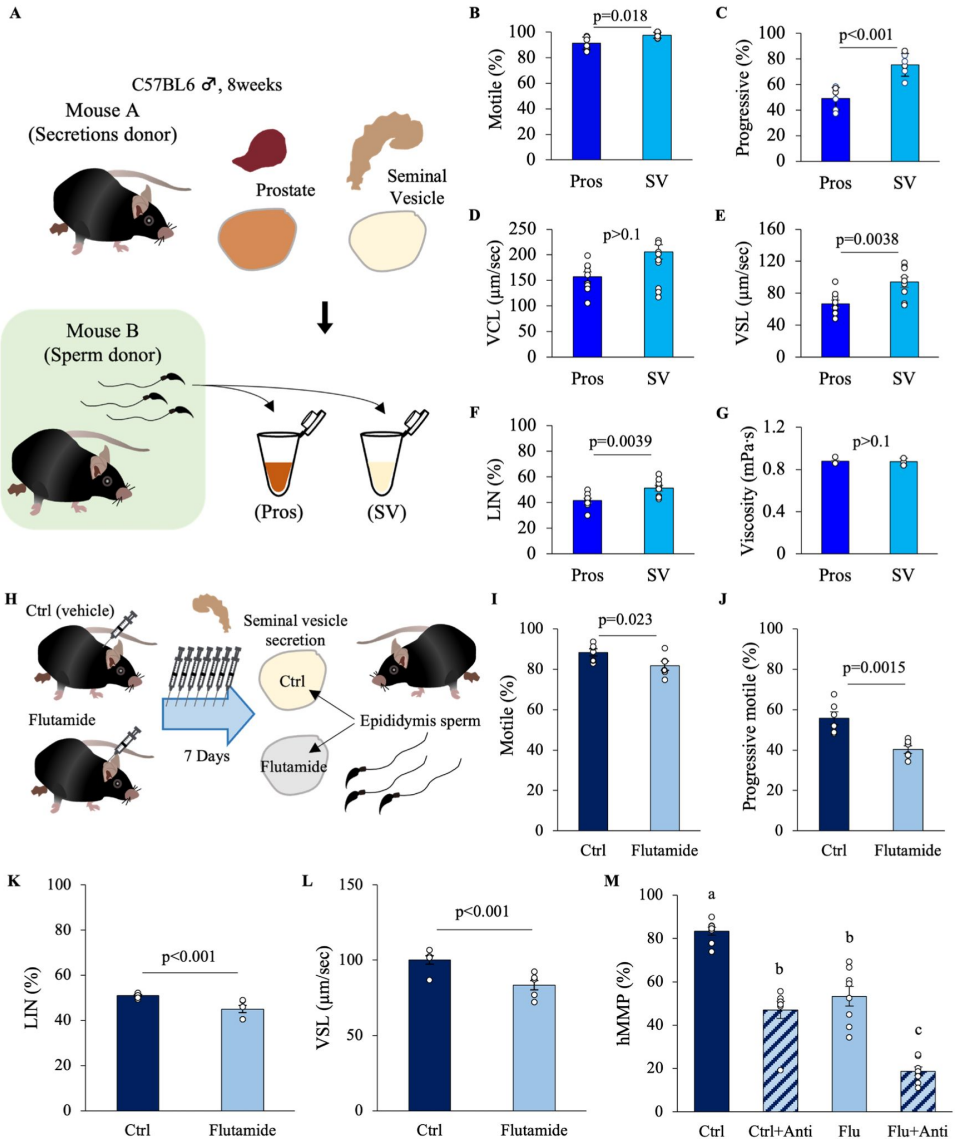


Figure 1.

Effects of seminal vesicle fluid and prostate fluids on sperm motility

(A) The effects of secretions from the prostate and seminal vesicles on sperm motility were directly compared. (B-F) The effects of secretions from the prostate (Pros) or seminal vesicles (SV) on the motility parameters of epididymal sperm were tested: (B) Motile, (C) Progressive motile, (D) Curvi-Linear Velocity; VCL, (E) Straight-Line Velocity; VSL and (F) Linearity; LIN is the ratio of VSL to VCL of sperm that was incubated with the mixture of seminal vesicle secretions or prostate extracts. (G) Viscosity of a solution containing prostate or seminal vesicle secretion with a protein concentration of 10 mg/mL. (H) Experimental design to evaluate the effect of seminal vesicle secretions collected from male mice treated with or without flutamide (50 mg/kg subcutaneously every day for 7 days) on sperm. Note that the donor mice for sperm and seminal vesicle secretions were different. (I-L) Performed bioassay using seminal vesicle fluid after treatment with vehicle (Ctrl) or flutamide: (I) Motile, (J) Progressive motile, (K) LIN, (L) VSL. (M) The high mitochondrial membrane potential (hMMP) was checked by the JC-1 kit. Antimycin (Anti) was used as the negative control for hMMP. Data are mean ± SEM. n=3-9 independent replicates. The viscosity measurement data were from n=3 repeated experiments using pooled prostate or seminal vesicle extracts from at least 20 mice. Percentage data were subjected to arcsine transformation before statistical analysis. (B-G, I-L) Significance was tested in comparison using Student's t-test. (M) Since the results of the Bartlett test were significant, a two-way ANOVA using a generalized linear model was performed. Both the flutamide administration and antimycin addition were significant. The interaction was not significant. Games-Howell was performed as a post hoc test. Different letters represent significantly different groups.

secretions from young adult mice (3 months old mice) (**Supplemental Fig.2A,B**). Furthermore, JC-1 staining to measure mitochondrial membrane potential, which is important for sperm linear motility, showed a significantly lower percentage of high mitochondrial membrane potential in sperm treated with the secretions of seminal vesicles from flutamide-treated mice and over 12 months old mice (**Fig.1M**, **Supplemental Fig.1C,D**, **Supplemental Fig.2C**). Since flutamide treatment and aging also affect spermatogenesis and sperm motility, it should be noted that epididymal sperm donors differ from seminal vesicle donors (**Fig. 1H**). The baseline information for the epididymal sperm used in the experiment showed that the sperm were normal (**Supplemental Fig.1A**). These results indicated that androgen-dependent changes in seminal vesicle functions alter the levels/activity of seminal vesicle secretion factors, which regulate sperm linear motility. The changes in seminal vesicle functions were also induced by aging.

Testosterone-androgen receptor activity suppresses the proliferation of seminal vesicle epithelial cells

To understand the difference between seminal vesicles that secrete factors for enhancing sperm linear motility and those that are impaired in this function, we performed histological analysis of seminal vesicles recovered from mice treated and non-treated with flutamide. In control mice, the seminal vesicle epithelial cells were in a single layer, and androgen receptors were localized in the nuclei of seminal vesicle epithelial cells (**Fig.2A**). However, in mice treated with continuous administration of flutamide, seminal vesicle epithelial cells were multilayered, and the strong accumulation of androgen receptors in nucleus was not observed (**Fig.2A**). To investigate the cause of this multilayering of cells, we performed Ki67 staining, a marker of cell proliferation, and apoptosis detection by TUNEL staining (**Fig.2B**). The results showed that the percentage of Ki67-positive cells increased significantly after treatment with flutamide, but the percentage of TUNEL-positive cells did not change, suggesting that the testosterone-androgen receptor pathway is responsible for the inhibition of proliferation of seminal vesicle epithelial cells (**Fig.2C**). These changes of abnormal morphology and high proliferation activity were also observed in seminal vesicle of more than 12 months old mice that showed the low level of testosterone in serum (**Supplemental Figure 2D-F**). Therefore, seminal vesicle epithelial cells were collected and cultured in primary culture, as shown in **Fig. 2D**. The changes in cell number were counted, and the cell cycle was analyzed by flow cytometry. When cultured without testosterone (c), the number of seminal vesicle epithelial cells increased day-dependent manner. However, the addition of testosterone significantly decreased the number of seminal vesicle epithelial cells during the 8-day culture period in a concentration-dependent manner (**Fig.2E**). Flow cytometric analysis of the cell cycle on day 7 showed that the G0/G1 phase was significantly increased. The G2/M1 phase was significantly decreased by the addition of testosterone (**Fig. 2F,G**). These results indicate that the testosterone-androgen receptor pathway inhibits seminal vesicle epithelial cells in cell division.

Testosterone alters metabolic gene expression in seminal vesicle epithelial cells

To better understand the roles of testosterone in suppressing the proliferation of seminal vesicle epithelial cells, RNA sequencing was done using the epithelial cells cultured with or without testosterone. DEG (Differential Expressed Genes) analysis revealed that a total of 23,459 genes were identified, including 997 upregulated genes and 3463 downregulated genes in seminal vesicle epithelial cells by testosterone relative to control (**Fig.3A**). Genes largely upregulated by testosterone included those already reported in seminal vesicles (17, 22, 31), such as the Seminal Vesicle Secretion (*Svs*) family (*Svs1~6*), *Pate4*, *Spinkl*, *Ceacam10*, and *Sva* (**Fig.3B**,C). Therefore, the primary culture of seminal vesicle epithelial cells used in this experiment was considered to maintain the characteristics of seminal vesicle epithelial cells *in vivo*. Many genes were downregulated by testosterone, and those with a high rate of variation included collagen synthase and *Mmp2*, which are involved in extracellular matrix formation (**Fig.3C**). However,

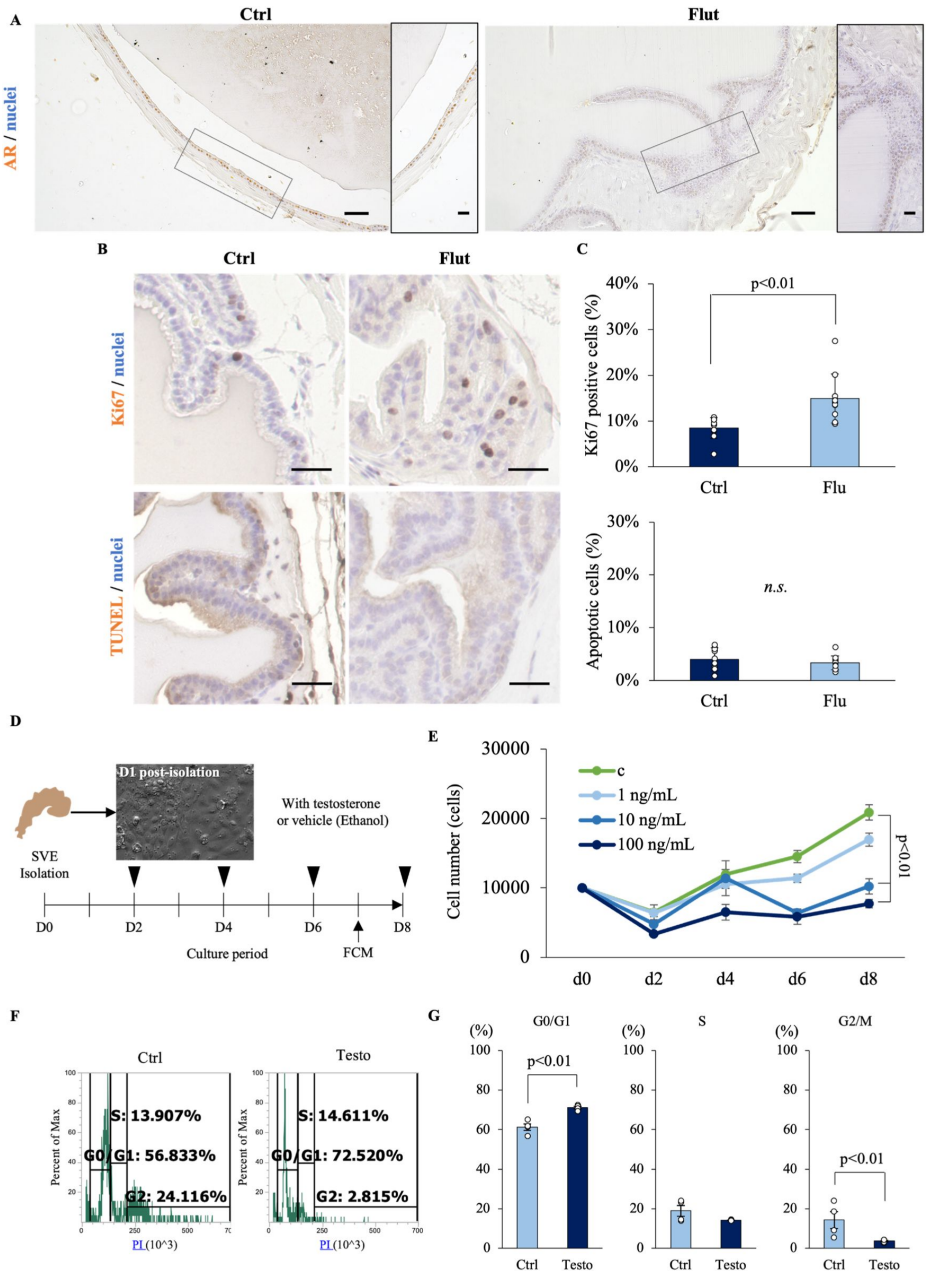


Figure 2.

The testosterone-androgen receptor pathway inhibits the cell proliferation of seminal vesicle epithelial cells.

(A) The localization of the androgen receptor (AR; NR3C4) in seminal vesicle of control (Ctrl) mice and those treated with flutamide (Fult). Scale bar=50 μm from 10× magnification for left panel and 20 μm from 20× magnification for right panel in each group. (B,C) Cell proliferation and apoptosis in seminal vesicle of control (Ctrl) mice and those treated with flutamide (Fult): (B) Representative images of Ctrl and Flut staining for Ki67 (top) and TUNEL (bottom). Scale bar=20 μm. (C) Percentage of Ki67 positive cells (top) and TUNEL positive (bottom) in Ctrl and Flut-treated seminal vesicle sections. (D) Experimental design to evaluate the effects of testosterone on the proliferation of seminal vesicle epithelial cells *in vitro*. The epithelial cells were culutured with or without testosterone for 8 days. (E) Growth curves of seminal vesicle epithelial cells that cultured with 0 (c), 1, 10 and 100 ng/mL of testosterone. (F,G) Cell cycle status was determined by flow cytometry. Data are mean ± SEM. n=3-5 mice or independent replicates. Each replicate experiments with 3-6 wells containing pooled cells from 3-5 mice. Percentage data were subjected to arcsine transformation before statistical analysis. Significance was tested in comparison to Ctrl using Student's t-test. The cell number data at d8 was compared with the control using Dunnett's test.

KEGG analysis, rather than individual genes, was used to predict functions altered by testosterone. It was found that the expression of genes involved in the metabolic pathway was the most highly affected by testosterone (**Fig.3D**). Because there is a close relationship between cell proliferation and metabolic activity and between metabolic substrates in seminal plasma and sperm motility, we hypothesized that testosterone induced changes in seminal vesicle epithelial cell metabolism alter the components of seminal plasma to mediate the enhancement of sperm motility.

Testosterone shifts the metabolic activity of seminal vesicle epithelial cells

In the presence of testosterone, the addition of glucose significantly activated the extracellular oxidation rate (ECAR) normalized by the total number of live cells compared to that in epithelial cells cultured without testosterone (**Fig.4A**-C). However, there was no difference in the Oligomycin-sensitive ECAR, indicating that testosterone may increase glucose metabolism but does not enhance the expression of a group of enzymes involved in the glycolytic pathway. To determine the metabolic fate of glycolytic end products, seminal vesicle epithelial cells were cultured with and without testosterone. Subsequently, the concentrations of pyruvate and lactate in the culture supernatant normalized by the total number of live cells were measured. The addition of testosterone significantly decreased the pyruvate concentration; however, the lactate concentration did not change significantly (**Fig.4D**,E). Therefore, we analyzed mitochondrial metabolism using a flux analyzer, predicting that more glucose is metabolized, pyruvate is synthesized in response to testosterone, and is further metabolized in the mitochondria. Unexpectedly, when measuring mitochondrial respiratory metabolism normalized by the total number of live cells with a Flux analyzer, the basal respiratory rate (the electron transport chain activity) was significantly decreased by testosterone (**Fig.4F**-J). Oligomycin-sensitive respiration was also significantly suppressed by exposure to testosterone. Furthermore, the FCCP uncoupled respiration and spare respiration capacity were also significantly decreased in response to testosterone treatment. These results suggest that testosterone does not reduce transient enzyme activity in mitochondria but rather weakens the metabolic pathway of the mitochondrial TCA cycle and/or the electron transport chain due to the changes in gene expression patterns in seminal vesicle epithelial cells.

Testosterone causes changes in the expression of genes encoding enzymes involved in glucose catabolism and anabolism

The expression of genes involved in glucose catabolism and anabolism in the *in vitro* culture system of seminal vesicle epithelial cells was analyzed by qPCR (**Fig.5**). The gene expression of glucose transporter (*Glut*) and enzymes involved in the glycolytic pathway was not significantly affected by testosterone, consistent with the results of metabolic flux analysis, which showed that the glycolytic pathway was not enhanced despite the activation of the glycolysis. Furthermore, testosterone did not significantly affect the expression of genes encoding enzymes involved in the TCA cycle in mitochondria. However, the expression of enzymes involved in the electron transport chain was significantly decreased. Interestingly, the expression of *Acly*, which encodes an enzyme that reconverts citric acid formed in the TCA cycle to Acetyl CoA and induces cholesterol and fatty acid synthesis, was significantly increased by testosterone. Although *Hmgcr*, which encodes the rate-limiting enzyme involved in cholesterol synthesis, was unchanged, the expression of *Acc*, which encodes the rate-limiting enzyme for fatty acid synthesis, was significantly upregulated by testosterone. These characteristic testosterone-induced changes in gene expression observed in cultured cells were also detected by ACLY immunostaining of *in vivo* seminal vesicle samples (**Supplemental Figure 3**). Specifically, the expression of genes encoding a group of enzymes of the electron transport chain was significantly upregulated by continuous flutamide administration *in vivo*, whereas the gene expression of *Acly* and *Acc* was significantly decreased by flutamide administration. Immunostaining results showed that ACLY was detected specifically in seminal

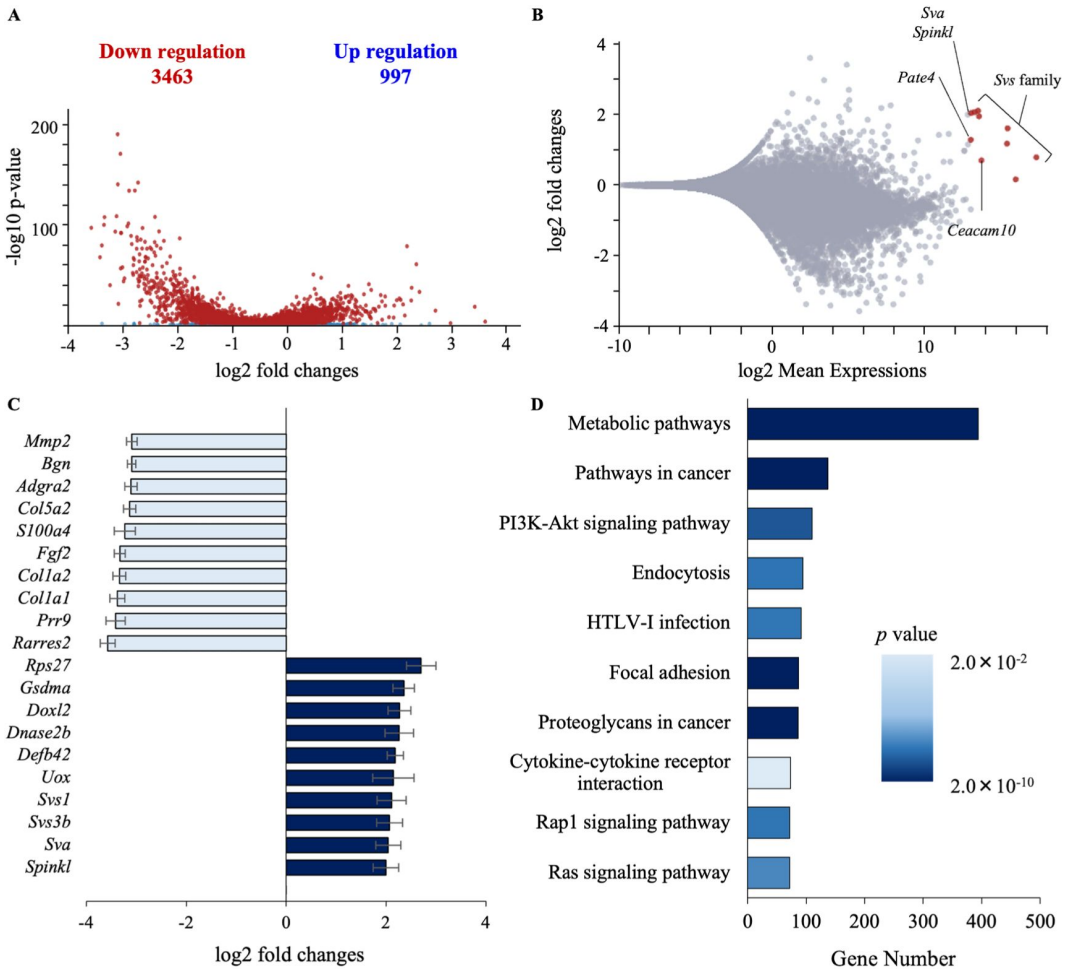


Figure 3.

Testosterone changes the expression of genes involved in metabolic pathway in seminal vesicle epithelial cells.

(A) Volcano plot of differentially expressed genes. RNA sequencing was performed using RNA extracted from the seminal vesicle epithelial cells cultured with or without 100 ng/mL testosterone. Genes with a significant expression change are highlighted as red dots. It found 4460 significant genes for a cutoff of P<0.05. (B) MA plot of differential expression results. The seminal vesicle specific genes are highlighted as red dots rather than significant genes. (C) Top 10 genes upregulated or downregulated by testosterone, respectively, for altered gene expression. (D) Conducted KEGG analysis to identify differences in pathway enrichment by testosterone, identifying the most variability in metabolic pathway genes. It shows the number of differential expressed genes annotated to Gene Ontology shows (x axis). p-value which measure the statistical significance of a possible functional enrichment for each term.

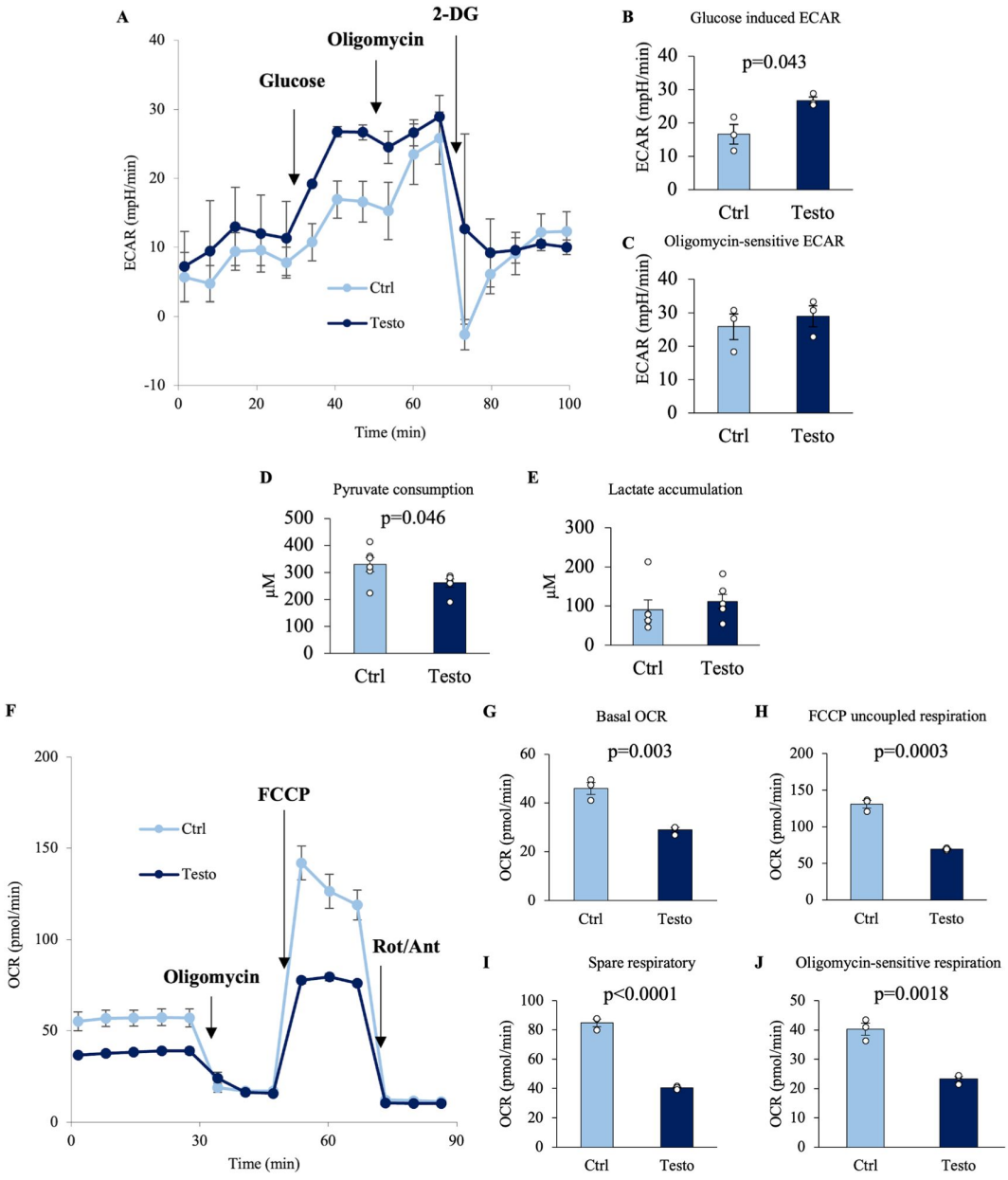


Figure 4.

Testosterone regulates glucose metabolism and mitochondrial ATP production in seminal vesicle epithelial cells.

(A-C) Extracellular acidification rate (ECAR) analysis by an extracellular flux analyzer in seminal vesicle epithelial cells cultured with 100 ng/mL testosterone (Testo) or vehicle (Ctrl) for 7 days: (A) ECAR kinetics of seminal vesicle epithelial cells using an extracellular flux analyzer. (B) Glucose induced ECAR and (C) Oligomycin-sensitive ECAR. (D,E) The concentrations of pyruvate and lactate in the culture supernatant. These measurements were normalized based on cell count and viability. (D) Pyruvate concentration in the medium where seminal vesicle epithelial cells were cultured with or without testosterone for 24 hours. (E) Lactate concentration. (F-J) Mitochondrial respiration measurement by an extracellular flux analyzer: (F) Oxygen consumption rate (OCR) kinetics of seminal vesicle epithelial cells with or without 100 ng/mL testosterone. (G) Basal OCR, (H) FCCP uncoupled respiration, (I) Spare respiratory capacity and (J) Oligomycin-sensitive respiration. Data are mean ± SEM of n = 3 replicate experiments with 3-6 wells containing pooled cells from 3-5 mice or medium. Student's t-test was used to compare Ctrl and Testo. The cells were normalized to 10,000 viable cells/well immediately before analysis. The viability of the cells before experiments was 86-93%.

vesicle epithelial cells, and their staining appeared to be reduced by flutamide treatment. The lower level of ACLY was also observed in seminal vesicle epithelial cells of over 12 months old mice (**Supplemental Figure 3B**).

Mechanism of testosterone-induced enhancement of glucose uptake and, thereby, fatty acid synthesis

Since it is known that testosterone regulates the intracellular localization of GLUT4, we focused on the intracellular localization of GLUT4 and its glucose uptake capacity (32, 33). The effect of testosterone on glucose uptake in seminal vesicle epithelial cells was analyzed using fluorescence-labeled glucose. As expected from the results of flux metabolic analyses, testosterone treatment significantly increased glucose uptake (**Fig.6A**). Indinavir, a functional inhibitor of GLUT4, significantly suppressed glucose uptake and significantly decreased fatty acid synthesis (the amount of fatty acids accumulated in the cell), which was increased by testosterone (**Fig.6A**,B).

Furthermore, immunostaining analysis of the localization of GLUT4 in seminal vesicles revealed that GLUT4 was translocalized outside the nucleus of seminal vesicle epithelial cells under the presence of testosterone (**Fig.6C**). These data suggest that GLUT4 was partially localized at the plasma membrane and contributed to glucose uptake. In contrast, in seminal vesicles from mice treated with flutamide continuously, no strong signal was detected at the presumed location of GLUT4 near the plasma membrane of seminal vesicle epithelial cells, even though the protein expression level was not affected (**Fig.6C**,D). The same characteristics were observed in aged mice.

Qualitative and quantitative analysis of the fatty acids synthesis was performed by GC-MS. In seminal vesicles, C16:0, C18:0, and C18:1 were detected, in which C18:1 was decreased to an undetectable level in seminal vesicles of mice treated with the flutamide (**Supplemental Figure 4A**). In the cultured seminal vesicle epithelial cells, C18:1, oleic acid, was significantly increased by the addition of testosterone as observed in the seminal vesicle (**Fig.6E**). The expression of *Elovl6* encoding ELOVL6, which desaturates C18:0 saturated fatty acids to C18:1, was significantly up-regulated by testosterone (**Fig.6F**,G). The testosterone-induced change was also detected in *in vivo* samples (seminal vesicle), where the expression of *Evovl6* was decreased by the flutamide treatment (**Supplemental Figure 4B**).

Oleic acid (18:1) is taken up by sperm and promotes their linear motility

We observed that seminal vesicles secrete testosterone-dependent factors that induce sperm to move linear and that seminal vesicle epithelial cells synthesize and secrete oleic acid in a testosterone-dependent manner. In order to examine whether oleic acid is a factor that induces sperm linear motility, we examined whether or not oleic acid is taken up by sperm and then changes in sperm motility pattern. When epididymal sperm were incubated with fluorescently labeled oleic acid, the fluorescent signal was selectively detected in the midpiece of the sperm after quenching of extracellular fluorescence (**Fig.7A**). The fluorescence intensity of sperm detected by flow cytometry significantly increased in a dose-dependent manner (**Fig.7B**, C). Oleic acid did not affect sperm and progressive motile at any concentration compared to the control (**Fig.7D**,E). In oleic acid-treated sperm, VSL was significantly increased in a dose-dependent manner in the 0 to 50 nM (**Fig.7F**). LIN values were highest when 10 nM oleic acid was added (**Fig.7G**). A flux analyzer was used to monitor real-time changes in mitochondrial β-oxidation normalized by the number of sperm to quantify metabolic changes in mitochondrial activity. The injection of 10 nM oleic acid induced a significant increase in OCR. Etomoxir, a β-oxidation inhibitor, significantly decreased the OCR that was induced by oleic acid (**Fig.7H**, Supplemental Figure 5A). In situ ATP detection by BioTracker ATP-Red revealed that the

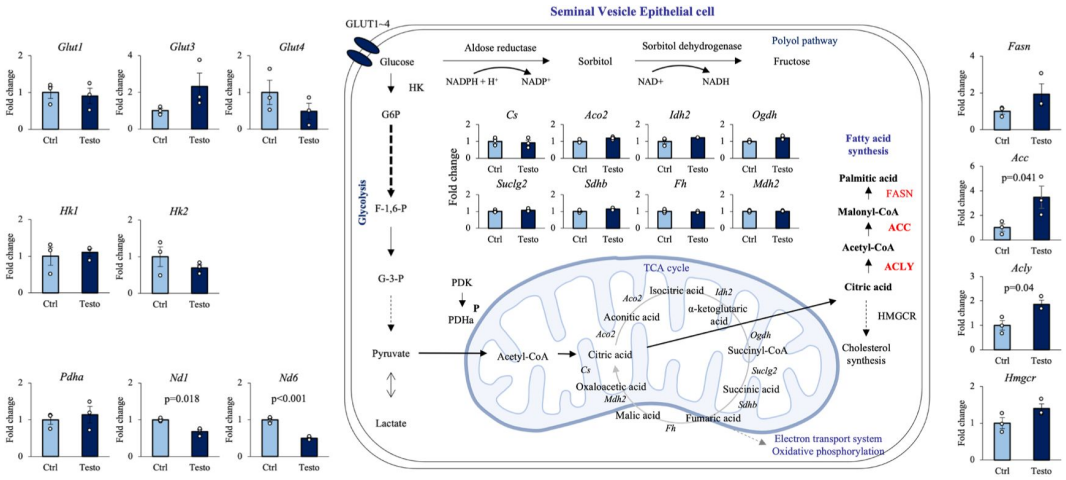


Figure 5.

Effect of testosterone on gene expression of enzymes involved in the glucose metabolic pathway in seminal vesicle epithelial cells.

To elucidate the effects of testosterone on gene expression of enzymes involved in glucose catabolism and anabolism in seminal vesicle epithelial cells. ctrl: 7 days of culture with vehicle. Testo: 7 days of culture with 100 ng/mL testosterone. Data are mean ± SEM of n = 3 replicate experiments with 3-6 wells containing pooled cells from 3-5 mice. Student's t-test was used to compare Ctrl and Testo.

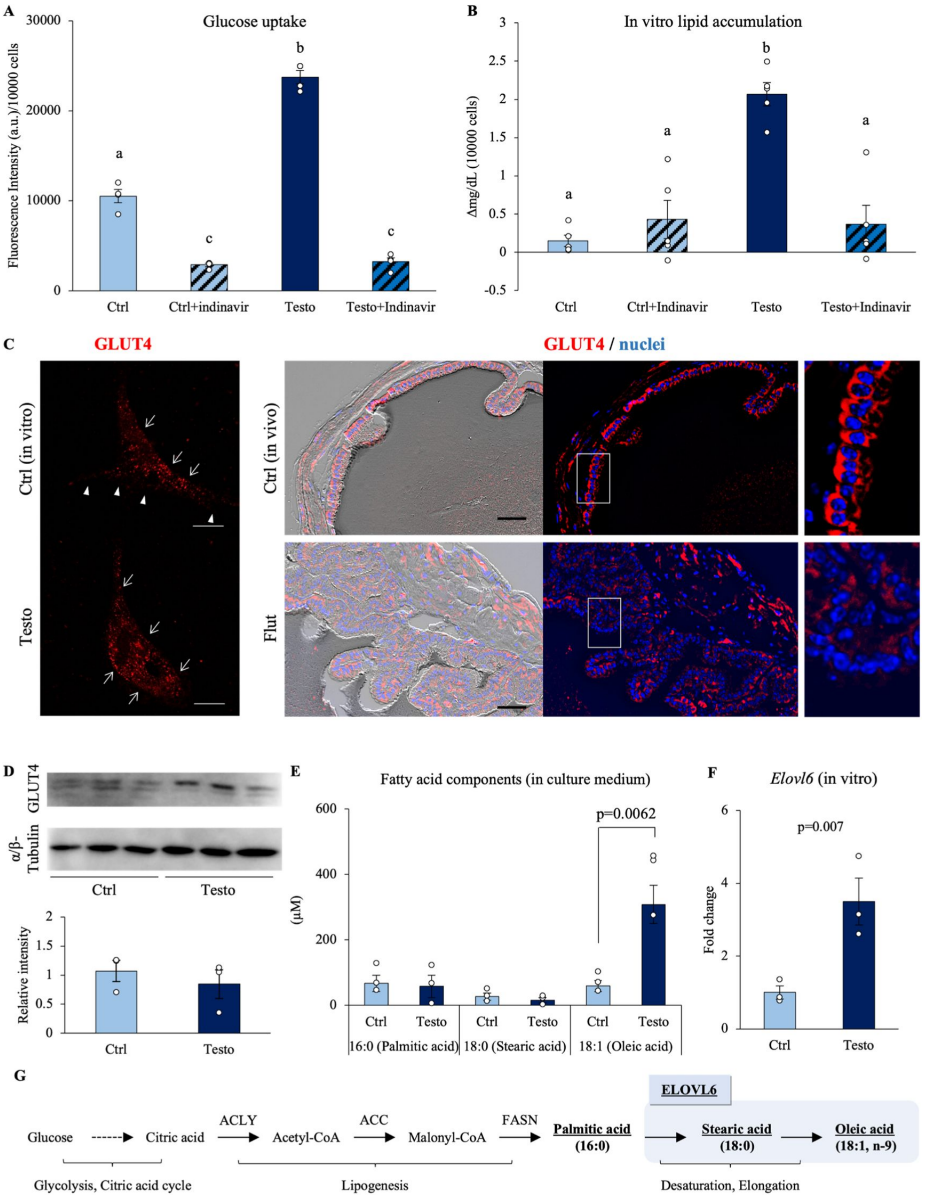


Fig 6.

Testosterone enhances glucose uptake and fatty acid synthesis via GLUT4 trans-localization in seminal vesicle epithelial cells.

(A) The glucose uptake ability of seminal vesicle epithelial cells was detected using fluorescence-tagged glucose. The cells were cultured with 100 ng/mL testosterone and/or indinavir (100 μM) for 7 days and then further treated with glucose uptake probe-green for 15 min, and intracellular fluorescence was measured by flow cytometry (n=4). (B) Lipid accumulation in the medium where seminal vesicle epithelial cells were incubated for 24 hours (n=6). (C) Immunostaining of GLUT4 in flutamide-treated or vehicle mice derived seminal vesicle. Scale bar is 50 μm. (D) Western blot images of GLUT4 and α/β-tubulin in three sets of seminal vesicle epithelial cells cultured with 100 ng/mL testosterone (Testo) or in vehicle (Ctrl) for 7 days. Quantitative analysis of GLUT4 relative to α-tubulin obtained from Western blot. (E) Fatty acid composition in the medium was analyzed using gas chromatography. (F) Quantification of *Elovl* family gene expression by RT-qPCR. (G) Pathway of glucose assimilation to oleic acid. Data are mean ± SEM. n=3-6 independent replicates. Each replicate experiments with 3-6 wells containing pooled cells from 3-5 mice or medium. (A,B) The glucose uptake and lipid measurements were normalized based on cell count and viability. A two-way ANOVA was performed. Both the Indinavir and testosterone treatment were significant. The interaction was significant. Tukey's Honest Significant Difference test was performed as a post hoc test. Different letters represent significantly different groups. Student's t-test was used for comparison between the two groups.

percentage of sperm with high ATP production was significantly increased under the presence of oleic acid (**Fig.7I**). To investigate the effect of oleic acid on the linear motility of sperm and its effect on the fertilization ability of sperm, *in vitro* fertilization (IVF) was performed. When the sperm number was sufficient, there was no change in the cleavage rate after *in vitro* fertilization, but when the sperm count was reduced to less than one-tenth of the conventional level, the cleavage rate increased compared to the control (**Fig.7J,K**). Due to the strong effect of the decapacitation factor, we found that seminal vesicle fluid reduces the fertilization rate in IVF (**Supplemental Figure 5B**). This results from inhibiting tyrosine phosphorylation of sperm and acrosome reaction by seminal vesicle secretions. However, oleic acid did not have this inhibitory effect (**Supplemental Figure 5C-F**).

To examine whether the addition of oleic acid to low quality seminal plasma (collected from flutamide-treated mice) restores *in vivo* the fertilization potential of epididymal sperm, sperm treated with seminal plasma collected from control or flutamide-treated mice was used for artificial insemination. The *in vivo* fertilization rate was about 70% when sperm was treated with the seminal vesicle secretions from control mice (SV); however, the fertilization rate was significantly lower in the group that sperm was treated with the seminal vesicle secretions from flutamide-treated mice (Flu) (SV: 73.1 ± 3.0%, Flu: 44.9 ± 4.5%). Although not significant, adding oleic acid to the seminal secretions of flutamide-treated mice (Flu+OA) increased the fertilization rate to 61.3 ± 3.9% (**Fig.7L**). These results indicated that the physiological role of oleic acid is to increase the probability of fertilization by keeping the linear sperm motility. This increases the likelihood of the sperm passing through the female reproductive tract and environments that are unfavorable to sperm survival.

ACLY expression is upregulated by testosterone and is essential for the metabolic shift of seminal vesicle epithelial cells that mediates sperm linear motility

ACLY is upregulated in seminal epithelial cells as a factor that enables the utilization of citric acid from the TCA cycle for fatty acid synthesis outside the mitochondria. Therefore, we performed siRNA knockdown experiments on ACLY to clarify the impact of ACLY on the cellular metabolic shift. The addition of testosterone significantly increased the amount of ACLY protein in seminal epithelial cells (**Fig.8A**,B). Viral infection with siRNAs encoding *Acly*- targeted siRNAs markedly decreased the amount of ACLY (**Fig.8C**). When ACLY was knocked down using shRNA, the proliferation arrest caused by testosterone was released, and the cells began to proliferate again (**Fig.8D**). This knockdown of ACLY increased the oxygen consumption of seminal epithelial cells in the control and testosterone-treated group (**Fig.8E**,F). Thus, ACLY expression is associated with mitochondrial metabolic pathway activity in seminal epithelial cells. Furthermore, the knockdown of ACLY also significantly suppressed testosterone-induced fatty acid synthesis in seminal epithelial cells (**Fig.8G**). According to the GC-MS quantification results, the amount of fatty acids secreted into the culture medium was also reduced by ACLY knockdown. In particular, the enhancement of oleic acid secretion by testosterone was inhibited by ACLY knockdown (**Fig.8H**-J). Supernatants from epithelial cells of the testosterone-added group induced motility and linear motility (Highest VSL and LIN; **Fig.8K**-N). However, the knockdown of ACLY significantly reduced the effects of the culture supernatant on sperm motility and LIN (**Fig.8K**-N). These results found that cell proliferation stops when a metabolic shift mediated by ACLY occurs, and glucose is reallocated to fatty acid synthesis. Fatty acid synthesis is an important function of the seminal vesicles, and it was found that when testosterone is present, fatty acid synthesis enhancement and cell proliferation stop simultaneously. However, it is not known what effects occur when cell proliferation stops.

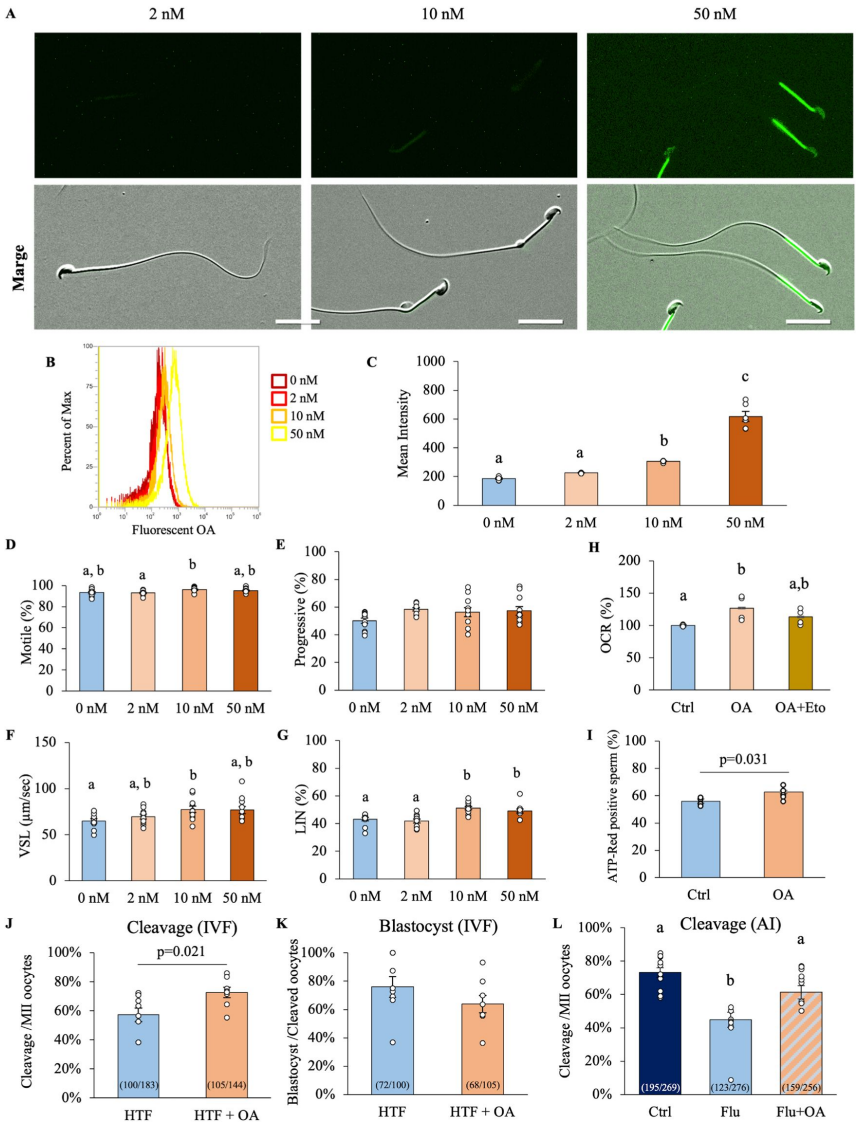


Figure 7.

Oleic acid is incorporated to sperm, which enhances linear motility and mitochondrial activity.

(A) Epididymal sperm were incubated with fluorescently labeled oleic acid (OA) and the fluorescence intensity after quenching was observed by a fluorescence microscope. (B) The fluorescence intensity of fluorescence-conjugated OA in sperm was detected by flow cytometry. (C) Average fluorescence intensity of sperm at different concentrations of fluorescence-conjugated OA. (D-G) OA improved sperm motility parameters. Sperm collected from mouse epididymis was incubated with OA for 60 min: (D) Motile, (E) Progressive motile, (F) Straight-Line Velocity; VSL and (G) Linearity; LIN (H) Increase in the Oxygen consumption rate (OCR) of cultured sperm was measured using flux analyzer after treatment with 10 nM OA or 10 nM OA + 40 nM Etomoxir (Eto). Ctrl: the sperm cultured in HTF medium. Each well was seeded with 180 μL of NaHCO3-Free HTF medium containing 3,000,000 sperm. (I) The percentage of ATP-Red positive sperm after 1 hour incubation with or without 10 nM OA, measured using BioTracker ATP-Red staining and flow cytometry. (J,K) The sperm, after 1 hour of incubation with or without 10 nM OA, were used for the IVF test and analyzed for the rate of oocytes reaching cleavage. and blastocyst stage. (J) Cleavage rate. (K) Blastocyst rate. (L) Artificial insemination using sperm treated for 1 hour with “pseudo” seminal plasma from control (Ctrl), flutamide-treated (Flu) mice and supplement of 10 nM OA (Flu+OA) followed by an evaluation of the fertilization rates. Note that the donor mice for sperm and seminal vesicle secretions were different. Data are mean ± SEM. n=3-11 independent replicates. Percentage data were subjected to arcsine transformation before statistical analysis. Differences between groups were assessed by one-way analysis of variance (ANOVA). When ANOVA was significant, differences among values were analyzed by Tukey’s Honest Significant Difference test for multiple comparisons. Different letters represent significantly different groups. Student’s t-test was used for comparison between the two groups.

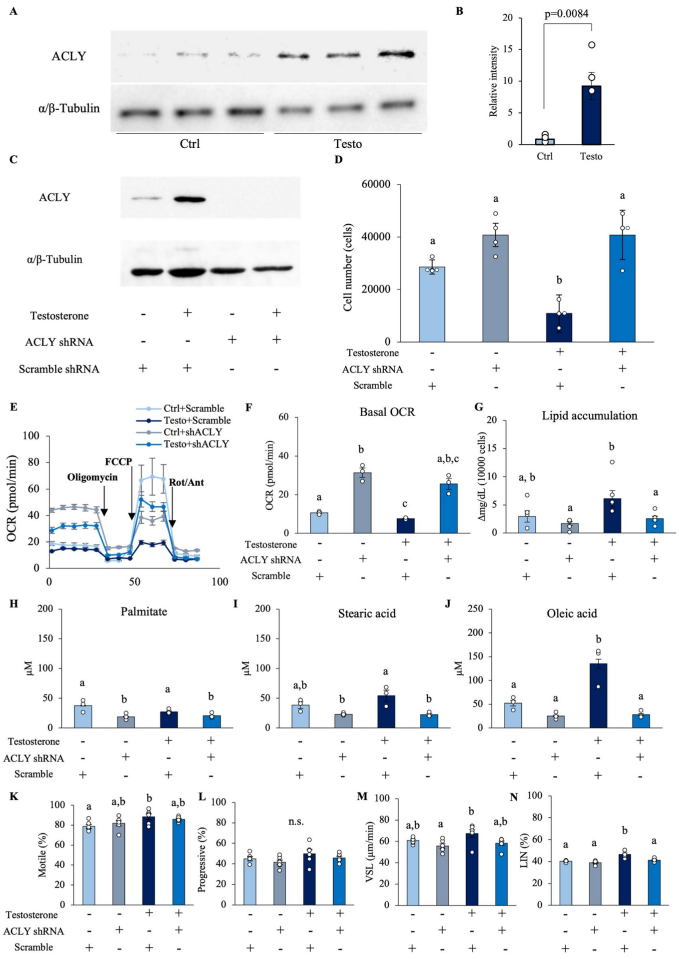


Figure 8.

Testosterone-regulated ACLY induces metabolic shifts in seminal vesicle epithelial cells.

(A) Western blot images of ACLY and α/β-tubulin in three sets of seminal vesicle epithelial cells cultured with 100 ng/mL testosterone (Testo) or in vehicle (Ctrl) for 7 days. (B) Quantitative analysis of ACLY relative to α-tubulin obtained from Western blot. (C) siRNA knockdown experiments of ACLY in seminal vesicle epithelial cells. ACLY protein levels in scrambled shRNA or ACLY shRNA-transfected seminal vesicle epithelial cells cultured with or without 100 ng/mL testosterone were determined by Western blot. (D) Number of cells at 7 days after incubation. (E,F) Changes in oxygen consumption of seminal epithelial cells were analyzed by a flux analyzer: (E) Oxygen consumption rate (OCR) kinetics of seminal vesicle epithelial cells transfected with scrambled shRNA or ACLY shRNA. (F) Basal OCR. (G) The impact of ACLY knockdown on testosterone-induced fatty acid synthesis was measured. These measurements were normalized based on cell count and viability. The viability of the cells before experiments was 67-81%. (H-I) Fatty acid composition in the cultured supernatants was analyzed using gas chromatography. (H) Palmitate (C16:0). (I) Stearic acid (C18:0). (J) Oleic acid (C18:1). (K-N) The effect of culture supernatants of testosterone-treated cells on sperm motility was evaluated, especially after ACLY knockdown. (K) Motile. (L) Progressive motile. (M) Straight-Line Velocity; VSL. (N) Linearity; LIN. Data are mean ± SEM. n=3-6 independent replicates. Repeated experiments were performed with cells recovered from 3 wells containing pooled cells from 3 to 5 mice. Student's t-test was used for comparison between the two groups. Percentage data were subjected to arcsine transformation before statistical analysis. (B) Significance was tested in comparison using Student's t-test. (D,G-M) A two-way ANOVA was performed. Tukey's Honest Significant Difference test was performed as a post hoc test. (D) Both the ACLY knockdown and testosterone treatment were significant. The interaction was significant. (G) Both the ACLY knockdown and testosterone treatment were significant. The interaction was not significant. (H,I) Only the ACLY knockdown were significant. The interaction was not significant. (J) Both the ACLY knockdown and testosterone treatment were significant. The interaction was significant. (F,N) Since the results of the Bartlett test were significant, a two-way ANOVA using a generalized linear model was performed. Games-Howell was performed as a post hoc test. (E) Only the ACLY knockdown were significant. The interaction was not significant. (N) Testosterone treatment were significant. The interaction was significant. Different letters represent significantly different groups.

This characteristic metabolic mechanism induced by testosterone is also observed in human seminal epithelial cells

To determine whether these findings in mice could be applied to humans, the commercially available human seminal vesicle epithelial cells were used for following studies (**Fig.9A**). The growth curves showed that cell proliferation was inhibited by 100 ng/mL of testosterone compared to the control; a pattern similar to that observed in murine seminal vesicle epithelial cells (**Fig.9B**). The characteristics of metabolic activity of human seminal epithelial cells were also similar to those of mice, with testosterone significantly enhancing the ECAR response (**Fig.9C**-E), but significantly reducing the capacity for oxygen consumption in mitochondria (**Fig.9F**-H). Moreover, fluorescent-glucose uptake was increased by testosterone and decreased by the inhibitor of GLUT4 function (**Fig.9I**). Additionally, while testosterone also significantly increased fatty acid synthesis, this increase was completely abolished by the GLUT4 inhibitor (**Fig.9J**). According to the quantitative results obtained using GC-MS, the amount of fatty acids secreted into the culture medium also decreased due to the inhibition of GLUT4 by indinavir. In particular, it was shown that oleic acid secretion is completely dependent on glucose via GLUT4 (**Fig.9K**-M).

Discussion

Seminal plasma has a variety of functions, including sperm transport, nutritional support, interactions with the female reproductive tract, and especially the regulation of its relationship with the female immune system. Mammalian seminal plasma is secreted mainly from the seminal vesicle and is rich in cytokines, prostaglandins, and SVS family members (34). For this reason, recent studies have focused on the immunomodulatory function of seminal plasma in the female reproductive tract. On the other hand, fructose and lipids in seminal plasma are the major energy sources for sperm (35). However, although metabolomic analyses of seminal plasma have shown that metabolic substrates are present at higher concentrations in seminal plasma than in blood (18, 24, 25), how these substrates are synthesized and specifically how they alter sperm motility has not been determined. Moreover, though the motility of ejaculated spermatozoa is known to decrease with aging, few reports have revealed age-related changes in the seminal vesicles, which are responsible for seminal plasma synthesis.

In this study, we focused on the reproductive organs that synthesize seminal vesicle secretion as target organs for testosterone and found that factors formed by testosterone-dependent metabolic changes in seminal vesicles activate sperm mitochondria and enhance their linear motility. Testosterone is known to activate intracellular ARs and alter intracellular metabolic pathways in skeletal muscle (36), prostate cells (37) and cardiomyocytes (33). ARs are known to directly regulate the expression of glycolytic and lipid metabolic genes (33, 38, 39). In the present study, the nuclear localization of AR in seminal vesicle epithelial cells (i.e., functioning as a transcription factor) and the activation of the glycolytic system by testosterone were similar to those in the above cell types. In seminal vesicle epithelial cells, an increased trend in the expression of *Glut3* and an enhancement of GLUT4 function were observed in a testosterone-dependent manner. Such effects of testosterone on glucose metabolism have also been reported in skeletal muscle, liver, and adipose tissue, where testosterone leads to the phosphorylation of GLUT4 and enhances its translocation to the plasma membrane (40, 41). This is consistent with the results of our metabolic flux analyses, which show that testosterone increases the rate of glycolysis in seminal vesicle epithelial cells by increasing glucose uptake. We further confirmed that GLUT4 is the transporter that mediates this glucose uptake using indinavir, a GLUT4- specific inhibitor.

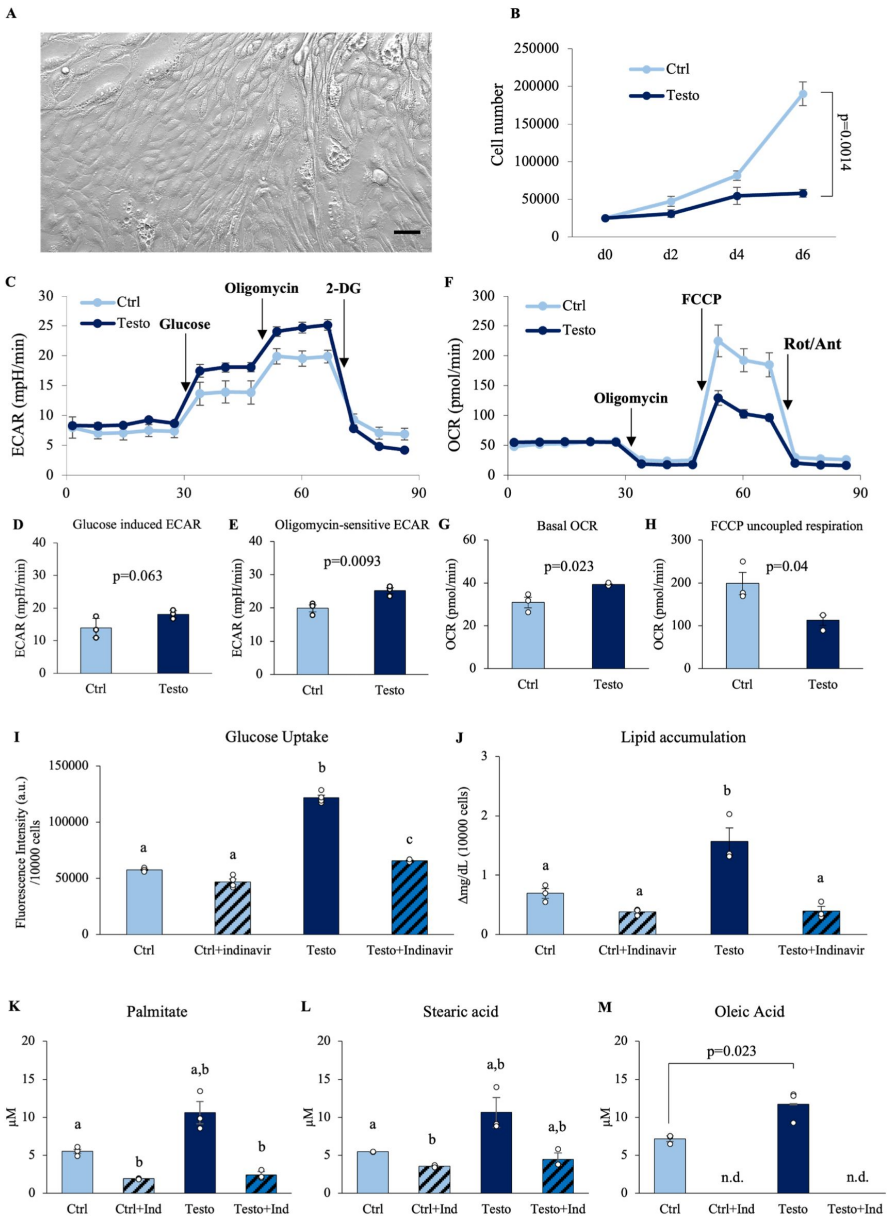


Figure 9.

Testosterone regulates the metabolic activity of human seminal epithelial cells.

(A) Representative image of human seminal vesicle epithelial cells. (B) Growth curves of seminal vesicle epithelial cells with 100 ng/mL testosterone (Testo) or without (Ctrl). (C-H) Extracellular acidification rate (ECAR) kinetics and Oxygen consumption rate (OCR) kinetics of human seminal vesicle epithelial cells after 6 days of culture with or without 100 ng/mL testosterone using a flux analyzer. (D) Glucose induced ECAR and (E) Oligomycin-sensitive ECAR. (G) Basal OCR, (H) FCCP uncoupled respiration. (I) The glucose uptake ability of seminal vesicle epithelial cells was detected using fluorescence-tagged glucose. (J) Lipid accumulation in the medium where human seminal vesicle epithelial cells were incubated for 24 hours. (H-I) Fatty acid composition in the cultured supernatants was analyzed using gas chromatography. (H) Palmitate (C16:0). (I) Stearic acid (C18:0). (J) Oleic acid (C18:1). Data are mean ± SEM. n=3 independent replicates. Student's t-test was used for comparison between the two groups. (C-J) These measurements were normalized based on cell count and viability. The viability of the cells before experiments was 88-96%. (I,K,L) Since the results of the Bartlett test were significant, a two-way ANOVA using a generalized linear model was performed. Games-Howell was performed as a post hoc test. Both the Indinavir and testosterone treatment were significant. The interaction was significant. (J) A two-way ANOVA was performed. Tukey's Honest Significant Difference test was performed as a post hoc test. Both the Indinavir and testosterone treatment were significant. The interaction was significant. Different letters represent significantly different groups.

This study suggests that fatty acid synthesis is an important function of the seminal vesicles, which provide an environment where the ejaculated sperm can carry out active metabolism. The shift to fatty acid synthesis was consistent with the cessation of cell proliferation in the presence of testosterone. This mechanism could prevent the seminal vesicles from excessively consuming the substrates necessary for sperm motility. Manipulation of ACLY expression in mouse myoblasts and embryonic stem cells has been shown to alter the TCA cycle into a "non-normal cycle" (42). One advantage of the non-normal TCA cycle is its ability to retain carbon instead of combusting it and to regenerate the cytosolic NAD+ needed to maintain glycolysis (42, 43). It suggests that ACLY, activated by testosterone, may suppress proliferation by skipping several steps in the mitochondrial TCA cycle and minimizing ATP production associated with aerobic respiration in seminal vesicle epithelial cells.

Testosterone has been reported to cause hypertrophy of cardiac myocytes (44, 45). Cell hypertrophy is partly due to the accumulation of fatty acids in the cytoplasm, which causes significant damage to cellular functions (46, 47). On the other hand, normal cell hypertrophy, especially in myofibers, involves the activation of signaling molecules such as Myc, hypoxia-inducible factor, and Pi3k-Akt-mTor, which regulate metabolic reprogramming in cancer by increasing glucose uptake in the presence of oxygen (48–50). In the present study, induction of ACLY expression by testosterone not only decreased cycling in the TCA cycle, but also promoted glucose assimilation, which played an important role in fatty acid synthesis. Thus, in tissues where fatty acid synthesis is promoted, either β-oxidation-dependent ATP production in mitochondria or the secretion of fatty acids occurs to maintain cellular function (51–53), providing evidence to explain why the presence of fatty acids in the cell has no adverse effect. In accessory reproductive glands, such as the prostate and seminal vesicles, the glucose uptake is activated in a testosterone dependent. However, mitochondrial respiration is not enhanced, cell proliferation is not induced, and the metabolites are used as a substrate for sperm motility.

This may be because, in the prostate, pyruvate synthesized by the glycolytic system in response to testosterone is converted to acetyl CoA, which is then converted to citric acid in the TCA cycle, citric acid is released as the main component of seminal plasma (37).

The testosterone-dependent growth of cancerous prostate epithelial cells may be due to genetic mutations, such as elevated expression of the gene encoding aconitase, which converts citric acid to isocitrate, which blocks the TCA cycle and inhibits citric acid secretion (54). On the other hand, in the analysis of seminal epithelial cells, testosterone treatment induced the expression of *Acc*, which encodes the rate-limiting enzyme for fatty acid synthesis, and *Elovl6*, which desaturates to oleic acid, significantly increasing total saturated fatty acid and oleic acid content. Therefore, testosterone promotes glucose conversion into fatty acids, especially oleic acid, in seminal epithelial cells. Although there is a commonality in the activation of the glycolytic system testosterone target tissues, the different expression patterns of genes involved in metabolism in mitochondria may exert their specific functions in each tissue. In addition to genes involved in fatty acid synthesis such as *Fasn* and *Elovl6*, *Acly* is also encoded in a region in close proximity to human chromosome 17 and mouse chromosome 11, suggesting that in seminal vesicle epithelial cells, this region is targeted to promote gene expression. The co-regulation of these genes in seminal vesicle epithelial cells leads to synthesizing oleic acid that ensures sperm motility and function.

Decreased testosterone synthesis in the testes is a phenomenon observed with aging associated with decreased spermatogenesis(55). However, the probability of obtaining sperm without ICSI or IVF is extremely low, despite the presence of sufficient numbers of sperm with normal-morphology (56–58). This indicates that testosterone plays a major role not only in spermatogenesis but also in the functional maturation of sperm. In this study, metabolic abnormalities induced by inhibition of androgen receptor function caused abnormal proliferation of seminal vesicle epithelial cells in aged mice. In other words, the findings of the seminal vesicle

epithelial cell culture experiments and flutamide treatment may mimic the changes that occur with aging. Oleic acid synthesis by testosterone-dependent metabolic pathways was regulated in seminal vesicle epithelial cells and the oleic acid stimulated sperm motility and enhanced *in vivo* fertilization success. It could be assumed that a decrease in testosterone levels causes abnormalities in the components of human semen. Specifically, the results open the possibility of translational studies to understand human male infertility, especially in cases of failure to conceive by natural mating or artificial insemination.

Because seminal plasma contains components specific to certain male reproductive organs, differences in protein composition may reflect pathological processes in these specific organs (59). However, although proteomic and metabolomic analyses of seminal plasma have been performed (31, 59), the functional changes in seminal plasma, especially the effects on *in vivo* fertilization potential, are not fully understood. Differences in fatty acid concentrations, including oleic acid, in seminal plasma may provide a predictive marker for the fertilization potential of both human and domestic animal semen. Furthermore, the addition of oleic acid to low-quality semen improved fertilization rates associated with artificial insemination, offering a strategy for improving artificial insemination. In our previous study, the addition of fatty acids to the thawing medium of frozen bull sperm improved linear motility and viability (21). The similarities between mouse and human seminal vesicle epithelial cells demonstrated in this study have strong potential for the clinical application of these observations.

Overall, the results of the studies described herein strongly support the hypothesis that testosterone-dependent glucose to oleic acid conversion processes are required for normal seminal plasma functions in the seminal vesicle and normal reproductive performance in males (**Fig.10**). This study advances our understanding of the role of testosterone in glucose metabolism and fatty acid synthesis and lays the groundwork for future research and potential therapeutic interventions in reproductive biology and fertility.

Materials and methods

Study design

The objective of this study was to elucidate the mechanisms of sperm plasma synthesis that enhance sperm fertility. Testosterone-dependent changes in the seminal plasma function were examined by comparative analysis of adult mice and flutamide-treated mice. The bioassays of extracts from male accessory glands by epididymal sperm motility analysis were used to examine the seminal plasma components important for sperm fertilization. We also isolated the mouse seminal vesicle epithelial cultured cells, and investigated the synthesis mechanism of the seminal plasma components using RNA-seq, FluxAnalyzer, and shRNA knockdown. Finally, we performed validation using the human epithelial cells.

Chemicals and Animals

Unless otherwise noted, chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Nacalai tesque (Osaka, Japan). Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Asuka Seiyaku (Tokyo, Japan). All procedures during animal experiments were reviewed and approved by the Animal Care and Use Committee of Hiroshima University (Hiroshima, Japan; C21-9-3) and conducted according to regulations. Specific pathogen-free C57BL/6NCrl male mice or Crl: CD1 (ICR) female mice were purchased from Jackson Laboratory Japan (Kanagawa, Japan) and housed in an environmentally controlled room with a 12-hour light/dark cycle, a temperature of 23 ± 3°C, and free access to laboratory food (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. 8- to 16-week-old male or female mice were used for the experiments. Flutamide-treated mice were injected

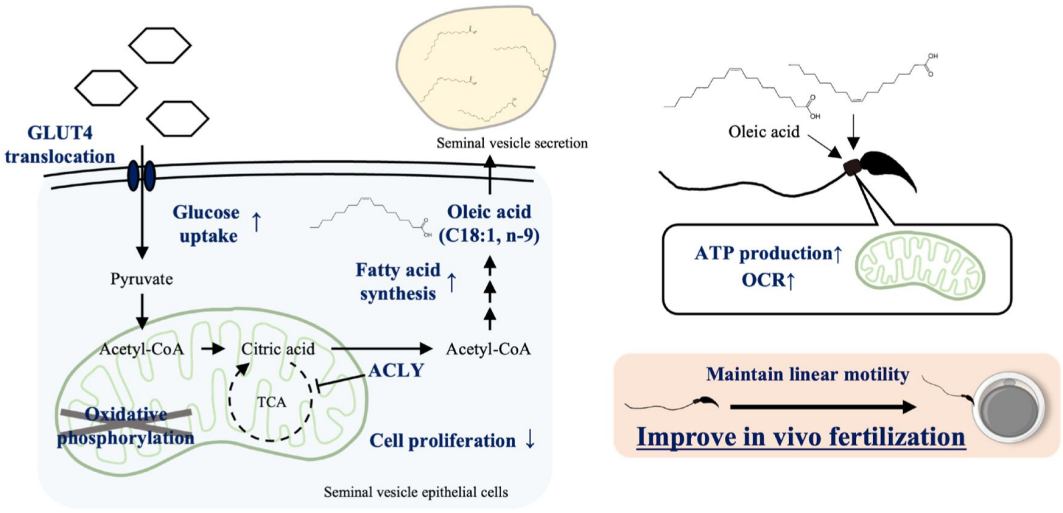


Figure 10.

Metabolic changes in the seminal vesicle epithelial cells by testosterone improve the fertility of sperm.

Left part: Testosterone promotes glucose uptake by changes in the localization of GLUT4 in seminal vesicle epithelial cells. ATP-citrate-lyase (ACLY) activated by testosterone promotes fatty acid synthesis by skipping some tricarboxylic acid (TCA) cycle steps. Simultaneously, cell proliferation in the epididymal epithelial cells is suppressed. Testosterone also promotes the synthesis of oleic acid from acetyl-CoA synthesized by ACLY. Right part: Oleic acid, incorporated into the sperm midpiece, increases the oxygen consumption rate (OCR) in sperm mitochondria and increases ATP production. Therefore, oleic acid in seminal plasma is important for male fertility because it improves sperm linear motility and fertilization ability.

subcutaneously for 7 days with 50 μg/g (body weight) of flutamide dissolved in corn oil. Vehicle-treated mice received 100 μL of corn oil subcutaneously for 7 days. The aged males were 12 to 16 months old.

Bioassay using pseudo-seminal plasma and epididymal sperm

Prostate (mixed anterior, dorsal, lateral, and ventral) and seminal vesicles were collected from adult C57BL/6NCrl mice. Seminal vesicle secretions were obtained by pressing the seminal vesicles immediately after euthanasia. Prostatic extracts were obtained by homogenizing the prostate immediately after euthanasia. Seminal vesicle secretions and prostate extracts collected from one male mouse were dissolved in 500 μL and 200 μL of mHTF medium without BSA (60), respectively, then centrifuged at 2000 × g for 5 min at room temperature, and the supernatant was collected, and protein levels were determined. The seminal vesicle secretions from flutamide-treated mice were also collected similarly.

Cauda epididymis sperm recovered from male mouse were dispersed in 500 μL of mHTF without BSA drops and centrifuged at 300 × g, 3 min to wash out epididymis-derived factors. The final protein concentration of prostate and seminal vesicle secretion was adjusted to 10 mg/mL.

An aliquot (2 mL) of the pooled prostate or seminal vesicle extracts was conducted the viscosity measurement data using Tuning Fork Vibro Viscometers (SV-1A, A&D Company, Tokyo, Japan). The epididymis sperm was mixed with seminal vesicle secretion and/or prostate extract as shown in **Fig.1** and Supplementary Figure 1. The samples were incubated at 37°C for 60 min in a humidified atmosphere of 5% CO2 in air. Sperm motility patterns were then examined using computer-assisted sperm analysis (CASA; HT CASA-Ceros II, Hamilton Thorne, Beverly, MA, USA), with software version 1.11.9. The following sperm motility parameters were analyzed: percentage of motile sperm (denoted by a path velocity >40 μm/s and a VSL >20%), percentage of motile sperm with progressive motility (denoted by a path velocity >40 μm/s and a straightness ratio >60%). Straight-Line Velocity; VSL: The distance between the first and last tracking points of the sperm trajectory divided by the elapsed time. Curvi-Linear Velocity; VCL: The sum of the distances between the centers of brightness in each frame divided by the elapsed time. Linearity; LIN: Measures the straightness of the path. LIN is derived from the ratio of VSL/VCL multiplied by 100.

Serum testosterone levels

The serum was separated from the clot by centrifugation at 1,500 × g for 15 min. Testosterone levels in the serum samples were determined by a rodent testosterone ELISA test kit (ERKR7016, Endocrine Technologies, Inc., Newark, CA, USA) according to the manufacturer's manual.

Mitochondrial activity

Mitochondrial activity of sperm was measured using a MitoPT® JC-1 Assay Kit (911, Immuno Chemistry Technologies, LLC, Bloomington, MN, USA) according to our previous study (20). Briefly, sperm were incubated with 200 μL of working solution containing 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) dye at 37 °C for 30 min in the dark. Using 10 μM of antimycin control (A8674; Sigma) under the same incubation conditions, we confirmed that the JC-1 reaction is sensitive to changes in membrane potential. The sperm suspension was centrifuged and washed twice with the base medium. After washing, the sperm pellet was resuspended in the base medium and analyzed with an Attune® NxTAcoustic Focusing Cytometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) using a 488 nm laser and a filter with a bandwidth of 574/26 nm. The intensity of the average value was analyzed as the mean fluorescence intensity (MFI) of JC-1 orange aggregates. A total of 100,000 sperm events were analyzed. The sperm population is shown in Supplementary Figure 6.

In vitro fertilization (IVF)

3 weeks old CD1 female mice were injected intraperitoneally with 4IU eCG to stimulate follicle development and 5 IU hCG at 48 hours later. COCs collected from the oviductal ampulla 16 h after hCG injection were placed into 100 μL of human tubal fluid (HTF) medium. Sperm were collected from the cauda epididymis of 15 weeks or older C57BL/6N male mice in 100 μL of HTF medium. After 60 min of incubation with or without treatments, the sperm were added into HTF medium at a final concentration of 2×10^5 spermatozoa/mL for conventional experiments or 1×10^4 10 nM OA treated sperm/mL, and coincubated with the COCs. After 6h incubation, oocytes were washed three times thoroughly and cultured in 250 μL of KSOM medium (MR 106-D; Sigma). The cleavage rate was determined on Day 1 (Day 0=day of IVF), and the appearance of blastocysts was recorded on Day 5.

Immunohistochemistry and immunofluorescence

Seminal vesicles were fixed overnight in 4% (w/v) paraformaldehyde/PBS, dehydrated in 70% (v/v) ethanol, and embedded in paraffin. Paraffin-embedded fixed sections (4 μm) were deparaffinized and hydrated with xylene and ethanol, respectively. Some slides were stained with hematoxylin and eosin (HE). For antigen retrieval, sections were placed in citrate buffer (pH 6.0), microwaved until boiling and allowed to stand at the temperature of pre-boil (95°C) for 15 minutes. Sections were incubated with 3% (v/v) hydrogen peroxide in methanol to block endogenous peroxidase and then incubated with Normal Goat Serum Blocking Solution for rabbit antibody (S-1000, Vector Laboratories, Newark, CA, USA) to block nonspecific sites. The sections were then incubated overnight with primary rabbit antibodies: anti-AR antibody (1:100; ab133273; Abcam, Cambridge, UK), anti-Ki67 antibody (1:1000; ab15580; Abcam) or anti- ACLY antibody (1:200; ab40793; Abcam). Positive signals were visualized by DAB (3,3'- Diaminobenzidine, 040-27001, Fujifilm Wako, Osaka, Japan) using the VECTASTAINE LifeABC kit (PK-6101, Vector Laboratories) according to the manufacturer's protocol. Apoptotic cells (including fragmented DNA) were detected using the In Situ Cell Death Detection Kit POD (11684817910, Roche Ltd., Basel, Switzerland). To avoid false-positive or false-negative results, DNAse-treated sections were used as positive controls, and TdT-untreated sections were used as negative controls for apoptosis.

Nuclei were visualized with hematoxylin (Sakura Finetek, Tokyo, Japan). Ki-67 and apoptotic positive cells were quantified using ImageJ software (National Institutes of Health; Bethesda, MD, USA), with software version 2.1.0/1.53u. Randomly selected sections were stained, and images were obtained from randomly selected fields in each section. The positive percentage is defined as the percentage of positively stained cells in the total number of epithelial cells evaluated. Only positive staining was considered, regardless of staining intensity. For immunofluorescence, after blocking nonspecific sites, the sections were incubated with a primary antibody: anti-GLUT4 antibody (1:200; ab33780; Abcam). After washing with 0.3% (v/v) Triton X-100 in PBS (-), Cy3-labeled goat anti-rabbit IgG (1:200, C-2306, Sigma) and DAPI (VECTESHIELD Mounting Medium with DAPI, H-1200, Vector Laboratories) were used to visualize the antigen and nuclei. Digital images were taken using an APX100 Digital Imaging System and CellSens imaging software (EVIDENT, Tokyo, Japan), with software version 4.1.1.

Isolation and culture of seminal vesicle epithelial cells

Seminal vesicles were removed from euthanized mice, cut open vertically to expose the seminal vesicle cavity, and incubated with Hanks' balanced salt solution (HBSS; $CaCl_2 \cdot 2H_2O$ 0.185 g/L, $MgSO_4$ 0.098 g/L, KCl 0.400 g/L, KH_2PO_4 0.060 g/L, NaHCO3 0.350 g/L, NaCl 8.000 g/L, Na_2HPO_4 0.048 g/L, D-Glucose 1.0 g/L, dissolved in ultrapure water). Tissues were digested with 10 mL of HBSS containing 25 mg/mL pancreatin and 0.25% (v/v) trypsin EDTA for 1 hour at 4°C, 45 minutes at room temperature, and 15 minutes at 37°C, and epithelial cells were obtained. These were passed through a 70 μm nylon filter (352350, Corning Inc., Corning, NY, USA) and seeded into collagen-

coated 12-well plates (4815-010, IWAKI, Shizuoka, Japan). Cells were cultured in DMEM/F-12, 10% fetal bovine serum (FBS; 10438-018, Thermo Fisher Scientific Inc.), 100 U/mL penicillin, 100 µg/mL streptomycin. The culture condition was 37°C, 5% CO_2, 95% air, and humidity.

Human seminal vesicle epithelial cells (4460, ScienCell Research Laboratories, Carlsbad, CA, USA) were purchased, seeded in 2 µg/cm^2 poly-L-lysine-coated plates, and cultured in epithelial cell medium (4101, ScienCell Research Laboratories). The cells were seeded at 10000 cells/cm^2 on collagen-coated plates the day before 100 ng/mL testosterone treatment. Testosterone was dissolved in ethanol, and the final concentration of ethanol was adjusted to 0.1% (v/v) when used for cultured cells. The culture medium was replaced every 48 hours.

Cell proliferation curve

The seminal vesicle epithelial cells were seeded at 10000 cells/cm^2 and then were cultured in various concentrations of testosterone. The cells were washed with HBSS (without $CaCl_2$-$2H_2O$/$MgSO_4$) at the time of cell counting and collected using 0.25% (v/v) trypsin. The recovered cells were suspended in DMEM/F-12 medium, and cell numbers were determined using an Automated Cell Counter TC20TM (BIO-RAD Laboratories, Hercules, CA, USA) to calculate the number of cells contained per well (4 cm^2).

Cell cycle analysis

The recovered cells as described above were fixed in 2-5 ml of cold (4°C) 70% methanol for at least 30 minutes on ice. After centrifugation at 500g for 5 minutes at 4°C to obtain pellets, the cell pellet was suspended in 50 µM propidium iodide and 20 × diluted RNase (19101, QIAGEN Inc., Venlo, Netherlands) and then were incubated at 37°C for 30 minutes. After twice washing with 1 ml of PBS at 300 g for 3 min at 4°C, the cells were analyzed by flow cytometry. The cell population is shown in Supplementary Figure 8. A total of 20,000 events were analyzed.

RNA-sequencing

Total RNA was extracted from cells using the RNeasy Mini Kit (74106, QIAGEN). Three independent samples were assayed to evaluate the reproducibility of the experimental procedures. The quality of total RNA (1 µg) submitted for sequencing was checked using a Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA), and all RNA integrity numbers were >7.0. The libraries for RNA sequencing were sequenced with 2 × 150-bp paired-end reads on a NovaSeq6000 (Illumina Inc., San Diego, CA, USA). The obtained sequencing data were analyzed using RaNA-seq (61), an open bioinformatics tool for rapid RNA-seq data analysis. FASTQ files were preprocessed in the pipeline using the Fastp tool, and expression was quantified using Salmon. DEseq2 was used to identify differentially expressed genes (DEGs) with adjusted p- values less than 0.05 between the testosterone-treated groups in transcripts per million (TPM) between control and testosterone-treated cells. The pathways were identified from the KEGG database on RaNA-seq.

Extracellular flux analysis

Oxygen consumption rate (OCR) and ECAR were measured using an extracellular flux analyzer (XF HS Mini; Agilent Technologies). Eight-well plates for the XF HS Mini were coated with fibronectin (5 µg/mL, F1141, Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS), incubated overnight, and then washed with Roswell Park Memorial Laboratory medium for XF (Roswell Park Memorial Institute medium; RPMI, 103576-100, Agilent Technologies; containing 1% FBS) for XF before analysis. Mouse seminal vesicle epithelial cells or human seminal vesicle epithelial cells treated with 100 ng/mL testosterone for 7 days were suspended in RPMI medium at a concentration of 10,000 cells/180 µL, or mouse seminal vesicle epithelial cells infected with shRNA were suspended at a concentration of 2000 cells/180 µL in each well. After centrifugation at 300 × g for 3 minutes, the plates were placed in an incubator at 37°C and 100% air and used for analysis within 1 hour.

For glucose metabolism assessment, cells were sequentially treated as indicated with D- Glucose (10 mM; 103577-100, Agilent Technologies), Oligomycin (1 µM; O4876, Sigma- Aldrich), and 2-Deoxy-Glucose (50 mM; D8375, Sigma-Aldrich).

For mitochondrial respiration measurement, cells were sequentially treated as indicated with Oligomycin (1 µM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 5 µM, SML2959, Sigma-Aldrich), and Rotenone (1 µM; R8875, Sigma-Aldrich)/Antimycin A (1 µM; A8674, Sigma-Aldrich). Cellular metabolism readouts, such as glycolysis, glycolytic reserve, basal OCR, Maximum respiration, ATP Production, Spare respiratory capacity, were determined using the Agilent Seahorse Wave software with software version 3.0.0.41. However, to avoid many ambiguities and problems with interpretation, “glycolysis” is written as “Glucose-induced ECAR”, “glycolytic reserve” as “Oligomycin-sensitive ECAR”, “maximum respiration” as “FCCP uncoupled respiration”, and “ATP production” as “Oligomycin-sensitive respiration”.

For mouse sperm analysis, NaHCO3-free HTF medium was prepared according to Balbach et al. (62). Plates were coated with concanavalin A (0.5 mg/mL, Fujifilm Wako Chemicals) overnight the day before assay. Each well was seeded with 180 µL of NaHCO3- Free HTF medium containing 3,000,000 sperm cells. Sperm were injected with oleic acid (final concentration: 10 nM) with or without etomoxir (final concentration: 40 nM, E1905, Sigma-Aldrich).

Measurement of pyruvate and lactic acid

For measurements, mouse or human seminal vesicle epithelial cells were treated with 100 ng/ml testosterone for 6 days. The culture supernatant was collected after 24 hr of additional culture. Pyruvate consumption was measured using the Pyruvate Assay kit (MET-5029, Cell Biolabs, Inc., San Diego, CA, USA). The fluorescence intensity was measured using a microplate reader (ex. 570 nm/em. 615 nm). Lactate released into the medium was measured using the Lactate Assay kit (L256, DOJINDO LABOLATORIES Co., Ltd., Kumamoto, Japan). The absorbance was measured using a microplate reader (wave length=450nm).

qPCR

The cDNA was synthesized from 40 ng of total RNA using oligo(dT)$_{15}$ primers (3805, Takara Bio Inc., Shiga, Japan) and an Avian myeloblastosis virus (AMV) reverse transcriptase from Promega (M5101, Madison, WI, USA). The cDNA and primers were added to Power SYBR Green PCR Master Mix (4367659, Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 15 µl. Conditions were set to the following parameters: 10 min at 95°C, 15 sec at 95°C, and 1 min at 60°C. Cycles were repeated 45 times. Specific primer pairs were selected and analyzed, as shown in **Table S1**. Expression was first normalized to housekeeping gene *Rpl19*, and fold change was calculated relative to the mean of the control samples.

Glucose uptake test of seminal vesicle epithelial cells and lipid determination of culture supernatants

Mouse or human seminal vesicle epithelial cells treated with or without 100 ng/mL testosterone for 6 days were used for this experiment. Cells were treated with or without the GLUT4 inhibitor indinavir (100 nM) and 100 ng/mL testosterone for an additional 24 hours. Collected 15,000 cells were subjected to Glucose Uptake Assay Kit-Green (UP02; DOJINDO), and fluorescence intensity was measured using a microplate reader (ex. 485 nm/em. 535 nm).

Total lipid content in cultured medium was quantified using a lipid quantification kit (STA-617 Fluorometric, Cell Biolabs, Inc.) according to the manufacturer’s protocol. The fluorescence intensity was measured using a microplate reader (ex. 485 nm/em. 535 nm).

Gas chromatography-mass spectrometry analysis

Fatty acid measurements in culture supernatant and seminal vesicle secretions were done according to our previous work (21). Fatty acids in the samples were extracted and methylated using a fatty acid methylation kit (06482-04, Nacalai Tesque) and then purified using a fatty acid methyl ester purification kit (06483-94, Nacalai Tesque). The methylated sample (3.0 mL) was dried and dissolved in 50 µL of elution solution. Samples were subjected to GC-MS.

Fatty acids were determined on an Agilent 7890A GC system coupled with a JMS-T 100 GCv mass detector at the Natural Science Research Center, Hiroshima University (N-BARD). Sample (1.0 µL aliquot) was injected to SP®-2560 capillary GC column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; 24056, Sigma-Aldrich). For measurements, helium was used as the carrier gas at a constant flow rate of 2.0 mL/min. The GC oven temperature was gradually increased from 100°C to 300°C at a rate of 10°C/min and held at 300°C for 20 minutes. Ionization was performed using electron ionization with an electron energy of 70 eV. Mass spectra were obtained in scan mode (mass scan range 29-800 m/z). NIST MS Search v.2.0 was used to detect and identify of fatty acids.

Quantification of sperm oleic acid uptake by flow cytometry

To detect the fluorescence intensity of each sperm sample using flow cytometry, sperm collected from the epididymis were incubated with 1, 10 or 100 µM of fluorescently labeled oleic acid (F-OA-01, NANOCS, New York, NY, USA). After washing, fluorescence were analyzed by flow cytometry using a 530/30 nm bandwidth filter and measured as mean intensity. A total of 100,000 sperm events were analyzed. The localization of fluorescence in sperm was determined using an APX100 Digital Imaging System.

Evaluation of acrosome damage

The acrosome status of sperm was assessed using 25 µg/mL fluorescein isothiocyanate- conjugated peanut agglutinin (FITC-PNA; L7381, Sigma Aldrich, St. Louis, MO) and 2.4 nM propidium iodide (PI; L 7011, Invitrogen) were used for evaluation. After incubation in the dark at 37°C for 10 min. The fluorescence of FITC and PI were analyzed by flow cytometry using a 530/30 and 695/40 nm bandwidth filter, respectively. Damaged sperm were analyzed—FITC fluorescence positive, but PI negative gate (Q4). A total of 100,000 sperm events were analyzed. The sperm population is shown in Supplementary Figures 5 and 6.

ACLY knock down experiment using shRNA

The U6 shRNA lentiviral vector of the *Acly* gene (mAcly[shRNA#1]; a virus packaging service, VectorBuilder, Chicago, IL, USA) was used for knockdown experiments. The ACLY sequence targeted by the shRNA vector used in this study was 5'-GCAGCAAAGATGTTCAGTAAA-3'. Lentivirally transduced cells were prepared according to manufacture protocol. Immediately after isolation, the mouse seminal vesicle epithelial cells were cultured overnight in a medium containing 5 µg/mL polybrene (VectorBuilder) and lentiviral particles expressing an shRNA targeting ACLY or a scramble (Multiplicity of Infection of 10). After an additional overnight incubation in a fresh culture medium, the cells were incubated for 5 days in a medium containing 1 µg/ml puromycin (19752-64, Nacalai tesque). 100 ng/mL of testosterone was added to all procedures for the testosterone-treated group. Infection efficiency, calculated based on the number of GFP-positive cells using an APX100 Digital Imaging System, ranged between 70% and 90%.

Western blot analysis

Protein samples of mouse seminal vesicle epithelial cells, seminal vesicle tissue, and sperm were prepared by homogenization in cell lysis buffer (04719964001, cOmplete™ Lysis-M EDTA-free, Roche) and diluted with an equal volume of sample buffer solution containing 2-ME (2 ×) for SDS-PAGE (30566-22, Nacalai tesque). The extracts (10 μg protein) were separated by SDS-polyacrylamide gel (10%) electrophoresis and transferred to polyvinylidene fluoride membranes (10600069, Cytiva, Tokyo, Japan). The membranes were then treated with Tris- buffered saline and Tween 20 (TBST, 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% (w/v) Non-Fat Dry milk (MORINAGA MILK INDUSTRY Co., Ltd., Tokyo, Japan) for the nonspecific reaction was blocked. When detecting phosphotyrosine, containing 5% (w/v) bovine serum albumin was used as a blocking agent. The membranes were incubated overnight at 4°C with primary antibodies: anti-phosphotyrosine antibody (1:10,000; 8959; Cell Signaling), anti-GLUT4 antibody (1:100; ab33780; Abcam), anti-ACLY antibody (1:10,000; ab40793; Abcam) or anti-α/β-Tubulin antibody (1:1,000; 2148S; Cell Signaling). The next day, membranes were washed with TBST and incubated with HRP-labeled antibody specific for rabbit IgG (1:4000; 7074S; Cell Signaling). The bands were visualized by using Enhanced Chemiluminescence detection systems (RPN2232, Cytiva) and ChemiDoc^{TM} MP Imaging System (BIO-RAD Laboratories). The bands were analyzed by using the software ImageJ. Densitometry analysis was performed using the ImageJ Gel Analysis tool, and the background of the gel was also removed individually for each band. The full-length blotting images are shown in Supplementary Figure 7.

ATP production in sperm

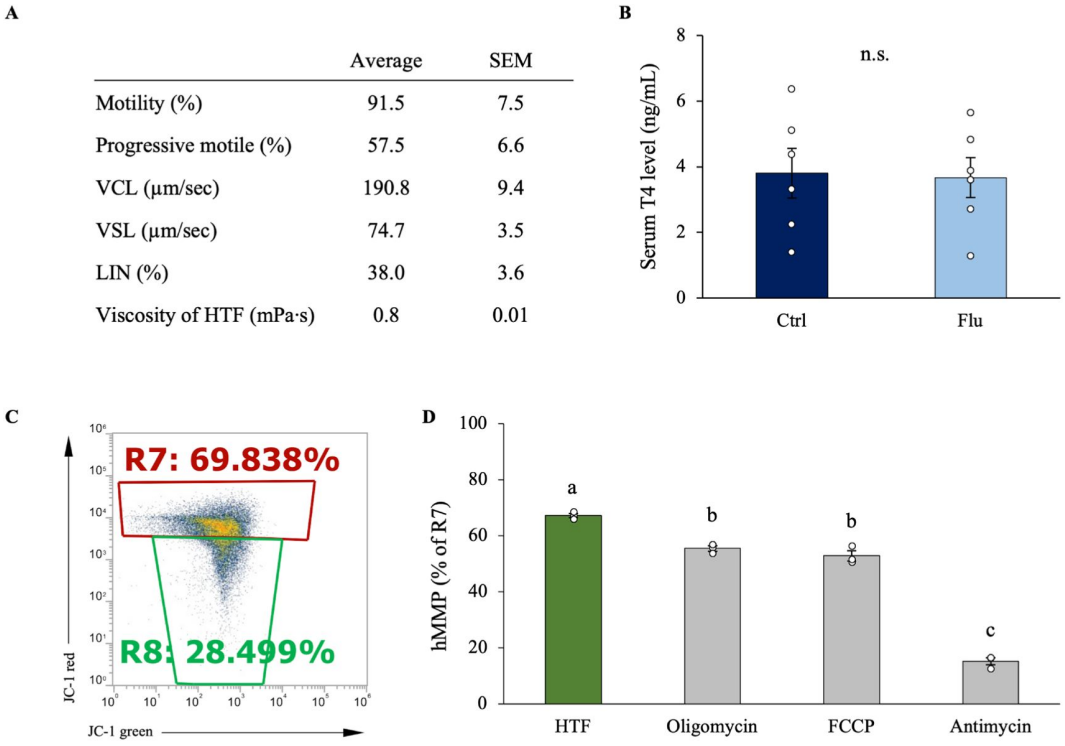
After 1 hour of incubation in the presence or absence of oleic acid (10 nM), ATP in sperm was visualized by BioTracker ATP-Red (SCT045, Sigma-Aldrich). The sperm were incubated with 10 nM of working solution containing BioTracker ATP-Red dye at 37 °C for 60 min in the dark. After washing, the sperm pellet was resuspended in the base medium and analyzed with the flow cytometry using a 488 nm laser and a filter with a bandwidth of 574/26 nm. A total of 100,000 sperm events were analyzed. The sperm population is shown in Supplementary Figure 6.

Artificial insemination

To examine the involvement of seminal vesicle secretions in fertilization, sperm from C57BL/6N mice were injected directly into the uterine bodies of superovulated CD1 mice by syringe, as previously described with minor modifications (17, 22). CD1 female mice were injected intraperitoneally with 8 IU eCG to stimulate follicle development and used as recipients for artificial insemination 48 hours later. Artificial seminal fluid was prepared in HTF medium to a final concentration of 10 mg/mL of collected control or frutamide-derived seminal vesicle secretions. As rescue experiment, 10 nM of oleic acid was added to the frutamide-derived seminal secretions. Cauda epididymis sperm was suspended with above artificial seminal fluid (1×10^6 sperm/mL), and a total of 40 μL (20 μL each per uterus) was injected through the cervix of the female mice. After artificial insemination, 7.5 IU of hCG was injected to stimulate ovulation. Subsequently, a mixture of vaseline and paraffin with a ratio of 10:1 was injected to form an artificial plug around the cervix. After 36~48 hours of sperm injection, the putative eggs were retrieved from the oviducts. The number of two-cell embryos and mature unfertilized eggs were counted and used to estimate the fertilization success rate.

Statistical analysis

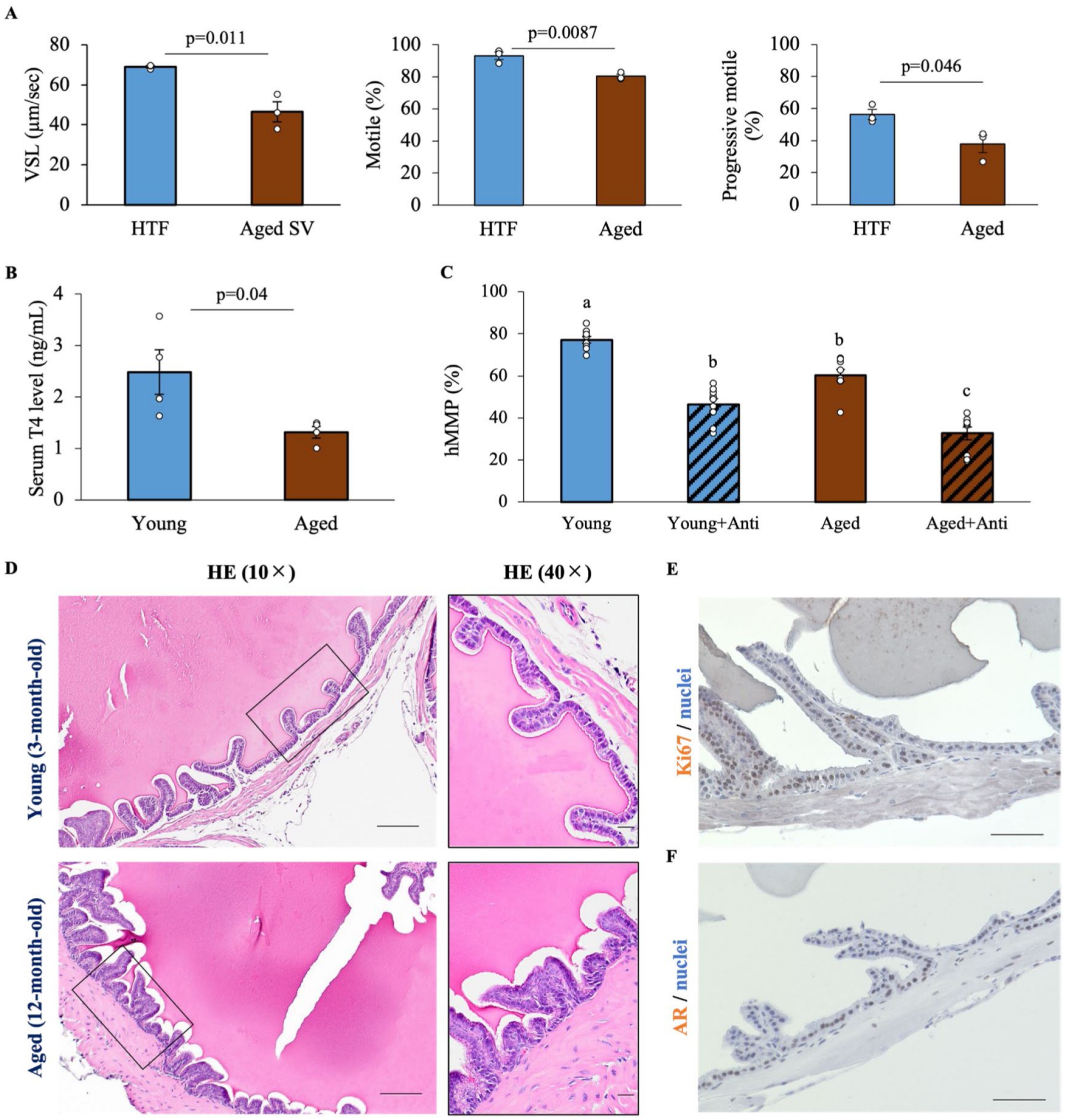
Quantitative data were presented as means ± SEM. The Bartlett's test was used to confirm whether the variances were equal for each group in experiments consisting of three or more groups. All statistical details of the experiments are given in the figure legends. R [(version 4.3.1 (2023-06-16)] was used for statistical analysis. Data were considered statistically significant at $p < 0.05$.



Supplemental Figure 1.

Sperm quality control data and exploratory research into the effects of seminal vesicle secretions on sperm fertilization

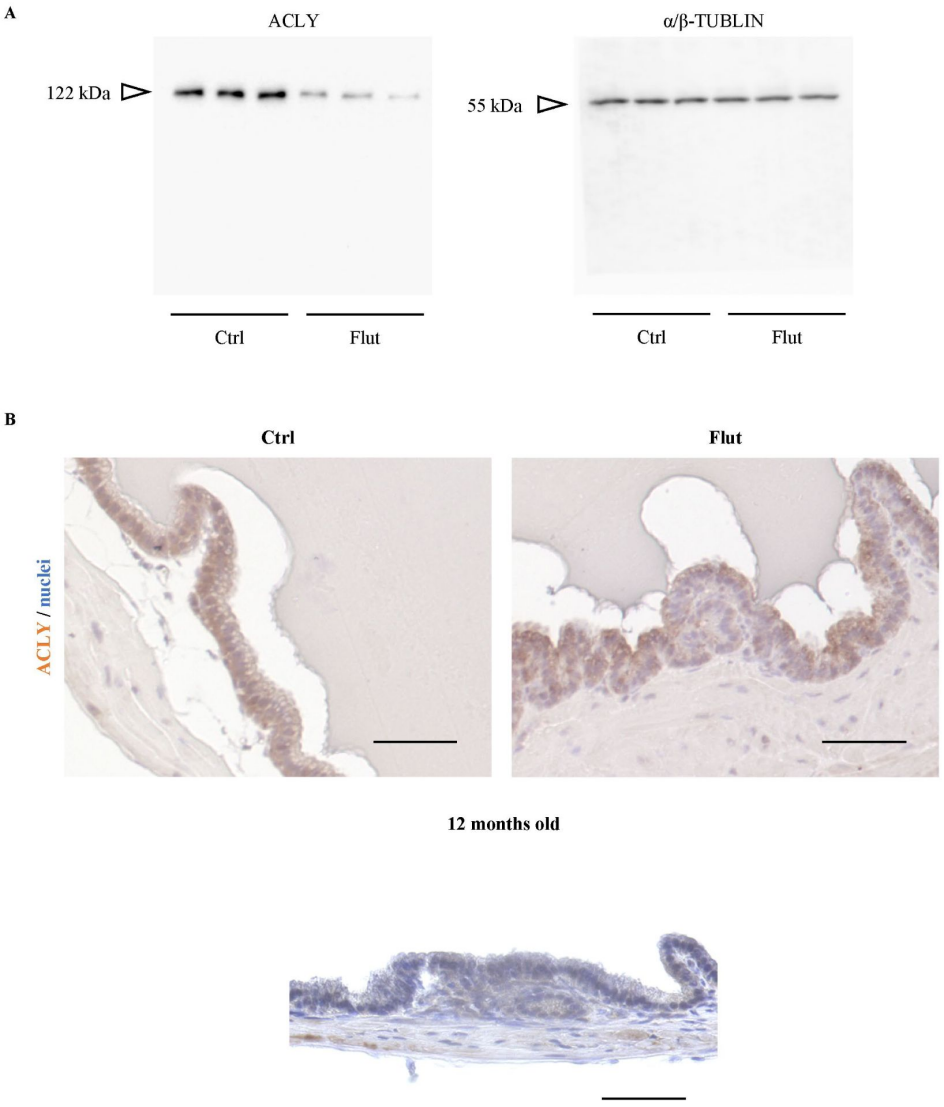
(A) The baseline information for the epididymal sperm used in **Fig 1** and Supplemental Fig 2A. Curvi-Linear Velocity; VCL, Straight-Line Velocity; VSL and Linearity; LIN is the ratio of VSL to VCL of sperm incubated in HTF medium one hour. (B) Serum testosterone levels in seminal vesicle secretion donors. (C) A representative flow cytometry pattern of JC-1 staining of sperm that was not treated with drugs (HTF). (D) The effect of various drugs on the proportion of high mitochondrial membrane potential (hMMP; R7). Data are mean ± SEM. At least three independent replicates. (B) Significance was tested in comparison using Student's t-test. (D) Percentage data were subjected to arcsine transformation before statistical analysis. Differences between groups were assessed by one-way analysis of variance (ANOVA). When ANOVA was significant, differences among values were analyzed by Tukey's Honest Significant Difference test for multiple comparisons. Different letters represent significantly different groups.



Supplemental Figure 2.

Characteristics of the seminal vesicle in aging mice

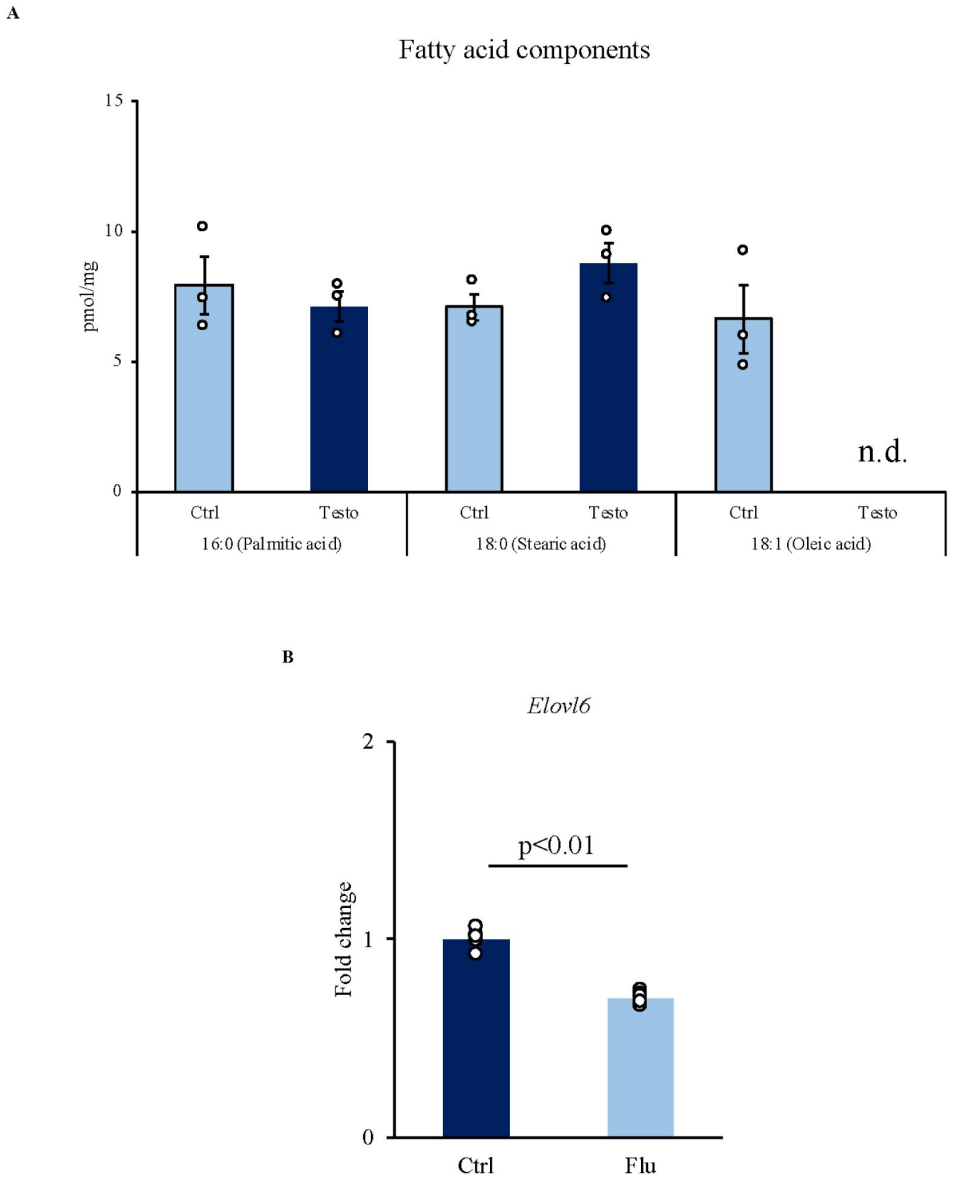
(A) The effects of secretions from aged seminal vesicle fluid on sperm motility. Straight-Line Velocity; VSL, Motile, and Progressive motile. (B) Serum testosterone (T4) levels in young (3 months old) or aged (12 months) mice. (C) The high mitochondrial membrane potential (hMMP) was checked by the JC-1 kit. (D) Hematoxylin/Eosin (HE) staining in seminal vesicles of young and aged mice. Scale bar=50 μm for left panel and 20 μm for right panel. (E) Representative images staining for Ki67. Scale bar=50 μm. (F) The localization of the androgen receptor (AR; NR3C4) in the seminal vesicles of aged mice. Scale bar=50 μm. (E,F) Images were obtained at 10× magnification. Data are mean ± SEM. At least three independent replicates. Percentage data were subjected to arcsine transformation before statistical analysis. (A,B) Significance was tested in comparison using Student's t-test. (C) A two-way ANOVA was performed. Tukey's Honest Significant Difference test was performed as a post hoc test. Both the age and antimycin treatment were significant. The interaction was not significant. Different letters represent significantly different groups.



Supplemental Figure 3.

ACLY expression in in vivo seminal vesicle

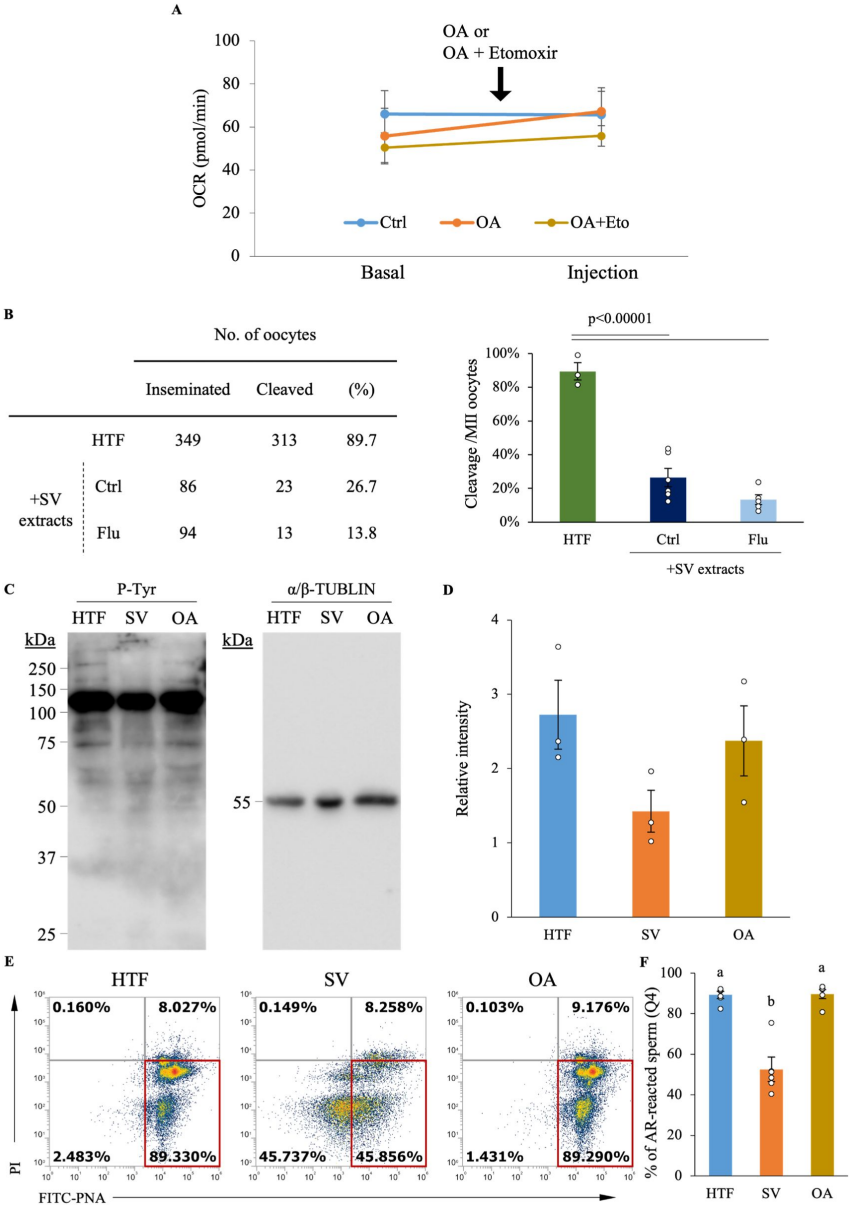
(A) Representative Western blot images of ACLY and α-tubulin in three sets of seminal vesicle collected from 50 mg/kg flutamide subcutaneously for 7 days (Flut) or vehicle treated mice (Ctrl). (B) ACLY immunostaining: seminal vesicles from wild-type mice, flutamide-treated mice, and 12-month-old aging mice. Scale bar is 50 μm.



Supplemental Figure 4.

Oleic acid synthesis in vivo regulated by the testosterone-androgen receptor system

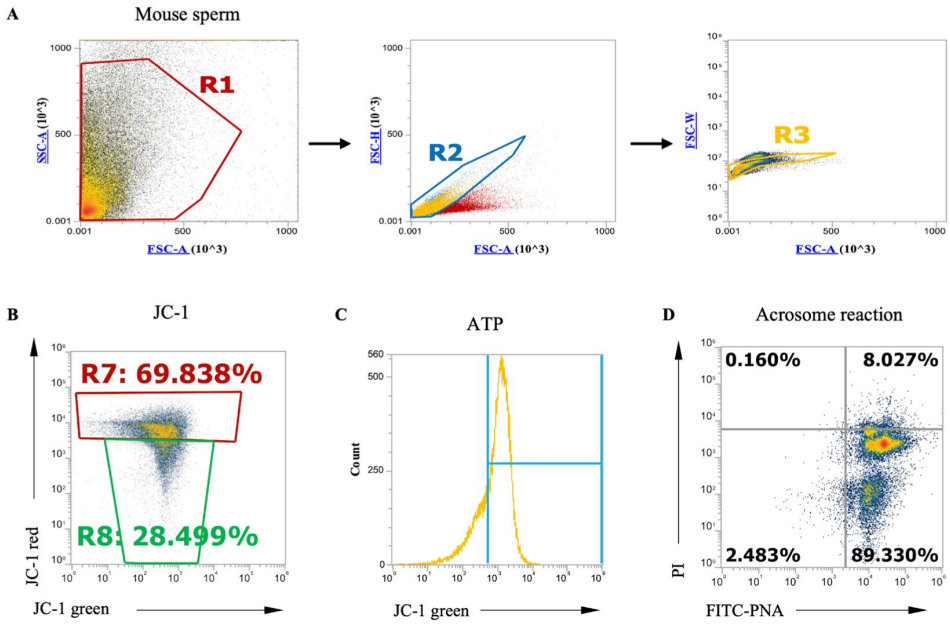
(A) Fatty acid composition of seminal vesicle extracts in flutamide-treated (Flu) or vehicle mice (Ctrl) pretreated with a methylation kit was analyzed by gas chromatography. *Moles contained per unit wight of seminal vesicle secretions. (B) Quantification of *Elovl6* gene expression by RT-qPCR. Data are mean ± SEM. Student's t-test was used for comparison between the two groups (n=5).



Supplemental Figure 5.

The effects of oleic acid and seminal vesicle secretions on sperm function

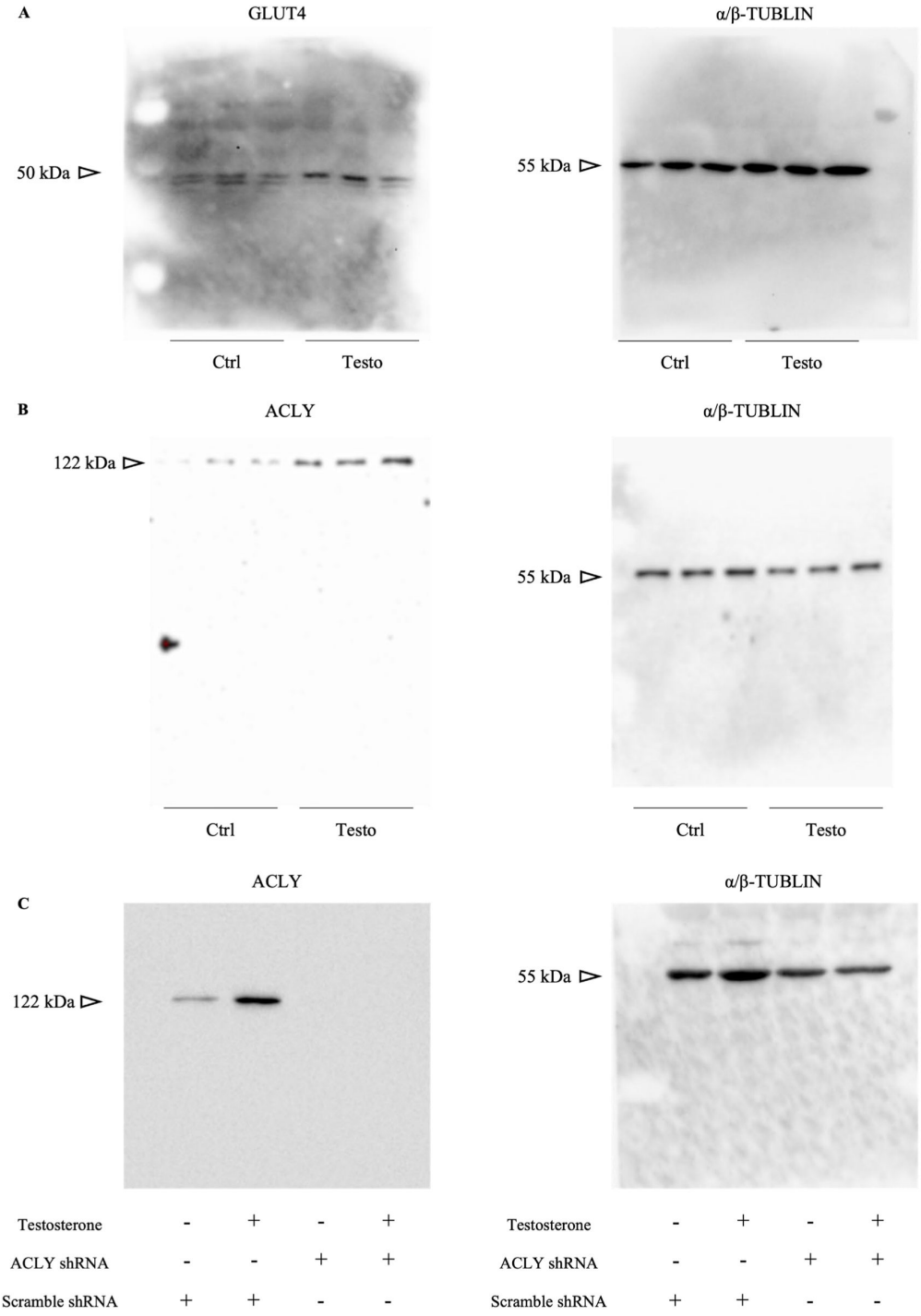
(A) Tracing of oxygen consumption rate (OCR) basal and injection with 10 nM oleic acid (OA) or OA + 40 nM Etomoxir (Eto). (B) The sperm, after 1 hour of incubation with or without seminal vesicle secretions (SV) from nontreated (Ctrl) or flutamide-treated mice(Flu), were used for IVF, and the cleavaged oocytes were observed. The absolute number of oocytes collected from the oviduct and the cleavage rate are shown. (C) Western blot analysis for phosphotyrosine (P-Tyr) in capacitated spermatozoa (Ctrl; 1-hour incubation in HTF medium) and treated with SV from healthy mice or 10 nM oleic acid (OA). (D) Quantitative analysis of GLUT4 relative to α-tubulin obtained from Western blot. (E) Flow cytometric analysis of the sperm after 1 hour incubation in control medium (HTF) and medium containing SV or 10 nM OA, using fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC; to distinguish between acrosome-reacted and non-reacted cells) and propidium iodide (PI; to distinguish between dead and viable cells). (F) The percentage of viable sperm with an acrosome reaction (PI-, PNA-FITC+) was evaluated by flow cytometry in the 4th quadrant (4Q; red square). Data are mean ± SEM. At least three independent replicates. Percentage data were subjected to arcsine transformation before statistical analysis. (B) Dunnett's test was used to analyze the cleavage rate. Different letters represent significantly different groups. (D,F) Differences between groups were assessed by one-way analysis of variance (ANOVA). When ANOVA was significant, differences among values were analyzed by Tukey's Honest Significant Difference test for multiple comparisons.



Supplemental Figure 6.

Gating strategy of flow cytometry for sperm

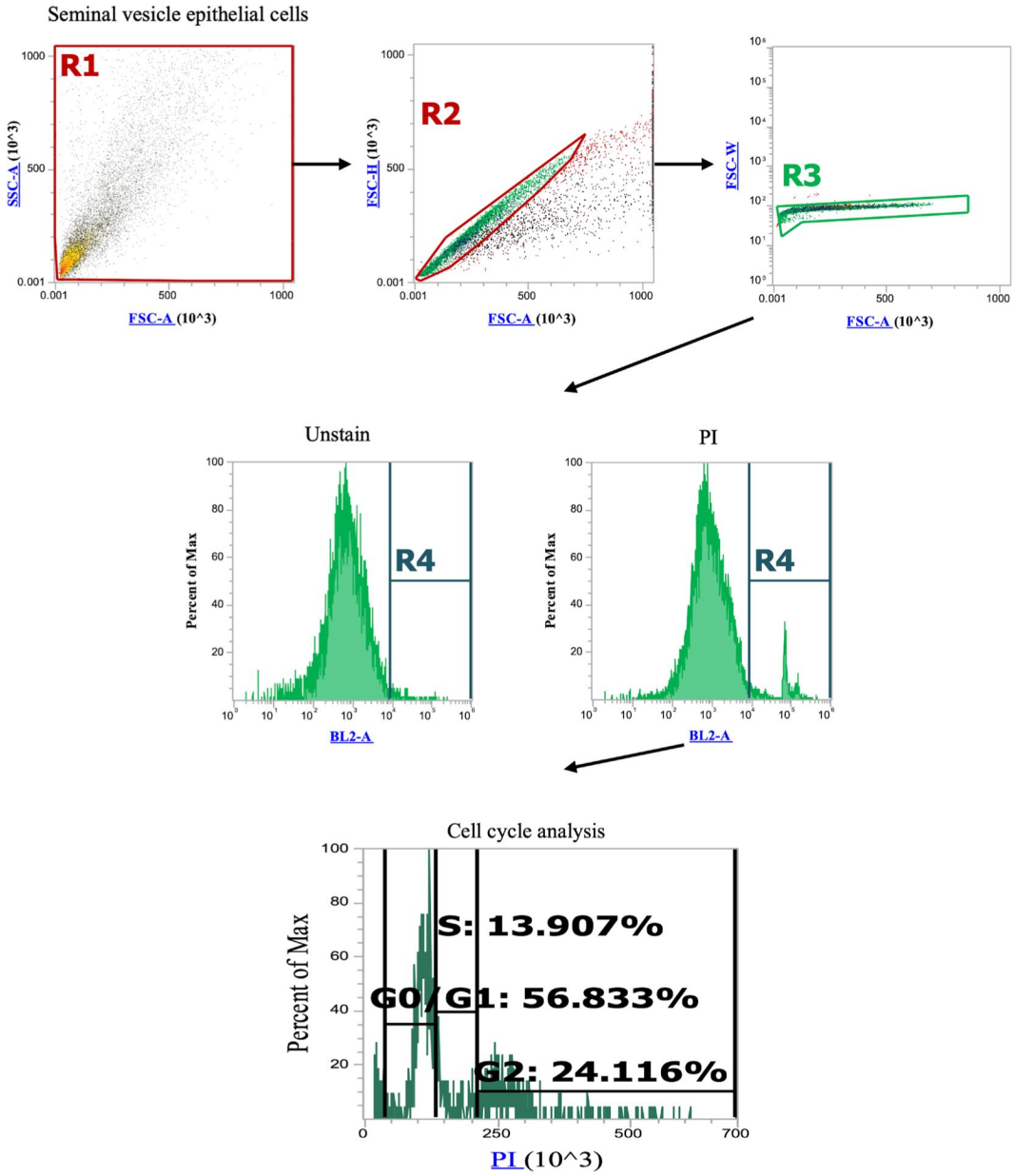
(A) Gating strategy for the selection of single sperm. Using forward scatter (FSC)-A and side scatter (SSC)-A dot plots, similar size and complexity cells were firstly selected (R1). In FSC-A and FSC-H dot plots and FSC-A and FSC-W dot plots, similar-size cells were accumulated near area; thus, using these plots, again similar-size cells were selected (R2, R3). The cells in R3 were used for below analysis. (B) The dot plots of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) green (x-axis) and red (y-axis). The percentage of JC-1 red-positive sperm (R7) was used for the analysis. (C) Histograms of BioTracker Red staining. Blue line indicates the range of positive cells used in **Figure 7I**. (D) The dot plots of fluorescein isothiocyanate-conjugated peanut agglutinin (PNA-FITC; x-axis) and propidium iodide (PI; y-axis).



Supplemental Figure 7.

Full-length blotting images of western blotting

(A) Full-length blotting images of GLUT4 and α/β-TUBLIN in seminal vesicle epithelial cells cultured with 100 ng/mL testosterone (Testo) or in vehicle (Ctrl) in **Figure 6D**. (A) Full-length blotting images of ACLY and α/β-TUBLIN in seminal vesicle epithelial cells cultured with 100 ng/mL testosterone (Testo) or in vehicle (Ctrl) in **Figure 8A**. (B) Full-length blotting images of ACLY and α/β-TUBLIN in seminal vesicle epithelial cells in **Figure 8C**. ACLY protein levels in scrambled shRNA or ACLY shRNA-transfected seminal vesicle epithelial cells cultured with or without 100 ng/mL testosterone were determined by Western blot.



Supplemental Figure 8.

Gating strategy of flow cytometry for seminal vesicle epithelial cells

Gating strategy for the selection of single sperm. Using forward scatter (FSC)-A and side scatter (SSC)-A dot plots, similar size and complexity cells were firstly selected (R1). In FSC-A and FSC-H dot plots and FSC-A and FSC-W dot plots, similar-size cells were accumulated near area; thus, using these plots, again similar-size cells were selected (R2, R3). The cells in R3 were applied to the propidium iodide (PI) histogram plot. Labelling DNA with PI allows fluorescence-based analysis of the cell cycle. The intensity of PI fluorescence correlates with the amount of DNA in each cell. Comparing the G1 and G2 phases, the amount of DNA doubles in the G2 phase, so the fluorescence intensity of the cell population also doubles.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Acc	GCCTCTTCCTGACAAACGAG	TGACTGCCGAAACATCTCTG
Acly	AGGTCTCTCTGCAGCCATGT	AAGCTTTCCTCGACGTTTGA
Aco2	CTAGCCTCAGCCCAGTGAAC	CCTTCAGCCACCAGTGAAAT
Cs	CAGATGCCCACAGAGGAACA	AGCTTGGCAATGAGGTCCAT
ElovI6	CCCGAACTAGGTGACACGAT	CCGCAAGGCGTAGTAAGAGT
Fasn	AGCACACATCCTAGGCATCC	TGTCGTGTCAGTAGCCGAGT
Fh	AGCAATGCATATTGCTGCTG	CGCATACTGGACTTGCTGAA
Glut1	AAACATGGAACCACCGCTAC	AGGCCAACAGGTTCATCATC
Glut2	GCCTGTGTATGCAACCATTG	TGGCCCAATCTCAAAGAAAC
Glut3	TGTCACAGGAGAAGCAGGTG	GCTCCAATCGTGGCATAGAT
Glut4	ACTCTTGCCACACAGGCTCT	AATGGAGACTGATGCGCTCT
Hk1	TATCGGTCCAGCACGTATGC	ACATCGTGACCCACACAGTC
Hk2	GGGTAGCCACGGAGTACAAA	TGGATTGAAAGCCAACTTCC
Hmgcr	TGGAGATCATGTGCTGCTTC	GCGACTATGAGCGTGAACAA
Idh2	CCGTCTTCAGAGAGCCAATC	GAAATGGACTCGTCGGTGTT
Mdh2	GCTTTGTCTTCTCCCTCGTG	CAAAGTCCTCGCCTTTCTTG
Nd1	CAGGATGAGCCTCAAACTCC	CCGGTTTGTTTCTGCTAGGG
Nd6	GGTTGGTTGTCTTGGGTTAGC	TAGATCCCCAAGTCTCTGGA
Ogdh	ACATGGCACAGTGCATCATT	ACATCTGCAGAAACCGCTCT
Pdha	GGGGACGTCTGTTGAGAGAG	TGTGTCCATGGTAGCGGTAA
Sdhb	ACTGGTGGAACGGAGACAAG	GTTAAGCCAATGCTCGCTTC
Suclg2	CTTTGGTGGGATCGTCAACT	AACAGCTTTCTTGGCTGCAT
L19	GGCATAGGGAAGAGGAAGG	GGATGTGCTCCATGAGGATGC

Table S1.

Primer sequences used for quantitative real-time PCR.

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Additional information

Data availability

Nucleotide sequence data reported are available in the DDBJ Sequenced Read Archive under the accession numbers DRA017090. Additional information needed to reanalyze the data reported in this paper is available from the principal contact upon request.

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Author contributions

Conceptualization: TY, TU, MS

Methodology: TY, ZX, NT, MA, TU

Investigation: TY, ZX, NT, MA, TU

Visualization: TY, MS

Funding acquisition: MS Supervision: MS

Writing – original draft: TY

Writing – review & editing: TY, TU, MS

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Reviewer #1 (Public review):

Summary:

In this revised report, Yamanaka and colleagues investigate a proposed mechanism by which testosterone modulates seminal plasma metabolites in mice. The authors identify oleic acid as a particularly important metabolite, derived from seminal vesicle epithelium, that stimulates linear progressive motility in isolated cauda epidydimal sperm in vitro. The authors provide additional experimental evidence of a testosterone dependent mechanism of oleic acid production by the seminal vesicle epithelium.

Strengths:

Often, reported epididymal sperm from mice have lower percent progressive motility compared with sperm retrieved from the uterus or by comparison with human ejaculated sperm. The findings in this report may improve in vitro conditions to overcome this problem, as well as add important physiological context to the role of reproductive tract glandular secretions in modulating sperm behaviors. The strongest observations are related to the sensitivity of seminal vesicle epithelial cells to testosterone. The revisions include addition of methodological detail, modified language to reflect the nuance of some of the measurements, as well as re-performed experiments with more appropriate control groups. The findings are likely to be of general interest to the field by providing context for follow-on studies regarding the relationship between fatty acid beta oxidation and sperm motility pattern.

Weaknesses:

Support for the proposed mechanism is stronger in this revised report than in the previous report, but there are many challenges in measuring sperm metabolism and its direct relationship with motility patterns. This study is no exception and largely relies on correlations between various experiments in lieu of direct testing. Additionally, the discussion is framed from a human pre-clinical perspective, and it should be noted that the reproductive physiology between mice and humans is very different.

https://doi.org/10.7554/eLife.95541.2.sa2

Reviewer #2 (Public review):

Using a combination of in vivo studies with testosterone-inhibited and aged mice with lower testosterone levels as well as isolated mouse and human seminal vesicle epithelial cells the authors show that testosterone induces an increase in glucose uptake. They find that testosterone induces a difference in gene expression with a focus on metabolic enzymes. Specifically, they identify increased expression of enzymes regulating cholesterol and fatty acid synthesis, leading to increased production of 18:1 oleic acid. The revised version strengthens the role of ACLY as the main regulator of seminal vesicle epithelial cell metabolic programming. 18:1 oleic acid is secreted by seminal vesicle epithelial cells and taken up by sperm, inducing an increase in mitochondrial respiration. The difference in sperm motility and in vivo fertilization in the presence of 18:1 oleic acid and the absence of testosterone, however, is small. Additional experiments should be included to further support that oleic acid positively affects sperm function.

https://doi.org/10.7554/eLife.95541.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *In this report, the authors investigated the effects of reproductive secretions on sperm function in mice. The authors attempt to weave together an interesting mechanism whereby a testosterone-dependent shift in metabolic flux patterns in the seminal vesicle epithelium supports fatty acid synthesis, which they suggest is an essential component of seminal plasma that modulates sperm function by supporting linear motility patterns.*

> *Strengths:*
>
> *The topic is interesting and of general interest to the field. The study employs an impressive array of approaches to explore the relationship between mouse endocrine physiology and sperm function mediated by seminal components from various glandular secretions of the male reproductive tract.*

Thank you for your positive evaluation of our study's topic and approach. We are pleased that you found our investigation into the effects of reproductive secretions on sperm function to be of general interest to the field. We appreciate your positive feedback on the diverse methods we employed to explore this complex relationship.

> *Weaknesses:*
>
> *Unfortunately, support for the proposed mechanism is not convincingly supported by the data, and the experimental design and methodology need more rigor and details, and the presence of numerous (uncontrolled) confounding variables in almost every experimental group significantly reduce confidence in the overall conclusions of the study.*
>
> *The methodological detail as described is insufficient to support replication of the work. Many of the statistical analyses are not appropriate for the apparent designs (e.g. t-tests without corrections for multiple comparisons). This is important because the notion that different seminal secretions will affect sperm function would likely have a different conclusion if the correct controls were selected for post hoc comparison. In addition, the HTF condition was not adjusted to match the protein concentrations of the secretion-containing media, likely resulting in viscosity differences as a major confounding factor on sperm motility patterns.*

We appreciate you highlighting concerns regarding our weak points and apologize for our unclear description. We revised the manuscript to be as rigorous and detailed as possible. In addition, some experimental designs were changed to simpler direct comparisons, and additional experiments were conducted (New Figure 1A-F, lines 103-113). We have made our explanations more consistent with the provided data, which includes further experimentation with additional controls and larger sample sizes to increase the reliability of the findings.

To address the multiple testing problem, a multiple testing correction was made by making the statistical tests more stringent (Please see Statistical analysis in the Methods section and the Figure legends). Based on different statistical methods, the analysis results did not require significant revisions of the previous conclusions.

Because the experiments on mixing extracts from the seminal vesicles were exploratory, we planned to avoid correcting for multiple comparisons. Repeating the t-test could lead to a Type I error in some results, so we apologize for not interpreting and annotating them. In the revised version, we removed the dataset for experiments on mixing extracts from the seminal vesicles and prostate, and we changed the description to refer to the clearer dataset mentioned above.

The viscosity of the secretion-containing medium was measured with a viscometer, confirming that secretions did not significantly affect the viscosity of the solution. In addition, as the reviewer pointed out, we addressed the issue that the HTF condition could not be used as a control because of the heterogeneity in protein concentration (New Fig.1G, lines 110-111).

Overall, we concluded that seminal vesicle secretion improves the linear motility of sperm more than prostate secretion.

> *There is ambiguity in many of the measurements due to the lack of normalization (e.g. all Seahorse Analyzer measurements are unnormalized, making cell mass and uniformity a major confounder in these measurements). This would be less of a concern if basal respiration rates were consistently similar across conditions and there were sufficient independent samples, but this was not the case in most of the experiments.*

We apologize for the many ambiguities in the first manuscript. Cell culture experiments in the paper, including the flux analysis, were performed under conditions normalized or fixed by the number of viable cells. The description has also been revised to emphasize that the measurement values are standardized by cell count (lines 183-185, 189-190, 194-197). We emphasize that testosterone affects metabolism under the same number of viable cells (New Fig.4). This change in basal respiration is thought to be due to the shift in the metabolic pathway of seminal vesicle epithelial cells to a “non-normal TCA cycle” in which testosterone suppresses mitochondrial oxygen consumption, even under aerobic conditions (New Figs.3, 4, 5).

> *The observation that oleic acid is physiologically relevant to sperm function is not strongly supported. The cellular uptake of 10-100uM labeled oleic acid is presumably due to the detergent effects of the oleic acid, and the authors only show functional data for nM concentrations of exogenous oleic acid. In addition, the effect sizes in the supporting data were not large enough to provide a high degree of confidence given the small sample sizes and ambiguity of the design regarding the number of biological and technical replicates in the extracellular flux analysis experiments.*

Thank you for your important critique. As you noted, the too-high oleic acid concentration did not reflect physiological conditions. Therefore, we changed the experimental design of an oleic acid uptake study and started again. We added an *in vitro* fertilization experiment corresponding to the functional data of exogenous oleic acid at nM concentrations (New Fig.7J,K, Lines 274-282).

For the flux data to determine the effect of oleic acid on sperm metabolism, we have indicated in the text that the data were obtained based on eight male mice and two technical replicates. Pooled sperm isolated and cultured from multiple mice were placed in one well. The measurements were taken in three different wells, and each experiment was repeated four times. We did not use the extracellular flux analyzers XFe24 or XFe96. The measurements were also repeated because the XF HS Mini was used in an 8-well plate (only a maximum of 6 samples at a run since 2 wells were used for calibration).

> *Overall, the most confident conclusion of the study was that testosterone affects the distribution of metabolic fluxes in a cultured human seminal vesicle epithelial cell line, although the physiological relevance of this observation is not clear.*

We thank the comments that this finding is one of the more robust conclusions of our study. Below we have written our thoughts on the physiological relevance of the observation results and our proposed revisions. In the mouse experiments, when the action of androgens was inhibited by flutamide, oleic acid was no longer synthesized in the seminal vesicles. The results of the experiments using cultured seminal vesicle epithelial cells showed that oleic acid was not being synthesized because of a change in metabolism dependent on testosterone. We have also added IVF data on the effects of oleic acid on sperm function (New Fig.7 and Supplementary Fig. 5, lines 274-282).
As you can see, we have obtained consistent data *in vitro* and *in vivo* in mice. Our data also showed that the effects of testosterone on metabolic fluxes *in vitro* are similar in mouse and human seminal vesicle epithelial cells (New Fig.9). Therefore, it can be assumed that a

decrease in testosterone levels causes abnormalities in the components of human semen. However, the conclusion was overestimated in the original manuscript, so we changed the wording as follows: It could be assumed that a decrease in testosterone levels causes abnormalities in the components of human semen. (lines 422-423)

> *In the introduction, the authors suggest that their analyses "reveal the pathways by which seminal vesicles synthesize seminal plasma, ensure sperm fertility, and provide new therapeutic and preventive strategies for male infertility." These conclusions need stronger or more complete data to support them.*

We appreciate your comments about the suggestion presented in the introduction.

We also removed our conclusions regarding treatment and prevention strategies for male infertility (lines 96-98). We wanted to discuss our findings not conclusively but as future applications that could result from further research based on our initial findings.

The last sentence of the introduction has been revised to tone down these assertions as follows: These analyses revealed that testosterone promotes the synthesis of oleic acid in seminal vesicle epithelial cells and its secretion into seminal plasma, and the oleic acid ensures the linear motility and fertilization ability of sperm.

We are grateful for your suggestions, which have prompted us to refine our manuscript.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Using a combination of in vivo studies with testosterone-inhibited and aged mice with lower testosterone levels, as well as isolated mouse and human seminal vesicle epithelial cells, the authors show that testosterone induces an increase in glucose uptake. They find that testosterone induces differential gene expression with a focus on metabolic enzymes. Specifically, they identify increased expression of enzymes that regulate cholesterol and fatty acid synthesis, leading to increased production of 18:1 oleic acid.*
>
> *Strength:*
>
> *Oleic acid is secreted by seminal vesicle epithelial cells and taken up by sperm, inducing an increase in mitochondrial respiration. The difference in sperm motility and in vivo fertilization in the presence of 18:1 oleic acid and the absence of testosterone is small but significant, suggesting that the authors have identified one of the fertilization-supporting factors in seminal plasma.*

Thank you for your positive comments regarding our work on the role of testosterone in regulating metabolic enzymes and the subsequent production of 18:1 oleic acid in seminal vesicle epithelial cells. We are pleased that the strength of our findings, particularly identifying oleic acid as a factor influencing sperm motility and mitochondrial respiration, has been recognized.

> *Weaknesses:*
>
> *Further studies are required to investigate the effect of other seminal vesicle components on sperm capacitation to support the author's conclusions. The author's experiments focused on potential testosterone-induced changes in the rate of seminal vesicle epithelial cell glycolysis and oxphos, however, provide conflicting results and a potential correlation with seminal vesicle epithelial cell proliferation should be confirmed by additional experiments.*

Thank you very much for your valuable criticism. Although we fully agree with your comment, conducting experiments to investigate the effects of other seminal vesicle components on the fertilization potential of sperm would be a great challenge for us. This is because it has taken us the last three years to identify oleic acid as a key factor in seminal plasma. We are considering a follow-up study to explore the effect of other seminal vesicle components on sperm capacitation. Therefore, we have revised the Introduction and conclusions to tone down our assertions .

The revised manuscript also includes additional data showing a correlation between changes in metabolic flux and the proliferation of seminal vesicle epithelial cells using shRNA. As a result, it was shown that cell proliferation is promoted when mitochondrial oxidative phosphorylation is promoted by ACLY knockdown (New Fig.8D, lines 303-305). This shows a close relationship between the metabolic shift in seminal vesicle epithelial cells and cell proliferation. The revised manuscript includes an interpretation and discussion of these results (lines 369-379).

We are grateful for your suggestions, which have prompted us to refine our manuscript.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Male fertility depends on both sperm and seminal plasma, but the functional effect of seminal plasma on sperm has been relatively understudied. The authors investigate the testosterone-dependent synthesis of seminal plasma and identify oleic acid as a key factor in enhancing sperm fertility.*
>
> *Strengths:*
>
> *The evidence for changes in cell proliferation and metabolism of seminal vesicle epithelial cells and the identification of oleic acid as a key factor in seminal plasma is solid.*
>
> *Weaknesses:*
>
> *The evidence that oleic acids enhance sperm fertility in vivo needs more experimental support, as the main phenotypic effect in vitro provided by the authors remains simply as an increase in the linearity of sperm motility, which does not necessarily correlate with enhanced sperm fertility.*

We appreciate the positive feedback on the solid evidence of cell proliferation and metabolic changes in seminal vesicle epithelial cells and the identification of oleic acid as an important factor in seminal plasma. We fully agree with the assessment that the evidence linking oleic acid and increased sperm fertility *in vivo* needs further experimental support. To address this concern, we changed the experimental design of an oleic acid study and started again to be more physiological regarding the effect of oleic acid on fertility outcomes, increased the replicates of artificial insemination, and added *in vitro* fertilization assessments (New Fig.7 and supplementary Fig.5, lines 274-282). The revised manuscript describes these experiments and discusses the association between oleic acid and fertility.

We are grateful for your suggestions, which have prompted us to refine our manuscript.

> ***Recommendations for the authors:***

> ***Reviewing Editor's note:***
>
> *As you can see from the three reviewers' comments, the reviewers agree that this study can be potentially important if major concerns are adequately addressed. The major concern common to all the reviewers is the incomplete mechanistic link between the physiological androgen effect on the production of oleic acid and its effect on sperm function. Statistical analyses need more rigor and consideration of other important capacitation parameters are needed to address these concerns and to improve the manuscript to support the current conclusions.*

Thank you for summarizing the reviewers' feedback and for your insights regarding the major concerns raised. We appreciate the reviewers' understanding of the potential importance of our work and have addressed the issues highlighted to strengthen the manuscript. We believe these changes will improve the quality of the manuscript and provide a clearer and more complete understanding of the role of androgens and oleic acid in sperm function.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *The following comments are provided with the hope of aiding the authors in improving the alignment between the data and their interpretations.*

Thank you for allowing us to strengthen our manuscript with your valuable comments and queries. We have made our best efforts to reflect your feedback.

> *Major Comments:*
>
> *(1) The methodological detail is not sufficient to reproduce the work. For example:*
> *a. Manufacturer protocols are referred to extensively. These protocols are neither curated nor version-controlled. Please consider describing the underlying components of the assays. If information is not available, please consider providing catalog numbers and lot numbers in the methods (if appropriate for journal style requirements).*

We appreciate this suggestion, which we believe is important to ensure reproducibility. We described the catalog number in our Methodology and included as much information as possible.

> *b. Please consider describing the analyses in full, with consideration given to whether blinding was part of the design. For example- line 492: "apoptotic cells were quantified using ImageJ". How was this quantified? How were images pre-processed? Etc.*

Although blinding was not performed, experiments and analyses based on Fisher's three principles were conducted to eliminate bias (lines 549-552). In order to avoid false-positive or false-negative results, it is clearly stated that tissue sections treated with DNAse were used as positive controls, and tissue sections without TdT were used as negative controls for apoptosis. We have added detailed quantification methods (lines 544-546).

> *c. Please consider providing versions of all acquisition and analysis software used.*

We have added software version information in Materials and Methods.

> *(2) Please consider revisiting the statistical analyses. Many of the analyses don't seem appropriate for the design. For example, the use of a t-test with multiple comparisons for*

> *repeated measures design in Figure 2 and the use of t-test for two-factor design in Figure 8. etc.*

To address the multiple testing issues, the statistical methodology was changed to a more rigorous one. Details are given in the Statistical analysis in the Methods section and the Figure legends.

> *(3) The increase in % LIN in Figure 1 may be confounded by differences in viscosity between HTF and the fluid secretion mixtures. For this reason, HTF may not be an appropriate control for the ANOVA post hoc analysis. HTF protein was not adjusted to the same concentration as the secretion mixtures, correct? Ultimately, it does not appear that there would be a significant statistical effect of the different fluid mixtures if appropriate statistical comparisons were made. This detracts from the notion that the secretions impact sperm function.*
>
> *(4) Figure 1, the statistical analysis in the legend suggests that the experiments were analyzed with a t-test. Were corrections made for multiple comparisons in B-D? An ANOVA would probably be more appropriate.*

We used a viscometer to measure the viscosity of a solution of prostate and seminal vesicle secretions adjusted to a protein concentration of 10 mg/mL. The results showed that the secretions did not cause any significant viscosity changes (New Fig.1G, Lines 110-111).

As you pointed out, the protein levels in the HTF medium and the secretion mixture are not adjusted to the same concentration. In addition, the original manuscript was not a controlled experiment because the two factors, seminal vesicle and prostate extracts, were modified. Therefore, to investigate the effect of prostate and seminal vesicle secretions on sperm motility, we modified the experimental design to directly compare the effects of the two groups: seminal vesicle and prostate extracts (New Fig.1A-G, lines 101-113). To show the sperm quality used in this study, motility data from sperm cultured in the HTF medium are presented independently in New Supplemental Fig.1A.

> *(5) Additionally in Figure 1, there is no baseline quality control data to show that there are no intrinsic differences between sperm sampled from the two treatment groups. So baseline differences in sperm quality/viability remain a potential confounder.*

We thank you for this important point. Epididymal sperm were collected from healthy mice. We recovered only the seminal vesicle secretions from the flutamide-treated mice to pursue its role in the accessory reproductive glands, since testosterone targets the testes and accessory reproductive organs. So, there was no qualitative difference between the epididymal sperm before treatment. Nevertheless, incubation with seminal vesicle secretion for one hour altered the sperm motility pattern and *in vivo* fertilization results. Sperm function was altered by seminal vesicle secretion in a short period of culture time. We apologize for the confusion, and we have revised the text and figure to carry a clearer message (lines 128-132).

> *(6) Figure 1E, did the authors confirm that flutamide-treated mice had decreased serum androgens? How often were mice treated with flutamide? This is important because flutamide has a relatively short half-life and is rapidly metabolized to inert hydroxyflutamide.*

Serum testosterone levels were unchanged. Flutamide was administered every 24 hours for 7 consecutive days. Although there was no change in blood testosterone levels (New Supplemental Fig.1B), a decrease in the weight of the seminal vesicles, prostate, and

epididymis was confirmed. This is thought to be due to the pharmacological activity of flutamide.

> *(7) Figure 1H, the meaning of 'relative activity of mitochondria' isn't clear. JC-1 does not measure 'activity'. A decreased average voltage potential across the inner mitochondrial membrane may indicate that more of the sperm from the flutamide group were dead. Additionally, J-aggregates are slow to form, generally requiring long incubation periods of at least 90 minutes or more. Additional positive and negative controls for predictable mitochondrial transmembrane voltage potential polarization states would have improved the quality of this experiment.*

Thank you for pointing this out. We have replaced the relative activity of mitochondria with high mitochondrial membrane potential (New Fig.1M, lines 125-128). Actually, it is thought that the sperm cultured in seminal vesicle secretions from mice that had been administered flutamide died because the motility of the sperm was also significantly reduced. Since antimycin reduces mitochondrial membrane potential, we have added an experiment in which 10 μM antimycin-treated sperm were used as a control to confirm that the JC-1 reaction is sensitive to changes in membrane potential.

> *(8) Figure 4, the extracellular flux data appear to be unnormalized. The Seahorse instruments are extremely sensitive to the mass and uniformity of the cells at the bottom of the well. This may be a significant confounder in these results. For example, all of the observed differences between groups could simply be a product of differential cell mass, which is in line with the reduced growth potential of testosterone-treated cells indicated by the authors in the results section.*

We thank you for this important point. After correcting for cell viability, we seeded the same number of viable cultured cells into wells between experimental groups before measuring them in the flux analyzer. There were no significant differences in survival rates in all experiments. As a result, an increase in glucose-induced ECAR and a suppression of mitochondrial respiration were observed. We would like to emphasize that this difference based on metabolic data does not imply a reduction in the growth potential of the cells due to testosterone treatment.

We described that these measurements are normalized based on cell count and viability (lines 184, 190, 195).

> *(9) How did the authors know that the isolated mouse primary cells were epithelial cells? Was this confirmed? What was the relative sample purity?*

The cells were labeled with multiple epithelial cell markers (cytokeratin) and confirmed using immunostaining and flow cytometry. The percentage of cells positive for epithelial cell markers was approximately 80%. A stromal cell marker (vimentin) was also used to confirm purity, but only a few percent of cells were positive. The contaminating cell type was considered to be mainly muscle cells because the gene expression levels of muscle cell markers verified by RNA-seq were relatively high.

> *(10) It is misleading to include the lactate/pyruvate media measurements in the middle of the figure in Figure 4 D and E because it seems at first glance like these measurements were made in the seahorse media but they are completely unrelated. Additionally, these measures are not normalized and are sensitive to confounding differences in cell viability, seeding density, mass, etc.*

Thank you for pointing this out. We have placed the lactate and pyruvate measurement graphs after the flux data of ECAR. We noted that these measurements are normalized based on cell count and viability (lines 189-190). The doubling time of seminal vesicle epithelial cells was approximately 3 days, and testosterone inhibited cell proliferation. Therefore, the seeding concentration of cells was increased 4-fold in the testosterone-treated group compared to the control, and experiments were conducted to ensure that the confluency at the time of measurement after 7 days of culture was comparable between groups.

> *(11) The flux analyzer assays sold by Agilent have many ambiguities and problems of interpretation. Unfortunately, Agilent's interest in marketing/sales has outpaced their interest in scientific rigor. Please consider revising some of the language regarding the measurements. For example, 'ATP production rate' is not directly measured. Rather, oligomycin-sensitive respiration rate is measured. The conversion of OCR to ATP production rate is an estimation that depends on complex assumptions often requiring additional testing and validation. The same is true for other ambiguous terms such as 'maximal respiration' referring to FCCP uncoupled respiration, and glycolytic rate- which is also not measured directly. If the authors are interested in a more detailed description of the problems with Agilent's interpretation of these assays please see the following reference (PMID: 34461088).*

Thank you for your critical criticism and thoughtful advice, as well as for sharing the excellent reference. We agree with you on the flux analyzer ambiguities and data interpretation problems. The description of the measured values has been revised as follows.

We have replaced the “ATP production rate” with the “oligomycin-sensitive respiratory rate.” Similarly, we have replaced “maximal respiration” with “FCCP-induced unbound respiration.” (lines 197-202) We chose not to deal with the conversion of OCR to ATP production rate because it is outside the scope of interest in our study.

Avoid using the term "glycolytic capacity". We use “Oligomycin-sensitive ECAR.” (line 186) We recognize that the ECARs measured in this study reflect experimental conditions and may not fully represent physiological glycolytic flux *in vivo*. So, the main section includes a data set of glucose uptake studies to emphasize the significance of the changes obtained with the flux analyzer assay. (New Fig.6, lines 230-254)

> *Figure 6, it's not surprising to see the accumulation of labeled oleic acid in the cells, however, this does not mean that oleic acid is participating in normal metabolic processes. Oleic acid will have detergent effects at high (uM) concentrations. The observation that sperm 'take up' OA at 10-100 uM concentrations should also be validated against sperm function the health of the cells is very likely to be negatively impacted. Additionally, no apparent accumulation is noted in the fluorescence imaging at 1uM, but the authors insinuate that uptake occurs at low nM concentrations. The effects in Figure 6D-F are nominal at best and are likely a result of the small sample sizes.*

Thank you for your good suggestion. We agree with the reviewer that high concentrations of oleic acid had a detergent effect. To improve the consistency of functional data and observations, oleic acid uptake tests were performed under the same concentration range as the sperm motility tests (New Fig.7A-C). The oleic acid concentration at this time was calculated regarding the oleic acid concentration in seminal fluid recovered from mice as detected by GCMS to reflect *in vivo* conditions.

Epididymal sperm were incubated with fluorescently labeled oleic acid and observed after quenching of extracellular fluorescence. Fluorescent signals were detected selectively in the

midpiece of the sperm. The fluorescence intensity of sperm quantified by flow cytometry increased significantly in a dose-dependent manner (New Fig.7A-C, lines 261-264).

Furthermore, increasing the sample size did not change the trend of the sperm motility data. Although the effect size of oleic acid on sperm motility was small (New Fig.7D-G, lines 265-268), an improvement in fertilization ability was observed both *in vitro* (IVF) and *in vivo* (AI) (New Fig.7J-L, lines 274-282, 286-291). We conclude that the effect of oleic acid on sperm is of substantial significance. These data and interpretations have been revised in the text in the Results section.

> *(12) Figure 6H, I applaud the authors for attempting intrauterine insemination experiments to test their previous findings. That said, there is no supporting data included to show that the sperm from the treatment groups had comparable starting viability/quality. Additionally, it is difficult to tell if the results are due to the small sample sizes and particularly the apparent outlier in the flutamide-only group.*

Thanks for the praise and comments for improvement. As we answered in your comment #5 above, the epididymal sperm was collected from healthy mice. Therefore, there is no qualitative difference in the epididymal sperm before treatment. This is described in the figure legend (lines 1130-1131). We apologize again for this complication. We also more than doubled the number of replications of the experiment. The impact of the outlier would have been minimal.

> *(13) One final question related to Figure 6H: how did the authors know they were retrieving all of the possible 2-cell embryos from the uterus? Perhaps the authors could provide the raw counts of unfertilized eggs and 2-cell embryos so we can see if there were differences between the mice.*

We retrieved the pronuclear stage embryos from the fallopian tubes. It is not certain whether all embryos were recovered. Therefore, we added the number of embryos in the graph and in the supplementary data.

> *(14) Figure 7 has the same seahorse assay normalization problem as mentioned earlier. Without normalization, it is difficult to tell if the effects are simply due to differences in cell mass. Were the replicates indicated in the graphs run on the same plate? If so, it would be much more convincing to see a nested design, with technical replicates within plates, and additional replicates run on separate plates.*

As we answered in your comment #8 above, these measurements were normalized based on sperm count. This has been corrected to be noted in the text and the figure legend (lines 1123-1124).

Pooled sperm isolated and cultured from multiple mice were placed in one well. The measurements were taken in three different wells, and each experiment was repeated four times. We did not use the extracellular flux analyzers XFe24 or XFe96. The measurements were also repeated because the XF HS Mini was used in an 8-well plate (only a maximum of 6 samples at a run since 2 wells were used for calibration).

> *(15) The statistical test in Figures 8E and F described in the legend is inappropriate (t-test), this appears to be a two-factor design.*

Thank you for pointing this out. Differences between groups were assessed using a two-way analysis of variance (ANOVA). When the two-way ANOVA was significant, differences among values were analyzed using Tukey's honest significant difference test for multiple comparisons.

> *(16) The data in Figure 8 are interesting, and the effects appear to be a little more consistent compared with the mouse primary cells, potentially due to cell uniformity. However, the data are unnormalized, causing significant ambiguity, and there are no measures of cell viability to determine if the effects are due to cell death (or at least relative cell mass).*

As we answered in your comments #8 and #14 above, these measurements were normalized based on cell count and viability. This has been corrected to be noted in the figure legend (lines 1185-1186).

> *Minor Comments:*
>
> *(1) The section title indicating the beginning of the results section is missing.*

A section title has been added to indicate the beginning of the results section.

> *(2) There were several typos and confusingly worded statements throughout. Please consider additional editing.*

We used a proofreading service and corrected as much as possible.

> *(3) In the introduction, a brief description of seminal fluid physiology is provided, but the reference is directed toward human physiology. Given that the research is performed solely in the mouse, a brief comparative description of mouse physiology would be helpful. For example, what is the role of mouse seminal fluid in the formation of the mating plug? What are the implications of the relative size disparity in seminal vesicles in mice versus humans? Etc.*

The third paragraph of the introduction has been revised (lines 57-60).

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Thank you for allowing us to strengthen our manuscript with your valuable comments and queries. We have made our best efforts to reflect your feedback.*
>
> *(1) The abstract is confusing and partly misleading and should be revised to more clearly and accurately summarize the study.*

The abstract was revised to be clearer and more accurate (lines 20-34).

> *(2) The introduction should be revised to more accurately describe the sperm life cycle. Spermatogenesis, per definition, for example, exclusively takes place in the testis, sperm do not gain fertilization competence in the epididymis, sperm isolated from the epididymis cannot fertilize an oocyte unless in vitro capacitated, etc. In the last paragraph the connection between changes in fructose and citrate concentration, sperm metabolism and testicular-derived testosterone and AR remain unclear.*

The introduction was revised to be clearer and more accurate (lines 44-45).

Citric acid and fructose are chemical components that are the subject of biochemical testing and are commonly used as semen testing items for humans and livestock. This is because the secretory function of the prostate and seminal vesicles is dependent on androgens. The measurement of citric acid and fructose concentrations in semen is routinely used to indicate testicular androgen production function (ISBN: 978-1-4471-1300-3, 978 92 4 0030787).

> *(3) Throughout the manuscript the concept of (in vitro) capacitation is missing. Mixing sperm with seminal plasma is not the only way to achieve sperm that can fertilize the oocyte. Since media containing bicarbonate and albumin is the standard procedure in the field to capacitate epididymal mouse sperm rein vitro, the manuscript would gain value from a comparison between the effect of seminal plasma and in vitro capacitating media. Interesting readouts in addition to motility would i.e. be sAC activation, PKA-substrate phosphorylation, and acrosomal exocytosis.*

Thank you for pointing out this important point. As the reviewer points out, fertilization can be achieved in artificial insemination and *in vitro* fertilization using epididymal sperm which have not been exposed to seminal plasma. This has historically led to an underestimation of the role of accessory reproductive glands, such as the prostate and seminal vesicles. However, it has been reported that the removal of seminal vesicles in rodents decreases the fertilization rate after natural mating. This has been shown to be due to multiple factors affecting sperm motility rather than factors involved in plug formation (PMID: 3397934), but details of these factors and the whole picture of the role of the accessory glands were not known. This led us to become interested in the effects of sperm plasma on sperm other than fertilization and led us to begin research on the role of the accessory glands that synthesize sperm plasma.

Early in our study, we found that simply exposing sperm to seminal vesicle extracts for 1 hour before IVF dramatically reduced fertilization rates, even in HTF medium containing bicarbonate and albumin. The experiment was designed on the assumption that seminal plasma contains factors that inhibit sperm from acquiring fertilizing ability. Therefore, we conducted experiments using modified HTF without albumin to avoid unintended motility patterns.

However, we also respect the reviewer's opinion, and we have added our preliminary data related to IVF (New supplementary Fig.5).

> *(4) In the introduction and throughout the manuscript it is unclear what the authors mean by "linear motility". An increase in VSL doesn't mean that the sperm swim in a more linear or straight way, or even that the sperm are 'straightened', it means that they swim faster from point A to point B. Do the authors mean progressive or hyperactivated motility? Please clarify.*
>
> *For all conditions tested the authors should follow the standard in the field and include the % of motile, progressively motile, and hyperactivated sperm.*

Thank you for pointing this out. We appreciate your feedback regarding the terminology. In our manuscript, "linear motility" refers to the degree to which sperm move in a straight line. We have clarified this by explaining that VSL (Straight-Line Velocity) and LIN (Linearity) are used to quantify and describe linear motility in sperm analysis: Higher VSL values indicate more direct, linear movement. A higher LIN value indicates a straighter path, thus representing greater linear motility. These terms have been standardized, and explanations have been added to the main text (lines 111-113).

In response to your suggestion, we have included the percentage of motility and progressive motility for all conditions tested. However, since the experiment was performed using modified HTF without albumin, we have decided not to report the percentage of hyperactivation to avoid confusion.

> *(5) Did the authors confirm that the injection of flutamide decreases androgen levels? That control needs to be included in the experiment to validate the conclusion.*

Injection of flutamide did not reduce androgen levels (see reviewer #1, comment 6). This is because flutamide's mechanism of action is based on antagonizing androgen and inhibiting its binding to the androgen receptor (New Fig.2A).

> *(6) The role of mitochondrial activity in sperm progressive motility is still under investigation. PMID: 37440924 i.e. showed that inhibition of the ETC does not affect progressive but hyperactivated motility. The authors should either include additional experiments to confirm the correlation between mitochondrial activity and sperm progressive motility or tone down that conclusion.*

We have previously shown that treatment with D-chloramphenicol, an inhibitor of mitochondrial translation, significantly reduced sperm mitochondrial membrane potential, ATP levels, and linear motility (PMID: 31212063). Also, in the previous manuscript, we did not address progressive motility or hyperactivated motility in our analysis. We have chosen to discuss the effect of mitochondrial activity on linear motility rather than on progressive motility and hyperactivation of sperm.

> *Was mitochondrial activity also altered in epididymal sperm incubated with and without seminal plasma or in aged mice?*

The mitochondrial membrane potential of epididymal sperm cultured with seminal vesicle extract (SV) was higher than that of epididymal sperm cultured without seminal vesicle extract (without SV: 67.3 ± 0.8%, with SV: 83.4 ± 1.8%). On the other hand, the mitochondrial membrane potential of epididymal sperm cultured with seminal vesicle extract recovered from aged mice was decreased (SV from aged: 60.3 ± 2.7%). It should be noted that the epididymal spermatozoa used in these experiments were healthy individuals, different from those from which seminal vesicle extracts were collected. (See also the response to reviewer 1's comment #5.)

> *(7) The quality of the provided images showing AR, Ki67, and TUNEL staining should be improved or additional images should be included. Especially the AR staining is hard to detect in the provided images. The authors should also include a co-staining between AR and vesicle epithelial cells. That epithelial cells are multilayered does not come across in the pictures provided.*

We apologize for any inconvenience caused. The image has been replaced with one of higher resolution. The multilayered structure of the epithelial cells will also be seen.

> *For the 12-month-old mice, an age-matched control should be included to support the authors' conclusion.*

To clarify the seminal vesicle changes associated with aging, we included images of 3-month-old mice as controls (New Supplementary Fig.2D).

> *Overall, the rationale for the experiment does not become clear. How are the amount of seminal vesicle epithelial cells, testosterone, and AR expression connected to seminal plasma secretions? Why is it a disadvantage to have proliferating seminal vesicle epithelial cells? How is proliferation connected to the proposed switch in metabolic pathway activity?*

We have added some explanations and supporting data to the manuscript (New Fig.8D, lines 303-305, 315-319, 369-379). Cell proliferation stopped when the metabolic shift occurred, redirecting glucose toward fatty acid synthesis. Fatty acid synthesis is an important function

of the seminal vesicle, and in the presence of testosterone, fatty acid synthesis enhancement and arrest of proliferation occur simultaneously. The connection between metabolism and cell proliferation was further demonstrated when ACLY was knocked down by shRNA, which stopped fatty acid synthesis and released the proliferative arrest induced by testosterone, allowing the cells to proliferate again. However, we do not know what effects occur when cell proliferation is stopped.

> *(8) The experiments provided for glycolysis and oxphos are inconsistent and insufficient to support the authors' conclusion that testosterone shifts glycolytic and oxphos activity of seminal vesicle epithelial cells. Multiple groups (PMID 37440924, 37655160, 32823893) have shown that the increased flux through central carbon metabolism during capacitation is accompanied by an accumulation of intracellular lactate and increased secretion of lactate into the surrounding media. How do the authors explain that they see an increase in glucose uptake and ECAR but not in lactate and a decrease in pyruvate? Did the authors additionally quantify intracellular pyruvate and lactate? Since pyruvate and lactate are in constant equilibrium, it is odd that one metabolite is changing and the other one is not.*

Thank you for pointing this out. Since ECAR is often used as an alternative to lactate production but does not directly measure lactate levels, we measured changes in lactate and pyruvate concentrations in the culture medium. Under our experimental conditions, glucose appeared to be directed primarily towards anabolic processes, such as fatty acid synthesis, rather than the OXPHOS pathway, which may explain the lack of lactate production. The observed decrease in pyruvate might indicate its conversion to acetyl-CoA in the mitochondria, supporting both fatty acid synthesis and the TCA cycle. This shift would be consistent with the metabolic reprogramming toward anabolic activity.

> *What do the authors mean by "the glycolytic pathway was not enhanced despite the activation of glycolysis" Seahorse, especially using a series of pathway inhibitors, only provides an indirect measurement of glycolysis and oxphos since the instrument does not provide a distinction from which pathways the detected protons are originating. The authors should consider a more optimized experimental design, i.e. the authors could monitor ECAR and OCR in the presence of glucose over time with and without the addition of testosterone. That would be less invasive since the sperm are not starved at the beginning of the experiment and would provide a more direct read-out. Did the authors normalize cell numbers in their experiment? Alternatively, the authors could consider performing metabolomics experiments.*

I agree with the reviewer. Buzzwords such as “glycolytic capacity” simply do not make sense, so we have removed them from the phrases noted by the reviewer. Please refer to the response to some of reviewer 1's points regarding the ambiguity of the data measured by the flux analyzer. Nevertheless, the assay design of the flux analysis could be used as a good “starting point” and provide information on the glycolytic system and respiratory control. Therefore, the interpretation of the flux analysis is supported by subsequent data sets.

> *(9) The authors would strengthen their results by confirming their gene expression data by quantifying the expression of the respective proteins.*
>
> *Does testosterone treatment increase GLUT4 protein levels in isolated seminal vesicle epithelial cells? Or does it change the localization of the transporter? Are GLUT4 gene and protein levels altered in flutamide-treated cells? How do the authors explain that testosterone increases glucose uptake without changing Glut gene expression?*

We performed Western blot analysis to measure GLUT4 protein levels in seminal vesicle epithelial cells after testosterone treatment. The results showed that testosterone does not alter the expression of GLUT4 protein but simply changes its subcellular localization (New Fig.6C,D, lines 238-244).

The discussion includes the interpretation of the observation that testosterone increases glucose uptake by altering localization without altering GLUT4 gene expression, a phenomenon commonly seen in other cells, such as cardiomyocytes (lines 362-364). The revised main figure also includes a data set of changes in GLUT4 localization, including flutamide-treated data. See also Reviewer 3's main comment #1.

> *(10) Considering that the authors claim that SV secretions are crucial for sperm fertilization capacity, how do they explain that fertilization rates are still at 40 % when sperm are treated with flutamide?*

It is actually about 50% fertilized with HTF because it is fertilized without SV. Considering this baseline, we found that seminal vesicle secretions positively affect sperm *in vivo* fertilization. On the other hand, seminal plasma from flutamide-treated mice reduced the fertilization ability of healthy sperm. These are described in the text (lines 283-294).

> *(11) It would be beneficial for the reader to include a schematic summarizing the results.*

Thank you for your advice from the reader's point of view. We have visualized the summaries of this study and added them to the manuscript (New Fig.10).

> *Minor comments:*
>
> *Line 38: Male fertility, no article, please revise.*

I have changed “The male fertility” to “Male fertility” and added some references (lines 42-43).

> *Line 55: Seminal plasma or TGFb? Please clarify.*

Corrected as follows. “TGFβ, a component of seminal plasma, increases antigen-specific Treg cells in the uterus of mice and humans, which induces immune tolerance, resulting in pregnancy.” (lines 60-62)

> *Line 63: Why do the authors find it surprising that blood and seminal plasma have different compositions?*

This is because seminal plasma contains unique biochemical components that are not normally found in blood or only in small quantities. The intention was to emphasize the unique function of seminal plasma in supporting the physiological functions of sperm and to highlight its complex role by comparing it to blood. We clarified these intentions and reflected them in the revised text (lines 62-67).

> *Line 94: The headline causes confusion. Seminal plasma does not induce sperm motility, it increases progressive sperm motility.*

Corrected as follows. “The effect of androgen-dependent changes in mouse seminal vesicle secretions on the linear motility of sperm” (lines 101-102)

> ***Reviewer #3 (Recommendations For The Authors):***

Thank you for allowing us to strengthen our manuscript with your valuable comments and queries. We have made our best efforts to reflect your feedback.

> *Major:*
>
> *Figure 4 and Figure 5: The trend shows that GLUT3 is up-regulated and GLUT4 is downregulated although both of them are not statistically significant. However, GLUT4 is picked for all the following experiments based on protein localization. Providing other evidence/discussion why not to further consider other GLUTs will help to justify. Also, this reviewer suggests including GLUT4 localization data in the main figure as it is important data for the logical flow to link the following figures.*

We focused on GLUT4 because it was known that testosterone increases glucose uptake by changing the localization of GLUT4 without changing its expression (lines 230-231). In the revised manuscript, the increasing trend in *Glut3* gene expression was also mentioned in the discussion, in addition to GLUT4 (lines 360-362). In any case, the results showed that testosterone increased glucose uptake by regulating the function of glucose transporters.

Immunostaining of GLUT1~4 was performed to compare seminal vesicles from flutamide-treated mice with controls, and localization changes were observed only in GLUT4. Therefore, we hypothesized that GLUT4 is regulated by testosterone and performed the experiment. Fortunately, we were able to obtain a GLUT4-specific inhibitor, which dramatically inhibited the testosterone-dependent glucose uptake and subsequent lipid synthesis in seminal epithelial cells, leading us to believe that GLUT4 is a major glucose transporter.

> *Increasing sperm linearity by oleic acid is observed and interpreted as enhanced sperm fertilizing potential. It is not clear why and how sperm linearity can be a determinant factor for enhancing sperm fertility in vivo. Providing an explanation of the effect of oleic acid on another key motility parameter more proven to be directly correlated with fertility (i.e., hyperactivation), and more direct evidence of oleic acid on enhancing sperm linearity indeed increasing sperm fertilization using IVF, is strongly recommended to support the author's main conclusion.*

Thank you for pointing this out. It is known that proteins derived from the seminal vesicles inhibit the hyperactivation of sperm and the acrosome reaction. Therefore, we conducted an experiment to add oleic acid, focusing on fatty acid synthesis caused by the metabolic shift of the seminal vesicles, which had not been known until now.

Sperm were pretreated with an oleic acid-containing medium before IVF and oleic acid enhanced sperm linearity. When the sperm number was sufficient, there was no change in the cleavage rate after *in vitro* fertilization, but when the sperm count was reduced to one-tenth of the normal, the cleavage rate increased compared to the control (lines 274-282). In other words, the physiological role of oleic acid is to increase the probability of fertilization by keeping the sperm motility pattern linear or progressive. This increases the likelihood of the sperm passing through the female reproductive tract and environments that are unfavorable to sperm survival. Our research has uncovered significant insights into the role of seminal vesicle fluid and oleic acid in sperm fertilization. Due to the strong effect of the decapacitation factor, we found that seminal vesicle fluid reduces the fertilization rate in IVF. However, it does not interfere with the fertilization rate in *in vivo* during artificial insemination. This emphasizes the importance of oleic acid, along with other protein components of seminal plasma, in ensuring the *in vivo* fertilization ability of sperm.

> *Minor:*
>
> *Please correct a typo in Line 173: sifts to shifts*

All typographical errors have been corrected.

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