Title page

Title: S-adenosylmethionine and S-adenosyl-L-homocysteine metabolism is involved in the sperm motility and in vitro fertility rate in mouse

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Abbreviations

SAM: S-adenosylmethionine

SAH: S-adenosyl-L-homocysteine

SAHH: S-adenosylhomocysteine hydrolase

ADOX: Adenosine dialdehyde

VSL: Straight-line velocity

VCL: Curvilinear velocity

ALH: Amplitude lateral head displacement

GSH: glutathione

IVF: In vitro fertilization

Abstract

Increased fragmentation of sperm DNA has been implicated in male infertility. Folate deficiency results in impaired methionine synthesis, depletion of S-adenosylmethionine (SAM) levels, an increase in S-adenosyl-l-homocysteine (SAH) levels, and increased DNA fragmentation. Disruption of the dynamic balance between SAM and SAH may also contribute, although the details of this process are not yet fully understood. We investigated the localization of SAM, SAH, and S-adenosylhomocysteine hydrolase (SAHH), and whether SAM/SAH metabolism contributes to sperm motility and fertilization rate. SAM, SAH, and SAHH levels were assessed in the acrosome, midpiece, and tail of spermatozoa. Chemical inhibition of SAM/SAH metabolism and extracellular SAH significantly decreased the straight-line velocity (VSL), curvilinear velocity (VCL), and amplitude lateral head displacement (ALH) of sperm cells, which were thus unable to swim forward and perform oscillatory movements in place. This significantly reduced the fertilization rate. Therefore, the disruption of the SAM/SAH balance may contribute to male infertility.

Keywords: SAM/SAH metabolism, sperm motility, fertilization rate

Highlights:

- SAM and SAH accumulate in the acrosome and midpiece of sperm cells.
- Chemical inhibition of SAM/SAH metabolism affected sperm motility patterns and impaired the fertilization rate.
- Extracellular SAM and SAH affect sperm motility patterns.

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1. Introduction

Clinical practice has found that more than 90 % of male infertility cases are due to low sperm count, poor sperm quality, or both [1]. The number and motility of sperm are important factors in their fertilization ability [2, 3]. Reportedly, the concentration and motility of sperm in semen are affected by oxidative stress and reactive oxygen species (ROS), and that oxidative stress is a factor that causes DNA damage [4, 5, 6]. Reactive oxygen species (ROS) are byproducts of ATP production via the mitochondrial electron transport chain [7]. Excessive intracellular ROS target the electron transfer chain proteins and mitochondrial transcription factors, including specific RNA polymerase and mitochondrial transcription factor-A, and in turn, disrupt ATP generation and gene expression systems in the mitochondria [8]. Supplementation with exogenous mitochondria-targeted antioxidants reduces ROS levels in sperm and improves sperm linear motility by protecting gene transcription and ATP generation systems [8]. Furthermore, supplementation with antioxidants substantially reduces DNA fragmentation in sperm [9]. Thus, the reduction of oxidative stress caused by excess ROS is an essential strategy for maintaining sperm quality and promoting fertilization.

To reduce oxidative stress, sperm biosynthesize the endogenous anti-oxidant factor glutathione using glycine, cysteine, and glutamate [10, 11]. Furthermore, glutathione is synthesized from folate and homocysteine [12]. Folate deficiency impairs methionine synthesis, depletes S-

adenosylmethionine (SAM), and increases S-adenosyl-L-homocysteine (SAH) and DNA fragmentation [13]. S-adenosylmethionine (SAM), a major methyl donor in cellular methylation reactions, plays a central role in establishing and maintaining DNA methylation patterns and affects gene expression systems [14]. Li et al. (2023) systematically evaluated the effects of folic acid on sperm motility and *in vitro* fertilization-intracytoplasmic sperm injection (IVF-ICSI) outcomes of infertile males involving 2168 patients with an average age raging from 32.5 to 36.5 years. As a result, 5−15 mg/day folic acid notably improved sperm motility and IVF-ICSI outcomes [15]. However, a recent study has suggested that excess folic acid supplementation can cause DNA damage induced by oxidative stress [16]. Therefore, the dynamic balance between SAM and its metabolic by-product, SAH, is essential for rate-limiting proper intracellular methylation reactions and is related to oxidative stress, and its disruption may contribute to reduced sperm motility and male infertility, although the details of this process are not fully understood.

In this study, we investigated the localization of SAM and SAH in mouse sperm, and analyzed their roles in sperm motility and fertilization rates. We also analyzed the relationship between S-adenosylhomocysteine hydrolase (SAHH), which is the rate-limiting enzyme in the reaction that regulates intracellular levels of SAM and SAH in mouse sperm.

2. Materials and Methods

2.1. Materials

Equine and human chorionic gonadotropins (eCG and hCG) were purchased from Asuka Seiyaku (Tokyo, Japan). Bovine Serum Albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). KOSM containing amino acids was purchased from Merck Millipore (Darmstadt, Germany). Adenosine dialdehyde (ADOX, a SAHH inhibitor) was purchased from Selleck Biotech (Yokohama, Japan). S-adenosylhomocysteine (SAH) and SAM were purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). Routine chemicals and reagents were obtained from Nacalai Chemical Co. (Osaka, Japan), or Sigma Chemical Co..

2.2. Animals

Male (8−12 weeks old) C57BL/6J mice and immature female (3-week-old) C57BL/6J mice were obtained from Charles River Laboratories Japan (Yokohama, Japan) or Japan SLC Inc. (Shizuoka, Japan), housed under a 12-h light/12-h dark schedule in the Experimental Animal Center at Okayama University, and provided with food and water ad libitum. The animal study was approved by the Okayama University Animal Committee (OKU-2023861) and the mice were maintained in accordance with the Okayama University Guidelines for the Care and Use of Laboratory Animals.

2.3. Collection of sperm

Cauda of epididymis was cut by 27 G needle (Terumo corporation) under the stereo microscope to release sperm in 500 μL of HTF medium, and the total number of sperms were counted. These sperm were also used as samples for immunofluorescence staining and *in vitro* fertilization.

2.4. Immunofluorescence analyses

Immunofluorescence analysis of sperm cells was performed as previously described [17], with minor modifications. Briefly, sperm cells were mounted on glass slides and fixed with 4 % paraformaldehyde (PFA) (Wako, Osaka, Japan) for 30 min at room temperature (~25 °C), followed by blocking and primary antibody reactions using the STAIN*perfect* immunostaining kit (ImmuSmol, Bordeaux, France). The stained cells were mounted with DAPI (SouthernBiotech, Birmingham, MA, USA). Imaging was performed using a confocal laser-scanning microscope LSM780 (Carl Zeiss, Oberkochen, Germany). The following antibodies were used in this study: [Antigen/Source/Identifier/Dilution]: [S-adenosyl-homocysteine/Bio-RAD/0100-0026/1:100]; [S-adenosylmethionine/BIOMATIK/CAU22257/1:100]; [Alpha Tubulin/Proteintech/66031-1-Ig/1:500]; [AHCY/Proteintech/10757-2-AP/1:100]; [Normal Mouse IgG/Sigma-Aldrich/NI03/1:100]; [Normal Rabbit IgG/Sigma-Aldrich/NI01/1:100]; [Donkey anti-mouse IgG

Alexa Fluor Plus 488/Thermo Fisher Scientific/A32766/1:500]; [Donkey anti-rabbit IgG Alexa Fluor Plus 594/Thermo Fisher Scientific/A32754/1:500].

2.5. Assay for sperm motility using CASA system

To study sperm motility, a CASA system designated as the Sperm Motility Analysis System (SMAS, Ditect Co., Tokyo, Japan) was used. Sperm were collected and incubated in HTF medium containing 0 or 10 μM of ADOX, SAH, or SAM for 15, 30, 60, 90, and 120 min at 37 °C under 5 % CO_2 in air. Five microliters of sperm samples were placed in a pre-warmed counting chamber, and sperm tracks (1 s, 60 frames) were captured. At least three fields were randomly selected to assess sperm motility for each sample analyzed using SMAS. Track and kinematic parameters were also recorded.

2.6. Collection of ovulated oocytes and *in vitro* fertilization

Immature female mice (3-week-old) were injected intraperitoneally with 4 IU of eCG. After 48 h, the mice were treated with 5 IU hCG. At 16 h post-hCG, the ovulated oocytes with cumulus cells were collected from oviducts by 27 G needle (Terumo corporation) under the stereo microscope and placed in 50 μL of HTF medium (fertilization medium) for each treatment. Sperm were collected and cultured with 0 or 10 μM of ADOX for 60 min at 37 °C under 5 % CO_2 in air. After

60 min of incubation, sperm were washed with HTF medium and transferred to the fertilization medium at final numbers of 100 sperms/μL. Six hours after fertilization, the oocytes were examined for the number of pronuclei and cultured in development medium (KSOM medium with amino acids, Merck Millipore) for 3 d. The number of embryos that developed into the two-cell or blastocyst stage was also determined.

2.7. Statistics

The results obtained from ADOX-treated and -untreated data from three replicates were compared using Student's *t*-test (BellCurve for Excel, Social Survey Research Information Co., Ltd., Tokyo, Japan). For comparisons of multi-treatment groups, one-way ANOVA was used. The Tukey–Kramer test was used for post hoc analyses. Percentage values were transformed into normally distributed numbers by angle transformation and then analyzed using one-way ANOVA and the Tukey–Kramer post hoc test. For the sperm motility assay using the CASA system, we analyzed three biological replicates for each group. All the values are presented as the mean ± standard error (SE). Treatments were considered statistically significant at $p < 0.05$.

3. Results

3.1. S-adenosylmethionine (SAM) and S-adenosyl-L-homocysteine (SAH) accumulate in acrosome as well as midpiece, tail, and end piece

To determine the localization of SAM/SAH in sperm cells, immunostaining was performed on freshly isolated murine sperm using antibodies against SAM and SAH. Because SAM and SAH are low-molecular-weight compounds, staining was performed using the STAIN*perfect* Immunostaining Kit (immusmol), which is known to be useful in staining low-molecular-weight compounds, instead of the usual staining procedure. The SAM staining signals were observed in the acrosome, centriole, midpiece, tail, and endpiece, but not in the cell nucleus (Fig. 1A). The SAH staining signals were similar to those of SAM (Fig. 1B). Because these signals were not observed upon staining with nonspecific antibodies (Fig. 1C), they were considered to be staining signals specific for SAM and SAH. These data indicate that the SAM and SAH are widely localized in mouse spermatocytes at various sites, including the acrosome.

The enzymatic reactions that produce and degrade the SAM and SAH are shown in Fig. 1D. S-adenosylhomocysteine hydrolase (SAHH) is the only SAH-hydrolyzing enzyme in mammalian cells and is responsible for the reaction shown in Fig. 1D [18]. Interestingly, SAHH, similar to SAM and SAH, was localized to the acrosome, centriole, and tail (Fig. 1E). These data suggest that the conversion of SAM to SAH by SAHH occurs in mouse spermatocytes at various sites,

including the acrosomes.

3.2. Chemical inhibition of SAM/SAH metabolism affected the sperm motility patterns

To investigate whether chemical inhibition of SAM/SAH metabolism affects sperm motility, sperms were treated with ADOX, an inhibitor of SAHH, and dynamic changes in sperm parameters after 2 h (120 min) of incubation were detected using the SMAS. When sperm were treated with 10 μM of ADOX, straight-line velocity (VSL) were significantly decreased at the 15-min point of incubation compared to just after incubation (Fig. 2A(a)). At the 60-min point, the percentage of sperm with VSL higher than 60 μm/s were dramatically decreased from 27.9 % to 5.3 %. However, the percentage of sperm exhibiting a VSL of 1 μm/s to 10 μm/s was dramatically increased from 19.1 % to 44.1 % (Fig. 2A(b)). At the 60-min point, the curvilinear velocity (VCL) and amplitude lateral head displacement (ALH) also showed a significantly lower rate (Fig. 2B, 2C). In contrast, the beat cross frequency (BCF) at the 30- and 60-min point was significantly higher in ADOX-treated sperms (Fig. 2D). The ADOX-treated sperms were unable to swim forward and performed oscillatory movements in place when sperm motility tracks were observed at 90-min point (Fig. 2E, S1A-B). SMAS analytic data showed that the total motility was significantly decreased by the treatment of 10 μM of ADOX for 90- or 120-min (Fig. S1E).

3.3. Extracellular SAM and SAH affected the sperm motility pattern

To investigate whether extracellular SAM and SAH affect sperm motility patterns, sperms were treated with SAM or SAH, and dynamic changes in sperm parameters after 120-min of incubation were detected by SMAS. When sperms were treated with 10 μM of SAH, VSL were significantly decreased at the 60-min point of incubation compared to just after incubation (Fig. 3A(a)). At the 60-min point, the percentage of sperms with VSL higher than 60 μm/s decreased from 30.7 % to 15.6 %. In contrast, the percentage of sperm exhibiting a VSL of 1 μm/s to 10 μm/s increased from 25.3 % to 41.6 % (Fig. 3A(b)). At 60-min point, no significant differences were observed in either VCL or ALH; however, these parameters showed a significantly lower rate at 90- and 120-min point in SAH-treated sperms (Fig. 3B, 3C). Interestingly, sperms treated with SAM exhibited a VCL of more than 300 μm/s, which was not detected in the sperms of the control group (Fig. 3B(b)). In contrast, the BCF at 15-, 60-, and 90-min point was significantly higher in SAH-treated sperms than in SAM-treated sperms (Fig. 3D). Sperms treated with 10 μM of SAH were unable to swim forward and performed oscillatory movements in place when sperm motility tracks were observed at 90-min. Sperms treated with SAM were able to swim forward like the sperms in the control group (Fig. 3E, S1C-D). The SMAS data showed that the total motility was not significantly changed by treatment with SAM or SAH (Fig. S1F).

3.4. Chemical inhibition of SAM/SAH metabolism impaired the fertilization rate

Notably, hyperactivated sperm motility is a useful capacitation marker that can be identified before the acrosome reaction [19]. Capacitation is also associated with changes in sperm motility patterns, particularly an increase in flagellar amplitude. A higher ALH is an index measure of the flagellum amplitude because the head of the sperm moves from side to side due to the principal and reverse bends [20]. In addition, previous studies have shown that hyperactivated sperm motility induced after incubation in HTF medium, which contains capacitation activators, is associated with higher VCL [20]. As VCL and ALH were significantly decreased in sperms treated with ADOX or extracellular SAH, the effect of chemical inhibition of SAM/SAH metabolism on the fertilization rate was investigated. Sperm treated with 10 μM of ADOX for 60-min significantly reduced the percentage of two-cell stage embryos from 71.7 % ± 4.0 % to 25.6 % ± 5.5 % (Fig. 4A, B), and the percentage of blastocyst stage embryos also significantly decreased from 45.7 % ± 2.6 % to 7.4 % ± 4.3 % (Fig. 4A, C).

4. Discussion

S-adenosylmethionine (SAM) is biosynthesized from methionine and ATP and is used as a methyl donor in the methylation reactions of various biomolecules such as proteins, DNA, and lipids [21]. After SAM donates a methyl group, it becomes S-adenosylhomocysteine (SAH), however, since SAH also acts as a competitive inhibitor of the methylation reaction by SAM, it needs to be rapidly converted to homocysteine, precursor of cysteine or methionine, by SAHH [22, 23]. Reportedly, the concentration of SAM in the semen of healthy fertile males is significantly higher than that in males who are infertile because of oligospermia [24, 25]. Therefore, the dynamic balance and localization between SAM and its metabolic by-product, SAH, is essential for proper rate-limiting intracellular methylation reactions and is related to oxidative stress; its disruption may contribute to reduced sperm motility and male infertility. In the present study, SAM and SAH were strongly localized in the acrosome or midpiece of sperms, suggesting that their accumulated amount may contribute to sperm motility. Furthermore, SAHH, similar to SAM and SAH, was localized to the acrosome and midpiece of sperms, suggesting that conversion to homocysteine by SAHH in these locations may also contribute to sperm motility.

When we investigated the effects on sperm motility treated with ADOX, an inhibitor of SAHH, VSL, VCL, and ALH were significantly decreased. Sperms treated with ADOX were unable to swim forward or perform oscillatory movements in place. Homocysteine, converted from SAH

by SAHH, is converted to glutathione (GSH) by cystathionine β-synthase (CBS) and γ-glutamylcysteine ligase (GCLC) [26]. Lin et al. (2023) confirmed that 993 semen samples with low acrosin activity had reduced sperm motility and low *in vitro* fertilization rates compared with 1332 normal semen samples. They revealed that sperms with low acrosin activity were deficient in GSH biosynthesis because of the downregulation of GCLC. Their results suggested that in sperms with low acrosin activity, GSH deficiency and excessive oxidative stress induced premature acrosome release, which significantly reduced acrosin activity and sperm motility [27]. In the present study, because sperms treated with ADOX had a significantly lower rate of development into two-cell stage embryos and blastocyst stage embryos after fertilization, we hypothesized that ADOX treatment locally increased the accumulation of SAH or inhibited GSH synthesis in sperms, thereby decreasing their motility and fertilization ability.

When SAM or SAH were added to the culture medium and their effects on sperm motility was examined, VSL, VCL, and ALH levels were significantly decreased. Finally, sperms treated with SAH were unable to swim forward and performed oscillatory movements in place, similar to ADOX-treated sperms. Since SAM-treated sperms were able to swim forward like sperms in the control group, higher levels of SAH induced abnormal sperm motility. The inhibition of SAHH expression induces DNA damage and cytotoxicity due to impaired methionine metabolism, glutathione synthesis, and SAH accumulation [13, 28]. Additionally, males with higher level of

SAH in semen is associated with the abnormal sperm motility and function, resulting in infertility [29, 30]. This is caused by a genetic polymorphism in MTHFR, which is involved in methionine metabolism [29, 30]. In our study, the localization of SAM, SAH, and SAHH was clarified, indicating that the increase in SAH concentration was caused by abnormal acrosomal or mitochondrial function in sperms, which affected sperm motility. Furthermore, in our study, sperms treated with SAM exhibited a VCL of more than 300 μm/s, which was not detected in the sperms of control group. Significantly higher SAM concentrations in the semen of healthy fertile males than in the semen of infertile males because oligozoospermia may promote VCL by enhancing the function of the acrosome or midpiece in the sperms.

In conclusion, the present study demonstrated that SAM and SAH accumulated in the acrosome and midpiece of sperms and SAM/SAH metabolism by SAHH; extracellular SAM or SAH affected sperm motility and fertilization potential. The SAM/SAH metabolism may affect the function of acrosome or midpiece in sperms, which may explain why SAM or SAH concentrations in semen affect sperm motility and fertility. The development of therapies targeting these low-molecular-weight compounds may improve male infertility.

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CRediT authorship contribution statement

Tomoko Kawai: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing-Original Draft, Visualization, Funding acquisition. **Atsushi Fujimura**: Conceptualization, Validation, Investigation, Data curation, Writing-Original Draft, Writing-Review & Editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

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Figure Legends

Fig. 1. Intracellular distribution of S-adenosylmethionine (SAM), S-adenosyl-L-homocysteine (SAH), and S-adenosylhomocysteine hydrolase (SAHH) in murine sperms.

(A). Representative immunostaining images with anti-α-tubulin (green) and anti-SAM (red) antibodies in murine sperms. The nuclei were stained with DAPI (blue). Scale bar, 20 μm.

(B). Representative immunostaining images with anti-α-tubulin (green) and anti-SAH (red) antibodies in murine sperms. The nuclei were stained with DAPI (blue). Scale bar, 20 μm.

(C). Representative immunostaining images of negative controls. Mock antibodies were used instead of the specific antibodies. Scale bar, 20 μm.

(D). Diagram of methionine cycle and homocysteine metabolism.

(E). Representative immunostaining images with anti-α-tubulin (green) and anti-SAHH (red) antibodies in murine sperms. The nuclei were stained with DAPI (blue). Scale bar, 20 μm.

Fig. 2. Effect of chemical inhibition of S-Adenosylhomocysteine hydrolase (SAHH) by ADOX on sperm motility.

(A-E): Dynamic changes in motility parameters of sperm treated with 0 or 10 μM of ADOX for 2 h (120 min) at 37 ℃. (A). VSL, Straight-line velocity. (B). VCL, Curvilinear velocity. (C). ALH,

Amplitude of lateral head displacement. (D). BCF, Beat cross frequency. (E). The total distance. A-E (a): Values are represented as the mean ± SEM (n = 3 biological replicates). A-E (b): Histograms showing the frequency distribution of each sperm motility pattern at 60 min after treatment with 0 or 10 μM of ADOX. Data from three replicates for comparison were analyzed using Student's *t*-test. *Significant differences were induced by 10 μM of ADOX compared with 0 μM of ADOX ($p < 0.05$).

Fig. 3. Effect of extracellular S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) on sperm motility.

(A-E): Dynamic changes in motility parameters of sperm treated with 10 μM of SAM or 10 μM of SAH for 2 h (120 min) at 37 ℃. (A). VSL, Linear velocity. (B). VCL, Curvilinear velocity. (C). ALH, Amplitude of lateral head displacement. (D). BCF, Beat cross frequency. (E). The total distance. A-E (a): Values are represented as the mean ± SEM (n = 3 biological replicates). A-E (b): Histograms showing the frequency distribution of each sperm motility pattern at 60 min after treatment with 10 μM of SAM or 10 μM of SAH. C: No additional chemicals (control, DMSO). The statistical analyses were also carried out using one-way ANOVA and the Tukey-Kramer post hoc test. *Significant differences were observed between the control group (DMSO) and the SAM or SAH treatment groups ($p < 0.05$).

Fig. 4. Effect of chemical inhibition of S-Adenosylhomocysteine hydrolase (SAHH) by ADOX on the fertilization rate.

(A). Photomicrographs of two-cell stage embryos and blastocyst stage embryos after *in vitro* fertilization.

(B and C). Effect of ADOX on fertilization. (B). Percentage of two-cell stage embryos. The percentage of two-cell stage embryos was defined as the number of two-cell stage embryos per approximately 30 ovulated oocytes. (C). Percentage of embryos at blastocyst stage. The percentage of blastocyst stage embryos was defined as the number of blastocyst stage embryos per approximately 30 ovulated oocytes. Significant differences in percentages were transformed to normally distributed numbers by angle transformation and then analyzed by one-way ANOVA. The Tukey-Kramer test was used for post hoc analyses. *Significant differences were induced by 10 μM of ADOX compared with 0 μM of ADOX ($p < 0.05$).

Supplemental Figure Legends

Fig. S1. SMAS derived changes in the sperm motility tracks and the percentage of sperm motility.

(A-D). SMAS-derived changes in the motility tracks of sperm treated with ADOX, SAM, or SAH

for 60 or 90 min. (A, C). 60 min. (B, D). 90 min. Control: No additional chemicals (control, DMSO).

(E-F). Percentage of sperm motility detected by SMAS in Figure 2 and Figure 3. (E). Figure 2, (F). Figure 3. * Significant differences were induced by 10 μM of ADOX compared with 0 μM of ADOX ($p < 0.05$). Values are represented as the mean ± SEM (n = 3 biological replicates).

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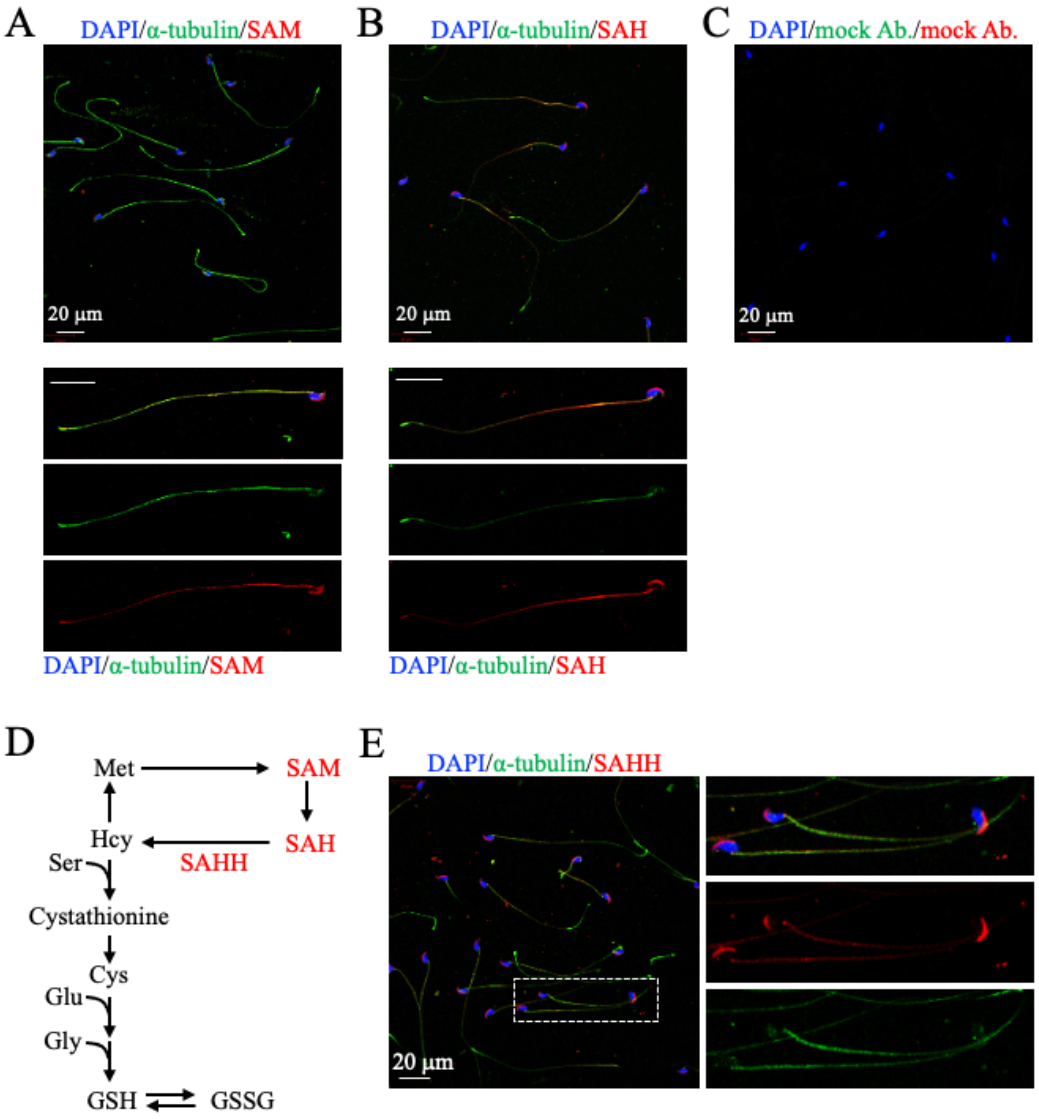

A
DAPI/α-tubulin/SAM
20 μm
B
DAPI/α-tubulin/SAH
20 μm
C
DAPI/mock Ab./mock Ab.
20 μm
DAPI/α-tubulin/SAM
DAPI/α-tubulin/SAH
D
Met
SAM
Hcy
SAH
SAHH
Ser
Cystathionine
Cys
Glu
Gly
GSH
GSSG
E
DAPI/α-tubulin/SAHH
20 μm

Figure 1

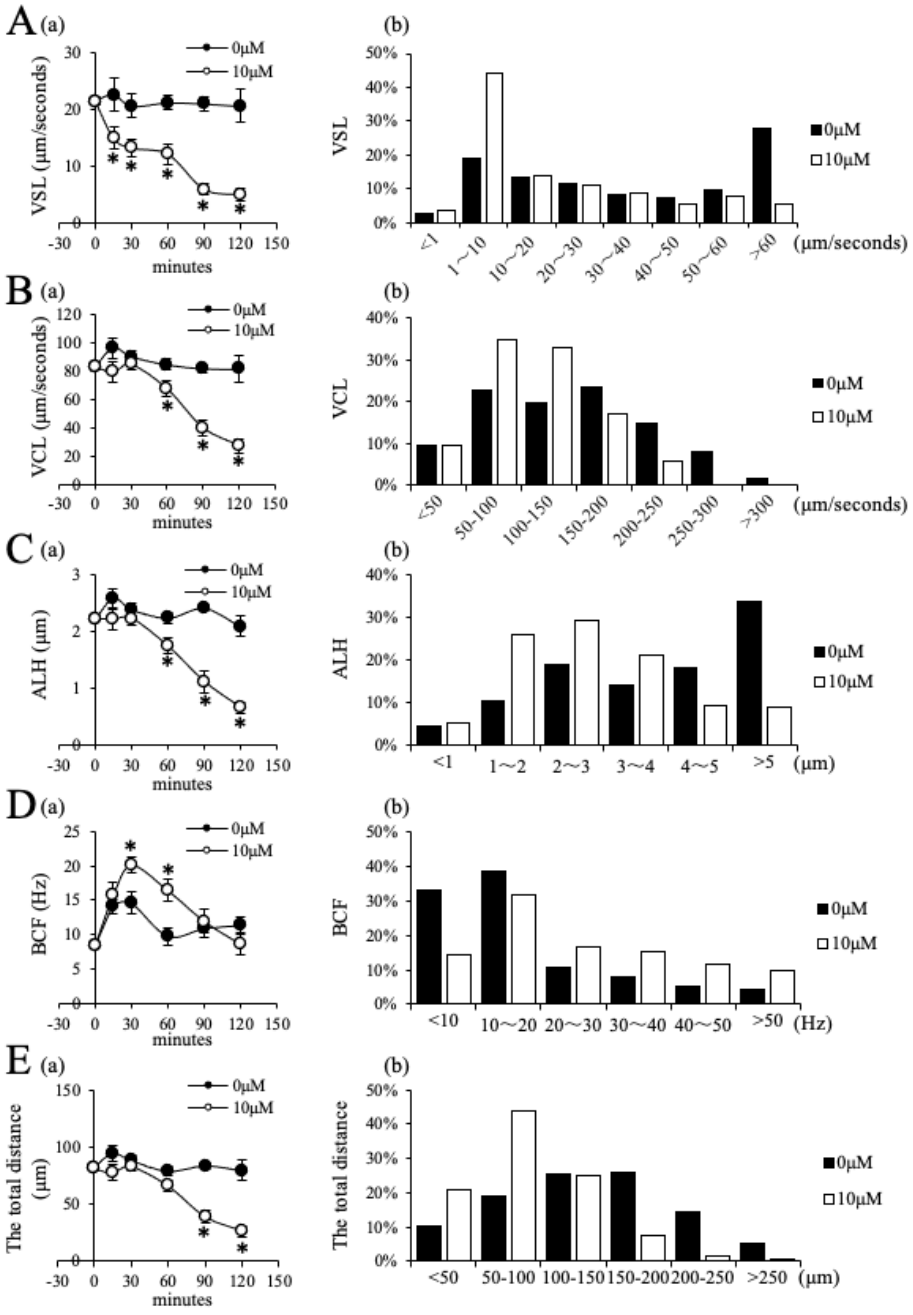

A (a)
VSL (μm/seconds)
0μM
10μM
minutes
(b)
VSL
(μm/seconds)
B (a)
VCL (μm/seconds)
VCL
C (a)
ALH (μm)
ALH
(μm)
D (a)
BCF (Hz)
BCF
(Hz)
E (a)
The total distance (μm)
The total distance

Figure 2

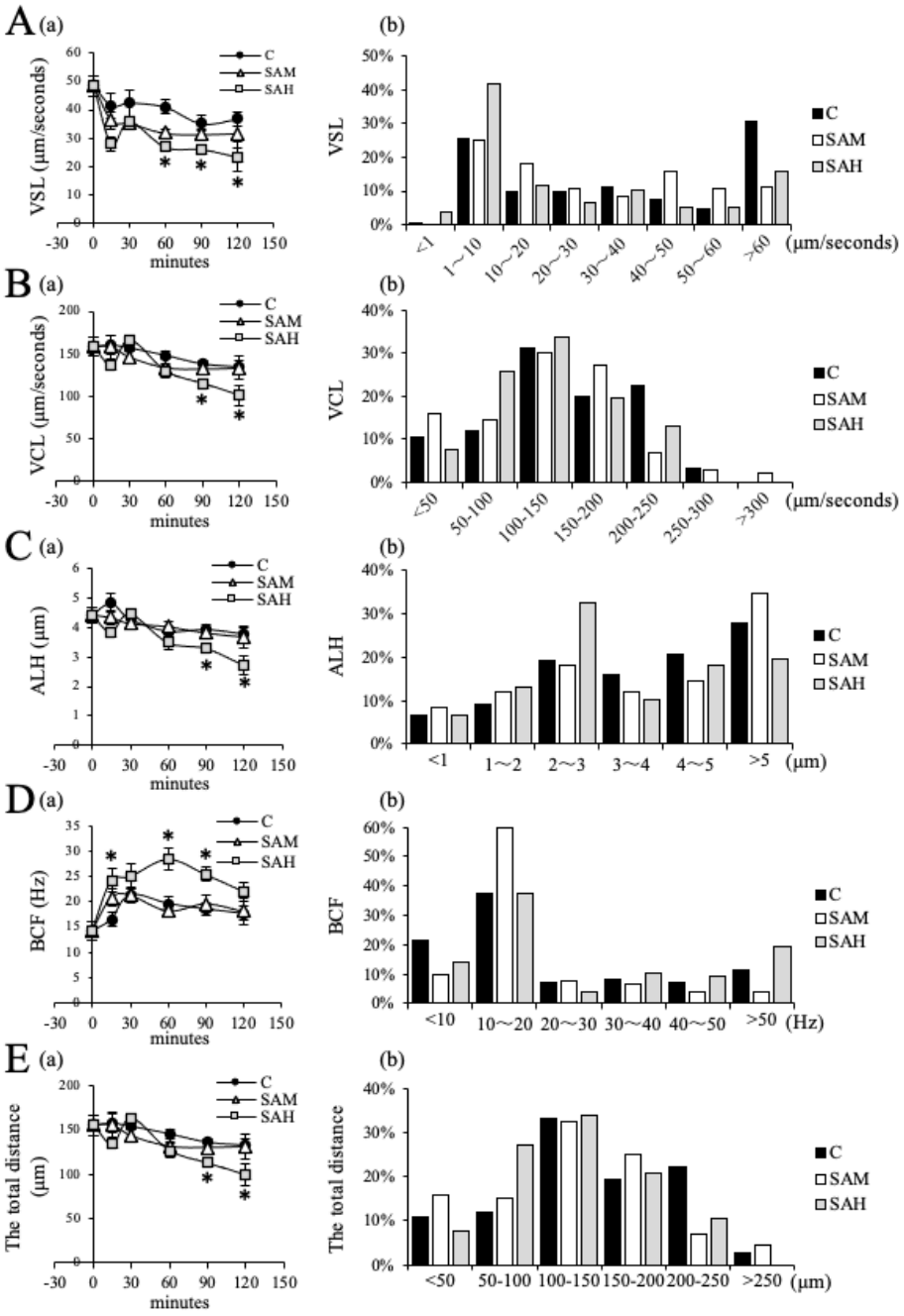

A (a)
VSL (μm/seconds)
minutes
C
SAM
SAH
(b)
VSL
<1
1～10
10～20
20～30
30～40
40～50
50～60
>60
(μm/seconds)
B (a)
VCL (μm/seconds)
(b)
VCL
<50
50-100
100-150
150-200
200-250
250-300
>300
C (a)
ALH (μm)
(b)
ALH
<1
1～2
2～3
3～4
4～5
>5
(μm)
D (a)
BCF (Hz)
(b)
BCF
<10
10～20
20～30
30～40
40～50
>50
(Hz)
E (a)
The total distance (μm)
(b)
The total distance
<50
50-100
100-150
150-200
200-250
>250
(μm)

Figure 3

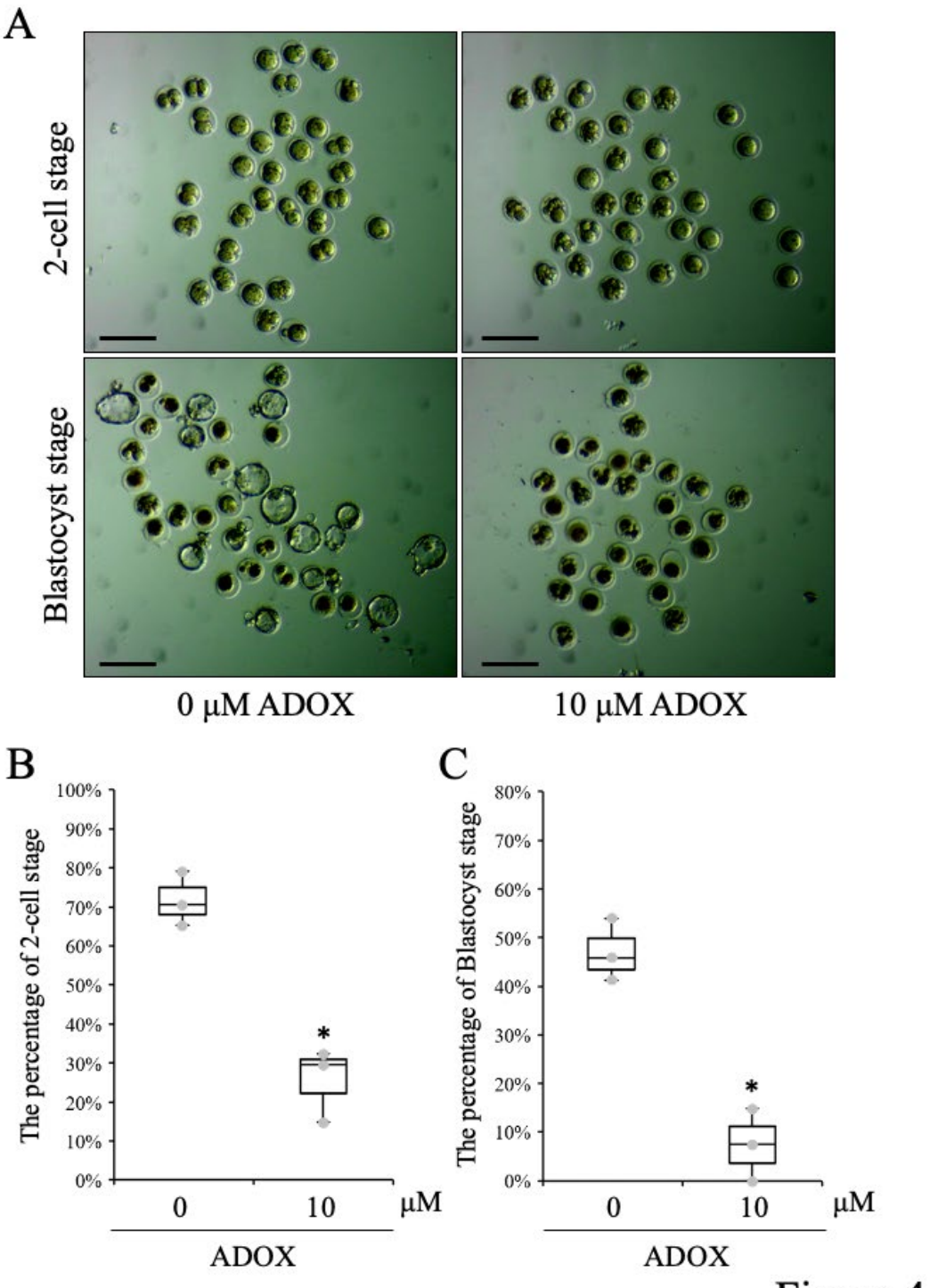
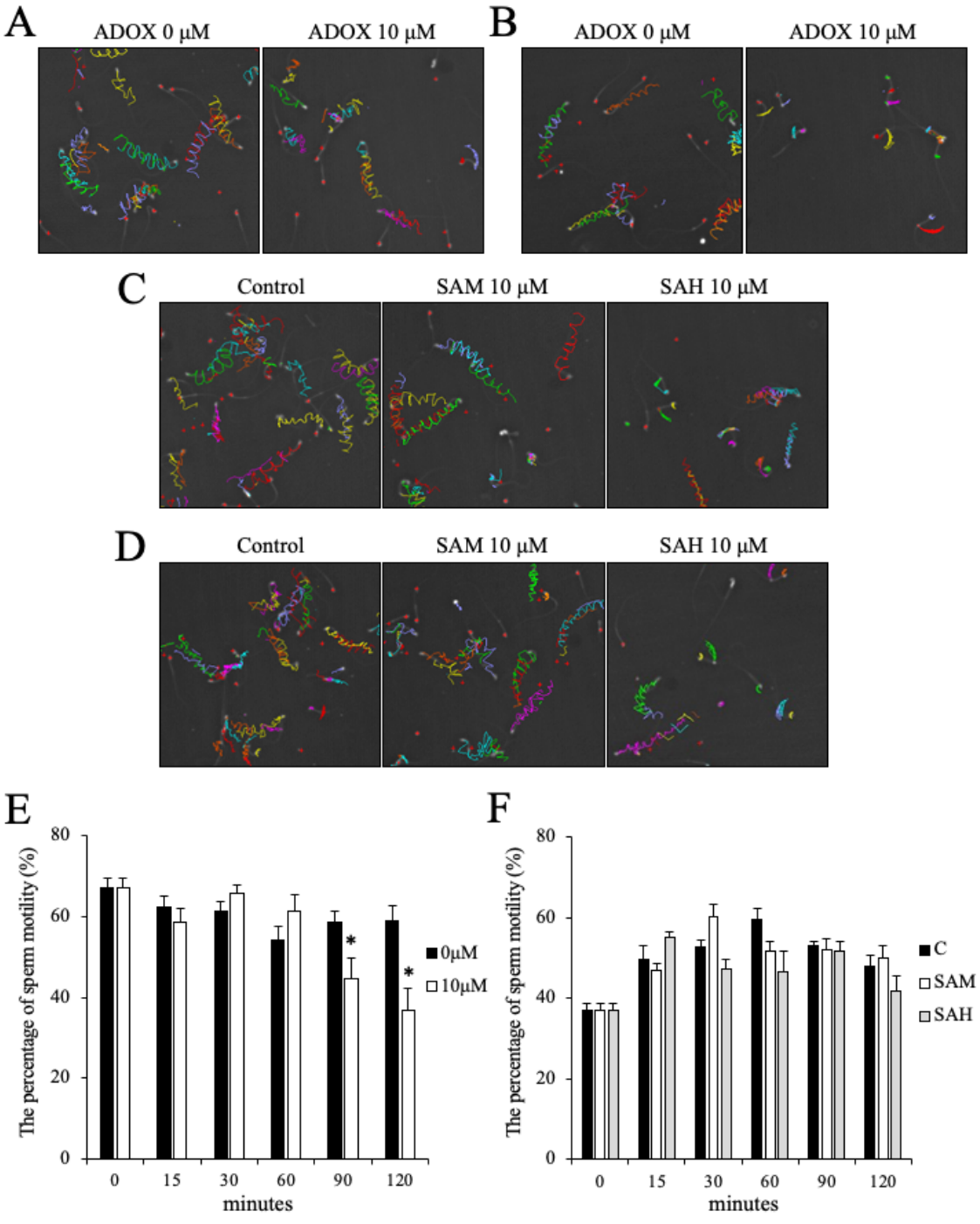

A
2-cell stage
Blastocyst stage
0 μM ADOX
10 μM ADOX
B
The percentage of 2-cell stage
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
0
10
μM
ADOX
C
The percentage of Blastocyst stage
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
0
10
μM
ADOX

Figure 4



Supplemental Figure 1