

## ORIGINAL ARTICLE

# Effects of aging and oviductal hormones on testes, epididymides, and sperm of hamster

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**Abstract**

**Purpose:** Aging is a major cause of decreased fertility. Using hamster, we examined the effects of aging on testes, epididymides, and sperm. Additionally, we examined whether progesterone ( $P_4$ ), melatonin (Mel) and 5-hydroxytryptamine (5-HT) mitigated effects of aging on sperm.

**Methods:** Young (10–16 weeks), Adult (5–7 months), Aged (13–15 months), and Old (19–22 months) hamsters were used. Weights of bodies, testes, and epididymides were measured. Testes and epididymides were studied by histological microscopy. Sera were obtained to determine testosterone concentrations. Sperm were analyzed by video-microscopy.

**Results:** By aging, body weights increased but weights of testes and epididymides decreased. Most hamsters were normozoospermia, although several old hamsters were azoospermia. In testes and epididymides, desquamation and structures resembling residual bodies (SRRBs) were observed. Although desquamation was not always related to aging, SRRBs occurred by aging. Testosterone concentrations were not changed in normozoospermic hamsters, but it was significantly reduced in old azoospermic hamster. Aging significantly reduced sperm ability to exhibit hyperactivation. Additionally, aging significantly increased the straight-line velocity (VSL).  $P_4$ , Mel, and 5-HT lessened the reduction in sperm hyperactivation and the increasing of VSL.

**Conclusion:** Aging reduces qualities of testes, epididymides, and sperm, and  $P_4$ , Mel, and 5-HT recover reduced quality of sperm.

**KEYWORDS**

aging, hyperactivation, motility, motility kinetics, sperm

## 1 | INTRODUCTION

It is generally known that the reproductive ability of young men is higher than that of old men because testosterone concentration decreases with aging.<sup>1–4</sup> In addition, it has been reported that the volume of semen also decreases with aging.<sup>3,5,6</sup> However, several

reports have shown that the volume of semen is not affected by aging.<sup>7–9</sup> Since spermatogenesis is affected by testosterone concentration,<sup>10</sup> spermatogenesis and sperm production reduce with aging.<sup>1,11</sup> In contrast, several reports<sup>12,13</sup> have shown that aging induced hypospermatogenesis but unaffected testosterone concentration. Sperm DNA damage increases with aging.<sup>3,4,11</sup> Sperm motility is also

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believed to reduce with age.<sup>3,6,7,13</sup> However, several reports have shown that sperm motility is unaffected by age.<sup>5,8,9</sup>

Capacitation is an important event for mammalian sperm in order to be fertilized in vivo and in vitro.<sup>14</sup> Capacitated sperm show two reactions: acrosome reaction and hyperactivation.<sup>14</sup> The acrosome reaction, which occurs at the sperm head, is an exocytotic event that releases proteases to digest an envelope of the oocyte.<sup>14</sup> Hyperactivation, which occurs at the sperm flagellum, is the specialized motility observed when sperm passes through an envelope of the oocyte.<sup>14-16</sup> Albumin,  $\text{Ca}^{2+}$ , and  $\text{HCO}_3^-$  are essential components for sperm to be capacitated in vitro.<sup>14-16</sup> In the absence of albumin, sperm are not hyperactivated.<sup>17,18</sup> Both  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  activate enzymes, which induced hyperactivation, for example soluble adenylate cyclase, protein kinases and protein phosphatases.<sup>14-16,19,20</sup>

It has been reported that oviductal hormones control sperm hyperactivation.<sup>21</sup> In humans, progesterone ( $\text{P}_4$ ) induces hyperactivation.<sup>22-24</sup> In hamsters,  $\text{P}_4$ , melatonin (Mel), and 5-hydroxytryptamine (5-HT) enhance hyperactivation via specific receptors.<sup>17,18,25,26</sup> In mice, 5-HT enhances hyperactivation.<sup>27</sup> In contrast, estradiol ( $\text{E}_2$ ) reduces the effects of  $\text{P}_4$  and Mel on hamster sperm hyperactivation.<sup>28-30</sup> Although  $\gamma$ -aminobutyric acid (GABA) reduces the effects of  $\text{P}_4$  and 5-HT on hamster sperm hyperactivation,<sup>31,32</sup> it induces human sperm hyperactivation.<sup>33</sup> Recently, it was suggested that the capability for hyperactivation is correlated with the success of in vitro fertilization (IVF) in humans.<sup>24</sup> Moreover, 5-HT increases the success of IVF by enhancing sperm hyperactivation in mice.<sup>27</sup>

In the present study, we examined the effects of aging on testes, epididymides, sperm motility, hyperactivation, and motility

kinetics. We also examined whether  $\text{P}_4$ , Mel, and 5-HT mitigated the effects of aging on sperm hyperactivation and motility kinetics.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

Mel,  $\text{P}_4$ , 5-HT, 5-methoxytryptamine (MT), and  $\alpha$ -methylserotonin maleate salt (MS) were purchased from Merck KGaA. Testosterone ELISA kit was purchased from COSMO BIO CO., LTD. Other reagent-grade chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation.

### 2.2 | Animals

Syrian hamsters (*Mesocricetus auratus*) were bred at the Research Center for Laboratory Animals, Comprehensive Research Facilities for Advanced Medical Science, School of Medicine, Dokkyo Medical University, Mibu, Japan. All hamsters were housed at 25°C and 12/12 light-dark period during the study. Generally, the lifespan of hamster is 2 or 3 years,<sup>34</sup> although over 1 year old hamsters are easy to die. Eight weeks old hamsters have fertility, although over 10 weeks old hamsters have stable fertility. Over 1-year-old hamsters show low fertility or subfertility. Around 1.5 years old hamsters are aged and show very low fertility or infertility. Around 2 years old hamsters are old and show infertility. All hamsters used in the present experiment were checked up fertility by mating with female

	Body weight (g)	Testis weight (g)	Epididymis weight (g)
Young hamster (n = 27)	126.77 ± 29.09	1.76 ± 0.22	0.58 ± 0.12
Adult hamster (n = 26)	129.03 ± 19.73	1.40 ± 0.24***	0.58 ± 0.13
Aged hamster (n = 27)	147.55 ± 18.90*	1.39 ± 0.40***	0.61 ± 0.10
Old hamster (n = 14)	142.76 ± 21.19*	0.89 ± 0.63#	0.43 ± 0.24 <sup>§</sup>
Normozoospermia (n = 7)	154.54 ± 16.70*	1.44 ± 0.40##	0.64 ± 0.13
Azoospermia (n = 7)	130.99 ± 19.25**	0.33 ± 0.11###	0.22 ± 0.07 <sup>§§</sup>

Note: Data are presented as mean ± standard deviation. n is numbers of animals.

\*Indicates significant difference compared with young and adult hamster ( $p < 0.05$ ).

\*\*Indicates significant difference compared with normozoospermia hamster ( $p < 0.05$ ).

\*\*\*Indicates significant difference compared with young hamster ( $p < 0.05$ ).

#Indicates significant difference compared with young, adult, aged, normozoospermia and azoospermia hamster ( $p < 0.05$ ).

##Indicates significant difference compared with young, old and azoospermia hamster ( $p < 0.05$ ).

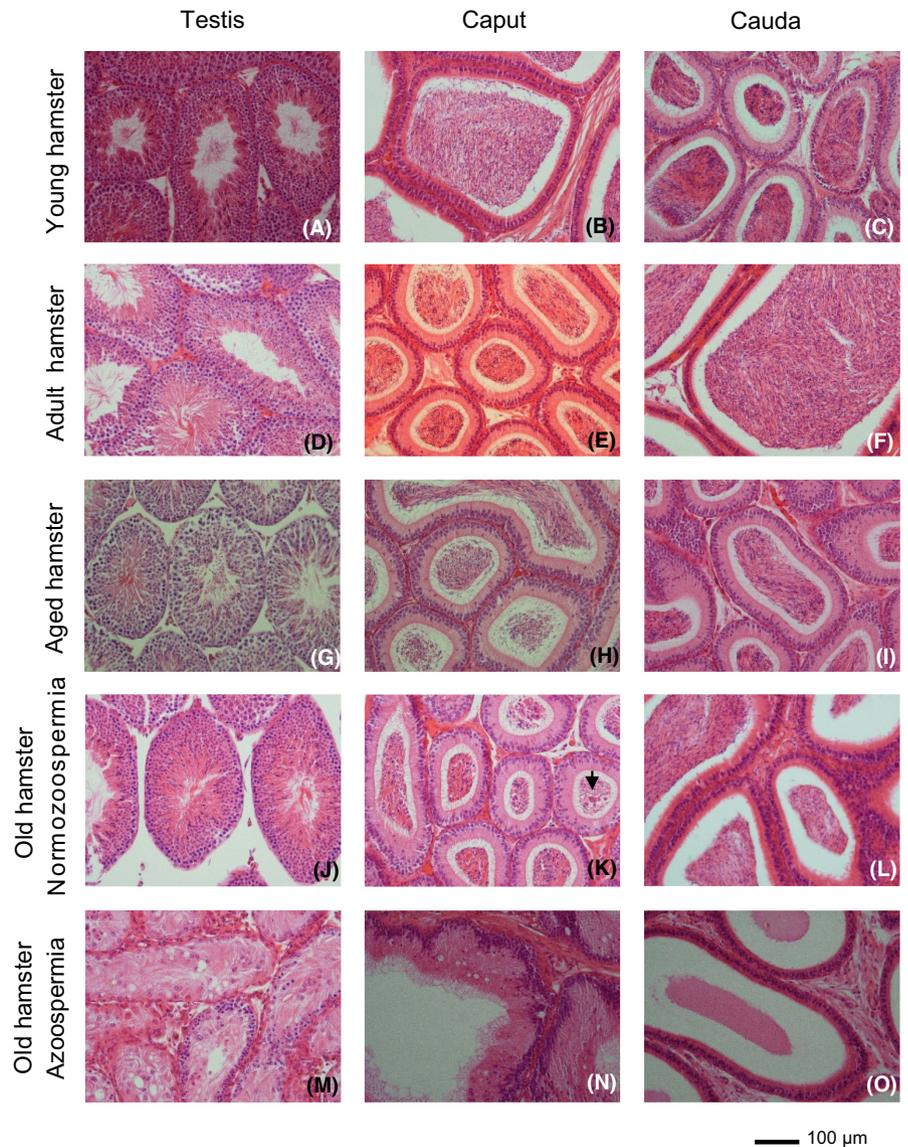
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TABLE 1 Effects of aging on body, testis, and epididymis weights

**FIGURE 1** Testicular and epididymal tissues stained with HE stain. Testis (A, D, G, J, M), carpus epididymis (B, E, H, K, N), and cauda epididymis (C, F, I, L, O) were obtained from young, adult, aged, old normozoospermic, and old azoospermic hamsters. Arrow shows structures resembling residual bodies. Bar = 100  $\mu$ m



hamsters. In the present experiment, we defined 10–16 weeks old hamsters, 5–7 months old hamsters, 13–15 months old hamsters and 19–22 months old hamsters as young hamsters, adult hamsters, aged hamsters, and old hamsters. Young hamsters are matured and stable fertile hamster. Adult hamsters show high fertile ability. Aged hamsters show low fertility or subfertility. Old hamsters show infertility. The present study was approved by the Animal Care and Use Committee of the university (experimental permission number: 0107) and performed in accordance with the University's Guidelines for Animal Experimentation.

### 2.3 | Measurements of weights of bodies, testes, and epididymides

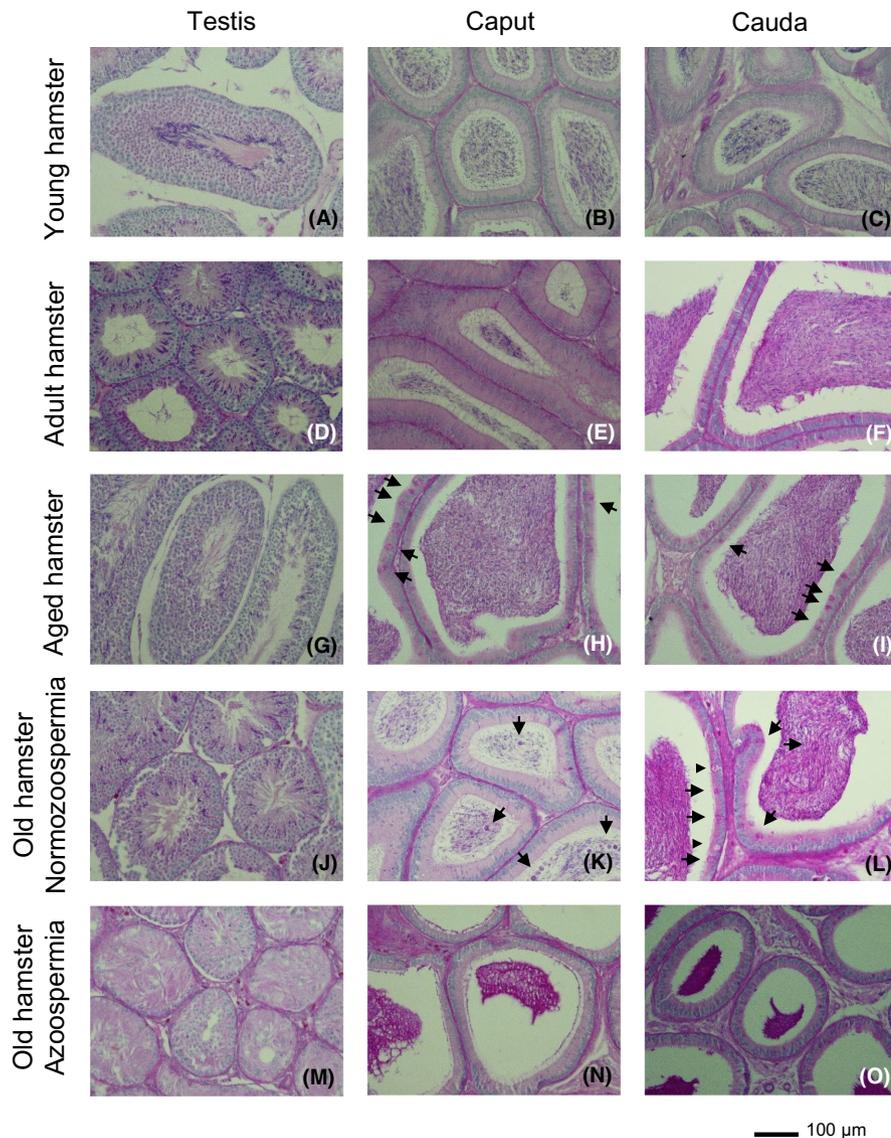
After humanely euthanizing the hamsters by isoflurane, body weights were measured using a weighing scale. After isolating the testis and epididymis, their weights were also measured on a scale.

### 2.4 | Measurements of serum testosterone concentrations

Immediately after death, blood was collected from the heart of hamster and were coagulated on a flat petri dish at room temperature. After incubation for overnight at 4°C, sera were carefully collected and were centrifuged at 15000g for 5 min at 4°C. After centrifugation, the supernatant was collected and was used as a serum sample. Testosterone concentrations were measured by testosterone ELISA kit with a sensitivity of 5.67 pg/ml. Serum samples were obtained at the same time of the day in order to avoid any possible variability due to circadian rhythms.

### 2.5 | Observation of tissue sections

Testes and epididymides were fixed in Bouin's solution for overnight, dehydrated in ethanol and processed for embedding in



**FIGURE 2** Testicular and epididymal tissues stained with PAS stain. Testis (A, D, G, J, M), caput epididymis (B, E, H, K, N), and cauda epididymis (C, F, I, L, O) were obtained from young, adult, aged, old normozoospermic, and old azoospermic hamsters. Arrow and arrow head show structures resembling residual bodies and desquamation, respectively. Bar = 100  $\mu\text{m}$

paraffin. Sections 6  $\mu\text{m}$  thick were stained with hematoxylin and eosin (HE) stain, periodic acid-Schiff stain (PAS) or toluidine blue (TB) stain (pH 4.1 and 7.0) for observation under a light microscope. Each observation was repeated using four different hamsters.

## 2.6 | Preparation of hyperactivated sperm

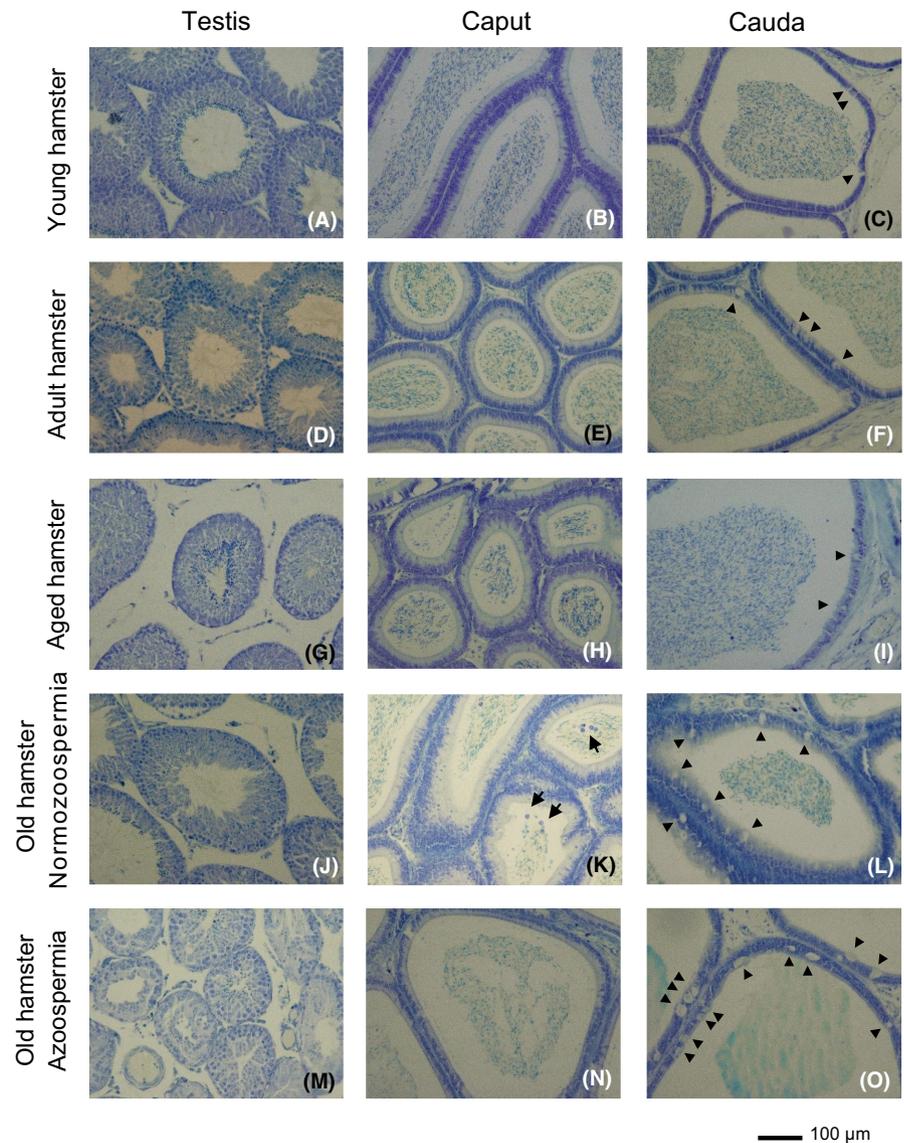
Sperm were collected from the cauda epididymis of male hamsters. Hyperactivated sperm were prepared as described previously.<sup>19</sup> Modified Tyrode's albumin lactate pyruvate medium<sup>35</sup> was used as the capacitation medium. Sperm were placed in a drop (approximately 5  $\mu\text{l}$ ) on a culture dish (35 mm diameter). After adding 3 ml medium to the dish, the sperm were incubated for 5 min at 37°C to

be activated. The supernatant containing motile sperm was placed in a new dish containing vehicle,  $\text{P}_4$ , Mel, 5-HT, MS, or MT, and was incubated for 4 h at 37°C under 5%  $\text{CO}_2$  to induce hyperactivation. As a stock solution, 5-HT (100  $\mu\text{M}$ ) and MS (100 pM) were dissolved in pure water. Mel (10 mM), MT (10 nM), and  $\text{P}_4$  (20  $\mu\text{g}/\text{ml}$ ) were dissolved in ethanol. In all the experiments, the maximum concentration of the vehicle was 0.1%.

## 2.7 | Measurements of motility and hyperactivation

Motility and hyperactivation were measured as described previously.<sup>26</sup> Motile sperm were recorded on a DVD recorder (RDR-HX50; Sony Corp.) using a CCD camera (Progressive

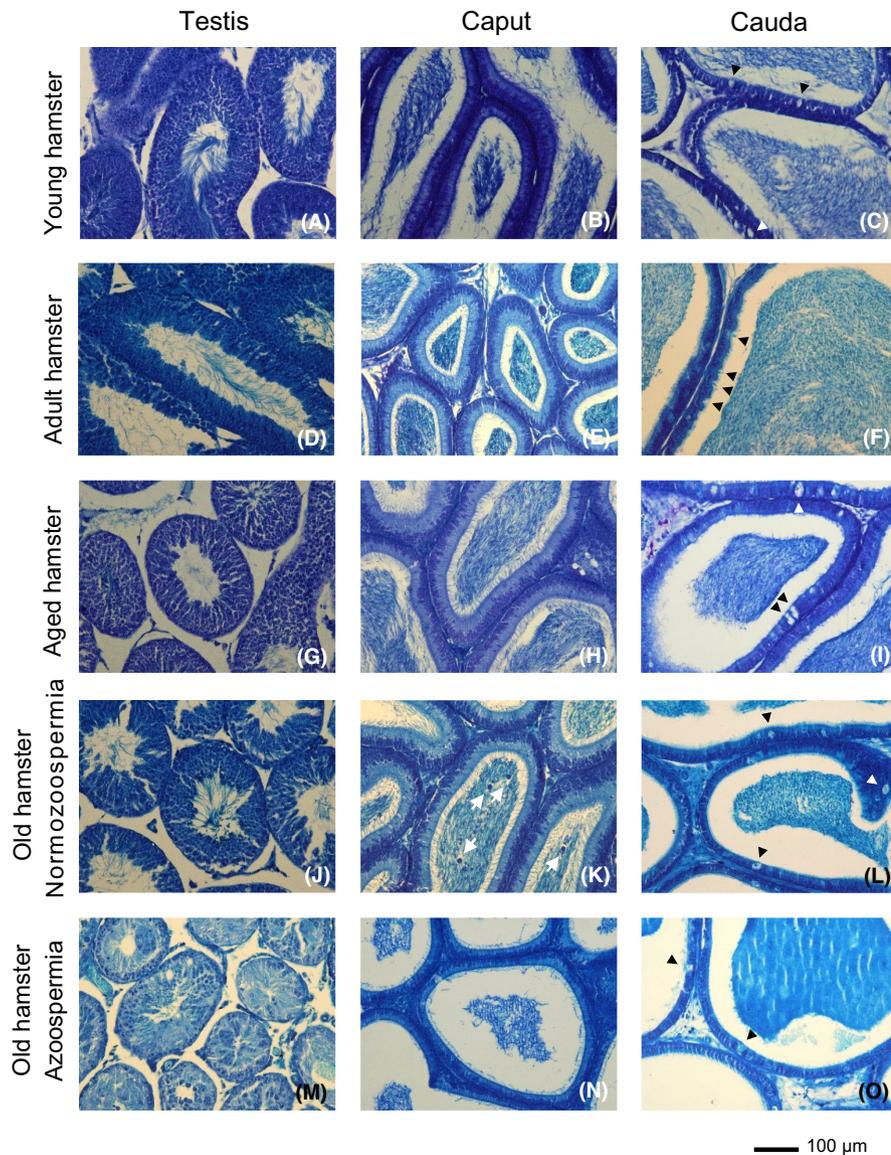
**FIGURE 3** Testicular and epididymal tissues stained with TB (pH 4.1) stain. Testis (A, D, G, J, M), carpus epididymis (B, E, H, K, N), and cauda epididymis (C, F, I, L, O) were obtained from young, adult, aged, old normozoospermic, and old azoospermic hamsters. Arrow and arrow head show structures resembling residual bodies and desquamation, respectively. Bar = 100  $\mu$ m



3CCD, Sony) attached to a microscope (IX70, Olympus Corp.) with phase-contrast illumination and a small CO<sub>2</sub> incubator (MI-IBC, Olympus). Observations were performed at 37°C for 1 min. The analyses comprised manual counts of the numbers of total sperm, motile sperm, and hyperactivated sperm, in four technical replicates. Visual analyses were conducted blind with respect to the treatment group. Motile sperm that exhibited asymmetric and whiplash-like flagellar movements were defined as hyperactivated.<sup>21,26</sup> Percentages of motility and hyperactivation were defined as: the number of motile sperm divided by the number of total sperm  $\times$  100, and the number of hyperactivated sperm divided by the number of total sperm  $\times$  100, respectively. The experiments were performed four times using four hamsters. When the proportion of motile sperm was equal to or below 80%, the experiment was repeated.

## 2.8 | Motility kinetics

Motility kinetics were evaluated using the Sperm Motility Analysis System (SMAS) for animals (Ver. 3.18) with the loaded parameter file mouse\_BM10 $\times$ \_640nm\_Bright59\_150fps-shutter200.ini (Ditect Co., Ltd.), as described previously.<sup>26</sup> The suspension containing motile sperm (20  $\mu$ l) was transferred to an observation chamber (0.1 mm deep, 18 mm wide, and 18 mm long) made of mending tape attached to a glass slide in two parallel strips, and then covered with a cover glass. Sperm movement was recorded for 1 s on the hard disk drive of the SMAS with a warm plate (MP10DM; Kitazato). The straight-line velocity (VSL,  $\mu$ m/s), curvilinear velocity (VCL,  $\mu$ m/s), average path velocity (VAP,  $\mu$ m/s), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH,  $\mu$ m), and beat-cross frequency (BCF, Hz) were



**FIGURE 4** Testicular and epididymal tissues stained with TB (pH 7.0) stain. Testis (A, D, G, J, M), carpus epididymis (B, E, H, K, N), and cauda epididymis (C, F, I, L, O) were obtained from young, adult, aged, old normozoospermic, and old azoospermic hamsters. Arrow and arrow head show structures resembling residual bodies and desquamation, respectively. Bar = 100  $\mu$ m

**TABLE 2** Effects of aging on serum testosterone concentrations

	Serum testosterone (pg/ml)
Young hamster ( $n = 7$ )	835.00 $\pm$ 217.89
Adult hamster ( $n = 10$ )	1007.14 $\pm$ 532.53
Aged hamster ( $n = 12$ )	1460.00 $\pm$ 1004.55
Old hamster ( $n = 10$ )	1571.00 $\pm$ 849.18
Normozoospermia ( $n = 7$ )	2051.43 $\pm$ 470.73
Azoospermia ( $n = 3$ )	450.00 $\pm$ 302.43*

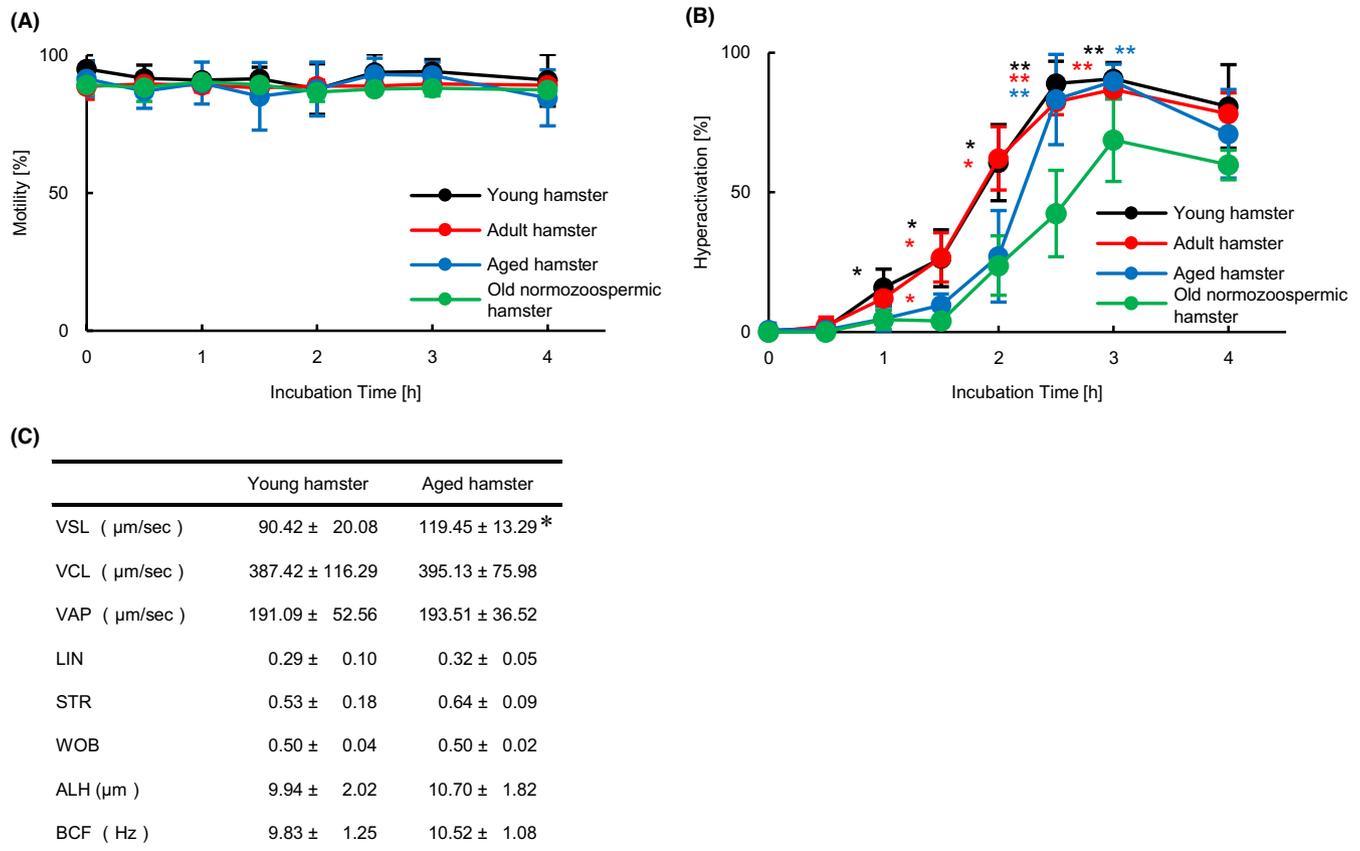
Note: Data are presented as mean  $\pm$  standard deviation.  $n$  is numbers of animals.

\*Indicates significant difference compared with normozoospermia hamster ( $p < 0.05$ ).

automatically calculated by the SMAS; wobbler coefficient (WOB; defined as VAP/VCL) was calculated manually.<sup>36</sup> The motility kinetics were repeated four times on four different hamsters. In each experiment,  $\geq 300$  spermatozoa were detected. Only motile sperm judged to be significant were analyzed.

## 2.9 | Statics

Data were statistically analyzed by Student's  $t$ -test or a repeated measures Analysis of Variance (ANOVA) post-hoc test, using Microsoft Excel (Microsoft Japan) with a ystat2018 (Igakutoshu Shuppan) add-on. Statistical significance was set at  $p < 0.05$ .



**FIGURE 5** Effects of aging on sperm motility, hyperactivation and motility kinetics. The percentages of motility (A) and hyperactivation (B) were detected after spermatozoa were cultured for 4 h. Spermatozoa were obtained from young, adult, aged, and old normozoospermic hamsters. (C) Motility kinetics were detected when spermatozoa were cultured at 2 h. Spermatozoa were obtained from young and aged hamsters. Data represent the mean  $\pm$  standard deviation. In (A) and (B), \* indicates significant differences compared with “Aged hamster” and “Old normozoospermic hamster” ( $p < 0.05$ ); \*\* indicates significant differences compared with “Old normozoospermic hamster” ( $p < 0.05$ ). In (C), \* indicates significant differences compared with young hamster ( $p < 0.05$ )

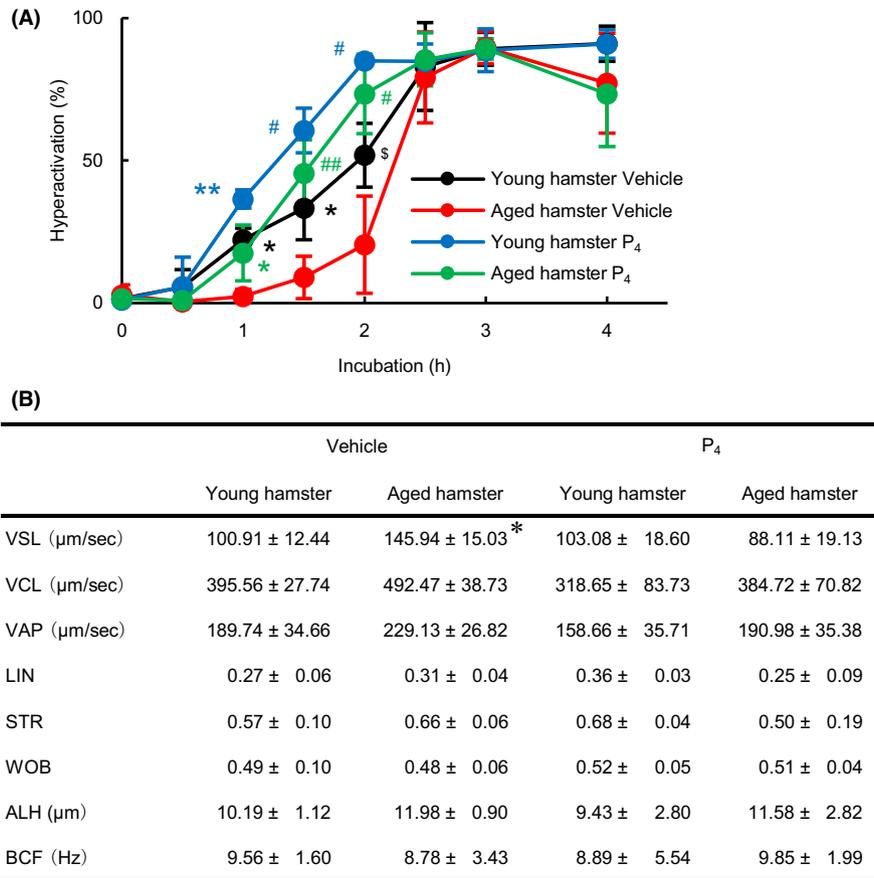
### 3 | RESULTS

#### 3.1 | Effects of aging on weights of bodies, testes, and epididymides

As shown in Table 1, the body, testis and epididymis weights of young, adult, aged, and old hamsters were measured. Body weights of aged and old hamsters were significantly heavier than the weights of young and adult hamsters. The testis weights of young hamsters were significantly heavier than others. In addition, the testis weights gradually decreased according to aging. As for the epididymis weights, old hamsters were significantly lighter than others. In old hamsters, moreover, normozoospermia and azoospermia were observed. As for weights of bodies, testes, and epididymides, old normozoospermic hamsters were similar to aged hamsters. But, testes and epididymides of old azoospermic hamsters were very light.

#### 3.2 | Histological effects of aging on testes and epididymides

In the next step, it was examined histological effects of aging on testes and epididymides (Figures 1–4). Testes and epididymides of young, adult, aged, and old hamsters were stained by HE (Figure 1), PAS (Figure 2), TB (pH 4.1) (Figure 3) and TB (pH 7.0) (Figure 4). In testes, histological effects of aging were not observed. In contrast, structures resembling residual bodies (SRRBs) and desquamations were slightly observed in epididymides stained by PAS, TB (pH 4.1) and TB (pH 7.0). In old normozoospermia hamster, SRRBs and desquamations were observed in epididymides stained by HE, PAS, TB (pH 4.1) and TB (pH 7.0). In testis of old azoospermic hamster, spermatogenesis did not happen. In epididymides of old azoospermic hamster, no sperm were observed at all but only fluids were observed. In addition, desquamations were observed in cauda epididymides stained by TB (pH 4.1) and TB (pH 7.0).



**FIGURE 6** Mitigation of aging-effects by P<sub>4</sub>. (A) The percentage of hyperactivation was detected after spermatozoa were cultured for 4 h in the presence of P<sub>4</sub>. (B) Motility kinetics were detected when spermatozoa were cultured at 2 h in the presence of P<sub>4</sub>. Spermatozoa were obtained from young and aged hamsters. Data represent the mean ± standard deviation. In (A), \* indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster P<sub>4</sub>” ( $p < 0.05$ ); \*\* indicates significant differences compared with “Aged hamster Vehicle”, “Young hamster Vehicle”, and “Aged hamster P<sub>4</sub>” ( $p < 0.05$ ); # indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster Vehicle” ( $p < 0.05$ ); ## indicates significant differences compared with “Aged hamster Vehicle” ( $p < 0.05$ ); \$ indicates significant differences compared with “Aged hamster Vehicle”, “Young hamster P<sub>4</sub>”, and “Aged hamster P<sub>4</sub>” ( $p < 0.05$ ). In (B), \* indicates significant differences compared with young hamster of vesicle ( $p < 0.05$ )

### 3.3 | Effects of aging on serum testosterone concentrations

It was examined whether aging affect testosterone concentrations (Table 2). Aging did not affect testosterone concentrations. Moreover, testosterone concentration of old azoospermic hamster was significantly lower than it of old normozoospermic hamster.

### 3.4 | Effects of aging on motility, hyperactivation, and motility kinetics

As shown in Figure 5, motility and hyperactivation were measured in sperm obtained from young, adult, aged, and old normozoospermic hamsters. As for motility, no effects of aging were observed (Figure 5A). However, aging-reduced hyperactivation (Figure 5B). In young and adult hamsters, no effects of aging on sperm

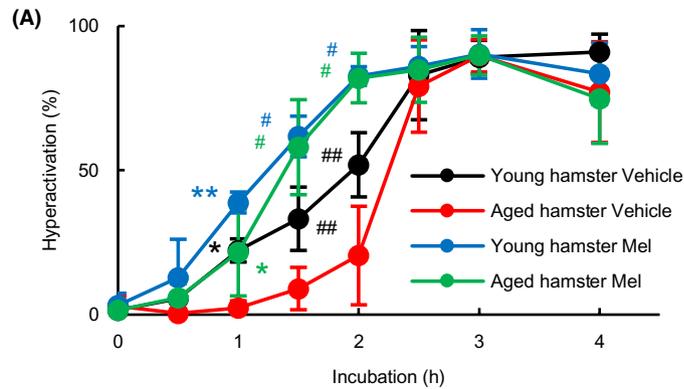
hyperactivation were observed. Sperm hyperactivation of aged and old normozoospermic hamsters were lower than it of young and adult hamsters at incubation for 1, 1.5 and 2 h. Moreover, sperm hyperactivation of old normozoospermic hamsters were lowest than others at incubation for 2.5 and 3 h.

In the next step, it was examined motility kinetics of young and aged hamster sperm at incubation for 2 h (Figure 5C). VSL of aged hamster sperm was significantly higher than it of young hamster sperm. The other kinetic parameters were not significantly different between young hamster sperm and aged hamster sperm.

### 3.5 | Mitigation of aging-reduced sperm hyperactivation by oviductal hormones

It was showed that P<sub>4</sub>, Mel, and 5-HT enhanced sperm hyperactivation in hamsters.<sup>17,18,25</sup> As shown in Figure 6A, P<sub>4</sub> significantly increased

**FIGURE 7** Mitigation of aging-effects by Mel. (A) The percentage of hyperactivation was detected after spermatozoa were cultured for 4 h in the presence of Mel. (B) Motility kinetics were detected when spermatozoa were cultured at 2 h in the presence of Mel. Spermatozoa were obtained from young and aged hamsters. Data represent the mean  $\pm$  standard deviation. In (A), \* indicates significant differences compared with "Aged hamster Vehicle" and "Young hamster Mel" ( $p < 0.05$ ); \*\* indicates significant differences compared with "Aged hamster Vehicle", "Young hamster Vehicle", and "Aged hamster Mel" ( $p < 0.05$ ); # indicates significant differences compared with "Young hamster Vehicle" and "Aged hamster Vehicle" ( $p < 0.05$ ); ## indicates significant differences compared with "Aged hamster Vehicle", "Aged hamster Mel", and "Young hamster Mel" ( $p < 0.05$ ). In (B), \* indicates significant differences compared with young hamster of vesicle ( $p < 0.05$ )

**(B)**

	Vehicle		Mel	
	Young hamster	Aged hamster	Young hamster	Aged hamster
VSL ( $\mu\text{m}/\text{sec}$ )	100.92 $\pm$ 12.44	145.94 $\pm$ 15.03*	91.81 $\pm$ 18.24	94.24 $\pm$ 7.39
VCL ( $\mu\text{m}/\text{sec}$ )	395.56 $\pm$ 27.74	492.47 $\pm$ 38.73	424.03 $\pm$ 166.50	304.76 $\pm$ 28.14
VAP ( $\mu\text{m}/\text{sec}$ )	189.74 $\pm$ 34.66	229.13 $\pm$ 26.82	177.17 $\pm$ 73.99	158.49 $\pm$ 18.66
LIN	0.27 $\pm$ 0.06	0.31 $\pm$ 0.04	0.24 $\pm$ 0.06	0.34 $\pm$ 0.02
STR	0.57 $\pm$ 0.10	0.66 $\pm$ 0.06	0.58 $\pm$ 0.15	0.62 $\pm$ 0.04
WOB	0.49 $\pm$ 0.10	0.48 $\pm$ 0.06	0.42 $\pm$ 0.08	0.54 $\pm$ 0.10
ALH ( $\mu\text{m}$ )	10.19 $\pm$ 1.12	11.98 $\pm$ 0.90	11.65 $\pm$ 3.20	8.34 $\pm$ 2.37
BCF (Hz)	9.56 $\pm$ 1.60	8.78 $\pm$ 3.43	9.53 $\pm$ 1.67	9.64 $\pm$ 3.17

sperm hyperactivation in young and aged hamsters and lessened the reduction in sperm hyperactivation caused by aging. As for motility kinetics (Figure 6B), VSL of aged hamster sperm was significantly higher than it of young hamster sperm in the absence of  $P_4$ . However,  $P_4$  decreased VLS of aged hamster sperm and canceled the aging-effect. The other kinetic parameters were not affected by aging and  $P_4$ . As shown in Figure 7A, Mel significantly increased hyperactivation of young and aged hamster sperm and canceled the reduction of sperm hyperactivation by aging. As for motility kinetics (Figure 7B), in the absence of Mel, VSL of aged hamster sperm was significantly higher than it of young hamster sperm. However, Mel canceled the increase in VSL caused by aging. The other kinetic parameters were not affected by aging and Mel.

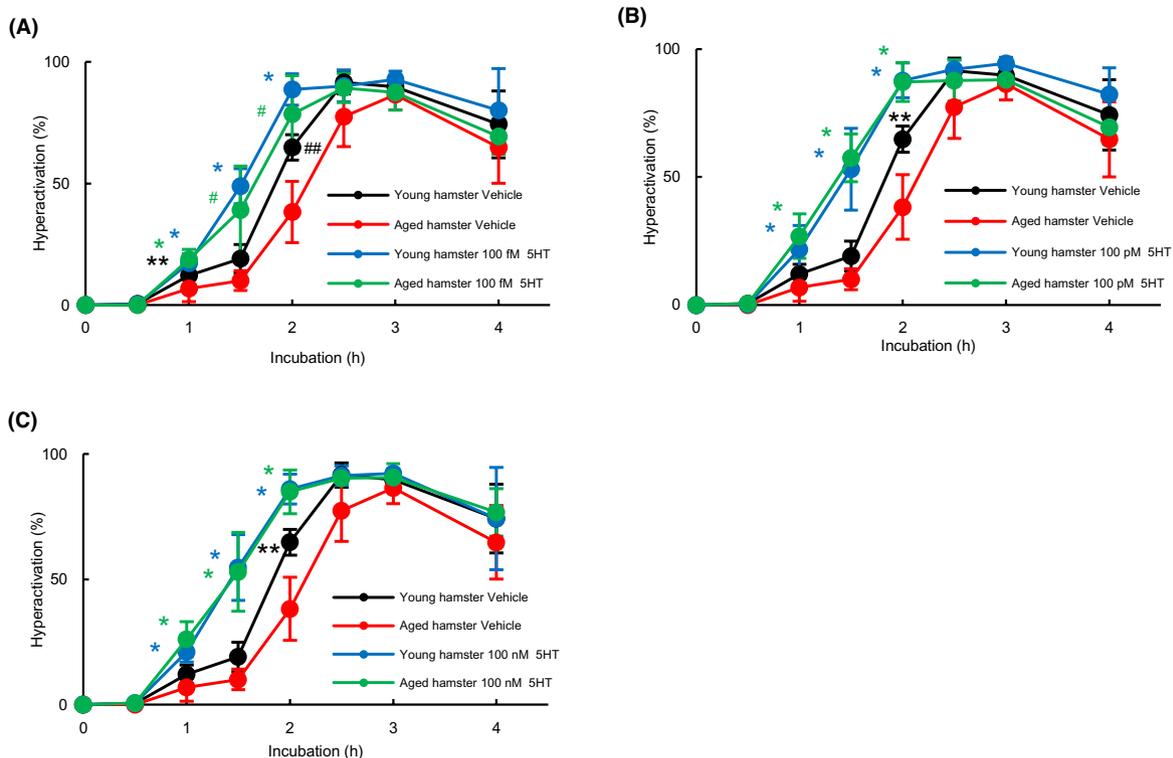
As shown in Figure 8A–C, 100fM, 100pM, and 100nM 5-HT significantly increased hyperactivation of young and aged hamster sperm and prevented the reduction of sperm hyperactivation caused by aging. As for motility kinetics (Figure 8D), in the absence of each 5-HT, VSL of aged hamster sperm was significantly higher than it of young hamster sperm. However, each 5-HT mitigated the increase in VSL caused by aging. Other kinetic parameters were not affected by aging and 5-HT.

In previous studies,<sup>25,26</sup> 5-HT has been reported to enhance hamster sperm hyperactivation via the 5-HT<sub>2</sub> and 5-HT<sub>4</sub> receptors. As shown in Figure 9A, MS (5-HT<sub>2</sub> receptor agonist) significantly

increased hyperactivation of young and aged hamster sperm and mitigated the reduction of sperm hyperactivation caused by aging. As for motility kinetics (Figure 9B), in the absence of MS, VSL of aged hamster sperm was significantly higher than it of young hamster sperm. However, MS lessened the increase in VSL with aging. Other kinetic parameters were not affected by aging and MS. As shown in Figure 10A, MT (5-HT<sub>4</sub> receptor agonist) significantly increased hyperactivation of young and aged hamster sperm and mitigated the reduction of sperm hyperactivation caused by aging. As for motility kinetics (Figure 10B), in the absence of MT, VSL of aged hamster sperm was significantly higher than it of young hamster sperm. However, MT mitigated the increase in VSL caused by aging. Other kinetic parameters were not affected by aging and MT.

## 4 | DISCUSSION

Generally, it has been suggested that aging significantly reduces male reproductive functions because of decreasing testosterone concentrations.<sup>1–4</sup> Many studies have suggested that aging increases DNA damage and decrease sperm motility, although several studies have shown that aging does not decrease sperm motility.<sup>3–9,11</sup>



(D)

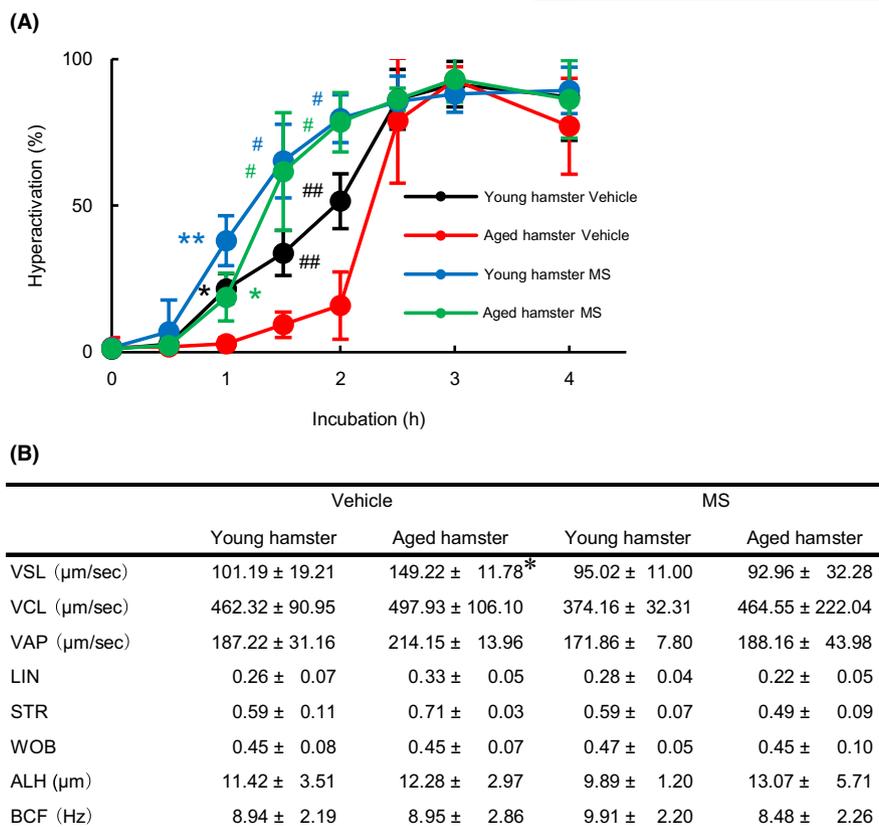
	Vehicle		100 fM 5-HT		100 pM 5-HT		100 nM 5-HT	
	Young hamster	Aged hamster	Young hamster	Aged hamster	Young hamster	Aged hamster	Young hamster	Aged hamster
VSL ( $\mu\text{m}/\text{sec}$ )	89.97 $\pm$ 12.57	152.56 $\pm$ 15.68 *	85.84 $\pm$ 13.03	86.11 $\pm$ 25.23	91.55 $\pm$ 14.81	79.45 $\pm$ 32.53	103.07 $\pm$ 13.23	82.34 $\pm$ 31.87
VCL ( $\mu\text{m}/\text{sec}$ )	408.68 $\pm$ 197.87	410.93 $\pm$ 88.70	390.32 $\pm$ 230.18	319.37 $\pm$ 68.16	356.49 $\pm$ 119.03	385.36 $\pm$ 77.99	392.13 $\pm$ 43.18	231.46 $\pm$ 100.02
VAP ( $\mu\text{m}/\text{sec}$ )	199.94 $\pm$ 85.25	232.39 $\pm$ 32.62	185.60 $\pm$ 96.99	155.87 $\pm$ 37.09	165.57 $\pm$ 60.33	160.34 $\pm$ 103.20	182.74 $\pm$ 10.35	130.86 $\pm$ 64.73
LIN	0.26 $\pm$ 0.08	0.39 $\pm$ 0.06	0.27 $\pm$ 0.14	0.28 $\pm$ 0.10	0.29 $\pm$ 0.06	0.21 $\pm$ 0.08	0.29 $\pm$ 0.07	0.36 $\pm$ 0.02
STR	0.49 $\pm$ 0.13	0.66 $\pm$ 0.08	0.54 $\pm$ 0.25	0.58 $\pm$ 0.15	0.61 $\pm$ 0.10	0.57 $\pm$ 0.22	0.58 $\pm$ 0.07	0.66 $\pm$ 0.15
WOB	0.51 $\pm$ 0.04	0.59 $\pm$ 0.08	0.50 $\pm$ 0.04	0.49 $\pm$ 0.11	0.49 $\pm$ 0.10	0.40 $\pm$ 0.19	0.49 $\pm$ 0.05	0.56 $\pm$ 0.10
ALH ( $\mu\text{m}$ )	10.24 $\pm$ 2.48	8.88 $\pm$ 1.86	9.58 $\pm$ 3.81	7.54 $\pm$ 1.50	10.73 $\pm$ 2.27	10.78 $\pm$ 4.44	10.50 $\pm$ 1.07	7.99 $\pm$ 4.22
BCF (Hz)	8.08 $\pm$ 2.65	7.39 $\pm$ 1.38	7.71 $\pm$ 1.74	5.81 $\pm$ 1.59	6.59 $\pm$ 1.45	9.88 $\pm$ 3.70	7.72 $\pm$ 1.16	4.46 $\pm$ 1.07

**FIGURE 8** Mitigation of aging-effects by 5-HT. The percentage of hyperactivation was detected after spermatozoa were cultured for 4 h in the presence of 100 fM 5-HT (A), 100 pM 5-HT (B), and 100 nM 5-HT (C). (D) Motility kinetics were detected when spermatozoa were cultured at 2 h in the presence of each 5-HT. Spermatozoa were obtained from young and aged hamsters. Data represent the mean  $\pm$  standard deviation. In (A), \* indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster Vehicle” ( $p < 0.05$ ); \*\* indicates significant differences compared with “Aged hamster Vehicle”, “Young hamster 100 fM 5-HT”, and “Aged hamster 100 fM 5-HT” ( $p < 0.05$ ); # indicates significant differences compared with “Aged hamster Vehicle” ( $p < 0.05$ ); ## indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster 100 fM 5-HT” ( $p < 0.05$ ). In (B), \* indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster Vehicle” ( $p < 0.05$ ); \*\* indicates significant differences compared with “Aged hamster Vehicle”, “Young hamster 100 pM 5-HT”, and “Aged hamster 100 pM 5-HT” ( $p < 0.05$ ). In (C), \* indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster Vehicle” ( $p < 0.05$ ); \*\* indicates significant differences compared with “Aged hamster Vehicle”, “Young hamster 100 nM 5-HT”, and “Aged hamster 100 nM 5-HT” ( $p < 0.05$ ). In (D), \* indicates significant differences compared with young hamster of vesicle ( $p < 0.05$ )

In the present study, aging decreased testis weights, although it did not affect epididymis weights and testosterone concentrations (see Tables 1 and 2). In contrast, previous hamster studies<sup>12,13</sup> suggested that aging did not affect testis weights and testosterone concentrations. Moreover, they suggested that aging induced desquamation and hypospermatogenesis in testes and epididymides.<sup>12,13</sup> In the present study, aging was not related to the desquamation and hypospermatogenesis in testes and epididymides,

although aging induced SRRBs in epididymides (see Figures 1–4). It has been reported that SRRBs are observed in testes and epididymides, which were exposed to reproductive toxic chemicals.<sup>37–41</sup> Thus, these observations might be damages that were affected by aging.

In the present study, some of old hamster were azoospermia. Their testes and epididymides were very small and did not have sperm (see Table 1 and Figures 1–4). Testosterone concentration of



**FIGURE 9** Mitigation of aging-effects by MS. (A) The percentage of hyperactivation was detected after spermatozoa were cultured for 4 h in the presence of MS. (B) Motility kinetics were detected when spermatozoa were cultured at 2 h in the presence of MS. Spermatozoa were obtained from young and aged hamsters. Data represent the mean  $\pm$  standard deviation. In (A), \* indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster MS” ( $p < 0.05$ ); \*\* indicates significant differences compared with “Aged hamster Vehicle”, “Young hamster Vehicle”, and “Aged hamster MS” ( $p < 0.05$ ). # indicates significant differences compared with “Aged hamster Vehicle” and “Young hamster Vehicle” ( $p < 0.05$ ); ## indicates significant differences compared with “Aged hamster Vehicle”, “Aged hamster MS”, and “Young hamster MS” ( $p < 0.05$ ). In (B), \* indicates significant differences compared with young hamster of vesicle ( $p < 0.05$ )

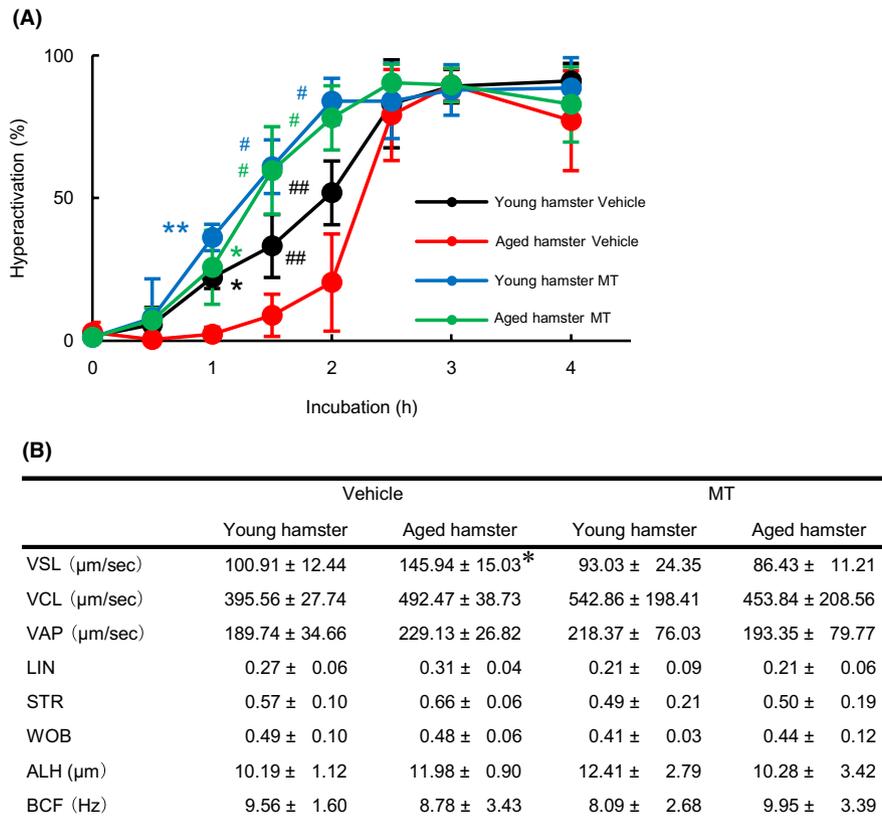
old azoospermic hamster was also very low (see Table 2). Because old hamsters were fertile when they were young, it is likely that hamsters became azoospermia by aging. However, it is not clear whether these results were caused by only aging, because all old hamster were not always azoospermia.

Previous hamster study<sup>13</sup> suggested that aging-reduced sperm progressive motility and decreased sperm quality. However, motility kinetics analysis show that aging does not affect progressive motility because VSL increased by aging and VCL, VAP, LIN and STR were not affected by aging (see Figure 5B). VSL, VCL, VAP, LIN and STR are kinetics parameters associated with progressive motility.<sup>36</sup> Although motility kinetics analysis was not carried out in the previous study,<sup>13</sup> it is likely that the previous study<sup>13</sup> show that aging-reduced sperm motility activity. In the present study, aging significantly reduced hyperactivation and significantly increased VSL, although it did not affect sperm motility (see Figure 5). Since VSL decreases when sperm are hyperactivated,<sup>26,36</sup> it seems that increasing of VSL by aging was related to the reduction of hyperactivation by aging. Moreover, previous human and mouse studies<sup>24,27</sup> suggest that the ability of hyperactivation is positively

correlated with the success of IVF. Therefore, it is likely that aging reduces sperm capacitation and is the likely cause of male infertility.

Hyperactivation is enhanced by several oviductal hormones.<sup>21</sup> In hamsters, these hormones  $P_4$ ,<sup>17</sup> Mel,<sup>18</sup> and 5-HT (in a dose-dependent manner)<sup>25</sup> enhance hyperactivation; where high concentrations of 5-HT enhanced hyperactivation via 5-HT<sub>4</sub> receptors and low concentrations enhanced hyperactivation via the 5-HT<sub>2</sub> receptor.<sup>25</sup> In the present study,  $P_4$ , Mel, 5-HT, and 5-HT receptor agonists mitigated effects of aging on sperm qualities (Figures 6–10). Since previous mouse study<sup>27</sup> showed that 5-HT enhanced sperm hyperactivation and increased the success of IVF, oviductal hormones likely increase the hyperactivation of aged animal sperm and lessen infertility caused by aging.

In conclusion, aging decreased qualities of the testes and epididymides and reduced hyperactivation of hamster sperm. Moreover,  $P_4$ , Mel, 5-HT, and 5-HT receptor agonists mitigated the age-related reduction of hyperactivation. Therefore, aging reduces sperm quality, although oviductal hormones may help assuage this reduction.



**FIGURE 10** Mitigation of aging-effects by MT. (A) The percentage of hyperactivation was detected after spermatozoa were cultured for 4 h in the presence of MT. (B) Motility kinetics were detected when spermatozoa were cultured at 2 h in the presence of MT. Spermatozoa were obtained from young and aged hamsters. Data represent the mean  $\pm$  standard deviation. In (A), \* indicates significant differences compared with "Aged hamster Vehicle" and "Young hamster MT" ( $p < 0.05$ ); \*\* indicates significant differences compared with "Aged hamster Vehicle", "Young hamster Vehicle", and "Aged hamster MT" ( $p < 0.05$ ). # indicates significant differences compared with "Aged hamster Vehicle" and "Young hamster Vehicle" ( $p < 0.05$ ); ## indicates significant differences compared with "Aged hamster Vehicle", "Aged hamster MT", and "Young hamster MT" ( $p < 0.05$ ). In (B), \* indicates significant differences compared with young hamster of vesicle ( $p < 0.05$ )

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

## HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

This work does not contain any studies with human subjects.

## ANIMAL STUDIES

This work was performed in according to guidelines of Dokkyo Medical University and nation for the care and use of laboratory animals.

## APPROVAL BY ETHICS COMMITTEE

The study was approved by the Animal Care and Use Committee at Dokkyo Medical University, Tochigi, Japan (experimental permission number: 0107).

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