—Original Article—

Estrogen suppresses melatonin-enhanced hyperactivation of hamster spermatozoa

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Abstract. Hamster sperm hyperactivation is enhanced by progesterone, and this progesterone-enhanced hyperactivation is suppressed by 17β-estradiol ($17\beta E_2$) and γ-aminobutyric acid (GABA). Although it has been indicated that melatonin also enhances hyperactivation, it is unknown whether melatonin-enhanced hyperactivation is also suppressed by $17\beta E_2$ and GABA. In the present study, melatonin-enhanced hyperactivation was significantly suppressed by $17\beta E_2$ but not by GABA. Moreover, suppression of melatonin-enhanced hyperactivation by $17\beta E_2$ occurred through non-genomic regulation via the estrogen receptor (ER). These results suggest that enhancement of hyperactivation is regulated by melatonin and $17\beta E_2$ through non-genomic regulation.
Key words: Estradiol, Hyperactivation, Melatonin, Non-genomic regulation, Spermatozoa

In mammals, only capacitated spermatozoa can fertilize an egg. Capacitated spermatozoa generally show two major responses, known as "acrosome reaction" and "hyperactivation" [1–3]. The acrosome reaction is an exocytosis occurring at the sperm head; and is required for penetration of the zona pellucida (ZP) [1]. Hyperactivation is a modification of flagellar movement to create the driving force for penetrating the ZP [1–3]. Moreover, the ability of spermatozoa to be hyperactivated correlates with the success of *in vitro* fertilization [4].

It has been recently reported that hyperactivation is regulated by several hormones and transmitters (e.g., progesterone [5–10], 17β-estradiol ($17\beta E_2$) [8, 10], melatonin [11, 12], serotonin (5-HT) [13] and γ-aminobutyric acid (GABA) [14–18]). In the hamster, progesterone, melatonin and serotonin enhance hyperactivation in a dose-dependent manner [7, 10, 11, 13]. Moreover, progesterone enhances hyperactivation through non-genomic regulation associated with a progesterone receptor (PR), phospholipase C (PLC), inositol 1,4,5-tris-phosphate receptor (IP_3R), protein kinases and tyrosine phosphorylations [7, 9]. Melatonin enhances hyperactivation via melatonin receptor type 1 (MT1) [11]. Serotonin enhances hyperactivation via the 5-HT_2 and 5-HT_4 receptors [13]. In humans, progesterone and melatonin change motility parameters and enhance hyperactivation [5, 6, 12]. It has also been shown that $17\beta E_2$ and GABA dose-dependently suppress progesterone-enhanced hyperactivation in the hamster [8, 9, 18]. Furthermore, $17\beta E_2$ suppresses progesterone-enhanced hyperactivation through non-genomic regulation associated with the estrogen receptor (ER) and tyrosine dephosphorylations

[8]. GABA suppresses progesterone-enhanced hyperactivation via the $GABA_A$ receptor [18]. Interestingly, in humans, rams and rats, GABA increases hyperactivation via the $GABA_A$ receptor [14–17].

In hamster spermatozoa, there are three enhancers of hyperactivation: progesterone [7, 9, 10], melatonin [11] and serotonin [13]. Moreover, there are two suppressors of progesterone-enhanced hyperactivation: $17\beta E_2$ [8, 10] and GABA [18]. In order to understand the regulatory mechanisms of sperm hyperactivation caused by enhancers and suppressors, interactions among them need to be examined. Therefore, in the present study, we examined whether melatonin-enhanced hyperactivation of hamster spermatozoa is suppressed by $17\beta E_2$ and GABA.

Materials and Methods

Chemicals

Hypotaurine, (–)epinephrine, 17α-estradiol ($17\alpha E_2$), $17\beta E_2$, fluorescein isothiocyanate and bovine serum albumin (BSA)-conjugated $17\beta E_2$ (BSA-$17\beta E_2$), GABA, melatonin, sodium taurocholate, sodium metabisulfite, and tamoxifen were purchased from Sigma-Aldrich (St Louis, MO, USA). BSA fraction V was purchased from Merck KGaA (Darmstadt, Germany). Other reagent-grade chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals and preparation of hyperactivated spermatozoa

Spermatozoa were obtained from the posterior epididymis of sexually mature male golden hamsters (*Mesocricetus auratus*). The experimental plan was approved by the Animal Care and Use Committee of the Dokkyo Medical University (Experimental permission number: 0107), and the experiment was carried out according to the Guidelines for Animal Experimentation of the University.

Hyperactivated spermatozoa were prepared using the modified Tyrode's albumin lactate pyruvate (mTALP) medium [20] containing 101.02 mM NaCl, 2.68 mM KCl, 2 mM $CaCl_2$-$2H_2O$, 1.5 mM $MgCl_2$-$6H_2O$, 360 μM NaH_2PO_4-$2H_2O$, 35.70 mM $NaHCO_3$, 4.5
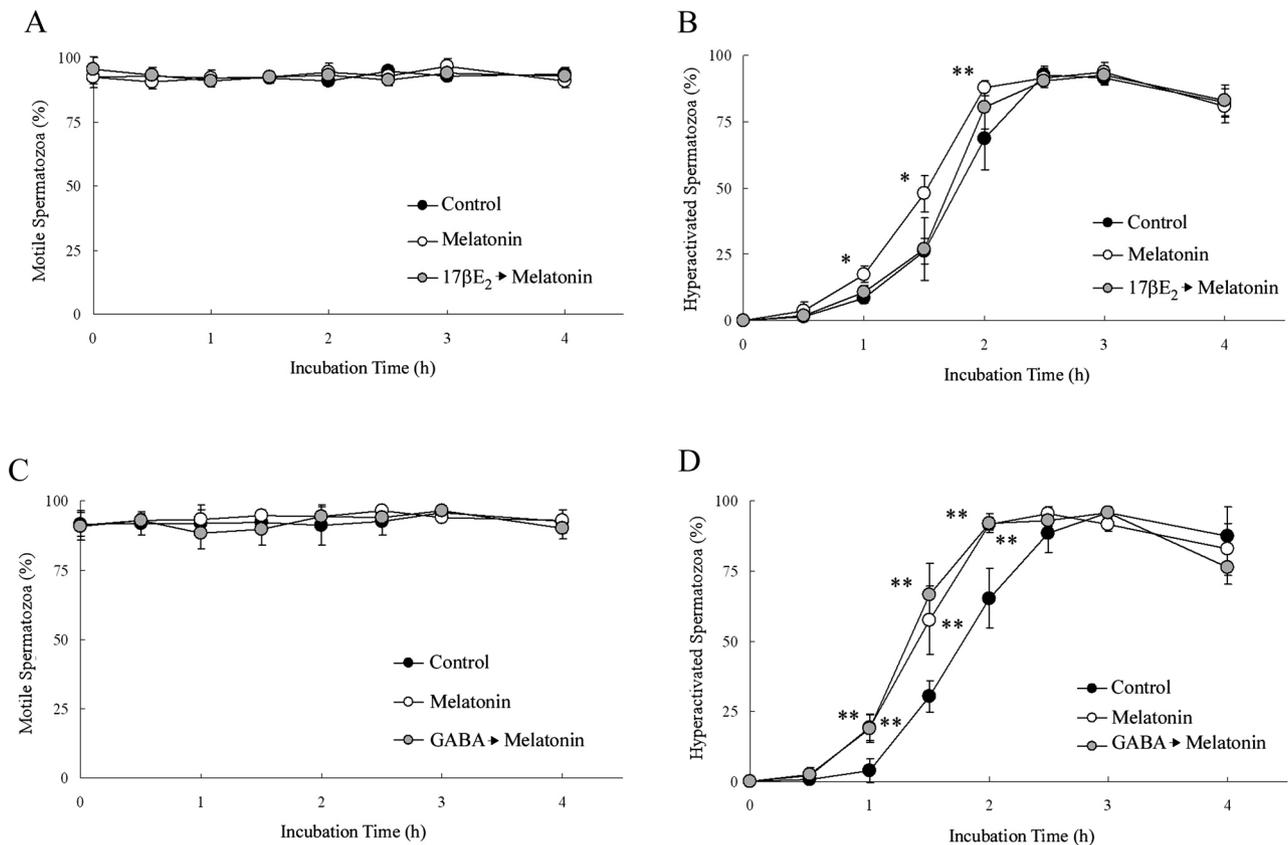
Fig. 1. Suppression of melatonin-enhanced hyperactivation by $17\beta E_2$ and GABA. The percentages of motile (A, C) and hyperactivated (B, D) spermatozoa are shown for when spermatozoa were exposed to melatonin and $17\beta E_2$ (A, B) or GABA (C, D). After swim up, $17\beta E_2$, GABA or vehicle was added to the mTALP medium containing motile spermatozoa. After incubation for 5 min, melatonin or vehicle was added to the same medium. Data are expressed as the mean ± SD. (A, B) (Control): mTALP medium with 0.2% (v/v) EtOH; (Melatonin): mTALP medium with addition of 1 nM melatonin and 0.2% (v/v) EtOH; ($17\beta E_2 \to$ Melatonin): mTALP medium with addition of 1 nM melatonin, 20 ng/ml $17\beta E_2$ and 0.2% (v/v) EtOH. (C, D) (Control): mTALP medium with 0.1% (v/v) EtOH; (Melatonin): mTALP medium with addition of 1 nM melatonin and 0.1% (v/v) EtOH; (GABA $\to$ Melatonin): mTALP medium with addition of 1 nM melatonin, 5 μM GABA and 0.1% (v/v) EtOH. * Significant difference compared with "Control" and "$17\beta E_2 \to$ Melatonin" ($P < 0.05$). ** Significant difference compared with "Control" ($P < 0.05$). GABA, γ-aminobutyric acid; $17\beta E_2$, 17β-estradiol; EtOH, ethanol; mTALP medium, modified Tyrode's albumin lactate pyruvate medium.

mM D-glucose, 90 μM sodium pyruvate, 9 mM sodium lactate, 500 μM hypotaurine, 50 μM (-)epinephrine, 200 μM sodium taurocholate, 5.26 μM sodium metabisulfite, 0.05% (w/v) streptomycin sulfate, 0.05% (w/v) potassium penicillin G and 15 mg/ml BSA (pH 7.4 at 37 C under 5% (v/v) CO_2 in air) according to the method described previously [19]. A drop (~ 5 μl) of posterior epididymal spermatozoa was placed in a culture dish (diameter, 35 mm) with 3 ml of the mTALP medium, followed by incubation at 37 C for 5 min to allow spermatozoa to swim up. All of the mTALP medium containing motile spermatozoa was placed in a new culture dish and incubated for 4 h at 37 C under 5% (v/v) CO_2 in air to allow hyperactivation to occur. As stock solutions, melatonin (1 μM), $17\alpha E_2$ (20 μg/ml) and $17\beta E_2$ (20 μg/ml) were dissolved in ethanol; tamoxifen was dissolved at 1 mM in dimethyl sulfoxide; and GABA (5 mM) and BSA-E_2 (7.4 μM) were dissolved in pure water. GABA, $17\alpha E_2$, $17\beta E_2$, tamoxifen or vehicle was added to the mTALP medium after swim up, and after 5 min of incubation, melatonin or vehicle was added to the mTALP medium (Figs. 1, 2, 3A, 3B, 5A, 5B). Tamoxifen

or vehicle was added to the mTALP medium after swim up, and after 5 min incubation, $17\beta E_2$, BSA-$17E_2$ or vehicle was added to the mTALP medium again. After additional incubation for 5 min, melatonin or vehicle was added to the mTALP medium (Figs. 3C, 3D, 4). Mixtures of $17\beta E_2$ and melatonin or vehicle were added to the mTALP medium after swim up (Figs. 5C, 5D). Melatonin or vehicle was added to the mTALP medium after swim up, and after 5 min of incubation, $17\beta E_2$ or vehicle was added to the mTALP medium (Figs. 5E, 5F). In all experiments, the maximal concentration of vehicle was 0.3% by volume.

Measurement of the percentage of hyperactivated spermatozoa

The percentage of hyperactivated spermatozoa was measured according to the method described previously [19]. Motile spermatozoa were recorded on videotape using a phase-contrast illumination unit attached to a microscope (IX70, Olympus, Tokyo, Japan) with a CCD camera (Progressive 3CCD, Sony, Tokyo, Japan) and a small CO_2 incubator (MI-IBC, Olympus). Each observation was performed
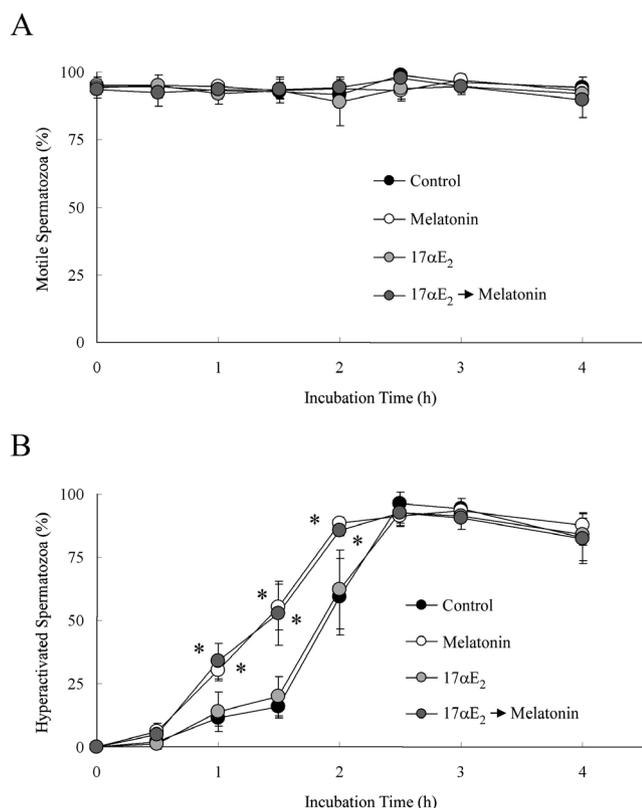
A

B

Fig. 2. Effect of 17αE₂ on melatonin-enhanced hyperactivation. The percentages of motile (A) and hyperactivated (B) spermatozoa are shown for when spermatozoa were exposed to melatonin and 17αE₂. After swim up, 17αE₂ or vehicle was added to the mTALP medium containing motile spermatozoa. After incubation for 5 min, melatonin or vehicle was added to the same medium. Data are expressed as the mean ± SD. (Control): mTALP medium with 0.2% (v/v) EtOH; (Melatonin): mTALP medium with 1 nM melatonin and 0.2% (v/v) EtOH; (17αE₂): mTALP medium with 20 ng/ml 17αE₂ and 0.2% (v/v) EtOH; (17αE₂ → Melatonin): mTALP medium with 1 nM melatonin, 20 ng/ml 17αE₂ and 0.2% (v/v) EtOH. * Significant difference compared with "Control" and "17αE₂" (P < 0.05). 17αE₂, 17α-estradiol; EtOH, ethanol; mTALP medium, modified Tyrode's albumin lactate pyruvate medium.

at 37 C, recorded for 2 min, and analyzed by slow-motion playback and manual counting of the numbers of total spermatozoa and hyperactivated spermatozoa in four different fields per point of analysis. The analysis was done in a blinded manner. Motile spermatozoa that exhibited asymmetric and whiplash flagellar movement and a circular and/or octagonal swimming locus were defined as hyperactivated [3, 21]. The percentage of motile spermatozoa was defined as the number of motile spermatozoa / total number of spermatozoa × 100 (%). The percentage of hyperactivated spermatozoa was defined as the number of hyperactivated spermatozoa / total number of spermatozoa × 100 (%). One hamster was used per experiment, and experiments were repeated four times.

Statistical analysis
Data are expressed as the mean ± standard deviation (SD). Statistical analysis was carried out using Tukey's test of analysis of variance. P < 0.05 was considered significantly different.

Results

Effects of 17βE₂ and GABA on melatonin-enhanced hyperactivation

We examined whether melatonin-enhanced hyperactivation is suppressed by 17βE₂ and GABA in a manner similar to progesterone-enhanced hyperactivation [8, 18]. After swim up, 20 ng/ml 17βE₂ or 5 μM GABA was added to the mTALP medium containing motile spermatozoa, and after additional incubation for 5 min, 1 nM melatonin was added to the same medium. After supplementation with 1 nM melatonin, spermatozoa were incubated for 4 h in order to induce hyperactivation. As shown in Fig. 1A, the percentage of motile spermatozoa was not affected by addition of 1 nM melatonin and 20 ng/ml 17βE₂ when spermatozoa were exposed to 1 nM melatonin after exposure to 17βE₂. In contrast, as shown in Fig. 1B, melatonin-enhanced hyperactivation was significantly suppressed by 20 ng/ml 17βE₂. After incubation for 1 or 1.5 h, melatonin-enhanced hyperactivation was significantly suppressed by 17βE₂ (1 h, Melatonin: 17.4% ± 3.17, 17βE₂ → Melatonin: 10.67% ± 2.45; 1.5 h, Melatonin: 47.89% ± 6.86, 17βE₂ → Melatonin: 26.98% ± 11.72) but not significantly suppressed by 17βE₂ after incubation for 2 h. GABA did not affect the percentages of motile spermatozoa, hyperactivated spermatozoa and melatonin-enhanced hyperactivation (Figs. 1C, 1D).

Estradiol consists of 17αE₂ and 17βE₂. Generally, 17αE₂ is a stereoisomer of 17βE₂ and is at least 200-fold less active as a hormone [22, 23]. After swim up, 20 ng/ml 17αE₂ was added to the mTALP medium containing motile spermatozoa. After additional incubation for 5 min, 1 nM melatonin was added to the same medium. After supplementation with 1 nM melatonin, spermatozoa were incubated for 4 h. As shown in Fig. 2, 20 ng/ml 17αE₂ did not affect the percentages of motile spermatozoa, hyperactivated spermatozoa and melatonin-enhanced hyperactivation.

Effect of tamoxifen on suppression of melatonin-enhanced hyperactivation by 17βE₂

Because melatonin-enhanced hyperactivation was suppressed by 17βE₂ (Fig. 1), we examined whether the mechanism acted via the ER. In order to examine whether the ER was associated with suppression of melatonin-enhanced hyperactivation caused by 17βE₂, we used tamoxifen, which is a popular ER antagonist [24–26]. It was reported that several micromoles of tamoxifen acted as an ER antagonist [27–29], although a high concentration of tamoxifen inhibited protein kinase C (PKC) in some cases [30, 31]. In a previous study [8], 1 μM tamoxifen inhibited only suppression of progesterone-enhanced hyperactivation caused by 17βE₂. After swim up in the present study, 1 μM tamoxifen was added to the mTALP medium containing motile spermatozoa, and after additional incubation for 5 min, 1 nM melatonin was added to the same medium. After supplementation with 1 nM melatonin, spermatozoa were incubated for 4 h. As shown in Figs. 3A and 3B, 1 μM tamoxifen did not affect the percentages of motile spermatozoa, hyperactivated spermatozoa and melatonin-enhanced hyperactivated spermatozoa.
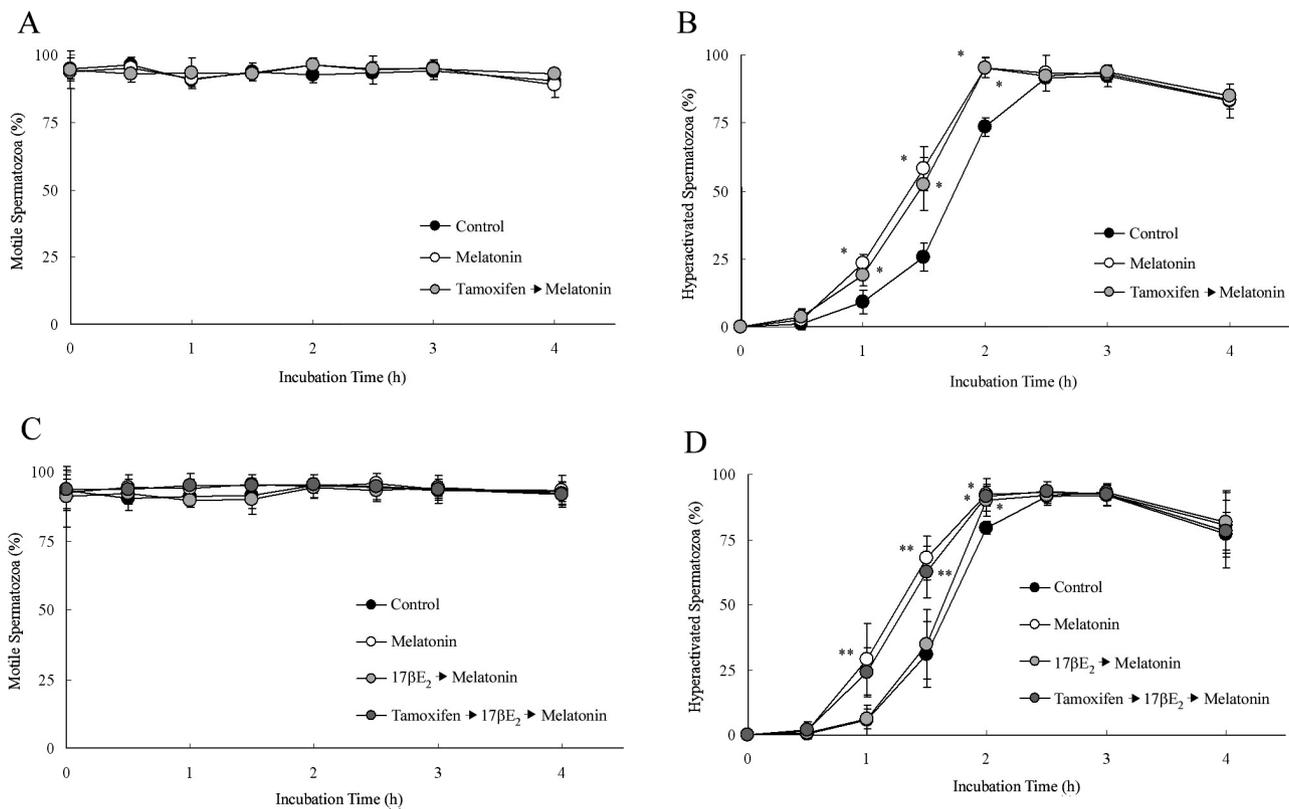
Fig. 3. Inhibition of suppression of melatonin-enhanced hyperactivation by 17βE$_2$. The percentages of motile (A, C) and hyperactivated (B, D) spermatozoa are shown (A, B) for when spermatozoa were exposed to melatonin and tamoxifen, and (C, D) for when spermatozoa were exposed to melatonin, 17βE$_2$ and tamoxifen. In A and B, after swim up, tamoxifen or vehicle was added to the mTALP medium containing motile spermatozoa. After incubation for 5 min, melatonin or vehicle was added to the same medium. In C and D, after swim up, tamoxifen or vehicle was added to the mTALP medium containing motile spermatozoa. After incubation for 5 min, 17βE$_2$ or vehicle was added to the same medium. After incubation for 5 min again, melatonin or vehicle was added to the same medium. Data are expressed as the mean ± SD. (A, B) (Control): mTALP medium with 0.1% (v/v) EtOH and 0.1% (v/v) DMSO; (Melatonin): mTALP medium with addition of 1 nM melatonin, 0.1% (v/v) EtOH and 0.1% (v/v) DMSO; (Tamoxifen → Melatonin): mTALP medium with addition of 1 nM melatonin, 1 μM tamoxifen, 0.1% (v/v) EtOH and 0.1% (v/v) DMSO. (C, D) (Control): mTALP medium with 0.2% (v/v) EtOH and 0.1% (v/v) DMSO; (Melatonin): mTALP medium with addition of 1 nM melatonin, 0.2% (v/v) EtOH and 0.1% (v/v) DMSO; (17βE$_2$ → Melatonin): mTALP medium with addition of 1 nM melatonin, 20 ng/ml 17βE$_2$, 0.2% (v/v) EtOH and 0.1% (v/v) DMSO; (Tamoxifen → 17βE$_2$ →Melatonin): mTALP medium with addition of 1 nM melatonin, 20 ng/ml 17βE$_2$, 1 μM tamoxifen, 0.2% (v/v) EtOH and 0.1% (v/v) DMSO. * Significant difference compared with "Control" (P < 0.05). ** Significant difference compared with "Control" and "17βE$_2$ → Melatonin" (P < 0.05). DMSO, dimethyl sulfoxide; 17βE$_2$, 17β-estradiol; EtOH, ethanol; mTALP medium, modified Tyrode's albumin lactate pyruvate medium.

After swim up, 1 μM tamoxifen was added to the mTALP medium containing motile spermatozoa, and after additional incubation for 5 min, 20 ng/ml 17βE$_2$ was added to the same medium. After additional incubation for 5 min again, 1 nM melatonin was added to the same medium. After supplementation with 1 nM melatonin, spermatozoa were incubated for 4 h. As shown in Figs. 3C and 3D, tamoxifen significantly inhibited suppression of melatonin-enhanced hyperactivation by 17βE$_2$ after incubation for 1 or 1.5 h (1 h, Melatonin: 28.94% ± 13.64, 17βE$_2$ → Melatonin: 6.01% ± 3.81, Tamoxifen → 17βE$_2$ → Melatonin: 23.94% ± 9.52; 1.5 h, Melatonin: 67.89% ± 8.37, 17βE$_2$ → Melatonin: 34.71% ± 13.41, Tamoxifen → 17βE$_2$ → Melatonin: 62.57% ± 9.77), but it did not affect the percentage of motile spermatozoa.

Non-genomic suppression of melatonin-enhanced hyperactivation by 17βE$_2$

Because 17βE$_2$ suppresses progesterone-enhanced hyperactivation through non-genomic regulation via the membrane ER [8], we examined whether melatonin-enhanced hyperactivation was also suppressed by 17βE$_2$ through non-genomic regulation using BSA-17βE$_2$, which binds only to the membrane ER and not to the intracellular ER, thereby blocking entry of BSA-17βE$_2$ into the cell [32, 33].

After swim up, 1 μM tamoxifen was added to the mTALP medium containing motile spermatozoa, and after incubation for 5 min, 7.4 nM BSA-17βE$_2$, which is converted into approximately 20 ng/ml 17βE$_2$, was added to the medium. After additional incubation for 5 min again, 1 nM melatonin was added to the medium. After supplementation with 1 nM melatonin, spermatozoa were incubated for 4 h. As shown in Fig.
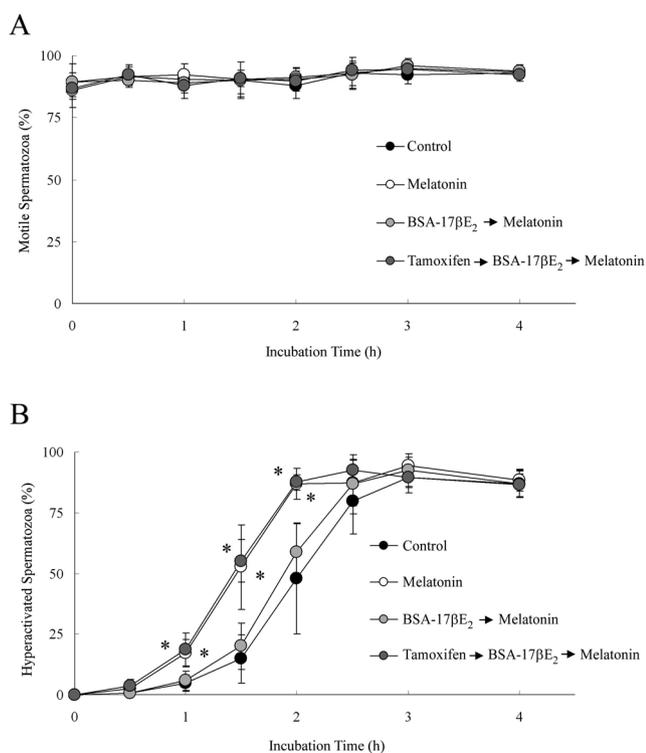
A

B

Fig. 4. Non-genomic suppression of melatonin-enhanced hyperactivation by 17βE$_2$. The percentages of motile (A) and hyperactivated (B) spermatozoa are shown for when spermatozoa were exposed to melatonin, BSA-17βE$_2$ and tamoxifen. After swim up, tamoxifen or vehicle was added to the mTALP medium containing motile spermatozoa. After incubation for 5 min, BSA-17βE$_2$ or vehicle was added to the same medium. After incubation for 5 min again, melatonin or vehicle was added to the same medium. Data are expressed as the mean ± SD. (Control): mTALP medium with 0.1% (v/v) EtOH and 0.1% (v/v) DMSO; (Melatonin): mTALP medium with addition of 1 nM melatonin, 0.1% (v/v) EtOH and 0.1% (v/v) DMSO; (BSA-17βE$_2$ → Melatonin): mTALP medium with addition of 1 nM melatonin, 7.4 nM BSA-17βE$_2$, 0.1% (v/v) EtOH and 0.1% (v/v) DMSO; (Tamoxifen → BSA-17βE$_2$ → Melatonin): mTALP medium with addition of 1 nM melatonin, 7.4 nM BSA-17βE$_2$, 1 μM tamoxifen, 0.1% (v/v) EtOH and 0.1% (v/v) DMSO. * Significant difference compared with "Control" and "BSA-17βE$_2$ → Melatonin" (P < 0.05). DMSO, dimethyl sulfoxide; 17βE$_2$, 17β-estradiol; EtOH, ethanol; BSA-17βE$_2$, fluorescein isothiocyanate and bovine serum albumin (BSA)-conjugated 17βE$_2$; mTALP medium, modified Tyrode's albumin lactate pyruvate medium.

4A, BSA-17βE$_2$ did not affect the percentage of motile spermatozoa. In contrast, BSA-17βE$_2$ significantly suppressed melatonin-enhanced hyperactivation after incubation for 1, 1.5 or 2 h (1 h, Melatonin: 17.31% ± 5.42, BSA-17βE$_2$ → Melatonin: 5.82% ± 3.74; 1.5 h, Melatonin: 52.72% ± 17.39, BSA-17βE$_2$ → Melatonin: 20.06% ± 9.53; 2 h, Melatonin: 87.03% ± 6.32, BSA-17βE$_2$ → Melatonin: 58.87% ± 12.08). Moreover, suppression of melatonin-enhanced hyperactivation by BSA-17βE$_2$ was significantly inhibited by 1 μM tamoxifen as in the case of 17βE$_2$ (1 h, Tamoxifen → BSA-17βE$_2$ → Melatonin: 18.61% ± 6.87; 1.5 h, Tamoxifen → BSA-17βE$_2$ → Melatonin: 55.11% ± 8.85; 1.5 h, Tamoxifen → BSA-17βE$_2$ →

Melatonin: 87.54% ± 3.24).

Dose-dependent effect of 17βE$_2$ on melatonin-enhanced hyperactivation

In the next step, we examined whether 17βE$_2$ suppressed melatonin-enhanced hyperactivation in a dose-dependent manner (Fig. 5) because our previous studies [8, 10] reported that progesterone-enhanced hyperactivation was dose dependently suppressed by 17βE$_2$.

Firstly, we observed sperm movement which were beforehand exposed to estrogen (Figs. 5A and 5B). After swim up, 2 pg/ml to 20 ng/ml 17βE$_2$ were added to the mTALP medium containing motile spermatozoa, and after additional incubation for 5 min, 1 nM melatonin was added to the medium. After supplementation with 1 nM melatonin, spermatozoa were incubated for 4 h. As shown in Fig. 5A, 17βE$_2$ did not affect the percentage of motile spermatozoa at the concentration tested. By contrast, 17βE$_2$ significantly suppressed melatonin-enhanced hyperactivation in a dose-dependent manner (Fig. 5B). After incubation for 1 h, 200 pg/ml to 20 ng/ml 17βE$_2$ significantly suppressed melatonin-enhanced hyperactivation (Melatonin: 21.38% ± 3.49, 200 pg/ml 17βE$_2$ → Melatonin: 6.32% ± 4.22, 2 ng/ml 17βE$_2$ → Melatonin: 5.1% ± 4.32, 20 ng/ml 17βE$_2$ → Melatonin: 4.77% ± 5.06), whereas 2 pg/ml 17βE$_2$ did not. Moreover, the effect of 20 pg/ml 17βE$_2$ was also not significantly different compared with the control and melatonin (Control: 4.48% ± 4.08, Melatonin: 21.38% ± 3.49, 20 pg/ml 17βE$_2$ → Melatonin: 13.85% ± 6.59). After incubation for 1.5 h, 20 ng/ml 17βE$_2$ significantly suppressed melatonin-enhanced hyperactivation (Melatonin: 83.34% ± 2.74, 20 ng/ml 17βE$_2$ → Melatonin: 48.66% ± 20.79), whereas 2 pg/ml to 20 pg/ml 17βE$_2$ did not. The effects of 200 pg/ml and 2 ng/ml 17βE$_2$ were not significantly different compared with the control and melatonin (Control: 45.95% ± 23.47, Melatonin: 83.34% ± 2.74, 200 pg/ml 17βE$_2$ → Melatonin: 58.85% ± 14.95, 2 ng/ml 17βE$_2$ → Melatonin: 52.13% ± 18.52). After incubation for 2 h, 17βE$_2$ did not suppress melatonin-enhanced hyperactivation.

In the next step, hamster spermatozoa were exposed to the mixture of 1 nM melatonin and 2 pg/ml to 20 ng/ml 17βE$_2$ after swim up and incubated for 4 h in order to mimic the physiological action of estrogen in the spermatozoa (Figs. 5C and 5D). As shown in Fig. 5C, 17βE$_2$ did not affect the percentage of motile spermatozoa. By contrast, 17βE$_2$ suppressed melatonin-enhanced hyperactivation in a dose-dependent manner (Fig. 5D). After incubation for 1 h, melatonin slightly enhanced hyperactivation, but the enhancement of hyperactivation caused by melatonin was not significantly different compared with the control (Control: 9.65% ± 2.84, Melatonin: 25.87% ± 8.86). Moreover, 20 pg/ml to 20 ng/ml 17βE$_2$ slightly suppressed the effect of melatonin, but the level of hyperactivation was not significantly different compared with that with melatonin only (Melatonin: 25.87% ± 8.86, Melatonin + 20 pg/ml 17βE$_2$: 18.47% ± 11.8, Melatonin + 200 pg/ml 17βE$_2$: 13.99% ± 7.39, Melatonin + 2 ng/ml 17βE$_2$: 12.73% ± 3.57, Melatonin + 20 ng/ml 17βE$_2$: 10.99% ± 6.28). After incubation for 1.5 h, 2 ng/ml and 20 ng/ml 17βE$_2$ significantly suppressed melatonin-enhanced hyperactivation (Melatonin: 65.55% ± 2.98, Melatonin + 2 ng/ml 17βE$_2$: 36.68% ± 9.41, Melatonin + 20 ng/ml 17βE$_2$: 39.35% ± 7.56), whereas 2 pg/ml to 200 pg/ml 17βE$_2$ did not. After incubation for 2 h, 17βE$_2$ did not suppress melatonin-enhanced hyperactivation.
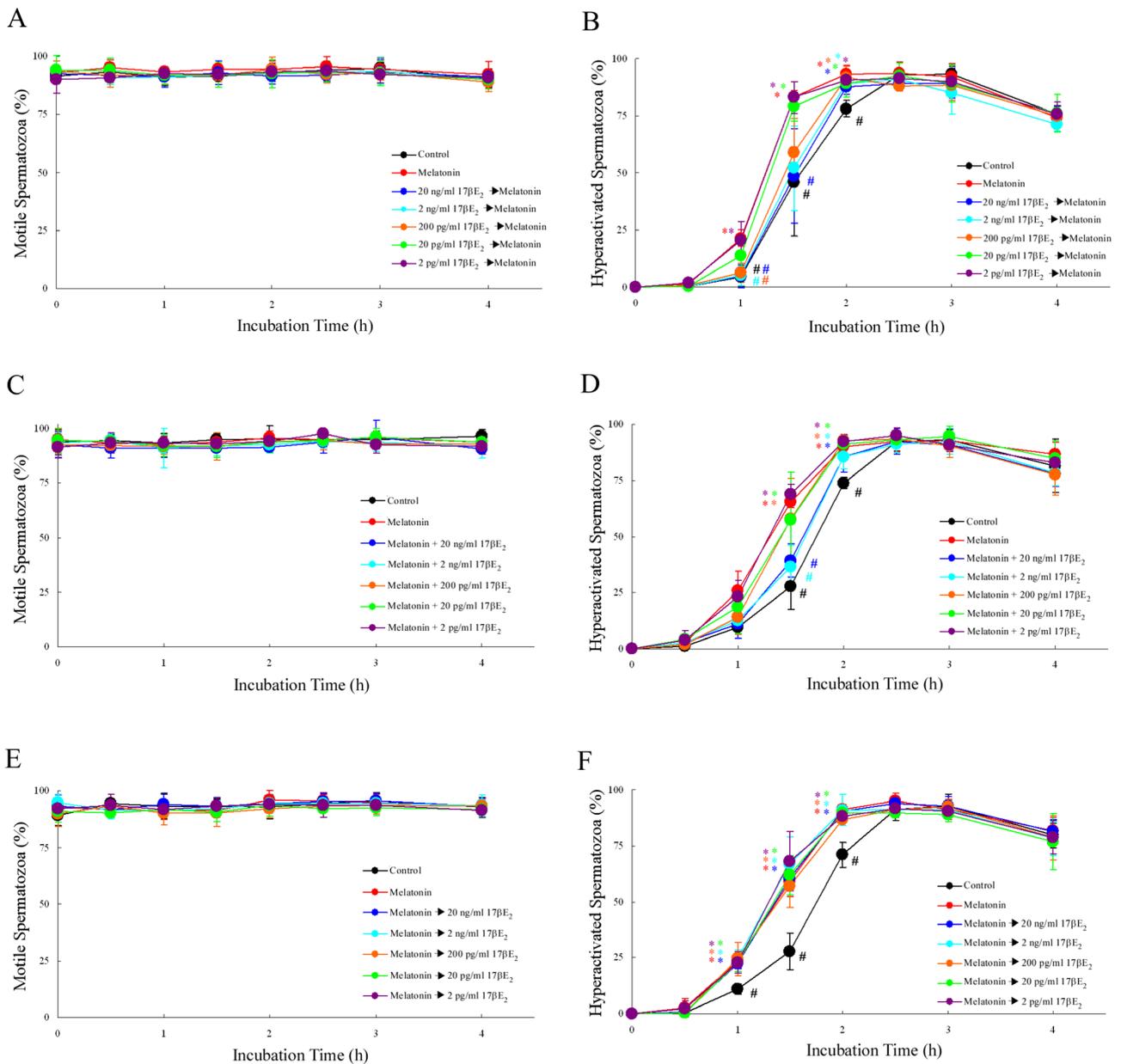
Fig. 5. Dose-dependent effects of 17βE₂ on melatonin-enhanced hyperactivation. The percentages of motile (A, C, E) and hyperactivated (B, D, F) spermatozoa are shown (A, B) for when spermatozoa were exposed to melatonin after exposure to 17βE₂, (C, D) for when spermatozoa were simultaneously exposed to melatonin and 17βE₂, and (E, F) for when spermatozoa were exposed to melatonin before exposure to 17βE₂. In A and B, after swim up, 17βE₂ or vehicle was added to the mTALP medium containing motile spermatozoa. After incubation for 5 min, melatonin or vehicle was added to the same medium. In C and D, after swim up, mixtures of melatonin and 17βE₂ or vehicle were added to the mTALP medium containing motile spermatozoa. In E and F, after swim up, melatonin or vehicle was added to the mTALP medium containing motile spermatozoa. After incubation for 5 min, 17βE₂ or vehicle was added to the same medium. Data are expressed as the mean ± SD. (Control): mTALP medium with 0.2% (v/v) EtOH; (Melatonin): mTALP medium with added 1 nM melatonin and 0.2% (v/v) EtOH; (20 ng/ml 17βE₂ → Melatonin, Melatonin + 20 ng/ml 17βE₂ or Melatonin → 20 ng/ml 17βE₂): mTALP medium with addition of 1 nM melatonin, 20 ng/ml 17βE₂ and 0.2% (v/v) EtOH; (2 ng/ml 17βE₂ → Melatonin, Melatonin + 2 ng/ml 17βE₂ or Melatonin → 2 ng/ml 17βE₂): mTALP medium with addition of 1 nM melatonin, 2 ng/ml 17βE₂ and 0.2% (v/v) EtOH; (200 pg/ml 17βE₂ → Melatonin, Melatonin + 200 pg/ml 17βE₂ or Melatonin → 200 pg/ml 17βE₂): mTALP medium with addition of 1 nM melatonin, 200 pg/ml 17βE₂ and 0.2% (v/v) EtOH; (20 pg/ml 17βE₂ → Melatonin, Melatonin + 20 pg/ml 17βE₂ or Melatonin → 20 pg/ml 17βE₂): mTALP medium with addition of 1 nM melatonin, 20 pg/ml 17βE₂ and 0.2% (v/v) EtOH; (2 pg/ml 17βE₂ → Melatonin, Melatonin + 2 pg/ml 17βE₂ or Melatonin → 2 pg/ml 17βE₂): mTALP medium with addition of 1 nM melatonin, 2 pg/ml 17βE₂ and 0.2% (v/v) EtOH. *Significant difference compared with "Control" (P < 0.05). # Significant difference compared with "Melatonin" (P < 0.05). Items with significant differences are indicated by symbols in the same color. 17βE₂, 17β-estradiol; EtOH, ethanol; mTALP medium, modified Tyrode's albumin lactate pyruvate medium.

Finally, we observed sperm movement which were beforehand exposed to melatonin (Figs. 5E and 5F). Motile spermatozoa were exposed to 2 pg/ml to 20 ng/ml 17βE$_2$ after exposure to 1 nM melatonin for 5 min. After addition of 17βE$_2$ to the mTALP medium, spermatozoa were incubated for 4 h. As shown in Figs. 5E and 5F, 2 pg/ml to 20 ng/ml 17βE$_2$ did not affect the percentages of motile spermatozoa, hyperactivated spermatozoa and melatonin-enhanced hyperactivation.

Discussion

Under capacitation conditions, sperm hyperactivation spontaneously occurs *in vivo* and *in vitro* [1, 3, 34–36]. Spontaneous hyperactivation time-dependently occurs during capacitation processes [1, 3, 34–36]. Recent studies using human and hamster spermatozoa have shown that hyperactivation is enhanced by progesterone, melatonin and serotonin [5–7, 11–13]. Moreover, it has been also shown that progesterone-enhanced hyperactivation of hamster spermatozoa is suppressed by 17βE$_2$ and GABA [8, 10, 18]. Steroids of these hormones, such as progesterone and 17βE$_2$, regulate sperm hyperactivation via non-genomic regulation [2, 7, 8, 37]. In genomic regulation, generally, steroids bind to an intracellular receptor and induce gene expression, whereas in non-genomic regulation, the steroids bind to a membrane receptor and increase the concentration of a second messenger such as Ca^{2+} and/or cAMP [7, 9, 37]. In order to examine whether the regulatory effects of steroids are non-genomic, a BSA-conjugated steroid was used in previous studies [7, 8, 18] and in the present study (Fig. 4). Because BSA blocks entry of a BSA-conjugated steroid into the cell, the steroid is unable to bind to the intracellular receptor but can bind to the membrane receptor [32, 33]. Therefore, it follows that the effects of a BSA-conjugated steroid will occur through non-genomic regulation. The results obtained from the present study (Fig. 4) suggest that enhancement of hyperactivation by melatonin was suppressed by 17βE$_2$ through non-genomic regulation via a membrane ER.

Progesterone regulates hyperactivation through non-genomic regulation associated with two types of Ca^{2+} signaling: Ca^{2+} influx and release of Ca^{2+} from the Ca^{2+} store [2, 7, 36–40]. Ca^{2+} influx is induced by progesterone through the CatSper, which is a sperm-specific Ca^{2+} channel located in the principal piece of the flagellum [41, 42]. The release of Ca^{2+} from the Ca^{2+} store by progesterone is associated with both the PR and PLC [7]. Activation of PLC produces IP$_3$ and diacylglycerol (DAG) from phosphatidylcholine and/or phosphatidylinositol. IP$_3$ releases Ca^{2+} from the IP$_3$R-gated Ca^{2+} store localized at the base of flagellum [36–40]. Ca^{2+} and DAG regulate hyperactivation through activation of calmodulin-dependent protein kinase II and PKC [9, 43]. After Ca^{2+} signaling is stimulated by progesterone, many tyrosine phosphorylations, especially the 80- and 85-kDa tyrosine phosphorylations of the fibrous sheath (FS), are increased and enhanced [7, 8, 36]. The 80- and 85kDa tyrosine phosphorylated FS proteins were identified as the A-kinase anchoring protein, which is a major component of the FS [44]. In general, tyrosine phosphorylation is a very important event during capacitation/hyperactivation [1, 2, 45–47]. It has been suggested that the 80- and 85-kDa tyrosine phosphorylations of the FS are closely associated with capacitation/hyperactivation [19, 46, 48];

and regulated by Ca^{2+}/calmodulin-dependent signals [49] and protein phosphatase 1 [50]. In non-genomic regulation, progesterone also activates adenylate cyclase to increase the cAMP concentration [37, 51, 52]. cAMP is an essential molecule for hyperactivation; and regulates the tyrosine phosphorylations, especially the 80- and 85-kDa tyrosine phosphorylations of the FS, through protein kinase A (PKA) signals [1, 45, 47]. Moreover, progesterone enhances hyperactivation through cAMP–PKA signals [9]. In contrast, 17βE$_2$ suppresses progesterone-enhanced hyperactivation through non-genomic regulation associated with the ER and tyrosine dephosphorylations except for the 80- and 85-kDa tyrosine phosphorylations of the FS [8]. GABA also suppresses progesterone-enhanced hyperactivation via the GABA$_A$ receptor [18]. However, the regulatory mechanisms associated with suppression of progesterone-enhanced hyperactivation by 17βE$_2$ and GABA are still unclear.

It has been shown that melatonin enhances hyperactivation of hamster spermatozoa via MT1 [11]. Moreover, in ram and human spermatozoa, it has been shown that melatonin increases sperm quality, motility, capacitation, fertility rate and the activities of antioxidant enzymes, and decreases nitric oxide (NO) [12, 53–56]. NO at low concentrations increases capacitation through a mitogen-activated protein kinase cascade and tyrosine phosphorylation, especially the 80- and 85-kDa tyrosine phosphorylations of the FS [57–59], whereas at high concentrations, it negatively affects sperm function [58, 60].

It has been shown that progesterone and 17βE$_2$ bind to the head of hamster spermatozoa [7, 8], but it has not been shown where melatonin binds [11]. Ram spermatozoa have two melatonin receptors (MT1 and MT2), which are localized at various sites [61]. In humans, the MT1 receptor is located in the equatorial region of the sperm head [62]. In the present study, we did not show the regulatory mechanism of sperm hyperactivation caused by melatonin and 17βE$_2$. One of the possibilities is the suppression of binding of melatonin to the melatonin receptor by 17βE$_2$. Another is the suppression of intracellular melatonin signals by 17βE$_2$. In a study investigating the regulation of sperm hyperactivation caused by progesterone and 17βE$_2$, neither steroid interfered with binding to their respective receptors [8]. In contrast, one of the intracellular progesterone signal, such as tyrosine phosphorylation, was suppressed by 17βE$_2$ [8]. We expect that 17βE$_2$ affects intracellular melatonin signals in a manner similar to the regulatory mechanism of progesterone and 17βE$_2$ because we previously noted that melatonin enhanced sperm hyperactivation through enhancement of tyrosine phosphorylations [11]. In future studies, we will show the detailed interaction between melatonin and 17βE$_2$.

Progesterone enhances hamster sperm hyperactivation in a dose-dependent manner [7], and progesterone-enhanced hyperactivation is dose-dependently suppressed by 17βE$_2$ and GABA [8, 10, 18]. The concentrations of progesterone and 17βE$_2$ in blood and tissues fluctuate in association with the estrous or menstrual cycle [63]. The concentration of GABA in the female genital tract also fluctuates in association with the estrous cycle [64]. Therefore, we previously proposed that hamster sperm hyperactivation was regulated by balances among the concentrations of progesterone, 17βE$_2$ and GABA [8, 10, 18]. Additionally, picomole or higher concentrations of melatonin enhance hamster sperm hyperactivation [11]. It was reported that picomole or higher concentrations of melatonin were

detected from the follicular fluid of humans [65, 66]. Based on these reports, we tried to examine the interactions among melatonin, $17\beta E_2$ and GABA; in the present study and found that $17\beta E_2$ suppressed melatonin-enhanced hyperactivation in a dose-dependent manner, whereas GABA did not (Figs. 1, 4 and 5). $17\beta E_2$ suppressed melatonin-enhanced hyperactivation through non-genomic regulation via the ER (Figs. 3 and 4). In another recent study [13], it was reported that serotonin also enhanced hamster sperm hyperactivation. These results and results of previous studies [7, 8, 10, 11, 13, 18] suggest that in the hamster, progesterone, melatonin and serotonin act as enhancers of hyperactivation; and that $17\beta E_2$ and GABA act as suppressors. Although we did not investigate relationships among the regulation by progesterone, $17\beta E_2$ and GABA, the regulation by melatonin and $17\beta E_2$ and the regulation by serotonin, we propose that materials in the follicular fluid, such as progesterone, melatonin, serotonin, $17\beta E_2$ and GABA, regulate hyperactivation of hamster spermatozoa.

It has been suggested that spermatozoa are capacitated/hyperactivated in response to changes in the environment of the oviduct [7, 8, 10, 11, 13, 18, 35]. During capacitation/hyperactivation of hamster spermatozoa, progesterone, melatonin and serotonin act as enhancers and $17\beta E_2$, and GABA acts as a suppressor [7, 8, 10, 11, 13, 18]. Although GABA acts as a suppressor of hamster spermatozoa [18], it is an enhancer of other mammalian spermatozoa such as those of humans, rams and rats [14–17]. Because the concentrations of hormones vary during the female's estrous cycle [63], it seems that mammalian spermatozoa are capacitated/hyperactivated in response to the changing environment of the oviduct [8, 10, 18, 35]. In particular, the results of the present study suggest that mammalian spermatozoa, at least hamster spermatozoa, are capacitated/hyperactivated according to changing concentrations of melatonin and $17\beta E_2$ in the oviduct.

There are two regulatory mechanisms of hyperactivation in mammalian or at least hamster spermatozoa. One is a spontaneous regulatory mechanism associated with Ca^{2+}, HCO_3^-, protein phosphorylations and so on [1–3, 45, 47]. Another is a ligand-dependent modulatory mechanism associated with progesterone, $17\beta E_2$, melatonin, serotonin and GABA [7–11, 13, 18] (Fig. 1). In both mechanisms, Ca^{2+} signals, cAMP–PKA signals and tyrosine phosphorylations are very important [1–3, 7–9, 19, 45, 47]. However, the ligand-dependent modulatory mechanism differs from the spontaneous regulatory mechanism because hyperactivation itself is not suppressed by inhibition of the ligand-dependent modulatory mechanism [7–9, 11, 13, 18] (Fig. 1).

In conclusion, we propose that spermatozoa start to be capacitated/hyperactivated through the spontaneous regulatory mechanism in the oviduct. After that, it seems that capacitation/hyperactivation of spermatozoa is enhanced through a modulatory mechanism associated with changes in the environment of the oviduct.

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