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ORIGINAL ARTICLE

Regulation and disruption of hamster sperm hyperactivation by progesterone, 17β-estradiol and diethylstilbestrol

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Abstract
Purpose Hyperactivation of hamster sperm is dose-dependently enhanced by progesterone (P) and 17β-estradiol (E). In the first part of the present study, enhancement of hyperactivation in response to the concentrations of P and E was examined in detail and in the second part, it was examined whether enhancement of hyperactivation by P and E was disrupted by diethylstilbestrol (DES).
Methods Hamster spermatozoa were hyperactivated by incubation in modified Tyrode's albumin lactate pyruvate medium with P, E and/or DES. After spermatozoa were recorded using a video-microscope, observations were quantified by manually counting the numbers of total, motile and hyperactivated spermatozoa.
Results Hyperactivation was enhanced in response to the concentrations of P and E. When spermatozoa were exposed to DES with E, moreover, DES significantly and strongly suppressed P-enhanced hyperactivation by accelerating the effect of E, but DES itself only weakly suppressed P-enhanced hyperactivation.
Conclusions Enhancement of hyperactivation was regulated by the concentrations of P and E, suggesting that in vivo hamster spermatozoa are hyperactivated through "monitoring" these concentrations in the oviduct. DES in combination with E suppressed P-enhanced hyperactivation, suggesting that DES significantly disrupts hyperactivation by acting as an accelerator of the effect of E.

Keywords Diethylstilbestrol · Estradiol · Hyperactivation · Progesterone · Spermatozoa

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Introduction

Mammalian spermatozoa must be capacitated before they can fertilize an oocyte [1, 2]. Capacitated spermatozoa exhibit an acrosome reaction (AR) in the head and hyperactivation in the flagellum, both of which are required for penetration of the zona pellucida of the egg [1]. Capacitation can be made to occur in vitro using a specialized culture medium containing albumin, bicarbonate, calcium and energy sources [e.g., modified Tyrode's albumin lactate pyruvate medium (mTALP medium)]. Albumin removes cholesterol from the sperm membrane, changing its fluidity [3]; bicarbonate stimulates adenylate cyclase (AC) to increase the concentration of cyclic adenosine monophosphate, which in turn activates protein kinase A (PKA), and induces protein phosphorylation [4–11]; calcium is also involved in many of the signal pathways associated with protein phosphorylation [5, 12–16]. Albumin and calcium are essential for sperm to be capacitated [17–19].

The AR and hyperactivation are induced/enhanced by progesterone (P), and these effects of P are suppressed by 17β-estradiol (E) [17, 20–29]. However, the regulatory mechanisms are not the same, because the effective concentrations of P and E differ for the AR and hyperactivation: μg/mL or μM versus ng/mL or nM, respectively [17, 30]. In the hamster, 20 ng/mL P is the concentration in oviductal fluid, which effectively increases/enhances penetration and hyperactivation, but does not induce the AR [17, 30, 31]. Moreover, P and E dose-dependently regulate the enhancement of hyperactivation [17, 28].

Endocrine-disrupting chemicals [e.g., diethylstilbestrol (DES)] have estrogenic effects and bind to an estrogen receptor (ER). Antenatal and neonatal treatment of animals with DES significantly affects their reproductive systems

[32, 33], but it is not well known whether DES directly affects gametic functions such as oocyte maturation, sperm capacitation and fertilization.

Therefore, in the 2-stage experiment reported here, the first part of the study examined whether enhancement of hyperactivation depended on the concentrations of P and E, then, secondly, whether DES disrupted the enhancement of hyperactivation by P and E.

Materials and methods

Chemicals

DES, E, P and tamoxifen were purchased from Sigma Chemical Company (St. Louis, MO, USA), bovine serum albumin (BSA) fraction V was purchased from Merck KGaA (Darmstadt, Germany), and all other reagent-grade chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of hyperactivated spermatozoa

Spermatozoa were obtained from the caudal epididymis of sexually mature, male golden hamsters (*Mesocricetus auratus*). The experimental protocols were approved by the Animal Care and Use Committee of the Dokkyo Medical University, and the experiment was carried out under the control of the Guidelines for Animal Experimentation in the Dokkyo Medical University.

Hyperactivated spermatozoa were prepared according to the method described previously [11] using mTALP medium containing 101.02 mM NaCl, 2.68 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2{\cdot}6H_2O$, 360 μM $NaH_2PO_4{\cdot}2H_2O$, 35.7 mM $NaHCO_3$, 4.5 mM D-glucose, 90 μM sodium pyruvate, 9 mM sodium lactate, 500 μM hypotaurine, 50 μM (−)epinephrine, 200 μM sodium taurocholic acid, 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]. An aliquot (~5 μL) of spermatozoa was placed on a culture plate (35-mm dish), 3 mL of the medium were carefully added and the mixture was incubated for 5 min to allow spermatozoa to swim up. The supernatant containing motile spermatozoa was re-plated onto a new culture plate and incubated for 4 h at 37 °C under 5 % CO_2 in air to accomplish hyperactivation. P, E, DES and tamoxifen were added to the medium after re-plating motile spermatozoa onto a new culture plate. When multiple chemicals were added to the medium, the additional chemical was added to the medium every time motile spermatozoa were re-plated onto a new culture plate after exposure to the primary chemical for 5 min. Each experimental condition is shown in Tables 1 and 2. P, E and DES were dissolved in ethanol. Tamoxifen was dissolved in dimethyl sulfoxide. In all experiments, the maximal concentration of the vehicle was 0.3 %.

Table 1 Experimental condition about dose-dependent regulation of enhancement of hyperactivation by progesterone and 17β-estradiol as shown in Fig. 2

	Concentration of steroids	
	Progesterone (P) (ng/mL)	17β-Estradiol (E)
Experiment 1 (Fig. 2a, b)		
Vehicle	–	–
10 ng/mL P	10	–
P + 1 pg/mL E	10	1 pg/mL
P + 10 pg/mL E	10	10 pg/mL
P + 100 pg/mL E	10	100 pg/mL
P + 1 ng/mL E	10	1 ng/mL
P + 10 ng/mL E	10	10 ng/mL
Experiment 2 (Fig. 2c, d)		
Vehicle	–	–
20 ng/mL P	20	–
P + 2 pg/mL E	20	2 pg/mL
P + 20 pg/mL E	20	20 pg/mL
P + 200 pg/mL E	20	200 pg/mL
P + 2 ng/mL E	20	2 ng/mL
P + 20 ng/mL E	20	20 ng/mL
Experiment 3 (Fig. 2e, f)		
Vehicle	–	–
40 ng/mL P	40	–
P + 4 pg/mL E	40	4 pg/mL
P + 40 pg/mL E	40	40 pg/mL
P + 400 pg/mL E	40	400 pg/mL
P + 4 ng/mL E	40	4 ng/mL
P + 40 ng/mL E	40	40 ng/mL

Measurement of motility and hyperactivation of spermatozoa

Motility and hyperactivation measurements were performed according to the method described previously [11] with some modifications. Motile spermatozoa were recorded on videotape via a CCD camera (Progressive 3CCD, Sony Corp., Tokyo, Japan) attached to a microscope (IX70, Olympus Corp., Tokyo, Japan) with phase-contrast illumination and a small CO_2 incubator (MI-IBC, Olympus). Each observation was performed at 37 °C, recorded for 2 min, and quantified by manually counting the numbers of total spermatozoa, motile spermatozoa and hyperactivated spermatozoa in 8 different fields. Motile spermatozoa that exhibited large bend amplitude, asymmetric and whiplash flagellar movement and a circular and/or octagonal swimming locus were defined as hyperactivated [1, 27, 34–36].

Table 2 Experimental conditions for dose-dependent regulation of enhancement of hyperactivation by progeteone, 17β-estradiol and diethylstilbestrol as shown in Figs. 6 and 7

	Concentration of steroids		
	Progesterone (P) (ng/mL)	17β-Estradiol (E) (pg/mL)	Diethylstilbestrol (DES) (pg/mL)
Experiment 1 (Fig. 6a, b)			
Vehicle	–	–	–
20 ng/mL P	20	–	–
P + E (2 pg/mL)	20	2	–
P + DES (2 pg/mL)	20	–	2
P + mixture of E and DES	20	2	2
Experiment 2 (Fig. 6c, d)			
Vehicle	–	–	–
20 ng/mL P	20	–	–
P + E (20 pg/mL)	20	20	–
P + DES (2 pg/mL)	20	–	2
P + mixture of E and DES	20	20	2
Experiment 3 (Fig. 6e, f)			
Vehicle	–	–	–
20 ng/mL P	20	–	–
P + E (20 pg/mL)	20	20	–
P + DES (20 pg/mL)	20	–	20
P + mixture of E and DES	20	20	20
Experiment 4 (Fig. 7)			
Vehicle	–	–	–
20 ng/mL P	40	–	–
P + E (4 pg/mL)	40	4	–
P + DES (4 pg/mL)	40	–	4
P + mixture of E and DES	40	4	4

The percentage of motile spermatozoa was defined as the number of motile spermatozoa/number of total spermatozoa × 100. The percentage of hyperactivated spermatozoa was defined as the number of hyperactivated spermatozoa/number of total spermatozoa × 100. Experiments were performed four times using four hamsters.

Statistical analysis

Data were analyzed using the post hoc analysis of variance (ANOVA) test. $P < 0.05$ was considered significant.

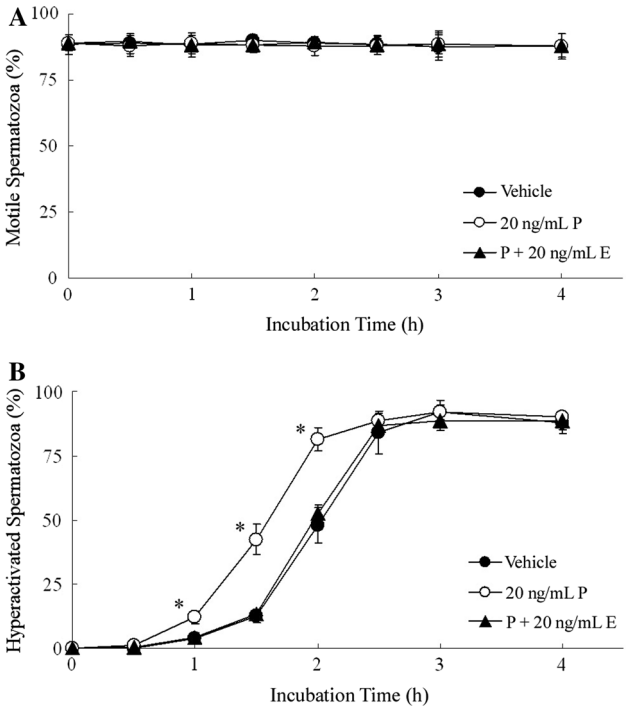

Fig. 1 Effects of progesterone (P) and 17β-estradiol (E) on sperm motility and hyperactivation. It shows an effect of P and E on percentages of motile spermatozoa (**a**) and hyperactivation (**b**) when spermatozoa were exposed to 20 ng/mL P after exposure to 20 ng/mL E. Data are expressed as mean ± SD. In **a** and **b**, "Vehicle" refers to mTALP medium + 0.2 % ethanol. "20 ng/mL P" is vehicle + 20 ng/mL P. "P + 20 ng/mL E" is vehicle + 20 ng/mL P + 20 ng/mL E. *Significant difference compared with "Vehicle" ($P < 0.05$)

Results

Regulation of enhancement of hyperactivation by concentrations of P and E

As shown in Fig. 1 and a previous study [28], E suppressed P-enhanced hyperactivation after incubation for 1, 1.5 and 2 h, although neither P nor E affected the percentage of motile spermatozoa. So, first the dose-dependent regulation of enhancement of hyperactivation by P and E was examined in detail (Fig. 2). Because it has been suggested that the effective concentration of P to enhance hyperactivation is 10–40 ng/mL and the most effective concentration of P is 20 ng/mL [17], the effects of 10–40 ng/mL P on hyperactivation were examined. The effects of E at 1 pg/mL to 40 ng/mL on P-enhanced hyperactivation were examined because the effective concentration of E to suppress P-enhanced hyperactivation was >1/1000 of the concentration of P [28]. As shown in Table 1, three types of experiment were performed in order to examine the dose-dependent regulation of enhancement of hyperactivation by P and E. In the first experiment, spermatozoa were exposed to 10 ng/mL P after exposure to 1 pg/mL to

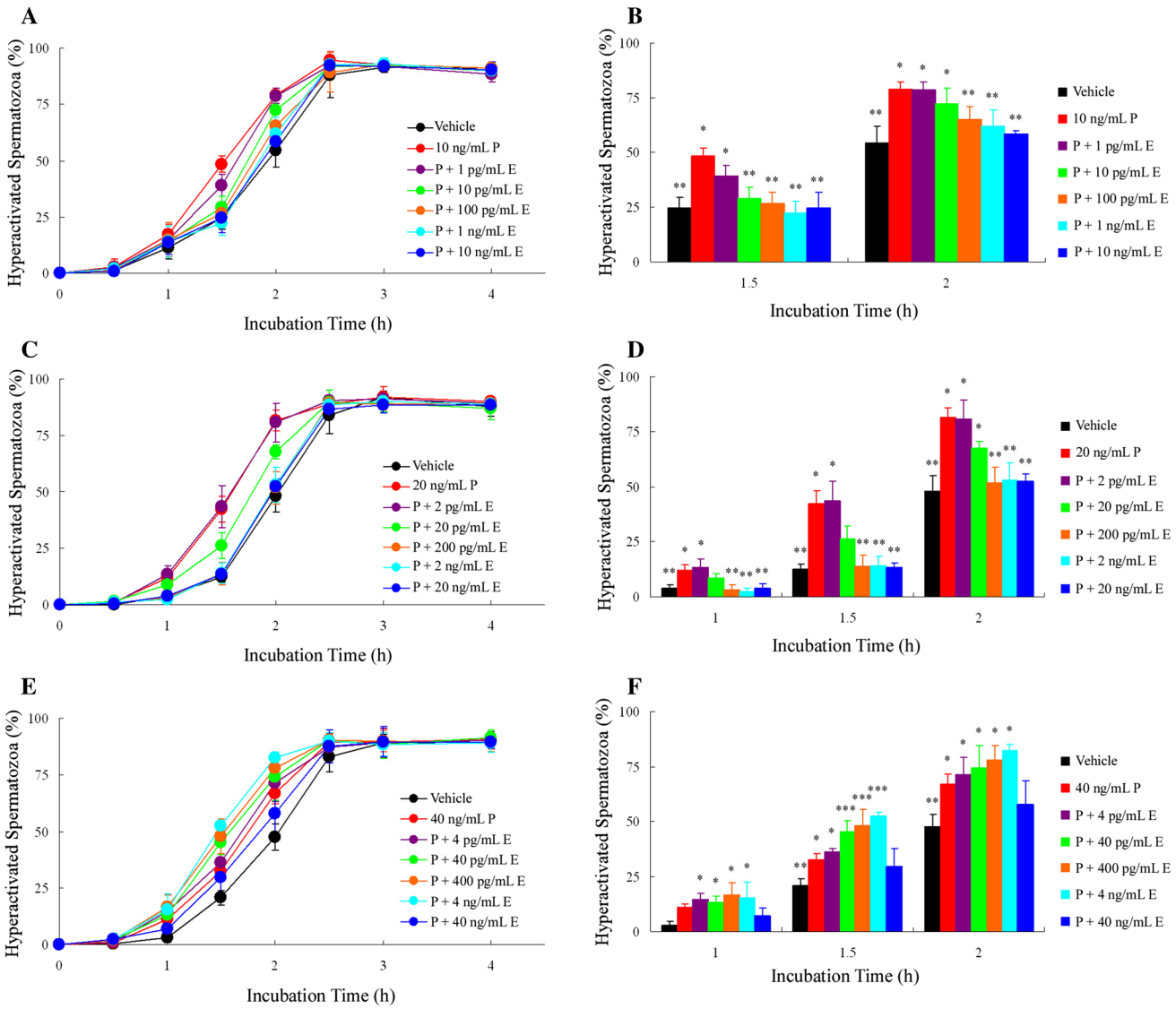

Fig. 2 Effects of 17β-estradiol (E) on progesterone (P)-enhanced hyperactivation when hamster spermatozoa were exposed to P after exposure to E for 5 min. Each experimental condition is shown in Table 1. It shows an overview of the effects of P and E on the percentage of hyperactivation (**a**, **c**, **e**) and details of significant effects (**b**, **d**, **f**). Data are expressed as mean ± SD. In all graphs, "Vehicle" refers to mTALP medium + 0.2 % ethanol. In **a** and **b**, "10 ng/mL P" is vehicle + 10 ng/mL P. P + all concentrations of E are vehicle + 10 ng/mL P + respective concentration of E. In **c** and **d**, "20 ng/mL P" is vehicle + 20 ng/mL P. P + all concentrations of E are vehicle + 20 ng/mL P + respective concentration of E. In **e** and **f**, "40 ng/mL P" is vehicle + 40 ng/mL P. P + all concentrations of E are vehicle + 40 ng/mL P + respective concentration of E. *Significant difference compared with "Vehicle" ($P < 0.05$). **Significant difference compared with "10 ng/mL P", "20 ng/mL P" or "40 ng/mL P" ($P < 0.05$). ***Significant difference compared with "Vehicle" and "40 ng/mL P" ($P < 0.05$)

10 ng/mL E for 5 min (Table 1; Fig. 2a, b). In the second experiment, spermatozoa were exposed to 20 ng/mL P after exposure to 2 pg/mL to 20 ng/mL E for 5 min (Table 1; Fig. 2c, d). In the third experiment, spermatozoa were exposed to 40 ng/mL P after exposure to 4 pg/mL to 40 ng/mL E for 5 min (Table 1; Fig. 2e, f).

As shown in Fig. 2a, enhancement of hyperactivation by 10 ng/mL P was weakly but significantly suppressed by 1 pg/mL to 10 ng/mL E in a dose-dependent manner after incubation for 1.5 and 2 h. After incubation for 1.5 h (Fig. 2b), 10 pg/mL to 10 ng/mL E significantly suppressed the enhancement of hyperactivation by 10 ng/mL P. After incubation for 2 h, 100 pg/mL to 10 ng/mL E significantly suppressed the enhancement of hyperactivation by 10 ng/mL P.

As shown in Fig. 2c, enhancement of hyperactivation by 20 ng/mL P was also significantly suppressed by 20 pg/mL to 20 ng/mL E in a dose-dependent manner after incubation for 1, 1.5 and 2 h. After incubation for 1, 1.5 and 2 h, 200 pg/mL to 20 ng/mL E clearly suppressed the enhancement of

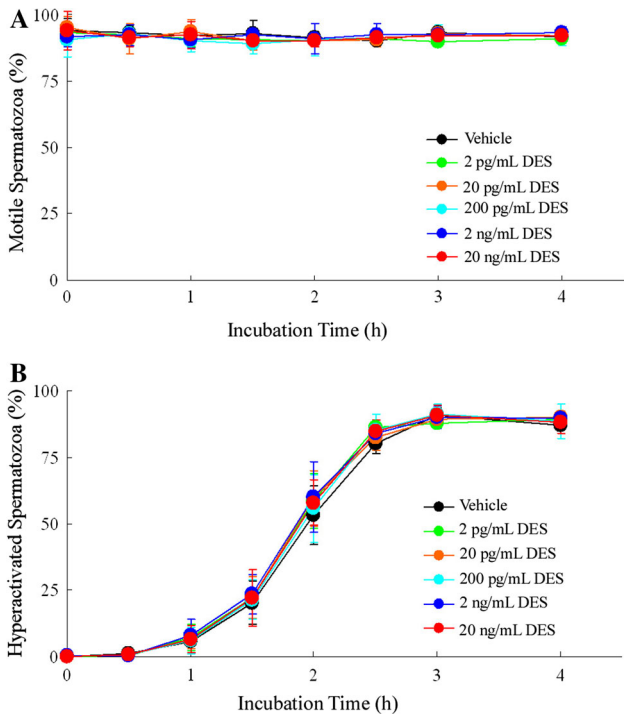

Fig. 3 Effects of diethylstilbestrol (DES) on sperm motility and hyperactivation. It shows an effect of DES on percentages of motile spermatozoa (**a**) and hyperactivation (**b**) when spermatozoa were exposed to 2 pg/mL to 20 ng/mL DES. Data are expressed as mean ± SD. In all graphs, "Vehicle" refers to mTALP medium + 0.1 % ethanol. All concentrations of DES are vehicle + respective concentration of DES

hyperactivation by 20 ng/mL P (Fig. 2d). Although 20 pg/mL E did not significantly suppress the enhancement of hyperactivation by 20 ng/mL P after incubation for 1 and 1.5 h, it did not suppress the enhancement of hyperactivation by P after incubation for 2 h.

When spermatozoa were exposed to 40 ng/mL P after exposure to 4 pg/mL to 40 ng/mL E, there was no suppression of the enhancement of hyperactivation (Fig. 2e). Enhancement of hyperactivation by 40 ng/mL P was significantly enhanced by E in a dose-dependent manner. Although 40 ng/mL P did not significantly enhance hyperactivation after incubation for 1 h, it significantly enhanced hyperactivation after incubation for 1.5 and 2 h (Fig. 2f). On the other hand, 40 ng/mL E weakly suppressed the enhancement of hyperactivation by 40 ng/mL P, although its suppression was not significantly different compared with vehicle and P after incubation for 1, 1.5 and 2 h (Fig. 2f). After incubation for 1 and 2 h, 4 pg/mL to 4 ng/mL E did not affect the enhancement of hyperactivation by 40 ng/mL P. After incubation for 1.5 h, 4 pg/mL E did not affect the enhancement of hyperactivation by 40 ng/mL P, but 40 pg/mL to 4 ng/mL E significantly enhanced the hyperactivation by 40 ng/mL P.

Effects of DES on hyperactivation and P-enhanced hyperactivation

Because P and E regulated the enhancement of hyperactivation in a dose dependent manner (Fig. 2), the study also examined whether endocrine-disrupting chemicals (i.e., DES) disrupted this regulation. As shown in Fig. 3, 2 pg/mL to 20 ng/mL DES did not affect the percentages of motile and hyperactivated spermatozoa. However, when spermatozoa were exposed to 20 ng/mL P after exposure to 2 pg/mL to 20 ng/mL DES, the enhancement of hyperactivation by 20 ng/mL P was weakly but significantly suppressed by DES after incubation for 1 and 1.5 h in a dose-dependent manner (Fig. 4a). After incubation for 1 and 1.5 h (Fig. 4b), 2 and 20 ng/mL DES significantly suppressed the enhancement of hyperactivation by 20 ng/mL P, whereas 2–200 pg/mL DES did not affect it.

Because it has been suggested that DES binds to an ER to exert its estrogenic effects [32, 33], tamoxifen (an ER

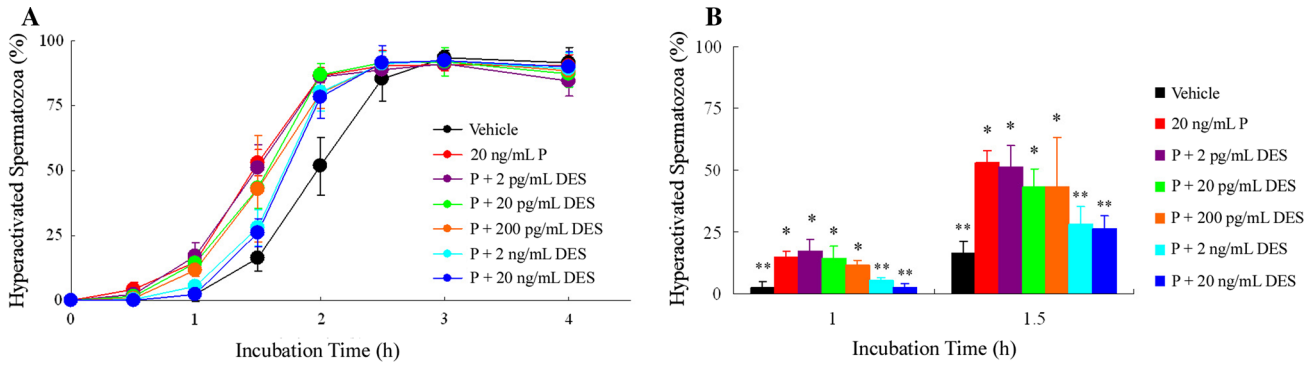

Fig. 4 Effects of diethylstilbestrol (DES) on progesterone (P)-enhanced hyperactivation. It shows an overview of effects of P and DES on the percentage of hyperactivation (**a**) and detailed percentages of hyperactivation after incubation for 1 and 1.5 h (**b**). Spermatozoa were exposed to 20 ng/mL P after exposure to 2 pg/mL to 20 ng/mL DES for 5 min. Data are expressed as mean ± SD. In all graphs, "Vehicle" refers to mTALP medium + 0.2 % ethanol. "20 ng/mL P" is vehicle + 20 ng/mL P. P + all concentrations of DES are vehicle + 20 ng/mL P + respective concentration of DES. *Significant difference compared with "Vehicle" ($P < 0.05$). **Significant difference compared with "20 ng/mL P" ($P < 0.05$)

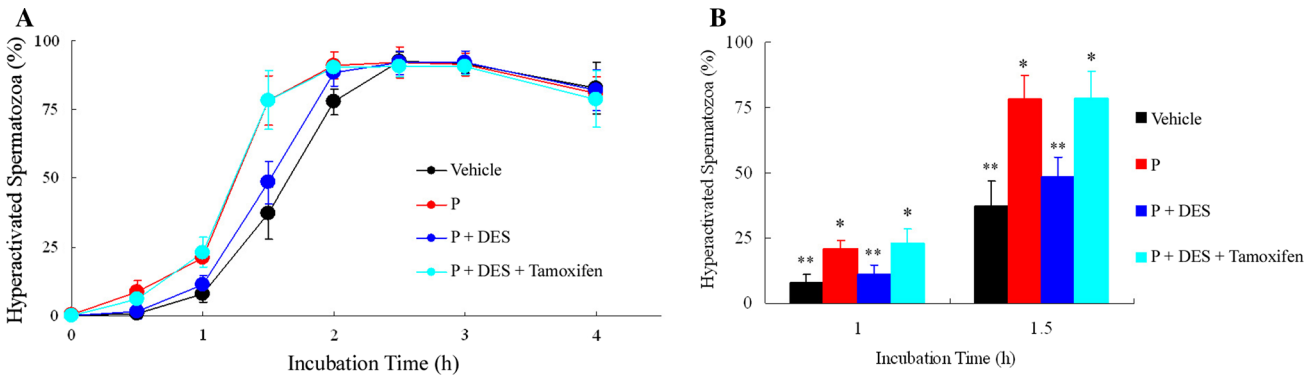

Fig. 5 Inhibition of the effect of diethylstilbestrol (DES) on progesterone (P)-enhanced hyperactivation by tamoxifen. It shows an overview of inhibition by tamoxifen on an effect of DES on P-enhanced hyperactivation (**a**) and detailed percentages of hyperactivation after incubation for 1 and 1.5 h (**b**). Spermatozoa were exposed to 20 ng/mL DES after exposure to 1 μM tamoxifen for 5 min. After incubation for 5 min, they were exposed to 20 ng/mL P. Data are expressed as mean ± SD. In all graphs, "Vehicle" refers to mTALP medium + 0.1 % ethanol + 0.1 % dimethyl sulfoxide. "P" is vehicle + 20 ng/mL P. "P + DES" and "P + DES + tamoxifen" are vehicle + 20 ng/mL P + 20 ng/mL DES and vehicle + 20 ng/mL P + 20 ng/mL DES + 1 μM tamoxifen. *Significant difference compared with "Vehicle" ($P < 0.05$). **Significant difference compared with "P" ($P < 0.05$)

antagonist) was used to examine whether DES suppressed P-enhanced hyperactivation via the ER (Fig. 5). After spermatozoa were exposed to 1 μM tamoxifen for 5 min, they were exposed to 20 ng/mL DES. After incubation for 5 min, they were exposed to 20 ng/mL P. Tamoxifen significantly inhibited the suppression of P-enhanced hyperactivation by DES after incubation for 1 and 1.5 h.

Acceleration of the estrogenic effect of DES on the suppression of P-enhanced hyperactivation

Although DES significantly suppressed P-enhanced hyperactivation, the effect was very weak (Fig. 4). However, in vivo DES would affect sperm function together with E, so the next stage of the study examined whether DES accelerated the effect of E on P-enhanced hyperactivation. As shown in Table 2, four types of experiment were performed in order to examine the effects of DES on the dose-dependent regulation of enhancement of hyperactivation by P and E. In the first experiment, spermatozoa were exposed to 20 ng/mL P after exposure to 2 pg/mL E, 2 pg/mL DES or a mixture of them for 5 min (Table 2; Fig. 6a, b). In the second experiment, spermatozoa were exposed to 20 ng/mL P after exposure to 20 pg/mL E, 2 pg/mL or a mixture of them for 5 min (Table 2; Fig. 6c, d). In the third experiment, spermatozoa were exposed to 20 ng/mL P after exposure to 20 pg/mL E, 20 pg/mL DES and a mixture of them for 5 min (Table 2; Fig. 6e, f). In the fourth experiment, spermatozoa were exposed to 40 ng/mL P after exposure to 4 pg/mL E, 4 pg/mL DES and a mixture of them for 5 min (Table 2; Fig. 7).

As shown in Fig. 6a, 2 pg/mL E and 2 pg/mL DES did not affect the enhancement of hyperactivation by 20 ng/mL P whereas the mixture of them slightly suppressed the enhancement of hyperactivation by 20 ng/mL P after incubation for 1 and 1.5 h. After incubation for 1 h (Fig. 6b), suppression of P-enhanced hyperactivation by the mixture of 2 pg/mL E and 2 pg/mL DES was not significantly different compared with vehicle and P. The mixture did not suppress the enhancement of hyperactivation by 20 ng/mL P after incubation for 1.5 h (Fig. 6b).

When spermatozoa were exposed to 20 ng/mL P after exposure to 20 pg/mL E, 2 pg/mL DES or a mixture of them, as shown in Fig. 6c, d, 20 pg/mL E and 2 pg/mL DES did not significantly suppress the enhancement of hyperactivation by 20 ng/mL P, but the mixture significantly suppressed the enhancement of hyperactivation by 20 ng/mL P after incubation for 1 and 1.5 h. After incubation for 1, 1.5 and 2 h, 20 pg/mL E was not significantly different compared with vehicle and P (Fig. 6d). Moreover, the mixture was not also significantly different compared with vehicle and P after incubation for 2 h.

When spermatozoa were exposed to 20 ng/mL P after exposure to 20 pg/mL E, 20 pg/mL DES or a mixture of them, the mixture significantly suppressed the enhancement of hyperactivation by 20 ng/mL P after incubation for 1, 1.5 and 2 h (Fig. 6e, f). After incubation for 1 and 1.5 h, 20 pg/mL E was not significantly different compared with vehicle and P (Fig. 6f). After incubation for 2 h, 20 pg/mL E did not affect the enhancement of hyperactivation by 20 ng/mL P. On the other hand, 20 pg/mL DES did not affect the enhancement of hyperactivation by 20 ng/mL P (Figs. 4, 6e).

In the fourth experiment (Fig. 7), spermatozoa were exposed to 40 ng/mL P after exposure to 4 pg/mL E, 4 pg/mL DES or a mixture of them. As shown in Fig. 7a,

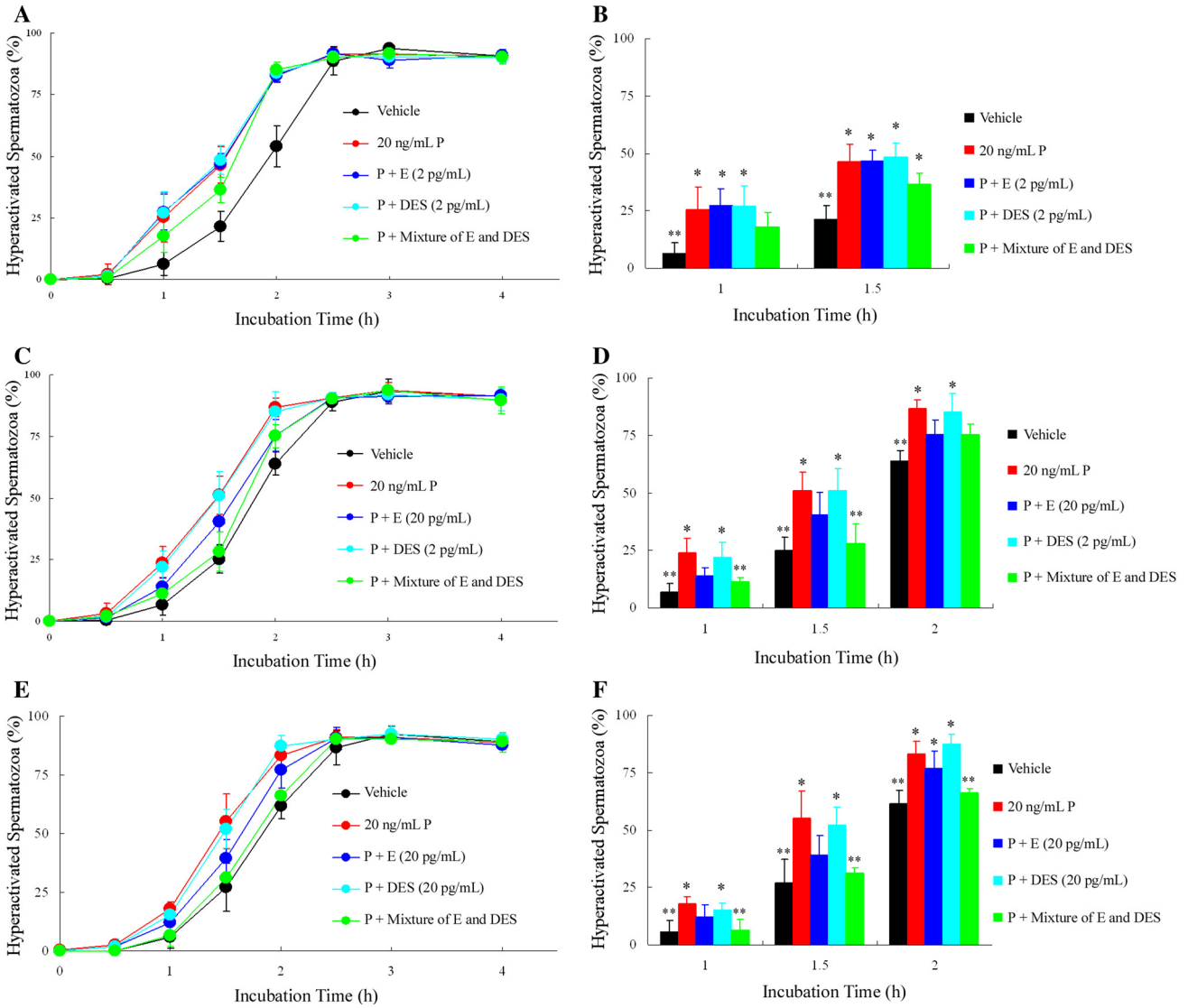

Fig. 6 Effects of 17β-estradiol (E) and diethylstilbestrol (DES) on progesterone (P)-enhanced hyperactivation when spermatozoa were exposed to P after exposure to E, DES or a mixture of them for 5 min. Each experimental condition is shown in Table 2. It shows an overview of effects of P, E, DES and the mixture on the percentage of hyperactivation (**a**, **c**, **e**) and details of significant effects (**b**, **d**, **f**). Data are expressed as mean ± SD. In all graphs, "Vehicle" refers to mTALP medium + 0.3 % ethanol. "20 ng/mL P" is vehicle + 20 ng/mL P. In **a** and **b**, "P + E (2 pg/mL)", "P + DES (2 pg/mL)" and "P + mixture of E and DES" are vehicle + 20 ng/mL P + 2 pg/mL E, vehicle + 20 ng/mL P + 2 pg/mL DES and vehicle + 20 ng/mL P + a mixture of 2 pg/mL E and 2 pg/mL DES. In **c** and **d**, "P + E (20 pg/mL)", "P + DES (2 pg/mL)" and "P + mixture of E and DES" are vehicle + 20 ng/mL P + 20 pg/mL E, vehicle + 20 ng/mL P + 2 pg/mL DES and vehicle + 20 ng/mL P + a mixture of 20 pg/mL E and 2 pg/mL DES. In **e** and **f**, "P + E (20 pg/mL)", "P + DES (20 pg/mL)" and "P + mixture of E and DES" are vehicle + 20 ng/mL P + 20 pg/mL E, vehicle + 20 ng/mL P + 20 pg/mL DES and vehicle + 20 ng/mL P + a mixture of 20 pg/mL E and 20 pg/mL DES. *Significant difference compared with "Vehicle" ($P < 0.05$). **Significant difference compared with "20 ng/mL P" ($P < 0.05$)

neither 4 pg/mL E nor 4 pg/mL DES affected the enhancement of hyperactivation by 40 ng/mL P. However, the mixture of 4 pg/mL E and DES significantly enhanced the hyperactivation by 40 ng/mL P after incubation for 1 and 1.5 h (Fig. 7b). After incubation for 2 h, the mixture did not affect the hyperactivation enhanced by 40 ng/mL P.

Discussion

Recent studies suggest that hyperactivation is regulated by P and E through non-genomic pathways [17, 28]. In the hamster, regulation of penetration and hyperactivation by P and E is very strict [17, 28, 30]. The most effective concentration of P for penetration and hyperactivation is

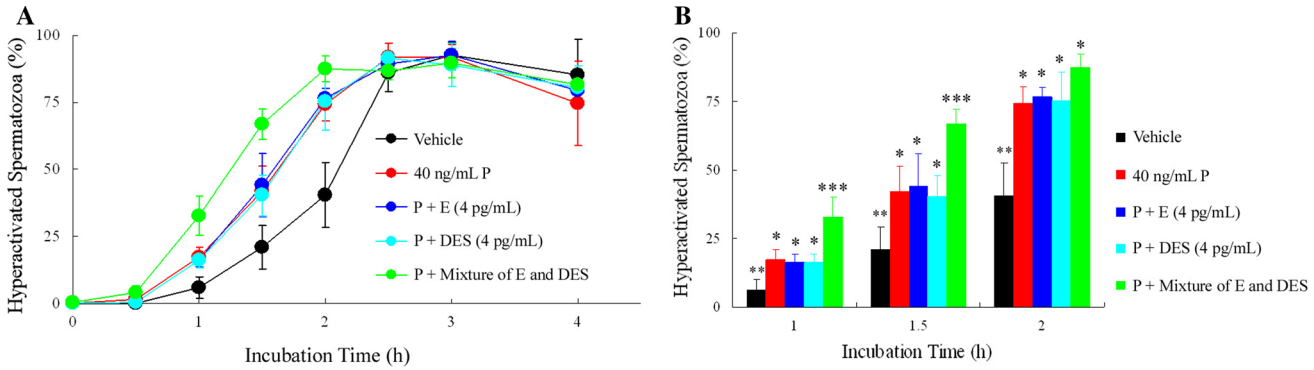

Fig. 7 Effects of 17β-estradiol (E) and diethylstilbestrol (DES) on progesterone (P)-enhanced hyperactivation when spermatozoa were exposed to 40 ng/mL P after exposure to 4 pg/mL E, 4 pg/mL DES or a mixture of them for 5 min as shown in Table 2. It shows an overview of effects of P, E, DES and the mixture on the percentage of hyperactivation (**a**) and detailed percentages of hyperactivation after incubation for 1, 1.5 and 2 h (**b**). Data are expressed as mean ± SD. In all graphs, "Vehicle" refers to mTALP medium + 0.3 % ethanol. "40 ng/mL P" is vehicle + 40 ng/mL P. "P + E (4 pg/mL)", "P + DES (4 pg/mL)" and "P + mixture of E and DES" are vehicle + 40 ng/mL P + 4 pg/mL E, vehicle + 40 ng/mL P + 4 pg/mL DES and vehicle + 40 ng/mL P + a mixture of 4 pg/mL E and 4 pg/mL DES. *Significant difference compared with "Vehicle" ($P < 0.05$). **Significant difference compared with "40 ng/mL P" ($P < 0.05$). ***Significant difference compared with "Vehicle" and "40 ng/mL P" ($P < 0.05$)

20 ng/mL [17, 30]. At this concentration, P does not induce the AR, although a concentration of several micromolar do induce the AR [30].

P-enhanced hyperactivation is dose-dependently suppressed by E (Figs. 1, 2) [28]. In the present study, when spermatozoa were exposed to 20 ng/mL P after exposure to E, >20 pg/mL E significantly suppressed P-enhanced hyperactivation, but not <20 pg/mL (Fig. 2c, d) [28]. When spermatozoa were exposed to 10 ng/mL P after exposure to E, >10 pg/mL E significantly suppressed P-enhanced hyperactivation, but not <10 pg/mL (Fig. 2a, b). In contrast, when spermatozoa were exposed to 40 ng/mL P after exposure to E, >40 pg/mL E significantly enhanced P-enhanced hyperactivation but <40 pg/mL did not affect P-enhanced hyperactivation (Fig. 2e, f). Only 40 ng/mL E was not significantly different compared with vehicle and 40 ng/mL P (Fig. 2f).

The present results in Fig. 2 and previous experiments [28] show that the effect of E varies according to the concentration of P, and that essentially E decreases the effect of P on the enhancement of hyperactivation. When spermatozoa were exposed to 40 ng/mL P after exposure to >40 pg/mL E, except to 40 ng/mL E, the hyperactivation enhanced by 40 ng/mL P was more enhanced (Fig. 2e, f). Because the hyperactivation enhanced by 40 ng/mL P was shown to be less than that of 20 ng/mL P [17], it is likely that enhancement of sperm hyperactivation changes from the lesser enhancement by 40 ng/mL P to the greater enhancement by 20 ng/mL P when spermatozoa are exposed to 40 ng/mL P after exposure to 4 pg/mL to 40 ng/mL of E. Although regulatory mechanisms of change of P-enhanced hyperactivation are not still clear enough, regulation of hyperactivation by P and E occurs through non-genomic mechanisms [17, 28]. After P binds to the progesterone receptor in the sperm head, phospholipase C is activated and produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylcholine and phosphatidylinositol [17]. IP_3 binds to its receptor, releasing calcium from the calcium store and enhancing hyperactivation [13–15, 37, 38]. DAG, on the other hand, is activated by protein kinase C (PKC) and also enhances hyperactivation [38]. P also enhances hyperactivation through activation of PKA [38]. After the protein phosphorylations are increased by PKA and PKC and so on, hyperactivation is enhanced by P [17, 28]. In contrast, E binds to the ER in the sperm head and suppresses P-enhanced hyperactivation by inhibiting the tyrosine phosphorylations [28].

In general, the sexual behavior of mammals and release of the oocyte from the ovary are controlled by the estrous cycle [2]. In the present study, hamster spermatozoa were hyperactivated in association with changing concentrations of P and E (Fig. 2), as occurs during the estrous cycle [2]. Therefore, it is likely that reproductive events are regulated by the concentrations of P and E associated with the stages of the estrous cycle. In addition, it seems that spermatozoa are hyperactivated by indirectly "monitoring" the concentrations of P and E to ensure congruity with the release of the oocyte from the ovary and/or maturation of the oocyte.

It is known that DES exhibits estrogenic effects via an ER. Reproductive processes, such as sex differentiation, spermatogenesis, etc., are disrupted by antenatal and neonatal treatment of animals with DES [32, 33], but it is not well known whether DES directly affects gametic functions. Because P and E dose-dependently regulated hyperactivation via non-genomic mechanisms in the hamster (Fig. 2) [17, 28, 38], the present study examined whether

DES affected the non-genomic regulation of hyperactivation by P and E. When spermatozoa were exposed to 20 mg/mL P after exposure to 2 and 20 ng/mL DES, there was weak but significant suppression of P-enhanced hyperactivation (Fig. 4) although DES itself did not affect sperm motility and hyperactivation at all when spermatozoa were exposed to 2 pg/mL to 20 ng/mL DES (Fig. 3). Because suppression of P-enhanced hyperactivation by DES was significantly inhibited by tamoxifen (Fig. 5), it is suggested that DES suppressed P-enhanced hyperactivation through binding to an ER. From these results, it seems that DES did not affect sperm motility and hyperactivation, but weakly suppressed the effect of P on sperm hyperactivation similar to E.

DES together with E would affect the reproductive system in vivo (Figs. 6, 7). When spermatozoa were exposed to 20 ng/mL P after exposure to 2 pg/mL E with 2 pg/mL DES, the mixture of E and DES weakly but not significantly suppressed the enhancement of sperm hyperactivation by 20 ng/mL P (Fig. 6a, b). When spermatozoa were exposed to 20 ng/mL P after exposure to 20 pg/mL E with 2 pg/mL DES, the mixture of E and DES significantly suppressed P-enhanced hyperactivation (Fig. 6c, d). When spermatozoa were exposed to 20 ng/mL P after exposure to 20 pg/mL E with 20 pg/mL DES, the mixture of E and DES significantly suppressed P-enhanced hyperactivation (Fig. 6e, f). When spermatozoa were exposed to 40 ng/mL P after exposure to 4 pg/mL E with 4 pg/mL DES, the mixture of E and DES significantly enhanced hyperactivation by 40 ng/mL P (Fig. 7). Because the hyperactivation enhanced by 40 ng/mL P was significantly enhanced by 40 pg/mL E (Fig. 2e, f), it is likely that E in cooperation with DES changed the enhancement of hyperactivation from the effect of 40 ng/mL P to that of 20 ng/mL P. These results suggest that DES accelerated or increased the effect of E. Although previous studies [32, 33] suggested that DES disrupts reproductive systems by acting as a substitute for E, the present study's results suggest that DES disrupted hyperactivation by acting as an accelerator of the effect of E.

In conclusion, because hyperactivation creates the propulsive force for penetration [1, 36], P and E regulate penetration through enhancement of hyperactivation [17, 28, 31]. After penetration, spermatozoa are able to fertilize the oocyte [1]. Furthermore, quite recent study [39] suggested that the ability of spermatozoa to be hyperactivated closely correlated with in vitro fertilization. Therefore, if penetration is also decreased by DES when it disturbs hyperactivation, then the effect of DES is to decrease the chance of fertilization.

Conflict of interest I have no conflict of interest.

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