Inhibitory effect of phosphate on in vitro development of 2-cell rat embryos is overcome by a factor(s) in oviductal extracts

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Abstract It has been found that inorganic phosphate (P) at concentrations as low as 10 μM markedly inhibits in vitro development of early rat embryos at the 1-cell or 2-cell stage to the blastocyst stage, although P is present at concentrations of 0.37–1.19 mM in oviductal fluid, in which the development of early embryos occurs. We show here that fractions (PTF, 50–250 μg/ml) of rat oviductal extracts (OVEs) passed through a Blue CL6B affinity column have the ability to overcome the inhibitory effects of P on the development of 2-cell rat embryos in a dose-dependent manner, whereas 250 μg/ml OVE or 250 μg/ml of the bound fractions (BF) induced degenerative changes in the embryos at the 2-cell stage. Moreover, PTF at concentrations of ≥ 100 μg/ml stimulated the hatching of blastocysts both in medium with and without P, although none of the blastocysts in medium without PTF hatched from their zona pellucida.
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Key words: Phosphate; Oviductal extract; Embryo development; Hatching; Rat

1. Introduction

In vitro development of mammalian preimplantation embryos from the 1-cell through the blastocyst stages is known to be arrested at the stage when zygotic gene activation occurs, which differs depending on the species [1,2]. Recently, it has been found that inorganic phosphate (P) in the medium is a critical factor for the developmental inhibition in hamster, rat and mouse embryos [3–7]. In the rat, successful culturing of embryos from the 1-cell through the blastocyst stages was achieved by complete elimination of P from the medium [4]. Furthermore, it was found that addition of P to medium at concentrations as low as 10 μM induces the developmental inhibition, and the higher rate of development to the blastocyst stage was attained by improvement of P-free medium, for example, by lowering osmolarity and the concentration of NaCl [5,8]. However, there is still the problem that most of the resulting blastocysts from 1-cell or 2-cell rat embryos cultured in P-free medium are not capable of hatching from their zona pellucida, which may account for the very low incidence of implantation of such embryos after they are transferred into pseudopregnant females [5,8,9]. Alternatively, it is known that in vivo oviductal fluid, composed of mainly oviductal epithelial secretions and maternal blood exudates [10], in which development of preimplantation embryos is physiologically supported, contains P at concentrations of 0.37–1.19 mM [11,12]. Therefore, these findings raised the question of

why P at concentrations of ≥ 10 μM has inhibitory effects on in vitro development of rat embryos at the 1-cell or 2-cell stage, and then led us to hypothesize that a factor(s) permitting the development of early rat embryos from the 1-cell through the blastocyst stages in the presence of P might exist in the oviductal environment.

Thus, in the present study, to test for the presence of such a factor(s), we examined the effects of rat oviductal extracts (OVEs) on in vitro development of rat embryos at the early 2-cell stage to the blastocyst stage and on the rates at which the blastocysts hatched from their zona pellucida completely, in medium with or without P.

2. Materials and methods

2.1. Collection and culture of embryos

Female Wistar rats (3–5 months old) maintained under controlled lighting conditions (14 h light: 10 h darkness; light on at 7:00 h) were superovulated according to a modification of the method described previously [13]; that is, i.m. injection of 40 IU of eCG at 20:00 h of estrus followed by i.p. injection of 40 IU of hCG 68 h later, and then females were placed with males of the same strain overnight for mating. The next morning (day 1), rats were examined for the presence of a vaginal plug or spermatozoa in the vagina. Early 2-cell embryos were collected at 15:00 h to 17:00 h on day 2 by flushing dissected oviducts with basal medium through the fimbrial opening. Embryos were washed three times with basal medium and then placed in 50 μl droplets of the respecting culture medium, which was then covered with a layer of mineral oil, in each experiment and then cultured for 100 h at 37°C, 5% O_2, 90% N_2 and 5% CO_2. The basal medium used was composed of 85.0 mM NaCl, 0.4 mM KCl, 1.19 mM $MgSO_4$, 1.71 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 1.0 mM L-glutamine, 0.1 mM sodium pyruvate, 23.3 mM sodium lactate and 0.01% polyvinylpyrrolidone with modified Krebs ringer bicarbonate (mKRB) medium [14]. Embryos were scored daily for developmental progress. Embryos showing blastocytic formation were classified as blastocysts. Blastocysts which completely hatched from their zona pellucida were classified as hatched blastocysts.

2.2. Preparation of oviductal extracts (OVEs) and fractionation of OVE

Oviducts were dissected from female Wistar rats (3–5 months old) at estrus and metestrus, and then stored at −80°C until use. One hundred frozen oviducts were thawed at room temperature in 15 ml of 5 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 5 mM benzamidine hydrochloride and 0.1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA), and then minced with surgical scissors and extracted by freeze/thaw cycling (three times). The oviductal extract was centrifuged at 3000 rpm at 4°C for 30 min, and then the supernatant was further centrifuged at $100\,000 \times g$ at 4°C for 1 h. The supernatant from the final centrifugation was used as whole OVE. Fourteen ml of OVE (15.4 mg protein/ml) was applied to a Blue CL6B affinity column (HiTrap Blue, 5 ml of bed volume; Pharmacia Fine Chemicals Co Ltd., Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer, pH 7.4. The column was washed extensively with the equilibration buffer, and then bound materials were eluted with the same buffer containing 2 M NaCl. Absorbance was monitored at 280 nm (Fig. 1). As shown in Fig. 1, the groups of fractions that passed through the column (fractions 2–20) and that bound to the gel (frac-

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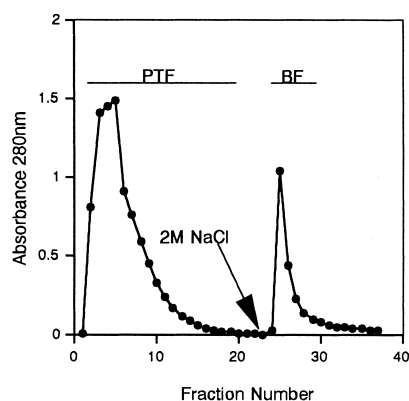E-mail: yamada@jkans.jkans.kais.kyoto-u.ac.jp

Fig. 1. Blue affinity chromatography of rat oviductal extracts (OVEs). OVE (14 ml) was applied to a Blue CL6B affinity column (5 ml of bed volume) pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.4. The column was washed with equilibration buffer, and the bound materials were eluted with 2 M NaCl (arrow). The fraction size was 7 ml/tube. Pass-through fractions (fractions 2–20) and bound fractions (fractions 24–30) were separately pooled, and the pooled fractions were tentatively referred to as PTF and BF, respectively.

tions 24–30) were separately pooled. They were dialyzed extensively in a dialysis membrane (Spectra/Pore CE Membrane: molecular weight cut-off ≤1000 Da; Spectrum, Houston, TX, USA) against 1 mM NH_4HCO_3 buffer, and then lyophilized. Each lyophilized fraction was resuspended in 5 ml of 20 mM Tris-HCl buffer, pH 7.4, and then centrifuged at $100\,000 \times g$ for 30 min at 4°C to remove insoluble materials. The bound fraction and the pass-through fraction were tentatively referred to as BF and PTF, respectively. OVE, BF and PTF were used for embryo culture after sterilization by filtration (Millex GV, 0.22 μm pore size; Millipore, Japan). All the samples were stored at −80°C before use. The concentration of each sample was expressed as the protein content. The protein concentration was estimated using BCA protein assay reagent (Perce, Rockford, IL, USA), according to the manufacturer's instructions.

2.3. Statistical analysis

Each experiment on embryo development was repeated four times, and in each replicate experiment, the proportions of embryos developing to each stage were subjected to arc-sin transformation before statistical analysis. Statistical analysis for comparisons between treatments was carried out by ANOVA and the Bonferroni/Dunn test. Differences were considered significant at $P < 0.05$.

Table 1
Effects of OVE and its fractions separated by Blue affinity chromatography on in vitro development of 2-cell rat embryos* in the presence of 10 μM P

Fraction** of OVE (μg/ml)***	No. of 2-cell embryos examined	% of embryos 100 h after commencement of culture	
		Blastocysts	Hatched blastocysts
Control (0)	83	25[a]	0[a]
OVE (250)	83	0[b]	0[a]
BF (250)	83	0[b]	0[a]
PTF (250)	83	67[c]	25[b]

*Embryos were cultured in medium containing OVE, BF, or PTF in the presence of 10 μM KH_2PO_4 (P). Control embryos (control) were cultured in medium with 10 μM P alone.
**Rat oviductal extract (OVE) was separated into a pass-through fraction (PTF) and a bound fraction (BF) by Blue affinity chromatography as shown in Fig. 1.
***Concentration is expressed as protein content.
[a,b,c]Within a column, values with different superscripts are significantly different ($P < 0.05$).

Table 2
Effects of various concentrations of PTF on in vitro development of 2-cell rat embryos* in the presence of 10 μM P

PTF (μg/ml)**	No. of 2-cell embryos examined	% of embryos 100 h after commencement of culture	
		Blastocysts	Hatched blastocysts
0	71	27[a]	0[a]
50	71	44[b]	0[a]
100	71	75[c]	33[b]
250	71	69[d]	30[b]

*Embryos were cultured in medium containing pass-through fractions (PTF) of OVE separated by Blue affinity chromatography as shown in Fig. 1, in the presence of 10 μM KH_2PO_4 (P).
**Concentration is expressed as protein content.
[a,b,c,d]Within a column, values with different superscripts are significantly different ($P < 0.05$).

3. Results

When early 2-cell rat embryos were cultured for up to 100 h in medium with 10 μM P, the developmental rate (25%) to the blastocyst stage markedly decreased compared to that (90%) in medium with no P (Table 4). Next, to determine the effects of OVE, BF and PTF on the developmental inhibition by P, embryos at the early 2-cell stage were cultured for 100 h in 10 μM P containing medium with or without OVE, BF and PTF at 250 μg/ml (Table 1). When embryos were treated with OVE, the development was arrested at the 2-cell stage, the cytoplasm of the embryos became granulated, and finally the embryos underwent lysis. However, we were able to eliminate the substance(s) in OVE detrimental for embryonic development by partitioning them into the fraction (BF) of OVE which adsorbed to Blue Sepharose CL6B beads. When PTF was added to medium with 10 μM P, the developmental rate (67%) to blastocysts of 2-cell embryos greatly increased, compared to that (25%) in medium with 10 μM P alone, whereas embryos treated with BF arrested at the 2-cell stage and then underwent degenerative changes similar to those induced by OVE treatment. Morphological observation revealed that most of the blastocysts that did form in medium containing P and PTF exhibited full expansion, whereas most of them in medium containing P alone were morphologically abnormal and exhibited poor expansion, with many degenerative cells extruded into the perivitelline space (Fig. 2). These results indicate that factor(s) which overcome the inhibitory effects of P on in vitro development of 2-cell rat embryos exist in OVE. Interestingly, 35% of the blastocysts resulting from embryos cultured in 10 μM P containing medium with PTF

Table 3
Effects of heat and acetic acid treatments on PTF

Treatments of PTF (100 μg/ml)	No. of 2-cell embryos* examined	% of blastocysts
Control	76	24[a]
Untreatment	76	67[b]
Heat (100°C, 5 min)	76	40[a]
Acetic acid (1 M, 2 h at 20°C)	76	34[a]

*Embryos were cultured in medium with 10 μM KH_2PO_4 in the presence or absence (control) of untreated, heat or acetic acid treated PTF for 100 h.
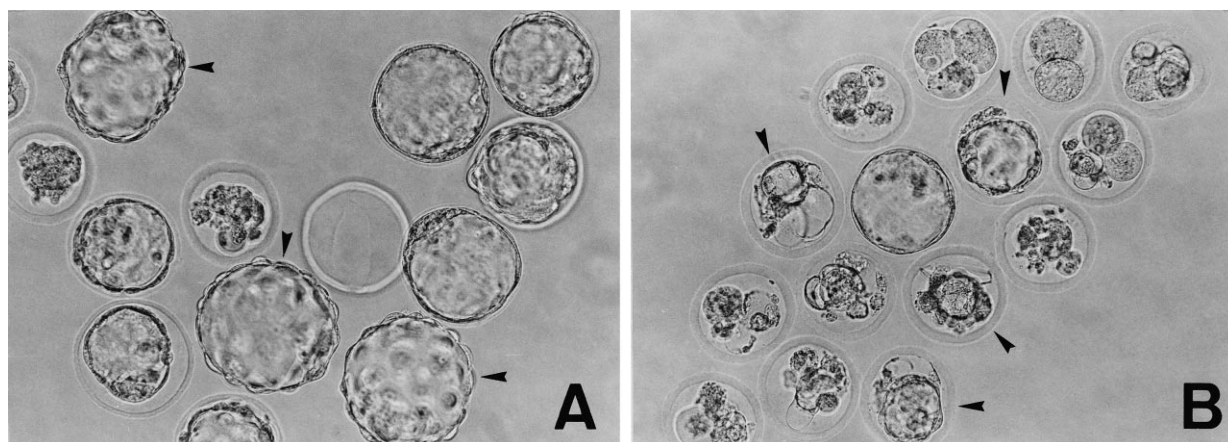[a,b]Within a column, values with different superscripts are significantly different ($P < 0.05$).

Fig. 2. Photomicrographs of rat embryos cultured for 96 h from the 2-cell stage in medium with (A) or without (B) 250 μg/ml PTF, in the presence of 10 μM KH$_2$PO$_4$. A: Arrowheads indicate hatched blastocysts. B: Arrowheads indicate morphologically abnormal blastocysts with poor expansion and many degenerating cells extruded into the perivitelline space.

developed further to hatched blastocysts (Table 1 and Fig. 2). As shown in Table 2, PTF showed a dose-dependent stimulation of rat embryo development in medium containing 10 μM P. The rate of blastocyst formation increased significantly in the presence of 50 μg/ml PTF (44%), compared to that in the absence of PTF (27%) and reached a plateau at 100 μg/ml (75%), with 43% of the resulting blastocysts hatching from their zona pellucida. Table 3 shows that heat and acetic acid treatments markedly reduced the stimulating activity of PTF, suggesting that the activity in PTF is associated with proteinous components.

As shown in Table 4, in medium without P, although the developmental rates of 2-cell embryos to the blastocyst stage were not significantly different in medium with and without 100 μg/ml of PTF (74% and 90%, respectively), the development to hatched blastocysts was found only in embryos treated with PTF (hatching rate; 16%). However, the rate of formation of hatched blastocysts with PTF treatment was significantly higher in medium with P (27%) than that in medium without P (16%).

4. Discussion

In the present study, we showed for the first time that developmental inhibition of 2-cell rat embryos by P is over-come by addition to the medium of fractions (PTF) of OVE passed through a Blue CL6B affinity column. Moreover, it was found that, in addition to increasing the rate of development to blastocysts, PTF stimulates the development of the resulting blastocysts to the hatched stage.

Recently, there have been reports that in vitro development of early embryos from the 1-cell to the blastocyst stage in many mammalian species, with the exception of rats, is significantly enhanced by coculturing the embryos with dissected oviduct organs or cultured primary or passaged oviductal cells from either homologous and heterologous species [15–20]. However, the mechanism of the enhancement of embryonic development in coculture systems remains controversial. Coculture systems might support embryonic development by improving the suboptimal culture conditions rather than stimulating development directly, because it has been reported that counterparts of embryos in coculture abolish the hazardous effects of harmful substances such as reactive oxygen species, so as to promote embryonic development [21]. On the other hand, an embryotrophic factor produced by oviductal cells has been purified from serum-free conditioned media of bovine oviductal epithelial cell cultures and identified as the tissue inhibitor of metalloproteinase-1 (TIMP-1) [18]. Moreover, the production of high molecular (> 10 kDa) and low molecular (< 10 kDa) weight embryotrophic factor(s) by human and mouse oviductal cells, respectively, has been suggested [15,19,20], although the molecular nature of these factors is not yet understood. In our previous experiments, in which early 2-cell rat embryos were cocultured in medium with 10 μM P with dissected rat oviductal ampulla according to the method described elsewhere [15,16], all the embryos cultured showed developmental arrest at the 2-cell stage, and subsequent cytoplasmic swelling and granulation of the embryos followed by lysis was observed (unpublished data). These degenerative changes were similar to those observed in embryos cultured in medium with OVE.

Fortunately, we were able to partition the embryotrophic activity from OVE into the fractions (PTF) passed through a Blue CL6B affinity column. It is of interest that this fraction has the ability not only to overcome the developmental inhibition by P of early 2-cell rat embryos, allowing the embryos to develop to the blastocyst stage at a high rate but also to increase the hatching rate of the resulting blastocysts. It has

Table 4
Effects of PTF on in vitro development of 2-cell rat embryos* in the presence or absence of 10 μM P

P (μM)	PTF (μg/ml)**	No. of 2-cell embryos examined	% of embryos 100 h after commencement of culture	
			Blastocysts	Hatched blastocysts
0	0	62	90[a]	0[a]
0	100	62	72[a]	16[b]
10	0	62	25[b]	0[a]
10	100	62	81[a]	27[c]

*Embryos were cultured in medium with or without 100 μg/ml pass-through fractions (PTF) of OVE separated by Blue affinity chromatography as shown in Fig. 1, in the presence or absence of 10 μM KH$_2$PO$_4$ (P).
**Concentration of PTF is expressed as protein content.
[a,b,c]Within a column, values with different superscripts are significantly different ($P < 0.05$).

also been found that hatching rate of bovine blastocysts is promoted by culturing them in oviduct-conditioned medium [20]. However, it is not yet known whether or not the various biological activities of PTF are mediated by different molecules, and whether the molecule(s) are related to embryotrophic factors from oviductal cells previously identified, such as TIMP-1 [18]. To further understand the molecular nature of the activities of PTF, and whether or not they are derived from oviductal fluid, which is mainly composed of the secretory substances from oviductal epithelial cells and exudates from maternal blood [10], additional studies are underway to characterize them.

Finally, the present findings might indicate the usefulness of oviductal factors for the development of in vitro culture systems for preimplantation embryos in humans as well as in experimental and livestock animals.

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