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0014-4754/86/040394-05$1.50 + 0.20/0

Effect of glucose and phloretin-2′-β-D-glucose (phloridzin) on in vitro fertilization of mouse ova

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Summary. The fertilization ratio of mouse ova in vitro decreased when glucose concentration in the medium was lowered. However, the addition of phloretin-2′-β-D-glucose (phloridzin), known as a glucose uptake inhibitor, restored the fertilization ratio back to the control level. The glucose moiety of the phloridzin seemed to be responsible for this effect.
Key words. In vitro fertilization; mouse ova; capacitation; glucose; phloretin-2′-β-D-glucose (phloridzin).

The physiological nature of sperm in vitro was greatly influenced by the energy source which was added to the medium[2–4]. In rat and mouse, in vitro fertilization was achieved only in glucose (or mannose)-containing media but not in fructose or other hexoses[5,6]. It could therefore be predicted that glucose uptake inhibitors would prevent the fertilization of mouse ova in vitro. Phloretin-2′-β-D-glucose (phloridzin; known as a glucose uptake inhibitor in intestine as well as in kidney cells), however, did not decrease the fertilization ratio in vitro. Rather, it actually improved the fertilization ratio in a low glucose concentration medium.

Materials and methods. In vitro fertilization of mouse ova. Media used were modified Krebs-Ringer bicarbonate buffer supplemented with glucose[7] (glucose-KRB) or fructose (fructose-KRB). The sperm were obtained from the cauda epididymis of mature ddy mice (weighing 35–40 g) as previously reported[7]. 4–5-week-old ddy mice were injected with 5 IU of PMSG (Teikoku Zoki) 48 h before the administration of 5 IU hCG (Teikoku Zoki) i.p. 14–16 h after the hCG injection, the ova in cumulus clots were collected by puncturing the ampullar portion of the oviduct with a needle. Ova collected from 10–20 female mice were mixed by pipetting and were divided and placed in 0.4 ml of glucose-KRB in test tubes. More than 25 ova were put into each tube. The right epididymis from each of two male mice was chopped in glucose- or fructose-KRB, while the procedure was repeated in fructose-KRB for the left half. Sperm were preincubated for 40 min in these media at a concentration of approximately $1.0–1.5 \times 10^6$ sperm/ml. 40 µl of the sperm suspension was introduced to the ova placed in 0.4 ml of glucose-KRB. Sperm were diluted to an appropriate concentration for in vitro fertilization and exposed to glucose following this procedure. After various intervals of sperm addition, hyaluronidase was added to the ova and the penetration of sperm through the zona pellucida layer of the ova was observed under a phase contrast microscope. Chemicals used were phloretin-2′-β-D-glucose (phloridzin), phloretine (guaranteed reagent grade; Nakarai Chem.) and 3-O-methyl-D-glucose and 2-deoxy-D-glucose (Sigma Chem.). The reagents were dissolved or suspended in distilled water and were stored at −20 °C. Stock solutions were diluted with fructose-KRB and used for the experiment.

For the statistical analysis, Student's t-test was employed to assess the significance of differences between the mean values for the control group and the sample added group.

Results and discussion. Epididymal sperm need time before penetrating the zona pellucida. Therefore, to investigate the capacitation stage, the sperm preincubated in fructose-KRB were introduced to the ova in glucose-KRB, and the time required for the zona penetration was observed. As shown in table 1, none of the sperm preincubated in fructose-KRB could penetrate the zona pellucida within 20 min, while capacitated sperm (preincubated in glucose-KRB) penetrated without a time lag. The addition of fructose instead of glucose to the medium preserved sperm motility, indicating that fructose may have been utilized as an energy source for motility. However, it was obvious that sperm were not capacitated during incubation in the fructose-containing medium (table 1).

The decrease of glucose concentration (fructose remained present in all media at a concentration of 6 mM) lowered the fertilization ratio. This effect was most pronounced between 1.2 and 0.6 mM of the glucose concentration (table 2). Considering the fact that fructose and glucose share almost the same metabolic pathway, the authors assumed the possibility that glucose serves as a triggering signal for capacitation apart from func-

Table 1. Effect of sperm preincubation on the time required for the penetration of zona pellucida

Preincubation	10 min	20 min	30 min	40 min	50 min
0 min in glucose-KRB	0	0	25 ± 8	57 ± 9	63 ± 4
40 min in glucose-KRB	56 ± 11	63 ± 7	65 ± 9	ND	ND
40 min in fructose-KRB	0	0	42 ± 12	52 ± 6	69 ± 10

Values are mean ± SE of four independent experiments. ND: not done.

tioning as an energy source. The effect of glucose derivatives such as 3-O-methyl-D-glucose and 2-deoxy-D-glucose on the fertilization of mouse ova in vitro was therefore examined.

No effect was observed in the case of 3-O-methyl-D-glucose (table 2, exp. 1). On the other hand, 2-deoxy-D-glucose (1.2 mM) suppressed fertilization when the glucose concentration was reduced to 1.2 mM while no effect was observed in the presence of 6 mM glucose (table 2, Exp. 2). It was assumed that the latter worked as a glucose uptake inhibitor.

However, phloridzin, known as a glucose uptake inhibitor, showed no fertilization inhibitory effect, but stimulated fertilization in low glucose concentrations. Even in the presence of 0.15 mM glucose, in which no fertilization was observed in the control group, the addition of the reagent to the medium enabled the sperm to penetrate the ova. Since phloridzin is a glucoside of phloretine, the authors suspected the release of a glucose moiety from the reagent. However, phloridzin per se failed to induce sperm capacitation. Furthermore, the effect of phloretin on in vitro fertilization of mouse ova was also examined, and only a detrimental effect was observed. It was interesting that these two reagents had an opposite effect on sperm capacitation, because the only difference in the structures of phloridzin and phloretin is the glucose moiety. The fact that a small amount of glucose had to be contained in the medium in order for phloridzin to express its activity might be related to the observation of Fraser and Quinn[8]: 'Once the spermatozoa had been primed by glucose, the removal of exogenous glucose did not block fertilization.'

In the guinea pig, it was reported that glucose retarded both the sperm acrosome reaction[2] and sperm respiration[3]. In human sperm, glucose was more effective than fructose in enabling them to fuse with zona-free hamster ova[4]. Although the requirement of glucose in sperm capacitation might vary from species to species, the mechanisms of how the exogenously added energy source affect the physiological function of sperm are important aspects of the capacitation process. Phloridzin will serve as a useful reagent for the investigation to clarify the roler of glucose in mouse sperm capacitation.

Table 2. The effect of glucose concentration and glucose uptake inhibitors on mouse fertilization in vitro

Glucose concentration (mM)	Ova penetrated (%)	
Exp. 1	Control	3-O-Methyl-D-glucose (1.2 mM)
6.0	60 ± 6	63 ± 2
2.4	56 ± 3	57 ± 11
1.2	56 ± 8	46 ± 10
0.6	28 ± 9	21 ± 4
Exp. 2	Control	2-Deoxy-D-glucose (1.2 mM)
6.0	68 ± 10	59 ± 5
2.4	48 ± 6	23 ± 7*
1.2	45 ± 6	1 ± 1**
0.6	17 ± 3	1 ± 1**
Exp. 3	Control	Phloretin-2'-β-D-glucose (500 μM)
6.0	77 ± 10	79 ± 2
1.2	68 ± 7	86 ± 7
0.6	7 ± 4	69 ± 5**
0.3	2 ± 1	63 ± 5**
0.15	0	53 ± 10***
0	0	0
Exp. 4	Control	Phloretin
6.0	60 ± 3	(500 μM) 0***
		(100 μM) 5 ± 3**
		(5 μM) 26 ± 2**

Values are mean ± SE of 3–7 independent tests. *$p < 0.05$, **$p < 0.01$. ***Student's t-test was not applicable. Epididymal sperm prepared in fructose-KRB were added to the ova. The assessment of fertilization was done at 90 min after the sperm addition.

Acknowledgment. We are grateful to S. Cook for her assistance in preparing the manuscript.

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0014-4754/86/040398-02$1.50 + 0.20/0

Gametes contain angiotensin converting enzyme (kininase II)[1]

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Summary. The localization of angiotensin converting enzyme (ACE) in the gonads of the normal rabbit was studied by immunofluorescence and immunoelectron microscopy. The enzyme is present in the cytoplasm of testicular spermatids and of epididymal and ejaculated spermatozoa, and on the surface of follicular and tubal cocytes. These findings support the hypothesis that ACE has a role in gamete maturation and in fertilization.

Key words. Angiotensin converting enzyme; rabbit, gametes; rabbit, Leydig cells.

Angiotensin converting enzyme (ACE) (kininase II; EC 3.4.15.1) is a dipeptidyl carboxypeptidase that catalyzes the conversion of angiotensin I to angiotensin II, a vasopressor, inactivates bradykinin, a vasodepressor, and is important in homeostasis of blood pressure. The enzyme is located on the luminal membrane of endothelial cells of vessels throughout the body[4,5]. In addition, ACE has been demonstrated on the brush border of renal and intestinal epithelial cells[6,7] and in neuroepithelial cells of the brain[8,9]. However, measurement of ACE activity in a variety of animal species and in man indicates that the highest concentration of ACE is present in the testis, the epididymis, and the seminal fluid[10,11]. In the rat the rise in ACE activity in the testis parallels sexual maturation[10,11], while in man a correlation has been observed between the activity of ACE in seminal plasma and semen quality, such as density and motility[12]. Recently it has been demonstrated by three different techniques that in rat[13,14], and swine[15] ACE is associated with male gametes. In one report[15], the precise localization of ACE in male swine