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—Original Article—

Glycosaminoglycans Improves Early Development of Zona-free 8-cell Rat Embryos to Blastocysts in a Chemically Defined Medium, but Not the Pregnancy Rate Following Transfer of the Blastocysts

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Abstract. The objective of the present study was to clarify the possible role of the zona pellucida (ZP) in early development of rat embryos and to determine the effect of glycosaminoglycans on the development of ZP-free 8-cell embryos before or after embryo transfer at the blastocyst stage. Eight-cell embryos were divided into three groups comprised of, 1) intact controls, 2) embryos with the ZP was removed with acidic solution and 3) pairs of ZP-free 8-cell embryos aggregated in a small hollow. These embryos were cultured in a chemically defined mR1ECM for 24 h. Developmental ability to the blastocyst stage and mean cell number in the blastocyst was lower in ZP-free embryos than in intact controls. When these blastocysts were transferred, the farrowing rate and efficiency of embryos developed to term were also lower in ZP-free embryos, but not in the aggregated ones. Supplementation with hyaluronan (HA; 63–250 μg/ml) or heparan sulfate proteoglycan (HS; 15 μg/ml) significantly improved blastocyst formation of ZP-free embryos and the cell number in the blastocyst by reducing the incidence of apoptosis. However, there were no beneficial effects of HA or HS on farrowing and newborn rates after transfer of the blastocysts. In conclusion, the ZP plays roles in maintaining successful development of early rat embryos at least from the 8-cell stage not only to the blastocyst stage but also to posttransfer stages. Glycosaminoglycans, such as HA or HS, appear to contribute to successful cleavage during early development to the blastocyst stage but may be insufficient to maintain the posttransfer survival of ZP-free embryos.

Key words: Blastocyst, Embryo culture, Glycosaminoglycans, Rat, Zona pellucida

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Mammalian oocytes, zygotes and early- to blastocyst-stage embryos are enclosed by a thick layer of glycoproteins, called the zona pellucida, which is produced in the early stage of oogenesis. The zona is known to regulate sperm binding to the ovulated oocytes and the monospermic penetration, to prevent separation of blastomeres or aggregation of different embryos during early development, and to protect against viral, bacterial and fungous pathogens or immune cells in the female reproductive tract [1, 2].

Recent developments in embryo culture technology have made it possible, in many species, for cleavage embryos to develop efficiently to the blastocyst stage in a chemically defined medium with successful results to term following transfer of the blastocysts [3–5]. In rats, a chemically defined culture medium, rat 1-cell embryo culture medium (R1ECM), has been developed and succeeded in achieving early development of *in vivo* and *in vitro* fertilized rat zona-intact zygotes to the blastocyst stage [6, 7]. Gametes and embryos of the rat, one of the standard experimental rodents, have been utilized not only for biomedical research but also for biotechnological attempts including *in vitro* fertilization [7–9], embryo culture [6, 10] and manipulation of gametes and embryos [11–13]. Currently, micromanipulation technologies to produce cloned, chimeric and transgenic embryos insure or remove the zona pellucida during the process. Although the zona pellucida appears to play a role in accumulating autocrine ligands in the perivitelline space during early development [14], it is not clear if insuring or removing the zona pellucida during micromanipulation of rat embryos affects subsequent development.

Addition of the glycosaminoglycan hyaluronan (HA), which is not only present in ovidactal and uterine fluids [15] but is also synthesized and secreted by oocytes [16], is known to improve developmental competence to the blastocyst stage in rodents [17, 18] and domestic animals [19–21]. The surface receptor for HA is known to express on preimplantation embryos [22]. The presence of HA in culture medium has also been shown to increase implantation rates of mouse embryos and fetal development after embryo transfer [18]. Furthermore, a basement membrane matrix including heparan sulfate proteoglycans (HS) allowed a larger number of mouse embryos to reach the blastocyst stage and to hatch [23]. It has been known that expression of the HS on the external trophectodermal cell surfaces of mouse blastocysts increases during acquisition of attachment competence [24, 25] and plays an important role in the initial stage of embryo attachment to the uterine surface [26]. Since these exogenous extracellular matrix molecules enhance the developmental competence and quality of zona-intact embryos, these molecules, such as HA and HS, should also be essential for zona-free embryos to maintain developmental competence and

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quality. Therefore, the zona pellucida may play an important role in supplying a suitable microenvironment to the blastomeres by retaining the extracellular glycans, such as HA and HS.

The objective of the current study was to clarify the possible role of the zona pellucida during early development of rat embryos and to determine the effect of HA or HS on the development of zona-free 8-cell embryos before or after embryo transfer at the blastocyst stage.

Materials and Methods

Chemicals and culture media

Sodium chloride, KCl, $MgCl_2$ and paraffin oil were purchased from Nacalai Tesque (Kyoto, Japan). Equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) were purchased from ASUKA pharmaceutical (Tokyo, Japan). Unless specified, other chemicals were purchased from Sigma-Aldrich (St. Luis, MO, USA). The medium used for embryo culture was modified rat 1-cell embryo culture medium (mR1ECM, Table 1) [6]. The medium used for collecting, washing and transferring embryos was HEPES-buffered mR1ECM (HEPES-mR1ECM, Table 1) [6, 27]. The solution used for zona removal was an acidified Tyrode's solution (Table 1) [28].

Table 1. Composition of mR1ECM, Hepes-mR1ECM and acidic Tyrode's solutions

Composition (mM)	Media		
	mR1ECM	Hepes-mR1ECM	Acidic Tyrode's
NaCl	76.7	76.7	136.9
KCl	3.2	3.2	2.7
$CaCl_2$	2.0	2.0	1.6
$MgCl_2$	0.5	0.5	0.5
$NaHCO_3$	25.0	4.0	-
HEPES	-	20.0	-
Glucose	7.5	7.5	5.6
Glutamine	0.1	0.1	-
Na-pyruvate	0.5	0.5	-
Na-lactate	10.0	10.0	-
EAA	2% (v/v)	2% (v/v)	-
NEAA	1% (v/v)	1% (v/v)	-
Polyvinyl alcohol	0.1% (w/v)	0.1% (w/v)	0.1% (w/v)

Preparation and culture of rat embryos

All rats used in the current study were treated according to the guidelines and permission for animal experiments at Okayama University.

Sexually mature female Wistar rats (16–20 weeks old) maintained under controlled lighting conditions (14 h light: 10 h darkness; light on at 0600) and with a proestrous vaginal smear were naturally mated from around 1700 for overnight with mature fertile males of the same strain. Between 1400 and 1600 h, three days after observation of sperm or a plug in the vagina, 8-cell embryos were recovered by flushing the excised oviduct-uterus with approximately 0.3 ml of HEPES-mR1ECM through the fimbrial opening. The embryos were washed three times with fresh HEPES-mR1ECM and divided into the following three treatment groups for culture. 1) In the first group, five to twenty intact embryos were washed three times with fresh mR1ECM, placed into a 400-µl droplet of the same medium and cultured at 37 C in an atmosphere of 5% CO_2 in air for 24 h (control). 2) In the second group, after zona removal by exposure to acidic Tyrode's solution for 30 sec (5 embryos per treatment), five to twenty zona-free 8-cell embryos were washed three times with fresh mR1ECM and cultured at 37 C in an atmosphere of 5% CO_2 in air for 24 h. 3) In the third group, after zona-removal as described above, pairs of zona-free 8-cell embryos were gently washed three times with fresh mR1ECM, placed into a 10-µl droplet of the same medium in a small hollow made with an iron needle to aggregate and cultured at 37 C in an atmosphere of 5% CO_2 in air for 24 h.

Assessment of total cell number and apoptosis cells in blastocysts

To examine the total cell number, blastocysts obtained after culture were exposed to a solution of 2 µM SYBR-14 (Invitrogen, Carlsbad, CA, USA) and 20 µg/ml propidium iodide (Invitrogen) in Hepes-mR1ECM at 37 C for 15 min and then observed under a fluorescence microscope (Eclipse 80i, Nikon, Tokyo, Japan; excitation at 450–490 nm, emission at 520 nm). Live cells were labeled with a proprietary cell-permeant nucleic acid stain, SYBR-14, and fluoresced green. Dead cells were labeled with both SYBR-14 and propidium iodide and fluoresced red-orange.

To observe apoptotic cells in blastocysts, according to a previous report [29], the embryos were treated with Hoechst 33342 and an In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). After culture, the blastocysts obtained after culture were fixed in 4% (w/v) paraformaldehyde solution at room temperature for 30 min. Then the embryos were washed with PBS (–) containing 0.1% (w/v) polyvinyl alcohol three times and permeated with 0.1% (v/v) Triton X-100 in PBS (–) containing 0.1% (w/v) polyvinyl alcohol at room temperature for 15 min. The embryos were processed with TUNEL solution at 37 C for 60 min, according to the manufacturer's protocol. After washing three times again, the blastocysts were stained with 5 µg/ml Hoechst-33342 for 30 min and then observed under a fluorescence microscope (Eclipse 80i, Nikon; excitation at 450–490 nm and emission at 520 nm for apoptotic cells and excitation at 346 nm and emission at 420 nm for total cell number).

Surgical embryo transfer

Basically, surgical embryo transfer was performed as described by Miyoshi *et al.* [30]. Briefly, four days (between 14:30 and 16:30 h) after the stimulation to induce pseudopregnancy by inserting a glass rod connected to an electric vibrator into the vagina between 19:00 and 20:00 h on the day of estrus (day 0), the recipient females were anesthetized with an injection of Avertin (0.012 ml/g body mass) into the peritoneal cavity. Avertin contains 1.8% (w/v) 2,2,2-tribromoethanol (Sigma-Aldrich) in 1.23% (v/v) 3-methyl-1-butanol solution. The uterus was exposed through a dorsal incision. The smooth tip of a mouth-controlled glass pipette (about 150 µm in diameter) with seven to fifteen morphologically normal

blastocysts in 1–2 μl of HEPES-buffered mR1ECM was inserted into an opening made previously by inserting a 26-gauge needle through the uterine wall at the oviductal side. The embryos were delivered by gentle pressure, and the development to term was examined. Twenty-one days after embryo transfer, all recipients were mercifully euthanized and the number of fetuses and implantation traces in the uterus were anatomically examined.

Experimental designs

Experiment 1: *In vitro* development of zona-free 8-cell embryos to blastocysts was compared with zona-intact 8-cell (control) and zona-free aggregated embryos (two of zona-free 8-cell embryos were aggregated). These embryos were cultured in mR1ECM at 37 C in an atmosphere of 5% CO_2 in air for 24 h, and then the percentage of those that developed to the blastocyst stage and mean number of live cells in single blastocyst were examined. Some of the blastocysts in each group were surgically transferred to synchronized recipient females (five to nine blastocysts per uterine horn). The percentages of embryos implanted and developed to term were examined.

Experiment 2: Zona-free 8-cell embryos were cultured in the absence or presence of HA (63, 125 and 250 μg/ml) or HS (15 μg/ml) in mR1ECM at 37 C in an atmosphere of 5% CO_2 in air for 24 h. As controls, zona-intact embryos were cultured in the absence of HA and HS under the same culture conditions. After culture, the percentage of embryos that developed to the blastocyst stage and mean number of live cells in a single blastocyst were examined.

Experiment 3: Zona-free 8-cell embryos were cultured in the absence or presence of HA (125 μg/ml) or HS (15 μg/ml) in mR1ECM at 37 C in an atmosphere of 5% CO_2 in air for 24 h. As controls, zona-intact embryos were cultured in the absence of HA and HS under the same culture conditions. Furthermore, as positive controls, blastocysts were collected from the uteri of females at 4.5 days after mating. After culture or collection, the numbers of total cells and apoptotic cells per blastocyst were examined in blastocysts derived from each experimental group.

Experiment 4: Zona-free 8-cell embryos were cultured in the absence or presence of HA: (125 μg/ml) or HS (15 μg/ml) in mR1ECM at 37 C in an atmosphere of 5% CO_2 in air for 24 h. As controls, zona-intact embryos were cultured in the absence of HA and HS under the same culture conditions. After culture, blastocysts from each experimental group were surgically transferred to synchronized recipient females. The percentages of embryos implanted and developed to term were examined.

Statistical analysis

Statistical analyses of results from more than three replicated experiments were used for treatment comparisons and were carried out by one-way analysis of variance (ANOVA) using the StatView program (Abacus Concepts, Berkeley, CA, USA). If the P value was less than 0.05 in ANOVA, Tukey's HSD test was performed using the StatView program. All percentage data were subjected to arcsine transformation before statistical analysis. For showing percentage data in tables, data were transformed back to the original percentages. All data were expressed as means ± SEM. $P<0.05$ was considered to be statistically significant.

Results

Experiment 1: When early development of zona-free 8-cell embryos to blastocysts was compared with those of zona-intact 8-cell embryos and zona-free aggregated embryos, the incidence of blastocyst formation was significantly lower ($P<0.05$) in zona-free 8-cell embryos than in the other embryos (Table 2). However, the incidence of zona-free aggregated embryos that developed to the blastocyst stage was not different from that of zona-intact controls. The mean number of live cells in a blastocyst was also lower in zona-free embryos than in zona-intact embryos. The live cell number in a blastocyst derived from aggregated zona-free embryos (successful aggregation rate was 72.8%) was almost twice that in a blastocyst from zona-free 8-cell embryos (Table 2). As shown in Table 3, the percentages of embryos farrowed and developed to term were much higher ($P<0.01$) when the blastocysts derived from zona-intact 8-cell embryos were transferred into uterine horn of synchronized recipients than when zona-free embryos were transferred. The percentages of blastocysts derived from zona-free aggregated embryos did not differ from those derived from zona-intact embryos (Table 3).

Experiment 2: As shown in Table 4, the presence of HA and HS during culture of zona-free 8-cell embryos for 24 h significantly increased the incidence of embryos that developed to the blastocyst stage and the mean number of live cells per blastocyst. Although the HA concentrations examined in the present study (63, 125 and 250 μg/ml) did not affect the incidence of blastocyst formation, the presence of 125 and 250 μg/ml HA improved the incidence to a level comparable with that of zona-intact embryos. The number of live cells in a blastocyst derived from zona-free 8-cell embryos was significantly improved, to the same level as zona-intact embryos, by adding HA at 63 μg/ml or higher concentrations, whereas the number was higher at 125 μg/ml than that in blastocysts derived from zona-intact embryos. Supplementation of culture medium with 15 μg/ml HS improved both the incidence of development of zona-free 8-cell embryos to the blastocyst stage and the number of live cells in the blastocyst, but the incidence was still lower than that of zona-intact embryos.

Experiment 3: As shown in Table 5, the total cell number and incidence of apoptotic cells in blastocysts were significantly improved, to the same level as *in vivo* derived blastocysts, when zona-free 8-cell embryos were cultured in the presence of HA (at 125 μg/ml) or HS (at 63 μg/ml). In particular, the presence of HA significantly reduced the incidence of apoptotic cells in blastocysts derived from zona-free embryos compared with zona-intact controls during *in vitro* development from the 8-cell stage.

Experiment 4: When the blastocysts developed *in vitro* from the 8-cell stage were transferred into the uteri of recipients, there were no differences in development to term of zona-free blastocysts, regardless of the absence or presence of HA (at 125 μg/ml) or HS (at 63 μg/ml) during culture from the 8-cell stage (Table 6). Both incidences were significantly lower than those of zona-intact embryos (Table 6).

Table 2. Effect of zona removal and aggregation of 8-cell rat embryos on *in vitro* development to the blastocyst stage in rats

Treatments		No. of embryos examined	% of blastocyst formation	No. of live cells in blastocyst
ZP	Aggregation			
+	–	68	100[a]	31.7 ± 2.6[a]
–	–	105	68.8 ± 6.3[b]	22.6 ± 1.6[b]
–	+	70	81.7 ± 3.6[a]	40.1 ± 0.9[c]

Data are given as means ± SEM from seven to ten replicated experiments. Values with different superscripts within a column are significantly different (P<0.05).

Discussion

In the present study, we found that removal of the zona pellucida of rat embryos at the 8-cell stage significantly reduced the incidence of embryos developing to the blastocyst stage and the number of cells in a blastocyst. In the rabbit [31] and pig [32], removal of the zona pellucida has been reported to significantly compromise early development of embryos, whereas it was not detrimental in the hamster [33] and bovine [34]. In mice, the critical effect of the zona pellucida appears to be stage specific. A previous study showed that mouse embryos did not develop normally to the blastocyst stage when the zona was removed at the 2- to 4-cell stages, but the embryos with the zona removed at the 8-cell or later stages

Table 3. Effect of zona removal and aggregation of 8-cell embryos on *in vivo* development following transfer of the embryos at the blastocyst stage in rats

Treatments		No. of embryos recipients	No. of recipients transferred	No. (%) of delivered	%[1] of embryos implanted	%[1] of embryos developed to term
ZP	Aggregation					
+	–	9	66	7 (78)	54.9 ± 11.2[a]	54.9 ± 11.2[a]
–	–	9	66	4 (44)	9.6 ± 4.2[b]	6.8 ± 3.4[b]
–	+	8	53	8 (100)	67.7 ± 1.6[a]	58.5 ± 3.2[a]

[1] Percentages are presented based on the total number of embryos transferred. Data are given as means ± SEM from seven to ten recipients. Values with different superscripts within a column are significantly different (P<0.05).

Table 4. Effect of hyaluronan or heparan sulfate proteoglycan on *in vitro* development of zona-free 8-cell embryos to the blastocyst stage in rats

Treatments			No. of embryos examined	%[2] of blastocyst formation	No. of live cells in blastocyst
ZP	Suppl. [1]	Conc. (μg/ml)			
+	–	0	44	92.6 ± 3.2[a]	30.3 ± 0.6[a]
–	–	0	44	67.8 ± 2.7[b]	22.0 ± 0.7[b]
–	HA	63	44	79.5 ± 6.1[c]	31.1 ± 0.5[ac]
–	HA	125	52	82.1 ± 3.9[ac]	32.6 ± 0.7[c]
–	HA	250	48	83.6 ± 1.9[ac]	30.2 ± 0.7[a]
–	HS	15	48	81.0 ± 2.4[c]	31.1 ± 0.6[ac]

[1] HA, hyaluronan; HS, heparan sulfate proteoglycan. [2] Percentages are presented based on the total number of embryos examined. Data are given as means ± SEM from five replicated experiments. Values with different superscripts within a column are significantly different (P<0.05).

Table 5. Effect of hyaluronan or heparan sulfate proteoglycan on the incidence of apoptotic cells zona-free rat blastocysts cultured from the 8-cell stage in rats

Treatments		No. of embryos examined	No. of cells in a blastocyst	%[2] of apoptotic cells in blastocyst
ZP	Suppl.[1]			
+	–	45	30.4 ± 0.5[a]	13.2 ± 0.9[a]
–	–	39	25.7 ± 0.4[b]	16.7 ± 1.1[b]
–	HA	53	32.2 ± 0.5[c]	9.9 ± 0.7[c]
–	HS	25	30.8 ± 0.6[ac]	11.2 ± 0.8[ac]
+	*In vivo* derived	36	30.7 ± 0.5[ac]	8.9 ± 0.8[c]

[1] HA, 125 μg/ml hyaluronan; HS, 15 μg/ml heparan sulfate proteoglycan. [2] Percentages are presented based on the total cell number of blastocyst. Data are given as means ± SEM from three to five replicated experiments. Values with different superscripts within a column are significantly different (P<0.05).

Table 6. Effect of hyaluronan or heparan sulfate proteoglycan during culture of zona-free 8-cell embryos for 24 h on *in vivo* development following transfer of the embryos at the blastocyst stage in rats

Treatments		No. of recipients	No. of embryos transferred	No. (%) of recipients delivered	%[1] of embryos implanted	%[2] of embryos developed to term
ZP	Suppl.[1]					
+	−	10	66	10 (100)	83.0 ± 4.3[a]	69.7 ± 5.6[a]
−	−	10	74	2 (20)	21.0 ± 8.1[b]	4.7 ± 3.2[b]
−	HA	10	68	3 (30)	23.7 ± 7.4[b]	8.6 ± 4.9[b]
−	HS	7	52	1 (14)	20.0 ± 7.8[b]	4.1 ± 4.1[b]

[1] HA, 125 μg/ml hyaluronan; HS, 15 μg/ml heparan sulfate proteoglycan. [2] Percentages are presented based on the total number of embryos transferred. Data are given as means ± SEM from seven to ten recipients. Values with different superscripts within a column are significantly different ($P<0.05$).

developed to the blastocyst stage [35]. Since methods to remove the zona pellucida, including acidic Tyrode's solution, did not affect both the incidence of golden hamster zygotes developing to the blastocyst stage and the total cell number in the blastocyst as compared with zona-intact embryos [33], acidic treatment itself does not appear to be deleterious. Furthermore, in the present study, the number of cells in blastocysts from which the zona pellucida was removed at the 8-cell stage was significantly lower than that of zona-intact controls. Even when two of the zona-free 8-cell embryos were aggregated in a small hollow, the number of blastomeres of the aggregated blastocyst was almost twice that of zona-free single embryos, whereas the culture conditions for aggregated embryos (in a small hallow) were different from those of zona-free and intact ones (on the bottom surface of the culture dish). The presence of the zona pellucida may supply a suitable environment for healthy cleavage in rats. Therefore, the current results demonstrate that in rats, the zona pellucida appears to play an important role in supporting the embryos during early development, at least from the 8-cell stage to the blastocyst stage.

Furthermore, we found that supplementation of culture medium with at least 63 μg/ml HA significantly increased both the incidence of zona-free 8-cell embryos developing to the blastocyst stage and the number of cells in the blastocyst. The presence of 125 or 250 μg/ml HA improved both the incidence and cell number to levels similar to those of zona-intact embryos. HA is a simple glycosaminoglycan with a molecular mass of approximately $3–7 \times 10^6$ Da and is composed of 2000–25000 carbohydrates, N-acetylglucosamine and D-glucuronic acid [36]. It exists as a polyanion and not in an acid form, is involved in cell-to-cell adhesion [37], promotes differentiation of extraembryonic tissues of mouse embryos [38] and is one of the abundant glycosaminoglycans in the uterine, oviductal and follicular fluids in several mammalian species [15, 39–41]. HA secreted by cumulus cells is released not only into the perivitelline space of oocytes [42, 43], but also into the oviduct (40–1830 μg/ml) [44–46]. The optimal concentration of HA in sequential culture media for mouse embryo has been reported to be 250 μg/ml [47], whereas physiological HA concentrations in bovine uterine fluid are 320–590 μg/ml [15]. The effective concentration of HA to improve early development of zona-free rat 8-cell embryos in the current study was similar to these concentrations. Supplementation of defined medium with 1 mg/ml HA improves the development of IVM/IVF bovine embryos to the blastocyst stage, without affecting embryo quality [20]. In mice, on the other hand, HA did not improve embryo development to the blastocyst stage [18], but did improve blastocyst quality as determined by total cell numbers [47]. In fact, CD44, the surface HA receptor, is expressed throughout preimplantation human embryo development [48], whereas the expression of CD44 was highest at the oocyte stage and a second highest level at the compacted 8-cell and morula stages in mice [17]. The CD44 molecule exhibited high expression around contact areas between the trophectoderm and zona pellucida during blastocyst hatching [17]. HA also acts as an antioxidant, protecting against radical oxygen species caused by enzymatically produced hydroxyl radicals [49]. In the present study, we cultured zona-free embryos in an atmosphere of 5% CO_2 in air, which is a relatively high partial pressure of oxygen. Combining the current evidence with previous reports, one of the possible advantages of supplementing with HA may be to reduce the oxidative stress around embryos. Interestingly, supplementation with 125 μg/ml HA beginning at the 8-cell stage significantly reduced the incidence of apoptotic cells in zona-free embryos to the same level as *in vivo* derived embryos and consequently improved the total number of cells in the blastocyst. The number of cells in blastocysts from which zona-free 8-cell embryos were cultured in the presence of HA was higher than that of zona-intact blastocysts developed in HA-free medium, whereas the number was not significantly different from that of *in vivo* derived blastocysts. Recently, HA has been shown to indicate the abundance of some developmentally important gene transcripts [50, 51]. The current results are consistent with previous studies showing that HA downregulates the expression of an apoptosis-inducing gene (*Bax*) in cattle [50, 51]. Since HA also upregulates the expression of a glucose transporter gene (*Glut-1*) in cattle [51], the presence of HA surrounding an embryo may facilitate survival and development of the embryo during culture from the 8-cell to blastocyst stages. Therefore, the zona pellucida makes a suitable microenvironment in which HA contributes to the optimal development of gametes, zygotes and embryos. Supplementation of culture medium with HA is strongly recommended for culture of zona-free or zona-insured embryos.

In the current study, we demonstrated that supplementation of culture medium with 15 μg/ml HS significantly increased both the incidence of zona-free 8-cell embryos developing to the blastocyst stage and the number of cells in the blastocyst to levels similar to when HA was supplemented into culture medium at 63–250 μg/

ml. In the female reproductive tract, HS and HA, are abundant [15]. The presence of HS in culture medium has been reported to increase the number of inner cell masses in blastocysts [47]. Culture of mouse zygotes in a BSA-free medium containing 250 µg/ml HA and 15 µg/ml HS, resulted in comparable development to the expanding to hatching blastocyst stages of development and comparable or better differentiated cell numbers in the resulting blastocysts compared with culture in the presence of 2 mg/ml BSA [47]. HS supplementation of sequential culture media supported the development of 1-cell mouse embryos to the blastocyst stage in a manner comparable with BSA-supplemented controls [47]. HS mRNA has also been shown to be first found in mouse *in vivo*-derived blastocysts at day 4, and both proteoglycan message and product progressively increase post hatching as the blastocyst becomes attachment competent [24, 25].

When blastocysts developed *in vitro* from zona-free 8-cell embryos in the absence of HA and HS were surgically transferred into the uterus, the incidence of embryos implanted or developed to term was significantly lower than that of zona-intact embryos, whereas transfer of aggregated blastocysts derived from pairs of 8-cell embryos improved both incidences to levels comparable with zona-intact blastocysts. From these results, the cell number in transferred blastocysts appears to be one of the critical factors for posttransfer survival of the embryos. On the other hand, *in vitro* development of zona-free 8-cell embryos in the presence of HA or HS significantly decreased the incidence of apoptotic cells and consequently increased the mean cell number in the blastocyst, but could not improve the incidences of embryos implanted and developed to term after embryo transfer. In mice and cattle, culture of intact embryos in the presence of HA (0.5–1.0 mg/ml) increased implantation rates and fetal development following transfer [18, 52]. In the present study, although the presence of HA or HS improved the cell number of embryos, due to the absence of the zona pellucida, the number may be still insufficient for successful implantation after transfer of the embryos. From this point of view, the zona pellucida may play important roles not only supporting healthy cleavage and survival of blastomeres but also improving the competence of blastocysts to implant and survive. Successful implantation results from reciprocal crosstalk between an implantation-competent blastocyst and a receptive uterus [53, 54]. During this time, the blastocyst is drastically activated with the expression of a number of genes associated with the cell cycle, metabolism/energy pathway, calcium signaling, chromatin remodeling, adhesive molecules and HB-EGF [55]. The presence of the zona pellucida during blastocyst formation may reflect this "blastocyst activation." To clarify the detailed role of the zona pellucida, further research will be required.

It can be concluded that the zona pellucida in the rat plays roles supporting successful development of early embryos from at least the 8-cell stage not only to the blastocyst stage but also to postimplantation stages. Although supplementation of culture medium with glycosaminoglycans, such as HA or HS, significantly contributes to successful development to the blastocyst stage, it may not be sufficient to maintain the posttransfer survival of zona-free rat embryos.

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