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Media optimization to promote rat embryonic development to the blastocyst stage *in vitro*

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ABSTRACT

Efficient model production in rats that incorporates newly developed genetic editing and embryo transfer tools, such as CRISPR/Cas9 technology and non-surgical embryo transfer, requires availability of an optimal embryo culture system. However, current technologies for *in vitro* manipulation of rat gametes, including embryo culture techniques, are less advanced compared to those in mice. In this study, we (1) identified a culture medium that was able to support optimal rat embryonic development by comparing two rat culture media: mR1ECM (modified rat 1-cell embryo culture medium) and KSOM-R (modified potassium simplex optimized medium for rats), and (2) evaluated the effect of glutamine dipeptides: alanyl-L-glutamine and glycyl-L-glutamine, on rat embryonic development. We also investigated the possibility of simplifying the KSOM-R culture procedure by increasing the volume of culture medium, reducing the need for daily medium changes. The results showed that rat embryos cultured in KSOM-R developed faster than those cultured in mR1ECM. Both alanyl-L-glutamine and glycyl-L-glutamine showed detrimental effects on rat embryonic development when supplemented in KSOM-R at the same concentration as glutamine. By increasing the volume of KSOM-R, rat zygotes were able to develop without daily medium refreshment at a similar rate and developmental competence as those in smaller volumes with daily medium changes. These results represent important improvements to rat embryo culture methods and will assist in more efficient production of rat models.

1. Introduction

The rat has been a major experimental model in biomedical research for more than a century because of the similarities of physiological characteristics with humans. The creation of rat models with targeted genetic alterations has been greatly accelerated due to the availability of germline competent embryonic stem cell lines and engineered nucleases-based genomic editing tools, such as zinc finger nucleases, transcription activator-like effector nucleases and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology [1–4]. With the availability of these tools, improvements in rat embryo manipulation techniques, such as *in vitro* culture and embryo transfer, are needed to further improve the efficacy and efficiency for rat model creation.

Currently, there are two culture media, mR1ECM and KSOM-R, that can support rat embryonic development *in vitro* and allow embryos transferred to surrogate dams to develop to pups *in vivo*. mR1ECM was developed from modifications of a hamster embryo culture medium with the elimination of phosphate and reduced osmolality [5–12]. KSOM-R was modified from mouse KSOM with the elimination of phosphate and the supplementation of additional amino acids according to their relative richness in rat oviduct fluid [13]. Both media support rat embryo development from the 1-cell stage (zygote) to the blastocyst stage *in vitro* and result in embryos that retain the ability to undergo full term development *in vivo* after embryo transfer [7,12,13]. However, there have been no side-by-side comparisons of the two media to measure their respective abilities to support rat embryonic development *in vitro* and *in vivo*.

It is possible to introduce modifications to rodent embryo culture media to optimize embryonic development. For example, due to the potential toxic effect of ammonium resulting from metabolism of glutamine in culture [14], the dipeptides Glycyl-L-

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glutamine (GlyGln) and alanyl-L-glutamine (AlaGln) were examined as a replacement of glutamine in KSOM. Results demonstrated that supplementation of GlyGln in KSOM is beneficial for mouse embryo development *in vitro* [15]. However, the effects of glutamine dipeptides on rat embryonic development have not been investigated. Currently, optimal rat embryo development in KSOM-R involves daily replacement of the medium [13]. This is inconvenient for staff when embryos need to be cultured over the weekend. Therefore, the ability to minimize the frequency of media changes would be highly desirable.

The goal of our study was to identify an optimized culture media system that would allow *in vitro* development of rat embryos to the blastocyst stage. Therefore, we 1) compared mR1ECM and KSOM-R for the ability to support rat embryo development, 2) used AlaGln or GlyGln as a glutamine replacement in KSOM-R and evaluated the effects on the development of rat zygotes *in vitro*, and 3) tested the hypothesis that rat embryos can develop in a larger volume of KSOM-R without daily medium replacement at a rate and developmental competence comparable to embryos cultured in smaller volumes of KSOM-R with daily medium change.

2. Materials and methods

2.1. Animals

Sprague Dawley (SD) rats were purchased from Envigo (Indianapolis, IN, USA) and were housed in microisolator caging on ventilated racks in an environmentally controlled room with a temperature of 22 °C, 14 h light/10 h dark cycle with access to food (Purina 5008), and water *ad libitum*. This study was conducted in strict accordance with the recommendations in the Guide for Animal Care and Use of Laboratory Animals of the National Institutes of Health. The protocols for animal care and surgical procedures were approved by the Animal Care and Use Committee of the University of Missouri.

2.2. Embryo collection and culture

Unless specifically stated, all chemicals were purchased from MilliporeSigma (St Louis, MO, USA). To collect zygotes from immature (4–5 weeks old) female SD rats, females were superovulated by intraperitoneal administration of 20 IU PG600 (Merck, Kenilworth, NJ, USA) followed by intraperitoneal administration of 40 IU human chorionic gonadotropin (HCG) (Calbiochem, San Diego, CA, USA) 50 h later. Females were then paired with individually housed stud males. Presumptive zygotes were collected at 23–24 h after HCG administration from plug-positive females using mR1ECM-Hepes (~310 mOSM) + 4 mg/mL fatty acid free BSA [12]. After CO_2 euthanasia, oviducts were excised by sterile technique and cumulus enclosed zygotes were released from the oviduct by tearing the swollen ampulla using fine forceps and an insulin needle. Presumptive zygotes were then denuded by briefly exposing to 1 mg/mL hyaluronidase in mR1ECM-Hepes.

Zygotes from mature (8–10 weeks old) female rats were collected from females synchronized by intraperitoneal administration of luteinizing hormone-releasing hormone agonist ((LHRHa, MilliporeSigma, L 4513) at 40 μg per rat and were paired individually with stud males 4 days later. Presumptive zygotes were collected the next day from plug-positive females and denuded as described above. For controls, *in vivo* derived embryos at the blastocyst stage were collected from pregnant females 4.5 days post coitum (dpc) by flushing both oviducts and uteri using mR1ECM-Hepes-10% FBS (Life Technologies Incorporated, Grand Island, NY, USA).

2.3. Embryo development assessment, embryo transfer and pup evaluation

Zygotes (with visible pronuclei) were randomly allocated into treatment groups with a maximum of 30 zygotes per 30 μL medium under mineral oil in 35 mm Petri dish or a maximum of 50 zygotes per 500 μL medium in NUNC 4-well culture plate under mineral oil and cultured at 37 °C with 5% O_2, 5% CO_2, 90% N_2 with maximal humidity for 96 h (Experiment #1 & 3) or 120 h (Experiment #2). Rat embryonic development at 24 h, 96 h or 120 h was assessed and the embryonic stages were recorded. For assessment of the *in vivo* developmental potential of the cultured embryos, embryos were surgically transferred into 3.5 dpc pseudo-pregnant SD females. These recipients were generated by synchronizing mature 8–10 week old female rats with LHRHa at 40 μg/rat and then pairing them individually with vasectomized males 4 days later. After mating overnight, copulation plug-positive females were selected as recipients. A total of 20 embryos (morula/blastocysts) were transferred into the uterine horns (10 embryos per uterine horn) of 3.5 dpc pseudo-pregnant SD females. The number of pups from each group were counted at birth. Pups were evaluated visually for any gross abnormalities and individual weights were recorded 7 days post-delivery.

2.4. Experimental design

Experiment 1 was designed to compare rat embryo culture of zygotes to the blastocyst stage in mR1ECM and KSOM-R, and then compare their subsequent development *in vivo* relative to uncultured embryos. In this experiment, embryos cultured in MR1ECM, KSOM-R and control were from three independent donor groups. For culture, zygotes (30 or less) from mature rats were placed in 30 μL modified mR1ECM or KSOM-R respectively. For embryos cultured in mR1ECM, zygotes were cultured in mR1ECM (~310 mOSM)+ 4 mg/mL fatty acid-free BSA for 24 h and then were moved into mR1ECM (~246 mOSM) + 0.01% PVA. When embryos progressed into stages with 16-cells or greater, 10% FBS was supplemented in mR1ECM [10]. This was done at around 92 h (around 8 o'clock of Day 4 of culture) due to the non-uniformity of individual embryonic development *in vitro* and the toxic effect of FBS to early stage rat embryos [10]. For embryos cultured in KSOM-R, the medium was changed daily as previously described [13]. FBS was also supplemented to KSOM-R medium when embryos reached stages with 16-cells or greater. After 96 h of culture, up to 20 morula and blastocysts from either mR1ECM or KSOM-R culture were surgically transferred equally into both uterine horns of a 3.5 dpc pseudo-pregnant SD rat. *In vivo* derived blastocysts were surgically transferred as a control. The resulting pups from each group were counted and evaluated for visible abnormalities and weight at 7 days old.

Experiment 2 was designed to investigate the effects of AlaGln and GlyGln on the development of rat embryos *in vitro*. Rat zygotes from mature rats were allocated into three KSOM-R groups with different glutamine sources: 1 mM AlaGln, 1 mM GlyGln, or 1 mM Gln (control). Medium was changed daily in each group. FBS was supplemented into the culture media when embryos progressed to stages of 16-cells or greater. Embryos that progressed to the 2-cell stage by 24 h and blastocyst stage by 120 h were recorded.

Experiment 3 was designed to investigate the effects on effects of embryos of both the volume of KSOM-R and the schedule of medium replacement. Because of genomic editing reagents are usually introduced into zygotes from superovulated immature rats, rat zygotes used in this experiment were collected from immature rats (40–50 g). Zygotes were randomly allocated into three culture groups: 1) up to 30 embryos in 30 μL KSOM-R with medium

replacement every 24 h; up to 30 embryos in 30 μL KSOM-R with medium replacement every 48 h, and up to 50 embryos in 500 μL KSOM-R without medium replacement. Embryos in 30 μL drops were cultured in 35 mm petri dishes and embryos in 500 μL KSOM-R were cultured in Nunc 4-well plates. By 96 h, resulting morula and blastocysts were surgically transferred into 3.5 dpc pseudo-pregnant recipients to assess their *in vivo* development. Similar to Experimental design 1, the resulting pups from each group were counted and evaluated for visible abnormalities and weight at 7 days old.

2.5. Statistical analysis

Each experiment was repeated at least three times. The percentage data are expressed as mean ± SEM. Data used to compare efficiency of embryo development in mR1ECM and KSOM-R (Table 1) were analyzed by student t-test analysis. For all other data analyses, one-way ANOVA was used for comparing data between groups after arcsine transformation followed by either post-hoc Tukey's test or Kruskal–Wallis test. A p value less than 0.05 was considered to be significant.

3. Results

Embryonic development *in vitro* from zygote to blastocyst stage was evaluated in triplicate for two different culture media (Table 1). By 96 h of culture, 64.3 ± 7.1% of rat embryos cultured in KSOM-R medium reached the blastocyst stage compared to 18.6 ± 4.1% cultured in mR1ECM. The majority (63.1 ± 10.0%) of embryos in mR1ECM were still at the morula stage.

Twenty embryos from each replicate were transferred into 3.5 dpc pseudo-pregnant female rats to assess their developmental competence *in vivo*. Rat embryos cultured in either mR1ECM or KSOM-R were able to develop into full term pups (Table 2). No gross physiological abnormalities were noted based on visual inspection of the pups.

Developmental effects of substituting AlaGln and GlyGln for Gln in KSOM-R during embryo culture are shown in Table 3. KSOM-R with either 1 mM AlaGln or 1 mM GlyGln supplemented significantly compromised the ability of rat embryos to develop from zygotes into blastocysts by 120 h of culture compared to KSOM-R with Gln ($p < 0.05$). 27.3 ± 7.3% of rat embryos cultured in KSOM-R with GlyGln developed to blastocyst stage, compared to only 4.5% ± 2.0% of those cultured in KSOM-R with AlaGln. However, zygotes to blastocysts development in KSOM-R was significantly increased to 88.6 ± 4.9% with supplementation of Gln.

The effects of culture medium volume and frequency of medium replacement on *in vitro* development of rat zygotes from immature SD rats are shown in Table 4. Daily replacement of KSOM-R medium (control) resulted in 91.5 ± 5.1% of zygotes developing to morula and blastocyst stage embryos *in vitro*. However, by increasing the volume of KSOM-R to 500 μL, 83.9 ± 2.8% of zygotes were able to develop into morula and blastocysts by 96 h without a medium replacement. In addition, by replacing the medium at 48 h interval, 77.1 ± 11.3% of zygotes developed to blastocyst stage embryos. Statistical analysis of the data showed no significant difference in embryo recovery between the three sets of culture conditions ($P > 0.05$).

The *in vivo* developmental competence of the cultured rat embryos was assessed by the recovery of pups after surgical embryo transfer into 3.5 dpc pseudo-pregnant rats. The effect of culture medium volume and replacement intervals is shown in Table 5. Embryos from all three culture groups were able to develop into full term pups with no overt physical abnormalities based on visual examination.

Table 1
Rat embryo development from zygote to blastocyst in mR1ECM and KSOM-R.

Treatment	0 h	24 h		96 h	
	Zygotes	1-cell	2-cell	morula (%±SEM)	blastocysts (%±SEM)
mR1ECM	98	4	94	61 (63.1 ± 10.0)[a] *	18 (18.6 ± 4.1)[c]
KSOM-R	71	0	71	18 (25.9 ± 7.6)[b]	46 (64.3 ± 7.1)[d]

*Different superscripts indicate statistical difference ($p < 0.05$).

4. Discussion

The present study represents the first direct comparison of the rat embryo culture media mR1ECM and KSOM-R on the development of zygotes to blastocysts *in vitro*. The results demonstrated that both media were able to support rat embryonic development. However, significantly more embryos developed to blastocysts in

Table 2
Effect of rat embryo culture in mR1ECM or KSOM-R on pup recovery.

Embryo source	Recipient #	Embryo transferred	Embryo transferred		Pups (%) at		Average weight 7 days (g±SEM)
			morula	blastocysts	DOB	one week	
mR1ECM	1	20	16	4	8(40.0)	8(40.0)	23.0 ± 0.7
	2	20	12	8	8(40.0)	8(40.0)	17.0 ± 1.7
	3	20	14	6	4(20.0)	4(20.0)	21.8 ± 3.9
KSOM-R	1	20	2	18	11(55.0)	10(50.0)	19.2 ± 0.7
	2	20	9	11	0	0	N/A
	3	20	3	17	14(70.0)	13(65.0)	16.1 ± 0.9
In vivo	1	16	0	16	10(62.5)	10(62.5)	17.9 ± 0.4
	2	16	0	16	7 (46.7)	7(46.7)	21.0 ± 0.5
	3	15	0	15	4(26.7)	4(26.7)	27.0 ± 1.4

*Different superscripts within a column indicate statistical difference ($p < 0.05$).

Table 3
Effect of glutamine source on the development of rat zygotes in KSOM-R.

Glutamine source in KSOM-R	Zygotes (0 h)	2-cell (%) (24 h)	Blastocysts (% ± SEM) (120 h)
Gln	44	44 (100)	39 (88.6 ± 4.9)[a] *
AlaGln	44	42 (95.5)	2 (4.5 ± 2.0)[b]
GlyGln	44	43 (97.7)	12 (27.3 ± 7.3)[c]

Table 4
Effect of medium replacement interval and volume on rat embryo development in KSOM-R.

Medium replacement Interval	Medium volume (μl)	Zygotes	2-cell	Morula and blastocysts at 96 h (% + SEM)
24 h	30	117	117	107 (91.5 ± 5.1)[a] *
48 h	30	118	118	91 (77.1 ± 11.3)[a]
None	500	186	186	156 (83.9 ± 2.8)[a]

*No statistical difference was found between groups ($p > 0.05$).

Table 5
Effect of medium replacement interval and volume on pup recovery from rat embryos cultured in KSOM-R.

Media change interval	Recipient #	Embryos transferred	Embryos transferred		Pups (%) at		Average body weight (g±SEM)
			morula	blastocysts	DOB	one week	
24 h	1	20	2	18	5 (25.0)	5(25.0)	21.2 ± 0.66
	2	20	0	18	12(60.0)	12(60.0)	18.9 ± 0.77
	3	12	2	10	5(41.7)	5 (41.7)	20.4 ± 0.51
48 h	1	20	6	14	12(60.0)	10(50.0)	17.1 ± 1.22
	2	12	2	10	8(66.7)	8(66.7)	20.25 ± 0.25
None	1	20	0	20	8(40.0)	7(35.0)	21.1 ± 0.74
	2	11	0	11	1(9.1)	1(9.1)	14.0
	3	12	0	12	8(66.7)	8(66.7)	19.75 ± 0.49
	4	12	0	12	6(50.0)	6(50.0)	21.3 ± 1.67

KSOM-R by 96 h than those cultured in mR1ECM. Embryos from both groups were able to develop into live pups after surgical embryo transfers. These pups had no gross abnormalities in morphology and growth. We also tested two media parameters: 1) substitution of glutamine with dipeptide glutamines, and 2) frequency of media changes. Replacing glutamine with either AlaGln or GlyGln in KSOM-R had a detrimental effect on rat embryonic development *in vitro*. By increasing the volume of KSOM-R to 500 μL, no medium changes were required for rat embryos to develop at a rate comparable to embryos cultured in the 30 μL drops with daily medium refreshment.

mR1ECM was the first medium that is able to support rat embryos from zygotes to blastocysts *in vitro*. It was initially described in 1994 as a modification of hamster embryo culture medium 1 [5,6]. Since then, this medium has been optimized for rats by several modifications to the medium to tailor embryo's nutritional needs during different stages of preimplantation development [7,10–12]. Although the pup rates remained low after transfer of embryos cultured in mR1ECM [7,12], mR1ECM has remained the medium of choice for culturing rat preimplantation embryos for over two decades. KSOM-R is a modification of mouse KSOM based on the profiles of amino acids found in the rat reproductive tract [13]. Significant more rat embryos developed from zygotes to blastocysts by 96 h of culture in KSOM-R than those in mR1ECM in this experiment. This simulation of the rat reproductive tract amino acid profile may contribute to the better support of rat embryonic development *in vitro*. Nevertheless, embryos cultured in both media had the ability to develop into live full-term pups with normal morphology and growth. Because KSOM-R more effectively supported embryo development to blastocyst stage, two culture parameters were chosen for further study with this medium.

First, Gln was substituted with dipeptide glutamines to reduce potential toxic effects of ammonium accumulation in the medium. Gln is an important energy source for both cells and embryos in culture. However, the toxic effect of ammonium resulting from its metabolism in culture is well established [14,16]. To reduce the deleterious effect of Gln in culture media, Gln in the dipeptide form, such as AlaGln or GlyGln, has been used widely as a substitute [17,18]. It has been demonstrated that GlyGln is able to promote mouse embryonic development by replacing Gln in culture medium [15]. However, in our studies, both AlaGln and GlyGln were detrimental to rat embryonic development *in vitro* when either of them replaced Gln in KSOM-R. There are several possibilities for the observed detrimental effect. For example, AlaGln and GlyGln were supplemented at a concentration of 1 mM in this experiment. This concentration was chosen because 1) KSOM-R normally contains 1 mM of Gln and 2) the maximal blastocyst formation rate for mouse embryo culture was achieved with supplementation of KSOM with 1 mM GlyGln [15]. However, this concentration of Gln-based dipeptide may not be appropriate for rat embryo development since the optimal concentration of dipeptide in culture is known to be cell type-specific [17]. It may also be that rat preimplantation stage embryos are unable to use Gln-based dipeptides effectively due to the low affinity of rat dipeptidases to both AlaGln and GlyGln [18]. Further research is needed to investigate if glutamine dipeptides can be used to replace Gln without negatively impacting rat embryonic development.

In KSOM-R, optimal rat embryonic development is achieved by daily replacement of media [13]. The positive effect of daily media changes may be due to the removal of toxic metabolites, including ammonia. We hypothesized that toxic effect of metabolite accumulation may be reduced by increasing the volume of culture medium. Therefore, the second parameter studied was toxin minimization by an increase in medium volume. In previous studies, using 100 μL KSOM-R with 30–40 zygotes per trial, daily medium change resulted in a significant increase in embryos reaching blastocyst stage by 120 h than those without daily medium refreshment [13]. In our studies, by increasing the volume of KSOM-R to 500 μL with a maximum of 50 zygotes per well, no medium replacement was required for efficient rat embryo development *in vitro*. Embryos cultured in KSOM-R with daily medium replacement and embryos cultured in a larger volume of medium all retained the ability to develop into live full-term pups with normal morphology and growth. Use of larger medium volume during embryo culture is compatible with recovery of healthy animals and requires less time and use of resources than the requirement for daily medium changes.

The work described in this manuscript was performed as an effort to implement a non-surgical embryo transfer (NSET) technique into rat model generation after engineered nuclease-based genomic editing. NSET provides an alternative to traditional surgical methods for the transfer of blastocyst stage embryos into pseudopregnant recipient rodents. In mice, NSET can significantly alleviate the cost, time and level of animal distress associated with

surgical embryo transfer while achieving similar transfer efficiencies [19,20]. An NSET device and protocol for the transfer of blastocyst stage embryos is also available for rats (ParaTechs Corporation, Lexington, KY, USA). Given that genomic editing reagents are typically introduced into early stage embryos [3,4,21] and the NSET technique is only effective for transferring embryos at the blastocyst stage [20], it is necessary to culture genetically modified embryos for several days prior to NSET. Therefore, an efficient rat embryo culture system is necessary to take advantage of the NSET technique when using genome editing methods to produce genetically engineered rats. This work will assist in the implementation of NSET technique for an efficient production of rat models.

Based on our experimental results, we recommend the use of KSOM-R for reliable and optimal rat embryonic development *in vitro* for studies requiring culturing of rat embryos to the blastocyst stage. Additionally, the need to replace the medium on a daily basis can be eliminated by increasing the volume to 500 μL per every 50 embryos being cultured. The replacement of Gln with either AlaGln or GlyGln is not recommended. These rat embryo culture modifications have particular relevance for procedures where embryos that are genetically modified at the zygote or 2-cell stage are cultured to the blastocyst stage for downstream analysis or applications involving NSET.

CRediT authorship contribution statement

Hongsheng Men: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - review & editing. **Barbara J. Stone:** Funding acquisition, Writing - review & editing. **Elizabeth C. Bryda:** Funding acquisition, Resources, Conceptualization, Methodology, Formal analysis, Project administration, Writing - review & editing.

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