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KSOM-R supports both mouse and rat preimplantation embryo development *in vitro*

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ABSTRACT

A modified KSOM for rat embryo culture (KSOM-R), which has enriched taurine, glycine, glutamic acid, and alanine, promoted rat embryo development *in vitro*. Since mice and rats share similar amino acid profiles in their female reproductive tracts, this study explored whether KSOM-R would also have a positive effect on mouse embryo development and if KSOM-R modifications could extend its shelf time at 2–8 °C for consistency. We first examined the effects of newly made (≤1 month at 2–8 °C) antibiotics-free KSOM-R (mKSOM-R), antibiotics-free KSOM (mKSOM) and KSOM on the development of *in vivo* or *in vitro* derived C57BL/6NJ zygotes. We then investigated the effect of extended shelf life (6 months at 2–8 °C) of mKSOM-R and mKOSM on the development of C57BL/6NJ mouse and Sprague Dawley (SD) rat embryos. The results showed that there were no significant differences in cleavage, blastocyst, and hatching rates of C57BL/6NJ embryos among the three freshly made media. After 6 months of storage at 2–8 °C, mKSOM-R and mKSOM were still able to support the development of *in vivo* C57BL/6NJ zygotes at comparable rates seen with newly made (≤1 month at 2–8 °C) KSOM (control) in terms of cleavage, blastocyst formation and hatching. There were also no significant differences in total cell numbers in day 4 blastocysts among the three groups. After surgical embryo transfers, C57BL/6NJ blastocysts cultured in mKSOM-R (6 months at 2–8 °C) and newly made (≤1 month at 2–8 °C) KSOM culture developed into live pups. These pups had no gross abnormalities in animal morphology and growth. SD zygotes cultured in mKSOM-R stored at 2–8 °C for 6 months developed at comparable rates in cleavage, blastocyst and hatching rates when compared to those cultured in newly made mKSOM-R (≤1 month at 2–8 °C). The data showed that, although no significant beneficial effects were observed on mouse embryo development, mKSOM-R was able to support both mouse and rat embryo development *in vitro*. Additionally, mKSOM-R and mKSOM can be stored at 2–8 °C for at least 6 months without significantly compromising quality. This study suggests that it is possible to reduce the media inventory by using only mKSOM-R to culture both mouse and rat embryos, and quality media with extended shelf life can be made through modifications.

1. Introduction

With the increasing understanding of their physiology and metabolism of mammalian preimplantation embryos, advanced culture media that can successfully grow embryos *in vitro* have been developed in various species [1–3]. However, current *in vitro* culture systems are still suboptimal compared to their *in vivo* counterparts as abnormalities including perturbed gene expression, especially imprinting genes, have been associated with *in vitro* culture [4,5]. Therefore, media optimization is needed to increase the efficiency and efficacy of mammalian embryo culture. Amino acids exist in female oviductal tracts and are also critical for the optimal development of embryos cultured *in vitro* [6,7]. Supplementation of Eagle's essential and non-essential amino acids to culture media improved the *in vitro* development of early-stage embryos from various species including mouse and rat [1,4,8,9].

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The additional supplementation of taurine, glycine, glutamic acid, and alanine, which are rich in rat oviducts, in a modified KSOM for rat (KSOM-R) media further improved rat embryo development *in vitro* [10,11]. Since the concentrations of taurine, glycine, glutamic acid, and alanine are also rich in mouse oviducts [12], KSOM-R may also be optimal for mouse preimplantation embryo development *in vitro*. Therefore, this study investigated the capacity of KSOM-R to support mouse early embryo development in *vitro*.

The quality and consistency of media significantly impact the optimal development of embryos in culture. Embryo culture media often contain components, such as glutamine and antibiotics, that are chemically unstable and readily undergoing degradation after extended storage even at 2–8 °C [13]. The degradation of essential gradients not only reduces their concentrations needed for optimal embryo development, but also produces substances that may be detrimental to embryo growth. Glutamine is an essential amino acid for mouse embryos and important in overcoming the 2-cell block [14,15]. However, ammonia from its spontaneous and metabolic breakdown is toxic to embryos in culture [16–18]. Its toxicity can be alleviated by substituting glutamine with its dipeptide form: Alanyl-L-Gln or Glycyl-L-Gln [19,20]. However, both Alanyl-L-Gln and Glycyl-L-Gln are toxic to early rat embryos [11]. Therefore, glutamine in its original form is needed in KSOM-R. Additional unstable components in KSOM-R formulation include penicillin and streptomycin [21]. Therefore, to make quality KSOM-R to further optimize the single medium culture system, KSOM-R stock solution is made without glutamine (added to working aliquots using 200 mM glutamine stock prior to use) and antibiotics, thus this mKSOM-R stock has a longer shelf life at 2–8 °C and better quality as it can be made in larger quantity (e.g., 1 litter) with less variation in individual components for consistency.

Currently, mouse and rat embryo culture use very different culture systems [8,9,22,23]. Of note, rat embryos require stage-specific media to support optimal embryo development *in vitro* [23]. Mouse models remain to be the most popular models in the biomedical community because the ability to generate mice with targeted mutation was more than two decades ahead of rats [24–26]. Consequently, rat embryo culture media may not be in the routine use at many transgenic core facilities. New orders for either media or chemicals are necessary for infrequent new rat models. Therefore, an optimized single medium (mKSOM-R) that can support both mouse and rat embryo development *in vitro* would reduce the media inventory and improve efficiencies of core facilities intending to work with both species.

The objectives of current study were to investigate if mKSOM-R will provide better support for mouse embryo development *in vitro*. The effect of both mKSOM-R and mKSOM with extended shelf life at 2–8 °C on their ability to support mouse embryo development *in vitro* was also investigated. Similarly, the effects of mKSOM-R with extended shelf life at 2–8 °C on rat embryo development *in vitro* were also investigated.

2. Materials and methods

2.1. Animals

C57BL/6NJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Sprague Dawley (Crl:CD) rats were purchased from the Charles River Laboratories (Wilmington, MA, USA) and animals were housed in microisolator caging on ventilated racks in an environmentally controlled room with a temperature of 22 °C, 12-h light/12-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 and under a protocol approved by the Animal Care and Use Committee of the University of Missouri.

2.2. Media used for flushing and culture

Unless specifically stated, all chemicals were purchased from MilliporeSigma (St Louis, MO, USA). A modified flushing-holding medium (mFHM), with supplementation of 20 mM HEPES and reduced $NaHCO_3$ in KSOM-R as described previously for mouse flushing-holding medium [22], was used for both mouse and rat embryo collection. Both KSOM [8,19] and KSOM-R [10] were modified by eliminating antibiotics. KSOM-R stock was further modified by eliminating glutamine to prolong their shelf life at 2–8 °C. These media were named mKSOM and mKSOM-R, respectively. Standard KSOM made monthly was used as a control. Prior to use, appropriate amount of 200 mM glutamine stock was aseptically added to an mKSOM-R aliquot to make a working solution with 1 mM glutamine. The detailed components of each medium were listed in Table 1.

2.3. Zygote production

Immature C57BL/6NJ mice (4–5 weeks) were superovulated by intraperitoneal administration of 5 IU PMSG (ProSpec, Rehovot, Israel) at 11:00 a.m.-1:00 p.m. followed by 5 IU HCG (Calbiochem, San Diego, CA, USA) 48 h later. Immature (4–5 weeks old) female SD rats were superovulated by intraperitoneal administration of 25 IU PMSG at 9:00 a.m.-10:00 a.m. followed by intraperitoneal administration of 40 IU HCG 50–52 h later. After HCG injections, females (either mouse or rat) were paired with individually housed stud males. At 23–24 h after HCG administration, females were killed by carbon dioxide inhalation following the AVMA guidelines for euthanasia. Oviducts were excised using sterile technique and cumulus enclosed zygotes were released from the oviduct by tearing the swollen ampulla using fine forceps and an insulin syringe with a 29-gauge needle. Presumptive zygotes were denuded by briefly exposing to 1 mg/mL hyaluronidase and then washed using mFHM (Table 1) for both mouse and rat zygotes.

To collect mouse oocytes for *in vitro* fertilization (IVF), both 5 IU PMSG and 5 IU hCG were administered between 4:30–5:00 p.m. with a 48 h interval. IVF was conducted as described previously [27]. Briefly, frozen-thawed C57BL/6NJ sperm were cultured in 200 μL TYH (Toyoda, Yokoyama, Hoshi) medium supplemented with 0.75 mm MBCD (Methyl-β-Cyclodextrin) for 60 min at 37 °C, 5% CO_2 with maximal humidity for capacitation. Cumulus-oocytes-complexes (COCs) were collected from females 15–15:30 h after HCG administration. After cervical dislocation of the females, oviducts were excised by sterile technique and COCs were released from the oviduct by tearing the swollen ampulla using fine forceps and an insulin syringe with a 29-gauge needle, and then COCs were introduced into 90 μL mHTF droplets. An approximately 10 μL sperm suspension was introduced into each mHTF drop (2–5 X 10^5 sperm/mL final). Sperm and COCs were then co-cultured for 6 h for fertilization *in vitro* in an incubator at 37 °C, 5% CO_2 with maximal humidity.

2.4. Embryo development assessment, embryo transfer and pup evaluation

Zygotes (with visible two pronuclei) produced either *in vitro* or *in vivo* were randomly allocated into treatment groups with a maximum of 50 zygotes per 500 μL medium in NUNC 4-well culture plates under mineral oil and cultured at 37 °C with 5% CO_2 in air with maximal humidity. Embryonic development was assessed: Cleavage at 24 h for both mouse and rat embryos, blastocysts at

Table 1
Media composition and their concentrations in stocks used in this study.

Chemicals	KSOM (mM)	mKSOM (mM)	mKSOM-R (mM)	mFHM (mM)
NaCl	95	95	95	95
KCl	2.5	2.5	5	5
KH_2PO_4	0.35	0.35	–	–
$MgSO_4 \cdot 7H_20$	0.2	0.2	0.2	0.2
$NaHCO_3$	25	25	25	4
$CaCl_2 \cdot 2H_2O$	1.71	1.71	1.71	1.71
D-Glucose	0.2	0.2	0.2	0.2
Na Lactate (60% syrup)	10	10	10	10
Sodium Pyruvate	0.2	0.2	0.2	0.2
EDTA	0.01	0.01	0.01	0.01
GlutaMax	1	1	–	–
Glutamine[a]	–	–	1.0	1.0
Penicillin G Potassium[b]	0.06 mg/mL	–	–	–
Streptomycin Sulfate[b]	0.05 mg/mL	–	–	–
MEM NEAA 100X	0.5 X	0.5 X	1 X	1 X
MEM EAA 50X	0.5 X	0.5 X	1 X	1 X
Taurine	–	–	1	1
Glycine	–	–	1	1
Glutamate	–	–	1	1
Alanine	–	–	1	1
HEPES	–	–	–	20
BSA	1 mg/mL	1 mg/mL	1 mg/mL	1 mg/mL

[a] Glutamine was excluded in mKSOM-R stock media and added to working solution using 200 mM glutamine stock.
[b] Antibiotics are excluded in mKSOM, mKSOM-R and mFHM.

96 h (mouse) or 120 h (rat) and hatching at 120 h (mouse) and 144 h (rat). A portion of mouse blastocysts (Day 4) resulting from KSOM, mKSOM and mKSOM-R culture were mounted on microscopic slides using ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher Scientific, Waltham, MA, USA) and the cells in each embryo were counted by counting the cell nuclei under a Zeiss Axiophot fluorescence microscope (Carl Zeiss Microscope GmbH, Jena, Germany).

The *in vivo* developmental potential of mouse embryos resulting from culture of *in vivo* derived zygotes in mKSOM-R was also assessed. A total of 16–18 day 4 blastocysts were surgically transferred into the uterine horns (each horn received 8–9 embryos) of one 2.5 dpc pseudo-pregnant CD female. These recipients were generated by pairing them individually with vasectomized males two days before embryo transfer. After mating overnight, copulation plug-positive females were selected as recipients. The number of pups from each group were counted and evaluated visually for any gross abnormalities at 7–9 days after birth and individual weights were recorded.

2.5. Experiment design

Experiment 1 investigated the effects of mKSOM-R on the development of C57BL/6NJ zygotes derived either *in vivo* or *in vitro*. Zygotes in a group of 50 or less were randomly allocated into 500 μL mKSOM-R, 500 μL mKSOM and 500 μL KSOM in wells of NUNC 4-well culture plates. Embryos that progressed to the 2-cell stage by 24 h, blastocyst stage by 96 h and hatched blastocysts by 120 h were recorded. A total of 15 (3 replicates X 5 animals/replicate) immature C57BL/6NJ females were used to collect *in vivo* zygotes and a total of 30 (6 replicates X 5 animals/replicate) immature C57BL/6NJ were used to collect oocytes.

Experiment 2 investigated the effects of mKSOM-R and mKSOM after 6 months storage at 2–8 °C on *in vitro* development of C57BL/6NJ zygotes derived *in vivo*. The procedures were the same as those in experiment 1. A portion of day 4 blastocysts from each group were mounted on microscopic slides using ProLong™ Gold Antifade Mountant with DAPI for cell number count. A total of 20 (4 replicates X 5 animals/replicate) immature females were used for zygote collection in this experiment. Day 4 blastocysts from mKSOM-R and KSOM made monthly (standard control media) were also transferred into synchronized surrogates to assess their development potential *in vivo*. A total of 15 (3 replicates X 5 animals/replicate) immature C57BL/6NJ females were used to obtain zygotes.

Experiment 3 investigated the effects of mKSOM-R with extended storage at 2–8 °C on the developmental potential of rat zygotes. Rat *in vivo* zygotes from immature Crl:CD were randomly allocated in 500 μL mKSOM-R with 6 months storage at 2–8 °C and in 500 μL mKSOM-R with less than one month storage at 2–8 °C (control) in NUNC 4-well plates. Their subsequent development was assessed by their cleavage, blastocyst formation and hatching. A total of 30 (3 replicates X 10 animals/replicate) immature Crl:CD females were used to obtain zygotes.

2.6. Statistical analysis

Each experiment was repeated at least three time and for each replicate, five C57BL/6NJ immature females or 10 Crl:CD immature females were used. Percentage data was obtained from each replicate resulting in an "n" of 3–6. These data were expressed as mean% ± SEM and arcsine transformed for use for statistical analysis. For rat studies when two groups were compared, data were analyzed with a Student T test. For mouse studies where three or more groups were compared, data were analyzed by one-way ANOVA or, if data were not found to be normally distributed, Kruskal–Wallis ANOVA on ranks. If the ANOVA F value was found to be significant, a Student-Newman-Keuls post-hoc test was employed. A p value less than 0.05 was considered to be significant.

3. Results

The results of *in vivo* C57BL/6NJ zygotes cultured in newly made (≤1 Month) mKSOM, mKSOM-R and KSOM (control) were shown in Table 2. There were no significant differences in cleavage, blastocyst and hatching rates among the three groups ($P > 0.05$).

We also examined C57BL/6NJ zygotes from IVF cultured in newly made (≤1 Month) mKSOM, mKSOM-R and KSOM (control)

Table 2
Development of C57BL/6NJ embryos from *in vivo* zygotes in mKSOM-R, mKSOM, and KOSM with short term shelf life at 2–8 °C.

Medium	Zygotes	Cleavage (% ± SEM)	Blastocysts (% ± SEM)	Hatched Blastocysts (% ± SEM)
mKSOM-R (≤1 Month)	176	166 (94.32 ± 1.65)	141 (84.94 ± 4.41)	101 (71.63 ± 3.70)
mKSOM (≤1 Month)	181	172 (95.03 ± 1.39)	154 (89.53 ± 3.15)	104 (67.53 ± 6.33)
KSOM (≤1 Month)	177	169 (95.48 ± 1.34)	149 (88.17 ± 2.85)	97 (65.10 ± 2.62)

There were no differences in cleavage, blastocyst and hatching among three groups (P > 0.05).

Table 3
Development of C57BL/6NJ embryos from *in vitro* zygotes in KSOM or KSOM-R with short term shelf life at 2–8 °C.

Medium	Zygotes	Cleavage (% ± SEM)	Blastocysts (% ± SEM)	Hatched Blastocysts (% ± SEM)
mKSOM-R (≤1 Month)	148	134 (91.10 ± 1.49)	116 (86.73 ± 2.08)	49 (36.84 ± 4.46)
mKSOM (≤1 Month)	145	130 (89.99 ± 1.03)	107 (83.76 ± 3.03)	46 (45.25 ± 6.77)
KSOM (≤1 Month)	147	132 (90.79 ± 2.24)	111 (84.24 ± 1.03)	42 (39.04 ± 4.18)

There were no differences in cleavage, blastocyst and hatching among three groups (P > 0.05).

(Table 3). There were no significant differences in cleavage, blastocyst and hatching rates among the three groups (P > 0.05).

At sixth month of storage at 2–8 °C, both mKSOM and mKSOM-R were able to support C57BL/6NJ zygotes development *in vitro* with rates in cleavage, blastocyst and hatching comparable to those with KSOM made monthly (Table 4). The average total cell numbers in each day 4 blastocyst resulting from mKSOM-R or mKSOM were not significantly different from those in control KSOM (made monthly): 64.4 ± 2.16 (average from 61 total blastocysts) in mKSOM-R, 64.8 ± 2.2 (average from 61 total blastocysts) in mKSOM vs 66.19 ± 2.02 (average from 62 total blastocysts) in control KSOM (P > 0.05).

The *in vivo* developmental competence of the C57BL/6NJ embryos cultured in mKSOM-R with extended shelf life and those in KSOM with short shelf life at 2–8 °C (control) was assessed by pups born after surgical embryo transfer into 2.5 dpc pseudo-pregnant CD mice (Table 5). Embryos from both groups were able to develop into full term pups with no overt physical abnormalities based on visual examination.

The ability of mKSOM-R with extended shelf life (6 months) to support rat preimplantation embryo development was also investigated. Zygotes from Crl:CD were able to develop *in vitro* in mKSOM-R with extended shelf life with rates in cleavage, blastocyst and hatching comparable to those cultured in mKSOM-R with short shelf life (Table 6).

4. Discussion

In the present study, we investigated the effect of the mKSOM-R, which has similar amino acid profile as mouse oviduct fluid, on mouse embryo development *in vitro*. We also investigated the effects of mKSOM and mKSOM-R with extended shelf life (6 months) on mouse and rat embryo development *in vitro,* respectively. The results showed that, though there were no significant beneficial effects on mouse embryo development, mKSOM-R was able to support the development of mouse embryos derived either *in vivo* or *in vitro* similar to KSOM. After extended storage at 2–8 °C, mKSOM-R still maintained its capacity in supporting mouse and rat embryo development, respectively. Similarly, mKSOM did not lose its capacity in supporting mouse embryo development after 6 months of shelf life at 2–8 °C.

Oviducts provide an unique nutritional environment for fertilization and early embryo development with a dynamic profile of carbohydrates and amino acids [28]. Amino acids have been identified to be essential in regulating embryo physiology and metabolism, and amino acids have become an essential part of embryo culture media formulation [29–31]. To this end, essential amino acids (EAA) and non-essential amino acids (NEAA), originally used in Eagle's minimal essential medium (MEM), are commonplace in embryo media formulation [4,8,29]. Although supplement of embryos with MEM EAA and NEAA significantly improved embryo development *in vitro* from various species, it may not be optimal for embryos from all species. By examining the amino acids profile in rat oviducts, taurine, glycine, glutamate, and alanine were found to be rich in rat oviducts [10]. Supplementation of the four amino acids at a concentration of 1 mM have a positive effect on rat embryo development [9–11]. However, the same positive effect on mouse embryos were not observed, though these amino acids are also relatively abundant in mouse oviducts [12]. One possible explanation for this observation is that mouse embryos development *in vitro* does not require enriched amino acids as it does with rat and bovine embryos. This supposition is supported by the fact that MEM EAA and MEM NEAA at half strength is able to support mouse embryo development *in vitro* whereas the full strength of MEM EAA and MEM NEAA is required for optimal growth of rat and bovine embryos *in vitro* [8–10,32].

Quality media are essential for the success of assisted reproductive procedures. Media from commercial sources are usually preferred. However, commercial media are usually complete with all the ingredients incorporated into the media. Therefore, some media need to be frozen during shipping and storage since some ingredients are unstable in liquid form, even at 2–8 °C. As a result, the recommended storage time at 2–8 °C for these media are usually short after thawing. For example, the recommended shelf life of EmbryoMax® Advanced KSOM Embryo Medium (Cat #: MR-101-D) from MilliporeSigma at 2–8 °C is 14 days after thawing

Table 4
Development of C57BL/6NJ embryos from *in vivo* zygotes in mKSOM or mKSOM-R with extended shelf life and in KSOM with short-term shelf life at 2–8 °C.

Medium	Zygotes	Cleavage (% ± SEM)	Blastocysts (% ± SEM)	Hatched blastocysts (% ± SEM)
mKSOM-R (≤6 months)	177	168 (94.92 ± 0.69)	150 (89.29 ± 4.24)	77 (51.33 ± 1.28)
mKSOM (≤6 months)	174	169 (97.13 ± 1.67)	151 (89.35 ± 2.36)	84 (55.63 ± 2.73)
KSOM (≤1 month)	175	166 (94.86 ± 1.35)	152 (91.57 ± 3.16)	85 (55.92 ± 7.63)

There were no differences in cleavage, blastocyst and hatching among three groups (P > 0.05).

Table 5
Pups resulting from surgical transfer of day 4 C57BL/6NJ blastocysts cultured in mKSOM-R with extended shelf life and in KSOM with short-term shelf life at 2–8 °C.

Medium	Recipients #	Embryo transferred	Pup (%)	Age (days) at weighing	Average weight by litter (g±SEM)
mKSOM-R	1	18	3 (16.7)	9	8.9 ± 1.30
(≤6 months)	2	18	3 (16.7)	7	5.9 ± 0.29
	3	18	4 (22.2)	7	5.0 ± 0.30
	4	16	6 (37.5)	8	8.6 ± 0.16
KSOM	1	18	4 (22.2)	7	7.9 ± 0.08
(≤1 month)	2	18	3 (16.7)	8	6.3 ± 0.15

Table 6
Development of Crl:CD (SD) embryos in mKSOM-R with short and extended shelf life at 2–8 °C.

Shelf life	Zygotes	Cleavage (% ± SEM)	Blastocysts (% ± SEM)	Hatched blastocysts (% ± SEM)
mKSOM-R (≤1 month)	165	161 (97.58 ± 1.42)	143 (88.82 ± 3.19)	90 (62.94 ± 4.99)
mKSOM-R (≤6 months)	162	156 (96.30 ± 0.22)	137 (87.82 ± 2.32)	78 (56.93 ± 0.49)

There were no differences in cleavage, blastocyst and hatching between the two groups ($P > 0.05$).

(www.emdmillipore.com). In addition, precipitations of unknown nature in culture drops are frequently observed in culture drops made from frozen/thawed media (unpublished data). Therefore, we investigated the possibility of making media in stock solution that excluded unstable components and storing the stock media at 2–8 °C for extended period of time (6 months). The effectiveness in supporting mouse or rat embryo development was then assessed. Glutamine, penicillin and streptomycin were excluded from mKSOM-R stock. Since glutamine is an essential amino acid for the development of early mammalian embryos including overcoming 2-cell block [14,15,33], it was then added to a working solution at a final concentration of 1 mM using 200 mM glutamine stock solution. After these modifications, mKSOM-R could be stored at 2–8 °C for at least 6 months without significantly compromising its quality in supporting *in vitro* development of both mouse and rat embryos. Transfer of mouse embryos derived from culture in mKSOM-R stored at 2–8 °C for 6 months developed into full-term pups normal in morphology and growth similar to those derived from KSOM made monthly. Similarly, antibiotics-free KSOM with 6 months of shelf life at 2–8 °C was of similar quality to KSOM made monthly. This result will benefit facilities with preference for KSOM other than mKSOM-R for their mouse embryo culture by using mKSOM with a longer shelf life at 2–8 °C.

In this study, we have demonstrated that mKSOM-R is able to support both mouse and rat embryo development *in vitro*. The shelf life of both mKSOM and mKSOM-R can be extended for at least 6 months at 2–8 °C without significantly compromising their quality. The ability to culture both mouse and rat embryos with one medium will benefit facilities with both mouse and rat model creation and/or embryo cryorecovery.

CRediT authorship contribution statement

Hongsheng Men: Conceptualization, Methodology, Investigation, Data curation, Formal analysis. **James M. Amos-Landgraf:** Formal analysis, Funding acquisition, Resources, Writing – review & editing. **Elizabeth C. Bryda:** Formal analysis, Funding acquisition, Resources, Writing – review & editing. **Craig L. Franklin:** Formal analysis, Funding acquisition, Resources, Writing – review & editing.

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