Published in final edited form as:

Theriogenology. 2011 September 1; 76(4): 729–736. doi:10.1016/j.theriogenology.2011.04.005.

Treatment of sperm with extracellular adenosine 5'-triphosphate improves the in vitro fertility rate of inbred and genetically modified mice with low fertility

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Abstract

In vitro fertilization (IVF) is one of the most important techniques used for assisted reproduction in mouse colony management. As with natural mating, where mice have varying fertility indices, fertility rates of genetically modified (GM) [transgenic (Tg), knock out (KO) and congenic (Cg)] mice are influenced by their genetic background. Lines of GM mice that have poor fertility have a concomitant poor IVF outcome. Treatment of mouse sperm with extracellular adenosine 5'-triphosphate (ATPe) enhanced in vitro fertilization rates in outbred and hybrid mice. The objective of this study was to analyze the effects of using extracellular adenosine 5'-triphosphate-treated sperm for IVF of inbred wild type, and genetically modified mouse lines, for which standard IVF did not work well. The IVF was performed using the GM mice on C57BL/10SnJ, C57BL/6J, BALB/cJ and NFS/N background strains and wild type (WT) mice such as C57BL/6N, BALB/cAnN, and B6129SF1 strains. Oocytes from superovulated females were fertilized in vitro with sperm from the same background strain, and either treated or not treated with ATPe. The ATPe treatment enhanced IVF outcome in most of the GM and some WT strains, as indicated by the percentage of embryos that progressed to the two-cell stage. There was no marked difference between ATPe treated and control groups for the development rate of two-cell embryos to blastocysts in culture, or in the number of pups born after transfer of two-cell embryos into recipient females. The observed improvement of the IVF results following ATPe treatment of transgenic and KO mouse sperm were a potential solution for improving the outcome of assisted reproduction techniques used for rederivation or for gamete banking.

Keywords

Genetically modified mice; In vitro fertilization; Extracellular ATP; Fertility

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1. Introduction

Assisted Reproductive Technology (ART), which includes embryo and gamete manipulation, are powerful and essential tools for facilitating maintenance of laboratory animals for biomedical research and for reproductive applications in domestic animals. In mice, IVF is the most popular technique among ART's. Because of the enormous number of mutant, transgenic and KO mice, IVF has become an essential technique to harvest numerous embryos for use in colony expansion and cryopreservation. Today, researchers studying reproductive processes, laboratories creating genetically modified animals, and repositories archiving cryopreserved embryos routinely use and often combine several ARTs to meet their needs.

Genetic background influenced the success of IVF and defined the reproductive performance of the mouse strain used [1–5]. The sensitivity of embryos and gametes to manipulation, embryo culture and cryopreservation varies depending on the genotype and the conditions used for culture, capacitation and fertilization [1–3,5–7]. Furthermore, when using these techniques, limited and unexpected outcomes are often encountered with some inbred, transgenic and knockout mice [8]. Therefore, fertilization and developmental competence need to be assessed on a strain-by-strain basis. Successful IVF is important to subsequent embryo manipulations such as cryopreservation or rederivation; consequently, knowing optimal IVF conditions is essential. One of the reasons for low fertility rate in any IVF procedure is poor quality sperm. In that regard, sperm morphology influenced the rate of successful IVF in mice [9–11] and in human infertility studies low sperm motility, in addition to other sperm abnormalities, influenced fertilization rate [12]. Several pharmacological agents (e.g. calcium ionophore, glycosaminoglycans, and heparin) have been used to stimulate sperm function in order to improve fertilization rates in standard IVF techniques [13–15]. However, most of these agents required prolonged incubation [16], and some had adverse effects on mouse oocytes at concentrations commonly used to activate sperm in human IVF [17]. In addition, platelet-activating-factor was also used to improve the IVF outcome. A recent study demonstrated that platelet-activating factor enhanced the success rate of intrauterine insemination when normal sperm was used, but did not improve the outcome when sperm from men with reduced sperm motility was used [18].

A pharmacological agent that has gained much attention recently for increasing IVF success rates is extracellular adenosine 5'-triphosphate (ATPe). Adenosine triphosphate (ATP) plays a critical role in cellular metabolism and energy production in every cell of the organism. It has also been demonstrated to play a role in mediating important physiological functions in various cell types [19]. Sperm are naturally exposed to ATPe, which is present in vaginal fluids and likely in the female upper reproductive tract, with the highest concentrations in the periovulatory period [20]. Extracellular ATPe increased the percentage of human [21] and bovine sperm [22] that underwent acrosomal exocytosis; it also increased fertilization rates of IVF in humans [23]. Recently extracellular ATPe improved the IVF rate in mice. Rodriguez-Miranda et al. [24] published extensive data on the mechanism of action of ATPe in improving the fertilizing capability of mouse sperm; however, these data were obtained using outbred and hybrid mice and made no mention regarding developmental ability and live-born from murine embryos generated with ATPe-treated sperm when compared to a control. To date, there are apparently no published studies regarding the effects of ATPe on the IVF efficiency of lines of genetically modified mice on various background strains. We previously demonstrated that the reproductive performance of inbred wild type mice and the transgenic mice built on the same background strain were different [8]. Therefore, the objective of the present study was to determine the effect of ATPe treatment during the IVF procedure of several genetically modified lines, where standard IVF did not work well, due to poor sperm quality.

2. Materials and methods

2.1. Animals

Inbred wild type mice B6129SF1, BALB/cAnN and C57BL/6N were purchased from Taconic (Hudson, NY, USA). The GM mice used in this study (Table 1) were maintained by brother X sister mating under a centralized breeding program, in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). The animal housing included a controlled light and dark cycle (14-10 h), *ad libitum* food, Purina 5058 irradiated high fat rodent diet, ultra-filtered water, ventilated caging systems, and standardized environmental enrichment. Male mice were housed individually for at least 5 d prior to sperm collection. All experiments were conducted under the approval of the ACUC/NIAID/NIH, Maryland. As field application, part of this study was carried out with mouse strains sent to our unit (ARTiC/NIAID/NIH) for rederivation.

2.2. Media

The IVF culture medium, Research Vitro Fert (K-RVFE-50; COOK Medical, Inc. Bloomington, IN, USA), was used for sperm incubation, IVF, and culture of zygotes. Two-cell embryos were cultured in modified KSOM (½ amino acids and glucose; Specialty Media, Millipore, Billerica, MA, USA).

2.3. In vitro fertilization

In vitro fertilization was performed using the method described by Vasudevan et al. [8]. Culture dishes were prepared the night before IVF and allowed to equilibrate at 37 °C under 5% CO₂ in a humidified incubator. The IVF dish (60 mm × 15 mm, BD Falcon, Fisher Scientific, Pittsburgh, PA, USA) contained five 250 µL drops of IVF medium; all were covered with embryo-tested mineral oil (Sigma Chemical Co., St. Louis, MO, USA). The sperm collection dishes (35 mm × 10 mm BD Falcon 1008, Fisher Scientific) contained a single 300 µL drop of IVF medium covered with embryo-tested mineral oil. For two-cell embryo culture, embryos were cultured from the two-cell stage in 300 µL drops of modified KSOM placed in 35 mm dishes and covered with embryo-tested mineral oil. Dishes were prepared on the day of IVF. ATPe was obtained from Sigma, Cat # A6419 and used at a final concentration of 1 mM. Sperm were collected from the caudae epididymides of 3- to 5-mo old male mice, as described by Sztejn et al. [5]. After euthanasia with CO₂, both caudae epididymides and vas deferentia were aseptically removed. Sperm from one epididymis was released into 300 µL drop of K-RVFE-50 medium and the sperm from the other epididymis was released into 300 µL drop of K-RVFE-50 medium containing 1 mM ATPe. The tissue was incised four or five times with the edge of a 30 gauge injection needle to allow the sperm to swim out and disperse for 60 min at 37 °C under 5% CO₂ in the humidified incubator. Sperm motility and concentration were confirmed by visual observation at the edge of the drop under a stereo dissecting microscope.

Female mice (21–35 d of age) were superovulated with 5 IU of equine chorionic gonadotropin (eCG; PMSG; Sigma) intraperitoneally. Forty six to 50 h post eCG, the mice were treated with 5 IU of human chorionic gonadotropin (hCG; Sigma) intraperitoneally. Thirteen to 14 h post-hCG treatment, female mice were euthanized with CO₂ and the oviducts were aseptically removed and dissected free from surrounding fat and tissues and placed in a Petri dish containing 2 mL of M2 medium (Specialty Media, Millipore) warmed to 37 °C. Cumulus oocyte complexes were removed from the ampullae using micro dissecting forceps and subsequently used for IVF. Oocytes from the superovulated females were pooled for each IVF and distributed equally between control and ATPe dishes.

After sperm incubation in the presence or absence of ATPe, 10 μ L of the sperm suspension was added, using a wide-bore pipette tip, to a 250 μ L drop of medium covered with embryo-tested mineral oil. The isolated oocytes complexes were then transferred to the drop of media containing the sperm and incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air for 5 h. The eggs were then washed to eliminate excess sperm and cultured overnight in a 250 μ L drop of IVF medium under the same conditions. The following day, the number of two-cell embryos were counted and prepared for either transfer into recipient females or for culturing in modified KSOM. The IVF experiment was repeated three or more times, on at least three different days for each line, except for the lines submitted for rederivation.

2.4. Embryo transfer

Embryo transfer was performed to assess whether there was a difference in the number of live births from embryos produced by IVF with or without ATPe. Female CD1 mice (Charles River Laboratories International, Inc. Wilmington, MA, USA), 9 to 13 wk of age were mated with vasectomized CD1 (Harlan Laboratories, Inc. Frederick, MD, USA) males to induce pseudopregnancy. The following morning, females with vaginal plugs were used as embryo transfer recipients. Embryos (12 to 17) were surgically transferred into the left oviduct of each female, as described by Nagy et al. [25], and the number of pups born was counted.

2.5. Statistical analyses

Fertility was considered as the percentage of two-cell stage embryos scored 24 h after insemination. For statistical analysis, percentages were transformed into arcsine values and then evaluated by a paired Student's *t*-test (two tailed). Differences were considered significant when a P value of <0.05 was obtained. Error bars on figures are SEM. Data were analyzed using the Graph Pad Prism version 5.0 computer program (Graph Pad Software, San Diego, CA, USA).

3. Results

The results of the IVF procedures are summarized in the following tables and figures. Average fertilization rates (percentage of zygotes which progressed to the two-cell stage are shown (Tables 2 and 3). Fertility was scored at the two-cell stage and was calculated by dividing the number of two-cell embryos by the total number of oocytes exposed. Treatment with ATPe increased fertility in all lines tested.

The percentage of eggs fertilized by control and ATPe treated sperm was 59 $\pm$ 10% vs 65 $\pm$ 8% (BALB/c AnN WT), 82 $\pm$ 1% vs 86 $\pm$ 0.8% (C57BL/6N WT), 36 $\pm$ 13% vs 70 $\pm$ 7% (B6129SF1 WT), 72 $\pm$ 7% vs 84 $\pm$ 5% (PrP KO), 27 $\pm$ 8% vs 46 $\pm$ 7% (Rgs 14 floxed KO), 54 $\pm$ 11% vs 79 $\pm$ 2% (TCRA51.233 Tg) and 35 $\pm$ 9 vs 69 $\pm$ 9% (C58v1 Cg) as shown (Table 2, Fig 1 and Fig 2).

For lines submitted to our unit for rederivation (Table 3, Fig 3), the percentage of eggs fertilized with control and ATPe treated sperm was 60 vs. 86% (Retnla KO), 33 vs 57% (IL-5 Tg), 27 vs 43% (IL-13Ra1 KO), 42 vs 78% (IL-5 Tg), 52 vs 72% (Eotaxin Tg), 31 vs 87% (MCP-3 KO), 18 vs 76% (Ola HPrP Tg NSE leu KO), and 50 vs 69% (gp49B KO). Statistical analyses were not done for these GM lines, since the experiment was conducted only once due to the limited number of mice available.

3.1. In vitro culture of embryos derived from ATPe treated and control sperm (no ATPe treatment)

Because many embryos tend to have developmental arrest immediately after the first cleavage to the two-cell stage, the percentage of two-cell embryos that progressed to the blastocyst stage in culture was examined for embryos, that were produced by IVF with either ATPe-treated or control sperm (Table 4). The results show that there was no difference in blastocyst development between the two groups.

3.2. Newborn mice derived from control sperm and sperm treated with ATPe

To assess whether there was a difference in the number of live births from embryos produced by IVF using either control or ATPe treated sperm, 12–17 embryos for control and ATPe treated groups were transferred to pseudopregnant CD1 foster mothers, and the number of pups born was counted. There was no significant difference between control and ATPe groups. The following numbers of offspring were produced with control and ATPe treated sperm, respectively: PrP KO, 33% (10/30) and 37% (11/30); RGS14 floxed KO, 40% (6/15) and 46% (11/24); TCR (A51.233)Tg, 26% (9/35), and 31% (11/35).

4. Discussion

The use of IVF to produce embryos for rederivation and/or embryo banking can shorten the interval required to complete the process. However, it is well known that the success of the IVF is influenced by the genetic background and the reproductive performance of the mice used [1–3,5,8], with differences in fertility rate being of high importance [8,26]. The addition of various chemicals to the IVF medium to aid capacitation or the nutritional aspects of the incubation media have been tried in an attempt to improve the in vitro fertilization rates [13,27–29]. The majority of the data published were obtained from studies using hybrid or outbred mice [24,30]. Although those reports are important information, they often overestimate or misinterpret the actual capability of the method when used in practice, because the strains studied do not have any fertility problems. Indeed, the demand to improve IVF comes from the desire to achieve higher fertilization rates for GM mouse lines (predominantly knockouts and transgenic) submitted to repositories and core facilities for cryopreservation or rederivation [8].

This study was designed in an attempt to improve the fertility rate of GM strains that did not respond well using the conventional IVF technique due to poor sperm quality. In that regard, ATPe was chosen to improve sperm fertility based on several features. 1) It has been successfully used in human fertility clinics [23]; 2) the percent of acrosome reaction in bovine sperm was similar, if not higher, to that obtained with the best sperm activating agents known; (e.g. calcium ionophore, heparin, and glycosaminoglycans) [16,31] although mouse sperm seems to lack this reaction [24]; 3) the effect of ATPe was rapid, compared to all other chemicals [21]; 4) human sperm treated with ATPe had a very high success rate in the hamster egg fertilization test [21]; and 5) except at a higher doses, ATPe had no side effects on sperm cell motility or fertilization potential [21].

Adenosine triphosphate is a physiological compound present in all living cells, and in recent years it has been demonstrated that it has a role in mediating important physiological functions in many cell types [19]. In mouse sperm, concentrations of ATPe as high as 2.5mM stimulated fertilizing ability, without any toxic or detrimental action on sperm motility or viability [24].

We had previously demonstrated that some GM mouse lines had a unique response to in vitro fertilization and that seemed to be independent of the outcome expected for their genetic background [8].

In the present study, ATPe was used to improve the fertility rate of GM strains where conventional IVF did not work well. In most cases, the fertilization rate was higher for ATPe-treated sperm than for untreated sperm, with comparable blastocyst development and live births after embryo implantation into recipient females. In some groups, the statistical analysis detected no difference between ATPe treated and not-treated groups; however, fertilization rate was higher in all groups treated with ATPe. In practice, producing more embryos resulted in use of fewer females, less time to complete the cryopreservation or rederivation project, less time for housing the colony, and therefore less total cost to obtain the same goal.

The mechanism by which ATPe improved fertilization in vitro was unclear. Edwards et al. [12] reported that ATPe did not induce motility in nonmotile sperm. However, it significantly increased the hyper-activation percentage of sperm with poor motility. For computerized sperm analysis (CASA), there was a significant increase in straight-line velocity (VSL), curvilinear velocity (VCL) and percentage of rapid sperm [12]. Miranda et al. [24] demonstrated that ATPe did not induce acrosomal exocytosis in neither noncapacitated nor capacitated mouse sperm, and it did not affect protein tyrosine phosphorylation of capacitated sperm. However, they reported that ATPe caused significant increases in VSL and VCL in mouse sperm, and that at least some of the effects of ATPe were mediated by P2 purinergic receptor-mediated intracellular calcium increases. Perhaps these motility changes accounted for, at least in part, the improvement in fertilizing capability when ATPe treated sperm were used in IVF procedures.

Treatment with ATPe did not enhance the fertility rate in some of the inbred WT and GM lines used in this study. Perhaps motility of the sub-optimal sperm population was improved by ATPe, but this response was at the same level of effect to that of a population of sperm with high motility. The beneficial effect of ATP could have been due to alterations in sperm motility parameters, faster and straighter movement of sperm after the treatment [24], whereas these effects were different in normal motility sperm.

In this study, the ATPe sperm treatment improved the outcome of IVF for some GM lines for which standard IVF did not work well. Although the results of the GM lines listed in Table 3 were not statistically analyzed, fertility rate was demonstrably higher for the ATPe treated group when compared to the control group. This situation reflected the real working condition in reproductive laboratories, where the number of IVF attempts is often restricted due to the limited number of mice available for a given procedure; therefore, even a slight improvement in fertility may result in the procedure's success. It is imperative that every attempt be made to improve the outcome when rederiving or rescuing a line with low fertility. This study highlighted an option for IVF procedures involving murine sperm with suboptimal performance, to improve rederivation or gamete banking success.

Acknowledgments

The authors thank Chris McGregor for technical assistance and Dr. Kathy Perdue for critical reading of the manuscript. We also thank all scientists that allowed us to use their mice for this study. This research was supported by Comparative Medicine Branch and the Intramural Research Program of National Institutes of Allergy and Infectious Diseases/National Institutes of Health.

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Fig. 1.

In vitro fertilization of wild type mice using sperm incubated in the absence (control) or presence (treated) of extracellular 1mM ATPe. Fertility was scored at the two-cell stage after overnight culture in IVF medium. Each bar represented the mean IVF rate from a total of three or more IVF procedures for each line.

*Significant difference between control and ATPe-treated groups.

Fig. 2.

In vitro fertilization of genetically modified mice using sperm incubated in the absence (control) or presence of extracellular 1mM ATPe. Fertility was scored at the two-cell stage after overnight culture in IVF medium. Each bar represents the mean IVF rate from a total of three or more IVF procedures for each line.

*Significant difference between control and ATPe-treated groups.

Fig. 3. In vitro fertilization rate of genetically modified mice submitted for rederivation. Sperm had been incubated in absence (control) or presence of extracellular 1mM ATPe. Fertility was scored at the two-cell stage after overnight culture in IVF medium.

Table 1

List of genetically modified mice used in this study, including strains, type of transgene and target organs.

Name	Gene	Target	Reference
PrP KO ¹	Prion	<u>Ubiquitous</u>	Chesebro et al. (2010)
RGS14 KO ²	RGS 14	Brain	Not published
TCRA51.233 ³	TCR TXA51	Lymphocytes	Candon et al. (2004)
NFS.C58v1 ⁴	C58v1;Emv32	Spleen	Hartley et al. (2000)
Retnla KO ²	FIZZ1/Relma	Lung	Pesce et al. (2009)
IL-5 ²	IL-5	Lung	Domachowske et al. (2002)
IL-13Ra1 KO ³	Il13ra1	Liver	Ramalingam et al. (2008)
IL-5 ³	IL-5	Lung	Kopf et al. (1996)
Eotaxin ³	Eotaxin	Lung / GI tract	Rothenberg et al. (1997)
MCP3 KO ²	MCP3	Bone marrow	Tsou et al. (2007)
NSEleu(KO) ^{5*}	NSE & Prion	Neurons	Not published
Gp49B KO ²	gp49B	Lung / eye	Norris et al. (2007)

¹⁻⁵ Genetic backgrounds

¹ C57BL/10SnJ;

² C57BL/6J;

³ BALB/cJ;

⁴ NFS/N;

⁵ B6;129

* Complete strain nomenclature: Ola HPrP Tg NSE leu KO

Table 2

Effect of treatment of murine sperm with ATPe on IVF.

Strain	Control			ATPe		
	No. oocytes	No. 2-cell embryos	IVF (%)	No. oocytes	No. 2-cell embryos	IVF (%)
B6129SF1WT	256	98	36±13	290	202	70±7 ^a
BALB/cAnN WT	191	92	59±10	217	150	65±8
C57BL/6N WT	221	180	82±1	305	263	86±0.8 ^a
PrP KO ¹	478	323	72±7	607	483	84±5
TCRA51.233 Tg ²	337	205	54±11	384	299	79±2 ^a
RGS14floxed KO ³	551	158	27±8	610	320	46±7 ^a
C58v1 Cg ⁴	428	159	35±9	370	268	69±9 ^a

Data represent the cumulative results of three or more IVF experiments. Average fertilization for each IVF was calculated by dividing the number of two-cell embryos obtained, by the total number of oocytes exposed to IVF.

^aIVF was greater for ATPe than for the control (P<0.05).

¹⁻⁴ Genetic background

¹ C57BL/10SnJ;

² BALB/cJ;

³ C57BL/6J;

⁴ NFS/N

Table 3

In vitro fertilization rate in mice, using control sperm and sperm treated with ATPe for lines submitted to our unit for rederivation.

Strain	Control			ATP		
	No. oocytes	No. 2-cell embryos	IVF (%)	No. oocytes	No. 2-cell embryos	IVF (%)
Remla KO ¹	116	70	60	125	107	86
IL-5 Tg ¹	60	20	33	97	55	57
IL-13Ra1 KO ²	245	64	27	148	56	43
IL-5 Tg ²	55	23	42	102	80	78
Eotaxin Tg ²	40	21	52	43	31	72
MCP-3 KO ¹	16	5	31	15	13	87
Gp49B KO ¹	40	20	50	93	64	69
NSEleu(KO) ^{3*}	29	4	18	81	62	76

All IVF procedures were done under similar conditions. No statistical analysis was done, since the experiment was performed only once due to the limited number of mice.

¹⁻³ Genetic background

¹ C57BL/6J;

² BALB/cJ;

³ B6;129

* Complete strain nomenclature – HPP-P Tg NSE leu KO

Table 4

Results of in vitro blastocyst development rate for mice in the present study.

Strain	Control			ATP		
	No. oocytes	No. 2-cell embryos	IVF (%)	No. oocytes	No. 2-cell embryos	IVF (%)
RGS14flox KO	96	30	43±25	159	86	60±8
PrP KO	167	119	70±10	277	217	75±8
TCRA51.233 Tg	189	114	60±5	249	170	64±7

Two-cell embryos were cultured in KSOM with ½ amino acids and glucose at 37 °C under 5% CO₂ in a humidified environment. Blastocysts were scored 48 h after cleavage and culture in KSOM/AA.