Cryopreservation of Rat Sperm

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1. Introduction

The laboratory rat, *Rattus norvegicus*, was the first mammalian species domesticated for scientific research, which work dating back to before 1850. From this auspicious beginning, the rat has become the most widely studied experimental animal model for biomedical research (Jacob, 1999). Since the development of the first inbred rat strain by King 1909, over 500 inbred rat strains have been developed for a wide range of biochemical and physiological phenotypes and different disease models (Aitman et al., 2008, Canzian. 1997). In the last decade, there has been an extraordinary increase in rat genomic resources (Gibbs et al., 2004, Pennisi. 2004), and the advent of knock-out technology allow the insertion or deletion of individual genes into the rat by advances in stem-cell technology (Geurts et al., 2009, Izsvak et al., 2010, Tong et al., 2010). Thus, a wide array of research opportunities now open up, especially in studies involving the laboratory rat (Hamra. 2010). However, protocol for sperm cryopreservation and oocytes fertilized *in vitro* by using cryopreserved sperm are still under development for preservation of most rat strains. Therefore, greater use of the cryopreservation of rat sperm may provide an essential resource to preserve and increase the number of valuable genetic strains for research and application.

In this chapter, we will introduce several of these approaches to cryopreserving the rat sperm. It will be valuable for developing new freezing extender for cryopreservation of rat sperm, and might be applied to other reproductive technologies in this species for preservation of valuable rat strains.

2. Reporting studies of rat sperm cryopreservation

The first live-born rat derived from frozen-thawed sperm were successfully reported artificial insemination (AI) by using frozen-thawed rat sperm, and also reported cryopreservation of several strains of rat sperm, including those from mutant and transgenic rats (Nakatsukasa et al., 2001, Nakatsukasa et al., 2003). More recent publication by same group confirmed that cryopreserved rat sperm can be revitalized and result in the birth of live offspring through embryo transfers after in vitro fertilization (*IVF*) (Seita et al., 2009a). Although the authors mentioned by another publication that intracytoplasmic sperm injection (ICSI) is the only way to routinely obtain offspring routinely derived from oocytes

fertilized in vitro using fresh and cryopreserved sperm (Seita et al., 2009b). Hagiwara et al, (2009) also pointed out that only one group has reported successful cryopreservation of rat sperm, subsequently used for AI and yielding live offspring. This result has not yet been repeated by other investigators and labs, and further investigation of the ability to yield viable rat sperm after cryopreservation is urgently needed. Further, each of cryopreservation procedures has not been completed for several mammalian species to date, the basic science and technology required to do so is rapidly becoming available and this should be completed for a number of species, in the future (Agca and Critser, 2002). Those of the information will also allow further improvements in cryopreservation of rat sperm from various mammalian species.

3. Cryodiluent for rat sperm

The characteristics of rodent sperm *in vitro* differ from that of other mammalian sperm largely due to differences in sperm membrane lipid content or composition (Parks and Lynch, 1992). The morphology of rodent sperm shows a longer tail when compared to that of sperm from domestic animals (Cardullo and Baltz, 1991). Rat sperm is extremely sensitive to a number of environmental changes, such as centrifugation, pH, viscosity, osmotic stress (Varisli et al., 2009a: Nakatsukasa et al., 2003: Chularatnatol, 1982: Si et al., 2006). Rat sperm have therefore proven to be more difficult to cryopreserve than other mammalian sperm, including that of the mouse, and current survival rates for sperm cryopreservation are still inadequate for AI, *IVF* and safe preservation of most rat strains. Important factors affecting sperm cryopreservation are cooling, freezing, thawing, and the composition of cryoprotectant in the freezing extender. In the rat, Nakatsukasa et al. (2001) employed a freezing medium that contained lactose monohydrate, Equex STM, and egg yolk solution. Based on the experimental conditions and extender components described in the current study, we offer the following suggestions to those attempting to cryopreserve epididymal rat sperm. Moreover, Varisli et al., (2009b) investigated that effect of chilling on the motility and acrosomal integrity of rat sperm in the presence of various extenders. They found that the addition of glycerol or propylene glycol to either Tris–citrate or TEST extender or of DMSO into lactose monohydrate, Tris–citrate, or TEST extender resulted in optimal motility rates.

4. Identified optimal energy substrates and other components of rat sperm cryodiluent

4.1 Freezability of rat sperm induced by raffinose in modified Krebs–Ringer bicarbonate (mKRB) based extender solution.

We first studied to develop an ideal freezing extender and method for rat sperm cryopreservation (Yamashiro et al., 2007). Experiments were conducted to study its post-thaw characteristics when freezing with raffinose-free buffer or various concentrations of raffinose and egg yolk dissolved in distilled and deionised water, PBS, or mKRB based extender. Different concentrations of glycerol, or Equex STM dissolved in either PBS or mKRB containing egg yolk were also tested. Based on the data from these experiments, further experiments tested how different sugars such as raffinose, trehalose, lactose, fructose, and glucose dissolved in mKRB with Equex STM and egg yolk supplementation affected the post-thaw characteristics of cryopreserved sperm. Beneficial effects on the post-

thaw survival of sperm were obtained when raffinose in mKRB was used with Equex STM, and egg yolk (Fig.1).

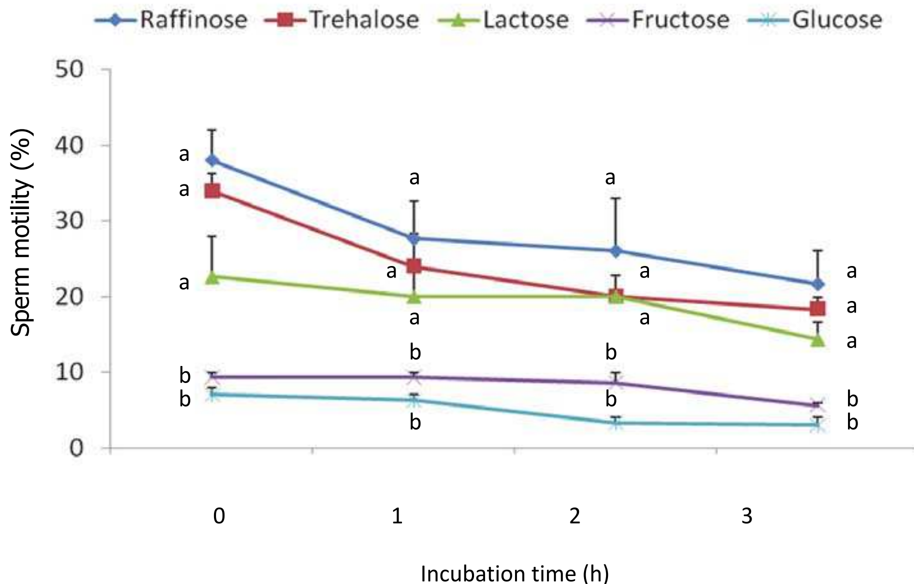

Fig. 1. Motility of frozen-thawed rat sperm which were frozen in different sugars dissolved in an mKRB egg yolk, and then subjected to a thermal resistance test at 37°C. Values are the mean ±SEM (n=3). [a-b] Different superscripts within the same column denote significant differences ($P < 0.05$).

Mammalian sperm provide energy for metabolic requirements by both mitochondrial oxidative respiration and glycolysis, and sperm motility is driven by the flagellum and is dependent on the availability of an adequate and continued supply of ATP (Cummins and Woodall, 1985). ATP is used by the dynein ATPases that function as the flagellar motors and in protein kinase A-mediated signal transduction pathways to regulate motility throughout the tail (Cao et al., 2006). In the mouse sperm, Mukai and Okuno (2004) reported that glycolysis has an important role in providing the ATP required for mouse sperm motility than mitochondrial respiration throughout the length of the flagellum. Bunch et al. (1998) also suggested that mouse sperm utilize glycolysis to generate ATP in the principal piece of the tail. While, Odet et al., (2011) demonstrated that lactate dehydrogenase (LDH) is responsible for the maintenance of energy metabolism in progressive and hyperactivated in mouse sperm. In rat sperm, Gallina et al. (1994) was studied the operation of shuttle functions for the ATP reconstituted systems in the mitochondria present in the middle place of rat, mouse and rabbit. They showed that the redox couple lactate/pyruvate and lactate dehydrogenase are active with rat and rabbit mitochondria, and it does not work with mouse. From these examining the energy metabolism of sperm, the glycolytic activity is exclusively responsible for the generation of mouse sperm metabolism, and mitochondrial metabolism seems to interacted with motility activity of rat sperm. These findings in conjunction with the present study indicate that successful cryopreservation of rat sperm in

the presence of glucose, lactate and pyruvate in mKRB egg yolk extender solutions may be achieved through the ability to synthesize ATP, which could have profound effects on sperm metabolism and thereby impart a greater endurance against freeze–thawing damage.

4.2 Lactate and adenosine triphosphate in the extender enhance the cryosurvival of rat sperm

On the basis of the results of the previous experiments, we hypothesized that the metabolic state of sperm before cryopreservation would influence their survival during this stressful process (Yamashiro et al., 2010a). We evaluated the cryosurvival of rat sperm preserved in raffinose–mKRB egg yolk extender supplemented with various energy-yielding substrates (glucose, pyruvate, lactate, and ATP) and assessed the effect on sperm oxygen consumption. The incubation of sperm in lactate-free extender decreased sperm motility and oxygen consumption before and after thawing compared with those of sperm in glucose- and pyruvate-free mediums. We then focused on the effect of supplementing the extender with lactate and found that sperm frozen and thawed in extender supplemented with lactate exhibited the highest motility (Fig. 2). When we supplemented extender containing lactate with ATP, sperm frozen and thawed in the extender supplemented with ATP exhibited considerably higher motility and viability than those of sperm frozen and thawed in ATP-free extender (Fig. 3). Especially, exogenous ATP was observed that it dramatically induced the cryosurvival of rat sperm (Fig.4). Moreover, this may involve a lactate-transport system

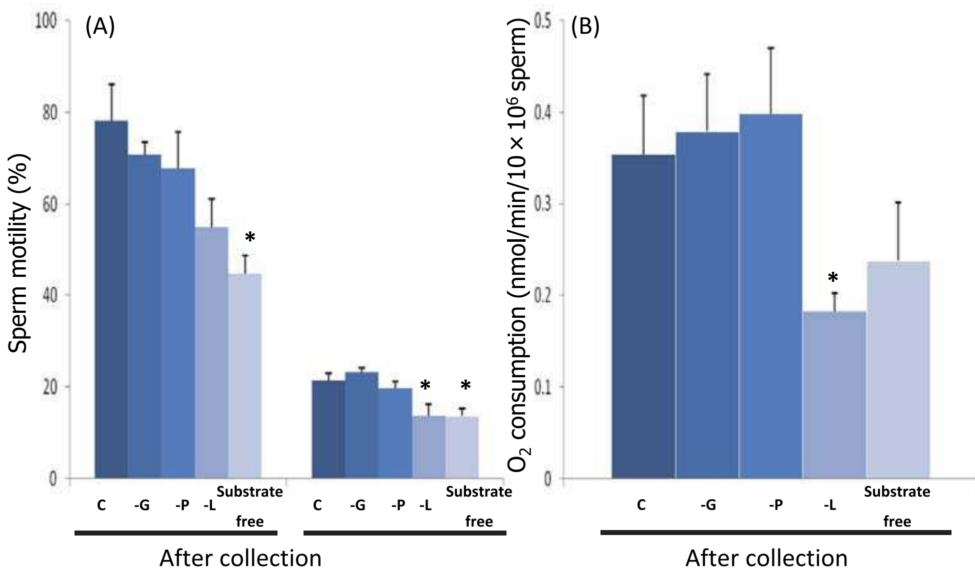

Fig. 2. Effect of the substrates in raffinose-mKRB egg yolk extender on the characteristics of after collected and frozen-thawed rat sperm (A) and oxygen consumption of after collected sperm during incubation at 37°C for 10 min (B). Indicates C; control, G; glucose, P; pyruvate, L; lactate. Values are mean ± SEM (n=5), respectively. Statistical difference (P<0.05) in comparison to the control is indicated by asterisk (*).

for regenerating cytoplasmic ATP throughout the principal piece of rat sperm in Fig.5 (Yamashiro et al., 2009). This thought is also in concert with the presence of a unique pathway that utilizes lactate and extracellular ATP in the rat sperm; this suggests new possibilities for energy production and translocation mechanisms related to motility, fertility, and freezability of rat sperm. These results provide the first evidence that supplementation of the raffinose–mKRB egg yolk extender with lactate and ATP increases of number of motile sperm before freezing and enhances the cryosurvival of rat sperm.

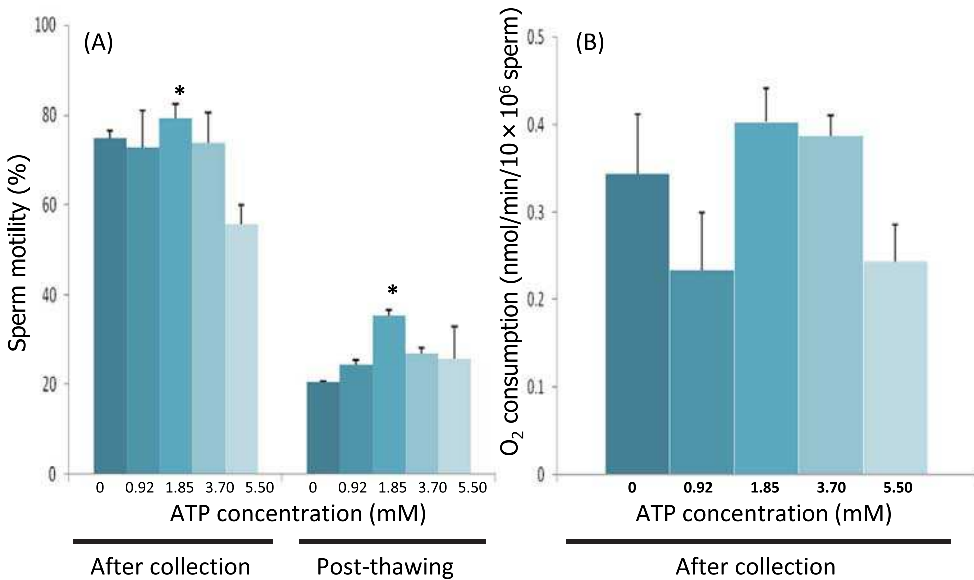

Fig. 3. Effect of different concentrations of ATP in raffinose-mKRB- gg yolk extender containing lactate on the characteristics of after collected and frozen-thawed rat sperm (A) and oxygen consumption of after collected sperm during incubation at 37°C for 10 min (B). Values are mean±SEM (n=5), respectivaly. Statistical difference (P < 0.05) in comparison to the control is indicated by asterisk (*).

The sperm-specific enzyme LDH isozyme C4 is located in the cytosol and the matrix of the mitochondria in the midpiece of rat sperm. Further, a study (Gallina et al., 1994) has revealed that both a shuttle involving the redox couple lactate–pyruvate and LDH C4 are active in rat sperm mitochondria. In another study (Harris et al., 2005), the lactate concentration in oviductal fluids was 10-fold higher than the glucose concentration, and the lactate concentration in the uterine fluids was 15-fold higher than the glucose concentration during the murine estrous cycle. Therefore, it is very likely that lactate is used by rat sperm as an essential substrate to maintain highly regulated ATP production and dissipation: lactate in the cytosol and mitochondrial matrix is oxidized to pyruvate by mitochondrial LDH isozyme C4, and pyruvate is oxidized through the Krebs cycle and electron transport chain (Brooks et al., 1994, Brooks. 2002, Montamat et al., 1988, Poole and Halestrap, 1993). To our knowledge, our findings are the first evidence showing that rat sperm can use

exogenous lactate in the cryodiluent as an essential substrate to maintain highly regulated metabolic capacity and that this lactate acts as an energy substrate for mitochondria to the mobilization of fresh and frozen–thawed sperm.

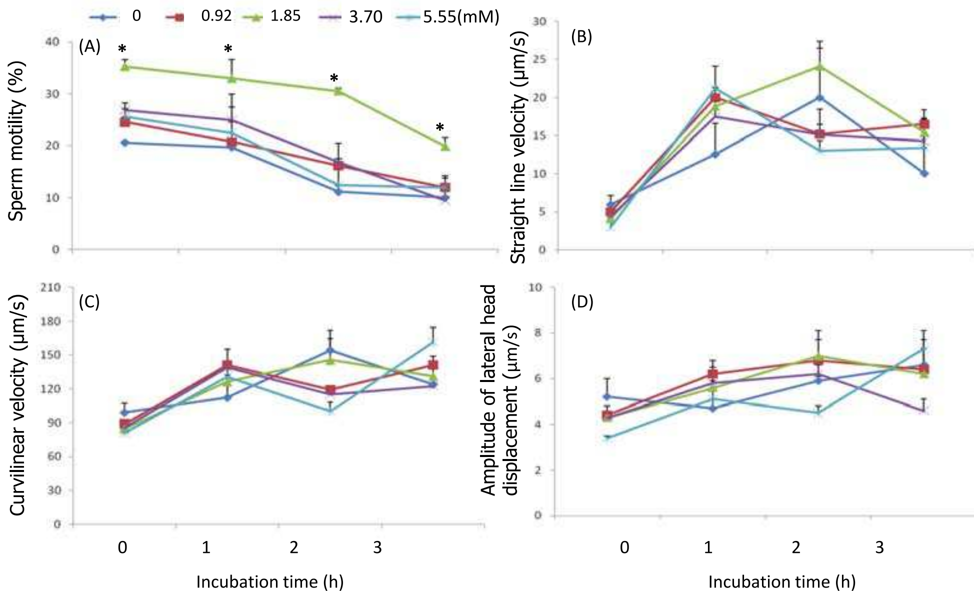

Fig. 4. Effect of different concentrations of ATP in raffinose-mKRB egg yolk medium containing lactate on the motility (A), straight line velocity (B), curvilinear velocity (C), and amplitude of lateral head displacement (D) of frozen-thawed rat sperm during incubation at 37 °C for 3 h. Values are represented as mean ± SEM (n = 3). Statistical difference ($P<0.05$) in comparison to the control is indicated by asterisk (*).

Mitochondria, the site of ATP generation due to oxidative phosphorylation, are localized solely in the midpiece of sperm (Millette et al., 1973). The oxidative production of ATP through the Krebs cycle is an essential function of the midpiece mitochondria for motility (Suarez et al., 2007). The mitochondrial biochemical pathways of oxidative phosphorylation are 15 times more efficient than is anaerobic glycolysis for ATP production (Cardullo and Baltz. 1991, Ruiz-Pesini et al., 2007). These findings also support our arguments that the energy production and dissipation in rat sperm are highly dependent on the mitochondria.

The present study showed that supplementation of raffinose–mKRB egg yolk extender with lactate and exogenous ATP considerably increases sperm motility before freezing, thus improving the survivability of sperm after cryopreservation. Exogenous ATP in the freezing medium may be responsible for the generation of multiple metabolic signals that appear to be related to the sperm motility through a rise in calcium levels (Gibbons.1963, Kinukawa et al., 2006, Litvin et al., 2003, Luria et al., 2002, Ren et al., 2001, Rodriguez-Miranda et al., 2007); this reaction increases de novo ATP synthesis before freezing and may contribute to the remobilization of sperm after freezing–thawing. The motility of ram sperm was restored by exogenous ATP that crossed plasma membrane when the membrane was damaged by cryopreservation (Holt et al., 1992). In light of that finding, we cannot discount that our

result is caused by the facultative transport of ATP across plasma membrane because of damage during freezing, thereby allow ing substrates to directly access ATP and allowing adenosine triphosphatase to use ATP directly to generate energy for the mobilization of rat sperm.

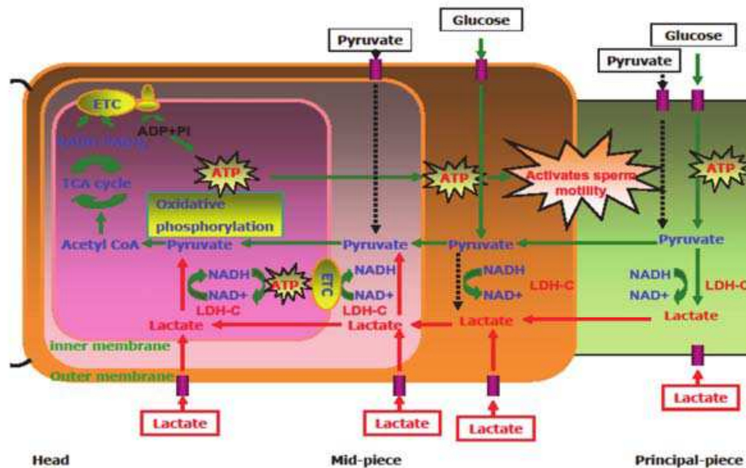

Fig. 5. Hypothesis of lactate-transport system for energy production and translocation in the rat sperm.

4.3 Extracellular ATP and dibutyryl cAMP enhance the freezability of rat sperm

The basic mechanochemical event underlying sperm motility is ATP-induced microtubule sliding (Brokaw. 1972). ATP associated with the dynein arms on outer-doublet microtubules provides the energy required for this process (Warner and Mitchell, 1980). ATP, calcium, and cAMP have received considerable attention as potential primary regulators of sperm motility in several species of animals (Aoki et al., 1996, Lindemann. 1978, Lindemann and Gibbons. 1975). Extracellular ATP acts on sperm by triggering a purinergic receptor-mediated increase in the intracellular calcium level; this increase may produce several downstream effects that enhance sperm motility (Luria et al., 2002, Rodriguez-Miranda et al., 2008). Increased calcium levels presumably activate soluble adenylyl cyclase, thereby increasing the cAMP concentration in sperm (Cook and Babcock. 1983, Garbers. 2001, Ren et al., 2001). cAMP induces protein phosphorylation by activating protein kinase A (Fujinoki et al., 2004, Kinukawa et al., 2006) and mediates calcium influx into sperm via the CatSper calcium ion channels (Cook and Babcock, 1983, Garbers. 2001, Ren et al., 2001). In addition, cAMP may elevate mitochondrial calcium levels (Degasperi et al., 2006), thereby activating the calcium-dependent dehydrogenases involved in the Krebs cycle and providing ATP required for sperm motility.

Previously, we showed that rat sperm become freezable when diluted in ATP-containing raffinose–mKRB egg yolk extender (Yamashiro et al., 2010a). This finding indicates the existence of a unique pathway that utilizes extracellular ATP in rat sperm and suggests that

extracellular ATP produces several downstream effects that improve sperm motility by increasing calcium levels or by activating cAMP signal transduction pathways. Further elucidation of the role of extracellular ATP in the energy-synthetic processes and motility-regulation system of rat sperm could lead to improved motility, freezability, and fertilizing ability of the sperm. We, therefore, evaluated the freezability of rat sperm preserved in raffinose–mKRB egg yolk extender with ATP, ionomycin (a calcium ionophore), and dibutyryl cAMP (dbcAMP; a membrane-permeable cAMP analog) under various conditions. We also determined the effects of these agents on oxygen consumption by sperm. Sperm cryopreservation was considered successful if frozen–thawed sperm fertilized oocytes. To improve the effectiveness of in vitro fertilization (*IVF*), we determined whether ATP- and dbcAMP-supplemented *IVF* media improve the fertilizing ability of sperm. We also attempted artificial insemination with frozen–thawed rat sperm.

Results showed that rat sperm become freezable when diluted in ATP, and dbcAMP-containing raffinose–mKRB egg yolk extender (Yamashiro et al., 2010b). This finding indicates the existence of a unique pathway that utilizes extracellular ATP in rat sperm and suggests that extracellular ATP produces several downstream effects that improve sperm motility by increasing calcium levels or by activating cAMP signal transduction pathways. Further elucidation of the role of extracellular ATP in the energy-synthetic processes and motility-regulation system of rat sperm could lead to improved motility, freezability, and fertilizing ability of the sperm (Fig. 6). The results showed that the cryopreservation of rat

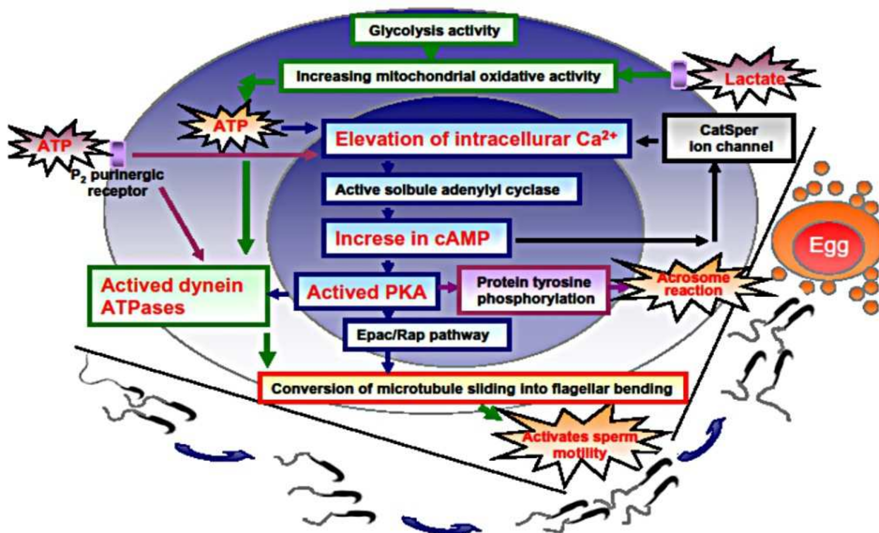

Fig. 6. Hypothesis of utilization of exogenous ATP pathway in the rat sperm.

sperm in raffinose–mKRB egg yolk extender supplemented with ATP and dbcAMP rendered sperm from rats freezable. This finding indicates that ATP- and dbcAMP-containing extenders improved the postthaw motility and fertilizing ability of cryopreserved rat sperm. Moreover, the *IVF* medium developed in the current study may be effective for the *in vitro* production of embryos from cryopreserved rat sperm. More recently, Vasudevan et al., (2011) reported that treatment of mouse sperm with extracellular ATP enhanced *IVF* rates in outbred and hybrid mice. Thus, this chapter was introduced how we identified optimal energy substrates and of rat sperm cryodiluent and *IVF* medium.

5. Concluding remarks

In this chapter described in detail the effects of the various components of cryodiluent that are used for rat sperm cryopreservation. We found that lactate, ATP and dbcAMP conferred freezability on rat sperm and enhanced oocyte fertilization by frozen-thawed sperm. In addition, if the rat sperm can be controlled flagellar movement in a way that changed by energy-yielding substrates such as lactate, it would be possible to enhance their fertilizing ability of rat sperm for *in vitro* fertilization by using a both of the fresh and frozen-thawed sperm (Fig. 7). However, there is still a lack of information for physiological trigger

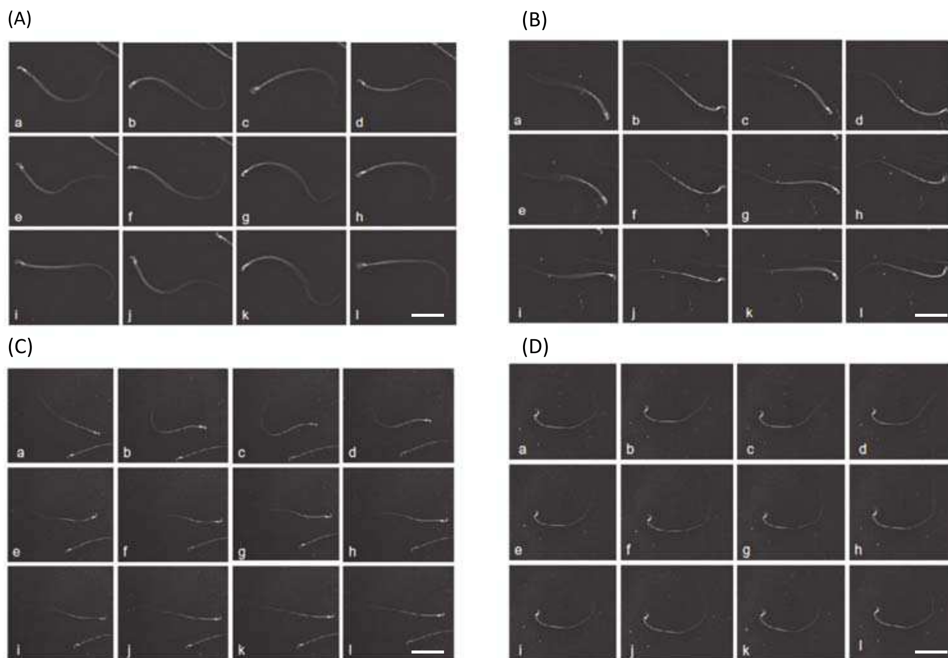

Fig. 7. Representative patterns of rat sperm movement which were extended in the presence （A) or absence （C and D) of lactate in raffinose-mKRB egg yolk solution. In B, sperm was incubated at 37°C for 3 h in the lactate containing solution. Panels a to l indicate the turn. Scale bars = 50μm

regarding how rat sperm switches on their flagellar movement at the real-time of fertilization, such as "hyper-activation" or "ultra-activation" (Yamashiro et al., 2009). Further, we believe that the *IVF* medium developed in our study is effective for the *in vitro* production of embryos from cryopreserved rat sperm. These results not only indicate that the cryopreservation of rat sperm with the present method can be applied to reproductive technologies but also indicate that exogenous lactate, rather than glucose and pyruvate, exerts a mediating effect on energy-dependent synthetic processes.

In conclusion, this is one of the very few information in the field that actually test the components of a cryodiluent in a logical manner for rat sperm cryopreservation. From this point of view, the chapter is likely to be important not only for freezing of rat sperm but also for freezing of sperm of other species in general.

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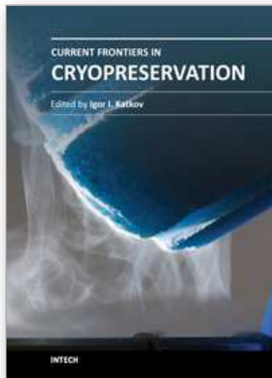

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Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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