—Brief Note—

A Trial of Oocyte Maturation and In Vitro Fertilization by Frozen-Thawed Spermatozoa in the Red-Bellied Tamarin (Saguinus labiatus)

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Abstract: *Freezing of epididymal spermatozoa from red-bellied tamarins (Saguinus labiatus) was achieved by using a semen-diluting medium called TTE (Tes, Tris, egg-yolk base) containing 5% glycerol. When the frozen spermatozoa were suspended and incubated in m-TYH medium (TYH medium supplemented with 1mM caffeine and 1mM dibutyryl cyclic AMP), the frozen-thawed spermatozoa showed a good sperm survival rate and hyperactive movement after 30 min of incubation. A total of 59 germinal vesicle (GV) stage oocytes were collected from 4 ovaries of 4 female red-bellied tamarins regardless of their menstrual cycles. At 24, 48, and 72 h after incubation in TCM-199 medium containing 10% fetal calf serum (FCS), 30%, 53%, and 71% oocytes, respectively, either showed germinal vesicle breakdown (GVBD) or had extruded the first polar body. Although the oocytes matured in vitro were inseminated by frozen-thawed spermatozoa, no eggs became fertilized.*

Key words: *Follicular oocyte, In vitro maturation, Epididymal spermatozoa, Sperm freezing, Red-bellied tamarin.*

Of over 200 species of nonhuman primates only a few have been used for studying *in vitro* fertilization (IVF) [1, 2]. In this area of research in monkeys, the key is obtaining a high number of good quality oocytes and spermatozoa. *In vitro* maturation (IVM) of germinal vesicle (GV) stage oocytes obtained from ovaries [3, 4] and the freezing of spermatozoa [5–7] are useful for studies such as IVF, early embryology and spermatology in the monkey genus *Macaca*. We previously succeeded in IVM of follicular oocytes and IVF by frozen-thawed spermatozoa in cynomolgus monkeys [7, 8]. To our knowledge, there is no report on IVM, IVF or sperm freezing for monkeys belonging to the genus *Saguinus*. Here we report on the trial of IVF of red-bellied tamarins (*Saguinus labiatus*) (Fig. 1) with IVM follicular oocytes collected from ovaries regardless of their menstrual cycles, and on the freezing of epididymal spermatozoa.

Four sexually mature male red-bellied tamarins, 6–10 years old, were used as donors for the freezing of epididymal spermatozoa. Four sexually mature females, 3–11 years old, were used for the IVM of follicular oocytes. The males and females were born and raised in our primate center [9]. After the animals were euthanized, we removed an epididymis and an ovary from one side of each male and female, respectively, through an abdominal incision (the remaining 4 epididymides and 4 ovaries were used for another experiment). For euthanasia, animals were phlebotomized under deep anesthesia by an intramuscular injection of ketamin hydrochloride (Ketalar; Sankyo, Tokyo, Japan).

The removed epididymides were rinsed with TYH medium [10]. The tails of the epididymides were hashed in 3 ml of the medium and kept for approximately 10 min in a humidified 5% CO_2-95% air atmosphere. The sperm suspension was layered on 3 ml of 90% Percoll (Pharmacia, Uppsala, Sweden) in a 15-ml disposable tube. This isoosmotic 90% Percoll solution was prepared by mixing a 9% NaCl solution with Percoll. The tube was centrifuged at 800 g for 10 min.

The sediment spermatozoa with good motility were frozen by the method previously developed for the cynomolgus monkey [7] and the Japanese monkey [11] spermatozoa in which TTE (Tes, Tris and egg yolk base) medium containing 5% glycerol was used for dilution.

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The spermatozoa were sealed into cryostraws and then preserved in liquid nitrogen.

Immediately before use, five straws containing frozen spermatozoa were thawed by placing them in 37°C water for 30 sec. This thawed sperm suspension was diluted with TYH medium and washed by the 90%-Percoll method.

Fresh unfrozen spermatozoa and frozen-thawed spermatozoa with good motility were incubated in a droplet of either supplemented or unsupplemented TYH medium under mineral oil (Squibb & Sons, Princeton, NJ, USA). For convenience, we designate these media as m-TYH and TYH medium, respectively. The supplement was 1 mM caffeine (Wako Chemical, Osaka, Japan) and 1 mM dibutyryl cyclic AMP (Yamasa Syoyu, Chiba, Japan). At different times during the incubation, we determined the ratio of motile spermatozoa and the presence of hyperactivated spermatozoa.

Fig. 1. Red-bellied tamarins (*Saguinus labiatus*) in the Tsukuba Primate Center, The National Institute of Health.

The removed ovaries were rinsed with TCM-199 (tissue culture medium-199; Flow Laboratories, Virginia, VA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 10 IU/ml eCG (equine chorionic gonadotrophin; Teikoku Hormone, Tokyo, Japan), and then hashed in the medium. The oocytes in the medium were collected, transferred to a 50-μl droplet of the medium, and then kept in a humidified 5% CO_2-95% air atmosphere. At different times during the incubation, these oocytes were examined under a Nomarski interference-contrast microscope (Nikon Optical, Tokyo, Japan) for the presence of either the GV, the first polar body or loosened cumulus cells.

Oocytes that showed germinal vesicle breakdown (GVBD) or had extruded the first polar body were transferred to Whitten's medium [12]. Frozen-thawed spermatozoa were suspended in m-TYH medium and incubated 1–2 h. Five μl of the sperm suspension was

Table 1. Comparison of survival rate and motility of fresh and frozen-thawed epididymal spermatozoa[a] from red-bellied tamarins during incubation

Spermatozoa	Anim. No. (Preservation period)	Medium	Incubation time (h)					
			0	2	4	6	8	
Fresh	5228809008	TYH	80+++	80+++	80+++	70+++	50+++	
		m-TYH[b]	80+++	80+++	80+++	80+++	70+++	
	5228703002	TYH	90+++	90+++	70+++	70++	50++	
		m-TYH[b]	90+++	90+++	80+++	70+++	50+++[c]	
			Incubation time (h)					
			0	0.5	1	2	3	9
Frozen-thawed	5228809008 (88 days)	TYH	70+++	70+++	70+++	70+++	60+++	30++
		m-TYH[b]	70+++	70+++[c]	50+++[c]	50++[c]	50++[c]	30+
	5128504001 (1 day)		50+++					
	5128506007 (65 days)		60++					

[a] The percentage of motile spermatozoa and the intensity of sperm motility are shown. The degree of sperm motility was expressed by the following motility scores: +++, active progressive motility; ++, sluggish progressive motility; +, mostly non-progressive motility. [b] TYH supplemented with caffeine and dbcAMP. [c] Hyperactivated motion spermatozoa were observed.

then transferred to a 50-μl droplet of Whitten's medium containing the oocytes. The final concentration of spermatozoa was approximately 3×10^5/ml. After 18 h of insemination, the eggs were examined under the Nomarski microscope for the presence of male and female pronuclei or for the second polar body.

Table 1 shows the survival rate, grade of motility and appearance of hyperactivated motion of the fresh and frozen-thawed spermatozoa at various times during incubation.

Fresh spermatozoa washed by the Percoll method showed an 80% and 90% survival rate, and frozen-thawed spermatozoa showed a 70% survival rate. When the fresh spermatozoa with the good survival rate were incubated with either m-TYH or TYH medium, the spermatozoa showed a 70–80% survival rate for 6 h, and showed a 50–70% survival rate after 8 h of incubation. Frozen-thawed spermatozoa (Anim. No. 5228809008) incubated with TYH medium showed a 70% survival rate after a 2-h incubation, whereas spermatozoa incubated with m-TYH medium showed that rate after 0.5 h. After a 9-h incubation, however, spermatozoa in either media showed a low survival rate of 30%.

Fresh spermatozoa showed active progressive motility during an 8-h incubation, regardless of the media. Frozen-thawed spermatozoa, however, showed this motility for 3 h in TYH medium and for 1 h in m-TYH medium. In TYH medium, neither fresh nor frozen-thawed spermatozoa showed hyperactivated motion. In m-TYH medium, fresh spermatozoa from 1 of 2 males showed hyperactivated motion after an 8-h incubation, whereas frozen-thawed spermatozoa showed this motion after a 0.5-h incubation.

Table 2 summarizes the results of IVM of follicular oocytes. A total of 59 good quality GV stage oocytes were collected from the 4 ovaries. At 24, 48 and 72 h after cultivation, 30% (18/59), 53% (24/45) and 71% (32/45) of the oocytes, respectively, either showed GVBD or had extruded the first polar body (Fig. 2). Degeneration of oocytes during the cultivation occurred within 48 h.

Nine oocytes after a 24-h cultivation and 32 oocytes (including oocytes that had extruded the first polar body at 24 h or 48 h of the cultivation) after a 72-h cultivation were then inseminated with frozen-thawed spermatozoa, but no fertilization occurred.

In our primate center, sperm freezing has been successful in cynomolgus monkeys [7] and Japanese monkeys [11], both of which belong to the genus *Macaca*. IVF has been also successful in these *Macaca* monkeys [7, 11] and African green monkeys [13] belonging to the genus *Cercopithecus*. Here we carried out these two processes in red-bellied tamarin, which belong to the genus *Saguinus*. In this study, frozen-thawed red-bellied tamarin spermatozoa maintained active progressive motility in TYH medium and sluggish progressive motility in m-TYH medium during 3 h of incubation. Hyperactivated motion spermatozoa were present only when incubated in m-TYH medium, and also within a short term (0.5 h) incubation. Though the motility of fresh red-bellied tamarin spermatozoa differed slightly from that reported for fresh *Macaca* monkey spermatozoa, the motility was similar to that reported for frozen-thawed *Macaca* monkey spermatozoa [7]. This means that this freezing method is effective for red-

Table 2. *In vitro* maturation of follicular oocytes from red-bellied tamarins

Culture time (h)	Number of oocytes examined	Stages of oocytes			
		GV	GVBD	1st PB	D
0	59	59			
24	59	36	10 (16.9)[a]	8 (13.6)[a]	5[b]
48	45	13	13 (28.9)	11 (24.4)	8
72	45	5	17 (37.8)[c]	15 (33.3)[c]	8

[a] Four of GVBD stage oocytes and five of 1st PB-extruded oocytes were used for insemination. [b] Culture of five degenerated oocytes was discontinued. [c] All GVBD stage oocytes and 1st PB-extruded oocytes were used for insemination. GV: germinal vesicle, GVBD: germinal vesicle breakdown, 1st PB: first polar body, D: degeneration ( ): % to oocytes examined.

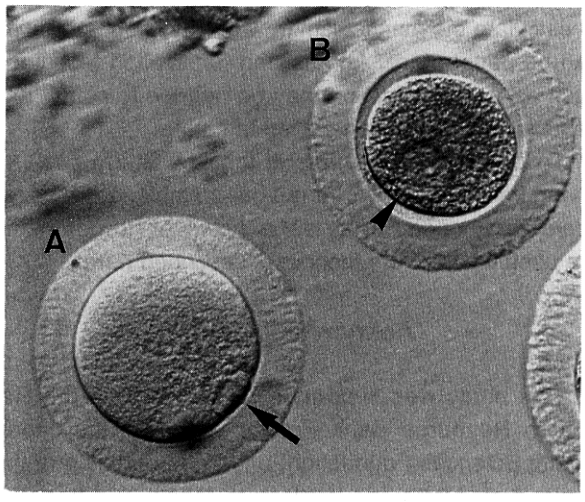

Fig. 2. Red-bellied tamarin oocytes 24 h after the start of incubation. (A) An oocyte that had extruded the first polar body (arrow) *in vitro*. (B) A degenerated oocyte that was underdeveloped at the GV stage (arrowhead).

bellied tamarin spermatozoa.

IVM in the rhesus monkey succeeded when TALP medium containing FCS was used, and the oocytes matured *in vitro* became fertilized after insemination [3, 4]. In our study, an average of 15 oocytes at the GV stage per ovary were collected from ovaries in utter disregard of the menstrual cycles in red-bellied tamarins. When TCM-199 medium containing FCS and eCG was used, the oocytes matured *in vitro* at a fast rate (38% oocytes showed GVBD and 33% extruded the first polar body after a 72-h incubation), but the maturation time varied greatly (24–72 h). Before the method can be used in IVF research, we therefore need to establish a maturation method in which the maturation time is known precisely. In the squirrel monkey during the non-breeding season, Yeoman *et al.* [14] successfully used a low dose of follicle-stimulating hormone to stimulate oocytes matured with Ham's F-10 medium containing bovine serum albumin and fertilized *in vitro*, but negligible maturation was observed in immature oocytes from unstimulated ovaries. Sankai *et al.* [11] collected follicular oocytes from Japanese monkey ovaries stimulated with eCG and hCG during the non-breeding season, and then successfully used them in IVF. In red-bellied tamarin as well, synchronization of oocyte stages is influenced by the endocrine system.

In our study, although oocytes matured *in vitro* were inseminated by frozen-thawed spermatozoa, no eggs were fertilized, possibly due to inaccurate timing of insemination. The red-bellied tamarin oocytes (Fig. 2) have a thick zona pellucida. Further study is required to elucidate the mechanism in which this zona pellucida can be penetrated. An assisted fertilization technique such as a microinjection may be needed to clarify and establish IVF in the red-bellied tamarin.

To our knowledge, this is the first study on IVM, sperm freezing and IVF for red-bellied tamarins. A large-scale study needs to be done, however, to determine the usefulness of the technique described here.

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References

1) Dukelow, W.R., Pierce, D.L., Roudebush, W.E., Jarosz, S.J. and Sengoku, K. (1990): *In vitro* fertilization in nonhuman primates. J. Med. Primatol., 19, 627–639.
2) Wolf, D.P., Thomson, J.A., Zelinski-Wooten, M.P. and Stouffer, R.L. (1990): *In vitro* fertilization-embryo transfer in nonhuman primates; the technique and its applications. Mol. Reprod. Dev., 27, 261–280.
3) Bavister, B.D. (1987): Oocyte maturation and *in vitro* fertilization in the rhesus monkey. In: The Primate Ovary (Stouffer, R.L., ed.), pp. 119–137, Plenum Press, New York.
4) Alak, B.M. and Wolf, D.P. (1994): Rhesus monkey oocyte maturation and fertilization *in vitro*; roles of the menstrual cycle phase and of exogenous gonadotropins. Biol. Reprod., 51, 879–887.
5) Mahone, J.P. and Dukelow, W.R. (1978): Semen preservation in *Macaca fascicularis*. Lab. Anim. Sci., 28, 556–561.
6) Tollner, T.L., VandeVoort, C.A., Overstreet, J.W. and Drobnis, E.Z. (1990): Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). J. Reprod. Fertil., 90, 347–352.
7) Sankai, T., Terao, K., Yanagimachi, R., Cho, F. and Yoshikawa, Y. (1994): Cryopreservation of spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). J. Reprod. Fertil., 101, 273–278.
8) Sankai, T., Sakakibara, I., Cho, F. and Yoshikawa, Y. (1993): *In vitro* maturation and *in vitro* fertilization of eggs recovered from ovaries of cynomolgus monkeys (*Macaca fascicularis*) at necropsy in a indoor breeding colony. J. Mamm. Ova Res., 10, 161–166.
9) Honjo, S. (1985): The Japanese Tsukuba Primate Center for Medical Science (TPC): an outline. J. Med. Primatol., 14, 75–89.
10) Toyoda, Y., Yokoyama, M. and Hoshi, F. (1971): Studies on the fertilization of mouse eggs *in vitro*. I. *In vitro* fertilization of eggs by fresh epididymal sperm. Jpn. J. Anim. Reprod., 16, 147–151.
11) Sankai, T., Shimizu, K., Cho, F. and Yoshikawa, Y. (1997): *In vitro* fertilization (IVF) of follicular oocytes by frozen-thawed spermatozoa in Japanese monkey. Lab. Anim. Sci., 47, 58–62.
12) Whitten, W.K. (1971): Nutritional requirements for the culture of preimplantation embryos *in vitro*. Adv. Biosci., 6, 129–139.
13) Sankai, T., Cho, F. and Yoshikawa, Y. (1997): *In vitro* fertilization and preimplantation embryo development of African green monkeys (*Cercopithecus aethiops*). Am. J. Primatol., 42 (in press).
14) Yeoman, R.R., Helvacioglu, A., Williams, L., Aksel, S. and Abee, C.R. (1994): Restoration of oocyte maturational competency during the nonbreeding season with follicle-stimulating hormone stimulation in squirrel monkeys (*Saimiri boliviensis boliviensis*). Biol. Reprod., 50, 329–335.