Comparison of the Media for Isolation and Storage of Round Spermatid Nuclei Before Intracytoplasmic Injection

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Purpose: This study investigated whether K^+ -rich medium is better than pure NaCl solution or Na^+ -rich cell culture medium for handling round spermatid nuclei prior to injection into oocytes (ROSNI).

Methods: Round spermatids of the mouse were isolated and stored in isotonic NaCl, a cell culture medium (CZB), or a nucleus isolation medium (NIM) before injection into oocytes. The rates of normal fertilization, embryonic development in vitro, and birth of normal offspring after transfer of embryos to foster mothers were determined.

Results: In vitro development of ROSNI-produced zygotes to blastocysts was the same when "naked" spermatid nuclei were exposed briefly to three media. However, a long (60-min) exposure of the nuclei to Na^+ -rich medium was detrimental. In K^+ -rich NIM "naked" spermatid nuclei best retained their ability to participate in normal embryonic development.

Conclusion: NIM was better than Na^+ -rich medium for retaining isolated spermatids competent to participate in normal embryonic development.

KEY WORDS: intracytoplasmic injection; mouse; ROSI; ROSNI; spermatid.

INTRODUCTION

The fact that the nucleus of a round spermatid is ready to participate in normal embryonic development was first demonstrated in the mouse by electrofusion of a spermatid with an oocyte (1). Subsequently mechanical injection of spermatid nuclei was used to produce

normal mouse offspring (2,3) as well as healthy human offspring (4). Injections of round spermatids or spermatid nuclei into oocytes are commonly called ROSI and ROSNI.

According to Kimura and Yanagimachi (2), the highest incidence of normal fertilization following ROSNI in the mouse was obtained when the spermatid nucleus was freed from the surrounding cytoplasm before it was injected into a previously activated oocyte. Because the nuclear "denudation" was performed in a cell culture medium (CZB), the spermatid nuclei must be temporarily exposed to the medium with a high (approximately 110 mM) $[Na^+]$ and a low (approximately 5 mM) $[K^+]$. Normally, any cell nuclei are suspended in the cytoplasm with a low (5–15 mM) $[Na^+]$ and a very high (approximately 140 mM) $[K^+]$. Although spermatid nuclei apparently tolerated the Na^+ -rich and K^+ -poor medium, prolonged exposure to such a medium could be detrimental to the nuclei. The present study was carried out to determine how the ionic composition of the medium affects the ability of spermatid nuclei to participate in normal fertilization and embryonic development.

MATERIALS AND METHODS

Reagents

All inorganic and organic reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Media

The medium used for culturing oocytes after microsurgery was CZB medium (5,6) supplemented with 5.5 mM D-glucose and 3 mg/ml bovine serum albumin (BSA; fraction V; Calbiochem, La Jolla, CA). The medium used for collection of oocytes from

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oviducts was modified CZB with 20 mM HEPES and reduced NaHCO_3 (5 mM) and 0.1 mg/ml polyvinyl alcohol (30–70 kDa; cold water-soluble PVA), instead of BSA. We called this medium HEPES-CZB. Media for preparing round spermatids for injection were HEPES-CZB, isotonic (150 mM) NaCl, and nucleus isolation medium (NIM). NIM consisted of 123.0 mM KCl, 2.6 mM NaCl, 7.8 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , 3 mM EDTA disodium salt, and 0.5 mM PMSF. A stock solution of 100 mM PMSF in 100% ethanol was kept at 4°C. It was added to NIM (final concentration, 0.5 mM) immediately before use. The pH value of NIM was adjusted to 7.2 by the addition of a small quantity of 1 M KOH. Isotonic NaCl, HEPES-CZB, and NIM, each supplemented with 12% (w/v) polyvinylpyrrolidone (PVP; 360 kDa), were used to suspend spermatids, disrupt their plasma membranes, and store denuded nuclei. They were called PVP-NaCl, PVP-HEPES-CZB, and PVP-NIM, respectively. PMSF-free NIM containing 12% PVP was used to rinse denuded nuclei immediately before injection. The medium used for parthenogenetic activation of mouse oocytes was Ca^{2+} -free M16 containing 5 mM SrCl_2 (7–9). This medium was called Sr^{2+} -M16.

Preparation of Oocytes

B6D2F₁ female mice (7–11 weeks old) were each induced to superovulate by intraperitoneal injection of 7.5 IU eCG followed by injection of 7.5 IU hCG 48 hr later. Oocytes were collected from oviducts approximately 16 hr after hCG injection. They were freed from the cumulus by treatment with 0.1% (w/v) bovine testicular hyaluronidase (300 USP U/mg; ICN Biochemicals, Costa Mesa, CA) in HEPES-CZB for 3–5 min. The oocytes were rinsed thoroughly and kept in CZB at 37°C under 5% CO_2 in air for up to 2 hr.

Preparation of Spermatids

Round spermatids were collected from B6D2F₁ male mice (8–12 weeks old). A testis was isolated and placed in cold (4–10°C) HEPES-CZB, isotonic NaCl, or NIM. After removal of the tunica, seminiferous tubules were cut into minute pieces using a pair of fine scissors. One part of the suspension containing fragments of seminiferous tubules was mixed thoroughly with 2 parts of PVP-HEPES-CZB, PVP-NaCl, or PVP-NIM. Thus, the final concentration of PVP in these media was approximately 8% (w/v). A small drop (approximately 2 μl) of this cell suspension was kept under mineral oil (Squibb and Sons, Princeton, NJ) in a plastic petri dish on the microscope stage (see

below). When viewed under an interference-contrast or phase-contrast microscope, mouse round spermatids were easily recognized by their small size and centrally located chromatin mass (10,11).

Preparation of Oocyte Holding and Injection Pipettes

To prepare oocyte holding and spermatid injection pipettes, borosilicate glass capillary tubing (1.0-mm o.d., 0.75-mm i.d.; Sutter Instruments, Novato, CA) was drawn with a pipette puller (Model P-97; Sutter Instruments). The holding and injection pipettes were both prepared as described in detail by Kimura and Yanagimachi (2).

Activation of Oocytes Prior to Spermatid Injection

The oocytes to be injected with spermatids were parthenogenetically activated by incubating them for 1 hr (37°C) in Sr^{2+} -M16 medium. Incubation was carried out under mineral oil in a plastic petri dish in an atmosphere of 5% CO_2 in air. Most Sr^{2+} -treated oocytes were at telophase of the second meiosis (telophase II) 1 hr after the initiation of treatment. They were thoroughly rinsed with HEPES-CZB before transfer onto the microscope stage (see below).

Spermatid Injection into Oocytes

The cover (10 mm in depth) of a plastic dish (100 $\times$ 15 mm; Falcon Plastics, Oxnard, CA; Cat. No. 1001) was used as a microinjection chamber. A row consisting of three round droplets and one elongated drop was placed along the center line of the dish. The first droplet (5 μl ; 2 mm in diameter) was for pipette washing (PVP-HEPES-CZB, PVP-NaCl, or PVP-NIM). The second droplet (5 μl ; 2 mm in diameter), either PVP-HEPES-CZB, PVP-NaCl, or PVP-NIM, was for denudation of spermatids (see below). The third droplet (5 μl ; 2 mm in diameter) was the same medium as the second droplet, but for the 1-hr storage of spermatid nuclei prior to injection. When spermatid denudation and storage were carried out in PVP-NIM, denuded nuclei were rinsed with PMSF-free NIM to avoid introduction of PMSF into oocytes. The fourth elongated droplet (10 μl ; 2 mm wide and 6 mm long) was HEPES-CZB medium, in which spermatid injection into oocytes was carried out. After these droplets had been covered with mineral oil, the dish was placed on the cooled (17–18°C) stage of an inverted microscope, with Nomarski differential interference optics (Leitz Labovert FS, Foster City, CA) equipped with a

Leitz micromanipulator. This low temperature was beneficial for the "wound-healing" of mouse oocytes punctured by an injection pipette (2).

Nuclei of round spermatids were injected into telophase II oocytes in two ways. In the first, each spermatid was drawn in and out of the injection pipette (approximately 5 μ m in the inner diameter) until its plasma membrane was broken and the nucleus became completely separated from the cytoplasm. The "naked" nucleus was immediately injected into a Sr^{2+} -activated oocyte using the technique described by Kimura and Yanagimachi (2). In the second, spermatid nuclei were separated from the cytoplasm as mentioned above, but they were left in PVP-HEPES-CZB, PVP-NaCl, or PVP-NIM for 1 hr at 17–18°C before injection into activated oocytes.

Incubation and Examination of Spermatid-Injected Oocytes

Spermatid-injected oocytes were kept in the operation medium (HEPES-CZB) for approximately 20 min on the cooled (17–18°C) stage of the microscope. They were then transferred to a dish of HEPES-CZB medium at room temperature (24–25°C). Approximately 10 min later, the oocytes were transferred into 50 μ l CZB medium under mineral oil in a plastic dish (35 $\times$ 10 mm; Falcon Plastics; Cat. No. 3001) and incubated at 37°C under 5% CO₂ in air. Five to seven hours later they were examined under an inverted microscope. Eggs with distinct signs of degeneration were counted and discarded. Those with two distinct pronuclei and the second polar body were considered normally fertilized and were cultured continuously. Embryos developing in vitro were examined at 24-hr intervals for up to 120 hr after sperm injection.

Embryo Transfer to Foster Mothers

Two-cell embryos developed from those normally fertilized were selected at random. On average six embryos (range, 5–10) were transferred into the oviducts of each recipient female (Swiss-Webster, albino) as described by Kimura and Yanagimachi (2). Mothers were allowed to deliver and raise their own pups (red eyes and white coat) as well as foster pups (black eyes and grey/brown/black coat). Some of their own pups were removed during lactation, but all foster pups were allowed to develop and mature. Offspring sex was determined 4 weeks after birth.

Statistical Analysis

Results were compared using the chi-square test.

RESULTS

We injected a single spermatid nucleus into a previously activated oocyte (see Materials and Methods). When we injected a spermatid nucleus into an oocyte immediately after its denudation, the majority of the oocytes was fertilized normally regardless of the three types of the medium (NaCl, HEPES-CZB, or NIM) and the duration of nucleus storage in the medium (0 or 1 hr) (Table I). The majority of the normally fertilized eggs developed into blastocysts when nucleus injection was done immediately after denudation (Table II). When the nuclei were denuded and stored for 1 hr in NaCl or HEPES-CZB, less than 20% of the eggs developed into blastocysts, whereas about 50% of the eggs developed into blastocysts when the nuclei were stored for 1 hr in NIM (Table II). Normal live offspring were obtained from the oocytes injected with spermatid nuclei stored in NIM, but not from those injected with the nuclei stored in isotonic NaCl (Table III).

Table I. Fertilization of Mouse Oocytes Injected with Spermatid Nuclei Stored in Various Media

Spermatid nucleus storage medium	Storage time (hr)	Total No. oocytes injected in 5 expts	No. (%) oocytes survived	No. (%) oocytes normally fertilized ^a
NaCl	0	57	49 (86)	31 (63)
	1	59	50 (85)	36 (72)
HEPES-CZB	0	64	57 (89)	37 (65)
	1	58	47 (81)	33 (70)
NIM	0	80	66 (83)	45 (68)
	1	65	56 (86)	43 (77)

^aNo significant difference between groups.

Table II. In Vitro Development of Mouse Zygotes Produced by Injection of Spermatid Nuclei

Spermatid nucleus storage medium	Storage time (hr)	Total No. zygotes cultured in 5 expts	No. (%) zygotes developed to*	
			2-cell	Blastocyst
NaCl	0	31	31 (100)	24 (77) ^a
	1	36	32 (88)	5 (14) ^b
HEPES-CZB	0	37	37 (100)	28 (75) ^c
	1	33	30 (91)	7 (21) ^d
NIM	0	45	45 (100)	35 (77) ^e
	1	43	40 (93)	20 (47) ^f

*No significant differences between superscripts a–c, a–e, c–e, and b–d. Statistical differences between superscripts d–f ($P < 0.05$), b–f ($P < 0.01$), e–f ($P < 0.01$), a–b ($P < 0.001$), and c–d ($P < 0.001$).

DISCUSSION

In the present study we used three media, isotonic NaCl, HEPES-CZB and NIM, to store isolated spermatid nuclei. Concentrations of Na^+ and K^+ in these solutions were approximately 150 and 0, 117 and 6, and 27 and 125 mM, respectively. Brief exposure to isotonic NaCl and HEPES-CZB was not harmful to the nuclei as evidenced by the development of the large proportion of the eggs to blastocysts (Table II). A prolonged (1-hr) exposure to these solutions was apparently harmful because fewer eggs could develop into blastocysts (Table II). Although the number of our embryo transfer experiments was rather small, the results nevertheless indicated that K^+ -rich NIM was superior to pure NaCl solution (and perhaps CZB) in retaining isolated spermatid nuclei competent to participate in embryonic development (Table III).

As of today only a few successful human pregnancies have been obtained following ROSI and ROSNI (4, 12–14). Schoysman *et al.* (13) were the only ones to report that plasma membrane of spermatids was partially disrupted before injection. No other investigators specifically mentioned the status of the spermatid's plasma membrane at the time of injection. Currently the rate of human pregnancy following spermatid injection is very low. There may be several reasons for this. First, identification of round spermatids from other types of testicular cells is rather difficult. Unlike mouse spermatids (10, 11), human spermatid nucleus does not have a distinct, centrally located chromatin mass that can be easily recognized under the interference-contrast or phase-contrast microscope. The acrosomal granule on the nucleus may or may not be a reliable marker of the spermatid (14–16). The cells thought to be round spermatids might not be spermatids, or even if they are, they might be "dead" for many hours before injection.

Second, spermatids may not activate oocytes as efficiently as mature spermatozoa, even though Tesarik *et al.* (4, 12) maintain that human round spermatids, unlike mouse spermatids, contain a factor (or factors) capable of activating oocytes. It is possible that all human spermatids do not contain sufficient amounts of the oocyte-activating factor. Additional stimulation may be necessary to induce full activation of the oocytes in human ROSI.

Third, in humans, the introduction of one sperm centrosome during fertilization appears to be essential for successful union of the male (sperm or spermatid) pronucleus and the female (oocyte) pronucleus (17, 18). It is not certain at present if all investigators engaged in ROSI/ROSNI introduced one centrosome

Table III. Results of Two-Cell Embryo Transfer Following Injection of Mouse Oocytes with Spermatid Nuclei Stored in Isotonic NaCl or NIM

Spermatid nucleus storage medium and period	Recipient	No. embryos transferred	No. (%) live offspring		
			Recipient's own (albino)	Foster (colored)	Sex of foster offspring
NaCl, 1 hr	a	8	11	0	
	b	5	9	0	
	c	6	13	0	
NIM, 1 hr	d	10	10	0	
	e	6	10	1	1 F
	f	6	13	2	1 F, 1 M
	g	6	10	1	1 F
	h	6	10	1	1 F

together with the nucleus. Centrosomes in round spermatids may or may not be ready to assemble microtubules for the pronuclear union of pronuclei.

Fourth, synchronization of cell cycle stages between the oocyte and the spermatid may be important to obtain consistently successful ROSI/ROSNI. In the mouse, the best ROSNI results were obtained when the oocyte (M phase) was activated first (e.g., by an electric shock), then a spermatid nucleus (G phase) was injected at about the time that the oocytes were about to complete the second meiosis (G_1 phase) (11). In all human ROSI/ROSNI in the past, spermatids were injected into unactivated oocytes. If the oocytes were activated first by sperm-borne oocyte activating factors such as oscillin (19) or a parthenogenetic agent such as calcium ionophore, then injected with spermatids during the G_1 phase of the oocyte, the chance of successful ROSI/ROSNI might have been higher.

Fifth and last, it is possible that some human spermatids injected into oocytes might have had intact plasma membranes. As for spermatozoa, local disruption or removal of spermatid's plasma membrane immediately before injection may facilitate mingling of spermatid's nucleus (and centrosome) with the ooplasm. As reported already, K^+ -rich media such as NIM perhaps less traumatic to "naked" spermatid nuclei than pure NaCl solution or Na^+ -rich cell culture media. The chance for successful ROSI/ROSNI may increase if a K^+ -rich medium is used to handle spermatids or spermatid nuclei.

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