Article

Progressive elimination of microinjected trehalose during mouse embryonic development

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Abstract

Recently, sugars such as trehalose have been introduced into mammalian cells by overcoming the permeability barrier of cell membranes, and have provided improved tolerance against stresses associated with freezing and drying. However, the fate of the intracellular sugars has remained an open question. To address this issue, mouse oocytes were microinjected with 0.1 mol/l trehalose, and intracellular trehalose and glucose concentrations were determined during embryonic development using a high performance liquid chromatography and pulsed amperometric detection protocol. Trehalose was not detected in non-injected controls at any stage of development. In the microinjection group, the amount of intracellular trehalose progressively decreased as embryos developed. There was a corresponding increase in intracellular glucose concentration at the two-cell stage, suggesting cleavage of trehalose to two glucose molecules. In summary, this study presents a simple, highly sensitive protocol to determine intracellular sugars. The data reveal rapid elimination of microinjected trehalose during embryonic development. These findings have implications for designing osmolarity-optimized culture media for sugar-injected oocytes.

Keywords: *cryopreservation, freezing, glucose, HPLC, oocyte, sugar, trehalose*

Introduction

The first successful cryopreservation of mammalian embryos and oocytes was achieved in the 1970s (Whittingham *et al*., 1972; Whittingham, 1977). By the mid-1980s, the successful cryopreservation of human embryos and oocytes had been reported (Trounson and Mohr, 1983; Zeilmaker *et al*., 1984). Of these two options, oocyte cryopreservation is the preferred approach because it avoids many of the legal and ethical issues that are encountered in human embryo freezing today. Furthermore, human oocyte cryopreservation may be beneficial to women who are anticipating loss of gonadal function due to radiation, chemotherapy or extirpative therapy. Current statistics suggest that 1 in 52 females under the age of 40 are diagnosed with cancer (Jemal *et al*., 2002). Successful cryopreservation of the oocytes before treatment could alleviate the emotional consequences of cancer therapy for women afflicted not only with a devastating disease, but possible impairment of their reproductive potential as well. However, oocyte cryopreservation proved to be a difficult task, while embryo freezing became a clinically established procedure in fertility clinics around the world (Veeck, 2003). The use of intracytoplasmic sperm injection (ICSI) with cryopreserved oocytes overcame some of the problems, and encouraging results have been reported recently (Porcu *et al*., 1997; Tucker *et al*., 1998; Kuleshova *et al*., 1999; Yoon *et al*., 2000; Quintans *et al*., 2002; Fosas *et al*., 2003). Nevertheless, human oocyte cryopreservation is still experimental, and there is a need for alternative approaches.

In nature a variety of organisms, including some frogs and nematodes, salamanders, tardigrades, insects, and brine shrimp, effectively use sugars such as trehalose, sucrose, and

glucose to cope with extreme freezing and desiccation (Crowe *et al.*, 1992; Potts, 1994). Results from several studies suggest that sugars such as trehalose afford their protection (i) by stabilizing lipid membranes and proteins as a result of direct interactions with polar residues through hydrogen bonding ('water replacement hypothesis') and (ii) by their excellent glass forming properties (Crowe *et al.*, 1993a,b, 1994). In general, freeze-concentrated solutions of trehalose and similar sugars have a higher glass transition temperature (~–30°C) compared with conventional penetrating cryoprotectants such as ethylene glycol (–85°C), propylene glycol (–67.5°C), and glycerol (–65°C)(Levine and Slade, 1988). The high glass transition temperature permits cells to be brought more quickly and safely to the storage temperature and has many other implications, as discussed in detail elsewhere (Eroglu *et al.*, 2002). Furthermore, non-reducing sugars are not toxic like conventional penetrating cryoprotectants, and are used as osmolytes by many organisms against osmotic (Somero, 1986; Singer and Lindquist, 1998), chemical (Sola-Penna *et al.*, 1997) and hypoxic (Chen and Haddad, 2004) stresses. Unfortunately, mammalian cell membranes are practically impermeable to sugars, and sugars were therefore used as extracellular additives in earlier studies (Mazur *et al.*, 1969; Leibo *et al.*, 1970). In recent years, several groups have overcome the permeability barrier of cell membranes to the sugars using different approaches such as thermotropic lipid-phase transition (Beattie *et al.*, 1997), genetically engineered reversible pores (Eroglu *et al.*, 2000), and transfection (Guo *et al.*, 2000; Puhlev *et al.*, 2001). All of these studies showed that intracellular sugars such as trehalose provide improved tolerance against freezing and drying stresses. More recently, the protective effect of intra- and extracellular trehalose during cryopreservation has also been demonstrated using both mouse and human oocytes (Eroglu *et al.*, 2001, 2002; Wright *et al.*, 2004). However, the fate of intracellular trehalose has remained an open question. This is important in terms of trehalose's safety as a cryoprotectant and further optimization of the procedure.

The objective of the present study was therefore to determine the fate of microinjected trehalose during embryonic development. To this end, a high performance liquid chromatography and pulsed amperometric detection (HPLC–PAD) protocol was developed to determine minute amounts (<1 ng) of sugars in a single oocyte/embryo.

Materials and methods

Reagents and media

All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. HEPES-buffered Dulbecco's Modified Eagle Medium (DMEM)/F-12 mixture (Gibco, Grand Island, NY, USA) containing 4 mg/ml bovine serum albumin (BSA) and 50 mg/ml gentamycin was used for manipulation of oocytes and embryos under air. Microinjection was also performed in this medium after supplementation with 0.1 mol/l trehalose. A concentrated trehalose solution (0.8 mol/l) was prepared for microinjection into oocytes by dissolving pure trehalose in 15 mmol/l HEPES (pH 7.4). For the culture of microinjected oocytes, hypermedium with an osmolality of 320 mOsmol was used in order to partially compensate for the increased intracellular osmolality due to injected trehalose. Previous experiments revealed that mouse zygotes cultured to the 2-cell and blastocyst stage in the hypermedium show normal development to day 16 fetuses, as well as to term at similar rates to those published in the literature (Eroglu *et al.*, 2003; unpublished data). Before use, drops of the hypermedium were overlaid by embryo-tested mineral oil and equilibrated overnight under a humidified atmosphere of 5% CO_2 in air.

Oocyte isolation

Metaphase II (MII) oocytes were obtained from 4- to 8-week-old B6D2F1 hybrid mice (C57BL/6 × DBA/2; Charles River Laboratories, Wilmington, MA, USA). This was approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital (accession no. 99–4053). Superovulation was induced by a combination of 5 IU pregnant mare serum and 2.5 IU human chorionic gonadotrophins (PG 600; Intervet, Millsboro, DE, USA) followed by 7.5 IU human chorionic gonadotrophin (HCG) alone 48–49 h later. Both hormone solutions were given by the intraperitoneal route. To collect MII oocytes, the oviducts were excised from killed mice 13–14 h after HCG injection and oocyte–cumulus masses were released from the ampulla. To remove cumulus cells, the oocyte–cumulus masses were exposed to 120 IU/ml of bovine testis hyaluronidase (type IV-S) at ambient temperature for 3–4 min. Next, the oocytes were washed in DMEM/F-12 twice and then transferred to the hypermedium (320 mOsmol) for recovery before experimentation.

Microinjection of trehalose

Quantitative microinjection of sugars has been described in detail elsewhere (Eroglu *et al.*, 2003). Briefly, microinjection and holding pipettes were manufactured from 1 mm borosilicate thin-wall (B100–75–10; Sutter, Novato, CA, USA) and thick-wall (B100–50–10; Sutter) glass capillaries, respectively. Both types of pipettes were pulled using a horizontal micropipette puller (model P-97; Sutter). The holding pipettes were broken off at an external diameter of 70–80 μm, and then fire-polished by an MF-900 microforge (Narishige, East Meadow, NY, USA). To obtain a sharp tip with an inside diameter of 0.5–0.7 μm, the injection pipettes were bevelled at an angle of 40° on a modified Sutter micropipette beveller (BV-10; Sutter) and their tip diameter was determined by bubble pressure measurement to calibrate them. Microinjection was accomplished using two identical sets of MMN-1 coarse mechanical and MMO-202D fine hydraulic micromanipulators (Narishige). Both sets were mounted on an inverted Diaphot 300 microscope (Nikon, Melville, NY, USA), which was equipped with Nomarski Interference Optics. To minimize vibration during microinjection, the micromanipulation system was set up on a vibration isolation table (TCM, Peabody, MA, USA). A modified Stoelting piezo injector (PM-20; Stoelting, Chicago, IL, USA) was used to facilitate puncture of the plasma membrane by a controlled sudden movement of the injection pipette. To obtain 0.1 mol/l intracellular trehalose concentration, a PLI-100 Pico-Injector (Medical Systems Co., Greenvale, NY, USA) was used to deliver an injection volume of ~22–26 pl from 0.8 mol/l trehalose solution, and this was confirmed by volumetric response of microinjected oocytes as