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Optimization of Cryoprotectant Loading into Murine and Human Oocytes

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ABSTRACT

Loading of cryoprotectants into oocytes is an important step of the cryopreservation process, in which the cells are exposed to potentially damaging osmotic stresses and chemical toxicity. Thus, we investigated the use of physics-based mathematical optimization to guide design of cryoprotectant loading methods for mouse and human oocytes. We first examined loading of 1.5 M dimethylsulfoxide (Me_2SO) into mouse oocytes at 23°C. Conventional one-step loading resulted in rates of fertilization (34%) and embryonic development (60%) that were significantly lower than those of untreated controls (95% and 94%, respectively). In contrast, the mathematically optimized two-step method yielded much higher rates of fertilization (85%) and development (87%). To examine the causes for oocyte damage, we performed experiments to separate the effects of cell shrinkage and Me_2SO exposure time, revealing that neither shrinkage nor Me_2SO exposure single-handedly impairs the fertilization and development rates. Thus, damage during one-step Me_2SO addition appears to result from interactions between the effects of Me_2SO toxicity and osmotic stress. We also investigated Me_2SO loading into mouse oocytes at 30°C. At this temperature, fertilization rates were again lower after one-step loading (8%) in comparison to mathematically optimized two-step loading (86%) and untreated controls (96%). Furthermore, our computer algorithm generated an effective strategy for reducing Me_2SO exposure time, using hypotonic diluents for cryoprotectant solutions. With this technique, 1.5 M Me_2SO was successfully loaded in only 2.5 min, with 92% fertilizability. Based on these promising results, we propose new methods to load cryoprotectants into human oocytes, designed using our mathematical optimization approach.

INTRODUCTION

Oocyte cryopreservation may help to preserve future fertility of women who face cancer/extirpative therapy or want to delay childbearing years. It would also avoid many legal and ethical issues associated with embryo freezing. Furthermore, cryobanking of oocytes may help conservation of endangered species and improvement of livestock breeding.

Although the first successful cryopreservation of mammalian and human oocytes was achieved in the 1970s [55; 76] and mid-1980s [13], respectively, oocyte cryopreservation has proven to be challenging due to the diversity of mechanisms leading to cryoinjury. Known manifestations of oocyte cryoinjury include intracellular ice formation [45], cell lysis [6], osmotic stress [1], disruption of cytoskeleton and spindle microtubules [18; 75], premature exocytosis of cortical granules and zona hardening [12; 64], parthenogenetic activation [65; 68; 73], and polyploidy [2; 18; 25]. Only after a decade of additional research and implementation of intracytoplasmic sperm injection (ICSI), it has become possible to mitigate some of these cryoinjury mechanisms, and to reproduce the initial success of human oocyte cryopreservation [40; 60; 71]. Subsequently, increasingly encouraging results have been reported with both ice-assisted slow-cooling techniques [9; 10; 11; 27; 39; 56] and ice-free vitrification methods [15; 41; 66; 77]. More recently, clinical success rates similar to those of unfrozen controls have been reported by three groups using an open-system vitrification approach requiring minimum sample volume and extremely fast cooling/warming rates [4; 17; 63]. However, this vitrification approach is particularly prone to handling issues and devitrification due to the minimal sample volume (less than 1 µl) and low concentrations (~30%) of intracellular cryoprotectant additive (CPA), while direct contact with LN_2 in open systems poses a serious biosafety risk [8; 28; 46; 70]. Moreover, the open-system vitrification approach is highly dependent on operator skill, and thawed oocytes must be fertilized by ICSI [4; 17; 63]. Although slow-cooling techniques are usually safer and not associated with a biosafety risk, their overall success rates in multicenter studies remain significantly lower than that of controls [11; 56]. Therefore, further research is

90 needed to improve the efficiency, reliability, and biosafety of the currently used cryopreservation
91 techniques.
92 One of the critical steps in cryopreserving oocytes is the loading of permeating CPAs such
93 as dimethyl sulfoxide (Me_2SO), propylene glycol (PROH), and ethylene glycol (EG), which may
94 result in severe osmotic perturbations and CPA toxicity depending on the specifics of the
95 experimental protocol. It is known that such insults compromise oocyte viability and
96 developmental capacity [1; 32; 34; 53; 65; 68]. Consequently, optimization of CPA loading
97 procedures is of importance to maximize the probability of success in oocyte cryopreservation.
98 Although it has long been recognized that mathematical models of membrane transport can
99 be used to guide the design of CPA loading and removal methods, the conventional approach to
100 protocol development has been limited to prevention of osmotic stress (i.e., avoiding excessive
101 cell volume excursions) without consideration of chemical toxicity due to prolonged CPA
102 exposure [5; 23; 33; 48; 50; 52]. Moreover, these early studies focused on the formulation of
103 CPA solutions used for step-wise addition and elution, whereas the exposure time to each
104 solution was not optimized [5; 23; 33; 48; 50; 52]. In contrast, we recently introduced an
105 optimization cost function to estimate the accumulation of toxicity damage, and used a Nelder-
106 Mead simplex algorithm to automatically select the optimal times of exposure to each CPA
107 solution as well as the optimal solution compositions [37]. Thus, we were able to design step-
108 wise addition and removal procedures for rhesus monkey oocytes, resulting in protocols with
109 total CPA exposure time comparable to or faster than that of one-step methods, but with
110 significantly reduced osmotic stress [37]. However, we were unable experimentally evaluate our
111 computer-optimized procedures, because primate oocytes are scarce, and all rhesus monkey
112 oocytes collected in our previous investigation were used for measurement of the biophysical
113 properties required to simulate the cell response.
114 Thus, in the present study, our goal was to experimentally test the hypothesis that oocyte
115 viability and function will be higher when CPA addition is performed using a two-step process

optimized by simultaneously limiting osmotic stress and CPA toxicity, than when CPA addition is performed in a single step (exposing oocytes directly to the full-strength CPA solution). To accomplish this, we employed our optimization approach to develop minimally damaging CPA addition procedures for mouse metaphase II (M II) oocytes. The computer-generated CPA loading protocols were realized experimentally, allowing the predictions to be validated, and demonstrating that optimized CPA addition methods can yield fertilization and embryonic development rates similar to those of untreated controls. Moreover, additional experimental tests were performed to shed light on the mechanisms of oocyte damage resulting from non-optimized CPA loading. Given the success demonstrated with mouse oocytes, we also used the computer models to design optimal processes for loading of PROH into human oocytes.

MATERIALS AND METHODS

Reagents and Media

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Bicarbonate-buffered Hypermedium [19; 20] supplemented with 4 mg/ml bovine serum albumin (BSA) and 50 mg/ml gentamycin served as a culture medium. For manipulation of oocytes and embryos under air, Hypermedium was buffered with 15 mM HEPES. Our previous studies showed that the Hypermedium supports mouse embryonic development and fertilized mouse eggs cultured in Hypermedium can develop into healthy mice when transferred to pseudo pregnant females [19; 20]. Before culturing oocytes and embryos, drops of the Hypermedium were overlaid by embryo-tested mineral oil and equilibrated overnight under a humidified atmosphere of 5% CO_2 in air at 37°C.

Galactose solutions used in osmotic shock experiments were prepared by adding 0.1 M, 0.3 M, or 0.5 M galactose to isotonic Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) containing 4 mg/ml BSA.

To dilute Me_2SO to specified concentrations as required for CPA loading (0.738M, 0.750 M, 0.976 M, 1.40 M, and 1.50 M), we used either the BSA-supplemented isotonic Ca^{2+}/Mg^{2+}-free PBS or one of two hypotonic buffers as the aqueous diluent. One hypotonic buffer formulation, designated "hypo-PBS", comprised Ca^{2+}/Mg^{2+}-free PBS that was diluted in ddH_2O as well as fetal bovine serum (FBS) to yield a calculated osmolarity of ~55 mOsmol/L (and a final FBS content of 10% v/v). Another hypotonic diluent, designated "hypo-NaCl", was prepared by adding NaCl and either FBS (10% v/v) or BSA (4 mg/ml) to a 15 mM Hepes buffer until the calculated osmolarity reached ~55 mOsmol/L. These hypotonic buffer formulations were designed so that the final salt osmolarity would be 50 mOsmol/L after addition of 1.4 M (i.e., 9.98% v/v) Me_2SO. To check this, the buffer osmolalities were measured by freezing-point depression osmometry after addition of an equivalent volume (9.98% v/v) ddH_2O, and confirmed to be 50 ± 5 mOsmol/kg (which is equal to 50 ± 5 mOsmol/L). For CPA removal, diluted Me_2SO solutions (0.5 M and 1.0 M), either with or without 0.25 M sucrose, were prepared using the isotonic Ca^{2+}/Mg^{2+}-free PBS with BSA only.

Oocyte Isolation

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Medical College of Georgia/Georgia Regents University. Metaphase II (M II) oocytes were obtained from 4-8 week-old B6D2F1 (C57BL/6NCr X DBA/2NCr; NCI, Frederick, MD) hybrid mice. Superovulation and collection of M II oocytes were carried out as described elsewhere [20]. 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 HEPES-buffered Hypermedium twice and then transferred to the Hypermedium for recovery before experimentation. For each experiment, M II oocytes were typically isolated from three or more female mice, pooled, and then randomly distributed among the experimental groups.

Simulation of Oocyte Response to Cryoprotectant Solutions

Oocyte volume and intracellular cryoprotectant concentration changes in response to molecular transport across the oolemma were simulated using custom software developed by one of us (JOMK) in the MATLAB® programming language (The MathWorks, Inc., Natick, MA). As previously described [37], the coupled transport of water and cryoprotectant additives (Me_2SO or PROH) across the cell membrane during cryoprotectant loading was described by a Jacobs-type two-parameter model [38], in which water chemical potential (i.e., the osmotic driving force) was estimated using an ideal-solution approximation.

For simulation of the response of mouse oocytes exposed to Me_2SO at 23°C or 30°C, published biophysical properties were used [57]. Oocytes were assumed to remain spherical during shrinking and swelling, and the isotonic (290 mOsmol/L) cell diameter was taken to be 74 µm, representing the average size of the mouse oocytes observed in our experiments. Because the reflection coefficients reported by Paynter et al. were near unity [57], the published Kedem-Katchalsky permeabilities were used directly in the two-parameter transport model, without modification to account for difference in model structure [38].

For simulation of human oocytes exposed to PROH at 24°C or 30°C, we used membrane permeability parameters that had been measured by Paynter et al. [58] and converted from the Kedem-Katchalsky to the two-parameter model format by Chuenkhum and Cui [16]. The oocytes were assumed to be spherical with an isotonic (290 mOsmol/L) volume of 1×10^6 μm^3, of which 20% was taken to be osmotically inactive [52].

Optimization of Cryoprotectant Addition Protocols

Protocols for step-wise addition of cryoprotectant additives (Me_2SO or PROH) to a final intracellular concentration of 1.5 M were designed using computer-aided optimization algorithms that simultaneously minimize the probability of oocyte damage due to osmotic shock and to so-

called solution effects (e.g., cryoprotectant toxicity). For a given set of process parameters $\mathbf{p} = \{p_1, p_2, p_3, \ldots\}$, the objective of the optimization algorithm was to search for the combination of values of the parameters **p** that would minimize a cost function $C(\mathbf{p})$ representing the probability of cell damage. To model solution-effects injury, the value of C was taken to be the cumulative (integrated) damage intensity:

$$C(\mathbf{p}) = \int_{t=0}^{t=t^*} \lambda dt$$

where λ is the instantaneous rate of damage accumulation; t, time; t^*, the total CPA loading time (i.e., the time required to reach the target intracellular CPA concentration). The target level of intracellular CPA was defined as 95% of the amount of CPA within an oocyte at isotonic volume, in which the osmotically active volume fraction contains CPA at a concentration of 1.5 M.

Following our previous approach [37], we have approximated the rate of damage accumulation (λ) as constant, so that the cost function is proportional to the total loading time, t^*; as a result, the optimization objective is to minimize the protocol duration. This is consistent with experimental observations of cell damage during cryopreservation, which indicate that the probability of damage attributable to solution effects (e.g., cryoprotectant toxicity) is strongly correlated with protocol duration [31; 36]. For purposes of comparison, we also considered an alternative cost function definition, in which the instantaneous rate of damage accumulation is assumed to have a power-law dependence on the molality of intracellular CPA [7].

For both cost function definitions, to avoid oocyte damage due to osmotic shock, any cell volume change greater than a critical threshold (conservatively defined as ±25% of the isotonic cell volume) were prohibited by adding a penalty value to the cost function [37].

To systematically search a multi-dimensional parameter-space **p** for the set of process parameter values that will minimize the cost function $C(\mathbf{p})$, the Nelder-Mead simplex algorithm

was used, as previously [37]. All computer-aided optimizations were performed using MATLAB® programs written by one of us (JOMK).

Preliminary simulations suggested that using three or more CPA loading steps did not significantly reduce the predicted cost, compared to two-step protocols (data not shown). Thus, we focused on designing two-step loading methods. To develop optimal two-step protocols, we allowed the optimization algorithm to adjust the CPA concentration in the first loading step, as well as the time of oocyte exposure to this initial loading solution. The second (and final) loading solution always contained 1.5 M of the CPA, while the exposure time to the final solution was taken as the time required to reach the target level of intracellular CPA. For both the first and second loading step, it was assumed that solutions would be prepared by mixing pure CPA with an isotonic (290 mOsmol/L) salt solution. However, to develop alternative approaches to CPA loading, we also performed separate optimizations in which the computer algorithm was allowed to adjust the extracellular salt concentration in the initial loading step (independently of the CPA concentration); nonetheless, because extracellular electrolytes are important for cell function, a lower bound of 50 mOsmol/L was imposed on the salt osmolarity.

Me_2SO Loading Experiments at Room Temperature

The Nelder-Mead simplex algorithm predicted an optimal two-step Me_2SO loading protocol consisting of the following steps: (1) exposure to 0.738 M Me_2SO for 7.1 min; and (2) equilibration in 1.500 M Me_2SO for 11.3 min. In addition to this optimized two-step loading method, the experimental groups for loading of Me_2SO at room temperature included non-optimized one-step loading, simplified two-step loading, and simplified two-step loading with a longer second step. Controls consisted of an untreated oocyte group as well as a group exposed to osmotic stress in the absence of CPA (see Supplementary Table 1 for details).

Non-optimized one-step loading was achieved by exposing the oocytes directly to 1.5 M Me_2SO for 15 min. The optimized two-step loading of 1.5 M Me_2SO was carried out as predicted

by the computer algorithm. Whereas the simplified two-step loading method (i.e., step #1: 0.75 M Me_2SO for 5 min and step #2: 1.5 M Me_2SO for 10 min) was testing a more practical version of the optimized two-step loading protocol, the objective of the last experimental group was to address the question of whether a longer exposure (i.e., 15 min, selected to match the exposure time in the one-step loading protocol) of oocytes to the final Me_2SO concentration (1.5 M) single-handedly changes the fertilization and development rates. To test the alternative possibility (i.e., alteration of the fertilization and development rates by solely osmotic stresses in the absence of chemical toxicity of Me_2SO), mouse oocytes in the osmotic stress control group were exposed to a 0.5-M galactose solution at 23°C for 15 min, a procedure that is expected to induce volume excursions similar in magnitude to those resulting from direct exposure to 1.5 M Me_2SO.

At the end of each final exposure time, the oocytes were transferred to successively decreasing concentrations (1.0 M, 0.5 M, and 0.0 M) of Me_2SO at ambient temperature with 5-min intervals, to reduce osmotic stresses during Me_2SO removal. Similarly, 0.5 M galactose was diluted by transferring oocytes to its successively decreasing concentrations (i.e., 0.3 M, 0.1 M, and 0.0 M) at ambient temperature with 5-min intervals.

Me_2SO Loading Experiments at Elevated Temperature

Next, we allowed our theoretical transport models to guide the development of alternative strategies for CPA addition, which were tested in experiments using mouse oocytes. The theoretical models indicate that the rates of permeation of water and Me_2SO across the cell membrane increase with increasing temperature, which suggests that the total exposure time can be reduced if CPA addition is carried out above room temperature. It is often assumed that the benefits of faster CPA loading at elevated temperatures would be negated by a concomitant acceleration of cytotoxicity kinetics. However, this assumption is rarely tested, and to our knowledge, there have been no previous attempts to increase temperature while also re-

263 optimizing the CPA loading procedure to account for the temperature-dependence of membrane
264 permeability parameters. Hence, we hypothesized that high survival, fertilizability and
265 developmental capacity can be achieved if CPA addition is performed slightly above room
266 temperature (30°C), provided that the CPA loading protocol is designed to minimize CPA
267 exposure and prevent excessive volume excursions.

268 The experiments involving loading of Me_2SO at 30°C were performed on a stage warmer
269 (MATS-U4020WF, Tokai Hit, Fujinomiya-shi, Japan) and the temperature of the Me_2SO
270 solutions was monitored using a thin (0.8 mm) thermocouple wire that was immersed into one of
271 the Me_2SO solutions in a 4-well dish. The temperature fluctuation was $\pm$0.4°C. Initially, all
272 Me_2SO solutions were prepared by dilution using isotonic PBS. The CPA loading protocol that
273 was predicted by the Nelder-Mead simplex algorithm and experimentally tested in this series of
274 experiments had two steps (i.e., step #1: 0.976 M Me_2SO for 1.7 min; and step #2: 1.500 M
275 Me_2SO for 5.4 min) and was compared to the non-optimized one-step loading protocol
276 consisting of 6.5-min exposure to 1.5 M Me_2SO. Untreated oocytes were used as controls. After
277 the final exposure periods, the removal of Me_2SO was carried out in a step-wise manner at
278 room temperature as described earlier.

279 The Nelder-Mead simplex algorithm also predicted a CPA loading approach that
280 prescribed the use of hypotonic salt buffers to dilute the Me_2SO. The computer-generated
281 protocol consisted of two-steps (i.e., step #1: 1.404 M Me_2SO with 50 mOsmol/L salt for 2.4 min;
282 and step #2: 1.500 M Me_2SO in isotonic saline for 8 s). To implement the first step of the
283 predicted protocol, the Me_2SO solution was prepared using one of two hypotonic diluent buffers
284 ("hypo-PBS" or "hypo-NaCl", see above), for testing of the effect of salt composition in
285 combination with the hypotonic condition. Furthermore, to examine the effect of buffer
286 supplements (macromolecule vs. salts+proteins+others), we added either BSA or FBS to the
287 hypo-NaCl buffer, whereas the hypo-PBS buffer was supplemented with FBS only. The step-

wise removal of 1.5 M Me_2SO was achieved by exposing the oocytes to decreasing concentrations (1.0 M, 0.5 M, and 0.0 M) of Me_2SO at ambient temperature as described earlier; however, the diluting solutions also contained 0.25 M sucrose this time, because in our preliminary experiments, the CPA removal in the absence of sucrose gave poor fertilization results following the loading of Me_2SO by using the hypotonic diluent buffers.

Partial Dissection of Zona Pellucida

To test for the possibility of hardening of the zona pellucida (ZP) in oocytes that had undergone one-step Me_2SO loading, a subset of such oocytes were subjected to partial dissection of the ZP, as described previously [30; 51]. Briefly, to attain sufficiently large perivitelline space, M II oocytes that had undergone one-step Me_2SO loading and subsequent Me_2SO elution were transferred to a 80-µl drop of 0.3 M sucrose in BSA-free PBS covered with mineral oil. Once the oocytes were equilibrated with the sucrose solution and attached to the culture dish, a 30-gauge sterile needle (BD, Franklin Lakes, NJ) was used to make a slit in the ZP of each oocyte under a stereomicroscope. Thereafter, 20 µl of 0.3 M sucrose solution containing 4% BSA was added to the dissection drop to detach the oocytes, after which the oocytes with partially dissected ZP were washed in PBS containing 0.4% BSA.

Recovery and Viability Assessment

After loading and removal of 1.5 M Me_2SO (and zona slitting, if applicable), the oocytes were rinsed in a fresh drop of Hepes-buffered Hypermedium plus BSA before being transferred to the culture medium for a recovery period of 40–60 min. Following the recovery period at 37ºC, the post-treatment survival was assessed using morphological criteria (i.e., translucent appearance of cytoplasm, integrity of the plasma membrane and the ZP, and the size of the perivitelline space) and the rate of the survival was calculated based on the number of oocytes

exposed to Me_2SO solutions. Subsequently, both treated and control oocytes were inseminated as descibed next to evaluate fertilization and embryonic development.

In Vitro Fertilization and Embryo Culture

In vitro fertilization (IVF) and culture of inseminated oocytes were carried out in Hypermedium at 37°C under a humidified atmosphere of 5% CO_2 in air as described previously [20]. Cleavage to the two-cell stage was examined after overnight culture while development to the blastocyst stage was evaluated after 5 days of culture. Fertilization and blastocyst rates were calculated based on the number of surviving and fertilized oocytes, respectively.

Statistical Analysis

Experiments in each series were repeated at least three times. Data reported are means of experimental repeats involving survival, fertilization, and development rates with error bars representing standard error of mean (SEM). The data were analyzed by ANOVA /Tukey's multiple comparison test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA) except the experiments involving exposure to 0.5 M galactose and zona slitting, which were analyzed by Fisher's exact test. Before ANOVA, arcsine transformation was performed on proportional data. Differences between the groups were considered statistically significant when the p-value was less than 0.05.

RESULTS

Optimization of Me_2SO Loading into Mouse Oocytes at Room Temperature

As explained previously, the Nelder-Mead simplex algorithm generated an optimal two-step Me_2SO loading protocol (See Materials and Methods). The predicted intracellular CPA concentrations and normalized volume excursions for this protocol are shown in Fig. 1A and 1B,

along with simulations of a conventional one-step loading protocol. The non-optimized one-step loading protocol is predicted to take 14.3 min to reach the target intracellular Me_2SO concentration, but results in a 40% volume excursion. In contrast, the optimized two-step loading procedure requires 18.5 min but only results in a 25% volume excursion.

Next, we experimentally tested the optimized and non-optimized Me_2SO loading methods. A total of 401 oocytes were used in this set of experiments, which were repeated more than three times each. As shown in Fig. 1C, although neither Me_2SO loading method had adverse effects on oocyte survival, there was significant sublethal damage in oocytes subjected to non-optimized one-step loading of Me_2SO. In particular, fertilization was significantly reduced after one-step Me_2SO loading in comparison to untreated controls (34% vs. 95%, $p<0.0001$). Furthermore, the one-step loading method significantly lowered the embryonic development (i.e., blastocyst formation) rate (60%) compared to the control group (94%, $p<0.0005$). Thus, the net blastocyst yield per Me_2SO-loaded oocyte in the non-optimized group was only 20% (compared to 89% for untreated controls). In contrast to the poor result with one-step loading, the fertilization rate after using the mathematically optimized two-step loading method (85%) was significantly higher than after one-step loading, and similar to that of untreated controls. The optimized two-step loading method also resulted in an improved embryonic development rate (87%), which was comparable to that of untreated controls.

To explore possible reasons for the differences in fertilization and embryonic development rates described above, we performed an additional set of experiments, the results of which are shown in Fig. 1D. The first experimental group was subjected to a simplified version of the mathematically optimized protocol in which the computer-generated values of the process parameter were rounded. Computer simulations indicated that this simplified protocol would result in a maximum volume excursion of 28%, and result in a final intracellular Me_2SO concentration of 1.47 M. To dissect the role of chemical CPA toxicity vs. CPA-induced volume

359 excursions in poor fertilization and embryonic development, we extended the 2nd step of the
360 simplified two-step protocol to 15 min similar to the one-step protocol and exposed oocytes to
361 0.5 M galactose in the second and third group, respectively. Each treatment and control
362 experiment was repeated more than three times, and a total of 496 oocytes were used for this
363 set of experiments. As shown in Fig. 1D, none of the tested CPA loading protocols induced
364 oocyte degeneration. Moreover, the simplified version of the optimized two-step loading yielded
365 fertilization (89%) and embryonic development (92%) rates similar to those of the control group,
366 indicating that the our minor modifications of the computer-generated protocol did not adversely
367 affect outcome. When subjecting oocytes to a 15-min exposure to 1.5 M Me_2SO in the second
368 step of the simplified two-step method, a slight decrease of the rates of fertilization (80%) and
369 embryonic development (88%) were observed compared to controls; however, these differences
370 were not statistically significant. This indicates that the Me_2SO chemical toxicity associated with
371 a 15-min exposure at 1.5 M is not sufficient to explain the sublethal oocyte damage manifesting
372 in the non-optimized loading group. Interestingly, the osmotic stress control group (exposed to
373 0.5 M galactose in the absence of Me_2SO) also did not significantly lower the rates of
374 fertilization or embryonic development (95% and 88%, respectively) compared to untreated
375 controls, even though the maximum volumetric excursion in this group was at least as large as
376 in the non-optimized loading group; i.e., osmotic shock alone is not sufficient to explain the
377 reduced function of oocytes subjected to one-step loading. Taken together, these results
378 suggest that the decreased fertilization and development rates after non-optimized one-step
379 CPA loading are due to an interaction between chemical and osmotic effects of the Me_2SO.

380 Based on our recent study [69], we reasoned that the significantly lower fertilization rate
381 caused by one-step Me_2SO loading might be related to zona hardening. To test this possibility,
382 we partially dissected the ZP of oocytes that had undergone one-step Me_2SO loading; these ZP-
383 dissected oocytes were inseminated along with untreated controls. A total of 102 oocytes were

used in this set of experiments, of which 6 degenerated during the dissection process. As hypothesized, the partial ZP-dissection greatly improved the fertilization rate (77%) compared to that of oocytes that were not ZP-dissected after one-step Me_2SO loading (34%), although the former was still lower than the fertilizability of the untreated controls (99%). The blastocyst formation rates in the ZP-dissection (92%) and control (97%) groups were similar, and these values were higher than the developmental capacity observed in non-dissected oocytes (60%). Overall, these results suggest that zona hardening is part of the mechanism by which non-optimized one-step loading of 1.5 M Me_2SO adversely affects the fertilization rate.

Development and Testing of Alternative Strategies for Me_2SO Loading

To optimize Me_2SO loading at 30ºC, we again used the Nelder-Mead simplex algorithm to systematically search for conditions that result in the fastest CPA loading without exceeding 25% volume excursions, thus mitigating deleterious osmotic shock and potential CPA toxicity. We considered two different optimization approaches that differed in their assumptions about the salt concentration in the first loading step. Our initial approach assumed that all Me_2SO solutions would be prepared using an isotonic salt buffer as the diluent solution; this is the same assumption that was used to develop room-temperature protocols for Me_2SO addition. The resulting computer-optimized two-step loading protocol (which allows loading of 1.5 M Me_2SO in 7.1 min without exceeding 25% volume excursion) was experimentally compared to the non-optimized one-step addition of Me_2SO, which requires 6.5 min for loading and is predicted to result in a 34% volume excursion, as shown in Fig. 2A and 2B. Untreated oocytes served as controls. A total of 241 oocytes were used for these experiments, and the results are summarized in Fig. 2C. None of the tested Me_2SO loading protocols caused degeneration of oocytes. However, only a few oocytes (8%) were fertilized when the non-optimized one-step loading protocol was used. In contrast, the optimized two-step loading protocol yielded a significantly higher fertilization rate of 86% ($p<0.0001$) similar to that of untreated controls

409 (96%). The blastocyst formation rates were over 89% and not significantly different between the
410 groups.

411 To remove unnecessary constraints on the search for optimal loading procedures, we
412 repeated the computer-aided optimization after relaxing the assumption that Me_2SO solutions
413 should be prepared using isotonic saline as the diluent. In particular, we used the sequential
414 simplex method to optimize a two-step Me_2SO loading protocol, this time allowing the
415 optimization algorithm to simultaneously adjust three process parameters, corresponding to the
416 concentration of salt in the first loading solution, the Me_2SO concentration in the first solution,
417 and the exposure time to the first solution. The only restriction imposed on the optimized
418 solution composition was that the saline osmolarity should be at least 50 mOsmol/L (because
419 pilot experiments indicated that some minimal presence of electrolytes was required to maintain
420 homeostasis during CPA addition). Using this approach, our computer simulations identified the
421 following general strategy for CPA addition: in the first exposure step, the solution should be
422 hypotonic, with a minimal salt content. Specifically, for two-step addition of Me_2SO, the optimal
423 saline osmolarity in the first step was predicted to be 50 mOsmol/L, equal to the imposed lower
424 bound.

425 As shown in Fig. 3A, this alternative CPA loading strategy (using a hypotonic diluent buffer
426 in the first step) yielded an optimized protocol for mouse oocytes that allows loading of 1.5 M
427 Me_2SO within a remarkably short time period of 2.5 min. We performed experimental tests to
428 evaluate this CPA loading protocol with respect to untreated controls. Moreover, to further refine
429 the efficacy of the new loading protocol, we also compared the effect of salt composition (NaCl
430 vs. PBS) and the effect of supplementation of the hypotonic NaCl solution with BSA or FBS. The
431 results of these experiments (which were repeated more than three times using a total of 616
432 oocytes) are summarized in Fig 3B. None of the loading protocols induced cell degeneration.
433 The loading of 1.5 M Me_2SO using the optimized two-step protocol with hypo-PBS+FBS

resulted in fertilization (92%) and blastocyst (88%) rates similar to those (96% and 93%, respectively) of untreated controls. The fertilization and blastocyst rates were also high in the two-step hypo-NaCl+FBS (85% and 86%, respectively) and two-step hypo-NaCl+BSA (85% and 82%, respectively) groups, but significantly lower compared to those in the control group ($p<0.01$). There was no significant difference between the experimental groups in terms of fertilization and embryonic development. Taken together, these results suggest that (i) the duration of CPA loading can be significantly shortened without adversely affecting fertilization and embryonic development when a hypotonic diluent buffer is used, and (ii) the presence of a balanced salt composition in the diluent buffer is beneficial during CPA loading.

Optimization of PROH Loading into Human Oocytes

Currently, PROH is the most commonly used CPA for slow cooling cryopreservation of human oocytes [9; 11; 59]. Encouraged by our success with mouse oocytes, we also used our mathematical optimization algorithm to predict procedures for loading of 1.5 M PROH into human oocytes. Fig. 4 shows predictions for the optimized two-step loading at 24°C, along with predictions for a conventional one-step loading method. In the non-optimized single-step protocol, 14.5 min is required for the intracellular PROH content to reach its target value, and the maximum cell volume excursion is 36%, exceeding the critical threshold value. In contrast, the optimized two-step protocol requires only 6 min to reach the target intracellular PROH level and the maximum volume excursion is 25%. These reductions in protocol duration and volume excursions would be expected to reduce damage due to chemical toxicity and osmotic stresses, respectively. The optimized protocol specifies an initial loading solution that contains 1.3 M PROH and 50 mOsmol/L salt. After a 5.5 min exposure to this hypotonic loading solution, the oocytes only require a 29 s exposure to the final CPA solution (1.5 M PROH prepared using isotonic buffer) before the target level of intracellular PROH is achieved.

458 We also used model predictions to evaluate PROH loading into human oocytes at 30°C,
459 as shown in Fig. 5. A conventional one-step loading method at 30°C is predicted to result in a
460 34% volume excursion (Fig. 5 dash-dotted lines), and to require a total of 3.5 min CPA exposure
461 time to reach the target intracellular PROH content. In contrast, the optimized two-step protocol
462 (Fig. 5, solid lines) is significantly faster (1.4 min) than the one-step method (which is expected
463 to reduce toxicity), while avoiding cell volume excursions in excess of ±25% (which is expected
464 to reduce osmotic damage). The optimized protocol specifies an initial loading solution that
465 contains 1.4 M PROH and 50 mOsmol/L salt. After a 1.3-min exposure to this hypotonic loading
466 solution, the oocytes only require a 4-s exposure to the final CPA solution (1.5 M PROH
467 prepared using isotonic buffer) before the target level of intracellular PROH is achieved. In
468 comparison to the loading methods at 24°C, PROH loading at 30°C is more than fourfold faster,
469 which is expected to reduce damage associated with CPA toxicity.

470 One of us (AZH) has proposed an alternative optimization approach in which the rate of
471 damage accumulation was assumed to have a power-law dependence on the CPA molality [7].
472 For purposes of comparison with the present algorithm (in which the concentration-dependence
473 of damage accumulation was approximated as negligible), the results of optimization using a
474 concentration-dependent cost function are also shown in Fig. 5 (dashed lines). The protocol
475 optimized using this alternative algorithm specifies an initial loading solution that contains 0.88
476 M PROH and 50 mOsmol/L salt. After a 1.5 min (89 s) exposure to this hypotonic loading
477 solution, the oocytes only require a 15-s exposure to the final CPA solution (1.5 M PROH
478 prepared using isotonic buffer) before the target level of intracellular PROH is achieved. Thus,
479 the total duration of the protocol is 1.7 min (103 s). This protocol is slightly longer than the
480 design generated by the original cost function (1.7 min vs. 1.4 min) but offers the potential
481 advantage of a lower PROH concentration in the first loading step (0.88 M vs. 1.4 M).

DISCUSSION

Our strategy for minimizing oocyte damage is based on computer-aided design of CPA addition protocols that are predicted to simultaneously reduce chemical cytotoxicity and osmotic shock. Whereas toxicity was minimized by decreasing exposure times as much as possible, osmotic stress was prevented by limiting the magnitude of cell volume changes during CPA loading. Our results are consistent with previous studies which show that multi-step CPA loading methods designed to avoid osmotic damage are superior to single-step loading methods [14; 23; 54]. However, our approach has the additional benefit of minimizing the duration of exposure to potentially toxic CPAs.

To identify the osmotic tolerance of oocytes, researchers have measured cell viability and function after exposure to hypertonic solutions containing saccharides (e.g., galactose and sucrose) in increasing concentrations [47; 72]. In such experiments, mature human oocytes were found to tolerate shrinkage by up to ~60% of the isotonic cell volume [47], while a study on in vitro matured human oocytes concluded that osmotic insults causing volume reductions up to ~70% were tolerated [72]. These results are consistent with our present finding that M II mouse oocytes tolerate exposure to 0.5 M galactose (yielding cell shrinkage >40%).

Although oocytes appear to tolerate osmotic stresses reasonably well in the absence CPAs, it is important to estimate osmotic tolerance limits of oocytes in the presence of CPA, due to the potential for interaction between chemical toxicity effects and osmotic shock. Songsasen et al. performed such a study by exposing rhesus monkey M II oocytes to EG solutions of increasing molar concentrations for 5 or 10 min, which was followed by a single-step dilution in an isotonic medium [67]. Analyzing the membrane integrity data of Songsasen et al. in the context of our mathematical model of coupled water- and CPA-transport, we estimated that rhesus monkey oocytes can tolerate volume excursions up to ~50% [37]. While the post-exposure survival results in the present study (i.e., no oocyte degeneration after one-step

507 loading of 1.5 M Me_2SO involving ~40% volume excursion) are in agreement with the findings of
508 Songsasen et al., we found that the fertilization and blastocyst rates were adversely affected by
509 one-step loading of 1.5 M Me_2SO, suggesting that the membrane integrity as a sole criterion is
510 not sufficient to determine the sensitivity of oocytes to osmotic stresses and CPA toxicity. The
511 potential for osmotic stresses to cause sublethal injuries affecting oocyte function has previously
512 been documented [1; 47; 49]. Some studies have suggested that cell volume excursions in
513 excess of ±30% should be avoided when loading CPAs into human oocytes [52; 58; 59].
514 Considering the variability in oocyte quality [67; 72] and difference in toxicity of various CPAs
515 [68], in the present study, we implemented an additional safety margin and limited volume
516 excursions of oocytes to ±25% when optimizing CPA loading protocols. It should be noted that
517 the simplified version of our two-step protocol for room-temperature loading exceeded our
518 preset cell volume constraints and resulted in a volume excursion of 28%, but still delivered
519 excellent results similar to controls, suggesting that mouse oocytes can tolerate volume
520 excursions beyond ±25% in the presence of Me_2SO. In contrast, non-optimized one-step
521 Me_2SO loading at 30ºC, which induces a 34% volume excursion, resulted in significantly lower
522 fertilization rates. Taken together, these results suggest that it might be a good idea to avoid
523 volume excursions beyond 30% in the presence of potentially toxic CPAs, although the exact
524 value of the osmotic tolerance limit is expected to vary depending on species, type of CPA,
525 composition of cryopreservation medium, and loading temperature.

526 In the present study, the primary deleterious effect of the non-optimized Me_2SO loading
527 was on fertilization. It is important to note that 1.5 M Me_2SO solution was prepared in PBS
528 supplemented with BSA (i.e., in the absence of any appreciable Ca^{2+} and Mg^{2+}). In our previous
529 study, when mouse M II oocytes were exposed to 1.5 M Me_2SO prepared in PBS containing
530 10% FBS and thus small amounts of extracellular Ca^{2+} and Mg^{2+}, no significant adverse effect
531 was observed [68]. We have performed similar experiments with PROH (i.e., exposure of

mouse oocytes to 1.5 M PROH in the presence and absence of extracellular Ca^{2+}/Mg^{2+}) and obtained similar results in our recent study, suggesting that extracellular presence/absence of Ca^{2+}/Mg^{2+} modulates cell injury induced by CPA loading [69]. Additional experiments in that study indicated that non-optimized loading of 1.5 M PROH perturbs intracellular Ca^{2+} homeostasis, leading to various injuries including fertilization failure. It has been shown that exposure to penetrating CPAs (including Me_2SO, PROH and EG) causes a transient rise in intracellular Ca^{2+}, which in turn induces premature cortical granule exocytosis [42; 43]. It is also known that premature cortical granule exocytosis in response to CPA exposure may result in hardening of the ZP, leading to fertilization failure [12; 24; 35; 43; 64; 74] and necessitating the use of work-around strategies such as ICSI. By showing a significant improvement in the fertilization rate of mouse oocytes that underwent zona slitting after one-step Me_2SO loading, the present results lend further support to a mechanism of CPA-induced cell injury that is triggered by perturbation of intracellular Ca^{2+} homeostasis. Significantly, all previous studies of this mechanism used non-optimized protocols when exposing oocytes to the penetrating CPAs. In contrast, the present experiments demonstrated that when osmotic stress was constrained (by limiting volume excursions to ≤25% in the optimized protocol, and ≤28% in the simplified versions), Me_2SO exposure did not significantly reduce fertilization rate in mouse oocytes. The absence of deleterious sequelae of Me_2SO exposure was also confirmed when the total exposure time to 1.5 M Me_2SO was increased to 15 min (equal to the duration of the non-optimized one-step protocol). These results indicate that the zona hardening mechanism described above is not caused by Me_2SO chemical toxicity alone, but due to a combined effect of Me_2SO exposure and osmotic stress. Therefore, the use of rigorous optimization methods to design CPA addition protocols that simultaneously minimize CPA toxicity and osmotic shock may reduce the need for ICSI after oocyte cryopreservation.

556 It is well known that the toxicity of penetrating CPAs is more pronounced at high
557 temperatures [68]. Our results are consistent with this general trend in that non-optimized
558 Me_2SO loading resulted in substantially lower fertilization rates at 30°C than at room
559 temperature. Because of this temperature-dependence of toxicity, it is commonly argued that
560 improved results can be obtained by loading CPA at a hypothermic temperature, typically at
561 room temperature or below [21; 61; 62]. However, CPA loading takes longer at reduced
562 temperatures due to decreased membrane permeability [57; 72]; the extended exposure to CPA
563 in the loading solution may, paradoxically, increase damage caused by chemical cytotoxicity.
564 Alternatively, it might be possible to minimize the toxicity effects by loading penetrating CPAs at
565 modestly elevated temperatures, thus minimizing the duration of CPA exposure [26]. This
566 approach offers additional benefits in terms of avoiding chilling injury. In the present study, we
567 developed CPA loading methods at 30°C to realize the benefits mentioned above. Indeed, our
568 two-step optimized protocol at 30°C achieved loading of 1.5 M Me_2SO in 7.1 min and delivered
569 survival, fertilization and blastocyst rates similar to those of controls. This positive result
570 indicates that mathematically optimized protocols may help safe loading of CPAs at elevated
571 temperatures by controlling volume excursions and reducing the total duration of CPA exposure.

572 Typically, CPA solutions are prepared by adding CPA to isotonic solutions or media.
573 Consequently, we initially constrained our mathematical optimization algorithm to consider only
574 loading solution compositions corresponding to mixtures of neat CPA and isotonic saline. Mullen
575 et al. [50] have suggested that a reduced-osmolality diluent (~175 mOsmol/kg) may be
576 beneficial during CPA loading, but they did not attempt to optimize the time of exposure. In a
577 previous study [37] we have shown that mathematically optimized CPA removal procedures can
578 be improved by allowing the computer algorithm to vary both the concentration of CPA and the
579 concentration of non-permeating solute. Thus, in the current study we also explored the
580 possibility of obtaining improved results by allowing the optimization algorithm to vary the non-

permeating solute concentration (in addition to the CPA concentration and exposure time). This optimization approach produced a CPA loading strategy based on preparation of CPA solutions in a hypotonic buffer (50 mOsmol/L, the lowest salt osmolarity permitted by the optimization algorithm constraints). The resulting computer-generated protocol was predicted to yield remarkably rapid loading of 1.5 M Me_2SO at 30°C within 2.5 min (almost three times faster than one-step loading at this temperature), without exceeding critical volume excursion thresholds. Experimental tests of this prediction confirmed that survival, fertilization and blastocyst rates for oocytes loaded using this 2.5-min protocol were similar to those of untreated controls, when the diluent solution consisted of hypotonic PBS. Interestingly, when the hypotonic diluent was prepared using NaCl, the rates of fertilization and embryonic development were slightly reduced compared to controls (albeit still satisfactory); this demonstrates the importance of an appropriate salt composition during CPA loading. Encouraged by the success of this approach with mouse oocytes, we also used our computer algorithms to predict optimal protocols for loading of 1.5 M PROH into human oocytes. Our simulations showed that it should be possible to introduce the CPA into the oocytes in <90 s, by using the hypotonic diluent approach at 30°C. Significantly, Ca^{2+}-mediated oocyte damage induced by PROH loading has been shown to be time-dependent, with >2 min exposure required for manifestation of injury [42]. Thus, the extremely rapid CPA addition protocols made possible by the hypotonic diluent strategy may prevent such time-dependent mechanisms of oocyte damage. Our experiments with mouse oocytes indicate that transient volume increases up to 25% during CPA loading using a hypotonic diluent have no negative impact on subsequent survival, fertilization and embryonic development.

Whereas in this study we have focused on loading of CPA at relatively low concentrations (for use in slow-cooling cryopreservation methods), it is important to point out that CPA toxicity is known to be more prominent at high concentrations (such as those required for vitrification);

indeed, the need to overcome toxicity damage is a well-recognized challenge in the design of vitrification procedures [3; 22; 29; 44]. Thus, our present optimization approach, which is able to simultaneously reduce toxicity and osmotic stress, has the potential to result in significantly improved vitrification methods. It is likely that the use of more sophisticated cost functions, which take into account the concentration-dependence of the rate of damage accumulation [7], will prove beneficial when adapting our approach for vitrification applications.

In conclusion, our results show that inappropriate loading of moderate concentrations of penetrating CPAs may induce significant cell injury through a combined action of chemical toxicity and osmotic perturbations. Such cell injuries can be mitigated by efficiently optimizing CPA loading protocols using physics-based computer simulations, as demonstrated in the present study. In particular, we showed that exceptionally fast CPA loading can be achieved by preparing the CPA loading solution using a hypotonic balanced salt buffer. Such rapid CPA loading processes may significantly improve the cryopreservation outcome of mammalian oocytes by minimizing exposure to potentially toxic CPAs while simultaneously limiting volume excursions.

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FIGURE LEGENDS

Figure 1. Optimization of Me_2SO loading at room temperature (23°C). Simulated intracellular CPA concentration (A) and normalized cell volume (B) for addition of 1.5 M Me_2SO to mouse M II oocytes. These simulations used an isotonic buffer for preparation of the Me_2SO solution. Results are shown for the predicted optimal two-step loading protocol (solid lines), as well as a non-optimized one-step loading protocol (dashed lines). Symbols mark the time at which the target intracellular CPA level has been reached. The critical values of the cell volume excursion (±25%) are demarcated by dotted reference lines (B). Survival, fertilization, and development of mouse oocytes after addition and removal of 1.5 M Me_2SO using non-optimized one-step loading and optimized two-step loading protocols (C), and two simplified versions of the optimized protocol (D), as well as after exposure to 0.5 M galactose (D). Data represent mean±SEM. Bars with different letters are significantly different ($p<0.05$).

Figure 2. Optimization of Me_2SO loading at 30°C. Simulated intracellular CPA concentration (A) and normalized cell volume (B) for addition of 1.5 M Me_2SO to mouse M II oocytes. These simulations used an isotonic buffer for preparation of the Me_2SO solution. Results are shown for the predicted optimal two-step loading protocol (solid lines), as well as a non-optimized one-step loading protocol (dashed lines). Symbols mark the time at which the target intracellular CPA level has been reached. The critical values of the cell volume excursion (±25%) are demarcated by dotted reference lines (B). (C) Survival, fertilization, and development of mouse oocytes after addition and removal of 1.5 M Me_2SO using the non-optimized one-step loading and optimized two-step loading protocols. Data represent mean±SEM. Bars with different letters are significantly different ($p<0.05$).

Figure 3. Optimization of Me_2SO loading at 30°C using a hypotonic diluent buffer. (A) Simulated intracellular CPA concentration (dashed line) and normalized cell volume (solid line)

for a novel two-step method for addition of 1.5 M Me_2SO to mouse M II oocytes, requiring only 2.5 min of Me_2SO exposure. Symbols mark the time at which the target intracellular CPA level has been reached. (B) Survival, fertilization, and embryonic development of mouse oocytes after addition and removal of 1.5 M Me_2SO that had been prepared using hypotonic diluent. Data represent mean±SEM. Bars with different letters are significantly different ($p<0.05$).

Figure 4. Prediction of an optimized PROH loading procedure for human oocytes at 24°C using a hypotonic diluent buffer. Simulated intracellular CPA concentration (A) and normalized cell volume (B) for addition of 1.5 M PROH to human M II oocytes at room temperature (24°C). Results are shown for the predicted optimal two-step loading protocol (solid lines), as well as a conventional single-step loading protocol (dashed lines). The critical values of the cell volume excursion (±25%) are demarcated by dotted reference lines (B). Symbols mark the time at which the target intracellular CPA concentration has been reached. The computer generated optimal two-step protocol specifies the use of a hypotonic diluent buffer for the initial CPA solution (see text for details).

Figure 5. Prediction of optimized PROH loading procedures for human oocytes at 30°C. Simulated intracellular CPA concentration (A) and normalized cell volume (B) for addition of 1.5 M PROH to human oocytes at 30°C using either a conventional one-step loading protocol (dash-dotted lines) or an optimized two-step method (solid lines). The critical values of the cell volume excursion (±25%) are demarcated by dotted reference lines (B). For comparison, the results using an alternative optimization algorithm (Benson et al., 2012) are also shown (dashed lines). Symbols mark the time at which the target intracellular CPA level has been reached (not shown for one-step loading). For both optimization methods, the computer-generated loading

protocols specified the use of a hypotonic diluent buffer for the initial CPA exposure (see text for details).

Figures 1AB

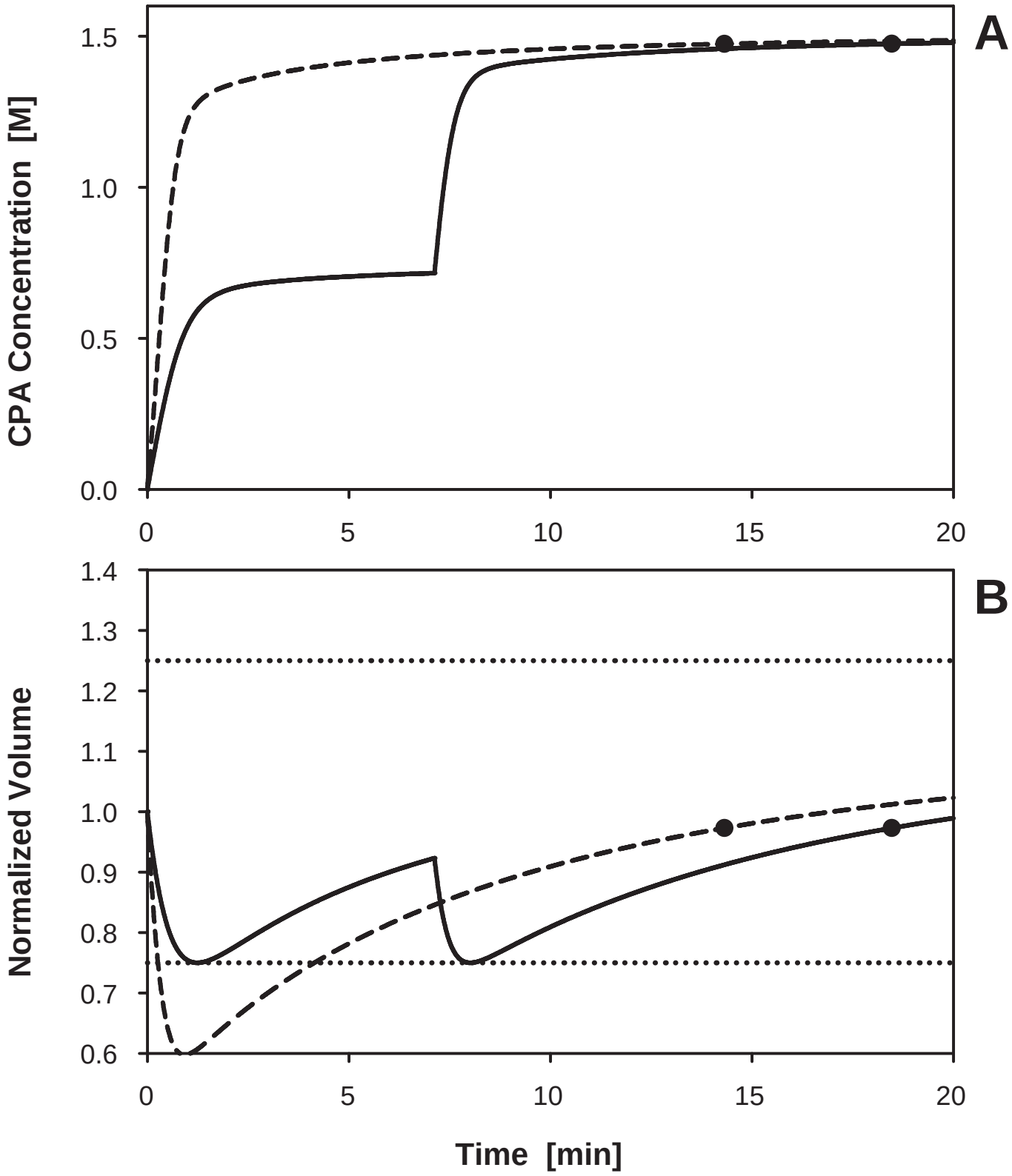

A
CPA Concentration [M]
1.5
1.0
0.5
0.0
0
5
10
15
20
B
Normalized Volume
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0
5
10
15
20
Time [min]

Figure 1C

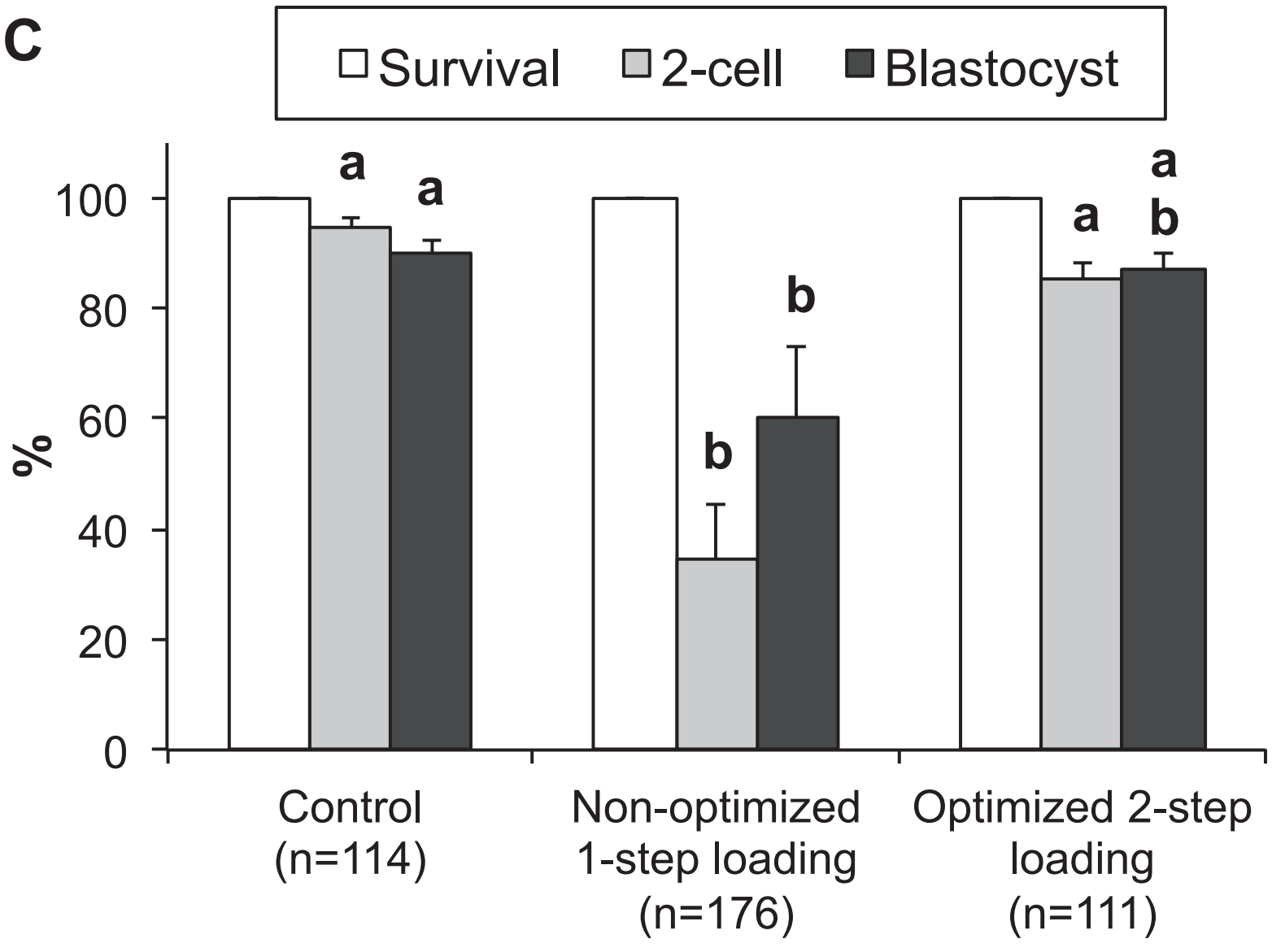
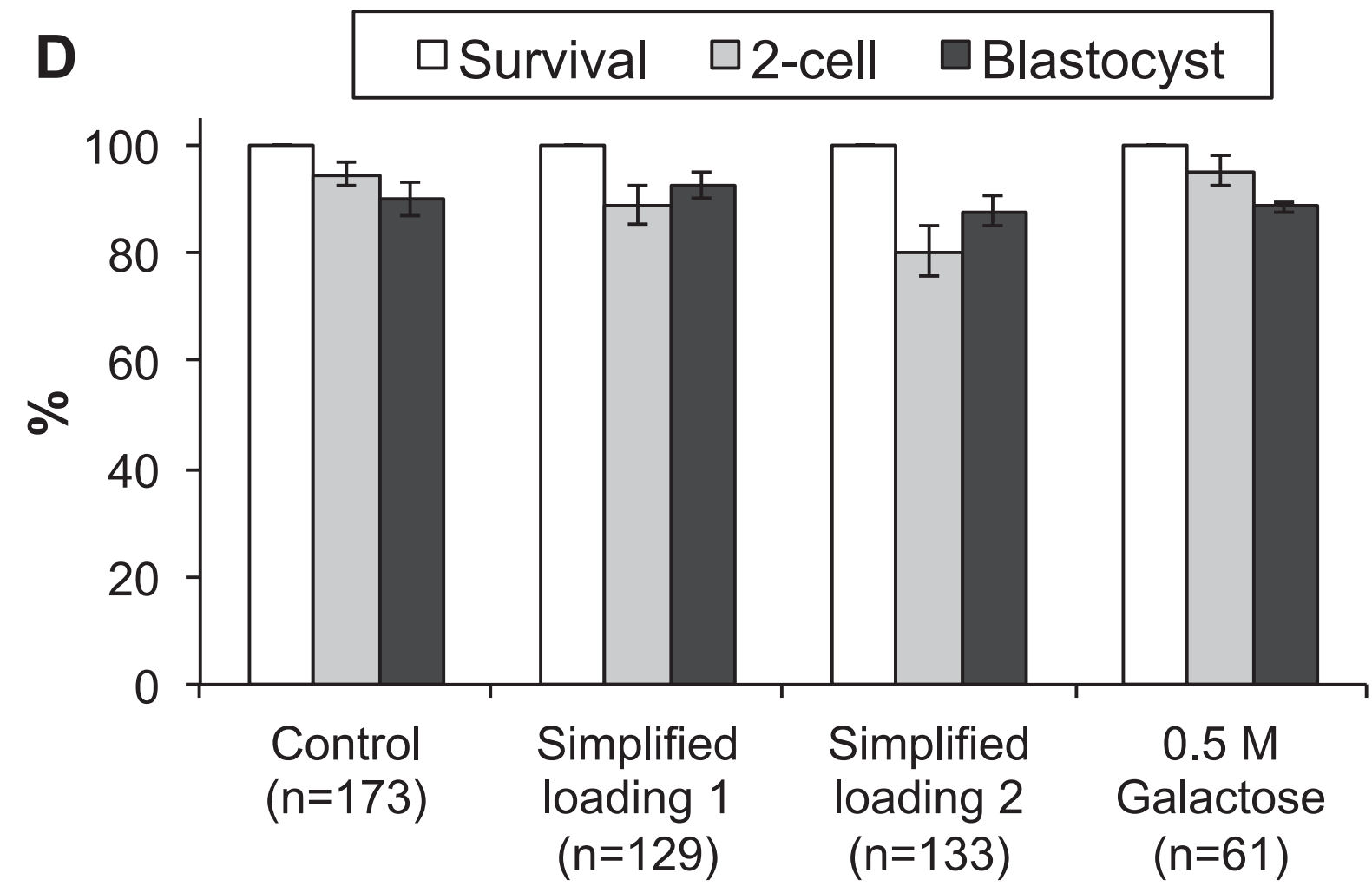

C
Survival
2-cell
Blastocyst
%
100
80
60
40
20
0
a
a
b
b
a
b
a
b
Control
(n=114)
Non-optimized
1-step loading
(n=176)
Optimized 2-step
loading
(n=111)

Figure 1D


D
Survival
2-cell
Blastocyst
100
80
60
40
20
0
%
Control (n=173)
Simplified loading 1 (n=129)
Simplified loading 2 (n=133)
0.5 M Galactose (n=61)

Figures 2AB

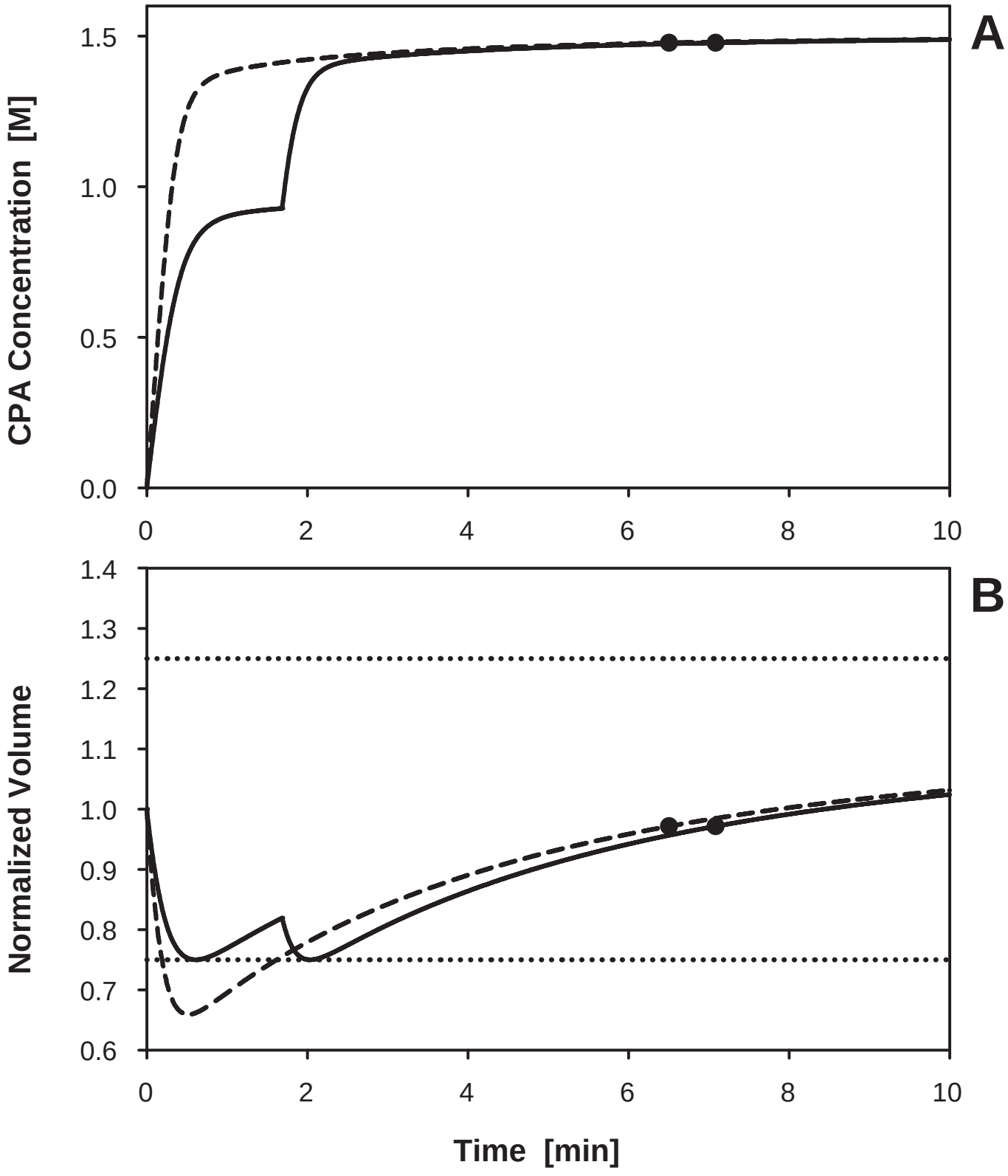

A
CPA Concentration [M]
1.5
1.0
0.5
0.0
0
2
4
6
8
10
B
Normalized Volume
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0
2
4
6
8
10
Time [min]

Figure 2C

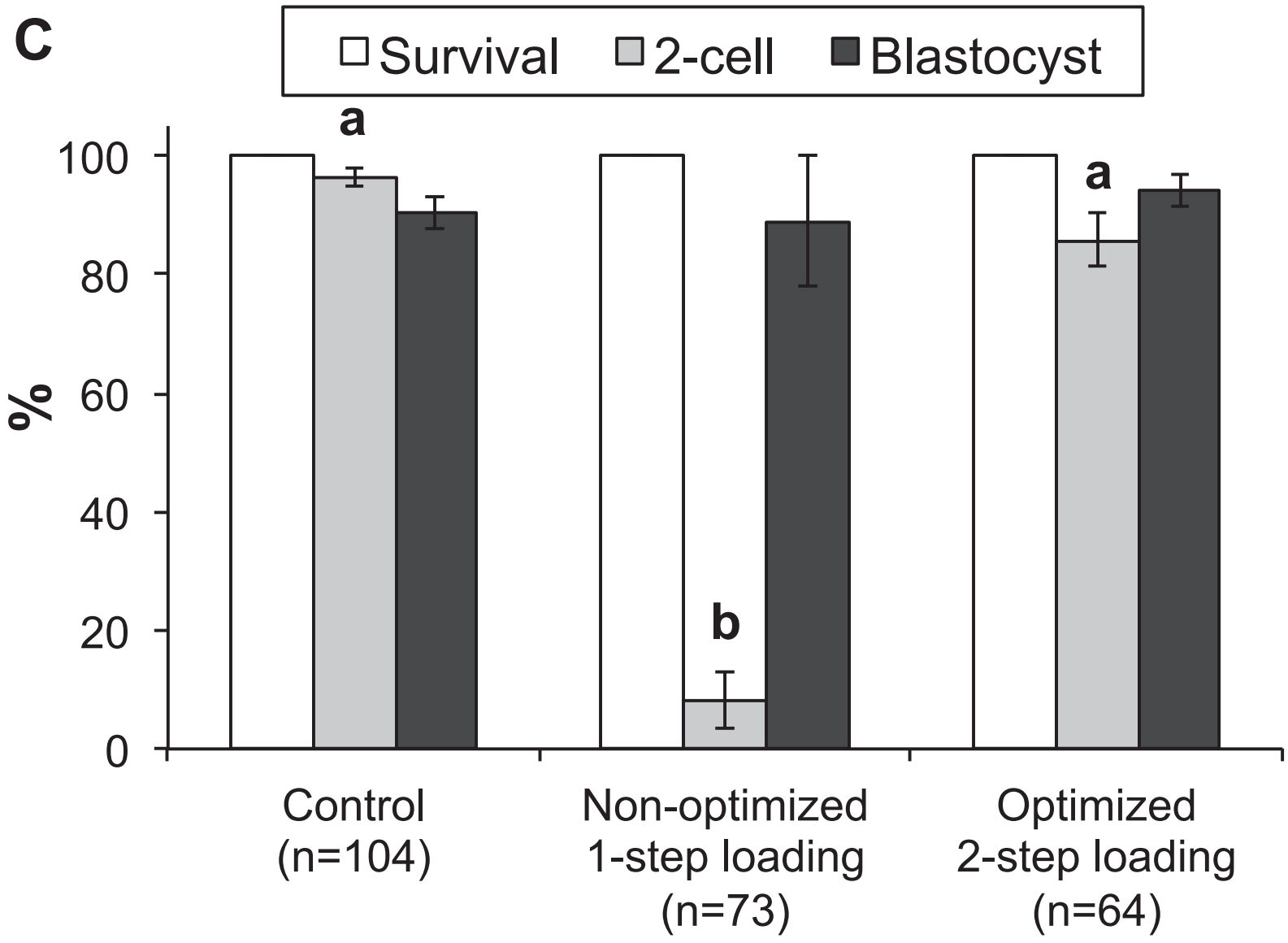

C
Survival
2-cell
Blastocyst
a
b
a
100
80
60
40
20
0
%
Control
(n=104)
Non-optimized
1-step loading
(n=73)
Optimized
2-step loading
(n=64)

Figure 3A

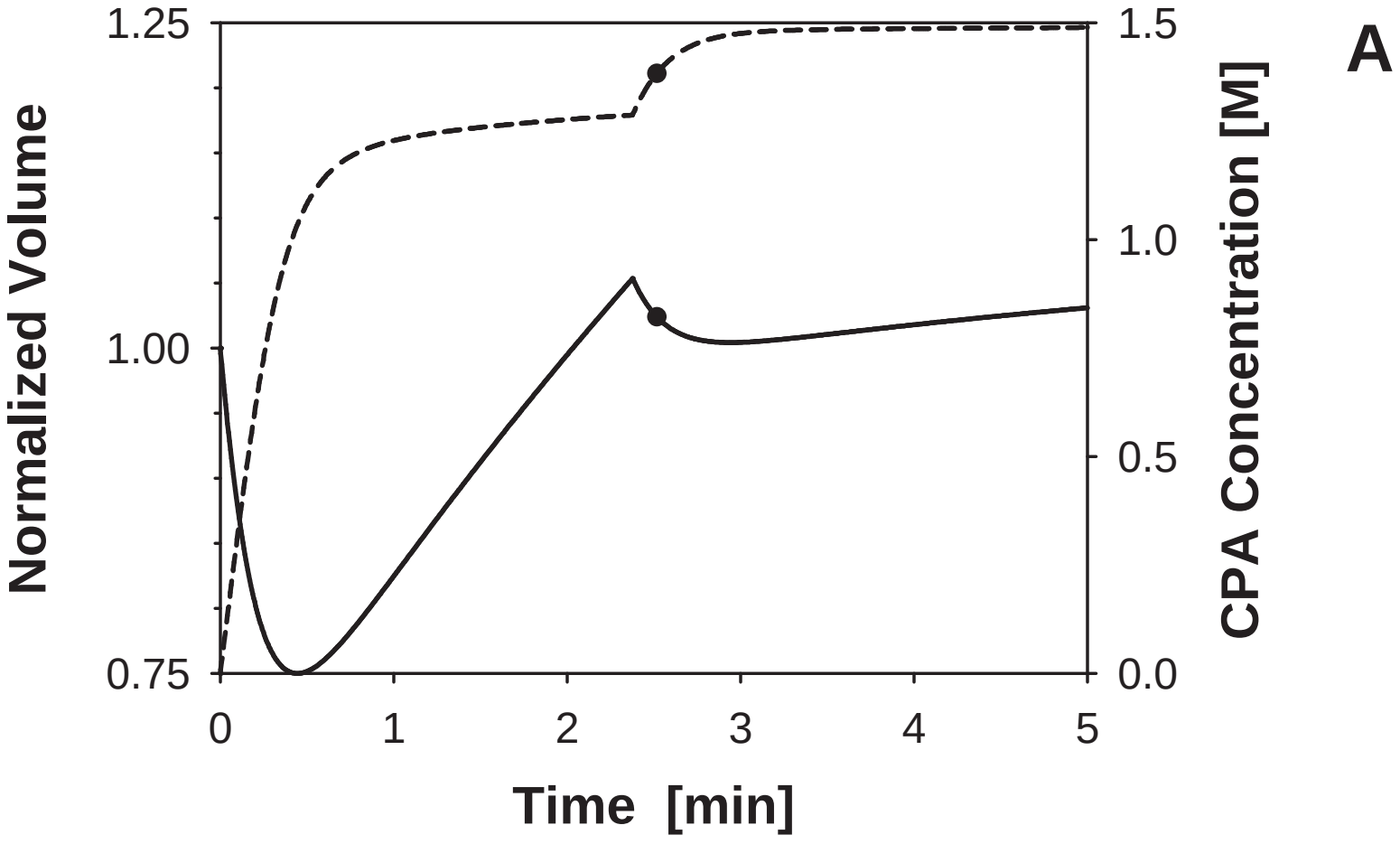
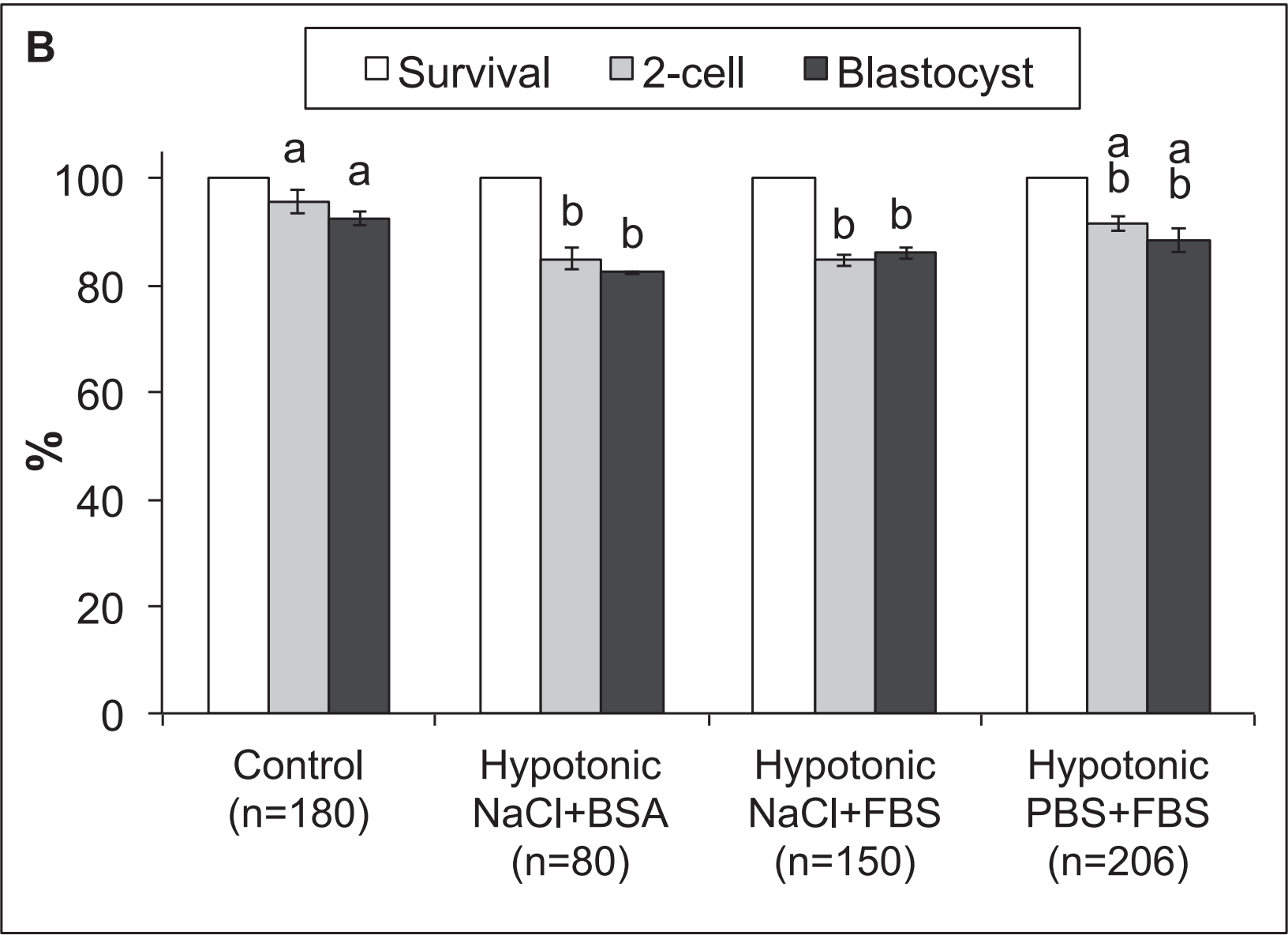
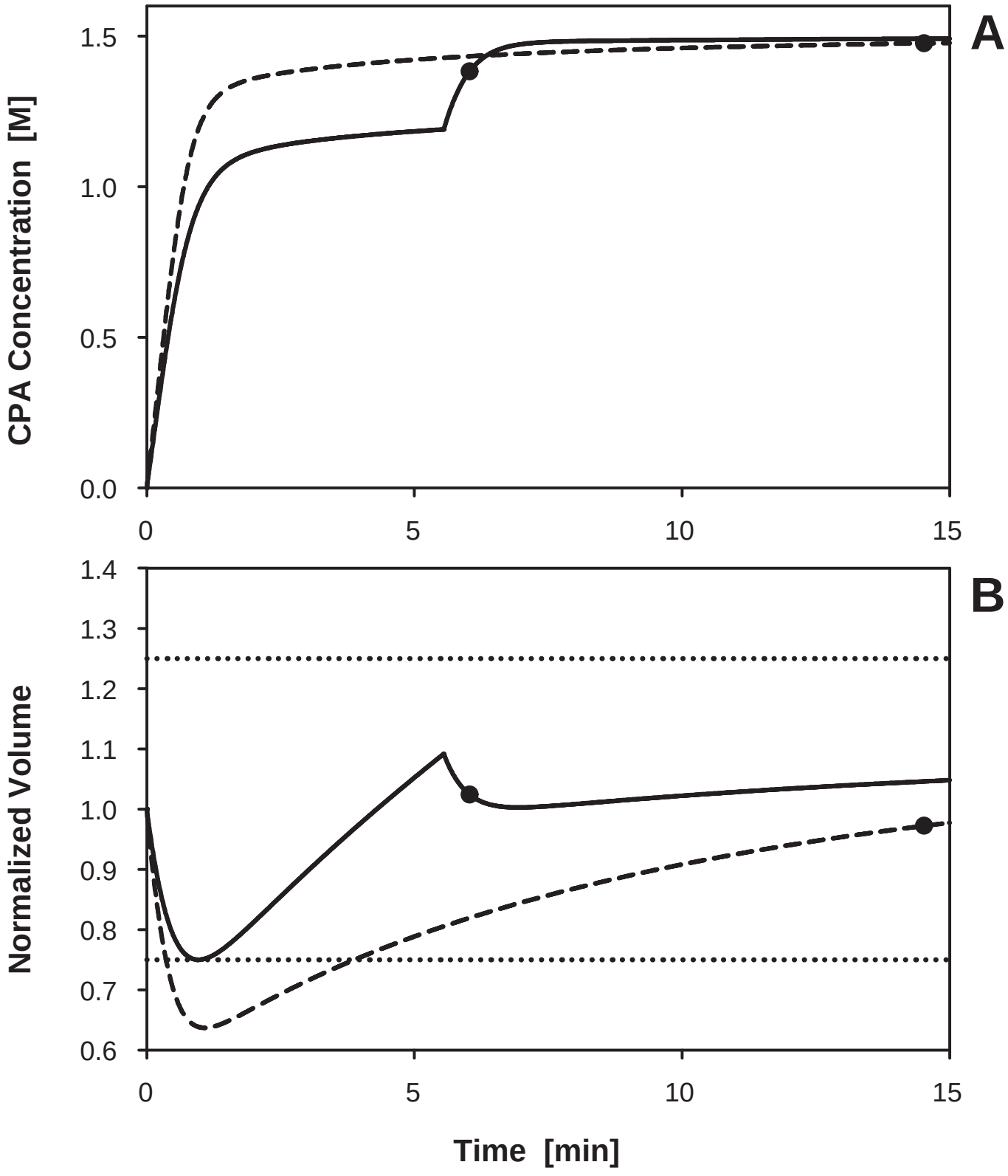

A
1.25
1.00
0.75
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Normalized Volume
CPA Concentration [M]
Time [min]

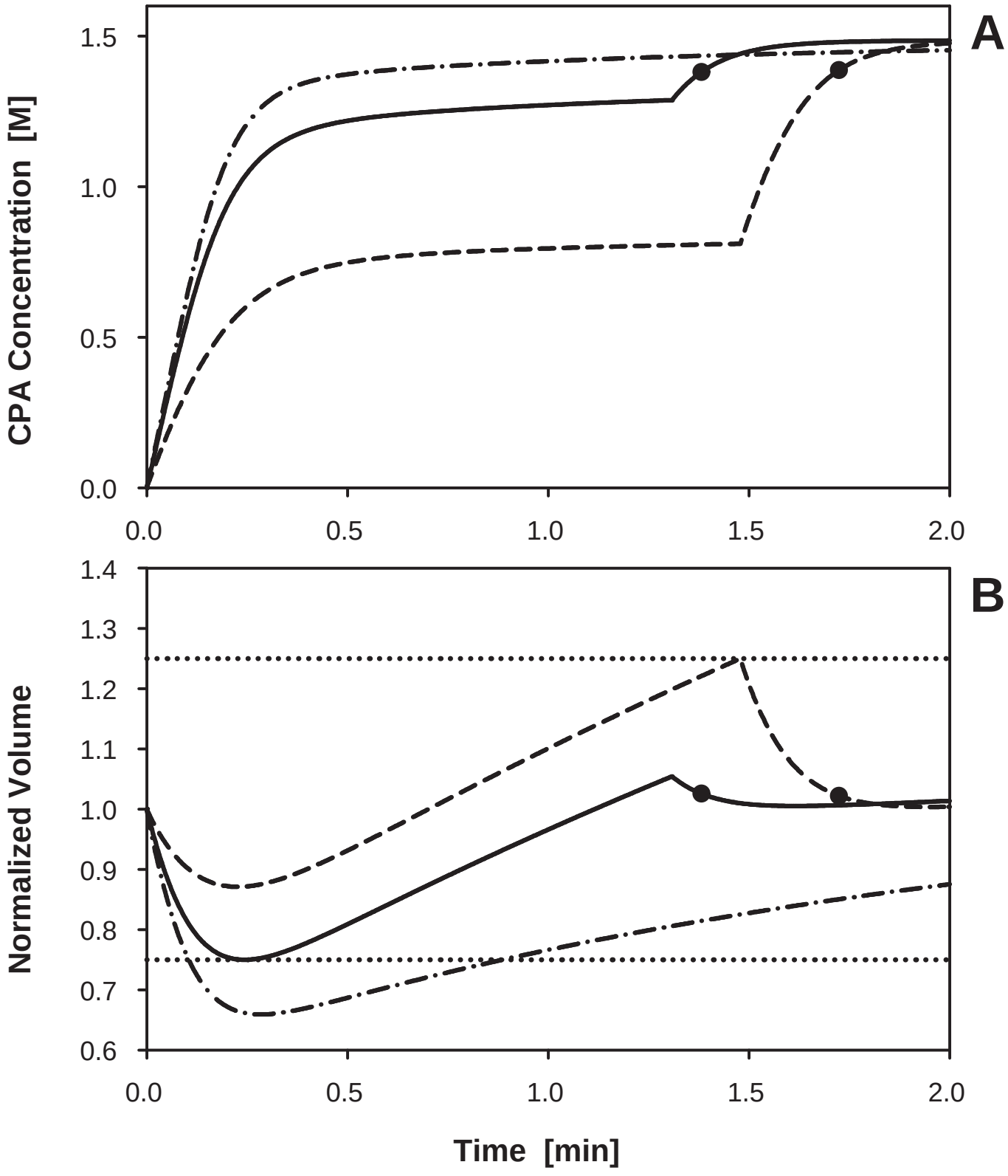
Figure 3B


B
Survival
2-cell
Blastocyst
100
80
60
40
20
0
%
a
a
b
b
b
b
a
b
a
b
Control
(n=180)
Hypotonic
NaCl+BSA
(n=80)
Hypotonic
NaCl+FBS
(n=150)
Hypotonic
PBS+FBS
(n=206)

Figures 4AB


A
CPA Concentration [M]
1.5
1.0
0.5
0.0
0
5
10
15
B
Normalized Volume
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0
5
10
15
Time [min]

Figures 5AB


A
CPA Concentration [M]
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
B
Normalized Volume
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.0
0.5
1.0
1.5
2.0
Time [min]

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