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Relationship Between Development, Metabolism, and Mitochondrial Organization in 2-Cell Hamster Embryos in the Presence of Low Levels of Phosphate

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Abstract

The effect of low concentrations of inorganic phosphate (P_i) on development, metabolic activity, and mitochondrial organization in the same cohorts of cultured hamster embryos was evaluated. Two-cell embryos were collected from eCG-stimulated golden hamsters and cultured in HECM-10 with 0.0 (control), 1.25, 2.5, or 5.0 μM KH_2PO_4. Glucose utilization through the Embden-Meyerhof pathway (EMP) and tricarboxylic acid (TCA)-cycle activity were determined following 5 h of culture. Mitochondrial organization in living embryos was evaluated using multiphoton microscopy at 6 h of culture. Development was assessed at 27 h (on-time 8-cell stage) and 51 h (on-time blastocyst stage) of culture. Total cell numbers, as well as cell allocation to the trophectoderm and inner cell mass were determined for morula- and blastocyst-stage embryos. Culture with P_i did not alter TCA-cycle activity. However, culture with ≥2.5 μM P_i significantly increased ($P < 0.01$) EMP activity compared to control. Mitochondrial organization was significantly ($P < 0.01$) disrupted by P_i in a dose-dependent manner. Development to the 8-cell, morula/blastocyst, and blastocyst stages was significantly reduced ($P < 0.05$) in the presence of ≥2.5 μM P_i compared to both control and 1.25 μM P_i. This study clearly demonstrates that, for hamster embryos, inclusion of even exceptionally low concentrations of P_i in culture medium dramatically alters embryo physiology. Additionally, although 2-cell embryos can tolerate some structural disruption without concomitant, detrimental effects on development or metabolic activity, metabolic disturbance is associated with decreased developmental competence.

Keywords

developmental biology; early development; embryo

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INTRODUCTION

The successful application of many new technologies, including genetic engineering, cloning, and xenotransplantation, are dependent, in large part, on our capacity to support the development of preimplantation mammalian embryos in culture. The ability to remove embryos from the female reproductive tract and to manipulate them in vitro has made these advances possible. Currently, a major limitation to widespread application of these technologies is our inability to consistently produce high-quality, developmentally competent embryos in vitro. The developmental competence (ability to produce a viable fetus) of cultured embryos is significantly reduced compared to in vivo-produced counterparts [1, 2]. In addition to enhancing our knowledge regarding basic developmental processes, increasing our understanding of how culture media components affect preimplantation embryos will facilitate the design of more suitable culture conditions, resulting in overall improved developmental competence of in vitro-cultured embryos. This, in turn, will allow us to maximize the benefit of technologies that are dependent on embryo culture.

An adequate supply of energy is vital for the developmental competence of preimplantation embryos. The dynamic period of development between formation of the zygote and of the blastocoele is metabolically very costly for the embryo [3]. Alterations in the metabolic profile could result in abnormal development or death of the embryo. Media components that alter the metabolic profile of the embryo can dramatically alter developmental competence of the embryo [4, 5]. Inclusion of oxidative phosphorylation inhibitors in the culture medium inhibits development of early cleavage stages in rats [4] and of all stages in mice [6]. Furthermore, studies of embryonic oxygen consumption in mice [7] and hamsters [8] show that conditions altering the rate of oxygen consumption also decrease the developmental competence of the embryo.

One pair of media components eliciting interest is glucose and inorganic phosphate (P_i). Glucose is an energy substrate that is present in the reproductive tract of most species [9–13], and P_i is essential for the production of ATP, the basic energy source for cell activities [14]. Considerable evidence, however, shows that inclusion of glucose/P_i in the culture medium is inhibitory to embryo development in a variety of species, including rodents [15–18], domestic animals [19, 20], and humans [21, 22]. In hamster embryos, glucose/P_i arrests development [23, 24], reduces respiration [8], and disrupts mitochondrial organization [25, 26].

Recent studies have shown, however, that glucose in the absence of P_i does not inhibit embryo development [27], suggesting that P_i alone may be responsible for the developmental inhibition previously attributed to the glucose/P_i combination. Whereas P_i alone is not inhibitory to hamster embryo development from the 8-cell stage [24], studies with earlier cleavage-stage embryos show that, even in the absence of glucose, P_i concentrations as low as 500 nM inhibit development in both hamsters and mice [23, 28–31]. Even with striking improvements in media formulations, a more recent report confirmed that concentrations of P_i as low as 2.5 μM alter ionic homeostasis and inhibit development of the embryo in culture [32]. The mechanism for this action remains to be elucidated.

It has been suggested that the mechanism for developmental inhibition in cultured hamster embryos may result from perturbed energy metabolism of the early embryo and/or disrupted mitochondrial organization, but such studies have only been performed in the presence of glucose [25]. Glucose can drive its own metabolism [14], so changes in metabolic profile in the presence and the absence of glucose in the medium are not surprising. Additionally, to our knowledge, only concentrations of P_i known to cause complete developmental inhibition (350 mM) have been examined. This makes it impossible to determine whether the observed developmental inhibition is the cause or the result of changes in the metabolic profile or mitochondrial organization of the embryo. Furthermore, although the developmental block in the presence of glucose/P_i is associated with disrupted mitochondrial organization [25], no study, to our knowledge, has examined the relationship between the mitochondrial organization and the metabolic profile of the early embryo. In the present study, we determined the effect of 0.0, 1.25, 2.5, and 5.0 μM P_i in the absence of glucose on development, mitochondrial organization, and metabolic profile within the same cohorts of embryos. Because this approach analyzed multiple parameters in the same experiment, it maximized the ability to correlate physiological parameters. Additionally, the use of multiple concentrations of P_i, including ones that are known not to cause developmental inhibition, permitted determination of a hierarchy and, thereby, answered the question of whether changes in developmental competence are preceded by alterations in metabolic profile or mitochondrial distribution.

MATERIALS AND METHODS

Culture Media

The base medium used in this study was a protein-free, chemically defined medium HECM-10 [33]. The formulation was as follows: 113.8 mM NaCl, 3.0 mM KCl, 1.0 mM $CaCl_2{\cdot}2H_2O$, 2.0 mM $MgCl_2{\cdot}6H_2O$, 25.0 mM $NaHCO_3$, 4.50 mM DL-sodium lactate, 0.01 mM asparagine, 0.01 mM aspartate, 0.01 mM cysteine, 0.01 mM glutamate, 0.20 mM glutamine, 0.01 mM glycine, 0.01 mM histidine, 0.01 mM lysine, 0.01 mM proline, 0.01 mM serine, 0.5 mM taurine, 3 μM pantothenate, and 0.1 mg/ml of polyvinyl alcohol. Treatments were prepared by modifying the base medium to contain varying concentrations (0.0, 1.25, 2.5, or 5.0 μM) of P_i (KH_2PO_4). Media were prepared the day before use from stock solutions and stored at 4°C. All salts, carbohydrates, amino acids, and vitamins were purchased from Sigma Chemical Co. (St. Louis, MO).

Measuring pH and Osmolarity of the Media

Culture medium was divided into 5-ml aliquots in 10-ml culture tubes (Falcon Plastics, Becton-Dickinson, Franklin Lakes, NJ) and treated with 0.0 or 0.35 mM NaH_2PO_4 or 0.35 mM KH_2PO_4. Osmolarity of each medium was determined using an Osmette osmometer (Precision Scientific, Natick, MA). The tubes, with caps loosened, were equilibrated in a humidified atmosphere of 10% (v/v) CO_2, 5% O_2, and 85% N_2 at 37.5°C for 2 h. The pH meter (Corning Life Sciences, Acton, MA) was adjusted and calibrated for reading 37°C solutions. Tubes were randomly removed from the incubator and placed into a 37°C hot block, and the pH measurement was obtained immediately.

Embryo Collection

These investigations were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and in accordance with the local guidelines of the Research Animal Resources Center at the University of Wisconsin-Madison (Animal Use Protocol A-07-5600-A00502-3-03-97). Embryos were collected from 3- to 6-mo-old, cycling golden hamsters. Multiple ovulations were induced by an i.p. injection of 10–20 IU (indexed to body weight) of eCG (Gestyl, Houston, TX) on the morning of the postestrus discharge (Day 1 of cycle). Females were mated to fertile males on the evening of Day 4. Two-cell embryos were collected at 29 h post-egg activation (PEA) as described by Barnett and Bavister [34]. Embryos were flushed from the oviduct with warmed and equilibrated (10% CO_2, 5% O_2, and 85% N_2) HECM-10 medium (no P_i). All embryos were then washed twice in HECM-10 and once in the appropriate treatment culture medium before being placed into culture.

Embryo Culture

All embryos were cultured in 35-μl drops of medium under mineral oil (Sigma) in groups of 5–12 embryos for either 5 h (for metabolic studies), 6 h (for cytoplasmic studies), or 51 h (for development to the blastocyst stage). Culture dishes, including media and oil overlay, were pre-equilibrated for at least 5 h before addition of embryos. Three replicates were performed. Each replicate assessed all three parameters in the same cohort of embryos. Embryos were cultured at 37.5°C in a humidified atmosphere of 10% CO_2, 5% O_2, and 85% N_2. Embryos from individual females were distributed among treatments so that each treatment group (i.e., each concentration of P_i) for the assessment of each parameter (i.e., development, metabolism, and mitochondrial organization) contained an equivalent number of embryos from any given female. Embryos from multiple females were pooled in each culture drop [35].

Morphology Assessment

Development to the 8-cell stage was assessed after 27 h of culture, and morula and blastocyst development was assessed after 51 h of culture. Development to the morula and blastocyst stages was expressed as a single endpoint, in addition to blastocyst development, due to difficulties in accurately distinguishing morula- from blastocyst-stage hamster embryos. The hamster blastocyst in vitro undergoes repeated cycles of expansion and collapse [36], and following collapse, a blastocyst is indistinguishable from a morula using low-power microscopy. Therefore, embryos identified as morula and blastocyst (morula/blastocyst)-stage embryos include all postcompaction embryos, whereas embryos identified as blastocyst-stage embryos include only those embryos exhibiting a distinct blastocoele at the time of examination.

Differential Staining of Inner Cell Mass and Trophectoderm Cells

Embryo cell number and allocation of cells to the inner cell mass (ICM) and trophectoderm (TE) were determined by differentially staining the cell nuclei using the technique described by Lane et al. [33]. The base medium used for staining procedures was a modified BM-3 medium [35] in which 20 mM $NaHCO_3$ was replaced with 20 mM Hepes (pH 7.4; HBM-3). Blastocysts were incubated in 0.5% (w/v) pronase in H-BM-3 for 30 sec to dissolve the zona

pellucida. Embryos were then washed in H-BM-3 and incubated in 0.25% (v/v) picrylsulfonic acid (Sigma) for 10 min at 4°C before a further wash and 10-min incubation in 0.1 mg/ml of Anti-DNP BSA (ICN Technologies, Costa Mesa, CA) at 37°C. Following incubation with the antibody, embryos were again washed in H-BM-3 and incubated in a 1:5 (v/v) dilution of guinea pig serum (ICN Technologies) containing 25 μg/ml of propidium iodide (Sigma) in H-BM-3 for 5 min. Embryos were subsequently placed in 25 μg/ml of bisbenzimide (Hoechst 33258; Sigma) in ethanol overnight at 4°C. The following morning, differential staining of nuclei was examined using a Nikon optiphot epifluorescence microscope (Nikon, Melville, NY).

Metabolic Assessment

A modification of the hanging-drop technique [37, 38], as described by Krisher et al. [39], was used to determine simultaneously the metabolism of D-5[^{3}H]glucose (specific activity, 15.9 Ci/mmol; Amersham Life Science, Buckinghamshire, England) and L-[^{14}C(U)]glutamine (specific activity, 250 Ci/mol; ARC, St. Louis, MO). The production of $^{3}H_2O$ from glucose was used as a measure of glucose metabolism through the Embden-Meyerhof pathway (EMP), and the production of $^{14}CO_2$ from glutamine was used to determine oxidation of glutamine through the tricarboxylic acid (TCA) cycle.

Assessment of Cytoplasmic Organization

Staining—Mitochondria of cultured embryos were labeled with the active mitochondria-specific dye Mitotracker-X-Rosamine (Molecular Probes, Eugene, OR) as described by Squirrell et al. [40]. Live embryos were incubated for 15 min in 330 nM Mitotracker in HECM-10 under culture conditions. Embryos were rinsed twice in 35-μl drops of HECM-10, then placed into 10-μl drops in imaging dishes [41] with up to six embryos per drop and one drop for each treatment in each dish. Because embryos had to be transported in culture drops to the imaging facility, all imaging drops were made with HECM-10 to eliminate the risk of contamination of medium from one treatment drop to another during transport. Embryos were transported in a warmed, gassed, covered chamber. The dishes were placed in an environmentally controlled minichamber on the microscope stage [42].

Multiphoton laser scanning microscopy—The multiphoton laser scanning microscopy (MPLSM) system, which was designed and used at the Integrated Microscopy Resource at the University of Wisconsin, has been previously described [43]. Briefly, the system utilized a 1047-nm fixed wavelength, femtosecond diode-pulsed, yttrium:lithium:fluoride laser (DPM 1000-PC; Microlase/Coherent, Santa Clara, CA) with a Nikon Eclipse microscope and a 100× oil-immersion objective. Digital images were recorded using the Lasersharp software (BioRad, Hercules CA). Single optical cross-sections through the nuclei were collected from each of the embryos in the control drop, then from each of the embryos in each of the P_i-containing drops. To confirm that changes observed in mitochondrial distribution were due to the experimental treatments and not an artifact of either the imaging itself or the time spent on the microscope stage, single optical sections were again collected from each control embryo. Embryos were returned to the incubator, and development to the morula and blastocyst stages was assessed at the same

time point as the developmental study to confirm that no adverse effects from the transportation and imaging had occurred.

Quantitation of mitochondrial distribution—The pattern of distribution of mitochondria labeling in 2-cell embryos was quantified using the method developed by Barnett et al. [25] and described by Squirrell et al. [44]. All measurements were made on the MPLSM digital images using NIH Image software (Bethesda, MD). The data analyzed were the ratios of the average pixel intensity in a 5-μm diameter circle set 4 μm in from the cortex (intermediate region) and a 5-μm diameter circle adjacent to the nuclear membrane (perinuclear region). Two such ratios were collected from each blastomere. The location for collection of the regional pixel intensity was assigned using a straight line bisecting the nucleus and parallel to the junction of the two blastomeres. The circles for pixel intensity collection were centered on this line in their respective regions.

Statistical Analysis

A Student *t*-test was used to compare the pH of culture media. For development and metabolic experiments, data were subjected to least-squares analysis of variance (ANOVA) using the general linear models (GLM) procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Percentage data were arcsine transformed and weighted for the number of embryos in each experiment before analysis. The ANOVA was performed for a randomized, complete-block design structure, with block being the day on which the replicate was performed and treatment being increasing concentrations of P_i. All tests of hypotheses were performed using appropriate error terms according to the expectation of the mean squares. Differences were detected by least-squares means. For mitochondrial organization studies, data were compared using the GLM procedure in SAS [45], blocking on day, with embryo as the experimental unit. Treatment differences were compared using the least-squares means analysis.

RESULTS

Effect of P_i on Osmolarity and pH of Medium

Previous work describing the developmental inhibitory effects of P_i was conducted with medium containing 0.35 mM P_i. Therefore, the pH of the equilibrated embryo culture medium, with or without 0.35 mM P_i, was measured. The pH of the equilibrated control medium was 7.26 ± 0.06. The addition of 0.35 mM P_i had no significant effect ($P > 0.1$) on the pH of the medium compared to control (NaH_2PO_4 = 7.24 ± 0.04, KH_2PO_4 = 7.20 ± 0.02). The osmolarity of the medium without P_i, with 0.35 mM NaH_2PO_4, or with 0.35 mM KH_2PO_4 was 310, 310, and 311 mOsm, respectively.

Effect of Low Levels of P_i on Development

Embryos were assessed at 27 h of culture to determine the effect of low levels of P_i on on-time development to the 8-cell stage. No effect of day on treatment was found. Culture with 1.25 μM P_i had no significant effect on mean cell number or percentage of embryos reaching the 8-cell stage at 27 h of culture (Table 1). However, culture with ≥2.5 μM P_i significantly decreased ($P < 0.05$) both the percentage of embryos reaching the 8-cell stage and the mean

cell number at 27 h of culture in a dose-dependent manner. None of the embryos cultured with 5.0 μM P_i reached the 8-cell stage at this time point. Formation of a presumptive blastocoele cavity was observed in 4% of embryos cultured from the 2-cell stage for 27 h with 1.25 μM P_i.

The effect of P_i on development to the morula/blastocyst stage was assessed at 51 h of culture. Culture of 29-h PEA, 2-cell embryos with 1.25 μM P_i had no significant effect on either morula/blastocyst or blastocyst development compared to control (Fig. 1). However, addition of ≥2.5 μM P_i significantly decreased morula/blastocyst and blastocyst development compared to control ($P < 0.01$). Additionally, culture with 5.0 μM P_i prevented development to the morula and blastocyst stages.

Whereas culture with 0.0 and 1.25 μM P_i resulted in statistically equivalent percentages of blastocysts, the morphology of the resulting blastocysts differed depending on the treatment. At visual examination, blastocysts cultured with 1.25 μM P_i had larger and more prominent blastocoele cavities than those cultured without P_i (Fig. 2A).

Cell counts were obtained to determine the effect of P_i on total mean cell number of morula/blastocyst-stage embryos as well as on cell allocation to the TE and ICM. In morulae and blastocysts derived from 2-cell embryos cultured without P_i, approximately 57% of the total mean cell number were TE cells, and 43% were ICM cells (Table 2). The presence of P_i significantly ($P < 0.01$) reduced total mean cell number and TE cell number in a dose-dependent manner. Culture with 2.5 mM P_i also significantly ($P < 0.01$) reduced ICM cell number compared to control. No morulae or blastocysts were obtained from culture of embryos with 5.0 mM P_i.

Effect of P_i on Metabolic Profile

The effect of low P_i concentrations on the metabolic profile of 2-cell embryos was determined. Culture of 2-cell embryos with 1.25 μM P_i for 5 h had no significant effect on EMP activity as determined by utilization of D-5[^{3}H]glucose (Fig. 3A). However, culture with both 2.5 and 5.0 μM P_i significantly ($P < 0.01$) increased EMP activity. In contrast, culture with up to 5 μM P_i had no observable effect on TCA-cycle activity as determined by utilization of L-[^{14}C(U)]glutamine (Fig. 3B).

Effect of P_i on Mitochondrial Organization

Two-cell embryos cultured in control medium exhibited a perinuclear distribution of mitochondria (Fig. 4A), as previously described [25, 26]. This pattern was maintained throughout the imaging period (Fig. 4E). With the addition of 1.25 μM P_i, the mitochondria were not only in the perinuclear region but also increasingly in the intermediate cytoplasm (Fig. 4B). This mitochondrial relocalization was more dramatic with increasing concentrations of P_i (Fig. 4, B–D). This observation was confirmed when the pattern of mitochondrial distribution was quantitated (see *Materials and Methods*). Culture with P_i resulted in a pattern of mitochondrial organization that was significantly ($P < 0.05$) different from the control (Fig. 4F). No difference was found in the pattern of mitochondria in the control embryos imaged at the beginning (Fig. 4A) and again at the end (Fig. 4E) of the imaging period.

DISCUSSION

The developmental competence of the embryo is related to its metabolic profile [4, 5, 15, 46] and, possibly, to its subcellular organization [25, 47–49]. Any specific relationship or hierarchy has remained unclear, however, because no single study has examined all parameters within the same embryos. Therefore, the present study was designed to allow direct comparisons to be made of the changes in different physiological parameters within one experiment. To our knowledge, this is the first time that the same cohort of embryos has been used to simultaneously assess developmental competence, metabolic profile, and mitochondrial organization. Culture with 350 mM P_i is known to halt development [23, 28, 29, 50], to have dramatic effects on mitochondrial organization [25], and to reduce oxygen consumption [8] in hamster embryos. Therefore, we used P_i to analyze the relationship between these physiological parameters. By using lower concentrations of P_i, including those that do not cause severe developmental inhibition, we were able to analyze subtle but potentially important changes in the developing embryo. Because equal numbers of embryos were taken from donor females to assess each parameter, and because these assessments were run concurrently, variation due to donor was eliminated within each replicate. This allowed us to be confident that any effects seen within a replicate were due to the treatment and, thus, increased the strength of the conclusions drawn regarding the interaction between development, metabolism, and cytoplasmic organization. Similar to genetic epistatic studies, use of this approach also allowed us to determine a hierarchy of changes.

Although embryo physiology can be affected by changes in the pH or osmolarity of the medium [51–53], neither the pH nor the osmolarity of HECM-10 was altered by the inclusion of even relatively high levels of P_i. Furthermore, the early hamster embryo can compensate for small changes in external pH [54]. Therefore, the mechanism of action for P_i on embryo physiology as described in the experiments presented here is not due to alterations in the pH or osmolarity of the culture medium.

Mitochondrial organization was significantly affected by treatment with P_i. Even concentrations of P_i that did not alter development to the blastocyst stage or the metabolic profile significantly disrupted mitochondrial organization in the 2-cell embryo. Subcellular localization is important for the function of a number of somatic cell types, and cytoplasmic reorganization is an important and necessary part of embryonic development in invertebrates and lower vertebrates [55–58]. Although much remains to be learned concerning the significance of changes in cytoplasmic organization during early mammalian development, a number of studies have suggested a relationship with embryo developmental competence [47–49, 59]. In cultured hamster embryos, perturbed mitochondrial distribution has been associated with developmental block, and considerable evidence suggests that the spatial positioning of mitochondria may be a prerequisite for normal development [25, 26, 44, 60]. The present study marks the first instance, to our knowledge, that the disruption of mitochondrial organization in hamster embryos was not associated with a developmental block, and it shows that embryos can compensate for some level of disruption in mitochondrial organization without concomitant developmental inhibition. However, disturbances in cytoplasmic organization may be a precursor to developmental and/or metabolic disruption and, therefore, could be an important marker. Our data indicate that

cytoplasmic organization is more sensitive than enzymatic pathways to culture conditions, because effects on mitochondrial distribution occurred at lower P_i concentrations than did detectable effects on embryo metabolism. Furthermore, this study makes the important observation that subcellular organization of embryos can change dramatically and is exquisitely sensitive to culture conditions. A more thorough understanding of the subcellular organization of mammalian embryos in culture may be particularly important for determining the effects of physically invasive procedures such as intra cytoplasmic sperm injection (ICSI) and nuclear transfer.

The metabolic profile of 2-cell hamster embryos was also altered following culture with P_i. Although no detectable difference in TCA-cycle activity of embryos treated with up to 5 μM P_i was found, treatment with as little as 2.5 μM caused a significant increase in EMP activity (a measure of glycolytic activity). Previous studies have shown that changes occur in the metabolic profile of the embryo in the presence of P_i. Seshagiri and Bavister [8] noted what they termed a “Crabtree-like effect,” referring to a decrease in oxygen consumption in the presence of P_i (and glucose) at the 8-cell stage [8]. However, this does not appear to be the case in the hamster 2-cell embryo, which is not surprising. Although some limited TCA-cycle activity occurs at this stage, mitochondria are probably immature at this point [59, 61, 62] and, therefore, potentially unable to perform additional oxidative phosphorylation. Low TCA-cycle activity at this stage may not be critical to appropriate embryo development, because studies that have omitted P_i from the culture media for embryo development [22, 35] suggest that the early embryo is able to recycle its intracellular P_i to meet its energy demands. However, if the end product of the increased glycolysis (pyruvic acid) seen in the presence of P_i is not being processed through the TCA cycle, then we can assume that it is, instead, being shunted into lactate production [63]. This would result in a decrease of ATP production (2 ATPs vs. 36 ATPs per glucose molecule) rather than the increase that might be expected to result from increased glycolysis. A significant shift in the amount of lactic acid being produced could also alter the intracellular ionic homeostasis and decrease the viability of the cell. Previous studies have demonstrated that “blocking strains” of mice (i.e., those in which embryos exhibit a 2-cell block in culture) have increased lactate production [64]. Furthermore, alterations in intracellular pH disrupt mitochondrial organization [44] and embryo development [65].

The increased glycolytic activity observed in this study was associated with decreased developmental competence. Although culture with 1.25 μM P_i altered mitochondrial organization, only those concentrations of P_i with a significant effect on glycolytic activity altered on-time development to the 8-cell and the morula/blastocyst stages. Development timing to both the 8-cell and blastocyst stages has been correlated with fetal viability [66–68]. Surprisingly, the percentages of embryos developing to the morula/blastocyst stage were the same in the 1.25 μM P_i treatment and the control, but the mean cell numbers of these morulae and blastocysts was not. Culture with 1.25 μM P_i decreased total mean cell number and TE cell number at the morula/blastocyst stage compared to controls, indicating that some developmental differences occur even at the lowest concentrations of P_i examined. Cell number further decreased as the P_i concentration in the medium increased, concomitant with a decrease in development, confirming that the effect of P_i on the developing hamster embryo is dose dependent.

The decrease in cell number in the presence of P_i is especially interesting considering the presence of precocious blastocoele cavities at the 8-cell stage (blastocoele cavities have not previously been noted at this early time point in any medium or in vivo) and the visual difference in the embryos at the blastocyst stage when compared to control embryos. One generally expects that formation of earlier and larger blastocysts translates into increased cell numbers, but this clearly was not the case in the present study. The presence of precocious blastocysts at the 8-cell stage and larger blastocoele cavities at the morula/blastocyst stage, despite reduced cell numbers, may provide insight regarding the mechanism of action for P_i in the preimplantation embryo. As the embryo clearly is not developing faster in the presence of P_i, the difference in visual morphology is probably due to an increase in fluid transport into the blastocoele cavity. Formation of the blastocoele cavity is dependent on the Na/K-ATPase [69]. Because the control medium already contains 3.0 mM KCl, it seems highly unlikely that the minute increase in K^+ contributed by the addition of 1.25 μM KH_2PO_4 would result in the precocious blastulation that was observed. More likely, this effect is due to increased intracellular calcium ($[Ca^{2+}]_i$). In his 1988 review, Yingst [70] noted that changes in $[Ca^{2+}]_i$ can affect the function of the Na/K-ATPase, with the specific effect (increase or decrease) being dependent on the cell type. Previous reports from our laboratory demonstrate that levels of $[Ca^{2+}]_i$ increased in the presence of P_i [32]. Therefore, P_i may work to enhance blastocoele formation in preimplantation hamster embryos by increasing $[Ca^{2+}]_i$ and, thereby, stimulating Na/K-ATPase in blastocysts. Additionally, changes in $[Ca^{2+}]_i$ can affect numerous cellular events, including protein synthesis, DNA replication, mitochondrial function, and cell-to-cell communication [71–74], all of which are necessary for normal development and appropriate metabolic profile. Therefore, the results presented here, in addition to previous work in our laboratory [32], increasingly suggest that the deleterious effect of P_i on preimplantation hamster embryos may be mediated by increasing levels of $[Ca^{2+}]_i$.

In summary, inclusion of even exceptionally low concentrations of P_i dramatically alters the physiology of cultured hamster embryos. Mitochondrial distribution is most sensitive to changes in media composition and may be an important marker for assessing developmental competence. Finally, whereas embryos can tolerate some level of organizational disruption without concomitant changes in blastocyst development or metabolism, increased glycolytic activity caused by P_i is associated with decreased developmental competence. This observation may have implications in other species for embryos developing in culture.

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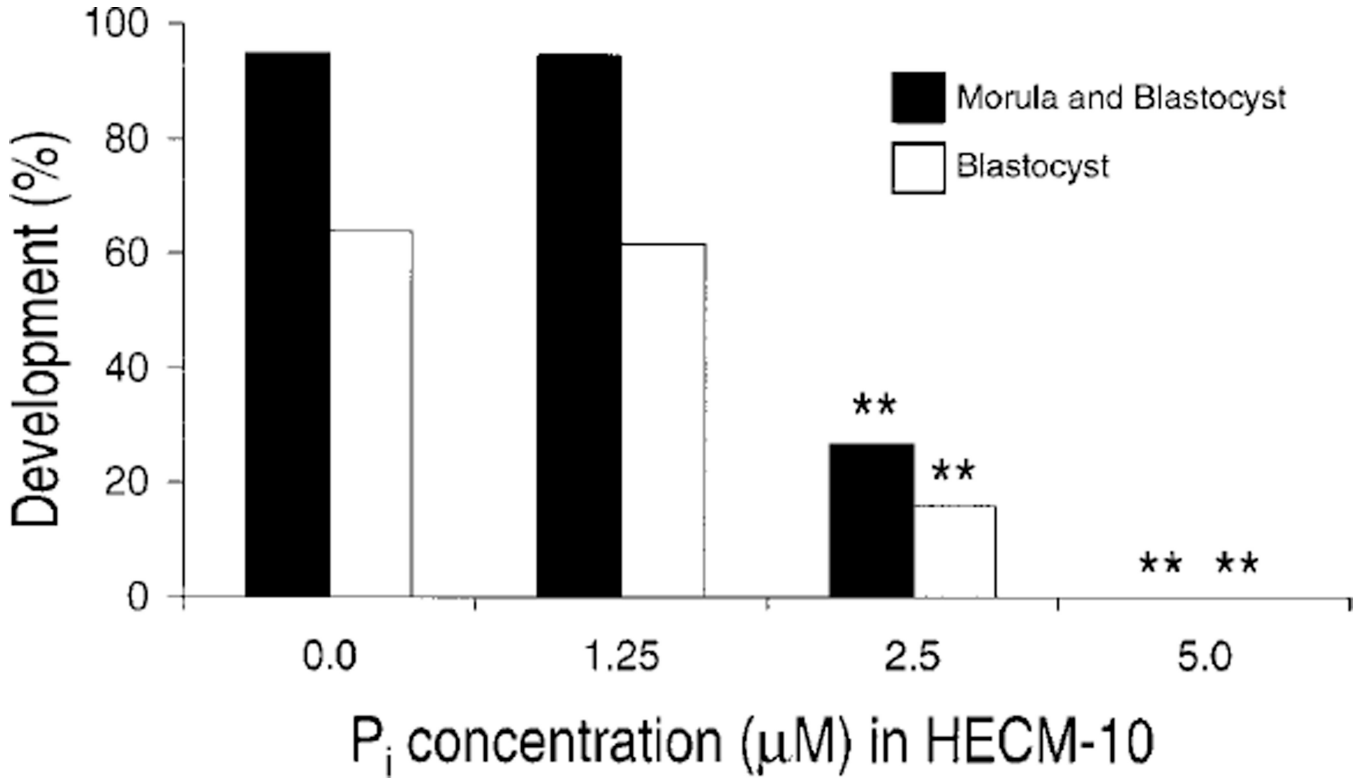

FIG. 1.

Effect of P_i on development to the morula and blastocyst stage in embryos cultured from the 2-cell stage (n = 12–24 embryos/treatment [depending on replicate], three replicates). **Significantly different from control ($P < 0.01$).

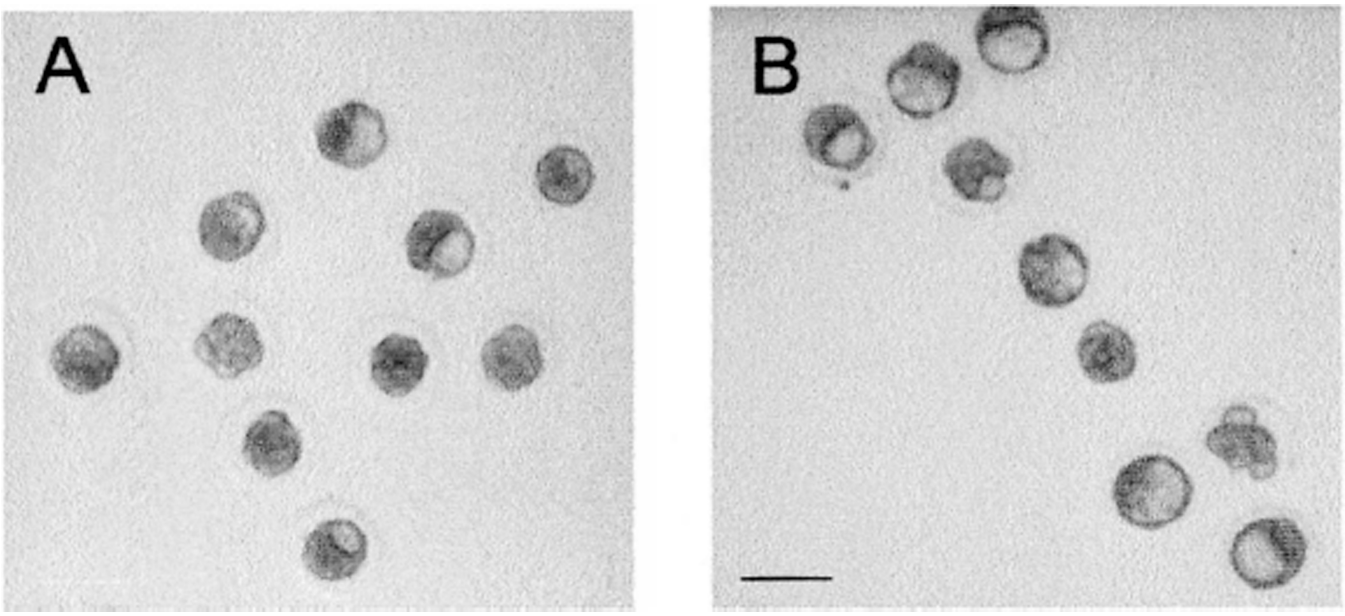

FIG. 2.

Morphological differences in blastocoele cavity formation caused by culture with P_i. These photographs show embryos cultured for 48 h from the 2-cell stage in **A**) HECM-10 or **B**) HECM 10 + 1.25 μM P_i. Bar = 100 μm. Note that blastocoele cavities were clearly larger and more prominent in the embryos cultured with low levels of P_i.

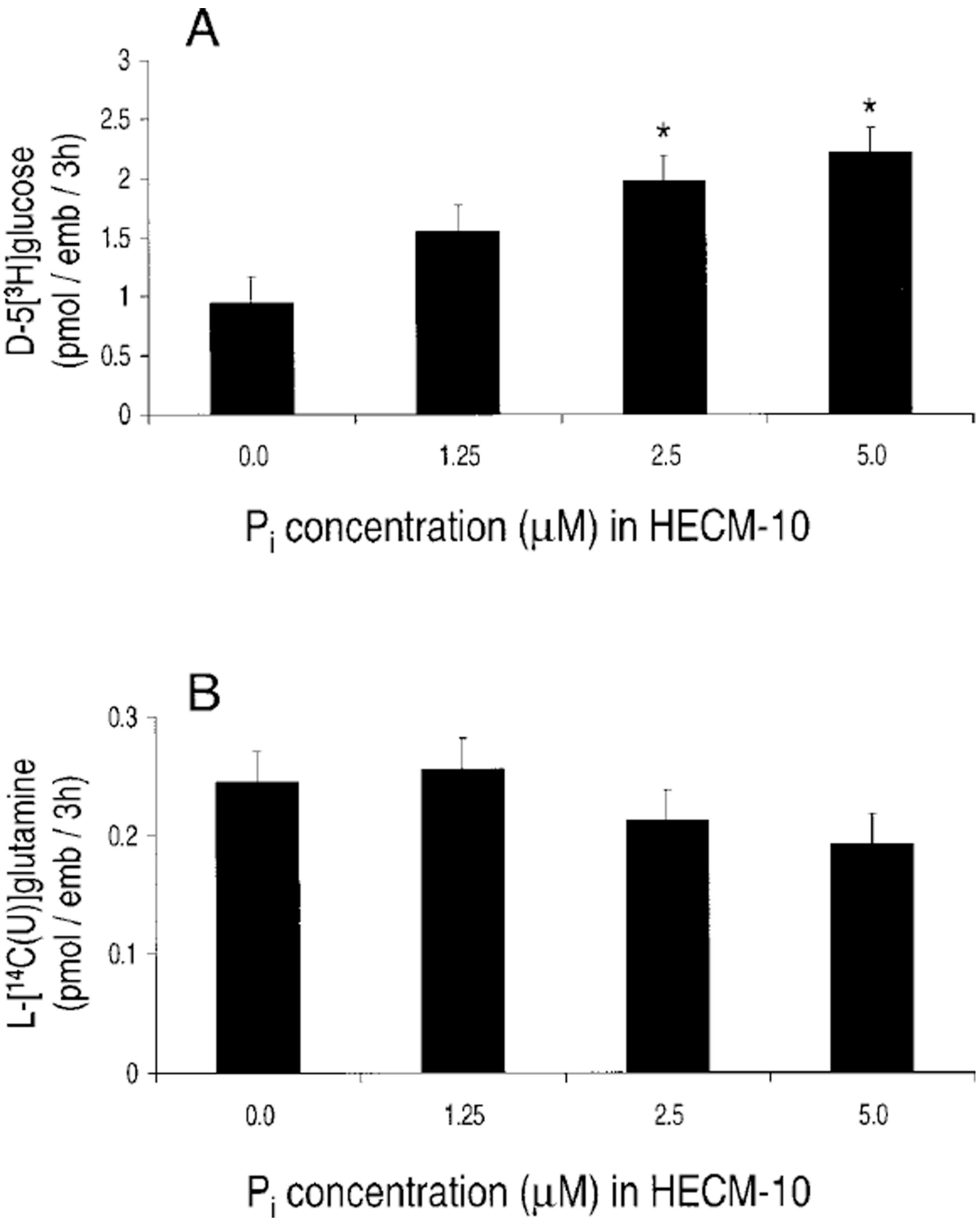

FIG. 3.

Effect of P_i on metabolism of **A**) D-5[^{3}H]glucose through the EMP and **B**) L-[^{14}C(U)]glutamine through the TCA cycle (n = 6–8 embryos/treatment [depending on replicate], three replicates). *Significantly different from control ($P < 0.01$).

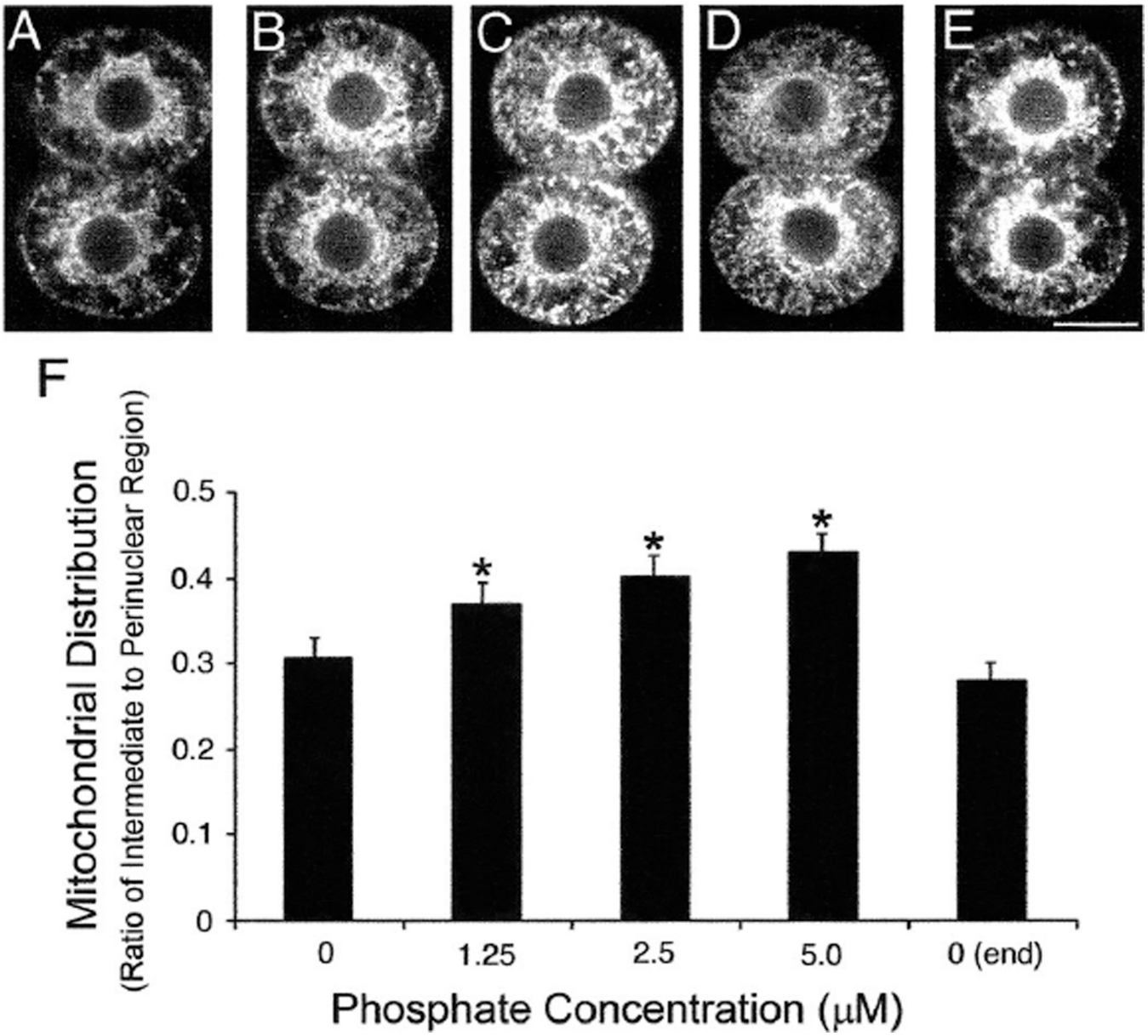

FIG. 4.

Distribution of mitochondria in 2-cell embryos cultured with increasing concentrations of P_i. These MPLSM micrographs illustrate the mitochondrial organization in 2-cell embryos labeled with Mitotracker and cultured for 5.5 h in HECM-10 containing different concentrations of P_i: **A**) 0 μM, **B**) 1.25 μM, **C**) 2.5 μM, **D**) 5.0 μM, and **E**) a second image of the same embryo shown in **A** collected following the imaging of all other embryos in all other treatments. All embryos shown are from the same replicate; bar = 25 μm. **F**) The graph shows the quantitation of the mitochondrial distribution (see Materials and Methods for explanation of quantitation). *Significantly different from control ($P < 0.01$).

TABLE 1

Development of embryos cultured from the 2-cell stage in phosphate at 27 h of culture.*

Concentration of P_i (μM) in HECM-10	% 8-cell embryos at 27 h of culture	Mean cell number (± SEM) at 27 h of culture
0.0	84.8[a]	7.3 ± 0.2[a]
1.25	80.4[a]	7.4 ± 0.2[a]
2.5	45.6[b]	5.6 ± 0.2[b]
5.0	0.0[c]	3.0 ± 0.2[c]

*Values within a column with different superscript letters are significantly different ($P < 0.05$).

TABLE 2

Effect of phosphate on morula/blastocyst (M/B) mean cell number and allocation (± SEM) of embryos cultured from the 2-cell stage.*

Concentration P_i (μM) in HECM-10	Total mean M/B cell number	Mean TE cell number	Mean ICM cell number	Mean ICM % of total cells
0.0	31.8 ± 1.6[a]	18.4 ± 1.1[a]	13.4 ± 1.0[a]	42.9 ± 2.7[ab]
1.25	26.4 ± 1.4[b]	13.8 ± 1.0[b]	12.6 ± 0.9[a]	47.1 ± 2.3[a]
2.5	17.0 ± 2.8[c]	11.0 ± 2.0[c]	6.0 ± 1.8[b]	32.5 ± 4.8[b]

*Values within a column with different superscript letters are significantly different ($P < 0.01$).