Improvement of mouse embryo quality by myo-inositol supplementation of IVF media

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Abstract

Objective Myo-inositol (myoIns) has a positive role in mammalian development and human reproduction. Since experiments on farming species suggest a similar role in preimplantation development, we evaluated the hypothesis that the inclusion of myoIns in human embryo culture media would produce an increase in embryo quality in IVF cycles, using the mouse embryo assay.

Methods To determine the effect of myoIns on completion of preimplantation development in vitro, one-cell embryos of the inbred C57BL/6N mouse strain were produced by ICSI, cultured in human fertilization media in the presence of myoIns

(myoIns+) or in its absence (myoIns−) and evaluated morphologically. Daily progression through cleavage stages, blastocyst production and expansion and blastomere number at 96 hours post fertilization were assessed.

Results Compared to myoIns− embryos, myoIns+ embryos displayed a faster cleavage rate and by the end of preimplantation development, the majority of myoIns+ blastocysts was expanded and formed by a higher number of blastomeres.

Conclusion The presence of myoIns resulted in both an increase in proliferation activity and developmental rate of in vitro cultured early mouse embryos, representing a substantial improvement of culture conditions. These data may identify myoIns as an important supplement for human embryo preimplantation culture.

Capsule We have evaluated whether the inclusion of myo-inositol (myoIns) in human embryo culture media may ameliorate embryo quality in IVF cycles by assessing the effect of this molecule on preimplantation development using the mouse embryo assay. One cell mouse embryos were produced by ICSI, cultured in human fertilization media in the presence of myoIns (myoIns+) or in its absence (myoIns−) and evaluated morphologically for four days. Compared to myoIns− embryos, myoIns+ embryos displayed a faster cleavage rate. On developmental day 4, the majority of myoIns+ blastocysts was expanded and formed by a higher number of blastomeres. In light of the increase in proliferation activity and developmental rate of in vitro cultured early mouse embryos observed, myoIns may represent an important supplement for human embryo preimplantation culture.

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Introduction

Myo-inositol (myoIns) is the major component of a group of exahydrocyclohexanes (C₆H₁₂O₆) with defined roles in eukaryotic cells, including mammalian oocytes and early embryos, as a precursor of phosphoinositides, membrane components, anchor molecules (reviewed by [35]), signaling molecules (reviewed by [3,11]), and as hyperosmotic stress protectant [46].

MyoIns appears particularly important for early development (reviewed by [2]): its concentration in the serum of foetuses and newborn infants is several times higher than in adults [45] and its administration during pregnancy reduces the chance to develop gestational diabetes [9,10] and, in the foetuses of *curly tail (ct)* mutant mice [17], the natural occurrence of spina bifida. It finally has a positive role in reproduction: its levels in the follicular fluid are positively correlated

with oocyte quality and pregnancy outcome in humans [5]; myoIns pre-treatment of women before the onset of hormonal stimulation in IVF cycles increases oocyte [6,42,48] and embryo quality [6,7], and possibly the implantation rate [34]. Finally, it reduces the amount of FSH and days needed for proper stimulation, parameters directly linked to the chance of pregnancy [41].

Experiments on farming species suggest a role for myoIns in mammalian preimplantation development, since myoIns supplementation of culture media improves rabbit and bovine blastocyst formation, expansion and hatching [20,51], and allows development to term of healthy animals [20]. These data prompted the hypothesis that inclusion of this molecule in human embryo culture media would produce an increase in the number of high quality embryos produced in IVF cycles. To test this hypothesis, we have analyzed myoIns effects on preimplantation embryos of the mouse, which represent the key model system for studies concerning experimental approaches to human assisted reproduction. As a matter of fact, a mouse embryo assay has long been conceived and is currently used to test whether a specific experimental condition, potentially applicable to human embryo culture, may lead to impaired development. In this test, mouse embryos are treated and cultured under the same conditions to be applied to our species and the effects are evaluated by analysis of the rate of development [16,44].

MyoIns is transported into mammalian cells, including oocytes and early embryos, by a sodium co-transporter [29] and partly by a sodium independent transporter [19]. During mouse preimplantation development, activity of these transporters produces a robust uptake, progressively increasing from the one-cell to the blastocyst stage [19,22]. However, information on the effects of myoIns on mouse development is limited to oocyte meiotic maturation and earliest embryonic stages. Indeed, Chiu et al. [4] showed that culture of fully grown, cumulus-enclosed oocytes at germinal vesicle stage in myoIns increases their rate of maturation to metaphase II (MII), and when these oocytes are fertilized in vitro and the resulting 2-cell embryos are transferred to foster mothers, they implant at a higher rate and display a higher postimplantation viability than control embryos.

In the present study we have sought to investigate the effects of myoIns supplementation of clinically used media throughout mouse preimplantation development. To model human ART techniques, we performed ICSI in standard human IVF medium. In addition, to detect possible beneficial effects of myoIns, we produced mouse embryos using gametes from the C57BL/6N mouse strain, in which the success rate of this procedure is lower than that normally observed on hybrid mice (i.e. B6D2F1), in terms of both oocyte survival (60 % vs. 80 %) and blastocyst development (40 % vs. 70 % of 2-cell embryos) [23]. We cultured the resulting embryos in a cleavage supporting human medium in the presence or

absence of myoIns (myoIns⁺ and myoIns[−], respectively) and monitored their daily progression to the expanded blastocyst stage. At this time we also determined the number of blastomeres in the resulting embryos as a measure of proliferation activity.

At each time interval post-fertilization (p.f.), myoIns⁺ embryos displayed a faster cleavage rate, with a higher percentage of embryos at the most advanced stage. The developmental difference between embryos cultured in the presence or absence of myoIns was particularly evident 96 h p.f., when the majority of myoIns⁺ blastocysts were expanded. These blastocysts showed higher numbers of blastomeres, suggesting that the faster developmental rate observed throughout cleavage and blastula stages reflects a higher proliferation activity. We conclude that myoIns supplementation resulted in an improvement of culture conditions and may therefore represent an important supplement for human embryo preimplantation culture.

Materials and methods

Animals

Animals (Charles River Italia, Calco, VA, Italy) were housed in a temperature-controlled facility (22±1 °C) on a 12/12 h light/dark cycle, inside standard cages with unlimited access to food and water. Forty-to-60 day old C57BL/6N mice were used in all experiments as donors of oocytes and sperm cells. All experimental procedures were conducted in accordance with the official European guidelines for the care and use of laboratory animals (86/609/EEC). Experimental protocols and related procedures were approved by the Italian Ministry of Public Health.

Culture media

In order to model human assisted reproduction, all procedures were performed in commercial Quinn's Advantage media and supplements (BioCare Europe, Napoli, Italia) for human embryo fertilization and preimplantation culture, which are specifically formulated to support development to the blastocyst stage (see for example [30]) and do not contain myoIns.

Animal treatment, gamete collection and ICSI

Female mice were hormonally induced by intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (NHPP, Torrence CA, USA) and, 46–48 h later, of 5 IU human chorionic gonadotropin (Corulon, Intervet Italia, Aprilia, LT, Italy). After an additional 13 to 15 h, cumulus–oocyte complexes were collected from the oviducts, freed from cumulus cells by treatment with 0.1 % hyaluronidase in Hepes buffered

Quinn's Protein Plus Advantage HTF Medium containing 5 % human serum albumin, rinsed in HTF-Medium and incubated at 37 °C in Quinn's Protein Plus Advantage Fertilization Medium (F-Medium) under a humidified atmosphere of 5 % CO₂ in air.

At the time of oocyte collection, sperm cells were collected from cauda epididymides of one male mouse in 1 mL Quinn's Sperm Washing Medium inside a Falcon 3008 plastic dish. The sperm suspension was left at 37 °C for 15 min in air, to allow cell dispersion into the medium.

ICSI was carried out by the procedure described [26,28], under a Nikon (Nikon Corp., Japan) invertoscope equipped with Leitz (Wild Leica, Wetzlar, Germany) mechanical micromanipulators, Märzhäuser (Wetzlar, Germany) piezoelectric apparatus and using Humagen (Charlottesville, VA, USA) holding and ICSI micropipettes. A 1 µL aliquot of sperm suspension was mixed thoroughly with a droplet of 50 µL Quinn's Sperm Washing Medium containing 10 % (w/v) polyvinylpyrrolidone (Mr 360,000). Single sperm heads were separated from the tails and injected into each MII oocyte in a droplet of HTF-Medium under mineral oil.

Embryo culture

Surviving oocytes were recovered at 37 °C in F-Medium under a humidified atmosphere of 5 % CO₂ in air for 30 min, and then divided in two groups. One group was further cultured in 14 µL/mL myo-inositol (10 mmolL⁻¹, Andrositol®LAB Lo.Li. Pharma, Italy) in F-Medium, the other one in 14 µL/mL phosphate buffered saline in F-Medium. From this time on, embryos were continuously maintained in either the presence of 10 mmolL⁻¹ myoIns (myoIns+) or its absence (myoIns-) in the culture media. Embryo culture was performed in 500 µL medium inside Falcon 4 well IVF plates without medium replacement.

Six hours after ICSI, zygotes containing two distinct pronuclei were transferred to myoIns+ or myoIns- Quinn's Protein Plus Cleavage Medium (C-Medium) and kept in culture for a total of 4 days at 37 °C under 5 % CO₂ in air, as described [40]. Developing embryos were scored daily between 11:00 a.m. and 1:00 p.m. for morphology and progression through cleavage stages.

Nuclear staining of blastocysts for cell count

After scoring on day 4, blastocysts were fixed in 5 µL ethanol/acetic acid (3:1) solution [20] on a glass slide. The drop of medium was allowed to dry out and embryos were impregnated with 2 µL Hoechst stain (10 mg/mL Hoechst 33343 in 2.3 % sodium citrate) for 5 min. The slide was coverslipped and the total number of stained nuclei in each blastocyst was counted under a Nikon 80i (Nikon Italia, Torino, Italy) microscope (excitation wavelength 320 nm) by a digital camera

connected to a personal computer. Images were analyzed using ImageJ software (Rasband, W.S., U.S. National Institutes of Health, USA. <http://imagej.nih.gov/ij/>).

Chemicals

Where not stated otherwise, chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Data analysis

Data were analyzed by using χ^2 tests with Yates continuity correction and ANOVA. Statistical analyses were performed using R: A language and environment for statistical computing (R development core team, R foundation for statistical computing, ISBN 3-900051-07-0, 2008, Vienna, Austria. <http://www.R-project.org>).

Results

To investigate the effect of myoIns on preimplantation mouse embryos, culture in myoIns+ medium was initiated 30 min post fertilization and performed continuously to the blastocyst stage. The effect of 10 mmolL⁻¹, 20 mmolL⁻¹ and 30 mmolL⁻¹ myoIns, as employed by Chiu et al. [4] on maturing mouse oocytes, was evaluated in preliminary experiments (not shown). Since optimal culture conditions were obtained with 10 mmolL⁻¹, we used this concentration in all further experiments.

Effect of myo-inositol on pronuclear formation and embryonic development through cleavage stages

Six hours p.f., most of the eggs cultured in either the presence (97/106) or the absence (84/97) of myoIns had formed pronuclei, indicating a similar rate of activation. After 1 day of development, however, zygotes cultured in the two experimental conditions displayed the first significant difference: those maintained in the presence of myoIns presented a proportion of 4-cell embryos larger than control embryos (55/106 vs 34/96; $p=0.0379$), with a corresponding decrease in embryos at the previous stage (not shown).

On developmental day 2, all embryos had progressed beyond the 2-cell stage, regardless of the culture conditions. However, while 60 % of myoIns+ embryos (63/106) had reached the 8-cell stage, less than 40 % of myoIns- embryos (36/96) was at the same stage ($p=0.0072$). This difference was maintained by day 3, when the proportions of embryos developed to the 16-cell stage were approximately 60 % for those cultured in myoIns+ C-medium (61/106) and only 30 % for the control ones (32/96) ($p=0.0111$).

Effect of myo-inositol on blastocyst formation and expansion

On developmental day 4, approximately 70 % of myoIns⁺ embryos (73/106) had progressed to the blastocyst stage, with 56 % (59/106) already expanded. On the other hand, the percentages of total and expanded blastocysts observed among control embryos were 47 % (45/96) and 27 % (26/96), respectively ($p=0.0151$) (Fig. 1).

Effect of myo-inositol on number of blastomeres in day 4 embryos

The faster developmental rate of myoIns⁺ embryos corresponded to an increase in the number of blastomeres displayed by blastocysts on day 4 of development. At this time the mean number of cells detected by Hoechst 33343 nuclear staining (Fig. 2) in non-expanded blastocysts developed in myoIns⁺ medium and control blastocysts was 31.8 ± 0.15 and 30.5 ± 0.13 , respectively, while that relative to expanded blastocysts was 80.4 ± 0.20 and 79.3 ± 0.69 , respectively, with no significant difference. This suggested that myoIns supplementation did not influence the number of blastomeres typically observed in blastocysts at the end of preimplantation development. However, when total blastocysts were compared, a difference emerged between cell numbers displayed by embryos of the two groups (myoIns⁺ blastocysts: 64.2 ± 0.42 ; control blastocysts: 49.5 ± 0.59 ; $p=0.0003$), reflecting

Fig. 1 Effect of myo-inositol medium supplementation on blastocyst formation and expansion on day 4 of in vitro development. Completion of preimplantation development by embryos cultured in the absence (grey bars) or the presence (solid bars) of 10 mmolL^{-1} myo-inositol. Embryos were scored 96–98 h p.f. Bars represent the mean $\pm$ SEM of 7 independent experiments of the fraction of embryos at each indicated stage. N, myoIns[−] embryos, 96; myoIns⁺ embryos, 106. One-cell stage, two-cell stage and four-cell stage bars are not shown. Exp. blastocyst: expanded blastocyst. Asterisk indicates difference between treatments, calculated by χ^2 test with Yates correction for continuity: $p=0.0151$

Fig. 2 Effect of myo-inositol medium supplementation on the number of blastomeres present in non-expanded and expanded blastocysts after 96 h of in vitro development. Representative Hoechst 33343 nuclear staining of day 4 embryos: (1, 3) Non-expanded blastocysts; (2, 4) expanded blastocysts; (1, 2) embryos cultured in the absence myoIns; (3, 4) embryos cultured in the presence of 10 mmolL^{-1} myoIns; magnification: $200\times$. Bars, $100 \mu\text{m}$

the larger fraction of expanded blastocyst among those developed in myoIns⁺ medium (60 % versus 30 %) (Table 1).

Discussion

Assisted reproduction techniques are gaining widespread use in the treatment of human infertility. However, the association of hormonal induction of ovulation with manipulation and culture of early embryos prior to uterine transfer, both in humans and in other mammals, has long represented a negative factor with regard to the success rate of these techniques. In the laboratory mouse, for example, it produces a delay in preimplantation development of 2 to 7 h per cycle [18,39]. Depending on the strain of mice analyzed, during in vivo

Table 1 Blastomere no. present in embryos after 96 h of in vitro culture

Culture condition	Blastocysts	Expanded blastocysts	Total blastocysts
MI [−] medium	30.5 ± 0.13	79.3 ± 0.69	49.5 ± 0.59
MI ⁺ medium	31.8 ± 0.15	80.4 ± 0.20	64.2 ± 0.42^a

Values represent the mean $\pm$ SEM of 4 independent experiments. N, myoIns[−] blastocysts, 23; myoIns[−] expanded blastocysts, 16; myoIns[−] total blastocysts, 39; myoIns⁺ blastocysts, 19; myoIns⁺ expanded blastocysts, 37; myoIns⁺ total blastocysts, 56

^a $p=0.0003$, calculated by ANOVA

development, the first two cell cycles range in length from 14 to 20 h [21,39], and from 18 to 22 h [39], respectively. From the four-cell stage to the blastocyst stage the cell cycle is relatively invariant with a length of approximately 10 h [24]. In light of these data, it has been calculated that embryos maintained in vitro throughout preimplantation development may be delayed by an entire day at the blastocyst stage [12,37,49]. The processes of compaction, blastulation and blastocyst expansion are thus completed in vitro with progressively increasing temporal delays, likely leading to a loss of developmental competence and a reduction in the chance of pregnancy.

It has been suggested that the culture process may also alter normal development [1,36,50] and perturb global patterns of gene expression [25] leading, among others, to a deficiency in auto/paracrine factors necessary for optimal embryo growth. As an example, one-cell mouse embryos express *hsp70.1* when cultured in vitro, but not when maintained in vivo, as a consequence of the osmotic stress to which they are exposed in consequence of isolation from the tubal fluid environment [13].

To overcome these deficiencies, besides reducing the impact of ovarian stimulation [41], one of the main goals of human assisted reproduction-related research is the improvement of culture conditions. This has led on one side to the use of chemically defined media containing growth factors [33], on the other side to the development of culture systems employing co-culturing with growth factor-releasing cells [47], so that dramatic improvements in the composition of embryo culture media have been made in the last 15 years allowing the efficient production of viable human blastocysts.

Among others, supplementation of culture media with myoIns has produced positive effects on the development of rabbit [51] and bovine [20,33] preimplantation embryos. We hypothesized that similar effects may be exerted by myoIns on human preimplantation embryos, resulting in an increase in embryo viability and possibly representing a step forward in the evolution in culture media used in clinical IVF. In light of the unfeasibility of empirical studies on human embryos, we evaluated this hypothesis on the mouse model. The mouse embryo assay was originally conceived to identify the toxicity of materials and suboptimal components of culture systems used in human assisted reproduction. We have here employed this assay under experimental conditions that allowed us to evaluate potentially toxic as well as positive effects of myoIns on preimplantation mouse embryos.

As shown here, media supplementation with myoIns resulted, as observed 96 h p.f., in: a) an increase in the percentage of embryos progressed to the most advanced stage of development; b) an overall increase in the percentage of embryos developed to the expanded blastocyst stage; and c) an increase in the average number of blastomeres forming the embryos at the blastocyst stage. With regard to the

observations of Kaneko and Ohno [23], myoIns medium supplementation increased the rate of in vitro production of ICSI-derived blastocysts of the inbred C57BL/6 strain to the levels typically observed in B6D2F1 hybrid embryos. All together, these results represent proofs of increased quality of the culture conditions.

While the rate of pronuclei formation observed after fertilization was independent of the culture conditions, being already very high in the zygotes maintained in the absence of myoIns, in agreement with the observations of Kaneko and Ohno [23], the lengths of cell cycles throughout preimplantation development were shortened by the presence of myoIns, probably as a direct consequence of its action inside the cells. This was expected by data on myoIns uptake in mouse preimplantation embryos and its incorporation into phosphatidylinositides (PtdIns) [22]. A higher availability of PtdIns may increase the production of intracellular second messengers and upregulate a number of cellular functions including proliferation [3,15,38,43]. Since myoIns has been reported to increase PKB/Akt phosphorylation in mouse skeletal muscle cells [8], among signal transduction mechanisms possibly induced as a consequence of myoIns medium supplementation and by increased PtdIns availability, is the phosphorylation of this enzyme [27], the activation and nuclear mobilization of which promotes proliferation activity of mouse embryo blastomeres [14].

Cell compaction was appropriately reached at the eight-cell stage, regardless of the culture conditions (data not shown), suggesting that exogenous myoIns does not influence the occurring of such differentiative steps. However, since embryos cultured in the presence of myoIns underwent cleavage at a faster rate, most of these embryos reached the eight-cell stage and the compact morula stage several hours earlier than control embryos.

In conclusion, these results exclude apparent toxic effects and show that myoIns allows a reduction in the developmental gap that typically characterizes embryos obtained and cultured in vitro compared to those developed in vivo, strengthening previous observations on the positive effects exerted by culture medium supplementation with myoIns on early development of mammalian embryos.

This appears of particular relevance when referred to assisted human reproduction. In fact, among factors known to influence the outcome of IVF/ICSI treatment, morphology and developmental speed are considered the most reasonable and common parameters as markers of embryo quality prior to uterine transfer. It has been recently shown that early onset-of-cleavage in human embryos is a predictor of normal development and high chance of live birth when compared with late onset-of-cleavage embryos in IVF/ICSI cycles [31]. Successful implantation of early cleaving embryos may be the result, among others, of a more optimal synchronization

between embryonic development and the preparation of a receptive uterine environment [32].

Although there is divergence in the overall timing of preimplantation development between human and mouse, including zygotic genome activation and other differentiative steps, probably reflecting differences in the overall gestational periods of the two species [52], core transcriptional and functional programs are shared and metabolic requirements are similar [30]. Therefore, if embryo media supplementation with myoIns positively affects preimplantation development by increasing its speed in the mouse, it is reasonable to expect a similar result on human embryos with a positive impact on live birth outcome. In order to further characterize myoIns action in early development, we are currently evaluating its effects on postimplantation development and investigating biochemical mechanisms underlying morphological results.

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