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ANIMAL

Mitochondria transfer can enhance the murine embryo development

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Abstract

Purpose To evaluate the effect of mitochondrial transfer on embryonic development.

Materials and methods Mitochondria concentrates were collected from murine hepatocytes and fertilized murine zygotes from young and older mice in the 2PN stage were subjected to mitochondrial transfer and cultured in vitro to evaluate the embryonic development.

Results After extended in vitro culture, 37.65% and 20.91% embryos from the young mice developed to the blastocyst stage in the injected and control groups respectively, which is statistically significant. There was no difference in terms of hatching rates (1.76% and 1.82% respectively). Zygotes from the older mice (>20 weeks old) that received mitochondrial transfer also had a better developmental outcome than the control group (54.35% and 18.92% developed to morula stage, 43.48% and 8.11% developed to the blastocyst stage respectively), which is statistically significant.

Conclusions Our results for the murine model provide direct scientific evidence that mitochondrial transfer improves embryonic development. However, potential risks such as mitochondrial heteroplasmy, nuclear-mitochondrial interaction and epigenetic aspects all deserve further evaluation before mitochondrial transfer is applied clinically.

Keywords Mitochondria · Embryo · Zygote

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Introduction

With advances of assisted reproduction techniques, successful pregnancy rates have increased to nearly 40–50% in some centers, however, there are still some patients who suffer repeatedly from failed trials, especially those with poor ovarian reserves and poor embryonic development. There have been various attempts to improve results, including alteration of stimulation regimens, ICSI, co-culture, alternative culture methods, assisted hatching and fragment removal etc. But the results are still disappointing. It has been suggested that the introduction of a small amount of ooplasm from a healthy donor oocyte might rescue the function of oocytes that may have certain ooplasm deficiencies [1]. Ooplasm transfer has been attempted in humans and a few pregnancies have resulted [2–4]. However, it is not yet fully clear how ooplasm transfer works. In recent years, adenosine triphosphate (ATP)-producing capacity of germ line cells has been correlated with reproductive performance [5]. Perez et al. [6] microinjected a small number of mitochondria into mouse oocytes and found that the procedure prevented

oocytes from undergoing apoptosis. Their data provides a direct correlation between ATP-producing capacity and reproductive performance and helped, at least partly, in elucidating the ooplasmic factors. In the same report, they also showed the occurrence of a common mitochondrial DNA (mtDNA) deletion in unfertilized human oocytes are more frequent as compared with fertilized oocytes, this indicates the status of mtDNA integrity is associated with potential fertility of oocytes. Later, the concept of mitochondrial transfer was proposed and practiced in women who suffered from repeated pregnancy failure and resulted in clinical pregnancies [7]. However, there is still no proper pre-clinical evaluation about the efficiency of mitochondrial transfer on embryonic development; this also limits its application. To further evaluate the effects of mitochondrial transfer on embryonic development, we injected the mitochondria extracted from mice hepatocytes into zygotes from young or older mice to compare the developmental outcome of zygotes with or without mitochondrial transfer.

Materials and methods

Preparation of fertilized mouse oocytes

Four- to 6-week-old young female ICR mice and "older mice " consisted of female ICR mice older than 20 weeks received intraperitoneal injection of 10 IU PMSG (Sigma, St. Louis, MO, US) followed by intraperitoneal injection of 10 IU HCG (Serono) 48 h later. They were mated with male mice and successful mating was checked by vaginal plug. The mated female mice were sacrificed by CO2 inhalation followed by cervical dislocation. The oviducts were dissected in 10 mmol HEPES buffered Human Tubal Fluid (HTF) and apparently healthy fertilized oocytes in the 2PN stage were collected and cultured in HTF supplemented with 0.5% human serum albumin (HSA, Sigma).

Preparation of mitochondria

The mitochondria were prepared by a modified method described by Pinkert et al. [8]. Briefly, the mice liver tissues measured as 2×2×1 mm were minced in a solution of 5 mM MOPS (pH7.4), 250 mM sucrose and 0.5 mM EDTA. (maintained at 4°C), minced tissues were rinsed twice, homogenized with a glass/Teflon homogenizer at 10^6 cells per ml buffer. The crude homogenate was centrifuged twice for 15 min at 800 g and the pellets were discarded. The supernatant was again centrifuged for 20 min at 9,800 g. The pellet was re-suspended very slowly by drop-wise dilution with 200 μl of IVF30 medium (Vitrolife, Kungsbacka, Sweden). It was then maintained at 4°C until used for microinjection.

Preparation of micropipettes and micromanipulation

The micropipettes were made from thin-walled borosilicate glass tubes with an outer diameter of 1.0 mm and an inner diameter of 0.75 mm (Drummond Scientific Company, Broomall, PA, US). The holding pipette was made with an outer diameter of 60 (60–80) μm and polished to an inner diameter of 20 (20–30) μm. The transfer pipette was made with an outer diameter of 5 μm and inner diameter of 3 μm for the mouse embryo. The pipettes were bent at an angle of 20–25 degree to allow horizontal movement. The pipettes were sterilized using dry heat at 160°C for 2 h.

Five droplets of 5 μl 10 mmol HEPES-buffered HTF medium (for mouse oocytes) were placed on one line in the middle of the cover of a Falcon dish 3002. One droplet of mitochondrial suspension was placed in the right side of HEPES-buffered droplets. The droplets were covered with paraffin oil.

Mitochondrial transfer procedure

The holding and transfer pipettes were mounted on the microscope (Nikon Diaphot 200) of the micromanipulator system (RI TDU500 Micromanipulator, Falmouth, Cornwall, UK) in a similar way as for ICSI.

Up to five zygotes at the 2PN stage were placed in the dish, one in each HEPES droplet. The dish was placed on the heated stage of the microscope and the droplet of the mitochondrial suspension was visualized. The transfer pipette was lowered into the droplet of mitochondrial suspension and the mitochondrial suspension was aspirated for about 1800–2000 μm. The aspiration was stopped and the dish was moved to visualize the first zygote. The holding pipette was lowered into the droplet and the zygotes were fixed on the holding pipette. The zygotes were immobilized by slight negative pressure. The polar body was held at 6 o'clock. The transfer pipette was then lowered. The transfer pipette was pushed through the zona pellucida and the oolemma into the ooplasma at 3 o'clock. The mitochondrial suspension about 4 pl was injected into the ooplasma. The transfer pipette was withdrawn gently and the injected zygotes were released from the holding pipette. The number of mitochondria transferred per zygote was estimated about 4000. The transfer procedure was repeated for all the zygotes in the dish.

Evaluation of subsequent fertilization and embryo development

The manipulated zygotes were cultured individually in a micro-droplet environment for 5 more days. They were observed each day for further division, formation of morula, blastocyst and hatching. The developmental rates

in each experimental and control group were calculated and statistically compared.

Statistics

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS v 12.0 for Windows, Chicago, IL). Statistical significance was assessed using the t test. At $P<.05$, the difference was considered to be statistically significant.

Results

For experiments with the young mice, we performed mitochondrial transfer in 13 replicates, a total of 170 zygotes were successfully injected, and another 110 zygotes were cultured at the same time as the control. During extended in vitro culture for another five days, 37.65% and 20.91% embryos developed to blastocysts in the injected and control groups respectively, which is statistically significant. There was no difference in terms of hatching rates. (1.76% and 1.82% respectively) (Table 1)

We also performed mitochondrial transfer in older mice (>20 weeks old) in another four replicates, there were better developmental outcomes in the injected than control group (54.35% and 18.92% developed to morula stage respectively, 43.48% and 8.11% developed to the blastocyst stage respectively), which is statistically significant (Table 2).

Discussion

With the use of assisted fertilization techniques, a successful pregnancy rate per treatment cycle of approximately 40% can be expected in most centers. However, repeated failures still occur in some patients even after many treatment cycles. In 1997, Cohen et al. [9] reported the first successful pregnancy following the transfer of cytoplasm from donor oocytes into the oocytes of a patient with a history of poor embryo development and recurrent implantation failure, yet the live birth of five healthy children was reported by the end of 1998 [2]. However, there are still some concerns about the risk of cytoplasmic transfer [10], including the transmission of infectious agents or other unfavorable apoptotic factors [11], the sources of donated cytoplasm also causes problems. To further delineate the possible mechanism of cytoplasm transfer on recipient oocytes and seek solutions, there have been many studies and different hypothesis about the beneficial effects of cytoplasm transfer on embryo development [12]: one is the correction of imbalances between anti-apoptotic and pro-apoptotic factors, another is correction of a defective mitochondrial membrane potential. Healthy mitochondria are essential for accurate chromatid segregation at the time of mitotic division; they are also responsible for respiratory processes and ATP production. The idea of mitochondrial transfer to increase ATP production was proposed in 1998 [13], and microinjection studies using purified mitochondria from granulosa cells showed suppression of apoptosis in cultured mouse oocyte by 50% [6] indicating the "rescue effect" of mitochondria in a compromised mouse oocyte developed in vitro. Mitochondria transfer into oocyte from autologous cumulus granulosa cells (cGCs) was practiced in infertile women with repeatedly failed IVF trials and resulted in a clinical pregnancy by 2001 [7]. This encouraging result revealed that mitochondria might play an important role in embryo development and implantation.

Recently, studies showed the average mitochondrial DNA (mtDNA) copy number was significantly lower in oocytes suffering from fertilization failures compared to cohorts with a normal rate of fertilization [14], and developmentally arrested embryos may contain mtDNA contents that are large enough to achieve a good grading but may not be able to support further development and implantation [15]. These findings about mtDNA amount and embryo development [16, 17] support the theoretical

Table 1 Development outcomes of injected and non-injected young mice zygotes

	Morula formation rate (%)	Blastocyst formation rate (%)*	Hatching rate (%)
Mitochondrial injected (170)[a]	38.8% (66)	37.65% (64)	1.76% (3)
Non-injected control (110)	27.27% (30)	20.91% (23)	1.82% (2)
P value	0.06	0.04	0.72

[a] Embryo numbers are in the parenthesis. Values are presented as the mean ± SD.

$*P<0.05$

Table 2 Development outcomes of injected and non-injected mice zygotes in elder mice

	Morula formation rate (%)*	Blastocyst formation rate (%)*	Hatching rate (%)
Mitochondrial injected (46)[a]	54.35% (25)	43.48% (20)	0% (0)
Non-injected controls (37)	18.92% (7)	8.11% (3)	0% (0)
P value	0.045	0.01	N.S.

[a] Embryo numbers are in the parenthesis. Values are presented as the mean ± SD.

$*P<0.05$

benefits of mitochondrial transfer on pregnancy outcomes. While clinical trials of mitochondrial transfer in women with recurrent pregnancy failures showed good results, there is still no case-control or randomized study about the real effects of mitochondrial transfer on embryonic development, so the beneficial effects of mitochondrial transfer are only "hypothetic". This also limits its further application. In these studies, we performed autologous mitochondrial transfer in mice zygotes, and the results showed more recipient zygotes developed to the blastocyst stage than those without mitochondrial transfer in both young and older mice. These results provide direct scientific evidence that mitochondrial transfer is efficient in improving embryonic development.

In humans, germ-line cells are derived from primordial germ cells, which are conspicuous in the developing embryo by the third week after conception. Quiescent primordial follicles might not enter meiotic division for decades, and therefore they are expected to accumulate abundant mutant mtDNA in oocytes [18, 19], accumulation of mtDNA deletions may contribute to mitochondrial dysfunction and impaired ATP production and thus impaired oocyte fertilization and further embryonic growth [20]. Studies of mitochondrial DNA (mtDNA) mutations have also revealed that the accumulation of mtDNA rearrangements or deletions may contribute to mitochondrial dysfunction and interfere with fertilization of human oocytes and further embryonic development [21]. Taken together, insufficient amounts of mitochondria, altered mitochondrial function by accumulated mutation, inadequate redistribution of mitochondria, unsuccessful mitochondrial differentiation, or decreased mitochondrial transcription [22] may result in poor oocyte fertilization and compromised embryo development [23]. In the other words, successful fertilization and embryo development need adequate functional mitochondria in terms of both quantity and quality. The mitochondrial transfer provides extra amounts of mitochondria with relative good quality, which not only correct the quantity deficit, but may also correct the quality deficit by diluting the pre-existing mutated mtDNA accumulated in compromised oocytes and may improve the embryonic developmental potential. Interestingly, in our study, the mitochondrial transfer can improve morula formation rate in zygotes from older but not young mice. Though we cannot jump to a definite conclusion that mitochondria transfer are more effective for embryo development in older mice than young mice from this limited data in the meanwhile, previous studies have shown that the mitochondrial gene transcription levels are significantly decreased in unfertilized oocytes and arrested embryos compared with 3PN embryos [22], and embryos may fail to achieve further development due to insufficient mitochondrial amounts [15]. Thus, it is reasonable to postulate that there may be "thresholds' present that gate each step of embryonic development, and the zygotes from young mice may contain sufficient intrinsic mitochondrial pools to support morula development; the zygotes from older mice may not contain enough functional mitochondria to support morula development, partly due to accumulated mtDNA rearrangement, hence the mitochondrial transfer can improve morula formation rates in elder but not young mice. The failure to improve hatching rate, suggests the mitochondria pool, even after mitochondrial transfer, still cannot overcome the "threshold". To further delineate the efficiency of mitochondria transfer, we plan to perform further experiments to study the effects of mitochondria transfer in mice zygotes cohorts with different degree of mitochondrial DNA rearrangements.

In conclusion, the current study provides evidence that mitochondrial transfer can improve embryonic development. However, the adequate mitochondria amounts required for transfer, and risks such as mitochondrial heteroplasmy [7, 24], nuclear-mitochondrial interaction and epigenetic aspects all deserve further evaluation before mitochondrial transfer can be applied clinically.

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