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Oogonial Precursor Cell-Derived Autologous Mitochondria Injection to Improve Outcomes in Women With Multiple IVF Failures Due to Low Oocyte Quality: A Clinical Translation

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Abstract

Background: Mitochondrial dysfunction has been suggested as a major cause of age-induced decline in oocyte quality. In the past, donor oocyte cytoplasmic transfer showed some success but was abandoned due to the concerns with heteroplasmy. Recent studies indicated presence of oogonial precursor cells (OPCs) in the human ovary, which could be an autologous source of "healthy mitochondria." We sought to investigate the clinical efficacy of OPC-derived autologous mitochondrial injection (AMI) to improve oocyte quality in women with multiple in vitro fertilization (IVF) failures. **Methods:** The OPCs were isolated from laparoscopically obtained ovarian cortical pieces by cell sorting using a monoclonal anti-vasa homolog (anti-DDX) antibody. They were then disrupted and mitochondria were isolated. Reconstituted mitochondria were injected into each oocyte during intracytoplasmic sperm injection. Paired comparisons were made between the first failed cycles and the post-AMI cycles. **Results:** Of the 15 women undergoing ovarian stimulation, 2 were canceled and 3 decided to pool oocytes for later AMI. In remaining 10 (mean age 34.7 $\pm$ 4.1), AMI significantly improved fertilization rates (49.7 $\pm$ 31.3 vs 78.3 $\pm$ 18.9; $P = .03$) with a trend for better embryo grades (2.3 $\pm$ 0.3 vs 3.1 $\pm$ 0.7; $P = .08$). Four of 10 women conceived after single frozen embryo transfer and 3 after confirmation of diploidy via array comparative genomic hybridization (aCGH) (clinical pregnancy/embryo transfer = 4/10). **Conclusion**: These data show encouraging results for AMI in comparison to previous failed IVF cycles.

Keywords

oogonial precursor cells, mitochondria, embryo transfer, pregnancy, oocyte quality, IVF

Introduction

A root cause of age-induced infertility is the decline in oocyte quality. The proportion of couples attempting to conceive in the later period of reproductive life span has increased significantly due to various socioeconomical reasons in the recent years. This fact brought oocyte quality-related research to the forefront. Among the mechanisms behind the age-related decline in oocyte quality, mitochondrial dysfunction has been suggested as a major player.[1]

In the course of human life, mitochondrial function loss occurs as a result of the accumulation of mitochondrial DNA mutations and deletions.[2] Because oocyte meiosis is highly energy dependent, declining intracellular energy production due to mitochondrial DNA damage may cause faulty meiosis and aneuploidy and may also affect other critical cellular functions.[3] In the past, cytoplasmic transfer from young donor oocytes to aged oocytes showed some success but was abandoned due to the concerns with heteroplasmy.[4] Recent studies in rodents and humans indicated the presence of OPCs in the ovary, which could be an autologous source of "healthy"

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mitochondria.[5] This approach would avoid heteroplasmy in oocytes and resultant embryos. In the present study, our objective was to investigate the clinical efficacy of OPC-derived autologous mitochondrial injection (AMI) to improve oocyte and embryo quality and to assess pregnancy outcomes after embryo transfer (ET) in human. To our knowledge, this is the first controlled study reporting on in vitro fertilization (IVF) outcomes after OPC-derived autologous mitochondrial injection.

Materials and Methods

The protocol was approved by the Ankara University Medical School Ethics Committee, and the study was conducted in its entirety in Ankara, Turkey. Written informed consent form was obtained from all participants.

Patient Selection

Seventeen women with $\geq$2 IVF failures and poor oocyte/embryo quality were evaluated initially (Figure 1). The exclusion criteria were (1) male factor infertility, (2) history of ovarian surgery or chemotherapy, (3) day 2/3 follicle-stimulating hormone (FSH) levels >20 mIU/mL. Two were excluded due to unavailability of prior IVF records. Fifteen underwent ovarian stimulation. Two patients did not undergo ET, one due to embryo arrest at 2-pronuclei stage and the other due to not having a diploid embryo available for transfer. Three patients elected to freeze and pool oocytes to increase the number of oocytes available for OMI in the future. In the end, 10 women aged between 27 and 41 years completed an AMI cycle with ET and fulfilled an informed consent requirement.

Ovarian Biopsy

Three 5 $\times$ 5-mm ovarian cortical biopsies were obtained via 3-puncture laparoscopy using 5-mm ports. The tissues were cryopreserved with a previously described slow-freezing process until processed.[6] Patients were then suppressed with an oral contraceptive pill for timing purposes.

Mitochondrial Isolation and Preparation of OPC

Previously cryopreserved autologous ovarian cortical tissue was thawed based on previously reported methodology.[6] The tissue was immersed in an enzymatic solution and physically dissociated to form a single cell suspension. The single cell suspension was then incubated with a proprietary anti-vasa antibody (DDX4). Labeled cells were then isolated by fluorescence-activated cell sorting. The batch of OPC obtained from each patient was then cryopreserved with standard slow freeze methodology until use.[7] On the day of intracytoplasmic sperm injection (ICSI), the cryopreserved OPCs were thawed and mitochondria were isolated by differential centrifugation and maintained in a respiration buffer until injected.

Intracytoplasmic Sperm Injection, AMI, and Culture

Oocytes were prepared for ICSI following standard procedures for ICSI. Prior to ICSI, the mitochondrial solution was concentrated into a $\sim$250 nL droplet on the ICSI plate. The autologous mitochondrial solution was then drawn into the ICSI pipette along with the selected sperm. An approximate volume of 1 to 2 pL was delivered to the MII oocyte cytoplasm together with sperm during ICSI. Injected oocytes were cultured per standard embryological procedures at GenArt.

Ovarian Stimulation

Ovarian stimulations were performed using a gonadotropin-releasing hormone (GnRH) antagonist protocol. Daily injections of recombinant FSH or human menopozal gonadotropin (hMG) were started on cycle day 3 after OCP withdrawal. The initial dose of recombinant FSH or hMG ranged from 150 to 300 IU/day. Patients were monitored by estradiol levels and transvaginal ultrasound every 1 to 2 days until the day of oocyte retrieval.

Embryo Freezing

Embryos were vitrified at the cleavage or blastocyst stage. Cryopreservation of all embryos was undertaken with vitrification. After thawing, embryos were classified as fully intact (100% cells survived), partially damaged ($\geq$50% cells survived), or degenerated (<50% cells survived) after thawing. Only intact and partially damaged embryos were transferred.

Sperm Preparation

Semen was collected on the day of oocyte retrieval after an abstinence period of 2 to 5 days. A sterile container was used, and the collection of the semen occurred in a private room via masturbation. Samples were evaluated according to the values established by the World Health Organization in 2010.[8] Sperm samples were prepared using a 2-layered density gradient centrifugation technique.[9]

Evaluation of Embryo Grade

On day 3, all embryos were evaluated for cell number and embryo morphology. Each embryo transferred was evaluated for blastomere size and fragmentation. Embryos exhibiting equal blastomere size and no fragmentation were considered G4. G3 embryos had blastomeres of equal size with slight fragmentation (<20%), while G2 embryos had blastomeres of unequal size but no fragmentation. G1 embryos had blastomeres of equal or unequal size with moderate to heavy fragmentation. Arrested embryos were given a score of "0." Embryos were allowed to grow to blastocyst stage and either cryopreserved after trophoectoderm biopsy for preimplantation genetic screening (if there were multiple embryos and the patient agreed) or freshly transferred (if there was a single embryo or the patient declined embryo biopsy).

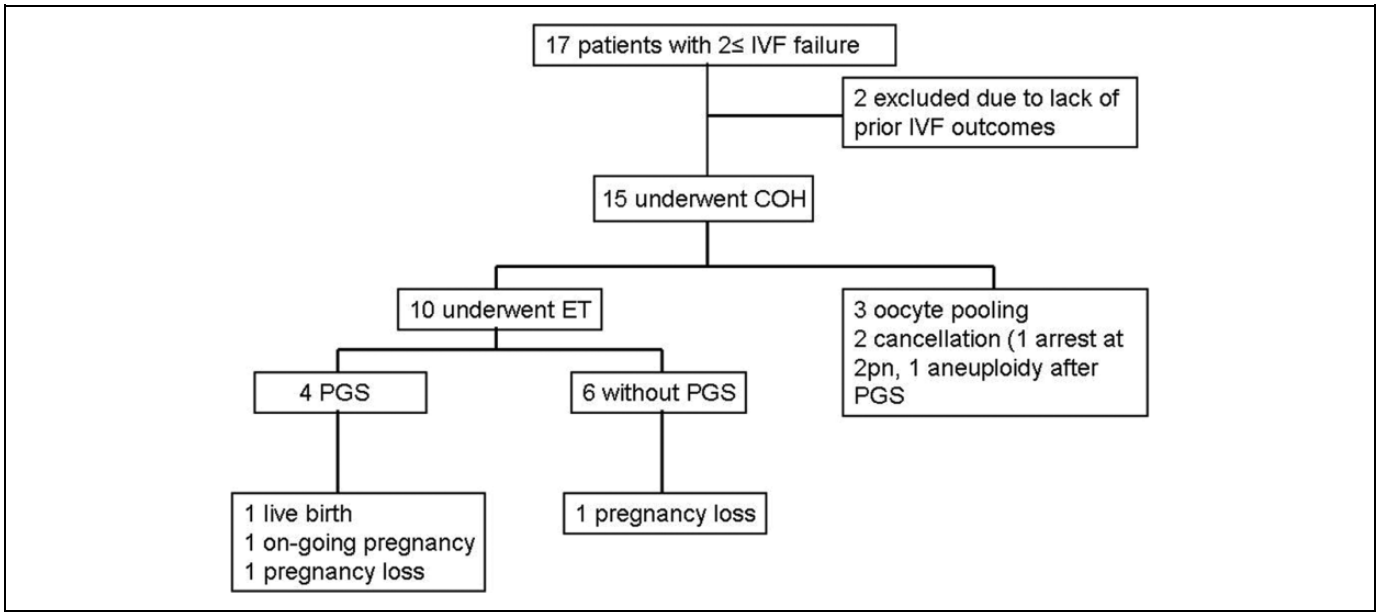

Figure 1. Study Design.

Embryo Transfer and Luteal Phase Support

In Turkey, number of embryos transferred in an IVF-ET cycle is limited to 1 to 2 by legislation. Embryo transfer was performed 3 to 5 days later under abdominal ultrasound guidance in fresh cycles. Cryopreserved embryos were thawed and replaced during natural cycle. All patients received 50 mg progesterone in oil intramuscularly daily for luteal support, which was initiated on the day after oocyte retrieval and was continued until fetal heart rate was documented, and the serum progesterone levels remained >30 ng/mL.

Clinical Outcome

A clinical pregnancy was defined as the presence of at least 1 gestational sac during first ultrasound examination between 6 and 8 weeks of gestation. Ongoing pregnancy was defined as an appropriately grown fetus with documented fetal heart activity on at least two 1-week apart ultrasound examinations.

Statistics

Statistical analysis was performed using the SPSS (release 15.0; SPSS Inc, Chicago, Illinois). The variables were investigated using visual (histograms and probability plots) and analytic methods (Kolmogorov-Smirnov and Shapiro-Wilks tests) to determine whether they were distributed normally. Paired *t* test was used to compare 2 related samples in a single group. Data were presented as mean $\pm$ standard deviation. A P value of .05 was considered statistically significant.

Outcome Measures

The primary outcome measures were patients' pre- and post-AMI fertilization rates and embryo quality which were compared in a paired analysis. To avoid any bias, the comparisons were made between the first failed cycles prior to AMI and the post-AMI cycle.

Results

Characteristics and cycle outcomes of all patients who underwent AMI are summarized in Table 1. These patients had undergone 42 previous cycles without a single pregnancy prior to AMI. Patients were older at the time of AMI and produced fewer oocytes compared to pre-AMI. This latter finding is expected, given the older age at the time of AMI. The AMI treatment resulted in a significant improvement in fertilization rates ($P = .036$). In parallel, there was a noteworthy trend for higher embryo grades on day 3 ($P = .082$; Table 2 and Figure 2).

Pregnancy Outcome

Of the 10 women undergoing ETs, 4 conceived with a clinical pregnancy rate of 40%. Of note, all pregnancies were from single frozen embryo transfers (FETs). One pregnancy, which was achieved after 7 IVF failures in a 34-year-old, resulted in a term live birth. A healthy baby girl delivered at 39th week of gestation via Cesarean section with Apgars of 9/9 and birth weight of 2950 gm. A second patient has an ongoing pregnancy at the time of this report.

Two other patients also conceived but experienced first-trimester spontaneous abortions. One of the losses was after the transfer of a diploid embryo as tested by aCGH from a trophoectoderm biopsy. However, the patient discontinued her luteal support after 6 weeks by medication error, which presumably led to the pregnancy loss.

Table 1. Characteristics and Cycle Outcomes of Patients Undergoing AMI Treatment.

Patients	Age, years	D2-3 FSH, IU/mL	D2-3 E2, pg/mL	AMH, ng/mL	N of IVF Failures	Fresh/Frozen	N of Oocyte Retrieved	N of Mature Oocyte	Fertil. Rate	N of Embryos Obtained	PGS (N of Embryos)	N of Embryos Transferred	Pregnancy Outcome
#1	35	7.4	47	1.8	5	Fresh	6	5	100%	5	NP	2	–
#2	34	15.2	100	1.4	7	Frozen-thawed	6	4	75%	3	1 normal of 3	1	Live birth
#3	27	6.1	57	2.5	6	Fresh	8	7	59%	4	NP	2	–
#4	31	7.2	39	3.5	3	Frozen-thawed	10	10	70%	7	2 normal of 7	2	–
#5	36	5.5	134	1.1	3	Frozen-thawed	8	7	43%	3	NP	2	–
#6	35	6.9	NA	NA	4	Fresh	8	6	83%	5	NP	2	–
#7	41	5.6	39	0.7	3	Frozen-thawed	6	3	100%	3	NP	1	Pregnancy loss
#8	40	9	NA	NA	7	Fresh	14	11	73%	7	NP	2	–
#9	32	6.3	NA	NA	2	Frozen-thawed	16	16	88%	14	4 normal of 8	1	Pregnancy loss
#10	36	7.2	NA	NA	2	Frozen-thawed	10	7	100%	7	1 normal of 7	1	Ongoing pregnancy

Abbreviations: AMH, anti-Mullerian hormone; E2, estradiol; FSH, follicle-stimulating hormone; IVF, in vitro fertilization; PGS, preimplantation genetic screening; NP, not performed; NA, not available.

Table 2. Comparison of Patient Characteristics and Cycle Outcomes Between Pre- and Post-AMI.

n = 10	Pre-AMI: First Cycle	Post-AMI	*P* Value
Age, years	30.3 ± 5.4	34.7 ± 4.1	.001
No. of oocytes	14.6 ± 4.8	9.2 ± 3.4	.018
No. of mature oocytes	10.6 ± 5.2	7.6 ± 3.8	.143
2PN embryos	5.9 ± 5.3	5.8 ± 3.3	.961
Fertilization rate (%)	47.9 ± 38.1	78.3 ± 18.9	.036
D3 embryo grade	2.3 ± 0.3	3.1 ± 0.7	.082

Abbreviations: 2PN, 2-pronuclei; AMI, autologous mitochondria injection.

Discussion

In 2004, a self-renewing population of cells in rodent ovary, purported to be oogonial stem cells, were reported by Johnson et al.[10] Although the initial reports created some significant discussion as this was against the preexisting notion of nonrenewal of primordial follicle population, later studies supported these findings in rodents and humans though others have continued to question the existence of these cells.[11,12.]

Augment is a proprietary treatment introduced by a public company named Ovascience. Based on the original patent that these OPCs produce higher adenosine triphosphate compared to other stem cell lines and ovarian stromal cells as well as the fact that they do not exhibit a common mitochondrial DNA mutation found in aging oocytes, they were utilized as an autologous source of mitochondria to enhance oocyte quality.[13]

Here we reported the first observations on the AMI via the Augment technique in a patient population with multiple IVF failures, which based on the clinical data from our center with similar IVF failures, had an anticipated baseline clinical pregnancy rate of 10% or less per ET. We found noteworthy improvements in primary outcome measures, fertilization rates and the embryo morphology. These improvements occurred, despite the fact that the patients were 3 to 4 years older at the time of the AMI cycle, compared to when they had their failed IVF cycles. Also of significance, all pregnancies occurred after single FETs.

In this patient population with poor prognosis for IVF success, 4 of 10 women conceived after AMI. The first healthy livebirth already occurred, from a patient who had previously experienced 7 IVF failures. Another patient with poor embryo quality and arrest in two previous failed IVF cycles is currently pregnant after AMI. Both patients had undergone preimplantation genetic screening (PGS) and a single diploid embryo was transferred after being frozen-thawed. Though the remaining two pregnancies were lost, one was after a FET of a diploid embryo. This patient accidentally discontinued her luteal support, most likely provoking the loss. The remaining pregnancy loss was in a 39-year-old women who had low ovarian reserve (anti-Mullerian hormone: 0.7 ng/mL) and experienced 3 prior IVF failures. This patient had not undergone PGS.

The relatively higher fertilization rates and embryo grades post-AMI indirectly indicate improvement in oocyte quality. While the importance of energy requirements during meiosis, fertilization, and embryo development have been documented in previous studies,[5] mitochondria play critical role in numerous other cellular functions. They are involved in key cellular processes such as beta oxidation, cell signaling, programmed cell death, oxygen sensing, and calcium hemostasis.[14] From this current study and preexisting laboratory studies, it is not possible to determine the exact mechanism of improvement in oocyte and embryo quality by autologous mitochondria supplementation. Further basic and translational research are needed to identify exact pathways that tie mitochondrial health to reproductive success.

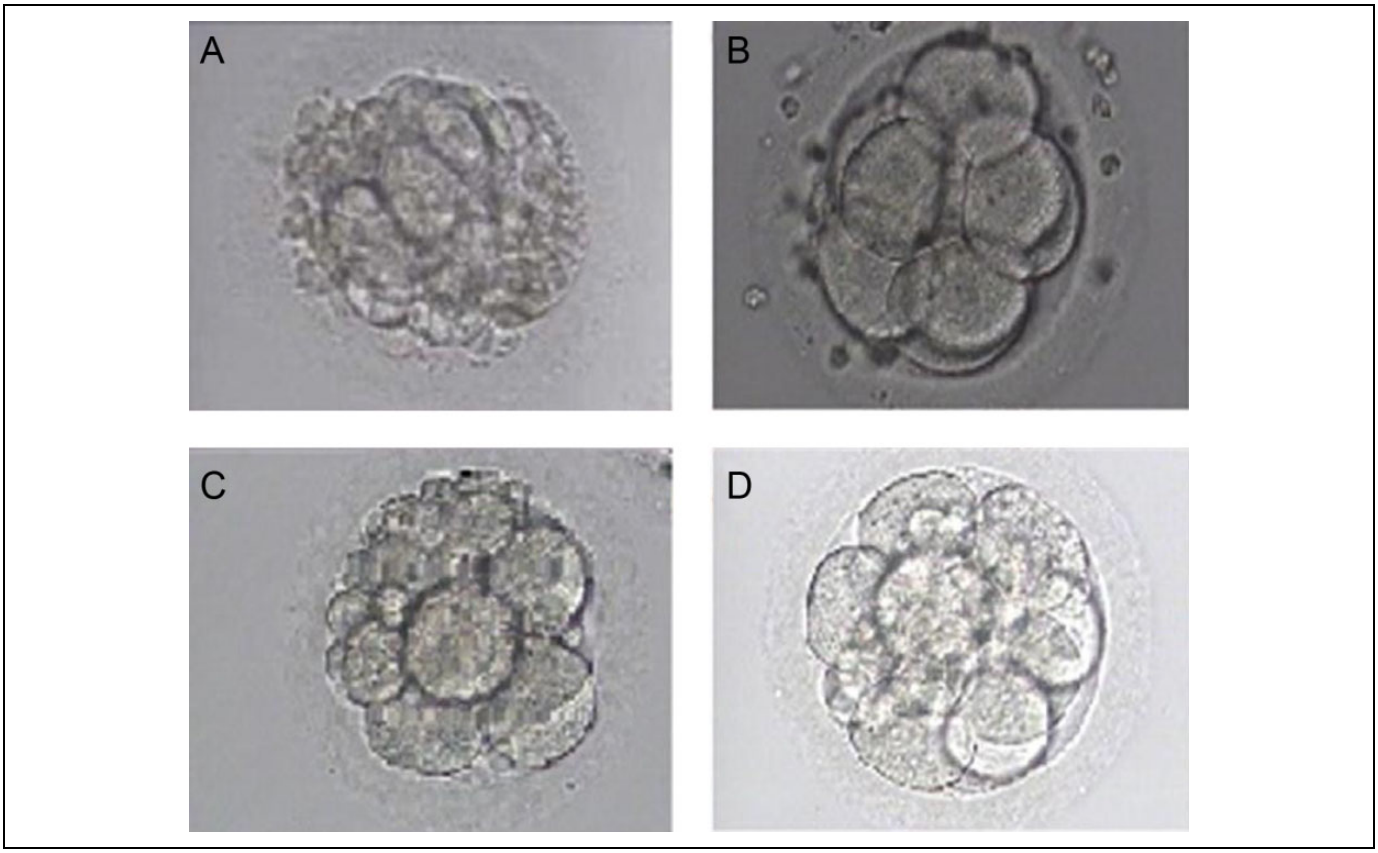

Figure 2. Improvement in embryo morphology post-autologous mitochondria injection (AMI). (A) A representative embryo from patient # 2 before AMI, and (B) improvement in embryo morphology in the same patient after AMI. This patient had a live birth. (C) A representative embryo from patient # 7 before AMI, and (D) improvement in embryo morphology in the same patient; this embryo resulted in a pregnancy which later miscarried.

The limitation of the current report is the sample size. Because of the relatively small sample size, limited follow-up, and lack of a prospective control group, we could not provide a comparison for live birth rates. However, in our center, live birth rates for a comparable group of women with multiple IVF failures after SET is <5%. Furthermore, the improvement in fertilization rates and the trend for better embryo morphology suggests biological plausibility.

Because of the innovative nature of this technology and the fact that it has already entered practice in number IVF centers around the world, we felt compelled to report the initial pregnancies and experience. Undoubtedly, as the reports from other centers accumulate, a bigger picture will emerge regarding the efficacy and safety of the AMI treatments derived from OPCs. In the meantime, our report provides the first scientific report on the initial experience.

Author Note

This work was performed in its entirety in Turkey, and presented at the 2015 annual meeting of the Society of Reproductive Medicine as a late breaking abstract.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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e-Corrigendum

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In the article published in the December 2015 issue, the corresponding author, Kutluk Oktay would like to declare his Medical Advisory Board Membership at Ovascience under Declaration of Conflicting Interests.