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Opinion

Cell-Free Mitochondrial DNA in Embryo Spent Culture Medium as Biomarker for *In-Vitro* Fertilization Outcome and its Potential Clinical Use

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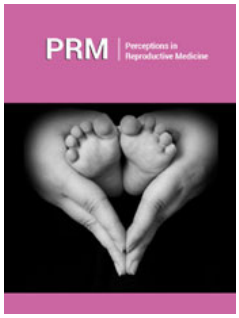

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Opinion

On 2013 for the first time, we discovered both cell-free genomic DNA (cf-gDNA) and cell-free mithocondrial DNA (cf-mtDNA) in spent culture media of human cleavage-stage embryos [1]. Analysis of DNA profiles of day 3 spent media demonstrated that cf-mtDNA/cf-gDNA ratio was correlated with embryo quality, and in particular it was significantly associated with degree of embryo fragmentation. Based on our results, the higher cf-mtDNA/cf-gDNA ratio was associated with blastocyst's developmental competence, trophoectoderm quality, and implantation [2]. In a prospective, blinded, multi-centric study we confirmed that the cf-mtDNA/cf-gDNA ratio in day 3 spent culture medium, combined with embryo morphology, improved the prediction upon blastulation compared to morphology alone, without any influence from patient and treatment-related factors [3].

Our opinion is that the cf-mtDNA in the embryo spent culture media would inversely mirror the overall embryonic mtDNA content, in line with recent studies suggesting that mtDNA abundance within blastomers may be correlated to aneuploidy, low embryo viability and development [4-6]. Undoubtedly, a direct comparison of mtDNA content between embryo and medium would provide a definitive demonstration of this hypothesis. The first criticism about the utility of cf-mtDNA as embryo biomarker was the potential genetic contamination from embryo-associated structures (*i.e.,* cumulus cells, polar bodies or sperm) and human serum albumin supplement of the culture medium [7]. In reality, the levels of cf-mtDNA were considerably higher for media co-cultured with embryos respect to the low baseline level of control samples [7,8]. Because of mtDNA has a cytoplasmatic localization, the developing embryo may release it into the culture media through mechanisms that are independent of the nuclear DNA.

It is reasonable that the cf-mtDNA could be generated from dead or fragmented blastomeres-that's why higher cf-mtDNA was associated with increased levels of day 2-3 embryo fragmentation [1] or actively secreted throughout pre-implantation development in a stage-specifically way - here because cf-mtDNA increased from the cleavage to the blastocyst stage and the highest amount of cf-mtDNA was found in continuous single-culture media [7]. Such a stage-specifically mtDNA release by embryos could further explain the discrepant conclusions reported on the basis of which stage of embryo development the cf-mtDNA was evaluated [9,10]. We believe that cf-mtDNA could be a novel, non-invasive, easy accessible, molecular-based marker of optimal development potential, increasing the productivity of the merely morphological analysis on which embryo evaluation is traditionally based. Nevertheless, we are aware that some questions are still open and need to be answered to validate cf-mtDNA as a reliable predictor in clinical practice:

a. To uniquely define at what timing (i.e., on day 3, day 5) the cf-mtDNA quantification should be performed to be used as a biomarker, in combination with embryo morphology/morphokinetics parameters.

b. To identify for which embryological/clinical outcome (i.e., blastulation, euploidy, implantation) the combined morphology-cf-mtDNA data could be useful.

c. To optimize the best methodological approach, concerning the most appropriate target gene(s) to represent the copy number of cf-mtDNA and the most sensitive and specific technique (i.e., quantitative-PCR or digital PCR).

For this purposes, randomized controlled studies should be performed on large set of samples applying robust univariable and multivariable analyses and complex statistical methodologies, *i.e.,* a generalized estimating equation model to consider the issue of subsequent cycles in the same woman, embryos from the same woman, etc.

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