In this issue: Celebrating the 70th Anniversary of the Hungarian Society of Laboratory Medicine

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

Non-invasive assessment of viability in human embryos fertilized in vitro

Gábor L. Kovács[1,2,3], Gergely Montskó[1,2,3], Zita Zrínyi[1,2], Nelli Farkas[4], Ákos Várnagy[3,5], József Bódis[2,3,5]

[1] *Department of Laboratory Medicine, Faculty of Medicine, University of Pécs*
[2] *Szentágothai Research Centre, University of Pécs*
[3] *MTA-PTE Human Reproduction Scientific Research Group, University of Pécs*
[4] *Institute of Bioanalysis, Faculty of Medicine, University of Pécs*
[5] *Department of Obstetrics and Gynecology, University of Pécs*

ARTICLE INFO

Corresponding author:
Univ. Prof. Gábor L. Kovács MD, PhD, D.Sc.
Department of Laboratory Medicine
Faculty of Medicine, University of Pécs, Hungary
Phone: +3672536120
Fax: +3672536121
E-mail: kovacs.l.gabor@pte.hu

Key words:
in vitro fertilization, embryo viability assessment, morphology, biomarker, mass spectrometry, non-invasive diagnostics

Ethical approval and supervision:
The study protocol was approved by the Committee of Human Reproduction, National Science Council of Hungary (5273-2/2012/HER), and later supervised by the Public Health Officer, Hungarian Government Office in Baranya County (BAR/006/58-2/2014).

Acknowledgements:
The presented publication was supported by the NKFI-EPR K/115394/2015 (Early biochemical indicators of embryo viability).

ABSTRACT

Human reproduction is a relatively inefficient process and therefore the number of infertile couples is high. Assisted reproductive technologies (ART) have facilitated the birth of over five million children worldwide. ART, however, superimposes its own relative inefficiency on the preexisting inefficiency of normal reproduction. The efficiency (expressed as pregnancy rate) is generally not more than 30%. Modern reproductive medicine is gradually moving from multiple embryo transfer to the transfer of a single embryo, mainly because of obvious and unwanted side effects of multiple embryo transfer (e.g. „epidemic" multiple pregnancies). This concept, however, requires a fast, professional selection of the most viable embryo during the first few days of ART. Thus the aim of a modern ART is the safe transfer of a healthy, viable, single embryo. Accurate and rapid methods of quantifying embryo viability are needed to reach this goal. Methodological advances have the potential to make an important contribution, and there has been a drive to develop alternative non-invasive methods to better meet clinical needs. Metabolic and genetic profiling of spent embryo culture (SEC) media should offer an exceptional opportunity for the assessment

of embryo viability. The current review focuses on the latest non-invasive diagnostic approaches for pre-implantation viability assessment of in vitro fertilized embryos.

❖❖❖❖❖

INTRODUCTION

Infertility has been recognized as a public health issue worldwide (1) leading to an increasing need to the use of assisted reproductive technologies (ART), including in vitro fertilization (IVF). After the first reported case of IVF in 1978 (2) ART enabled millions of people to have their own children in cases when pregnancy did not occur under natural circumstances. ART has advanced significantly and became more and more widespread resulting in ca. 700,000 cycles a year in the USA and Europe together (3,4). Despite of evolving intracytoplasmatic sperm injection (ICSI) technique the rate of the successful embryo implantations is surprisingly low (5,6). A success rate of 25% and 28% has been reported in 2005 (7) and 2008 (8), respectively. Nowadays, this rate went up to 32% (9), which cannot be considered as a significant development. Earlier clinical protocols preferred multiple embryo transfer, but multiple gestations can result in the increased risk of preterm delivery (10-16). Other studies report that multiple gestations also increased the risk of low birth weight cerebral palsy (17). In the US alone, preterm births resulting from multiple pregnancies during IVF cause a 1 billion USD extra cost to the social insurance (18). In order to exclude the discussed risk factors, single embryo transfer becomes the standard of care for all. It is imperative, however, that accurate and economical methods should be developed to ensure that the most viable euploid embryo is selected for transfer. Ideally, such tests would be noninvasive, lessening the risks to the embryo and reducing costs and workload in the embryology laboratory (19). The biggest issue with pre-implantation viability assessment is that due to ethical reasons any assay should be completely non-invasive because no one can predict what kind of interference would be the unwanted result in the later embryonic development.

THE MORPHOLOGICAL APPROACH

The most apparent – and routinely applied - way of the assessment of viability is the morphological evaluation of in vitro fertilized embryos using microscopy. There are several morphological features described which could be used for viability assessment purposes, these are dependent on the time spent after fertilization. Right after fertilization in the 1-cell embryo the size and symmetry of the two pronuclei can be examined. The time of the first cell division is also a good predictor of later implantation potential, as zygotes that divide early tend to develop more frequently to the blastocyst stage. Criteria as cleavage rate and blastomere shape and symmetry, an adequate trophectoderm layer (TE) and an inner cell mass (ICM) is a morphological marker of the later stages (5,20). Not only can the morphology of the fertilized embryo be used for further prediction of implantation potential, but morphological defects of the retrieved oocyte as well. Fertilization and pregnancy rate correlates with the grade of cumulus-oocyte complexes, and embryos originating from dysmorphic oocytes show a larger grade of pregnancy loss (21-23). The cleavage stages of morulae and blastocysts or the symmetry and patterns of cell division are also notable and frequently used aspects, and are often examined during the prediction of embryo viability (23). The biggest issue of morphological assessment is that it is still a highly subjective method (20). The reason is partly due to the fact that the final decision is made by a clinician, and not by an objective test result, and secondly it is does matter how important are the individual

morphological features in the final conclusion (24-26). To overcome the different practice of laboratories worldwide in 2011 an international consensus (Istanbul Consensus) has been reached on embryo viability assessment (27). The selected morphological markers of respective stage embryos, the weighing of individual features and a scoring system has been set up. The limitations due to static time-point observation, is now solved with the use of time-lapse microscopy (28,29). Time-lapse microscopy also enables the observation of dynamics of cytoplasmic movements and cytokinesis, reflecting the functionality of microtubule and actin cytoskeleton, which is critical for proper development. In our laboratory, we aimed to improve the success rate of implantation by adopting and further optimizing the Istanbul consensus. This score has been called as the optimized criteria system (OCS). According to this scoring, 3-Day old embryos were divided into two subgroups: the subgroup with low blastomere number (less than 7) and with high blastomere number (7 or more). Symmetric position of blastomeres indicates the rate of symmetry of holoblastic cleavage along the embryo axis. It was classified as good (full symmetry); fair (light asymmetry); or poor (evident asymmetry). The percent values of fragmentation are based on the ratio of fragmented to total cell numbers. As a further modification to the Istanbul consensus, the assessment of fragmentation was slightly changed. Embryos were considered as good if the fragmentation rate was <15% (instead of the original 10%). This shift from 10 to 15% was the result of our observation that a fairly high proportion of the embryos between 10-15% appeared to be viable. In summary the optimized criteria system (OCS) highlights 3 modified or new parameters: fragmentation (with a more permissive criterion of <15% in the "good" category); symmetry and the blastomere number. In addition, the blastomere size was evaluated according to the original Istanbul consensus. A scoring-map was created to facilitate the evaluation (Table 1) As far as the 5-Day old embryos are concerned, we modified the original Istanbul Consensus for blastocysts by leaving out the hatched stage from the evaluation. The Istanbul Consensus for the 5-day old embryos has a shortcoming, i.e. it does not express the viability of embryos with a single category (good, fair, poor). We tried to overcome this by using a scoring map (Table 2). In conclusion, we constructed a composite score for Day-3, as well as Day-5 old embryos, based on morphological parameters. As it is evident from the results, this composite score is sensitive to evaluate viability (Figure 1)

THE BIOCHEMICAL APPROACH

Another possibility for non-invasive embryo viability assessment is the metabolomic examination of the culture medium surrounding the in vitro fertilized embryo. Metabolomic, (proteomic) profiling of spent embryo culture (SEC) offers an exceptional, non-invasive opportunity for the assessment of embryo viability (30,31). The metabolomic profiling (32,33) of early embryo development might mean the analysis of the total metabolome by following the changes of several selected compounds, metabolomic analysis using unidentified, but significantly differing metabolomic changes, or by the analysis of a limited population of nutrients or end products. The common feature in all three concepts is that they are concentrating on the metabolomic alterations caused by differently developing embryos in the culture medium. Very simple idea is the monitoring of glucose consumption or pyruvate formation, since this would directly indicate the metabolism of the developing embryo and it is an obvious conclusion that a metabolically active embryo would have higher implantation potential. Some authors reported that the identification of these parameters

Table 1 The composite score of the "optimized scoring system" for Day-3 old embryos

ICCS for cleavage stage embryos		
Good	**Fair**	**Poor**
<10% fragmentation	10–25% fragmentation	Severe fragmentation (>25%)
Stage-specific cell size	Stage-specific cell size for majority of cells	Cell size not stage specific
No multinucleation	No evidence of multinucleation	Evidence of multinucleation

OCS for cleavage stage embryos							
Fragmentation		**Blastomere size**		**Number of blastomeres**		**Symmetry**	
1	Good (<15%)	1	Stage specific	1	≥7	1	Symmetric cleavage
2	Fair (15-25%)	2	No stage specific	2	<7	2	Light asymmetry
3	Fair (15-25%)	-	-	-	-	3	Evident asymmetry

Scoring map							
Good		**Fair**			**Poor**		
1111	1121	1112	1112	1131	2132	2212	2221
1211	1221	1132	1212	1222	2222	2231	2232
2111	2121	1231	1232	2112	3131	3132	3211
2211	-	2122	2131	3111	3212	3221	3222
-	-	3112	3121	3122	3231	3232	-

resulted in successful prediction of embryo implantation potential, but other research groups describe contradictory results (34, 35). The amino acid profile of culture media is also used in the prediction of implantation potential, though not exclusively as an independent parameter, rather in combination with morphological features (36). The detection of unidentified metabolomic changes using near infra-red (NIR) or Raman spectroscopy (37, 38) is a very interesting and challenging possibility.

More complicated is the concept when unknown, new biomarker molecules of embryo viability are searched for, assuming that these biomarkers were secreted by the embryo. The difficulty of the concept is that only 4-8 cells are present in the culture medium; thus a very sensitive analytical tool is required. Mass spectrometry (MS)

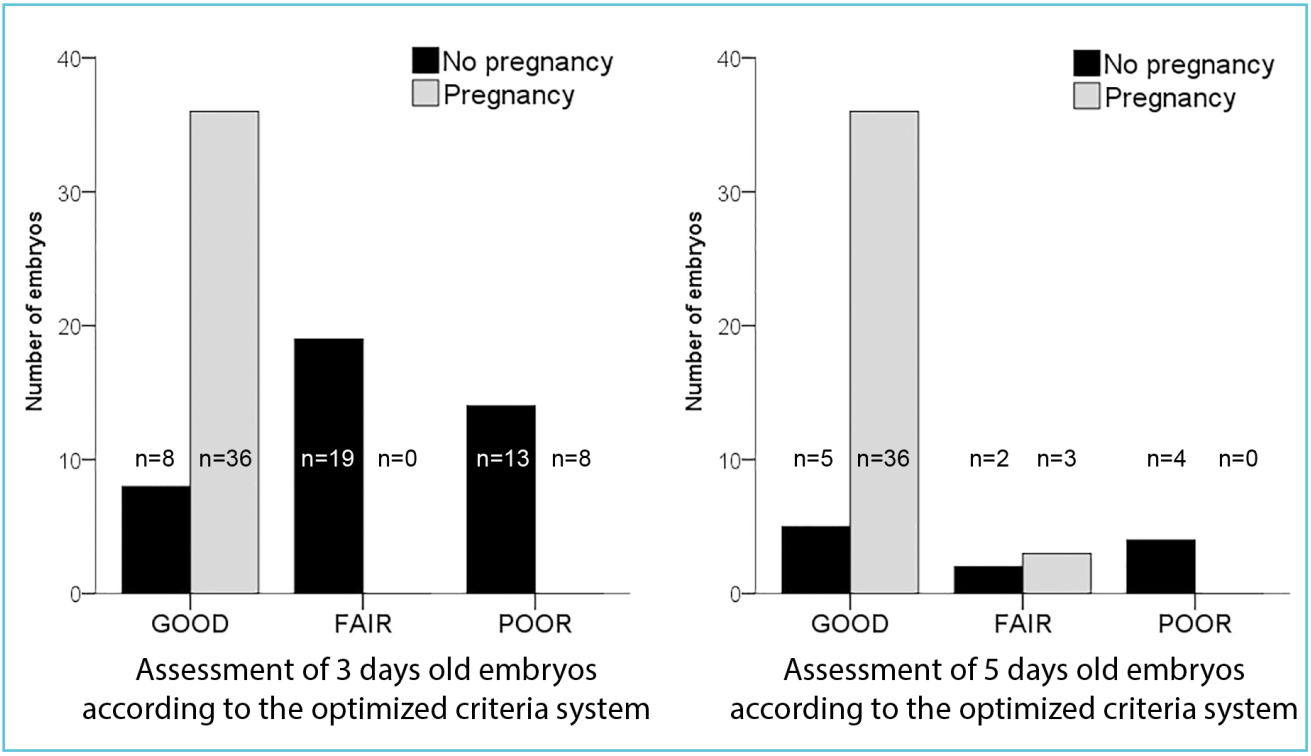
Table 2 The composite score of the "optimized scoring system" for Day-5 old embryos

ICCS for blastocysts (Day-5)	
Stage	
1	Early blastocyst
2	Blastocyst
3	Expanded blastocyst
4	Hatched/hatching
ICM	
Good	Prominent, easily discernible, with many cells that are compacted and tightly adhered together
Fair	Easily discernible, with many cells that are loosely grouped together
Poor	Difficult to discern, with few cells
TE	
Good	Many cells forming a cohesive epithelium
Fair	Few cells forming a loose epithelium
Poor	Very few cells

OCS for blastocyst (Day-5) – scoring map					
Good		**Fair**		**Poor**	
111	112	122	132	133	223
113	121	213	222	231	232
123	131	313	322	233	323
211	212	-	-	331	332
221	311	-	-	333	-
312	321	-	-	-	-

has the potential of specific and sensitive quantification in a wide spectrum of molecular mass ranges and therefore suites well the needs of metabolomic or proteomic fingerprinting and quantification. In parallel to the spreading of mass spectrometry, proteomics is also an emerging field in the understanding of embryo development (39,40). The analysis of the embryonic secretome (41,42) provides information of the total transcriptome of the developing embryos. Mass

Figure 1 The sensitive "optimized scoring system"



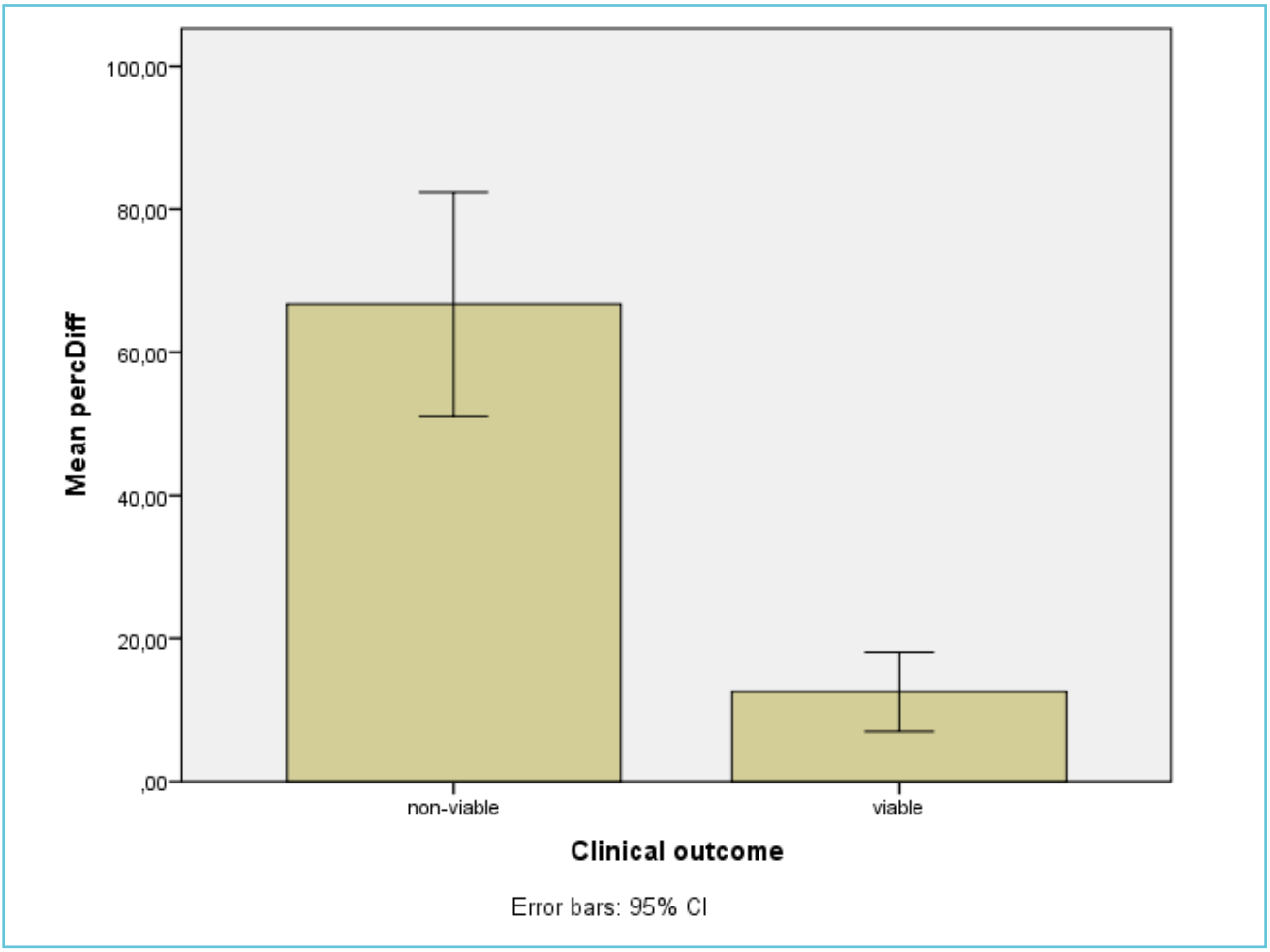
Day-3 old embryos (left panel): Pregnancy occurred only in the group evaluated as good quality embryo. No pregnancies occurred if the embryos were assigned to the fair or poor quality groups. It should also be noted the good quality embryo does not necessarily mean pregnancy (maternal causes of infertility may be present).

Day-5 old embryos (right panel): With the exception of a very few cases, most pregnancies occurred in the good quality group.

spectrometry can be used both in targeted and discovery analysis with accurate quantification of identified biomarkers after molecular identification by bottom-up or top-down proteomics using tandem or multiple MS (43-46).

In a recent publication from our laboratory (47) using liquid chromatography coupled mass spectrometry (LC-MS), a fragment of the human haptoglobin molecule was identified in the culture medium. Rather than analyzing the embryonic secretome, the aim this experiment was to use preexisting molecules present in the cell culture media as biomarkers. Haptoglobin - which was detected in the culture medium - is not a product of the developing embryo; the polypeptide is a contaminant of the human serum albumin standard used to supplement the culture medium (47,48). During the first three days of embryo development the formation of a subunit (alfa-1) of the human haptoglobin molecule was observed. This subunit similar to the total haptoglobin molecule was detectable in the blank control medium samples as well. The differentiation of the viable and non-viable embryos was done using the observation that compared to blank controls the samples of embryos which later did not resulted in pregnancy contained the alpha-1 subunit in a much larger quantity than the samples of embryos which did (Figure 2). 160 samples of 77 Day-3 old embryos were analyzed. Clinical statistical analysis of the results revealed that the specificity of the diagnostic test was 64%, while the sensitivity was 100%. It is more informative that the

Figure 2 The mean difference in the amount of the haptoglobin alpha-1 peptide fragment between viable and non-viable embryos



The mean difference in the amount of the haptoglobin alpha-1 peptide fragment between viable and non-viable embryos.

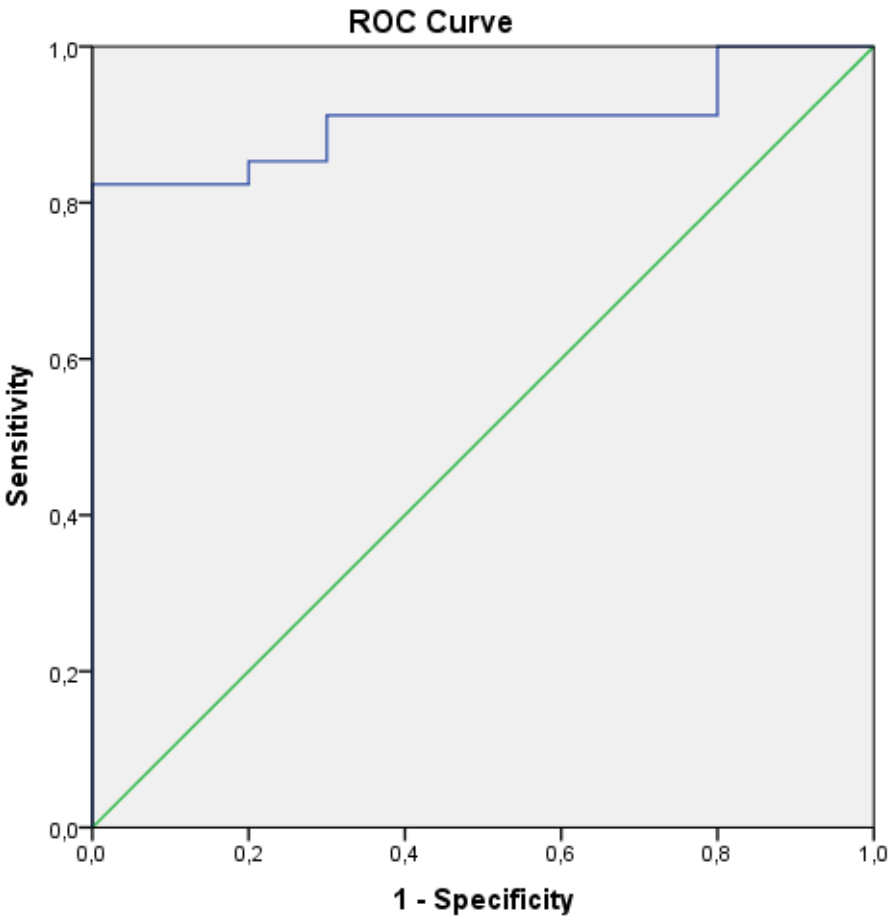
The quantification of the biomarker (n=160) was carried out by mass spectrometric detection following reverse-phase HPLC separation; the analyzed sample volume was 25 µl. In the samples of the non-viable embryos (no pregnancy) the fragment was present in a significantly larger (p<0.001) content than in the sample of the viable (live birth) embryos.

positive predictive value of the assay was 51% and – maybe more importantly – the negative predictive value was 100%.

Receiver operating characteristic (ROC) analysis provides tools to select possibly optimal models and to discard suboptimal ones. ROC analysis is related in a direct and natural way to cost/benefit analysis of diagnostic decision-making. The ROC curve of the morphological versus metabolomic approach in relation to the correct prediction of pregnancy outcome is illustrated in Figure 3. It is obvious that our biochemical investigation method enables a selection of the embryos by sorting out the non-viable ones. The test selected with 100% potential the embryos, which did not lead to successful implantation at all.

One of the areas of collaboration between clinicians, the clinical laboratory and the research laboratory at the University of Pécs is related to the research of infertility. Since the clinical background gives the beauty and the medical

Figure 3 Receiver operating characteristics (ROC) curve of the blinded, retrospective cohort study



Embryo viability prediction was made using the data of the mass spectrometric haptoglobin alpha-1 chain quantification. n=160, the area under the curve (AUC) is 0.906.

A ROC curve (blue line) visualizes a calculation by graphing the sensitivity on the y-axis (vertical) and the false positive rate (1-specificity) on the x-axis (horizontal) for all possible cut-off values of the diagnostic test. The green line is the reference line. The AUC is used as a measure of the performance of a diagnostic test against the ideal and may also be used to compare different tests.

importance of laboratory research, it was of outstanding importance for us to receive the EFLM-Abbott Diagnostics Award for Excellence in Outcomes Research in Laboratory Medicine (Paris, 2015), the award given to the best published paper (47), as judged by an independent panel of experts, which demonstrates improved outcomes arising out of the application or improved utilization of an in-vitro diagnostics test. This short review summarizes some of our recent findings and views on this field.

REFERENCES

1. Boivin J, Bunting L, Collins JA, Nygren KG. International estimates of infertility prevalence and treatment-seeking: potential need and demand for infertility medical care. Human Reproduction 2007;22(6):1506-1512.

2. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet 1978;12;2(8085):366.

3. Seli E, Sakkas D, Scott R, Kwok SC, Rosendahl SM, Burns DH. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. Fertil Steril. 2007;88(5):1350-1357.

4. Ferraretti AP, Goossens V, de Mouzon J, Bhattacharya S, Castilla JA, Korsak V et al. Assisted reproductive technology in Europe, 2008: results generated from European registers by ESHRE dagger. Hum Reprod. 2012;27(9):2571-2584.

5. Hardy K, Stark J, Winston RML. Maintenance of the inner cell mass in human blastocysts from fragmented embryos. Biol of Reprod. 2003;68(4):1165-1169.

6. Fancsovits P, Toth L, Takacs ZF, Murber A, Papp Z, Urbancsek J. Early pronuclear breakdown is a good indicator of embryo quality and viability. Fertil Steril. 2005;84(4):881-887.

7. Dawson KJ, Conaghan J, Ostera GR, Winston RM, Hardy K. Delaying transfer to the third day postinsemination, to select non-arrested embryos, increases development to the fetal heart stage. Hum Reprod. 1995;10(1):177-182.

8. Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study. Fertil Steril. 2008;90(1):77-83.

9. Ferraretti AP, Goossens V, Kupka M, Bhattacharya S, de Mouzon J, Castilla JA et al. Assisted reproductive technology in Europe, 2009: results generated from European registers by ESHRE. Hum Reprod. 2013;28(9):2318-2331.

10. Ghobara TS, Cahill DJ, Ford WCL, Collyer HM, Wilson PE, Al-Nuaim L, Jenkins JM. Effects of assisted hatching method and age on implantation rates of IVF and ICSI. Reprod Biomed Online. 2006;13(2):261-267.

11. Halliday J. Outcomes of IVF conceptions: are they different? Best Pract Res Clin Obstet Gynaecol. 2007;21(1):67-81.

12. Hansen M, Bower C, Milne E, de Klerk N, Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects - a systematic review. Hum Reprod. 2005;20(2):328-338.

13. Kissin DM, Schieve LA, Reynolds MA. Multiple-birth risk associated with IVF and extended embryo culture: USA, 2001. Hum Reprod. 2005;20(3):2215-2223.

14. Criniti A, Thyer A, Chow G, Lin P, Klein N, Soules M. Elective single blastocyst transfer reduces twin rates without compromising pregnancy rates. Fertil Steril. 2005;84(6):1613-1619.

15. Pinborg A, Loft A, Ziebe S, Andersen AN. What is the most relevant standard of success in assisted reproduction? Is there a single 'parameter of excellence'? Hum Reprod. 2004;19(5):1052-1054.

16. Göçmen A, Güven Ş, Bağci S, Çekmez Y, Şanlıkan F. Comparison of maternal and fetal outcomes of IVF and spontaneously conceived twin pregnancies: three year experience of a tertiary hospital. Int J Clin Exp Med. 8(4):6272-6276.

17. Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study. Fertil Steril. 2008;90(1):77-83.

18. Kovalevsky G, Patrizio P. High rates of embryo wastage with use of assisted reproductive technology: a look at the trends between 1995 and 2001 in the United States. Fertil Steril. 2005;84:325-330.

19. Steer CV, Mills CL, Tan SL, Campbell S, Edwards RG. The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. Hum Reprod. 1992;7(1):117-119.

20. Ajduk A, Zernicka-Goetz M. Quality control of embryo development. Mol Aspects Med. 2013;34(5):903–918.

21. Sela R, Samuelov L, Almog B, Schwartz T, Cohen T, Amit A et. al. An embryo cleavage pattern based on the relative blastomere size as a function of cell number for predicting implantation outcome. Fertil Steril. 2012;98(3):650-656.

22. Ng ST, Chang TH, Wu TC. Prediction of the rates of fertilization, cleavage, and pregnancy success by cumulus-coronal morphology in an in vitro fertilization program. Fertil Steril. 1999;72(3):412-417.

23. Boiso I, Veiga A, Edwards RG. Fundamentals of human embryonic growth in vitro and the selection of high-quality embryos for transfer. Reprod Biomed Online. 2002;5(3):328-350.

24. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. Curr Opin Obstet Gynecol. 1999;11(3):307-311.

25. Gardner DK, Reed L, Linck D, Sheehan C, Lane M. Quality control in human in vitro fertilization. Semin Reprod Med. 2005;23(4):319-324.

26. Stephenson EL, Braude PR, Mason C. International community consensus standard for reporting derivation of human embryonic stem cell lines. Regen Med. 2007;2(4):349-362.

27. Balaban B, Brison D, Calderon G, Catt J, Conaghan J, Cowan L et al. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Reprod Biomed Online. 2011;22(6):632-646.

28. Lemmen JG, Agerholm I, Ziebe S. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. Reproductive Reprod Biomed Online. 2008; 17(3):385–391.

29. Brown R, Harper J. The clinical benefit and safety of current and future assisted reproductive technology. Reprod Biomed Online. 2012;25(2):108–117.

30. Nagy ZP, Sakkas D, Behr B. Symposium: innovative techniques in human embryo viability assessment. Non-invasive assessment of embryo viability by metabolomic profiling of culture media ('metabolomics'). Reprod Biomed Online. 2008;17(4):502–507.

31. Seli E, Robert C, Sirard MA. OMICS in assisted reproduction: possibilities and pitfalls. Mol Hum Reprod. 2010;16(8):513–530.

32. Rødgaard T, Heegaard PM, Callesen H. Non-invasive assessment of in-vitro embryo quality to improve transfer success. Reprod Biomed Online. 2015;31(5):585-592.

33. Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. Fertil Steril. 2013;99(4):1062-1072.

34. Devreker F, Hardy K, Van den Bergh M, Winston J, Biramane J, Englert Y. Noninvasive assessment of glucose and pyruvate uptake by human embryos after intracytoplasmic sperm injection and during the formation of pronuclei. Fertil Steril. 2000;73(5):947-954.

35. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertil Steril. 2001;76(6):1175-1180.

36. Sturmey RG, Brison DR, Leese HJ. Symposium: innovative techniques in human embryo viability assessment. Assessing embryo viability by measurement of amino acid turnover. Reprod Biomed Online. 2008;17(4):486-496.

37. Vergouw CG, Botros LL, Judge K, Henson M, Roos P, Kostelijk EH et. al. Non-invasive viability assessment of day-4 frozen-thawed human embryos using near infrared spectroscopy. Reprod Biomed Online 2011;23(6):769-776.

38. Seli E, Sakkas D, Scott R, Kwok SC, Rosendahl SM, Burns DH. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. Fertil Steril. 2007;88(5):1350-1357.

39. Katz-Jaffe MG, Gardner DK, Schoolcraft WB. Proteomic analysis of individual human embryos to identify novel biomarkers of development and viability. Fertil Steril. 2006;85(1):101-107.

40. Nyalwidhe J, Burch T, Bocca S, Cazares L, Green-Mitchell S, Cooke M, Birdsall P, Basu G, Semmes OJ, and Oehninger S. The search for biomarkers of human embryo developmental potential in IVF: a comprehensive proteomic approach. Mol Hum Reprod. 2013;19(4):250–263.

41. Katz-Jaffe MG, Schoolcraft WB, Gardner DK. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. Fertil Steril. 2006;86(3):678-685.

42. Cortezzi SS, Garcia JS, Ferreira CR, Braga DP, Figueira RC, Iaconelli A Jr, Souza GH, Borges E Jr, Eberlin MN. Secretome of the preimplantation human embryo by bottom-up label-free proteomics. Anal Bioanal Chem. 2011;401(4):1331-1339.

43. Combelles CM, Holick EA, Racowsky C. Release of superoxide dismutase-1 by day 3 embryos of varying quality and implantation potential. J Assist Reprod Genet. 2012;29(4):305-311.

44. Sher G, Keskintepe L, Fisch JD, Acacio BA, Ahlering P, Batzofin J, Ginsburg M. Soluble human leukocyte antigen G expression in phase I culture media at 46 hours after fertilization predicts pregnancy and implantation from day 3 embryo transfer. Fertil Steril. 2005;83(5):1410-1413.

45. Mains LM, Christenson L, Yang B, Sparks AE, Mathur S, Van Voorhis BJ. Identification of apolipoprotein A1 in the human embryonic secretome. Fertil Steril. 2011;96(2):422-27.

46. McReynolds S, Vanderlinden L, Stevens J, Hansen K, Schoolcraft WB, Katz-Jaffe MG. Lipocalin-1: A potential marker for noninvasive aneuploidy screening. Fertil Steril. 2011 30;95(8):2631-2633.

47. Montskó G, Zrínyi Z, Janáky T, Szabó Z, Várnagy Á, Kovács GL, Bódis J. Noninvasive embryo viability assessment by quantitation of human haptoglobin alpha-1 fragment in the in vitro fertilization culture medium: an additional tool to increase success rate. Fertil Steril. 2015;103(3):687-693.

48. Darcel CL, Kaldy MS. Further evidence for the heterogeneity of serum albumin. Comp Biochem Physiol B. 1986;85(1):15-22.