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REVIEWS
AND THEORETICAL ARTICLES

Preimplantation Genetic Diagnosis by Blastocentesis: Problems and Perspectives

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Abstract—The discovery of cell-free DNA in blastocoele fluid opens new perspectives for the development of preimplantation genetic diagnosis of human chromosomal and genetic diseases. In this review we analyzed the results of the first studies, which made it possible to evaluate the effectiveness of the application of a new source of biological material and showed a high degree of agreement between the results of molecular karyotyping with cell-free DNA and blastocyst cells. The results suggest the possibility of developing a noninvasive method of preimplantation genetic diagnosis, which may open a new round of progress in the field of assisted reproductive technologies and the genetics of early stages of human ontogenesis.

Keywords: blastocentesis, cell-free DNA, preimplantation genetic diagnosis, chromosomal mosaicism, *in vitro* fertilization

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INTRODUCTION

Cell-free DNA was detected in blastocoele fluid in 2013. The amount of DNA was enough to perform whole-genome amplification and PCR [1]. This discovery supported the possibility of using this DNA for preimplantation genetic diagnosis. It is worth mentioning that such an approach would make it possible to solve simultaneously several problems that are regularly faced by specialists performing in vitro fertilization. For example, there are cases when, due to insufficient blastocyst quality, the use of the trophectoderm cells for preimplantation diagnosis of diseases becomes impossible, because the trophectoderm biopsy is possible only for blastocysts of good quality [2]. Moreover, the intake of cells even from good-quality blastocysts can be accompanied by a risk of embryo injury, which may interrupt its further development and decrease the ability of implantation. Therefore, the use of DNA from blastocoele fluid in clinical practice seems to be extremely attractive. However, a wide range of questions remains open [3], making its application difficult.

Does the cell-free DNA in a blastocoele reflect the karyotype or genotype of all embryonic cells? If so, then to what extent?

Which type of DNA makes the largest contribution to cell-free DNA—the DNA from trophectoderm cells or that from the developing inner cell mass? How can it influence the effectiveness of chromosomal mosaicism diagnosis, which is achieved by tissue-specific compartmentalization of cells with chromosomal mutations?

Are abnormal or apoptotic cells the source of cell-free DNA? Is it mainly the cells with chromosomal mutations that undergo apoptosis? In this case, is it possible to use cell-free DNA analysis to judge the chromosomal constitution of cells remained in the blastocyst?

Does the fluid-removal procedure avoid the damage to a portion of cells and the contamination of the analyzed cell-free DNA sample with cellular contents?

Can the DNA fragments appear not only in blastocoele fluid but also in the culture medium? In this case culturing of the embryos in the minimally acceptable medium volume and cell-free DNA analysis in these media would be an option for noninvasive diagnosis.

The first steps in answering these questions have recently been undertaken by Gianaroli et al. They aimed to determine the degree of karyotype similarity detected in an analysis of blastocoele DNA, with chromosome sets obtained by karyotyping polar bodies, blastomeres, trophectoderm cells, and the whole blastocyst [4]. They demonstrated a high level of agreement with the results of molecular cytogenetic

analysis. However, a whole range of questions arose. It became obvious that the methods of blastocoele fluid removal are not as simple as had been suggested. It was shown that the analysis results in some cases only partially coincide and are sometime even totally different. These questions cannot remain open, and they require further intense study by cytogeneticists and embryologists.

CELL-FREE BLASTOCOELE DNA AS A SOURCE OF MATERIAL FOR PREIMPLANTATION GENETIC DIAGNOSIS

Blastocyst formation is related to the origin of the trophectoderm epithelium and begins after the appearance of ion gradients and the osmotic accumulation of fluid coming through the cell layer [5]. Membrane ion transporters and channels, particularly Na/K-ATPase, are actively involved in this process [6]. Fluid coming into the blastocyst through aquaporins (AQP) accumulates and thereby widens the inner space of the blastocyst, resulting in cavity formation—the blastocoele [7]. These processes take place 4–5 days after fertilization. The *zona pellucida* then becomes thin, and the blastocyst hatches. At this moment the embryo is ready for implantation in the uterus.

Embryo cryopreservation by vitrification is used in the modern clinical practice of in vitro fertilization (IVF). Before this procedure is carried out, the blastocoele fluid is removed or the blastocyst is collapsed in order to increase embryo survival at the blastocyst stage. It has been shown that collapsing before the vitrification has a positive influence on blastocyst viability, which makes it possible to increase the chance of pregnancy onset after the IVF cycle [8, 9]. This procedure can be performed with different methods, including laser pulse, micropipetting, microneedle puncture, and fluid removal. The obtained 0.3–0.5 nL of the fluid is not used. However, Palini et al. for the first time detected the presence of cell-free DNA in the fluid [1].

How does DNA appear in the blastocoele fluid? The most likely source of cell-free DNA is found in embryonic cells undergoing apoptosis. Programmed cell death occurs even at the preimplantation stage of development [10], as well as at the beginning of implantation [11] and during placenta creation [12]. However, it is yet not fully clear why and which cells undergo apoptosis. It is possible that apoptosis is necessary for the elimination of functionally deficient cells and thus becomes involved in cellular differentiation during the early stages of ontogenesis [13]. The question of the role of apoptosis in the embryogenesis remains open. However, regardless of the causes of cellular apoptosis, DNA is indeed present in blastocoele fluid [1]. Using the discovery of Palini and colleagues, researchers can make cell-free DNA at the preimplantation stages of development the object of their focused attention and investigation.

The main methods used by Palini and her colleagues for the genetic analysis of cell-free DNA were Whole-Genome Amplification (WGA), PCR, and Array Comparative Genomic Hybridization (array-CGH). WGA was performed for five samples, which resulted in an increased amount of DNA from the fluid. To confirm the WGA efficiency, PCR for two regions of *MAP1LC3B* and *TBC1D3*, which are located on the chromosomes 16 and 17 respectively, genes was performed. Amplification of the *TSPY1* gene, which is located on the Y chromosome, was also performed. WGA appeared to be successful for four samples, two of which during PCR showed *TSPY* gene amplification. DNA was not detected in one case. This could be due to the initial absence of DNA in the blastocyst fluid. Moreover, some methodological problems of WGA associated with such a small initial amount of DNA cannot be excluded. For example, it is worth mentioning that the WGA quantitative parameters in all samples were comparable, including the sample in which DNA was not found to be present and in the negative control. The authors explain it with a WGA artifact that is related to the occasional origin of primer dimers. Molecular cytogenetic analysis with array-CGH was performed for two samples. It was shown that both samples are aneuploid and have the following karyotypes: 47,XY,+22 and 46,XY,–1,–10, +11,+16. However, it should be noted that these karyotypes do not reflect the chromosomal set of cells containing the DNA that appeared in the blastocyst fluid. In particular, it is not possible to say if aneuploidies for the four chromosomes were present in the second sample in each cell at the same time or if the karyotype was determined due to the analysis of cell-free DNA from different cells with numerical abnormalities of different chromosomes.

The application of PCR in this work was also intended to estimate the number of copies of the single-copy *GAPDH* gene and multiple-copy *TBC1D3* gene. The blastocoele fluid was investigated by *GAPDH* amplification in 16 samples. Amplified product was detected in only nine samples. Two types of products with different melting temperatures were observed. It was shown that such results are the consequence of nonspecific amplification of the *GAPDH* pseudogene (*GAPDHP1*), which is related to the characteristics of the used primer. Though the obtained results indicate that in 9 of 16 samples (56%) of blastocoele fluid, DNA was present and it was successfully amplified. Moreover, DNA of blastocoele fluid from 31 blastocysts was tested via the amplification of *TBC1D3* gene sequence. Two samples were excluded from the analysis due to the methodological reasons. The frequency of cell-free DNA detection appeared 89.7% (26/29).

The authors consider that the blastocoele fluid DNA can be used to determine the fetus sex in case of

X-associated diseases in families [1]. The PCR-test was proposed based on amplification of the *TSPY1* gene from the Y chromosome and the *TBC1D3* control gene from chromosome 17. The amplification efficiency during PCR was 98 and 95% for the *TSPY1* and *TBC1D3* genes respectively. Of the 26 samples showing *TBC1D3* gene product, 17 samples also demonstrated *TSPY1* amplification. It is interesting that the *TSPY1* amplicon was detected in 17 of 26 samples, which demonstrates some excess of male embryos. A sexual imbalance could originate for several reasons. First, a small number of samples were included in the analysis, which could lead to sample bias. Second, such a sex ratio can be related to the fact that male embryos have a higher cleavage rate than female [14]; therefore, they are selected as being of better quality.

The average amount of genomic DNA in the fluid was 9.9 pg per sample. It should be noted that a concentration of DNA less than 5 pg did not allow performance of the analysis. Although PCR was successful for most of samples (89.7%), the overall effectiveness of this approach was lower than of the analysis performed for one or several blastomeres (98–99%) [15]. It also should be emphasized that Palini et al. did not verify the karyotyping of blastomeres corresponding to the blastocoele fluid samples.

Thus, for the first time it was shown with a high degree of probability that DNA can be isolated from blastocoele fluid, followed by the whole-genome amplification and analysis with PCR and array-CGH. If it were possible to increase the frequency of successful WGA by the modified methods, then the introduction of such approach into the clinical practice would be quite possible. However, here the central question appears: how does this DNA reflect the karyotypes of embryonic cells? There are currently very few publications in the literature related to DNA analysis involving blastocoele fluid. This is partly due to the relatively recent detection of DNA in the fluid, as well as the need to use rather complex and expensive methods for its study. Moreover, a mandatory condition of performing analysis in this field is collaboration with embryologists and, consequently, with clinics of reproductive medicine. Thus, the first and the only study to date demonstrated extremely interesting results in this question.

At the end of 2014, a published paper by Gianaroli et al. reported on pilot DNA analyses from blastocoele fluid [4]. Their goal was to compare the results of cell-free DNA analysis with karyotypes of trophectoderm cells, polar bodies, or whole blastocysts. Seventeen pairs undergoing preimplantation genetic diagnosis with array-CGH were included in the analysis. This method makes it possible to detect within a single study all of the unbalanced numerical and structural chromosome abnormalities, in contrast to FISH-analysis, in which aneuploidy diagnosis is performed only for some chromosomes. The use of comparative genomic hybridization for blastocoele cell-free DNA analysis can, in theory, solve the problem of chromosomal mosaicism diagnosis, because the analysis results would simultaneously reflect the karyotypes of all dead blastocyst cells; therefore, this analysis can provide information on all or most of the abnormalities in the blastocyst. However, the limitations of array-CGH cannot be forgotten. Comparative genomic hybridization cannot detect balanced chromosomal rearrangements (translocations and invesrtions) and polyploidy, as well as changes in the DNA sequence unrelated to the copy number variation. In comparison with FISH, which is currently more often used in clinical cycles of IVF and PGD, CGH is rather laborious and can last for 72 h when metaphase chromosomes are used as the hybridization matrix. Such terms do not allow the transferral of an embryo into the uterus within five days, which makes it difficult to perform the IVF cycle. This problem is solved with blastocyst cryopreservation. However, this deficiency, on the contrary, becomes an advantage when a method based on blastocoele-fluid DNA analysis is introduced, because blastocoele fluid removal is more often associated with cryopreservation. Moreover, the modern protocols of array comparative genomic hybridization make it possible to perform the whole analysis during 24 h, including the whole genome amplification. Obviously, the array-CGH was the most suitable method for Gianaroli's group, since the main focus in the work was on numerical chromosome abnormalities.

In the study 71 blastocysts were obtained from 17 patients, i.e. 69% of the total number of fertilized oocytes. This figure did not changed after the polar body and blastomere biopsy, confirming the results of studies showing no harm to embryos from the biopsy. Blastocoele fluid was obtained from 51 blastocysts. For 37 of them, a preliminary polar body biopsy was performed; for 14, a biopsy of one blastomere at the cleavage stage from 6–8-cell morula was carried out. The blastocyst fluid was removed with a micropipette injected into the blastocyst at the point of contact between two trophectoderm cells in order to exclude cytoplasm aspiration. After WGA, DNA was detected in 39 fluid samples (76.5%). The average amount of DNA after whole-genome amplification was 900.38 ng/mL (876.3–939.5 ng/mL).

Blastocoele fluid samples obtained from blastocysts with the preliminary polar body biopsy were tested by array-CGH 30, and nine samples from blastocysts underwent blastomere biopsy. It was shown that blastocoele DNA reflects the karyotype predicted by the polar body analysis in 93.3% of cases and that from blastomere analysis in 100% of cases. The results did not coincide only in two cases (5.1%). In one case multiple aneuploidies were observed (the copy number increased for chromosomes 5, 8, 11, 12, 15, and 19, and the copy number decreased in for chromosomes 3, 9, and 16) only after blastocoele-fluid DNA analysis, while the first and second polar bodies, as well as trophectoderm cells, had normal karyotypes.

In another case the first and second polar bodies analysis predicted trisomy 6, but the blastocyst appeared euploid based on DNA analysis from blastocoele fluid and trophectoderm cells.

The study of chromosome abnormalities in 30 samples showed that the analysis results for polar bodies and blastocoele fluid fully matched for 21 cases (70%). A partial match was observed in seven samples (23.3%), and the results did not match in two more cases (6.7%). One of them may be of special interest for the cytogenetics of embryo development. The point is that the analysis of the first polar body showed an increased copy number for chromosomes 4, 5, 6, 7, 9, 11, 12, 15, 19, 20, and X and a decreased copy number for chromosomes 1, 2, 3, 8, 10, 13, 14, 16, and 18, i.e., almost all chromosomes, except 17, 21, and 22, were involved in aneuploidy. Surprisingly, the analysis of the second polar body revealed reciprocal aneuploidies, namely an increased copy number for chromosomes 1, 2, 3, 8, 10, 13, 14, 16, and 18 and a decreased copy number for chromosomes 4, 5, 6, 7, 9, 11, 12, 15, 19, 20, and X. It is probable that due to two consecutive chromosome segregation errors in the first and second meiotic divisions, normalization of the chromosomal set occurred in the oocyte, and the karyotype determined during DNA analysis on the blastocyst fluid and trophectoderm cells appeared to be euploid. In the authors' opinion, such chromosome segregation errors can be explained by the preliminary segregation of chromatids in the first meiotic division. However, the cytological mechanism of complete correction of such multiple aneuploidies in the second meiosis remains unclear. At the same time, such an example demonstrates the necessity of karyotyping both polar bodies to exclude the rejection of some part of euploid embryos. However, this point is also debatable, since it was shown in earlier investigations that only 18% of blastocysts formed from fertilized oocytes, which demonstrated similar consecutive chromosome segregation abnormalities (at least, 13, 16, 18, 21, and 22) in the first and second meiosis divisions (restoring thereby normal karyotype) and are able to keep the normal karyotype during cleavage division [16]. In all other cases, new chromosome mutations continue to appear at the postzygotic stages of cleavage, possibly reflecting an unbalance in the mechanisms of chromosome segregation.

Comparative analysis of blastomere and cell-free DNA from blastocoele fluid showed a full match of the results for nine samples (88.9%), while a partial match was in only one case (11.1%). In this case the blastomere had trisomies 5, 10, 17, and 21 and monosomies 1, 6, 14, 15, and 22, while the cell-free DNA analysis showed the presence of trisomies 8, 13, and 21 and monosomies 1, 5, 17, 18, and X. Thus, some abnormalities detected in the blastomere matched those determined by molecular karyotyping in cell-free DNA, while the chromosomal aberrations of chromosomes 6, 10, 13, 14, 15, 22, and X were observed only in one of the analyzed samples. Such examples clearly demonstrate the need of further comparative investigation of cell-free DNA in blastocoele and blastomeres, since they indicate the probability of false results if applied in clinical practice. It is important to determine the real rate of accordance and discordance and to test sensitivity and specificity by analyzing large samples.

A comparison of the results of karyotyping with cell-free DNA and trophectoderm cells showed agreement for 38 of 39 samples (97.4%). A full match was observed in 32 cases (82%). Partial discordance was detected in six cases (15.4%), whereas an absolute difference between the results was shown only in one case (2.6%). A full discordance was due to the detection in cell-free DNA of trisomies of chromosomes 5, 8, 11, 12, 15, and 19 and monosomies 3, 9, and 16, while the trophectoderm cells were euploid. Such results can be explained by the fact that the embryonic cells with chromosome abnormalities were eliminated by apoptosis and, thus, their DNA appeared in the blastocoele fluid. This raises a question: how common are blastocysts that would be similar to the described case upon analysis. If the portion of such blastocysts would be large enough and exceed the error of the currently used PGD methods in relation to detection of chromosomal mosaicism, then the need to develop a method for preimplantation genetic diagnosis based on blastocentesis may become questionable.

When calculated for one chromosome, the agreement of the karyotyping results was 93.5% for cell-free DNA and the polar body biopsy, 94% with blastomere biopsy, and 96.6% with trophectoderm cells biopsy. The authors suppose that blastocoele-fluid DNA can become a valuable material for clinical investigation and preimplantation diagnosis. However, it is necessary that the portion of samples with informative DNA from blastocyst fluid would increase up to the level of informativeness observed during the blastomere biopsy.

The work of Gianaroli et al. is very important for the development of methods of preimpalntation diagnosis by blastocentesis. They showed a high rate of agreement for the analysis of blastocoele-fluid DNA and embryonic cells. Therefore, further investigations in this field would be reasonable and would possibly lead to the creation and introduction in clinical practice of diagnosis technology. This investigation demonstrated a great advantage of cell-free DNA use, since the real opportunity appeared to register all chromosomal abnormalities in the embryo. In light of the high frequency of mosaic karyotypes in embryos, there is some hope for a solution to the problem of diagnosing chromosomal mosaicism.

Moreover, the goal of the new studies described above was the analysis of blastocoele fluid properties in order to show the possibility of its use in practice. For example, not long ago the probability was shown to use blastocyst-fluid DNA for preimplantation genetic

diagnosis in horses. Herrera and colleagues reported on the successful application of cell-free DNA fraction for detection of the sex in 11 of 13 (84.6%) horse embryos. Also, a high survival of embryos was observed after fluid removal. The authors suggest that this approach does not violate embryo viability [17]. However, two cases when the sex was determined incorrectly made it necessary to consider further research aimed at the detection of the frequency and causes of incorrect results.

THE PROBLEM OF CHROMOSOMAL MOSAICISM IN PREIMPLANTATION GENETIC DIAGNOSIS

Mosaicism at the early stages of embryo development is a phenomenon in which one portion of embryo cells is euploid and another portion can have different chromosomal abnormalities. It was shown that 15–50% of monospermic embryos at the cleavage stage are mosaic [18]. The frequency of blastocysts with chromosomal mosaicism varies, according to various authors, from 17.6 to 95% [19–22]. The real frequency of mosaicism in embryos can be even higher, but the applied methods of preimplantation genetic diagnosis does not always allow an estimation of the presence of mosaic karyotype. This is mainly due to the minimal amount of material usually used for the analysis, for example, one blastomere.

There is a probability that the selection against mosaic embryos begins at the preimplantation stage. This early embryo death may contribute to a large number of losses upon implantation at 2–3 days after embryo transfer into the uterus of a woman [18]. Los et al. investigated the dynamics of the frequency of mosaic karyotypes in embryos and proposed a model, according to which the frequency of mosaicism at the 8-cell stage was 59.8% and it gradually decreased during the embryo development [23]. The authors suggest that the decrease of the frequency of mosaic embryos after the morula stage verifies the realization of the conception of lethality and viability of cells under the cell cycle control. Thus, beginning from the morula stage, the frequency of mosaicism in embryos gradually decreases.

It is worth noting that a small number of aneuploid cells at early stages of embryo development may not be fatal for consecutive cleavage division. If chromosomal abnormalities are compatible with implantation, then the embryo would have an opportunity for further development, especially if there are cells with normal karyotypes, which will lead to the origin of tissue-specific chromosomal mosaicism [24, 25]. Moreover, it was shown that a significant portion of abnormalities observed in a mosaic state at earl stages of development are polyploidy, which may well be a part of the normal differentiation of trophoblast [18].

Returning to the results of Gianaroli et al. [4], we see a high rate of agreement between karyotyping with cell-free DNA and single blastomeres (88.9%) or trophectoderm cells (97.4%). It is fundamentally important to note that a time shift in the preimplantation diagnosis to a later date apparently favorably effects a decrease in the frequency of false-negative results due to the natural elimination of mosaic embryos. In this respect the prospects for fluid removal from the blastocoele before cryopreservation at the later stages of preimplantation development seems attractive for an increased reliability of preimplantation genetic diagnosis and a decreased probability of diagnostic errors due to the presence of chromosomal mosaicism.

Thus, while the cytogenetic mechanisms of chromosomal mosaicism origin at early stages of embryo development are actively studied, a lot of questions remain open. It is extremely important for the preimplantation genetic diagnosis to find a way to determine the mosaic embryos and to obtain information on karyotypes of all aneuploid cells. To a great extent, the problems of diagnosis are related to the methods of obtaining of biological material for cytogenetic investigation, most of which do not allow an estimation of the presence of chromosomal mosaicism in an embryo.

MEANS OF OBTAINING BIOLOGICAL MATERIAL FOR PREIMPLANTATION GENETIC DIAGNOSIS

The currently used methods for obtaining material include polar body biopsy, blastomere biopsy at the cleavage stage, and the biopsy of trophectoderm cells at the blastocyst stage. Each of these approaches has obvious advantages; however, they also have a number of significant disadvantages, especially regarding the diagnosis of chromosomal mosaicism.

The use of the polar bodies is the least invasive and is a quite effective method when it is necessary to select oocytes free of maternal aneuploidies. It is worth noting that most (more than 90%) human aneuploidies are of maternal origin [26]. Despite this, such an analysis is not informative for the karyotype of blastocyst cells, and it excludes the possibility of embryo mosaicism assessment.

Blastomere biopsy on the third day of development is performed in about 94% of cases of preimplantation genetic diagnosis [27]. It is worth mentioning that analysis of a single blastomere is also sufficiently informative due to the possibility of embryo mosaicism [28]. There are still a number of methodological problems, including the absence or loss of a nucleus during blastomere fixation, a decrease subsequent blastocyst formation that results from the micromanipulations of a number of cells, and others. However, it was shown that the biopsy of a single blastomere does not influence the viability and the probability of embryo implantation [29].

A trophectoderm biopsy on the fifth day of embryo development is a more suitable way to obtain material

for the diagnostics, since it is possible to use more cells. This reduces the probability of a false-negative result due to chromosomal mosaicism. McArthur et al. showed a high blastocyst viability after the trophectoderm biopsy, and the probability of pregnancy appeared to be more than 40% [30]. However, the biopsy of more than 25% of blastomeres can be dangerous and can reduce the probability of implantation and embryo viability [31]. While most studies indicate a slight decrease in the probability of implantation after different types of biopsy, these approaches are still invasive. When they are used, there is a probability for embryo injury.

CONCLUSIONS

The idea of using DNA from blastocoele fluid as a material for preimplantation genetic diagnosis is original and quite promising. The first studies make it possible to draw some conclusions.

First, the presence of such a fraction of cell-free DNA was confirmed. It is observed in 76.5–90% of blastocysts, and the DNA content per sample varies from 8.7 to 9.9 pg. DNA analysis becomes impossible if its concentration is less than 5 pg per sample.

Second, it was shown that the embryo karyotype can be correctly determined with the use of cell-free DNA at the preimplantation stages of ontogenesis in 97.4% of cases on average, and the diagnostic accuracy increases with the course of development.

Third, the high agreement of karyotyping results using cell-free DNA and embryo cells supports the idea of using of a new source of biological material to solve the problem of chromosomal mosaicism diagnosis.

As can be seen, the frequency of DNA detection in blastocoele fluid is rather high, as is, as a whole, the extent to which the karyotypes correspond to each other after the analysis of cell-free DNA and embryo cells. Though it is obvious that methodological improvement of the processes of isolation and whole-genome amplification of DNA is needed to increase the number of blastocysts for which it would be possible to perform such analysis. Comparative analysis of the karyotype of cells of trophectoderm, inner-cell mass, and cell-free DNA in blastocoele fluid for a more detailed understanding of the origin of this fraction of DNA and for increased effectiveness of chromosomal mosaicism diagnosis seems to be relevant.

A number of other uncertain moments exist in this sphere. Thus, the question of the probability of embryo injury during the removal of blastocoele fluid remains open. Does the micropipette injection for fluid removal between neighboring trophectoderm cells make it possible to fully exclude the probability of getting of DNA from embryo cells in the analyzed sample? It would be interesting to know exactly which cells undergo apoptosis. Does it happen only when DNA from cells with a certain karyotype (normal or abnormal) or specific localization in the blastocyst (in trophectoderm or in the inner cell mass) appear in blastocoele fluid?

The first studies of cell-free DNA from blastocoele fluid may become the starting point of the beginning of new era of preimplantation genetic diagnosis. However, we should not forget that the development of new approaches for introduction in clinical practice is a very important process demanding long and laborious work of various research groups all over the world.

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