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Preimplantation Development of Human Embryos with Numerical Chromosome Abnormalities in vitro

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Abstract—The study was focused on morphokynetic characteristics of in vitro cultured human embryos that were considered to be aneuploid or euploid according to the preimplantation genetic screening results. Among all the embryos examined, only 34.2% were chromosomally balanced. Although morphological features of cleaving pathologic and euploid embryos did not differ significantly, on the fifth day of culture, a chromosomally balanced specimen formed an "expanded" blastocyst twice more frequently than abnormal ones. Moreover, development of 38.4% of aneuploid embryos was compromised before the initiation of cavitation. Thus, prolonged embryo culture advances selection of samples with the highest implantation potential for the transfer on the basis of the morphokynetic characteristics and helps to avoid additional genetic testing.

Keywords: preimplantation genetic screening, chromosome abnormality, human embryo, morphological features

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INTRODUCTION

The development and wide practical use of in vitro fertilization draws the attention of scientists towards the study of peculiarities of preimplantation development of human embryos, especially the influence of different regulatory factors on the process of cell division and differentiation [1]. Detailed studies of human embryological development will improve the efficiency of application of assisted reproductive technologies (ART) for infertility treatment and allow discovering of important genetic, biochemical, and physiological factors at the key stages of human embryogenesis.

A human being has a unique characteristic that requires the review of available embryological data. It deals with the decreased fertility in comparison with other mammals [2]. One of the most probable explanations of this feature concerns the high frequency of embryos with quantitative or structural chromosome abnormalities, most of which are not compatible with normal fetus development [3]. Thus, chromosomal aberrations can hinder implantation of the embryo in the uterine cavity, cause early interruption of pregnancy, and birth of children with congenital abnormalities of different systems and mental retardation [4].

Implementation of preimplantation genetic screening (PGS), aiming to reveal genetic abnormalities of embryos before the implantation of the latter, is important for observation of in vitro development of embryos with known chromosomal status. The findings provided important data regarding abundance and spectrum of chromosome abnormalities in early human ontogenesis [5]. Thus, according to some scientists, approximately 50% of all conceptuses are exposed to genome mutations [6]; at the same time, only 4.5–7.0% of fetuses at 10–12 weeks of gestation have chromosome abnormalities [7], and chromosomal aberrations make up 0.6–0.7% among newborn babies [8]. The decrease of the number of embryos with chromosomal disorders during the embryogenesis implies the existence of severe natural selection that acts against pathological embryos. Presumably, key moments of such selection are preimplantation stage and the process of implantation itself.

The evaluation of morphological characteristics of embryos with particular chromosomal complement at the stage of cleavage and blastulation will help us to detect the influence of aneuploidies on the process of early human development and to establish the efficiency of their natural selection. Concerning the infertility treatment cycles this may help to select euploid embryos for the following transfer to the uterine cavity according to their morphological features, without invasive genetic diagnostics. Efficient selection of samples with the highest implantation potential for embryotransfer will ensure obtaining of high pregnancy rate and simultaneous decrease of number of embryos transferred in particular cycle of treatment. This reduces the risk of the most widespread compli-

cation of ART—multiple pregnancy [9], whose frequency after fertilization in vitro exceeds the indices of natural conception [10].

Thus, the aim of our study was to analyze morphokinetic characteristics of human embryos, whose genetic status was established during the preimplantation genetic screening. Discovering of the connection between embryo morphology and its chromosomal status may increase the efficiency of noninvasive selection of euploid samples for embryotransfer on the basis of their morphology evaluation.

MATERIALS AND METHODS

The study included the data analysis of 84 cycles of infertility treatment that included the preimplantation genetic screening of embryos obtained during the in vitro fertilization of oocytes. In the period from July 2010 to December 2012, the analysis of morphokinetic and genetic characteristics of 735 embryos was carried out. The average age of patients was 34.0 ± 5.03 years. In case of oocyte donation, the donor's characteristics were taken into consideration, since it was proved that there is a direct correlation between women's age and probability of gamete formation defects [11, 12]. All the women had satisfactory state of somatic health and normal karyotype. To obtain the greater number of oocytes, they were exposed to the procedure of hormonal stimulation of superovulation. In general, the study did not contradict with ethic norms, Ukrainian legislation, and international laws.

The fertilization of mature oocytes obtained with the use of puncture of ovarian follicles was carried out by intracytoplasmic sperm injection. The success of procedure was estimated after 16–18 hours on the basis of presence of two pronuclei in cytoplasm and the second polar body in perivitelline space of the zygotes. Embryos were cultured in vitro in marked drops of medium (Global, United States) during 120–144 hours at 37°C and 6.0% level of carbon dioxide.

On the third day of culture after mechanical dissection of zona pellucida, one blastomere was biopsied in order to conduct chromosomal analysis. It is known that all cells of the embryo are totipotent at the cleavage stage. This means that the loss of one cell, probably, does not negatively affect the further development of the embryo [3]. Removed cells were treated with hypotonic solution (0.3% bovine serum albumin, 1% sodium citrate), and the obtained nuclei were fixed with a mixture of ethanol and acetic acid in 3 : 1 ratio. The diagnostics was conducted by the fluorescent in situ hybridization with centromeric and locus-specific probes to autosomes 13, 16, 18, 21, 22 (PB Multivysion, Vysis, United States) and sex chromosomes (CEPX/CEPY, Vysis, United States). Numerical abnormalities of studied chromosomes significantly decrease the implantation potential of embryos. Most often, they cause fetal wastage (aneuploidies of autosomes 16 and 22) [13] or lead to the development of key chromosomal syndromes (13, 18, 21, X, Y) [14].

The samples were denatured at 69°C and incubated for at least 4 hours in a humidified box at 37°C. Posthybridization processing included washing in 0.7× SSC/0.4% NP-40 at 73°C and 2× SSC/0.1% NP-40 at room temperature. Hybridization signals were estimated according to the requirements of the European Society of Human Reproduction and Embryology [15] and registered with the use of ISIS software (MetaSystems, Germany). If it was impossible to establish the number of homologues of a particular chromosome using the existing signals, an additional round of hybridization with probes, specific to other areas of chromosomes of interest, was carried out.

From the moment of fertilization, morphological characteristics of embryos were evaluated daily under 400x magnification. During the first three days of culture, the number of existing cells and morphological abnormalities (uneven cleavage, fragmentation or multinucleation) were determined. On the fourth day we observed the rate of compaction of blastomeres. Cell compaction reached its maximum at the stage of morula, when distinguishing of blastomeres was impossible [16]. At the stage of blastulation (fifth day of embryo development), implantation potential of the embryo was estimated according to Gardner's classification (Table 1). Each embryo was related to the particular category on the basis of its morphological characteristics. Embryos, considered to be euploid according to the results of PGS and possessing satisfactory morphological characteristics, were transferred to the recipient uterus or frozen.

RESULTS AND DISCUSSIONS

Preimplantation genetic screening of seven chromosomes was successfully accomplished for 735 embryos. According to the results of diagnostics, each sample was related to one of the given categories [18].

(1) Euploid samples had two copies of studied autosomes and appropriate to male or female set of sex chromosomes (XY or XX).

(2) Samples with disorders of the ploidy, which is characterized by simultaneous decrease or increase of number of all studied chromosomes.

(3) Aneuploid samples, namely: (a) embryos with isolated mono- or nullisomy (when the signal from one of two homologues of chromosomes of interest is absent); (b) embryos with isolated polysomy (when additional copies of studied chromosomes are present); (c) embryos with complex chromosomal pathology (in case of deviations of number of homologues of ≥2 studied chromosomes).

In general, combinations of hybridization signals corresponding to euploid chromosome complement

Table 1. Classification of human embryos on the basis of morphology at the stage of blastulation [17]

Quality class	Blastocyst morphology and its quiality grade
	Embryo status
1	Blastocele of "cavitated" embryo does not exceed a half of its volume
2	Cavity constitutes more than a half of embryo volume
3	Blastocoele fills the embryo
4	"Expanded" blastocyst with thin and stretched fertilization membrane
5	Hatching blastocyst
6	Hatched blastocyst
	Morphology of inner cell mass
A	Many densely packed cells, clear shape of embryoblast
B	Small number of loosely grouped cells
C	Very few cells
	Trophectoderm characteristics
A	Many densely packed cells
B	Few loosely packed cells
C	Very few huge cells, there can be intervals between them

were found in 246 embryos. This makes up 34.2% of studied samples. Thus, in every diagnostical cycle, on average, 2.87 ± 1.1 samples were appropriate for embryotransfer. The established frequency of euploid embryos is lower than the data provided by other authors. Thus, during preimplantation genetic screening conducted by fluorescent hybridization in situ Blockeel et al. [19] found 50.3% of examined embryos to be normal, while in paper of De Vos et al. [20] 61% of embryos were announced as euploid. The lower rate of chromosomally balanced embryos established in our study may be due to the general approach to formation of study group without separation of groups of patients according to age, indications for treatment, number of previous attempts, etc.

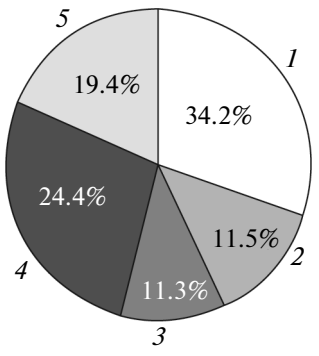

Fig. 1. Distribution of embryos according to their chromosome complements on the basis of the preimplantation genetic screening results: (*1*) embryos considered to be euploid; (*2*) samples from isolated polysomy; (*3*) samples with isolated monosomy; (*4*) embryos with complex chromosome abnormalities; (*5*) embryos with disorders of ploidy level.

The other 489 studied embryos were characterized by numerical aberrations of one or some chromosomes. A significant part of group—29.4%—included samples with complex chromosomal pathology (Fig. 1). Disorder of ploidy level was found in 13.6% embryos. For example, 56 embryos had only one copy of each chromosome of interest, that corresponded to haploidy, and 42 samples were polyploid having three or four chromosome sets. The rates of embryos with isolated mono- and polysomies were similar and comprised 11.3 and 11.5% of the studied group.

To estimate the influence of numerical chromosome abnormalities on early development of embryos, the morphological characteristics of embryos were analyzed on the second–fifth day of culture. The study of morphokinetic markers of embryos with pathological chromosome complement is based on the negative effect of aneuploidies on the process of division, interaction and differentiation of cells in early embryogenesis. Probably, it will allow us to discover the particular characteristics of development of aneuploid embryos in order to isolate them in vitro.

It was found that the majority of embryos contained four blastomeres on the second day of culture (Fig. 2). This is an optimal number of cells at the given stage and indirectly indicates their high implantation potential of embryos [21]. The develop-

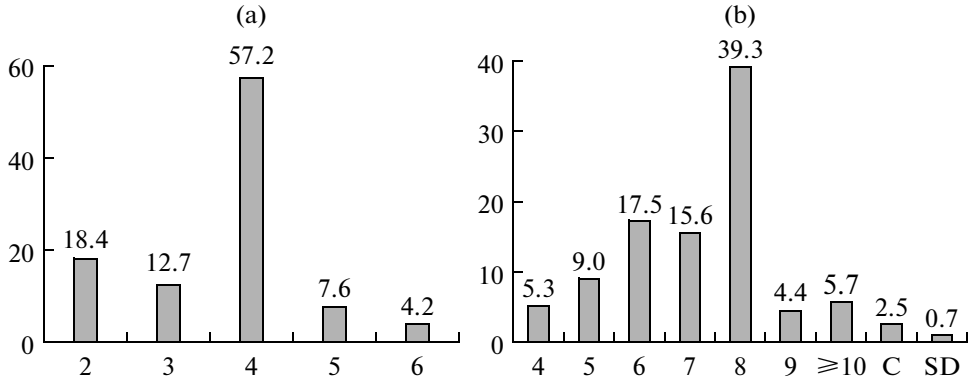

Fig. 2. Distribution of studied embryos according to the quantity of existing blastomeres in (a) 40–42 hours and (b) 64–66 hours following fertilization: down—embryos with particular number of blastomeres, %; across—number of embryonal cells; (C) samples that started compaction, (SD) embryos that stopped development.

ment of 223 embryos was suppressed, and cell division of the remaining samples was accelerated. Some scientists [22] consider this to be a negative prognostic factor. On average, on the second day of development, studied embryos contained 3.66 ± 1.02 cells, and 7.1% of them had some morphological defects, in particular: presence of nucleus-free fragments (21 cases) and uneven blastomeres (31 cases).

In 64–66 hours after fertilization, eight blastomeres contained 39.3% of analyzed samples, that is a sign of their high quality according to the known criteria [23, 24] (Fig. 2); 103 embryos from the studied group were backward in development and had only three to five blastomeres. At the same time, 18 studied samples started the process of compaction. In total at the given stage of cleavage, embryos contained 7.15 ± 1.59 blastomeres on average (without compacted samples). Also, 48.1% of embryos had morphological defects. The prominent morphological abnormalities were fragmentation and blastomere unevenness (49 and 207 cases, correspondingly).

Taking into consideration chromosome complement, the analysis of cleavage rate of embryos showed that euploid samples had 3.68 ± 1.02 cells in 40–42 hours after fertilization, and pathological ones had 3.66 ± 1.02 blastomeres in the same time period. However, we did not observe the statistically significant difference in the number of blastomeres in both groups ($p > 0.5$). On the third day of development, chromosomally balanced samples averagely consisted of 7.28 ± 1.43 cells, while abnormal embryos had 7.09 ± 1.66 blastomers. We did not reveal the differences between morphokinetic characteristics of studied groups of embryos. Thus, the kinetics of development of abnormal and euploid embryos did not differ significantly at the cleavage stage. This means that during the first 2 days of culture the evaluation of morphological features of embryos does not allow us to distinguish the samples with balanced chromosome complement.

While analyzing the frequency of euploid samples among embryos with different morphokinetic characteristics, we found that most chromosomally balanced samples contained an even number of cells and were characterized by moderate rate of cleavage on the second day of development. In other words, 80.9% of euploid embryos had two or four blastomeres. At the same time, the highest probability of detection of chromosomally balanced samples was 37.5% and was discovered in the group of four-cell embryos (Fig. 4). The high frequency of genetically balanced specimen was found among embryos with five cells (32.8%). It is necessary to admit that in the group of embryos with a high rate of cleavage, which contained more than six blastomeres on the second day of development, chromosomally balanced embryos comprised 26.7%. The lowest rate (23.1%) of euploid embryos was inherent to samples that contained three cells in 40–42 hours after fertilization.

On the third day of culture, the highest frequency of chromosomally balanced samples (40.1%) was found among embryos with eight cells. In general, propotion of chromosomally balanced embryos was almost similar and the frequency of euploid embryos was low only in groups with suppressed and accelerated cleavage (23.7, 21.5, 24.4%). These results correspond with distribution discovered on the second day of development: chromosome abnormalities were more typical for the samples with low or accelerated cleavage. It is important to admit that the rate of normal samples was 38.9% in groups of embryos that had signs of compaction on the third day of development. Probably, early formation of close intercellular contacts is observed in the case of efficient realization of the program of embryo development and can be considered as a positive prognostic parameter. However, the group of embryos with preliminary compaction of

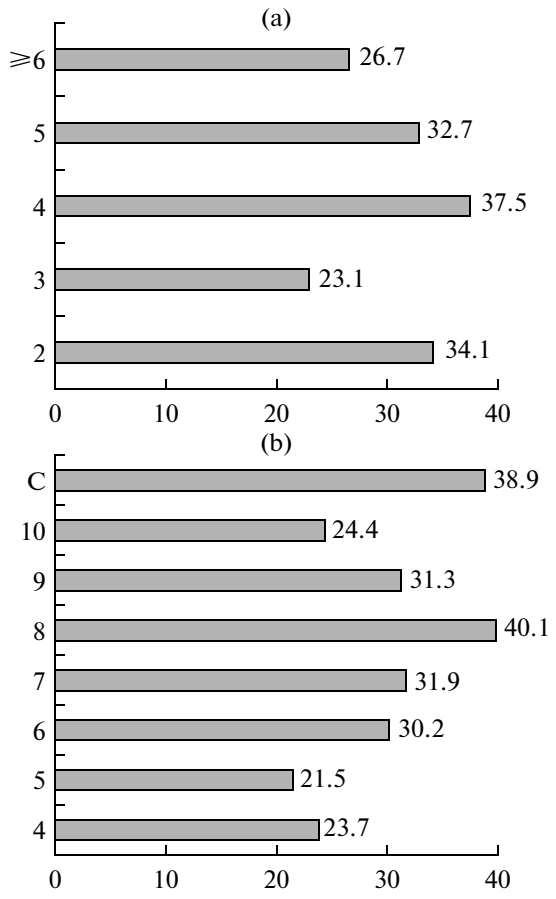

Fig. 3. Frequency of detection of euploid samples among embryos with appropriate number of blastomeres in (a) 40–42 hours and (b) 64–66 hours after fertilization: (C) embryos with signs of compaction; down—number of embryonal cells; across—euploid embryos among samples with particular number of cells, %.

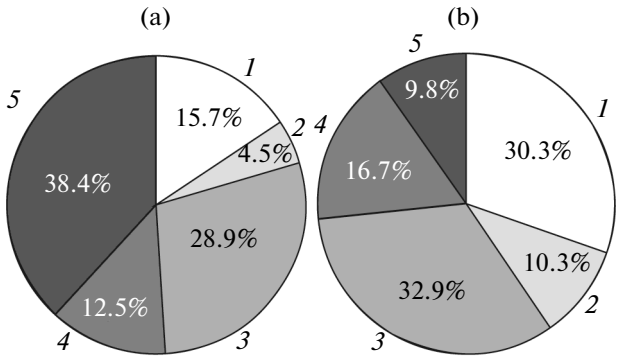

Fig. 4. Relative distribution of (a) abnormal and (b) chromosomally balanced embryos on the basis of development rate after 112–114 hours of culture; (*1*) blastocysts that started hatching, (*2*) expanded blastocysts, (*3*) early blastocysts, (*4*) morulae, (*5*) development of embryos is blocked.

the blastomeres was not numerous ($n = 18$), and the discovered tendency for concentration of euploid samples among such embryos should be confirmed with the additional studies.

The process of blastulation is one of the most important stages of natural selection of genetically normal embryos. At the given stage of development, an embryo passes the primary differentiation. At this stage it should be determined which cells form the fetus (inner cell mass) or the extraembryonic membranes (trophectoderm) [17]. Besides, on the third day of embryo development, newly formed embryonal genome is activated and the synthesis becomes more important than breakdown reactions, which requires transfer to anabolic metabolism. At the given stage of development, the check-points of the cell cycle are activated, that blocks cell division of abnormal embryos, including the samples with genetics defects [1]. Thus, the numerical abnormalities of chromosomes can cause the deviations of cell compaction and differentiation of cultured in vitro human embryos.

The studies of morphological characteristics of embryos after 112–114 hours of their culture showed that 73.5% of euploid embryos underwent cell differentiation successfully (Fig. 4). At the same time, an "expanded" blastocysts were formed by 64 chromosomally balanced samples; 32.9% of euploid group had signs of cavity initiation and trophectoderm formation, and 16.7% of chromosomally balanced embryos were related to morulae and developed slowly but progressively. Generally, 23 euploid embryos stopped their development until the fifth day of culture, which comprises only 9.8% of the euploid group.

Also, 49.0% of embryos considered to be abnormal according to the preimplantation genetic screening results successfully passed initial cell differentiation. The rate of "expanded" blastocysts that started hatching was equal to 15.7% and was two times lower comparing to euploid embryos, while 28.9% of pathological samples had signs of cavity formation. Probably, the decreased number of embryos of the abnormal group, which formed full blastocyst, is caused by the great number of samples that stopped their development at the stages of cleavage, compaction, and initiation of blastulation. Thus, general frequency of chromosomally balanced embryos, whose cell division was blocked after 112–114 hours of culture, was 38.4%. It exceeds appropriate indices of euploid group by four times (Fig. 4).

To conduct integral estimation of development of embryos with known chromosome complement at the stage of blastulation, we created a combined system of their classification according to morphological features (Table 2). Every embryo referred to a particular category in accordance with the development stage on the fifth day of culture and the structure of embryo at the moment of analysis. The samples whose develop-

Table 2. Combined classification of human embryo development stages after 88–114 hours of culture in vitro

Quality class	Morphological characteristics of embryo
1	Signs of progressive development were absent during the last 24 hours of culture
2	Embryo has 8–12 cells, its development is slow, but the attributes of cell degeneration are not observed
3	Compaction
4	Morula: embryo contains 16–32 densely packed cells whose boundaries are not visible
5	Cavitation—initial stage of blastocyst formation
6	Cavity covers more than a half of embryo volume. Surrounding cells become flat
7	Formation of cavity is complete, trophectoderm and embryoblast are clearly separated. Fertilization membrane stretches
8	Expanded blastocyst with thin zona pellucida
9	Blastocyst that started to hatch
10	Embryo left fertilization membrane and is ready for implantation in uterine cavity

Classes 5–10 of the given combined classification correspond to classes 1–6 of classification of blastocysts according to Gardner [17].

ment was blocked during the last 24 hours of observation belonged to the lowest quality class "1." Blastocysts that left the fertilization membrane at the moment of analysis obtained the highest rank of classification: "10."

At the stage of blastulation, the development rate of aneuploid embryos was impaired in comparison with normal samples: average rank in pathological group was 4.07 ± 2.05. Chromosomally balanced embryos corresponded to grade 5.88 ± 0.96. According to these findings, on the fifth day of culture, aneuploid embryos were at the stage of morula with gradual transition to early blastula, and chromosomally balanced samples mostly formed blastocysts successfully.

Thus, embryos with abnormalities of the chromosome complement show significant aberrations during transition to advanced stages of preimplantation development, especially during the realization of program of primary cell differentiation. But the analysis of morphological characteristics of embryos with progressive development did not reveal considerable differences between quality of pathological and euploid samples: 88–90 hours after fertilization, their average rank was 3.33 ± 0.87 and 3.18 ± 0.8 correspondingly (Fig. 5). On the fifth day of culture, euploid embryos had rank 6.41 ± 2.2 on average, and pathological samples had rank 5.96 ± 2.1. So, we did not find statistically significant differences between structure characteristics of abnormal and chromosomally balanced samples with progressive development.

In total, the frequency of blastocyst formation in euploid group was 73.5%. It exceeds the proper indices of pathological group, where 49.0% of embryos had signs of conversion to initial cell differentiation. Among all chromosomally balanced blastocysts 30.3% started the process of hatching. Among pathological embryos, hatching was typical only for 15.7%. Considering data about the frequency of prenatal occurence of aneuploid embryos, we can assume that blastulation is one of the most important stages of natural selection against embryos with constitutional chromosome abnormalities. Thus, on the fifth day of development, the number of aneuploid embryos decreased by two times. Almost 49.0% of pathological samples overcome this barrier and their further selection probably takes place at the stage of implantation and further development of pregnancy.

CONCLUSIONS

According to the results of our study, we found numerical abnormalities of chromosomes 13, 16, 18, 21, 22, X, and Y in 65.8% of preimplantation human embryos cultured in vitro, which significantly exceeds the frequency of aneuploidy in pre- and postnatal period. Thus, from the moment of zygote formation to the moment of childbirth, there is a tendency to decrease in number of embryos with chromosome aberrations. Furthermore, the most effective selection against chromosome pathologies occures at early stage of embryogenesis. Natural elimination of a great number of pathological embryos before the moment of their invasion into the uterine cavity can be an explanation of low human fertility.

Probably, one of the key stages of selection against pathological embryos is primary cell differentiation. Thus, according to the results of our research, morphological characteristics of euploid and pathological samples at the stage of cleavage did not differ signifi-

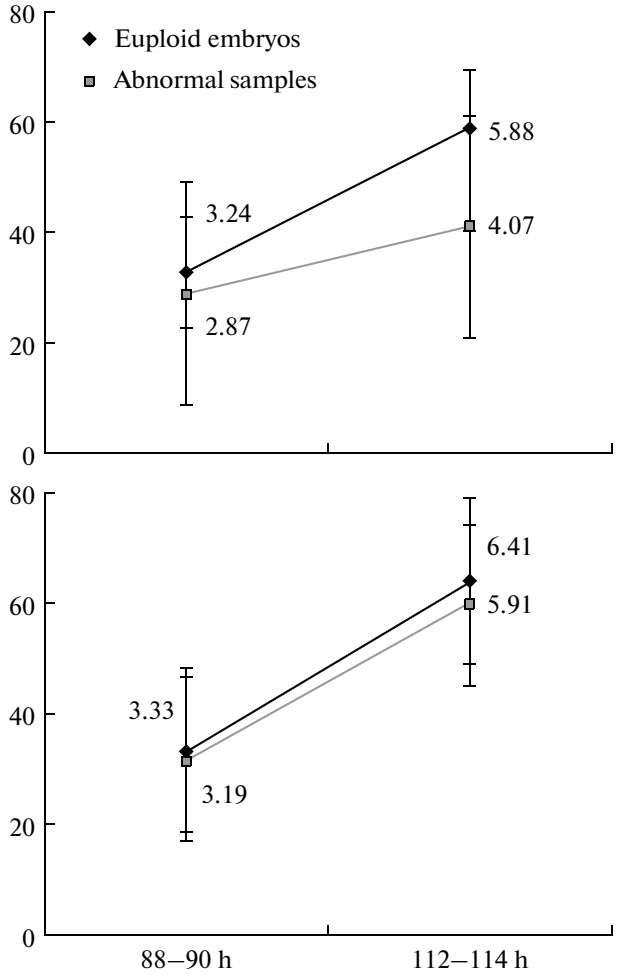

Fig. 5. Characteristics of preimplantation development of chromosomally balanced and pathological embryos according to the suggested integral system of morphological evaluation at the stage of blastulation (a) considering (b) not considering samples whose cleavage is blocked: down—grade of embryos; across—duration of culture.

cantly. But before the moment of cavitation, 38.4% of chromosomally unbalanced embryos stopped developing. This means that prolonged culture of embryos allows us to estimate implantation potential of an embryo according to its morphokinetic characteristics more precisely than at the cleavage or compaction stage.

Despite the fact that blastulation is an important natural barrier for embryos with chromosome abnormalities, the samples that overcame it successfully do not differ from euploid embryos by the morphological characteristics. Thus, culture of embryos during 88–114 hours helps us to accomplish natural negative selection of pathological samples, but it does not allow to identify the samples with balanced chromosome complement. Prolonged culture, probably, can help us to differentiate euploid embryos with the highest implantation potential indirectly. However, it still can enhance the likelihood conception without increase in number of embryos transferred and without the necessity to carry out additional genetic and physiological tests.

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