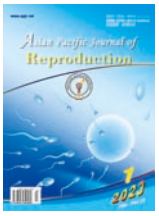

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Embryo quality and chromosomal abnormality in embryos from couples undergoing assisted reproductive technology using preimplantation genetic screening

Mina Niusha[1], Seyed Ali Rahmani[2]✉, Leila Kohan[1], Ladan Sadeghi[1], Mohammad Nouri[3], Hamid Reza Nejabati[4]

[1]*Department of Biology, Arsanjan Branch, Islamic Azad University, Arsanjan, Iran*

[2]*Department of Medical Genetics, Tabriz University of Medical Sciences, Tabriz, Iran*

[3]*Department of Reproductive Biology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences Tabriz, Iran*

[4]*Department of Biochemistry and Clinical Laboratories, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran*

ABSTRACT

Objective: To detect common chromosomal aneuploidy variations in embryos from couples undergoing assisted reproductive technology and preimplantation genetic screening and their possible associations with embryo quality.

Methods: In this study, 359 embryos from 62 couples were screened for chromosomes 13, 21, 18, X, and Y by fluorescence in-situ hybridization. For biopsy of blastomere, a laser was used to remove a significantly smaller portion of the zona pellucida. One blastomere was gently biopsied by an aspiration pipette through the hole. After biopsy, the embryo was immediately returned to the embryo scope until transfer. Embryo integrity and blastocyst formation were assessed on day 5.

Results: Totally, 282 embryos from 62 couples were evaluated. The chromosomes were normal in 199 (70.57%) embryos and abnormal in 83 (29.43%) embryos. There was no significant association between the quality of embryos and numerical chromosomal abnormality (P=0.67).

Conclusions: Embryo quality is not significantly correlated with its genetic status. Hence, the quality of embryos determined by morphological parameters is not an appropriate method for choosing embryos without these abnormalities.

KEYWORDS: Assisted reproductive technology; Preimplantation genetic screening; Aneuploidy; Fluorescence in-situ hybridization; Chromosomal abnormalities; Embryo quality; Blastomere; Blastocyst

1. Introduction

Preimplantation genetic screening (PGS) is a widely accepted technology to screen embryos generated through *in vitro* fertilization (IVF) for genetic and chromosomal disorders[1,2]. It is an early prenatal diagnosis technique for transferring embryos that are chromosomally normal and disease-free, to the uterus. Unlike prenatal genetic diagnosis, which involves chorionic villus sampling and amniocentesis on an ongoing intrauterine pregnancy, PGS is a non-invasive method, performed on embryos developed in the IVF laboratory[3]. PGS allows couples who in risk of transmitting genetic disorders to have healthy children and it prevents complications such as the birth of a child with physical and developmental disabilities or psychological problems resulting from the termination of a pregnancy. It has been shown that the reduction of the competence of the oocyte in advanced age women could increase

Significance

Nowadays, selecting the best embryo for transfer can play an important role in the success rates of assisted reproductive technology in infertility centers. Hence, conducting high quality genetic studies to address this issue is essential. This study showed that the quality of embryos determined by morphological parameters may not be an appropriate method for choosing the embryos without these abnormalities. Accordingly, it is important to routinely examine the aneuploidy of embryos generated by ICSI method for all couples in infertility centers.

✉To whom correspondance may be addressed. E-mail: rahmaniseyedali@yahoo.com

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the risk of mosaicism[3]. However, high chromosomal abnormalities are not solely observed in high maternal age and also reported in embryos from young women[4]. Since chromosomal abnormalities are one of the important reasons for early pregnancy loss and could also cause infertility, many technologies are available and used to screen and diagnose chromosomal abnormalities in embryos[5]. Fluorescent in situ hybridization (FISH) technique is a gold standard method to screen embryos for common chromosomal abnormalities and improves the efficiency of IVF[6]. The FISH can be used for detecting social sexing or X-linked genetic diseases, inherited chromosome rearrangements, and chromosomal aneuploidy[7]. The most frequently used indications for PGS include female infertility with advanced maternal age (defined as ≥35 years), couples with normal karyotypes but recurrent pregnancy loss, or with repeated implantation failure, and severe male factor infertility[7]. Assessment of embryo morphology is broadly used for embryo selection around the world based on some well-known criteria such as the number of cells, polar bodies, and quality of trophectoderm[8–10]. However, given the lack of any strong correlation between the embryo morphology and implantation success, the embryo transfer based on the morphology of embryo is still remains limited[7,8]. Furthermore, high-quality embryos may be genetically abnormal, while low-quality embryos are represented as normal[11]. Previous reports showed that the embryo quality has limited effects on implantation rate[12]. The aim of this study was to investigate the prevalence of chromosomal aneuploidy in embryos from couples undergoing assisted reproductive technology, and association of chromosomal aneuploidy with embryo morphological parameters, and maternal age.

2. Materials and methods

2.1. Study design

In this retrospective study, the variations in 13, 18, 21, X, and Y chromosomal copy numbers were analyzed in embryos from couples undergoing assisted reproductive technology treatments in Milad Fertility Clinic and Omid Fertility Clinic in Tabriz, Iran from August 2020 to July 2021.

2.2. Patients

The cycle and PGS for chromosomes 13, 18, 21, X, and Y were performed at Milad Fertility Clinic and Dr. Rahmani Genetic Laboratory in Tabriz. All couples were complaining from primary infertility. Couples (age range > 30 years old) with a history of recurrent miscarriage, repeated implantation failure, and male infertility, were enrolled in the study, and couples with secondary infertility or who used donated eggs and sperm were excluded from the study. All couples received genetic counseling and written informed consent was obtained from all couples in this study.

2.3. Sample size

In this study, 359 embryos created by IVF/ICSI method for 62 couples were studied by PGS-FISH. The number of included embryos was based on related articles[2,10].

2.4. IVF/intracytoplasmic sperm injection (ICSI) program

In ICSI procedure, females went through controlled ovarian hyperstimulation to mature several oocytes at the same time. The development of the follicles in the ovaries was monitored during stimulation and when there were at least three follicles ≥17 mm, maturation of the oocyte and luteinization of the follicles were induced and oocyte retrieval was done after about 37 h. The fertilization of the metaphase Ⅱ oocytes was carried out by ICSI procedure. The embryos were observed at 200× and 400× magnification on an inverted microscope (Nikon) and classified by experienced embryologists according to the following morphological criteria at the cleavage and blastocyst stages. The embryos were classified into three grades (A, B, and C) at the cleavage stage and into 3 groups (high, middle and weak) at the blastocyst stage and all evaluations were implemented just as Gardner's classification. The blastocysts grades were classificated into 3 groups: high (expanded AA, hatching AA, hatched AA, early AA, mid AA, expanded AB, BA, hatching AB, BA, hatched AB, BA), middle (F; early AB, BA, mid AB, AC, BA, BB, expanded AC, BB, CA, hatching AC, BB, CA, hatched AC, BB, CA), and weak (P; early AC, BB, BC, CA, CB, CC, mid BC, CA, CB, CC, expanded BC, CB, CC, hatching BC, CB, CC, hatched BC, CB, CC)[13]. The day 5 embryo grading was evaluated two inner cell mass types (A and B) and two trophectoderm types (A and B), and the top blastocyst stage of expansion was hatching[13].

2.5. Biopsy

Among embryos, only those with at least five blastomeres (grades A, B, and C on day 3) were chosen. First, the embryos were incubated in a biopsy medium (Ca^{2+} and Mg^{2+} free) for 10 to 30 min to stop the compaction of the cells. The embryos were washed twice and moved into a fresh medium just before the time of transfer. The blastomeres were spread on slides coated with poly-L-lysine.

2.6. Single cell spreading

A circle was drawn on the bottom of the slide and filled with 10 μL spreading solution (0.1 N hydrochloric acid (HCl)/0.05% Tween-20, Sigma-Aldrich, UK). A 10 μL drop of phosphate buffer saline (PBS) was located near the spreading drop and was used for washing. The blastomere was transferred from the culture dish to the PBS drop and from the PBS drop, the blastomere was transferred to the spreading drop and the slide was transferred on an inverted microscope (Olympus, UK). The spreading solution was disturbed

carefully and the blastomere was monitored for the cell membrane to start to lyse and the cytoplasm to be washed away. The slides were left to dry and used for FISH procedure. Then, the embryos were chosen for transfer according to FISH results of chromosomes 13, 18, 21, X, and Y and their quality.

2.7. Genetic analysis

2.7.1. FISH procedure

A FISH procedure was done, which permitted for the assessment of chromosomes X, Y, and 18 in the first round and chromosomes 21 and 13 in the second round. The FISH Prenatal 13, 18, 21, X, and Y Probe Kit was provided by Cytocell Company (Oxford United Kingdom).

2.7.2. FISH protocol

Based on some scoring criteria, FISH signals were defined by the existence of two sets[14]. In the first round, the X-chromosome specific signal presented as green, Y-chromosome specific signal as red, and 18 chromosome specific signals as blue. The second probe set was applied with fluorescent colors of green and red for chromosomes 13 and 21, respectively. The slides were immersed in saline sodium citrate (2× SSC) (pH 7.0) buffer for 2 min at room temperature without agitation. Then, they were each dehydrated in an ethanol series (70%, 85%, and 100%) for 1 min at room temperature, and the slides were allowed to dry. Next, 10 μL of probe set 1 was spotted onto the cell sample and a coverslip was carefully applied. The slides were on a hotplate at 37 ℃ for 5 min and then incubated at 75 ℃ for 2 min. The slides were placed in a humid lightproof container at 37 ℃ for 4 h. After carefully removing the coverslip, the slides were immersed in 0, 4×SSC (pH 7.0) buffer at 72 ℃ for 2 min and then immersed in 2×SSC, 0.05% Tween-20 at room temperature (pH 7.0) for 30 s. Finally, the slides were drained and 10 μL of 4′, 6-diamidino-2-phenylindole (DAPI) was applied to each. Then, they were covered with a coverslip after 10 min and studied for X, Y, and 18 chromosomes abnormalities with a fluorescent microscope. The slides of the second round were washed again with a PBS solution for 10 min and dehydrated through an ethanol series for 1 min at each. The FISH process was repeated with probe set 2 and the slides were studied for 13 and 21 chromosomes abnormalities with a fluorescent microscope.

2.7.3. Fluorescence microscope analysis

Analysis of chromosomes was carried out using an epi-fluorescence microscope (Motic BA 410). Nuclei were first located under the blue filter as they were stained with DAPI. The scoring of each nucleus was done according to color-changing of the filters. The nuclei that did not generate clear bright signals were not considered.

2.8. Statistical analysis

Data were analysed by using IBM SPSS (Statistical Package for the Social Sciences version 26.0, SPSS Inc., Chicago, IL, USA). The categorical data were presented as percentages. The comparison of frequency between groups was done using *Chi*-square test. Also, the relationships between the quality of embryos, the studied chromosomal abnormalities, and the age of patients were measured by Spearman correlation coefficient with the IBM SPSS.

2.9. Ethics statement

This study was approved by the Research Ethics Committee of Islamic Azad University-Arsanjan branch (code: IR.IAU.A.REC.1400.001). Written informed consent was obtained from all participants.

3. Results

3.1. Clinical characteristics of couples

In this study, 359 embryos from 62 infertile couples (age range > 30 years old) generated by ICSI method was obtained. The flowchart of screning of the study is presented in Figure 1.

Table 1 represents the clinical characteristics of all studied couples (n=62); all couples used their own eggs and sperm. Approximately, 67.7% (42/62) of females were aged less than 35 years old, and 32.3% (20/62) of them had an advanced maternal age (≥35 years), and 69.4% (43/62) of males were aged less than 40 years old and 30.4% (19/62) of them had an advanced paternal age (≥40 years).

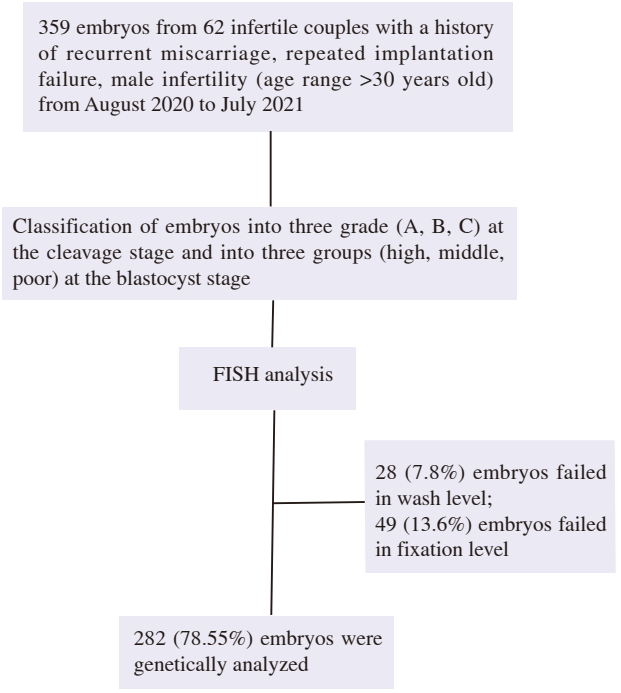

Figure 1. Flowchart of the study. FISH: fluorescent in situ hybridization.

Moreover, 48.4% of couples had a previous unsuccessful assisted reproductive technology, all of which had been treated with ICSI/IVF. All couples were normal for karyotypes and showed primary infertility. Furthermore, 30 (48.4%) couples had a history of failed IVF. The 359 embryos were gathered from 62 couples and 282 embryos were genetically analyzed (Table 2).

3.2. Embryo characteristics

In this study, among the 359 embryos obtained by assisted reproductive technology from 62 couples, 282 embryos were included in the genetic study. The embryos were genetically analyzed by PGS-FISH, and 70.57% (199/282) embryos showed normal results.

Table 2 shows the results of FISH test. The aneuploidy rate of embryos was 23.8% (67/282), and the main aneuploidy was complex aneuploidy of sex chromosomes, which was seen in 17.0% (48/282) embryos. Aneuploidy of chromosomes 13, 18, 21 was observed in 1.1% (3/282), 3.2% (9/282), and 2.5% (7/282) of cases, respectively.

3.3. Association of maternal age with quality and aneuploidy of embryos

Table 3 represents the clinical parameters of embryos quality. In addition, 69.2% (74/107) of high-quality, 76.2% (64/84) of middle quality, 71.8% (79/110) of low quality and 72.4% (42/58) arrested embryos were related to females under 36 years old. No significant relationship was observed between the quality of embryos and the age of the mother (P=0.40), and there was no correlation between these variables by Spearman correlation analysis (P=0.55). In addition, the most normal and abnormal embryos were related to women between 30-32 range ages. According to Table 4, no significant relationship was observed between the age of the mother and aneuploidy (P=0.08), and there was no correlation between these variables by Spearman correlation analysis (P=0.68).

3.4. Association of male factors with embryo quality

Among the couples, 40.3% of males had reproductive problems, including low sperm production and abnormal sperm function. Besides, 24.1% (33/137) of the embryos from infertile males and 33.3% (74/222) of the embryos from normal males had high quality. Table 5 shows the quality of normal and infertile men and by comparing the quality of embryos from normal and infertile men; no significant correlation was obtained between male factors and embryo quality (P=0.13) and there were weak correlation between these variables by Spearman correlation analysis (r=-0.092).

3.5. Association of embryo quality with euploidy/aneuploidy status

A total of 282 embryos from 62 females aged above 30 years old were evaluated by FISH analysis for chromosomes 13, 18, 21, X,

Table 1. Characteristics of couples included in the study.

Parameters	Data
Maternal age, years, median (range)	33 (30-42)
<35, *n* (%)	42 (67.7)
≥35, *n* (%)	20 (32.3)
Paternal age, years, median (range)	38 (30-48)
<40, *n* (%)	43 (69.4)
≥40, *n* (%)	19 (30.6)
Cytogenetics, *n*	124
Normal maternal karyotype, *n* (%)	62 (100)
Normal paternal karyotype, *n* (%)	62 (100)
Male factor, *n* (%)	25 (40.3)
Primary infertility, *n* (%)	62 (100)
Previous ART failure, *n* (%)	30 (48.4)
Number of total cycles ICSI/IVF, *n* (%)	62 (100)
Number of arrested embryo, *n* (%)	58 (16.2; 58/359)
Number of nucleus of embryos loss in the fixed stage, *n* (%)	49 (13.6; 49/359)
Number of nucleus of embryos loss in the wash stage, *n* (%)	28 (7.8; 28/359)
Number of genetically analyzed embryo, *n* (%)	282 (78.6; 282/359)

ART: reproductive assistance technology; ICSI: intracytoplasmic sperm injection; IVF: *in vitro* fertilization.

Table 2. Results of FISH in genetically analyzed embryos (n=282).

Results	Frequency	Percent
xx normal	125	44.3
xy normal	74	26.2
xo	21	7.4
xxx	15	5.3
xxy	8	2.8
Monoploidy	7	2.5
Tetraploidy	5	1.8
xx trisomy 18	4	1.4
xyy	4	1.4
Triploidy	4	1.4
xx monosomy 18	3	1.1
xy trisomy 21	2	0.7
xy trisomy 18	2	0.7
xx monosomy 21	2	0.7
xy monosomy 21	2	0.7
xx monosomy 13	1	0.4
xy monosomy 13	1	0.4
xx trisomy 13	1	0.4
xx trisomy 21	1	0.4

Table 3. Distribution of maternal age and quality of embryo (n=359).

Quality of embryo	Age of women, years						Total
	30-32	32-34	34-36	36-38	38-40	40-42	
High	44 (41.10)	22 (20.56)	8 (7.48)	16 (14.95)	11 (10.28)	6 (5.61)	107 (29.70)
Middle	33 (39.30)	21 (25.00)	10 (11.90)	12 (14.30)	2 (2.40)	6 (7.10)	84 (23.50)
Weak	36 (32.72)	34 (30.90)	9 (8.18)	16 (14.54)	9 (8.18)	6 (5.45)	110 (30.50)
Arrest	23 (39.65)	9 (15.51)	10 (17.24)	7 (12.01)	3 (5.17)	6 (10.34)	58 (16.20)
Total	136 (37.88)	86 (23.95)	37 (10.30)	51 (14.20)	25 (6.96)	24 (6.68)	359 (100.00)

Chi square test is used. Data are expressed as *n* (%). No significances between these variables are found.

Table 4. Distribution of maternal age and aneuploidy of genetically analyzed embryos (n=282).

Parameters	Age of women, years					
	30-32	32-34	34-36	36-38	38-40	40-42
xx normal	45 (38.13)	32 (47.05)	11 (39.28)	19 (54.28)	6 (46.15)	12 (60.00)
xy normal	39 (33.05)	14 (20.59)	9 (32.14)	6 (17.14)	3 (23.07)	3 (15.00)
abnormal	34 (28.81)	22 (32.35)	8 (28.57)	10 (28.57)	4 (30.76)	5 (25.00)
Total	118	68	28	35	13	20

Chi square test is used. Data are expressed as *n* (%). No significances between these variables are found.

Table 5. Distribution of male factors and embryo quality.

Quality	Infertile men		Total
	Yes	No	
High	33(24.08)	74 (33.33)	107 (29.80)
Middle	33 (24.08)	52 (23.42)	85 (23.67)
Weak	49 (35.76)	60 (27.02)	109 (30.36)
Arrest	22 (16.05)	36 (16.21)	58 (16.15)
Total	137	222	359

Chi square test is used. Data are expressed as *n* (%). No significances between these variables are found.

Table 6. Distribution of aneuploidy and quality of genetically analyzed embryos.

Quality of embryo	Results of FISH			Total
	Normal	Aneuploidy	Euploidy	
A	52 (26.13)	19 (28.35)	3 (18.75)	74 (26.24)
B	49 (24.62)	14 (20.89)	6 (37.50)	69 (24.46)
C	67 (33.66)	24 (35.82)	3 (18.75)	94 (33.33)
Stop growing	31 (15.57)	10 (14.92)	4 (25.00)	45 (15.95)
Total	199	67	16	282

Chi square test is used. Data are expressed as *n* (%). No significances between these variables are found.

and Y. 26.1% (52/199) of normal embryos and 28.4% (19/67) of aneuploid embryos had high quality (grade A). The frequency of normal and aneuploid embryos in weak quality embryos (grade C) was 33.7% (67/199) and 35.8% (24/67), respectively, and 15.6% (31/199) of normal embryos, 14.9% (10/67) of aneuploid embryos stopped growing. According to the Table 6, no significant correlation between quality of embryos and chromosomal status (P=0.67).

4. Discussion

In this study, all couples were above 30 years old and all of them underwent IVF treatment and PGS for common chromosomal abnormalities. Some studies have suggested that the rate of meiotic errors may increase in eggs during ovulation induction protocols used for standard IVF procedures. Therefore, it can lead to the increase of aneuploidy in the resulting embryos[14,15]. The detection of chromosomal abnormalities has promoted the success rate of embryo transfer and healthy live births[16]. In this study, 62 couples had aneuploidy in 67 (23.8%; 67/282) analyzed embryos. It has been indicated that PGS improves clinical outcomes by successful embryo implantation in embryos from women of advanced maternal age; however, recurrent implantation failure is common because of unknown paternal factors[14,17].

Our data demonstrated that there is no significant correlation between the quality of embryos and the genetic status. This finding is in agreement with the studies conducted by Bazgar *et al*[18] and Fesahat *et al*[10], reporting inconsistency between embryo morphology and the results of genetic in embryos obtained through assisted reproductive technology. Alfarawati *et al*[2] reported a weak association between morphology of embryos and rate of aneuploidy and they concluded that obtaining high-quality embryo does not warrant the euploidy of the embryo. However, such results differ from the study by Majumdar *et al*[19] who demonstrated a significant correlation between the morphology of blastocyst and the euploidy rate. Chamayou *et al* demonstrated that a large percentage of chromosomally abnormal embryos are able to reach normally to blastocyst stage with a high possibility of implantation and pregnancy[20]. Besides, Cárdenas *et al* showed that embryo quality is not associated with its genetic status[21]. Ziebe *et al* indicated that the number of embryos (scored as containing <6 cells at 68 h after insemination) is not significantly correlated with chromosomal abnormality rate[22], which is inconsistent with the results of some other studies. Anderson *et al* showed a significant difference between embryo quality and euploidy and aneuploidy blastocysts at day 5[23]. Braga *et al* compared the embryo morphology between euploid and aneuploid embryos and found a significant difference between embryo quality at day 3 and euploidy and aneuploidy blastocysts[24]. Considering that in the studies that had conflicting results with our results, biopsy of embryos was performed at the blastocyst stage and chromosomal abnormalities were examined at this stage, while in our study chromosomal abnormalities were examined at the cleavage stage. This difference can be related to the difference in the developmental stage of the embryos during the studies.

Based on the results of several previous reports, the embryo aneuploidy rate increases with a woman's age[25–28]. However, in the current study, 75% of abnormal embryos were from mothers aged below 36 years old. Our observation supports the findings by Fesahat *et al*[10] that reported 37.1% chromosomal abnormalities in embryos from women under 35 years old. Capalbo *et al*[29] reported 55.5% aneuploidy rate in embryos with a maternal age between 26 and 44 years. In the current study, no significant association was achieved between aneuploidy rate and maternal age. This result was in accordance with the study of Eaton *et al*[30], which concluded that maternal age does not significant effect on embryo morphology. Overall, there are limitations in our study and similar studies. Ovarian reserves are different in age groups, and patients over 35 years of age may have little ovarian reserve and a small number of embryos will be formed for them, in which case the number of embryos must be increased many times by using embryo freezing,

and then PGS is performed. This method takes much more time and is not suitable for short-term study. More prospective long-term studies, including a larger number of patients with low ovarian reserve, could influence these results and consistent pregnancy rates, suggesting that PGS may be more beneficial for older women who are over 35 years of age and have a lower chance of pregnancy[31].

In our study, there was no significant correlation between male factors and the genetic status of embryos. This observation was in line with the study by Mazzilli *et al*[32] that reported no significant correlation between male factors and embryo genetic status.

As far as the researchers of this study investigated, this is the first study from East Azerbaijan in Iran. However, the results of the current study are comparable with other published reports. For future works, it would be better to increase the sample size with a multicentric approach to create significant associations between the studied parameters. Furthermore, the number of chromosomes could be increased and the results of FISH technique could be compared with other techniques to identify the advantages and disadvantages of the techniques for PGS.

The main limitations of this study are the using PGS instead of other newly developed advanced technologies and also relatively small sample size.

The current study assists the use of PGS-FISH technology to improve the outcome of assisted reproductive technology; however, other novel molecular techniques, such as next-generation sequencing and array-based comparative genomic hybridization, have their own clinical and technical advantages like high-throughput mutation screening and genome-wide copy number analysis over FISH, but these methods also have some drawbacks like low specific signal, lack of directly detection of polyploidies and balanced chromosomal rearrangements, and higher costs and time[33]. Although PGS-FISH is not a novel technique, it can be useful to analyze all embryos for infertile couples without any known genetic indications or consequences, for X-linked genetic diseases, inherited chromosome rearrangements, and chromosomal aneuploidy before the transfer of embryo to the uterus.

In conclusion, the rate of abnormal embryos from the infertile couples is 29.4% (83/282) in this study. Since there is no statistically significant correlation between embryo quality and chromosomal abnormality, the appearance of the embryo could not be considered as a criterion for choosing a healthy embryo for transfer to the uterus.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Authors' contributions

Mina Niusha and Dr Seyed ali Rahmani designed the study, collected all the data, and drafted the manuscript. Mina Niusha and Dr Hamid Reza Nejabati drafted the manuscript. Dr Mohammad Nouri analysed and interpreted the scientific data/results. All authors read and approved the final manuscript.

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