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Lapatinib Decreases the Preimplantation Aneuploidy Rate of in vitro Fertilized Mouse Embryos without Affecting Completion of Preimplantation Development

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In vitro fertilization · Preimplantation · Aneuploidy · Lapatinib · Fluorescent in situ hybridization

Abstract
One of the major reasons for implantation failure and spontaneous abortion is a high incidence of preimplantation chromosomal aneuploidy. Lapatinib simultaneously inhibits EGFR and HER2, leading to apoptosis. We hypothesized a higher sensitivity for aneuploid cells in preimplantation embryos to lapatinib based on reports of aneuploid cell lines being sensitive to some anticancer drugs. Late 2-cell mouse embryos were treated with lapatinib after determining a nontoxic dose. Morphologies were recorded 24, 48, and 60 hours later. The effect of lapatinib on the aneuploidy rate was evaluated by studying blastocyst cells using FISH. Although the rate of development to 8-cell and morula stage was higher in the control group ($p < 0.05$), there was no difference in development to the blastocyst stage at the same studied intervals between lapatinib-treated and control groups ($p = 0.924$). The mean number of cells in morula and blastocyst stages were not different between the groups ($p = 0.331$ and $p = 0.175$, respectively). The frequency of aneuploid cells and diploid embryos was, respectively, significantly lower and higher in lapatinib-treated embryos, ($p < 0.001$). Since lapatinib treatment reduced the aneuploidy rate without impact on the development of mouse preimplantation embryos to the blastocyst stage and number of total cells, lapatinib seems useful for prevention of preimplantation aneuploidy in in vitro fertilization.

Introduction

Assisted reproductive technology has been employed as an approach for treatment of infertility after the birth of the first baby named Louise Brown in 1978 [Steptoe and Edwards, 1978] using in vitro fertilization (IVF). One of the major reasons for implantation failure and spontaneous abortion of embryos obtained from IVF is the high incidence of chromosomal abnormalities, especially aneuploidy in the preimplantation stage [Lee and Kiessling, 2017]. Also, when an established pregnancy fails to progress to a live birth, one of the most important known contributing factors is aneuploidy [Forman et al., 2018]. Presence of aneuploid cells reduces the rate of IVF success

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[Sugawara et al., 2006] and induces profound changes in gene expression, as well as the proliferation and tumorigenicity of human pluripotent stem cells which results in the sensitivity of aneuploid pluripotent cells to some anticancer drugs [Ben-David et al., 2014]. Embryo selection based on morphology is routine in assisted reproductive technology [Nasiri and Eftekhari-Yazdi, 2015]. However, it does not seem to be a reasonable criterion [Gleicher and Orvieto, 2017]. Culture media influences mammalian oocytes and preimplantation embryos [Scott and Whittingham, 1996; Martinez et al., 2017]. Genes which are essential for the growth, proliferation, and survival of mouse preimplantation embryo cells such as erb-b2 receptor tyrosine kinase 2 (Erbb2, HER2, Neu or c-erbB2) and baculoviral IAP repeat-containing 5 (Birc5) play important roles in the regulation of cell proliferation and the inhibition of apoptosis [Haouzi et al., 2018]. The mentioned genes are highly expressed in some cancers and preimplantation embryos [Alroy and Yarden, 1997; Mull et al., 2014]. We hypothesized that aneuploid cells in preimplantation embryos would show a higher sensitivity to the anticancer drug lapatinib based on reports for more sensitivity of aneuploid cell lines to anticancer drugs [Ben-David et al., 2014; Dobbelstein and Moll, 2014]. Lapatinib (GW572016 or Tykerb) is a potent reversible ATP-competitive inhibitor, which simultaneously inhibits the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTKs) including Egfr (Errb1) and Erbb2 (HER2, Neu, c-erbB2) which are involved in cell survival, proliferation and mobility [Wang and Hung, 2012] by binding to the mentioned receptors' intracellular tyrosine kinase domain and influencing intracellular signaling pathways including the PI3K and MAPK pathways, giving rise to a marked inhibition of cell division and cell cycle with subsequent apoptosis [Moy et al., 2007]. The effect of lapatinib on survivin, which regulates cell division and inhibits apoptosis, is mediated by the inactivation of HER2 and the PI3K pathway [Caldas et al., 2005]. Survivin and HER2 as 2 key proteins in the early development of the embryo belonged to proto-oncogenes that induce cell growth and proliferation and inhibit apoptosis. The occurrence of chromosomal instability by disruption of these 2 proto-oncogenes causes the cells to grow out of control, resulting in more chromosomal abnormalities, which manifests itself in the form of cancer in humans and abortions in fetuses resulting from IVF. Previous studies have shown that lapatinib has a more specific inhibitory effect on HER2 in the family of EGFR receptors [Rusnak et al., 2001], and the cancer cells with overexpression of HER2 are more sensitive to lapatinib [Wainberg et al., 2010]. This drug has been approved by the FDA for the first time in 2007, and later for the treatment of HER2-positive metastatic breast cancers [Sannino and Brodsky, 2017].

Materials and Methods

Animals

The Naval Medical Research Institute (NMRI) mice (Royan Institute, Iran) with 6–8 weeks of age and 25–35 g in weight were kept at 23°C, humidity of 40–50% and 12 h of light (6 a.m–6 p.m.).

Sample Preparation

Superovulation was induced using 7.5 IU of pregnant mare's serum gonadotropin (Intervet, UK), followed by 7.5 IU of human chorionic gonadotropin (Intervet, UK) 48 h later. After 18 h, the cauda epididymis of male mice was dissected and sperms were added to cumulus-oocyte complexes in Tyrode (T6) medium. The dishes were then transferred to an incubator (37°C, 5% CO_2). Fertilization was confirmed 16–20 h after insemination by the presence of 2 pronuclei (2 PN) and extrusion of the second polar body. Zygotes at the 2 PN stage were transferred to G1 medium containing human serum albumin (HSA; Vitrolife, Sweden). In order to find the appropriate nontoxic dose of lapatinib, 150 late 2-cell embryos were randomly included in either the control or the following treatment groups: 0.05, 0.1, 0.2, and 0.5 μM of lapatinib (Biovision, USA) for 24 h. The number of embryos which developed to the 4-cell stage was compared with the control and treated groups for each concentration. The highest dose in which there was no statistically significant difference between the survival rate of embryos in the lapatinib-treated group and the control group was chosen as the appropriate nontoxic dose. Afterward, late 2-cell embryos were treated with the nontoxic dose of lapatinib, and their development toward blastocyst was compared with controls following transfer of embryos to G2 medium (Vitrolife, Sweden) containing HSA and daily recording of the developmental stages 24, 48 and 60 h after the late 2-cell stage (equating to days 1.5, 2.5, and 3.5 following the formation of 2 PNs postfertilization, respectively).

Embryo Fixation and FISH

Fixation of embryos at the morula/blastocyst stages and FISH were performed according to a previously described method [Bazrgar et al., 2016] with some changes. Embryos were exposed to a hypotonic solution, 1 mg/mL bovine serum albumin (Sigma, USA) in distilled water, before carrying out the mentioned method. Specific probes for chromosomes 2 (2qH3) and 11 (11qE2) (Kreatech, The Netherlands) were used. FISH analyses were performed based on the protocol described by Munne et al. [1998], including the diameter of the nuclei, the distance between same-chromosome signals, domains apart (the domains in which 2 signals are separated), and the size of signals related to each homologue chromosome in interphase.

Embryo Classification

Each embryo was classified based on its chromosomal constitution, according to previously published criteria [Elaimi et al., 2012].

Table 1. In vitro development of mouse preimplantation embryos in lapatinib and control groups after removing the drug from the medium day 1.5, days 2.5 and 3.5 since late 2-cell, *N* (%)

Stage Time	<8-cell lapatinib	 control	8-cell lapatinib	 control	Morula lapatinib	 control	Blastocyst lapatinib	 control
Day 1.5 L (*n* = 299) C (*n* = 281) *p* value	238 (79.6) 0.024	201 (71.5)	61 (20.4) 0.030	79 (28.1)	0 0.302	1 (0.4)	0 	0
Day 2.5 L (*n* = 249) C (*n* = 246) *p* value	103 (41.4) 0.201	88 (35.8)	30 (12) 0.051	17 (6.9)	104 (41.8) 0.022	128 (52)	12 (4.8) 0.813	13 (5.3)
Day 3.5 L (*n* = 251) C (*n* = 234) *p* value	107 (42.6) 0.107	83 (35.5)	27 (10.8) 0.089	15 (6.4)	28 (11.2) <0.001	54 (23.1)	89 (35.5) 0.924	82 (35)

Statistical Analysis

For statistical analysis of various parameters, including morphological outcomes and the comparison of development between the studied groups as well as the rate of normal cells and the average percentages of normal and aneuploid cells, the χ^2 test and independent *t* test were used. The statistical significance of the results was analyzed using SPSS software version 22 (Chicago, IL, USA). *p* values <0.05 were considered statistically significant.

Results

Nontoxic Dose of Lapatinib

There was no significant difference between the viability of embryos treated with 0.2 μM of lapatinib and controls. Accordingly, it was selected as the nontoxic dose. Viability in the other conditions was significantly less in the treated group compared to controls.

Preimplantation Embryo Development

The development of 580 late 2-cell embryos was assessed, and toward the 8-cell stage, 281 were placed in the control group and 299 treated with 0.2 μM of lapatinib (named the lapatinib group). After 24 h, the number of embryos developed up to the 8-cell stage, based on their morphology, was higher in the control group (79/281, 28.1%) than the lapatinib group (61/299, 20.4%) (p = 0.030).

The number of embryos that passed the cleavage stage was significantly higher in the lapatinib group compared to the controls with 238/299 (79.6%) versus 201/281 (71.5%) (p = 0.024), and they were allowed to continue their growth for further studies. After a 2-day period, of the 495 embryos that had passed the 2-cell stage, 52% (128/246) reached the morula stage in the controls compared to 41.8% (104/249) in the lapatinib group (p = 0.022). There was no difference in the early blastulation rate at this time with 12/249 (4.8%) of treated embryos in comparison to 13/246 (5.3%) for the controls (p = 0.813) (Table 1).

Embryo Development after 60 h

Of the 495 abovementioned embryos, after 60 h, 485 were reliable for morphological classification: 28/251 (11.2%) and 54/234 (23.1%) were at the morula stage in lapatinib and control groups, respectively (p < 0.001), while 89/251 (35.5%) and 82/234 (35%) were in the blastocyst stage (p = 0.924) in lapatinib and control groups, respectively (Table 1). It is notable that other embryos were in either <8- or 8-cell stages. A total of 57 embryos in lapatinib group and 39 in the control group were analyzed by FISH and were also compared based on their mean number of cells. There was no significant difference between the groups in either of the morula or blastocyst stages (Table 2).

Effect of Lapatinib on Aneuploidy Rate

In total, 96 embryos were cultured for 60 h (up to day 3.5 after fertilization) and analyzed using FISH. In the 39 embryos from the control group (7 morula and 32 blastocysts), 846 of the 1,721 total cells were analyzed. From

Table 2. The mean number of cells in embryos developed for 60 h

Group (number of embryos)	Morula	Blastocyst
Lapatinib (57)	16±6.28[a]	44.65±14.17[a]
Control (39)	20.14±7.33[a]	48.96±13.81[a]
p value	0.331	0.175

[a]Values are means±SD.

Table 3. Embryo classification based on diploid cell percentages after 60 h culture, *N* (%)

Embryo classification	Diploid cells, %	Lapatinib	Control	*p* value
Normal	100	22 (38.6)	1 (2.6)	<0.001
	90–99	14 (24.6)	5 (12.8)	0.156
		36 (63.2)	6 (15.4)	<0.001
Abnormal	0	1 (1.7)	4 (10.2)	0.066
	1–10	2 (3.5)	0	0.237
	11–89	18 (31.6)	29 (74.4)	<0.001
		21 (36.8)	33 (84.6)	<0.001
Total		57 (100)	39 (100)	

the 57 embryos of the lapatinib group (5 morula and 52 blastocysts), 1,262 of the 2,414 total cells were analyzed. The number of aneuploid cells was 178 (14.1%) versus 277 (32.7%) in the lapatinib and control groups, respectively ($p < 0.001$). The frequency of normal and fully diploid embryos was higher in the lapatinib group compared to controls ($p < 0.001$) (Table 3).

Of the 1,262 analyzed nuclei related to 57 lapatinib-treated embryos, 85.9% (1,084) had 2 signals for chromosomes 11 and 2. Figure 1 presents chromosome-specific probe FISH for a mosaic embryo. The same analysis in the control group showed that of the 846 analyzed nuclei related to 39 embryos, 67.3% (569) were normal in terms of these 2 chromosomes ($p < 0.001$). In the case of abnormal cells, there were 178 (14.1%) cells in the lapatinib group, of which 41 (3.2%) cells were abnormal for chromosome 2 but not for chromosome 11, 38 (3.0%) cells were abnormal for chromosome 11 but not for chromosome 2, and 99 (7.8%) cells were abnormal for both chromosomes. Of the 277 aneuploid cells in the control embryos, 101 (11.9%) cells were abnormal for chromosome 2 but not for chromosome 11, 51 (6.0%) cells were abnormal for chromosome 11 but not for chromosome 2, and 125

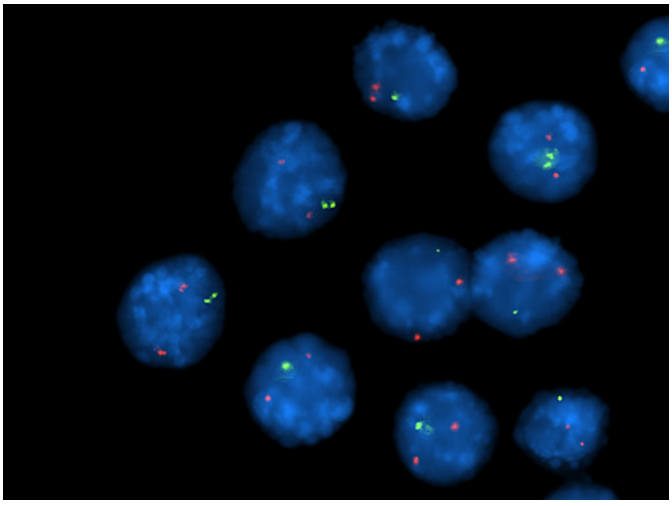

Fig. 1. Normal and aneuploid cells in a mosaic embryo according to FISH by chromosome-specific probes for chromosomes 2 (green) and 11 (red).

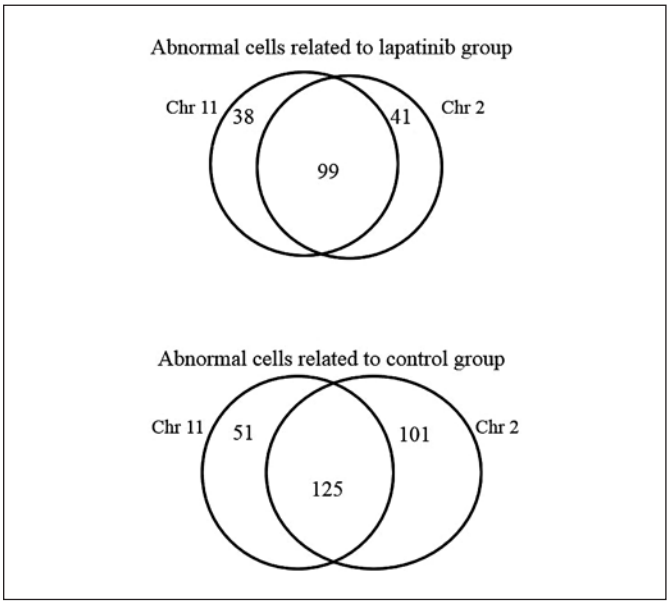

Fig. 2. Distribution of aneuploidy in abnormal cells of lapatinib (top) and control (bottom) groups according to chromosomes detected as aneuploid.

(14.8%) cells were abnormal for both chromosomes ($p < 0.001$). Figure 2 represents the distribution of aneuploidy in abnormal cells of lapatinib and control groups according to chromosomes detected as aneuploid.

The number of normal cells was higher in the embryos treated with lapatinib which reached the blastocyst stage by day 3.5 compared to the control group with 1,043/1,209

(86.3%) versus 509/734 (69.3%), respectively ($p < 0.001$). Furthermore, the number of normal cells in the lapatinib group was higher (45/53, 84.9%) than the control group (63/112, 56.3%) in embryos which were in the morula stage on day 3.5 ($p < 0.001$).

Discussion

Considering the activation of the mouse embryo genome at the 2-cell stage which is crucial for preimplantation development to continue beyond the 2-cell stage [Latham and Schultz, 2001; Abe et al., 2018], treatment with lapatinib was carried out at late 2-cell stage so that the desired drug would be in the culture media from the beginning of genome activation. In terms of embryo survival, 0.2 μM for 24 h was determined to be the appropriate treatment conditions as it was the highest dose without any negative effect on embryo development. This is in accordance with our recent study on reduction of chromosomal instability in mouse embryonic stem cells (unpublished data) and evaluation of cancer cell lines with chromosomal abnormality [Wetterskog et al., 2014; Xiang et al., 2018]. According to a previous study, 40.9% of 2-cell embryos developed into a blastocyst [Summers et al., 2005]. In the present study, following the exposure of 2-cell embryos to lapatinib, their subsequent development was lagged, so as comparisons between groups at the same time revealed a lower frequency of 8-cell and morula embryos, cultured for 1.5 and 2.5 days postfertilization, in the lapatinib group (20.4 vs. 28.1%, $p = 0.030$ and 41.8 vs. 52%, $p = 0.022$). Lapatinib slows down cell cycle progress by inhibiting cell division via survivin which is involved in the proper targeting of chromosome passenger proteins to the kinetochores and the formation of a bipolar spindle needed for proper chromosome segregation [Mita et al., 2008] as well as cell proliferation through HER2 [Wang and Hung, 2012] in cancer cell lines. After 60 h postfertilization, there was no difference between the development of embryos to the blastocyst stage in both the lapatinib (35.5%) and control (35%) groups ($p = 0.924$) (Table 1). We compared blastocyst the rate of NMRI mouse strain in our study with 3 other studies [Moshkdanian et al., 2011; Dehghani-Mohammadabadi et al., 2014; Toori et al., 2014]; 1 of them was comparable with the current study at the same postfertilization time, while 2 others had reported a higher rate of blastocyst than ours. These differences could be explained by differences in the genetic characteristics of the mice used in the outbred strain in addition to extrinsic factors.

Interestingly, in day 3.5, when we expect embryos to pass the morula stage, a lower portion of treated embryos were in the morula stage compared to the controls (11.2 vs. 23.1%), ($p < 0.001$).

This means that despite the initial lag in the lapatinib group, they continually maintained their fairly desirable morphology. All in all, it seems that lapatinib treatment did not interfere with the completion of preimplantation development of the mouse embryos.

The demand for human IVF is increasing, but the success rate as measured by live births has not increased as much as expected. One of the leading causes of IVF failure and pregnancy loss is aneuploidy [Minasi et al., 2016]. To determine the quality of the embryos in addition to their development, the total number of morula- and blastocyst-stage cells as well as the rate of aneuploidy were compared between 2 groups in the present study. The mean number of cells was comparable with a previous report on IVF mouse preimplantation embryos, with no significant difference between groups, although they were naturally lower than in vivo-developed embryos [Forman et al, 2018] (Table 2). The high frequency of aneuploidy in embryos obtained from IVF is one of the reasons related to the inhibition of embryo development, failure in embryo implantation, and ultimately abortion [Nagaoka et al., 2012; Scott et al., 2012; Lee and Kiessling, 2017]. Sabhnani et al. [2011] declared a 31% prevalence of aneuploidy in in vitro-cultured mouse blastocysts, according to FISH assays for chromosomes 11 and 2. In the current study, the aneuploidy rate was clearly lower in the lapatinib group compared to controls and what Sabhnani et al. [2011] reported. The culture conditions do affect the rate of preimplantation aneuploidy [Simopoulou et al., 2018]. Aneuploidy may create different cell lines resulting in chaotic or mosaic embryos, albeit raising the live birth in some cases. For instance, mosaic embryos with a low aneuploidy percentage (<50%) have higher chances of resulting in the birth of healthy babies similar to euploid embryos compared to embryos with higher mosaicism levels (≥50%), and the extent of the mosaicism influences the IVF success rate [Bolton et al., 2016; Spinella et al., 2018]. It seems that stoichiometric imbalance of protein copy numbers in aneuploid cells activates mechanisms and pathways in these cells [Tang et al., 2011; Amano et al., 2015] that possibly play a role in lineage-specific depletion of aneuploid cells and a shift of the mosaic embryos toward normality, i.e.,selection against abnormal cells in the embryo [Bazrgar et al., 2013; Spinella et al., 2018]. It should be noted that aneuploidy is a hallmark of cancer, and the occurrence of chromosomal abnormali-

ties in mouse cell line amplifies the ability of these cells to proliferate as cancer cells [Sugawara et al., 2006]. Moreover, screening of 89 anticancer drugs revealed that trisomy 12 raises the sensitivity of human pluripotent stem cells to several replication inhibitors compared to normal cells [Ben-David et al., 2014]. Lapatinib was one of the tested drugs in this study, but they could not classify it as a lethal or hit drug based on sensitivity of used aneuploid cell line to each of the tested drugs in their experimental condition. Together, these findings suggested that applying some of anticancer drugs will reduce the instability of cancer cells or prevent the growth of abnormal cells [Negrini et al., 2010]. Anticancer drugs are classified in 3 groups in terms of their mechanism of action. They either target (1) DNA replication and repair, (2) cellular signaling pathways, or (3) cellular machineries that are essential for tumor growth and survival, such as chromatin modifiers, protein chaperones, or the proteasome [Dobbelstein and Moll, 2014]. The possible detriments of enriching culture media with "promising" elements, such as the mentioned drugs to act on embryos during development, should always be considered. With that in mind, the effect of the anticancer drug lapatinib on the reduction of aneuploidy in mouse preimplantation embryos obtained from IVF was investigated in the current study.

According to a recent review article [Tšuiko et al., 2019], aneuploidy and mosaicism have been reported in preimplantation stage in human and different animal models; although some of the reviewed studies report a high rate of spontaneous aneuploidy in mouse, some others report it in a low rate. Preimplantation development in mouse and human are different in some aspects, certainly in the time of genome activation, murine model might not always be perfect for human preimplantation studies. The diploid cell rate in the control group of the current study was comparable with its rate in human blastocysts [van Echten-Arends et al., 2011] that significantly increased in the lapatinib group. We first classified embryos according to previously published criteria [Elaimi et al., 2012] that embryos with more than 10% aneuploidy should not be counted as normal; this analysis resulted in normality in 15.4 and 63.2% of embryos in control and lapatinib, respectively, ($p < 0.001$). Obviously, classification based on the mosaicism rate is highly dependent on the cutoff for interpretation of an early embryo as normal. Since mouse model of mosaicism revealed live birth following 50% aneuploidy [Bolton et al., 2016], we also compared groups with a 50% cutoff for aneuploidy; according to this comparison, the rate of embryos with ≥50% diploid cells in the treatment and control groups was 89.5 and 76.9%, respectively ($p = 0.962$). It should be noted that embryonic aneuploidy is not a fixed fate [Bazrgar et al., 2013; Tšuiko et al., 2019] as transfer of mosaic embryo in mouse [Bolton et al., 2016] and human [Greco et al., 2015; Spinella et al., 2018] resulted in live birth.

We used chromosome-specific FISH for a limited number of chromosomes due to the inherent limitation of the FISH technique. We faced a lot of overlapping signals in our preliminary experience with a pancentromeric probe, which made chromosome enumeration difficult (data not shown). Array comparative genome hybridization (aCGH) and next-generation sequencing (NGS) can provide a comprehensive view of the cell's genomic status, but it should be considered that the most frequent finding of our study was embryos containing both diploid and aneuploid cells which was consistent with previous preimplantation reports [Blagosklonny et al., 1997; Lee et al., 2005]. Study of a pool of cells using aCGH or NGS could lead to masking of aneuploidy by normal cells in cases of mosaicism at levels lower than the mosaicism detection power of these techniques. Therefore, using aCGH or NGS could be helpful in cases of studying at the single cell level. Obviously, analysis of numerous cells of morula and blastocysts using aCGH or NGS makes the study very expensive to conduct. We thus turned to FISH as a more cost-effective technique. The decision to study chromosomes 2 and 11 was based on evidence about the aneuploidies of these chromosomes in different somatic [Yoshida et al., 2007; Olme et al., 2013] and germinal [Cushman et al., 2007; Champroux et al., 2018] murine tissues. Kreatech has offered probes for these chromosomes together with ready-to-use dual-color vials in this regard. Increased incidence of mosaicism for chromosomes 2 and 11 detected by FISH has been reported in murine blastocysts cultured in vitro [Sabhnani et al., 2011].

FISH analyses of cells with good quality signals revealed that the percentage of aneuploidy for chromosomes 2 and 11 in the lapatinib group was clearly less than the controls in both embryo classification and total cell aneuploidy evaluations ($p < 0.001$). Lapatinib selectively affects cells with overexpression of HER2 [Wainberg et al., 2010] and moves them toward apoptosis by depleting survivin in Erbb2-overexpressing breast cancer cells [Xia et al., 2006]. One open question in the current study is whether the reduced aneuploidy originates from superior genome stability (e.g., less frequent chromosome malsegregation) or from enhanced elimination of blastomeres that carry aneuploidy. Despite a temporary developmental delay in cleavage stage passage, there was no signifi-

cant difference in total cells of the treatment and control groups (Table 2), it appears that the mechanism lies in a superior genome stability supported by lapatinib.

It is important to mention that according to previous reports by Elaimi et al. [2012] as well as Gleicher and Orvieto [2017], morphologically normal and degenerated embryos obtained from IVF can be chromosomally abnormal and normal, respectively. Regarding recent reports that mosaic embryos with more than 50% diploid cells will have the ability to lead to live births [Bolton et al., 2016; Spinella et al., 2018], it seems that the reduction of aneuploidy via lapatinib treatment is a promising finding to decrease the preimplantation aneuploidy rate as a common phenomenon that affects assisted reproductive technology success rate.

Conclusion

In conclusion, the treatment of preimplantation embryos with lapatinib does not affect preimplantation development completion, despite a temporary delay in embryo development, and could be effective in reducing the rate of aneuploidy. Since there was no significant difference in total cells of the treatment and control groups, it seems that the mechanism lies in a superior genome stability supported by lapatinib.

Statement of Ethics

This study was approved by the Ethics Committee of Royan institute (ID: IR.ACECR.ROYAN.REC.1394.88).

Conflict of Interest Statement

The authors declare no conflict of interests.

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Author Contributions

Conceptualization: P.M., M.B., H.G., B.E. Data curation: P.M., H.G., M.T., A.G.N., M.B. Formal analysis: P.M., H.G., M.T., A.G.N., M.B. Funding acquisition: M.B., H.G. Investigation: P.M., H.G., M.T., A.G.N., M.B. Methodology: P.M., H.G., M.T., A.G.N., M.B. Project administration: M.B., H.G. Resources: P.M., M.B., H.G. Supervision: P.M., H.G., M.T., M.B. Validation: P.M., H.G., M.T., A.G.N., M.B. Visualization: P.M., H.G., A.G.N., M.B. Original draft: P.M., M.B. Review and editing of the manuscript: P.M., H.G., M.T., A.G.N., M.B.

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