CRISPR/Cas9-mediated gene editing in human zygotes using Cas9 protein

Lichun Tang1,2 · Yanting Zeng3 · Hongzi Du3 · Mengmeng Gong1 · Jin Peng1 · Buxi Zhang1 · Ming Lei3 · Fang Zhao4 · Weihua Wang5 · Xiaowei Li6 · Jianqiao Liu3

Abstract Previous works using human tripronuclear zygotes suggested that the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system could be a tool in correcting disease-causing mutations. However, whether this system was applicable in normal human (dual pronuclear, 2PN) zygotes was unclear. Here we demonstrate that CRISPR/Cas9 is also effective as a gene-editing tool in human 2PN zygotes. By injection of Cas9 protein complexed with the appropriate sgRNAs and homology donors into one-cell human embryos, we demonstrated efficient homologous recombination-mediated correction of point mutations in HBB and G6PD. However, our results also reveal limitations of this correction procedure and highlight the need for further research.

Keywords CRISPR/Cas9 · Homology-directed repair (HDR) · Cas9 protein · Gene modification · Human zygotes

Introduction

There are many hundreds of disease-causing single-gene mutations and often there are no cures for these diseases. Correcting disease-causing genetic defects in human zygotes was previously unthinkable because the efficiency would be too low to be of any practical value. CRISPR/Cas9 offers, for the first time, a tangible potential to allow the correction of genetic defects (Cong et al. 2013; Mali et al. 2013; Niu et al. 2014). Separately from the debate of the merit and ethics of germline editing in humans, the feasibility of correcting genetic defects via CRISPR technology in human zygotes has not been really tested. Two recent reports used 3PN (3 or more pronuclei) human zygotes to test gene editing efficiency and the reported HDR efficiencies were only around 10% (Liang et al. 2015; Kang et al. 2016).

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Gene editing has been demonstrated in many cells including cultured human cells and fertilized rodent and non-human zygotes and is easily achieved using a specific single guide RNA (sgRNA) to guide and target the Cas9 protein to the sequence position to be modified (Li et al. 2013; Shen et al. 2013; Niu et al. 2014). The Cas9/sgRNA complex creates a DNA double-strand break (DSB) at the targeted site, which triggers the endogenous DNA repair system, resulting in targeted genome modifications. DSB can be repaired in the cell by two major pathways: homology-directed repair (HDR) or non-homologous end joining (NHEJ) depending on the cell state and the presence of a repair template (Lieber 2010; Chapman et al. 2012). Precise genome editing is required to repair genetic defects and largely depends on the HDR repair pathway, which functions in the late S–G2 phase (Heyer et al. 2010). The efficiency of HDR is determined by the time of generation of the DSB site, the concentration of donor DNA, and the length of the homology arms of the donor DNA (Hasty et al. 1991; Lin et al. 2014a). The Cas9 protein can be provided from a Cas9 expression plasmid or Cas9 mRNA to bypass the transcription or translation steps to generate protein, and can generate DSB site quickly at designed sites. By controlling the time of injection, we can easily control the time of generation of DSB site (Lin et al. 2014a). Cas9 protein is degraded rapidly in cells, which may reduce off-target effects (Kim et al. 2014). A number of investigators have used Cas9 protein (instead of its mRNA) to edit genes in other models (Cho et al. 2013; Lee et al. 2014; Chen et al. 2016; Hashimoto et al. 2016). Here we show that Cas9-mediated gene editing is highly efficient in human 2PN zygotes. Homology-directed repair occurred in both the embryos carrying the G6PD G1376T mutation. Our work shows the feasibility of the correction of disease-causing genetic defects in human zygotes.

Materials and methods

Cas9/sgRNA production and injection

In vitro transcription and production of sgRNAs were performed as described (Zhou et al. 2014). Briefly, sgRNA oligos were annealed and ligated into the pUC57-sgRNA expression vector (Niu et al. 2014; Zhou et al. 2014), transcribed in vitro using the MEGAshortscript Kit (Ambion), and purified with the MEGAclean Kit (Ambion). The sgRNAs were concentrated by ethanol precipitation. Recombinant Cas9 protein was purchased from PNA Bio (Thousand Oaks, CA). Cas9 mRNA was purchased from ViewSolid Biotech (Beijing, China).

The Cas9 protein and sgRNAs were diluted into injection buffer (0.25 mM EDTA/10 mM TrisHCl, pH7.4) and incubated for 10 min at 37°C before injection. The final concentration of Cas9 was 50 ng/µl, and that of sgRNA was 30 ng/µl. The donor oligos were present at 100 ng/µl for the injection. The oligo sequences are listed in the supplemental material. Microinjection was performed under an inverted microscope equipped with micromanipulators and a microinjector (FemtoJet, Eppendorf).

Source of embryos

Patients undergoing IVF treatment at the Center for Reproductive Medicine, at the Third Affiliated Hospital of Guangzhou Medical University, gave signed informed consent for the use of their 3PN embryos for research. They also gave signed informed consent for use of their immature oocytes and leftover sperm to produce normal (2PN) zygotes for research. The collection of oocytes and the culture of early embryos were performed routinely in the clinic using standard procedures. The zygotes were observed under an inverted microscopy 16–18 h after the insemination and the 3PN embryos were selected for experiments.

To obtain 2PN zygotes, the immature oocytes were transferred to maturation medium (Cooper Surgical/SAGE, Trumbull, CT) supplemented with 75 mIU/ml FSH and LH. After in vitro maturation, the oocytes were fertilized by intracytoplasmic sperm injection (ICSI). The fertilization was confirmed by formation of two pronuclei 16–18 h later.

Molecular analyses

Genomic DNA from cultured embryos was amplified with the REPLI-g Single Cell Kit (QIAGEN) according to the manufacturer’s instructions. Genomic regions of interest were further amplified from the WGA (whole genome amplified) DNA with KOD FX DNA polymerase (Toyobo) under the following PCR conditions: 95 °C for 5 min followed by 40 cycles of (98 °C for 10 s, 60 °C for 30 s, and 68 °C for 1 min), and then 68 °C for 5 min. The amplified fragments were gel-purified, cloned into the pGEMT-easy vector (Promega) for sequencing, or subjected to the T7E1 assay (Niu et al. 2014). PCR primers are listed in Table S1 (see supplemental material).

Whole genome sequencing (WGS) and WGS data analysis were performed by Beijing Genomics Institute (BGI). In brief, the amplified DNA was sequenced as paired-end 90-nucleotide reads to a target of 30X haploid coverage on an Illumina HisSeq2000 sequencer. The pair-end reads were aligned onto the hg19 (GRCh37v.71) human reference genome after removing reads containing sequencing adapters and low-quality reads with more than five ambiguous bases using BWA (v0.5.9) with default parameters. The variants identified were then further filtered through

**Results**

**Gene editing in human 3PN using Cas9 protein**

We tested the efficiency of Cas9 protein-mediated gene editing in human fertilized oocytes. We collected the would-be-discarded one-cell embryos because of their triploid (3PN, 3 pronuclei) nature for experiments. The embryos were collected in our reproductive clinic in Guangzhou, China, and the patients consented for the use of their 3PN embryos in research. In our routine practice, 16–18 h after insemination (when the pronucleus becomes prominent and visible), the embryos are observed under a microscope and the 3PN embryos are separated. We produced an sgRNA that targeted RAG1 (Fig. 1a) in vitro and mixed it with the purified Cas9 protein and injected the mixture into the cytoplasm of the 3PN one-cell embryos. We found concentrations of 50 ng/μl Cas9 with 30 ng/μl sgRNA were effective and did not interfere with the development of the injected embryos (Fig. 1b). The embryos were cultured for 48 h in vitro after injection. By then, most of them, as expected, had reached the early morula stage (8–13 cells) (Fig. 1b). The embryos were harvested and their genomic DNA was extracted,

![Diagram of RAG1 locus. The red-colored sequence is the sgRNA, and the green-colored sequence is the PAM motif. Sequencing results of three embryos are also shown. Numbers in the parentheses indicate the type of editing (“−19” means deletion of 19 bp and “+3” is the insertion of 3 bp) and its apparent frequency (“3/20” means 3 times in 20 clones sequenced).](image)

![Images of the embryos 3 days after the injections. Scale bar 100 μm.](image)

![A typical result of the T7E1 assay used to detect editing in targeted loci.](image)

![The number of independent edited alleles observed by sequencing. For each gene we randomly selected 6 T7E1 assay-positive embryos (E1–E6), cloned, and sequenced the PCR products. (Color figure online)](image)
amplified with a whole genome amplification (WGA) kit, and subjected to molecular analyses. A pair of primers flanking the target site in RAG1 was used to amplify the targeted region. The PCR-amplified products were denatured, re-annealed, and digested with T7 endonuclease I (T7E1), which cuts at mismatched sites (Fig. 1c). Of a total of 21 injected 3PN embryos, 12 (60%) showed cleavage products, indicating RAG1 targeted editing. This RAG1 targeting efficiency is comparable with the reported efficiency of 9/15 (60%) observed for Cas9 mRNA/sgRNA injection into cynomolgus monkey one-cell embryos (Niu et al. 2014). We also cloned the PCR products and sequenced 20 clones for 6 of the 12 samples that were positive in the T7E1 assay. Typical indels were observed (Fig. 1a). Some embryos contained up to 3 different indels, indicating independent editing of different alleles (Fig. 1d).

Having demonstrated efficient indel creation in RAG1 (60%) by NHEJ editing, we next tested editing two additional loci, G6PD and NEK1, to assess the general applicability of this method. 2 sgRNAs targeting G6PD (1 bp overlap on different strands, Fig. 2a) and 1 targeting NEK1 (Table S1) were tested. Again, up to three independent editing events were observed at the two loci (Fig. 1d). The G6PD sgRNA1 showed about 80% efficiency and G6PD sgRNA2 and NEK1 sgRNA were close to 70% (Table 1). This overall efficiency was comparable with the efficiencies reported previously in rodents of between 50 and 90% (Wang et al. 2013; Ma et al. 2014).

**Homologous recombination in human 3PN embryos**

Encouraged by the high observed efficiencies of generating an indel mutation (NHEJ editing) at the selected loci using Cas9 protein, we next attempted to determine the HDR efficiency in human 3PN embryos using Cas9 protein. As shown previously, when Cas9 protein is introduced to human cells via electroporation, DSB generated by Cas9 can be achieved within 3 h (Kim et al. 2014). The 3PN embryos were collected about 16–18 h after insemination, at which time the S phase had commenced or even completed (Capmany et al. 1996) and, therefore, the embryos were in a cell cycle phase that was permissive for HDR. We chose two loci to determine Cas9-mediated HDR efficiency, G6PD and HBB (β-globin). It was reported previously that the use of a single-strand oligo donor (ssODN) as a source of homologous sequence for HDR required 20–50 bases of homology on each side of the DSB site (Chen et al. 2011). Therefore, for G6PD,
we designed a ssODN of 90 nt (ssODN1) in length containing two additional bases relative to the wildtype (to create a HindIII site for easy identification). This was injected into 3PN embryos with the Cas9 protein and G6PD sgRNA1 (Fig. 2a) at about 20 h post-insemination and the injected embryos were harvested for analyses after 2 days of culture when they had reached the early morula stage. Of the ten 3PN embryos injected, nine of them showed editing at the G6PD locus, and two of those contained the designed HindIII site, indicating a HDR efficiency of 20% (Fig. 2b). Sequencing of the region did not detect any additional sequence changes besides HDR (Fig. 2c). The two extra bases in the donor were within the region targeted by the G6PD sgRNA1, which likely prevented re-editing of the site after HDR. We also performed injection using Cas9 mRNA (100 ng/μl) instead of Cas9 protein and analyzed the HDR efficiency at the same locus. Of a total of 20 3PN embryos injected, 16 (80%) showed cleavage in the T7E1 assay, but none of them were sensitive to digestion with HindIII, indicating no HDR occurred. These data suggest that using Cas9 protein instead of mRNA promotes precise HDR genome modifications in our conditions.

For the second locus, HBB, we tested if HDR could occur at a site positioned some distance from the DSB generated by Cas9. We designed an sgRNA (HBB sgRNA1) and a 200 nt oligo donor (ssODN2) so that the intended genetic alteration (a HindIII site) was positioned 39 bases 3’ of the predicted Cas9 cutting site (Fig. 3a). Of the 10 3PN embryos that were injected, 7 showed editing and one of them resulted from HDR (Fig. 3b). Thus, at this locus, the HDR efficiency was 10%, which is lower than the 20% observed for injection with G6PD sgRNA1 and ssODN1. This result demonstrates that genetic modifications can be successfully introduced via HDR some distance away from the DSB in human embryos, but with decreased HDR efficiencies (Ran et al. 2013). However, sequencing of the targeted region showed the presence of both NHEJ repair (within the HBB sgRNA1 sequence) and HDR on the same chromosome (Fig. 3c), indicating that re-editing occurred after HDR. In addition, this embryo also contained another independent edited allele (Fig. 3c). Thus, sgRNAs must be designed carefully to avoid the introduction of unintended alterations in the attempt to correct genetic defects.

### Correcting disease-causing mutations in human 2PN embryos

Having established that CRISPR/Cas9-mediated gene editing (NHEJ and HDR) could be achieved efficiently in human 3PN embryos, we decided to determine the editing efficiencies directly in human 2PN to assess the possibility of using CRISPR/Cas9 in correcting disease-causing mutations. To obtain human 2PN zygotes, wildtype immature oocytes were collected and subjected to in vitro maturation and ICSI procedures. We examined the disease-causing mutation β41–42 (TCTT) in HBB. This is a common β-thalassemia mutation that results in a frameshift and generates a stop codon (TGA) at the position of the new 59th codon (Kimura et al. 1983). We collected sperm from a male patient who suffers from β-thalassemia and is heterozygous for the β41–42 mutation, as determined with a HBB genotyping kit. We designed an sgRNA (HBB sgRNA2) that specifically targets the mutant allele and a single-strand oligo donor (ssODN3) 90 nt in length as the template for HDR (Fig. 4a). We generated ten zygotes. Theoretically, half of the zygotes should be heterozygous for the corresponding mutation. However, at this stage it was not possible to determine which zygote carried the mutation. Thus, all fertilized embryos were injected with the Cas9/sgRNA/donor oligo mixture at the concentrations previously demonstrated to be safe for 3PN embryos. The injection was performed 18 h after ICSI. At this time point, the pronuclei were visible. The embryos were cultured for 2 days and then harvested for analyses. To assess the correction efficiency of the mutation in HBB, the genomic

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Table 1 CRISPR/Cas9-mediated gene editing in human embryo samples

<table>
<thead>
<tr>
<th>Group</th>
<th>Injected zygotes</th>
<th>NHEJ editing (% embryos)</th>
<th>HDR editing (% embryos)</th>
</tr>
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<tbody>
<tr>
<td>Cas9+RAG1 sgRNA</td>
<td>21</td>
<td>12 (60)</td>
<td>–</td>
</tr>
<tr>
<td>Cas9+NEK1 sgRNA</td>
<td>13</td>
<td>9 (69)</td>
<td>–</td>
</tr>
<tr>
<td>Cas9+G6PD sgRNA1</td>
<td>15</td>
<td>12 (80)</td>
<td>–</td>
</tr>
<tr>
<td>Cas9+G6PD sgRNA2</td>
<td>12</td>
<td>9 (75)</td>
<td>–</td>
</tr>
<tr>
<td>Cas9+G6PD sgRNA1+ssODN1</td>
<td>10</td>
<td>9 (90)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Cas9 mRNA+G6PD sgRNA1+ssODN1</td>
<td>20</td>
<td>16 (80)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cas9+HBB sgRNA1+ssODN2</td>
<td>10</td>
<td>7 (70)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Cas9+HBB sgRNA2+ssODN3</td>
<td>4a</td>
<td>2 (50)</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Cas9+G6PD sgRNA3+ssODN4</td>
<td>2b</td>
<td>1 (50)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

a,b The number of embryos carrying sgRNA target mutation allele
region containing the mutation was amplified and subjected to T7E1 assay, HDR-diagnostic HindIII digestion, and sequencing. The expectation was that about half of the embryos would be heterozygous for the mutation and positive in the T7E1 assay. Indeed, four embryos were positive (2, 4, 6, and 8) by the T7E1 assay but only one (2) was digestible by HindIII (Fig. 4b). Sequencing analysis indicated that embryo #2 contained 3 \(\text{HBB} \) alleles: wildtype, corrected, and the \((-\text{TCTT}, -\text{C})\) mutant, at a ratio of 2:1:1 (Fig. 4c). Embryo #6 also contained three \(\text{HBB} \) alleles, but without HDR: wildtype, a \(-8\) bp mutant, and a \(-174\) bp mutant, at roughly 2:1:1 (Fig. 4c). Further sequencing of embryos #4 and #8 revealed that all the mutant alleles contained an additional C deletion within \(\text{HBB} \) sgRNA2 targeting site, which cannot be generated by DSB repair by the embryos. We then PCR amplified and sequenced the \(\text{HBB} \) sgRNA2 targeting site from the patient’s genomic sample and confirmed that the \(\text{HBB} \) mutant allele genotype is \((-\text{TCTT}, -\text{C})\). Thus, the editing efficiency was 50% (two out of four embryos), slightly lower than that found for other loci (Table 1), and the HDR efficiency was 50% (one out of two edited embryos). These data indicated that the CRISPR/Cas9 system may also tolerate a one base indel at the sgRNA targeting site in human zygotes using Cas9 protein (Lin et al. 2014b). The one base deletion in the \(\text{HBB} \) \(\beta 41-42\) mutation \(\text{HBB} \) sgRNA2 targeting site could be an explanation of the observed lower targeting efficiency.

We next decided to test correction of the \(G1376T\) mutation at the X-linked \(\text{G6PD} \) locus in human 2PN zygotes, one of the two most common point mutations affecting the Chinese Han population (Du et al. 1999). First, we injected eight 3PN embryos to test the ability of the \(\text{G6PD} \) sgRNA3 to target the wildtype allele. None of the embryos was positive for T7E1 digestion, suggesting that the \(\text{G6PD} \) sgRNA3 specifically targeted the \(G1376T\) mutant allele. Next, we generated ten human 2PN zygotes using wildtype oocytes and sperm carrying the \(\text{G6PD} \) \(G1376T\) mutation. After injection, culturing, and testing, only two embryos were positive for T7E1 and HindIII digestion (Fig. 4e). Sequencing of the region indicated that embryo #2 contained one wildtype and one corrected \(\text{G6PD} \) allele at a roughly 1:1 ratio, and embryo #7 contained three different alleles, wildtype, corrected, and edited, at a ratio of 2:1:1 (Fig. 4f).
Further determination of the sex of the ten embryos showed that all embryos but #2 and #7 contained a Y chromosome (Fig. 4g). Thus, only these two embryos were XX, receiving one X chromosome from the sperm that contained the G1376T mutation. Thus, the sequencing of embryos #2 and #7 indicated HDR occurred, so the HDR efficiency was 100% (two out of two edited embryos). We further analyzed off-targeting in embryo #2. None of the 13 potential off-target sites identified with the CRISPR design tool (http://tools.genome-engineering.org) showed off-target effect. Sequencing data of the T7E1 assay-positive samples revealed no editing but polymorphisms at these sites (Fig. S1). Next, we sequenced the whole genome of embryo #2 to x30 coverage via next-generation sequencing. There were no large-scale alterations such as translocations and deletions. By allowing for ungapped alignments with up to four mismatches in the 20 bp G6PD sgRNA3 target sequence, and by scanning locus sequences that included 40 nt upstream and downstream of the on- and off-target

![Diagram of the HBB locus, HBB sgRNA2 sequence (red), PAM motif (green), and the sequence of the single-strand donor oligo (ssODN3, silent mutations are in brown color).](Image)

![Analyses of targeted HBB β41–42 (-TCTT) mutant locus in ten embryos. M, DNA molecular weight marker, 1000, 750, 500, and 250 bp (top to bottom). *T7E1 assay-positive samples, and **HDR.](Image)

![Sequencing results of embryos #2 and #7.](Image)

**Fig. 4** CRISPR/Cas9-mediated genome editing in one-cell human 2PN zygotes. **a** Diagram of the HBB locus, HBB sgRNA2 sequence (red), PAM motif (green), and the sequence of the single-strand donor oligo (ssODN3, silent mutations are in brown color). **b** Analyses of the targeted HBB β41–42 (-TCTT) mutant locus in ten embryos. M, DNA molecular weight marker, 1000, 750, 500, and 250 bp (top to bottom). *T7E1 assay-positive samples, and **HDR. **c** Sequencing results of T7E1 assay-positive embryo samples. These data indicate that the HBB mutant type is β (-TCTT, -C). **d** Diagram of the G6PD locus, G6PD sgRNA3 sequence (red), PAM motif (green), and the sequence of the single-strand donor oligo (ssODN4, silent mutations are in blue color). **e** Analyses of targeted G6PD G1376T mutant locus. M, DNA molecular weight marker, 1000, 750, 500, and 250 bp (top to bottom). *T7E1 assay-positive samples, and **HDR. **f** Sequencing results of embryos #2 and #7. **g** PCR detection of SRY. ***The absence of SRY. (Color figure online)**

<table>
<thead>
<tr>
<th>Candidate off-target sites</th>
<th>SNP</th>
<th>Indel</th>
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<tbody>
<tr>
<td>sgRNA CTCCTACGAGCGAGCTCCT</td>
<td>TG AGG</td>
<td></td>
</tr>
<tr>
<td>Chr1 TCTACGAGCGAGCTCCT</td>
<td>TG AAA</td>
<td></td>
</tr>
<tr>
<td>Chr2 CTCCTACGAGCGAGCTCCT</td>
<td>TG AAA</td>
<td></td>
</tr>
<tr>
<td>Chr2 CTCCTACGAGCGAGCTCCT</td>
<td>TG AAA</td>
<td></td>
</tr>
<tr>
<td>Chr3 CTCTACGAGCGAGCTCCTAG</td>
<td>TGT</td>
<td></td>
</tr>
<tr>
<td>Chr9 CTCCTACGAGCGAGCTCCTAG</td>
<td>TGT</td>
<td></td>
</tr>
<tr>
<td>Chr12 GCCGGCGAGCGAGCTCCTAG</td>
<td>AGG</td>
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site (a total of 100 bp), we obtained seven candidate off-target sites. However, these are all known SNPs and InDels (Table 2).

Discussion

We demonstrate here that the CRISPR/Cas9 system is quite effective in correcting point mutations in human zygotes. For G6PD, only two mutation-carrying embryos were created and both underwent HDR on the mutant allele, so HDR occurred at 100%. One embryo was completely corrected and another became mosaic, with half of its cells corrected and another half suffered a 4-bp deletion around the mutation (Fig. 4f). For HBB, HDR occurred at 50% (one out of two edited embryos) (Fig. 4c; Table 1). However, HDR efficiency was much lower in 3PN embryos (20% for the G6PD sgRNA1-ssODN1 pair and 10% for the HBB sgRNA1-ssODN2 pair) (Fig. 2; Table 1). The explanation for these differences is currently unclear, but experimental data from a higher numbers of embryos are required to be meaningful.

Through whole genome sequencing, we did not find clear evidence of off-targeting in one of the two embryos with the G1376T-mutation corrected. However, the use of CRISPR/Cas9-mediated gene editing in reproductive clinics is not a current option due to both ethical and technical issues (safety, mosaicism, and other factors).

A potential application of CRISPR/Cas9 system in human zygotes is to study gene function in preimplantation development, an area of research with very limited numbers of molecular tools for humans, unlike the situation in other species. For example, one could introduce inactivating mutations and even point mutations to probe gene function in early human embryos.

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Compliance with ethical standards

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Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This study was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University, numbered [2015] No. 068. The methods used in the present study closely followed the guidelines legislated and posted by the Ministry of Health of the People’s Republic of China. The patients involved in this study knew about and understood the usage of tripronuclear zygotes, immature oocytes and leftover sperm, and voluntarily donated them after providing informed consent.

Informed consent Informed consent was obtained from all individual participants included in the study.

References


