Heated spermatozoa: effects on embryonic development and epigenetics

Shi-Bin Chao1,2, Lei Guo1, Xiang-Hong Ou1,3, Shi-Ming Luo1, Zhen-Bo Wang1, Heide Schatten4, Guo-Lan Gao5, and Qing-Yuan Sun1,*

1State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China  2Center of Reproductive Medicine, The First Affiliated Hospital of NanChang University, NanChang, China  3Center of Reproductive Medicine, Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, GuangZhou, China  4Department of Veterinary Pathobiology, University of Missouri, Columbia, MO, USA  5Department of Obstetrics and Gynecology, Aviation General Hospital of China, Beijing, China

*Correspondence address. E-mail: sunqy@ioz.ac.cn (Q.-Y.S.)

Submitted on September 21, 2011; resubmitted on December 21, 2011; accepted on January 5, 2012

INTRODUCTION

The phenomenon of sperm capacitation was independently reported by Chang and Austin and this discovery allowed detailed studies to uncover the mechanisms of fertilization (Yanagimachi and Chang, 1963). Successful IVF requires thousands of motile and capacitated sperm, but the development of ICSI has allowed fertilization to be achieved with a single sperm. ICSI is highly effective both as an assisted reproductive technology (ART) technique to treat infertile patients and as a way to study fertilization (Kimura and Yanagimachi, 1995b; Yanagimachi, 2005). The single sperm used for ICSI does not necessarily need to be motile or even viable. Previous studies demonstrated that fertilization could be achieved by sperm exposed to various treatments, including decapitation by sonication, freeze-thawing without cryoprotectant, freeze-drying, lysolecithin and pronase treatment, alkyl(trimethyl)ammonium bromide and dithiothreitol treatment (Wakayama and Yanagimachi, 1998; Ward et al., 1999; Kaneko et al., 2003; Kwon et al., 2004; Jiang et al., 2005; Yan et al., 2008; Yazawa et al., 2009).

The special chromatin structure of the mammalian spermatozoon provides the foundation for its resistance to such physical or chemical treatments. The association of DNA with sperm-specific basic proteins, protamines, is important for this specific chromatin condensation. Sperm DNA is further condensed and stabilized by an extensive cross-linking of protamines by disulfide bonds (Miller et al., 2009). Studies have shown that sperm nuclei of hamster, mouse and human treated with high temperature (90°C, 30 min) were able to form pronuclei (PN) when microinjected into hamster oocytes (Yanagida et al., 1991). Subsequent studies have shown that embryos generated with mouse spermatozoa heated at 56°C for...
Sperm tolerance to heat

30 min were able to support full-term embryonic development and produce live offspring (Cozzi et al., 2001). However, the blastocyst rates and implantation rates were significantly lower compared with those produced with fresh spermatozoa, although the underlying reasons for these differences have not been studied so far. These results inspired us to investigate the maximal temperature for sperm treatment that would still allow live offspring and the mechanisms that are involved in the poor rate of full-term embryonic development.

Previous studies also showed that gametes and preimplantation embryos were sensitive to environmental factors or in vitro manipulations that could affect developmental potential and epigenetic modifications (Fleming et al., 2004). In the present study, oocyte activation and embryonic development were investigated after fertilization with heat-treated spermatozoa using ICSI. Epigenetic reprogramming including DNA methylation, histone H3K4 trimethylation as well as karyotypes of embryos produced with heat-treated sperm were compared with those produced with fresh sperm. Finally, full-term development of embryos derived from heat-treated sperm was demonstrated by production of live offspring after embryo transfer into recipient females.

Materials and Methods

Unless otherwise stated, chemicals used in this research were purchased from Sigma (St. Louis, MO, USA). All animals were maintained in accordance with the Animal Experiment Standard of the State Key Lab of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Preparation of mouse oocytes

Metaphase II oocytes were collected from oviducts of 8- to 12-week-old Institute of Cancer Research (ICR) female mice that had been induced to superovulate with 5 IU equine chronic gonadotrophin (Ningbo Animal Hormone Factory, Ningbo, China), followed by 5 IU hCG (Ningbo Animal Hormone Factory) 48 h later. Oocytes were collected from oviducts 14 h after hCG injection, and treated with 0.3% hyaluronidase in HEPES-buffered human tubal fluid medium (mHTF; SAGE ART-1023, CA, USA) in order to disperse cumulus cells. The cumulus-free oocytes were rinsed thoroughly, then placed in drops of HTF medium (SAGE ART-1020) containing 10% serum protein substitute (SAGE ART-3010, CA, USA) and covered with mineral oil for up to 1 h at 37°C under 5% CO2 before ICSI.

Spermatozoa preparation

Cauda epididymides were excised from C57BL/6 male mice and spermatozoa were collected by squeezing them in a 600-μl drop of mHTF under mineral oil. To allow even dispersion of spermatozoa, they were incubated in medium for 10 min at 37°C. Next, this suspension was placed in a polyethylene microcentrifuge tube containing PBS and heated in a water bath (50, 65, 85 and 95°C) for 30 min. The samples were then immediately cooled to 4°C. To separate heads from tails, the sperm suspension was sonicated 2–4 times for 15 s at an interval of 15 s using an ultra sonicator. With this treatment, >90% sperm were decapitated.

Mouse ICSI

ICSI was carried out as described previously (Kimura and Yanagimachi, 1995a; Yoshida and Perry, 2007; Yazawa et al., 2009). Sperm heads were used for ICSI immediately after treatment. Briefly, a single sperm head was sucked into an injection pipette (6–9 μm inner diameter at the tip) that was attached to a piezo-electric driving unit (Model PMAS-CT150; Prime Tech Ltd., Japan). A mature unfertilized mouse oocyte was stabilized using a holding pipette and the zona pellucida was penetrated by applying several piezo pulses. When the needle had advanced deep enough into the ooplasm, the oolemma was punctured with a single piezo pulse and the sperm head was slowly released into the ooplasm, before gently removing the pipette. After injection, oocytes were washed three times in HTF and incubated at 37°C under 5% CO2. For artificial oocyte activation, oocytes were incubated in Ca2+-free mHTF (SAGE ART-4100) containing 10 mM SrCl2 for 1 h immediately after ICSI and then transferred in HTF.

Immunofluorescent detection of DNA methylation and H3K4 trimethylation

Zygotes with two distinct PN and two polar bodies at 6 h of ICSI were washed in phosphate-buffered saline (PBS), fixed for 20 min in 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Subsequently, zygotes were blocked for 1 h in a blocking solution containing 2% bovine serum albumin/0.05% PBS-Tween 20. Samples were incubated with anti-H3K4 trimethylation (diluted 1:500; Millipore, Bedford, MA, USA) for 1 h and washed with 0.05% PBS-Tween 20 for 30 min. Subsequently, samples were incubated for 1 h at room temperature with a fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (ZhongShan Goldenbridge Biotechnology, Beijing, China). After several washes in 0.05% PBS-Tween 20, samples were chromatin-labeled for 10 min with 10 μg/ml propidium iodide (PI) and washed again with 0.05% PBS-Tween 20. Samples were mounted in 90% glyceral, 0.1 M Tris–HCl, pH 8.0 and 2.3% 1,4-diazobicyclo-(2,2,2)-octane (DABCO, Sigma, MO, USA), followed by examination with Confocal Laser Scanning Microscopy (Zeiss LSM 510 META, Germany).

For detection of DNA methylation, zygotes collected 6 h after ICSI were treated with 4 M HCl at room temperature for 50 min and later neutralized for 10 min with 100 mM Tris–HCl buffer (pH 8.5) before blocking. After several washes with 0.05% Tween 20, embryos were incubated with 5-methyl cytidine antibody (diluted 1:100; Abcam, Cambridge, MA, USA), followed by the procedures described for H3K4 trimethylation staining. For quantitative analysis of pronuclear DNA methylation levels, fluorescence images were subjected to densitometric analysis using Image J software (National Institute of Health, Bethesda, MA, http://www.ncbi.nlm.nih.gov/ij, 1997–2007). Each pronucleus was outlined manually at its greatest diameter, and the total fluorescence intensity emitted from each pronucleus was calculated after subtraction of the cytoplasm background fluorescence in the Alexa Fluor-488-stacked image. The relative methylation (RM) of the paternal PN to the maternal PN in each zygote was calculated using the following equation: \[ RM = \frac{\text{total fluorescence intensity in the paternal PN}}{\text{total fluorescence intensity in the maternal PN}} \]

Karyotype analysis

Chromosome spreads of the 1-cell zygotes were performed according to procedures described previously (Kamiguchi and Mikamo, 1986; Akiyama et al., 2006) with some modifications. Briefly, 12 h after ICSI, zygotes were transferred into HTF containing 0.1 μg/ml colcemid to arrest the cells at the first metaphase. Seven to nine hours later, the zygotes were treated hypotonic solution (0.9% w/v sodium citrate) for 10 min at room temperature and then exposed to a freshly prepared fixative mixture of 3:1 methanol/acetic acid. Chromosomes were air-dried and stained with 10 μg/ml PI. Samples were examined with a Confocal Laser Scanning Microscope (Zeiss LSM 510 META). Two groups of metaphase chromosomes were observed: one group from oocytes and one group from sperm. It has been reported that <1% of normal mouse oocytes have...
abnormal chromosomes (Kusakabe et al., 2001; Kaneko et al., 2003). Therefore, all aberrant chromosomes were assumed to originate from sperm.

### Embryo transfer

About 6 h after ICSI, eggs with two distinct PN and a second polar body were cultured in HTF. Within 20–24 h of ICSI, embryos that had developed to the 2-cell stage were transferred into oviducts of ICR females that had been made pseudo-pregnant by mating with vasectomized males.

### Data analysis

Statistical analyses were conducted by analysis of variance (ANOVA). Differences between treated groups were analyzed by ANOVA using SPSS software (SPSS Inc., Chicago, IL, USA) followed by Student–Newman–Keuls test. Data are expressed as mean ± SEM and \( P < 0.05 \) is considered significant.

### Results

#### Oocyte activation capacity of heated sperm after ICSI without artificial activation

To determine whether the heat-treated sperm had maintained oocyte activation capacity, sperm were injected into mature ICR mouse oocytes. Oocyte activation rates decreased significantly from 91.8 to 6.1% when the temperature was increased from 37°C (control) to 65°C. No pronucleus was observed in oocytes after ICSI with sperm heated at 80°C (Table I).

#### Oocyte activation and embryonic development after ICSI with heat-treated sperm combined with artificial activation

It is well known that the oocyte activation capacity by sperm is heat sensitive (Perry et al., 1999). After heat treatment, the sperm’s capacity to activate the oocyte is lost, but the oocytes can be activated artificially by treatments such as strontium or electro-stimulation treatment (Bos-Mikich et al., 1997; Cozzi et al., 2001; Yan et al., 2008). We artificially activated oocytes injected with heat-treated sperm in Ca\(^{2+}\)-free medium containing 10 mmol/l SrCl\(_2\). Surprisingly, even after 95°C treatment, 31.7% of oocytes still developed two PN. However, only 9% were able to develop to the 2-cell stage and none developed to blastocyst stages. When temperatures between 50 and 80°C were applied, the two PN rate increased to >50%. The blastocyst rate remained at a relatively high level when the temperature was <65°C, but decreased dramatically (10.4%) when the temperature was raised to 80°C (Table II).

#### DNA methylation in the zygote

After fertilization, the paternal genome is subjected to DNA replication-independent, genome-wide active demethylation within a few hours (Mayer et al., 2000; Dean et al., 2001; Santos et al., 2002; Metivier et al., 2008). To determine whether heat treatment of sperm changed active demethylation in the zygotes they fertilized, we used a well-documented 5-methyl cytidine antibody to visualize pronuclear genomic methylation (Santos et al., 2002; Zaitseva et al., 2007). As shown in Fig. 1A, the results clearly indicated that the demethylation of paternal chromatin, as assessed by 5-MeC labeling, occurred in all groups (Fig. 1A: A1–D1) except for the 95°C treatment group (Fig. 1A: E1). Analysis with confocal microscopy revealed an incompletely decondensed sperm head in the 95°C treatment group (Fig. 1A: E1). Analysis with confocal microscopy revealed an incompletely decondensed sperm head in the 95°C treatment group (Fig. 1A: E1–E3 white frames indicating the male PN; uncondensed male PN). We measured the methylation density of the zygotes’ two PN and compared the RM of each group. The results showed that there was no statistical difference among the groups from control to the 80°C treatment group.

---

### Table I  Oocytes activation after ICSI with heated sperm without artificial activation.

<table>
<thead>
<tr>
<th>Heat treatment (°C)</th>
<th>No. of surviving oocytes (no.exp)</th>
<th>No. of oocytes activated (%)(^*)</th>
<th>No. of oocytes activated (2PN)(%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>85 (3)</td>
<td>78 (91.8)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>78 (3)</td>
<td>16 (20.5)</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>98 (4)</td>
<td>6 (6.1)(^*)</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>107 (4)</td>
<td>0 (0)(^*)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Activated oocytes are the oocytes with two well-developed PN and second polar body.

\(^*\) \( P < 0.01 \) compared with 37°C group.

### Table II  Oocyte activation and embryonic development after ICSI by heated sperm combined with artificial activation.

<table>
<thead>
<tr>
<th>Heat treatment (°C)</th>
<th>No. of surviving oocytes (exp)</th>
<th>No. of oocytes activated (2PN + 2PN)(%)(^a)</th>
<th>No. of zygotes developed to (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Two-cell</td>
</tr>
<tr>
<td>37(^b)</td>
<td>127 (4)</td>
<td>115 (90.5)</td>
<td>111 (87.4)</td>
</tr>
<tr>
<td>50</td>
<td>169 (5)</td>
<td>111 (65.7)(^*)</td>
<td>100 (59.2)(^*)</td>
</tr>
<tr>
<td>65</td>
<td>189 (6)</td>
<td>107 (56.6)(^*)</td>
<td>94 (49.7)(^*)</td>
</tr>
<tr>
<td>80</td>
<td>183 (6)</td>
<td>104 (56.8)(^*)</td>
<td>88 (48.1)(^*)</td>
</tr>
<tr>
<td>95</td>
<td>167 (5)</td>
<td>53 (31.7)(^*)</td>
<td>15 (9.0)(^*)</td>
</tr>
</tbody>
</table>

PB, polar body.

\(^a\) The percentage is calculated from oocytes survived.

\(^b\) ICSI of oocytes with sperm incubated in 37°C for 30 min as control group (no oocyte activation).

\(^*\) \( P < 0.01 \) compared with control group (37°C).
Histone H3 lysine 4 trimethylation in the zygote

Sperm chromatin is decondensed immediately after fertilization; then protamines are replaced by histones (Lepikhov and Walter, 2004). The male pronucleus undergoes dynamic histone H3 methylation (Lachner and Jenuwein, 2002). We compared the histone H3K4 trimethylation patterns of zygotes derived from different heat-treated groups. The female pronucleus showed H3K4-trimethylation staining, while staining in the male pronucleus was hardly detectable in each group (Fig. 2: A1–E1). An incomplete decondensation pattern of sperm chromatin was also detected in the 95°C treatment group (Fig. 2: E1–E3, m′). Scale bar = 10 μm. (B) Relative methylation (RM; male/female pronucleus) of each group. Mean ± SEM (control n = 27, 0.601 ± 0.146; 50°C n = 21, 0.548 ± 0.154; 65°C n = 29, 0.604 ± 0.2; 80°C n = 22, 0.693 ± 0.159). No data are shown for the 95°C group due to incomplete decondensation of sperm chromatin.

Embryo transfer

Yanagida et al. (1991) demonstrated that mature mammalian sperm nuclei are moderately heat stable. Cozzi et al. (2001) obtained live pups from 56°C-treated sperm. In the present study, there were still 10.4% embryos derived from 80°C-treated sperm that developed to blastocysts. Therefore, we explored whether we could obtain live pups from temperatures higher than 56°C and we set out to determine the maximal temperature in our series of experiments that

Karyotype analysis

Morozumi et al. (2004) found chromosome damage in spermatozoa following 56°C treatment for 30 min. In the present study, we analyzed the karyotypes of the zygotes derived from heat-treated spermatozoa. In the control group, two intact haploid groups of metaphase chromosomes were observed (Fig. 3A: a). Multiple chromosome fragments (Fig. 3A: c, s′ white arrows) in a male pronucleus of a 1-cell zygote derived from a heated sperm head were observed (the sample shown here was from the 65°C-heated group). We found an incompletely decondensed sperm head and one group metaphase chromosomes in the 95°C treatment group (Fig. 3A: b). The aberrant chromosome rate (aberrant samples/tested samples: control, 0 of 28; 50°C, 5 of 31; 65°C, 15 of 33; 80°C, 32 of 40 and 95°C, 25 of 25) increased from 16.3 to 100% when the temperature was raised from 50 to 95°C (Fig. 3B).
would allow obtaining live pups. Two cell-stage embryos were transferred into oviducts of recipient mice. Table III shows that 55% (27 of 42) of embryos derived from ICSI with 50°C-treated spermatozoa and 23.5% (21 of 89) from ICSI treated with 65°C-treated spermatozoa supported full-term development, whereas only 4.3% (5 of 115) of those embryos obtained from ICSI with 80°C-treated spermatozoa did so (Fig. 4). None of the recipients became pregnant in the 95°C-treated sperm group. All live offspring survived and were healthy.

Discussion

Fertilization is a complex process comprising sperm capacitation, acrosome reaction, sperm penetration of the oocyte and oocyte activation (Yanagimachi, 2011), and many of the mechanisms involved in fertilization are only incompletely understood (Ikawa et al., 2010). To initiate normal embryonic development, an egg must receive a signal from sperm to become activated at fertilization. Recent studies implicate a sperm-specific phospholipase C zeta as the responsible factor that triggers oocyte activation (Saunders et al., 2002). The soluble factor originates from sperm head submembrane matrices and is heat sensitive (Stricker, 1996; Kashir et al., 2010). In the present study, the oocyte activation rate decreased from 91.8 to 0% when the temperature with which the spermatozoa were treated increased from 37°C (fresh sperm) to 80°C. In contrast to the results of Cozzi et al. (2001) and Jiang et al. (2005) who observed no fertilization with sperm heated at 56 or 58°C, we obtained 6.1% (6 of 98) fertilization rates with sperm treated with 65°C for 30 min. The difference in mouse strains used for these experiments may account for the different results. Our results confirmed that oocyte activation capacity of sperm was heat liable but indicated that treatment at 80°C for 30 min was required to inactivate completely the sperm’s oocyte activation capacity.
During the final post-meiotic phases of spermatogenesis, the sperm chromatin is tightly condensed and the normally nucleosome-bound histone is almost completely replaced by protamines (Braun, 2001) and the extensive disulfide bonds cross-linking nuclear protamine render sperm resistant to physical and chemical disruption. In our studies, the sperm underwent successively double disruptions, decapitated by sonication and subsequent heat-treatment. Isolated sperm heads decapitated by sonication were diagnosed as ‘dead’ after assessment with the sperm viability test (Yazawa et al., 2009). The fresh ‘dead’ sonicated sperm heads maintained a high fertilization capacity (>90%, Table I and II) and embryonic full-term development (64.3%, Table III). After heat treatment, the sperm heads progressively lost their oocyte activation ability with increasing temperature. However, the heat-treated sperm that had lost fertilization ability could form a male pronucleus after using strontium to artificially activate oocytes. But compared with the non-heated group, full-term embryo development significantly decreased and was negatively correlated with the temperature increase (Table III) probably due to chromosomal damage by heat treatment. In the present study, by karyotype analysis of each group, we showed that an aberrant chromosome rate was positively correlated to the increased temperature. The results showed that the destroyed integrity of sperm chromatin was the reason for poor full-term embryonic development and the effect was temperature dependent.

After fertilization, the paternal genome undergoes dramatic structural and epigenetic reprogramming. Sperm chromatin is decondensed, protamines are replaced by histones and DNA is rapidly and actively demethylated (Feng et al., 2010). Epigenetic reprogramming marked by DNA methylation and the covalent modification of the core histones create molecular landmarks that differentiate between active and inactive chromatin, which is likely to be needed for totipotency and correct initiation of embryonic gene expression (Morgan et al., 2005). It has been reported that aberrant epigenetic reprogramming leads to aberrant fetal growth and development (Khosla et al., 2001). Epigenetic reprogramming plays a vital role in embryonic development (Feng et al., 2010). Defective epigenetic reprogramming is associated with fetal growth abnormalities and many kinds of diseases such as cancers and diabetes (Fleming et al., 2004; Ballestar, 2011).

DNA methylation is an enzyme-mediated chemical process that includes transferring a methyl group from S-adenosylmethionine to C5 positions of the cytosine in the CpG (CG island) dinucleotides by different categories of methyltransferase enzymes (Wilkins, 2005; Wossidlo et al., 2011). DNA methylation plays an important role in the control of gene expression, chromosomal structure in mammalian cells and contributes significantly to genomic imprinting and X chromosome inactivation. It plays a crucial role in the earliest stages of embryogenesis (Jones and Takai, 2001; Li, 2002; Kiefer, 2007). Aberrant DNA methylation was found in cloned embryos and was identified as one of the causes for the low efficiency in the cloning success rate (Pick et al., 2009). In our study, we compared the active demethylation pattern of the paternal pronucleus in each group. The results showed that the pattern of active DNA demethylation in the pronuclear stage of each group did not change except in the 95°C treatment group which showed incomplete decondensation of sperm chromatin (Fig. 1). This finding indicates that 95°C treatment is detrimental to sperm and affects decondensation of sperm chromatin.

In addition to DNA methylation, covalent modifications of nucleosomal histone are also involved in the processes of epigenetic reprogramming (Wang et al., 2007). Histone modification (including methylation, acetylation, phosphorylation, ubiquitination and sumoylation) is another important epigenetic modification to the chromatin and widely regulates gene transcription and silencing (Klose and Zhang, 2007; Kouzarides, 2007; Surani et al., 2007).
Histone modifications do not act alone, but interact with one another. Methylation at histones H3K4 and K9 plays opposite roles in structuring repressive or accessible chromatin domains, with K4 methylation associated with transcriptionally active chromatin and K9 methylation with inactive chromatin in higher eukaryotes (Lachner and Jenuwein, 2002). These lysine residues can be mono-, di- or tri-methylated. Histone H3K4 special histone methyltransferase is quite active shortly after fertilization. H3K4 monomethylation could be detected at the beginning of PN1 and was only slightly delayed compared with core histone replacement. Histone H3K4 trimethylation becomes detectable starting at the PN4 stage (Nishioka et al., 2002; Lepikhov and Walter, 2004). The histone modifications are epigenetic marks that control the developmental pattern of the preimplantation mouse embryo and are influenced by many factors, such as the status of sperm, oocyte quality and in vitro manipulation. Defective histone modification of the mouse paternal zygotic genome is associated with microinjection of round spermatids (Kishigami et al., 2006). This might be one of the most important reasons for the significantly lower success rate with round spermatid injection compared with mature sperm injection (Kishigami et al., 2004). In the present study, the distribution of histone H3K4 trimethylation in the pronuclear-stage zygotes at 6 h of fertilization was analyzed. In each group except for the 95°C treatment group, very low density of H3K4 trimethylation was detected which was in agreement with previous studies (Nishioka et al., 2002; Lepikhov and Walter, 2004). The results also showed that except for the 95°C-treatment group, there was no change in the distribution of histone H3K4-trimethylation in the pronuclear stage.

Mammalian sperm including human sperm have similar chromatin structure which can resist heat treatment. Our results may be beneficial for practical applications aimed at protecting extinct animals and preserving human fertility in specific cases. For instance, one may be able to retrieve spermatozoa from tissue exposed to burns or other heat damages and to restore fertility by ICSI. More importantly, this

<table>
<thead>
<tr>
<th>Heat treatment (°C)</th>
<th>No. of embryos transferred</th>
<th>No. of recipients</th>
<th>No. of pregnant recipients</th>
<th>Living pups (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>42</td>
<td>2</td>
<td>2</td>
<td>27 (64.3)</td>
</tr>
<tr>
<td>50</td>
<td>69</td>
<td>4</td>
<td>4</td>
<td>38 (55)**</td>
</tr>
<tr>
<td>65</td>
<td>89</td>
<td>6</td>
<td>5</td>
<td>21 (23.5)*</td>
</tr>
<tr>
<td>80a</td>
<td>115</td>
<td>7</td>
<td>4</td>
<td>5 (4.3)*</td>
</tr>
<tr>
<td>95b</td>
<td>149</td>
<td>9</td>
<td>0</td>
<td>0 (0)*</td>
</tr>
</tbody>
</table>

*a*Except the five living pups, there were another three dead pups, including one stillbirth and one bit to death and one retention of dead fetus.

*b*The embryos transfered were two-pronuclear stage due to the poor development rate of 2-cell stage.

*P < 0.01 compared with control group (37°C).

**P > 0.05 compared with control group (37°C).
knowledge may also be of importance for the development of a sperm processing procedures in ART to prevent vertical transmission of pathogens such as human immunodeficiency virus (HIV) and hepatitis viruses. Special sperm washing protocols have been used to decrease the risk of transmission of chronic viral diseases (Englert et al., 2004; Garrido et al., 2009). However, Baccetti et al. (1994) reported that semen washing could not entirely eliminate HIV-1 RNA from HIV-1-positive semen. Since it is well known that inactivation of HIV can be achieved by heating at 56°C (Ball, 1987), it may be possible to explore a heat-treatment sperm processing protocol to eliminate vertical transmission of HIV and other chronic viral factors for these specific patients.

In conclusion, we have obtained live offspring generated by ICSI of oocytes fertilized with 80°C-treated sperm. To our knowledge, this is the highest reported temperature to which mammalian sperm can be exposed and still produce live offspring. In addition, we further showed that once it becomes modified into a pronucleus, heat-treated sperm chromatin can undergo normal active DNA demethylation and histone methylation. We have also demonstrated that incomplete sperm chromatin decondensation occurs when sperm is exposed to high temperatures, and that destruction of sperm chromatin integrity is the cause for decreased embryonic development.

Acknowledgements
We thank Dr Xingjiang-Yu’s for the generous gift of histone H3K4-trimethylation antibody. We are grateful to Shi-Wen Li, Yi Hou and Yingchun-Ouyang for their technical assistance.

Authors’ roles
S.-B.C. performed the mouse ICSI, most of the immunofluorescent, karyotype of mouse zygotes and took the lead in the writing of the manuscript. L.G. participated in embryo transfer and data collection. X.-H.O. participated in oocyte preparation and data analysis. S.-M.L. performed some of the mouse oocyte activation tests and manuscript writing. Z.-B.W. participated in embryo culture and data analysis. H.S. performed critical review. G.-L.G. and Q.-Y.S. was responsible for the study design, overseeing the completion of the study, editing and finalizing of the manuscript.

Funding
This study was supported by National Basic Research Program of China (2011CB944501 and 2012CB944404) to Q.-Y.S.

Conflict of interest
We declare there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

References