Epigenetic analysis of human spermatozoa after their injection into ovulated mouse oocytes

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BACKGROUND: The epigenetic status of human spermatozoa is difficult to analyse. The method of interspecies fertilization can be used for different purposes. The aim of our work was to adopt this approach for the detailed analysis of epigenetic status of human spermatozoa injected into mouse oocytes. METHODS: Human spermatozoa were injected into ovulated mouse oocytes. When both parental pronuclei were formed, the zygotes were fixed and labeled with antibodies against histones methylated or acetylated at different positions (residues). RESULTS: Our results show that human spermatozoa injected into mouse oocytes fully respond to oocyte cytoplasmic factors and form analysable pronuclei. The labeling of zygotes showed that as in other species, the paternal chromatin is extensively epigenetically remodeled. CONCLUSIONS: The interspecies ICSI may be a powerful tool for the analysis of sperm epigenetic status even with a very low number of spermatozoa available. This analysis could be used as an additional approach for the assessment of certain forms of human infertility, as well as for testing the normality of male gametes obtained from embryonic stem cells.

Keywords: spermatozoa; epigenetics; chromatin; acetylation; methylation

Introduction

Recent experiments in different species, including humans, clearly demonstrate that very soon after fertilization dramatic changes accompany the formation of both paternal and maternal pronuclei. Thus, e.g. the paternal DNA is rapidly demethylated, whereas the maternal genome remains methylated. This demethylation wave can be detected even before the onset of pronuclear DNA replication, and it is thought that this phase represents an active process (Morgan et al., 2005). What is more important however is that abnormal patterns of epigenetic changes closely reflect further embryonic development. These abnormalities are, e.g. typical for cloned embryos, in which the developmental potential is known to be rather compromised (Santos and Dean, 2004). The same seems to be true for cultured embryos. Shi and Haaf (2002) demonstrated that the labeling pattern in mouse embryos and their further developmental potential are in close relation. This may be influenced by a number of factors, including culture conditions and embryo handling. Moreover, recent results in the rat show that epigenetic remodeling program can be disrupted by chronic paternal cyclophosphamide exposure and this also negatively influences further embryonic development (Barton et al., 2005).

The data in humans are rather fragmentary. Fulka et al. (2004) showed that in about half of one-cell stage embryos, the paternal genome is less methylated than the maternal genome. Similar results were obtained by Xu et al. (2005), who stained triprominal human embryos. On the other hand, Beaujean et al. (2004a) observed almost complete human paternal genome demethylation. A detailed analysis of the epigenetic status in germ cells, gametes and embryos in humans is rather limited due to ethical reasons and the insufficient quantity of biological material. It is, however, evident that abnormal epigenetic regulation may have serious consequences and may contribute to a decline of fertility (Kelly and Trasler, 2004). For example, Benchaib et al. (2005) reported that a decreased level of sperm methylation negatively influences the pregnancy rate in humans.

From a technical point of view, the paternal epigenetic abnormalities are extremely difficult to analyse. To overcome these obstacles, we have adapted the technique of interspecies intracytoplasmic sperm injection, where human spermatozoa were injected into mouse oocytes, for the study of epigenetic and morphological changes occurring soon after fertilization. The aim of our experiment was to answer the question of whether interspecies ICSI can be used as an
approach for the evaluation of the epigenetic status of human spermatozoa.

Materials and Methods

Isolation of mouse oocytes

Mouse oocytes were isolated from oviductal ampulae of previously superovulated CD1 females. Mice were injected intraperitoneally with 5 IU of PMSG (Intervet, Boxmeer, Holland) and 40 h later with 5 IU of hCG (Intervet). The oocytes were released from oviducts ∼14–15 h post-hCG injection into HTF–HEPES medium (Cambrex, Verviers, Belgium) and immediately transferred into HTF–HEPES supplemented with hyaluronidase (1 mg/ml) to disperse the enclosing cumulus cells. These cells were finally removed by vigorous pipetting, the oocytes were transferred into HTF–HEPES medium, and only healthy looking oocytes were selected for ICSI.

Spermatozoa treatment

Spermatozoa were obtained from healthy fertile donors, after informed consent was obtained. The sperm were washed several times with M199 (M4530) supplemented with bovine serum albumin (BSA) (4 mg/ml), Na-pyruvate (0.2 mM) and gentamicin (50 μg/ml) and then immediately used for ICSI.

Sperm injection was done basically as described by Kimura and Yanagimachi (1995) with the help of PMM piezo injector (PrimeTech, Ibaraki, Japan). Briefly, the oocytes and spermatozoa were transferred into HTF–HEPES droplets under paraffin oil. Spermatozoa were then immobilized and directly injected into oocytes. Injected oocytes were then transferred into M199 and cultured for 8–9 h or 14–15 h at 5% CO2 and 37°C. The oocytes were then evaluated under Olympus inverted microscope IX71. Then their zona pellucidae were dissolved in acid Tyrode’s solution and oocytes were fixed for 15 min in 3.7% paraformaldehyde before labeling with different antibodies.

Identification of the sperm derived pronucleus

To identify the paternal pronucleus, the spermatozoa were first incubated for 30 min with MitoTracker Green FM (Molecular Probes, Eugene, OR, USA; 100 nM) to label mitochondria located in the mid-piece, then washed several times in HTF–HEPES and injected into oocytes as described above. The labeled mid-piece could be then detected under fluorescence microscope in the vicinity of the paternal pronucleus.

The second method employed to identify the paternal pronucleus was fluorescence in situ hybridization (FISH) where human Cot1 DNA was used to detect specifically DNA of human origin. Human Cot1 DNA (Invitrogen, Carlsbad, CA, USA) was labeled by fluorescein-HighPrime random priming labeling mix (Roche, Prague, Czech Republic) and used as a probe. Briefly, the interspecies zygotes were placed on Super Frost Plus slides (Menzel Glaser, Braunschweig, Germany) in a drop of 0.01 M HCl/0.1% Tween-20 spreading solution, fixed by methanol:acetic acid (3:1) and air-dried. The slides were dehydrated through ethanol series and co-denatured with the labeled Cot1 probe in 50% formamide, 10% dextran sulphate in 2× SSC and left to hybridize overnight. After hybridization, the slides were washed 3 × 10 min in 2× SSC/50% formamide, 0.1× SSC and 4× SSC/0.1% Tween-20 at 42°C. After this, the slides were mounted in Vectashield anti-fade medium containing DAPI (Vector Labs, Peterborough, UK) and observed under an Olympus IX71 microscope.

Labeling, examination and evaluation of zygotes

The antibodies used were: anti-5-methylcytosine (5-MeC) (Eurogentec, Seraing, Belgium), anti-nuclear pore complex (anti-NPC) (Covance, Berkeley, CA, USA), anti-HP1β (Abcam, Cambridge, UK), anti-trimethyl H4/K20 (Abcam), anti-trimethyl H3/K4 (Abcam), anti-acetyl H3/K9 (Upstate, Lake Placid, NY, USA), anti-acetyl H4/K12 (Upstate), anti-dimethyl H3/K9 (Upstate), anti-trimethyl H3/K9 (Upstate), anti-trimethyl H3/K27 (Upstate) and anti-pan histone (Roche).

After fixation, the zygotes were permeabilized in 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 20 min at room temperature.

For anti-5-MeC labeling, the zygotes were washed 3 × 10 min in PBS/1% BSA, then transferred to 2 M HCl for 30 min (room temperature) and washed again 3 × 10 min in PBS/BSA. The samples were blocked for 2 h in 0.2% Triton X-100/5% Normal Goat Serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS. After blocking, the samples were incubated with the primary antibody in the blocking buffer at 4°C overnight.

For anti-acetyl H4/K12 and anti-acetyl H3/K9 the samples were blocked in PBS/BSA/0.1% Triton X-100 at 4°C overnight. The next day, they were incubated with one of the primary antibodies for 1 h at 37°C and then washed in PBS/BSA (3 × 10 min).

For all other antibodies, after fixation and permeabilization, the samples were blocked either in PBS/BSA or PBS/BSA/5% Normal Goat Serum. After blocking, the samples were incubated with one (single labeling) or two (double labeling) of the above mentioned primary antibodies appropriately diluted in the blocking buffer at 4°C overnight.

After incubation with the primary antibody, the samples were washed thoroughly in PBS/BSA and incubated with secondary antibody conjugated with FITC or a mixture of FITC and Texas Red conjugated secondary antibodies (Santa Cruz Biotechnology) for 2 h at room temperature. After incubation with a secondary antibody, the samples were washed again and mounted in Vectashield mounting medium (Vector Labs) and examined. The images were captured using an Olympus IX71 fluorescence microscope equipped with the OptiGrid system (Thales Optem, UK) and processed by ImagePro (Media Cybernetics, Silver Spring, USA) and Adobe Photoshop software.

Unless stated otherwise, all chemicals were purchased from Sigma, Prague, Czech Republic. The study was approved by our local institutional ethics board.

Results

In total, we injected 929 ovulated mouse oocytes, from which 752 (81%) survived the injection procedure. When evaluated after 8–9 h in culture, in 679 (679/752; 90%) of surviving zygotes, we found fully formed pronuclei with clearly visible nucleoli. The same evaluation has also been performed after 14–15 h post-ICSI. As no differences between these two groups were detected, we thereafter used only the zygotes fixed at 8–9 h post-ICSI. Typically, one pronucleus was much larger than the other one. As this is a typical situation for mouse zygotes, we assumed that the larger pronucleus originated from the decondensed sperm head, whereas the smaller pronucleus originated from decondensed oocyte chromosomes. This has been confirmed by DNA FISH with human Cot1 DNA as a probe, which showed a positive signal in the larger pronucleus (Fig. 1a–c), and no signal in the smaller pronucleus (25 zygotes), and also by labeling the spermatozoa mid-piece.
with Mitotracker Green FM before ICSI (Fig. 1d). This piece was always detected in the vicinity of the larger pronucleus (20 zygotes). Thus, these experiments confirm that the larger pronucleus contains DNA of human origin. All zygotes with pronuclei also extruded the second polar body. The smaller (maternal) pronuclei were usually located near the second polar body. This further confirms that the larger pronuclei were of paternal origin.

The pronuclei were enclosed with a distinct nuclear membrane (Fig. 2a). This has been verified after labeling against

Figure 1: Verification of the male pronucleus origin
(a–c) The interspecies zygotes were hybridized with human FITC labeled Cot1 DNA. The positive signal was detectable only over the paternal (larger) pronuclei (a). Parallel staining with DAPI confirms the presence of both pronuclei (b). A merged picture is presented in (c). The Mitotracker labeled sperm mid piece could be also detected in the vicinity of the paternal pronucleus (d). ♂, Paternal pronucleus; ♀, maternal pronucleus; 2PB, second polar body, ×600

Figure 2: Demonstrations of some pronuclear characteristics in interspective zygotes
(a) The interspecies zygote produced by ICSI of human sperm into metaphase II mouse oocyte was labeled against NPC. The labeling could be seen in both pronuclei (PN). The second polar body is also labeled (2PB), ×400. (b) The interspecies zygote labeled with anti-Pan Histone antibody. The positive labeling in all pronuclei documents the exchange of sperm protamines with oocyte histones. ♂, paternal pronuclei; ♀, maternal pronuclei; 2PB, second polar body, ×400
NPC (all 21 examined zygotes showed positive labeling). The exchange of sperm protamines with histones was verified after labeling against histones (Fig. 2b). The oocytes without visible pronuclei were not used for labeling and were discarded.

**Anti-5-MeC labeling**

Of all of the antibodies used in our study, the anti-5-MeC antibody was by far the most frequently used and best characterized. For these reasons, we present the labeling results for 5-MeC separately. In this group, we have evaluated 43 interspecies zygotes which contained one large and one smaller pronucleus. As mentioned above, we confirmed that the larger pronucleus originated from the injected sperm and in 20 cases, this pronucleus showed only a weak labeling which was visible under the pronuclear membrane (Fig. 3a), whereas in 17 zygotes, the weak labeling was rather homogenous covering the entire pronuclear area (Fig. 3b). In the remaining six zygotes, the male pronucleus showed no labeling at all (Fig. 3c and d). Contrary to this, the other (smaller), presumably maternal, pronucleus as well as the second polar body were heavily and homogenously labeled. This confirms the previous results demonstrating the demethylation of paternal DNA within a very short time interval post-fertilization. Moreover, these results also indicate an intensive mouse oocyte demethylation activity.

**Anti-acetylated H3/K9 and -acetylated H4/K12 labeling**

These antibodies were reported to localize symmetrically in both parental pronuclei in the mouse (see Table I.). Thus we have tested, whether pronuclei in interspecies ICSI produced zygotes also show symmetrical localization of labeling. In almost all cases (>90%; at least 30 zygotes from 3 independent experiments were evaluated with the selected antibody), both the maternal and paternal pronuclei were intensively and homogenously labeled. The anti-Pan Histone labeling indicates that soon after ICSI, the paternal pronucleus is associated with histones that are readily modified by acetylation, as shown by the anti-acetylated H3/K9 and anti-acetylated H4/K12 antibodies (Fig. 4a and b).

**Anti-HP1β, -trimethyl H4/K20, -trimethyl H3/K4, -dimethyl H3/K9, -trimethyl H3/K9 and -trimethyl H3/K27 labeling**

These antibodies were selected because of their asymmetrical localization between parental pronuclei (see Table I). Almost all pronuclei (>90%) also showed an asymmetrical labeling in interspecies zygotes (≥30 zygotes from at least three

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Figure 3: Examples of the most common patterns after anti-5-MeC labeling of interspecies zygotes
(a-d) The labeling of interspecies zygotes against 5-MeC showed an intensive demethylation of the paternal DNA (c'). On the other hand, the maternal pronuclear DNA (c) as well as both polar bodies (1PB, 2PB) remained heavily methylated. The two most typical patterns are showed in (a, b). In (a) (×400), the labeling is detectable only under the paternal pronuclear membrane whilst the homogenous weak labeling can be seen over the whole paternal pronuclear area in (b) (×350). The paternal pronucleus sometimes showed an absolute absence of labeling (c). Parallel propidium iodide labeling (d), ×400
independent experiments were evaluated for each antibody). In this group, only the maternal pronucleus was intensively labeled, whilst the paternal pronucleus showed no or only very weak labeling (Fig. 4a and d). As reported in the mouse, also in interspecies zygotes, the anti-trimethyl H4/K20 and anti-trimethyl H3/K9 (not shown) label only a thin rim of heterochromatin associated with the maternal nucleolus.

**Discussion**

The interspecies ICSI technique is a powerful approach that can be used also for studies related to assisted human reproduction (Yanagimachi, 2005). For example, Araki _et al._ (2005) demonstrated the possibility of human sperm karyotype analysis after the injection of spermatozoa into enucleated mouse oocytes. The activation capacity of human sperm can also be

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**Table 1.** Chromatin characteristics of parental pronuclei in interspecific ICSI produced zygotes and intraspecific mouse zygotes.

<table>
<thead>
<tr>
<th>Antibody (symmetrical or asymmetrical localization/total evaluated)</th>
<th>Human × mouse</th>
<th>Mouse</th>
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<tbody>
<tr>
<td></td>
<td>M</td>
<td>P</td>
</tr>
<tr>
<td>MeC (37/43)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Trimethyl H3/K27 (52/57)</td>
<td>+</td>
<td>–</td>
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<tr>
<td>HP1 β (31/33)</td>
<td>+</td>
<td>–</td>
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<tr>
<td>NPC (21/21)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pan Histone (36/36)</td>
<td>+</td>
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M, maternal pronucleus; P, paternal pronucleus.

+, Positive labeling; –, weak/asymmetrical labeling; ±, intermediate labeling.

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**Figure 4:** Examples of the symmetrical and asymmetrical labeling in interspecies zygotes

(a, b) An interspecies zygote labeled against acetyl H4/K12 (a) shows a positive labeling in both pronuclei as well as in second polar bodies. (b) Shows parallel DNA labeling with DAPI, ♀ paternal pronucleus; ♂ maternal pronucleus; 2PB, second polar body, × 700. (c, d) An interspecies zygote labeled against trimethyl H3/K27 shows the asymmetric pattern of labeling where only the maternal DNA is positively labeled, whereas the paternal chromatin is without labeling (b). Parallel DAPI staining documents the presence of the paternal pronuclei (d). ♂ paternal pronucleus; ♀, maternal pronucleus; 2PB, second polar body, × 600
tested by their injection into mouse oocytes (Heindryckx et al., 2005). Terada et al. (2004) injected human sperm into rabbit oocytes and analysed the sperm centrosomal function. A clear relation has been found between centrosomal characteristics and the fertility of sperm donors. Thus, the interspecies ICSI may serve as an additional approach for the assessment of certain forms of infertility and our results clearly show that this approach can also be used for the evaluation of the epigenetic status of human spermatozoa.

Soon after fertilization, a dramatic transformation of the sperm head must occur in order to establish a fully developmentally competent embryo (McLay and Clarke, 2003; Kimmins and Sassone-Corsi, 2005). Interestingly, from the epigenetic point of view, in a single zygote, two completely distinct sets of chromatin (maternal and paternal) co-exist. It has been shown, in some mammals, that dramatic demethylation of paternal DNA occurs soon after the sperm penetrates the oocyte. This has been confirmed for the mouse, rat, pig, cattle, humans, but not for sheep and rabbit paternal DNA (Santos and Dean, 2004; Young and Beaujean, 2004). In human zygotes, the process of paternal genome demethylation has been detected in about half of the embryos examined. Here, the paternal pronucleus was less labeled when compared to the maternal pronucleus (Fulka et al., 2004). Basically, similar results were obtained by Xu et al. (2005), who analysed tripolar paternal human zygotes. On the other hand, complete paternal genome demethylation has been observed by Beaujean et al. (2004a).

In our experiment, we have injected human sperm from fertile donors into ovulated mouse oocytes. The survival of injected oocytes was quite high and about three quarters of the surviving zygotes formed well developed pronuclei with protamines replaced by histones as shown by labeling with anti-Pan Histone antibody. These pronuclei were enclosed by a prominent nuclear membrane as shown by anti-NPC labeling. Interestingly, whilst in normal human zygotes both pronuclei are of the same size, in interspecies zygotes the paternal pronucleus was always much larger when compared with the maternal pronucleus. This indicates that the pronuclear size regulation is the property of the cytoplasm into which the spermatozoon is introduced.

The high efficiency of this technique enables us to analyse the response of human sperm chromatin to the mouse oocyte cytoplasmic activities. As we expected, the results convincingly showed that the response of human sperm was exactly the same as in the case of intraspecific (mouse × mouse) fertilization. This means that the paternal chromatin was rapidly demethylated, as shown by anti-5-MeC labeling and was associated with highly acetylated histones, as shown by anti-acetylated H3/K9 and acetylated H4/K12 labeling.

The human paternal pronucleus also showed the absence of methylated histones as demonstrated by anti-trimethyl H4/K20, trimethyl H3/K4, dimethyl H3/K9, trimethyl H3/K9 and trimethyl H3/K27 labeling. The same situation has been observed in the mouse (see Table I).

The only exception was the HP1β antibody. Arney et al. (2002) reported a total absence of labeling in the paternal pronucleus. On the other hand, Santos et al. (2005) reported the labeling in both parental pronuclei with the paternal pronucleus being slightly less intensively labeled when compared to the maternal pronucleus; our results are in agreement with these findings.

It may be suggested that a similar response will be obtained even when epigenetically abnormal spermatozoa from infertile patients are used, because the oocyte plays an essential role in the regulation of these epigenetic processes. However, the possibility of sperm involvement cannot be completely excluded as some data contradict the above suggestion. For instance, Beaujean et al. (2004b) demonstrated that the process of paternal genome demethylation is influenced by both the origin of spermatozoa injected as well as the origin of oocytes into which the spermatozoa are injected. Thus, mouse spermatozoon DNA is almost completely demethylated in the mouse oocyte, but evidently less demethylated after injected into the ovine oocyte. On the other hand, sheep sperm DNA is not demethylated in its natural environment (ovine oocyte cytoplasm), but evident demethylation can be detected when injected into a mouse oocyte. Also nuclear DNA from a somatic cell is only slightly demethylated in zygotes produced by nuclear transfer (Santos et al., 2003; Beaujean et al., 2004c). As mentioned above, Barton et al. (2005) detected an aberrant paternal epigenetic reprogramming when males were exposed to cyclophosphamide. These abnormalities included, e.g., the hypomethylation of paternal pronuclear DNA and hyperacetylation of histones associated with it. Similarly, Anway et al. (2005) showed transgenerational effects of environmental toxins upon epigenetic alterations in the germ cells. These effects correlate with an altered DNA methylation pattern in the germ line. Benchabib et al. (2005) analysed the level of methylation in human spermatozoa and found that lower values correspond to a decreased pregnancy rate (33.3% versus 8.3%). Thus, we can expect that certain epigenetic abnormalities could be detected for some specific forms of human male infertility. This has been confirmed very recently by Kishigami et al. (2006) who analysed the male pronucleus methylation pattern in mouse zygotes produced either by intracytoplasmic injection of mature spermatozoa, elongated spermatids or round spermatids, respectively. Although the paternal genome from mature sperm remained demethylated throughout the first mitotic cell cycle, a completely different situation was observed after injection of round spermatids. Here, the demethylation wave was followed by a remethylation wave that was dependent on DNA replication. The intermediate pattern was detected when more developmentally advanced cells, i.e., elongated spermatids, were used. The authors concluded that this aberrant pattern reflects the much lower developmental potential of embryos produced by injection of round spermatids. This has been confirmed by Yamazaki et al. (2007) who compared the pronuclear methylation pattern zygotes produced by ICSI or round spermatid injection (ROSI) in the mouse. Although in the first group, the paternal pronuclear DNA was homogenously demethylated, in the ROSI group bright dots of methylated DNA were detected. Consequently, these ROSI oocytes cleaved to the two-cell stage at a significantly lower rate. As these techniques are now used even in humans, it may be assumed that a similar
situation will exist here as well (Lee et al., 2006). Moreover, some other new approaches such as the testicular stem cell transplantation and the production of spermatozoa from embryonic stem cells clearly demonstrate that the gametes thus produced differ from those produced naturally (Goossens et al., 2006). This influences the number of cells in the blastocyst and the IC/M ratio (inner cell mass/trophectoderm ratio) and, more importantly, the genotype and phenotype of offspring. Nayernia et al. (2006) injected sperm cells that were derived from embryonic stem cells and obtained few offspring that were in most cases abnormal and died soon or later after birth. The abnormalities were very similar to those seen in cloned animals and it is commonly accepted that these problems are epigenetic in nature (Luciferio and Reik, 2006). It may be the case that similar approaches are developed sooner or later for human assisted reproduction as well.

Taken together, our results show that interspecies ICSI can serve as a useful additional approach for the eventual assessment of aberrant epigenetic changes in human sperm. As for the next step, it is necessary to select, in extensive tests in fertile patients, the most convenient antibody that will indicate the potential abnormalities. Moreover, we do believe that our approach gives an unrivalled opportunity to test the safety of potential abnormalities. Moreover, we do believe that our approach, where a limited number of sperm cells that were derived from embryonic sperm cells (Aflatoonian and Moore, 2006). Moreover, here we can expect that a very limited number of germ cells will be produced and this will be further complicated by analyses to evaluate if they are indeed normal. Thus we do believe that our approach, where a limited numbers of spermatozoa are necessary, will enable at least their partial evaluation.

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