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Decline in fertility of mouse sperm with abnormal chromatin during epididymal passage as revealed by ICSI

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BACKGROUND: Recent studies showed that ICSI with cauda epididymal or ejaculated sperm of infertile mice or men, respectively, was less effective in fertilization and normal embryo development than ICSI using sperm from the testes. These studies suggested that sperm nuclear quality declined after release from the testis, but the site where this loss of fertility occurs has not been localized. METHODS: We performed ICSI with testicular, caput, and cauda epididymal sperm from infertile *Tnp1–/–Tnp2+/–* mutant mice, which have a minimal level of transition nuclear proteins and are sterile by natural mating. RESULTS: When the heads of motile sperm from the testis or caput epididymis of *Tnp1–/–Tnp2+/–* males were injected into enucleated mouse oocytes, sperm chromosomes showed no difference from those of wild-type mice, but the chromosomes from sperm taken from the cauda epididymis of mutant males showed increased abnormalities. Injection of testicular or caput epididymal sperm from *Tnp1–/–Tnp2+/–* males into intact oocytes resulted in normal embryonic and fetal development and yields of liveborn equivalent to wild-type, but cauda sperm from *Tnp1–/–Tnp2–/–* mice produced lower implantation rates and yields of liveborn than did those from wild-type mice. CONCLUSIONS: These results demonstrate that in mice with sperm chromatin abnormalities, the decline in fertility of sperm with ICSI occurs after the caput epididymis. The advantage of using caput epididymal sperm for ICSI in certain situations may be considered as an approach to be tested in human assisted reproduction.

Key words: embryo development/epididymal sperm/ICSI/testicular sperm/transition nuclear proteins

Introduction

The major steps in chromatin remodelling during the final stages of spermiogenesis involve the replacement of histones by transition nuclear proteins (TP), of which TP1 and TP2 are the major forms, and their subsequent replacement by protamines in spermatids (Meistrich, 1989). Further maturation of the chromatin occurs in the epididymis, as the thiols in the protamines form cross-linked disulphide bonds. This tightly compacted protamine structure is believed to be required to fully stabilize and protect the DNA from damage during epididymal passage, without which the DNA may be more susceptible to damage (Bianchi *et al.*, 1993), perhaps by endogenous nucleases (Szczygiel and Ward, 2002) or reactive oxygen species (Irvine *et al.*, 2000).

Recently higher pregnancy rates have been reported with ICSI using testicular versus ejaculated sperm from infertile individuals (Greco *et al.*, 2005). The observation of a higher frequency of sperm showing detectable terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining in the ejaculated than in the testicular sperm suggested that the poorer outcome with ejaculated sperm was a result of increased DNA damage between release from the testis and ejaculation.

To investigate this phenomenon, a well-characterized animal model, preferably with a defined chromatin defect, is needed. Mice carrying mutations in the *Tnp* genes (Yu *et al.*, 2000; Zhao *et al.*, 2001, 2004a,b; Meistrich *et al.*, 2003; Shirley *et al.*, 2004) meet these criteria and various combinations of heterozygous or homozygous mutations in both *Tnp1* and *Tnp2* can be produced to modulate the severity of the defect. In mice lacking one of the TP, the DNA of cauda epididymal sperm is more accessible to intercalating dyes and shows increased strand breakage (Zhao *et al.*, 2001; Meistrich *et al.*, 2003). Furthermore, although testicular sperm from mice completely lacking TP were just as capable of fertilizing oocytes by ICSI and progressing through normal development as were sperm from wild-type mice (Zhao *et al.*, 2004a), the cauda epididymal sperm from these *Tnp* mutant mice had reduced ability to fertilize oocytes and bring about progression of the fertilized oocytes to the 2-cell stage. Others have similarly shown that the cleavage rate after ICSI with sperm from infertile mice was lower when cauda epididymal rather than testicular sperm were used (Baart *et al.*, 2004).

An important question in the study of the mechanism of this process is whether the fertilizing ability of mutant sperm was

compromised during release from the testis or during epididymal transport. Mice lacking both TP could not be used because of the extremely low numbers of epididymal sperm and the very poor motility of their sperm (Shirley *et al.*, 2004; Zhao *et al.*, 2004a). We therefore used *Tnp1–/–Tnp2+/–* mice, which have only one copy of the gene for TP2, which is the lower abundance protein of the two TP. These mice are sterile by natural mating, and so ICSI is thus required to study the developmental potential of the sperm nucleus of these mice within the oocyte. We have already shown that ICSI with cauda epididymal sperm from these mice resulted in lower fertilization and embryonic development than did ICSI with sperm derived from wild-type mice (Shirley *et al.*, 2004).

In this study we compared the ability of sperm from the testis and different regions of the epididymis of these infertile mice to fertilize oocytes by ICSI, the integrity of the sperm chromosomes at the first cleavage division, and the ability of the fertilized oocytes to undergo complete development.

Materials and methods

Animals

Sperm were collected from testes and caudae epididymides of wild-type 129Sv male mice and mutant 129Sv males deficient in TP (*Tnp1–/–Tnp2+/–*) bred in the same colony. The mutants were produced by crossing *129S-Tnp1*^{tm1Mlm} mice (Yu *et al.*, 2000) with *129S-Tnp2*^{tm1Mzh} mice (Zhao *et al.*, 2001). *Tnp1–/–Tnp2+/–* males are infertile after natural mating (Shirley *et al.*, 2004). B6D2F$_1$ (C57BL/6×DBA) females were used as oocyte donors. All were maintained in a temperature- and light-controlled room (14 h light:10 h dark) for ≥2 weeks before use. B6D2F$_1$ females were 6–12 weeks old at the time of the experiment, and *Tnp1–/–Tnp2+/–* and 129Sv males were 6–10 months old. All animals were maintained in accordance with the guidelines of the Laboratory Animal Service at University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council. The animal handling and treatment protocols were reviewed and approved by the animal care and use committees at the M.D.Anderson Cancer Center and the University of Hawaii.

Reagents and media

Polyvinyl alcohol (PVA, cold water-soluble, mol. wt ~10 000) was purchased from Sigma Chemical Co. (St Louis, MO, USA), polyvinyl pyrrolidone (PVP, mol. wt ~360 000) and bovine testicular hyaluronidase (200 UPS IU/mg) from ICN Pharmaceuticals (Costa Mesa, CA, USA), bovine serum albumin (BSA, fraction V) from Calbiochem (La Jolla, CA, USA), and mineral oil from Squibb & Sons (Princeton, NJ, USA). All other reagents were obtained from Sigma unless specifically stated otherwise. CZB medium (Chatot *et al.*, 1990), supplemented with 5.56 mmol/l glucose and 4 mg/ml BSA, was used for culturing mouse oocytes after microsurgery. The medium used for collecting oocytes from oviducts and micromanipulating of oocytes was a modified CZB (HEPES–CZB) (Kimura and Yanagimachi, 1995a) containing 20 mmol/l HEPES–HCl, a reduced amount of $NaHCO_3$ (5 mmol/l), and 0.1 mg/ml PVA instead of BSA. CZB was used under 5% CO_2 in air, and HEPES–CZB was used under air.

Preparation of oocytes and sperm

Collection of oocytes and sperm and microsurgical manipulation of oocytes were performed according to Kimura and Yanagimachi (1995a,b). Briefly, oocytes were collected from oviducts of superovulated females soon after ovulation, freed from cumulus cells by hyaluronidase treatment, and kept in CZB medium at 37°C for <2 h.

For analysis of chromosomal aberrations in sperm, a group of oocytes (usually ~20) was transferred into a droplet of HEPES–CZB containing cytochalasin B at 5 μg/ml located in the operation chamber on the microscope stage. They were kept there for 5–10 min and then enucleated by aspirating the metaphase II chromosome–spindle complex into a pipette (8–10 μm inner diameter) with minimal volumes of oocyte cytoplasm (Wakayama *et al.*, 1998). Enucleated oocytes were transferred to cytochalasin B-free CZB and kept there for up to 2 h at 37°C before they were injected with sperm.

Epididymal sperm from normal (wild-type) males were allowed to disperse in HEPES–CZB for 10–20 min at 37°C before transfer of a 2–5 μl droplet into 20 μl HEPES–CZB containing 12% (w/v) PVP (PVP–HEPES). The resulting sperm suspension was kept under mineral oil in a micromanipulator dish at room temperature for <1 h. Epididymal sperm from *Tnp1–/–Tnp2+/–* males and testicular sperm from both wild-type and mutant males were kept in HEPES–CZB with 1% PVP for <1 h at room temperature before they were injected into oocytes. A drop (10–20 μl) of testicular sperm suspension was transferred to the micromanipulation chamber on the microscope stage. Light microscopy of live cells indicated that high proportions (70%) of testicular and epididymal sperm from *Tnp1–/–Tnp2+/–* mice had abnormal head morphology, as previously described in detail for cauda epididymal sperm (Shirley *et al.*, 2004). They were classified into normal (Figure 1A), quasi-normal (not shown), and markedly abnormal sperm (Figure 1B and C). Only sperm with normal and markedly abnormal head morphology were used in this study. Haematoxylin staining of dried smears revealed that many of the markedly abnormal sperm (Figure 1E) had nuclei with relatively normal shapes,

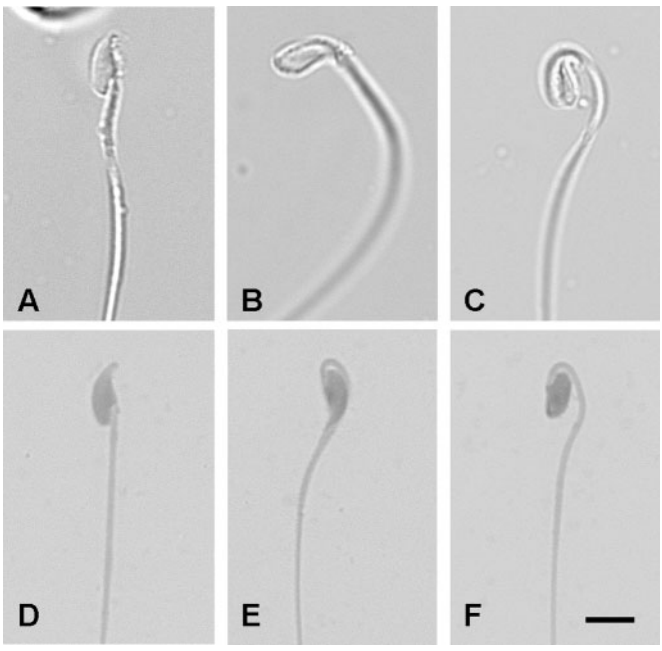

Figure 1. Sperm from *Tnp1–/–Tnp2+/–* mice. (**A**–**C**) Fresh sperm examined at high resolution with light microscopy with a ×100 objective. Some appear to have (**A**) normal nuclear morphology and others (**B**, **C**) appear to have markedly abnormal head shapes. Further examination of the stained sperm in dried smears (**D**–**F**) reveals a few sperm with (**D**) normal morphology and many (**E**, **F**) with the nucleus reflected back along midpiece of the tail. Some of these have normal nuclear morphology and some of them have abnormal morphology, often involving a blunted tip of the nucleus. Bar = 5 μm.

that reflected back along the midpiece portion of the tail so that they appeared to have a rounded anterior end and a tail emanating from the tapered part of the head. A smaller number of abnormal sperm also had a deformed nucleus, typically a blunted anterior tip (Figure 1F) (Yu *et al.*, 2000; Shirley *et al.*, 2004). The motility of sperm in PVP-containing medium was visually assessed immediately before ICSI. Sperm with discernible flagellar motion, regardless of their patterns and degrees, were considered motile.

ICSI

ICSI was carried out according to Kimura and Yanagimachi (1995a) with some modifications. A single motile or immotile spermatozoon was drawn tail-first into the injection pipette and moved back and forth until the head–midpiece junction (i.e. the neck) was at the opening of the injection pipette. The head was separated from the midpiece and tail by applying one or a few piezo pulses (Kimura and Yanagimachi, 1995b). After the tail had been discarded, the head was redrawn into the pipette and injected into an oocyte. The whole procedure was performed at room temperature in HEPES–CZB within 1 h after mixture of the sperm suspension with PVP medium. Sperm-injected oocytes were cultured in 50 μl droplets of CZB medium under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air. They were examined with an interference-contrast microscope 5–6 h after ICSI. Those with two distinct pronuclei and the second polar body were recorded as normally fertilized.

Examination of sperm chromosomes

Sperm karyotyping was performed since it is a direct measure of genomic damage. A single spermatozoon was injected into an enucleated oocyte, allowed to transform into metaphase chromosomes at the first cleavage division, and then examined for chromosomal integrity (Akutsu *et al.*, 2001). Seven to 8 h after ICSI, activated oocytes, characterized by a single large sperm pronucleus, were transferred into another droplet (0.2 ml) of CZB containing vinblastine at 0.006 μg/ml, which prevented the formation of an ooplasmic microtubule network. Between 18 and 21 h after ICSI, oocytes arrested at metaphase of the first cleavage were treated with 0.25% (w/v) pronase (Kaken Pharmaceuticals, Tokyo, Japan) for 5 min to dissolve their zonae pellucidae and then treated with a hypotonic solution (1:1 mixture of 1% sodium citrate and 30% fetal bovine serum) for a few minutes at room temperature. Oocytes were fixed and chromosomes spread according to Mikamo and Kamiguchi (1983). The chromosome spreads on slides were stained with 2% Giemsa solution for 8 min to detect aneuploidy and structural chromosome aberrations such as chromatid breaks, chromatid fragmentations, and chromatid exchanges.

Embryo development and embryo transfer to surrogate mothers

Oocytes fertilized normally by ICSI were allowed to develop in CZB at 37°C. Embryos reaching the morula/blastocyst stages were transferred to the oviducts of pseudopregnant CD-1 females that had been mated the previous night with vasectomized males of the same strain. Morula/blastocysts thus transferred enter the uterus and implant 3–4 days later. Pregnant females were euthanized at 19.5 days post-coitum and all implantation sites were counted. Live full-term fetuses were delivered by Caesarean section and raised by lactating CD-1 foster mothers.

Acridine Orange staining of sperm nuclei

Sperm smears were fixed in acetic acid–alcohol and stained with Acridine Orange (AO) in a citrate–phosphate buffer (pH 2.5) as described by Tejada *et al*. (1984) and Kosower *et al*. (1992). The stained samples were examined by fluorescence microscopy immediately after washing and mounting in distilled water. At least 300 sperm, irrespective of head or tail morphology, were examined for each experimental group and the colour of the fluorescence was determined. In one experiment, the ductus efferentia (the junction between the testis and caput epididymis) was ligated and animals were killed 7 days later to collect sperm from the rete testis.

It has been previously shown (Kosower *et al.*, 1992) that when sperm are stained with AO under these conditions after fixation on slides, those with high levels of free thiols in the protamines show red nuclear fluorescence and those with high levels of protamine disulphide bonds show green fluorescence; sperm nuclei with intermediate levels of protamine disulphide bonding fluoresce yellow.

Statistical analysis

The data were analysed with the χ^2-test using Yates' correction for continuity or Fisher exact test.

Results

Chromosomes and reproductive potential of sperm

Motile testicular and epididymal sperm from normal (wild-type) male mice activated the majority (92–98%) of enucleated oocytes by ICSI. Only ~15% of these oocytes fragmented, and 2% were arrested in the pronuclear stage, the remainder being available for sperm karyotyping. Only 10–13% had abnormal chromosomes (Table I). Similar results with respect to oocyte activation, development to cleavage mitosis, and chromosome integrity were obtained with motile testicular and caput epididymal sperm from *Tnp1–/–Tnp2+/–* males, whether they had normal or abnormal head morphology. However, motile sperm from the cauda epididymis of *Tnp1–/–Tnp2+/–* males showed increases in the percentage of oocytes that were fragmented or did not progress to mitosis, when compared to wild-type or to sperm from either the testis or caput epididymis of these mutants (Table I). There were no significant differences between sperm with normal or abnormal head morphology.

The results summarized in Table I indicate that motile sperm from the cauda epididymis of *Tnp1–/–Tnp2+/–* males had a higher incidence of structural chromosome aberrations than those of wild-type or testicular and caput epididymal sperm of the same males. Although the increase in structural abnormalities in live, normal-looking sperm from the cauda epididymis of *Tnp1–/–Tnp2+/–* males was not statistically significant compared with those of wild-type or testicular and caput epididymal sperm of the same males (Table I), the incidence of chromosomal abnormalities in the abnormally shaped sperm from the cauda epididymis of *Tnp1–/–Tnp2+/–* males was higher than in the normally shaped sperm from wild-type mice ($P < 0.05$). Since development to the first cleavage division and the incidence of chromosomal abnormalities were similar and not significantly different between the normal and abnormal sperm, the two classes were pooled. The percentage of structural chromosomal aberrations resulting from fertilization by cauda epididymal sperm (normal and abnormal morphology) from *Tnp1–/–Tnp2+/–* males (24–33%) was significantly higher ($P < 0.05$) than both the percentages of such aberrations resulting from cauda epididymal sperm (normal) from wild-type males (10%) or testicular sperm (normal and abnormal) from *Tnp1–/–Tnp2+/–* males (10–13%).

Table I. Development and chromosomal analysis of nuclei of motile sperm and from *Tnp1–/–Tnp2+/–* wild-type males after injection into enucleated oocytes

Genotype of mice	Source of sperm	Head morphology	No. of oocytes surviving ICSI (no. of experiments)	No. of activated oocytes (% of surviving)	No. of oocytes fragmented next morning (% of surviving)	No. of non-fragmented oocytes analysed	No. of pronuclear arrested oocytes (% of analysed)	No. of oocytes with analysable sperm chromosomes	No. of oocytes with indicated sperm chromosome abnormalities (% of those karyotyped)	
									Structural	Aneuploid[a]
Wild	Testis	Normal	110 (3)	108 (98)	18 (16)	92	2 (2)	90	12 (13)	0 (0)
	Caput epididymis	Normal	129 (3)	126 (98)	18 (14)	111	1 (1)	110	11 (10)	0 (0)
	Cauda epididymis	Normal	146 (3)	134 (92)†‡	25 (17)	121	5 (4)	116	12 (10)	0 (0)
Tnp1–/– *Tnp2+/–*	Testis	Normal	50 (2)	47 (94)	9 (18)	41	2 (5)	39	4 (10)	0 (0)
		Abnormal	61 (2)	56 (92)	13 (21)	48	3 (6)	45	6 (13)	0 (0)
	Caput epididymis	Normal	35 (2)	33 (94)	5 (14)	30	0 (0)	30	4 (13)	0 (0)
		Abnormal	48 (2)	45 (94)	7 (15)	41	0 (0)	41	6 (15)	1 (2)
	Cauda epididymis	Normal	35(4)	26 (74)**†‡	12 (34)*	23	6 (26)**†‡‡	17	4 (24)	0 (0)
		Abnormal	43(3)	32 (74)**†‡	16 (37)**‡	27	9 (33)***†††‡‡‡	18	6 (33)*	1 (6)
		Normal or Abnormal	78(4)	58 (74) ***†††‡‡‡	28 (36)*†††‡‡	50	15 (30)***†††‡‡‡	35	10 (29)* †	1 (3)

[a]Aneuploid sperm also had structural aberrations.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, versus same sperm source from wild-type.
†$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ versus sperm of same characteristics from testis.
‡$P < 0.05$, ‡‡$P < 0.01$, ‡‡‡$P < 0.001$ versus sperm of same characteristics from caput epididymis.

Table II summarizes the development of intact oocytes following ICSI. Motile sperm from *Tnp1–/–Tnp2+/–* males, regardless of whether they had normal or abnormal head morphology, supported the development of ICSI-fertilized oocytes to the morula–blastocyst stage, irrespective of the source of the sperm. The only category that showed a significantly reduced frequency of fertilization was the abnormal sperm from the cauda epididymis of *Tnp1–/–Tnp2+/–* males. There were no differences in the frequencies of implantation and development to term of embryos derived from testicular or caput epididymal sperm from *Tnp1–/–Tnp2+/–* males and from wild-type mice. However, the frequencies of embryo implantation and the overall yield of liveborn for embryos derived from cauda epididymal sperm from *Tnp1–/–Tnp2+/–* males were significantly lower than for those obtained after fertilization with sperm from the caput epididymis of these mice or with cauda sperm from wild-type mice. It was noted that irrespective of the genotype of the mice, the source of sperm, and the head morphology, some live offspring were produced and there were no differences in the sex ratio of these offspring (data not shown).

Chromatin properties of sperm

AO staining was used to partially characterize the chromatin of the sperm from *Tnp1–/–Tnp2+/–* males. Whereas only ~4% of sperm in the testes of wild-type mice showed green fluorescence (Figure 2), ~25% of sperm in the testes of *Tnp1–/–Tnp2+/–* mice fluoresced green. However, many sperm were not released from the seminiferous epithelium normally at stage VIII in *Tnp1–/–Tnp2+/–* mice but were still retained within the epithelium at stages XI and XII (data not shown), similar to observations previously made with other *Tnp* mutants (Zhao *et al.*, 2001, 2004a). To demonstrate that retention of sperm in the testis could cause increased disulphide bond formation, the ductus efferentia of wild-type mice was ligated for 7 days. The proportions of red, yellow and green nuclei observed in these sperm (Figure 2) were similar to those observed from testicular sperm from *Tnp1–/–Tnp2+/–* mice.

The proportion of green fluorescing sperm increased markedly in the epididymis in both genotypes supporting the relationship between AO fluorescence and protamine disulphide bond formation. In wild-type mice, the percentage of sperm showing green fluorescence increased from >90% in the caput epididymis and 100% in the cauda epididymis. *Tnp1–/–Tnp2+/–* mice had lower percentages of sperm that fluoresced green in the caput (60%) and cauda (90%) epididymides, indicating that fewer had high levels of disulphide bond formation.

Discussion

Motile sperm from the caput epididymis of *Tnp1–/–Tnp2+/–* males showed normal fertilization and activation of oocytes by ICSI, chromosomal integrity, resulting zygote development, implantation, and production of liveborn compared with either caput epididymal sperm from wild-type mice or testicular sperm from *Tnp1–/–Tnp2+/–* mice (Tables I and II). By contrast, when the motile sperm from the cauda epididymis of *Tnp1–/–Tnp2+/–* males were evaluated by ICSI, there were increases in chromosome abnormalities at the first cleavage division, and reductions in fertilization, development to the first cleavage division, and implantation of developing embryos compared to cauda sperm from wild-type mice and caput epididymal sperm from these *Tnp1–/–Tnp2+/–* mutants.

The observation of increased chromosomal structural aberrations when ICSI was performed with cauda epididymal sperm from *Tnp1–/–Tnp2+/–* mutants demonstrates that there is deterioration of the genomic integrity of the sperm during epididymal transit. In mice, it has been shown that conditions that promote DNA sperm degradation also increase chromosome aberrations after ICSI (Szczygiel and Ward, 2002; Sotolongo *et al*., 2005). Furthermore, when ICSI of enucleated oocytes was performed with cauda epididymal sperm from *Tnp1–/–Tnp2+/–* mutants there were defects in the development of the fertilized oocytes. However, when intact oocytes were used, this stage of development was normal. This result indicates that enucleated oocytes lack factors present in intact oocytes to support the initial development of the damaged sperm chromatin. With intact oocytes, the major developmental defect appeared later, at implantation (Table II). The latter is similar to a clinical observation that ejaculated sperm with DNA damage progress through preimplantation stages of embryonic development but fail to implant (Tesarik *et al*., 2004).

The minimal effect of sperm head morphology on the outcome of ICSI in the mouse is consistent with previous studies (Burruel *et al.*, 1996; Akutsu *et al.*, 2001) showing that several types of abnormal sperm head morphology did not affect embryonic development or production of live fetuses. Although some more severe abnormalities in nuclear shape or the configuration of the head relative to the tail did show an increased incidence of structural chromosome abnormalities at the first cleavage division (Kishikawa *et al.*, 1999; Akutsu *et al.*, 2001), this did not appear to be a significant factor in the case of the *Tnp1–/–Tnp2+/–* mutants.

The results of this study show that there is no immediate deterioration of the sperm nuclear function, as assessed by their ability to sustain development and form normal chromosomes after ICSI, as the sperm are released from the testis, pass through the efferent ducts, and enter the caput epididymis. However, deterioration occurs as they pass through the corpus and/or cauda epididymis. The observation of apparent deterioration of genomic integrity is consistent with the results of a previous study examining the DNA dye binding of epididymal sperm from *Tnp1–/–Tnp2+/+*, *Tnp1+/–Tnp2–/–*, and the *Tnp1–/–Tnp2+/–* mice used here (Shirley *et al*., 2004). In that study the percentages of sperm showing very low levels of DNA dye binding increased as the sperm passed from the caput to the cauda epididymis, indicating massive DNA degradation in these sperm. For example, 20% of sperm from the caput epididymis of *Tnp1–/–Tnp2+/–* mice showed very low levels of propidium iodide fluorescence but 42% of those from the cauda did, confirming that DNA damage can occur during epididymal transport.

The loss of genomic integrity of viable sperm of *Tnp1–/–Tnp2+/–* mice during passage from the caput to cauda epididymis could be related in part to abnormalities in the protection of the DNA by the protamine. In contrast to sperm with

Table II. Development of intact oocytes injected with motile sperm from *Tnp1–/–Tnp2+/–* and wild-type males

Genotype of mice	Sperm Source	Head morphology	No. of oocytes surviving ICSI (no. of experiments)	No. of normally fertilized (% of surviving)	No. of morulae –blastocysts (% of fertilized)	No. of embryos transferred (no. of recipients)[a]	No. of implantations (% of transferred)	No. of live offspring (% of implantations)	% of live offspring per oocyte injected
Wild	Testis	Normal	77(2)	73 (95)	57 (78)	57 (5)	41 (72)	27 (66)	33
	Caput epididymis	Normal	51(2)	49 (96)	44 (90)	44 (4)	36 (82)	25 (69)	44
	Cauda epididymis	Normal	63(2)	63 (100)	56 (89)	56 (5)	50 (89)	38 (76)	56††
Tnp1–/– *Tnp2+/–*	Testis	Normal	46(2)	44 (96)	35 (80)	35 (3)	28 (80)	16 (57)	31
		Abnormal	42(2)	39 (93)	30 (77)	30 (3)	24 (80)	14 (58)	30
	Caput epididymis	Normal	28(2)	27 (96)	25 (93)	24 (2)	20 (83)	15 (75)	46
		Abnormal	44(2)	43 (98)	38 (88)	38 (2)	33 (87)	27 (82)	55†
	Cauda epididymis	Normal	19(2)	18 (95)	15 (83)	15 (2)	6 (40)***†‡	4 (67)	17**‡
		Abnormal	34(2)	27(79)***‡	22 (81)	22 (2)	11(50)***†‡‡	8 (73)	21***‡‡

[a]All recipients were pregnant.
***P* < 0.01, ****P* < 0.001 versus same sperm source from wild-type.
†*P* < 0.05, ††*P* < 0.01, versus sperm from testis of same genotype.
‡*P* < 0.05, ‡‡*P* < 0.01, versus sperm from caput epididymis of same genotype.

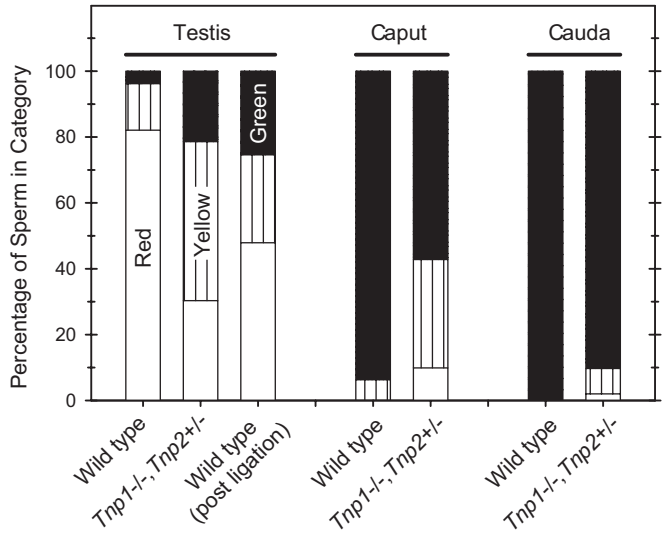

Figure 2. Acridine Orange fluorescence of the nuclei of sperm collected from the testis and different regions of the epididymis. Open bars represent the percentage of sperm nuclei with red fluorescence indicative of reduced sulphydryl groups in protamines; bars with vertical lines represent the percentage of sperm nuclei with yellow fluorescence, indicative of intermediate levels of protamine cross-linking; and solid bars represent the percentage of sperm with green fluorescence.

reduced amounts of protamine (Cho *et al.*, 2003), sperm from *Tnp1–/–Tnp2+/–* mice have approximately normal levels of protamines and a normal ratio of protamines 1 and 2 (Shirley *et al.*, 2004). Yet the number of caput or cauda epididymal sperm fluorescing red or yellow, indicating free thiol groups, remained higher in *Tnp1–/–Tnp2+/–* mice than in wild-type mice. Among the possible explanations for the lower disulphide cross-linking and reduced DNA protection in sperm from *Tnp1–/–Tnp2+/–* is the deficiency in protamine 2 processing. Protamine 2 is synthesized as a 106 amino acid precursor but is processed to the 63 amino acid mature form, which is the only form found in wild-type mouse sperm (Yelick *et al.*, 1987; Shirley *et al.*, 2004). However, in the cauda epididymal sperm from *Tnp1–/–Tnp2+/–* mice, only 11% of the protamine 2 is processed to the mature form, the rest remaining in the precursor or intermediate forms (Yelick *et al.*, 1987; Shirley *et al.*, 2004). Since the extra 43 amino acids of the protamine precursor lack cysteine, they cannot participate in cross-linking but instead may act as a steric hindrance to reduce intermolecular disulphide bond formation.

The failure to fully protect the DNA during epididymal passage may result from the presence of protamine 2 precursors, slightly higher levels of residual histones (estimated at 9% of the total basic proteins associated with the chromatin), less disulphide bond formation, and decreased compaction of the sperm nuclei (Zhao *et al.*, 2001, 2004a; Shirley *et al.*, 2004). This may leave the DNA more exposed and susceptible to degradation by endogenous nucleases (Szczygiel and Ward, 2002; Sotolongo *et al.*, 2003) or reactive oxygen species (Ollero *et al.*, 2001). Sperm produce reactive oxygen species (Baker and Aitken, 2004), which is enhanced by the presence of residual cytoplasm (Gil-Guzman *et al.*, 2001). Since sperm from the *Tnp* mutants contain more residual cytoplasm than do wild-type sperm (Shirley *et al.*, 2004; Zhao *et al.*, 2004a), they may produce more DNA-damaging reactive oxygen species than do wild-type sperm. Direct measurements of the damage to DNA of these mutant sperm must be the subject of future investigations.

We suggest that this loss of sperm nuclear integrity during epididymal passage can also occur in other mouse mutants involving sperm chromatin defects in which only testicular and/or cauda epididymal sperm were analysed (Cho *et al.*, 2003; Shirley *et al.*, 2004; Zhao *et al.*, 2004a) . Thus, the use of testicular or caput epididymal sperm extraction in conjunction with ICSI might yield better results than the use of cauda epididymal or ejaculated sperm. In one clinical study, the use of ICSI with testicular sperm has been demonstrated to improve the pregnancy rate in cases when there was a poor pregnancy rate with ejaculated sperm and a higher percentage of TUNEL staining with ejaculated than with testicular sperm (Greco *et al.*, 2005). The present results in mice suggest that caput epididymal sperm, being more mature than testicular sperm, have higher yields of offspring (50%, Table II) and may be even better than testicular sperm for successful ICSI. The possible extension of these preliminary studies in mice to the use of caput epididymal sperm in human ICSI should focus on cases in which the human sperm show defects similar to those of the *Tnp* mutant mice. Such defects include cytoplasmic retention, and chromatin defects such as the presence of high levels of unprocessed or incompletely processed protamine 2 precursors, incomplete nuclear condensation, the presence of DNA strand breaks (Evenson *et al.*, 1999), and incomplete disulphide cross-linking of protamine.

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