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Species Difference in the Expression of Fas and Fas Ligand in Mature Mammalian Spermatozoa

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ABSTRACT

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Although it has been proposed that the Fas and Fas ligand (FasL) may protect ejaculated spermatozoa against apoptosis induced by lipoperoxidative damage and against lymphocytes present in the female genital tract, studies reported conflicting results on the presence of Fas receptors in ejaculated human spermatozoa. Furthermore, the expression of Fas/FasL on mature spermatozoa has not been observed in several important mammals. Using seven species, we observed the possibility for species difference in Fas/FasL expression on mature spermatozoa by both immunofluorescence microscopy and western blot analysis. Whereas intensive signals of Fas immunolabelling were detected in sperm head and middle piece and weak signals observed in the tail in 86–100% of the mouse, rat, bull, ram, and buck spermatozoa, only weak signals were detected on the whole body of 27% boar spermatozoa and in the head of 21% human spermatozoa. The pattern of FasL localization was identical to that of Fas in spermatozoa from human, mouse, rat, ram, and buck, but boar and bull spermatozoa showed weak and intensive FasL signals, respectively, only in the head. Western blotting further confirmed the Fas and FasL expression in mouse, rat, bull, ram, and buck, but not in human and boar spermatozoa. Taken together, the results revealed a marked species difference in Fas/FasL expression and an extensive co-expression of Fas and FasL among mature mammalian spermatozoa, suggesting that whereas spermatozoa from most species may be protected by Fas/FasL, those from human and boar may not use the Fas system for protection.

Keywords: sperm; Fas antigen; FasL; apoptosis; mammals

The Fas/Fas ligand (FasL) system has been implicated in the apoptosis of germ cells. Within the mammalian testis, FasL is expressed by Sertoli cells (Suda et al. 1993) and induces apoptosis in Fas-bearing germ cells under different physiological and pathological conditions (Lee et al. 1997, 1999; Richburg et al. 2000). The presence of Fas on ejaculated sperm was first reported in human

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by Sakkas et al. (1999) who explained it by the abortive apoptosis theory. This theory suggests that in some cases of infertility, the normal apoptotic mechanisms have failed to eliminate the Fas positive sperm during spermatogenesis (Sakkas et al. 1999). Subsequently, Meggiolaro et al. (2006) observed the expression of Fas in bovine ejaculated spermatozoa. Chang et al. (2016) and Zhang et al. (2017) observed the expression of Fas in boar sperm. Studies also demonstrated the expression of FasL in mature spermatozoa of rodents (D'Alessio et al. 2001; Riccioli et al. 2003) and cattle (Porcelli et al. 2006).

As for the function of the Fas/FasL in mature spermatozoa, it has been proposed that whereas the Fas antigen in ejaculated spermatozoa may protect the spermatozoa against apoptosis induced by lipoperoxidative damage that can occur spontaneously in the male gamete at various stages of its lifetime, the FasL may provide immunoprotection for the spermatozoa in the female genital tract as the FasL may represent a self-defense mechanism against lymphocytes present in that tract (Riccioli et al. 2003). However, although the hypotheses are tempting, recent studies have reported different results. For example, Perticarari et al. (2008) detected no Fas receptors on ejaculated human spermatozoa. Furthermore, Fas and/or FasL expression in mature spermatozoa has not been reported in other important mammalian species such as goat and sheep. Thus, a special study including multiple species is necessary to observe whether there is a species difference in Fas/FasL expression on mature spermatozoa, to confirm its role in sperm protection.

MATERIAL AND METHODS

All chemicals and media used were purchased from Sigma Chemical Co. (USA), unless otherwise specified.

Semen collection and handling

Mouse and rat. Healthy and fertile male mice of Kunming breed (n = 6) and rats of Sprague-Dawley breed (n = 6) were used at the age of 10–12 weeks. Cauda epididymides and vasa deferentia were collected in 2 ml Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and were carefully dissected to remove blood vessels and fat. Then, several small cuts were made with iridectomy scissors, and sperm were allowed to swim out in 1 ml PBS for 10 min. The supernatant was then removed for evaluation of sperm motility and concentration. Motility was evaluated based on both the percentage of motile spermatozoa and the quality of their progressive movement under a microscope. Supernatants containing more than 60% of progressively moving spermatozoa and with a concentration of $2–4 \times 10^7$ total sperm/ml were used for experiments.

Human. The study was approved by the Institutional Review Board of the Central Hospital of Tai'an City, P.R. China. Semen samples were obtained from three healthy donors undergoing routine infertility investigations at the Assisted Reproduction Unit of the Central Hospital of Tai'an City. Semen samples were collected by masturbation into sterile containers after 3–4 days of sexual abstinence. After the semen were liquefied for 30 min at room temperature, routine analysis was performed according to World Health Organization (1999) standard guidelines. Only healthy and normozoospermic semen were used in our study. Semen samples that had a sperm concentration of more than 20×10^6 sperm/ml were diluted 1 : 2 with PBS and centrifuged for 5 min at 2000 rpm/min at room temperature, then the pellet was resuspended with the same solution as above.

Boar and bull. Semen of the Large White boar (n = 3) and Simmental bull (n = 3) was purchased from the Department of Veterinary Drugs, Jin-tai New Technology Research Institute in Tai'an City. Whole ejaculates were collected from 1.5–3-year-old boars, and the gel portion was removed using double gauze. After dilution with a commercial extender (CXM-ZHL dilution-powder dissolved in distilled water), the semen was transported to laboratory in a thermos flask at 25°C in 2 h. Semen samples with a sperm motility of over 70% were used in this study. Cryopreserved bull semen with confirmed fertility for artificial insemination was thawed at the Department of Veterinary Drugs by placing the straws in 37°C water. Then, the semen was transported to the lab in a thermos flask at 37°C in 40 min. The sperm motility of bull semen was over 30% when checked before use.

Ram and buck. Ram and buck semen was collected from fertile male bucks of Lubei White breed (n = 4) and rams of Dorper breed (n = 3) at the age of 1.5–4 years. Semen collection was conducted every 4 days from each animal using an artificial vagina. The ejaculate was collected into a pre-warmed tube and was diluted with pre-warmed

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PBS. Ejaculates with a volume of more than 0.7 ml, sperm concentration of at least 2 × 10^9 sperm/ml, and motility of at least 90% progressively motile spermatozoa were used for experiments.

Assessment of sperm motility by CASA. Sperm motility was determined by assessing at least 200 cells per semen sample in a minimum of four fields by a computer-assisted sperm analyzer (CASA) (SCA, Microptic Ltd., Spain) using a sperm class analyzer (SCA®, V.4.001). 10 µl of sperm suspension was placed on a pre-warmed microscope slide, overlaid with a 22 mm^2 cover slip and the slide was maintained at 37°C during analysis with a thermal plate. Sperm motility was measured by a camera BASLER A312fc (Basler AG, Germany) attached to a Nikon Eclipse E200 microscope (Nikon Corp., Japan) equipped with a 10× negative phase-contrast objective and a 10× projector ocular. According to the World Health Organization (1999) classification, spermatozoa of a + b motility type (type a – rapidly progressive spermatozoa, red; type b – slowly progressive spermatozoa, green) were used.

Immunofluorescence microscopy. All the procedures were conducted at room temperature unless otherwise specified. Indirect immunofluorescence staining for Fas and FasL was performed as described by Perticarari et al. (2008) with slight modifications. Sperm were washed 3 times by centrifugation (2000 rpm/min; 3 min in each time at room temperature) in PBS between treatments. (1) After the initial wash, sperm were fixed with 3.7% paraformaldehyde in PBS for at least 30 min; (2) blocked for 1 h in PBS containing 3% bovine serum albumin (BSA); (3) incubated at 4°C overnight with primary antibodies (rabbit anti-Fas or anti-FasL IgG, 1 : 50 dilution; Abcam, UK) in PBS containing 3% BSA; (4) incubated for 1 h with Cy3-conjugated goat-anti-rabbit IgG (1 : 200; Jackson ImmunoResearch Laboratories Inc., USA) in PBS with 3% BSA; (5) incubated for 10 min with 10 µg/ml Hoechst 33342 stain (Sigma-Aldrich, USA) in PBS. Negative control samples in which the primary antibody was omitted were also processed. The stained sperm were mounted on glass slides and observed with a Leica laser scanning confocal microscope TCS SP2 (Leica Microsystems GmbH, Germany) with a 63× plan apochromatic objective and the appropriate filter sets. Helium/neon (He/Ne; 543 nm) lasers were used to excite Cy3, fluorescence was detected with bandpass emission filter (560–605 nm), and the captured signals were recorded as red.

Western blot analysis. Sperm samples (the deposits after centrifugation) were placed in a 0.5 ml microfuge tube containing 100 µl lysis buffer and lysed on ice. Brain and prostate tissue were used for positive control in Fas and FasL experiment, respectively. The positive control tissue isolated was put in a glass homogenizer with cooled lysis buffer and homogenized immediately on ice. The homogenates were then centrifuged (16 000 *g*, 10 min, 4°C), and the supernatant was recovered for use. After the total protein concentration in tissue and sperm samples was determined by using the bicinchoninic acid (BCA) kit (P0010S; Beyotime Institute of Biotechnology, China) and adjusted to 5 µg/µl, 20 µl were placed in each microfuge tube and frozen at –80°C until use.

For protein extraction, 6.66 µl of 4× SDS-PAGE loading buffer (P1015; Solarbio, China) was added to each tube, and the tubes were heated to 100°C for 5 min. The samples were separated on a 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were washed twice in Tris-buffered saline with Tween 20 (TBST) and blocked with TBST containing 3% BSA at room temperature for 1–1.5 h, which aimed at blocking of nonspecific sites on the membrane. The membranes were then incubated at 4°C overnight with primary antibodies at a dilution of 1 : 1000 in 3% BSA-TBST. After being washed three times in TBST (5 min each), the membranes were incubated for 1 h at 37°C with second antibodies diluted according to specifications in 3% BSA-TBST. After three washings in TBST, the membranes were detected by a BCIP/NBT alkaline phosphatase colour development kit (Beyotime Institute of Biotechnology).

The lysis buffer used was composed of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, l% Triton X-100, 0.25% sodium deoxycholate, and 1 mM phenylmethylsulphonyl fluoride. The TBST consisted of 150 mM NaCl, 2 mM KCl, 2 mM Tris, and 0.05% Tween-20, with pH adjusted to 7.4. The primary antibodies included rabbit polyclonal anti-Fas antibody (ab82419; Abcam); rabbit polyclonal anti-Fas Ligand antibody (ab15285; Abcam), and mouse anti-β-tubulin antibody (05-661; Merck Millipore, USA). The secondary antibodies included goat anti-rabbit IgG AP conjugated (CW0111, CWBIO Co. Ltd., China) and goat anti-mouse IgG AP conjugated (CW0110, CWBIO Co. Ltd.). Mice brain extracts were used as a positive control

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for the Fas receptors according to specifications and Park et al. (1998). Rat prostate extracts were used as a positive control for the Fas ligand according to specifications and Nastiuk et al. (2003).

RESULTS

Fas expression in mature spermatozoa of different species. When observed under a laser confocal microscope after immunolabelling with anti-Fas antibodies, intensive signals were detected in the head and in the middle piece of spermatozoa and weak signals were observed in the sperm tail in mouse, rat, bull, ram, and buck (Figure 1A–G, Table 1). Weak signals were observed in the head, the middle piece, and the tail in boar spermatozoa. However, human spermatozoa showed weak signals only in the head. Our calculation of percentages of Fas-positive sperm showed that whereas only

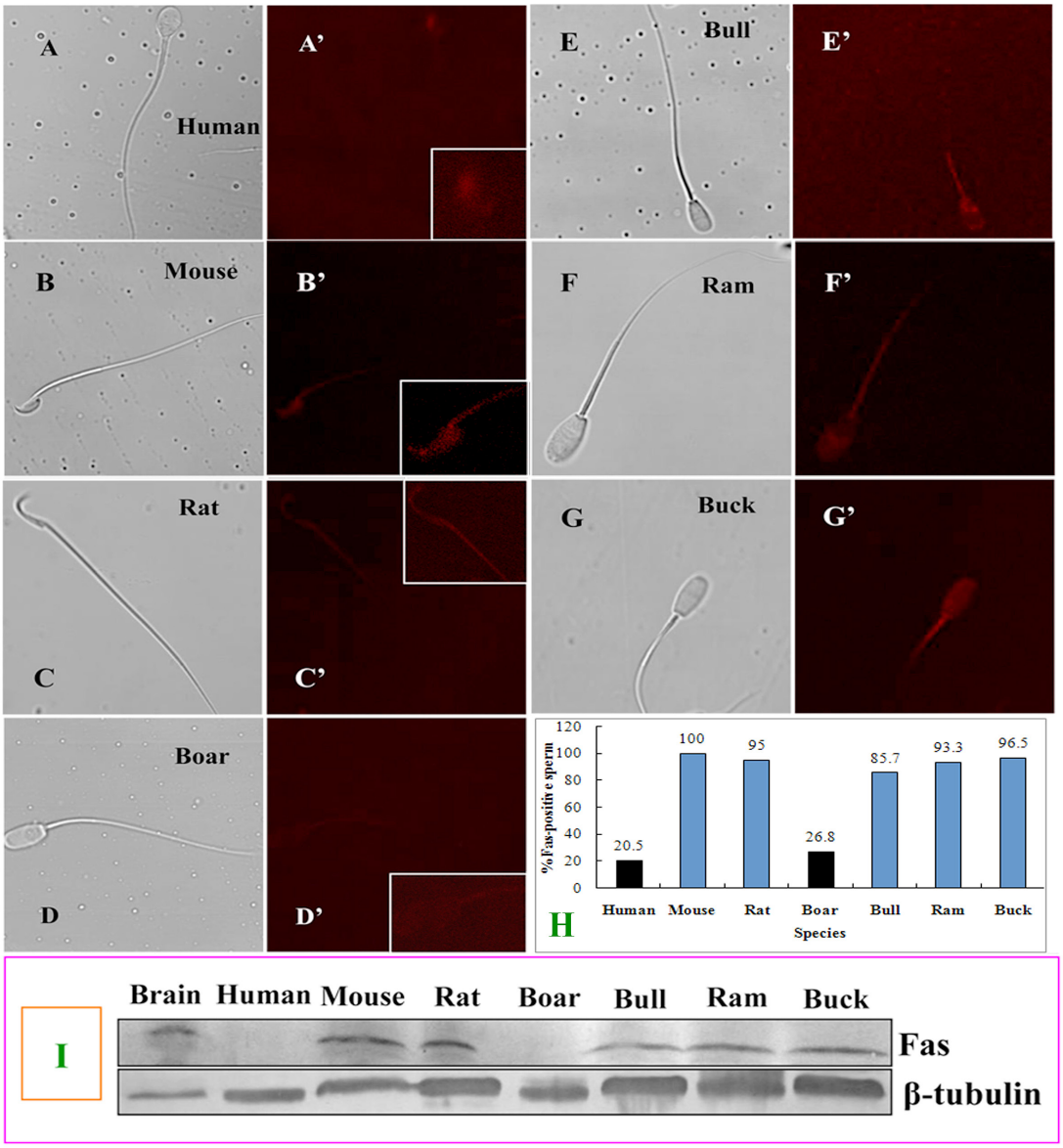

Figure 1. Expression of Fas receptors in spermatozoa of different species

micrographs (**A**–**G**) and (**A'**–**G'**) show spermatozoa observed under phase contrast and laser confocal microscope, respectively, following immunostaining with anti-Fas antibodies (original magnification ×630). (**H**) shows percentages of Fas-positive sperm in semen from different species. The Fas-positive percentages were calculated from about 40–60 sperm in each species. (**I**) shows results of western blotting

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about 21–27% of the human and boar spermatozoa showed weak signals, 86–100% of the spermatozoa from other species displayed intensive labelling (Figure 1H). Our analysis with western blotting further confirmed the Fas expression in mouse, rat, bull, ram, and buck spermatozoa, but not in sperm from human and boar (Figure 1I).

FasL expression in mature spermatozoa of different species. Among the 7 species observed, whereas spermatozoa from human, mouse, rat, ram, and buck showed a pattern of FasL localization identical to that of Fas, boar and bull spermatozoa showed weak and intensive FasL signals, respectively, only in the head (Figure 2A–G, Table 1). Our calculation of percentages of FasL-positive spermatozoa indicated that whereas only about 15–20% of the human and boar spermatozoa showed weak signals, 90–100% of the spermatozoa from other species displayed intensive labelling (Figure 2H). Similarly, our western blotting analysis confirmed

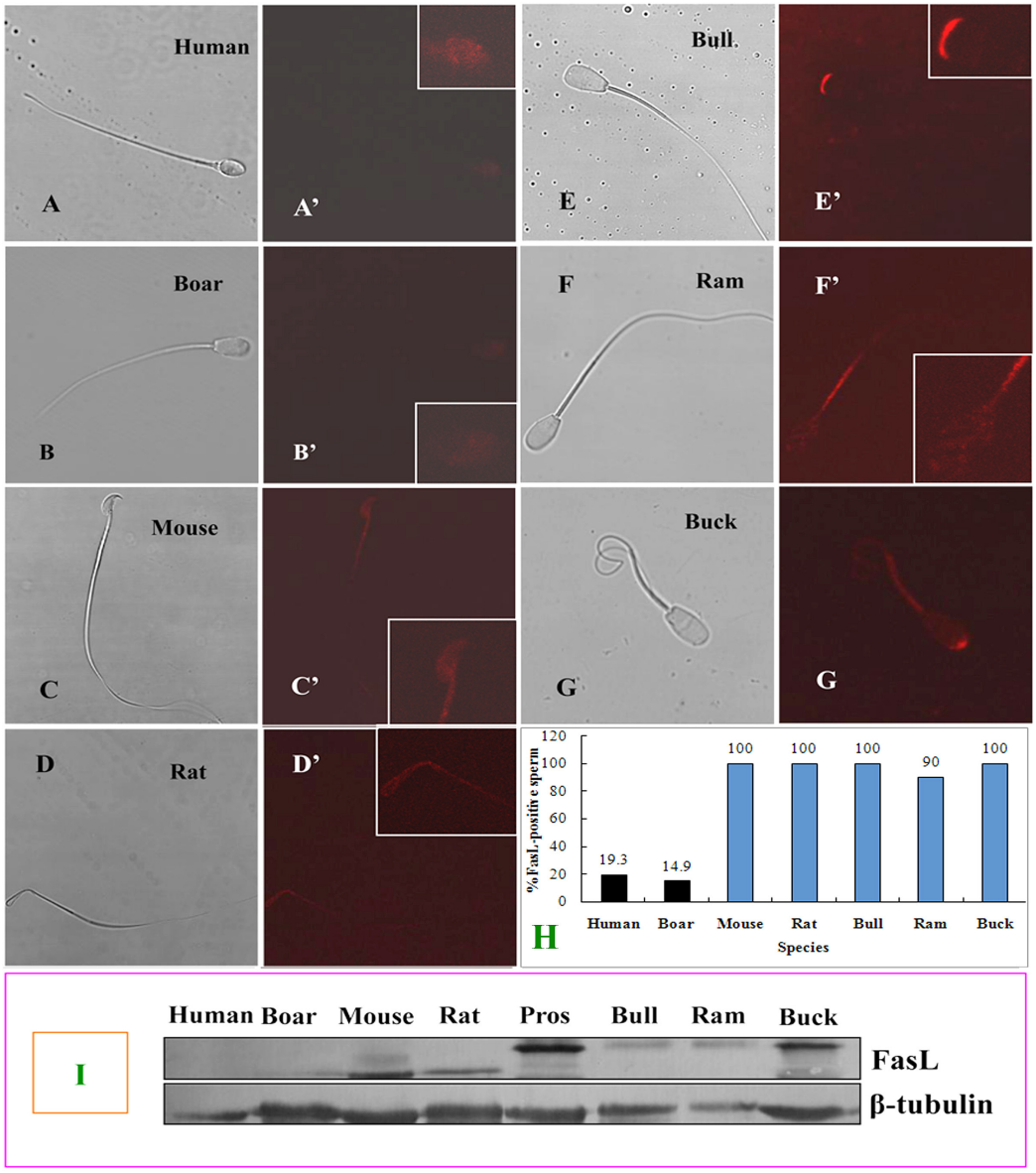

Figure 2. Expression of Fas ligands in spermatozoa of different species

micrographs (**A**–**G**) and (**A'**–**G'**) show spermatozoa observed under phase contrast and laser confocal microscope, respectively, following immunostaining with anti-FasL antibodies (original magnification ×630). (**H**) shows percentages of FasL-positive sperm in semen from different species. The FasL-positive percentages were calculated from about 40–60 sperm in each species. (**I**) shows results of western blotting

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Table 1. Localization of Fas and FasL immunolabelling in spermatozoa of different species

Species	Fas			FasL		
	head	mid-piece	tail	head	mid-piece	tail
Human	+			+		
Mouse	++	++	+	++	++	+
Rat	++	++	+	++	++	+
Boar	+	+	+	+		
Bull	++	++	+	++		
Ram	++	++	+	++	++	+
Buck	++	++	+	++	++	+

++ = intensive signals, + = weak signals

the FasL expression in mouse, rat, bull, ram, and buck spermatozoa, but not in spermatozoa from human and boar (Figure 2I).

DISCUSSION

Our immunofluorescence microscopy detected intensive signals of Fas and FasL immunolabelling in spermatozoa of mouse, rat, bovine, sheep, and goat, but only weak signals in spermatozoa of pig and human. Our western blot analysis confirmed the presence of Fas and FasL in mouse, rat, bull, ram, and buck spermatozoa, but not in human and boar spermatozoa. Among the 7 species observed in the present study, the presence of Fas receptors in mature spermatozoa has been reported in human (Sakkas et al. 1999; McVicar et al. 2004; Starace et al. 2009; Soleimani et al. 2010), bovine (Meggiolaro et al. 2006), sheep (Zan Bar et al. 2008), and mice (Starace et al. 2009), but it has not been observed in goat and rat, to our knowledge. Similarly, the presence of FasL in mature spermatozoa has been reported in human (Riccioli et al. 2005), mouse (D'Alessio et al. 2001; Riccioli et al. 2003), and cattle (Porcelli et al. 2006), but it has not been observed in pig, sheep, goat, and rat.

In the present study, intensive signals of Fas immunolabelling were observed in the sperm head and middle piece and weak signals were detected in the tail of the mouse, rat, bull, ram, and buck spermatozoa, but only weak signals were detected on the whole body of boar spermatozoa and in the head of human spermatozoa. The pattern of FasL localization was found similar to that of Fas in spermatozoa from human, mouse, rat, ram, and buck, but boar and bull spermatozoa showed weak and intensive FasL signals, respectively, only in the head. Reports on the localization of Fas and FasL in the mature spermatozoon are few and inconclusive. Strong Fas (Meggiolaro et al. 2006) and FasL (Porcelli et al. 2006) immunolabelling was observed in the head particularly in the periacrosomal domain of bovine spermatozoa. Although not mentioned by these authors, from the micrographs we could see the existence of signals in the middle piece and the tail. By means of an immunoelectron microscopy, Starace et al. (2009) observed that Fas localization in human and mouse sperm was closely associated with the perinucleus, mainly at the level of the inner acrosomal membrane, as well as in the inner compartment of mitochondria. The implications of the Fas and FasL localization in the mature spermatozoon are unclear but are worth exploring.

The current study revealed co-localization of Fas and FasL in most cases. In the adult mouse, several tissues clearly co-express both Fas and FasL genes (French et al. 1996). Because most tissues constitutively co-expressing Fas and FasL in the adult mouse are characterized by apoptotic cell turnover, and many of those expressing FasL are known to be immune privileged, French et al. (1996) thus deduced that the Fas system is implicated in both the regulation of physiological cell turnover and the protection of particular tissues against potential lymphocyte-mediated damage. Co-expression of Fas and FasL has also been reported in *ex vivo* pediatric brain tumor tissues, which were found to be resistant to apoptosis induction (Riffkin et al. 2001). According to Riffkin et al. (2001), tumor cells that co-express Fas and FasL may have acquired mechanisms of resistance to pathways of apoptosis not restricted to the Fas pathway to prevent Fas-induced cell death resulting from the contact with surrounding FasL-expressing malignant cells. Taken together, our results that Fas and FasL were co-localized in mature spermatozoa in multiple species strongly suggest that the Fas system may provide protection to spermatozoa against potential lymphocyte-mediated damage in the female genital tract.

The present results showed that whereas intensive signals of Fas and FasL immunolabelling were detected in 86–100% of the mouse, rat, bull, ram, and buck spermatozoa, only weak signals were detected in 15–27% of boar spermatozoa and in about 20% of human spermatozoa. Using a fluorescence-activated

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cell sorter, Sakkas et al. (1999) observed Fas-positivity in less than 10% of the human spermatozoa from normal males and in more than 10% of those from oligozoospermic samples. Similarly, by flow cytometry, Soleimani et al. (2010) confirmed the presence of Fas in ejaculated human sperm but concluded that the Fas expression was very low. Thus, it might be the low level of expression in few cells that had led to the conclusion that there was no detectable quantity of Fas on human ejaculated sperm (Perticarari et al. 2008). Our western blot analysis also showed no expression of Fas and FasL in human spermatozoa. In contrast, Meggiolaro et al. (2006) and Porcelli et al. (2006) demonstrated that a large percentage of normal ejaculated spermatozoa from fertile bulls were immunocytochemically positive for Fas and FasL.

CONCLUSION

In summary, by means of both immunofluorescence microscopy and western blot analysis, we have observed the expression of Fas/FasL in mature spermatozoa of seven mammalian species. Our immunofluorescence microscopy detected intensive signals of Fas immunolabelling in the head and middle piece, weak signals in the tail in 86–100% of the mouse, rat, bull, ram, and buck spermatozoa, but only weak signals on the whole body of 27% of boar spermatozoa and in the head of 21% of human spermatozoa. The pattern of FasL localization was identical to that of Fas in spermatozoa from human, mouse, rat, ram, and buck, but the boar and bull spermatozoa showed weak and intensive FasL signals, respectively, only in the head. Our western blotting further confirmed the presence of Fas and FasL in mouse, rat, bull, ram, and buck, but not in human and boar spermatozoa. Taken together, the results have revealed a marked species difference in the Fas/FasL expression and an extensive co-expression of Fas and FasL among mature mammalian spermatozoa, suggesting that whereas spermatozoa from most species may be protected by Fas/FasL, those from human and boar may not use the Fas system for protection. The data are important for further analysis of the role of the Fas/FasL system in human and animal reproduction.

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