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Short Communication

The Apoptotic Marker cPARP can be Detected in Ram Spermatozoa

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Contents

The presence of apoptotic features in spermatozoa has been related to lower quality and functional impairment. Members of the poly-ADP-ribose polymerases (PARP) familyare involved in both DNA repair and apoptosis, playing important roles in spermatogenesis. Poly-ADP-ribose polymerase can be cleaved by caspases, and the presence of its cleavage product (cPARP) in spermatozoa has been related to chromatin remodelling during spermatogenesis and to the activation of apoptotic pathways. There are no reports on immunodetection of cPARP in ram spermatozoa; thus, we have tested a commercially available antibody for this purpose. cPARP was microscopically detected in the acrosomal ridge of some spermatozoa (indirect immunofluorescence). A preliminary study was carried out by flow cytometry (direct immunofluorescence, FITC). Ram semen was extended in TALP and incubated for 4 h with apoptosis inducers staurosporine (10 μM) or betulinic acid (200 μM). Both inducers and incubation caused a significant increase in cPARP spermatozoa (0 h, control: 21.4±3.3%, inducers: 44.3±1.4%; 4 h, control: 44.3±2.4%, inducers: 53.3±1.4%). In a second experiment, we compared the sperm fractions after density gradient separation (pellet and interface). The pellet yielded a slightly lower proportion of cPARP spermatozoa (28.5±1.2% vs 36.2±2.0% in the interface; $p < 0.001$), and a 12-h incubation increased cPARP similarly in both fractions ($p < 0.001$). cPARP seems to be an early marker of apoptosis in ram semen, although its presence in untreated samples was weakly related to worse quality (pellet/interface). We suggest to study the relationship of PARP and cPARP levels with between-male differences on sperm fertility.

Introduction

Signalling pathways related to apoptosis or necrosis exist in spermatozoa, with a clear correlation between the detection of apoptotic markers and loss of resilience, increase in DNA damage and decreased fertility (Anzar et al. 2002; Martin et al. 2007; Martí et al. 2008; Martínez-Pastor et al. 2009; Espino et al. 2011; Gallardo Bolaños et al. 2012; Mendoza et al. 2013). Whereas apoptosis can be detected by supervising changes in the plasma membrane or mitochondria (Brugnon et al. 2009; Martínez-Pastor et al. 2010), the detection of activated members of the apoptotic/necrotic pathways could be used as sensitive endpoints of sperm quality. This might be convenient in sheep, because of the need to improve AI results (Anel et al. 2006) and the practical benefits of counting on tools to detect subtle sperm quality changes.

Several authors have focused on the poly-ADP-ribose polymerase (PARP) family. Some members of this family contribute to both DNA repair and cell death (Agarwal et al. 2009), and they also play important roles at different stages of spermatogenesis (Celik-Ozenci and Tasatargil 2013). Jha et al. (2009) confirmed the presence of at least three PARP members in human spermatozoa (1, 2 and 9) and suggested that PARP homologues could play a role by preventing sperm apoptosis.

Poly-ADP-ribose polymerase-1 can be cleaved by activated caspases. Mahfouz et al. (2009) used a fluorescence-tagged antibody and flow cytometry to detect the larger product of PARP-1 cleavage (termed cPARP) in human spermatozoa. Although no clear relation was found between cPARP levels and apoptosis-inducing or PARP-inhibition treatments, cPARP was correlated to the activation of caspase 3. This study is the first attempt to detect cPARP in non-human spermatozoa. We have tested whether a commercially available antibody could be used to detect cPARP in ram spermatozoa, and whether cPARP levels could be affected by apoptosis inducers or by density gradient separation.

Materials and Methods

Reagents were purchased from Sigma (St. Louis, MO, USA), except the PARP FITC Apoptosis kit (Invitrogen, Carlsbad, CA, USA), which contains the antibody PARP[214/215] CLEAVAGE Rabbit Polyclonal Antibody-FITC Conjugated, the IC Fix™ fixative solution and the IC Perm™ binding/permeabilizing solution. Flow cytometry equipment, software and consumables were purchased from Beckman Coulter (Brea, CA, USA).

Semen was obtained from three adult rams by artificial vagina, pooled and cryopreserved (Mata-Campuzano et al. 2015). Straws were thawed in a water bath at 37°C and washed with PBS. In a first experiment, the spermatozoa were resuspended in TALP medium (Domínguez-Rebolledo et al. 2009) at 50 ml and submitted to the apoptosis inducers staurosporine (10 μM) or betulinic acid (BTA) (200 μM). The tubes were

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incubated at 37°C and 5% CO_2 for 30 min and assessed for cPARP. In a second experiment, spermatozoa were selected in a 45/90 Percoll gradient (600 × *g*, 15 min) recovering the interface and the pellet, which were washed and resuspended at 50 ml with TALP. The tubes were incubated at 37°C and 5% CO_2 for 12 h and assessed for cPARP.

Flow cytometry detection of cPARP was carried out following the kit instructions. Briefly, washed spermatozoa were fixed with IC Fix in ice, double-washed with PBS and resuspended in IC Perm at 20 ml. A 50-µl aliquot was incubated with 10 µl of the antibody for 30 min at RT and in the dark. The samples were washed and resuspended in PBS with 5 mM of Hoechst 33342 (for discarding debris). Flow cytometry analyses were carried out with a CyAn ADP flow cytometer with semiconductor lasers emitting at 405 and 488 nm. Filters used for each fluorochrome were 450/50 (blue) for Hoechst 33342 and 530/40 (green) for FITC. The analysis of the flow cytometry data was carried out using WEASEL v. 3.1 (WEHI, Melbourne, Australia). Labelling was confirmed by fluorescence microscopy using a Nikon E400 equipped with a fluorescent lamp, appropriate filters and a Nikon DX-1200 camera. In both cases, specificity was confirmed using a negative control, in which the antibody was previously blocked with the PARP cleavage site peptide provided with the kit.

Western blot was carried out as described by Mendoza et al. (2013). Proteins were extracted, separated on 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. Membranes were blocked and incubated with the antibody (4°C overnight), followed by incubation with a secondary antibody IRDye 800-CW for 1 h at RT. The membranes were scanned using the Odissey CLx Imaging System (LI-COR Biosciences, Bonsai Adv. Tech., Madrid, Spain). HeLa cell extract was used as a reference.

Data were analysed in the R statistical environment using linear mixed-effects models. Results are shown as mean ± SEM of three replicates for each experiment.

Results

In the first experiment (Fig. 1a), both the application of apoptosis inducers and the incubation caused a significant increase in cPARP events evaluated by flow cytometry ($p < 0.001$). In the first analysis (0 h), 21.4±3.3% events were classified as cPARP. The application of BTA or STS approximately doubled the proportion of cPARP (44.3±1.4%). These two treatments showed a higher cPARP proportion at the three times, comparing to the control. However, the increase rate of the control was faster, with a lower difference at 4 h (44.3±2.4% vs 53.3±1.4%; $p = 0.003$).

In the second experiment (Fig. 1b), the proportion of cPARP events was also lower at 0 h. The pellet presented less cPARP (28.5±1.2%) than the interface (36.2±2.0%) with $p < 0.001$. The incubation

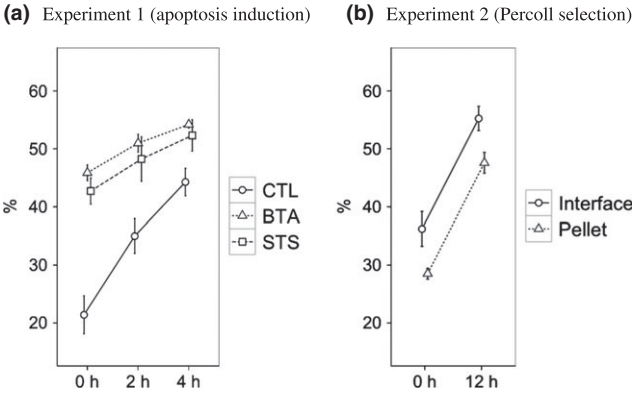

Fig. 1. (a) Summarizes the results of Experiment 1. Control (CTL) samples showed low cPARP presence at 0 h, but both staurosporine 10 µM (STS) and betulinic acid 200 µM (BTA) induced a large increase ($p < 0.001$). Incubation time increased cPARP, faster in the control samples (interaction $p = 0.003$). (b) The analysis of the two fractions obtained after density gradient selection (Percoll). Effect significance was $p < 0.008$ for the fraction and $p < 0.001$ for the incubation

significantly increased cPARP ($p < 0.001$), whereas the difference between the fractions remained similar (47.6±2.6% vs 55.3±6.1%, $p < 0.001$).

The cPARP labelling was microscopically detected at the acrosomal ridge of positive spermatozoa (Fig. 2a, b). Western blot analysis (Fig. 2c) evidenced a faint band by 90 kDa in control samples, coinciding with the HeLa lane. HeLa cells also yielded other two clear bands by 45 and 30 kDa, which were not present in control spermatozoa. BTA-treated samples did not present these bands, showing instead a clear band by 55 kDa.

Discussion

The presence of cleaved PARP-1 (cPARP) in mature and viable spermatozoa might be related to the activation of DNA repairing pathways in the different steps of spermatogenesis (Maymon et al. 2006). This might not be bad news by itself, but an excessive proportion of cPARP spermatozoa could be interpreted as a widespread occurrence of DNA damage during spermatogenesis. Moreover, one of the causes of PARP activation is the oxidative stress (Radovits et al. 2007), a major cause of spermatogenic failure and infertility (Celik-Ozenci and Tasatargil 2013).

The antibody used in this study, PARP[214/215] CLEAVAGE Rabbit Polyclonal Antibody-FITC, was produced against the N-terminus of the cleavage site of human PARP. We have successfully used it to detect cPARP in ovine spermatozoa. Indeed, we have also tested it against boar spermatozoa, with similar immunofluorescence results (unpublished). Fluorescence was detected in the apical part of the acrosomal region, which is compatible with the extrusion of the larger cPARP fragment from the nucleus to the cytoplasm (Soldani et al. 2001). Mahfouz et al. (2009) used the

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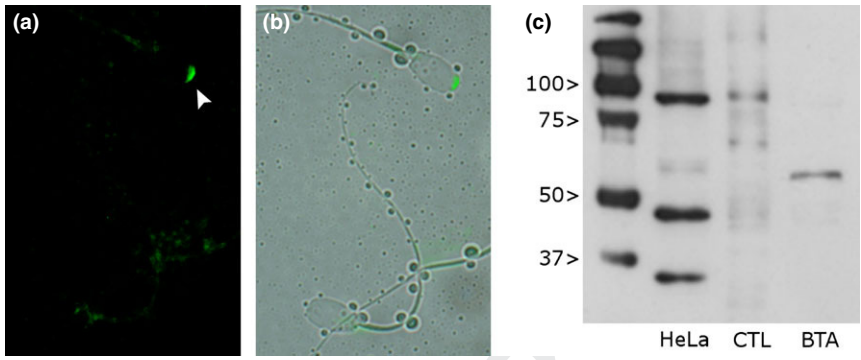

Fig. 2. (a) and (b) The localization of cPARP (FITC labelling) in ram spermatozoa (fluorescence and merged with bright field, 1000×). (b) A negative cell on the bottom. (c) A Western blot analysis using the same antibody in HeLa cells (positive), and spermatozoa, untreated (CTL) or treated with 200 μM betulinic acid (BTA)

same antibody in human sperm, but they did not report the location of the fluorescence labelling. However, Jha et al. (2009) indicated that PARP-1 showed localization near the acrosomal region. A hypothesis is that PARP-1 is sequestered within the apical part of the nucleus by the end of the spermatogenesis, thus resulting in cPARP being preferentially translocated to the cytoplasm of the apical part of the sperm head when PARP-1 is cleaved.

Our antibody does not detect uncleaved PARP-1, which might be found in all spermatozoa (Jha et al. 2009). Therefore, the quick increase in labelling that we have found in our experiments must be due to the activation of apoptotic/necrotic pathways, resulting in the cleavage of PARP-1 and in the translocation of cPARP to the cytoplasm. Ram spermatozoa present inactive and active caspases, and the apoptotic processes can progress quickly when challenged (Martí et al. 2008). In human semen (Mahfouz et al. 2009), cPARP labelling was similar after incubation with either staurosporine or hydrogen peroxide in both mature and immature fractions of ejaculated semen. Our results may reflect a different physiological response of ram spermatozoa to apoptosis induction and incubation. Indeed, they suggest that PARP-1 is one of the targets of apoptotic pathways in ram spermatozoa.

We also detected cPARP in unprocessed spermatozoa. This cPARP could have been created during chromatin remodelling during spermatogenesis (Maymon et al. 2006; Celik-Ozenci and Tasatargil 2013). Nevertheless, we cannot discard an effect of abortive apoptosis prior to carry out the experiments (spermatogenesis, maturation or sample preparation), resulting in PARP cleavage.

In our second experiment, we did not obtain a large difference between the high-quality (pellet) and the low-quality (interface) fractions, regarding cPARP presence. This supports that PARP cleavage could normally occur during spermatogenesis and maturation (Celik-Ozenci and Tasatargil 2013), resulting in both bad and good quality spermatozoa. This implies that the presence of cPARP would not necessarily indicate that a spermatozoon is of bad quality or apoptotic. Nevertheless, a threshold could be defined for a given sample, beyond which one could classify samples as low maturity or apoptotic, as we have demonstrated that adding apoptosis inducers cause a fast generation of cPARP.

In conclusion, cPARP is present in ovine spermatozoa, and its production (PARP-1 cleavage) can be induced by apoptosis inducers or incubation. However, its association with semen quality in untreated samples seems to be weak in our study. As PARP members are important in spermatogenesis and possibly related to sperm fertility (Agarwal et al. 2009), it might be worthy to study the variability of PARP and cPARP levels between rams of different fertility.

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Conflict of interest

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Author contributions

F. Martínez-Pastor and T. Muiño-Blanco designed the study and supervised the experiments. M. Mata-Campuzano, L. Ordás and F. Martínez-Pastor set up the experiments and carried out the flow cytometry analyses. A. Casao and J. A. Cebrián-Pérez carried out the immunofluorescence and Western blot analyses. T. Muiño-Blanco and F. Martínez-Pastor analyzed the data and wrote the manuscript. All authors revised the manuscript.

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