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SHORT NOTE

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Whole extra-charged DNA spermatozoa in the saltwater crocodile (*Crocodylus porosus*) ejaculate

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Saltwater crocodile spermatozoa examined in this study exhibited unusually high levels of extra DNA charged (2C and 4C) spermatozoa that can be explained by arrested cytokinesis at both meiotic divisions. From the ejaculates of five crocodiles, abnormal spermatozoa containing 2C (1–12%) and 4C DNA (0–2%) charges were discriminated after assessing 1500 spermatozoa using morphological characterisation under fluorescence microscopic and image analysis protocols. Individual crocodiles with a high proportion of 2C spermatozoa in their ejaculate, also showed a high level of DNA fragmentation in 1C sperm cells (8.7–12.7%) but there was no such relationship between DNA fragmentation and 2C sperm. The value of whole extra-charged sperm DNA in the ejaculate as a possible marker for fertility is discussed.

Key words: polyploid sperm, reptile, reproduction, saltwater crocodile, sperm DNA fragmentation

Sexual reproduction involving normal meiosis in diploid organisms is linked to the production of haploid spermatozoa in order to maintain the diploid state. Consequently, the production of diploid spermatozoa would only be expected to occur in polyploid organisms (Otto & Whinton, 2000; Devillard et al., 2002; Stenberg & Saura, 2013). If polyploid extra DNA charged spermatozoa are produced during spermatogenesis in diploid species, then this is typically considered to be a mutation, for example, linked to a cytokinesis arrest occurring when chromosome segregation is not fully accomplished. Mammalian species are typically free of polyploidy. However, extra DNA charged spermatozoa have been noted in humans, and its presence can be linked to low fertility (Wegner et al., 2001). In insects, the presence of extra chromosomes (B-chromosomes) can result in an increased frequency of extra DNA charged spermatozoa (diploid or tetraploid) as a consequence of B-chromosome delay during meiotic division (Gosálvez et al., 1985; Hewitt et al., 1987; Teruel et al., 2009). While the production of extra-charged DNA spermatozoa is detrimental for normal embryonic development, as triploid or pentaploid embryos are potentially produced, the presence of these cells in the ejaculate is problematic as they are likely to compete with normal spermatozoa for the fertilization of the oocyte and more generally, are considered evidence of meiotic failure.

The saltwater crocodile (*Crocodylus porosus*) is the largest of all living reptiles, with adult males between 4.3 and 5.2 m in length and weighing up to 400–1000 kg. Recent developments in assisted breeding technology have led to the establishment of successful techniques for semen collection and analysis in conjunction with studies of sperm physiology (Johnston et al., 2014a, b, c). When describing the seminal characteristics from a population of saltwater crocodiles in a crocodile farm, Johnston et al. (2014b) noted the regular presence of "macrocephalic" sperm heads in over 30% of the animals. The aim of the present investigation was to further analyse the nature of such spermatozoa with respect to their DNA charge (C) level, in order to examine the level of sperm DNA fragmentation co-incident in the ejaculate, and whether these extra DNA charged spermatozoa showed evidence of DNA damage. This information will be of interest when examining possible relationships between the prevalence of this germ line mutation and any putative loss of sperm characteristics that may induce a detrimental effect on fertility, since this mutation may be considered as linked to cryptic mechanisms connected with abnormal meiosis.

This project was approved by the University of Queensland Animal Ethics Committee (SAS/361/10) and conducted under the Queensland Government Scientific Purposes Permit, WISP09374911. Semen was collected by digital massage of the terminal ductus deferens from 5 sexually mature, clinically healthy saltwater crocodiles located at Koorana Crocodile Farm Coowonga, Australia (Latitude: -23.28333; Longitude: 150.71666) in December 2012; two of the five crocodiles were proven breeders (see Table 1). Crocodile capture, semen collection, and the semen cryopreservation procedure have previously been described by Johnston et al. (2014b, c).

Spermatozoa were prepared by mixing unfixed frozen-thawed cells into agarose and then into thin-layer microgels deposited on the surface of pretreated slides (López-Fernández et al., 2007). To assess sperm

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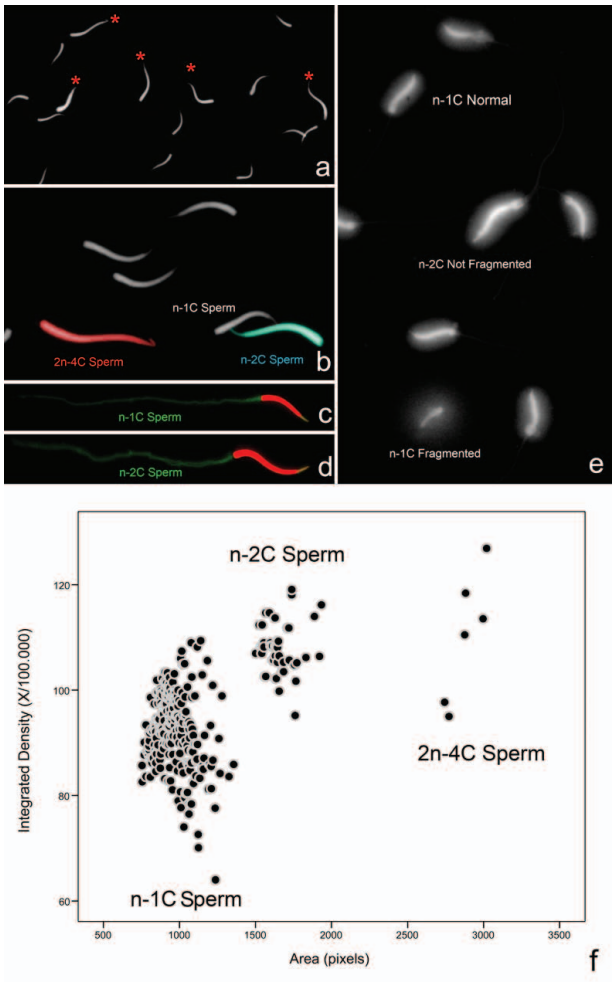

Fig. 1. Visualisation of whole extra-charged sperm DNA in *C. porosus*. (a) Normal and extra DNA charged (red asterisk) crocodile spermatozoa. (b) Range of sperm nuclei showing n-1C (grey level), n-2C sperm nuclei (blue) and 2n-4C (red) spermatozoa. (c) Normal n-1C sperm and (d) n-2C sperm showing equivalent morphological traits presenting different levels of DNA charge – note presence of double flagellum. (e) Crocodile spermatozoa following sperm chromatin dispersion test; note n-2C sperm with no fragmented DNA while some n-1C sperm nuclei exhibit fragmented DNA. (f) Graphic representation of sperm distribution after plotting integrated density and area. Three distinct sub-populations of spermatozoa corresponding to n-1C, n-2C and 2n-4C are delineated.

DNA damage, we used the Halomax® kit (Halosperm SL, Madrid, Spain) to assess DNA damage as described in Johnston et al. (2015). For image analysis, slides were directly stained using 10% Gel Red ™ (Biotium, Hayward, CA, USA) and mounted in VectaShield® mounting medium (Vector Laboratories, Burlingame, California, USA). Some slides were counterstained with mercuri-dibrom-fluorescein (Sigma-Aldrich Química SL, Madrid, Spain) for the staining of proteins primarily associated with the flagellum (Fig. 1). DNA fragmentation was also assessed using the microgels. Following a 5 min protein depletion treatment with the lysis solution (provided in the kit), spermatozoa were dehydrated and stained using the same fluorochrome as above.

Spermatozoa were examined using a Leica DMRB epifluorescence microscope (Leica Microsystems, Barcelona, Spain) equipped with single-band fluorescence block filters (Cy3 equivalent and FITC equivalent, Semrock, Rochester, New York, USA). A total of 1500 spermatozoa were analysed per sample to identify the percentage of spermatozoa with extra DNA charge. To assess the relative amount of DNA per sperm nucleus, a series of black and white images were captured using 12-bit format with a CCD (Leica DFC350 FX, Leica Microsystems) and stored as black and white tif files. All images were captured using a 40x plan-acromatic objective with a fixed exposure of 0.3 seconds onto the CCD after a previous pre-exposure to excitation light of 5 seconds to homogenise fluorochrome fading among different captures. Black and white images were pseudo-coloured using Adobe Photoshop CS3 (Adobe Systems Incorporated; CA, USA). Sperm DNA charge (C), associated with the level of ploidy (n), was assessed using image analysis protocols (ImageJ: <http://rsbweb.nih.gov/ij/download.html>, USA). After background subtraction, two different parameters (surface: number on pixel per region of interest – ROI, integrated density: product of area and mean gray value in the ROI) were used in the analysis, using a fixed threshold protocol for ROI selection.

Standard seminal characteristics of sperm concentration, motility and viability for each individual crocodile are shown in Table 1. The crocodile sperm nucleus has an elongated filiform appearance so that extra DNA charged spermatozoa were easily distinguished (Fig. 1a). All three sperm nuclear morphotypes, (1) normal n-1C (grey levels), (2) mutant n-2C sperm (blue), and (3) mutant 2n-4C sperm (red), are illustrated in Fig. 1b. When counter-stained using a protein directed fluorochrome, extra DNA charged spermatozoa usually possessed two flagella (Fig. 1d and compare with 1c); these spermatozoa showed normal head morphology except for the relative larger size of the nucleus. The distal extremity of the sperm nucleus was extended as a nuclear rostrum, which produced a characteristic orange fluorescence when stained with dual fluorochromes for DNA (red) and for proteins (green, Fig. 1c and 1d). The nuclear rostrum was similarly visualised in both types of spermatozoa (Fig. 1c and 1d).

Image analysis provided clear evidence of whole undivided genomes in the extra DNA charged spermatozoa. There was a strong correlation between the integrated density detected in each spermatozoa and the area observed in terms of pixels (R=0.332; p<0.001). When these data were plotted, it was possible to detect 3 sub-populations corresponding to the predefined sperm morphotypes n-1C, n-2C and 2n-4C (Fig. 1f). All three populations were easily differentiated based upon pixel area (p<0.001); however, based on integrated density n-C1 could be distinguished from n-C2 (p<0.001) but not n-2C from 2n-4C (p=0.771).

The incidence of extra DNA charged sperm in the ejaculate of 5 crocodiles is reported in Table 1, along with their corresponding proportion of DNA fragmentation. In 3 out of the 5 crocodiles, sperm DNA damage in n-2C

Table 1. Individual crocodile data and general seminal characteristics and incidence of sperm DNA charge (n-2C and 2n-4C) and sperm DNA fragmentation (SDF) in ejaculates of 5 crocodiles. SDF: sperm DNA fragmentation (data are expressed as percentages). For extra sperm DNA charge (C values) see text. *Proven breeders.

Crocodile	Snout/Tail Length (cm)	Volume (mL)	Concentration ($X10^6$ ml^{-1})	Progressive Motility (%)	Viability (%)
819*	285	0.7	3700	65	86
A95*	310	0.4	3600	80	85
Adam	350	1.5	2400	63	83
37A	310	2.5	3000	80	80
5AE	264	1.5	2800	54	85
	n-1C	n-2C	2n-4C	SDF(n-1C)	SDF(n-2C)
819*	99	1	0	1	0
A95*	98	2	0	0.33	0
Adam	89	9	2	8.7	1
37A	88	11	1	7.3	1
5AE	86	12	2	12.7	1

spermatozoa was approximately 10% (Table 1), while low frequencies of 2n-4C espèrmatozoa were observed. Spermatozoa with fragmented DNA displayed a large halo of spotty and dispersed chromatin (Fig. 1e). Sperm nuclei with non-fragmented DNA exhibited the typical baseline compact halo of relaxed chromatin following application of the sperm chromatin dispersion test. There was no correlation between the presence of extra-charged sperm and the incidence of DNA damage (Table 1). Moreover, n-2C and 2n-4C spermatozoa appeared to be less affected by DNA damage than n-1C spermatozoa (Fig. 1e).

Spermiogenesis can occur despite abnormalities of meiotic division (Perrin et al., 2008). As observed in our study, this typically gives rise to mature sperm that have a similar morphology to normal spermatozoa but with an increase in nuclear size. We suggest that the presence of whole extra-charged spermatozoa is likely to be associated with a failure of disjunction in either telophase 1 or telophase 2 for non-disjunction of the whole chromosome set during the formation of a germ cell (Nicholas, 2003). As crocodiles do not possess sex chromosomes (Bull, 1983; Ferguson-Smith, 2007), it is difficult to ascertain whether anaphase 1 or 2 has been involved in cytokinesis failure to produce the extra-charged nuclei (n-2C). While this phenomenon could be investigated using specific DNA probes for sex chromosomes (Devillard et al., 2002), the presence of 2n-4C spermatozoa in the crocodile ejaculate is strong evidence that both meiotic divisions appear to have failed.

Crocodiles that showed an elevated level of spermatozoa with extra DNA charge also showed a higher level of DNA damage in their n-1C spermatozoa. While sperm DNA damage has previously been correlated with fertility (Erenpreiss et al., 2006; Zhao et al., 2014), the incidence of whole extra-charged DNA spermatozoa is not usually considered to be correlated with reproductive success (Wegner et al., 2001). However, given the prevalence of this mutation in crocodiles (Johnston et al. 2014b), a high proportion of sperm with whole extra-charge could be characteristic of infertility. Further tests based on a larger sample size should reveal whether this parameter is an informative marker for reproductive success.

In cases where sperm only present partial DNA charges (aneuploidies), the incidence of sperm DNA damage affecting carrying individuals is typically higher (Wyrobek et al, 2006; Vendrell et al., 2014). Furthermore, the presence of extra chromosomes may also fourfold increase the level of DNA damage in the spermatozoa carrying the mutation (Muriel et al., 2007; Brahem et al., 2012). However, this does not appear to be the case in crocodiles, as the incidence of whole extra-charge spermatozoa (n-2C/2n-4C) was not associated with a high incidence of DNA damage in the same spermatozoa. On the contrary, while individual crocodiles with a high level of extra DNA charged spermatozoa, also exhibited high levels of damaged DNA, this phenomenon was predominantly associated with normal n-1C spermatozoa.

In conclusion, the presence of extra whole-DNA charged spermatozoa in the crocodile ejaculate is linked to a failure of normal spermatogenesis. The incidence and heritability of this phenomenon will need to be closely monitored when selecting crocodiles for breeding programs. Given that semen collection in the saltwater crocodile is now readily feasible, it should be possible to track its potential effect on fertility.

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