DNA fragmentation of epididymal freeze-dried ram spermatozoa impairs embryo development

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Introduction: Sperm freeze-drying is a revolutionary technique that resolves many of the drawback of long-term storage under liquid nitrogen. The first significant result of this method was provided by Wakayama and Yanagimachi in 1998, demonstrating for the first time the birth of healthy offspring from epididymal freeze-dried (mouse) spermatozoa. Besides mouse and rat models, which are the first small mammals born from epididymal lyophilized sperm by Intracytoplasmic Sperm Injection (ICSI), most studies in this field have used ejaculated sperm.

Aim: In this work, aiming to repeat the strong result of Wakayama and Yanagimachi, we tried to apply this technique to epididymal spermatozoa from a large mammal (ram). Moreover, we checked the correlation between freeze-dried spermatozoa DNA integrity and embryo development.

Material and Methods : To do this, epididymal sperm from four rams was lyophilized in a trehalose, glucose, KCl, HEPES, Trolox media. To evaluate DNA damage and fragmentation at rehydration, part of the sperm was processed for Sperm Chromatin Dispersion test (SCD) and Two-Tailed Comet Assay and the rest was used for ICSI.

Result 1: DNA fragmentation analysis allowed comparing the proportion of intact DNA and the type of DNA breaks, categorized into Single Strand Breaks (SSBs) and Double Strand Breaks (DSBs) in each ram. Ram #2 had considerably higher proportion of sperm with intact DNA compared to the other three rams and Ram #3 had the highest rate of DSBs and the lowest rate of sperm with intact DNA (Table 1).

	Normal DNA %	Fragmented DNA %	
		SSBs	DSBs
Ram 1	3.8	95.9	0.33
Ram 2	28	70	2
Ram 3	2.8	92.6	4.6
Ram 4	5	93	2

Table 1. Normal and fragmented DNA level in Ram #1, #2, #3 and #4

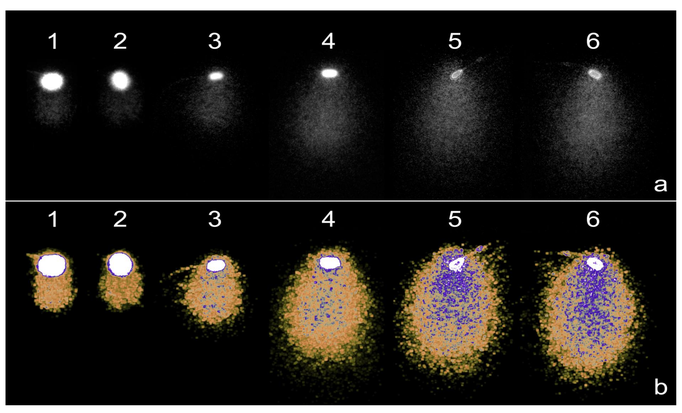

Fig 1. Structural Coment. 1) Fresh-frozen sperm. 2) Lyophylozed sperm. 3-6) Different degrees of single strand breaks in lyophylized sperm. b) Electroneic filtering in b is to show density of single strand breaks as the comet is larger.

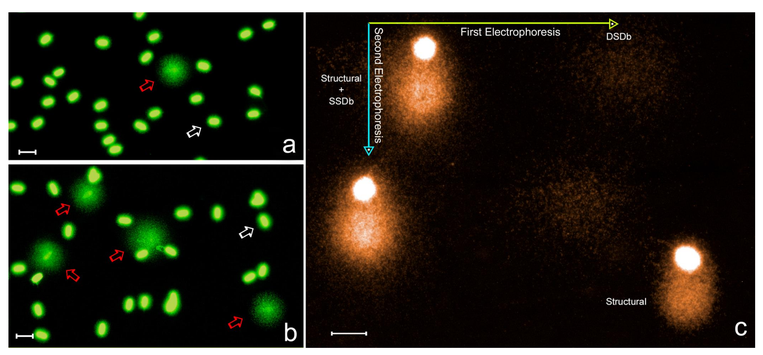

Fig 2. SCD. (a, b) and a 2-Tails comet assay (c). Low (a) and high (b) levels of sperm DNA fragmentation in two different rams. Red arrow: nucleoid with fragmented DNA). White arrow: nucleoid containing a fragmented DNA molecule. Selected nucleoid to show normal DNA molecule (structural) and mapping of the different SSBs or double DSBs

Result 2: Table 2 shows the outcome of embryo development. The surprising result was that only the semen from the Rams #2 and #4 were able to direct embryonic development to the expanded blastocyst stage on day 7 of embryo culture (Fig 3).

Groups	N. oocytes	Fragmented (%)	Not-Divided (%)	2-Cells (%)	Expanded Blastocyst (%)
Ram 1	72	6 (8.3)[a]	57 (79.2)[b]	9 (12.5)	0 (0)
Ram 2	83	22 (26.5)	38 (45.8)	23 (27.7)	5 (6)[f]
Ram 3	58	12 (20.7)	29 (50)	17 (29.3)[d]	0 (0)
Ram 4	64	16 (25)	36 (56.3)	12 (18.8)	4 (6.25)[g]
IVA	210	52 (24.8)	44 (21)[c]	114 (54.3)[e]	42 (20) [h]

Table 2. a) Ram #1 vs. Ram #2, Ram #4 and IVA, mean value P<0.05. b) Ram #1 vs. Ram #2, Ram #4 and IVA, mean value P<0.05. c) IVA vs. Ram #2, Ram #3 and Ram #4, mean value P<0.05. d) Ram #3 vs. Ram #1 and Ram #4, mean value P<0.05. e) IVA vs. Ram #1, Ram #2, Ram #3 and Ram #4, mean value P<0.05. f) Ram #2 vs. Ram #1 and Ram #3, mean value P<0.05. g) Ram #4 vs. Ram #1 and Ram #3, mean value P<0.05. h) IVA vs. Ram #1, Ram #2, Ram #3 and Ram #4, mean value P<0.05.

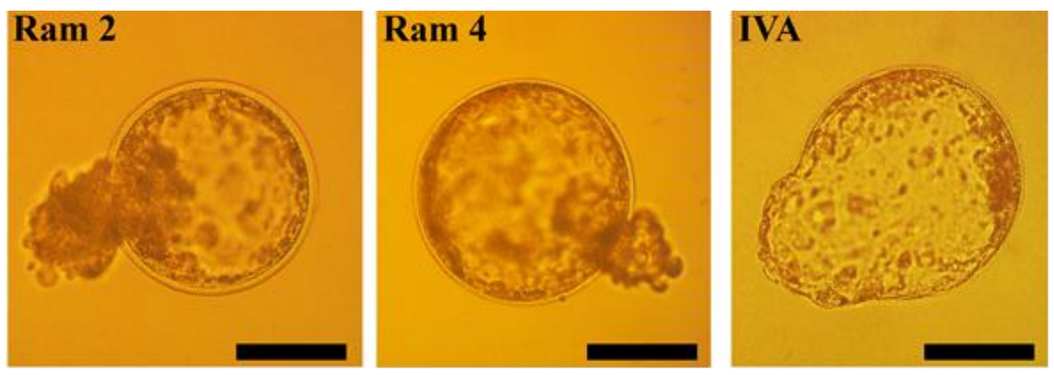

Fig 3. Pictures of expanded blastocyst of Ram 2 (left), Ram 4 (middle) and IVA (right). Scale bars represent 100 µm.

Conclusion:

Here we have demonstrated for the first time the ability of epididymal freeze-dried ram spermatozoa to direct embryonic development to the blastocyst stage. Moreover, the implication of sperm DNA damage in embryonic development should depend on the balance between the extent of sperm DNA fragmentation, the type of fragmentation (SSBs or DSBs), and the oocyte's repair capacity. Rams 2 and 4 were the only rams that produced blastocyst probably because they had considerably more sperm with normal DNA (Ram #2) or with SSBs not located at irreparable sites (Ram #4) and so it is important to select the spermatozoa with the best DNA quality to obtain healthy embryos from lyophilized spermatozoa through ICSI. It is evident that the extent and type of SSBs is as important as the lever of DSBs.

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