Reprod Nutr Dev (1990) 30, 59-64
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Original article

Effects of freezing-thawing on the spermatozoon nucleus: a comparative chromatin cytophotometric study in the porcine and human species

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(Received 21 October 1988; accepted 23 October 1989)

Summary — Freezing-thawing effects on the nuclei of porcine and human spermatozoa were studied by determining native DNA percentage from fluorescence after acridine orange (AO) staining and by analyzing chromatin structure by a quantitative microspectrophotometric study of Feulgen-DNA complexes before and after freezing. The study of boar spermatozoa revealed no alteration in native DNA percentage after freezing. However, native DNA percentage decreased significantly in human spermatozoa. Feulgen-DNA content and sperm nuclear surface area decreased in both species after freezing. These results prompted us to hypothesize an overcondensation of sperm chromatin after freezing-thawing. This overcondensation may be related to the lower conception rates obtained with human and porcine semen after cryostorage *via* defective decondensation.

freezing-thawing / spermatozoon / chromatin / acridine orange / Feulgen-DNA / boar / man

Résumé — Effet de la congélation sur le noyau des spermatozoïdes : Etude cytophotométrique comparative entre le verrat et l'homme. *L'effet de la congélation sur le noyau des spermatozoïdes a été étudié en déterminant le taux d'ADN natif, en microscopie à fluorescence, par le marquage à l'Acridine Orange et en analysant la structure de la chromatine par l'étude microspectrophotométrique quantitative de l'ADN-Feulgen, avant et après congélation de spermatozoïdes porcins et humains. L'étude réalisée chez le verrat ne montre pas d'altération du taux d'ADN natif après congélation. En revanche ce taux est significativement diminué dans les mêmes conditions chez l'homme. En outre pour ces 2 espèces, nous avons observé une réduction significative des complexes ADN-Feulgen après congélation, qui s'accompagne également d'une diminution de la surface des noyaux des spermatozoïdes. Ces résultats évoquent une compaction ou «surcondensation» des noyaux au-delà de l'état de condensation acquis en fin de maturation épididymaire. Le taux d'ADN natif semble, chez l'homme, apporter une information complémentaire sur l'appréciation du pouvoir fécondant du sperme avant et après congélation. Chez le verrat comme chez l'homme, l'état de «surcondensation» nucléaire provoqué par la congélation pourrait, en retardant la décondensation du pronucleus paternel, expliquer en partie la diminution du taux de conception lors d'insémination avec sperme congelé.*

congélation / spermatozoïde / chromatine / acridine orange / ADN-Feulgen / verrat / homme

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INTRODUCTION

Several studies have shown that the fertilizing ability of human and porcine spermatozoa is reduced by cryostorage (boar: Paquignon *et al*, 1980; Johnson, 1985; ma: Schwartz and Heuchel, 1982). This decrease is possibly due to changes in the structure of the acrosomal, plasma membranes and/or flagellum (boar: Paquignon, 1984; Johnson, 1985; Courtens and Paquignon, 1985; man: Pedersen and Lebech, 1971; Escalier and Bisson, 1980; Mahadevan and Trounson, 1984; Oettle and Soley, 1986). However, the possibility that the reduced fertilizing ability of cryostored sperm might also be associated with nuclear abnormalities has not been investigated. The purpose of the present study was to compare the effects of freezing-thawing on porcine and human chromatin of spermatozoa nuclei using acridine orange (AO) staining and quantitative Feulgen-DNA measurement. Results concerning the nuclear state of human spermatozoa have been partly reported elsewhere (Royère *et al*, 1988).

MATERIALS AND METHODS

Ejaculates were collected from 8 adult Large-White boars and from 10 men participating in our IVF programme. Half of each ejaculate was processed as described by Paquignon *et al* (1980) and by Behrman and Ackerman (1969) for boar and man respectively. Fresh and frozen semen from each ejaculate was washed twice by centrifugation with PBS, pH 7.3 (300 g, 10 min) and adjusted to 50 x 10^6 spermatozoa/ml in PBS. Spermatozoa were smeared on slides and fixed in methanol/glacial acetic acid (3/1, v/v). The AO staining was carried out according to Tejada *et al* (1984). Slides were observed on an epifluorescence microscope using a 490-nm excitation filter and a 530-nm barrier filter. 300–400 spermatozoa were observed on each slide and divided into green and orange/red spermatozoon heads.

Feulgen-DNA content was determined after Schiff-Feulgen staining. The Feulgen-DNA content and the surface area of the spermatozoon head were measured simultaneously, using a scanning method (Esnault and Nicolle, 1976) with an automatic microspectrophotometer (UMSP1 Zeiss). To avoid errors due to experimental technique, fresh and frozen sperm were treated together during the various stages of Feulgen staining. Feulgen-DNA and nuclear surface were measured in 4 of the 8 boars and in all of the men.

AO staining was expressed as the ratio of the spermatozoon heads, which stained green (native DNA: double-helix), to the total count. Feulgen-stained DNA content was expressed in arbitrary units (AU) as the mean extinction (± SEM) for 30 and 15 morphologically normal spermatozoa for each sample of boar and human semen, respectively. Freezing-thawing effects on native DNA percentage and Feulgen-DNA content were analyzed using the Wilcoxon paired signed-rank test (Siegel, 1956).

RESULTS

Freezing-thawing effects on the native DNA percentage of spermatozoa

The native DNA percentage of fresh and frozen ejaculated boar spermatozoa was high (> 90%). It showed relatively low inter-individual variability with 89–96% of the nuclei showing green fluorescence, indicative of the presence of the DNA in its native state in most sperm cells. After freezing, these values did not change significantly (fig 1a). Thus in the boar, freezing-thawing did not affect the spermatozoon DNA state.

In humans, as opposed to boar spermatozoa, the mean native DNA percentage of fresh ejaculated spermatozoa was lower (44.65%) and varied considerably between individuals (26–64%). Moreover, a significant decrease (≈ 10%) in mean na-

tive DNA percentage was observed after freezing-thawing ($P < 0.02$; fig 1b).

In contrast with the high human interindividual variability, we observed no significant intraindividual variation between 2 ejaculates of the same subjects in boars as in men : mean difference 1.5 ($P = 0.27$), and 0.81 ($P = 0.49$), respectively.

Freezing-thawing effects on the structure of chromatin

Four boars were selected for the Feulgen-DNA study, as their sperm presented the greater decrease in native DNA percentage after freezing-thawing. The Feulgen-DNA content of boar spermatozoa, except in 1 animal, decreased after freezing-thawing (– 23, – 7.2, + 1.3 and – 14.7%, respectively; fig 2a). Under the same conditions, a reduction was observed in the nuclear surface area of the sperm heads (– 4, – 1, – 1 and – 5%, respectively; fig 2c).

The effects of freezing on the chromatin of human spermatozoa were similar to those observed in the boars. Frozen spermatozoa showed a significant reduction (10%) of mean Feulgen-DNA content ($P < 0.001$; fig 2b) as the surface of the spermatozoa nuclei decreased (6%, $P < 0.01$; fig 2d).

No relationship was observed between Feulgen-DNA content and nuclear surface, nor any relationship between these 2 parameters and human *in vitro* fertilization rate.

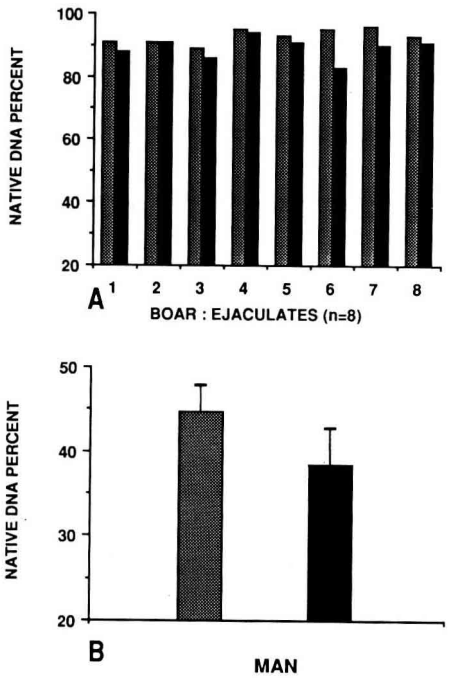

Fig 1. Freezing-thawing effects on native DNA percent in spermatozoa from 8 boars and 10 men. Results are represented before (▨) and after (■) freezing-thawing for boars (A) as individual values (400 spermatozoa/ejaculate), for men (B) as mean (± SEM). (1 ejaculate/subject).

DISCUSSION

Freezing-thawing effects on the nuclei of boar and human spermatozoa were studied using acridine orange staining and microspectrophotometric Feulgen-DNA measurements. Acridine orange, a fluorochrome stain which binds to double-stranded DNA and shows green fluorescence, also binds to single-stranded DNA causing a red fluorescence (Stockert and Lisanti, 1972). Whatever the population of spermatozoa (homogenous in the boar or heterogenous in man), the evaluation of abnormal chromatin by AO staining revealed the existence of spontaneous DNA denaturation in ejaculated semen. This denaturation was greater in man than in the boar. Moreover, the single-stranded DNA percentage increased in human spermatozoa after freezing and thawing. In our study, the percentage of spontaneously

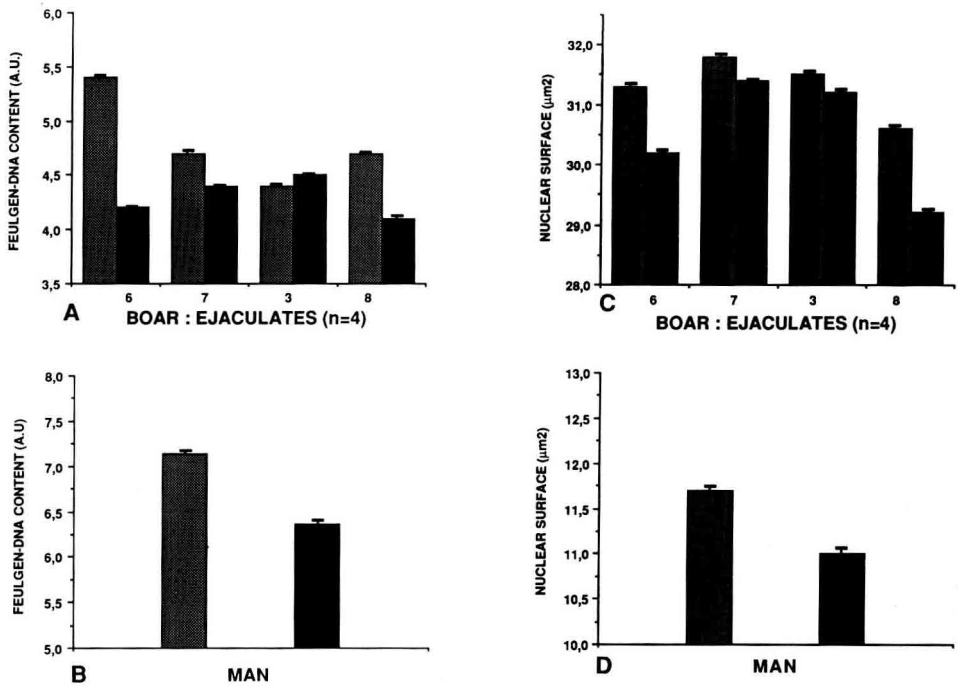

Fig 2. Freezing-thawing effects on spermatozoa Feulgen-DNA content (A = boars; B = men) and mean nuclear surface (C = boars; D = men). Values are represented before (▨) and after (■) freezing-thawing as individual values for boars, as mean (± SEM) for men. Individual results were expressed as a mean (± SEM) between 30 spermatozoa (boars). Same No for ejaculates as in fig 1.

denatured DNA observed in the boar was lower than in the bull and the mouse (Evenson *et al,* 1980), contrasting with the higher denaturation rates for man (Tejada *et al,* 1984; Royère *et al,* 1988). It is important to notice that in our study, we observed in the 2 species a low intraindividual variability, in spite of the great interindividual variability found only in human spermatozoa. Such results allow us to consider native DNA as a reliable parameter for each subject. Similarly, AO staining revealed no freezing effects on native DNA percentage of boar spermatozoon nuclei, contrasting with the decrease of native DNA percentage after freezing in man, indicating that, in this species, staining with AO should provide another parameter for estimating the quality of frozen spermatozoa. Tejada *et al* (1984) and Botes (1987) have already suggested native DNA percentage as an additional parameter of fertilizing ability of human spermatozoa. Furthermore, it would be of interest to determine spermatozoon nucleus quality in infertile subjects with apparently normal sperm.

In this study, the decrease of Feulgen-DNA content and nuclear surface area observed in boar as in man after freezing-thawing, argues for modified relationships

between DNA and nucleoproteins. Combined, these results suggest that spermatozoon chromatin might reach a degree of condensation greater than that which normally occurs at the end of epididymal maturation. Such chromatin alterations were observed during spermateliosis and epididymal maturation in the bull (Gledhill, 1971) and in the ram (Esnault and Nicolle, 1976) as well as *in vitro* under the effects of uterine secretions on ram corpus epididymal spermatozoa (Nicolle *et al,* 1985). Similar effects of freezing and thawing on spermatozoon chromatin have been described qualitatively in boar (Courtens and Paquignon, 1985) and quantitatively in man (Royère *et al,* 1988).

Variation in the stability of spermatozoon chromatin after freezing suggests that chromatin undergoes important changes which might involve variation, during the freezing-thawing procedure, in the content of elements such as zinc, known to be involved in chromatin condensation (man: Kvist *et al,* 1988).

Finally, it seems that the modifications in spermatozoon nuclei caused by freezing-thawing might result in defective decondensation of the nucleus during fertilization, leading to a delay in the formation of the male pronucleus and/or the first division events. One consequence of this might be early embryonic mortality or lower embryonic development, observed after artificial insemination with frozen spermatozoa (boar: Lwoff *et al,* 1987; man: Schwartz *et al,* 1983).

ACKNOWLEDGMENTS

We would like to thank Dr U Kvist and Dr JL Courtens for their very helpful comments on the manuscript, Dr A Mills for his help in the English translation, and Miss I Berdrin for the preparation of this manuscript. This research was supported in part by the Institut National de la Recherche Agronomique (cryobiologie, INRA 1247 A).

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