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Cytochemical Characterization of Sperm Nuclei during Epididymal Maturation in Mammals*

Caracterización citoquímica del núcleo de espermatozoides durante la maduración epididimaria en mamíferos

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Among numerous parameters of sperm maturation along the epididymal transit, the biochemical stabilization of the DNA-protein complex (probably due to formation of disulfide cross linkages in the histones), has been considered of paramount functional significance.

Teratospermic spermatozoa as well as male gametes obtained from seasonal breeders in the non-breeding season may show altered cytochemical findings in what concerns total nuclear proteins, SH-proteins and DNA.

Significant cytophotometric differences were found in DNA for normal (oval) *vs.* teratospermic (amorphous) heads human spermatozoa, with no difference for total proteins.

In maturing stallion spermatozoa, a clear tendency to show maximal cytophotometric values for both total proteins and DNA in cauda as compared to head and body of the epididymis or vas deferens, can be easily demonstrated.

Ejaculated stallion spermatozoa were cytochemically analyzed during the breeding and non-breeding season. It was found that the spermatozoal nuclei of out-of-breeding season samples were characterized by a lower relative SH-protein content, though the total protein did not change, and a lower DNA cytophotometric value. Both changes are consistent with the idea that out-of-breeding gametes are overmature, have lowered fertility and may lead to higher embryonic mortality. Teratospermic (human) spermatozoa may share these characteristics and display a faulty association of nuclear DNA to its proteins.

In eutherian mammals the final events of sperm maturation take place in the epididymis. These events deal with morphological traits of the spermatozoa, either microscopically or ultrastructurally identifiable; with cytochemical changes on the surface of the plasma membrane and the nuclear chromatin; with the middle piece organelles and the flagellar proteins, and with the pattern of swimming of the sperm, among other more subtle changes (1, 2).

Some of these changes are programmed in the spermatozoa, but evidently there are changes in the epididymal plasma that elicit modifications of the gametes. This interaction has been manifest since the early work of Young in 1931 (3) and has been

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studied in detail by many authors (4).

Probably, all these changes are important for the future eventual capacitation of the spermatozoon and thus, they play a role in its adquisition of full fertilizing ability. Therefore, an understanding of epididymal maturation is not only relevant to understand male infertility (5) but could, on the other hand, give new insight into male contraceptive procedures by inhibiting the production of functional spermatozoa without acting upon the gonad itself (1).

The biochemical modification or stabilization of the deoxyribonucleoprotein complex, perhaps due to formation of disulfide cross linkages in the histones, has been considered of paramount functional significance in sperm during epididymal maturation. Apparently, formation of S–S bridges starts in the caput epididymis and continues through the distal epididymal segments. However, not all SH-groups are utilized, since the nuclear chromatin of mature (cauda epididymis or ejaculated) spermatozoa may form additional S–S bridges if stored *in vitro* (6), in a process that may be called overmaturation of the male gamete (7).

As a consequence of the changing interaction of DNA and proteins in the chromatin of spermatozoa taken from different segments of the seminal excurrent pathway, variations in the Feulgen staining of sperm nuclei have been recorded, without true changes in the actual DNA content or total dry mass of the nuclei (8, 9).

Since stabilization of the chromatin occurs during epididymal transit due to the establishment of S–S bonds, caput spermatozoa can be differentiated from cauda spermatozoa by methods designed to permeate the gamete membrane and the subsequent action of a thiol reducing agent (1, 6).

Teratospermic spermatozoa have usually been considered a reflection of a faulty spermiogenesis, due to a primary testicular failure. These gametes probably do not undergo a normal maturation in the epididymis and should be cytochemically different (in terms of their DNA and nuclear chromatin) if compared to the normal ejaculated gametes. This hypothesis will be considered for human semen in the present review.

On the other hand, morphologically similar spermatozoa may differ in the relationship of DNA to proteins in the chromatin, depending on the site of collection of the gametes along the epididymis. It may also be surmised that spermatozoa from the ejaculate may differ in their degree of maturation (as assayed by cytochemistry of the nuclear DNA and proteins) in the breeding season. These latter postulates will be analyzed using stallion ejaculated sperm during the breeding and the out-of-breeding season.

MATERIAL AND METHODS

a) *Human semen: normal and teratospermic spermatozoa*

Human semen samples were obtained from patients referred to our Laboratory of Reproduction, once the routine spermiogram was prepared. Samples with abnormally high degree of teratospermia, showing a proportion of amorphous heads over 25%, were used to analyze cytophotometrically this type of abnormality. Normal spermiograms, in those cases where the infertility of the couple was adscribed to a female factor, were used for comparison. In these cases the normal oval heads were in excess of 80% (the figures chosen are the highest for either teratospermy or normality, respectively, according to a document from the W.H.O. 1979 (10).

Conveniently diluted semen samples were processed for staining with the Feulgen reaction, according to De Cosse and Aiello (11) and with the ninhydrin Schiff methods, modified by Yasuma and Ichikawa (12). A minimum of 50 spermatozoa per case were read in a Zeiss 01 cytophotometer, using a green filter (560 nm) for the Feulgen reaction and a cytofluorometric technique for proteins. The samples analyzed were 8 normal and 10 teratospermic semen (13).

b) *Stallion spermatozoa from different segments of the seminal pathway*

Sperm samples were obtained by either chopping the tissue into small pieces and filtering the resulting material through double gauze (used for head, body and tail of the epididymis) or gently squeezing the content of the vas deferens.

Smears were prepared, fixed in methanol-acetic acid and processed for cytophotometry, essentially as stated above for human semen (13), except that proteins were read in the visible range (560 nm) with or without trichloroacetic acid extraction for nucleic acids (14). At least 50 spermatozoa per segment and per stallion were read in the cytophotometer. Samples were obtained immediately after castration from 4 fertile stallions, 3 years old.

c) *Ejaculated stallion spermatozoa in the breeding and non-breeding season*

Semen from 4 fertile stallions (two of the light strain, average body weight 450 Kg and two of the heavy strain, 650 Kg) with an average age of 14.2 years, was used. Samples were collected using an artificial vagina during the breeding (early December) or the non breeding (late January) season.

Smears were prepared for quantitative cytochemistry of total SH-proteins (15), using the D.D.S. method; total (non histonic) proteins (16), using naphtol yellow S; DNA, using the Feulgen reaction modified according to De Cosse and Aiello (11). Spermatozoa were also submitted to a procedure for reducing the S–S proteins bonds, following a modification of a technique by Calvin and Bedford (6), that uses sodium dodecyl sulphate (SDS) to permeate the sperm membrane and dithiothreitol (DTT) as the reducing agent. The reaction, under appropriate conditions of pH, concentrations of the reagents, and time and temperature causes a swelling of the spermatozoal nuclei that is inversely proportional to the amount of S–S proteins present in the chromatin.

Cytophotometric determination for SH-proteins and DNA were done at 560 nm, and at 435 nm for total proteins. At least 40 cells were measured per stallion for each cytochemical method, both in the breeding and out-of-the breeding season. The cytophotometrical values were found not to differ significantly in condensed spermatozoal nuclei if compared to those that had undergone a swelling that duplicates the nuclear area, after reduction of S–S bonds.

RESULTS

a) *Human semen*

As shown in Table I, the cytophometric values for DNA differ significantly between normal oval spermatozoa and amorphous cells, whereas no significant difference is found (Table II) for the cytophotometric values for NH_2 of proteins in the same types of spermatozoa.

TABLE I

Cytophotometric DNA-Feulgen* values** in normal (oval)and teratospermic (amorphous) human spermatozoal nuclei

Oval Heads (n = 630)	Amorphous Heads (n = 400)
$\bar{x}$ = 3.782	$\bar{x}$ = 3.115
SD = 0.026	SD = 0.497
SE = 0.01	SE = 0.02
$p < 0.0001$	

* Feulgen reaction after De Cosse, J.J. and Aiello, N.(11)
** Arbitrary cytophotometric values

TABLE II

Cytofluorometric (NH2)–protein* values** in normal (oval) and teratospermic (amorphous) human spermatozoal nuclei

Oval Heads (n = 150)	Amorphous Heads (n = 150)
$\bar{x}$ = 42.58	$\bar{x}$ = 43.32
SD = 6.25	SD = 5.23
SE = 0.66	SE = 0.55
$p > 0.3$	

* Ninhydrine – Schiff reaction after Yasuma and Ichikawa (12).
** Arbitrary cytofluorometric values.

b) *Maturing stallion spermatozoa*

The same trend is observed for spermatozoa from caput, body and cauda epididymis and vas deferens in regard to total protein (Table III) and DNA (Table IV) and a clear tendency for both cytophotometric determinations to show maximal values at the level of the reservoir (cauda epididymis), once epididymal maturation is completed, can be easily seen.

TABLE III

Cytofluorometric (NH_2) – protein* values** in maturing stallion spermatozoal nuclei (n = 50 for each case)

		$\bar{X}$ ± S.D.	p values
Epididymis Head	(a)	35.75 ± 2.51	(a) vs (b) <0.001
Body	(b)	41.01 ± 2.87	(b) vs (c) <0.0001
Tail	(c)	46.29 ± 3.26	(c) vs (d) >0.3
Vas deferens	(d)	48.03 ± 3.14	

* and ** as in Table II.

TABLE IV

Cytophotometric DNA – Feulgen* values** in maturing stallion spermatozoal nuclei (n = 50 for each case)

		$\bar{X}$ ± S.D.	p values
Head	(a)	2.68 ± 0.19	(a) vs (b) <0.01
Epididymis Body	(b)	3.26 ± 0.10	(b) vs (c) >0.2
Tail	(c)	3.43 ± 0.18	(c) vs (d) <0.05
Vas deferens	(d)	2.95 ± 0.11	

* and ** as in Table I.

c) *Ejaculated stallion spermatozoa*

The cytophotometric measurements in condensed spermatozoal nuclei from ejaculates taken in the breeding and in the non-breeding season are shown in Tables V, VI and VII. They deal respectively with determination of total SH-protein, total (nonhistonic) proteins and DNA-Feulgen. All values, as in the two preceding sets of experiments, are expressed as arbitrary (cytophotometric) units.

TABLE V

Cytophotometric SH – protein* values** in stallion spermatozoal nuclei in the breeding and non-breeding season

Stallion N°		Breeding $\bar{X}$ ± S.D.		Non – breeding $\bar{X}$ ± S.D.	
1	(n = 37)	0.83 ± 0.11	(n = 51)	0.76 ± 0.11	
2	(n = 43)	0.98 ± 0.16	(n = 51)	0.65 ± 0.10	
3	(n = 31)	1.07 ± 0.20	(n = 50)	0.91 ± 0.18	
4	(n = 40)	0.98 ± 0.18	(n = 51)	0.76 ± 0.17	
	$\bar{x}$ =	0.97 ± 0.18	$\bar{x}$ =	0.77 ± 0.17	p <0.0001

* Barrnett and Seligman reaction (15).
** Arbitrary cytophotometric values.

TABLE VI

Cytophotometric total protein* values** in stallion spermatozoal nuclei in the breeding and non-breeding season

Stallion N°		Breeding $\bar{X}$ ± S.D.		Non – breeding $\bar{X}$ ± S.D.	
1	(n = 51)	3.77 ± 0.51	(n = 50)	4.02 ± 0.25	
2	(n = 53)	3.98 ± 0.43	(n = 51)	3.89 ± 0.31	
3	(n = 51)	3.83 ± 0.40	(n = 50)	3.95 ± 0.22	
4	(n = 51)	3.95 ± 0.32	(n = 54)	4.02 ± 0.22	
		$\bar{x}$ = 3.89 ± 0.42		$\bar{x}$ = 3.96 ± 0.26	$p < 0.02$

* After Deitch (16) at pH 3.
** Arbitrary cytophotometric values.

TABLE VII

Cytophotometric DNA – Feulgen* values** in stallion spermatozoal nuclei in the breeding and the non-breeding season

Stallion N°		Breeding $\bar{X}$ ± S.D.		Non – breeding $\bar{X}$ ± S.D.	
1	(n = 46)	3.06 ± 0.30	(n = 43)	1.58 ± 0.08	
2	(n = 41)	2.96 ± 0.19	(n = 42)	1.79 ± 0.10	
3	(n = 40)	3.06 ± 0.18	(n = 50)	2.16 ± 0.15	
4	(n = 40)	2.79 ± 0.12	(n = 52)	2.09 ± 0.12	
	$\bar{x}$ =	2.97 ± 0.24		$\bar{x}$ = 1.92 ± 0.26	$p < 0.0001$

* and ** as in Table I.

The swelling (or lack thereof) of spermatozoal nuclei is estimated using an ocular micrometer to analyze the smears from semen submitted to the procedure of Calvin and Bedford (6). A maximum area of $17\mu^2$ corresponds to the normal sperm nuclei and a 100% increase is defined as a significant swelling, both for ejaculates obtained in December (breeding season; Table VIII) and January (non-breeding season; Table IX).

TABLE VIII

Percentages of decondensed* stallion spermatozoal nuclei in ejaculates of December (breeding season)

	Stallion N° 1	2	3	4			
(A)	33.0	25.59	39.28	35.51	$\bar{X}$	=	33.34
(B)	67.0	74.41	60.72	64.49	$\bar{X}$	=	66.65
	n = 309	336	313	352	N	=	1.310

* 30 minutes treated by the SDS/DTT method of Calvin and Bedford (6).
(A) spermatozoal head area ca. 17 μ^2.
(B) spermatozoal head area ca. 34 μ^2.

TABLE IX

Percentages of decondensed* stallion spermatozoal nuclei in ejaculates of January (non-breeding season)

	Stallion Nº 1	2	3	4			
(A)	77.20	99.50	95.98	78.54	$\bar{X}$	=	87.80
(B)	22.80	0.50	4.02	21.46	$\bar{X}$	=	12.19
	n = 351	412	324	345	N	=	1.432

* and (A) (B) as in Table VIII.

DISCUSSION

Upon revision of published data (8, 9) no clear conclusions may by drawn regarding DNA content of spermatozoal nuclei and infertility, both in man and some domestic species. However, bovine spermatozoa, when stored under "optimal" conditions *in vitro* show a decreased Feulgen stain ability that correlates well with the deterioration of their fertilizing ability and the increased early embrionic mortality following insemination with such seminal samples (7).

Apparently, a normal DNA content is a prerequisite for an adequate capacitation of the spermatozoa. Rigorously speaking, no true alteration of DNA content has ever been firmly proven by Feulgen cytophotometry, as confirmed by independent methods, such as UV-absorption, dry mass determination, etc. Rather, the decreased Feulgen reaction implies a relative lack of accesibility to the DNA, due to a different degree of interaction with its associated proteins. This situation may be due to a rearrangement of the chromatin proteins since no net loss or synthesis has ever been substantiated (17). Among these rearrangements, S–S linkages formed in the nuclear histones are considered to be definitory for the degree of chemical maturation attained by the male gamete (18). It goes without saying that these S–S proteins will be reduced back to their SH–form once the male pronucleus starts to evolve in the oocyte cytoplasm, as will be described in another paper in this volume (19). Appropriate nuclear decondensation at this stage is of vital importance for normal embryonic development. It may well be that this process is not physiologically accomplished by "overmature" spermatozoa, be they obtained *in vitro* (as in bovine, see above) or *in vivo*, as with the non-breeding season stallion spermatozoa. The normal (sperm) nuclear decondensation during fertilization has been postulated to occur due to the action of free thiols in the ovular cytoplasm (see Kvist (20), for a review), but no clear distinction has been made between this "male pronuclear growth factor" (21, 22) and inherent, intrinsic properties of the S–S sperm chromatin proteins or the role of prostatic zinc in their stability (20).

The *in vitro* nuclear decondensation test (modified from Calvin and Bedford (6)), constitutes a good experimental approach to elucidate this point further, and to explore the overmaturation of spermatozoa, obtained either *in vitro* or *in vivo*.

Since amorphous head (as well as tapered) human sperm (23) show decreased DNA-Feulgen cytophotometric values, they may *a priori* be considered infertile spermatozoa. However, at least in a few cases, we have detected penetration of hamster zona-free oocytes by teratospermic human spermatozoa and a initial decondensation of the chromatin has been documented by electron microscopy in one such cases (24).

The higher percentage of decondensed sperm nuclei (after S–S reduction) in the breeding season implies that part of the nuclear protein already exists as SH–protein. The number of gametes with high SH–protein nuclear content may increase with a higher rate of seminal emissions, since under these circumstances spermatozoa from segments proximal to the cauda epididymis may be mobilized. Such a situation is known in the boar, where the

cytoplasmic droplet is used as a morphological criteria of sperm immaturity (25). In this situation not only is there an acceleration of the epididymal transit (which in itself could result in faultly maturation) but also a recruitment of immature cells, depending on the sperm reserve capacity of the seminal ducts in each species. Such a capacity is very low in human subjects.

The total SH–protein that can be obtained by maximal reduction in the sperm from non-breeding stallions is comparatively low, though the total nuclear protein has not changed. This implies that some S–S linkages are not exposed to the reagent, most probable due to a change in the tertiary structure of the DNA–protein complex. This extreme packing of the molecule can functionally be equated to an *in vivo* overmaturation. This observation agrees with the relative decrease of the DNA–Feulgen cytophotometric values observed in sperm nuclei from the non-breeding season.

A detailed analysis of the DNA–associated histones under the three experimental sets analyzed in the present work may provide further insight into the changes of the sperm chromatin that characterize normal and abnormal sperm maturation from a cytochemical point of view.

The obvious relationship to the fertilizing ability of the spermatozoon can advantageously be tested with overmature and out-of-breeding season male gametes exposed to foreign eggs *in vitro*; pronuclear formation and the rate and pattern of chromatin decondensation may be informative of the egg-spermatozoal interactions during this crucial step of reproduction.

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