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Induced Metachromasia in Bull Spermatozoa

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Summary. The availability of sequential DNA phosphates to bind toluidine blue molecules after acid hydrolysis was studied in normally shaped and misshaped spermatozoa from subfertile and highly fertile bulls. The aim was to associate induced spermatozoal metachromasia with infertility. Some few normally and abnormally shaped cells from highly fertile bulls exhibited an induced metachromasia after being treated with 4 N HCl for 10–30 min at 25° C prior to staining. Subfertile bulls contained 12 times as many metachromatic spermatozoa as highly fertile animals. The induced toluidine blue metachromasia is suggested as a rapid and simple method for detecting bull spermatozoa bearing an anomalous DNA-protein complex. This nucleoprotein complex was found to be more frequent in subfertile bulls.

Introduction

Sperm cells containing arginine-rich nuclear proteins display altered nuclear basophilia in comparison with somatic cells or with spermatozoa bearing lysine-rich histones (Lison 1955; Gledhill et al. 1966; Gledhill 1970; Mello and Vidal 1973, 1977). In addition to a low staining intensity, in toluidine blue (TB) – stained spermatozoal nuclei of the bull, the rat and the grasshopper, the spectral absorption peak is shifted to longer wavelengths ($\lambda = 630$–40 nm). Analysis of the response of bull spermatozoal chromatin subjected to Feulgen reaction, as hydrolysis times increase, reveals a special resistance of this chromatin to DNA depurination and solubilization of the apurinic acid fragments (Sandritter et al. 1965; Anderson and Kjellstrand 1975). An altered chromatin structure has been postulated for some morphologically abnormal and even normal bull spermatozoal heads which exhibit an increased Feulgen lability rate, although having a normal DNA content (Gledhill 1966, 1970). This has been explained by assuming that biochemical "immaturity" of the nuclear basic protein which associates with spermatozoal DNA is presented. Furthermore, a decrease in resistance to heat denaturation has also been demonstrated for the DNA of misshaped sperm heads and for that of many morphologically normal spermato-

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zoa of subfertile bulls, possibly promoted by changes in chromatin conformation (Evenson et al. 1980). The low chromatin resistance of certain bull spermatozoa to Feulgen hydrolysis might be associated with a relatively rapid removal of the protein element from the DNA during hydrolysis. Such being the case, the ability of sequential DNA phosphates to bind TB, in spermatozoa with altered chromatin conformation, would probably also be changed under certain Feulgen hydrolysis time conditions. This cytochemical characteristic was therefore investigated in normally shaped and misshaped spermatozoa from subfertile and highly fertile bulls in an attempt to associate acid hydrolysis-induced metachromasia with infertility.

Materials and Methods

Smears of bull semen samples from 5 fertile and 3 subfertile animals were obtained freshly or from frozen ampoules. Bulls of the Friesian Red and White and Black and White breed as well as of the Brazilian "Mantiqueira" breed were used. The bull fertility levels were determined by Cooperativa Central Agro-Pecuária de Campinas, Instituto de Zootecnia de Nova Odessa, and Propec. Com. Repres. Ltda., Campinas. All the bulls were healthy, and the causes of the lower fertility levels are not known.

The cells were fixed in acetic ethanol for 5 min and hydrolyzed in 4 N HCl for various periods at 25° C prior to staining with a 0.025% TB solution at pH 4.0 for 15 min (Lison 1960). The slides were then rapidly rinsed (5 s) in distilled water, air-dried, cleared in xylene and mounted in Canada balsam. Some preparations were subjected to nuclear protein removal with 4 M urea and 0.2 M 2 – mercaptoethanol at 0.8 M of NaCl (Marushige and Marushige 1974).

Microspectrophotometry was performed with a Zeiss photomicroscope equipped with an 01 photometer and an EMI 6256 photomultiplier. One plug point was taken nearly at the centre of the spermatozoon nucleus at various wavelengths. The area of the specimen measured was 2.16 μm^2.

Results

Figure 1 shows bull spermatozoa subjected to acid hydrolysis and stained with the TB solution. Figure 2 and Table 1 display the spectral absorption curves and parameters which define the characteristics of the nuclear basophilia. As acid hydrolysis progressed up to 1 h, there was a shift of the absorption maximum from $\lambda = 640$ nm towards shorter wavelengths. Then the peak shifted back to longer wavelengths. Although normally shaped, some few cells exhibited an atypical response to hydrolysis. While most of the spermatozoal nuclei were stained light green to blue at 10–30 min after the starting of the hydrolysis, some nuclei stained blue to violet (metachromasia) after the same period of hydrolysis (Fig. 1 A). In other words, the absorption maximum of the blue-violet group appeared shifted to shorter wavelengths with advancing hydrolysis as compared with the situation of the green-blue group (Fig. 2, Table 1). Eventually, misshaped sperm nuclei from highly fertile bulls also exhibited the metachromatic pattern (Table 1). In addition, the violet spermatozoal nuclei had the highest absorbances and metachromatic ratios, only surmounted by the values of the deeply metachromatic sperm nuclei deprived of nuclear protein (Figs. 1 D and 2, Table 1). A significant increase in number of spermatozoal nuclei giving metachromatic response was found in samples from bulls with low fertility. These showed nearly 12 times more metachromatic nuclei than the samples from highly fertile bulls (the proportion of metachromatic to non-metachromatic

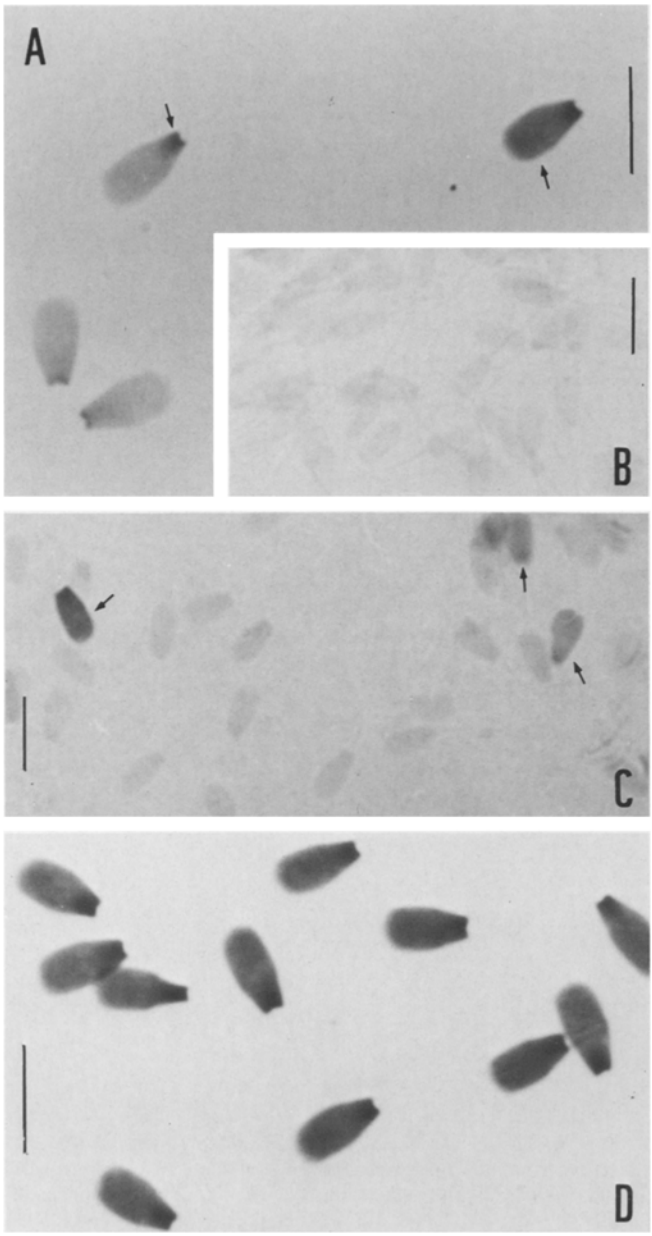

Fig. 1 A–D. Toluidine blue (TB) affinity levels in spermatozoa obtained from bulls of high fertility (conception rate higher than 80%) (**A, B, D**) and from one bull of unexplained low fertility (conception rate lower than 50%) (**C**). **A** and **C**. Cells hydrolyzed in 4 N HCl for 20 min prior to staining. **A** Most of the cells stained green as did those of the unhydrolyzed control (**B**). Some of the nuclei, however, stained completely or partly violet (metachromasia) (arrows). **C** The percentage of violet fully-stained nuclei was a rather larger than that shown in preparations from highly fertile bulls (**A**). Deep and slight violet intensities were found for the metachromatic nuclei (arrows). **D** Spermatozoa subjected to nuclear protein removal prior to TB staining. All the nuclei appeared deep violet. The bars equal 10 μm

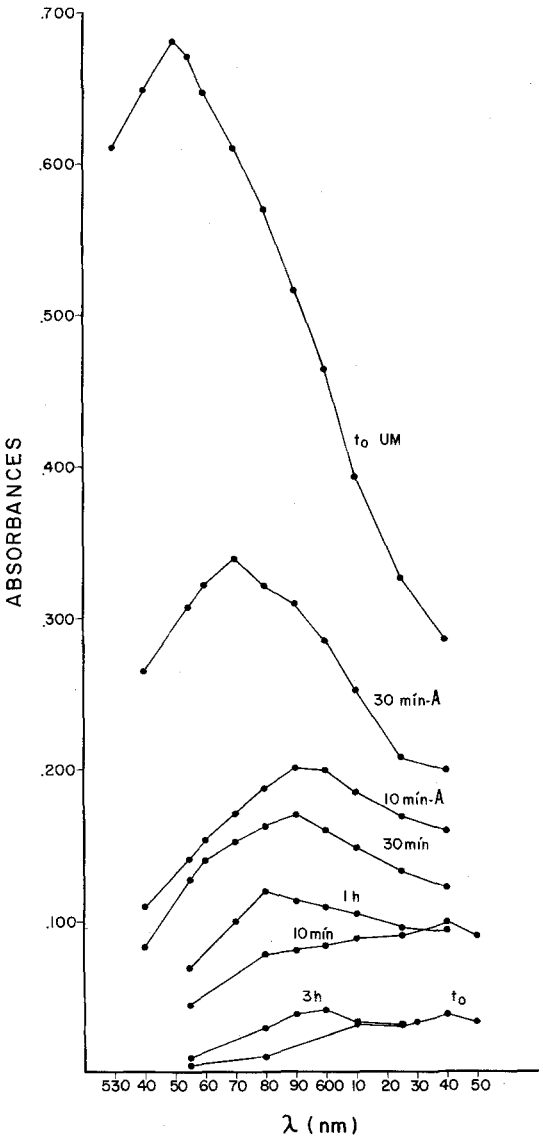

Fig. 2. Spectral absorption curves of TB-stained spermatozoal nuclei of 5 highly fertile bulls. The preparations were subjected to various periods of acid hydrolysis prior to staining. T_o = unhydrolyzed controls; UM = nuclear protein removal with urea plus mercaptoethanol. Each point in the curves in the arithmetic mean of 10 values except for the t_o and t_o UM curves (n=20). A = curves for spermatozoa with atypical response to hydrolysis

nuclei in the samples here analyzed was 36:63,007 (highly fertile bulls), and 85:12,429 (subfertile bulls) for an hydrolysis time of 20 min). The different affinity for the dye appeared only in preparations hydrolyzed for the time period ranging from 10 to 30 min. Some metachromatic nuclei of the subfertile bulls were found with a pale staining intensity (Fig. 1C). However, the metachro-

Table 1. Characteristics of the nuclear basophilia of spermatozoa from five highly fertile bulls. The material was subjected to acid hydrolysis with 4 N HCl at 25° C prior to TB staining (A = absorbances; t_o = unhydrolyzed controls; UM = nuclear protein removed with urea plus mercaptoethanol; * = abnormally-shaped spermatozoal heads). One (a), two (b) or four (c) absorption curves were determined for spermatozoa of each bull

Hydrolysis times	λ of the absorption peaks (nm)	Metachromatic ratios ($A_{\lambda=555\,nm}/A_{\lambda=625\,nm}$)	
		$\bar{X} \pm S$	n
10 min	590–600	0.86 ± 0.14	10[b]
	640	0.50 ± 0.10	10[b]
10 min*	590	0.87 ± 0.12	5[a]
20 min	570	1.47 ± 0.20	10[b]
	590–600	0.94 ± 0.15	10[b]
1 h	580	0.73 ± 0.12	10[b]
3 h	600	0.32 ± 0.05	10[b]
Controls t_o	640	0.16 ± 0.07	20[c]
t_o UM	550	2.05 ± 0.16	20[c]
t_o UM*	550	1.93 ± 0.13	5[a]

matic ratios and spectral localization of the absorption peak of all the metachromatic nuclei of the subfertile animals were practically the same as those of the highly fertile bulls.

Discussion

The metachromasia induced in the sperm heads reflects an increase in the overall number of sequential DNA phosphate groups available for dye binding after acid hydrolysis (Miura and Ohba 1967; Toepfer 1970; Mello and Vidal 1977), despite some loss of the nucleic acid (Kjellstrand 1977). This suggests that the DNA – protein relationship is altered, in certain spermatozoa probably due to immaturity of the associated protein. Normally, during spermiogenesis in the bull, somatic histones are replaced by an arginine and cystine – rich protein which tightly packages DNA (Gledhill et al. 1966; Marushige and Marushige 1974). Consequently, even after an acid hydrolysis, few DNA phosphate groups are available for TB binding. However, if some change in this protein has occurred affecting its interaction with DNA, then the DNP complex would be expected to become less stable than normal. The spermatozoal heads with the increased TB binding capacity are therefore assumed to possess an atypical DNP complex possibly related to a form of infertility, as their number, albeit small, appeared nearly twelve times increased in the sperm preparations from the subfertile bulls. However, not all the spermatozoa expected to exhibit a lower fertilizing potential displayed TB metachromasia induced after acid hydrolysis. Misshaped spermatozoa, for instance, represent 5 to 20% of the sperm cell population from highly fertile bulls (Gledhill 1966). Furthermore, sperm

nuclei bearing a DNA sensitive to thermal denaturation (abnormal chromatin conformation) represent 17% of the population from highly fertile bulls and 76% of that from subfertile bulls (Evenson et al. 1980). However, a small percentage of metachromatic spermatozoa appeared in the preparations from highly fertile and subfertile bulls. Even so, the acid – induced metachromasia is suggested as a simple tool for detection of an atypical DNA – protein complex, the frequency of which may appear enhanced as one of the expressions of a form of infertility in the bull.

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