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METHODOLOGY:

Methodological variants contributing to detection of abnormal DNA-protein complexes in bull spermatozoa*

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ABSTRACT

Efficient, simple and inexpensive methods for detecting abnormal DNA-protein complexes in sperm cells present in the semen of bulls with different fertility characteristics were tested cytochemically. The methods are based on nuclear metachromasy induced after toluidine blue staining and on different fluorescence emissions after acridine orange staining of spermatozoal nuclei bearing abnormal DNA-protein complexes. Treatments with 2-mercaptoethanol plus urea and NaCl, with SSC solution and with 4 N HCl were found to be the most efficient for inducing nuclear metachromasy. The 4 N HCl treatment was found to be the least efficient among these, though the simplest and cheapest. With the methods employing acridine orange as the dye solution, the frequency of sperm cell nuclei exhibiting high fluorescence intensity resembled that of the metachromatic nuclei highlighted with toluidine blue staining, if preceded by treatment with 4 N HCl or 2% citric acid. Although the animals with morphological defects associated with low fertility rates generally exhibited a higher frequency of metachromatic sperm cell nuclei and greater acridine orange (AO) fluorescence intensity, this could not be taken as a precise rule.

INTRODUCTION

Abnormalities in the DNA-protein complex of bull spermatozoa were first identified by Gledhill *et al.* (1966) when analyzing data obtained with the Feulgen reaction in comparison to data obtained with ultraviolet microspectrophotometry.

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The method named "induced nuclear metachromasy" was proposed to identify DNA-protein complex abnormalities highlighted when the spermatozoa were treated with 4 N HCl at 25°C for 15-20 min prior to staining with a toluidine blue (TB) solution (Mello, 1977, 1982). While chemically normal spermatozoa treated with the TB solution stained green, the sperm cell nuclei bearing abnormalities in the DNA-protein complex have been found to stain violet (metachromasy). The high availability and proximity of DNA phosphate groups unbound to protein amino groups and capable of binding TB molecules are responsible for the phenomenon of meta- chromasy (violet color) (Mello, 1982). The frequency of induced nuclear metachromasy in spermatozoa of subfertile bulls was found to be 12 to 20 times higher than that of highly fertile bulls (Mello, 1982).

Evenson and co-workers (1980) found that abnormal DNA-protein complexes in bull spermatozoa could be demonstrated with acridine orange (AO) staining, provided that instability of the complexes was enhanced by heat treatment. In this case, nuclei bearing a stable DNA-protein complex exhibited a green fluorescence, whereas those bearing unstable DNA-protein complexes, 17% of the sperm cell nuclei in highly fertile bulls, and 76% of the spermatozoa in subfertile bulls, showed a red fluorescence. The green fluorescence would result from the intercalation of the AO molecules within the DNA helix while the red fluorescence would reflect the electrostatic binding of AO molecules to DNA phosphates (Evenson *et al.*, 1980; Vidal, 1987).

Both the induced nuclear metachromasy and the AO fluorescence phenomenon, which rely on different chemical principles (Vidal, 1987), have thus been proposed to help with diagnosis of subfertility in the bull (Mello, 1977, 1982; Evenson *et al.*, 1980; Britto and Mello, 1988).

The efficiency of various treatments for highlighting the highly labile abnormal DNA-protein complexes in bull spermatozoa was tested here.

MATERIAL AND METHODS

Nineteen sets of frozen semen samples from several bull breeds (Nelore, Gir, Simmenthal, Holstein) were used. Eighteen of these were supplied by the Central de Tecnologia de Semen Pecplan Bradesco of Uberaba (MG) and one by the Central de Tecnologia de Semen Sembra of Barretos (SP). Fourteen lots were not commercialized due to a relatively high content of sperm cell abnormalities such as pouch formation, narrow base and knobbed acrosome. One lot was commercialized, but soon demonstrated an unexplained low conception rate, which was later attributed to a high frequency of spermatozoa bearing slightly narrow bases. Only four lots were commercialized. These gave a high conception rate (highly fertile bulls).

A list of codes and descriptions of the semen sets are shown in Table I.

Semen samples were thawed in water at 40°C for one minute and smears prepared. One part of the material was smeared right after thawing, whereas the other was smeared after a dilution of the sample and removal of the dilution medium used for freezing. Treatment was as follows: Semen (0.5 ml) was diluted in 9.5 ml of a solution containing 0.15 M NaCl and 0.001 M EDTA (v/v) and centrifuged at 3000 rpm for six minutes at room temperature (Evenson *et al.*, 1980). The

Table I - Codes and other characteristics of the semen samples.

Codes	Breed	Source	Cell abnormalities	Conception rate
1	Holstein	PECPLAN	Normal	High
2	Simmenthal	PECPLAN	Normal	High
3	Holstein	SEMBRA	Normal	High
4	Gir	PECPLAN	Normal	High
5	Nelore	PECPLAN	Pouch formation	Not tested
6	Nelore	PECPLAN	Pouch formation	Not tested
7	Nelore	PECPLAN	Pouch formation	Not tested
8	Limousin	PECPLAN	Pouch formation	Not tested
9	Nelore	PECPLAN	Pouch formation	Not tested
10	Gir	PECPLAN	Pouch formation	Not tested
11	Nelore	PECPLAN	Pouch formation	Not tested
12	Guzera	PECPLAN	Pouch formation	Not tested
13	Nelore	PECPLAN	Pouch formation	Not tested
14	Nelore	PECPLAN	Pouch formation	Not tested
15	Gir	PECPLAN	Pouch formation	Not tested
16	Simmenthal	PECPLAN	Narrow base	Not tested
17	Nelore	PECPLAN	Narrow base	Not tested
18	Nelore	PECPLAN	Pouch formation + knobbed acrosome	Not tested
19	Holstein	PECPLAN	Slightly narrow bases	Low

PECPLAN = Pecuária Planejada - Bradesco.
SEMBRA = Sêmen do Brasil Ltda.

supernatant was removed and the precipitate resuspended in 10 ml of the same solution and centrifuged again at 3000 rpm for six minutes. After being subjected three times to the procedure of dilution and centrifugation, the semen was resuspended in 0.5 ml of the solution.

The smears were fixed in an ethanol-acetic acid mixture (3:1, v/v) for one minute, washed in 70% ethanol for three minutes and air dried. The preparations were stained with a 0.025% TB solution in McIlvaine buffer either at pH 4.0 or 5.0, according to Vidal's method (Mello and Vidal, 1980), preceded by the following treatments:

a_1 - No previous treatment (control);

b_1 - 4 N HCl for 15 min at room temperature followed by rinsing in distilled water (Mello, 1982);

c_1 - 0.67% citric acid solution for 37 min followed by rinsing and washing in distilled water (modified from Roy *et al.*, 1982);

d_1 - 2 SSC solution at 60°C overnight followed by rinsing in distilled water;

e_1 - SSC solution at 60°C overnight followed by rinsing in distilled water;

f_1 - 0.2 M 2-mercaptoethanol, 0.1 M urea and 0.8 M NaCl solution for 20 min at room temperature followed by rinsing in distilled water (modified from Mello, 1982);

g_1 - 0.2 M 2-mercaptoethanol, 0.1 M urea and 0.8 M NaCl solution for 10 min at room temperature followed by rinsing in distilled water (modified from Mello, 1982).

The frequency of spermatozoa exhibiting nuclear metachromasy was determined in two samples of 1000 cells/slide/animal chosen at random at 400X magnification.

Part of the preparations were also stained with 2.67×10^{-5} acridine orange (AO) prepared in a 0.5 M NaCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl solution at pH 7.4, after the following procedure:

a_2 - No previous treatment (control);

b_2 - Equal to b_1;

c_2 - Equal to c_1;

d_2 - 2% citric acid solution for 30 min followed by rinsing in distilled water (modified from Roy *et al.*, 1982;

e_2 - 0.5 N HCl for one of six min at room temperature followed by rinsing in distilled water.

One minute after the preparations were covered with the AO solution, they were observed with a fluorescence microscope, using exciter filter I which transmits a 400 nm exciting light, and barrier filters 500 and 530 (Vidal, 1987). For the b_2 and d_2 preparations, the frequency of sperm cell nuclei exhibiting a brighter fluorescence intensity was detected in a sample of 1000 cells/animal chosen at random at 400X magnification.

RESULTS

Semen with no withdrawal of the dilution medium

The preparations in which staining with toluidine blue was preceded by treatments with 2 SSC (d_1) and SSC (e_1) solutions showed a loss of material in consequence of which they were considered to be not adequate for analysis. Also the preparations treated with 2-mercaptoethanol prior to staining (f_1 and g_1) were not considered, since there was formation of a pellicle over the material, hindering reliable analysis.

However, the preparations subjected to the other treatments showed part of the spermatozoal population with heads exhibiting totally or partly a metachromatic response, similarly to reports by Mello (1977, 1982). The frequency of metachromatic sperm cell nuclei under the a_1-c_1 experimental conditions for the various bull semens is reported in Table II.

The occurrence of a clear nuclear metachromasy in the area of the sperm head base was much more evident in the semen code No. 16, for all these treatments. In the semen of the other bulls this occurrence was occasional.

Nuclear metachromasy was also found for spermatozoa of some of the semen in which no treatment was used prior to staining (a_1), especially when staining was carried out with the dye solution at pH 5.0 and the animals were identified as subfertile. Generally speaking, however, the induced metachromasy was more frequent after an HCl treatment (b_1), especially when using the dye solution at pH 5.0 and for subfertile animals, although the frequency of this chemical abnormality was relatively low. The citric acid treatment was not found to be effective for inducing the nuclear metachromatic response, since the frequency with which metachromatic sperm heads appeared after this treatment was very similar to that found in controls (a_1).

As regards the smears stained with the AO solution, the sperm cell nuclei exhibited a variable fluorescence intensity, being orangeish-red when treated with 4 N HCl, and green after the other

Table II - Relative frequencies (%) of metachromatic sperm cell nuclei in bull semen with no withdrawal of the dilution medium and stained with toluidine blue at pH 4.0 and 5.0.

Bull code	Treatments					
	a_1 pH4	a_1 pH5	b_1 pH4	b_1 pH5	c_1 pH4	c_1 pH5
1	0.05	0.00	0.10	0.15	0.05	0.10
2	0.00	0.15	0.10	0.05	0.00	0.10
3	0.00	0.00	0.05	0.10	0.10	0.05
4	0.00	0.05	0.15	0.15	0.05	0.10
5	**0.15**	0.10	0.10	0.15	**0.15**	0.10
6	0.00	0.05	**0.25**	**0.30**	0.00	**0.15**
7	0.05	**0.25**	0.10	**0.25**	0.10	**0.15**
8	0.05	**0.25**	**0.25**	**0.30**	**0.20**	**0.15**
9	0.00	0.15	**0.25**	**0.20**	0.05	**0.20**
10	**0.10**	0.10	0.05	**0.20**	**0.15**	**0.15**
11	0.00	**0.20**	0.10	**0.30**	0.00	**0.30**
12	0.00	0.15	0.05	**0.20**	0.00	0.05
13	**0.10**	**0.25**	0.15	**0.25**	0.05	**0.15**
14	**0.10**	**0.20**	0.10	**0.25**	0.10	**0.15**
15	**0.10**	**0.35**	**0.25**	**0.20**	**0.25**	**0.20**
16	**0.10**	**0.30**	**0.30**	**0.35**	0.10	**0.40**
17	**0.15**	0.15	0.10	**0.30**	0.10	0.10
18	**0.20**	0.15	0.15	**0.25**	0.20	**0.15**
19	**0.10**	0.10	**0.20**	**0.20**	0.05	**0.20**

Bold - Frequencies above the maximum found in the semen of bulls with high conception rates (bulls 1-4). a_1 - Control; b_1 - 4 N HCl for 15 min at room temperature; c_1 - 0.67% citric acid for 37 min.

treatments. A quantitative analysis of the frequency of the different intensities of the emission colors was not carried out since they varied even in different slides prepared with the semen of the same bull.

Semen with withdrawal of the dilution medium

All the spermatozoa in smears subjected to treatment with the 2 SSC solution (d_1) and 2-mercaptoethanol plus 4 M urea and 0.8 M NaCl (f_1) exhibited nuclear metachromasy.

The preparations subjected to the other treatments showed part of the spermatozoal population with the nuclear metachromatic response which was more frequent than that observed in the semen smears with no withdrawal of the dilution medium (Tables II and III). The metachromasy exhibited by some of the spermatozoal heads was of a faint color.

Although all treatments used prior to staining were effective for inducing the metachromatic response, 2-mercaptoethanol (g_1) and SSC solution (e_1) were the most efficient (Table III). Some metachromatic response was also verified in the smears with no treatment prior to staining, similarly to the observations in the semen preparations with no withdrawal of the dilution medium (Tables II and III).

The spermatozoa of bulls No. 9 and 10, and even 5 and 6, although presenting a high frequency of the pouch formation pathology (Table I), displayed frequencies of the nuclear metachromatic response similar to those verified for bulls with a high conception rate (1-4) (Table III). The semen of the other bulls, especially that of bull No. 16, presented frequencies of nuclear metachromasy higher than those exhibited by the highly fertile bulls (Table III).

The smears in which no treatment (a_2) or 0.67% (c_2) and 2% (d_2) citric acid treatments were used prior to AO staining exhibited green fluorescent sperm cell nuclei, some of them with a higher fluorescence intensity, especially at the nuclear base. This was more evident in the smears treated with the 2% citric acid solution (d_2) and varied in frequency with the bull semen considered (Table III).

In the smears treated with 4 N HCl prior to AO staining (b_2) a red fluorescent color was found evenly distributed in the sperm cell nuclei or predominating in their nuclear base. A higher fluorescence intensity varied in frequency with the bull semen considered (Table III).

With varying exposure time to 0.5 N HCl treatment prior to AO staining, sperm cell nuclei with green and yellow or orangeish-red fluorescence could be found in the same preparation of different bull semen samples. However, the frequency with which these differently fluorescent cell nuclei appeared could not be evaluated since the time for appearance of two different colors varied from two to six minutes in the preparations of a same bull semen and the orangeish-red fluorescence emission faded quickly.

DISCUSSION

Among the chemicals used to induce nuclear metachromasy in chemically abnormal bull spermatozoa of semen smears deprived of the dilution medium, SSC solution and 2-mercaptoethanol plus urea and NaCl were found to be the most effective. Acid hydrolysis with 4 N HCl for 15 min showed to be comparatively less effective; however, it was the more practical and cheapest of the tested treatments. No

Table III - Relative frequencies (%) of metachromatic sperm cell nuclei exhibiting metachromasy (a_1-g_1) after staining with toluidine blue at pH 4 and 5, or an especially strong fluorescent response after AO staining (d_2, b_2) in bull semen after withdrawal of the dilution medium.

Bull code	Treatments											
	a_1 pH4	a_1 pH5	b_1 pH4	b_1 pH5	c_1 pH4	c_1 pH5	e_1 pH4	e_1 pH5	g_1 pH4	g_1 pH5	b_2 AO	d_2 AO
1	0.00	0.00	0.25	0.25	0.10	0.15	0.15	0.30	0.20	0.25	0.45	0.50
2	0.10	0.15	0.20	0.30	0.15	0.15	0.35	0.30	0.40	0.40	0.70	0.75
3	0.00	0.05	0.15	0.30	0.15	0.15	0.40	0.45	0.25	0.30	0.45	0.45
4	0.00	0.15	0.25	0.40	0.15	0.30	0.35	0.35	0.35	0.35	0.65	0.35
5	**0.15**	**0.20**	0.20	**0.45**	0.15	0.20	0.35	**0.55**	**0.50**	**0.70**	0.70	0.60
6	0.10	**0.20**	0.25	**0.55**	**0.20**	0.20	0.40	0.40	0.40	**0.50**	0.65	0.60
7	**0.25**	**0.20**	**0.60**	**0.60**	**0.35**	**0.35**	**0.50**	**0.55**	**0.60**	**0.60**	**0.75**	0.70
8	0.05	**0.20**	**0.35**	**1.15**	0.15	0.25	**0.55**	**0.65**	**0.60**	**1.10**	**1.15**	**1.25**
9	0.05	0.10	0.25	0.40	0.10	0.20	0.25	0.45	0.40	**0.50**	0.70	0.75
10	0.05	0.10	0.25	**0.45**	0.15	0.20	0.35	0.40	0.40	**0.55**	**1.05**	**1.10**
11	0.05	0.10	0.25	**0.55**	**0.25**	**0.25**	**0.55**	**1.00**	**0.75**	**0.80**	0.70	0.45
12	0.05	0.10	**0.35**	**0.80**	**0.25**	0.25	**1.05**	**1.00**	**0.95**	**1.00**	0.70	0.50
13	**0.20**	**0.20**	**0.45**	**0.60**	**0.25**	**0.35**	**1.35**	**1.35**	**0.55**	**0.60**	**0.80**	0.65
14	**0.15**	**0.20**	**0.40**	**1.00**	**0.35**	**0.50**	**1.65**	**2.85**	**0.65**	**0.75**	**1.35**	**1.40**
15	**0.20**	**0.25**	**0.40**	**0.60**	**0.30**	**0.35**	**0.80**	**0.65**	**0.45**	**0.75**	**1.00**	**1.05**
16	**0.20**	**0.35**	**1.35**	**7.45**	**0.35**	**0.75**	**5.65**	**16.7**	**33.2**	**38.8**	**29.9**	**40.1**
17	0.05	0.10	0.25	**0.55**	**0.30**	0.30	**0.60**	**0.55**	**0.45**	**0.75**	**0.75**	**1.00**
18	**0.20**	**0.25**	**0.65**	**1.75**	**0.35**	**0.90**	**1.55**	**1.50**	**2.95**	**3.55**	**1.75**	**1.65**
19	**0.15**	**0.20**	0.25	**0.60**	**0.40**	**0.40**	**0.65**	**1.05**	**0.75**	**0.50**	0.70	0.65

Bold - Frequencies above the maximum found in the semen of bulls with high conception rates (bulls 1-4). a_1 - Control; b_1 - 4 N HCl - 15 min room temperature; c_1 - 0.67% citric acid - 37 min; e_1 - SSC - 60°C overnight; g_1 - 0.2 M 2-mercaptoethanol, 0.1 M urea and 0.8 M NaCl - 20 min room temperature; b_2 - 4 N HCl - 15 min room temperature; d_2 - 2% citric acid - 30 min.

advantage at all was found when using the 0.67% citric acid solution prior to TB staining.

The efficiency of the HCl treatment was lower than that of the 2-mercaptoethanol plus urea and NaCl, or the SSC solution probably because of a certain DNA (apurinic acid?) loss with advancing acid hydrolysis (Savage and Plaut, 1958; Sandritter *et al.*, 1965; Kjellstrand, 1977; Mello, 1979), although the hydrolysis time used corresponded to the ascending branch of the Feulgen hydrolysis curve for this material (Silva and Mello, 1986). With the DNA loss, the number of DNA phosphates available for TB binding would be reduced and the phenomenon of nuclear metachromasy would be possibly affected. On the other hand, the treatment with 2-mercaptoethanol plus urea and NaCl dissociates partly or totally protein from DNA in bull spermatozoa (Marushige and Marushige, 1974; Mello, 1982) and does not affect DNA itself, increasing the number of binding sites for TB attachment. The same phenomenon, although at a lower intensity, would occur when using the SSC treatment (Roy *et al.*, 1982).

Staining with the TB solution at pH 5.0 was found to be more adequate than that at pH 4.0 for detection of metachromatic nuclei, probably because it turned the visual observation easier, besides promoting a partial dissociation of protein areas from the DNA and exposing amino-acid residues (glutamic acid?) reactive to TB (Coelingh *et al.*, 1972).

The spermatozoa that did not require pretreatment to exhibit nuclear metachromasy possibly had their histones not, or deficiently substituted by the usual spermatozoal basic protein (Gledhill, 1970). The frequency with which these spermatozoa appeared in the semen samples was, however, quite low.

The fact that the induced nuclear metachromasy in bull spermatozoa was found to be more frequent in subfertile animals in comparison with highly fertile animals is in agreement with previous data by Mello (1982). The frequency with which the nuclear metachromatic response appeared in the highly fertile animals was similar to that previously reported for an elite herd (Britto and Mello, 1988).

Since bulls for which subfertility was associated with a high frequency of spermatozoa bearing the pouch formation defect did not show simultaneously a high frequency of metachromatic spermatozoa, it is assumed that subfertility diagnosed on the basis of a morphologic spermatozoal head defect is not necessarily accompanied by abnormalities in the DNA-protein complexes. Furthermore, the finding of low levels of nuclear metachromasy in bull spermatozoa is thus not sufficient to disregard suspicion of subfertility.

As regards the fluorescence emitted by the AO-stained bull spermatozoa, the results obtained with the methods tested here were not comparable to those reported by Evenson and co-workers (1980) for heat-treated sperm cells. A certain relationship could be suggested between high fluorescence intensity in AO-stained cells and the exhibition of nuclear metachromasy in the TB-stained cells. The spermatozoal nuclei with deep orangeish-red fluorescence would have more DNA phosphate groups available for AO electrostatic binding on the surface of the DNA helix (Vidal, 1987). This situation would correspond to that in which nuclear metachromasy is induced in the bull spermatozoa subjected to TB staining. The spermatozoal nuclei with a green fluorescence, on the other hand, would have a greater number of AO molecules intercalated within the DNA double helix and very few free DNA phosphates available for AO electrostatic binding. This is the usual situation in normal DNA-protein complex of bull spermatozoa and would be corresponding to the lack of a metachromatic response in TB-stained preparations.

When analyzing the results concerned with TB or AO staining after treatment with 4 N HCl we may speculate that the conditions required for induction of the metachromatic response with TB staining (high frequency of available DNA phosphate groups close to each other) would be more stringent than those required for appearance of the orangeish-red AO fluorescence (DNA denaturation and possibly a smaller amount of free DNA phosphate groups). Consequently, while a few spermatozoa would exhibit nuclear metachromasy, a lot of sperm cells would exhibit the orangeish-red AO fluorescence. A higher intensity of this fluorescence, however, would be compatible with the nuclear metachromatic response.

Anyway, a more accurate study on the interpretation of AO fluorescence that fitted well with Evenson and coworkers (1980) data, present nuclear metachromasy and AO results and Mello's (1982) data is required. Considering only the feature related to high fluorescence intensity, it can be concluded that like induced metachromasy, it is more frequent in bulls with subfertility rates.

Since the medium in which the semen is commercially diluted was found to affect the results, it is recommended that for studies involving cytochemical responses and quantitative analysis, preparations free from this medium should be used.

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RESUMO

Esfregaços de sêmen de touros com níveis conhecidos de fertilidade foram submetidos a variantes metodológicas que visavam à identificação de anomalias no complexo DNA-proteína, baseando-se na metacromasia induzida após coloração com azul de toluidina (TB) e na diferença em fluorescência após coloração com o alaranjado de acridina (AO). Buscou-se o estabelecimento de métodos simples, eficazes e de baixo custo para a evidenciação de espermatozóides portadores de complexos DNA-proteína anômalos, no sêmen de touros com diferentes características de fertilidade.

Dos métodos testados, aqueles envolvendo pré-tratamento com 2-mercaptoethanol mais uréia e NaCl, com solução SSC e com HCl 4 N mostraram-se os mais eficientes na evidenciação da metacromasia induzida, sendo os dois primeiros mais eficazes que o último. Contudo, este mostrou-se mais simples e menos oneroso que os primeiros.

Nos preparados em que se realizou coloração com AO, a freqüência de núcleos de espermatozóides com maior intensidade de fluorescência foi semelhante à de núcleos metacromáticos (TB), desde que os esfregaços fossem pré-tratados com HCl 4 N ou ácido cítrico a 2%.

De maneira geral os animais com problemas de fertilidade apresentaram maior freqüência de espermatozóides metacromáticos e com maior intensidade de fluorescência com o AO, porém nem todos os animais se comportaram segundo esta regra.

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