Microphotokinesigraphic analysis of buffalo spermatozoa
I. Characteristic features and significance of the immobile buffalo spermatozoa

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ABSTRACT - The article discusses the results of a study of immobile spermatozoa of buffalo bulls from Bulgarian murrayah breed, based on photo images registered in various displays in conditions of dark background photokinesis. The analysis showed the way developer affects the brightness of kinesigrams. The study is based on samples of semen of six buffalos analysed by means of the microphotokinetic method developed by Tchakarov and Natchev. Brightness parameters of the cells and their photokinetic images have been established photometrically; their metric parameters have been defined by means of an eyepiece and object micrometer. It has been found out that the optimal exposure time for a photokinetic analysis of buffalo spermatozoa was 5 sec, and the recommended developer for treating of negative registration material is the pyrocatechin developer by Windisch.

key words: Sex tissue, Spermatozoa, Exposition.

INTRODUCTION - Microphotokinesigraphy is a method, used for graphic registration and analysis of biological objects' (mainly spermatozoa) kinetics, which was developed by Tchakarov and Natchev [1962]. The method was used to study 20 types of salmonella, as well as the spermatozoa of laboratory animals (rabbit, white mouse, rat, golden hamster, guinea pig and toad), rodents in wild (ordinary mouse, forest mouse, field mouse and snow mouse, common shrew), domestic farm animals (buffalo, ram and boar) and the human. Our study is concerned mainly with buffalo spermatozoa. The first publications regarding the graphic registration of mobile spermatozoa on dark background are by Rothschild Lord and Swann [1950], Rothschild Lord [1953, 1962], and Gray [1955], with the main object of their studies Psammechinus millaris and Echinus esculentus. Their experiments registered separate or small groups of spermatozoa with relatively short trajectories. A considerable number of trajectories and images of spermatozoa, caught on a single still called kinesigram, was registered by means of photokinetic method. The experiments were conducted in conditions of dark-field microscopy with an exposition suitable for the respective species. The trajectories had the necessary length and depth, sufficient for an extrapolation of the obtained results to the whole ejaculated sample. There were three distinctive cell groups of spermatozoa in the kinesigrams: immobile, vibrating and forward-moving. The aim of our study was to investigate each of these groups, and more specifically: a) the photokinetic im-
ages of immobile spermatozoa of the buffalo in different expositions, and b) the role of the developer in decreasing the halo around the highactinic parts of the cells in kinesigrams resulting from preexposition.

**MATERIAL AND METHODS** - The study was based on samples of semen of six buffalos of the Bulgarian murrah breed. The samples were obtained once a week, in a period of four weeks. Ten registrations were conducted from each sample, diluted with glucose-phosphate diluter for buffalo and 2% beef serum albumin. The microscope used was *Ergval* (*Zeiss*) with a standard phase-contrast condenser. Along the rays there was the wreath-shaped diaphragm 100, with a digital aperture of 0.80 to 1.00 and a screened inside ring. The optic system consisted of a-chrome object-glass 10X with digital aperture 0.20, aplanated eye-pieces 8X, MF – projective 3.2:1 and camera *Vera*. The filmed area diameter was 1.4 mm, and the expositions applied – 3, 4, 5, 6 and 7 sec. The filmed material of the first week (black and white *Konika* films) was treated with a standard developer, and the rest of the material – with Windisch (500 ml. distilled H$_2$O, 1.6 g. benzcatechin, 0.25g Na$_2$SO$_3$ and 5ml 10% NaON). The treatment lasted for 12–14 min. at 20°C. Positive kinesigrams’ dimensions were 16x16 cm. The length of the spermatozoa tail and its images was measured by using an object-and-eyepiece micrometer of smearscoloured according to Chakarov and Mollova’s method [1982], and also by using images obtained in conditions of dark-background microcopy. The brightness of the parts of immobile spermatozoa in conditions of dark-background kinesigraphics, and the permeability of the parts of their photokinesigraphic images on the negative kinesigrams developed with Windisch, were measured by means of the photometric head of *Fluoval photometer*, respectively *NU-2* (*Zeiss*).

**RESULTS AND CONCLUSIONS** - The kinesigraphic image of the immobile spermatozoa is characterised by a maximum proximity, yet not a complete identity with their image on dark background. (figure 1).

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**Figure 1.** Images of immobile spermatozoa: 1. Lightmicroscopic image in full-face position, 2. Dark-background image in full-face position, 3. Dark-background image in profile position, 4. Photokinesigraphic image in full-face position, 5. Photokinesigraphic image in profile position; a – head of the spermatozoon, b – connecting part [NAV, 1992] (neck) of the tail of the spermatozoon, c – middle part of the tail, d – main part of the tail, e – end part of the tail.

In the kinesigraphic images of this group of spermatozoa, compared to the dark-background and the optomicroscopic ones, both in their full-face and in their profile position, there was no narrowing in the connecting part of the tail (fig. 1.4b, 1.5b and figs. 5.2, 5.3, 5.4 and 5.5).
Since the forming of the kinesigraphic images depends on their brightness and the duration of the exposition, the photometric characteristics of their actinic parameters were the first to be defined. The brightness of the head in full-face position was found to be 1.67±0.12 along the scale of permeability; its brightness in profile – 48.13±3.16, the brightness of the medium part of the tail – 7.56±0.26, and that of the main part of the head – 2.23±0.11 correspondingly. From the analysed data it was clear that the head was the brightest in a profile position – its actinity was more than 30 times higher than it was in full-face position, and about 6.5 times higher than that of the middle part of the spermatozoon’s tail. On the other hand, the actinity of the middle and the main parts of the tail, which seemed too high in the microphotographs of the negative kinesigrams, turned out to be rather low – 7.56 and 2.23 correspondingly. The low actinity of the main part made us presume some reduction in the image of the end part of the tail, which corresponded to its overall length.

To check this presumption we conducted comparative metric measurements of smears of buffalo spermatozoa, coloured with active dyes as is in Chakarov and Mollova’s method [1982], and of immobile spermatozoa in conditions of dark-background kinesigraphics. The results indicated some shortening of the length in the image of the immobile spermatozoa tail in the conditions of kinesigraphics – 51.86±0.86 µm, compared to 60.10±1.19 µm in the rubbings (P<0.001). Increasing the time of exposure did not lead to the visualising of this part - it remained beyond the registering capacity of the negative material. The actinic characteristics of the images of buffalo immobile spermatozoa depending on the time of the exposition - 3, 4, 5, 6 and 7 sec., obtained from microphotographs of negative kinesigrams are shown in figure 1. With the increased time for the exposition, more grains amassed on the photographic plate, which in turn increased the transverse and the longitudinal dimensions of spermatozoon's middle part.

Figure 1. Microphotographs of buffalo immobile spermatozoa obtained in filming negative spermatokinesigrams. Optic enlargement of negative kinesigrams – 10x3.2. Optic enlargement of microphotographs – 16x3.2. Exposition time of kinesigrams: 1 – 3sec., 2 – 4sec., 3 – 5sec., 4 – 6sec., 5 – 7sec.

This effect was visualised as an actinic deformation – an exposition halo around the part of the cell, which blurred the visible in normal lighting and in dark-background microscopy contour of the connecting part of the tail and shortened the main part of the tail (figure 1.4 і 1.5. and figures 1.4 and 1.5). In expositions which lasted for 2 and 3 sec. (figure 1.1) the halo was low, the images – incomplete (fragmentary), and the registered trajectories of the forward-moving - short and insufficiently informative. In expositions lasting for more than 6 sec. the exposition halo was high, the trajectories - long and going beyond the margins of the filmed area, which made them practically immeasurable. Optimal halo and length of trajectory could only be achieved at an exposition of 5 sec. Not depending on the time of the exposition turned out to be the significant brightness differences of the parts of the sperma-
tozoon, which were registered photometrically. In order to make the negative kinesigrams more informative, we conducted a parallel developing using a standard and a pyrocatechin developer by Windisch. Compared to the standard one, the pyrocatechin developer significantly decreased the exposition halo in the area of the connecting part. (figure 2).

Figure 2. Positive kinesigrams of buffalo immobile spermatozoa (fragments), obtained from negative spermkinesigrams developed with standard (1) and pyrocatechin (2) developer. Optic enlargement of negative kinesigrams – 10x3.2. Exposition time: 5 sec. Size of the positive kinesigram – 16x16 cm.

The microdensitometric experiments, conducted with the aim of defining the qualitative characteristics of Windisch developer with regard to the picturing of objects of different brightness, proved that the curve of the negative material developed with the named agent, slanted less steeply than the curve of the one developed with a standard developer. In other words, Windisch developer turned out to have a substantial equalising effect. Densitometric data from the images of the immobile spermatozoa in the negative photographic materials treated with the Windisch showed less multiplicity of the difference in the light absorption of spermatozoon’s head in a profile and in a full-face position when treated with the Windisch developer.

In conclusion, we can state that: 1. The optimal exposition time in a photokinesigraphic study of buffalo spermatozoa was 5 sec.; 2. It's preferable to develop negative kinesigrams with high-equilizing developers. We recommend the one Windisch.

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