THE EFFECT OF STORAGE *IN VITRO* ON THE DNA CONTENT OF BULL SPERMATOZOA

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Abstract

Bull spermatozoa were aged *in vitro* for periods of up to 9 days. Microspectrophotometric measurements were made of the nuclear DNA content of the sperm heads using ultraviolet absorption, acridine orange fluorescence, and Feulgen staining and of the nuclear histone content using fast green staining. The surface area of the sperm heads was calculated from linear measurements of head length and width.

No significant alteration in any parameter was found after storage but marked variability in the Feulgen stain was evident.

I. Introduction

The DNA content of spermatozoa has been considered by many workers to be a biological parameter of constancy. Preparations of bull spermatozoa have been used as reference cells for microspectrophotometric estimations of DNA (Leuchtenberger *et al.* 1952; Ruch 1966).

During spermiogenesis in the bull, Gledhill *et al.* (1966) have shown that the average amounts of DNA, measured by ultraviolet absorption are constant but there are marked changes in Feulgen-DNA absorption and acridine orange fluorescence as the spermatids mature. Bouters *et al.* (1967) showed marked variations in Feulgen estimates of DNA in rabbit spermatozoa from the epididymis, ampullae, and the ejaculate, which were attributed to aging *in vivo*.

During aging *in vitro* of bull spermatozoa, Salisbury *et al.* (1961) and Hanada and Nagase (1968) have described a reduction in Feulgen-DNA content which has been related to decreased fertilizing capacity and increased embryonic mortality. However, Miller and Blackshaw (1968) could not show any significant changes in the Feulgen-DNA absorbance or acridine orange fluorescence of rabbit spermatozoa stored *in vitro*.

The techniques of measuring the DNA of individual cells have received much attention but comparatively little effort has been made to isolate sources of variation in the measurements (Bahr and Wied 1966).

In the present work the effects of aging bull spermatozoa *in vitro* on DNA and histone content and on the area of the sperm head have been studied and sources of variation in these measurements have been examined.

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II. Materials and Methods

(a) *Semen Preparation*

Bull spermatozoa from normal ejaculates and displaying good initial motility were extended 1 in 8 in yolk citrate diluent (20% egg yolk in 2·9% sodium citrate) containing 1000 units penicillin and 2000 μg dihydrosteptomycin sulphate per millilitre. Aliquots (1 ml) of diluted semen were placed in stoppered tubes (2 ml capacity) and cooled slowly to 5°C. The semen was stored at this temperature for periods of up to 9 days. At intervals samples were selected at random and washed twice in 0·9% NaCl to remove the egg yolk which interferes with the Feulgen staining.

Thin smears were made on quartz (ultraviolet measurements) or selected 0·9 mm glass slides (Feulgen, fast green, and acridine orange measurements), dried in air, and fixed for 10 min in absolute ethanol. The fixed smears were then air-dried and stored at either −196°C (ultraviolet and acridine orange) or −20°C (Feulgen and fast green) until the end of the experiment when the slides were prepared for evaluation.

(b) *Staining Methods*

(i) *Acridine Orange*

Numbered slides were stained in groups containing one slide for each day of storage. In the storage experiments four groups or staining runs constituted one replicate (bull). Staining was done in small (40 ml) plastic containers and fresh reagents were used for each staining run. The method of staining was essentially that of Bertalanffy and Bickis (1956) and the smears were mounted in 0·002% Tween 80 in water.

(ii) *Feulgen and Fast Green*

The semen smears were hydrolysed in 0·1N HCl at 60°C for 9 min (Salisbury, Lodge, and Baker 1964) and then stained by the Feulgen technique of Leuchtenberger (1958). Nuclear histone was stained by fast green (Deitch 1966) after removal of DNA by hot trichloroacetic acid (90°C for 15 min).

Following washing and dehydration the smears were mounted in Cargille immersion liquid ($\eta = 1{\cdot}554$) to minimize light scattering (Bahr and Wied 1966).

(c) *Measuring Equipment*

(i) *General*

The basic optical and photometric system has been described by Salisbury, Lodge, and Baker (1964). In the present experiments a xenon arc (Osram XBO-450) replaced the previous source; a Zeiss M4QII quartz monochromator was placed in the light path and a Zeiss narrow-field telescope was used to collimate the light beam.

(ii) *Fluorescence Measurements*

For fluorescence measurements the optical train comprised an achromatic–aplanatic condensor [numerical aperture (NA) 1·4], and a ×100 apochromatic oil-immersion objective (NA 1·32). Immediately before the substage mirror a BG 12 (2 mm) exciter filter (range 312–509 mm; peak 405 nm) was placed. Barrier filters (Zeiss 50 and 44) were housed in the filter intermediate tube to eliminate exciting wavelengths. An accessory tungsten lamp on a sliding base was used to locate and centre specimens for both fluorescence and ultraviolet measurements.

The instrument response was standardized for each slide; a sperm was focused and the monochromator slit adjusted to 0·2 mm at 405 mm. Neutral density filters in the intermediate tube and the BG 12 exciter filter were selected and the meter reading adjusted to a convenient value. Careful alignment and focusing of the condensor enabled the meter reading to be returned to this arbitrary setting for each slide without altering the sensitivity of the meter in any way.

Barrier filters were selected, the monochromator set to 2·0 mm, and the path to the photo-tube opened; exposure to exciting light was kept to a minimum. Ten cells and adjacent backgrounds were measured on each slide and the difference between the cell and background constituted one observation.

(iii) *Ultraviolet Absorption*

Measurements of ultraviolet absorption were made with essentially the same equipment. Filters were removed, a dry ultraviolet condensor (NA 0·8) stopped down to NA 0·3 and an Ultra-Fluar ×100 (NA 0·85) glycerol-immersion objective were substituted. The monochromator was set at 260 nm and slit 0·4 mm for measuring but preliminary search and focus of sperm was done at 545 nm and slit 2·0 mm.

Absorption measurements were made using a 2 μm plug in centre of the sperm head, which has been shown for Feulgen staining to closely approximate the mean density of the head (Baker, Bouters, and Salisbury 1964). The light absorption of the sperm plug and adjacent background was measured and the absorbance calculated. Non-specific absorption at 310 nm was slight and no correction was made.

(iv) *Measurements after Staining with Feulgen and Fast Green*

An integrating microdensitometer (Barr and Stroud, Glasgow) was used to estimate the absorbance of sperm heads stained with Feulgen (DNA) and fast green (histone). Conditions of measurement were: tungsten lamp 9 V; interference filter—560 nm for Feulgen, 625 nm for fast green; objective ×100, NA 1·25; ocular size ×10; extinction level 1·0. Integrated measurements of total relative absorption were made of the sperm heads and of an adjacent blank area, the difference giving the corrected absorbance. Duplicate measurements of 10 sperm on each slide were made.

(v) *Area of the Sperm Head*

A formula has been developed for estimating the area of bull spermatozoan heads from linear measurements (van Duijn 1960). The validity of the formula for the conditions of the storage experiments has been checked by Salisbury and van Dongen (1963). The formula is as follows:

$$A = l[1{\cdot}050-0{\cdot}225(w/W)](0{\cdot}36w+0{\cdot}69W),$$

where A = area in μm^2; l = total head length; w = width of base of sperm nucleus; W = maximum width of sperm nucleus; each measurement made in microns.

Linear measurements were made under phase-contrast illuminations using a ×100 oil-immersion objective and a Vickers image-splitting measuring eyepiece which was calibrated against a stage micrometer.

III. Results

The effect of aging *in vitro* on acridine orange fluorescence of bull sperm DNA is shown in Table 1. Storage effects were seen as a significant quadratic curvature. There were no differences between staining runs within replicates and variation between sperm on a slide was not significant, but differences between replicates were significant.

A split-plot design with semen from four bulls was used to test the effect of aging on Feulgen-DNA and fast green-histone absorbance of sperm heads (Tables 2 and 3). The only significant sources of variation were the staining techniques, the Feulgen method showing marked variability.

The measurement of the ultraviolet absorbance of sperm heads required quartz slides and the same slides were used to measure the linear dimensions of the heads. Tables 4 and 5 show the ultraviolet absorbance through a 2 μm plug in the centre of the head and the surface area of sperm heads calculated from the linear measurements. Storage of spermatozoa did not affect either parameter but there were significant differences between bulls and duplicate slides.

The standard error of a single observation was calculated using the mean square for spermatozoal variation in the analysis of variance. The mean, standard error, and 5% confidence limits of each parameter studied are given in Table 6.

TABLE 1

EFFECT OF AGING *IN VITRO* ON THE ACRIDINE ORANGE FLUORESCENCE OF BULL SPERM HEADS

Means in arbitrary units

(*a*) Storage period

Storage period (days)	1	2	3	5	9
Means	24·1	22·1	23·4	20·8	27·2

(*b*) Bulls and staining runs

Staining Runs	Bulls			
	1	2	3	4
1	24·6	24·6	20·4	23·9
2	32·3	20·3	17·7	21·0
3	31·2	22·0	17·0	18·4
4	34·0	30·2	19·5	18·5
Bull means	30·7	24·3	18·7	20·4

(*c*) Analysis of variance

Source of variation	D.F.	Sum of squares	Mean square	Variance ratio	*P*
Total	799	69502	—	—	
Replications (bulls)	3	17144	5714·7	21·86	<0·01
Days	(4)	3738	—	—	—
Linear	1	370	369·6	1·41	>0·05
Quadratic	1	1896	1896·0	7·25	<0·01
Cubic	1	531	531·3	2·03	>0·05
Quartic	1	941	940·8	3·60	>0·05
Replications × days	12	7914	659·5	2·52	>0·05
Runs in replications	12	6362	530·2	2·03	>0·05
Within-run variation (sperm)	720	21797	30·3	0·12	>0·05
Residual (error)	48	12547	*261·4*		

IV. DISCUSSION

The maturation of spermatids in the testis has been shown to be associated with marked changes in the capacity of DNA to bind certain dyes and with the content of nuclear histone (Gledhill *et al.* 1966). During the long maturation period in the epididymis Bouters *et al.* (1967) observed a decrease in the Feulgen staining of rabbit spermatozoa, but Gledhill (1966) found no significant quantitative change in DNA, in total dry mass, or in optical area associated with the passage of spermatozoa through the epididymis. Similar results were obtained by Pauffler and Foote (1968).

Nevertheless Gledhill (1966) believed that further qualitative changes were occurring in the spermatozoal deoxyribonucleoprotein complex in the epididymis.

There is ample evidence to show that *in vitro* aging of spermatozoa leads to a reduction in fertilizing capacity and to an increase in embryonic mortality (Salisbury, Bratton, and Foote 1952; Lanman 1968; Salisbury 1968; Salisbury and Hart 1970). It has not been clear, however, whether there are measurable changes in the quantity or quality of spermatozoan DNA during such storage.

TABLE 2

EFFECT OF AGING *IN VITRO* ON THE FEULGEN-DNA OF BULL SPERMATOZOA

Means of integrated arbitrary units

(*a*) Storage period

Storage period (days)	1	2	4	8
Means	7·8	7·9	8·4	7·9

(*b*) Bulls and staining runs

Staining Runs	Bulls				Run means
	1	2	3	4	
1	9·7	9·7	9·5	9·4	9·6
2	6·3	6·6	6·7	6·2	6·4
Bull means	8·0	8·2	8·1	7·8	

(*c*) Analysis of variance

Source of variation	D.F.	Sum of squares	Mean square	Variance ratio	*P*
Main plots					
Days	3	31·06	10·35	2·81	>0·05
Bulls	3	12·40	4·13	1·12	>0·05
Main plot error	9	61·00	6·78	—	
Subplots					
Runs	1	1581·31	1581·31	428·83	<0·01
Runs × days	3	35·20	11·73	1·84	>0·05
Sperm on slides	288	842·75	2·92	0·79	>0·05
Duplicates	320	474·00	1·48	0·40	>0·05
Error	12	44·25	3·69	—	

Chemical analyses of spermatozoan DNA have shown that live boar spermatozoa, in contrast to dead cells, did not lose DNA on storage although there were significant differences between boars in the DNA content of their spermatozoa (Anand and First 1968). Graves and Salisbury (1963, 1966), by use of ^{14}C-labelled glycine, fructose, and glucose have observed incorporation of the radioactive label in DNA of ejaculated bovine spermatozoa during incubation for 4 hr at 37°C. The label from the [2-^{14}C]-glycine, though found in all the bases, was found primarily in thymine. They interpreted their results as evidence for metabolic DNA turnover in the mature spermatozoa.

However, Koefoed-Johnsen, Fulka, and Kopecny (1968) could detect no loss of radioactivity during storage for 10 days of ejaculated rabbit spermatozoa labelled with [^{3}H]thymidine during spermatogenesis. Berchtold, Salisbury, and Graves (1971) studied DNA base ratios by determination of thermal denaturation curves for DNA isolated from ejaculated bovine spermatozoa stored at 4°C for up to 15 days. They could find no differences in the mean temperature (86·43±0·025) of denaturation, indicating no change in the adenine–thymine/cytosine–guanine base ratios due to aging on storage.

TABLE 3

EFFECT OF AGING *IN VITRO* ON THE FAST GREEN-HISTONE OF BULL SPERMATOZOA

Means of integrated arbitrary units

(*a*) Storage period

Storage period (days)	1	2	4	8
Means	6·9	6·6	6·6	6·4

(*b*) Bulls and staining runs

Staining Runs	Bulls				Run means
	1	2	3	4	
1	5·8	6·7	6·5	6·5	6·4
2	6·4	6·9	7·1	7·0	6·9
Bull means	6·1	6·8	6·8	6·8	

(*c*) Analysis of variance

Source of variation	D.F.	Sum of squares	Mean square	*F*	*P*
Main plots					
Days	3	21·88	7·29	0·82	>0·05
Bulls	3	59·32	19·77	2·22	>0·05
Error (*a*)	9	80·23	8·91	—	—
Subplots					
Staining runs	1	34·28	34·28	6·43	<0·01
Days × runs	3	8·70	2·90	0·54	
Sperm on slides	288	239·60	0·83	0·16	>0·05
Duplicates	320	36·25	0·11	0·02	
Error (*b*)	12	64·00	5·33	—	—
Total	639				

The present results can be easily summarized as they indicate that during storage *in vitro* there are no significant changes in DNA and histone content or head area of bull spermatozoa. These observations are not in agreement with the earlier work reported by Salisbury *et al.* (1961). Similar methods have been used by Miller and Blackshaw (1968) who found no changes in the DNA or histone content of rabbit

spermatozoa during *in vitro* storage. In these experiments there was a decrease in the percentage of motile sperm which was correlated with fertility, and this suggests that

TABLE 4

EFFECT OF AGING *IN VITRO* ON THE SURFACE AREA OF BULL SPERM HEADS

(*a*) Storage period

Storage period (days)	1	3	5	9
Surface area (μm^2)	34·05	33·82	34·14	34·15

(*b*) Bulls

Bull No.	1	2	3	4
Surface area (μm^2)	32·92	34·34	34·47	34·43

(*c*) Analysis of variance

Source of variation	D.F.	Sum of squares	Mean square	*F*	*P*
Bulls	3	135·50	45·16	6·61	<0·01
Days	3	5·53	1·84	0·27	>0·05
Bulls × days	9	159·70	17·74	2·60	>0·05
Duplicate slides	16	109·26	6·83	14·65*	<0·01
Sperm on slides	288	134·22	0·466		

* Mean square for duplicates used to test other variates.

TABLE 5

EFFECT OF AGING *IN VITRO* ON THE ULTRAVIOLET ABSORBANCE OF BULL SPERM HEADS

(*a*) Storage period

Storage period (days)	1	3	5	9
Mean absorbance (*A*)	0·163	0·166	0·166	0·165

(*b*) Bulls

Bull No.	1	2	3	4
Mean absorbance (*A*)	0·151	0·169	0·170	0·168

(*c*) Analysis of variance of coded data (10^3A-100)

Source of variation	D.F.	Sum of squares	Mean square	*F*	*P*
Bulls	3	19137	6379	10·63	<0·01
Days	3	393	131	0·21	>0·05
Bulls × days	9	14991	1666	2·98	>0·05
Duplicate slides	16	9595	600	2·31*	<0·01
Sperm on slides	288	74938	260·2		

* Mean square for duplicates used to test other variates.

physiological changes relating to aging in spermatozoa may be found in the midpiece and tail or from other evidence in the acrosome.

Salisbury *et al.* (1961) suggested that spermatozoa might contain a limited reserve of DNA to insure the stability of the genetic material. Bahr and Wied (1966) compared the variance of three spermatozoa measured 20 times each to that of 300 cells and found a significant distribution of DNA in the population of bull spermatozoa. From these data they postulated a characteristic distribution of redundant chromosomal DNA for spermatozoa similar to that of Salisbury *et al.* (1961).

Our analyses of variance show that variation between spermatozoa on a slide was not significant and that a distribution of redundant DNA did not occur under our conditions. The methods of preparation of spermatozoa for nuclear staining and photometry may affect DNA and histone content. Although Henle, Henle, and Chambers (1938) found spermatozoal nuclei to be very resistant to physical stress, the ultrasonic treatment used by Bahr and Wied (1966) to separate sperm heads from tails was very vigorous and may have caused losses from sperm heads leading to the observed variability of the nuclear parameters.

TABLE 6

MEANS, STANDARD ERRORS, AND 5% CONFIDENCE LIMITS FOR THE ESTIMATION OF SPERMATOZOAL DNA AND SURFACE AREA

Parameter	D.F.	Mean	Standard error*	5% confidence limits
Acridine orange fluorescence	720	23·5	5·50	17·5–41·1
Feulgen absorbance (560 nm)	288	8·0	1·71	4·6–11·4
Fast green absorbance (625 nm)	288	6·6	0·91	4·8–8·4
Ultraviolet absorbance (260 nm)	288	0·165	0·016	0·133–0·197
Surface area (μm^2)	288	34·0	0·68	32·7–35·3

* The standard error was calculated using the between sperm mean square of the analysis of variance.

The sources of error in the estimation of DNA by photometric means have been discussed generally by Sandritter (1966), and Bahr and Wied (1966) have examined the importance of some of these for the estimation of sperm DNA. The precision of the present results seems adequate although the coefficients of variation for Feulgen-DNA and ultraviolet absorbance are greater than those obtained by Bahr and Wied (1966). It appears that variations in staining techniques are important sources of error in photometry and for comparisons between treatments to be valid, slides representing each treatment must be stained at the same time. In this way differences in staining procedure can be detected. Photometric evaluation of DNA should be done promptly after staining and the slides in each staining batch presented in random order, observer bias being taken into account by strict anonymity of the slides during staining and evaluation.

V. References

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