Alteration of free sulphhydryl content of rat sperm heads by suppression of intratesticular testosterone

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Summary. Subcutaneous injections of testosterone propionate to adult male rats at a dose of 2.5 or 10 mg/kg body weight, 3 times per week for 7 weeks, resulted in a 75% reduction in serum LH and more than 50% reduction in intratesticular testosterone concentration, but serum FSH levels remained unchanged. The free -SH content, measured as iodo[¹⁴C]acetamide binding, increased by 70–100% in testicular sperm heads after suppression of testicular testosterone, and by 25–30% in caput epididymal sperm heads but was decreased by 70–80% in cauda epididymal sperm heads. These results demonstrate an alteration in the oxidative state of sperm nuclear basic proteins, suggesting incomplete nuclear maturation. These changes may be specific for the suppression of intratesticular testosterone, thus illustrating the androgen dependency of sperm head maturation. The contrast effects noted between the iodo[¹⁴C]iodoacetamide binding by the caput and the cauda epididymal sperm heads indicate that testosterone propionate treatment may affect the mechanisms regulating the oxidation of the sulphhydryl residues in sperm heads during epididymal transit. This alteration may not directly relate to the tissue androgen concentrations.

Introduction

During the final stages of mammalian spermatogenesis, spermatids undergo a series of morphological changes which include the expulsion of cytoplasm, condensation of cell nuclei and development of the species-specific sperm head morphology (Clermont, 1972). Concomitantly, the lysine-rich, somatic histones are replaced by arginine-rich sperm basic proteins which contain cysteine predominating at specific stages of spermiogenesis (Monesi, 1965; Vaughn, 1966; Gledhill, Gledhill, Rigler & Ringertz, 1966; Kopecny, 1970; Meistrich, Reid & Barcellona, 1976). The replacement of histones by the basic proteins and perhaps, to some extent, the formation of disulphide cross-linkage between basic protein molecules are thought to be responsible for the condensation of the sperm nucleus during the final stages of spermatogenesis (Marushige & Marushige, 1974; Meistrich *et al.*, 1976; Grimes, Meistrich, Platz & Hnilica, 1977).

Sperm heads become further stabilized during their transit through the epididymis (Calvin & Bedford, 1971). The binding of iodo-[¹⁴C]acetamide, an alkylating agent used for the measurement of the -SH content, decreases as sperm travel from the caput to cauda epididymidis (Calvin, Yu & Bedford, 1973). The stabilization of sperm heads during epididymal transit is considered a part of sperm maturation and may be important for fertility (Bedford, Bent & Calvin, 1973a).

Androgens are considered essential for the final stages of spermatogenesis (Steinberger, 1971; Vernon, Go & Fritz, 1975), as well as for epididymal function and sperm maturation (Orgebin-Crist, 1969; Bedford, 1975). However, the link between the androgen requirement and the

condensation of sperm nuclei has not been demonstrated. The present study was therefore designed to investigate the effect of the suppression of intratesticular testosterone concentration on sperm nuclei at different stages of maturation by examining the free sulphhydryl content in sperm heads.

Materials and Methods

Animals

Mature male Sprague–Dawley rats (300–350 g) were assigned to one of the three treatment groups to receive 3 times per week (Monday, Wednesday and Friday) for 7 weeks a subcutaneous injection of (1) 0, (2) 2.5 or (3) 10 mg testosterone propionate (Sigma, St Louis, MO, U.S.A.) per kg body weight in 0.1 ml sesame oil. All rats were caged individually in an air-conditioned, light-controlled animal room and were provided with standard rat pellets and water *ad libitum*.

Blood sampling

On the Monday of the 8th week, blood samples were taken from the retro-orbital sinus of each rat under light ether anaesthesia immediately before, and then 2 and 24 h after the last testosterone propionate injection. Animals were then killed by an overdose of ether.

Serum hormone measurement

Gonadotrophins. Serum FSH and LH were measured by radioimmunoassays using reagents provided by NIAMDD, Rat Pituitary Distribution programme. Rat-FSH-RP1, Rat-FSH-I4, anti-rat-FSH-S10 and rat-LH-RP1, rat-LH-I4 and anti-rat-LH-S5 were used for the FSH and LH assays respectively. The conditions of these assays have been reported previously (Huang & Hembree, 1979). The sensitivities of the assays were 50 ng FSH/ml serum and 2 ng LH/ml serum. The intra-assay coefficient of variation was 8.2% for FSH and 8.7% for LH. To avoid interassay variation, FSH or LH were measured in one assay for all samples.

Testosterone. Since serum DHT concentration in the male rat is 5% of the serum testosterone values (Frankel, Mock, Wright & Kamel, 1975) and measurement of testosterone in dilutions of unchromatographed serum extract yielded as good parallelism to the standard curve as did the chromatographed extracts (H. F. S. Huang, I. Dyrenfurth, G. Gunsalus & W. C. Hembree, unpublished observation), testosterone was measured in serum extracts by radioimmunoassay according to the method of Nieschlag & Loriaux (1972) without chromatography. The sensitivity of the assay is 15 pg/ml serum and the intra- and inter-assay coefficients of variation were 3.7% and 11.9%, respectively.

Testicular testosterone determination

Testicular tissues (80–120 mg) were homogenized in 1 ml 0.9% NaCl and extracted 3 times with 5 ml ether. Testosterone was separated from other steroids by thin-layer chromatography and then measured by radioimmunoassay (Nieschlag & Loriaux, 1972). An aliquant equivalent to 10–20% of the chromatographed testosterone fraction capable of replacing 30–70% of radioactive tracer was used in the assay. The recovery of [³H]testosterone after extraction and chromatography ranged between 70 and 80%. The coefficient of variation within the assay was 9.8%.

Epididymal androgen determination

Epididymal tissues (100–150 mg) were homogenized in 2 ml 0.9% NaCl. After the addition of 1000 c.p.m. [³H]testosterone and 1000 c.p.m. [³H]dihydrotestosterone (DHT) the homogenates

were extracted twice with 10 ml ether. Because of the high lipid content in the epididymal tissue, extra steps were used to remove the fat from the extract (Vreeburg, 1975). Briefly, after the ether extracts from each tissue were combined and dried, the residues were dissolved in 5 ml of an acetone–water mixture (70 : 30, v : v) and allowed to stand in a -30°C methanol bath for 30 min. The acetone–water fractions were removed and dried under a stream of air overnight. The residues were then dissolved in 1 ml *n*-heptane:chloroform mixture (8 : 1, v : v). The solution was removed and dried. The residues were dissolved in 100 μl methanol and then subjected to thin-layer chromatography and radioimmunoassay for testosterone and DHT according to the method of Nieschlag & Loriaux (1972). An aliquot equivalent to 20% or 5% of the chromatographed testosterone or DHT fraction, respectively, capable of replacing 30–70% of radioactive tracer, was used in the radioimmunoassay. The recovery of the radioactive tracer after extraction and chromatography was 70–80% and 40–60% for testosterone and DHT, respectively. The intra-assay coefficient of variation was 13.9% for testosterone and 12.7% for DHT.

Measurement of free -SH content

The free -SH residues of sperm heads were measured by the binding of iodo[^{14}C]acetamide according to the following procedure. The testes and the epididymides of each rat were dissected and kept in ice-cold buffer containing 0.025 M-sodium phosphate, 10 mM-Tris-HCl and 0.1 mM-EDTA (pH 7.0) until all rats had been killed. Two thirds of one testis and the caput or cauda epididymidis (separated at the middle point of the corpus) were each homogenized in 10 ml of the same buffer at $2-4^{\circ}\text{C}$. The homogenates were filtered through 3 layers of cheese cloth. The filtrates were centrifuged at 3500 *g* for 15 min at 4°C . The pellets were resuspended in 1 ml (testicular cells) or 0.5 ml (epididymal cells) of the same buffer and dispersed by brief sonication. A 400 μl aliquant of the cell suspension was incubated with 0.1 ml iodo[^{14}C]acetamide (100 nmol; sp. act. 838 000 d.p.m./nmol) in a 25°C water bath for 60 min. These conditions were chosen after a series of preliminary experiments. At the end of the incubation, the reaction was stopped by adding 0.1 ml of 1 mM non-radioactive iodoacetamide then 3 ml ice-cold distilled water. After the samples had been centrifuged at 3500 *g* for 15 min, the pellets were washed 3 times with ice-cold distilled water. The final pellets were stored at -30°C .

Sperm heads were isolated by discontinuous sucrose gradient sedimentation centrifugation after sonication (Calvin, 1976a). The sperm heads were then washed with phosphate buffer. The iodo[^{14}C]acetamide bound to sperm heads was determined by counting the radioactivity of 0.1 ml sperm head suspension in 4 ml scintillation counting solution (Aqualuma, Lumac B.V., The Netherlands). The data are expressed as pmol/ 10^6 sperm heads.

Morphological response of sperm heads to SDS–DTT treatment

An aliquant of 50 μl sperm head suspension was incubated with 500 μl 1% SDS in 0.05 M-sodium borate (pH 9.0) in the presence or absence of 0.002 M-DTT for 15–60 min (Calvin & Bedford, 1971). At the end of incubation 5 ml distilled water were added and the tubes were centrifuged at 330 *g* for 10 min. The pellets were dispersed by gentle pipetting and the extent of sperm head swelling was examined by phase contrast microscopy.

Statistics

Student's *t* test was used to compare the mean values between control and testosterone propionate-treated rats.

Results

Organ weights

In mature male rats 3 injections per week of 2.5 or 10 mg testosterone propionate per kg body weight for 7 weeks resulted in a significant reduction of testicular weights ($P < 0.001$), but epididymal weights were not affected (Table 1).

Table 1. Organ weights and serum hormonal concentration of rats after testosterone propionate treatment

	Dose of testosterone propionate (mg/kg body wt)		
	0 (control)	2.5	10
No. of rats	6	5	5
Testicular wt (mg)	1916 $\pm$ 30	1206 $\pm$ 11***	1501 $\pm$ 13*
Epididymal wt (mg)	629 $\pm$ 17	561 $\pm$ 42	663 $\pm$ 41
Serum hormone conc. (ng/ml)			
Testosterone			
0 h	2.2 $\pm$ 0.6	2.8 $\pm$ 0.5	3.85 $\pm$ 1.5
2 h	1.1 $\pm$ 0.1	12.6 $\pm$ 1.4***	27.18 $\pm$ 2.0***
24 h	1.1 $\pm$ 0.1	3.9 $\pm$ 0.6	11.18 $\pm$ 3.1**
FSH	189 $\pm$ 8	196 $\pm$ 25	180 $\pm$ 44
LH	17 $\pm$ 5	3 $\pm$ 1***	3 $\pm$ 1***
Epididymal androgen conc. (pg/mg)			
Caput			
Testosterone	14.2 $\pm$ 3.4	15.7 $\pm$ 4.1	14.2 $\pm$ 3.8
DHT	22.5 $\pm$ 2.4	29.5 $\pm$ 4.4	28.6 $\pm$ 3.0
Cauda			
Testosterone	5.5 $\pm$ 1.1	3.9 $\pm$ 0.9	5.8 $\pm$ 1.4
DHT	12.0 $\pm$ 1.8	11.7 $\pm$ 2.3	12.2 $\pm$ 2.0

Values are mean $\pm$ s.e.m.

Compared to control group values, * $P < 0.01$, ** $P < 0.05$, *** $P < 0.001$.

Serum hormone concentrations

Serum testosterone levels were comparable in the 3 groups before the last injection was given (Table 1). It increased by 10- or 25-fold, respectively, above that of the control values 2 h after the injection of 2.5 or 10 mg testosterone propionate per kg body weight ($P < 0.001$). The testosterone levels declined by 60–75% 24 h after the injection but remained significantly elevated compared to the control rat values ($P < 0.05$). By 24 h after the last testosterone injection serum LH levels were 75% lower than those of the controls ($P < 0.001$; Table 1), but serum FSH levels were not affected (Table 1).

Intratesticular testosterone concentration

The mean $\pm$ s.e.m. intratesticular testosterone concentrations were 36 ± 4 , 17 ± 3 and 12 ± 0.8 pg/mg tissue in the rats treated with 0, 2.5 or 10 mg testosterone propionate, respectively. The effect of testosterone propionate was significant ($P < 0.01$) for both doses and the degree of reduction appeared to be dose-dependent.

Epididymal androgen concentrations

The epididymal androgen concentrations are presented in Table 1. Testosterone and DHT levels in the caput and cauda epididymidis were not affected by the testosterone propionate treatments although the DHT levels in the caput epididymidis tended to be increased.

Iodo[^{14}C]acetamide binding by sperm heads

The iodo[^{14}C]acetamide binding by testicular sperm heads increased after testosterone propionate treatment (Text-fig. 1). Sperm heads from the caput epididymidis of testosterone propionate-treated rats also bound more iodo[^{14}C]acetamide than did the control rats but sperm heads from the cauda epididymidis showed decreased binding of iodo[^{14}C]acetamide after testosterone propionate treatment (Text-fig. 1).

Text-fig. 1. Binding of iodo[^{14}C]acetamide by sperm heads isolated from (a) testis, (b) caput epididymidis and (c) cauda epididymidis after testosterone propionate treatment. Values are mean $\pm$ s.e.m. for 5 or 6 rats (see Table 1). * $P < 0.05$; ** $P < 0.025$; *** $P < 0.001$ compared with value for control group (0 mg).

Sperm head response to SDS-DTT treatment

The sperm heads isolated by the procedures described are shown in Pl. 1, Figs 1–4. No difference was noted between samples from testosterone propionate-treated animals and those of control animals. After exposure to SDS with or without DTT, the degree of sperm head swelling was comparable between experimental and control rats (Pl. 2, Figs 5 & 6).

Discussion

The present study demonstrates that in mature male rats chronic treatment with testosterone propionate resulted in alteration of the iodo[^{14}C]acetamide binding of sperm heads at different stages of maturation. This result illustrates a change in the character of sperm heads. Cunningham & Huckins (1979) found that daily injections of 25 μg –1 mg testosterone propionate per kg body

weight suppressed testicular testosterone concentrations by 50–90%. In addition, both LH and FSH were suppressed significantly at the dose of 1 mg/kg body weight (Cunningham & Huckins, 1979). In the present study, injection of 2.5 or 10 mg testosterone propionate per kg body weight 3 times per week resulted in 75% reduction in serum LH levels. This decrease in LH was apparently responsible for the >50% reduction in intratesticular testosterone concentration in these rats. In contrast, serum FSH levels were normal at the time when animals were killed. The different serum LH and FSH responses observed in the present study, and the different FSH response between this study and the study of Cunningham & Huckins (1979) may be attributable to the alteration of GnRH pulsing by the fluctuating serum testosterone levels (Steiner, Bremner & Clifton, 1982) thereby modifying the pituitary sensitivity to the steroid negative feedback (Lincoln, 1979; Wildt *et al.*, 1981).

The increased iodo[¹⁴C]acetamide binding by testicular sperm heads indicates an increase in the free -SH content. Although sulphhydryl residues associated with other membrane structures (Bedford, Calvin & Cooper, 1973b) may also react with iodo[¹⁴C]acetamide during incubation, the procedure used to purify sperm heads (extensive sonication in distilled water) was probably sufficient to remove most, if not all, the -SH containing structures except the chromatin-bound basic proteins and, perhaps, the perforatorium. The change in -SH content may therefore reflect a modification in the sperm nucleus. Since the disulphide cross-linkage in testicular sperm heads is negligible (Marushige & Marushige, 1974), the higher free -SH content measured in the testicular sperm heads after testosterone propionate treatment cannot be explained by an insufficient disulphide cross-linkage. Furthermore, the normal sperm head morphology before and after SDS-DTT treatment suggests that the total -SH content or the component of the basic proteins in sperm heads probably was not affected by testosterone propionate treatment. Therefore, the higher -SH contents may result from an increase in the proportion of sperm heads containing S1 protein, which has 3 times more cysteine residue than other basic proteins (Kistler, Geroch & Williams-Ashman, 1973; Calvin, 1976b; Grimes *et al.*, 1977), due to a delay in the transport of spermatozoa from the testis to the epididymis or to the destruction of younger elongated spermatids (containing basic proteins with less cysteine residues) during the preparation of sperm heads. It is also possible that suppression of testicular testosterone concentration results in a modification in the folding of chromatin or the configuration of sperm nuclei, making the -SH residue more available to react with iodoacetamide. Although serum FSH may be suppressed temporarily after each testosterone propionate injection, the normal serum FSH at the time when the animals were killed suggests that the testes of the treated rats probably had been exposed to relatively normal serum FSH levels during the experimental period. Therefore, the change in the sperm head character may represent a specific effect of the reduction of intratesticular testosterone. This is compatible with the concept that androgen is required for the final stages of spermatogenesis (Steinberger, 1971; Vernon *et al.*, 1975).

Stabilization of sperm nuclei during epididymal transit is thought to be regulated by the epididymal environment (Bedford *et al.*, 1973a), which is androgen-dependent (Orgebin-Crist, 1969; Bedford, 1975). In the present study caput epididymal androgen concentrations remained unchanged after testosterone propionate treatments. Yet the binding of iodo[¹⁴C]acetamide by sperm heads recovered from this region had also increased. Since the caput epididymidis receives

PLATE 1

Photomicrographs of rat sperm heads. $\times 160$.

Fig. 1. Testicular sperm heads from control animals.

Fig. 2. Testicular sperm heads from rats receiving 10 mg testosterone propionate.

Fig. 3. Caput epididymal sperm heads from control rats.

Fig. 4. Caput epididymal sperm heads from rats receiving 10 mg testosterone propionate.

PLATE I

(Facing p. 36)

Photomicrographs of rat sperm heads after exposure to SDS-DTT for 60 min, showing similar degrees of swelling of sperm heads from the cauda epididymidis of control rats (Fig. 5) and rats treated with 10 mg testosterone propionate (Fig. 6).

newly released testicular spermatozoa bathed in rete testis fluid, it is not surprising that spermatozoa recovered from this region share the same character with those recovered from the testis. On the other hand, the reduction in iodo[^{14}C]acetamide binding by sperm heads from the cauda epididymidis after testosterone propionate treatment may reflect an enhanced disulphide cross-linkage formation. Since the treatment period (7 weeks) was 3–4 times longer than that required for spermatozoa to travel from the testis to the cauda epididymidis (10–14 days; Hamilton, 1972), the sperm heads recovered from different regions of the epididymis should have been exposed to a common intratesticular environment during their spermiogenic development. Thus, the difference in the iodo[^{14}C]acetamide binding by sperm heads at various phases of maturation probably is not related to the change in the total -SH content of sperm heads. It is more likely to be related to the alteration in the oxidative states of -SH residues of sperm heads. Since spermatozoa are transported in fluid originated from the rete testis or secretions from epididymal cells, the differences observed in the sperm heads from different regions of the epididymis may relate to an abnormal tubular milieu which is not directly related to the epididymal tissue androgen concentration.

Condensation of sperm nuclei requires phosphorylation and dephosphorylation of the sperm basic proteins (Marushige & Marushige, 1975). These modifications of the basic proteins may be important for the formation of disulphide cross-linkages during epididymal maturation (Marushige & Marushige, 1975). On the other hand, the presence of glutathione in rat testis (Calvin & Turner, 1982) and in the spermatozoa and seminal plasma of other species (Li, 1975; Smith, Senger, McCutchan & Landa, 1979) suggests a possible role for glutathione in the protection of sperm cells against oxidation which, in turn, may influence the oxidative state of the basic proteins (Mann & Lutwak-Mann, 1981; Calvin & Turner, 1982). A decrease in intratesticular testosterone concentration and a change in epididymal physiology under the influence of the fluctuating circulatory testosterone concentrations may alter the enzyme systems responsible for the regulation of sulphhydryl oxidation at various phases of sperm maturation, thus resulting in a change in free -SH content.

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