The metabolism and motility of human spermatozoa in the presence of steroid hormones and synthetic progestagens

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Summary. The metabolism and motility of human ejaculated spermatozoa incubated *in vitro* with steroids were studied. Progesterone and norethynodrel depressed the respiration, glycolytic metabolism and the motility of washed spermatozoa. Lynoestrenol did not affect the respiration or glycolysis of the spermatozoa, but did inhibit motility. Oestradiol did not cause any consistent alteration of the sperm metabolism, and did not affect the motility. Progesterone and norethynodrel appear to act on the plasma membrane of human spermatozoa to increase its permeability and hence to facilitate the loss of essential cofactors required for the glycolytic and oxidative processes.

Introduction

Some spermagglutinins, which induce head-to-head agglutination of human spermatozoa, represent a steroid attached to a carrier protein (Boettcher, 1974). This spermagglutinating activity can be induced *in vitro* by the addition of specific steroids such as progesterone to certain serum samples (Boettcher & Kay, 1973) or *in vivo* in the serum of some women taking oral contraceptives for the first time (Boettcher, Hay, Kay, Baldo & Roberts, 1970). Similar spermagglutinating activity can be produced *in vivo* in cervical mucus by intravenous injection of progesterone (Lindahl, Ingelman-Sundberg, Furuhielm & Nilsson, 1956). The naturally occurring spermagglutinating activity of cervical mucus from post-menopausal women can be decreased by the intravenous injection of oestradiol dipropionate (Lindahl *et al.*, 1956).

Several reports indicate that steroids can affect the migration of human spermatozoa *in vivo* (Kesserü-Koos, 1971) and *in vitro* (Beck, Herschel, Hungerschöfer & Schwinger, 1976; Kesserü, Camacho-Ortega, Laudahn & Schöpfli, 1975) and sperm metabolism (Hicks, Pedron & Rosado, 1972; Rosado, Hicks, Aznar & Mercado, 1974). Steroids administered to women by intravenous injection have been shown to pass into the genital tract and appear in cervical mucus (Gallegos, González-Diddi, Merino & Martínez-Manautou, 1970; Laumas, Murugesan & Hingorani, 1971; Reed, Fotherby & Steele, 1973; Reed, Fotherby, Peck & Gordon, 1973) and follicular fluid (Braunenberg, Irvine & James, 1967). It is therefore possible that steroids may exert a direct effect on spermatozoa as they pass through the female genital tract.

In preliminary experiments, an examination of the effect of a range of synthetic progestagens on human sperm migration and motility indicated that the progestagens lynoestrenol and norethynodrel had a strong inhibitory effect on spermatozoa. The present investigation was undertaken to evaluate the metabolism and motility of human spermatozoa in the presence of progesterone, oestradiol, lynoestrenol and norethynodrel.

Materials and Methods

Semen

Samples of semen from normal healthy donors were collected by masturbation into clean glass jars after a minimum of 48 h of sexual abstinence. The samples were allowed to liquefy in an incubator at 37°C before examination. Each semen sample was diluted with 5 volumes of diluent. An

aliquot from each diluted sample was placed on a warm glass slide, covered with a coverslip and examined under a phase-contrast microscope ($\times 500$). One hundred (100) spermatozoa were examined at random and the number of motile spermatozoa was recorded: motility was rated from 0% (no cells motile) to 100% (all cells motile). Sperm concentration was determined by diluting the semen in 10 volumes of 3% (w/v) NaCl solution and using a Petroff-Hausser bacterium counter. The cells were counted under a phase-contrast microscope ($\times 125$) and the final concentrations varied between 0.9×10^8 and 2.6×10^8 spermatozoa/Warburg flask.

Radioactive substrates

Radioactive substrates were obtained from the Radiochemical Centre, Amersham, England, and were added to a Ca-free Krebs-Ringer-phosphate (KRP) diluent (pH 7.4) (Deluca, 1972) to give the following concentrations/Warburg flask: 5 μ mol (500 nCi) D-[U- 14 C]glucose; 10 μ mol (500 nCi) [1- 14 C]acetate, sodium salt; 5 μ mol (50 nCi) α -keto-[5- 14 C]glutaric acid, sodium salt; 5 μ mol (500 nCi) [1,4- 14 C]succinic acid, and 5 μ mol (500 nCi) L-[U- 14 C]malic acid. The succinate and malate solutions were adjusted to pH 7.4 with 0.1 M-NaOH solution. The radiochemical purity of the citric acid cycle intermediates was established by descending paper chromatography with *n*-butanol: glacial acetic acid:water (120:30:50 by vol.) as the solvent system. The radioactive succinic acid and malic acid were used as supplied, but it was necessary to purify the radioactive α -ketoglutarate further by preparative paper chromatography with the above solvent system.

Steroids

Steroid hormones were dissolved in ethanol or in a benzene-ethanol (9:1 v/v) solution and were added separately to Warburg flasks in 0.1 ml solvent. Steroid-free solvent was added to control flasks. The solvent in each flask was evaporated under a stream of air before the addition of substrate and spermatozoa. Oestradiol (oestra-1,3,5(10)-triene-3,17 β -diol) and progesterone (pregn-4-ene-3,20-dione) were from Sigma Chemical Co.; lynoestrenol (17 α -ethynyl-oestr-4-en-17 β -ol) was from Organon (Australia) Pty Ltd, and norethynodrel (17 α -ethynyl-17 β -hydroxyoestr-5(10)-en-3-one) was from Searle Laboratories.

Experiments

Experiments were replicated 3 to 5 times with different pooled ejaculates from at least 5 donors for each replicate. After pooling the ejaculates, the spermatozoa were washed by diluting the semen with 3 volumes of the Ca-free KRP diluent, and centrifuging for 20 min at 400 g. After aspiration of the supernatant, the spermatozoa were again washed in 5 ml diluent and then suspended in 3 ml diluent. The motility of the spermatozoa in the washed preparations varied from 35 to 70%. Unless otherwise stated the washed sperm suspension (0.5 ml) was incubated at 37°C for 3 h with 0.5 ml of the appropriate substrate in 5 ml Warburg flasks containing 0.1 ml CO₂-free 20% (w/v) KOH in the centre well. The rim of the centre well was coated with a thin layer of Apiezon grease to prevent migration of the reaction mixture into the KOH. After allowing a 10-min period for temperature equilibration, the flasks were closed and oxygen utilization was measured manometrically with air as the gas phase and a shaking rate of 80 strokes/min. Substrate oxidation was determined from the assay of 14 CO₂ trapped in the KOH of the centre well. The KOH was transferred to a scintillation vial with a Pasteur pipette and the centre wells of the flasks were twice washed with 0.35 ml water. The combined solutions were counted by liquid scintillation techniques in 10 ml scintillation fluid consisting of toluene:Brydet (2:1 v/v) or toluene:Triton X-100 (2:1 v/v) (Patterson & Greene, 1965) containing 0.4% (w/v) 2,5-diphenyloxazole. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 3385) and all counts were corrected for background. The efficiency of counting was measured by using the external standard ratio method and was found to be constant for each 14 C-substrate. For experiments requiring anaerobic conditions, single side-arm Warburg flasks (6 ml capacity), containing 0.1 ml 20% (w/v) KOH in the centre well and 5 μ mol glucose in 0.5 ml diluent in the side arm, were gassed for 10 min with nitrogen. The stop-cocks were then closed and the glucose solution tipped into the sperm suspension.

In experiments with glucose as substrate, aliquots of the sperm suspension before incubation and the flask contents after incubation were deproteinized by the addition of 5% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1 ml) and 0.3 N-Ba(OH)₂ (1 ml); glucose and lactate were then estimated in the filtrates by enzymic methods (Huggett & Nixon, 1957; Barker & Britton, 1957).

In experiments designed to assess the effects of steroids on the integrity of the sperm membrane, 5 ml Warburg flasks were prepared to contain 0.5 ml sperm suspension, 0.5 ml diluent and 320 nmol steroid. Appropriate control flasks containing no added steroid were also employed. All flasks contained 0.1 ml 20% (w/v) KOH in the centre well and were incubated with air as the gas phase for 3 h at 37°C. At the end of the incubation period, the reaction mixture in each flask was centrifuged at 1500 g for 15 min and the supernatant retained. An aliquot of the supernatant (0.5 ml) was deproteinized with 5% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 ml) and 0.3 N-Ba(OH)₂ (0.5 ml), diluted with distilled water (1.5 ml), centrifuged at 1500 g for 10 min, and measured for absorbance at 260 nm. Allowances were made for absorbance at this wavelength due to the added steroids. The supernatant was also assayed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

Statistical analysis

The significance of the results was assessed by analysis of variance. The interaction mean square was used to calculate the standard errors (s.e.) of the differences between means as presented in the tables, and this was then used in *t* tests.

Results

Norethynodrel effects on the oxidative metabolism of glucose

Various concentrations of norethynodrel were tested to assess the minimum concentration at which the steroid affected the oxidative metabolism of the spermatozoa (Table 1). A concentration of 320 nmol norethynodrel/ml significantly depressed the oxygen consumption and the amount of glucose oxidized by the spermatozoa, but at 32 nmol/ml only the amount of glucose oxidized was affected. Preliminary experiments revealed that if acetate was used as substrate, norethynodrel at a concentration of 32 nmol/ml depressed both the amount of acetate oxidized and the oxygen consumed.

Table 1. The effect of various norethynodrel concentrations on the oxidative metabolism of D-[U-¹⁴C]glucose by washed human spermatozoa

Norethynodrel conc. (nmol/ml)	Oxygen uptake (μl)	Glucose oxidized (μmol)
0 (control)	9.1	0.043
0.32	9.0	0.041
3.2	9.3	0.043
32	9.0	0.038**
320	5.7***	0.022***
s.e.m.	0.18	0.001

Values represent the means of 4 replicates and are calculated per 10^8 spermatozoa per 3 h.

Significantly different from control value, ***P* < 0.01, ****P* < 0.001.

Effect of steroids on the aerobic metabolism of glucose

When low concentrations of steroids (32 pmol/ml) were used (Table 2, Exp. 2A), oestradiol and lynoestrenol had no significant effect on the four metabolic indicators measured. Progesterone decreased the oxygen uptake of the spermatozoa, and enhanced the rate of lactate production but had no effect on the amount of glucose utilized or glucose oxidized. Norethynodrel decreased only the amount of oxygen consumed by the spermatozoa.

The effects of high concentrations of steroids (320 nmol/ml) varied considerably (Table 2, Exp. 2B): oestradiol slightly depressed the oxygen uptake and increased the amount of lactate produced, lynoestrenol increased the amount of lactate produced, and progesterone and norethynodrel markedly depressed the oxidative and glycolytic metabolism of the spermatozoa.

Table 2. The effect of steroids (32 pmol/ml in Exp. 2A, 320 nmol/ml in Exp. 2B) on the aerobic metabolism of D-[U-¹⁴C]glucose by washed human spermatozoa

Exp.	Steroid	Oxygen uptake (μl)	Glucose oxidized (μmol)	Glucose utilized (μmol)	Lactate produced (μmol)
2A	None (control)	8.7	0.030	1.27	2.45
	Oestradiol	8.2	0.030	1.24	2.48
	Progesterone	7.7**	0.030	1.26	2.56*
	Lynnoestrenol	8.2	0.030	1.23	2.39
	Norethynodrel	7.6**	0.030	1.26	2.44
	s.e.m.	0.3	0.000	0.02	0.04
2B	None (control)	8.4	0.039	1.45	2.93
	Oestradiol	7.3*	0.033	1.48	3.36***
	Progesterone	3.1***	0.015***	1.15***	3.05
	Lynnoestrenol	8.5	0.036	1.47	3.17**
	Norethynodrel	4.0***	0.018***	0.92***	2.46***
	s.e.m.	0.49	0.003	0.05	0.07

Values represent the means of 5 replicates and are calculated per 10⁸ spermatozoa per 3 h.

Significantly different from control value, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Energy metabolism in the presence and absence of steroids

The spermatozoa were incubated under anaerobic conditions to determine whether the steroids were having a direct effect on the Embden–Meyerhof pathway. Progesterone and norethynodrel produced a slight inhibition of the rate of anaerobic glycolysis (Table 3, Exp. 3A). Oestradiol, however, resulted in a slight increase in the amount of lactate produced by the spermatozoa but had no significant effect on the amount of glucose utilized. Lynnoestrenol had no significant effect on the anaerobic glycolytic metabolism of the spermatozoa.

Table 3. The effect of steroids (320 nmol/ml) on the metabolism of washed spermatozoa

Experiment			Steroid						
No.	Conditions	Substrate	Reaction measured	None (control)	Oestra- diol	Proges- terone	Lyn- oestrenol	Norethy- nodrel	s.e.m.
3A	Anaerobic	Glucose	Glucose utilized (μmol)	1.63	1.78	1.30**	1.51	1.23***	0.09
			Lactate produced (μmol)	3.05	3.38*	2.57**	2.93	2.22***	0.12
3B	Aerobic	[1- ¹⁴ C]Acetate	Oxygen uptake (μl)	9.7	8.9	2.6***	9.1	4.4***	0.40
			Acetate oxidized (μmol)	0.057	0.052	0.035***	0.053	0.036***	0.003
3C	Endogenous	None	Oxygen uptake	10.4	10.4	2.9***	8.9*	2.9***	0.63

Values represent the means of 3 (Exp. 3A) or 4 (Exps 3B and 3C) replicates and are calculated per 10⁸ spermatozoa per 3 h.

Significantly different from control value, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

The influence of the steroids on the tricarboxylic acid (TCA) cycle was examined in Exp. 3B in which acetate (which directly enters the TCA cycle after conversion to acetyl-CoA) was the substrate. Only progesterone and norethynodrel had any effect (Table 3). Progesterone and norethynodrel also significantly depressed the oxygen consumption of the spermatozoa when incubated without any exogenous energy source (Table 3, Exp. 3C); lynoestrenol produced a small inhibition.

Effect of norethynodrel on the oxidation of labelled citric acid cycle intermediates

As shown in Table 4, norethynodrel depressed the oxygen consumption of spermatozoa incubated in the presence of all three substrates and inhibited the oxidation of the intermediates. α -Ketoglutarate was oxidized by the spermatozoa at a greater rate than either malate or succinate.

Table 4. The effect of norethynodrel (320 nmol) on the oxidation of α -[5- 14 C]ketoglutarate, [1,4- 14 C]succinate and L-[U- 14 C]malate by washed human spermatozoa

	Oxygen uptake (μ l) with:			Ketoglutarate oxidized (μ mol)	Succinate oxidized (μ mol)	Malate oxidized (μ mol)
	Ketoglutarate	Succinate	Malate			
Control	8.9	14.2	10.7	0.0357	0.0110	0.0096
Norethynodrel	1.5	4.9	2.3	0.0175	0.0041	0.0033

Summary of the analysis of variance

Source of variation	d.f.	Variance ratios	
		Oxygen uptake	Substrate oxidized
Effect of steroid (A)	1	396.82**	33.21**
Between substrates (B)	2	37.90**	52.55**
Interaction A $\times$ B	2	1.44	4.95*
Between replicates	3	7.64**	6.28**
Replicate interactions (error)	15	1.05	0.0000196

Values represent the means of 3 replicates and are calculated per 10^8 spermatozoa per 3 h.

* $P < 0.05$, ** $P < 0.01$.

Table 5. The effect of steroids on the motility (as % of motile spermatozoa) of washed human spermatozoa incubated at 37°C in 1 ml KRP diluent containing 10 mM-D-glucose

Exp.	Steroid	Incubation time			
		0 min	30 min	1 h	4 h
5A	None (control)	54	54	54	45
	Oestradiol (320 nmol)	54	54	54	41
	Progesterone (320 nmol)	54	50**	45***	24***
	s.e.m.	0	1.0	1.7	1.8
5B	None (control)	55	55	55	50
	Lynoestrenol (320 nmol)	55	33**	18***	8***
	Lynoestrenol (32 nmol)	55	13***	10***	0***
	Lynoestrenol (3.2 nmol)	55	50	53	35
	Norethynodrel (320 nmol)	55	2***	0***	0***
	Norethynodrel (32 nmol)	55	47	47	3***
	Norethynodrel (3.2 nmol)	55	55	53	32*
	s.e.m.	0	6.7	4.1	7.7

Values represent the means of 4 (Exp. 5A) or 3 (Exp. 5B) replicates.

Significantly different from control value, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Effect of steroids on the motility of spermatozoa

As shown in Table 5, oestradiol had no detectable effect on the motility of the spermatozoa over the 4-h incubation, but the three progestagens had a strong inhibitory effect.

Table 6. Loss of cell components from human spermatozoa caused by the addition of steroids (320 nmol)

Exp.	Steroid	Oxygen uptake (μ l)	Total absorbance of supernatant fluid after 6-fold dilution ($\lambda = 260$ nm)	Total protein in supernatant fluid (μ g/ml)
6A	None (control)	10.4	0.026	230
	Lyncestrenol	7.2**	0.012	280***
	Norethynodrel	3.5***	0.142***	276***
	s.e.m.	0.69	0.0104	9.0
6B	None (control)	11.7	0.042	265
	Oestradiol	11.0	0.004***	299
	Progesterone	2.9***	0.188***	302*
	s.e.m.	0.43	0.0100	16.2

Values represent the means of 3 replicates and are calculated per 10^8 spermatozoa per 3 h.
Significantly different from control value, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Effect of steroids on the integrity of the sperm plasma membrane

Cell components with an absorbance at 260 nm were lost into the supernatant fluid when steroids were added to the spermatozoa (Table 6). All 3 progestagens caused loss of soluble protein from the cell, but only norethynodrel and progesterone caused loss of components with absorbance at 260 nm. The effects of the steroid treatments on the oxygen uptake of the spermatozoa were similar to those of Exp. 3C (Table 3).

Discussion

The large range of steroid doses used in the present investigation varied from concentrations of steroids reported in the female genital tract of women given steroid contraceptives systemically (Laumas *et al.*, 1971) to those which may possibly be expected in the genital tract of women with a steroid releasing intrauterine or intravaginal device (Leone, 1974; Martínez-Manautou, 1975; World Health Organization, 1976). Rosado *et al.* (1974) have suggested the importance of a direct inhibition of sperm capacitation by the uterine secretions of women using progesterone-T devices. Since the maintenance of motility of spermatozoa appears to be important in several species for the passage of the spermatozoa through the egg investments (Yanagimachi, 1966, 1969, 1972), and since the processes of capacitation and the acrosome reaction of spermatozoa are possibly metabolically controlled (Rogers & Yanagimachi, 1975; Rogers, Ueno & Yanagimachi, 1977), the inhibition of sperm metabolism and motility by progesterone observed in the present investigation suggests that one contraceptive effect of the progesterone-T device could be the direct action of progesterone on the spermatozoa.

However, the results with the other progestagens in the present study suggest that they may influence human spermatozoa by two different mechanisms. Progesterone and norethynodrel inhibited both the motility and oxidative metabolism of the spermatozoa and produced a significant loss of 260 nm-absorbing materials from the cells, suggesting that they may act on the sperm plasma membrane to increase its permeability, and hence facilitate the loss of essential cofactors required for glycolytic and oxidative processes. Further work is required to assess whether these steroids directly affect catabolic reactions of spermatozoa.

Lynoeestrenol, however, inhibited motility but did not produce any significant change in oxidative metabolism or cause excessive loss of 260 nm-absorbing material from the spermatozoa. Its action may involve steroid binding sites on the plasma membrane (Hyne & Boettcher, 1977), leading to a disruption of a membrane function associated with sperm motility. The direct action of lynoeestrenol on spermatozoa allows its use as a biochemical probe to investigate a possible sperm-specific effect of progestagens on spermatozoa because flagellar motility is unique to the sperm cell in man.

Further investigations on the direct effect of steroids on spermatozoa may lead to the development of a sperm-specific steroid that would act directly on spermatozoa at low concentrations to affect sperm fertilizing capacity adversely. Such a compound could be released from a reservoir in an intravaginal or an intracervical device to prevent sperm migration.

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