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Effect of Testosterone, Estradiol-17β and Progesterone on the Oxygen Uptake by Bovine Semen, Washed Spermatozoa and Epididymal-Like Spermatozoa

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The effect of testosterone, estradiol-17β and progesterone on the oxygen uptake of bovine spermatozoa in Tris–HCl buffer and saline (NaCl) media containing 500 mg per 100 ml fructose was determined. Testosterone significantly inhibited the oxygen uptake by freshly ejaculated semen, washed spermatozoa and epididymal-like spermatozoa. The effect was greatest with washed and epididymal-like spermatozoa, both of which have the seminal plasma removed. This effect was dependent on the dose of testosterone used with the most effective inhibitory concentration being 25 μg/2.5 × 10^8 spermatozoa/ml.

Estradiol-17β was stimulatory to the oxygen uptake of bovine semen and washed spermatozoa at a concentration of 10 μg/2.5 × 10^8 spermatozoa/ml. Higher concentrations of estradiol-17β decreased the stimulatory effect and could become inhibitory.

Progesterone tended to reduce oxygen uptake by whole semen and washed spermatozoa, but significant inhibition was only obtained when 100 μg progesterone per milliliter was added to whole semen.

These steroids may have some direct effect on spermatozoa *in vivo* in addition to the indirect effects mediated through the reproductive systems.

Naturally occurring steroid hormones exert an important regulatory function over the male and female reproductive systems. Testosterone has been reported to be present in the fluids of the efferent ducts of the bull, ram, dog, rabbit and human (Voglmayr *et al.* 1966, 1970a; White and Hudson, 1968) and therefore in the immediate environment of spermatozoa. Information on steroids present in the luminal fluids of the female reproductive tract is limited. Short (1962) reported estradiol-17β to be the most prominent steroid in bovine follicular fluid.

Baker *et al.* (1949) first reported that testosterone in aqueous suspension depressed the oxygen uptake by bull semen. This observation was confirmed by Gassner and Hopwood (1955) for bull semen and by Mounib (1964) for washed bull spermatozoa, but Scott *et al.* (1963) found no effect of testosterone on bull semen and washed spermatozoa. Other reports show testosterone to depress respiration of ejaculated and testicular ram spermatozoa (Murdoch *et al.*, 1970; Voglmayr *et al.*, 1970b). Progesterone and estradiol-17β to a lesser extent, reduced respiration of washed bull (Mounib, 1964) and ram (Murdoch *et al.*, 1970) spermatozoa. Estradiol-17β also reduced respiration of bull semen (Gassner and Hopwood, 1955).

The purpose of this paper is to compare the effect of testosterone, progesterone and estradiol-17β on oxygen consumption by bovine semen, washed spermatozoa and epididymal-like spermatozoa.

MATERIALS AND METHODS

Bull semen samples used were collected by artificial vagina from bulls maintained for breeding and research at the University of Illinois dairy farm. The collected samples remained stoppered for approximately 1 hour at about 25 C prior to incubation. The spermatozoan concentration was determined by optical density (Salisbury *et al.*, 1943) and spermatozoan motility estimated microscopically prior to use.

Washed spermatozoa were obtained by removing the seminal plasma by centrifugation, resuspending the spermatozoa in sterile physiological saline (0.9% NaCl) and repeating the process once more. Epididymal-like spermatozoa were obtained as reported by Salisbury and Graves (1963). Pooled bull semen samples were used to reduce the problem of variability in metabolism among samples.

The range of steroid concentrations used were chosen to as nearly as possible include levels which other investigators had used, even when the levels exceeded maximum solubility coefficients (Eik-Nes *et al.*, 1954).

Steroids in methanol were added to 10-ml flasks and the methanol evaporated under nitrogen. Spermatozoa and suspension medium were added to the flasks to a final volume of 2 ml. Medium consisted of 0.06 M Tris–HCl buffer, pH 7.0 unless otherwise indicated and 0.9% NaCl containing 500 mg per 100 ml fructose. All flasks were then set at room temperature for 15–30 min before the oxygen uptake was measured to allow the spermatozoa time to assimilate the steroid.

Oxygen uptake was measured using a Clark type polargraphic electrode (Model YSI 5331 Oxygen Probe) and a Biological Oxygen Monitor (Model YSI 53), and recorded with a Sargent Recorder (Model SRLG). The order of samples to be assayed was determined by a table of random numbers.

The incubation procedure consisted of tipping a sample from a flask into a chamber of the bath assembly which had 37 C water circulating in the outside of the chamber. The sample was stirred magnetically and allowed to equilibrate to 37 C saturated with air (approximately 1 min) before the oxygen electrode was inserted. Oxygen consumption by the spermatozoa was linear during the 2-min incubation period recorded. Oxygen uptake by the sample was determined from the percent decrease per unit of time in the oxygen concentration of the saturated solution (YSI Biological Oxygen Monitor Instructions). Since oxygen uptake may be influenced by a number of factors, including spermatozoan concentration (Bishop and Salisbury, 1955), the values reported in this paper are oxygen uptake for the spermatozoan concentration used. Zo_2 values may be calculated from these data.

Statistical procedures and tests were according to Steel and Torrie (1960). The Bayes least-significant difference for multiple-comparison testing was applied according to Carmer (1968) and Duncan (1965).

Table 1

TESTOSTERONE INHIBITION OF THE OXYGEN UPTAKE BY WHOLE BOVINE SEMEN AND WASHED SPERMATOZOA

Spermatozoa[a]	Testosterone (μg/ml)	Mean oxygen uptake[b] (μl/ml/hr)	Percent deviation from control values
Semen	Control	20.1	
	10	20.6	+2.5
	25	19.0	−5.5
	100	16.5**	−17.9
Washed	Control	10.8	
	10	9.1	−15.7
	25	7.8*	−27.8
	100	9.2	−14.8

[a] 1.67×10^8/ml.

[b] Mean of three replicates.

* $P < 0.05$ significant from control.

** $P < 0.01$ significant from control. Error mean square 12.89 (14 df).

RESULTS

Testosterone effect on oxygen uptake by bovine spermatozoa. The data in Table 1 demonstrate testosterone inhibition of the oxygen uptake by whole bovine semen and washed spermatozoa. Analysis of variance showed testosterone had a significant linear effect for both semen and washed spermatozoa ($P < 0.05$). The interaction of testosterone and spermatozoa approached a significant quadratic effect, the inhibition by testosterone being dose dependent. Testosterone inhibited washed spermatozoa in a curvilinear fashion, the most inhibition (27.8%) found was at a concentration of 25 μg/ml. Washed spermatozoa resuspended in buffered saline had reduced oxygen uptake, probably due to the lack of potassium in the suspending medium (Mann, 1964).

In the previous experiment the largest percent inhibition by testosterone of oxygen uptake was with washed spermatozoa. It was then decided to examine the effect of testosterone on the oxygen uptake of epididymal-like spermatozoa since they are collected directly into a wash solution and have the least exposure to seminal plasma.

Table 2 shows testosterone inhibition of the oxygen uptake by bovine epididymal-like

spermatozoa. The analysis of variance showed a significant ($P < 0.05$) quadratic effect of testosterone on epididymal-like spermatozoa. Testosterone was inhibitory in a curvilinear fashion for concentrations from 1 to 100 μg/ml, with the greatest inhibition (36.2%) being at a concentration of 25 μg/ml ($P < 0.005$).

Testosterone was thus inhibitory in a curvilinear fashion with greatest inhibition at the same concentration (25 μg/ml) for both washed spermatozoa and epididymal-like spermatozoa.

Estradiol-17β effect on oxygen uptake by bovine spermatozoa. The first experiment with estradiol-17β was studied at pH 7.0 and at pH 8.5. The latter is closer to the optimum pH for conversion of estradiol-17β to estrone (Hathaway, 1967). Figure 1 shows estradiol-17β stimulation of the oxygen uptake by whole bovine semen and washed spermatozoa at pH 7.0 and 8.5. Analysis of variance showed a significant quadratic ($P < 0.05$) effect due to estradiol-17β. Estradiol-17β was only stimulatory to semen at a concentration of 10 μg/ml (65.2% at pH 7.0; 18.8% at pH 8.5), and had no effect or was inhibitory at 25 and 100 μg/ml. Estradiol-17β was stimulatory to washed

Table 2

TESTOSTERONE INHIBITION OF THE OXYGEN UPTAKE BY BOVINE EPIDIDYMAL-LIKE SPERMATOZOA (ELC)[a]

Testosterone (μg/ml)	Mean oxygen uptake[b] (μl/ml/hr)	Percent deviation from control value
Control	56.2	
1	40.4*	−28.1
5	43.5	−22.6
10	41.7*	−25.8
25	35.8***	−36.3
100	49.4	−12.1

[a] Spermatozoan concentration 2.9 × 10^8/ml.
[b] Mean of two replicates.
* $P < 0.05$ significant from control.
*** $P < 0.005$ significant from control. Error mean square 157.51 (6 df).

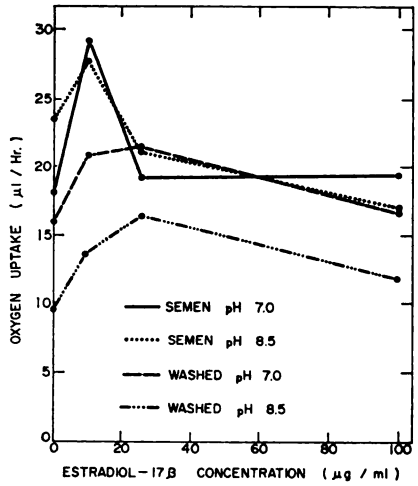

FIG. 1. Estradiol-17β stimulation of the oxygen uptake by bovine semen and washed spermatozoa at pH 7.0 and 8.5.

spermatozoa at 10 and 25 μg/ml (30% and 33%, respectively) at pH 7.0, and stimulatory at 10, 25 and 100 μg/ml (44.7%, 74.5% and 27.6%, respectively) at pH 8.5. The overall oxygen uptake by washed spermatozoa at pH 7.0 was greater than at pH 8.5.

The effect of estradiol-17β at concentrations of 0.5, 1, 5, 10 and 25 μg/ml was studied next. Estradiol-17β again stimulated oxygen uptake of whole semen at 10 μg/ml (34.9%) and at 25 μg/ml (18.6%), and stimulated the oxygen uptake of washed spermatozoa at 10 μg/ml (19.2%). Lower concentrations of estradiol-17β had no effect.

The stimulatory effect of estradiol-17β on bovine spermatozoan respiration and the stimulatory effect by low levels of CO_2 (Lodge and Salisbury, 1962) were examined to see if the lower level of oxygen uptake by washed spermatozoa was due to removal of CO_2 with the seminal plasma, and to find out if the estradiol-17β stimulation was in addition to CO_2 stimulation, as seen with oviduct fluid (Foley and Williams, 1967). Carbon dioxide was added in the form of 0.0038 M $NaHCO_3$ at the concentration found in bovine semen (Salisbury and Van-Demark, 1961).

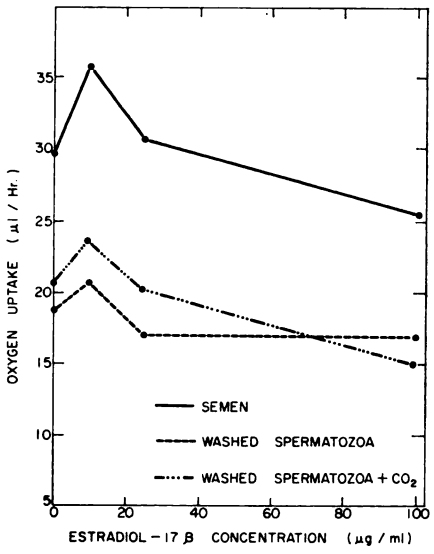

FIG. 2. Estradiol-17β stimulation of the oxygen uptake by bovine semen, washed spermatozoa and washed spermatozoa + CO_2.

Table 3

PROGESTERONE INHIBITION OF THE OXYGEN UPTAKE BY WHOLE BOVINE SEMEN AND WASHED SPERMATOZOA

Spermatozoa[a]	Progesterone (μg/ml)	Mean oxygen uptake[b] (μl/ml/hr)	Percent deviation from control values
Semen	Control	23.1	
	10	19.6	−15.2
	25	19.9	−13.8
	100	18.8*	−18.6
Washed	Control	17.0	
	10	17.7	+4.1
	25	17.0	0.0
	100	14.8	−12.9

[a] 2.5×10^8/ml.

[b] Mean of three replicates.

* $P < 0.05$ significant from control. Error mean square 31.46 (14 df).

Figure 2 demonstrates that there is a great difference in the oxygen uptake by whole semen and washed spermatozoa and that the addition of CO_2 at the level found in semen stimulated (10%) the oxygen uptake by washed spermatozoa. The CO_2 stimulation was insufficient to account for the difference between semen and washed spermatozoa. Estradiol-17β was again significantly ($P < 0.05$) stimulatory to the oxygen uptake by semen at 10 μg/ml (20.5%), by washed spermatozoa at 10 μg/ml (11.8%), and by washed spermatozoa with CO_2 at 10 μg/ml (14.6%). At other concentrations estradiol-17β was either not effective or inhibitory. The stimulatory action of CO_2 and estradiol-17β appear to be independent actions since neither appears to affect the action of the other.

Progesterone effect on oxygen uptake by bovine spermatozoa. Table 3 shows the effect of progesterone on the oxygen uptake by whole bovine semen and washed spermatozoa. Only 100 μg/ml progesterone significantly ($P < 0.05$) inhibited oxygen uptake and that was with whole semen.

DISCUSSION

Steroid effect on spermatozoan oxygen uptake. Testosterone was inhibitory to bovine spermatozoan oxygen uptake, which is in agreement with previous reports (Baker *et al.*, 1949; Gassner and Hopwood, 1955; Mounib, 1964). Inhibition was greatest when the seminal plasma was removed to obtain washed and epididymal-like spermatozoa. Testosterone was more effective at a lower concentration (25 μg/ml) with washed and epididymal-like spermatozoa than with whole semen. Progesterone was also inhibitory to bovine spermatozoan oxygen uptake, in agreement with Mounib (1964). In contrast to the testosterone effect, progesterone was more inhibitory with whole semen than with washed spermatozoa.

Estradiol-17β was stimulatory to the spermatozoan oxygen uptake of both whole semen and washed spermatozoa. The stimulatory effect was limited to 10–25 μg/ml. Concentrations less than 10 μg/ml had no effect. Higher concentrations were either not effective or somewhat inhibitory. The de-

crease in oxygen uptake with the higher concentration of estradiol-17β is in agreement with the report of Mounib (1964) in which a comparably high concentration of estradiol-17β was used. The report by Gassner and Hopwood (1955) showed estradiol-17β to be inhibitory. The dose dependency effect of estradiol-17β and the relationship of steroid concentration to spermatozoan concentration (Wester *et al.*, 1971) may explain the differences of the two reports.

The contrasting effects of testosterone and estradiol-17β on spermatozoan oxygen uptake found here have also been reported for both normal and cryptorchid rat testicular tissue. Vera Cruz *et al.* (1970) reported testosterone propionate significantly depressed oxygen consumption, whereas low concentrations of estradiol benzoate (15, 30 and 45 μg/ml) were stimulatory.

Some of the steroid hormone levels used exceeded their solubility in buffered media (Eik-Nes *et al.*, 1954). However, inclusion of 5% protein in the media increases solubility of the hormones to all of the values used in the present study. If the added spermatozoa bind steroids as Ericsson *et al.* (1967) showed with rabbit spermatozoa, then most of the added steroid could be in solution bound to the spermatozoa and seminal fluids. Preliminary studies (Wester and Foote, unpublished) with tritiated testosterone indicate that the addition of semen to the incubation flask increases the solubility of testosterone in the media. If this result is true for the other steroid hormones tested, the concentrations used would have been the effective concentrations in the medium during sperm incubation.

Environment of spermatozoa. Spermatozoa in the male reproductive system are in a quiescent state with minimum metabolism. The potential for metabolism and motility is present as shown with testicular (Voglmayr *et al.*, 1966) and epididymal (Graves and Salisbury, 1960) spermatozoa. Testosterone is present in testicular fluid (Voglmayr *et al.*, 1966, 1970a), and in epididymal fluid (White and Hudson, 1968). With its presence in the immediate environs of spermatozoa, testosterone may have some direct effect on the sperm cells in the male tract.

Seminal plasma may have reduced the testosterone effect by coating the spermatozoa with protein and reducing access of testosterone to spermatozoa. This seems unlikely since the effect of progesterone was more pronounced with whole semen than with washed spermatozoa.

At ejaculation spermatozoa are mixed with seminal fluids and immediately deposited in the environment of the female reproductive fluids. Information on steroids present in the female reproductive fluids is limited. Progesterone presumably would be relatively low at the time of mating. Estradiol-17β is the prominent steroid in bovine follicular fluid (Short, 1962) and would be expected to be released with the ovum at ovulation. Whether this release of estradiol-17β would be a stimulus to spermatozoa as found in the present *in vitro* study is unknown. Bovine female genital fluids are stimulatory to spermatozoan respiration, with follicular fluid giving the greatest stimulation (Olds and VanDemark, 1957). The stimulus factors for oviduct and uterine fluids have been attributed to substrates present in the fluids and to a nondialyzable, heat labile factor (Iritani *et al.*, 1969).

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