Ouabain Effect on Bovine Spermatozoan Motility and Testosterone Binding (36709)

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Testosterone stimulates motility of bull spermatozoa (3). Farnsworth (1, 2) reported that testosterone stimulated the ouabain sensitive Na^+-K^+ dependent ATPase formation in the microsomal fraction of rat prostate. Bovine spermatozoa also contain ouabain sensitive Na^+-K^+ dependent ATPase (4), where ouabain irreversibly binds to the cell membrane of the midpiece-tail fraction (5). Therefore, the present study was designed to examine possible competitive binding between ouabain and testosterone and the effects on spermatozoan motility.

Materials and Methods. Semen samples were collected from bulls maintained for breeding at Eastern Artificial Insemination Cooperative, Inc. Spermatozoan concentration was determined, and each incubation vial contained 2.5×10^8 spermatozoa/ml in physiological saline with appropriate additives.

Testosterone was added to incubation flasks in methanol and evaporated to dryness under a stream of nitrogen before adding the suspension of spermatozoa. Wester, Foote and Salisbury (3) reported that the concentration of testosterone required to stimulate spermatozoan motility decreased as the spermatozoan concentration decreased. From this relationship testosterone concentrations were chosen which were effective for the spermatozoan concentration used. Ouabain was dissolved in the physiological solution used for incubation. When spermatozoa received the ouabain–testosterone treatment sequence they were first incubated with ouabain, and at the appropriate time they were put in flasks containing only crystalline testosterone. In the testosterone–ouabain sequence spermatozoa were first incubated with testosterone in saline (1.9 ml); then 0.1 ml ouabain was added to give the final volume of 2.0 ml. This way spermatozoan concentration was kept relatively constant at 2.5×10^8/ml.

The semen was incubated aerobically at 37° and the samples were stirred hourly to assure a homogeneous suspension. The percentage of motile spermatozoa and rate of progressive movement (0–4) scale) was estimated visually at hourly intervals with the aid of a microscope equipped with a stage warmer. Motility was then expressed as the product of percentage $\times$ rate. Samples were evaluated in a random order and the identity was unknown to the evaluator.

The effect of ouabain on testosterone binding to spermatozoa was tested by preincubating 2.5×10^8 spermatozoa/ml with 10^{-4} *M* ouabain for 30 min before incubating with tritiated testosterone. O'Donnell and Ellory (5) reported that with 5×10^8 spermatozoa/ml a ouabain concentration of 5×10^{-6} *M* was high enough to saturate most of the transport sites. Since ouabain binds irreversibly (5) most of the transport sites in the present experiments should be ouabain bound before incubation with tritiated testosterone. After incubation, spermatozoa were separated from the media by centrifugation. Except where noted, spermatozoa were then washed by suspending the cells with 0.9% NaCl solution and centrifuged again. Aliquots of the media plus wash and spermatozoa were dissolved in NCS solubilizer (Nuclear Chicago) and counted in a Nuclear-Chicago Mark II liquid scintillation system. The decanted incubation tubes were rinsed with methanol for residual testosterone, the cpm of which were added to the media plus wash cpm. If ouabain and testosterone bind at the same site,

TABLE I. Effect of Ouabain on Bovine Spermatozoan Motility.

Ouabain (M)	Incubation time at 37° (hr)				
	1	2	3	4	5
	(motility = percentage × rate)[a]				
0	235.4	190.6	120.2	33.8	6.0
10^{-3}	209.0[b]	127.2[b]	48.2[b]	5.9[b]	0
10^{-4}	209.6[b]	141.4[b]	78.0[b]	6.7[b]	0
10^{-5}	211.2[b]	141.8[b]	56.2[b]	7.4[b]	0
10^{-6}	229.3	141.3[b]	71.6[b]	10.2[b]	0
10^{-7}	229.4	184.8	98.8[b]	25.6	6.0

[a] Mean of 10 replicates, 5 on each semen sample from 2 bulls.

[b] $p < .005$ significant from control within the same time period.

the preouabain treated spermatozoa should contain less tritiated testosterone than control spermatozoa not exposed to ouabain.

Statistical significance of the results was assessed by analysis of variance and differences between means were determined by Bayes least-significant difference for multiple-comparison testing (6).

Results. Ouabain effect on spermatozoan motility. The effect of different ouabain concentrations on spermatozoan motility is shown in Table I. By the first hour of incubation ouabain concentrations of 10^{-3} to 10^{-5} M reduced spermatozoa motility. The inhibitory range of ouabain was extended to 10^{-6} M by the second hour of incubation, and to 10^{-7} M by the third hour.

The effect of 10^{-4} M ouabain was further tested for an effect on spermatozoan motility with 20 replicates and the results presented in Table I were confirmed. The 10^{-4} M level of ouabain was selected for studies of possible ouabain–testosterone interaction, using the same spermatozoan concentration of 2.5 × 10^8 cells/ml.

Interaction of ouabain and testosterone. Motility was estimated for spermatozoa treated with 10^{-4} M ouabain and 10 μg/ml testosterone, singly and in combination (Table II). Ouabain again inhibited spermatozoan motility, and testosterone significantly ($p < .05$) stimulated motility. In combination there was a significant interaction ($p < .05$) between ouabain and testosterone which was also significant ($p < .01$) with time. Also, at the second hour of incubation the combination of ouabain and testosterone greatly ($p < .005$) inhibited motility, and the effect was greater than with ouabain alone. In this study the molar concentration of testosterone was one-third that of ouabain.

In the next experiment spermatozoa were incubated with equimolar amounts of testosterone (30 μg/ml) and ouabain (10^{-4} M) in various combinations (Table III). Ouabain significantly ($p < .005$) inhibited motility and testosterone showed a characteristic significant ($p < .005$) stimulation. When combined, the effect of equimolar amounts of ouabain and testosterone during the first hour of incubation was intermediate between the individual effects. Subsequently the effect was similar to ouabain alone. Preincubation with ouabain for 30 to 45 min followed by the addition of testosterone resulted in spermatozoan motility being inhibited throughout the incubation, as with ouabain alone. When the spermatozoa were preincubated with testosterone followed by ouabain, motility during the first hr was intermediate between the individual effects. The motility was similar to that obtained when ouabain and testosterone were added at the same time. After the first hour of incubation motility was inhibited as with ouabain alone.

TABLE II. Effect of Ouabain and Testosterone on Bovine Spermatozoan Motility.

Treatment	Incubation time at 37° (hr)			
	1	2	3	4
	(motility = percentage × rate)[a]			
Control	258.3	157.3	104.1	57.4
Testosterone (10 μg/ml)	245.5	175.2[d]	112.5	64.1
Ouabain (10^{-4} M)	242.8[d]	144.9	77.4[b]	42.3
Ouabain (10^{-4} M) and testosterone (10 μg/ml)	242.0[d]	118.6[b]	84.1[c]	35.6[d]

[a] Mean of 15 replicates, 5 on each semen sample from 3 bulls.

[b] Significant from control within the same time period, $p < .005$; [c] $p < .01$; [d] $p < .05$.

TABLE III. Effect of Ouabain and Testosterone on Bovine Spermatozoan Motility.

Treatment	Incubation time at 37° (hr)			
	1	2	3	4
	(motility = percentage × rate)[f]			
Control	199.6[b]	145.6[b]	60.8[b]	30.3[a]
Testosterone (30 μg/ml)	220.6[a]	156.6[a]	79.1[a]	34.0[a]
Ouabain (10^{-4} *M*)	159.4[d]	117.5[c]	44.7[c]	11.5[b]
Ouabain + testosterone	181.4[c]	115.6[c]	38.2[c]	21.7[ab]
Preouabain (30–45 min), then testosterone	139.9[e]	116.8[c]	37.2[c]	27.4[a]
Pretestosterone (30–45 min), then ouabain	182.8[c]	122.1[c]	44.8[c]	20.8[ab]

[f] Mean of 10 replicates, 5 on each semen sample of 2 bulls.

Any two means not followed by the same superscript *a*, *b*, *c*, *d*, or *e* are significantly ($p < .05$) different.

Distribution of testosterone between spermatozoa and media of semen pretreated with ouabain. Experiments were next designed to test whether ouabain was directly prohibiting testosterone action by binding to spermatozoa at a site common to both. Control and preouabain (10^{-4} *M*) treated spermatozoa were incubated with testosterone (30 μg/ml) containing trace amounts of tritiated testosterone. Spermatozoa and the media plus washing solution were separated by centrifugation and assayed for total radioactivity.

Table IV shows the proportion of testosterone associated with spermatozoa and media after a 2-hr incubation at 37°. Table V shows corresponding values after incubation for 30 min followed by centrifugation at different speeds to insure complete separation of spermatozoa and media. In none of the experiments did ouabain affect the uptake of testosterone. All of the radioactive testosterone was recovered bound to spermatozoa or in the media and washing. Testosterone binding by different semen samples varied significantly ($p < .005$). This would account for the small differences in levels of bound testosterone for the experiments in Tables IV and V. From these values the amount of testosterone bound by spermatozoa can be estimated on the order of 2–4 μg/10^8 spermatozoa.

Discussion. Concentrations of 10^{-4} to 10^{-7} *M* ouabain were inhibitory to bovine spermatozoan motility, with inhibition being more rapid at the higher concentrations. After 1 hr of incubation the lowest inhibitory level of ouabain was 10^{-5} *M*. O'Donnell and Ellory (7) reported that 5×10^{-7} *M* ouabain had no inhibitory action on motility of washed boar and ram spermatozoa after 30 min of incubation. Testosterone was stimulatory as previously reported (3).

TABLE IV. Tritiated Testosterone in Control and Preouabain Treated Sperm (2 hr).

Treatment	Testosterone (% ± SD)[a]			Motility (percentage × rate)
	Sperm	Media + washing	Total	
Control				140.3
Ouabain (10^{-4} *M*)				98.5[b]
Testosterone (30 μg/ml)	25.4 ± 3.3[NS]	81.9 ± 3.7	107.3	158.8
Preouabain	27.5 ± 3.5[NS]	73.6 ± 3.3	99.0	81.5[b]
Ouabain + testosterone	23.1 ± 2.1[NS]	75.9 ± 3.4	101.1	99.2[b]

[a] Mean of 8 replicates, 2 on each of 4 ejaculates. Centrifuged at 1200*g* for 30 min. NS = nonsignificant.

[b] $p < .005$ significantly different from control.

TABLE V. Tritiated Testosterone in Control and Preouabain Treated Sperm (30 min.).

Expt.[a]	Treatment	Testosterone (% ± SD)		
		Sperm	Media + washing	Total
I	Control	31.2 ± 2.5	74.2 ± 3.0	105.4
	Preouabain	31.2 ± 3.6	73.8 ± 3.2	105.0
II	Control	13.4 ± 2.0	94.8 ± 3.0	108.2
	Preouabain	12.2 ± 1.8	95.6 ± 3.4	107.8
III	Control	24.5 ± 3.0	69.6 ± 2.9	94.1
	Preouabain	25.0 ± 2.2	66.2 ± 3.0	91.1

[a] (I): Washed sperm centrifuged at 1200*g* or 6500*g* for 30 min. Means of 24 replicates from 8 ejaculates. (II): Washed sperm centrifuged at 12,000*g* for 45 min. Means of 8 replicates from 2 ejaculates. (III): Unwashed sperm centrifuged at 12,000*g* for 45 min. Means of 8 replicates from 2 ejaculates.

Pretreatment with ouabain completely blocked the stimulatory effect of equimolar testosterone added subsequently. Pretreatment with testosterone partly blocked the effect of ouabain during the first hour of incubation. This antagonistic effect of the two suggested a possible common site of action. However, pretreatment with ouabain failed to block the binding of testosterone to spermatozoa, clearly disproving a common site. Testosterone is reported to regulate the Na^+-K^+ dependent ATPase of rat prostate (1, 2). The ouabain sensitivity of the reaction probably represents a different mechanism. Yet, the sensitivity that ouabain appears to have on testosterone action and the partial effect that testosterone has on ouabain action suggest that they may be acting through similar metabolic pathways.

When testosterone and ouabain were incubated together the combined effect on spermatozoan motility in the first hr was intermediate between the individual effects only when equimolar amounts were used. With ouabain and one-third the amount of testosterone the effect was greater than with ouabain alone. This latter result satisfies the definition of synergism where a hormone in lower concentration increases the effectiveness of another compound that is present with it (8). Only the effects of individual steroid hormones on spermatozoa have been measured. Whether steroid hormones added in combination have a synergistic effect remains to be seen.

This report also shows that bovine spermatozoa have the capacity to bind testosterone. Ericsson, Cornette and Buthala (9) reported *in vitro* and *in vivo* binding of testosterone, progesterone and 17β-estradiol by rabbit spermatozoa. The ability of spermatozoa to bind steroids is a logical prerequisite for their demonstrated capacity to convert steroid hormones (10) and for the effects these hormones have on spermatozoan motility and energy metabolism (3).

Summary. Spermatozoan motility of whole bull semen incubated aerobically at 37° in 0.9% NaCl saline was inhibited by 10^{-3} to 10^{-7} *M* ouabain, an effect opposite to that observed for testosterone. Preincubation for 30 to 45 min with ouabain blocked the effect of testosterone, while preincubation with testosterone only partly blocked the effect of ouabain. When equimolar ouabain and testosterone were incubated together motility of spermatozoa initially tended to be intermediate between the individual effects of ouabain and testosterone. However, by the second hour of incubation ouabain inhibition was similar to that observed in ouabain treatment alone. Ouabain showed a synergistic effect when testosterone was added at only one-third the molar concentration of ouabain.

The ability of ouabain and testosterone to block the action of each other suggests that their mechanisms are interrelated. However, they do not appear to have common binding sites because pretreatment of spermatozoa with ouabain (which is irreversibly bound)

did not diminish the capacity of spermatozoa to bind testosterone.

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