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4(R) - 5, 10, SECO 19 – NORPREGNA 4,5 DIENE 3, 10, 20 TRIONE (SECO), AN INHIBITOR OF EPIDIDYMAL 5α–REDUCTASE ARRESTS SPERM FORWARD MOTILITY IN WISTAR RATS

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SUMMARY

The effect of 4(R) – 5, 10, Seco 19 – norpregna 4,5 diene 3, 10, 20 trione (SECO), a potent inhibitor of 5α-reductase, an intracellular enzyme that converts testosterone to dihydrotestosterone (DHT), on epididymal sperm count and motility was studied in Wistar rats. Administration of (i.m.) 250, 500, 750, 1000 µg SECO/Kg b.wt/day to mature rats for 45 days impaired sperm forward motility in a dose dependent manner. Epididymal weight and sperm number decreased in rats treated with 750 and 1000 µg SECO, whereas weight of other accessory organs decreased in rats treated with 1000 µg SECO only. The epididymal histology showed degraded sperm and increased number of clear cells indicating high endocytotic activity, to clear dead sperm, whereas no obvious change was observed in the testicular histology. SECO treatment increased testosterone concentration whereas, it decreased estradiol concentration in circulation and in the epididymal tissue. SECO treatment specifically decreased 5α-reductase in epididymis without affecting the same in the liver, indicating its targeted effect on the epididymis. Thus, the preliminary *in vivo* study points out that SECO impairs the acquisition of sperm forward motility.

Key words: Epididymis; 5α-reductase inhibitor; SECO; Sperm forward motility.

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INTRODUCTION

The epididymis, which plays a pivotal role in post-testicular sperm maturation and storage, has been rooted as an alternative site for intervening male fertility (1). Androgens are the major regulators of epididymal structure and function (2), and the epididymis derives a major portion of testosterone from the testis through the rete testicular fluid, bound to androgen binding protein (3). 5α-Dihydrotestosterone (DHT), the most potent androgen, which promotes sperm maturation and fertilizing ability (4) is formed predominantly in the epididymal principal cells from testosterone. Therefore, prevention of the action or synthesis of epididymal DHT is considered as one of the important areas of male fertility regulation (5). Inhibition of the enzyme 5α-reductase, which catalyzes the conversion of testosterone into DHT in the epididymal principal cells, is one such approach in this direction (6).

Many non-steroidal and steroidal compounds have been designed and suggested as competitive and non-competitive inhibitors of 5α-reductase (7, 8). 4 (R) 5, 10, Seco 19 norpregna 4, 5 diene 3, 10, 20 trione (SECO) is a non-competitive, irreversible inhibitor of 5α–reductase and was reported to be specific to the epididymis (9). In the present study, we report a dose dependent inhibitory effect of SECO on epididymal sperm count and motility *in vivo*.

MATERIALS AND METHODS

Chemicals : Charcoal and dextran were obtained from Sigma Chemical Co., St.Louis, USA. Diphenyloxazole (PPO), bis (5-phenyl-2-oxazole) phenyl-2, 2ß phenyl bis 5 –phenyl oxazole (POPOP), ethyl acetate; iso-octane (HPLC grade), benzene (analytical grade), ethylene glycol, propylene glycol and toluene (scintillation grade) were obtained from SRL, Mumbai, India. 2, 4, 6, 7, ^{3}H - estradiol (85-110 Ci/mmol) and 1, 2, 6, 7, ^{3}H - testosterone (70-105 Ci/mmol) were obtained from Amersham International Plc., U.K. ^{125}I was supplied by the Board of Radiation and Isotope Technology (BRIT), Mumbai, India. 4 (R) – 5, 10, Seco – 19 – norpregna 4, 5 diene 3, 10, 20 trione (SECO) was a gift from Prof. C. H. Robinson, Johns Hopkins University, Baltimore, U.S.A. Testosterone standards were supplied by World Health Organization under SMA programme. Testosterone and estradiol antibodies were a gift from Dr. Coralie Munroe, Endocrinology Lab, School of Veterinary Medicine, Davis, California, U.S.A. Rat RIA kits for LH and FSH were a gift from the National Institute of Arthritis, Kidney and Digestive Diseases (NIDDK), Bethesda, U.S.A.

Animals : Mature (120 days old) albino rats of Wistar strain, weighing 225 ± 10 g were used in the present study. They were maintained in an air conditioned animal quarter (25 ± 2°C) with a lighting schedule of twelve hours light and twelve hours dark. The rats were fed with a standard rat pellet diet (Gold Mohur, Hindustan lever, India) and drinking water ad *libitum*.

Experimental Design : Four groups of five rats each were administered SECO intramuscularly every day at a concentration of 250, 500, 750 and 1000 µg per kg body weight, respectively

for 45 days. The control rats were injected with 0.25 ml vehicle (propylene glycol) alone for 45 days. Body weight was recorded before, during and after the experimentation. Blood was collected in individual siliconised tubes, sera separated and stored at -20°C until assayed for hormones. The epididymis and other accessory sex organs were autopsied, cleaned from adhering fat, connective tissues and blood vessels and weighed. Epididymis was divided into caput, corpus and cauda (1) and used for various analyses, after removing sperm. Caput and caudal epididymidal sperm were removed by fine mincing with a sharp razor blade and gentle teasing in Kreb's Ringer phosphate buffer (KRP buffer; pH 6.9). The caudal sperm were separated by retrograde flushing through proximal vas deferens and then subjected to the assessment of sperm motility.

Assessment of sperm forward motility: The forward motility of spermatozoa was assessed after killing the animal following the method of Ratnasoorya (10). Epididymal fluid with sperm was transferred to an equivalent volume of physiological saline and mixed well. A drop of this suspension was transferred onto a clean dry glass slide immediately. Ten random fields were examined at 40X magnification using a light microscope with pre-cultivated oculometer. The time taken for a spermatozoon to move a distance of 20 µm in a straight line was recorded using a stopwatch. Motility of atleast 25 sperm in ten fields each was calculated.

Sperm count: The number of sperm collected from each epididymal region was counted as per the procedure described in WHO manual (11). One drop was added to each side of an improved Neubauer's blood cell hemocytometer. The number of spermatozoa in the appropriate squares of the hemocytometer was counted under the microscope at 10X. Both sides of the hemocytometer were counted and the average taken. The number of spermatozoa was calculated as follows:

Sperm concentration = No. of spermatozoa x multiplication factor x dilution factor Counted

Total sperm count = Sperm concentration x sperm volume

The sperm count was expressed as number of sperm per selected epididymal region.

Histological studies : One testis and one epididymis from each animal were fixed in 10% formalin for 24 hours and embedded in paraffin, sectioned and stained with haematoxylin-eosin. The slides were examined with light microscope.

Epididymal epithelial cell height, and mean diameter of the tubules were calculated by the method of Romppanen (12).

Seminiferous tubular and its lumen diameters were also measured with an oculometer in order to study the impact of SECO on testicular architecture (13).

5α-Reductase activity in the caput and cauda epididymidal and hepatic tissues were estimated by the radiometric method of Robaire *et al.* (14). In brief, tissues were homogenized in Kreb's Ringer Phosphate (KRP) buffer pH 6.9 and filtered through nylon mesh (93µm). The

filtrate was centrifuged at 1500 x g for 10 min and the pellet was washed thrice with KRP buffer. The washed pellet was used to prepare nuclear fraction. 5α-reductase activity was assessed by incubating appropriate volumes of nuclear fractions in KRP buffer containing 5 x 10^{-4}M NADPH, 5% glycerol, 5 x 10^{-7} M testosterone and 0.05 μCi (1, 2, 6, 7 ^{3}H) testosterone for one hour at 22°C. DHT was extracted with ethyl acetate and was separated with thin layer chromatography and the radioactivity was counted in a liquid scintillation counter. The protein content of the tissue was determined by the method of Lowry *et al.* (15).

Hormone assays : Serum testosterone and estradiol were assayed by standard RIA method. Testosterone and estradiol from epididymis were extracted by the method followed by Jean-Faucher *et al.* (16). Rat LH and FSH were iodinated by chloramine-T method with carrier free ^{125}I as described by Greenwood *et al.* (17) and used for RIA using specific antibodies and reference preparations. Sensitivity of these assays were: 5.0 pg/ml for testosterone, 0.03 pg/ml for estradiol, 0.14 ng/ml for LH and 0.20 ng/ml for FSH. Interassay variation was 6.2 – 10.2% for testosterone, 8.2 – 9.60% for estradiol, 9.9% for LH and 12.2% for FSH. Intraassay variation was 3.20 – 4.5% for testosterone, 4.0 – 6.0% for estradiol, 8.48% for LH and 8.90% for FSH.

Statistical Analysis : Statistical evaluation of the data was performed using Students 't' test and values were expressed as mean ± SEM.

RESULTS

SECO treatment did not modify the body weight. Most of the doses of the SECO used did not alter the weights of accessory sex organs, except 1000 μg dose which decreased the relative weight of epididymis (49.2%), ventral prostate (11.5%) and seminal vesicles (18%) ($P < 0.001$) (Table 1).

SECO treatment altered the histology of the caput and cauda epididymides. While SECO treatment did not affect the morphology of the principal or basal or halo cells of the caput and cauda epididymides, it increased the number of clear cells. The clear cell population was more in the cauda epididymidis of experimental rats as compared to controls (Plate 2A, 2B, 2C and 2D). Caput and cauda epididymidal lumen of rats treated with 750/1000 μg of SECO were having less number of sperm, which were predominantly dead and showed clumping and disintegration (Plate 1A, 1B, 1C and 1D).

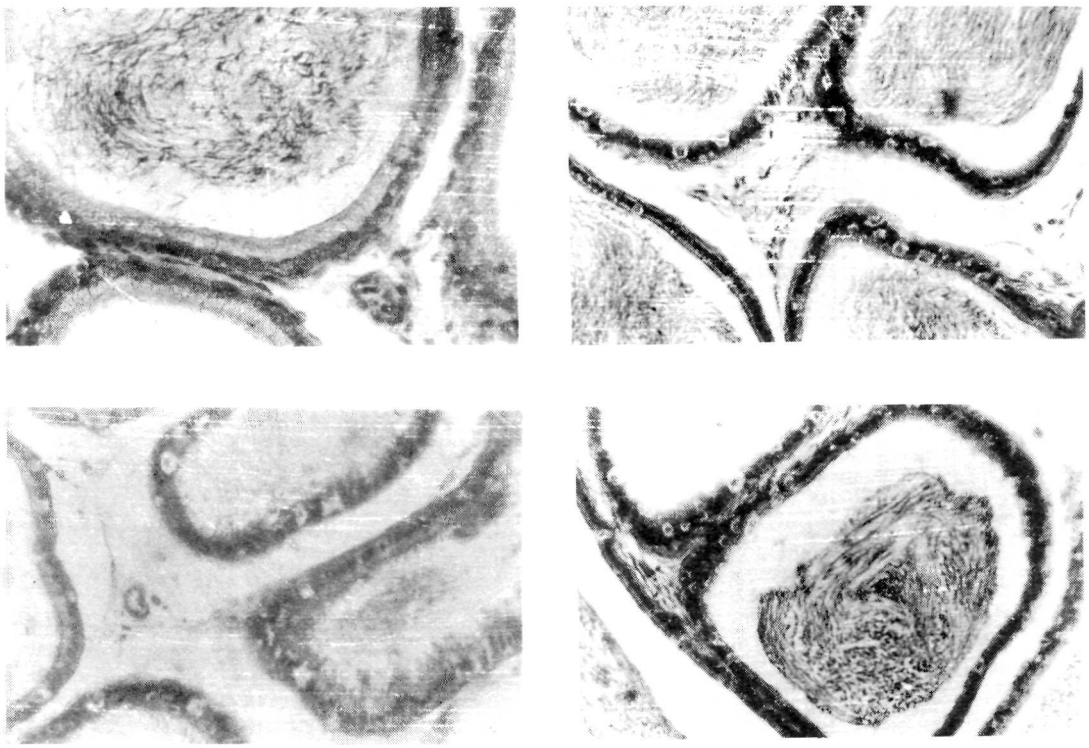

PLATE 1

A. **C.S. of caput epididymidis of control rats** (H & E; 50X).

Note the compact pseudostratified epithelium enclosing the lumen containing spermatozoa.

B. **C.S. of caput epididymidis of rats treated with 500 µg SECO** (H & E; 50X).

Note the loss of structural integrity of the epithelium and appearance of clear cells in the epithelium.

C. **C.S. of caput epididymidis of rats treated with 750 µg SECO** (H & E; 50X).

Note the appearance of clear cells.

D. **C.S. of the cauda epididymidis of rats treated with 1000 µg SECO** (H & E; 50X).

Note the loss of integrity of the epithelium with numerous clear cells and clumping of disintegrated spermatozoa in the lumen.

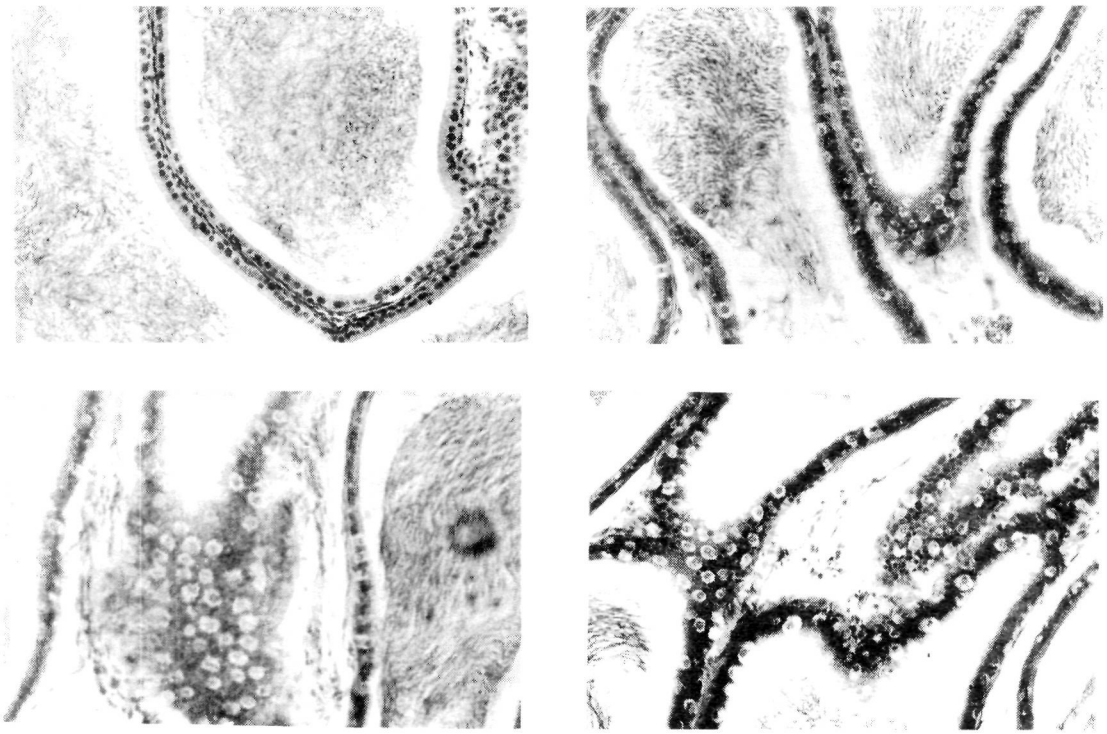

PLATE 2

A. **C.S. of the cauda epididymidis of control rats** (H & E; 50X).
Note the pseudostratified epithelium of the lumen containing abundant spermatozoa.

B. **C.S. of the cauda epididymidis of rats treated with 500 µg SECO** (H & E; 50X).
Note the appearance of clear cells in the epithelium.

C. **C.S. of the cauda epididymidis of rats treated with 750 µg SECO** (H & E; 50X).
Note the predominance of clear cells in the epithelium and proliferation of new clear cells.

D. **C.S. of the cauda epididymidis of rats treated with 1000 µg SECO** (H & E; 50X).
Note the predominance and a high proportion of clear cells in cauda epididymidal tubule.

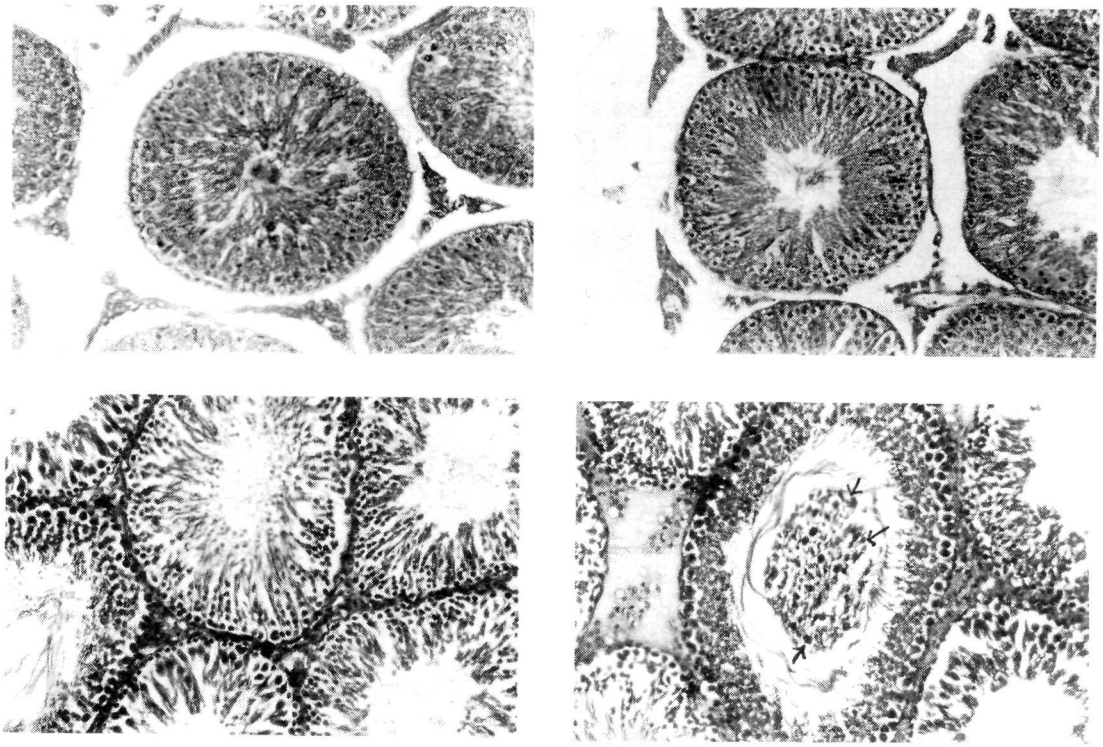

PLATE 3

A. **C.S. of the testis of control rats** (H & E; 50X).
Note normal spermatogenesis with lumen full of spermatozoa.

B. **C.S. of the testis of rats treated with 500 µg SECO** (H & E; 50X).
Note unaltered spermatogenesis.

C. **C.S. of the testis of rats treated with 750 µg SECO** (H & E; 50X).
Note the decrease in sperm concentration in the lumen.

D. **C.S. of the cauda epididymidis of rats treated with 1000 µg SECO** (H & E; 50X).
Note the appearance of apoptotic cells in the lumen.

Table 1: Effect of SECO treatment on body and organ weight of adult rats.

Parameters	Control	Dose of SECO			
		250 µg	500 µg	750 µg	1000 µg
Body weight (grams)	248.66 ± 5.93	249.20 ± 9.21	253.67 ± 8.58	248.00 ± 8.58	232.60 ± 7.2
Epididymis weight (mg/g body weight)	287.57 ± 17.0	241.83 ± 31.30	233.78 ± 12.5	257.52 ± 3.04	145.99 ± 3.2 ***
Dorsolateral prostate weight (mg/g body weight)	38.630 ± 2.06	46.01 ± 5.46	41.550 ± 4.21	40.71 ± 3.84	9.22 ± 3.08
Ventral prostate weight (mg/g body weight)	90.660 ± 2.24	77.24 ± 7.96	87.950 ± 2.08	83.19 ± 8.80	80.20 ± 6.05 ***
Seminal vesicle weight (mg/g body weight)	83. 070 ± 4.62	66.95 ± 6.83	71.100 ± 5.69	76.51 ± 2.40	68.02 ± 4.31 ***

Each value is mean ± SEM of 5 observations. *** P < 0.001 – Control vs Experimental

All doses of SECO treatment significantly reduced the epithelial cell height and the mean diameter of tubules of epididymis, when compared with control. The mean distance between the tubules was larger in SECO treated groups than the controls (Fig 1, 2 and 3).

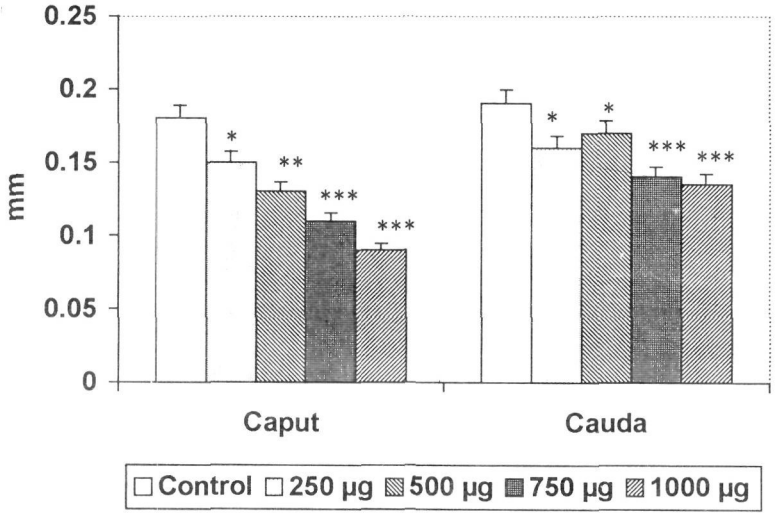

Figure 1 : Effect of SECO treatment for 45 days on epithelial cell height of caput and cauda epididymides of rats

Each histogram denotes mean ± SEM; n = 5.
*** p < 0.05; ** p < 0.01; *** p < 0.001; Control vs Experimental**

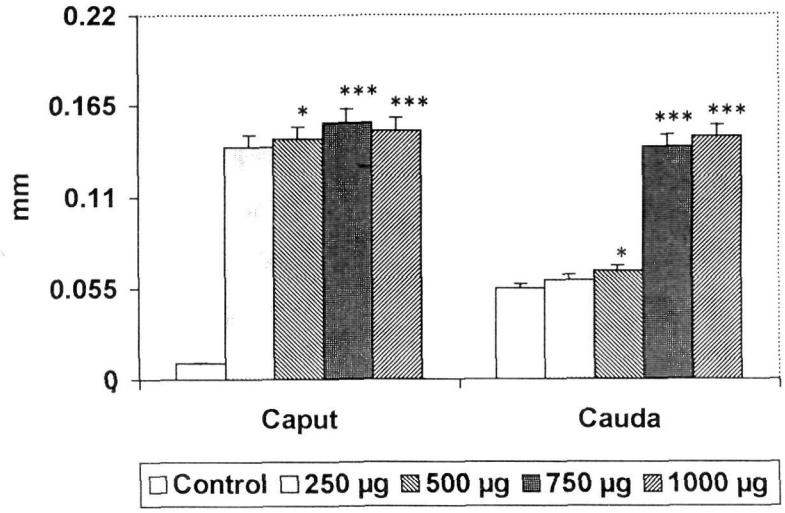

Figure 2 : Effect of SECO treatment for 45 days on mean distance between the tubules of caput and cauda epididymides of rats

Each histogram denotes mean ± SEM; n = 5.
* $p < 0.05$; *** $p < 0.001$; Control vs Experimental

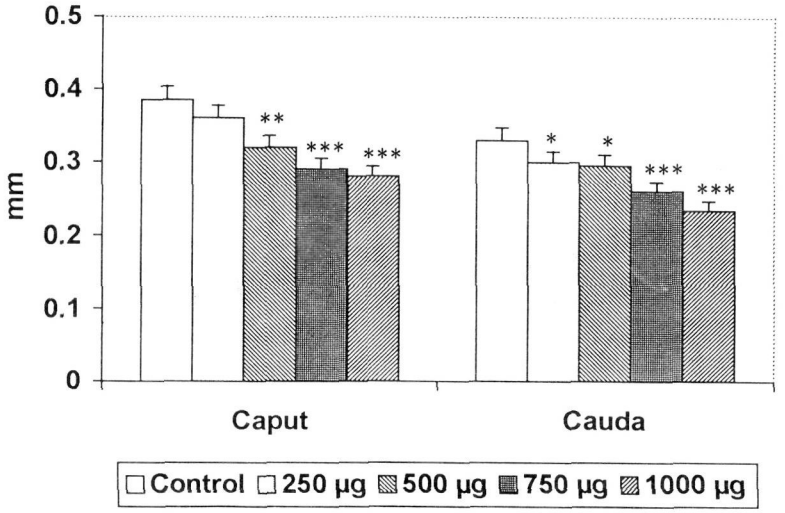

Figure 3 : Effect of SECO treatment for 45 days on mean diameter of caput and cauda epididymidal tubules of rats

Each histogram denotes mean ± SEM; n = 5.
* $p < 0.05$; *** $p < 0.001$; Control vs Experimental

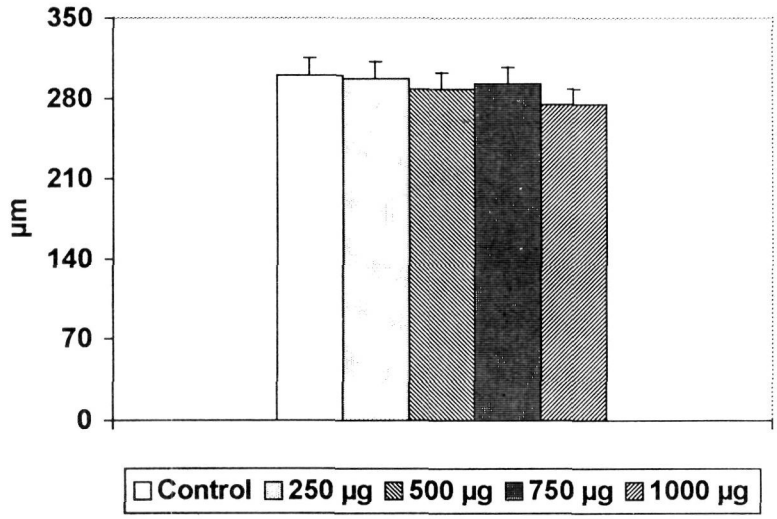

Figure 4 : Effect of SECO treatment for 45 days on seminiferous tubular diameter of rats

Each histogram denotes mean ± SEM; n = 5.

Low doses of SECO treatment did not produce any significant change in the seminiferous tubule and tubular lumen diameter (Fig 4 and 5). However, 1000 µg SECO treated rats showed disruption of spermatogenic activity and reduced number of sperm in seminiferous tubular lumen with a few apoptotic cells and cell debris (Plate 3).

Serum LH and FSH levels significantly decreased in all groups of SECO treated rats (Fig 6). Serum and epididymal testosterone titre significantly increased in rats treated with SECO, except in cauda epididymidis of rats treated with 250 µg SECO (Fig 7 and 8). An opposite trend was observed in estradiol level in SECO treated rats. The lower doses of SECO treatment did not produce any significant alteration in serum and epididymal (both caput and caudal regions) estradiol concentration (Fig 7 and 9). Nevertheless, the same was decreased in rats treated with 500, 750 and 1000 µg SECO (Fig 7 and 9).

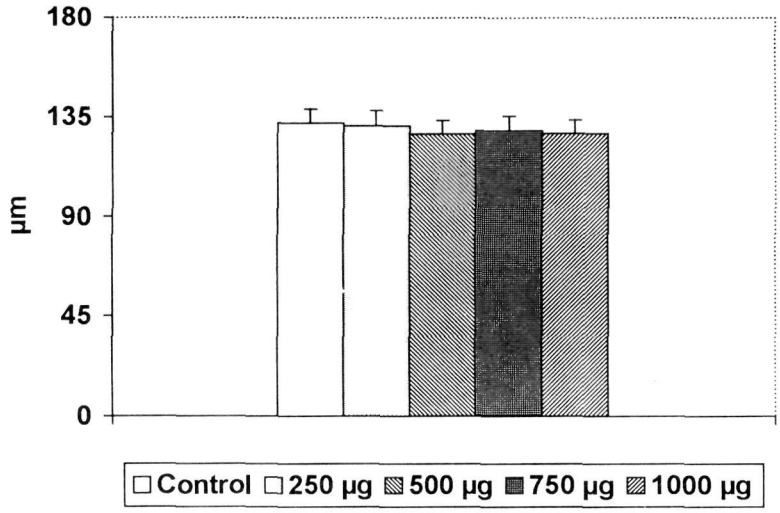

Figure 5 : Effect of SECO treatment for 45 days on seminiferous tubular lumen diameter of rats

Each histogram denotes mean ± SEM; n = 5.

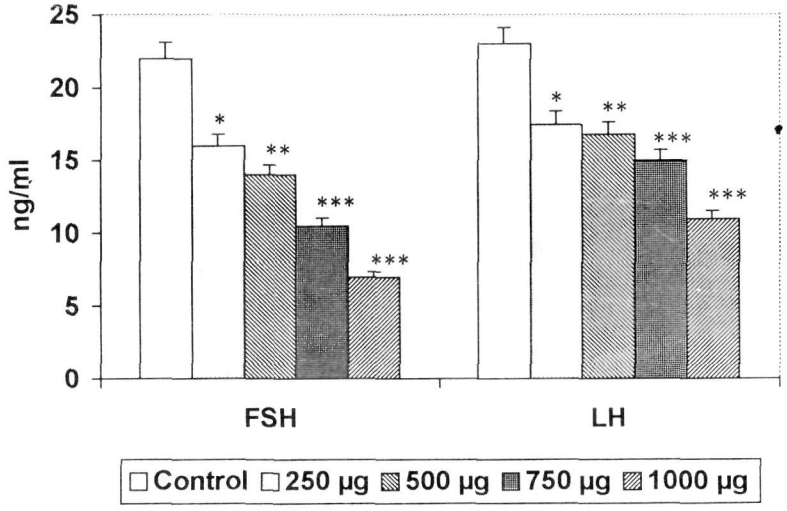

Figure 6 : Effect of SECO treatment for 45 days on serum FSH and LH in rats

Each histogram denotes mean ± SEM; n = 5.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Control vs Experimental

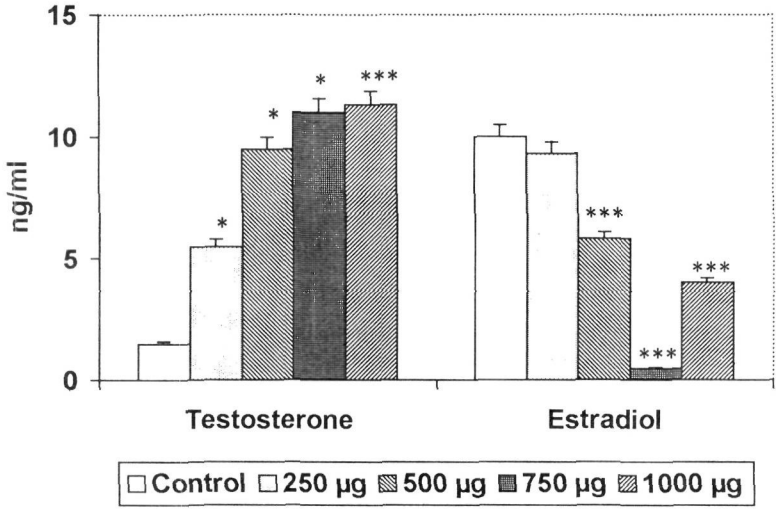

Figure 7 : Effect of SECO treatment for 45 days on serum testosterone and estradiol in rats

Each histogram denotes mean ± SEM; n = 5.
* P < 0.05; *** P < 0.001; Control vs Experimental.

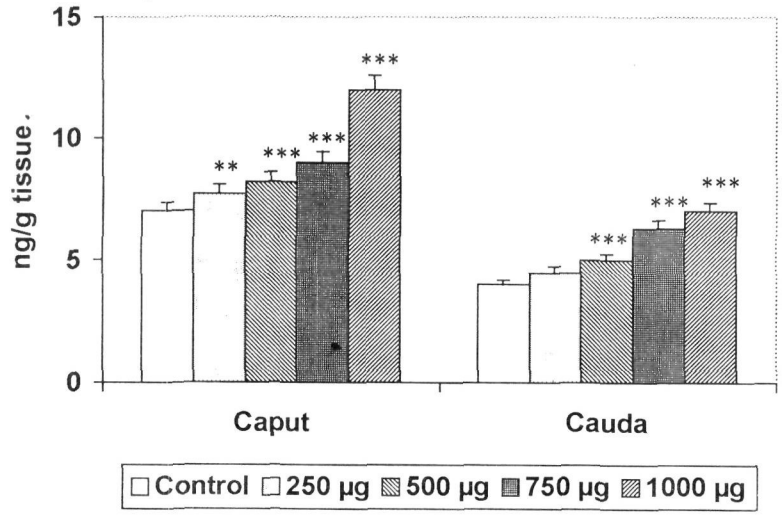

Figure 8 : Effect of SECO treatment for 45 days on caput and cauda epididymidal testosterone in rats

Each histogram denotes mean ± SEM; n = 5.
* P < 0.05; *** P < 0.001; Control vs Experimental

SECO treatment perceptibly reduced sperm forward motility, in a dose dependent manner, i.e., 23.38% in rats with 250 µg SECO; 67.03% reduction with 500 µg; 74.63% with 750 µg and 100% reduction in rats treated with 1000 µg SECO (Fig 10).

Among the four doses of SECO employed, 750 and 1000 µg SECO were effective in reducing the sperm count in caput and cauda epididymides when compared to controls (Fig 10).

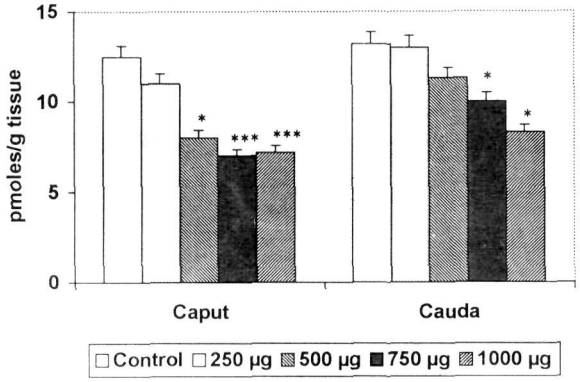

Figure 9 : Effect of SECO treatment for 45 days on caput and cauda epididymidal estradiol in rats

Each histogram denotes mean ± SEM; n = 5.

* P < 0.05; *** P < 0.001, Control vs Experimental.

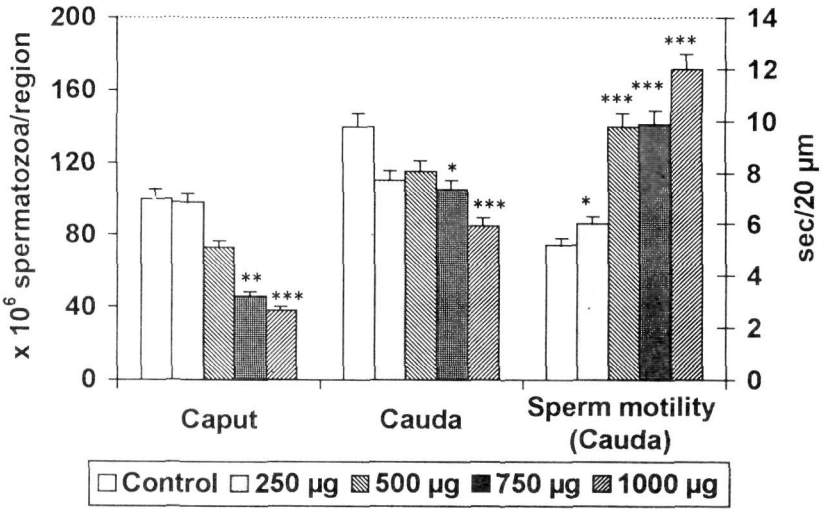

Figure 10 : Effect of SECO treatment for 45 days on cauda epididymidal sperm forward motility and caput and cauda epididymidal sperm count in rats.

Each histogram denotes mean ± SEM; n = 5.

* P < 0.05; ** P < 0.01; *** P < 0.001; Control vs Experimental

5α-Reductase activity diminished significantly in caput ($p < 0.001$) and cauda ($p < 0.01$) epididymides of rats treated with 750 µg or 1000 µg SECO but hepatic 5α-reductase activity was unaltered (Table II) (5α-reductase activity was assayed only in rats treated with higher doses of SECO which inhibited sperm motility maximally).

Table 2 : Effect of SECO treatment on 5α-reductase activity in epididymidal and hepatic tissues in rats

Tissue	Control	Dose of SECO	
		750 µg	1000 µg
Caput Epididymidis	86.0 ± 3.73	34.83 ± 0.73 ***	30.28 ± 1.22 ***
Cauda Epididymidis	43.2 ± 3.57	27.48 ± 1.36 **	28.33 ± 2.08 *
Liver	70.17 ± 5.96	68.29 ± 2.98	65.30 ± 3.20

Each value is mean ± SEM of 5 observations.
Values are expressed as pmoles of DHT formed/hour/mg protein.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ – Control vs Experimental.

DISCUSSION

The present study reveals inhibitory effect of SECO on sperm forward motility in a dose dependent manner. Among the various maturational changes in sperm during the epididymal sojourn, acquisition of progressive forward motility and fertilizing capacity are the most significant one (18). During the process of sperm maturation, numerous epididymal proteins are added to spermatozoa, which imparts fertilizing capacity and forward motility (19). The synthesis and secretion of these proteins depend on epididymal DHT (20). Data on epididymal 5α-reductase enzyme activity confirm the inhibitory effect of SECO on 5α-reducatse, leading to reduced production of DHT in the epididymis. Unaffected hepatic 5α-reductase activity suggests that SECO selectively inhibits the epididymal 5α-reducatse (9) and thus, sperm maturation.

The observed reduction in epithelial cell height and the mean diameter of tubules and increased mean distance between tubules of SECO treated rats suggests tissue regression. This may be attributed to the reduced availability of androgens, as these aspects of the epididymis have been shown to be androgen dependent (21).

A perceptible decrease in epididymal sperm count observed in rats treated with higher doses of SECO (750 µg and 1000 µg) might be attributed to sperm death in rats treated with high doses of the compound. An indirect evidence for this speculation is that SECO treated rats had increased number of clear cells in caput and caudal regions and a maximal increase in clear

cell number was seen in rats treated with higher doses of SECO (22). Clear cells are involved in endocytosis wherein they phagocytose the dead cells and debris (1). Therefore, it is suggested that the epididymis meets the situation of increased sperm death in response to high dose of SECO by enhancing the number of clear cells.

The observed elevated testosterone titre in serum and tissues of all four groups of SECO treated rats is attributed to the inhibition of 5α-reductase and thereby blockade of conversion of testosterone into DHT (23).

The observed reduction in serum LH and FSH titres of SECO treated rats may be the result of increased peripheral testosterone titre. Unaltered weights of accessory sex glands despite an elevated level of testosterone suggest normal androgen action. Suppression of estradiol titre may compensate the reduced action of androgen due to block in formation of DHT, as the former is a known antagonist of androgen action (24).

Reduction in serum estradiol titre of SECO treated rats may be due to a diminished peripheral conversion of testosterone into estradiol. Though it is not known whether epididymis contains the enzyme aromatase, mouse epididymal sperm were shown to have the same and it was hypothesized that sperm synthesized estrogen is important in the process of sperm maturation (25). However, it is not known whether SECO, a specific inhibitor of 5α-reductase also inhibits aromatase. Since there was a reduction in serum and tissue estradiol, there may be an inhibition of aromatase in SECO treated rats but this suggestion needs confirmation.

The results of the present study points out that SECO inhibits sperm maturation in the epididymis in a dose dependent manner. Sialic acid, a marker of epididymal function, is a kind of lubricant, which facilitates the forward motility of spermatozoa, and an optimal level of sialic acid is necessary for maturation and survival of spermatozoa in the epididymis (26). Epididymal sialic acid being an androgen (DHT) dependent parameters, we tested whether SECO treatment decreases epididymal sialic acid concentration and found that SECO does so (23). This shall point out that SECO impairs androgen dependent sperm maturational events culminating in forward motility arrest.

Thus, the present preliminary *in vivo* study clearly points out that SECO impairs the acquisition of sperm forward motility by specifically blocking the epididymal 5α-reductase activity.

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