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Research Article

HISTOMORPHOMETRIC ANALYSIS OF THE ANTI-SPERMATOGENIC AND ANTI-ANDROGENIC ACTIVITY OF *PERGULARIA DAEMIA* FORSK. IN MALE WISTAR RATS

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ABSTRACT
Pergularia daemia Forsk. known as "Dudhi bel" or Sagovani in Hindi is a perennial twining herb, which belongs to the Family Ascelpiadaceae. It is found throughout the hotter parts of India. Leaves and flowers are edible and juice of the leaves is used in catarrhal infections, infantile diarrhoea, asthma, applied to rheumatic swellings and in snake bite. The plant is described as antihelminthic, laxative, antipyretic and expectorant and effective in malarial intermittent fevers. The plant has also exhibited anti diabetic activity. The plant has the folkloric reputation of an antifertility agent in females and has therefore been used by rural people as an abortifacient. Some workers have also reported the antispermatogenic effect of the plant. The present study was undertaken to histologically determine the site of antifertility action of the plant in male rats. For this three doses (50,100 and 250 mg/kg.b.wt) of the 50% ethanolic extract of aerial parts of *Pergularia daemia* were administered orally for duration of 60 days. The diameter of the seminiferous tubule and leydig cell nuclear diameter was measured. There was decline in these diameters. As a result, there was increase in the interstitial spaces. Lesser number of total spermatocytes and very few spermatids, were observed. In the epididymis, there was a visible decline in size of the tubules and epithelial cell height and an increase in the interstitial stroma. Reduction in stereocilia height was also observed. As we know that the maintenance of structural and functional integrity of male reproductive organs requires a continuous presence of circulating androgen, all the above mentioned effects could have arisen from low plasma level of testosterone which was affected by the drug treatment.

Keywords*: Antifertility, Pergularia daemia, Testosterone, Histology, Leydig Cell*

INTRODUCTION
There is no more fundamental threat to the human race than it's own burgeoning numbers. Population explosion is the root cause of all kind of social and economic problems, which are widening their range day by day. Today we understand that our sheer numbers have increased so much that they are straining earth's capacity to supply food, energy and raw materials.

World population reached 6 billion mark in 1999 and it is expected that 8 billion people will populate our planet by 2020 (World Population Data Sheet, 2007). Responsibility for this enormous growth largely lies with the developing countries, as the population of industrial countries is largely stable. This population explosion creates hardly surmountable ecological and economic problems. India can be considered as a case in point where almost one –fifth of the world population (1.3 billion) (Population Reference Bureau) occupies 2.5% of earths land surface and where uncontrolled population wipes out countries economic growth, which has been spectacular. More than 18 million people are added every year, which is almost the entire population of Australia.

Although medical science has made tremendous progress, it offers limited possibilities of adjusting reproduction to altered social and cultural life circumstances.

As a consequence, of the 910000 conceptions taking daily 50% are unplanned and 25% unwanted. Out of them 150,000 are terminated daily worldwide by abortion. Of these 500 ends lethally for women and most of these are performed in countries where effective contraceptive methods are not available.

Research Article

Contraception has played a significant role in controlling population over the last fifty years. However, there is a paucity of male contraceptive methods despite their willingness (Stephanie *et al.,* 2008).
The development of safe, effective and reversible contraceptive methods for men is an important goal in expanding the choices available to couples to regulate their fertility.
Fertility regulation with plants or plant preparations and medicaments has been mentioned in ancient texts of indigenous systems of medicine of many countries. A lot of plants have been investigated for their antifertility potential. The present investigation has been taken up to study the male antifertility potential of *Pergularia daemia* Forsk. Syn. *Daemia extensa* R Br. (Asclepiadaceae) known as "Dustapu teega" in Telugu, "Uttaravaruni" in Sanskrit and "Utranajutuka", "Dudhi bel" or Sagovani in Hindi is a perennial twining herb, which belongs to the Family Ascelpiadaceae. It is found throughout the hotter parts of India. Leaves and flowers are edible and juice of the leaves is used in catarrhal infections, infantile diarrhoea, asthma, applied to rheumatic swellings and in snake bite (Singh *et al.*, 1990).
The decoction of the root and leaves is used as an emetic and to treat bronchitis (Mital *et al.,* 1962). The plant has the folkloric reputation of an antifertility agent in females and has therefore been used by rural people as an abortifacient.
Recently, Sadik *et al.,* (2001) showed that the ethanolic extract and its steroidal fraction in were able to prevent fertilization in female mice. Londonkor *et al.,* (2009) have reported antispermatogenic and antiandrogenic potential of Pergularia daemia in male rats. The present study has been taken up to histologically determine the site of antifertiltiy action of the plant.

MATERIALS AND METHODS

P. *daemia* was collected from University of Rajasthan campus. The specimen of the plant was authenticated at Department of Botany, University of Rajasthan, where voucher specimen is kept.
Pergularia daemia (Herbarium No. RUBL 19909).
***Animals*:** Colony bred, adult, healthy, male albino rats of Wistar strain were used in the present study. Animals weighing 160-180 g were used. The animals were housed in polypropylene cages under standard husbandry conditions (12 hrs light/dark cycle: 25 ± 3°C). Rats were provided water and nutritionally adequate pellet diet (Hindustan Lever Ltd., Delhi, India.) *ad libitum*.
***Extract Preparation*:** The aerial parts of *Pergularia daemia (Forssk)* was collected and dried in shade. The dried plant materials were pulverized into coarse powder and soxhalated with 50% ethanol at 55-60ºC for 36 hours. The crude extract was then filtered and evaporated to dryness under reduced pressure and low temperature. The residue obtained was utilized for evaluating antifertility activity by suspending in required volume of distilled water.
***Experimental Design*:** The Rats were divided into four different groups, each consisting of six animals and treated as follows:
Group I: Control group, receiving vehicle (0.5 ml distilled water/ rat) orally for 60 days.
Group II: Extract of *P. daemia* (low dose; 50 mg/kg b.wt.), orally, for 60 days.
Group III: Extract of *P. daemia* (Medium dose; 100 mg/kg. b.wt.), orally, for 60 days.
Group IV: Extract of *P. daemia* (High dose; 250 mg / kg b.wt.), orally, for 60 days.
Histopathology: Testes and epididymis were fixed in Bouin's fixative and cut into pieces and processed through ethanol-xylene series. The tissue, then were embedded in paraffin wax. The sections (5μ) of testis, and epididymis were cut and stained in haematoxylin-eosin and observed for histopathological aspects.
Histometry: The tubular diameter of seminiferous tubules were measured at X 100 by an ocular micrometer calibrated with a stage micrometer; 20-40 tubular profiles, that were round or nearly round were measured for each animal and a mean was determined.
Similarly, Leydig cell nuclear diameters (under X 1000 magnification) were determined using ocular micrometer under light microscope. The epithelial cell height of cauda epididymis was measured at X 400. Quantitative evaluation of germ cells includes enumeration of spermatogonia, preleptotene and pachytene primary spermatocytes and round spermatids in stage VII seminiferous tubules per cross

sections under X 100 magnification (Leblond and Clermont, 1952). Counting was performed in 10 tubule cross sections and mean was calculated. Crude counts so obtained were corrected /adjusted by the Abercrombie corrections factor (Abercrombie, 1946).

$$\text{True counts} = \frac{\text{Crude counts} \times \text{Section thickness } (\mu m)}{[\text{Section thickness } (\mu m) + \text{Nuclear diameter } (\mu m)]}$$

Statistical Analysis: All the data were calculated and statistically analyzed with SPSS/PC^{+TM} V. 13.0 computer software. The data were expressed as mean ± SEM and tested for variance. When variance was homogeneous, a one way analysis of variance (ANOVA) was carried out; when any significant difference was detected, the data were further analyzed with *post hoc test*. A probability (P) of < 0.05 was accepted as statistically significant. The percentage values were converted by arcsine transformations before the above analysis. All the other data were statistically analyzed with one way ANOVA for the comparison between groups, followed by Student Newman Keuls (SNK) multiple comparison as a *post hoc test*.
Serum Testosterone was assayed by the method of Spratt *et al.,* (1992).

RESULTS AND DISCUSSION

Body and Organ Weights (Table 1): There was a significant dose dependent decline in the weight of the Testes and the accessory reproductive sex organs such as epididimides, vas deferens and the seminal vesicles. However, the body weight gain was normal which possibly suggests non toxicity of the extract.

Table 1: Body and Organ Weight in Rats Treated with 50% Ethanolic Extract of *Pergularia daemia*

Determination		*P. daemia* Extract Treatment			
		Control	**50 mg/kg**	**100 mg/kg**	**250 mg/kg**
Body (g)	**Initial**	175.17 ±3.35	161 ± 4.47	182.5 ± 5.68	197.33 ± 4.99
	Final	201.17± 6.44	185 ± 4.16	200.33± 17.72	214.17± 14.57
Testis (mg/100g. Body wt.)		1168.17± 19.56[a]	1116.16 ±23.81[b]	941.83± 30.06[c]	788.5± 21.2[d]
Epididymis (mg/100g. Body wt.)		435.67± 13.88[a]	405.00 ± 6.80[b]	368.5± 14.92[c]	316.67± 8.71[d]
Seminal Vesicles (mg/100g. Body wt.)		617.17± 10.43[a]	585.66± 6.69[b]	508.83± 12.44[c]	452.67± 6.42[d]
Ventral prostate (mg/100g. Body wt.)		315.17± 7.29[a]	302.25 ± 4.65[a]	256.17 ± 14.3[b]	224.5 ± 5.6[c]
Vas Deferens (mg/100g. Body wt.)		116.17± 6.17[a]	113.83 ± 6.48[a]	100.5± 4.16[b]	96.16± 4.38[c]
Heart (mg/100g. Body wt.)		341.16± 19.96[a]	338.00± 17.08[a]	335.50± 15.88[a]	344±16.89[a]
Kidney (mg/100g. Body wt.)		600.67± 36.49[a]	598.33± 23.63[a]	608.5± 8.90[a]	588.83± 24.99[a]
Liver (mg/100g. Body wt.)		3229.5± 119.6[a]	3214.67± 120.2[a]	3276± 162.61[a]	3188.83± 176.67[a]

Values represent mean value ± SEM (n=6)
Values in a row with different letter in superscript indicate significant difference according to one way ANOVA and SNK multiple comparison procedure.

Sperm Parameters (Density, Motility and Viability) (Table 2): There was a dose dependent decline in the Sperm parameters viz. Density, Motility and Viability which also resulted in reduced fertility. The litter size also reduced significantly.

Table 2: Sperm Analysis in Rats Treated with 50% Ethanolic Extract of *Pergularia daemia*

Determination	*P. daemia* Extract Treatment			
	Control	50 mg/kg	100 mg/kg	250 mg/kg
Sperm Density (million/mm^3)	48.50 ± 1.78 [a]	41.50 ± 0.88 [b]	28.5 ± 1.78 [c]	18.00 ± 1.15 [d]
Sperm Motility (%)	78.00 ± 1.26 [a]	68.78 ± 3.42 [a]	29.33 ± 1.42 [b]	16.16 ± 0.98 [c]
Sperm Viability (%)	79.33 ± 2.10 [a]	70.26 ± 3.76 [b]	37.00 ± 1.52 [c]	26.33 ± 1.81 [d]
Fertility (Pregnant/Mated) (%)	92 (11/12) [a]	75 (9/12) [b]	25 (3/12) [c]	0 (0/12) [d]
Litter Size	9.62 ± 0.84 [a]	7.56 ± 0.68 [b]	3.45 ± 0.48 [c]	0.00 [d]

Values represent mean value ± SEM (n=6)
Values in a row with different letter in superscript indicate significant difference according to one way ANOVA and SNK multiple comparison procedure

Testicular Morphometry (Table 3): There was decline in these diameters for all the groups, however, it was only significant for the medium and high dose regimens, as the variation for the low dose group was not significantly different from the control group. (Figure 1) (Control value seminiferous tubular diameter 268.00 ± 8.09, Leydig cell nuclear diameter 7.13 ± 0.32).

Table 3: Testis Morphometry and Cauda Epididymal Epithelial Cell Height in Rats Treated with 50% Ethanolic Extract of *Pergularia daemia*

Determination	*P. daemia* Extract Treatment			
	Control	50 mg/kg	100 mg/kg	250 mg/kg
Seminiferous Tubule Diameter μm)	268.00 ± 8.09[a]	245.50 ± 4.61[b]	230.33 ± 8.30[c]	189.66 ± 4.63[d]
Leydig cell Nuclear Diameter μm)	7.13 ± 0.32[a]	6.31 ± 0.15[a]	5.72 ± 0.11[b]	5.45 ± 0.15[b]
Cauda Epididymal Epithelial Cell Height (μm)	25.42± 1.10 [a]	23.88± 0.98 [a]	18.35 ± 1.04 [b]	16.50 ± 0.78 [b]

Values represent mean value ± SEM (n=6)
Values in a row with different letter in superscript indicate significant difference according to one way ANOVA and SNK multiple comparison procedure

Table 4: Testicular Germ Cell Counts (no./Cross Section of Seminiferous Tubule) in Rats Exposed to *Pergularia daemia* at Different Doses

Determination	*P. daemia* Extract Treatment			
	Control	50 mg/kg	100 mg/kg	250 mg/kg
Spermatogonia	5.8 ± 0.24 [a]	5.62 ± 0.24 [a]	5.54 ± 0.18 [a]	4.9 ± 0.16 [a]
Preleptotene Spermatocyte	19.46 ± 0.62 [a]	17.80 ± 0.74 [a]	12.00 ± 0.45 [b]	10.22 ± 0.32 [c]
Pachytene Spermatocyte	22.42± 1.10 [a]	21.46± 1.08 [a]	18.35 ± 0.78 [b]	14.50 ± 0.65 [c]
Round Spermatid	67.36 ± 3.86 [a]	59.44 ± 4.40 [a]	30.82 ±1.34 [b]	16.65 ± 0.68 [c]

Values represent mean value ± SEM (n=6)
Values in a row with different letter in superscript indicate significant difference according to one way ANOVA and SNK multiple comparison procedure

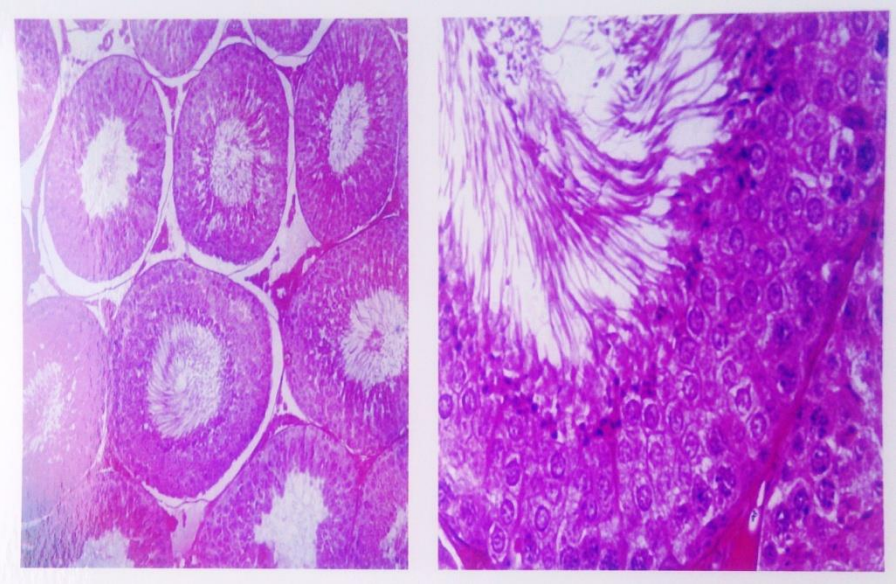


Figure 1: Control Testis

Epididymal Epithelial Cell Height (Table 3): The treatment brought down the epididymal epithelial cell height, however, it was non-significant for the low dose, but significant for the medium and the highest dose.

Serum Testosterone: The level of serum testosterone was brought down significantly in all the groups of rats receiving *P. daemia* plant extract (Figure A).

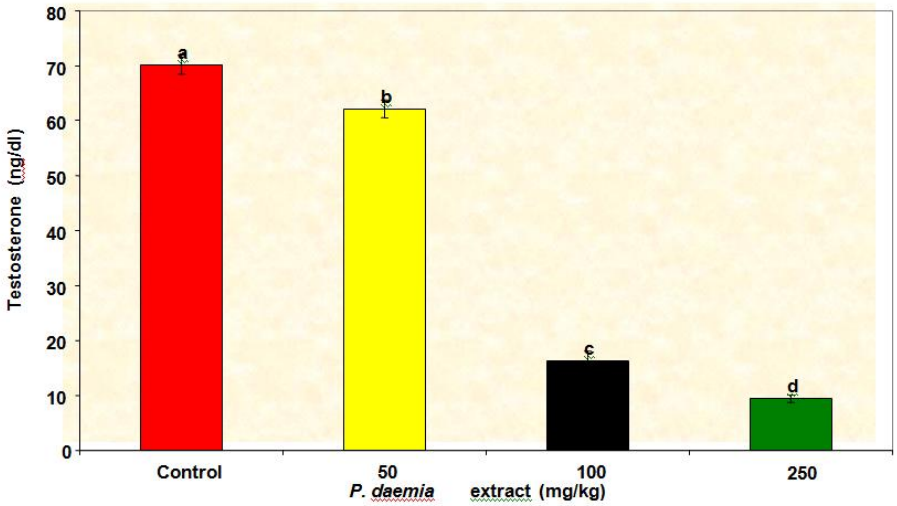


Figure A: Serum Testosterone of Rats Treated with Various Doses of 50% Ethanolic extract of *P. daemia.* Vertical Bars Indicate ± SEM and Different Letters above Bar Indicate Significant Difference According to SNK Multiple Test

Discussion

Histopathologically, it is well established that the maintenance of structural and functional integrity of male reproductive organs requires a continuous presence of circulating androgen (Chinoy *et al.*, 1982, Brooks, 1983). A fall in the concentration of androgen below the threshold levels required for the maintenance of differentiated cells, stimulates autolysis and removal of cells and alters the pattern of

cellular proliferation in these reproductive organs (Figure 2) (Prasad and Vijayan, 1987; Afolabi *et al.*, 2007).

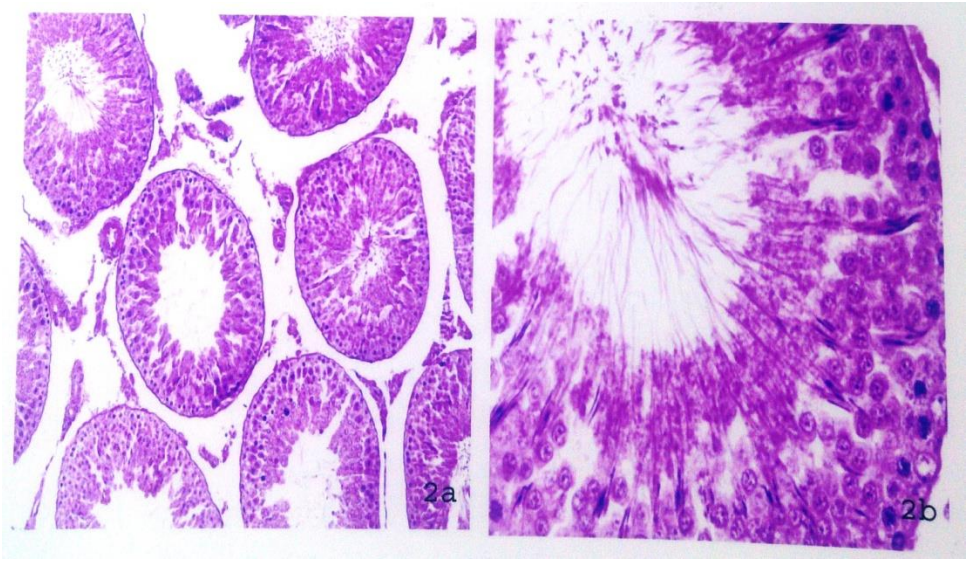


Figure 2: Treated Testis

Pergularia which belongs to Asclepiadaceae, contains many phytochemicals similar to C*alotropis procera* in phytoconstitution and also contains calotropin, a glycoside that has been implicated in the antifertility effect of the plant (Akinloye *et al.*, 2002). Besides, the kinds of histopathological effects that have been observed in our study are similar to the ones observed by Akinloye *et al*., (2002) who worked with Calotropis procera. Therefore, it can be inferred that the plant arrests spermatogenesis at primary or secondary spermatocyte or even spermatid stage like *Calotropis procera*. Moreover, triterpenoids and compounds like lupeol acteate that have been reported in the ethanolic extract of *Pergularia daemia* (Sathish *et al.*, 1998) have established effects on testicular histopathology (Gupta *et al*., 2005).

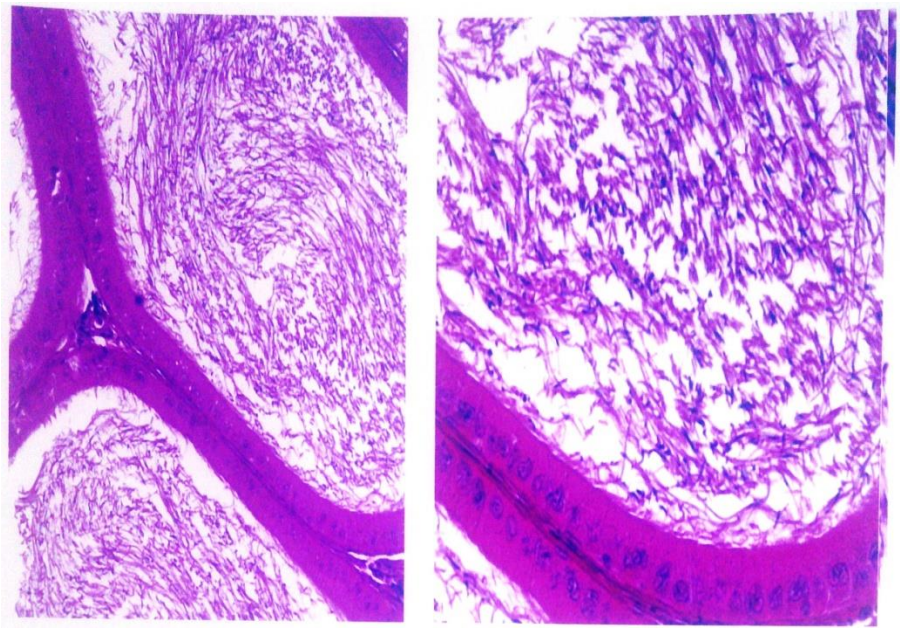
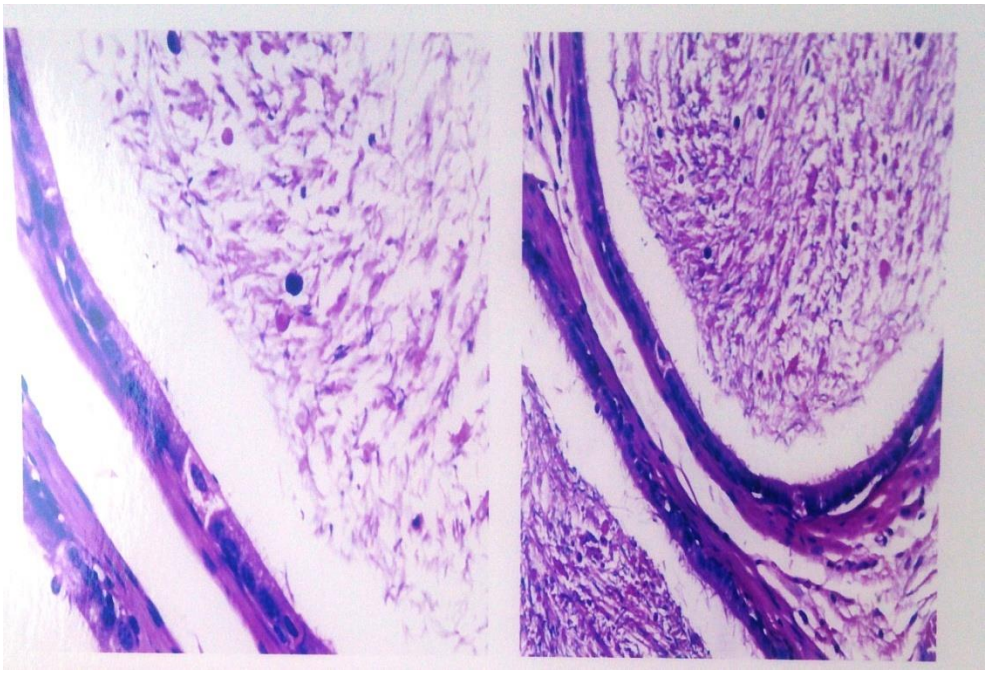


Figure 3: Control Epididymis

We also observed alteration in the histoarchitecture of the cauda epididymis. There was a dose dependent decline in the spermatozoa and presence of debris and leucocytes in the lumen of the cauda epididymis. Histologically, there was a visible decline in size of the tubules and epithelial cell height and an increase in the interstitial stroma. Reduction in stereocilia height was also observed. These histological changes were dose-dependent (Figure 4). These histopathological findings would have primarily arisen from lack of androgen and its metabolites. As we know the threshold of androgens in epididymis is relatively higher, the deficiency of it, would have caused inhibited metabolic activity of the epididymal epithelium resulting in altered luminal milieu making it non-conducive for sperm maturation and survival, ultimately leading to their disintegration (Smithwick and Young, 2001). Changes in the cauda epididymis have been reported after administration of many plant extracts by virtue of androgen deficiency (Vanitha Kumari *et al.*, 1989; Kasturi *et al*., 1997; Nusier *et al.*, 2007).



`Figure 4: Treated Epididymis

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