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Antifertility effects of fluphenazine in adult male rats[1]

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ABSTRACT. The underlying mechanisms in human infertility associated with hyperprolactinemia have yet to be established. Hyperprolactinemia is a known side-effect of fluphenazine, a broad spectrum, long-acting phenothiazine known to be D2 dopamine receptor antagonist. Dose-related effects of fluphenazine decanoate were ascertained on the fertility of 60-day treated, adult male rats. Significant increase in the serum levels of prolactin and decrease in the levels of LH and FSH were seen at doses of 1-3 mg/kg/day. No effect was evident on the serum testosterone (T) and estradiol. The tissue levels of Inhibins were not affected. The weights of testes, epididymides, seminal vesicles, ventral prostate, adrenal and pituitary glands were not affected. Testicular histology showed sloughing indicating the sensitivity of this parameter to FSH deficiency. Mating occurred within 10 days of cohabitation in the control and 1-2 mg/kg/day treated groups but delayed in the 3 mg/kg/day treated group with a significant effect on potency. Implantation sites, litter size and fertility index were significantly reduced at 2-3 mg/kg/day doses of fluphenazine. No effects however were seen on sperm counts or motility whereas morphological changes were apparent in the acrosome. Chromatin decondensation *in vitro* was enhanced and sperm chromatin structure assay revealed DNA denaturation. Hypothalamic tyrosine hydroxylase levels were increased in 1-3 mg/kg/day dose range. Hyperprolactinemic males sired fewer pups as compared to controls. Hypothalamic tyrosine hydroxylase was upregulated at all the doses. The antifertility effects of fluphenazine-induced hyperprolactinemia appeared to be unrelated to testosterone (T). In addition, FSH decrease might have affected the intrinsic sperm quality and thereby reduced litter size.

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INTRODUCTION

Prolactin has been reported to be involved in maintaining reproductive functions in males. It has been implicated in maintaining the functions of testes and accessory sex glands, release of testosterone and upregulation of gonadotropin receptors (1-3). Though the development of prolactin and prolactin receptor disrupted mice have largely discounted the relevance of prolactin to male fertility, hyperprolactinemia has been associated with prostate hyperplasia, gonadotropin suppression and impotence in males (4-9).

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Key-words: Fluphenazine, adult male rat, hyperprolactinemia, fertility, FSH.

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Several mechanisms have been proposed to explain the antifertility effects of prolactin. It has been reported to activate the adrenal glands and thereby suppress the release of T, stimulate tyrosine hydroxylase, the rate limiting enzyme for dopamine synthesis in tuberoinfundibular dopaminergic neurons (TIDA) and T-sensitive, incertohypothalamic dopaminergic neurons (IHDA) and suppress gonadotropins and copulatory behavior (10-12). Dopaminergic and opiatergic neurons sensitive to prolactin have been described in the hypothalamus (13). Dopaminergic and GnRH neurons have also been shown to be in anatomic proximity in the hypothalamus (14). Though lactotrophs and gonadotrophs have been observed to be in anatomic juxtaposition in the pituitary gland, suggesting the possibility of a paracrine relationship, the underlying mechanism through which prolactin suppresses gonadotropins and fertility, perhaps involving hypothalamic dopamine, opiatergic peptides and pituitary GnRH receptors has yet to be conclusively demonstrated (15-16). The present study was undertaken to induce hyperprolactinemia in adult

Table1 - *Effects of fluphenazine on fertility parameters, tissue weights and serum hormones in adult male rat*

Affected parameters	Fluphenazine treatment (mg/kg/day) 0	1	2	3
Potency (%)	100.00±00	83.30±11.24	100.00±00	66.7±14.21*
Fecundity (%)	100.00±00	66.70±21.09	83.30±16.67	50.0±22.36
Motility (%)	75.80±5.23	75.80±1.54	80.00±3.65	85.00±3.42
Sperm.counts (mil/cauda)	63.80±3.89	54.30±4.18	52.80±3.53	59.00±6.68
Implantation sites/rat	11.30±1.16	6.70±1.67	5.10±1.54*	5.40±1.69*
Litter size/rat	10.30±1.17	6.40±1.60	4.80±1.49*	5.70±1.73*
Fertility index/rat	0.90±0.08	0.60±0.14	0.50±0.15*	0.50±0.15*
Testes (g/100gbw)	0.88±0.02	0.91±0.02	0.79±0.06	0.88±0.03
Epididymis (g/100gbw)	0.33±0.01	0.35±0.01	0.31±0.00	0.32±0.01
Sem.Vesicles (g/100gbw)	0.14±0.01	0.04±0.01	0.12±0.00	0.13±0.01
V.prostate (g/100gbw)	0.17±0.02	0.17±0.02	0.17±0.01	0.17±0.02
Adrenals (mg/100gbw)	9.95±0.97	10.37±0.36	8.65±0.40	10.00±0.44
Pituitary (mg/100gbw)	2.740±0.10	2.68±0.11	2.59±0.10	2.51±0.10
Prolactin (ng/ml)	18.99±1.82	76.80±11.1*	82.53±10.6*	139.3±25.1*
LH (ng/ml)	1.13±0.26	0.23±0.04*	0.44±0.05*	0.54±0.05*
FSH (ng/ml)	7.72±0.26	5.43±0.44*	6.08±0.31*	6.00±0.22*
Testosterone (ng/ml)	2.60±0.14	2.17±0.17	2.40±0.20	2.32±0.24
Estradiol (ng/ml)	82.00±9.51	114.20±21.3	70.61±8.140	58.19±10.4

All values are mean±S.E. Dose "0" represents control values. * denotes significance at $p \leq 0.05$ as compared to control

male rat with fluphenazine (D2 dopamine receptor antagonist) and ascertain the effects on the mechanisms underlying fertility.

MATERIALS AND METHODS

Animals

Randomly bred 75 day old male rats and 90 day old virgin female rats of the Holtzman strain (inbred at IRR, Parel Mumbai), were maintained at a temperature of 22-23 C, 50-55% humidity and lighting cycle of 14 h light: 10 h dark. Commercial food pellets were available ad libitum. All experiments were carried out with the approval of the ethics committee of the institute.

Drugs

Fluphenazine decanoate injections (Anatensol decanoate) containing 25 mg fluphenazine per one mililitre of seasame oil were obtained from Sarabhai chemicals, Mumbai, (India). Fluphenazine was diluted in seasame oil and administered daily via sc route. Drug was given at doses of 1, 2, 3 mg/kg/day, between 10:00-12:00 h.

Experimental protocol

The study was designed to evaluate the effect of hyperprolactinemia on the reproductive performance and fertility of adult male rats treated with fluphenazine, a dopamine receptor blocker. The rats were assigned to four groups of six rats each. The control and treatment groups were administered seasame oil or fluphenazine at 1,2,3 mg/kg/day via sc route for a period of 60 days. At the end of the treatment period each group of rats was allowed to mate with cycling female rats.

Mating studies

Female rats were cohabited with male rats for 10–20 days. The rats in the control group mated within 10 days (within two cycles of estrus). Mating design was 1Male X 2 Females. The occurrence of mating was confirmed by the presence of copulatory plug, spermatozoa in the vaginal smears or persistent dioestrus for over 10 days. Successful matings culminated in sterility, pseudopregnancy or pregnancy. A successful mating indicates normal potency. The number of days taken by each group to mate was also observed.

Fertility parameters

Mated female rats were sacrificed on day 21 of gestation (in order to avoid ingestion by the mothers). Litter size of each female was recorded. Number of implantation sites in the two uterine horns was recorded. The ovaries were exposed to count the number of *corpora lutea*. Potency (% mated females), litter size (number of pups per female), fecundity (% male rats siring at least one viable pup) and fertility index (ratio of implantation sites to *corpora lutea*) were calculated as described earlier by Gill-Sharma *et al*., 1993 (17).

Autopsy of male rats

Male rats were killed by decapitation. Blood from the trunk was allowed to clot at 4 C. Serum was collected by centrifugation at 800g for 20 min and stored at –30 C for radioimmunoassays of prolactin, LH, FSH, T and estradiol. The weight of testes and accessory sex organs were recorded and relative organ weights were expressed as per 100g BW. *Caudae epididymides* were excised in saline. Motile sperms in the *caudae* were counted in a haemocytometer. Motility was expressed as percentage of motile sperms per *cauda* as described by Gill-Sharma *et al*., 2001(18). Sperms in

the saline extract of *caudae* were counted in a haemocytometer and sperm counts were expressed as million per *cauda* as described by Gill-Sharma *et al.*, 2001(18). Sperm morphology was seen by routine Gill's haematoxylin and eosin stain. Briefly, sperm smears on slides were air dried and fixed in 95% alcohol for 2hrs. Slides were hydrated through a series of graded alcohols 95%, 70%, 50%, 30% for 10min each. Slides were then immersed in distilled water for 10min, stained in Gill's haematoxylin for 15min and differentiated in 1% acid alcohol (70%) for 30secs. Color was allowed to develop under running tap water for 15min. Slides were stained in 1% aqueous eosin for 2min, washed in water, dehydrated through a series of graded alcohol, cleared in xylene and mounted in DPX. Hypothalami (4 mm thickness) were dissected out by cutting along the sulci, mammillary bodies and optic chiasm and stored under liquid nitrogen as described by Gill-Sharma *et al.*, 1985 (19).

Sperm nuclear chromatin decondensation test

Degree of sperm DNA compaction was ascertained by standard *in vitro* chromatin decondensation test (20). 5 million sperms from *caput* and *cauda* of each rat were suspended in 1ml of borate buffer (pH 8) containing 0.25ml of 3% SDS and 0.14ml of 50mM Dithio-threitol (DTT) (freshly prepared in borate buffer) at a final concentration of 0.5% and 5mM for SDS and DTT respectively. The sperms were incubated for 2, 3, 5 mins at 45 C and reaction stopped with 0.6ml of 2.5% glutaraldehyde. The fixed sperms were smeared on a slide and the length of 100 decondensed sperm heads per sample recorded with an occular micrometer under a phase contrast microscope.

Sperm chromatin structure assay

Structural integrity of sperm chromatin was ascertained by DNA Flow Cytometry (21). 2 million sperms from *caput* and *cauda* of each rat were suspended in 200ul of 0.01 M PBS buffer (pH 7.4). The sperms were made permeable by incubation with 400ul of chilled lysis buffer containing 0.1% Triton X, 0.08 N HCl, 0.15 M NaCl for 30 secs. Thereafter, the sperms were stained for 3min with 1.2ml of ice-cold Acridine orange stain containing 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, 0.1 M Citric acid (stock concentration of 6ug/ml). The flourescence of 5000 stained sperms laser excited at 488nm was recorded at both 630nm (green) and 530nm (red) in a Flow cytometer.

Histology

Testicular tissues from each group of rats were flxed in Karnovsky's fixative, washed in cacodylate buffer, post fixed in 1% osmium tetroxide, dehydrated in ascending series of acetone (30-100%), embedded in Araldite and semi-thin sections of 0.5um thickness were cut. The tissues were stained with toluidine blue and observed under bright-field optics at 40/100 X magnification (17).

Detection of testicular inhibin B α and β subunitis

Testis from all groups were fixed in Bouin's fixative, embedded in paraffin wax and processed for immunohistochemistry of Inhibins as described by Sharpe *et al.*, 1999 (22). Brieffy, 5 μm paraffin sections of testis fixed in Bouin's fixative were dewaxed in xylene, rehydrated in graded alcohol and washed with water and Tris buffered saline (TBS: 0.05 M Tris-HCl, 0.85% NaCl, pH 7.4). Sections were incubated with 0.1% Total Body Surface Area (TBSA) (0.1% BSA in 0.05 M TBS) for 30 min in order to block non-specific sites. Tissues were then incubated overnight in light proof box with monoclonal antibodies to Inhibin-a (Code R1) and B (Code C5) at concentrations of 14μg/ml and 4μg/ml in 0.1% TBSA respectively. Sections were then washed twice for 5 min each with TBS. Tissues were again incubated with biotinylated rabbit anti-mouse IgG at a dilution of 1:500 prepared in 0.1% TBSA for 30 min. The sections were washed twice with TBS for 5min each, these were incubated with avidin-biotin conjugated to alkaline phosphatase. Slides were washed twice with TBS for 5min each and finally with 100mM Tris-MgCl buffer (100mM Tris, 100mM NaCl, 50mM MgCl, pH 9.5). Antigens were visualized with chromogens nitro Blue tetrazolium (450μg/ml), 0.001% Levamisole in 10ml Tris-MgCl buffer and enzyme substrate 5-Bromo,4-Chloro, 3-Inddolyl phosphate (175μg/ml). The reaction was terminated by immersing the slides in distilled water. Negative controls were run alongside wherein the primary antibody was substituted with normal mouse serum/culture supernatant. Stained slides were dehydrated in absolute alcohol, cleared in xylene and mounted in DPX.

Detection of hypothalamic tyrosine hydroxylase by western blot

Tyrosine hydroxylase (the rate limiting enzyme for dopamine/norepinephrine biosynthesis, translocated to nerve endings) in the hypothalami of control and fluphenazine treated rats was detected by Western blotting (23). Hypothalamic tissues were homogenised in a buffer comprising (10 mM Tris pH 7.4, 1mM EDTA, 1mM DTT, 10% glycerol and protease inhibitor cocktail) on ice and centrifuged at 4 C at 15,000 rpm for 1 h. Aliquots of supernatant containing 20ug hypothalamic protein were separated on 10% gel by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The protein bands were electophoretically transferred to nitrocellulose membranes (Hybond C-Amersham) (24). Tyrosine hydroxylase was detected by immunoblotting with monoclonal antibody (Sigma). The nitrocellulose membrane was blocked with 5% nonfat dry milk powder in phosphate buffered saline (PBS 0.01M, pH 7.4) for 1 hr at room temperature. The membrane was incubated with tyrosine hydroxylase antibody (1:10000), biotinylated secondary antibody (1:1000) and avidin-biotinylated Horse Radish Peroxidase (HRP) complex (Vectastain ABC kit, Sigma) in Phenobarbital (PB) 0.01 M, pH 7.4, for 1 h each with thorough washings with Phosphate-Buffered Saline Gelatine (PBSG) containing 0.1% Triton X after each step. Tyrosine hydroxylase protein bands of 60 kDa (along with two non-specific bands due to the second antibody) were visualized by oxidation of diaminobenzidine (PB 0.01 M containing 0.05% DAB weight/volume) with hydrogen peroxide (0.015% in PB 0.01M). Tyrosine hydroxylase antibody was eliminated in the negative controls, which were run alongside the samples (showing absence of specific band for tyrosine hydroxylase). The relative concentration of tyrosine hydroxylase in the control and fluphenazine treated groups was compared by densitometery of the western blots using Gel Pro image analysis system and concentration represented as ng enzyme protein/20μg hypothalamic protein Mean±SEM.

Hormone assays

Protocol: LH, FSH, Prolactin were estimated by the double antibody radioimmunoassay as described by Gill-Sharma *et al.*, 1993 (17). Briefly, serum samples containing unlabelled antigens (max. 300ul) were incubated with 100ul (30,000cpm) NIADDK antigens (diluted in 0.5% BSA) labelled with I^{125}, 200ul of specific NIDDK

antibodies (diluted in normal rabbit serum and PBS in a ratio of 1:400), where the labelled and unlabelled antigens were allowed to compete for sites on specific antibodies for 24 h at 37 C. The standard curves for LH (NIADDK-rat LH-RP-3) FSH (NIADDK-rat FSH-RP-2) and Prolactin (NIADDK-RAT-Prl-RP-3) ranged from 0.01ng to 12.5 ng for LH, FSH, Prolactin, respectively. Total antigen binding tubes were set up containing only labelled antigens and primary antibodies diluted in 0.5% PBSA and PBS-NRS respectively. Separate tubes for non-specific binding were set up and contained only labeled hormone in 0.5% PBSA and PBS-NRS. The antigen-antibody complexes were precipitated by incubation with 200ul of anti-rabbit gamma Globulin (diluted in physiological saline) for 24 h at 37 C followed by 24 h at 4 C. The complexes were separated by centrifugation at 4 C for 30min at 2500g and air-dried. The I^{125} antigens bound to their respective antibodies were counted in a gamma counter. The sensitivities for LH, FSH, Prolactin assays were 0.09ng, 0.19ng, 0.78ng respectively. The Inter and intra assay coefficients of variation were 9% and 6% for LH, 10% and 6% for FSH and 14% and 5% for Prolactin respectively. All assays were run in duplicates.

Protocol: T and estradiol were estimated by dextran-coated, charcoal extraction assay as described by Gill-Sharma *et al.*, 1993 (17). Briefly, T and estradiol were extracted twice from 500ul of serum with 10 volumes of purified ether (passed through neutral aluminium oxide columns). Same volume of 0.01 M PBS buffer (pH 7.4) was also extracted simultaneously with ether to construct ether blanks. The ether extracts were allowed to evaporate overnight. The dried steroid residues were reconstituted in 1.5 ml of 0.01%, 0.01M PBSG (pH 7.4) and solubilised by heating in a water bath at 50 C for 1 h. Sample aliquots (diluted to 500ul with 0.1% PBSG buffer) were assayed in duplicate for T and estradiol, respectively. The assay mixture contained 100ul (10,000cpm) of ^{3}H antigens (diluted in 0.1% PBSG) and 100ul of specific antibodies (diluted in 0.1% PBSG) at the appropriate dilution. The standard curves for T and estradiol were in the range of 3.9pg to 1000pg respectively. Ether blanks were set up with 500ul of buffer extracts. The labelled and unlabelled antigens were allowed to compete overnight for antibody sites (16-18 h) at 4 C. The unbound antigens were precipitated with 500ul of dextran-coated charcoal suspension prepared in 0.1% PBSG at 0-4 C in ice for 10min. The soluble antigen-antibody complexes were rapidly separated by centrifugation at 4 C for 15 min at 2500g. The supernatants containing the bound ^{3}H antigens were solubilized in a toluene based scintillation fluid counted in a beta counter after 2 h of stabilization. The sensitivities for T and estradiol assays were 15.6pg and 31.25pg, respectively. Inter- and intra- assay coefficients of variation were 11% and 5.5% for T, 10% and 6% for estradiol, respectively.

Statistical analysis

Hormone concentrations, tissue weights and data of individual rats were subjected to ANOVA. Significant differences between groups were determined by Duncan's multiple range test. The fertility parameters (of individual rats) were subjected to non-parametric Kruskal- Wallis ANOVA. Significant differences within groups were calculated by Mann-Whitney-U-Wilcoxon and Duncan's tests. Significant differences between control and treated decondensed sperm lengths and mean flourescence intensity of dye attached to sperm chromatin were calculated by student's *t* test. Level of significance for each test was set at $p \leq 0.05$.

RESULTS

Effect of fluphenazine on potency, fecundity, sperm counts and motility (Table 1)

1-2 mg/kg/day: Matings occurred within 10 days of cohabitation in both the control and treatment groups, covering two estrus cycles (10 days). Reproductive behavior of the treated male rats was not affected at these doses. In spite of hyperprolactinemia at these doses, the fertility parameters were not affected. The sperm counts and motility in the control and treated animals were no different. The potency of the treated males was similar to that of control animals. Fecundity was equivalent in the treated and control groups. However, a normal fecundity does not ensure a normal number of progeny.

3 mg/kg/day : The treated males mated after a considerable delay, within 20 or more days of cohabitation. Therefore a significant reduction was seen in potency. However, the slight decrease in fecundity was not statistically significant (perhaps due to large standard deviation).

Effect of fluphenazine on implantation sites, liter size and fertility index (Table 1)

1 mg/kg/day: When the treated male rats were mated with normal females, the observed reduction in number of implantation sites, fertility index and litter size was not statistically significant (again due to small group size).

2–3 mg/kg/day: When the fluphenazine treated males were mated with normal females, a significant reduction was seen in pregnancy outcome. The number of implantation sites was significantly reduced and accordingly the fertility index was lower. There was a significant decrease in the litter size.

Effect of fluphenazine on tissue weights (Table 1)

1-3 mg/kg/day: At all the doses the weights of the pituitary glands were maintained. No changes were observed in the weights of testes and androgen-dependent tissues viz. epididymides, seminal vesicles, ventral prostate. The weight of the adrenal glands was also maintained.

Effect of fluphenazine on serum hormone profile (Table 1; Fig. 1)

1 mg/kg/day: A significant increase was observed in serum prolactin level after 60 days of treatment. A significant suppression was also evident in serum LH and FSH levels. Serum T and estradiol levels however remained unchanged.

2-3 mg/kg day: A significant, progressive and persistent increase was observed in serum prolactin level only at 3 mg dose. Interestingly, chronic hyperprolactinemia could maintain serum prolactin level seen at 1-2 mg doses but could not restore it

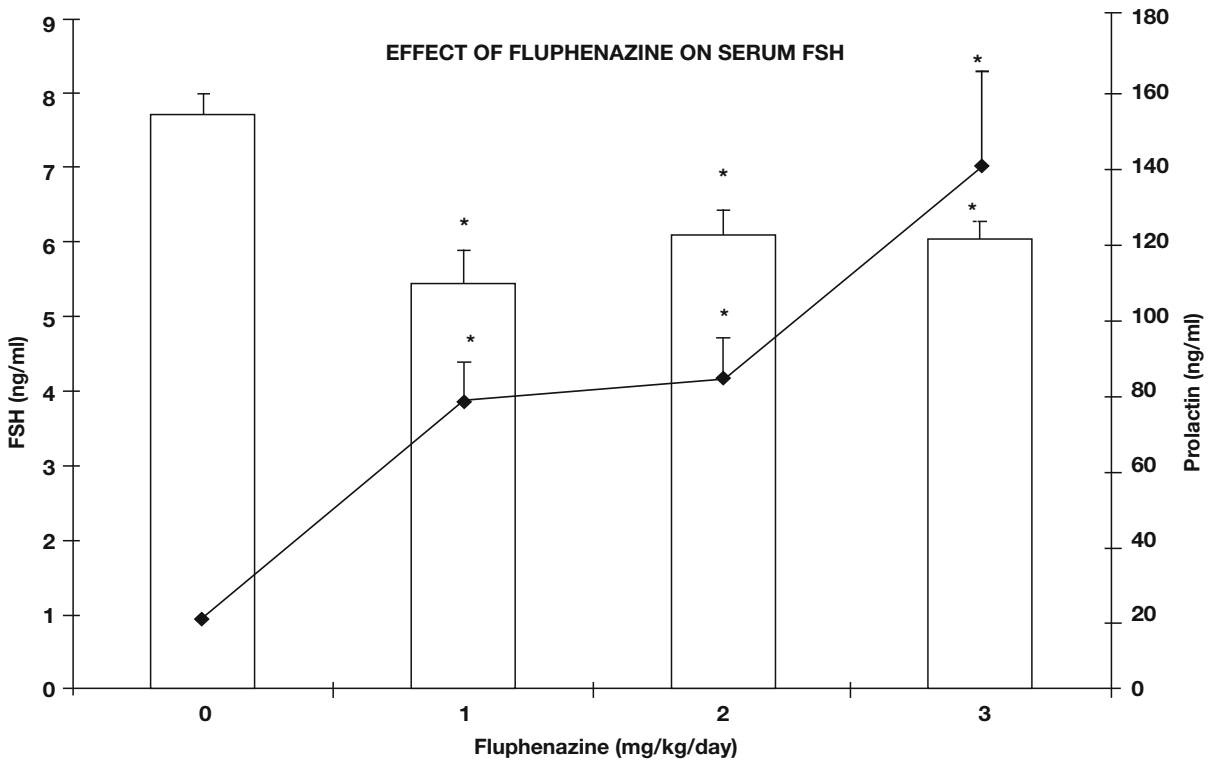

Fig. 1 - *The graph depicts correlative changes between serum FSH (bars) and Prolactin (line) at different doses of fluphenazine. The concentrations are represented as mean±S.E.M. Dose "0" represents control values. * represents significance w.r.t control.*

to control values via the normal autofeedback mechanism. The persistent suppression of serum LH and FSH levels did not show dose dependence. Serum T and estradiol levels remained constant .

Effect of fluphenazine on cauda *sperm morphology (Fig. 2a-b)*

1-3mg/kg/day: Fluphenazine treatment was observed to lead to formation of smaller sperm acrosomes concomitant with FSH reduction.

Effect of fluphenazine on testicular histology (Fig. 2c-d)

1-3mg/kg/day: Sloughing of immature germ cells was observed in the lumen of the seminiferous tubules of the testis, at all doses of fluphenazine treatment concomitant with FSH reduction.

Effect of fluphenazine on chromatin decondensation (Fig. 2e)

1-3mg/kg/day: Fluphenazine treatment altered the rates of *in vitro* chromatin decondensation of *caput* spermatozoa. The decondensed lengths of spermatozaoa taken from treated rats were increased at 2-3 minutes of incubation.

Effect of fluphenazine on chromatin structure (Fig. 2f)

1-3mg/kg/day: Fluphenazine treatment produced a statistically significant increase in the concentration of metachromatic acridine orange dye taken up by single stranded *caput* sperm DNA as can be seen from the characteristic increase in red fluorescence (530 nm) at 3mg/kg/day dose. Fluphenazine treatment appears to have increased single stranded DNA. No difference was evident in flourescence due to acridine orange intercalated in double stranded DNA (630 nm).

Effect of fluphenazine on testicular inhibin B immunoexpression (Fig. 3)

1-3mg/kg/day: Fluphenazine treatments did not appear to produce any detectable change in the immunoexpression of testicular inhibin B subunits.

Concentration of immunoreactive hypothalamic tyrosine hydroxylase protein (Fig. 4)

1-3 mg/kg/day: The hypothalamic tyrosine hydroxylase was upregulated after fluphenazine treatment indicating increased dopamine synthesis/release. The concentration of tyrosine hydroxylase protein

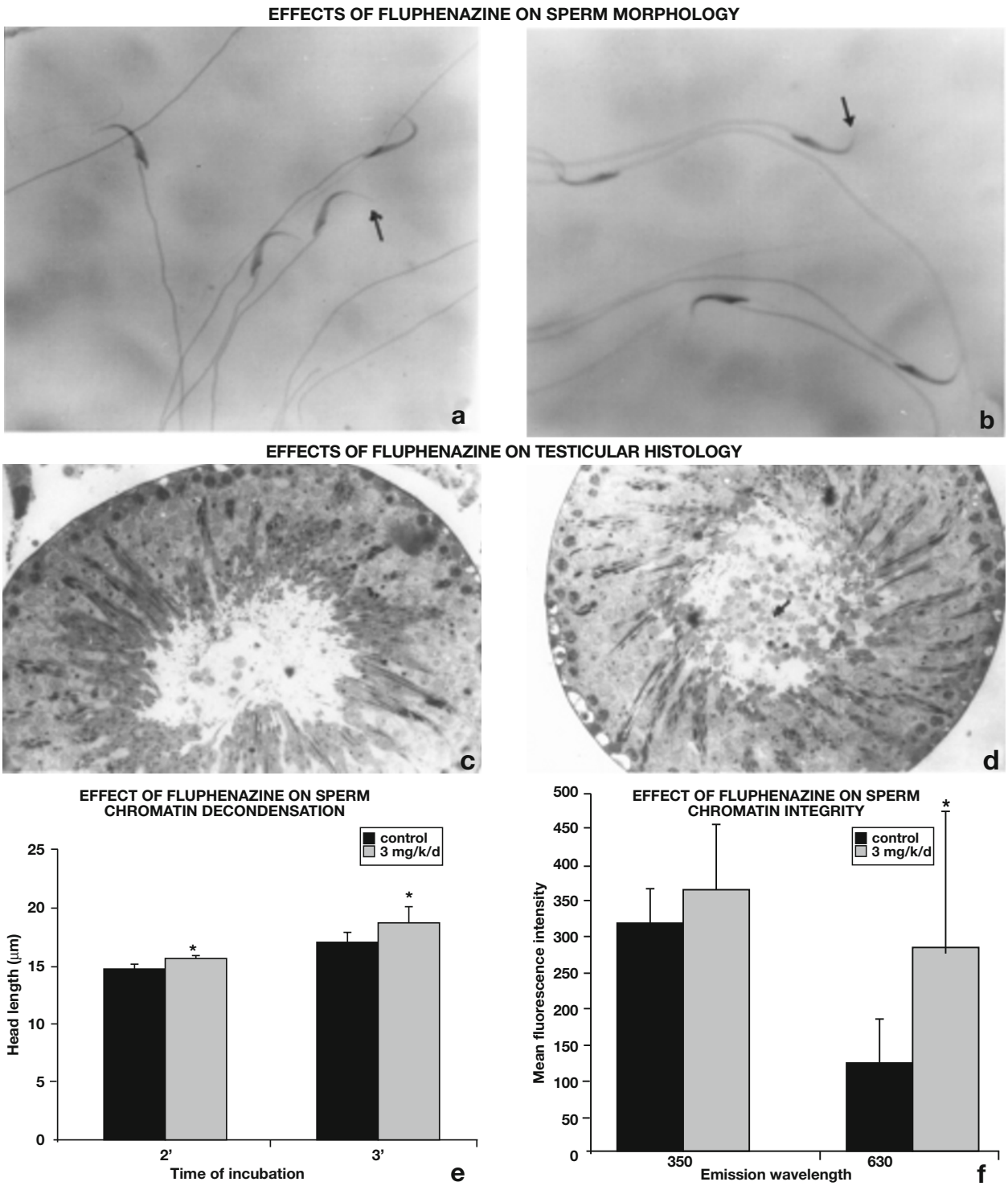

Fig. 2 - *Morphology of caudal sperm from control rat (a) and rats treated with fluphenazine 3 mg/kg/day (b). Arrows depict reduced size of sperm head acrosomal region. Histology of testis from control rat (c) and rats treated with fluphenazine 3 mg/kg/day (d). Arrows depict sloughed spermatids. Solid bars depict chromatin decondensation rate of* caput *sperms from control; Hatched bars depict rates after treatment with 3 mg/kg/day fluphenazine (e). Solid bars depict mean fluorescence intensity of acridine orange in control* caput *sperms; Hatched bars depict mean fluorescence intensity of acridine orange in 3 mg/kg/day fulphenazine treated sperms (f). All values are Mean±S.D. * depicts significance w.r.t control at* $p<0.05$.

in the hypothalamic samples in the three treatment groups viz. 7.74±0.49, 7.26±1.1, 8.89±0.36 ng/20µg hypothalamic protein was significantly higher than that in control group (4.15±0.11). The increased concentration of hypothalamic tyrosine hydroxylase persisted at 1-2 mg doses but did not show dose dependence. Further increase of newly synthesized protein was seen only at 3 mg dose in the estradiol-sensitive dopaminergic neurons, which were still responsive to prolactin.

DISCUSSION

Prolactin, secreted from lactotrophs in the pituitary gland of males does not have a well defined physiological role in male reproduction. It has been reported that prolactin can augment LH receptors on Leydig cells and thereby maintain serum T levels (3). However, the effects of prolactin can vary depending upon serum levels. (7, 11). Prolactin is a unique hormone which autoregulates its serum level by inducing tyrosine hydroxylase in the dopaminegic neurons through prolactin receptors on dopaminergic neurons in the hypothalamus (25). Dopamine released from the tuberoinfundibular dopaminergic nerve terminals, into the portal circulation of median eminence suppresses further release of prolactin from lactotrophs (26). The autoregulatory prolactin circuit manifests as the suppression of serum gonadotropins in certain experimental, physiological or pathological situations viz. circadian rhythms, photoperiods, antidopaminergic drugs, tumors (12, 27). The degree of hyperprolactinemia, which can vary from mild, modest to severe, ultimately determines the magnitude of endocrine disruption (11). Accordingly, the degree of hyperprolactinemia will determine the nature of effects on fertility of male rat. The reproductive behavior may be delayed or abolished leading to loss of libido or impotence (5). The mechanism underlying behavioral effects of prolactin might involve hypothalamic or central dopaminegic activation through cognate receptors (28).

The present study has demonstrated that fluphenazine, a D2 dopamine receptor blocker, at doses of 1-3 mg/kg/day, augments prolactin to render the adult male rat subfertile within 60 days of subcutaneous treatment. The serum endocrine profile however, shows a significant, dose-independent and persistent reduction in both LH and FSH, indicating suppression of GnRH neurons. The serum T and estradiol levels are within the normal range indicative of a compensatory mechanism to up-regulate testicular LH receptors or prolactin-dependent T synthesis. Moreover, potency is also significantly affected at the

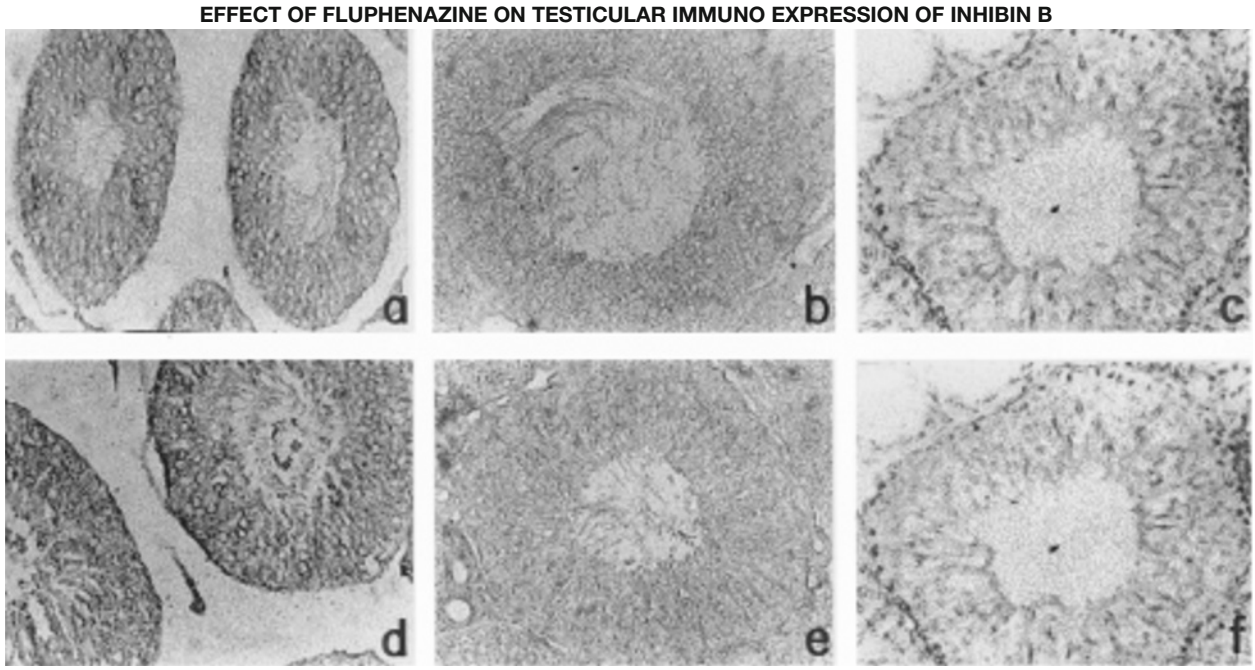

Fig 3 - *Immunoexpression of testicular Inhibin B α subunits from control (a) and rats treated with fluphenazine 3 mg/kg/day (b) and negative controls (c). Immunoexpression of testicular Inhibin B β subunits from control (d) and rats treated with fluphenazine 3 mg/kg/day (e) and negative controls (f).*

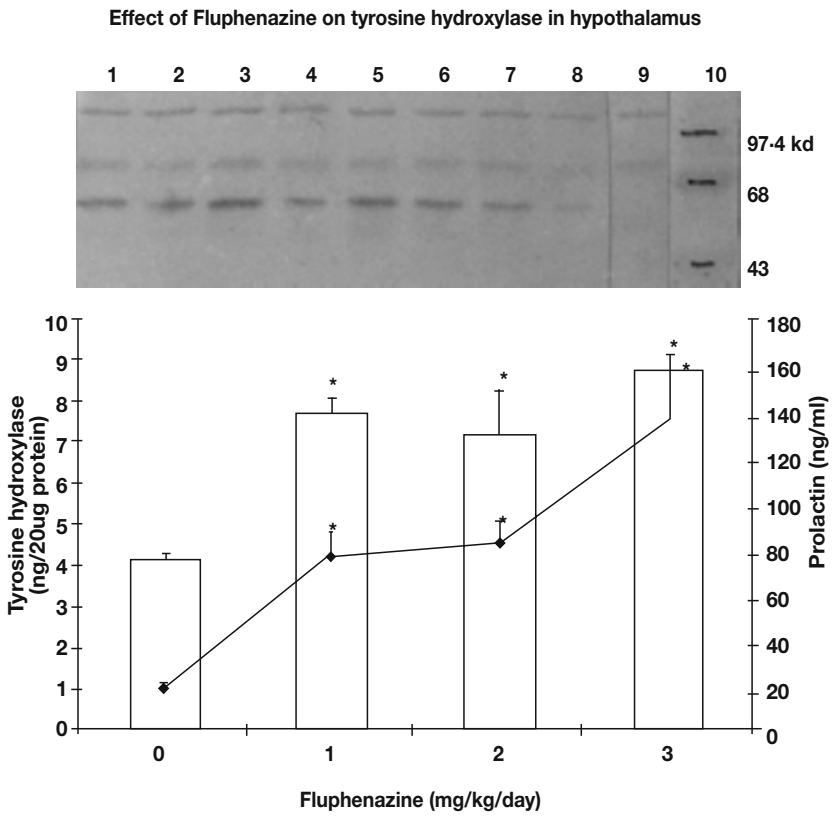

Fig. 4 - Upper panel: *Representative Immunoblot of 60 kDa tyrosine hydroxylase protein band in 20µg rat hypothalamic protein by SDS-PAGE analysis on 10% gel. Lane 1-2: after treatment with fluphenazine 3 mg/kg/d, Lane 3-4: after treatment with fluphenazine 2 mg/kg/d, Lane 5-6: after treatment with fluphenazine 1 mg/kg/d, Lane 7-8: band in untreated hypothalamus, Lane 9: tyrosine hydroxylase negative control showing absence of 60 kDa band, Lane 10: molecular weight markers.*

Lower panel: *The dose dependent concentration of tyrosine hydroxylase (ng) in the hypothalami of control and fluphenazine treated rats per 20µg of hypothalamic protein was calculated from integrated optical density of the corresponding bands and is represented as mean±SEM. Bars represent concentration of tyrosine hydroxylase. Line represents concentration of prolactin. Dose "0" represents control values. * represents significance w.r.t controls.*

3 mg dose due to delayed mating behavior. The fertility index but not fecundity, was also significantly decreased indicating reduced fertilizing ability and litter size. Normal fecundity, however, does not ensure a normal number of progeny. The adverse effects on fertility comprise therefore both central as well as testicular mechanisms. Both mating behavior and sperm fertilizing ability are compromised. Does a common hypothalamic mechanism involving differentially sensitive dopaminergic neurons, GnRH neurons and opiatergic peptides operate in eliciting both antigonadotropic and behavioral effects (29)?

Several D2 dopamine receptor antagonists have been shown to induce hyperprolactinemia by interfering with hypothalamic regulation of pituitary prolactin (27). In the present study, serum prolactin levels could not be restored via the physiological autofeedback mechanism. This did not indicate a failure of hypothalamic dopaminergic neurons to respond to prolactin. The persistent blockade of D2 dopamine receptors on the lactotrophs by the long-acting antagonist would have preempted the inhibitory response to dopamine hypersecretion (natural ligand). However, the degree to which prolactin can be suppressed by various treatments has already been shown to differentially affect gonadotropins and T levels (11). Thus, the circulating levels of prolactin during hyperprolactinemia would determine the degree of LH suppression and thereby that of T. The lack of effect of fluphenazine on T, estradiol and accessory organ weights in the present study therefore reflects medium level of hyperprolactinemia and would suggest up-regulation of testicular LH receptors and/ increased T synthesis, countering the effect of LH suppression (30). T has also been reported to be essential for maintaining spermatogenesis and sperm fertilizing ability (31). The normal T environment therefore ensured that both testis and androgen-dependent glands did not regress functionally. The lack of effect on sperm counts and motility reflect normal quantitative spermatogenesis and epididymal maturation. However, subtle changes in the intrinsic quality of spermatozoa, as a consequence of endocrine disruption induced by hyperprolactinemia, which would have led to the observed reduction in litter size, can not be ruled out.

Though fluphenazine induced hyperprolactinemia produced a significant suppression of both gonadotropins, suppression of FSH acquires significance in the present study as the effects of LH suppression

were counteracted by stimulation of testosterone synthesis. The important role of FSH in spermatogenesis and regulation of male fertility has been amply demonstrated in diverse experimental paradigms where it has been implicated in dynamic regulation of Sertoli cell morphology and functions, binding patency, and sperm counts (32-33). FSH has been specifically demonstrated to play a role in chromatin condensation through developmental activation of CREM tau protein (cyclic AMP response element modulator), necessary for synthesis of thiol rich transition proteins and protamines (34). FSH has been suggested to be involved in the elimination of aberrant testicular spermatozoa through activation of apoptosis (35). The dose-independent increase in the concentration of tyrosine hydroxylase observed at 1-2 mg doses reflects a dynamic compensatory effect. Further increase in tyrosine hydroxylase protein at 3 mg dose suggests synthesis of new protein and indicates that estradiol-sensitive dopaminergic neurons have not become refractory to prolactin. This could be attributed to prevailing normal serum levels of estradiol. The persistent up-regulation of tyrosine hydroxylase also suggests that opiatergic peptides, which can inhibit dopaminergic activity, may not have been elicited. Therefore, inspite of the increased dopaminergic activity, prolactin may not have led to gonadotropin suppression through autoregulatory activation of TIDA and concomitant activation of opiatergic neurons (36). Alternatively, prolactin could have either reduced gonadotropins by rendering GnRH neurons refractory through stimulatory D1 dopamine receptors or through inhibitory prolactin receptors or blockade of noradrenergic receptors (37-39). There is no evidence in literature to implicate serum inhibin in the mechanism through which prolactin regulates FSH. Inhibin B is an important testicular regulator of serum FSH in males (40). Increased synthesis of inhibin may not have led to FSH suppression. However, reduced testicular inhibin expression could reflect reduction in serum FSH. In the present study, although FSH concentrations were reduced, the levels appear not to have altered testicular inhibin B levels and were sufficient to sustain normal spermatogenesis as suggested by normal sperm counts and motility. Subtle changes in sperm morphology, structural integrity, testicular histology are however suggestive of testicular effects which could have produced intrinsic effects on sperm quality and reduction in litter size. Germ cell binding appears to be the most sensitive indicator of FSH deficits in fluphenazine treated male rat. Disorganization of tubular cytoarchitecture has been observed in FSH receptor knockout mutant male mice which display altered collagen organization adjacent to the basement membrane along with reduction in testicular weight and sperm counts (41). FSH has also been reported to alter the Sertoli cell cytoskeletal proteins concomitant with morphology (33). Aberrations in nuclear chromatin condensation have been linked to human infertility (42). FSH deficits have been reported to affect chromatin decondensation *in vitro* of non-human primate sperm (43). The enhanced rate of rat *caput* sperm chromatin decondensation *in vitro* as a result of fluphenazine treatment suggests a defect in nuclear chromatin condensation mechanism during spermiogenesis. FSH deficits have been reported to decrease the concentration of acrosomal proteins in non-human primates (44-45). FSH deficits have also been reported to affect sperm morphology with formation of malformed spermatids in FSH receptor knockout mutant male mice (41). The observed changes in acrosomal morphology of fluphenazine treated male rats could either be a consequence of sloughing or abnormal development.

An IHDA dopaminergic system, sensitive to T, has been implicated in suppression of mating behavior. Could the effects of hyperprolactinemia be mediated through a mechanism involving hypothalamic and central amygdaloidal dopaminergic cells? In the present study, up-regulation of tyrosine hydroxylase in the hypothalamus at all doses of fluphenazine, when T milieu is normal, indicates increased dopamine synthesis/turnover rate (46). Increased dopamine turnover in the hypothalamus and possibly amygdala could have progressively affected the mating behavior (11). Decreased potency as a consequence of delayed mating, is partially responsible for low fertility index in the 3 mg fluphenazine treated rat (7). In view of the reproductive consequences, hyperprolactinemia warrants in depth studies in a drug free model like transgenic mice in order to unequivocally demonstrate the underlying mechanism (8).

In conclusion, the present study has demonstrated that chronic exposure to fluphenazine induces hyperprolactinemia concomitant with gonadotropin suppression in adult male rat. The study indicates that hyperprolactinemia could have reduced the fertility of adult male rat mainly through an effect on sexual behavior but a FSH dependent testicular mechanism can not be excluded. Hypothalamic dopaminergic neurons could be mediating the effects of chronic hyperprolactinemia on gonadotropins and reproductive performance.

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