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Hyperprolactinemia affects spermiogenesis in adult male rats

M. Aleem[1], J. Choudhari[2], V. Padwal[2], N. Balasinor[2], P. Parte[2], and M.K. Gill-Sharma[2]

[1]Department of Urology, Medical College of Ohio, Toledo, Ohio, USA; [2]Department of Neuroendocrinology, National Institute for Research in Reproductive Health (ICMR), Parel, Mumbai, India

ABSTRACT. The mechanisms underlying the antifertility effects of hyperprolactinemia have yet to be established in an appropriate experimental model. Hyperprolactinemia is a known side effect of fluphenazine, a broad spectrum, long-acting phenothiazine known to be dopamine type-D2 receptor antagonist. In our earlier study in adult male rats, we reported that fluphenazine at a dose of 3 mg/kg/day suppressed serum FSH but not testosterone (T) through increasing dopamine (DA) metabolism in the pituitary gland, within 60 days. Fluphenazine treatment affected sperm quality and male rats treated with fluphenazine sired fewer litters. The effects of fluphenazine-induced hyperprolactinemia on sperm quality appeared to be related to reduced FSH. We now report that FSH suppression enhanced the uptake of acridine orange (AO), a DNA intercalating, fluorescent dye by the fluphenazine-treated caput epididymal sperms with concomitant reduction in the uptake of thiol-specific monobromobimane (mBBr) fluorescent dye *in vitro*, suggesting greater accessibility of DNA intercalating dye to sperm chromatin and reduction in free sperm protein thiols. The concomitant increase in AO and decrease in mBBr fluorescence was suggestive of loose chromatin packaging in caput epididymal sperms after treatment with fluphenazine at 3 mg/kg/day for 60 days. The suppression in levels of protamine (P1) in caput epididymal sperms suggested that chromatin hypocompaction was due to reduced deposition of protamines in sperm chromatin. Reduction in testicular levels of cyclic adenosyl 3', 5' monophosphate response element modulator (CREMτ) and P1 further suggested that reduced deposition was indeed due to reduced synthesis. The concomitant reduction in testicular levels of transition protein 1 (TP1) and transition protein 2 (TP2) also suggested that hypoprotamination was due to reduced synthesis of these proteins crucial for facilitating P1 deposition. The effect appeared to have occurred at the level of translation of CREMτ, since its transcript levels were unaffected whereas those of TP1, TP2 and P1 and protamine were upregulated. The study led to the view that the effects of FSH suppression were manifest on the posttranscriptional modifications of CREMτ, as also on transcript repression of TP1, TP2, P1, which do the RNA- binding proteins bring about. Reduction in FSH did not decrease ABP expression in the testis, which has recently been implicated in the expression of transition protein 1 *in vitro*. However, a significant reduction was evident after fluphenazine treatment, in the immunoexpression of testicular cAMP, the mediator of FSH effects in the Sertoli cells and putative mediator of ABP effects in the spermatids. The study suggests that fluphenazine-induced hyperprolactinemia suppressed FSH and affected a putative cAMP-dependent mechanism underlying posttranscriptional modification of spermatidal genes involved in chromatin condensation, presumably by reducing the availability/secretion of ABP, a paracrine regulator of spermiogenesis *in vitro*.

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

Correspondence: M. Gill-Sharma, Assistant Director, Department of Neuroendocrinology, National Institute for Research in Reproductive Health (ICMR), Parel, Mumbai, India.

E-mail: manjitgill_sharma@hotmail.com

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INTRODUCTION

Hyperprolactinemia has been associated with a host of antifertility effects that presented as structural abnormalities in sperms, testicular alterations as seminiferous epithelial disorganization, germ cell exfoliation, increased tubule wall thickness, abnormal Leydig cell lipid content, structural disruption

of Sertoli-germ cell junctional complexes and apical Sertoli cell cytoplasmic degeneration, increase in percentage of convoluted tubules with apoptotic spermatogonia and spermatocytes, abnormalities in reproductive behavior in rodents and humans (1-3). The underlying mechanisms also range from variations in LH, androgen, androgen-binding protein (ABP) and estradiol levels (2, 4, 5). In view of the diversity of hormonal variations involved in hyperprolactinemia, some exceptions have also been reported where either no correlation was observed between serum prolactin levels and infertility or correction of prolactin levels did not lead to an improvement in sperm morphology or functions (6, 7). In such cases hyperprolactinemia could have been a side effect induced by another hormone rather than the cause of infertility (5).

Though the antifertility effects of hyperprolactinemia are considered to be mediated primarily via suppression of testosterone (T), the role of FSH is also becoming increasingly clear since prolactin is also associated with a compensatory increase in T levels (8). The antifertility effects of moderate hyperprolactinemia could therefore also be attributed to FSH deficits. The effects of FSH deficits have been adequately demonstrated in FORKO mice where receptor deletion led to reduction of T and ABP concomitant with large irregularly shaped spaces within the seminiferous epithelium due to apparent accumulation of fluid in the Sertoli cell cytoplasm, extending from the base to the lumen, often separated by anastomotic cords of spherical germ cells or completely surrounded by elongating spermatids; the Sertoli organelles appeared intact and to be floating in the enlarged fluid-filled cytoplasm, with structural defects in sperms, increase in the rates of sperm chromatin decondensation and enhanced susceptibility to denaturation *in vitro* (9-11). Identical results on sperm chromatin decondensation have also been reported after treatment with fluphenazine, a dopamine receptor blocker, which induced hyperprolactinemia and FSH suppression, without any change in T in adult male rats (8). The poor sperm quality induced by FSH deprivation apparently led to reduction in litters sired by affected male rodents since the sperm counts were not reduced (8). It would thus appear that fluphenazine treatment predominantly affected the paracrine relationship between the Sertoli cells and developing spermatids in the testis.

Several genes expressed in Sertoli cells have been reported to be FSH-regulated, namely ABP, hyaluronidase, inhibins, androgen receptor, aromatase, and CREM (12-17). Although the genes mediating the FSH dependent paracrine effects on spermiogenesis are yet to be demonstrated, FSH bioneutralization studies have implicated ABP as a putative mediator of fertility in immature male rats (18). Reduced bioavailability of ABP has been reported to produce adverse effects on sperm fertilizing ability in mutant and hypophysectomized rats (19, 20). Rodent ABP, known to be upregulated during stage VII of spermatogenesis in rats, is involved in intratesticular transport of T to germ cells wherein it is internalized (21-23). Recently, ABP has been implicated in the *in vitro* expression of spermatidal transition protein 1, implicated in the process of sperm chromatin condensation and repair, suggesting it has a putative role in the paracrine regulation of spermiogenesis in rats (24-26). The transition proteins 1 and 2, which facilitate the deposition of protamines in sperm chromatin being cAMP dependent, lead to the obvious conclusion that ABP could be mediating paracrine effects through cAMP (24, 25, 27).

The present study was therefore extended to quantify the effects of fluphenazine-induced hyperprolactinemia on the free sperm thiols and expression of testicular genes involved in sperm chromatin condensation in adult male rats.

MATERIALS AND METHODS

Animals

Seventy-five-day-old male rats of the Holtzman strain (bred at NIRRH, Mumbai) were maintained at a temperature of 22-23 C, 50-55% humidity and by light/dark cycle of 14 h: 10 h. Commercial food pellets and water were available *ad libitum*. All experiments were carried out with the approval of the ethics committee of the institute.

Drugs

Fluphenazine decanoate injections (Anatensol deca-noate) containing 25 mg/ml sesame oil were obtained from Sarabhai Chemicals (Mumbai, India). Fluphenazine in sesame oil was administered daily to the animals subcutaneously at a concentration of 3 mg/kg body weight. The drug or the vehicle for control purposes was injected for 60 days between 10:00 and 12:00 h. The rats were randomly grouped to six animals each for the different sets of experiments.

EXPERIMENTAL PROTOCOL

Autopsy of male rats

Male rats were killed by decapitation (in view of the fact that anesthetics affect hormone levels). The testes were dissected under sterile conditions for RT-PCR, western blotting and acid-urea SDS-PAGE and stored frozen under liquid nitrogen. For immunohistochemical localization of cAMP, the testes were immersion fixed in 10% formalin in 0.01 M PBS and processed through a series of graded alcohols,

xylene, and embedded in paraffin wax for immunohistochemistry.

Sperm chromatin acridine orange dye uptake assay

The sperms were extracted from caput epididymis of treated and untreated rats as described earlier (5). Two million sperms in 0.2 ml of PBS (pH 7.4) were incubated with 0.4 ml of chilled lysis buffer (0.1% Triton X -100, 0.08 N HCl, 0.15 M NaCl) for 30 sec at room temperature (RT). 1.2 ml of ice-cold acridine orange dye (Sigma), at a critical concentration of 6 µg/ml in dye buffer (0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, and 0.1 M citric acid) was added. After 3 min at RT the sperms were washed twice with dye buffer. Sperms were then incubated in 2 ml of DNA lysis buffer (10mM Tris, 100mM NaCl, 25mM EDTA, 2% SDS, 150µm β- mercaptoethanol, 400µg/ml Proteinase K (Sigma) at 56 C for 3 h in order to dissolve and extract the dye intercalated in sperm DNA. A standard curve for AO stain was set up in parallel, in the range of 1.9-1000µg. The wavelengths of excitation and emission for dye were 490 and 530nm, respectively (Shimadzu). The concentration of the dye taken up by sperm DNA was estimated from the standard curve of AO and expressed as µg dye/mg sperm protein. Sperm protein in the lysis buffer was estimated from the absorbance at 280nm.

Sperm chromatin monobromobimane (mBBr) dye uptake assay

Caput sperms ($5x10^6$) from all rats were suspended in 0.5 ml of 0.01M PBS and incubated with monobromobimane (mBBr) (Sigma), fluorescent thiol-specific reagent, at a final concentration of 0.5mM, in the dark for 10 min (28). Labeled sperms were washed twice with 0.01M PBS and dissolved in 2 ml of sperm solubilizing solution containing 0.1M β-mercaptoethanol (Sigma) and 8M guanidine hydrochloride. For quantification of sperm thiols, a standard curve for mBBr stain was set up in parallel, in the range of 0.0285-14.82 nmoles. The wavelengths of excitation and emission for dye were 390 and 495nm, respectively. The concentration of the total dye reacted with sperm DNA in the samples was estimated in a Shimadzu flourometer, from the standard curve of mBBr and expressed as nmoles dye/mg sperm protein. Sperm protein in the lysis buffer was estimated from the absorbance at 280nm.

Detection of CREMτ by Western blotting in testes

Testicular tissues (100 mg) were homogenized in 1ml of TEDG buffer (10mM Tris, 1mM EDTA, 1mM DTT, 10% glycerol) in a mechanized homogenizer (Remi, India) and supernatants separated by centrifugation at 15,000rpm for 45 min at 4 C in a centrifuge (Heraus, Germany) and stored at –20 C. The protein concentration in the homogenates were estimated by the method of Lowry (29). The proteins (200µg) were resolved on 12.5% denaturing polyacrylamide gels at constant current of 70V according to the method of Laemmli (30). Molecular weight markers in the range of 17.5-218-kDa were run alongside. The proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham), treated with blocking solution [5% non fat dry milk (NFDM) in 0.01 M PBS] for 1 h at RT, incubated with the anti-rabbit polyclonal primary antibody to CREMτ (Santracruz biotechnology, USA), at a dilution of 1:1000 in diluting buffer [0.1% Tween 20 (Sigma), 5% NFDM in 0.01 M PBS] at 4 C O/N, washed five times for 10 min each with the wash buffer (0.1% Tween 20 in 0.01 M PBS). The membranes were then incubated with horse radish peroxidase, labeled, goat anti-rabbit secondary antibody for 1 h at RT at a dilution of 1:1000 in buffer comprising 0.1% Tween 20,5% NFDM in 0.01 M PBS, washed six times for 10 min each with wash buffer (0.1% Tween 20 in 0.01 M PBS). The proteins were visualized by chemiluminescence reagents. The protein bands on the films were quantified with Gel Pro 3.1 image analysis software (Macintosh) and expressed as IOD.

Detection of transition protein (TP)1, TP2, protamine (P1), by Western blotting in testes and sperms

The basic proteins were extracted as described by Kistler et al. (31). Testicular tissues (0.5 gm) from all rats were homogenized in 9 volumes of 0.5M HCl solution. The homogenates were chilled on ice for 20 min and then centrifuged at 8000rpm for 10 min in a Heraus centrifuge (Germany). The proteins in the supernatants were precipitated with trichloroacetic acid (TCA) at a final concentration of 20% and pelleted by centrifugation at 15000 rpm for 20 min. The protein pellets were washed with 1 ml of acetone and chilled for 10 min. The pellets were allowed to dry before solubilization in 0.2 ml of 5-5-5 solution (5M urea, 5% β- mercaptoethanol, 0.5% acetic acid). Caput epididymal sperms from the control and treated rats were suspended in 0.5 ml of 0.01M PBS and lysed with 2 ml of chilled lysis solution (6M guanidine hydrochloride, 50mM Tris HCl and 5% v/v - mercaptoethanol) and acidified with HCl at a final concentration of 0.5 M HCl. The acidic lysate was dialyzed (6kDa pore size dialysis bags, Pierce, USA) O/N in cold against dialysis buffer containing 0.25M HCl and 0.1% β-mercaptoethanol in DW, with two changes of the dialysis buffer. The basic proteins in the supernatants, obtained after centrifugation of lysate at 10.000 for 30 min, were precipitated with TCA at a final concentration

of 20%, separated by centrifugation at 8,000 rpm for 10 min and washed five times with acetone. The protein pellets were allowed to air dry and dissolved in 0.2 ml of 5-5-5 solution. The protein concentration in dissolved pellets was estimated from absorbance at 280nm.The positively charged basic proteins were resolved on continuous 15% acid urea polyacrylamide gels (after reversing the direction of current). The resolving gel was pre-electophoresed at 100 V for 2 and a half h, till a constant current was obtained, using 5% acetic acid as running buffer. Protein samples (100µg) were loaded in the wells and resolved at constant voltage of 150 V for 45 min. Methylene green was used as tracking dye in 5-5-5 solution. The basic proteins were transferred to nitrocellulose membranes and incubated in blocking solution [0.03% gelatin, 0.5% Tween 20.5% NFDM, 1% normal serum (goat serum for TPs and horse serum for protamine) in 0.01 M PBS] for 1 h at RT, followed by incubations with primary antibodies (generous gifts from Stephen Kistler and Rod Balhorn) to transition proteins 1, 2 (polyclonal) or protamine (1Hup1N, monoclonal), for 1 h at RT, biotinylated anti- rabbit for TPs and anti-mouse for protamine secondary antibody (1:1000) for 1 h at RT, and avidin-biotinylated HRP complex (Vector laboratories) in 0.01M PBS (pH 7.4) for 1 h at RT, with thorough washings with wash buffer (0.03% gelatin, 0.5% Tween 20 in 0.01M PBS) after each step. Specific bands of TP1, 2 and P1 were visualized with diaminobenzidine (DAB) (Sigma). 0.01M PBS was substituted for primary antibody in the negative controls for testicular and sperm proteins. The proteins were quantified with Gel Pro 3.1 image analysis software and expressed as integrated optical density (IOD).

Detection of TP1, TP2, P1, CREMτ, ABP transcripts by biplex RTPCR in testis

Testicular tissues (50-100 mg) were homogenized in 1 ml of Trizol reagent (Invitrogen) and RNA extracted with 0.2 ml chloroform (per ml of Trizol reagent). The mixture was allowed to stand for 5 min at RT and centrifuged at 12000 g for 15 min at 4 C. The RNA in aqueous phase was extracted with 0.5 ml of isopropanol (per 1 ml of Trizol used for initial homogenization) and allowed to precipitate at RT for 10 min, RNA pellet was separated by centrifugation at 12000 g for 10 min at 4 C and washed with 1 ml of 70% ice-cold ethanol, centrifuged at 7500 g for 5 min at 4 C and air-dried. The RNA pellet was dissolved in RNAse free water by warming at 65 C for 10 min and stored at –70 C. The concentration of RNA was determined by measuring the absorbance at 260 nm. Integrity of the RNA was checked by resolving on 1.5% agarose gel. RNA (1µg) was reverse transcribed at 48 C for 45 min using AMV reverse transcriptase and amplified by biplex polymerase chain reaction (PCR) using Titan one tube RT-PCR kit (Roche diagnostics) and forward and reverse primers for specific RNAs for TP1 (5'CCAGCCGCAAACTAAAGACTCATGG3'; 5'AGCTCATTGCCGCATTACAAGTGGG3'), TP2 (5'AGGAAAGGTGAGCAAGAGAAAGGCG3'; 5' CATTCCCCTAGTGATGGCTATCTCC3'),P1(5'AG CAAAAGCAGGAGCAGAAG3';5'GGCGAGATGC TCTTGAAGTC3'),CREMι(5'GATTGAAGAAGAAA AATCAGA3';5'TTGACATATTCTTTCTTCTTCTT3') ,ABP(5'GAGAAGGGAGAGGTGGCCT3';5'GCTC AAGGCTACTTTGAATAC3'), and mouse β-actin as internal control (5'CTGGCACCACACCTTCTA3'; 5'GGGCACAGTGTGGGTGAC3', Gene accession number 007393) in a 50 µl reaction volume (32-35). The conditions of PCR amplification for 30 cycles are shown in Table 1. For amplification of the CREMτ mRNA, the concentration of the $MgCl_2$ was increased to 2 mM instead of 1.5 mM. All PCR products in 20 µl of samples were resolved on 2% agarose gel (4% for protamine) containing 0.5 µg/ml ethidium bromide to check the product size. DNA markers were run along with the products. The resolved PCR products were quantified with Gel Pro 3.1 analyzer software. RNA concentrations in samples were expressed as the ratio of IODs of the specific PCR products and β-actin control product.

Table 1 - *PCR conditions.*

Transcripts	TP1	TP2	P1	CREMτ	ABP
Denaturation	94 CX30s	94 CX30s	94 CX30s	94 CX30s	94 CX30s
Annealing	59 CX1min	59 CX1min	55 CX1min	52.4 CX1min	60 CX1min
Primer extension	68 CX1min	68 CX1min	68 CX1min	68 CX1min	68 CX1min
Final extension	68 CX7min	68 CX7min	68 CX7min	68 CX10min	68 CX7min
Size	175bp	152bp	201bp	525bp	924bp

TP1=transition protein1; TP2=transition protein 2; ABP: androgen-binding protein.

IMMUNOLOCALIZATION OF cAMP IN TESTIS

cAMP was detected in the testis according to the method of Pascolini et al. (36). Paraffin sections of the testis (5µ) were dewaxed, cleared in xylene for 30 s, rehydrated through 100%, 95%, and 70% alcohol for 5 min each. Endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide (diluted in 70% methanol) for 30 min and washed for 5 min with 0.01 M PBS. To block non-specific sites, the sections were incubated for 30 min in the blocking solution [2% bovine serum albumin (BSA), 1.5% normal goat serum in 0.01M PBS]. The sections were incubated O/N at 4 C in a light proof box with cAMP polyclonal primary antibody (Sigma, USA) diluted in 0.01M PBS containing 0.1% BSA, at a dilution of 1:10. Sections were washed twice for 10 min with 0.01M PBS and incubated with goat anti-rabbit, biotinylated secondary antibody, at a dilution of 1:500 (0.1% BSA in 0.01M PBS) for 1 h at RT, followed by two washes of 10 min each. The slides were incubated with HRP conjugated avidin-biotin complex (Vector laboratories) for 1 h followed by two washes of 10 min each. The cAMP was visualized with DAB and the reaction terminated by immersing the slides in DW. Sections were counterstained with hematoxylin. The slides were dipped in absolute alcohol, air dried and left in xylene O/N and mounted with DPX. 0.01M PBS was substituted for primary antibody in negative controls. The concentration of cAMP in the tissue sections was estimated from the intensity of staining using BIOVIS 4.12 image analysis software. The sections were photographed under bright field optics at 40/100x magnification.

Statistical analysis

All results were expressed as mean±SEM (standard error of the mean). Significant differences between means of control and experimental group rats were calculated by Student's t -test (Microsoft Excel). Level of significance was set at $p<0.05$.

RESULTS

Effect of fluphenazine on AO uptake by sperms

Fluphenazine treatment led to a significant increase in the total AO uptake by the sperm chromatin taken from caput epididymis (60%) as compared to control rat sperms.

Effect of fluphenazine on mBBr uptake sperms

Fluphenazine treatment led to a significant reduction (47.5%) in the uptake of mBBr and concentration of free thiols in caput epididymal sperms as compared to control rat sperms. Such a significant decrease (49%) in dye uptake is normally seen after the control rat sperms transit from caput to cauda epididymis (result not shown).

Effect of fluphenazine on basic protein levels in sperms

Fluphenazine treatment suppressed the deposition of (Fig. 1c) protamine 1 (6KDa) in the caput epididymal spermatozoa. There was no evidence of abnormal persistence of either (Fig. 1a) TP1 (6.2Kda) or (Fig. 1b) TP2 (13Kda) in fluphenazine treated or untreated sperms.

Effect of fluphenazine on testicular cAMP levels

Fluphenazine treatment produced a significant decrease (13.88±0.131, IOD) in levels of testicular cAMP (hypothetical second messenger in germ cells) as compared to controls (14.42±0.09, IOD) and was immunolocalized in the cytoplasm of hematoxylin-stained, spermiogenic cells, spermatocytes and spermatids in particular, within the testes, irrespective of treatment.

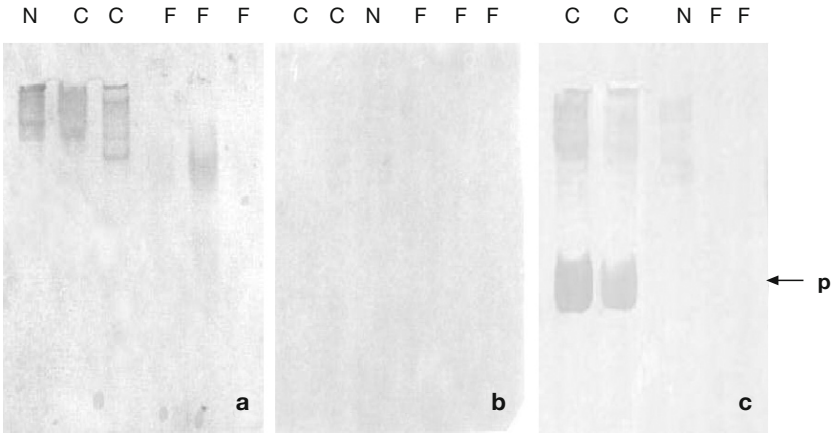

Fig. 1 - *Immunoblots showing the absence of transition protein 1 (a), transition protein 2 (b) and presence of protamine 1 (c) in sperm proteins. Lanes N: negative control showing absence of specific bands; Lane C: control sperms; Lanes F: fluphenazine treated.*

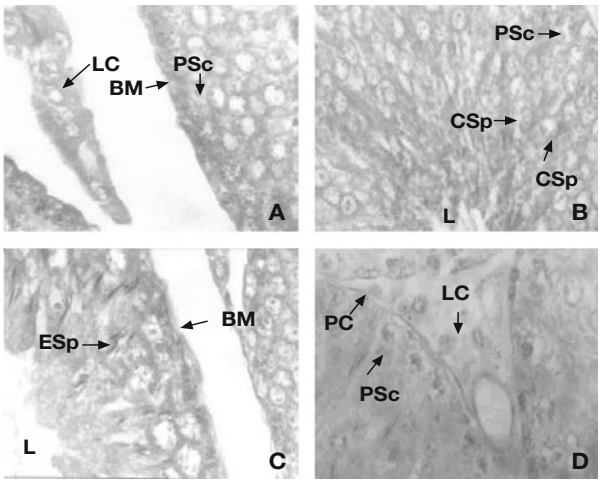

Fig. 2 - *cAMP immunoexpression in control and fluphenazine treated rat testis. Control (A) and (B-C) treated and (D) negative control. Positive staining seen in cytoplasm of germ cells. BM: basal membrane, CSp: cap phase spermatid, ESp: elongated spermatid, PSc: primary spermatocyte, PC: peritubular cell, LC: Leydig cell.*

Effect of fluphenazine on protein levels in testes

Fluphenazine treatment led to a complete suppression of protein bands corresponding to (Fig. 3) TP1 (6.2Kda), (Fig. 4) TP2 (13Kda), (Fig. 5) protamine (6KDa) and (Fig. 6) CREMτ (35Kda) as compared to control rat testes.

Effect of fluphenazine on testicular genes involved in chromatin condensation

Fluphenazine treatment produced a significant increase in (Fig. 3) TP1 (20%), (Fig. 4) TP2 (56.83%) and (Fig. 5) protamine (9.69%) transcripts as compared to control rat testis. No change was observed in (Fig. 6) CREMτ, (Fig. 7) ABP transcripts after fluphenazine treatment as compared to control rat testes.

DISCUSSION

Hyperprolactinemia has a long standing association with infertility (1-3). Nevertheless, definite proof of an underlying mechanism has not emerged so far. On the contrary, evidence exists which indicates that elevated prolactin levels in infertile patients are incidental and bear no correlation whatsoever to observed subnormal semen parameters (6, 7). Furthermore, hyperprolactinemia has a wide range from mild, moderate to acute, often associated with reduction in T levels where correction of latter hormone improves fertility status of the patients (4, 6). The causes of hyperprolactinemia, too, can be equally diverse ranging from stress induced hormonal imbalance to pathological causes like pituitary cancer. Therefore a pertinent question remains to be answered as to whether unphysiological prolactin levels do in fact lead to testicular infertility.

Our earlier studies with fluphenazine in adult male rats had indicated that the drug leads to elevated serum prolactin and suppression of FSH without any change in circulating T (8). Concomitantly, the drug also affected the packaging of caput epididymal sperm chromatin leading to enhanced rate of chromatin decondensation *in vitro*. The standard flow cytometric sperm chromatin structure assay had further revealed an enhanced propensity of chromatin to denaturation *in vitro*. The present study confirmed these results that the sperm were indeed loosely packaged as a result of

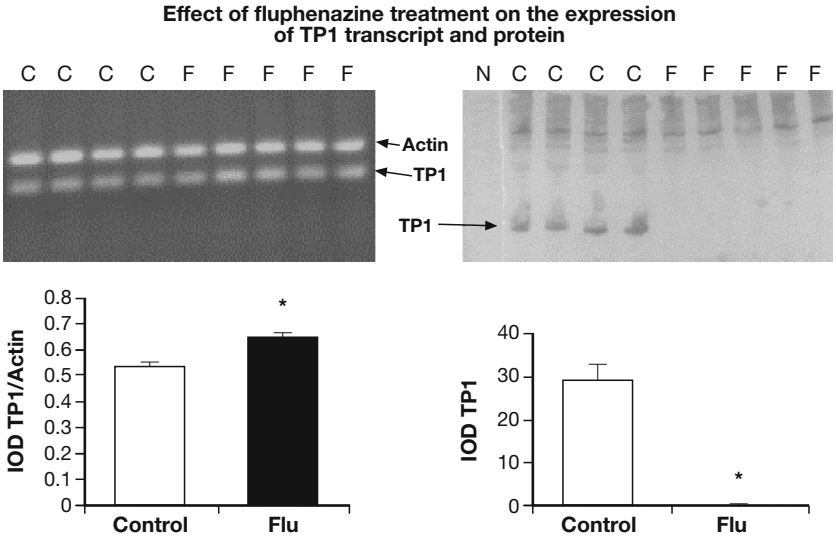

Fig. 3 - *Upper left panel: representative RT-PCR product of transition protein (TP) 1 transcript from rat testis. Lanes C: control testis, Lanes F: fluphenazine treated. Lower left panel: ratios of IOD of TP1/ β-actin before and after treatment. Upper right panel: representative TP1 protein band in rat testicular protein by acid-urea PAGE analysis on 15% gel. Lane N: TP1 negative control, Lanes C: control testis, Lanes F: fluphenazine treated. Lower right panel: IOD of TP1 protein bands before and after treatment. All values are mean±SEM. *depicts significance with respect to (w.r.t) control at* $p<0.05$.

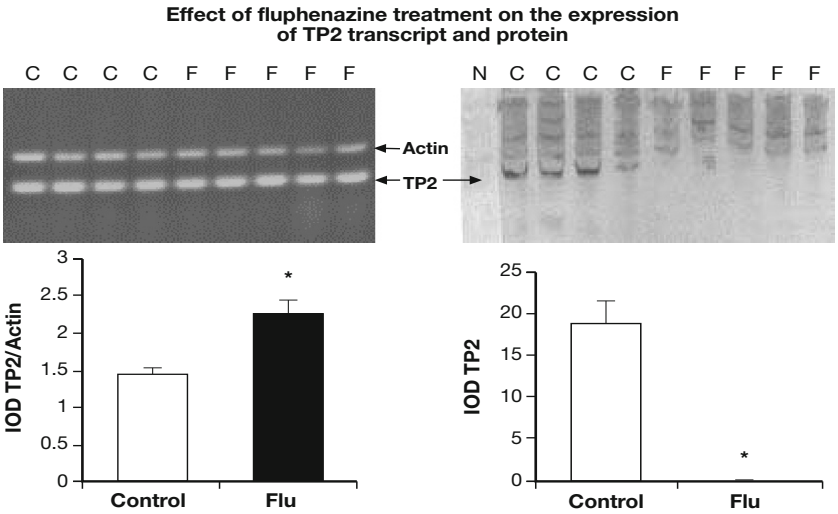

Fig. 4 - *Upper left panel: representative reverse transcription polymerase chain reaction (RT-PCR) product of transition protein (TP) 2 transcript from rat testis. Lane C: control testis, Lanes F: fluphenazine treated. Lower left panel: ratios of IOD of TP2/β-actin before and after treatment. Upper right panel: representative TP2 protein band in rat testicular protein by acid-urea PAGE analysis on 15% gel. Lane N: TP2 negative control, Lanes C: control testis, Lanes F: fluphenazine treated. Lower right panel: IOD of TP2 protein bands before and after treatment. All values are mean±SEM. *depicts significance w.r.t control at* p<0.05.

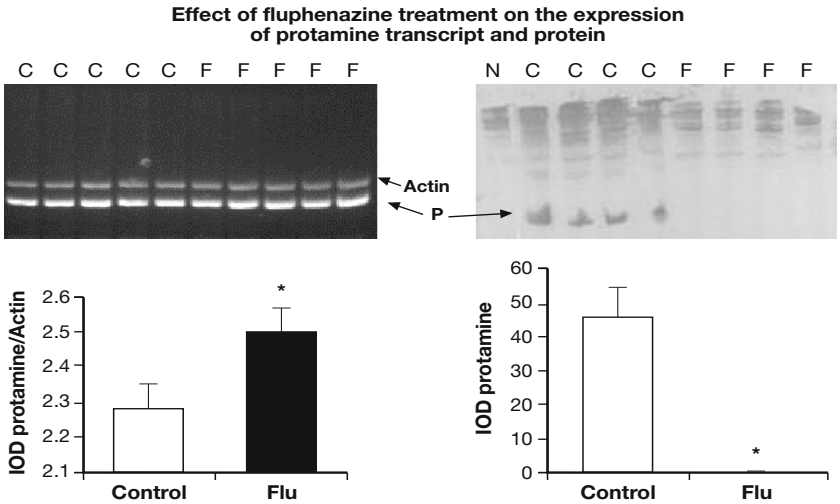

Fig. 5 - *Upper left panel: representative reverse transcription polymerase chain reaction (RT-PCR) product of protamine transcript from rat testes. Lanes C: control testes, Lanes F: fluphenazine treated. Lower left panel: ratios of IOD of P/β-actin before and after treatment. Upper right panel: representative protamine protein band in rat testicular protein by acid-urea PAGE analysis on 15% gel. Lane N: P negative control, Lanes C: control testis, Lanes F: fluphenazine treated. Lower right panel: IOD of P protein bands before and after treatment. All values are mean±SEM. *depicts significance w.r.t control at* p<0.05.

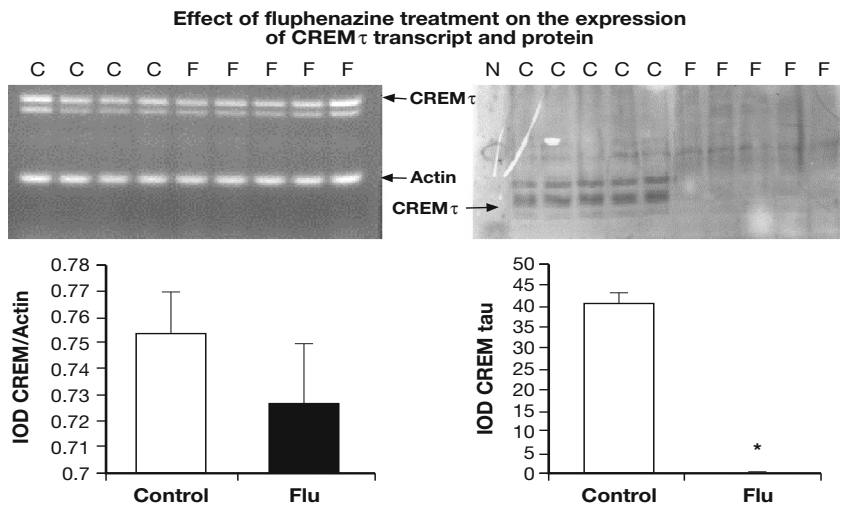

Fig. 6 - *Upper left panel: representative reverse transcription polymerase chain reaction (RT-PCR) product of CREMτ transcript from rat testis. Lanes C: control testis, Lanes F: fluphenazine treated. Lower left panel: ratios of IOD of CREMτ/β-actin before and after treatment. Upper right panel: representative CREMτ protein band in rat testicular protein by SDS-PAGE analysis on 12.5% gel. Lanes N: CREMτ negative control, Lanes C: control testis, Lane F: fluphenazine treated. Lower right panel: IOD of CREMτ protein bands before and after treatment. All values are mean±SEM. *depicts significance w.r.t control at* p<0.05.

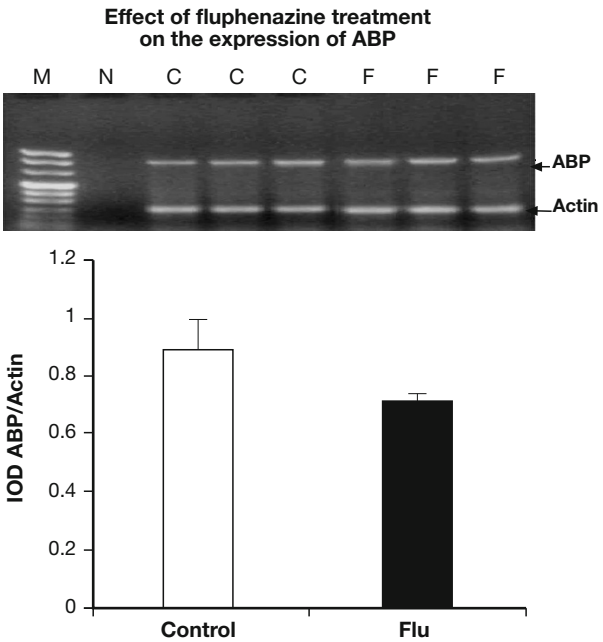

Fig. 7 - *Upper panel: representative amplified band of ABP transcript from rat testis. Lane M: molecular weight markers, Lanes C: control testis, Lanes F: fluphenazine treated. Lower panel: ratio of IOD of ABP/β-actin before and after treatment. All values are mean±SEM.*

fluphenazine treatment. The sperms from caput epididymis retained significantly higher amounts of a DNA intercalating AO dye *in vitro* as compared to controls, suggestive of a relatively greater accessibility of DNA to the dye. Concomitantly, fluphenazine treated caput epididymal sperms reacted with significantly lower amounts of a thiol group specific fluorescent dye as compared to control sperms. The loss of free sperm thiols after fluphenazine treatment suggested a reduction in thiol rich sperm protamines.

Indeed, a significant reduction was observed in the deposition of protamines in sperms as a result of treatment. The reduction was caused by reduced testicular synthesis of protamines. Protamine synthesis occurs in elongating spermatids after the removal of RNA binding proteins, which are involved in the stabilization and translational repression of this protein (37). Indeed, deletion of RNA binding protein tarbp2 in mutant mice revealed a premature translation of protamines leading to spermatogenic abnormalities (37). The results suggest that fluphenazine induced suppression of protamine synthesis could involve posttranscriptional modifications of the transcripts. The concomitant reduction in the testicular levels of transition proteins 1 and 2 further suggested that fluphenazine treatment had not only affected the synthesis of protamines but also suppressed the two proteins required for the removal of nuclear histones, a prerequisite for the deposition of the thiol rich protamines (38). Deletion of transition protein1 gene has been reported to lead to arrest of spermatogenesis, presumably due to premature deposition of protamines (39). Such an effect was not seen in our study indicating that spermiogenesis can proceed to completion in the concomitant absence of both transition proteins and protamines. The transition proteins and protamines are transcribed in the stage VII spermatids in rats and reported to be induced by CREMτ (40). A further analysis revealed that fluphenazine treatment had suppressed the testicular levels of CREMτ as well. Thus the entire mechanism involved in chromatin condensation appeared to have been adversely affected by fluphenazine. However, the CREMτ transcript levels were unaltered. CREMτ is considered to be induced by FSH prior to spermiogenesis (41).

Since our earlier studies had established that FSH was the major hormone suppressed by fluphenazine treatment, this indicated that alternative mechanisms operate to regulate this gene. Such a lack of effect on CREMτ expression has been reported in FORKO mice lacking a functional FSH receptor (42). The results suggest that reduced FSH adversely affected the translation of all four proteins required for chromatin condensation. This was further supported by the observed upregulation of the transition proteins and protamine genes in spite of the suppression of CREMτ protein.

The present study in fact suggested the existence of an alternative mechanism to CREMτ for the regulation/modulation of the expression of both transition proteins and protamine. In order to understand the CREMτ independent changes in gene expression, the effect of fluphenazine treatment was seen on the expression of the Sertoli cell marker, ABP, which has recently been implicated in facilitating the expression of spermatidal transition protein1 *in vitro* (24). Fluphenazine treatment, which suppresses FSH, did not increase the expression of ABP *in vivo*. In fact, deletion of FSH receptor has also been reported to reduce the secretion of seminiferous tubular fluid as has ABP, though experimental hyperprolactinemia induced by pituitary grafts in rats does not lead to reduction in epididymal

Table 2 - *Effects of fluphenazine on acridine orange (AO) uptake, thiol groups by caput epididymal sperms of adult male rats.*

Affected parameters	Control	Fluphenazine (3 mg/kg/day)
µg AO /mg protein	1.08±0.110	1.72±0.21*
nmoles mBBr/mg protein	95.19±11.21	49.88±14.6*

All values are mean±SEM. *: significance at $p<0.05$ as compared to control.

levels of secreted ABP (9, 10). This suggested that fluphenazine treatment could have reduced the secretion of ABP *in vivo*, which presumably affected the post-transcriptional modifications of transition proteins and protamine. However, since ABP has been implicated with upregulation of transition protein 1 and both T and estradiol with its suppression *in vitro*, it can be conjectured that the genes were upregulated due to reduced availability of these inhibitors *in vivo* after fluphenazine treatment (24).

In view of the lacuna that prevails pertaining to the receptor-linked downstream events for gene regulation in germ cells, the putative molecules involved in ABP action have not been identified yet. Several reports have suggested the presence of cognate binding sites for ABP in rat testes (43). Furthermore, sex hormone binding globulin (SHBG), an analogous human serum protein, is reported to increase cAMP levels in prostate cancer cells (44). Since both rat ABP and human SHBG proteins are expressed from the SHBG gene, a commonality of post-receptor events can be envisaged (45). In the present study, fluphenazine treatment reduced the cAMP immunoexpression in the testis though not that of ABP. Both cAMP and FSH, suppressed by fluphenazine, have been reported to facilitate the expression of ABP through cyclic adenosine 5′, 3′ monophosphate response element (CRE) in the promoter (12). Since cAMP-dependent ABP expression was unaffected, it can be averred that the decrease in cAMP occurred in the germ cells where it could be immunolocalized, rather than in Sertoli cells. Thus the fluphenazine study suggests that hyperprolactinemia-induced reduction in FSH levels could have affected a common ABP-regulated mechanism involving cAMP-dependent posttranscriptional modifications of the transcripts of genes involved in chromatin condensation during spermiogenesis.

In conclusion, the fluphenazine study suggests a mechanism underlying the antifertility effects associated with hyperprolactinemia. Thus, the low testicular CREMτ levels reported in some infertile human males could possibly be linked to hyperprolactinemia (46).

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