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·Original Article·

Effects of tamoxifen citrate on gene expression during nuclear chromatin condensation in male rats

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Abstract

Aim: To evaluate the effects of tamoxifen citrate on gene expression during nuclear chromatin condensation in male rats. **Methods:** The effects of an oral dose of 0.4 mg/(kg·d) tamoxifen citrate on rates of *in vitro* chromatin decondensation, acridine orange (AO) dye uptake, concentration of thiol-groups, levels and/or expression of transition proteins 1, 2 (TP1, TP2), protamine 1 (P1), cyclic AMP response element modulator-τ (CREMτ), androgen-binding protein (ABP) and cyclic adenosine 3', 5' monophosphate (cAMP) were evaluated after 60 days of exposure in adult male rats. Controls received the vehicle. **Results:** Tamoxifen citrate enhanced the rates of chromatin decondensation, increased AO dye uptake and reduced free thiols in caput epididymal sperms and reduced the levels of TP1, TP2, P1, and CREMτ in the testis, while cAMP was unaffected. P1 deposition was absent in the sperm. The transcripts of TP1, TP2 were increased, of P1 and ABP decreased, while those of CREMτ unaffected in the testis. **Conclusion:** Tamoxifen citrate reduced caput epididymal sperm chromatin compaction by reducing the testicular levels of proteins TP1, TP2 and P1 and the CREMτ involved in chromatin condensation during spermiogenesis. Tamoxifen citrate affects the expression of these genes at both the transcriptional and post-transcriptional levels. *(Asian J Androl 2005 Sep; 7: 311–321)*

Keywords: tamoxifen; nuclear chromatin condensation; gene expression; spermatogenesis

1 Introduction

In rats, spermatogenesis occurs in 14 well-defined stages leading to the differentiation of germ cells into morphologically normal, functionally competent spermatozoa. Chromatin condensation is initiated in stage VII round spermatids after upregulation of the follicle-stimulating hormone (FSH)-dependent cyclic AMP response element modulator-τ (CREMτ) protein and CREMτ-inducible transition proteins1 (TP1), 2 (TP2) and protamine 1 (P1) [1-2]. During spermiogenesis, TP1 and TP2 initiate the removal of histones from nucleosomal sperm chromatin in solenoidal conformation and facilitate the deposition of P1, leading to the torroidal conformation characteristic of condensed chromatin [3]. Spermatidal TP1 has been reported to be upregulated by the androgen binding protein (ABP) and suppressed by estradiol *in vitro* [4]. After fertilization chromatin decondenses *in vivo* at a species-specific rate and maintains the synchrony be-

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tween the formation of male and female pronuclei prior to syngamy [5].

Our earlier studies indicated that chronic oral tamoxifen citrate treatment, at a dose of 0.4 mg/(kg·d), led to reduction in viable litters sired by the treated male rats without a decrease in epididymal sperm counts after 60 days of exposure [6]. A low dose of tamoxifen citrate did not produce adverse effects on sperm morphology or fertilizing ability, but specifically reduced the motility and fertilizing potential [7]. The earlier studies also showed that tamoxifen-induced testosterone deficits did not lead to the expected arrest of spermatogenesis at the androgen-dependent stage VII [8]. The disorganization of the testicular cytoarchitecture and Leydig cell hypotrophy observed in our earlier studies with tamoxifen citrate suggested that reduced testosterone possibly affected the Sertoli-germ cell interaction, which could have led to subtle but irreversible changes in the lineages of differentiating germ cells within the testis. The affected functions in the morphologically normal sperm were perceived to be sensitive enough to have reduced the viability of the engendered progeny after fertilization. The molecular basis of reduced viability of the embryos can be complex and may involve sperm genome instability and the degree of chromatin compaction. The aim of the present study was to evaluate the effects of chronic oral tamoxifen citrate treatment on epididymal sperm chromatin compaction and the molecular mechanisms underlying nuclear chromatin condensation during spermiogenesis in rat testes.

2 Materials and methods

2.1 Animals

Randomly bred 75-day-old male rats of the Holtzman strain were maintained at 22 °C–23 °C, 50 %–55 % humidity and lighting cycle of 14 h light : 10 h dark. Commercial food pellets and water were available *ad libitum*. Control and experimental rats were divided in groups of six each.

2.2 Drugs

Tamoxifen citrate tablets containing 10 mg pure tamoxifen were obtained from the LYKA Lab Ltd. (Mumbai, India). The tablets were uniformly suspended in water by sonication and administered via a feeding tube daily, at a dose of 0.4 mg/(kg·d) for 60 days, between 10:00 AM and 12:00 AM. Control animals received vehicle (administered water instead of drug) for 60 days. All experiments were carried out with the approval of the ethics committee of the institute.

2.3 Autopsy of male rats

Male rats were killed by decapitation. One testis from each rat was immersed in Bouin's fixative, processed through a series of graded alcohols and xylene and embedded in paraffin wax for immunohistochemistry. Caudae epididymides from control and treated rats were excised in saline for sperm counts. The caput epididymides from control and treated rats were given a small cut and incubated in saline at 37 °C to release the sperm. Harvested sperm pellets were stored at –20 °C and used for sperm quality tests with the exception of a nuclear chromatin decondensation test, which was done on the same day [9].

2.4 Sperm nuclear chromatin decondensation assay

Five million caput sperms from each rat were suspended in 1 mL of freshly prepared borate buffer (0.02 mol/L boric acid, 0.05 mol/L borax, pH 8) containing sodium dodecyl sulphate (SDS) and dithiothreitol (DTT) at a final concentration of 0.5 % and 5 mmol/L respectively and incubated for 2, 3, 5 min at 45°C. The reaction was stopped with 0.6 mL of 2.5 % glutaraldehyde and the fixed sperms were smeared on a slide to measure the length of 100 decondensed sperm heads per sample with an ocular micrometer, under a phase contrast microscope [9].

2.5 Sperm chromatin structure assay (SCSA)

Structural integrity of sperm chromatin was ascertained by DNA flow cytometery [9]. Two million caput sperms from each rat were suspended in 0.2 mL of 0.01 mol/L phosphate buffered saline (PBS) (pH 7.4) and subjected to denaturation *in vitro* by incubating in 0.4 mL of chilled lysis buffer (0.1 % Triton-X, 0.08 mol/L HCl, 0.15 mol/L NaCl) for 30 s and stained for 3 min with 1.2 mL of ice cold acridine orange (AO) stain (stock concentration of 6 μg/mL) prepared in buffer containing 0.15 mol/L NaCl, 0.2 mol/L Na_2HPO_4, 1 mmol/L EDTA and 0.1 mol/L citric acid. The fluorescence intensity of 5000 stained sperms, laser excited at 488 nm, was estimated at both 530 nm and 630 nm in a flow cytometer because the AO reacted with double and stranded DNA emits fluorescence at these respective wavelengths.

2.6 AO dye uptake assay for sperm chromatin

The concentration of AO dye taken up by caput sperms was estimated in a fluorometer. Two million caput sperms suspended in 0.2 mL of 0.01 mol/L PBS (pH 7.4), were incubated for 30 s in 0.4 mL of chilled lysis buffer (0.1 % Triton-X, 0.08 mol/L HCl, 0.15 mol/L NaCl). The sperms were incubated with 1.2 mL of chilled AO dye (at a critical concentration of 6 μg/mL) for 3 min, and washed twice with dye buffer (0.15 mol/L NaCl, 0.2 mol/L Na_2HPO_4, 1 mmol/L EDTA, 0.1 mol/L citric acid). Intercalated dye was extracted from sperms by incubation in 2 mL of DNA lysis buffer (10 mmol/L Tris, 100 mmol/L NaCl, 25 mmol/L EDTA, 2 % SDS, 150 μmol/L β-mercaptoethanol, 400 μg/mL proteinase K) at 56 ºC for 3 h with constant shaking. A standard curve for AO stain (dissolved in 2 mL dye buffer) was set up in parallel, in the range of 1.9 mg–1000 mg. The wavelengths of excitation and emission for dye were 490 nm and 530 nm, respectively. The concentration of the total dye reacted with sperm DNA in the samples was estimated from the standard curve of AO and expressed as μg dye/mg sperm protein. Sperm proteins in the lysis buffer were estimated from the absorbance at 280 nm.

2.7 Sperm chromatin monobromobimane (mBBr) dye uptake assay

Caput sperms (5×10^6) from all rats were suspended in 0.01 mol/L PBS and incubated with mBBr, fluorescent thiol-specific reagent, at a final concentration of 0.5 mmol/L, in the dark for 10 min. Labeled sperms were washed twice with 0.01 mol/L PBS and solubilized in 2 mL of 0.1 mol/L β-mercaptoethanol containing 8 mol/L guanidine hydrochloride. A standard curve for mBBr stain was set up in parallel, in the range of 0.0285 nmol–14.82 nmol. The wavelengths of excitation and emission for dye were 390 nm and 495 nm, respectively. The concentration of the total dye reacted with sperm DNA in the samples was estimated in a fluorometer (Shimadzu), from the standard curve of mBBr and expressed as nmoles dye/mg sperm protein. Sperm proteins in the lysis buffer were estimated from the absorbance at 280 nm.

2.8 Detection of gene expression in testis and sperm

2.8.1 Detection of TP1, TP2, P1 by Western blotting in testis and sperm

The basic proteins were extracted as described by Kistler *et al.*[10]. Testicular tissues (0.5 g) from all rats were homogenized in nine volumes of 0.5 mol/L HCl solution, chilled on ice for 20 min and centrifuged at 6225 × *g* for 10 min. The proteins in the supernatants were precipitated with trichloroacetic acid (TCA) at a final concentration of 20 % and pelleted by centrifugation at 21885 × *g* 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 (5 mol/L urea, 5 % β-mercaptoethanol, 0.5 % acetic acid). Caput sperms from the control and treated rats were suspended in 0.5 mL of 0.01 mol/L PBS and lysed with 2 mL of chilled lysis solution 6 mol/L guanidine hydrochloride, 50 mmol/L Tris HCl and 5 % v/v β-mercaptoethanol and acidified with HCl at a final concentration of 0.5 mol/L HCl. The acidic lysate was dialyzed in 6 kDa pore size bags (Rockford, IL, USA) O/N in cold against dialysis buffer containing 0.25 mol/L HCl and 0.1 % β-mercaptoethanol in distilled water (DW), with two changes of the dialysis buffer. The basic proteins in the supernatants, obtained after centrifugation of lysates at 9727 × *g* for 30 min, were precipitated with TCA at a final concentration of 20 %, separated by centrifugation at 6225 × *g* for 10 min and washed five times with acetone. The protein pellets were allowed to air dry and were solubilized in 0.2 mL of 5-5-5 solution. The proteins were estimated from absorbance at 280 nm. The positively charged basic proteins were resolved on continuous 15 % acid urea polyacrylamide gels (after reversing the direction of the current). The resolving gel was pre-electophoresed at 100 V for 2.5 h, till a constant current was obtained, using 5 % acetic acid as the running buffer. Protein samples (100 μg) 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 % non fat dry milk [NFDM], 1 % normal serum [goat serum for TPs and horse serum for protamine] in 0.01 mol/L PBS) for 1 h at room temperature (RT), followed by incubation with primary antibodies (gifts from Drs Stephen Kistler and Rod Balhorn) to transition proteins 1, 2 (polyclonal) or P1 (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 in 0.01 mol/L PBS (pH 7.4) for 1 h at RT, with thorough washings with wash buffer (0.03 % gelatin, 0.5 % Tween 20 in 0.01 mol/L PBS) after each step. Specific bands of TP1, TP2 and P1

Table 1. Polymerase chain reaction conditions. TP: transition proteins; P1: protamine 1; CREMτ: cyclic AMP response element modulator-τ; ABP: androgen-binding protein.

Transcripts	TP1	TP2	P1	CREMτ	ABP
Denaturation	94 ºC × 30 s	94 ºC × 30 s	94 ºC × 30 s	94 ºC × 30 s	94 ºC × 30 s
Annealing	59 ºC × 1 min	59 ºC × 1 min	55 ºC × 1 min	52.4 ºC × 1 min	60 ºC × 1 min
Extension	68 ºC × 1 min	68 ºC × 1 min	68 ºC × 1 min	68 ºC × 1 min	68 ºC × 1 min
Final extension	68 ºC × 7 min	68 ºC × 7 min	68 ºC × 7 min	68 ºC × 10 min	68 ºC × 7 min
Size	175 bp	152 bp	201 bp	525 bp	954 bp

were visualized with diaminobenzidine (DAB). 0.01 mol/L 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 (MediaCybernetics, Silver Spring, MD, USA) and expressed as integrated optical density (IOD).

2.8.2 Detection of CREMτ by Western blotting in testis

Testicular tissues (100 mg) were homogenized in 1 mL of TEDG buffer (10 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L DTT, 10 % glycerol) and supernatants separated by centrifugation at 21885 × *g* for 45 min at 4 °C and stored at –20 °C. The proteins (200 μg) were resolved on 12.5 % denaturing polyacrylamide gels. Molecular weight markers in the range of 17.5–218 kDa were run. The proteins were transferred to nitrocellulose membrane, treated with blocking solution (5 % NFDM in 0.01 mol/L PBS) for 1 h at RT, incubated with the anti-rabbit polyclonal primary antibody to CREMτ (Santacruz Biotechnology, Santa Cruz, USA) at a dilution of 1:1000 (diluting buffer comprised 0.1 % Tween 20, 5 % NFDM in 0.01 mol/L PBS at 4 °C O/N, washed five times for 10 min each with the wash buffer [0.1 % Tween 20 in 0.01 mol/L PBS]). The membranes were then incubated with the horse radish peroxidase (HRP) 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 mol/L PBS, washed six times for 10 min each with wash buffer (comprising 0.1 % Tween 20 in 0.01 mol/L PBS). The proteins were detected by chemiluminescence. The protein bands on the films were quantified with Gel Pro 3.1 image analysis software and expressed as IOD.

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

Testicular tissues (50 mg–100 mg) were homogenized in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, California, USA) 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 12 000 × *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 12 000 × *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 ribonuclease (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. The integrity of the RNA was checked by resolving on 1.5 % agarose gel. RNA (1 μg) 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 (Roche Diagnastics, Basel, Switzerland) RT-PCR kit and forward and reverse primers for specific RNAs for TP 1, 5'-CCA GCC GCA AAC TAA AGA CTC ATG G-3'; 5'-AGC TCA TTG CCG CAT TAC AAG TGG G-3'; TP 2, 5'-AGG AAA GGT GAG CAA GAG AAA GGC G-3'; 5'-CAT TCC CCT AGT GAT GGC TAT CTC C-3'; P1, 5'-AGC AAA AGC AGG AGC AGA AG-3'; 5'-GGC GAG ATG CTC TTG AAG TC-3'; CREMτ, 5'-GAT TGA AGA AGA AAA ATC AGA-3'; 5'-TTG ACA TAT TCT TTC TTC TTC TT-3'; ABP, 5'-GAG AAG GGA GAG GTG GCC T-3'; 5'-GCT CAA GGC TAC TTT GAA TAC-3'; and mouse β-actin as an internal control 5'-CTG GCA CCA CAC CTT CTA-3'; 5'-GGG CAC AGT GTG GGT GAC-3' (gene accession number NM_007393) in a 50-μL reaction volume [2, 11–13]. 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 mmol/L instead of 1.5 mmol/L. The 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 software. RNA concentrations in samples were expressed as the ratio of IODs of the specific PCR products and β-actin control product.

2.9 Detection of Cyclic AMP (cAMP) in testis

cAMP was detected in the testis according to the method of Pascolini *et al.* [14]. Paraffin sections of the testis (5 μm) 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 mol/L 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 0.01 mol/L PBS). The sections were incubated overnight at 4 °C in a light proof box with cAMP polyclonal primary antibody (St. Louis, MO, USA) diluted in 0.01 mol/L PBS containing 0.1 % BSA, at a dilution of 1:10. Sections were washed twice for 10 min with 0.01 mol/L PBS and incubated with goat anti-rabbit, biotinylated secondary antibody, at a dilution of 1:500 (0.1 % BSA in 0.01 mol/L PBS) for 1 h at RT, followed by two washes of 10 min each. The slides were incubated with HRP conjugated avidin-biotin complex 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 counter-stained with hematoxylin solution. The slides were dipped in absolute alcohol, air dried and left in xylene O/N and mounted with oil. PBS (0.01 mol/L) 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 (Expert Vision Lab, Mumbai, India). The sections were photographed under bright field optics at 40/100× magnification.

2.10 Statistical analysis

All results were expressed as mean ± SEM. Significant differences between control and experimental group rats were calculated by Student's *t*-test. Level of significance was set at $P < 0.05$.

Table 2. Effects of tamoxifen citrate on acridine orange (AO) and monobromobimane (mBBr) uptake by caput epididymal sperm chromatin in adult male rats. All values are mean ± SEM. ($^bP < 0.05$, compared with controls)

Affected parameters in male sires	Control	Tamoxifen 0.4 mg/(kg·d)
μg AO/mg protein	2.07 ± 0.21	3.28 ± 0.40^b
AO uptake by double stranded chromatin	317.67 ± 36.5	449 ± 51^b
AO uptake by single stranded chromatin	176.17 ± 12.73	309.63 ± 19.57^b
nmol/L mBBr/mg protein	143.15 ± 16.87	86.46 ± 12.24^b

3 Results

3.1 Effect of tamoxifen treatment on sperm counts

No change was evident between the epididymal sperm counts of control ([62.16 ± 11.46] × 10^6/cauda) and tamoxifen treated rats ([60.0 ± 12.16] × 10^6/cauda) ($P > 0.05$).

3.2 Effect of tamoxifen treatment on the rate of sperm chromatin decondensation

A significant increase from (13.83 ± 0.23) μm to (14.82 ± 0.26) μm ($P < 0.05$) was observed in the length of decondensed caput sperm heads after 2 min of incubation as a result of tamoxifen treatment.

3.3 Effect of tamoxifen treatment on total AO dye uptake by sperm

Tamoxifen treatment led to a significant increase (55.8 %) ($P < 0.05$) in the AO uptake by the caput sperm chromatin as compared with control sperms (Table 2).

3.4 Effect of tamoxifen treatment on differential AO dye uptake by sperm chromatin

Tamoxifen treatment led to a significant increase (41 %) ($P < 0.05$) in AO intercalated in double stranded (ds) chromatin and (75 %) ($P < 0.05$) increase in AO reacted with single stranded (ss) chromatin of caput sperms as compared with control sperms (Table 2).

3.5 Effect of tamoxifen treatment on the content of free thiol groups in sperm

Tamoxifen treatment led to a significant reduction (39.6 %) ($P < 0.05$) in the concentration of free thiols in caput sperms as compared with control sperms (Table 2).

3.6 Effect of tamoxifen treatment on levels of cAMP in rat testes

Tamoxifen treatment did not produce a significant change in the levels of testicular cAMP (14.51 ± 0.10, IOD) (hypothetical second messenger in germ cells) compared with controls (14.42 ± 0.09, IOD). cAMP was immunolocalized in the cytoplasm of hematoxylin-stained spermiogenic cells, spermatocytes and spermatids in particular (Figure 1).

3.7 Effect of tamoxifen treatment on the sperm basic proteins

Tamoxifen treatment suppressed the deposition of protamines in the spermatozoa. There was no evidence of abnormal persistence of either TP1 or TP2 in tamoxifen treated sperms (Figure 2).

3.8 Effect of tamoxifen treatment on the testicular protein levels

Tamoxifen treatment led to a significant reduction in the testicular levels of TP1 (88 %), TP2 (56 %), P1 (63.6 %) and CREMτ (52.2 %) as compared with control rats (Figures 3–6).

3.9 Effect of tamoxifen treatment on the testicular expression of genes involved in nuclear chromatin condensation

Tamoxifen treatment led to a significant increase in TP1 (32 %), TP2 (49.1 %) and decrease in P1 (4.29 %) and ABP (51.9 %) transcripts relative to controls. No change was observed in CREMτ transcripts after tamoxifen treatment (Figures 3–7).

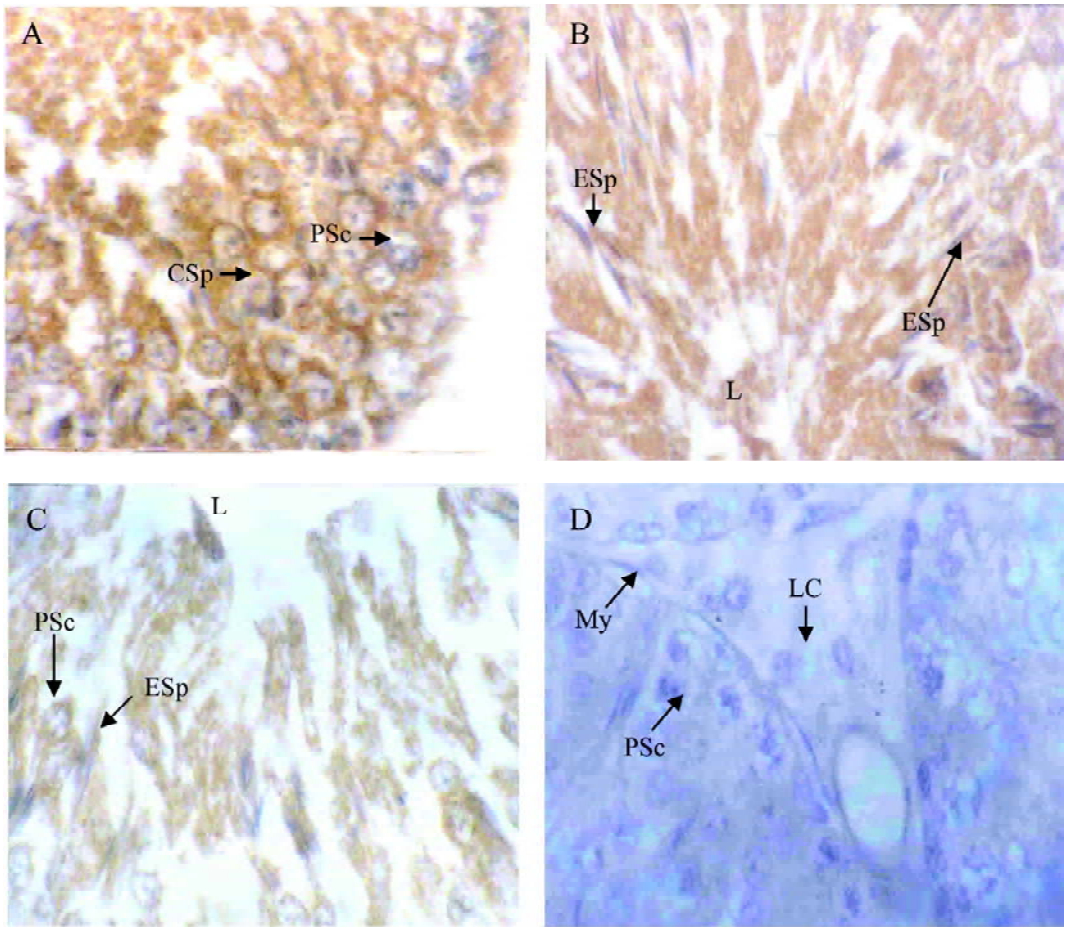

Figure 1. Cyclic AMP immunoexpression in control and tamoxifen treated rat testes. (A): Control, (B, C): treated and (D): negative control. Positive staining seen in cytoplasm of elongated spermatids (ESp), primary spermatocytes (PSc), cap phase spermatid (CSp), myoid cell (My), Leydig cell (LC) and Lumen (L).

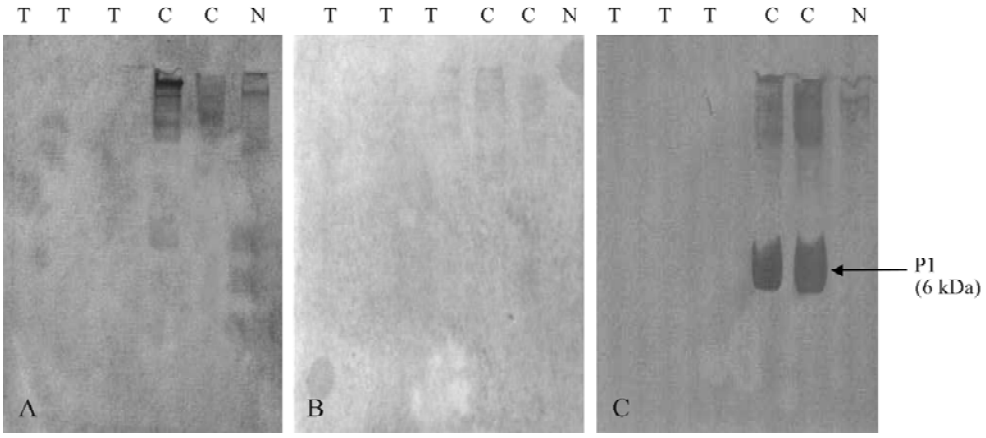

Figure 2. Immunoblots showing the absence of TP1 (A), TP2 (B) and presence of protamine1 (P1) (C) in sperm proteins. Lane N: negative control showing absence of specific bands; Lane C: control sperm; Lane T: tamoxifen treated.

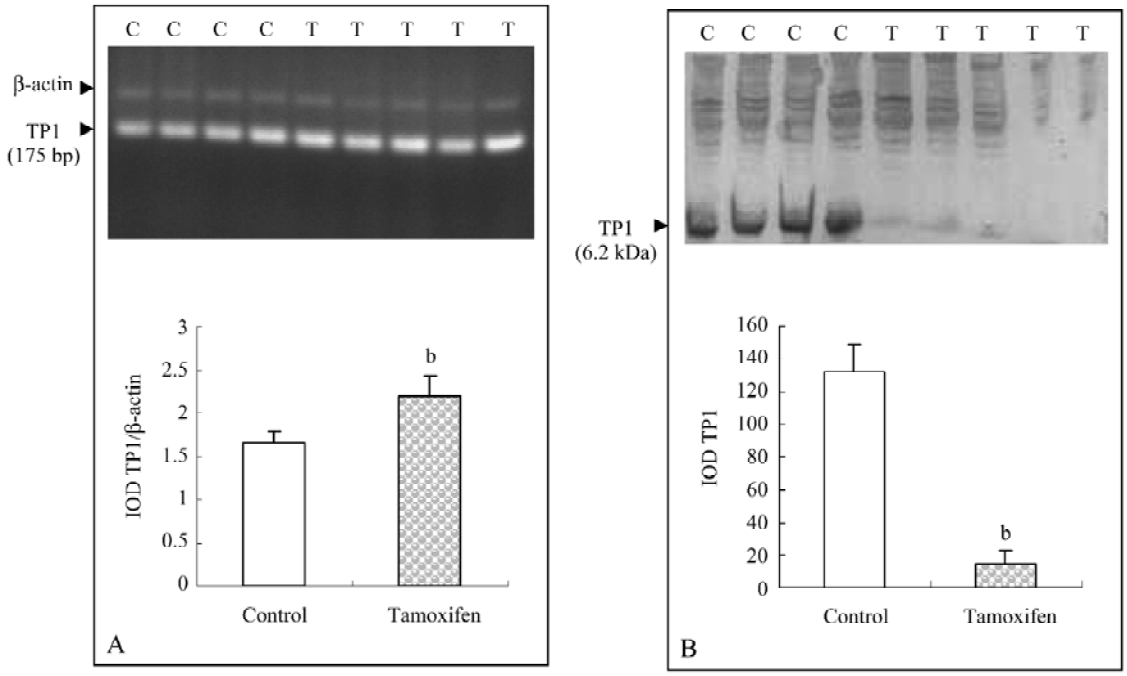

Figure 3. (A) Upper panel: RT-PCR product of TP1 transcript from rat testis. Lane C: control; Lane T: tamoxifen treated. Lower panel: ratios of IOD of TP1/β-actin before and after treatment. (B) Upper panel: representative TP1 protein band in rat testicular protein by acid-urea PAGE analysis on 15 % gel. Lane C: control; Lane T: tamoxifen treated. Lower panel: IOD of TP1 protein bands before and after treatment. All values are mean ± SEM. ($^bP < 0.05$, compared with controls).

4 Discussion

The dose-, duration- and species-specific biological effects of the antiestrogen, tamoxifen citrate are often paradoxical because of the residual estrogenicity of the drug molecule, presumably the consequence of gradual conversion to hepatic metabolites [6–7]. The anticancer, antifertility and fertility-regulating effects are perceived to occur by virtue of its property to associate with the estrogen receptors expressed in most tissues, including

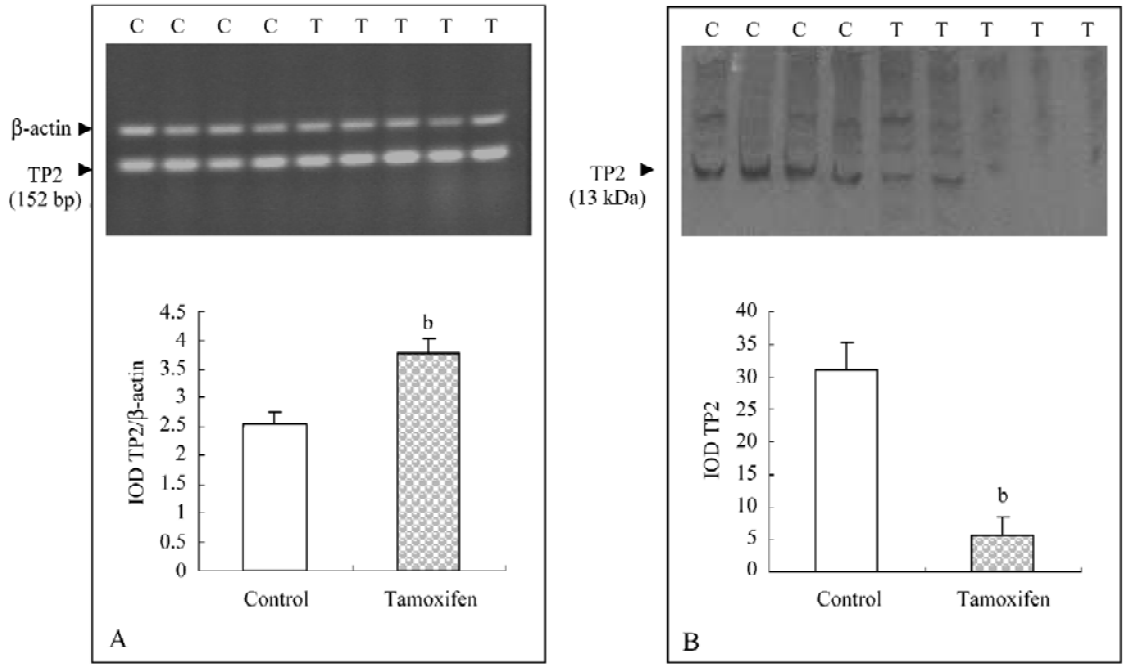

Figure 4. (A) Upper panel: RT-PCR product of TP2 transcript from rat testis. Lane C: control; Lane T: tamoxifen treated. Lower panel: ratios of IOD of TP2/β-actin before and after treatment. (B) Upper panel: representative TP2 protein band in rat testicular protein by acid-urea PAGE analysis on 15 % gel. Lane C: control; Lane T: tamoxifen treated. Lower panel: IOD of TP2 protein bands before and after treatment. All values are mean ± SEM. ($^bP < 0.05$, compared with controls).

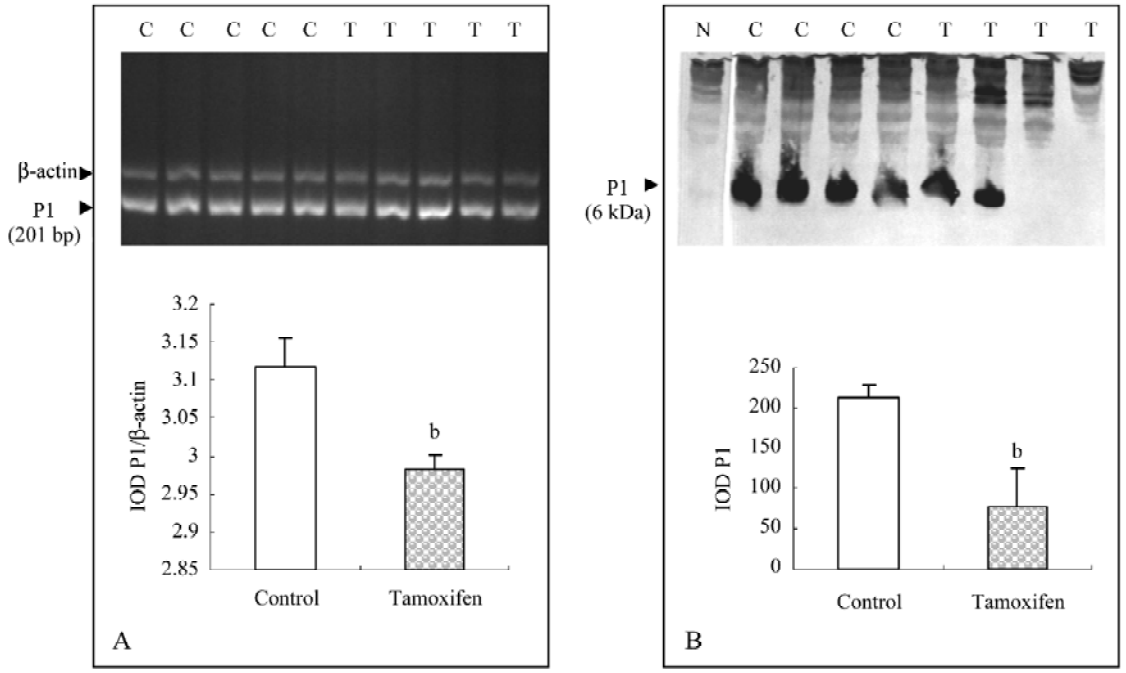

Figure 5. (A) Upper panel: RT-PCR product of protamine 1 (P1) transcript from rat testis. Lane C: control; Lane T: tamoxifen treated. Lower panel: ratios of IOD of P1/β-actin before and after treatment. (B) Upper panel: representative protamine protein band in rat testicular protein by acid-urea PAGE analysis on 15 % gel. Lane N: negative control; Lane C: control; Lane T: tamoxifen treated. Lower panel: IOD of P1 protein bands before and after treatment. All values are mean ± SEM. ($^bP < 0.05$, compared with controls).

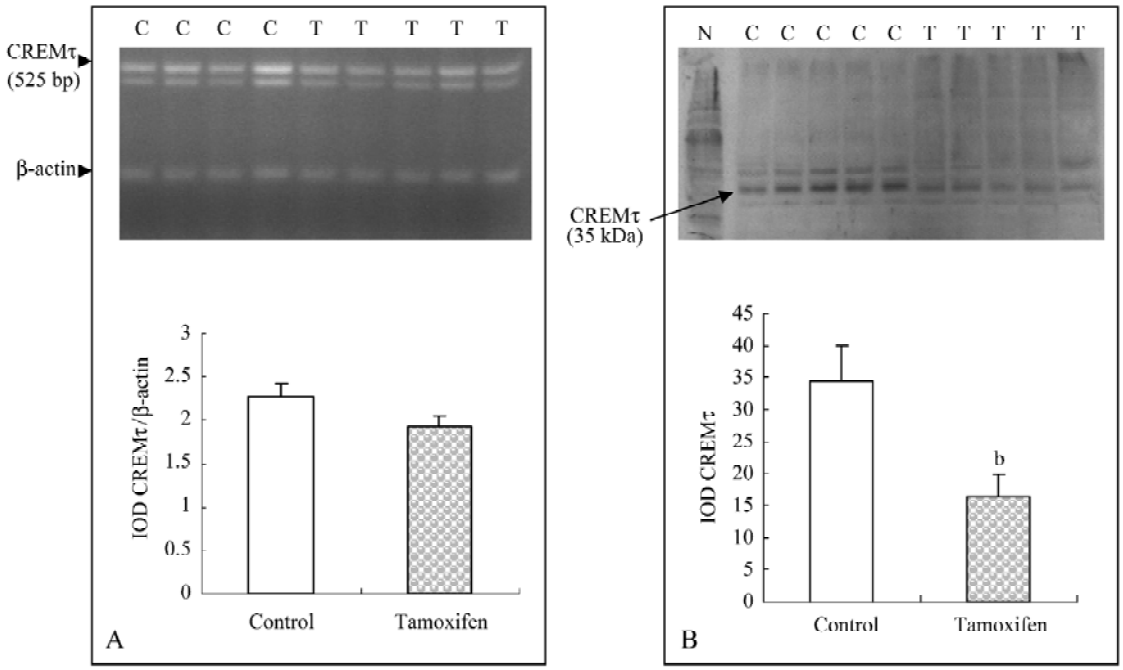

Figure 6. (A) Upper panel: RT-PCR product of CREMτ transcript from rat testis. Lane C: control; Lane T: tamoxifen treated. Lower panel: ratios of IOD of CREMτ/β-actin before and after treatment. (B) Upper panel: representative CREMτ protein band in rat testicular protein by SDS-PAGE analysis on 12.5 % gel. Lane N: CREMτ negative control; Lane C: control; Lane T: tamoxifen treated. Lower panel: IOD of CREMτ protein bands before and after treatment. All values are mean ± SEM. ($^bP < 0.05$, compared with controls).

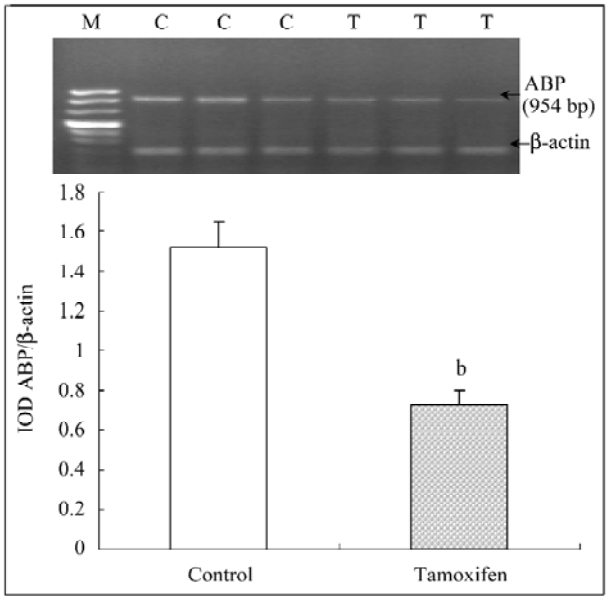

Figure 7. Upper panel: representative amplified band of ABP transcript from rat testes. Lane M: molecular weight markers; Lane C: control; Lane T: tamoxifen treated. All values are mean ± SEM. ($^bP < 0.05$, compared with controls).

somatic and germinal cells within the seminiferous tubules of rats [15]. Our earlier studies with low doses of tamoxifen citrate suggested that the estrogenic effects of tamoxifen on spermatogenesis were being mediated through cognate receptors in the pituitary, involved in the regulation of gonadotropins secretion [6–7]. In view of the neuroendocrine target receptors, the studies suggesting a direct testicular role of estradiol involving the estrogen receptor beta (ERbeta) expressed therein are understandably few [16].

The enhanced rate of caput sperm chromatin decondensation observed in this study is consistent with the reported enhancement of the rate of primate sperm chromatin decondensation obtained after treatment with aromatase inhibitors, expected to reduce the availability of estradiol [17]. The increased uptake of DNA intercalating AO dye by both double- and single-stranded sperm DNA observed after tamoxifen treatment is also consistent with the enhanced uptake of DNA intercalating dye by sperms taken from the caput epididymis of primates treated with the gonadotropin releasing hormone (GnRH)

antagonist, indicating that the sperms were indeed loosely packaged [18]. Thus, reported suppression of LH and thereby testosterone by low dose tamoxifen treatment led to loose packaging of sperm chromatin and increased the accessibility to DNA intercalating dyes.

The loose packaging of sperm chromatin observed after low dose tamoxifen treatment was a consequence of reduced concentration of caput sperm free-thiols, known to exist unoxidized in this region of the epididymis. A clear suppression of testicular P1 levels and transcripts further confirmed that reduction in sperm thiols was due to reduced deposition of protamines in sperm chromatin [10]. The significant reduction also observed in the levels of testicular transition proteins indicated that tamoxifen citrate treatment affected the deposition of P1 in the rat sperm chromatin through suppression of proteins that facilitate removal of spermatidal histones during chromatin condensation. However, the increase in levels of transition protein transcripts suggested that reduction in testosterone levels had not affected transcription of transition protein genes. Nonetheless, simultaneous loss of both transition proteins and protamines, in the prevailing hormonal milieu of reduced testosterone but normal circulating FSH, did not lead to spermatogenic arrest. Tamoxifen citrate treatment, which reduces testosterone specifically affected the mechanism underlying the synthesis, oxidation and deposition of thiol-rich sperm protamines which bring about chromatin compaction during epididymal maturation. The results also clearly suggested that ERbeta could also play a role in the transcriptional and post-transcriptional regulation of spermatidal transition proteins, known to be induced by CREMτ [2, 16].

The observed reduction in the testicular levels of CREMτ, though not in the levels of its transcripts or FSH, added to the conflicting views pertaining to the true import of the role of FSH in the regulation of induction of transition proteins and protamines by CREMτ. The results suggested that tamoxifen affected a putative post-transcriptional regulatory mechanism which operates to maintain spermatidal CREMτ levels, either through suppression of testosterone or mediated directly through cognate testicular estrogen receptor beta. Moreover, reduction in the levels of CREMτ could not have led to the decrease in the levels of transition proteins, in view of the observed upregulation of transcripts of transition proteins after tamoxifen treatment. Besides, only a slight, though significant, decrease occurred in the levels of protamine transcripts, not in proportion to the decrease in levels of CREMτ. It could be inferred from these results that tamoxifen citrate produced major adverse effects on a post-transcriptional regulatory mechanism that operates to regulate levels of these proteins crucial for chromatin condensation, mainly as a consequence of reduced testosterone levels, but a modulatory effect on transcription of transition proteins and protamine involving the testicular estrogen receptor beta could not be ruled out. This inference was based on the fact that substantial suppression observed in the levels of all the proteins was disproportionate or contradictory to the changes in the levels of their transcripts.

Moreover, the substantial decrease in the levels of testicular ABP transcripts, implicated in TP1 transcriptional regulation, did not agree with the upregulation of TP1 observed after tamoxifen treatment [4]. The reduced expression of ABP was clearly suggestive of a direct effect of the antiestrogen, tamoxifen, at the level of transcription since our results agreed with the down regulation of the TP1 gene observed after estradiol treatment in the same study [4]. We also evaluated the effect of tamoxifen treatment on the testicular cAMP levels, putative mediator of ABP signals in spermatids, since it is not possible to estimate the concentration of ABP secreted at the germ cells [19]. Though cAMP could be localized to the germ cells, the levels were not affected after tamoxifen treatment. ABP could, however, not be implicated in the observed reduction in the levels of proteins involved in chromatin condensation, since the decrease in its expression could not be correlated to any change in cAMP levels. It is likely that significant changes could have been masked by Sertoli cell cAMP linked to FSH, which is not suppressed by tamoxifen.

The study has demonstrated that tamoxifen exposure adversely affects Sertoli germ cell interactions within the testis and reduces the levels of crucial testicular proteins required for chromatin condensation. Since the transcripts of these genes are normally translationally repressed by binding proteins, it can be averred that the effect possibly occurs at the level of removal of RNA binding proteins affecting either translation or degradation [20]. The observed effects are a consequence of suppression of testosterone that possibly facilitates the upregulation of ERbeta, which could be playing a role in post-transcriptional mechanisms involved in preventing precocious translation or degradation of these proteins. Nevertheless, in view of the prevailing controversy per-

taining to the restricted expression of ERbeta and preferential expression of estradiol receptor alpha (ERalpha) in rat germ cells, a consensus needs to be arrived at before the unequivocal acceptance of this interpretation [15–16]. It would be pertinent to report that in an earlier study we had localized non-genomic ERalpha to rat spermatozoa and averred that it must have been expressed in the differentiating germ cells for eventual expression on membrane [21]. Furthermore, since the study clearly indicates that failure of protamine deposition within the testis is responsible for the observed effects of tamoxifen on chromatin condensation, the involvement of efferent duct estrogen receptors can be ruled out.

In conclusion, tamoxifen-induced reduction in the levels of CREMτ-inducible, TP1, TP2 and P1 proteins in rat testis, mediated mainly through testicular androgen receptors and ERbeta adversely affected the chromatin packaging during spermiogenesis.

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