Pakistan J. Zool., vol. 45(3), pp. 817-832, 2013

Pretreatment With GnRH Antagonist Causes Partial Restoration of Testicular Tissue After Kisspeptin-10 Induced Degeneration in Prepubertal Rats

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Abstract.- Hypothalamus-derived kisspeptins are critical regulators of reproduction in nearly all mammalian species. How kisspeptins regulate gonadal maturation in sexually immature male mammals remains elusive. For this purpose, prepubertal rats were assigned to four experimental groups namely: control (saline treated), kisspeptin alone, saline with acyline pretreatment (saline + acyline pretreated) and kisspeptin with acyline pretreatment (kisspeptin + acyline pretreated). Acyline was administered prior to kisspeptin treatment at the rate of 300 µg/kg body weight. Kisspeptin and saline were administered intraperitonealy at 1 µg/rat twice daily to prepubertal male rats. Effects on spermatogenesis, levels of testosterone, LH and FSH, histomorphology and ultrastructure of testicular tissue were studied. At the end of treatments plasma FSH levels were not altered in kisspeptin alone treatment group while it decreased (P<0.001) in the saline (acyline pretreated) treated group, and in the kisspeptin (acyline pretreated) treated group. LH (P<0.001) and testosterone (P<0.01) concentrations were reduced in all treated groups. Alone kisspeptin and saline (acyline pretreated) treatment adversely affected all testicular parameters. In the kisspeptin (acyline pretreated) treatment group, only type A spermatogonia decreased (P<0.001) significantly while other parameters remained unaffected. Histomorphology and ultrastructure showed atrophied germinal epithelium with alone kisspeptin and saline (acyline pretreated) treatment, while seminiferous tubules of rats treated with kisspeptin (acyline pretreated) revealed partial restoration of spermatogenesis. The present findings indicate that subchronic kisspeptin administration may act as a suppressor of pubertal maturation during non-pubertal states and a partial recovery of the testicular tissue occur with acyline pretreatment.

Key words: Kisspeptin, acyline, seminiferous tubules, testicular tissue, histomorphology, testosterone, GnRH antagonist.

INTRODUCTION

Lee and colleagues (1999) discovered a novel G protein-coupled receptor in the rat termed GPR54. The GPR54 G protein-coupled receptor mediates the actions of the kisspeptins, the peptide products of the Kiss1 gene. The Kiss1 gene encodes a 145-amino acid peptide that can be cleaved into peptides 54, 14, 13 ad 10 amino acids long. All these kisspeptins share the same amidated C-terminus and bind to the GPR5 (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001; Stafford *et al.*, 2002).

Kisspeptin-54 was originally identified as a metastasis suppresser peptide, and was thus named metastin (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001; Stafford *et al.*, 2002). Later, dysfunctional or deletional mutations in the gene encoding GPR54 in mice and in humans were shown to cause hypogonadotropic hypogonadism; a condition characterized by absent or delayed pubertal development. Neurons that express kisspeptin are present in the arcuate nucleus (Arc), the periventricular nucleus (PeN), and the anteroventral periventricular nucleus (AVPV) in rodent hypothalamus (Gottsch *et al.*, 2004, Smith *et al.*, 2005).

Kisspeptin-54 stimulates secretion of leutinizing hormone (LH) and follicle stimulating hormone (FSH) following central or peripheral administration, and this effect can be blocked by pretreatment with acyline, a potent GnRH antagonist (Gottsch *et al.*, 2004). Furthermore, chronic central administration of kisspeptin-10 to

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0030-9923/2013/0003-0817 $ 8.00/0

sexually immature female rats induced early vaginal opening, elevated uterus weight and increased level of serum LH and estrogen (Navarro *et al.,* 2004b). Similarly, it was found that the transcriptional activity of *Kiss-1* neurons in the Arc is increased significantly after castration and inhibited by testosterone replacement. Thus these neurons act as targets for the negative feedback regulation of GnRH secretion by forebrain, whereas activity of the *Kiss-1* neurons in the AVPV and PeN is reduced with castration and stimulated by testosterone, implicating that they may mediate other testosterone-dependent processes (Smith *et al.,* 2005).

The smallest endogenous kisspeptin, kisspeptin-10, binds to the receptor Kiss1R with a similar affinity to the full-length peptide, kisspeptin-54 (Curtis *et al.,* 2010). Kisspeptin-10 boluses potently evoke LH secretion in men, and continuous infusion increases testosterone, LH pulse frequency, and pulse size (George *et al.,* 2011). Central and peripheral administration of kisspeptin-10 increased plasma LH, FSH and total testosterone in adult male rats (Thompson *et al.,* 2004). Whitlock *et al.* (2008) suggested that reproductive steroids enhance the sensitivity of the somatotropic axis to physiologically relevant doses of Kp10, and support the possibility that Kp10 is an integrator of luteinizing hormone and GH release.

We have shown previously that intraperitoneal kisspeptin-10 administration causes dose dependent degenerative changes in testicular tissue of prepubertal rats (Ramzan and Qureshi, 2011). The aim of the present study was to understand the mechanism of kisspeptin-10-induced testicular degeneration. Therefore, we blocked the endogenous kisspeptin with a potent GnRH antagonist. The present study was carried out to investigate in sexually immature male rats, the effects of kisspeptin administration on testicular tissue following the subchronic intraperitoneal administration of mammalian kisspeptin-10 (KP-10) alone and after pretreatment with GnRH antagonist, acyline. We administered acyline to indirectly block endogenous kisspeptin to test whether the effects of kisspeptin on testicular degeneration are mediated either by endogenous or exogenous kisspeptin. We used kisspeptin-10, a 10-residue peptide, because it is the shortest and the most active Kiss1 gene product (Kotani *et al.,* 2001). It has been suggested that kisspeptin-54 is unstable and may be proteolytically cleaved into shorter products (Kotani *et al.,* 2001).

MATERIALS AND METHODS

Animals and maintenance

To investigate the role of puberty regulating peptide "kisspeptin" in prepubertal male Sprague Dawley rats, forty (n=40), five weeks old (postnatal day, PND 35) prepubertal male rats with an average weight of 100±10g were purchased from the National Institute of Health, Islamabad and maintained in the animal house facility of Quaid-i-Azam University, Islamabad, during the experimental period. To minimize crowding stress, five rats were housed per cage (15″ × 11″ × 9″, steel mesh cages) under standard conditions of 12L: 12D hrs photoperiod, 25±2ºC temperature controlled with automatic timers and adjustable controls for heating and cooling. Standard rat diet and water were provided *ad libitum.*

All animal handling and subsequent sacrifice were done according to the guidelines provided by the "Ethics Committee" of the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, for humane use of animals for scientific research. Animal handling was also in accordance with European Union guidelines for use of laboratory animals.

Dosage and treatment

Kisspeptin (metastin 45-54 or kisspeptin-10; 1mg lyophilized powder) was procured from Calbiochem (EMD Biosciences, Inc. La Jolla, CA) and was dissolved in 1 ml dimethylesulphoxide (DMSO) to give a stock solution of 1 mg ml^{-1} that was diluted further with distilled water (dH_2O). 100 ng kg^{-1} b.w. dose of kisspeptin was administered intraperitonealy (i.p.) for twelve days because a significant effect on hormones concentration and cellular populations was obtained at this dose in our previous experiments (Ramzan and Qureshi, 2011). Rats were arranged randomly to four groups each comprising ten rats (n=10). Control non-treated rats received 0.9% w/v physiological saline (DMSO was

added to saline at the same rate as it was added to kisspeptin stock, and was diluted further to concentration equivalent to the experimental doses), while the kisspeptin alone group received a dose of kisspeptin-10: 1µg rat^{-1} which is equal to 100ng Kg^{-1} body weight.

To block the action of endogenous kisspeptin indirectly, remaining two groups of (n=20) rats were pretreated with a single subcutaneous (s.c.) dose of acyline, (kind gift of Prof. Dr. M. Shahab, Animal Sciences Department, Quaid-i-Azam University, Islamabad), given one day prior to kisspeptin treatment (day 0) at the rate of 300 µg kg^{-1} b.w. Acyline dose was selected according to Herbst *et al.* (2004) who showed that a single 300 µg kg^{-1} b.w. dose of acyline suppressed gonadotropins and testosterone release to castrate levels for 15 days. To determine the plasma LH, FSH and testosterone levels post-acyline exposure, blood was drawn from the tail vein, at day one of the experiment. Soon after the required suppression of hormone concentrations was achieved; of these twenty rats, ten constituted the acyline group and were injected saline only, while the other ten were injected with 100 ng kisspeptin dose. Saline and kisspeptin were administered i.p. twice daily after every 12 h for 12 days to control and experimental rats respectively. Ten rats each from control and treated groups were sacrificed 3 h after the last dose of kisspeptin.

This sub-chronic treatment of 12 days was designed because the germ cells advance within the seminiferous epithelium, in a specific 12 to 13-day cycle that begins with mitotic division of spermatogonia and proceeds through meiosis and finally ends with the release of "sperm" (Leblond and Clermont, 1952).

Collection of blood and tissue samples

Animals were anesthetized (sodium pentobarbital: 60-80 mg kg^{-1} b.w. i.p.); blood was collected in EDTA vaccutainers directly from the left ventricle of heart. Blood was allowed to stand for 1 h and plasma was extracted by a 10 min centrifugation at 1258 ×*g* at 4°C. Plasma samples were aliquoted and stored at –20 ºC until assayed for hormone concentrations.

Of ten rats in each group, separate testes were taken for light and electron microscopy, elongated spermatid head count and DNA parameters. For light microscopy, testes from control and treated rats were excised, weighed, rinsed in phosphate buffered saline (PBS) and fixed in freshly prepared 4% paraformaldehyde (PFA) solution prepared in PBS. For electron microscopy, the tissues were fixed in 5% glutaraldehyde (BDH, Germany) solution prepared in pipes buffer.

For the determination of elongated spermatid head count and daily sperm production (DSP), the testes of control and kisspeptin treated rats were snap frozen in liquid nitrogen and stored at -70ºC. They were later homogenized and processed.

Similarly for investigation of the DNA fragmentation, the testicular tissues were frozen in liquid nitrogen and stored at -70ºC, to process at a later stage.

Testes were weighed. Gonadosomatic index (GSI %) was determined according to the formula:

$$\text{GSI (\%)} = \frac{\text{Testes weight}}{\text{Body weight}} \times 100$$

Testis length and width were determined using the digital vernier callipers. Testis volume was estimated from the equation of an ellipsoid according to Pochron and Wright (2002):

$$\text{Testis volume } (mm^3) = 4/3 \times \quad \times L \times W^2$$

where L is length, and W is width of the testis. The volumes of the left and right testis were then averaged.

Light microscopy

Tissue processing and staining

Tissue samples were fixed overnight at 4ºC, in freshly prepared 4% PFA (pH=7.2), dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraplast. Sections (5 µm) were cut on a rotary microtome (Shandon Finesse 325, Italy).

Testicular sections were stained with Periodic acid-Schiff (PAS) and Harris's hematoxylin. Sections were observed and photographed under a Nikon Optiphot BH 2 research microscope (Japan) using Fuji color 100 ASA films. Photographic

panels were prepared using the Adobe Photoshop Software (Version 7, Inc. Chicago, Illinois, USA).

Stereology and morphometry

Quantitative study of spermatogenesis was carried out by counting the number of each variety of germ cells at stage VII of the seminiferous cycle. Stage VII was selected for quantitative analyses of spermatogenesis as the androgen receptor protein expression is highest in stage VII in the case of rat, providing support to the contention that mid-spermatogenic stages are androgen responsive. In rats androgen receptor mRNA and immuno-expression is maximal in the midspermatogenic stages VII–VIII, with a clear-cut downregulation during stage VIII (Donnel, 2006).

Type-A spermatogonia (Asg), preleptotene spermatocytes (PlSc), pachytene spermatocytes (PSc) and step 7 spermatids (7Sd), were counted according to the method of Leblond and Clermont (1952). The nuclei of different germ cells were counted in 100 round tubules per treatment group (five slides were selected from one animal; two sections from each slide were taken for counting). Two notes were obtained from each transverse section, considering the representative measurement as the mean of both. All the counts (crude counts) of germ cells were corrected for section thickness and differences in the nuclear or nucleolar diameter according to the method of Abercrombie (1946) using the following formula:

$$P = A \frac{M}{L + M}$$

where P is the average number of nuclear points per section, A is the crude count of nuclei in the section, M is the section thickness (μm) and L is the average diameter (μm) of the nuclei. The results are expressed as cell number per cross section of seminiferous tubule. Ratios for coefficient of spermatogonial mitosis, meiotic index, Sertoli efficiency and total support capacity of each Sertoli cell were obtained from the corrected counts according to the method described by Segatelli *et al.* (2004) and explained elsewhere in detail (Ramzan and Qureshi, 2011).

The diameter of seminiferous tubules and germ cells was measured with an ocular micrometer calibrated with a stage micrometer, while sections were observed under immersion oil at 100× magnification to count Sertoli and germ cell nuclear profiles.

Cell identification and grouping

Sertoli and germ cells were readily identified by the appearance of their nuclei in conjunction with the stage of the cycle of the seminiferous epithelium according to the descriptions of Leblond and Clermont (1952), Clermont (1972), Hess (1990). Stage VII of seminiferous epithelium was recognized by the acrosome system which covers nearly one third of the step 7 spermatid nucleus and type B spermatogonia had been replaced by small preleptotene spermatocytes. Sertoli and germ cells were grouped as described previously in Ramzan and Qureshi (2011).

Testicular spermatid head count

Frozen testes were thawed for 2-3 min. A shallow incision was made in the tunica albuginea to remove the tunica and associated blood vessels. Homogenization was done for one min at 1000×*g*. Only one testis was transferred to homogenizer at a time. Spermatid heads were stained with 0.1 % trypan blue. Spermatid heads were counted using an improved Neubaur's chamber. Two notes were taken for each animal and the mean of both was considered as representative measurement. The total number of spermatid heads in a rat testis was calculated as described by Seung *et al.* (2001) and described formerly in Ramzan and Qureshi (2011).

Hormone analyses

Plasma LH, FSH and testosterone concentrations were determined through standard solid phase radioimmunoassay (RIA). Rat-specific RIA and IRMA (FSH) kits were purchased from commercial suppliers: LH and FSH from Biocode-Hycel (Liege, Belgium) and testosterone from Immunotech (Marseille, France). Assays were done according to the manufacturer's instructions. The radioactivity was counted in a 16-channel Gamma counter (Oakfield Sourcerer RIA counter, No. 238, Type SD 16, UK) for at least 60s. Mean sensitivities of the assays were 0.14 ng/ml, 0.2 ng/ml, 0.025

ng/ml for LH, FSH and testosterone, respectively. Intra and inter-assay coefficients of variation, respectively, were below or equal to 10.5% and 12.2% for LH, 2.7% and 7.2% for FSH, and 14.8% and 15% for testosterone.

Transmission electron microscopy (TEM)

Electron microscopy was conducted as described in Ramzan and Qureshi (2011).

DNA ladder assay

Tissues stored at -70°C were brought to room temperature. DNA was isolated according to the method of Gilbert *et al*. (2007). At least 30 mg testicular tissue was washed twice with 1 ml TE buffer (pH 7.5) and were mashed. Lysis buffer and 10% SDS were added then. Contents were vortexed gently and then incubated overnight at 45ºC in a water bath. Phenol was then added, shaken vigorously for 5 min, and the contents were centrifuged for 5 min at 1300x*g*. Supernatant was pipetted out into a new tube to which phenol and chloroform/isoamyl alcohol (24:1) were added, centrifuged for 5 min. The supernatant was pipetted out into a new tube and sodium acetate (pH 5.2) and ethanol were added and tubes were incubated overnight at -20°C and then centrifuged for 30 min. The supernatant was aspirated gently. DNA was washed in 70 % ethanol and dried in oven at 30ºC. Finally, TE buffer (pH 8.0) and RNase were added.

Gel electrophoresis

DNA samples from control and treated testis were analyzed on 2 % agarose resolving gel. 100 bp DNA ladder was loaded alongside the DNA samples to identify the size of the DNA fragment. Electrophoresis was performed for 45 min at 100 V (80 mA) in 1 × TBE buffer. The gel was viewed under Gel Doc system (Biorad, Germany) and photographed.

Quantification of DNA fragmentation

DNA quantification was done according to the method of Boraschi and Maurizi (1998). Frozen testis tissues (30 mg) were ground in one ml TTE solution (pH 7.4) to make cell suspension and kept overnight at 37ºC. Cells were centrifuged at 1000×*g* for 10 min. Supernatants were transferred carefully to new tubes labeled “S”. To the pellet in tubes “B”, TTE solution was added and thoroughly vortexed. To separate fragmented DNA from intact chromatin, tubes B were centrifuged at 20,000 × *g* for 10 min at 4°C. Supernatants were transferred carefully to new tubes labeled “T”. To the small pellet in tubes B, TTE solution was added. 25% TCA was added to tubes T, B and S and were vortexed vigorously. Precipitation was allowed to proceed overnight at 4°C. After incubation, precipitated DNA was recovered by pelleting for 10 min at 20,000 × *g* at 4°C. Supernatants were aspirated and DNA was hydrolyzed by adding 5% TCA to each pellet and heated for 15 min at 90°C in a heating block. A blank with 5% TCA alone was prepared. To each tube, 320 µl of freshly prepared diphenylamine (DPA) solution was added, vortexed and allowed to develop color for about 4 h at 37°C or overnight at room temperature. Optical density at 620 nm was read on a UV-visible spectrophotometer (HP8453, Agilent Technologies, USA). The percentage of fragmented DNA was calculated using the formula:

$$\% \text{ Fragmented DNA} = \frac{T \times 100}{T + B}$$

Statistical analysis

Results were expressed as mean ± SE. The results obtained were analyzed and compared by one way ANOVA followed by post hoc Tukey’s adjustment using the Statistical Package for Social Sciences (SPSS, version 16, Inc, Chicago, Illinois, USA). $P<0.05$ was considered to be statistically significant. Data have presented as mean and standard error of mean (SEM).

RESULTS

Body weight and testicular parameters

No difference was found in body weight, testicular weight, testis volume, Gonadosomatic index (%) and epididymes weights between control, alone kisspeptin, saline (acyline pretreated) and kisspeptin (acyline pretreated) treated groups

(Table I).

Seminiferous tubular diameter and epithelium height

Seminiferous tubular diameter was not effected at any of the treatment doses (Table I). Seminiferous tubular epithelial height decreased highly significantly ($P<0.001$) in treated groups as compared to control. Intergroup comparison showed no difference between kisspeptin (acyline pretreated) and alone kisspeptin treatment, whereas, it increased highly significantly ($P<0.001$) in kisspeptin treated (acyline pretreated) group as compared to saline treated (acyline pretreated) group (Table I).

Plasma hormone levels

Following acyline administration, a significant decrease was observed in plasma FSH ($P<0.001$) (Fig. 1B), LH ($P<0.001$) (Fig. 1E) and testosterone levels ($P<0.001$) (Fig. 1H), at day one of the experiment. After 12 days of treatment, following kisspeptin alone challenge, plasma FSH levels were unaltered. They decreased highly significantly ($P<0.001$) in the saline (acyline pretreated) treated and kisspeptin (acyline pretreated) treated groups as compared to the control rats (Fig. 1C). Plasma LH levels decreased significantly ($P<0.01$) in kisspeptin alone and saline (acyline pretreated) treated groups. On intergroup comparison, LH concentration showed non-significant decrease in kisspeptin (acyline pretreated) treated group as compared to the saline (acyline pretreated) control (Fig. 1F). Plasma testosterone concentration decreased significantly ($P<0.001$) with the kisspeptin alone treatment and where kisspeptin was given after acyline pretreatment ($P<0.01$) than the saline treated rats. Between group comparisons showed no difference in the kisspeptin (acyline pretreated) and saline (acyline pretreated) treated groups (Fig. 1I).

Quantitative data on spermatogenic cells

With kisspeptin alone and saline (acyline pretreated) treatment Type A spermatogonia ($P<0.001$), preleptotene spermatocytes ($P<0.05$), pachytene spermatocytes ($P<0.01$) and step 7 spermatids ($P<0.001$) decreased significantly when compared to control. Intergroup comparison showed that Type A spermatogonia ($P<0.01$), preleptotene spermatocytes ($P<0.01$), pachytene spermatocytes ($P<0.001$) and step 7 spermatids ($P<0.001$) increased significantly in kisspeptin (acyline pretreated) treated group as compared to saline (acyline pretreated) treated group although not approaching control values (Fig. 2).

Elongated spermatid heads and daily sperm production

Elongated spermatids (g^{-1} testis wt.) and daily sperm production (g^{-1} of testis weight day^{-1}) both decreased significantly ($P<0.01$) in the kisspeptin alone and saline (acyline pretreated) treated groups but in the kisspeptin (acyline pretreated) group, compared to control, no significant difference was found. Both elongated spermatids and daily sperm production increased significantly ($P<0.01$) in the kisspeptin (acyline pretreated) treated group as compared to the saline (acyline pretreated) treatment (Fig. 3).

Cell ratios from the corrected counts

Meiotic index, Sertoli efficiency and total support capacity of each Sertoli cell decreased at alone kisspeptin treatment ($P<0.01$, $P<0.001$, $P<0.001$) and saline (acyline pretreated) treatment ($P<0.05$, $P<0.01$, $P<0.001$), while coefficient of mitosis increased ($P<0.001$). Between group comparison demonstrated that meiotic index, Sertoli efficiency and total support capacity of each Sertoli cell increased ($P<0.05$, $P<0.01$, $P<0.01$), while coefficient of mitosis decreased ($P<0.001$).

Kisspeptin (acyline pretreated) treated group and control did not differ significantly in all cell ratios except coefficient of mitosis increased ($P<0.001$) in kisspeptin (acyline pretreated) treatment ($P<0.001$) (Fig. 4).

Effect on histomorphology of seminiferous tubules

Light microscopic examination of control saline treated testicular sections showed normal seminiferous tubules with intact epithelia and evenly spaced type A spermatogonia and primary spermatocytes. Dark stained small size preleptotene spermatocytes were arranged at the periphery, while pachytene spermatocytes containing large size nuclei were present next to these. Round spermatids

and elongated spermatids, as characterized by the

Table 1.- Effect of kisspeptin (KP) on testicular parameters in different experimental groups of prepubertal male rats.

	Control (n=10)	Kisspeptin alone (n=10)	Saline (Acyline pretreated) (n=10)	KP (Acyline pretreated) (n=10)
Body weight (g)	108.41±18.98	115.54±11.96	122.00±20.54	120.20±31.49
Testis weight (g)	1.02±0.18	1.03±0.16	1.05±0.10	1.04±0.07
Testis volume (mm^3)	1.06±0.25	0.96±0.13	1.01±0.08	1.04±0.07
Gonadosomatic index (%)	0.93±0.13	0.85±0.13	0.88±0.19	0.91±0.19
Epithelium height (µm)	63.06±7.97	53.25±5.69 [a] ***	42.54±5.79 [a,b]***	54.58±5.43 [a,c]***
Seminiferous tubular diameter (µm)	170.52±12.16	167.37±10.33	169.24±10.24	169.24±10.24

Mean±SD; Difference from control = *P<0.05; **P<0.01; ***P<0.001.
a, difference from control groups; b, difference from kisspeptin (KP) group; c, difference between saline (pre-treated with acyline) and kisspeptin (pre-treated with acyline) group.

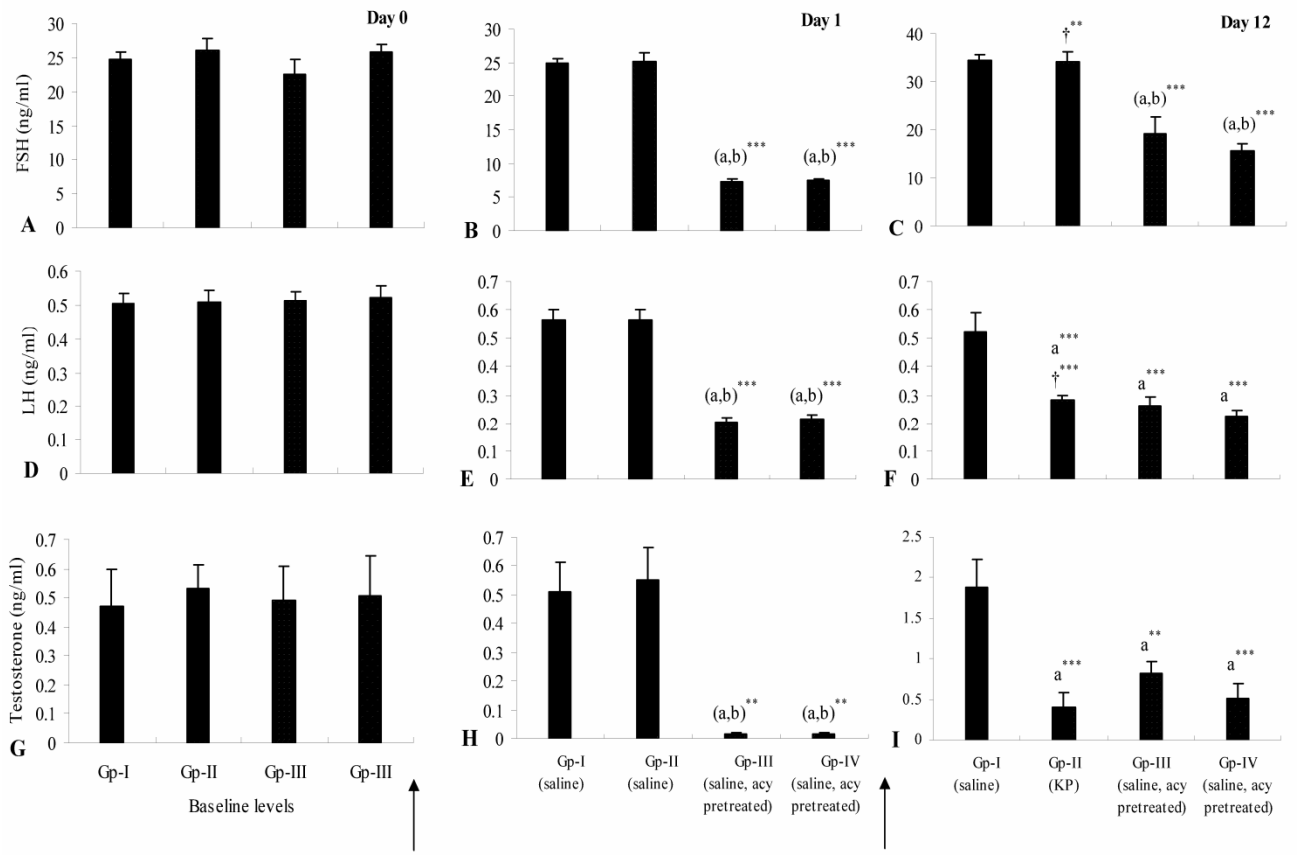

Fig. 1. Effect of kisspeptin and acyline treatment on plasma FSH (ng/ml), LH (ng/ml), and testosterone (ng/ml) concentrations. Left panel (day 0) represent baseline levels of hormones. Middle panel (day 1) represents the levels of hormones after treatment with saline (Gp I and Gp II) or acyline (Gp III and Gp IV). The levels of gonadotropins and testosterone were suppressed after treatment with acyline. Right panel represents the levels of hormones after 12 days of kisspeptin treatment to saline (Gp II) and acyline (Gp IV) treated animals. Arrow between left and middle panel represents administration of 300 µg kg^{-1} body weight acyline once at day 0 after taking blood samples for baseline levels. Arrow between middle and right panel represents administration of 1µg rat^{-1} kisspeptin at day 1 after taking blood samples to check the levels of hormones after 24 hrs of acyline treatment. Kisspeptin treatment was continued twice daily for 12 days. Gp= Group, a= difference from control group, b=difference from kisspeptin group,

†=difference from Kisspeptin group (day 1). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

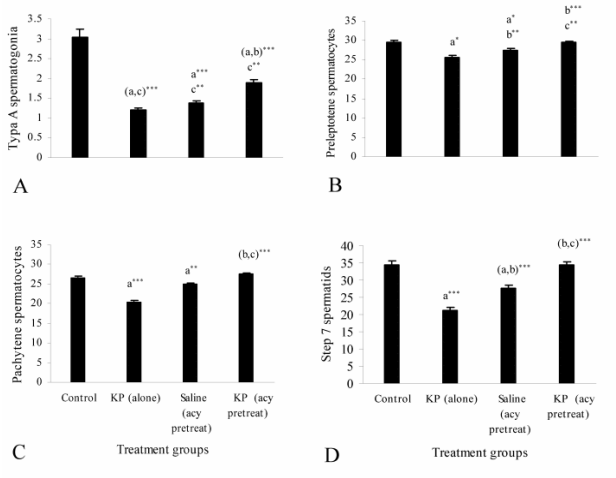

Fig. 2. Number of germ cells per 100 round seminiferous tubules at stage VII of spermatogenesis. Type A spermatogonia (A), preleptotene spermatocytes (B), pachytene spermatocytes (C) and step 7 spermatids (D) decreased significantly in saline (acyline pretreated) group. With kisspeptin (acyline pretreated) they increased as compared to saline treatment (acyline pretreatment). a, difference from control group; b, difference from kisspeptin group; c, difference between saline (acyline pretreated) group and kisspeptin (acyline pretreated) group. Values are expressed as ± SE. $^{**}P<0.01$, $^{***}P<0.001$.

acrosome, were present further toward the lumen (Fig. 5a,b). With kisspeptin alone treatment, marked degeneration of seminiferous tubules was readily noticeable. Spermatogonia were regressed and hyperchromatic. Round and elongated spermatids were scanty indicating maturation arrest. Luminal space was increased while the epithelial height decreased. Atrophy of germinal epithelium was also noticeable (Fig. 5c,d). Seminiferous tubules treated with saline (acyline pretreated) showed degenerative changes and intratubular vacuolizations. Germ cells were regressed, atrophied and showed severe necrosis. Tubules were characterized by the secretion of amorphous substance into the lumen and germ cell maturation arrest (Fig. 5e,f). Seminiferous tubules of rats treated with kisspeptin (acyline pretreated) revealed partial restoration of spermatogenesis (Fig. 5g,h).

Electron microscopic examination

The spermatogonia located at the boundary

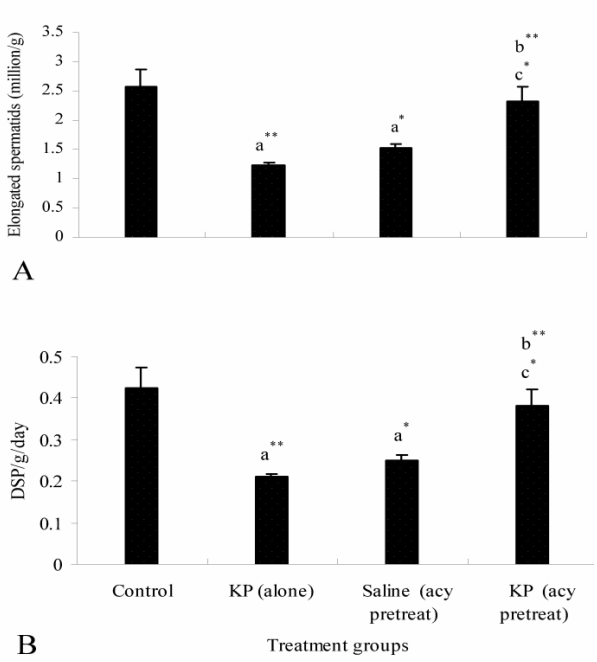

Fig. 3. Elongated spermatid heads per gram of testis (A) and daily sperm production (DSP) (B) after treatment with saline, kisspeptin alone, saline (acyline pretreated) and kisspeptin (acyline pretreated). a, difference from control group; b, difference from kisspeptin group; c, difference between saline (acyline pretreated) group and Kisspeptin (acyline pretreated) group. Values are expressed as ± SE. $^{*}P<0.05$, $^{**}P<0.01$.

tissue of the seminiferous tubules were characterized by large ovoid nuclei containing fine granular nucleoplasm. The nuclei were present with their long axis parallel to the boundary tissue. The primary spermatocytes had spherical nuclei with fine granulated nucleoplasm and chromatin accumulation. Sertoli cell nuclei had irregular shapes but the nucleoplasm was homogenous (Fig. 6a). After a pretreatment with GnRH antagonist, acyline, the seminiferous tubules showed altered cellular architecture compared to control tubules. Spermatogonia, spermatocytes and Sertoli cells contained vacuolated and swollen mitochondria and germ cells were regressed in shape (Fig. 6b). With kisspeptin (acyline pretreated) treatment, the testicular tissue showed recovery. Spermatogonia

were normal and basal laminae were intact. Enlarged intercellular spaces were still present.

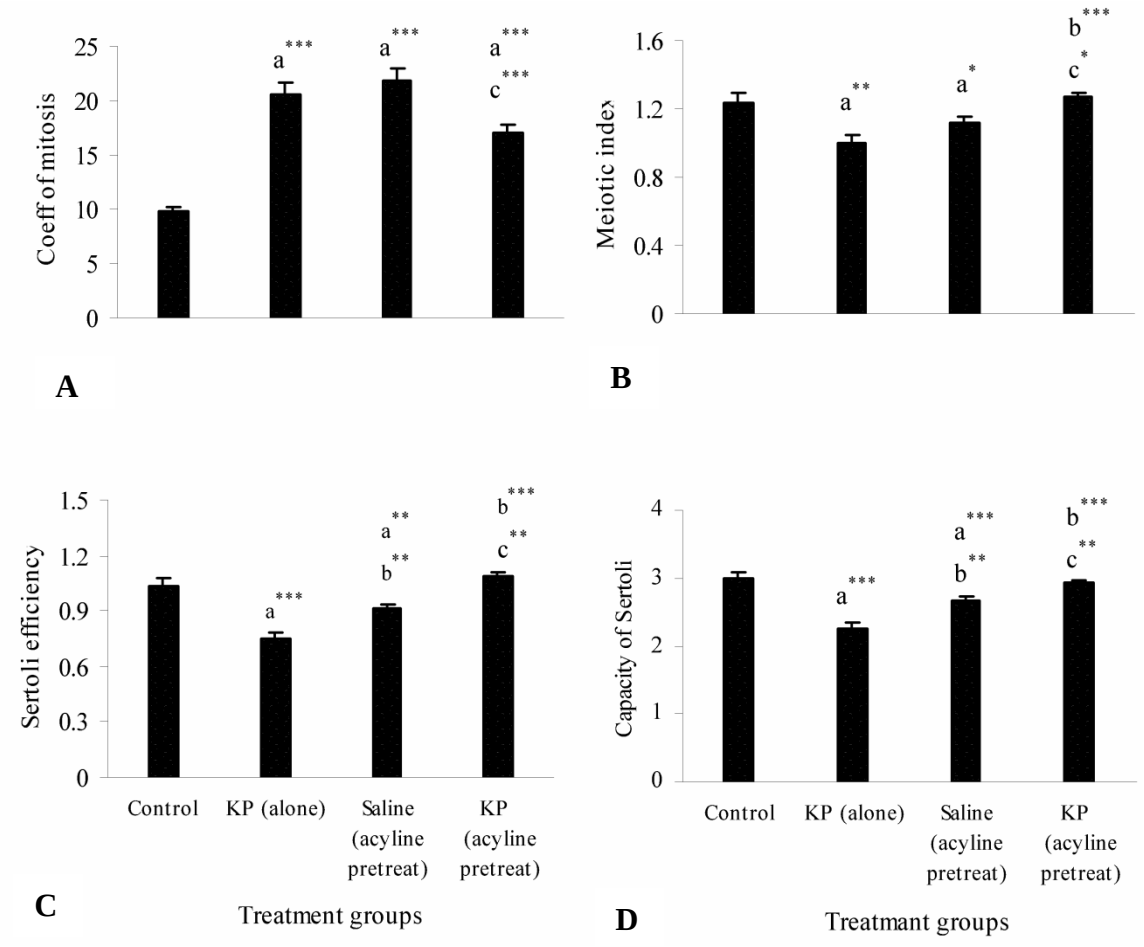

Fig. 4. Cell ratios from corrected counts following kisspeptin and acyline administration: **A**, Coefficient of efficiency of spermatogonial mitosis increased in all treated groups as compared to saline treated controls; **B**, Meiotic index (rate of germ cell loss during meiosis) decreased in kisspeptin alone and saline (acyline pretreated) group while kisspeptin (acyline pretreated) remained similar to control; **C**, Sertoli efficiency decreased in kisspeptin alone and saline (acyline pretreated) group while kisspeptin (acyline pretreated) remained similar to control; **D**, Capacity of Sertoli cell decreased in kisspeptin alone and saline (acyline pretreated) group while kisspeptin (acyline pretreated) remained similar to control. a= difference from control group, b= difference from kisspeptin group, c= difference between saline (acyline pretreated) group and KP (acyline pretreated) group. Values are expressed as ± SE. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Spermatogonia and spermatocytes still contain vacuolated organelles (Fig. 6c).

DNA fragmentation

DNA ladder assay demonstrated 180-bp and 400-bp fragments in saline (acyline pretreated) treated group, while in the kisspeptin (acyline pretreated) treated group, only 180-bp fragments were recognizable (Fig.7A). The degree of % DNA fragmention increased highly significantly ($P<0.001$) in kisspeptin alone and saline (acyline prereated) treated groups as compared to the control. In contrast, it remained similar to control values in the kisspeptin (acyline pretreated) treated group. Intergroup comparison showed a significant increase ($P<0.001$) in % DNA fragmention in the saline (acyline pretreated) group as compared to kisspeptin (acyline pretreated) group, however compared to kisspeptin alone treatment, the % DNA

fragmentation was lower (P<0.001) in all treated groups (Fig. 7B).

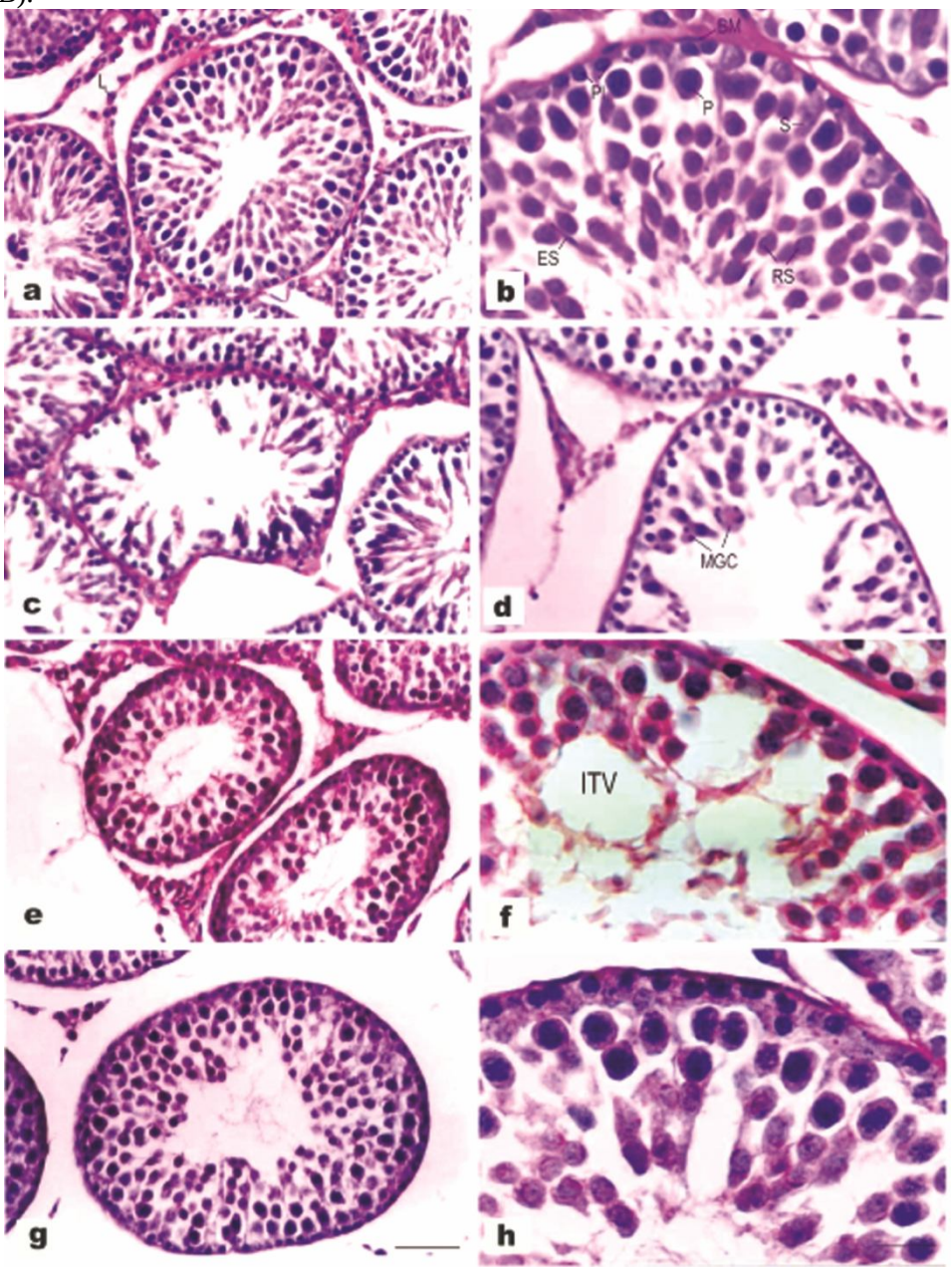

Fig. 5. Histological structure of seminiferous tubules of control, kisspeptin alone, saline (acyline pretreated), kisspeptin (acyline pretreated) rats: **a-b**, Control seminiferous tubules showing basement membrane (BM), spermatogonia (SG), preleptotene spermatocytes (Pl), pachytene spermatocytes (P), round spermatids (RS), elongated spermatids (ES), Sertoli cell nuclei (S) and Leydig cells (L); **c-d** Seminiferous tubules of rats treated with kisspeptin alone showing marked degeneration of seminiferous tubules. Note deshaped and regressed pachytene spermatocytes. Luminal space is increased while the epithelial height appears decreased; **e-f,** Seminiferous tubules of rats treated with saline (acyline pretreated) showing intratubular vacuolation (ITV), germ cell loss and abnormal cellular associations. Maturation arrest of germ cell is also evident; **g-h**, Seminiferous tubules of rats treated with kisspeptin (acyline pretreated) showing partial restoration of spermatogenesis.

Scale bar left panel = 50 µm, right panel = 20 µm.
Magnification left panel = ×400, right panel = ×1000

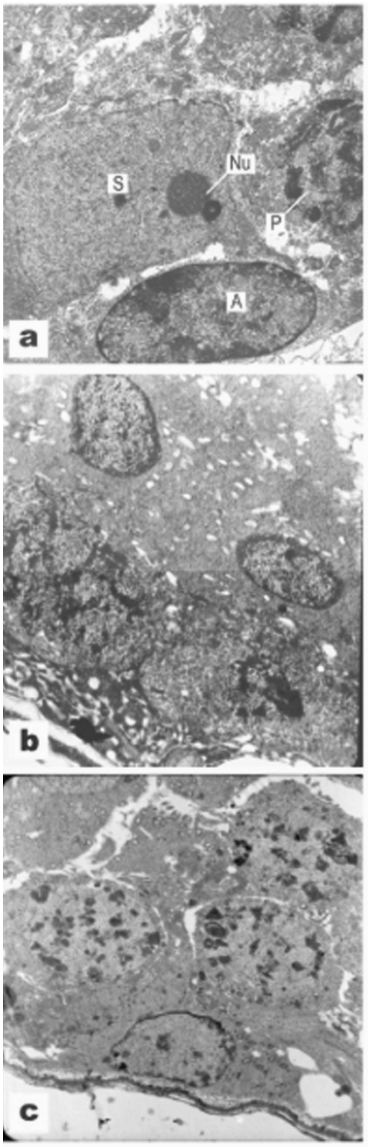

Fig. 6. Ultrastructure of testicular tissue of control, saline (acyline pretreated) and kisspeptin (acyline pretreated) treated rats: **a**, Seminiferous tubule from control rats showing Sertoli cell nucleus (S) with a prominent nucleolus (Nu), type A spermatogonia (A) and pachytene spermatocytes. ×8, 000; **b,** Seminiferous tubule of a rat treated with saline (acyline pretreated) showing Sertoli cell and primary spermatocytes containing vacuolated organelles. ×3,000; **c**, Seminiferous tubule of a rat treated with kisspeptin (acyline pretreated) showing recovery in the structure of basement membrane, primary spermatocytes and Sertoli cell cytoplasm. ×5, 000.

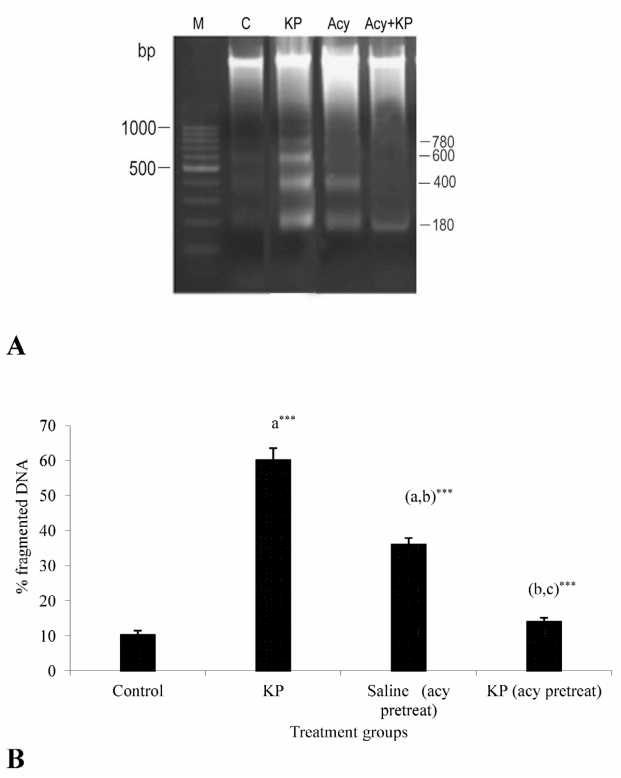

Fig.7. DNA damage to control, kisspeptin alone, Saline (acyline pre-treated), Kisspeptin (acyline pre-treated) treated testicular tissues: **A**, DNA ladder assay showing 180, 400, 600 and 780 bp bands of fragmented DNA, at alone kisspeptin dose, from rat testis. With acyline treatment 400 and 180 bp band were observed. Kisspeptin (acyline pre-treated) treatment demonstrated a single 180 bp fragment. Control testis showed intact DNA. M= 100-bp ladder (DNA size markers), C=control (saline treated), KP= kissprptin alone, Acy= Saline (acyline pre-treated), Acy+KP= Kisspeptin (acyline pre-treated); **B**, DNA fragmentation assay. Percentage of fragmented DNA relative to total DNA remained similar to control in acyline pretreatment followed by kisspeptin treatment while it increased significantly in alone kisspeptin and acyline pretreatment followed by saline treatment. Values are expressed as mean ± SE. $^{*}P<0.05$, $^{***}P<0.001$ compared with control. a= difference from control, b= difference from alone kisspeptin, c= difference

between saline (acyline pre-treated) and kisspeptin (acyline pre-treated).

DISCUSSION

The kisspeptin/GPR54 interaction has finally been identified in recent years as key regulator of the HPG axis (de Roux *et al.,* 2003, Seminara *et al.,* 2003). Although the acute effects of central and peripheral administration of kisspeptin in rats and mice (Gottsch *et al.,* 2004, Navarro *et al.,* 2004a, Navarro *et al.,* 2005), as well as the effects of long term kisspeptin administration on structure and functional aspects of testicular tissue in adult (Thompson *et al.,* 2009) and prepubertal testes (Ramzan and Qureshi, 2011) have been explored in detail. However, there is no report on kisspeptin effects on structural and functional aspects of testes, following blockade of endogenous kisspeptin actions. The present study investigated the gonadal maturation and reproductive hormones status following kisspeptin challenge in prepubertal male rats as well as by blocking the actions of endogenous kisspeptin using acyline as an antagonist for GnRH. The data showed a significant reduction in LH and testosterone level in the serum by acyline and kisspeptin (acyline pretreated); however, kisspeptin alone was unable to reduce FSH level. The germinal epithelium was found to be atrophied by kisspeptin and acyline; while, the combination partially restored spermatogenesis. Histological and ultastructural degeneration of the testicular epithelium in kisspeptin treated rats has been demonstrated in detail in our previous paper (Ramzan and Qureshi, 2011).

It has been shown that in prepubertal male rats, persistent administration of kisspeptin may lead to obvious dissociation of gonadotropin levels, with selective decrease of LH secretion only. We have previously shown that testicular degeneration followed by subchronic kisspeptin administration was dose dependent (Ramzan and Qureshi, 2011). It seems logical to presume that kisspeptin perhaps acted to suppress an early maturation of gonads as the male gonads have to develop fully at a later stage. Spermatozoa first appear in the testes around day 45 with optimal production occurring at 75 days (LD, 1992).

Expression of both KiSS-1 and GPR54 has been reported in the ovaries and testes of rodents (Kotani *et al.,* 2001, Ohtaki *et al.,* 2001, Terao *et al.,* 2004, Castellano *et al.,* 2006), a direct testicular effect may be speculated to provide a functional kisspeptin-GPR54 system for local regulation of reproductive status at the level of gonads. Some reports of the effects of GnRH agonists also suggest an extrapituitary direct inhibitory action on the gonads (Hsueh and Schaeffer, 1985, van Kroonenburgh *et al.,* 1986). However, Thompson *et al.* (2009) documented that a single i.c.v. injection of kisspeptin-54 caused testicular degeneration, suggesting that the action is GnRH-mediated. Similarly our finding that pretreatment with a GnRH-R antagonist prevented testicular degeneration caused by kisspeptin-10 also suggests that it is unlikely that a direct effect of kisspeptin-10 on the testes plays any major role in inducing testicular degeneration.

Presently, kisspeptin alone and saline (acyline pretreated) treatment significantly elevated internucleosomal DNA fragmentation. DNA fragmentation was detected concomitant with the decrease in organ weights and a compromise of histological status. Studies suggest that activation of endogenous endonucleases which fragment the DNA into oligonucleosomal fragments of 200 base pairs is the earliest and most characteristic biochemical event common to all processes of apoptosis (Wyllie, 1980, Compton, 1992). Apoptosis is characterized by chromatin condensation, activation of some caspases and fragmentation of DNA at internucleosomal linker sites giving rise to discrete bands of multiples of 180–200 bp (Carson and Ribeiro, 1993). Although exceptions do exist, this form of DNA degradation has been very widely observed in apoptosis. Different types of DNA fragmentation have been reported during apoptosis, in the presence or absence of the characteristic internucleosomal DNA cleavage (ladder-like) pattern (Bortner *et al.,* 1995). Our histomorphological data also appears to support apoptosis hypothesis, because germ cell nuclei were found regressed and pyknotic with kisspeptin alone and saline (acyline pretreated) treatment. At higher doses multinucleated germ cell formation was also

frequent.

The present study showed that in saline (acyline pretreated) treated rats there occurred significant decrease in the population of type A spermatogonia, preleptotene spermatocytes, pachytene spermatocytes and step 7 spermatids with a concomitant decrease in plasma LH and testosterone concentrations. These results are parallel to those of Zhengwei *et al.* (1998) who reported that proportion of the type A spermatogonia, preleptotene, leptotene, zygotene and pachytene spermatocytes were all reduced in cetrorelix (GnRH antagonist) treated monkey testes and suppressed serum testosterone concentrations to castrate levels. In the present experiments pretreatment of rats with a single injection of acyline protected against kisspeptin induced decline in spermatogenesis. This was indicated by quantitative assessment of testicular histology at stage VII of spermatogenesis, testicular spermatid head counts, ultrastructure of testicular tissue and DNA fragmentation assay. In instances of testicular damage produced by various chemical or physical agents, revival of spermatogenesis can be induced by suppression of testosterone and FSH by treatment with GnRH analogs (Kangasniemi *et al.,* 1995, Meistrich and Kangasniemi, 1997, Meistrich and Shetty, 2003, Blanchard *et al.,* 1998).

Meistrich *et al.* (2001) showed that GnRH antagonist is more effective at stimulating the recovery of tubule differentiation and sperm counts in irradiated rats than a GnRH agonist. The present study also suggests that pretreatment with acyline can recover testicular degeneration. The mechanisms causing failure of spermatogonial differentiation following cytotoxic therapies and how the suppression of testosterone restores this ability to differentiate are not yet known (Boekelheide *et al.,* 2005). Also, Meistrich and Shetty (2008) documented that mechanism behind the protective effect of GnRH antagonist in preventing testicular tissue damage after injury/ chemotherapy are not clear yet (Meistrich and Shetty, 2008). However, Meistrich *et al.* (2000) proposed that prevention of the block in differentiation of surviving stem spermatogonia in rat testes after exposure to cytotoxic agents is the mechanism by which hormone suppression appears to protect spermatogenesis from toxicant exposure.

Medical treatments required for life-threatening diseases or exposure to environmental toxicants may jeopardize the fertility of men and women of reproductive age. In men, such exposures can lead to effects ranging from temporary oligospermia to permanent azoospermia, and occasionally to androgen insufficiency. In women, such exposures can result in a range of effects from temporary amenorrhea to premature menopause and permanent amenorrhea, with the associated estrogen insufficiency. Methods to prevent these effects on fertility and to restore gonadal function after the toxic treatment are of particular importance to men and women of child bearing age. A variety of biochemical and biological approaches such as thiol radioprotectors, prostaglandin analogs, growth factors, blockers of apoptotic pathways, and reduction in blood flow, have been tested to protect the testes in experimental animal model systems against radiation and chemotherapy (Meistrich, 2007). However, the greatest research interest in nearly all clinical trials involved hormonal modulation in attempts to prevent or reverse damage to the germ line from radio- and chemotherapy. Given results of the present study it can be suggested that kisspeptin/GPR54 system may act as one of the targets for the pharmacological intervention of the reproductive system.

CONCLUSIONS AND FUTURE PROSPECTS

It is finally concluded that continuous kisspeptin administration to prepubertal rats causes severe testicular degeneration and a differential desensitization of the HPG axis, while pretreatment with GnRH antagonist restores kisspeptin induced testicular degeneration and that the action of kisspeptin appear to be centrally mediated.

Antagonist for kisspeptin is available now (Roseweir *et al.,* 2009). The development of kisspeptin antagonists provides a valuable tool for investigating the physiological and patho-physiological roles of kisspeptin in the regulation of reproduction and offers a unique therapeutic agent for treating hormone-dependent disorders of reproduction, including infertility, delayed and precocious puberty and metastatic prostate cancer.

Further pharmacological and pathological studies using kisspeptin antagonist are required to be carried out. There was no antagonist available at the time the present study was conducted therefore attempt was made to indirectly block kisspeptin actions via acyline, a potent GnRH antagonist.

Authors' contributions

FR has designed the study, conducted the experiments and drafted the manuscript. IZQ has supervised the study. MAK edited the manuscript. MJI helped in electron microscopy.

ACKNOWLEDGEMENTS

The work was funded by a graduate scholarship grant (B-065-041311401) to the first author by the Higher Education Commission, H-9, Islamabad, Pakistan and partly through a research grant (PSF/Res/C-QU/Med/255) offered to the second author by the Pakistan Science Foundation. Authors are highly indebted to Prof. Dr. M. Shahab, Chairman, Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan for gift of Acyline.

Competing interests

The authors have no conflict of interest of intellectual or financial nature with any individual or institution.

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(Received 28 February 2013, revised 21 March 2013)