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Direct effects of neuropeptide nesfatin-1 on testicular spermatogenesis and steroidogenesis of the adult mice

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ABSTRACT

Recent studies have revealed nesfatin-1 as a hypothalamic neuropeptide, regulating food intake, energy expenditure and reproduction primarily by acting on the hypothalamic-pituitary-gonadal axis. Nesfatin-1 is also localized in several peripheral tissues including testes. However, functional significance of nesfatin-1 in testicular activities is not yet well documented in mammals. Therefore, this study was aimed to elucidate the direct effects of nesfatin-1 on testicular markers for steroid productions, spermatogenesis, metabolic changes and oxidative stress. The results revealed the expression of both protein and mRNA of nesfatin-1 in the testes of adult mice. The testes treated *in vitro* with nesfatin-1 showed significant increase in testosterone production, which correlated significantly with increased expression of steroidogenic markers and insulin receptor proteins in the testes. Furthermore, the *in vitro* treatment with nesfatin-1 showed stimulatory effects on spermatogenesis by promoting cell proliferation (PCNA) and survival (Bcl2), while inhibiting apoptosis (caspase-3) in the testes. The nesfatin-1 treatment *in vitro* further increased the expression of insulin receptor and GLUT8 proteins, in parallel with increase in the intra-testicular transport of glucose and production of lactate. This nesfatin-1 induced enhanced transport of energy substrate (glucose and lactate) may be responsible for promoting spermatogenesis and steroidogenesis. Nesfatin-1 significantly reduced oxidative stress and nitric oxide, which may also be responsible for stimulatory effects on testicular activities. The present finding suggests that nesfatin-1 acts via paracrine manner to increase sperm count and fertility, thus promoting the testicular function.

1. Introduction

The reproductive activities require the presence of sufficient supply of energy substrates including glucose, and inadequate supply of these energy substrates to the testes leads to suppression of testicular activities (Boussouar and Benahmed 2004; Rato et al., 2012; Banerjee et al., 2014). The neuroendocrine regulation of energy balance (food intake) and that of testicular activities are also closely interlinked (Hill et al., 2008). However further studies warrant to ascertain the mechanism by which neuropeptides that regulate food intake also affect testicular functions. Usually, the neuropeptides that stimulate reproduction inhibit food intake and vice versa (Small et al., 2004). Recently several neuropeptides have emerged as modulators of both food intake and reproduction. Among them, nesfatin-1 shows ability to reduce food intake and to regulate onset of puberty (Garcia-Galiano et al., 2010b).

Nesfatin-1, the hypothalamic 82-amino acid neuropeptide, is derived from its precursor protein, nucleobindin 2 (NUCB2) (Oh-I et al., 2006). It is distributed in various areas of hypothalamus and brain, and implicated in multiple functions such as food intake (energy balance), locomotion, stress, thermogenesis and reproduction (Stengel et al., 2011; Gonzalez et al., 2011). The central treatment with nesfatin-1 resulted in dose-dependent reduction in food-intake and consequent decline in body weight in the rat (Oh-I et al., 2006). It's circulating level changes with metabolic disorders such as obesity and type-II diabetes mellitus (Abaci et al., 2013, Li et al., 2010). The follow-up studies established that nesfatin-1 modulates energy homeostasis in mammals (Goebel-Stengel and Wang, 2013). It has been shown that NUCB2/Nesfatin-1 is localized in pancreatic β-cells and that nesfatin-1 enhances glucose-stimulated insulin release from β-cells in mice (Nakata et al., 2011). The obesity and hyperglycemic state have also been shown to induce the synthesis and release of NUCB2/nesfatin-1. Based on these findings nesfatin-1 is considered an important metabolic hormone.

An earlier study has shown mRNA expression of NUCB2/nesfatin-1 in the testes of human, rat and mice as well as in the rat ovary (Garcia-Galiano et al., 2010a). This study also showed the involvement of nesfatin-1 in gonadotropin release (Garcia-Galiano et al., 2010b).

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Further studies have revealed the involvement of nesfatin-1 in reproduction by modulating the Hypothalamic-Pituitary-Gonadal (H-P-G) axis in male rats (Gao et al., 2016). Both protein and mRNA of nesfatin-1 are present in hypothalamus, pituitary and gonad but varies with the age of the rat. In hypothalamus nesfatin-1 is localized in paraventricular nuclei (PVN) and arcuate area (Arc), which are involved in secretion of kisspeptin (KP)-1 and GnRH. The presence of nesfatin-1 together with GnRH and KP-1 in hypothalamus suggests its role on gonadotrophin secretion. However, the adult rat treated *in vivo* with nesfatin-1 suppresses the hypothalamic mRNA of GnRH and Kiss, resulting in decreased LH and FSH release from pituitary and testosterone (T) synthesis in testis. In the testis, nesfatin-1 was found in the Leydig cells (Kim et al., 2014). It is thus suggested that nesfatin-1 acts as a local regulator of testicular functions based on studies carried out mainly in the rats. Despite the current advances in the studies on nesfatin-1, distinct role of nesfatin-1 in the testicular function has not been extensively studied in mammals. Therefore, this study has been conducted in adult mice to elucidate the direct effects of nesfatin-1 on spermatogenic, steroidogenic and metabolic changes together with changes in oxidative stress in the testes of adult mice.

2. Material and methods

2.1. Animal

Adult Park's strain mice weighed ~30 g aged ~90 days were used for this study. All the animals were housed in standard laboratory condition with 12 h light-12 h dark cycle and fed with pelleted mice food and water *ad libitum*. All experiments were conducted following the principles and procedures approved by the institutional animal ethical committee (F.Sc/88/IAEC/2016–17/63) of Banaras Hindu University, Varanasi, India.

2.2. Chemicals and antibodies

The nesfatin-1 peptide was provided by Dr. Toshihiko Yada, Japan. The lyophilized nesfatin-1 powder was dissolved in double distilled water. The two doses of nesfatin-1 peptide (Low Dose = 0.1 nM and High Dose = 10 nM) used for the *in vitro* studies were grossly selected from previous study by Gonzalez et al. (2011) with minor modification based on our preliminary study. Nesfatin-1 antibody (anti-NUCB2; N-terminal) used in this study is produced in rabbit using synthetic rat NUCB2 corresponding to amino acids 31–51 as immunogen conjugated to KLH. Anti-NUCB2 specifically recognizes rat and mouse nesfatin-1 and is specifically inhibited by the NUCB2 immunizing peptide. Antibody of nesfatin-1 was validated for use in the testis of mouse by immunoblotting taking mouse hypothalamus as positive control (Fig. 1f). All general chemicals used in the study were procured from the Merck, New Delhi, India. The details of various antibodies used in this study are shown in Table 2.

2.3. The in vitro study

Adult mice (n = 21) were sacrificed by decapitation under mild anesthesia and their testes were immediately collected out. The one side of testis from each mouse was cleaned and used for the *in vitro* study. Each testis was cut into 2–3 pieces and divided into three groups: Control testis, testis treated with low dose of nesfatin-1 and testis treated with high dose of nesfatin-1. Equal pieces (~10 mg) of testis were cultured according to protocol describes by Banerjee et al. (2012). Control group testes did not received any treatment. Culture media includes a mixture of DMEM (with sodium pyruvate and L-Glutamine) and Ham's F-12 (1:1; v: v) (Hi-media, Mumbai, India) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1% BSA (Sigma Aldrich, St. Louis, MO, USA). After providing preliminary incubation for 2 h at 37 °C, the culture media was discarded and cultured in fresh 1 ml medium (supplemented with nesfatin-1) with 95% air and 5% CO_2under humidified atmosphere for 12 h at 37 °C. The experiment was run in triplicate. During the culture, testes were healthy and without necrosis. Testes were collected at the end of culture, wash away with PBS and kept at −20 °C for immunoblot analysis. The media was stored at −20 °C for steroid and glucose measurement.

2.4. Immunohistochemistry

Immunohistochemical localization of nesfatin-1 was carried out in paraffin sections of testis of adult mice in accordance with our previous study (Anjum et al., 2012). The testicular sections were deparaffinized and rehydrated in graded ethanol. The antigen unmasking was done by microwave heating (750 W) in 10 mM Citrate buffer (pH 6.0) for 10 min, followed by washing the sections three times for 5 min with 0.1 M Tris-buffered saline (TBS) at pH 7.4. The endogenous peroxide of the sections were quenched with 0.3% H_2O_2 in methanol and then equilibrated in 0.1 M TBS. The tissue section was then kept in 5% normal goat serum in TBS for 5 min to reduce the background and incubated with primary antibody (Nesfatin-1, 1:250) for overnight at 4 °C. The sections were washed with PBS and then incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Bangalore Genei, Bangalore, India) for 2 h at room temperature. The peroxidase activity was detected with chromogen substrate (0.1% 3, 3′ diaminobenzidine tetra hydrochloride in 0.05 M Tris pH-7.6 and 0.01% H_2O_2) for 1–2 min. Nuclear counterstaining was done with Ehrlich's Hematoxylin. Finally, tissue sections were dehydrated and mounted with DPX. The negative control was performed by eliminating primary antibody and preabsorbed control was performed by replacing primary antibody with preabsorbed primary antibody with antigen. Photography was performed under a light microscope (Nikon, Tokyo, Japan).

2.5. RT-PCR

Total RNA was extracted from the testis of adult mice using trizol reagent (Sigma Aldrich, St. Louis, MO, USA). The RNA sample from hypothalamus of adult mice was used as positive control. The concentration of the isolated RNA of each samples were measured by NanoDrop (Epoch microplate Spectrophotometer, BioTek, Mumbai, India). The specific cDNA was synthesized from testis and hypothalamus RNA samples using commercially available cDNA kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instruction. A total of 2 μg of RNA in 20 μl of reaction mixture was used for reverse transcription reaction (RT-PCR). Information regarding primer and housekeeping (GAPDH) internal control are shown in Table 1. The RT-PCR was performed according to the procedure described in detail by Garcia-Galiano et al. (2012). The PCR program include denaturation at 97 °C for 5 min, following 30 cycles of denaturation at 96 °C of 30 s, annealing at 63 °C of 30 s and extension at 72 °C of 1 min. A final extension at 72 °C was done for 10 min. As internal control, GAPDH was also run in parallel with each sample. At last RT-PCR product was run in agarose gel electrophoresis. RT-PCR signal was quantified by scanning the band and analyzing using computer-assisted image analysis (Image J 1.38×, NIH, USA).

2.6. Western blotting

The pooled testicular slice were homogenized 20% (w/v) in suspension buffer (0.01 M Tris pH 7.6, 0.001 M EDTA pH 8.0, 0.1 M NaCl, 1 μg/ml aprotinin, 100 μg/ml PMSF). An equal volume of protein (50 μg) determined by Bradford's (1976) method was subjected to 10% SDS-PAGE for electrophoresis following the method described in detail previously (Banerjee et al., 2012). The proteins were transferred to polyvinylidene fluoride membrane (PVDF) (Millipore Pvt. Ltd., MA, USA) overnight at 4 °C. The blocking of PVDF membrane was done in Tris-buffered saline (TBS; Tris 50 mM (pH 7.5), NaCl 150 mM, 0.02%

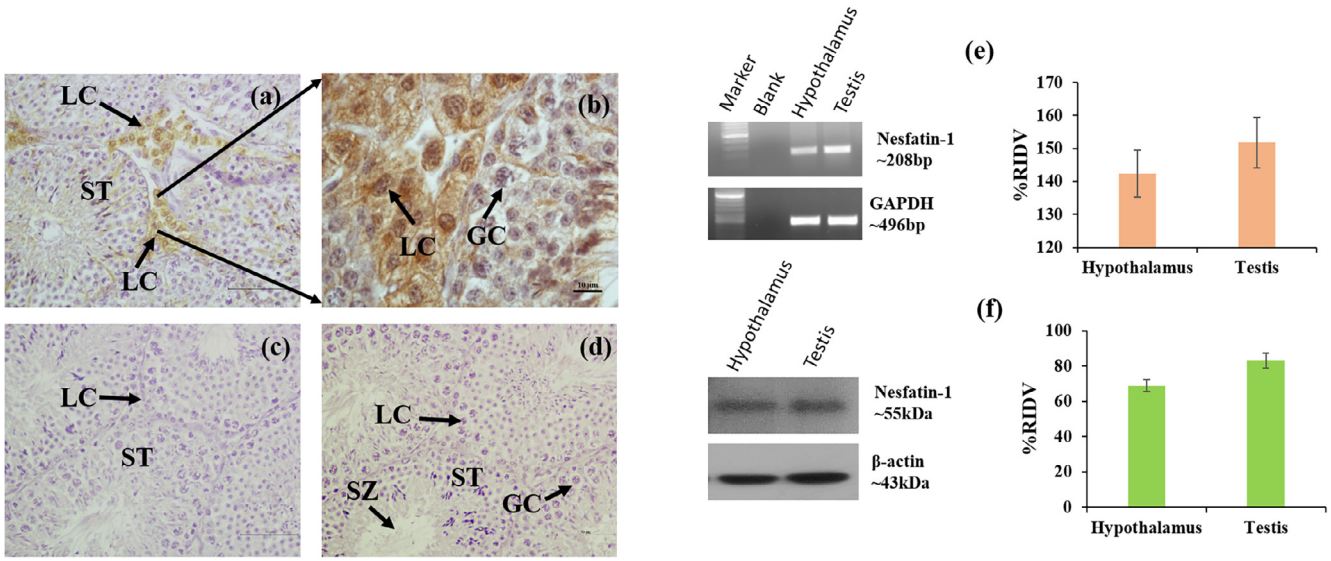

Fig. 1. (a) Immunolocalization of nesfatin-1 in the adult mice testis section. The nesfatin-1 showed positive immunoreactivity in the Leydig cells (LC) (black arrowhead). (b) The high magnification (100×) of the stained Leydig cells with nesfatin-1. (c) The negative control for nesfatin-1. (d) The preabsorbed control with nesfatin-1. Scale bars, 50 µm. (e) Representative expression and densitometric analysis of nesfatin-1 mRNA in mice hypothalamus and testis. (f) Immunoblot of nesfatin-1 protein in the mice hypothalamus and testis. %RIDV, percentage relative integrated density value. Values are denoted as mean ± SEM.

Tween 20) containing 5% fat-free dry milk and incubated with primary antibodies (details in Table 2) for 3 h at room temperature. Washing was done in three changes of PBS-Tween-20 and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Bangalore Genei, Bangalore, India) (1:4000) for 2 h at room temperature. Washing was performed in three changes of PBS-Tween-20. Immunodetection was performed using enhanced chemiluminescence (ECL) detection system (BioRad, Hercules, CA, USA). Each experiment was repeated three times. Densitometry of the band was done by scanning and analyzing using computer-assisted image analysis (Image J 1.38×, NIH, USA). The normalization of the intensity of protein bands measured by densitometry was done with β-actin (Sigma Aldrich, St. Louis, MO, USA) bands. All the data were presented as the mean of the integrated density value ± S.E.M.

2.7. ELISA for steroid measurement

The T and estradiol (E2) in culture media was measured using human ELISA kit purchased from Dia Metra, (Giustozzi, Foligno (PG) Italy; LOT No: DKO002/DKO003 respectively) in accordance with the manufacturer's protocol. Both of these kits were validated in our laboratory for the use in mice (Banerjee et al., 2012). Briefly, 25 µl of standard or samples were added in each well separately following the addition of enzyme conjugate in each well. The ELISA plate was kept for incubation at room temperature for 1 h under mild shaking. The wells were then washed with wash solution and incubated at room temperature for 15 min with 100 µl tetramethyl benzidine chromogen substrate. Finally, reaction was stopped using 100 µl of stop solution (0.2 M sulphuric acid) added to each well. Absorbance was determined at 450 nm using a microplate reader. For T assay, the standard curve ranging from 0.00 to 2000.00 pg/ml and samples of unknown concentrations were run within the narrow range representing the most linear part of the standard curve. The intra-assay coefficient of variation for T was less than 6.0%. For E2 assay, the standard curve ranging from 0 to 16 ng/ml and samples of unknown concentrations were run within the narrow range representing the most linear part of the standard curve. The intra-assay coefficient of variation for E2 was less than 8.0%.

2.8. Glucose estimation

Testicular glucose was measured by commercially available kit (Span Diagnostic Ltd, Mumbai, India) by quantitative colorimetric method as described by the manufacturer's instruction with minor modifications. Testicular homogenate 10% (w/v) was prepared in 6 N perchloric acid (PCA) under ice bath and was centrifuged at 10,000 rpm for 15 min. The supernatant collected was used for the glucose estimation. Briefly, 10 µl of supernatant was added with 1 ml of working glucose reagent (prepared by mixing 1 vial of glucose reagent with diluent) in a tube. The tube was incubated at 37 °C for 10 min. The absorbance was measured at 505 nm and result was calculated.

2.9. Testicular lactate dehydrogenase (LDH) assay

Testicular 10% (w/v) homogenate prepared in PBS (0.1 M, pH 7.6) was centrifuged at 7000 rpm at 4 °C. The supernatant collected was used for the LDH assay using the commercial available kit (LDH (P-L) Kit, Coral Clinical Systems, Goa, India) according to manufacturer's instructions. Briefly, 10 µl of sample (supernatant) and 1 ml of working reagent (prepared by mixing 4 parts of buffer reagent and 1 part of starter reagent) were mixed well in a test-tube. Initial absorbance (A_0) was recorded after 1 min at 340 nm and repeated the absorbance reading after every 1, 2 and, 3 min. The mean absorbance change (ΔA/

Table 1
List of forward (F) and reverse (R) primers based on NUCB-2 mRNA sequences used for RT-PCR analysis.

Name	Sequence	Tm (°C)	Gene	Amplicon size
r*Nef* F	5′-GAGGAGATAAGGAGCGGGAGGC-3′	61.1	NUCB-2	208b.p.
r*Nef* R	5′-ATGTGTCAGGATTCTGGTGGTTCA-3′	58.3	NUCB-2	
GAPDH F	5′-ACCACAGTCCATGCCATCAC-3′	59.4	GAPDH	496b.p.
GAPDH R	5′-TCCACCACCCTGTTGCTGTA-3′	59.4	GAPDH	

Table 2
Details of the antibodies used for Immunohistochemistry and Immunoblotting experiments.

S.No.	Antibody	Species raised in; Monoclonal/Polyclonal	Source	Sequence information	Cat. No.	Dilution (used for Western blot)
1	Nesfatin-1	Rabbit; Polyclonal	Sigma-Aldrich Co. LLC., USA	Gene ID 59,295	N9414	1:500 1:250 (IHC)
2	AMPK	Rabbit; Polyclonal	Genscript	Synthetic peptide TSPPDSFLDDHHLTR conjugated to KLH	A00893-40	1:1000
3	IR	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt P06213 - INSR_HUMAN	sc-711	1:1000
4	AKT	Rabbit; Polyclonal	Genscript	KLH conjugated human Akt (Q-F-S^{473}-Y-S)	A00959-SZ	1:500
5	pAKT	Rabbit; Polyclonal	Genscript	Human Akt (Q-F-S^{p}-Y-S)	A00965-SZ	1:300
6	GLUT8	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt Q9NY64 - GTR8_HUMAN	sc-30108	1:500
7	pERK1/2	Rabbit; Polyclonal	Genscript	Human ERK 1 (Swiss prot: P27361) and ERk 2 (Swiss prot: P28482)	A01386-SZ	1:500
8	PCNA	Rabbit; Polyclonal	Thermo Fisher Scientific Inc.	Recombinant human PCNA protein (Human P12004).	PA1-38424	1:1600
9	Bcl2	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt P10415 - BCL2_HUMAN	sc-492	1:1000
10	Caspase-3	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt P42574 - CASP3_HUMAN	sc-7148	1:250
11	STAR	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt P49675 - STAR_HUMAN	sc-25806	1:1600
12	3β-HSD	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	amino acids 231–373 mapping at the C-terminus of 3β-HSD of human origin.	sc-28206	1:500
13	LH-R	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt P22888 - LSHR_HUMAN	sc-25828	1:500
14	AR	Rabbit; Polyclonal	Sigma-Aldrich Co. LLC., USA	Human AR (367)	A 9853	1:1000
15	P450 SCC	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt P05108 - CP11A_HUMAN	sc-292456	1:500
16	17β-HSD	Rabbit; Polyclonal	Sigma-Aldrich Co. LLC., USA	APREST75491.	HPA021311	1:1000
17	Aromatase	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	UniProt P11511 - CP19A_HUMAN	sc-30086	1:1000

minute) and LDH activity was calculated using the formula: LDH Activity in U/L at 25 °C = ΔA/minute*3333.

2.10. Determination of anti-oxidant enzymes activities

2.10.1. Homogenate preparation

In order to find out the nesfatin-1-induced changes in oxidative stress in the testis, anti-oxidant enzymes (superoxide dismutase, SOD; catalase, CAT; Glutathione peroxidase, GPx) and Lipid peroxidation (LPO) was estimated. The 10% homogenate of testicular samples were prepared in ice-cold PBS (0.1 M, pH 7.6) and centrifuged at 12000*g* for 30 min at 4 °C. The supernatant was collected and used for the estimations. Protein content was estimated by Folin's method (Lowry et al., 1951).

2.10.2. Super-oxide dismutase activity assay

The SOD activity in the testis was measured by Das et al., (2000). The 100 µl of supernatant was treated with 1.4 ml of reaction mixture (1.11 ml phosphate buffer + 75 µl α-methionine + 40 µl triton-X + 75 µl 100 mM hydroxylamine hydrochloride + 100 µl EDTA). The mixture was incubated for 5 min at 37 °C. The reaction mixture was added with 80 µl of 100 µM riboflavin and incubated in an illuminated light box for 10 min. The reaction was stopped by addition of 1.0 ml Greiss reagent (1.0% nepthylethyenediamines + 1.0% sulphanilamide in 5.0% orthophosphoric acid, ratio 1:1). Blank test tube contains only distilled water, while in control test tube sample was replaced by distilled water. Optical density measurement was noted against blank at 543 nm. The activity of the enzyme was expressed in units per milligram protein.

2.10.3. Catalase activity assay

Catalase activity was determined according to Aebi (1974). The ten times diluted supernatant mixed well with 10 µl of absolute ethanol and incubation on ice for 30 min. This reaction mixture prepared was mixed with 50 µl of Triton X-100 to the final volume 500 µl. Then, 100 µl from the above mixture was mixed along with 2.8 ml of 50 mM PBS. The absorbance was measured at 240 nm with and without the addition of the substrate (6 mM of H_2O_2) using spectrophotometer. The enzyme activity was expressed in nano-Kat per milligram protein.

2.10.4. Lipid peroxidation assay by TBARS level estimation

Lipid peroxidation was estimated by the method of Ohkawa et al. (1979). In brief, testicular supernatant (0.2 ml) was mixed with 3.2 ml of Thiobarbituric acid (TBA) reagent containing (0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.8% TBA. This mixture was incubated for 60 min in hot water bath (95 °C). After cooling, the mixture was centrifuged at 500*g* for 10 min at 4 °C. The supernatant collected after centrifugation contained malonaldehyde (MDA)-TBA as by-product. Absorbance of this product was taken at 534 nm using spectrophotometer against a blank.

2.10.5. Estimation of glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity was measured using the protocol described by Mantha et al. (1993). Briefly, 50 µl of testicular homogenate was added to the reaction mixture containing 398 µl of phosphate buffer (pH 7.0, 50 mM), 2 µl of 1 mM EDTA, 10 µl of 1 mM sodium azide, 500 µl of 0.5 mM NADPH, 40 µl of 0.2 mM GSH and 1U glutathione reductase. Following incubated for 1 min at room temperature, the reaction was initiated by addition of 100 mM H_2O_2. The kinetic optical measurement was recorded at 340 nm for 3 min. The enzyme activity was expressed as nmol of NADPH oxidized/min/mg of protein. The extinction coefficient for NADPH was 6.22 mM^{-1} cm^{-1}.

2.10.6. Total testicular nitrate estimation

Total nitrate estimation was performed as previously described by Miranda et al. (2001) with minor modifications. Briefly, testicular

homogenate (10% w/v) prepared in phosphate buffer (0.1 M pH 7.6) was centrifuged at 7000 rpm for 15 min at 4 °C. The protein precipitation of the supernatant was done using ethanol and this mixture was kept on ice for 10–15 min and thereafter centrifuged at 7000 rpm for 15 min at 4 °C. The supernatant obtained was used for the assay. Standard of varying concentrations of 20 µM, 40 µM, 60 µM, 80 µM and 100 µM respectively was prepared with potassium nitrate. In a 96 well microtiter plate, 100 µl sample supernatant, 100 µl vanadium trichloride and 100 µl freshly prepared Greiss (1% sulphanilamide + 0.1% N-(1-Naphtyl) ethylenediamine hydrochloride (NEDD) reagent was added and color was allowed to develop for 45 min in dark and then optical density was read at 545 nm.

2.10.7. Statistical analysis

The densitometric data were presented as the mean of percentage related integrated density value (IRDV) ± SEM. The significance of difference between the groups were analyzed by one way ANOVA followed by Duncan's multiple range post hoc test using SPSS software 12 for Windows (SPSS Inc., IBM, Chicago, IL, USA) to compare the data from different groups. The correlation study was performed using SPSS software 12 to analyze how the changes in various parameters correlated with each other in control and nesfatin-1 treated group. The P value less than 0.05 were considered statistically significant.

3. Results

3.1. Immunolocalization and expression of nesfatin-1 protein and mRNA in the testes of adult mice

Immunohistochemical study showed positive immunostaining of nesfatin-1 in Leydig cells in the testis of adult mice (Fig. 1a & b). The negative and preabsorbed controls for nesfatin-1 are shown in (Fig. 1c & d). The expression of nesfatin-1 mRNA in the testis of mice was determined by RT-PCR and result showed amplification of specific DNA fragment at 208 bp (Fig. 1e). The immunoblot analysis of nesfatin-1 protein in the testes showed immunoreactive band at ~55 kDa (Fig. 1f). The mice hypothalamus was used as a positive control which showed the presence of nesfatin-1 mRNA and protein respectively.

3.2. Effect of in vitro treatment of nesfatin-1 on production of steroid hormones and expression of steroidogenic markers in the testes of adult mice

The testes treated *in vitro* with two different doses of nesfatin-1 showed significant ($p < 0.05$) increase in the synthesis of T, whereas significantly ($p < 0.05$) decline in E2 synthesis as compared with the control (Fig. 2). The effect of two different doses of nesfatin-1 on changes in the testicular expression of various steroidogenic markers, luteinizing hormone-receptor (LH-R), Steroid acute regulatory protein (StAR), p450 side chain cleavage enzyme (p450-scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase, were estimated by densitometric analysis of their Western blots and results are shown in Fig. 3. The results showed significant ($p < 0.05$) increase in the expression of LH-R, StAR, P450-scc, 3β-HSD and 17β-HSD proteins, but showed decreased expression of aromatase enzyme as compared with the control. The Western blot of LH-R, StAR, P450-SCC, 3β-HSD, 17β-HSD, and aromatase proteins showed a single immunoreactivity band of ~85, ~29, ~65, ~42, ~79, and ~58 kDa respectively.

3.3. Effect of in vitro treatment of nesfatin-1 on spermatogenic markers in the testes of adult mice

To study the effect of *in vitro* treatment of nesfatin-1 on spermatogenesis, changes in the rate of cell survival (expression of Bcl2), proliferation (expression of PCNA), apoptosis (expression of caspase-3) and expression of AR proteins were evaluated in the testes of mice. The changes in rate of expression of these spermatogenic markers were studied by Western blot followed by densitometric analysis. The results showed dose-dependent significant ($P < 0.05$) increase in the expression of Bcl2, PCNA and AR proteins, whereas significant ($P < 0.05$) decrease in the expression of caspase-3 protein as compared with the control (Fig. 4). The Western blot of PCNA, Bcl2, Caspase 3 and AR showed a single immunoreactivity band of ~29, ~29, ~29 and ~110 kDa respectively.

3.4. Effect of in vitro treatment of nesfatin-1 on the metabolic markers in the testes of adult mice

To study the effect of *in vitro* treatment of nesfatin-1 on metabolic activity, changes in the rate of expression of insulin receptor (IR) and glucose transporter 8 (GLUT8) proteins and intra-testicular concentration of glucose and LDH activity were evaluated in the testis of mice. The results showed significant ($P < 0.05$) increase in the expression of insulin receptor and GLUT8 proteins and in intra-testicular glucose concentration and LDH activity as compared with the control (Fig. 5).

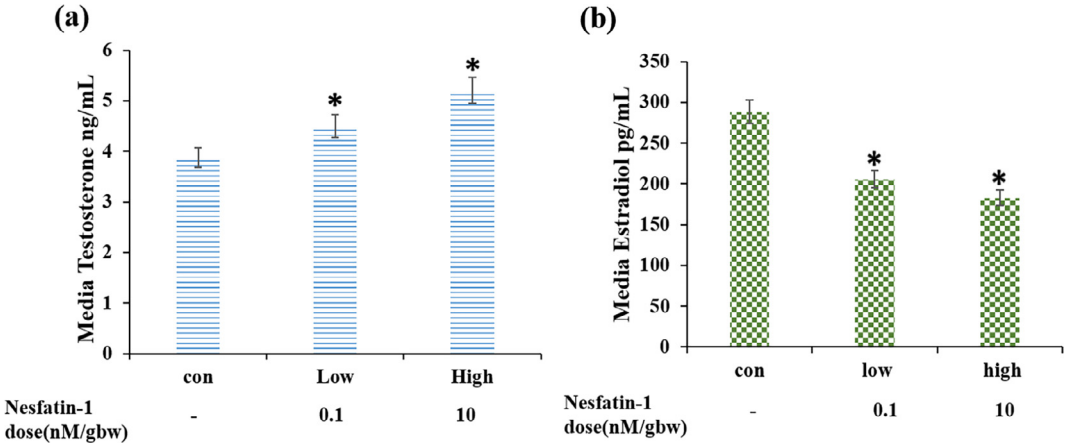

Fig. 2. Changes in media testosterone (T) and media estradiol (E2) level in the mice testis after *in vitro* nesfatin-1 treatment with two different doses of nesfatin-1 compared to control group. (a) T level increases significantly ($^{*}p < 0.05$) in both the doses compared with the control. (b) E2 level decreases significantly ($^{*}P < 0.05$) compared with the control. Values are mean ± SEM.

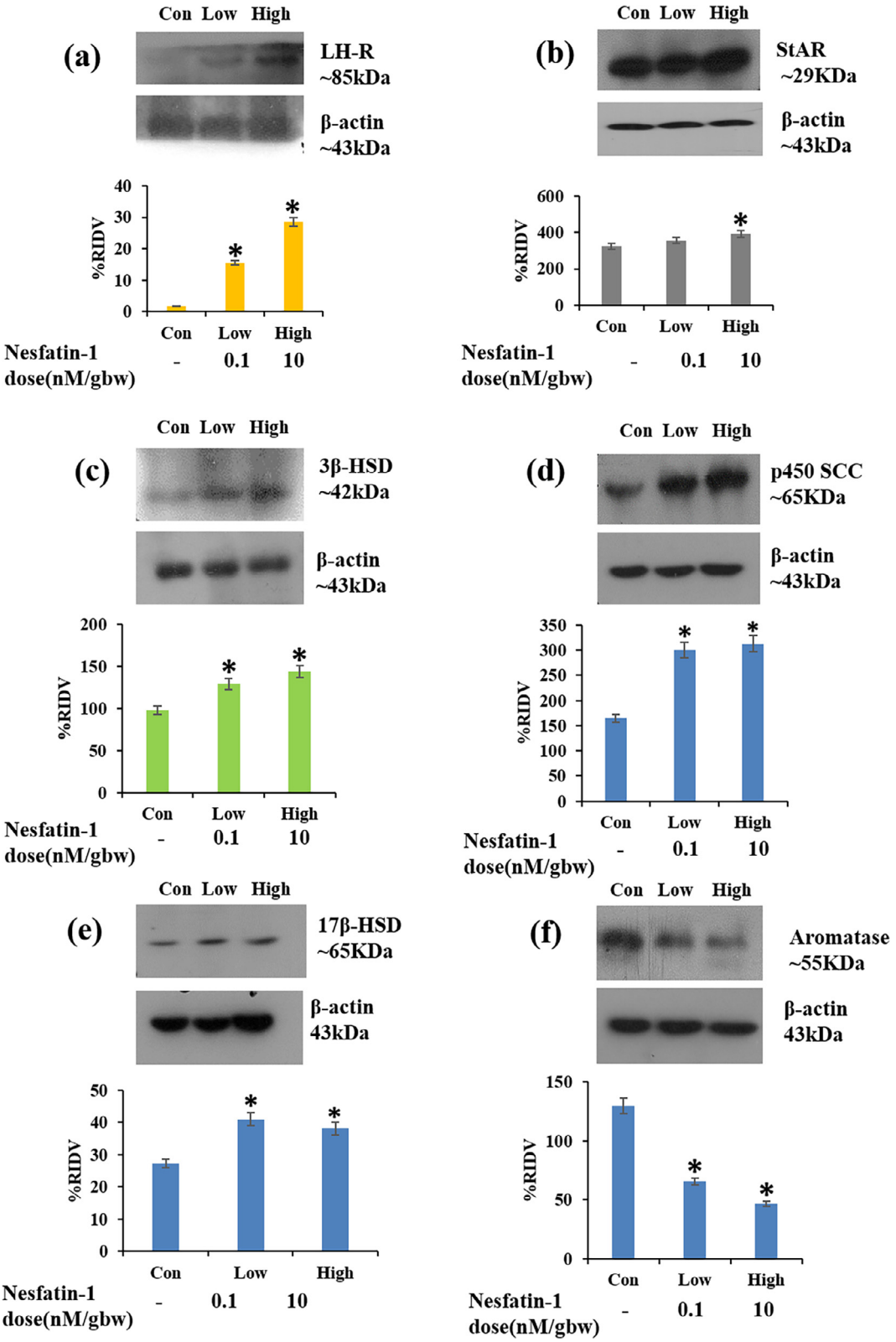

Fig. 3. Western blots and densitometric analysis of steroidogenic markers (a-f) in the mice testis after *in vitro* nesfatin-1 treatment with two different doses compared with the control group. The treatment showed significant ($^{*}p < 0.05$) increased expression of LH-R, StAR, 3β-HSD, p450-scc, and 17β-HSD compared with the control. The *in vitro* treatment with nesfatin-1 showed significant ($^{*}p < 0.05$) decreased expression of aromatase compared with the control. Values shown are mean ± SEM.

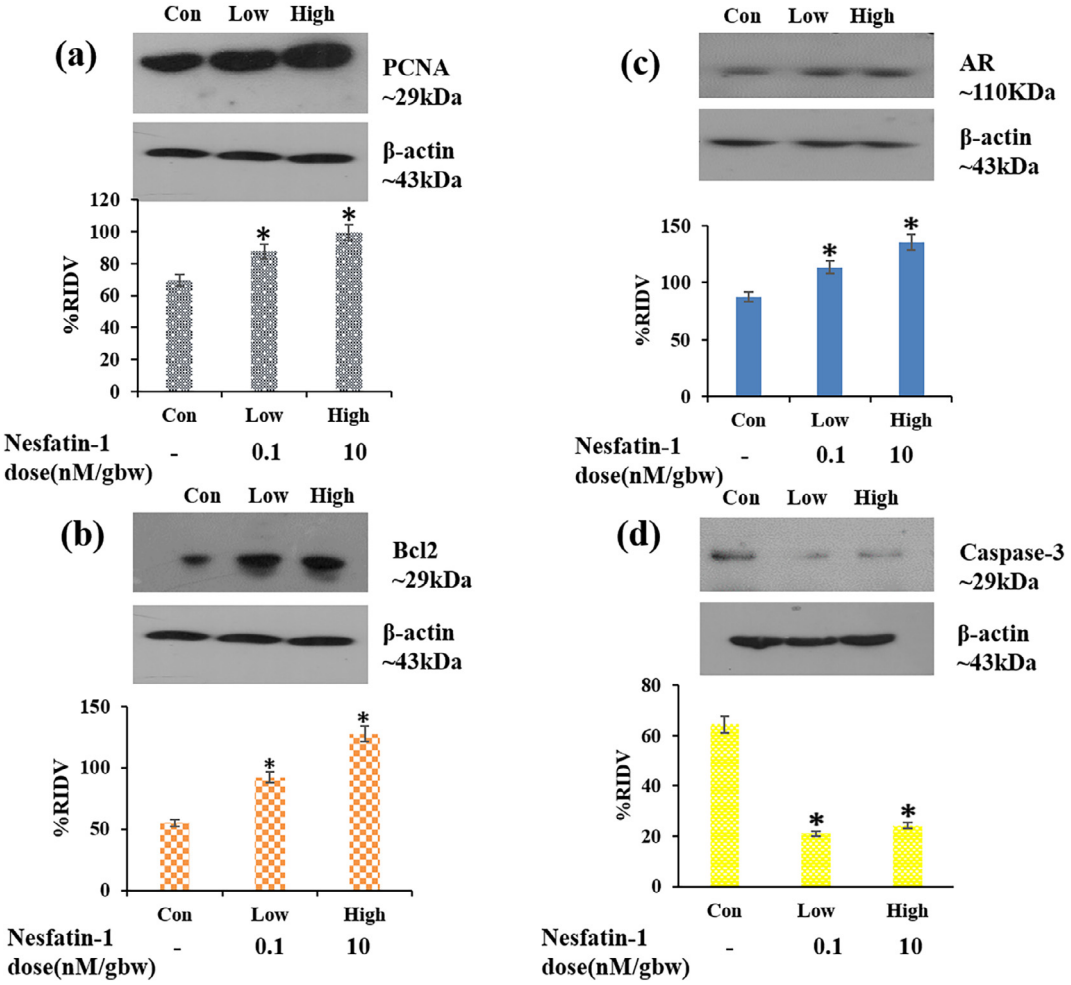

Fig. 4. Effect of *in vitro* treatment of nesfatin-1 on spermatogenic markers (a-d). Western blots and densitometric analysis of (a) PCNA (b) Bcl2, and (c) AR, proteins showed significant ($^{*}p < 0.05$) increased expression in the treated group compares with the control. (d) Caspase-3 proteins expression was decreased significantly ($^{*}p < 0.05$) in the treated group compared with the control. Data shown as mean ± SEM.

3.5. Effect of in vitro treatment of nesfatin-1 on the expression of extracellular signal-regulated kinases (pERK1/2), AMP-activated protein kinase (AMPK) and pAKT/AKT in the testes of adult mice

The testes treated *in vitro* with either low or high doses of nesfatin-1 showed a significant ($P < 0.05$) increase in the expression of ERK1/2, AMPK, and pAKT/AKT proteins as compared with the control (Fig. 6). The Western blot of pERK1/2, AMPK, pAKT, and AKT showed a single immunoreactivity band at ~42/44, ~62, ~57, and ~57 kDa respectively.

3.6. Effect of in vitro treatment of nesfatin-1 on the oxidative stress parameters in the testes of adult mice

The testes treated *in vitro* with two doses of nesfatin-1showed significantly ($P < 0.05$) increase in the concentration of testicular super oxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) enzyme activities, whereas, significantly ($P < 0.05$) decrease in the lipid peroxidation and nitric oxide levels as compared to the control (Fig. 7).

3.7. Correlation study

A correlation study was undertaken to evaluate the how the changes in steroidogenic, spermatogenic, metabolic and signaling parameters correlated with each other in the control and nesfatin-1 treated mice. A significant positive correlation was found between nesfatin-1 induced testosterone production with various steroidogenic markers, LH-R, StAR, 3β-HSD, and insulin receptor (Table 3a). A significant positive correlation was also found between nesfatin-1 induced changes in testicular expression of PCNA (cell proliferation) with intra-testicular concentration of glucose, GLUT8 and SOD (Table 3b). Furthermore, nesfatin-1-induced expression of insulin receptor significantly correlated with nesfatin-1-induced expressions of pAKT/AKT ratio and AMPK/ERK1/2 in the testes (Table 3c).

4. Discussion

This study was undertaken to provide the important insight into reproductive performances of nesfatin-1 on the testes of adult mice. This study corroborates with the earlier findings showing the presence of both mRNA and protein of nesfatin-1 in the testes of human, rat and mouse (Garcia-Galiano et al., 2012). Similar to other neuroendocrine regulators of energy balance, such as ghrelin, resistin, adiponectin and orexin (Tena-Sempere et al., 2002; Nogueiras, 2004; Caminos et al., 2008; Singh et al., 2018; Joshi and Singh, 2017), nesfatin-1 was expressed in the Leydig cells of mice testes. Based on these findings it is presumed that nesfatin-1 may be involved in energy equilibrium together with local paracrine/autocrine action on other testicular activities.

The testes treated *in vitro* with the two different doses of nesfatin-1 showed significant increase in the production of T, whereas significant

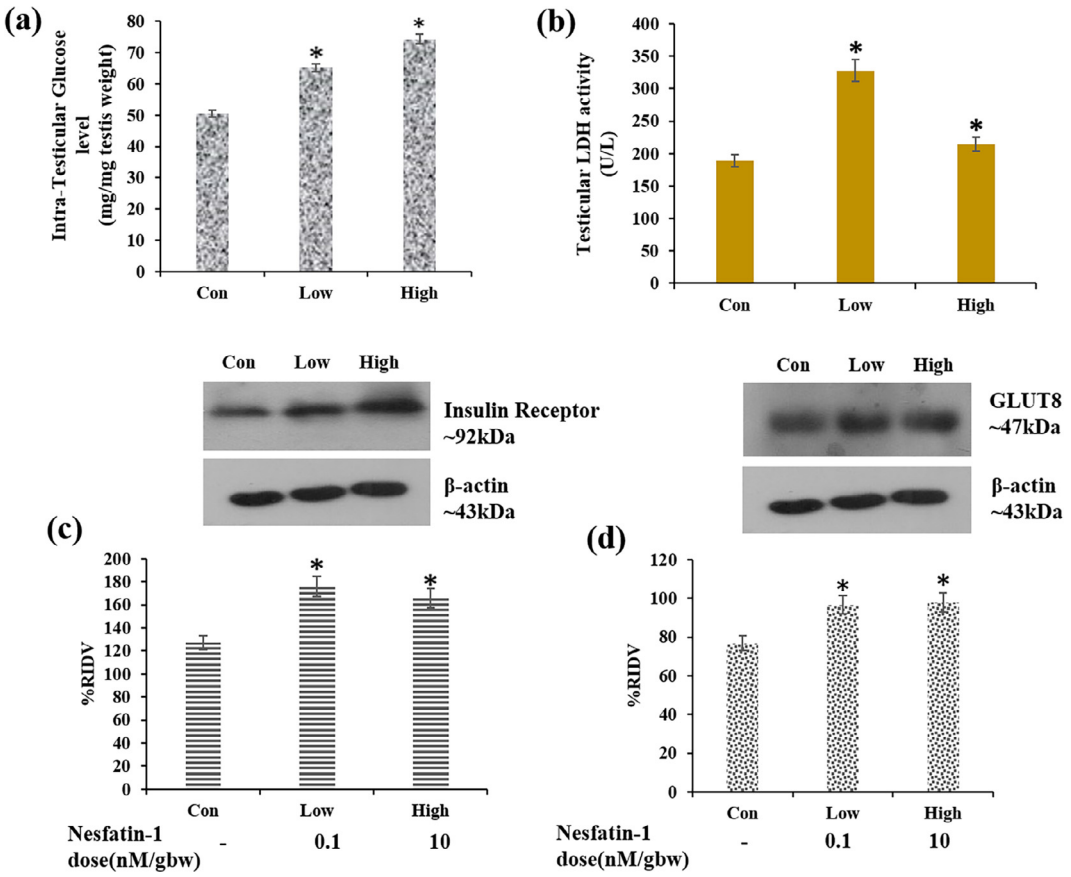

Fig. 5. Effect of *in vitro* treatment of nesfatin-1 on metabolic markers. (a) Changes in the intra-testicular glucose level and (b) testicular lactate dehydrogenase (LDH) activity. The intra-testicular glucose and LDH activity was increased significantly ($^{*}p < 0.05$) compared with the control. Representative Western blots and densitometric analysis of (c) Insulin receptor, and (d) GLUT 8 proteins in the mice testis showed significant ($^{*}p < 0.05$) increased expression compared with the control. Values are represented as mean ± SEM.

decline in the synthesis of E2 as compared with the control. This reciprocal effects may be due to the stimulatory effect of nesfatin-1 on testicular expression of LH-R, StAR, SCC, 3β-HSD and 17β-HSD proteins and the suppressive effect on the expression of aromatase as compared with the control. This finding thus suggests direct stimulatory action of nesfatin-1 on the expression of steroidogenic enzymes and LH-R in the testis of mice.

Further in this study, the testes treated *in vitro* with nesfatin-1 showed a significant increase in the expression of insulin receptor protein. This is in accordance with the earlier finding showing increased insulin sensitivity in the brain upon treatment with nesfatin-1 in the rat (Yang et al., 2012). Insulin receptor in the testes is mainly demonstrated in Leydig cells and Leydig cells culture treated with insulin was shown to stimulate T synthesis (Lin et al., 1986). Thus, it appears that nesfatin-1 stimulates T production by up-regulating insulin receptor in the testes. This hypothesis is further substantiated in this study by the observation of significant positive correlation between increased productions of T together with increased expression of insulin receptor protein in the testes treated with nesfatin-1. Further in this study, the testes treated *in vitro* with nesfatin-1 showed a significant increase in the expression of insulin receptor and GLUT8 proteins simultaneously with an increase in intra-testicular level of glucose. This observation is in accordance with our previous finding suggesting essential role of insulin in promoting transport of glucose to the testis which profoundly affect testicular activities. This observation is supported by the earlier findings that the glucose transport and other metabolic intermediates are key player in regulating testicular activities (Doege et al., 2000; Alves et al., 2013). Based on these observations, it is thus hypothesized that nesfatin-1 induced increase in synthesis of T might be due to insulin-mediated increased transport of glucose in the testes. The lower testicular concentration of glucose leads to low rate of T synthesis from Leydig cells (Banerjee et al., 2014), and increased glucose availability enhances the concentration of the steroidogenic enzyme, nicotinamide adenine dinucleotide phosphate (NADPH), in the testis (Bajpai et al., 1998).

The testes treated *in vitro* with two different doses of nesfatin-1, even in the absence of gonadotropin, showed a dose-dependent significant increase in expression of PCNA and Bcl2 while decrease in expression of caspase-3 as compared to the control. Further in this study, nesfatin-1 induced increased expression of PCNA correlated with the increased expression of GLUT8 ($r = 0.83^{*}$; $p < 0.05$) and level of glucose ($r = 0.91^{*}$; $p < 0.05$) in the testes. The present study together with the previous studies thus suggest that nesfatin-1 augment the transport of glucose in the testis which in turn may be responsible for increased cell proliferation and subsequently increased rate of spermatogenesis in the testis. The stimulatory effect of nesfatin-1 on spermatogenesis was further supported by a recent report, where steroidogenically active testes of adult rat showed several fold higher immunostaining of nesfatin-1 as compared with the steroidogenically less-active testes of pre-pubertal rat (Garcia-Galiano et al., 2012). However, the mechanism by which nesfatin-1 modulates spermatogenesis remains to be further investigated.

Spermatogenesis requires surplus amount of energy and that is fulfilled by glucose as primary energy source, thus its uptake is crucial for cellular metabolism, survival, and proliferation (Banerjee et al., 2014). Previous studies have demonstrated the importance of nesfatin-1 on energy balance in various peripheral tissues in mammals (Xun et al., 2013, Ayada et al., 2015; Shimizu et al., 2009; Stengel et al., 2009). The

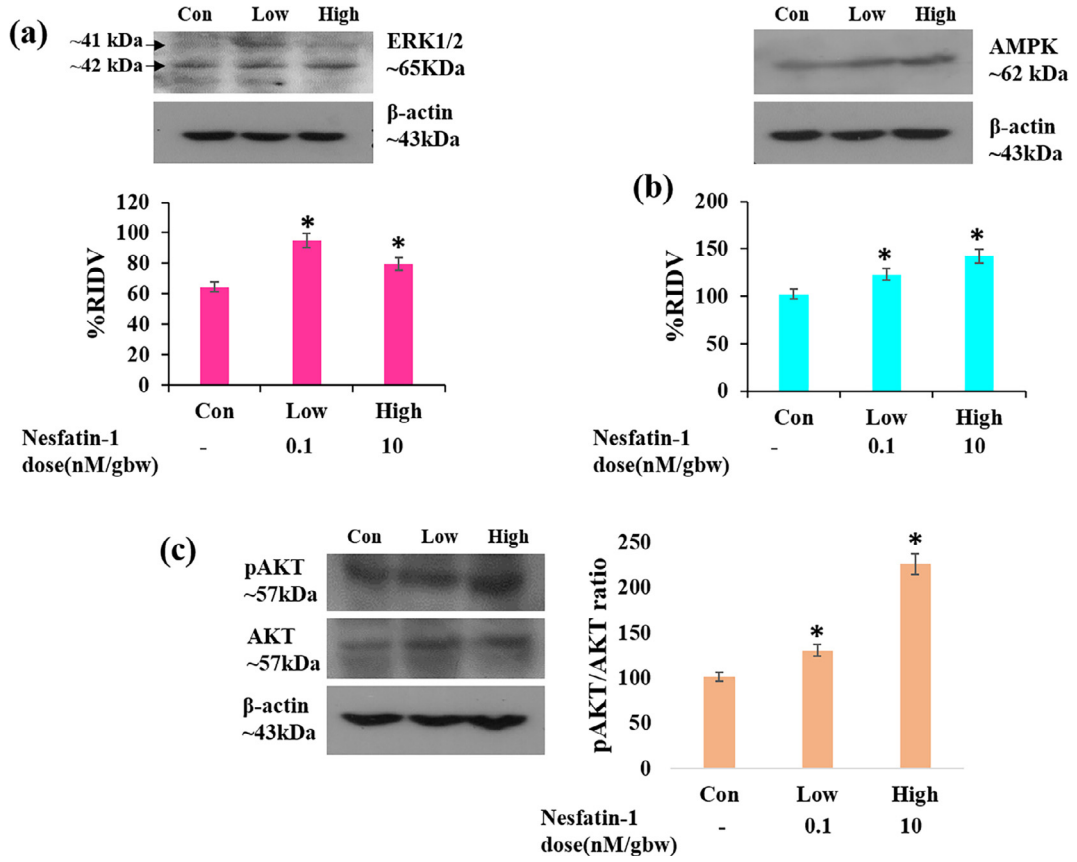

Fig. 6. Western blots and densitometric analysis of (a) pERK1/2, (b) AMPK, and (c) pAKT/AKT proteins after *in vitro* nesfatin-1 treatment with two different doses compared to control group. Values are represented as mean ± SEM. Value (*) is considered significant if ($P < 0.05$) in both the doses versus control.

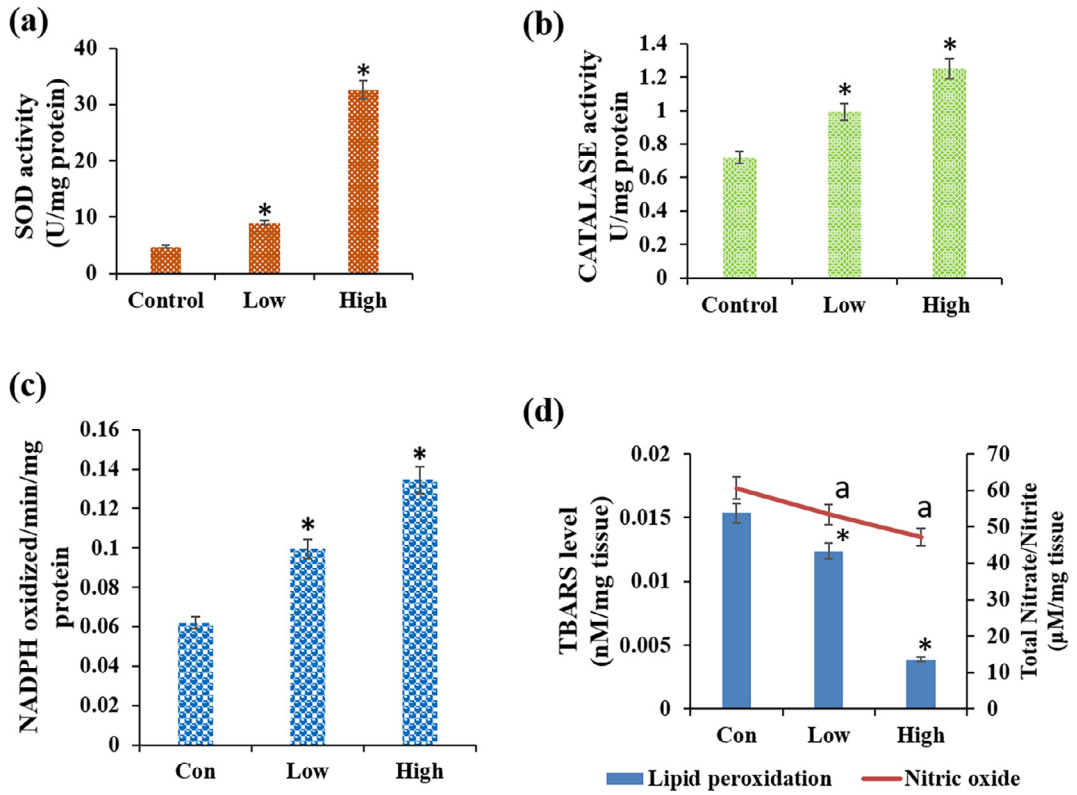

Fig. 7. Changes in the activities of testicular antioxidant enzymes (a) SOD, (b) Catalase, (c) GPx (d) lipid peroxidation (LPO), and total Nitrate/Nitrite after *in vitro* nesfatin-1 treatment with two different doses compared to control group. Values are represented as mean ± SEM. Value (*) & (a) is significantly different ($P < 0.05$) in both the doses versus control.

Table 3a
Correlation study. (a) Correlation of T with LHR, StAR, 3β-HSD, 17β-HSD, IR expression.

Parameters	LHR	StAR	3β-HSD	IR
T	r = 0.96*; p < 0.01	r = 0.84*; p < 0.05	r = 0.79*; p < 0.05	r = 0.85*; p < 0.05

* Values are significantly different at $p < 0.05$ and $p < 0.01$ level. r is Pearson's correlation coefficient.

present study clearly showed the involvement of nesfatin-1 in increased transport of glucose by insulin-mediated up-regulation of GLUT8 expression in the testis. This finding is consistent with the recent study that nesfatin-1 alters glucose metabolism by increasing insulin sensitivity and translocation of GLUTs in skeletal muscle, adipose tissue, and liver (Li et al., 2013). The GLUT8 transports glucose to Sertoli cells (Banerjee et al., 2014), where LDH enzyme catalyzes the conversion of glucose to lactate (Rato et al., 2012). Further in this study, the testes treated with nesfatin-1 showed significantly increased LDH enzyme activity. This suggests the involvement of nesfatin-1 in increasing production of lactate by up-regulating expression of LDH enzyme. Lactate produced by Sertoli cells provides energy to proliferating pachytene spermatocytes during spermatogenesis (Jutte et al., 1982). It is well known that the glucose to lactate conversion is critical for normal spermatogenesis (Alves et al., 2013) and is regulated by insulin dependent LDH enzyme synthesis in the Sertoli cells (Mita et al., 1982; Rato et al., 2012; Alves et al., 2013), whereas insulin deficiency causes decreased lactate synthesis by suppressing LDH enzyme in Sertoli cells (Blackshaw, 1969). The present study has demonstrated the significant increase in expression of insulin receptor protein with simultaneous increase in LDH enzyme activity. Thus it can be deduced from the above finding that nesfatin-1 regulates lactate production via insulin-mediated increased synthesis of LDH enzyme in the testis.

Both spermatogenesis and steroidogenesis are vulnerable to oxidative stress (Chandra et al., 2009). The apoptotic effect on testicular cells as a result of oxidative imbalance can be protected by anti-oxidant enzymes (Aitken and Roman, 2008). This study has clearly shown the role of nesfatin-1 in mitigation of testicular oxidative stress. The treatment with nesfatin-1 showed dose-dependent significant increase in the antioxidant enzymes (SOD, catalase, and GPx) activity, whereas significantly decrease in TBARS level (lipid peroxidation). This increase changes in anti-oxidant enzymes showed significant correlation with the changes in the expression of PCNA and Bcl2 proteins in the testis (Table 3b). These results support the previous finding, where increased Bcl2 expression protect cells against apoptosis by enhancing anti-oxidant enzyme activities (Hockenbery et al., 1993). The increased nitric oxide production may lead to tissue injury and cell death (Murphy, 1999). The *in vitro* treatment with nesfatin-1 showed significant decline in testicular nitric oxide. The elevated serum nitric oxide was found to be associated with regressive changes in testicular activity such as during aging (Banerjee et al., 2012). Thus, the result of this study suggested important role of nesfatin-1 in protecting testicular functions from anti-oxidative and anti-nitric oxide effects.

Since putative nesfatin-1 receptor is still not known, mechanism by which this neuropeptide induces these changes in the testes is not well documented. This study showed that nesfatin-1 increases expression of insulin receptor protein, which in turn may increase transport of glucose required for active cell proliferation in the testis spermatogenesis. To find out the likely signaling pathways of nesfatin-1 induced increased uptake of glucose, AKT/AMPK levels were evaluated in the testis. The testes treated *in vitro* with two different doses of nesfatin-1 showed significant increase in the pAKT/AKT ratio simultaneously with the increased expression of GLUT8 and insulin receptor (0.82*; $p < 0.05$) proteins. This finding corroborates with recent study that showed activation of AKT by nesfatin-1 (Yang et al., 2012). Based on this finding and together with earlier reports, it is likely that AKT may be the signaling mechanism for nesfatin-1 induced increased glucose trafficking (Huang et al., 2007). It was confirmed previously that AKT mediate cell survival by suppressing positive regulator of apoptotic cell death (Hers et al., 2011). Thus, increased expression of pAKT protein appears to be a pro-survival signal during spermatogenesis. Simultaneously with the increased expression of insulin receptor, the testis treated *in vitro* with the nesfatin-1 also showed significant increase in AMPK/ERK1/2 proteins. This study further showed a significant positive correlation between nesfatin-1 induced increased expression of insulin receptor and expression of AMPK/ERK1/2 in the testes ($r = 0.87$*; $p < 0.05$ and $r = 0.81$*; $p < 0.05$ respectively) (Table 3c). Thus it can be concluded that activation of AMPK/ERK1/2 system induced via nesfatin-1 could lead to insulin sensitivity in the testis. This possible mechanism needs to be confirmed by blocking these signaling molecule following treatment with nesfatin-1 and assessing physiological response.

In brief, the testes treated with nesfatin-1 showed a significant increase in testosterone production, which showed significant correlation with increased expression of steroidogenic enzymes and insulin receptor protein. This study also showed the direct stimulatory effect of nesfatin-1 on spermatogenic markers like proliferation (PCNA) and survival (Bcl2) and by suppressing cell apoptosis (caspase-3). The testes treated *in vitro* with nesfatin-1 also showed increased expression of insulin receptor and GLUT8 proteins together with the increased intratesticular level of glucose. This study further showed nesfatin-1 induced increased expression of lactate dehydrogenase enzyme, which may be responsible for increased production of lactate together with increased uptake of glucose by the testis. The results of this study for the first time provide the evidence for an active role of nesfatin-1 in increasing glucose uptake in the testis by up-regulating insulin sensitivity, thereby providing energy for increased spermatogenesis as well as steroidogenesis in testes of adult mice. This study further showed the likely role of AKT as a signaling mechanism for nesfatin-1 induced increased glucose trafficking and activation of AMPK/ERK1/2 system, a plausible mechanism through which nesfatin-1 increases insulin sensitivity in the testis. Summarizing the whole study, the results presented here suggests stimulatory role of nesfatin-1 in the regulation of various testicular functions, such as spermatogenesis and steroidogenesis. Further studies are warranted to determine the changes in testicular level of nesfatin-1 during type II diabetes mellitus, aging and subfertility in men.

Table 3c
Correlation study. (c) Correlation of IR with pAKT/AKT and AMPK/ERK1/2.

Parameters	pAKT/AKT	AMPK	ERK1/2
IR	r = 0.82*; p < 0.05	r = 0.87*; p < 0.05	r = 0.81*; p < 0.05

* Values are significantly different at $p < 0.05$ level. r is Pearson's correlation coefficient.

Table 3b
Correlation study. (b) Correlation of PCNA with GLUT8, intra-testicular glucose, SOD and Bcl2 with SOD.

Parameters	GLUT8	Testicular glucose level	SOD
PCNA	r = 0.83*; p < 0.05	r = 0.91*; p < 0.02	r = 0.84*; p < 0.05
Bcl2	NA	NA	r = 0.81*; p < 0.05

* Values are significantly different at $p < 0.05$ level. r is Pearson's correlation coefficient.

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Conflict of interest

Declared none

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.10.022.

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