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Role of adiponectin as a modulator of testicular function during aging in mice

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ABSTRACT

The mechanisms by which testicular functions decline with aging remain largely speculative. Our recent finding showed the importance of adiponectin in the regulation of testicular functions, whereas its concentration declines during male infertility. Thus, the aim of present study was to explore the potential role of adiponectin during aging. The changes in adiponectin, adiponectin-receptors, and insulin receptor proteins expression in the testis were evaluated and compared with the testicular parameters, mass, and testosterone level in the mice from early post-natal to late senescence period. Further, the current study has examined the effect of exogenous adiponectin treatment on testicular functions in aged mice. The results showed a significant decline in adiponectin/adiponectin-receptors expression simultaneously with a significant decline in testicular mass, insulin receptor expression and testosterone synthesis in the testis of aged mice. Exogenous treatment of adiponectin to aged mice resulted in marked improvements in testicular mass, histological features (cells proliferation), insulin receptor expression, testicular glucose uptake, anti-oxidative enzymes activity and testosterone synthesis as compared with the control. Based on these findings, it may be concluded that a marked decline in adiponectin synthesis and action results in decreased insulin sensitivity (development of insulin resistance) and increased oxidative stress which consequently suppresses testicular functions during aging. This study further showed that treatment with adiponectin ameliorates reduced testicular functions by enhanced expression of insulin receptor in the testis of senescent mice. It is thus hypothesized that systemic adiponectin treatment could be a promising therapeutic strategy for improvement of testosterone production and sperm counts during aging.

1. Introduction

The reproductive system is involved in the propagation of species, but also regulates longevity and metabolism of the organism [1]. Aging not only declines reproductive activities but also increases susceptibility to a number of diseases, such as type 2 diabetes mellitus, dyslipidemia, glucose intolerance, obesity, insulin resistance, hypertension etc. [2]. Thus, understanding the process of testicular aging might help to develop a therapeutic strategy that can delay or avoid age-associated diseases in the male. Several hypotheses have been proposed to explain aging in the male reproductive system. These studies suggest that aging affects both endocrine and metabolic factors of testes together with changes in the sensitivity of the hypothalamic-pituitary axis to a negative feedback regulation by gonadal hormones. Aging-dependent changes in hypothalamic-pituitary axis result in decreased gene expression and secretion of hypothalamic GnRH [3,4]. This, in turn, may be responsible for the decreased release of LH and FSH, which consequently declines gonadal activity in mice [5]. The age-dependent changes in testicular activities include decreased spermatogenesis, increased apoptosis of germ cells, and reduced steroidogenesis. Our earlier study showed a decrease in estrogen synthesis during aging which via increased production of nitric oxide causes decreased steroidogenesis and increased germ cells apoptosis in the testis of aging mice [6].

Further studies suggested an imbalance between increased reactive oxygen species (ROS) levels and insufficient antioxidant defense mechanism as a possible cause for the decreased sperm parameters during

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Table 1
Details of the antibodies used for immunohistochemistry and immunoblotting experiments.

S. No.	Antibody	Species raised in; Monoclonal/polyclonal	Source	Concentration (used for Western blot)
1	Adiponectin	Rabbit; polyclonal	Cell Signaling Technology, Inc. Sigma-Aldrich Co. LLC., USA	1:500 1:50 (IHC)
2	AdipoR1	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:300 1:50 (IHC)
3	AdipoR2	Rabbit; polyclonal	Thermo Fisher Scientific Inc.	1:100 1:25 (IHC)
4	IR	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:1000
5	AMPK	Rabbit; polyclonal	Genscript	1:1000
6	AKT	Rabbit; polyclonal	Genscript	1:500
7	pAKT	Rabbit; polyclonal	Genscript	1:300
8	GLUT8	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
9	MCT2	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
10	MCT4	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
11	pERK1/2	Rabbit; polyclonal	Genscript	1:500
11	PCNA	Rabbit; polyclonal	Thermo Fisher Scientific Inc. Thermo Fisher Scientific Inc.	1:1600 1:200 (IHC)
12	Bcl2	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:1000
13	Caspase-3	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:250
14	LH-receptor	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
15	StAR	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:1600
16	3β-HSD	Rabbit; polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
17	AR	Rabbit; polyclonal	Sigma-Aldrich Co. LLC., USA	1:1000
18	β-actin	Rabbit; monoclonal HRP-tagged	Sigma-Aldrich Co. LLC., USA	1:100000

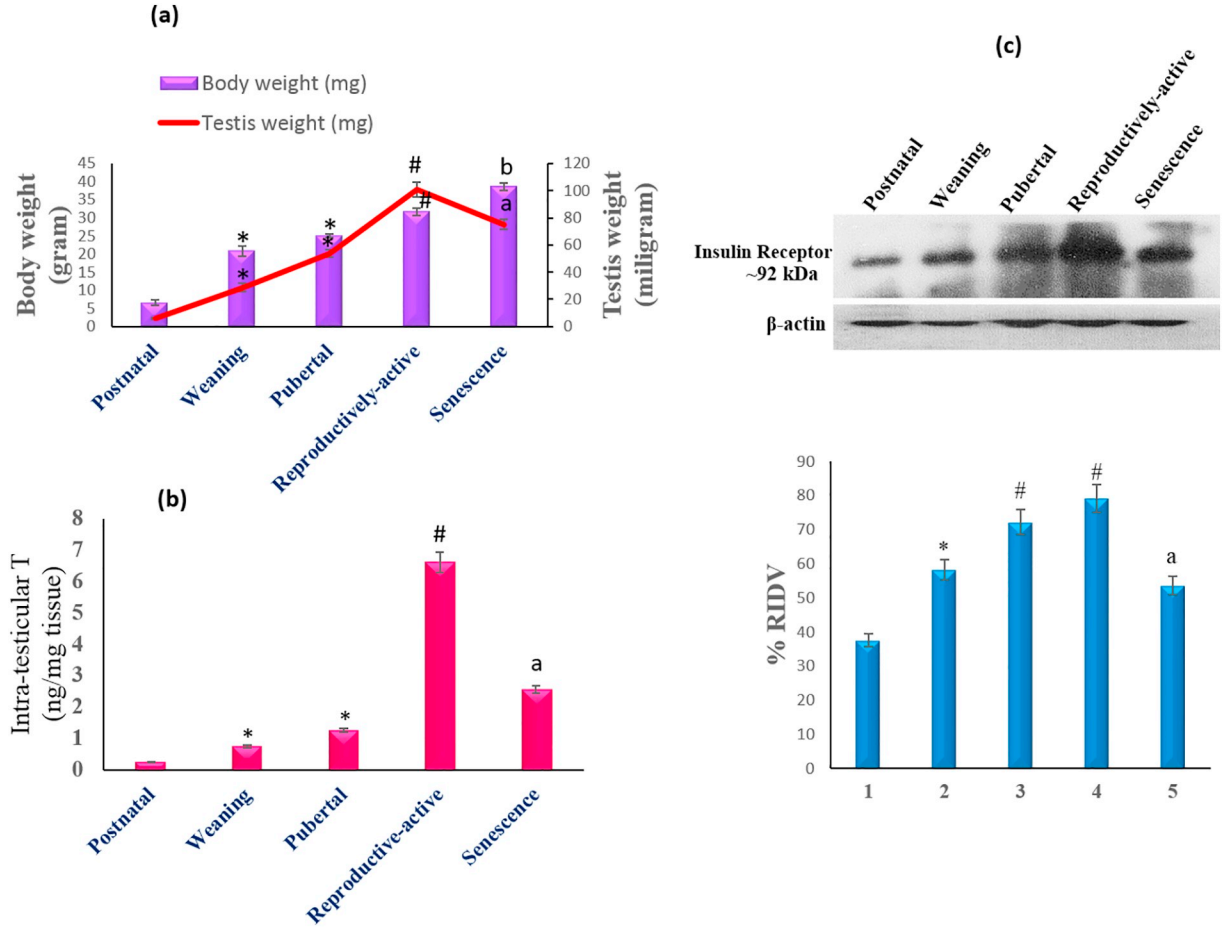

Fig. 1. (**a**) Age-dependent variation in body weight, testis weight, intra-testicular testosterone levels in mice during different stages of aging (Prenatal, Weaning, Pubertal, Reproductively-active, and Senescence). (**b**) Age-dependent variation in intra-testicular testosterone levels in mice during different stages of aging. (**c**) Age-dependent variation in the expression of insulin receptor (IR) protein in mice during different stages of aging. Values are represented as mean ± SEM. * represent values that are significantly ($P < 0.05$) increased in weaning and pubertal period versus postnatal. # represent values that are significantly ($P < 0.05$) increased during reproductively active stages versus postnatal, weaning, pubertal, and senescent mice. (a) represent values that are significantly ($P < 0.05$) decreased in senescence versus reproductively active mice and (b) represent values that significantly ($P < 0.05$) increased in senescence versus reproductively active mice.

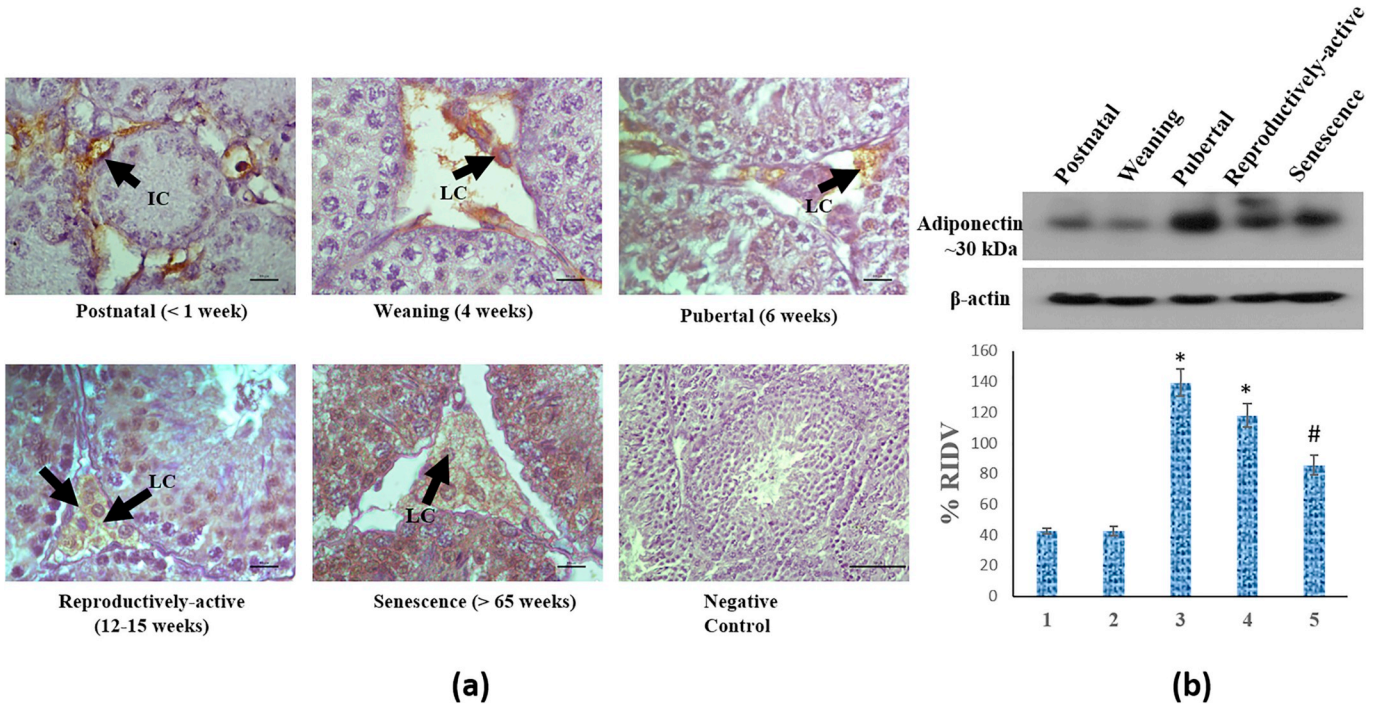

Fig. 2. (a): Immunolocalization of adiponectin in the testis of mice during different stages of maturation (Postnatal, Weaning, Pubertal, Reproductively-active) and aging (Senescence). Strong immunostaining of adiponectin was observed in the Leydig cell (LC) in the pubertal and reproductive active stage, whereas moderate immunostaining during infancy, weaning, and aging period. Black arrowhead showing immunostaining in the Leydig cell (LC). All figures are shown in 100 × magnification. (b): Immunoblot analysis of adiponectin protein in the testis of mice of different age groups. Expression of APN increased in the interstitium during the postnatal to reproductively active stage and decreased during aging condition. * represent values that are significantly ($P < 0.05$) increased during pubertal and reproductively active stages versus postnatal, weaning, and senescence mice. # represent values that are significantly ($P < 0.05$) decreased in senescence stage versus pubertal and reproductively active stages of mice.

aging [7]. Similarly, a recent study showed that the increase in oxidative stress in semen correlates with reduced sperm motility and increased DNA fragmentation of sperm in aging male [8]. Others suggested an impairment in insulin action and secretion due to progressive loss of pancreatic β-cell function because of increase in free fatty acid and glucose during aging [9,10]. It is now well established that the testicular activities are closely linked to glucose (metabolic fuel) availability [11,12]. Our recent study provided convincing evidence that a decline in glucose concentration leads to impairment in testicular steroidogenesis during aging in mice [5]. These findings thus suggest that the regulation of metabolism and energy production plays a crucial role in modulating longevity and cellular senescence. The factor responsible for decreased uptake of glucose by the testes during aging remains to be determined.

Aging promotes the accumulation of adipose tissue simultaneously with the progressive development of insulin resistance, and disruption of endocrine factors, which consequently contributes to metabolic alterations [13]. Recent studies suggested an inverse association between increased adiposity and impaired testicular activity [14,15]. These findings suggest that the gain in body mass observed during aging may be responsible for the decline in testosterone synthesis and release [16]. The obesity factor involved in decreased testicular functions during aging requires further investigation. It is recently observed that the adiposity hormone, adiponectin, is expressed and regulates both spermatogenesis and steroidogenesis in adult testis via its two receptors, AdipoR1 and AdipoR2 [12,17]. Earlier studies showed impairments in insulin action and secretion during aging as indicated by the increased level of free glucose during this period [18]. Another important role of adiponectin is to maintain insulin sensitivity by stimulating glucose uptake in the testes [12]. Some of the earlier studies showed a negative correlation between circulating adiponectin level and obesity, insulin resistance and metabolic disturbances [19]. Based on these observations, it is thus hypothesized that reduced circulating level of adiponectin and increased adiposity may be responsible for regression of testicular activity during aging. Therefore, the primary aim of this study was to evaluate, simultaneously the changes in testicular expression of adiponectin and its receptors (AdipoR1 and AdipoR2) and to compare this with the changes in expression of testicular insulin receptor and testosterone levels from birth to senescence in order to understand the significance of adiponectin during the aging process. A further study was conducted to find out whether adiponectin supplementation could reverse the regressive changes in the testis of aged mice.

2. Material and methods

2.1. Animal model

All the procedures involving animals were conducted in accordance with the ethical principles adopted by the committee for the purpose for control and supervision of experimental animals (CPCSEA), Government of India (No. 1802/G0/Re/S/15/CPCSEA) and were approved by the Institutional Animal Ethical Committee (No. F.Sc/88/IAEC/2017-18/1423-3), Institute of Science, Banaras Hindu University. Mice (*Mus musculus*) of Parkes strain were used and maintained under hygienic conditions in a well-ventilated room, housed in polypropylene cages (430 mm × 270 mm × 50 mm), with 12-hr photoperiod (6 AM to 6 PM, light) with controlled temperature (24 ± 2 °C) and humidity and were fed with standard pelleted commercial laboratory chow and drinking water ad libitum. Dry rice husk was used as the bedding material. General health condition and body weight of the animals were monitored regularly during the entire tenure of the experiment.

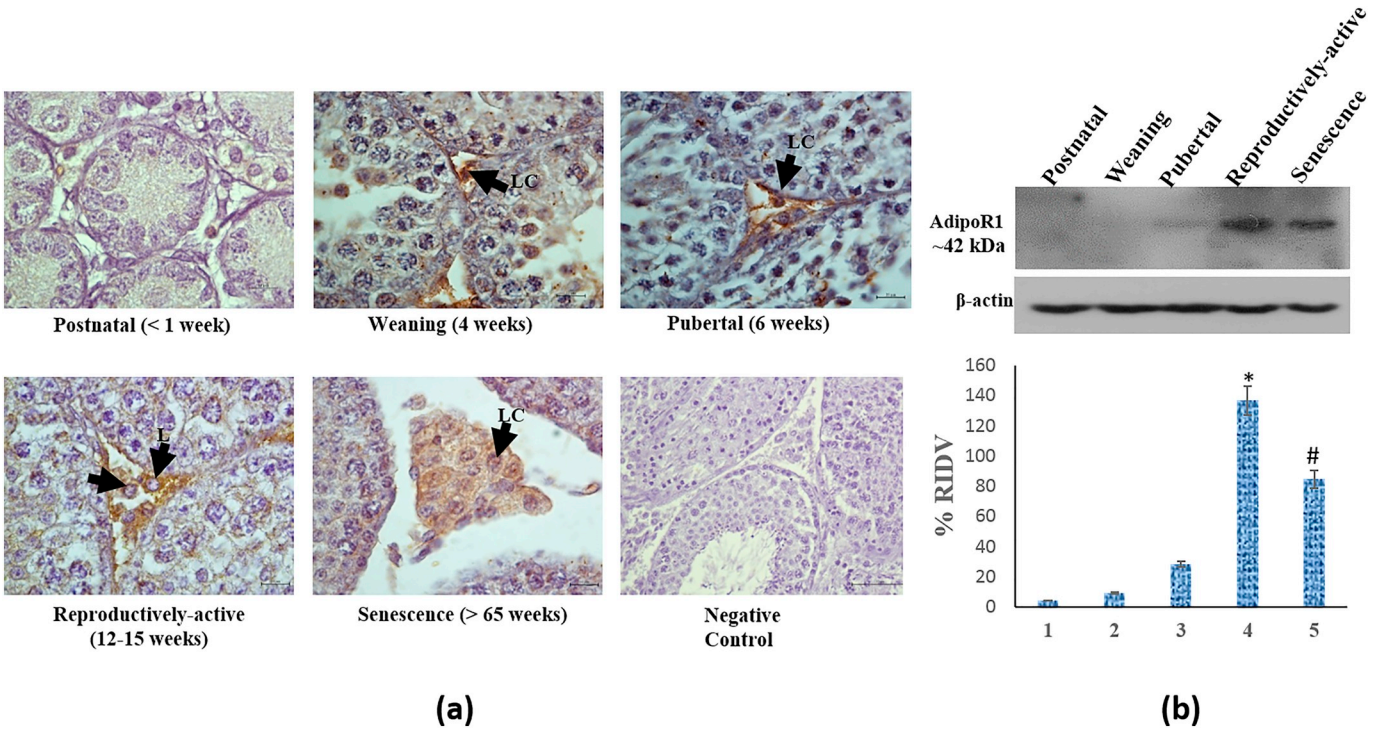

Fig. 3. (a): Immunolocalization of AdipoR1 in the testis of mice during different stages of maturation (Postnatal, Weaning, Pubertal, Reproductively-active) and aging (Senescence). Strong immunostaining of AdipoR1 was observed in the Leydig cell (LC) in the pubertal and reproductive active stage, whereas moderate immunostaining during prenatal, weaning, and aging period. Black arrowhead showing immunostaining in the Leydig cell (LC). All figures are shown in 100× magnification. (b): Immunoblot analysis of AdipoR1 protein in the testis of mice of different age groups. AdipoR1 protein expression increased in the Interstitium during weaning to the reproductively active stage and declined during aging condition. * represent values that are significantly ($P < 0.05$) increased during reproductively-active stages versus birth, pre-pubertal, pubertal, and senescent stages. # represent values that are significantly ($P < 0.05$) decreased in aging versus reproductively-active stage of the mice.

2.2. Sample collection

Mice were classified into following age groups: (a) Postnatal (day 1–7); (b) Weaning (4 weeks); (c) Pubertal (6 weeks); (d) Reproductively-active (15 weeks); and (e) Senescence or Aged (> 65 weeks). Mice were weighed and sacrificed at their respective age groups ($n = 5$) by decapitation under mild dose of anesthesia. Testis of one side of each animal was kept at −20 °C for immunoblot and the other side of the testis was fixed in Bouin's fluid for immunohistochemistry.

2.3. Adiponectin treatment: an in vivo study

This study was undertaken to find out the role of adiponectin treatment on spermatogenesis, steroidogenesis, anti-oxidative, and metabolic parameters in the testis of senescent mice. The aged male mice ($n = 15$) were divided into three groups (a) control mice treated with vehicle (PBS) (b) mice treated with low dose (1 μg/day/animal) of adiponectin and (c) mice treated with high dose (10 μg/day/animal) of adiponectin. The 1/5/10 μg/day dose of adiponectin showed physiological changes in the mice model. Thus, a lower physiological dose (1 μg/day) and a higher physiological dose of (10 μg/day) of adiponectin were selected in accordance with the previous studies [20,21]. The treatment was given intraperitoneally for 28 days as described previously [22]. After 24 h of the last dose, mice were sacrificed and blood and tissue samples were collected. The serum was separated out from blood within 2 h by centrifuging at 3500 rpm for 15 min at 4 °C and stored at −20 °C deep freezer until assayed for biochemical and hormonal estimation. The testis of each mice was dissected out, cleaned and weighed. The testis from one side was fixed in Bouin's fixative and processed for histological staining and immunohistochemistry. The remaining testis was stored at −20 °C for western blotting, biochemical studies, and oxidative stress parameters.

2.4. Histological analysis and quantitative measurements of spermatogenesis

The Bouin's fixed, paraffin-embedded aged mice testes with or without adiponectin treatment were serially sectioned at 6 μm. One set of the slide was used for hematoxylin and eosin (H&E) staining and the other set was used for PCNA immunostaining. The histological sections were assessed under a Nikon microscope and photographed. The germinal epithelium height and diameter of seminiferous tubules of control and treated testis was measured with motic image software using Nikon-E200. Quantitative analyses of spermatogenesis were carried out by counting the number of each type of germ cell at various stages, particularly at stage VII-VIII of the seminiferous cycle [23]. This stage was chosen because it is the most frequent stage of spermatogenesis and contains spermatogonia type A (Sg), preleptotene (Pl) spermatocytes, pachytene (P) spermatocytes, and step VII spermatids (S7). For this purpose, five slides were selected from one animal and two sections from each slide were taken for counting. Type A Sg, Pl spermatocytes, P spermatocytes, and S7 spermatids cells were counted according to the method of Meistrich and Hess [24]. The nuclei of different germ cells were counted in 50 round seminiferous tubules (STs) per treatment group. All the crude counts of germ cell were corrected for the section thickness and differences in the nuclear or nucleolar diameter using Abercrombie's formula [25] i.e. $P = A * [M / (L + M)]$; where P is the average number of nuclear points per section; A is the crude count of nuclei in the section; M the thickness (μm) of the section; and L is the average length (μm) of the nuclei. The results are expressed as a corrected count of germ cells.

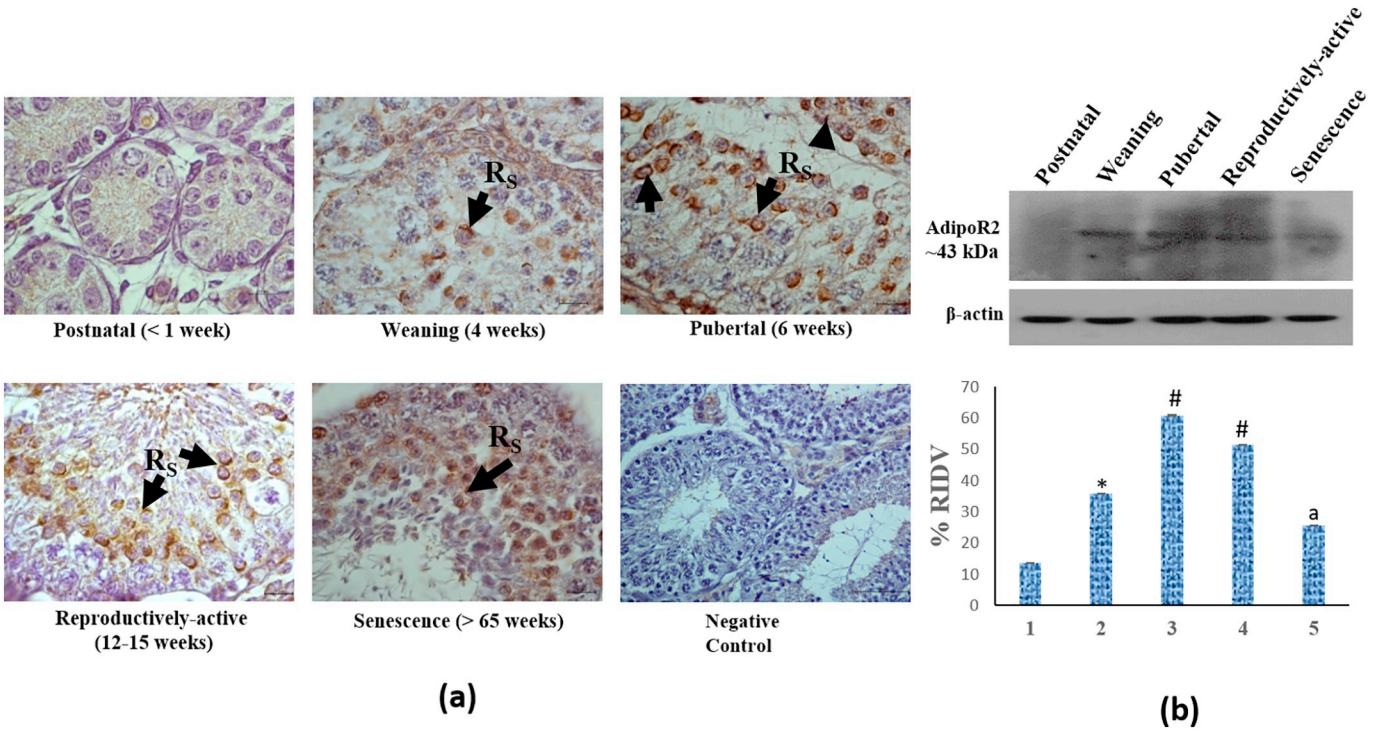

Fig. 4. (a): Immunolocalization of AdipoR2 in the testis of mice during different stages of maturation (Postnatal, Weaning, Pubertal, Reproductively-active) and aging (Senescence). Strong immunostaining of AdipoR2 was observed in the seminiferous tubules mainly in the round spermatids (SR) in the pubertal and reproductive active stage, whereas moderate immunostaining during prenatal, weaning, and senescence period. Black arrowhead showing immunostaining in the Leydig cell (LC). All figures are shown at 100 × magnification. (b): Immunoblot analysis of AdipoR2 protein expression in the testis of mice of different age groups. AdipoR2 protein expression increased in the Interstitium during development and decreased during aging condition. * represent values that are significantly ($P < 0.05$) increased during weaning versus postnatal stages. # represent values that are significantly ($P < 0.05$) increased during pubertal and reproductively active stages versus birth, weaning, and senescent mice. (a) represent a value that is significantly ($P < 0.05$) decreased in senescence versus pubertal and reproductively active stages of mice.

Table 2
Correlation studies of testis weight, intra-testicular testosterone and Insulin receptor with the testicular adiponectin, AdipoR1, and AdipoR2 proteins. * denotes data were correlated significantly.

Parameter	Adiponectin	AdipoR1	AdipoR2
Testis weight	0.564	0.943*	0.501
Intra-testicular testosterone	0.735*	0.903*	0.564
Insulin receptor	0.826*	0.673	0.810*

2.5. Immunohistochemistry (IHC)

IHC was performed according to the method described earlier [5]. Testis of different age groups of mice (postnatal, weaning, pubertal, reproductively-active and senescence) was paraffin embedded, and 6 μm sections were analyzed by IHC, for adiponectin, AdipoR1, and AdipoR2. The testicular sections were deparaffinized in xylene followed by hydration through graded alcohol. The sections were then treated with 3% H_2O_2 in methanol for blocking endogenous peroxidase activity. The sections were incubated with blocking serum for 1 h, followed by incubation with the primary antibody (dilution given in Table 1) for overnight at 4 °C. The sections were washed in PBS and incubated with the horseradish peroxidase tagged secondary antibody (dilution given in Table 1) for 2.5 h at room temperature. After incubation of secondary antibody, sections were washed and incubated with the chromogen substrate (0.1%; 3,3 diaminobenzidine tetrahydrochloride in 0.5 M Tris pH –7.6 and 0.01% H_2O_2) in dark for 1–2 min. Sections were then dehydrated and mounted with DPX. Slides were analyzed under a light microscope (Nikon, Japan) and photographed.

2.6. Western blotting

The testis were pooled and homogenized 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) to produce 20% (w/v) homogenate. Further, extraction of protein and immunoblotting was performed as described previously [12]. An equal amount of proteins (60 μg) as estimated by the method of Bradford [26] was loaded on to 10% SDS–PAGE for electrophoresis. Thereafter, proteins were transferred electrophoretically to PVDF membrane (Millipore India Pvt. Ltd.) overnight at 50 V, 4 °C. The membranes were blocked for 1 h with Phosphate buffer saline (PBS- 0.1 M, pH 7.4; NaH_2PO_4 –16 mM; Na_2HPO_4 64 mM; NaCl 154 mM; 0.02% Tween 20) containing 5% fat-free dry milk and incubated with primary antibodies (dilution given in Table 1) for 3 h at room temperature. Membranes were then washed with three changes of PBST over 10 min. Immunoreactive bands were detected by incubating the membranes with horseradish peroxidase tagged secondary antibody (at a dilution of 1:4000); for 1.5 h. Finally, the blot was washed three times with PBST and developed with enhanced chemiluminescence (ECL) detection system (BioRad, USA). Blot for each protein was repeated for three times. The densitometric analysis of the blots was performed by scanning and quantifying the bands for density value by using computer-assisted image analysis (Image J 1.38×, NIH, USA). The densitometric data were presented as the mean of the percentage relative integrated density value (%RIDV) ± SEM. The bands obtained from western blot were normalized to β-actin (Sigma Aldrich, St. Louis, MO, USA).

Table 3
Effect of adiponectin treatment on body mass, testicular mass, tubular diameter, germinal epithelium height, and corrected count of germ cells. Values are expressed as mean S.E.M. for five animals. * and # denotes significantly different from controls ($P < 0.05$) by one-way ANOVA followed by the Bonferroni test.

Treatment groups	Body weight (gm)	Testis weight (mg)	Tubular diameter (μm)	Germinal epithelium height (μm)	Type A spermatogonia	Preleptotene (PL) spermatocyte	Pachytene (P) spermatocyte	Stages 1–7 spermatids
Control	30 ± 0.57	63.67 ± 0.04	163.66 ± 1.85	52.65 ± 1.38	1.11 ± 0.16	6.39 ± 0.42	10.63 ± 0.64	40.14 ± 1.54
LD (1 μg APN)	33 ± 0.66	80.28 ± 0.99*	192.16 ± 2.31*	65.93 ± 1.53*	1.64 ± 0.04*	9.40 ± 0.67*	15.24 ± 0.86*	50.31 ± 0.63*
HD (10 μg APN)	35 ± 0.66	89.28 ± 3.07#	225.69 ± 2.96#	86.03 ± 1.25#	2.04 ± 0.13*	15.03 ± 0.45*	19.79 ± 0.96#	57.14 ± 0.89#

2.7. Biochemical parameters

Testicular glucose and Lactate dehydrogenase (LDH) were determined spectrophotometrically from testicular homogenate using an iMark™ Microplate Absorbance Reader (BioRad, USA).

2.8. Glucose estimation

Glucose content was measured from the testis samples using the commercial available glucose-reagent kit (Span Diagnostics Ltd., India) according to the manufacturer's protocol. Ten percent tissue homogenate (w/v) was prepared in triple distilled water under ice bath. The testicular homogenate was centrifuged at 10,000*g* and the supernatant was used for glucose estimation. To 10 μL of supernatant or serum sample or glucose standard (100 mg/dL), 1000 μL of working glucose reagent was added, mixed well, and incubated at 37 °C for 10 min. After incubation tubes were again thoroughly mixed and the color intensity was read at 505 nm against purified water (blank). All the tubes were run in triplicate. Intra-assay variation was < 3% and inter-assay variation was about 5%. Glucose content of the samples was calculated using formula Glucose (mg/dL) = (OD of sample/OD of the standard) ∗ 100.

2.9. Lactate dehydrogenase assay

Testicular LDH enzyme activity was estimated using commercially available LDH (P-L) KIT (Coral Clinical Systems, India) by the quantitative calorimetric method. Ten percent tissue homogenate was prepared in 100 mM Phosphate buffer saline (pH –7.8). The homogenate was centrifuged at 10,000*g* at 4 °C and the supernatant was used as the source of enzyme. 50 μL of sample was added to 1000 μL of working reagent, mixed well and incubated in 37 °C for 1 min, and the absorbance was read at 340 nm after the interval of every 1 min for 1, 2, & 3 min. All the test were run in triplicates. The mean absorbance change per minute was calculated (ΔA/min). Intra-assay variation was < 3% and inter-assay variation was about 5%.

2.10. Testosterone assay

Intra-testicular testosterone level was estimated using the testosterone ELISA kit (Diametra, USA) according to the procedures as described in the manufacturer manual. In brief, 25 μl of the standards, control or samples (testicular homogenate) were added to each well of the ELISA plate. Subsequently, the enzyme conjugate solution was added to each of these wells. The ELISA plate was then incubated at 37 °C for 1 h. The wells were then aspirated and washed 3 times with wash solution. Then, 100 μl of the tetramethylbenzidine chromogen (TMB) solution was added to each well and the plate was incubated at room temperature for 15 min. Finally, 100 μl of stop solution (0.2 M sulfuric acid) was added and the optical density (OD) was noted at 450 nm using an iMark™ Microplate Absorbance Reader (BioRad, USA).

2.11. Anti-oxidative enzymes activities and lipid peroxidation assay

The anti-oxidative enzyme activities for Superoxide dismutase (SOD), Catalase (CAT), and Glutathione peroxidase (GPx) in the testicular homogenate of control and treated mice were determined according to procedures described [27–29], with minor modifications as described earlier [12]. Lipid peroxidation measurement by thiobarbituric acid (TBARS) assay in the testicular homogenate of control and adiponectin-treated mice was performed in accordance with the procedure described previously [30] with minor modifications as published earlier [12].

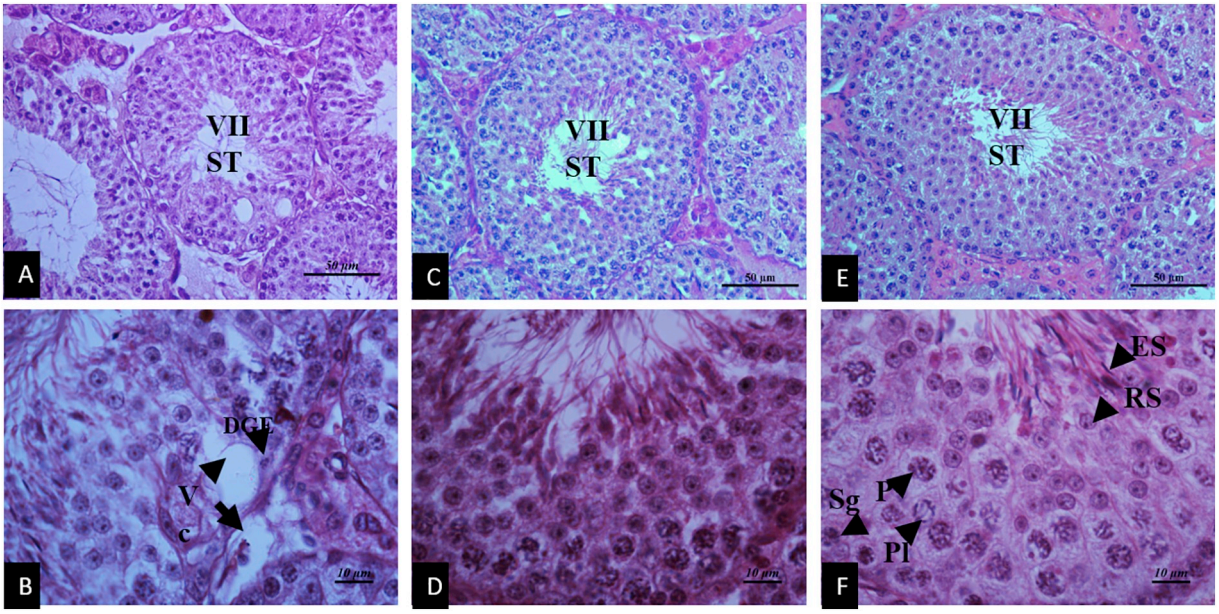

Fig. 5. Representative images showing histological changes in the testis after *in vivo* treatment of aged mice with adiponectin. The histological changes after adiponectin treatment of the following groups: (A,B) Group 1 (vehicle-treated control aged mice), (C,D) Group 2 (aged mice treated with low dose of APN-1 µg/animal/day) (E,F) Group 3 (Aged mice treated with high dose of APN-10 µg/animal/day). The testis of aged mice showed abnormal regressive changes such as the presence of vacuolization (V), degenerated nuclei in the germ cells (DGE), and loss of primary spermatogenic cells in the STs. Histomorphology of aged mice testis had undergone remarkable changes from control to adiponectin treated mice. Testis of aged (Fig. 5 a and b) mice showed fewer spermatogonia (Sg) with advanced spermatocyte (preleptotene (PL), pachytene (P) and dividing spermatocytes (S). Both the dose of adiponectin treatment showed enhanced round (RS) and elongated spermatids (ES), along with the preleptotene (PL), pachytene (P) and dividing spermatocytes (S). Black arrowhead showing Sg, PL, P, S and RS, ES. All the figures are shown in 40× and 100× magnification.

2.12. Statistical analysis

Data are expressed as mean ± SEM. The significance of the differences between groups was measured by using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test using SPSS software version 12 for Windows (SPSS Inc., IBM, Chicago, IL, USA) to compare the data from different groups. The correlation studies were performed by linear regression analysis using the Pearson's coefficient method in all the groups pooled together. The data were considered significant if $P < 0.05$.

3. Results

3.1. Changes in the testes during different stages of aging from post-natal to senescence periods in the mice

3.1.1. Body and testes mass

The result showed significant variation in the body and testes mass from birth to senescence. The body mass gradually increased from birth to senescence, whereas the testes mass increased gradually from birth to reproductively active stage but declined significantly during senescence (Fig. 1a).

3.1.2. Intra-testicular testosterone concentration and expression of insulin receptor

The intra-testicular concentration of testosterone was estimated during different (post-natal, weaning, pubertal, reproductively active and senescence) periods of mice. The intra-testicular testosterone level increased gradually from the post-natal period to reproductively active period. The testicular testosterone concentration declined significantly during senescence compared to the reproductively active period in mice (Fig. 1b). The densitometric analysis of immunoblots of testicular insulin receptor protein showed significant variation from post-natal to senescence. The expression of insulin receptor increased significantly from pubertal to the reproductively active period but decreased significantly from reproductively active to senescence period in mice (Fig. 1c).

3.1.3. Expression of adiponectin and its receptors (AdipoR1 and AdipoR2)

Immunohistochemical localization of adiponectin, AdipoR1, and AdipoR2 in the testis showed a distinct variation from the early post-natal period to senescence. The immunostaining of both adiponectin (Fig. 2A) and AdipoR1 (Fig. 3A) were mainly localized in the interstitial cells, whereas immunostaining of AdipoR2 (Fig. 4A) was localized mainly in round spermatid in seminiferous tubules of the mice testes. Mild to moderate immunostaining of adiponectin, AdipoR1 and AdipoR2 were noted in the testis from postnatal and weaning stages, but immunostaining of these proteins showed a distinct increase during pubertal and reproductively active periods and a marked decline during senescence. The pattern of changes in the immunostaining of adiponectin (Fig. 2B) and AdipoR1 (Fig. 3B) and AdipoR2 (Fig. 4B) in the testis of aging mice was later confirmed by densitometric analysis of the immunoblots of these proteins, which showed significant variation from postnatal to senescence. The expression of these proteins increased significantly during pubertal and reproductively active phases as compared with the post-natal and weaning phases. The expression of proteins decreased significantly during senescence as compared with the reproductively active phase.

3.1.4. Correlation between changes in adiponectin, AdipoR1, and AdipoR2 expression with the changes in testicular weight, expression of insulin receptor, and testosterone level during different stages of aging

The results of correlation studies are summarized in Table 2. The changes in adiponectin, AdipoR1, and AdipoR2 expression showed a significant correlation with the changes in testicular weight, and expression of insulin receptor and testosterone concentration in the testes of mice during different stages of aging.

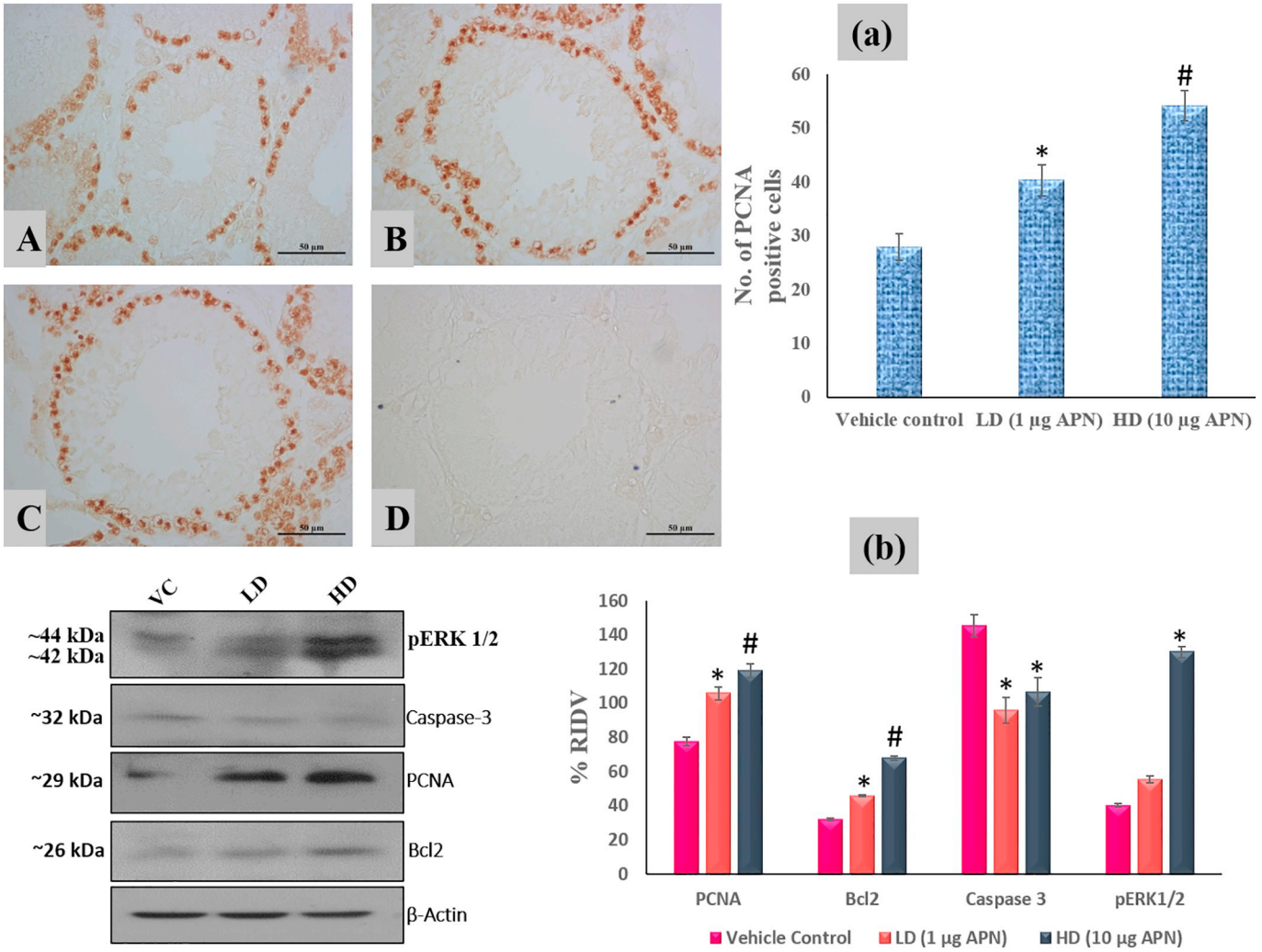

Fig. 6. (a) Immunohistochemical localization and proliferating cell nuclear antigen (PCNA) cell counting in the testis of control aged mice (A), low dose (1 µg/mice/day) adiponectin treated (B) and high dose (10 µg/mice/day) adiponectin treated aged mice (C). (b) The densitometric analysis of the immunoblots showed changes in the expression of testicular cell growth protein (pERK), proliferation protein (PCNA), survival protein (Bcl2), and apoptotic protein (Caspase-3) in the mice after *in vivo* adiponectin treatment with low and high dose. Values are represented as mean ± SEM. RIDV (relative integrated density value). Value (*) and (#) is significantly different ($P < 0.05$) in both the doses versus control.

3.2. Effect of in vivo treatment of adiponectin in the aged mice

3.2.1. Body and testes mass

The aged mice treated with adiponectin showed no significant changes in the body mass, whereas the testes mass showed a dose-dependent significant ($P < 0.05$) increase as compared with the control aged mice (Table 3).

3.2.2. Changes in testicular histology

The aged mice treated with adiponectin showed changes in testicular histoarchitecture and the development of germ cells. The testis of aged mice showed abnormal regressive histological changes such as the presence of vacuolization, degenerated nuclei in the germ cells (DGE), and loss of primary spermatogenic cells in the STs (Fig. 5A–B) while both low and high dose of adiponectin treated groups showed slight improvement in the spermatogenesis with presence of all stages of spermatogenesis (type A spermatogonia, PL spermatocytes, P spermatocytes, and step VII spermatids (S7) (Fig. 5C–F). Both the adiponectin treated (1 and 10 µg APN) aged mice showed dose-dependent significant ($P < 0.05$) increase in the tubular diameter, and germinal epithelium height compared to the control aged mice. Treatment with both the doses of adiponectin to aged rat showed dose-dependent significant ($P < 0.05$) improvement in testicular histoarchitecture as appearance of primary spermatocytes with fewer vacuole in seminiferous tubule and interstitium as well (Fig. 5C–F) and significant ($P < 0.05$) increased number of Sg, PL spermatocyte, P spermatocytes and S7 spermatids in the STs as compared with the aged mice testis (Table 3).

3.2.3. Testicular expression of pERK1/2 (a marker of germ cells growth), PCNA (proliferation), Bcl2 (survival) and caspase-3 (apoptosis)

Immunocytochemical study showed PCNA positive immunostaining mainly in the spermatogonial cells in the testis of control and adiponectin-treated mice (Fig. 6A–D). The number of PCNA-positive spermatogonial cells per seminiferous tubule increased significantly ($P < 0.05$) dose-dependently in the testes of adiponectin-treated mice as compared to control (Fig. 6a). The changes in levels of pERK1/2, PCNA, Bcl2, and caspase-3 proteins were studied by western blotting followed by densitometric analysis in the testis of mice treated with adiponectin. Western blot of pERK1/2, PCNA, Bcl2, and caspase-3 proteins showed a single immunoreactive band at ~42, ~29, ~26 and ~32 kDa respectively. The results showed significantly increased production of pERK1/2 in the testes treated with high dose of adiponectin. Both doses of adiponectin significantly increased the production of

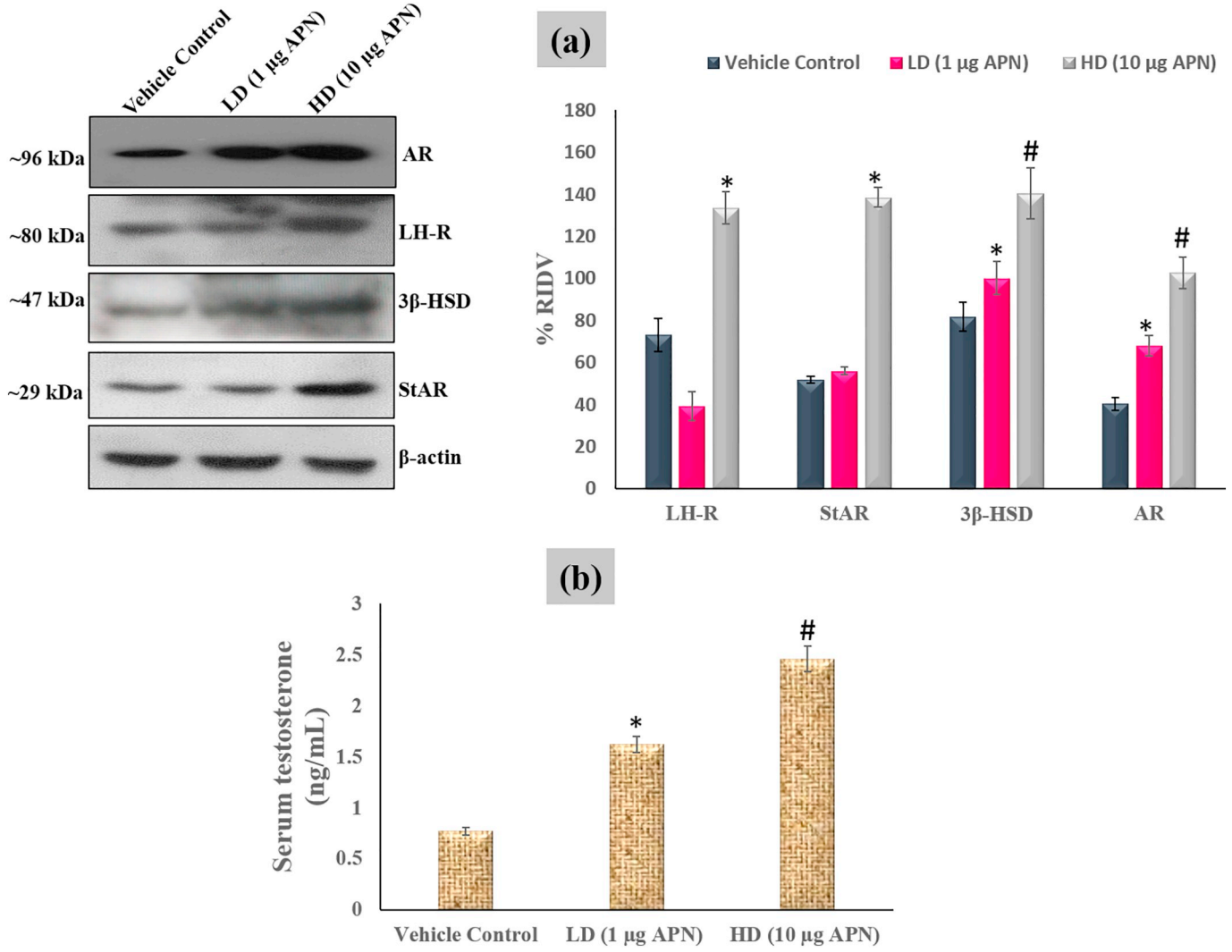

Fig. 7. (a) The densitometric analysis of the immunoblots showed changes in the expression of testicular steroidogenic markers Luteinizing hormone-receptor (LH-R), 3β-hydroxysteroid dehydrogenase (3β-HSD), and steroidogenic acute regulatory (StAR), and Androgen receptor (AR) proteins in the aged mice after *in vivo* adiponectin treatment with low and high dose. (b) Changes in the circulating testosterone (T) in the aged mice after *in vivo* adiponectin treatment with low and high dose. Values are represented as mean ± SEM. RIDV (relative integrated density value). Value (*) and (#) is significantly different ($P < 0.05$) in both the doses versus control.

PCNA and Bcl2 in the testes of treated mice as compared to the control, whereas caspase-3 production was declined significantly in adiponectin-treated mice as compared to the control (Fig. 6b).

3.2.4. Changes in circulating testosterone level and in the expression of LH-R, 3B-HSD, StAR and AR proteins in the testes

The changes in concentrations of LH-R, 3B-HSD, StAR and AR proteins were studied by western blotting followed by densitometric analysis in the testis of aged mice treated with adiponectin. Western blot of LH-R, 3B-HSD, StAR, and AR proteins showed a single immunoreactive band at ~80, ~47, ~29 and ~96 kDa respectively. The results showed dose-dependent significantly increased production of 3B HSD and AR proteins in the testes of aged mice treated with both low and high doses of adiponectin as compared to the control. However, only the high dose of adiponectin significantly increased the expression of LH-R and StAR proteins in the testes as compared to the control (Fig. 7a). The adiponectin treated aged mice showed a dose-dependent significant increase in circulating testosterone level as compared with the vehicle-treated control aged mice (Fig. 7b).

3.2.5. Changes in the expression of adiponectin (AdipoR1 & R2) and insulin (IR) receptors and their signaling molecules (AMPK and pAKT/AKT) in the testis

The changes in concentrations of AdipoR1, AdipoR2, IR, AMPK, and pAKT/AKT proteins were studied by western blotting followed by densitometric analysis in the testis of aged mice treated with adiponectin. Western blot of AdipoR1, AdipoR2, AMPK, IR, and pAKT proteins showed a single immunoreactive band at ~42, ~43, ~60, ~92, and ~59 kDa respectively. The results showed significantly increased expression of AdipoR1, AdipoR2 and AMPK proteins in the testes of aged mice treated with both low and high doses of adiponectin as compared with control (Fig. 8a). The aged mice treated with adiponectin also showed a significant increase in the expression of IR protein. Treatment with the high dose of adiponectin significantly increased the expression of pAKT/AKT protein in the testes as compared to the control (Fig. 8b).

3.2.6. Changes in the glucose concentration, expression of glucose transporter 8 (GLUT8) and monocarboxylate transporter (MCT 2 & 4) and LDH activity in the testis

The changes in concentrations of GLUT8, and MCT 2 and 4 proteins

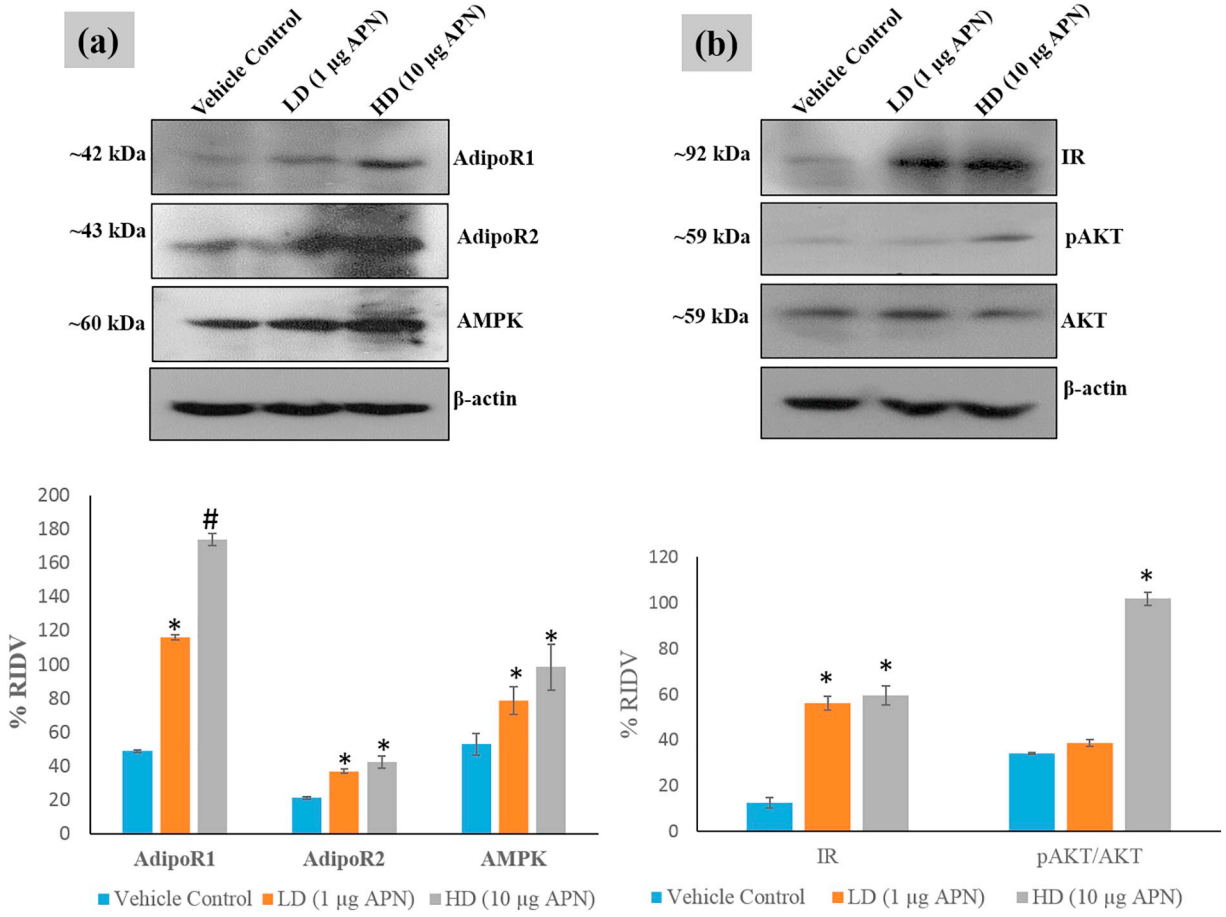

Fig. 8. (a) The densitometric analysis of the immunoblots showed changes in the expression of testicular adiponectin receptor (AdipoR1 and AdipoR2) and AMP-activated protein kinase (AMPK) proteins in the aged mice after *in vivo* adiponectin treatment with low and high dose. (b) The densitometric analysis of the immunoblots showed changes in the expression of testicular insulin-receptor (IR) and phosphorylated AKT (pAKT/AKT) proteins in the aged mice after *in vivo* adiponectin treatment with low and high dose. Values are represented as mean ± SEM. RIDV (relative integrated density value). Value (*) and (#) is significantly different ($P < 0.05$) in both the doses versus control.

were studied by western blotting followed by densitometric analysis in the testis of aged mice treated with adiponectin. Western blot of GLUT8, and MCT 2 and 4 proteins showed a single immunoreactive band at ~47, ~40 and ~43 kDa respectively. The results showed dose-dependent significantly increased expression of GLUT8, and MCT 2 and 4 proteins in the testes of aged mice treated with both low and high doses of adiponectin as compared with control (Fig. 9a). The aged mice treated with adiponectin also showed a dose-dependent significant increase in the intra-testicular concentration of glucose (Fig. 9b) and LDH enzyme (Fig. 9c) activity in the adiponectin treated mice as compared to the control.

3.2.7. Changes in the anti-oxidative markers (SOD, Catalase and GPx) and lipid peroxidation (LPO) in the testis of aging mice treated with adiponectin

The aged mice treated exogenously with low and high doses of adiponectin showed dose-dependent significant increase in levels of anti-oxidant markers (SOD, Catalase, and GPx) and a significant decline in the level of lipid peroxidation products (TBARS) as compared to the control (Fig. 10).

4. Discussion

Although a number of theories are described by which male reproductive organ undergo regressive changes leading to hypogonadism during senescence, an effective treatment which may ameliorate such age-related testicular changes has not been investigated in any animal model so far [31]. Earlier attempts to treat human aging by hormone replacement generally failed to show desired changes [32]. Our recent study together with others has clearly demonstrated that an adequate concentration of adiponectin and its receptors may be required for normal testicular functions [12,33]. It has also been demonstrated that the deficiency of adiponectin receptors leads to male subfertility with aspermia and atrophy of seminiferous tubules [34]. The present study was thus undertaken to find out whether a decline in adiponectin and its receptors may be responsible for impaired testicular functions in senescent mice.

This study has examined for the first time the changes in the expression of adiponectin and its receptors (AdipoR1 and AdipoR2) in the testis of mice during different stages of aging from early post-natal period to late senescence. The results showed significant variations in the expressions of testicular adiponectin and its receptors, which correlated significantly with the changes in testicular mass, expression of insulin receptors in the testis and testosterone levels during different stages of aging in the mice. The expression of testicular adiponectin and its receptors (AdipoR1 and AdipoR2), increased gradually from the early post-natal period and attained a peak level during puberty. The high pubertal expression of adiponectin persists during the reproductively active period but declines significantly in the testis of senescent mice. This observation corroborates with the earlier finding that demonstrated a significant increase in testicular expression of adiponectin mRNA from pre-puberty to puberty, with peak expression in early adulthood (Day 60) [17]. In the testis of chicken, the expression of

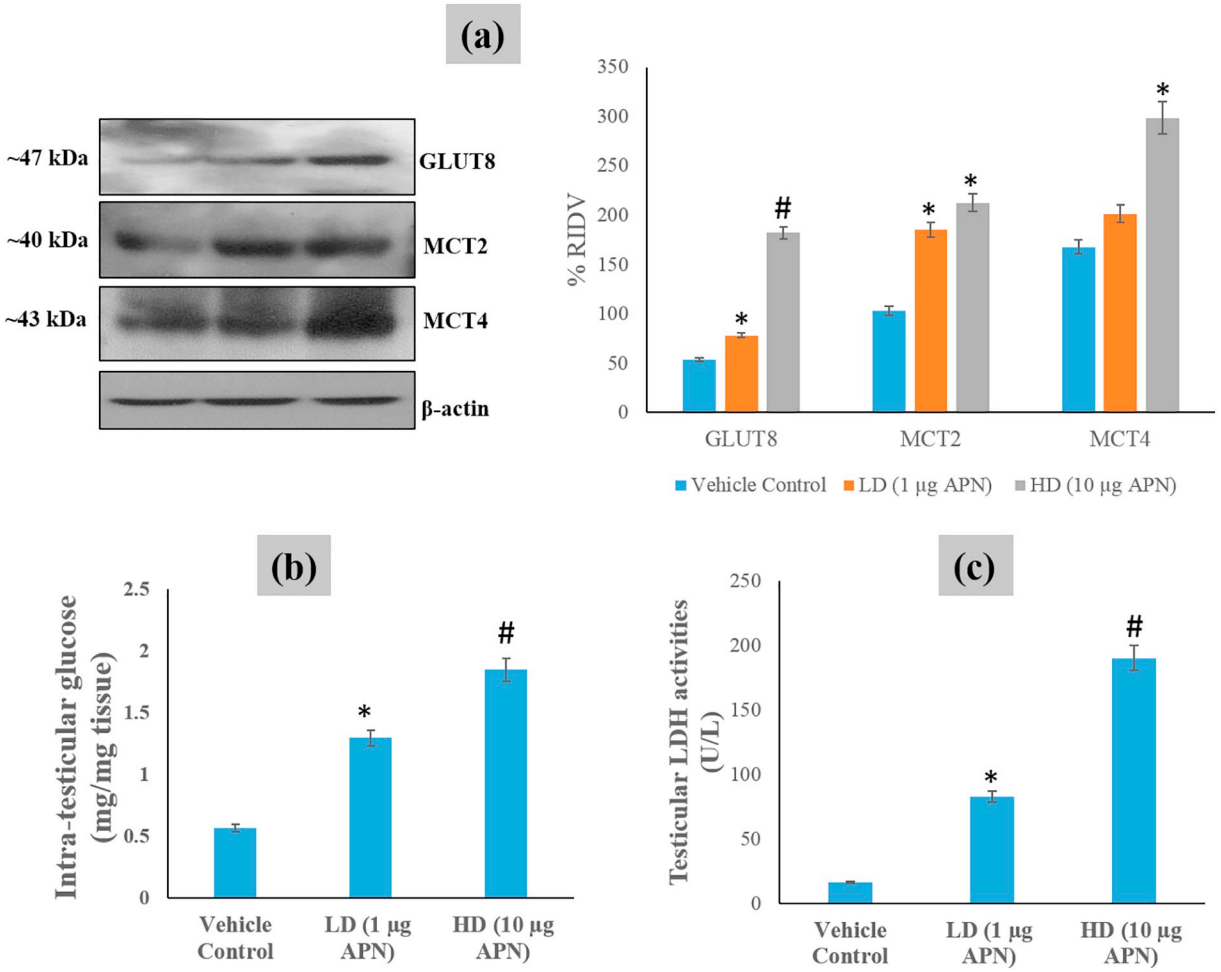

Fig. 9. (a) The densitometric analysis of the immunoblots showed changes in the expression of testicular metabolic marker glucose transporter 8 (GLUT8), monocarboxylate transporters 2 and 4 (MCT2 and MCT4) proteins in the aged mice after *in vivo* adiponectin treatment with low and high dose. (b) Changes in the intra-testicular glucose concentration in the aged mice after *in vivo* adiponectin treatment with low and high dose. (c) Changes in the testicular lactate dehydrogenase (LDH) activity in the aged mice after *in vivo* adiponectin treatment with low and high dose. Values are represented as mean ± SEM. RIDV (relative integrated density value). Value (*) and (#) is significantly different ($P < 0.05$) in both the doses versus control.

adiponectin mRNA was increased several folds from sexually immature to mature chicken [35]. These findings thus suggest that the up-regulation of adiponectin and its receptors genes expressions may be associated with sexual maturation of testes [33]. The current study also showed a simultaneous decline in the expression of testicular adiponectin and its receptors with the decline in testicular mass and production of testosterone during the senescent stage of life. This is in agreement with the earlier study showing decreased circulating adiponectin level with age [36]. In addition, decreased circulating adiponectin level was observed in aged mice as compared with the young mice [36,37]. These findings thus clearly suggest that decreased adiponectin/adiponectin receptors action may contribute to the impaired testicular functions (hypogonadism) in senescent mice.

The mechanism by which decline in adiponectin impairs testicular functions during aging is largely unknown. Our study showed a decline in adiponectin level during aging, which correlated significantly with the decline in the expression of insulin receptor (insulin sensitivity) in the testis of senescent mice. This finding supports earlier reports showing reduced insulin sensitivity (or development of insulin resistance) with aging [18,32]. It is well documented that adiponectin deficiency contributes to insulin resistance during aging [38]. These findings suggest that decreased adiponectin level during aging may be responsible for decreased insulin signaling, resulting in age-dependent metabolic changes in the testis. It is also well recognized that aging is characterized by the progressive loss of beta-cell function [9,10]. Thus it appears that decreased concentration of adiponectin associated decrease in insulin receptor in the testes of senescent mice may be responsible for impaired testicular functions or hypogonadism. The testis with insulin resistance was shown to synthesize testosterone at a lower rate [39].

The aged mice treated with adiponectin resulted in a dose-dependent significant increase in expression of AdipoR1 and AdipoR2 simultaneously with a significant increase in expression of insulin receptor and AMP-activated protein kinase (AMPK) protein in the testis. This finding corroborates our earlier study that activation of AMPK may be a possible signaling mechanism by which adiponectin increases insulin sensitivity in the testis [12,40]. The adiponectin-induced increased insulin receptor in the testis correlated with the increased concentration of circulating testosterone. This supports earlier studies showing the involvement of insulin in testosterone synthesis [41]. Thus, the treatment of adiponectin, by up-regulating adiponectin receptors and through AMPK signaling, increases insulin sensitivity in the testis, which subsequently may stimulate testosterone production [42].

The present study showed regressive changes in the seminiferous tubules characterized by a decrease in the number of germ cells, the presence of vacuolization and multinucleated cells in the testis of aged mice. The aged mice treated exogenously with adiponectin showed dose-dependent improvements in testicular histology as indicated by a

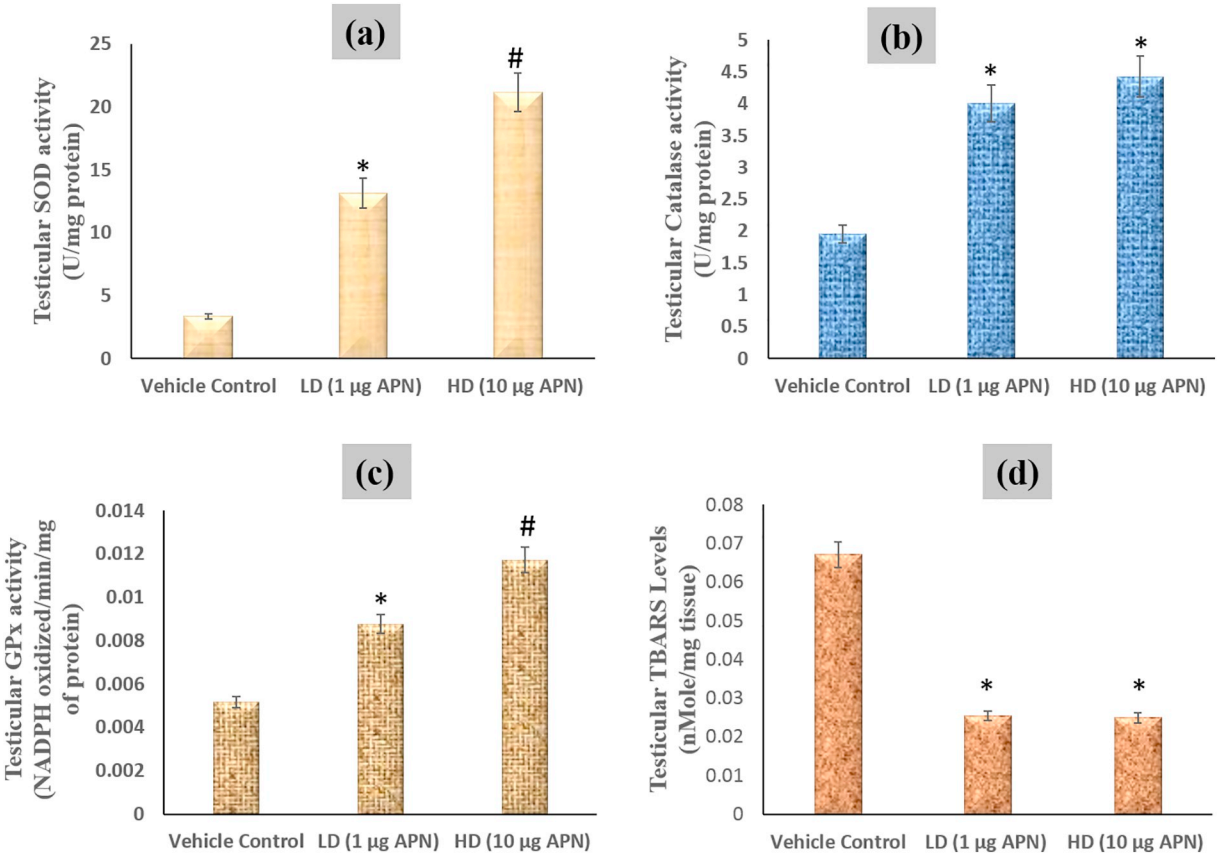

Fig. 10. Changes in the activity of testicular anti-oxidant enzymes superoxide dismutase (SOD), catalase (Cat), glutathione peroxidase (GPx), and lipid peroxidation (TBARS) level in the aged mice after *in vivo* adiponectin treatment with low and high dose. Values are represented as mean ± SEM. RIDV (relative integrated density value). Value (*) and (#) is significantly different ($P < 0.05$) in both the doses versus control.

dose-dependent significant increase in testicular mass due to increase in germinal epithelium height and tubular diameter. The number of type A spermatogonia, pre-leptotene, and leptotene spermatocytes showed a dose-dependent significant increase in the seminiferous tubules of treated as compared with the untreated aged mice (Table 3). Administration of adiponectin to the aged mice also showed stimulatory changes in spermatogenesis, as characterized by a significant increase in germ cells proliferation (PCNA) and survival (Bcl2) markers, while a decrease in apoptotic marker (caspase-3) as compared with the untreated control aged mice. Adiponectin treatment also showed a dose-dependent significant increase in the expression of the testicular phosphorylated extracellular signal-regulated kinase (ERK1/2) which correlated with the increase in the number of spermatogonial cells in the seminiferous tubules in the testis of treated-aged mice. This study is in agreement with an earlier study that showed that adiponectin treatment induces phosphorylation of ERK1/2, which may be responsible for self-renewal of spermatogonial stem cells [43,44]. The stimulatory effect of adiponectin on germ cell proliferation and survival is supported by a previous study demonstrating the presence of adiponectin receptors in the seminiferous tubules and complete lack of spermatogenesis in mice deficient of adiponectin receptor [45]. The current research is consistent with the earlier finding showing an increased rate of spermatogenesis simultaneously with a significant increase in the rate of expression of adiponectin receptor in the testis of chicken [35]. Thus, our study together with the earlier findings clearly suggests that increased adiponectin during puberty and reproductively active periods stimulates spermatogenesis, whereas decreased adiponectin during aging may be responsible for impaired spermatogenesis.

Testes are the major source of circulating testosterone, a marked decline in testosterone synthesis leads to reproductive aging in male [46]. In our current study, the aged mice treated with adiponectin showed a dose-dependent significant increase in circulating testosterone levels along with significant increase in the expression of various steroidogenic markers, such as LH-R, StAR, 3β-HSD, and AR proteins, in the testis. These findings suggest that the adiponectin is directly involved in the regulation of testosterone synthesis and may be involved in age-dependent decline in steroidogenesis. Adiponectin was previously shown to stimulate cholesterol transport in adrenocortical cells *in vitro* by increasing expression of StAR protein [47]. Treatment by adiponectin together with cAMP of cultured MA-10 Leydig cells resulted in increased mRNA expressions of StAR and the cholesterol side-chain cleavage enzyme CYP11A1 [48]. These findings suggest a direct involvement of adiponectin in the regulation of testicular steroidogenesis and a decline in its synthesis and action may be associated with reduced steroidogenesis as found during aging.

It is well known that an adequate amount of intra-testicular glucose is needed as a source of energy to achieve normal spermatogenesis and steroidogenesis [11,46]. Further, a marked decline in intra-testicular glucose level was shown to be associated with reduced testicular functions or hypoandrogenism during aging [46]. Our recent study on adult mice demonstrated that testis treated with adiponectin *in vitro* showed significantly increased glucose transport, which in turn leads to a series of metabolic changes that ultimately contribute to active spermatogenesis by promoting cell proliferation [12]. This study clearly showed a decreased synthesis and action of adiponectin in the testis of senescent mice. These findings thus led to the hypothesis that a decline in adiponectin may be responsible for decreased intra-testicular transport of glucose and impaired spermatogenesis during senescence. To examine this hypothesis, the aged mice were treated exogenously with adiponectin and results showed a dose-dependent significant increase

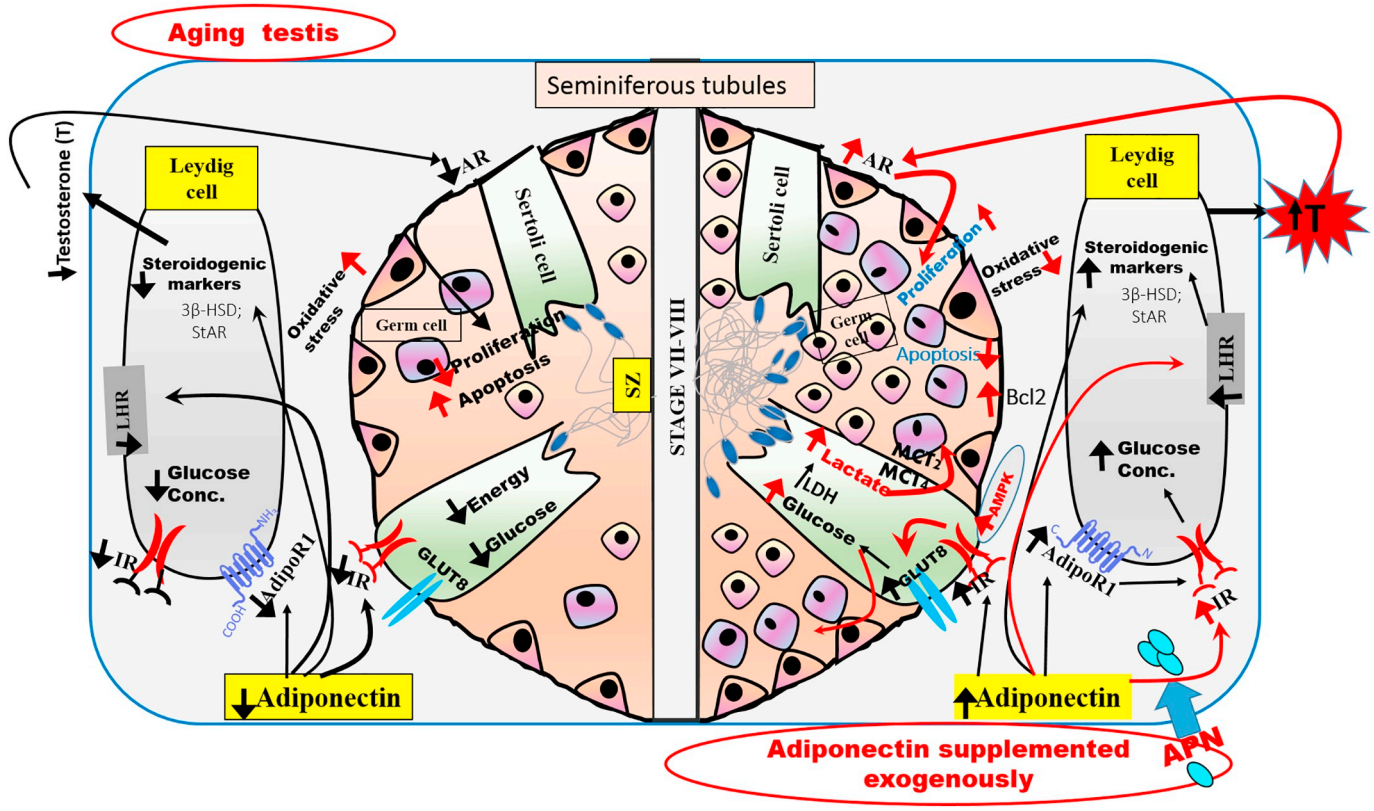

Fig. 11. Schematic illustration of how decreased adiponectin signaling during aging process resulted into decreased testicular metabolism (glucose and energy) via decreased insulin receptor action leading to disturbed germ cells proliferation, survival, apoptosis, metabolism, and oxidative stress status. Also, decreased testicular adiponectin level downregulate steroidogenic markers and hence a lower testosterone level which leads to lower expression of androgen receptor and hence it resulted in a regression in older testis. Exogenous supplementation of adiponectin during aging results in increased expression of testicular adiponectin receptors (AdipoR1 and AdipoR2) which triggers a cascade of signaling events resulting in activation of AMPK and insulin receptor (IR) which enhances testicular metabolic effects mediated through phosphorylation of AKT, leading to uptake of glucose in the Sertoli cells via GLUT8 transporter. Sertoli cells are proficient enough to metabolize a variety of energetic fuels, including glucose and lactate. Adiponectin-mediated stimulation of AMPK is the probable mechanism by which adiponectin enhances both glucose uptake and enzyme Lactate dehydrogenase (LDH) activity. LDH acts on pyruvate, a product from glucose metabolism, and converts it to lactate. Lactate is transported from Sertoli cell to germ cell through a proton-linked transporters MCT4 and MCT2. Lactate produced by Sertoli cell influences the survival of pachytene spermatocytes and prevents apoptosis during the normal process of spermatogenesis. Apart from this, activation of AdipoRs directly activates germ cell growth (pERK), proliferation (PCNA), survival (Bcl2) markers, and hence decreases apoptosis. Also, the increased expression of metabolic markers resulted in a decline in testicular oxidative stress level by upregulating the activity of antioxidant enzymes.

in expression of the testicular insulin receptor, phosphorylated AKT, GLUT8, and MCT 2 and 4 proteins together with an increase of the intra-testicular concentration of glucose and testicular LDH enzyme activity. These observations clearly suggest that aging mice treated with adiponectin have improved testicular uptake of glucose by up-regulating insulin receptors mediated increased expression of the GLUT8 transporter in the testis. This study supports the earlier observation that phosphorylated AKT is involved in increased glucose trafficking within the testis [12,49]. Adiponectin also promotes the production of lactate by insulin mediated increased synthesis of the LDH enzyme. The adiponectin-induced increase of MCT2 and 4 improves synthesis and transport of lactate from Sertoli cells to developing germ cells and promotes spermatogenesis in the testis. Further, in this study, the aged mice treated exogenously with adiponectin showed a positive correlation between the increased intra-testicular glucose levels with the increase in PCNA expression in testes. These findings thus suggest that adiponectin-induced increased uptake of glucose may be responsible for the increased proliferation of germ cells or increased rate of spermatogenesis.

It is well recognized that decreased concentration of anti-oxidative enzymes, SOD, Cat, and GPx, while the increased concentration of oxidative substance TBARS (lipid peroxidation) in the testes of aged mice result in overproduction of ROS. This increased production of ROS during aging then serve as a mechanism for decreased production of ATP and enhances apoptosis, leading to impaired spermatogenesis [50,51]. Further, studies showed a decline in insulin synthesis and action during aging [18]. Hence, we examined the effects of *in vivo* treatment of adiponectin on testicular oxidative stress in the aged mice. Our results clearly demonstrate for the first time that treatment with adiponectin improves insulin sensitivity and reduces the level of oxidative stress by significantly increasing the expression of testicular antioxidant enzymes (SOD, Cat, and GPx) while decreasing the oxidative stress by decreased lipid peroxidation in the testis of aged mice. These findings suggest that adiponectin-induced decline in oxidative stress may be responsible for the improvement in testicular functions. A recent review suggested a possible association between adiponectin, oxidative stress and insulin resistance as seen in our present study [52].

In brief, results from our current study showed a marked decline in adiponectin/ adiponectin-receptor expression, which correlated with the reduced testicular steroidogenesis, (testosterone synthesis), metabolism (glucose and lactate uptake), and enhanced oxidative stress in the aged mice (Fig. 11). Further, we showed that exogenous adiponectin treatment of aged mice resulted in a marked improvement in testicular functions. This may be accomplished by increasing insulin sensitivity and reducing oxidative stress. Our current findings are also supported by recent studies which showed shortened lifespan in AdipoR1/R2 double knockout mice [53] and also centenarians showed a higher serum concentration of adiponectin [54]. However, further

studies to determine, (a) what causes decline in adiponectin during aging; (b) whether the effects of adiponectin are through direct action on the gonads and/or through indirect actions involving other factors; (c) whether defective adiponectin action may lead to precocious aging, are required to support the current findings. Therefore, adiponectin treatment could be a promising anti-aging therapy promoting normal male reproductive function.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflict of interest

The authors declare that there is no conflict of interest.

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