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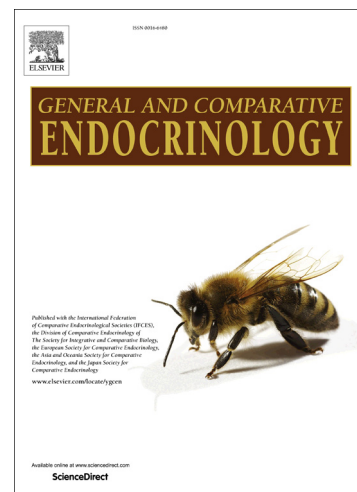
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**Direct actions of adiponectin on changes in reproductive, metabolic, and anti-oxidative enzymes status in the testis of adult mice**

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**Abstract**

Obesity is a major health problem that is linked to decreased sperm count. It is hypothesized that an obesity-associated reduction in adiponectin secretion may be responsible for impairment of spermatogenesis. Therefore, the aim of the study was to evaluate the direct role of adiponectin in spermatogenesis and steroid synthesis in adult mice. This study showed that adiponectin receptors (AdipoR1 and AdipoR2) were localized in Leydig cells and seminiferous tubules in the testis of adult mice. The result of the *in vitro* study showed the direct action of adiponectin on spermatogenesis by stimulating cell proliferation (PCNA) and survival (Bcl2) and by suppressing cell apoptosis. Treatment of testis with adiponectin also enhanced transport of the energetic substrates glucose and lactate to protect cells from undergoing apoptosis. Adiponectin treatment further showed a significant reduction in oxidative stress and nitric oxide. Our findings suggest that adiponectin effectively facilitates cell survival and proliferation, as well as protects from apoptosis. Thus, adiponectin treatment may be responsible for enhancing sperm counts. Interestingly, this study showed the stimulatory effect of adiponectin in spermatogenesis but showed an inhibitory effect on testosterone and estradiol synthesis in the testes. Based on the present study, it is hypothesized that systemic adiponectin treatment may be a promising therapeutic strategy for the improvement of spermatogenesis and sperm count.

Keywords: Adiponectin; Insulin receptor; Metabolism; Oxidative stress; Spermatogenesis; Testis

## Introduction

Obesity is a condition of excess accumulation of white adipose tissue (WAT) and has been associated with insulin resistance and hypogonadism (Phillips & Tanphaichitr, 2010). During the last few decades, a marked decline in male fertility due to a deterioration in semen quality was noted by a meta-analysis (Swan and Elken, 1999). The decline in male fertility has occurred in parallel with increasing rates of obesity. This suggests that obesity could be a possible reason for infertility and reduced fecundity in males. The molecular mechanism that links obesity with abnormal sperm production and infertility in males is not well understood. Adipose tissue is known to secrete a number of factors called adipokines that causes metabolic changes in various part of the body, including reproductive tissues. Adiponectin is most abundantly secreted adipokine (Scherer et al., 1995), and its circulating level decreases with obesity, but increases with the weight loss (Yang et al., 2001).

Adiponectin has many metabolic functions, such as the regulation of glucose and lipid metabolism, energy homeostasis, and potent insulin-sensitizing functions in various tissues, including testes. The obesity-associated decline in circulating adiponectin level also causes decreased insulin sensitivity (insulin resistance) and glucose intolerance in gonad cells (Rak et al., 2017). It is also possible that the obesity-associated, abnormal insulin signaling will induce an adverse effect on the testes (Papaetis et al., 2013). Glucose serves as the basic fuel molecule and provides important nutritional support to developing germ cells in the testes (Banerjee et al., 2014). The glucose transporter 8 (GLUT8) is one of the main glucose transporters in the testes (Gomez et al., 2006). The majority of glucose taken up by the testicular cells is converted to lactate, which is utilized as a key energy substrate by the developing germ cells and it also protects germ cells from undergoing apoptosis (Rato et al., 2012). Although adiponectin plays an important role in the

metabolic activities by regulating insulin sensitivity, the role of adiponectin in testicular energy homeostasis remains unknown.

Adiponectin and its receptors (AdipoR1 and AdipoR2) are demonstrated in different testicular cells, suggesting a possible regulation of testicular function by adiponectin (Kadivar et al., 2016). While the physiological relevance of adiponectin and its receptors in the male reproductive tract has received only a little attention, expression of AdipoR1 and AdipoR2 in the testicular cells suggests a role in various reproductive activities. In rats, adiponectin is found mainly in the Leydig cells, whereas AdipoR1 is expressed in seminiferous tubules (Caminos et al., 2008). Further, our study showed a positive correlation with total sperm concentration in the seminal plasma. Adiponectin receptors in the testes increased during sexual maturation in chickens (Ocon grove et al., 2008). These studies suggested a possible important contribution of adiponectin in testicular activities. However, obesity induces metabolic disturbances that lead to increased oxidative stress (Furukawa et al., 2004), and this increased oxidative stress has been reported to contribute to the decline in reproductive functions (Agarwal et al., 2014).

To date, a comprehensive understanding of the adiponectin action and underlying mechanism in the testes is not well investigated; we, therefore, sought to elucidate the direct role of adiponectin on spermatogenic, testicular steroidogenic, and metabolic activities in adult mice. To examine the effect of adiponectin on spermatogenesis, we assessed the changes in the expression rate of cell proliferation (PCNA), survival (Bcl2) and apoptosis (caspase-3). To determine the effect of adiponectin on steroidogenesis, we examined the changes in the expression rate of LH-R, StAR, and 3 $\beta$ -HSD and the changes in the levels of testosterone and estradiol. To determine the impact of adiponectin on metabolic activities, we determined the expression changes of insulin receptors, glucose and lactate transporters (GLUT8 and MCT2 & 4), and lactate dehydrogenase activity, tissue

glucose, and changes in antioxidant (SOD, Catalase, and GPx) and TBARS, nitric oxide levels were evaluated in the testis of adult mice.

## **Material and Methods**

### **Animals used for the study:**

The institutional animal ethics committee approved all animal studies (No. F.Sc/IAEC/2014-15/224). All experiments were conducted in accordance with principles and procedures approved by Departmental Research Committee, Banaras Hindu University. Mice (*Mus musculus*) of Parkes strain with average weight 30-35 g of 90-95 days were used for this study. Mice were maintained under hygienic conditions in a well-ventilated room, housed in polypropylene cages (430mm x 270mm x 50 mm), had 12-hour photoperiod (6 AM to 6 PM, light), controlled temperature ( $24\pm 2^{\circ}$  C) and humidity, and were fed standard pelleted commercial laboratory chow (Amrut Laboratory Animal Feeds, Pune, India) and drinking water ad libitum. Dry rice husks were used as the bedding material. Adult male mice (n=9) were euthanized by decapitation while under a mild-dose of anesthetic ether. Their testes were quickly dissected out and either fixed in Bouin's fluid for immunohistochemistry or immediately processed for *in vitro* study.

### **The peptide used for the study:**

The 18 amino acid, adiponectin peptide [NH<sub>2</sub>-LQVYGDGDHNGLYADNVN-COOH] dissolved in phosphate buffer saline (PBS - 0.1 M) was used in this study, which is synthesized in the laboratory of Dr. Puran S Bora. This peptide resembles mouse adiponectin peptide, having the globular domain (amino acid residues 216-233) at C-terminus of adiponectin protein (247 amino acid) that is highly conserved among mammalian species. This globular region of adiponectin shows a strong affinity with AdipoR1 but has moderate affinity for AdipoR2 (Yamauchi *et al.*, 2003).

### ***In vitro* study**

The dissected mice testes were cleaned of any adhered fat tissue in DMEM (Himedia, Mumbai, India) containing 250 U/ml penicillin and 250 mg/ml streptomycin sulfate. Each testis was cut into 2-3 pieces (~15-20 mg in weight) and cultured by the method described previously (Banerjee et al., 2012). The two different doses of adiponectin peptide (Low Dose = 0.1  $\mu\text{g}/\text{mL}$  and High Dose = 1  $\mu\text{g}/\text{ml}$ ) were selected in accordance with the previous study with some modifications (Caminos et al., 2008). The 1  $\mu\text{g}/\text{ml}$  dose was earlier suggested as a physiological dose of adiponectin (Rossi and Lord, 2013). The control group received 10  $\mu\text{l}$  of 0.1 M PBS per ml of medium per culture well. Each control and treatment group was run in triplicate. The culture medium was a mixture of DMEM (with sodium pyruvate and L-Glutamine) and Ham's F-12 (1:1; v:v) (Himedia) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1% BSA (Sigma). After an initial incubation period of 2 hours at 35° C, the culture medium was discarded and testis (one slice per well) were finally cultured in 1 ml medium in a humidified atmosphere with 95% air and 5% CO<sub>2</sub> to maintain pH 7.4 for 12 hours at 35° C. In the control group, testicular sections were only incubated in culture media without any treatment. Each treatment group was run in triplicate. Testis cultured under these conditions appears healthy and do not show any sign of necrosis. Testis slices were collected at the end of culture, washed several times with PBS, and stored at -20° C for immunoblot study, steroid assay, and biochemical estimations.

### **Immunohistochemistry (IHC)**

Immunohistochemical detection of adiponectin receptors (AdipoR1 and AdipoR2) was carried out in Bouin's fixed, paraffin-embedded adult mice testicular sections. The testicular sections (6  $\mu\text{m}$  thick) were deparaffinized in xylene followed by hydration through graded alcohol and subjected to antigen retrieval in a microwave oven. The sections were then treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for blocking endogenous peroxidase activity. The sections were incubated with blocking serum for

1 hour, followed by overnight incubation with the primary antibody (dilution given in **Table 1**) at 4° C. The sections were then washed in PBS and incubated with the horseradish peroxidase tagged secondary antibody (dilution 1:200) for 2.5 hours at room temperature. After incubation with the secondary antibody, sections were washed in PBS and incubated with the chromogen substrate (0.1%; 3,3 diaminobenzidinetetrahydrochloride in 0.05 M Tris pH -7.6 and 0.01% H<sub>2</sub>O<sub>2</sub>) in the dark for 1-2 minutes. After this sections were dehydrated and mounted with DPX. Slides were analyzed under a light microscope (Nikon, Tokyo, Japan) and photographed.

### **Western blotting**

The testicular slices 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 (Banerjee et al., 2012). An equal amount of proteins (60 µg) as estimated by the method of Bradford (1976) was loaded on to 10% SDS-PAGE for electrophoresis. Thereafter, proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane (Millipore India Pvt. Ltd.) overnight at 50V, 4° C. The membranes were blocked for 1 hour with phosphate buffer saline (PBS- 0.1 M, pH 7.4; NaH<sub>2</sub>PO<sub>4</sub> -16 mM; Na<sub>2</sub>HPO<sub>4</sub> 64 mM; NaCl 154mM; 0.02% Tween 20) containing 5% fat-free dry milk and incubated with primary antibodies (dilution given in **Table 1**) for 3 hours at room temperature. Membranes were then washed with three changes of PBST for 10 minutes. Immunoreactive bands were detected by incubating the membranes with horseradish peroxidase tagged secondary antibody (dilution 1:4000); for 1.5 hours. Finally, the blot was washed three times with PBST and developed with enhanced chemiluminescence (ECL) detection system (BioRad, USA). A Western blot for each protein was repeated 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 software (Image J 1.38x, NIH, USA). The densitometric data were presented as the mean of the integrated density value  $\pm$  SEM. The bands obtained from western blot were normalized to  $\beta$ -actin (Sigma Aldrich, St. Louis, MO, USA).

### **Biochemical assays**

Testicular glucose was estimated using commercially available biochemical kits (Autospan, India) while testicular lactate dehydrogenase (LDH) was measured using a commercially available LDH (P-L) Kit (Coral Clinical Systems, India). The total nitrate/nitrite level was estimated using vanadium trichloride method in accordance with the protocol of Miranda et al., 2001.

### **Antioxidative Enzyme Activities**

#### **Superoxide Dismutase (SOD) Assay**

SOD activity was measured in accordance with the method of Das et al., (2000). In brief, 10% testicular homogenate was prepared in PBS. The clear supernatant obtained was further diluted ten times with PBS. The supernatant (100  $\mu$ L) was mixed with 1.4 mL of a mixture containing 50mM phosphate buffer, 20 mM L-methionine, 10 mM hydroxylamine hydrochloride, 50 mM EDTA, and 1% Triton X-100 and were incubated at 37 $^{\circ}$  C for 5 minutes. After incubation, 80  $\mu$ L of 100  $\mu$ M riboflavin was added to each sample and kept under the SOD illuminated white light for 10 minutes. The reaction was stopped by the addition of 1 mL of Griess reagent (consisting of 0.1 % NED and 1 % sulphanilamide) to each sample. The absorbance was observed for 2 minutes at 543 nm with a UV-visible spectrophotometer. The enzyme activity was expressed as units per milligram protein.

#### **Catalase Assay**

Catalase activity was estimated in the samples according to the method of Aebi (1984). The ten times diluted supernatant sample was mixed with 10  $\mu$ L of absolute ethanol, vortexed and incubated

on ice for 30 minutes. Then, 450  $\mu\text{L}$  of the reaction mixture was mixed with 50  $\mu\text{L}$  of Triton X-100. An aliquot of 100  $\mu\text{L}$  of above sample was taken along with 2.8 mL of 50 mM PBS. The change in OD was measured at 240 nm with and without the addition of the substrate (6 mM of  $\text{H}_2\text{O}_2$ ) in the spectrophotometer. Catalase activity was expressed as nano-Kat per milligram protein. (One Kat is defined as 1 mole of  $\text{H}_2\text{O}_2$  consumed per second per milligram protein).

### **Glutathione Peroxidase (GPx) Assay**

The GPx activity in the supernatant was measured according to the method of Mantha et al., (1993). The 50  $\mu\text{L}$  of sample was mixed with assay mixture consisted of 398  $\mu\text{L}$  phosphate buffer (50 mM, pH 7.4), 2  $\mu\text{L}$  of EDTA (1 mM), 10  $\mu\text{L}$  of sodium azide (1 mM), 500  $\mu\text{L}$  of NADPH (0.5 mM), 40  $\mu\text{L}$  of GSH (0.2 mM) and 1 U glutathione reductase. The enzymatic reaction mixture was allowed to equilibrate at room temperature. The reaction was started by the addition of 100  $\mu\text{L}$  of 100 mM  $\text{H}_2\text{O}_2$ , and the absorbance was scanned kinetically for 3 minutes at 340 nm using UV-visible spectrophotometer. The GPx activity was expressed as nmol of NADPH oxidized per minute per milligram protein using an extinction coefficient [ $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ] for NADPH.

### **Lipid Peroxidation Assay**

Lipid peroxidation was estimated spectrophotometrically by thiobarbituric acid-reactive substances (TBARS) method as described earlier (Ohkawa et al., 1979). In brief, 10% testicular homogenates were prepared in PBS. The 0.2 mL supernatant was mixed with 3.2 mL of TBA reagent (containing 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% TBA). The mixture was incubated at 95° C in the water bath for 1 hour, cooled on ice, centrifuged at 500 g for 10 minutes at 4° C. The absorbance of the clear supernatant was measured at 534 nm using a spectrophotometer. It is expressed in terms of thiobarbituric acid reactive substances (TBARS) formed (nmol) per milligram protein.

**Steroid hormone assay: testosterone (T) and estradiol (E2)**

Testicular T and E2 level were estimated using the ELISA kit purchased from DiaMetra, Giustozzi (Foligno (PG), Italy; lot no.: DKO0002/DKO003) and validated by spiking control (Banerjee et al., 2012; Verma and Krishna, 2017). In brief, 25  $\mu$ l of the standards, control or samples (testicular homogenate or media) 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 hour. The wells were then aspirated and washed 3 times with wash solution. Then, 100  $\mu$ l of the tetramethyl benzidinechromogen (TMB) solution was added to each well and the plate was incubated at room temperature for 15 min. Finally, 100  $\mu$ l of stop solution (0.2 M sulfuric acid) was added and the optical density (OD) was noted at 450 nm using a microplate reader (BioRad). The standard curve ranged from 0.2 to 16 ng/ml for T and 20 to 2000 pg/ml for E2. The lowest detectable concentration of T and E2 that can be distinguished from the zero standards is 0.07 ng/ml and 8.68 pg/ml respectively at the 95% confidence limit. The coefficient of intra-assay variation was 5.8% for T and <9% for E2 and that of inter-assay variation was 10.5% for T and <10% for E2.

**Statistical analysis**

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

**Results****Immunolocalization of AdipoR1 and AdipoR2 protein in the testis of adult mice**

The presence of AdipoR1 and AdipoR2 protein was demonstrated immunohistochemically in the testis of adult mice. The AdipoR1 positive staining was mainly observed in interstitial Leydig cells. In contrast, negligible AdipoR1 immunostaining was observed in the seminiferous tubules (Sertoli and germ cells) (**Figure. 1, a and b**). Immunostaining of AdipoR2 was mainly found in the germ cells within seminiferous tubules of the testis particularly in round spermatids while no immunostaining was observed in the interstitium of the testis (**Figure 1, c and d**).

#### **Changes in the expression of AdipoR1, AdipoR2 and AMPK proteins in the testis treated *in vitro* with adiponectin**

The testis treated *in vitro* with the low and high doses of adiponectin showed a dose-dependent, significant ( $P < 0.05$ ) increase in the expression of AdipoR1, AdipoR2, and AMPK proteins as compared with the control. Immunoblot of AdipoR1, AdipoR2, and AMPK proteins showed the single immunoreactive band at ~42, 43, and 62 kDa (**Figure 2a-c**).

#### **Direct effect of adiponectin on the expression of insulin receptor (IR), pAKT/AKT, GLUT8, MCT2, and MCT4 proteins in the testis of adult mice**

The testis treated *in vitro* with either low or high dose of adiponectin showed a dose-dependent significant increase in the expression of IR, pAKT/AKT, GLUT8, MCT2, and MCT4 proteins compared to the control. Immunoblot of IR, pAKT, AKT, GLUT8, MCT2, and MCT4 proteins showed the single immunoreactive band at ~92, 57, 57, ~47, 40, 43 kDa respectively (**Figure 2d-e, Figure 3a-c**).

#### **Direct effect of adiponectin on the tissue glucose level and LDH activity in the testis of adult mice**

The testes treated *in vitro* with either low- or high-doses of adiponectin showed a dose-dependent significant ( $p < 0.05$ ) increase in the intra-testicular glucose concentration as well as LDH enzyme activity compared to the control (**Figure 3d-e**).

**The *in vitro* effect of adiponectin on the expression of markers of cell growth (pERK1/2), proliferation (PCNA), survival (Bcl2) and apoptosis (Caspase-3) in the testis of adult mice**

The testis treated *in vitro* with either low or high dose of adiponectin showed a dose-dependent significant increase in expression of markers of cell growth (pERK1/2) and proliferation (PCNA) (**Figure 4a**). Both the dose of adiponectin treatment showed significant ( $P < 0.05$ ) increase in the expression of cell survival marker, Bcl2 protein. Both the dose of adiponectin treatment showed dose-dependent significant ( $P < 0.05$ ) decrease in the expression of the cell apoptotic marker, caspase-3 protein (**Figure 4b**). Immunoblot of pERK1/2, PCNA, Bcl2, and Caspase3 proteins showed the single immunoreactive band at ~42, 29, 26 and 32 kDa respectively.

**Direct effect of adiponectin treatment on the activities of antioxidant enzymes (SOD, Catalase, and GPx), Lipid peroxidation, and nitric oxide level in the testes of adult mice**

The direct effect of low- and high-doses of adiponectin treatment showed a marked variation in testicular anti-oxidative enzyme activity. The testes treated *in vitro* with either low- or high-dose of adiponectin showed a dose-dependent significant ( $p < 0.05$ ) increase in the level of testicular SOD, Catalase, and GPx enzyme activities, whereas significant ( $p < 0.05$ ) decrease in the lipid peroxidation and nitric oxide (total nitrate/nitrite) levels compared to the control testis (**Figure 5**).

**The *in vitro* effect of adiponectin on steroid synthesis and on the expression of steroidogenic markers (LH-R, StAR, 3 $\beta$ -HSD, and Aromatase) in the mice testis**

The testes treated *in vitro* with either low- or high-dose of adiponectin showed a significant ( $P<0.05$ ) decline in the intra-testicular concentration of both T and E2 compared to the control (**Figure 6a**).

The testis treated *in vitro* with the high dose of adiponectin showed significantly ( $P<0.05$ ) decreased expression of the steroidogenic marker (LH-R, StAR,  $3\beta$ -HSD, and Aromatase) proteins compared to the control. The low dose of adiponectin generally decreased the expression of LH-R protein, but not the StAR,  $3\beta$ -HSD, and aromatase proteins as compared with the control (**Figure 6b**).

### Correlation analysis data

A correlation analysis was performed to find out how the various parameters correlated with each other in the control and adiponectin (Low dose,  $0.1 \mu\text{g/mL}$ ; High dose;  $1 \mu\text{g/mL}$ ) treated groups. A positive correlation was found between testicular expression of IR, GLUT8 with an expression of pAKT/AKT proteins (**Table 2a**); PCNA expression level correlated positively with expression of the GLUT8 protein and intra-testicular glucose level (**Table 2b**), and also a positive correlation was found between testicular anti-oxidant enzymes (SOD, Catalase, and GPx) with PCNA and Bcl2 proteins.

### Discussion

The aim of this study was to evaluate the hypothesis that obesity-associated decline in adiponectin level is responsible for impaired sperm production (infertility) in adult mice. The present immunohistochemical study has clearly demonstrated the presence of AdipoR1 and AdipoR2 in the testis, particularly in the Leydig and germ cells respectively. Our findings were consistent with the earlier reports that showed the presence of both adiponectin and its receptors in different testicular cells (Ocon-Grove et al., 2008; Kadivar et al., 2016). In rats, adiponectin is found mainly in Leydig cells, whereas AdipoR1 is expressed in seminiferous tubules (Caminos et al., 2008). The results of

the present study corroborate with earlier findings that suggest Leydig cells and seminiferous tubules are the major sites of adiponectin synthesis and action in the testes of mice (Rak et al., 2017). This study together with the earlier report (Martin, 2014) thus clearly suggests adiponectin as a potential regulator of testicular functions through endocrine and/or paracrine mechanisms.

The testis treated *in vitro* with adiponectin showed a significant dose-dependent increase in expression of PCNA (cell proliferation) and Bcl2 (cell survival), but they showed a significant decline in expression of caspase-3 (cell apoptosis). The results of this *in vitro* study suggest a direct action of adiponectin, even in the absence of gonadotropins, on spermatogenesis by stimulating cells proliferation and survival and by suppressing the rate of apoptosis. The direct action of adiponectin in the cells proliferation and survival is supported by the previous findings that showed the presence of adiponectin receptors in the seminiferous tubules and mice deficient of AdipoR2 resulted in atrophy of seminiferous tubule and aspermia (Bjursell et al., 2007). Simultaneously with a sharp increase in spermatogenesis, Sertoli cell function and sperm motility during sexual maturation in chicken were found a marked increase in expression of adiponectin receptors in the testis (Ocon-Grove et al., 2008). These findings suggest that increased adiponectin improves spermatogenesis, whereas the decline in adiponectin as found during obesity may be responsible for suppression of spermatogenesis (infertility). However, the mechanism by which adiponectin affects spermatogenesis is not yet fully elucidated.

The testis treated *in vitro* with adiponectin showed a significant, dose-dependent increase in expression of AdipoR1 and AdipoR2. This study also showed a dose-dependent significant increase in expression of insulin receptor and AMP-activated protein kinase (AMPK) proteins in the testis treated *in vitro* with adiponectin, simultaneously with the increase in the expression of adiponectin-receptors. Based on our findings it may be hypothesized that adiponectin affects testis by up-

regulating AdipoR1 and AdipoR2 through AMPK signaling (Wu et al., 2013), although our study requires further confirmation by blocking AMPK. A number of earlier studies have also shown that the insulin-sensitizing effect of adiponectin may be mediated by activation of AMPK (Yamauchi et al., 2002, Tomas et al., 2002; Kahn et al., 2005). These findings further suggest that activation of AMPK may be a probable mechanism by which adiponectin increases insulin sensitivity in the testes (Combs et al., 2004). Thus, the treatment of adiponectin may improve insulin sensitivity in the testes, which is required for fertility in male (Loeken, 2012; Schoeller et al., 2012).

Spermatogenesis is a high-energy dependent process that requires glucose for energy. There are multiple mechanisms and involvement of various factors that are associated with glucose transport to testis (Banerjee et al., 2014), but the role of adiponectin in this process is not yet elucidated. Interestingly, in our present study, the testes treated with adiponectin *in vitro* showed a significant, dose-dependent increase in the expression of GLUT8 and IR proteins as well as increased **uptake** of intra-testicular glucose level. This finding is consistent with our earlier report suggesting the significant role of insulin in up-regulating the expression of GLUT8, which subsequently increases the transportation of glucose in the testis (Chen et al., 2003; Gomez et al., 2006; Banerjee et al. 2014, Anjum et al., 2016). This study thus showed an increased **concentration** transport of glucose in the testes treated *in vitro* with adiponectin. These observations thus suggest that treatment of adiponectin may increase testicular **intake** of glucose level by insulin mediated increased expression of GLUT8 in the testes. To find out the signaling pathway of adiponectin and insulin-mediated glucose uptake, phosphorylated AKT levels were evaluated in the testis. The testis treated *in vitro* with a low and high dose of adiponectin showed a significant increase in the expression of pAKT protein simultaneously with increased expression of GLUT8 and IR proteins. Furthermore the adiponectin-induced changes in GLUT8 ( $r= 0.941$ ;  $p<0.05$ ; **Table 2a**) and IR ( $r= 0.929$ ;  $p<0.05$ ;



**Table 2a)** expression level correlated significantly with the changes in pAKT/AKT ratio in the testis. It is well recognized that AKT is involved in many signaling pathways including glucose trafficking (Huang et al., 2007). However, in this study, AKT may also function as a signaling molecule in adiponectin-induced changes in the expression of the GLUT8 protein in the testis.

Further, in this study, the testes treated *in vitro* with adiponectin showed a significant correlation between the increase in the expression of testicular GLUT8 ( $r= 0.885$ ;  $p<0.05$ ; **Table 2b**) and intratesticular glucose ( $r = 0.889$ ;  $p<0.05$ ; **Table 2b**) level with the increase in the expression of PCNA. These observations thus suggest that adiponectin-induced increased availability of glucose may be responsible for increased cell proliferation or increased rate of spermatogenesis in the testis. It has earlier been shown that the testicular glucose is involved in a series of metabolic changes that finally contribute to active spermatogenesis by cell proliferation (Villarroel-Espíndola et al., 2015). Glucose transports by GLUTs that occur across the blood-testis-barrier to the Sertoli cell are crucial events of spermatogenesis. The results of our study showed, for the first time, the active participation of adiponectin in increased transport of glucose in the testis of mice. ~~Sertoli cells convert glucose to lactate by enzyme lactate dehydrogenase.~~ The conversion of increased glucose level to pyruvate via glycolysis and further pyruvate got converted to lactate in the Sertoli cells, which is catalyzed by the enzyme LDH (Rato et al., 2012). The testes treated *in vitro* with adiponectin showed simultaneously the significant increase in the expression of insulin receptor and phosphorylation of AKT protein, together with the significant increase in LDH enzyme activity. This finding also corroborates with the earlier reports that Sertoli cells treated with insulin showed increased LDH protein and intracellular lactate content (Rocha et al., 2014) and insulin-deprived Sertoli cell down-regulate lactate synthesis by suppressing LDH-A enzyme (Blackshaw and Elkington, 1990). Based on these findings it is postulated that adiponectin regulates the production

of lactate by insulin-mediated increased synthesis of LDH in the testis. Lactate produced by Sertoli cell influences the survival of pachytene spermatocytes during the spermatogenesis (Jutte et al., 1983). Glucose metabolism, therefore, is critical for normal spermatogenesis (Alves et al., 2013). Lactate is transported to germ cells through a family of proton-linked plasma membrane transporters known as monocarboxylate transporter (MCT) 2 and 4 (Oliveira et al., 2011). MCT4 transports lactate from Sertoli cells to the extracellular spaces, whereas MCT2 transports lactate from the extracellular space to germ cells (Rato et al., 2012). Activation of AMPK is known as a stimulator of MCT4 expression. The testis treated *in vitro* with adiponectin showed a dose-dependent significant increase in the expression of MCT2 and MCT4 proteins. Our findings thus suggest that adiponectin, by increasing expression of MCT2 and MCT4, transport lactate to the germ cells and prevent apoptosis.

It is well known that oxidative stress can adversely affect spermatogenesis (Chandra et al., 2009); whereas anti-oxidant enzymes protect testicular germ cells against the apoptotic effects of oxidative stress (Aitken and Roman, 2008). Our study examined the attenuating effects of adiponectin on testicular oxidative stress. The results showed a significant, dose-dependent increase in the antioxidant enzymes (SOD, Catalase, and GPx) activity, but a significant decrease in TBARS level (lipid peroxidation) in the testis of mice treated *in vitro* with adiponectin. The adiponectin-induced increase in anti-oxidant enzymes (SOD, Catalase, and GPx) were significantly correlated with changes in the expression of PCNA ( $r= 0.904, 0.882, 0.78; p<0.05$ ; **Table 2b**) and Bcl2 ( $r= 0.83, 0.709, 0.772; p<0.05$ ; **Table 2b**) proteins in the testis. These results are consistent with the earlier published report, in which increased Bcl2 expression was shown to protect cells against apoptosis by enhancing anti-oxidant enzyme activities (Hockenbery et al., 1993). The increased nitric oxide can also mediate tissue injury and cell death (Murphy, 1999). Our study showed a

significant decrease in the testicular level of nitric oxide upon treatment with adiponectin *in vitro*. The increased serum level of nitric oxide was shown in the mice during decreased testicular activity such as during senescence (Banerjee et al., 2012). The result of our study thus suggests that increased oxidative stress may be one of the mechanisms for the manifestation of testicular impairment during obesity, whereas treatment of adiponectin due to its anti-oxidant and anti-nitric oxide effect may improve testicular functions.

The earlier literature showed species-specific variable effects of adiponectin on gonadal steroidogenesis. The granulosa cells of rats and humans treated with adiponectin showed the increased synthesis of progesterone and estradiol only in presence of IGF-1 (Chabrolle et al., 2007; 2009). Whereas, adiponectin inhibited steroidogenesis in bovine ovaries by inhibiting CYP11A1 and CYP17A1 in the theca cells, but showed no effect on granulosa cells (Lagaly et al., 2008). In swine, ovaries treated with adiponectin showed increased expression of StAR but decreased expression of CYP19 (Ledoux et al., 2006). The present *in vitro* study showed that adiponectin alone significantly decreases the testicular synthesis of T and E2. This is in accordance with an earlier study in the ovary of the bat that when treated with adiponectin saw a significant decline in androgen synthesis (Singh and Krishna, 2012). In our study, adiponectin treatment inhibited the steroid synthesis mainly by dose-dependent inhibition of the expression of LH-R protein in the testis. The testis treated *in vitro* with an only high dose of adiponectin showed a significant decrease in expression of steroidogenic enzymes, StAR, 3 $\beta$ -HSD, and aromatase, but a low dose of adiponectin showed no significant variation in the expression of steroidogenic enzymes. This study thus indicates that adiponectin suppresses T and E2 synthesis by suppressing the testicular expression of LH-receptor as well as other steroidogenic markers, StAR, 3B HSD, and aromatase.

In brief, our study showed the localization of adiponectin receptors (AdipoR1 and AdipoR2) in Leydig cells and seminiferous tubules in the testis of adult mice. The result of our *in vitro* study showed the direct action of adiponectin on spermatogenesis by stimulating cell proliferation (PCNA) and survival (Bcl2) and by suppressing cell apoptosis. Treatment of testis with adiponectin also enhances transport of energy substrate lactate to protect germ cells from undergoing apoptosis (**Figure 7**). Thus, adiponectin treatment may be responsible for enhancing sperm counts. The adiponectin treatment further showed a significant reduction in oxidative and nitrosative stress. Interestingly, our study showed the stimulatory effect of adiponectin in spermatogenesis but showed an inhibitory effect on steroid synthesis in the testis. In conclusion, the present results suggest a putative role of adiponectin pertaining to the male reproductive activities. Based on the present study, we hypothesize that systemic adiponectin treatment may be a promising therapeutic strategy for the improvement of sperm count.

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### **Conflict of interest**

The authors declare that there is no conflict of interest.

**References**

- Aebi, H., 1974. Catalase. In: *Methods of Enzymatic Analysis* (ed. HU Bergmeyer), Academic Press, New York. pp. 673–684.
- Agarwal, A., Virk, G., Ong, C., du Plessis, S.S., 2014. Effect of Oxidative Stress on Male Reproduction. *The World Journal of Men's Health* 32, 1. doi:10.5534/wjmh.2014.32.1.1.
- Aitken, R.J., Roman, S.D., 2008. Antioxidant systems and oxidative stress in the testes. *Oxidative Medicine and Cellular Longevity* 1, 15-24.
- Alves, M.G., Martins, A.D., Cavaco, J.E., Socorro, S., Oliveira, P.F., 2013. Diabetes, insulin-mediated glucose metabolism and Sertoli/blood-testis barrier function. *Tissue Barriers* 1, e23992. doi:10.4161/tisb.23992.
- Anjum, S., Krishna, A., Tsutsui, K., 2016. Possible role of GnIH as a mediator between adiposity and impaired testicular function. *Frontiers in Endocrinology* 7, 1–12. doi:10.3389/fendo.2016.00006.
- Banerjee, A., Anjum, S., Verma, R., Krishna, A., 2012. Alteration in expression of estrogen receptor isoforms alpha and beta, and aromatase in the testis and its relation with changes in nitric oxide during aging in mice. *Steroids* 77, 609–620. doi:10.1016/j.steroids.2012.02.004.
- Banerjee, A., Anuradha, Mukherjee, K., Krishna, A., 2014. Testicular glucose and its transporter GLUT 8 as a marker of age-dependent variation and its role in steroidogenesis in mice. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* 321, 490–502. doi:10.1002/jez.1881.
- Bjursell, M., Ahnmark, A., Bohlooly-Y, M., William-Olsson, L., Rhedin, M., Peng, X.R., Ploj, K.,

Gerdin, A.K., Arnerup, G., Elmgren, A., Berg, A.L., Oscarsson, J., Lindén, D., 2007. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes* 56, 583–593. doi:10.2337/db06-1432.

Blackshaw, A.W., Elkington, J.S., 1970. Developmental changes in lactate dehydrogenase isoenzymes in the testis of the immature rat. *J ReprodFertil* 22, 69-75; <http://dx.doi.org/10.1530/jrf.0.0220069>.

Bradford, MM., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72, 248-54.

Caminos, J.E., Nogueiras, R., Gayta, F., Pineda, R., Gonza, C.R., 2008. Novel Expression and Direct Effects of Adiponectin in the Rat Testis. *Endocrinology* 149, 3390–402. doi:10.1210/en.2007-1582.

Chabrolle, C., Tosca, L., Dupont, J., 2007. Regulation of adiponectin and its receptors in rat ovary by human chorionic gonadotrophin treatment and potential involvement of adiponectin in granulosa cell steroidogenesis. *Reproduction* 133, 719–731. doi:10.1530/REP-06-0244.

Chabrolle, C., Tosca, L., Ramé, C., Lecomte, P., Royère, D., Dupont, J., 2009. Adiponectin increases insulin-like growth factor I-induced progesterone and estradiol secretion in human granulosa cells. *Fertil. Steril.* 92, 1988–96. doi:10.1016/j.fertnstert.2008.09.008.

Chandra, a, Surti, N., Kesavan, S., 2009. Significance of oxidative stress in human reproduction. *Arch Med Sci* 5, 28–42.

Chen, Y., Nagpal, M.L., Lin, T., 2003. Expression and regulation of glucose transporter 8 in rat Leydig cells. *J Endourol* 179, 63–72.

- Combs, T.P., Pajvani, U.B., Berg, A.H., Lin, Y., Jelicks, L.A., Laplante, M., Nawrocki, A.R., Rajala, M.W., Parlow, A.F., Cheeseboro, L., Ding, Y.Y., Russell, R.G., Lindemann, D., Hartley, A., Baker, G.R., Obici, S., Deshaies, Y., Ludgate, M., Rossetti, L., Scherer, P.E., 2004. A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. *Endocrinology* 145, 367–383.
- Das, K., Samanta, L., Chainy, G.B.N., 2000. A modified spectrophotometric assay of superoxide dismutase using nitrite formation by superoxide radicals. *Indian Journal of Biochemistry and Biophysics* 37, 201–204.
- Furukawa, S., Fujita, T., Shumabukuro, M., Iwaki, M., Yamada, Y., Makajima, Y., Nakayama, O., Makishima, M., Matsuda, M., Shumomura, I., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., Shimomura, I., 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. *The journal of clinical investigation* 114, 1752–1761. doi:10.1172/JCI200421625.1752.
- Gomez, O., Romero, A., Terrado, J., Mesonero, J.E., 2006. Differential expression of glucose transporter SLC2A8 during mouse spermatogenesis. *Reproduction* 131, 63–70.
- Hockenbery, D., Oltvai, Z., Yin, X.M., Milliman, L., Korsmeyer, S.J., 1993. Bcl-2 functions in an antioxidant pathway of apoptosis. *Cell* 75, 241–251.
- Huang, S., Lifshitz, L.M., Jones, C., Bellve, K.D., Standley, C., Fonseca, S., Corvera, S., Fogarty, K.E., Czech, M.P., 2007. Insulin Stimulates Membrane Fusion and GLUT4 Accumulation in Clathrin Coats on Adipocyte Plasma Membranes. *Molecular and Cellular Biology* 27, 3456–3469. doi:10.1128/MCB.01719-06.

- Jutte, N.H., Jansen, R., Grootegoed, J. a, Rommerts, F.F., Clausen, O.P., van der Molen, H.J., 1982. Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells. *Journal of reproduction and fertility* 65, 431–8. doi:10.1530/jrf.0.0650431.
- Kadivar, A., Heidari Khoei, H., Hassanpour, H., Golestanfar, A., Ghanaei, H., 2016. Correlation of Adiponectin mRNA abundance and its receptors with quantitative parameters of sperm motility in rams. *International Journal of Fertility and Sterility* 10, 127–135.
- Kahn, B.B., Alquier, T., Carling, D., Hardie, D.G., 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metabolism*. 1, 15–25. doi: 10.1016/j.cmet.2004.12.003.
- Lagaly, DV., Aad, PY., Grado-Ahuir, JA., Hulsey, LB., Spicer, LJ., 2008. Role of adiponectin in regulating ovarian theca and granulosa cell function. *Mol. Cell. Endocrinol.* 284, 38–45. doi:10.1016/j.mce.2008.01.007.
- Ledoux, S., Campos, DB., Lopes, FL., Dobias-goff, M., Palin, M., Murphy, BD., 2006. Adiponectin Induces Perioovulatory Changes in Ovarian Follicular Cells. *Endocrinology* 147, 5178–86. doi:10.1210/en.2006-0679.
- Loeken, M.R., 2012. A new role for pancreatic insulin in the male reproductive axis. *Diabetes* 61, 1667–1668. doi:10.2337/db12-0539
- Mantha, S.V., Prasad, M., Kalra, J., Prasad, K., 1993. Antioxidant enzymes in hypercholesterolemia and effects of vitamin E in rabbits. *Atherosclerosis*. 101, 135-44.
- Martin, LJ., 2014. Implications of adiponectin in linking metabolism to testicular function *Endocrine*. 46, 16-28. doi: 10.1007/s12020-013-0102-0.



- Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A Rapid , Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite 5, 62–71. doi:10.1006/niox.2000.0319.
- Murphy, M.P., 1999. Nitric oxide and cell death. *Biochimica et biophysica acta* 1411, 401–414. doi:10.1080/15216540152845993
- Ocón-Grove, OM., Krzysik-Walker, SM., Maddineni, SR., Hendricks, GL., Ramachandran, R., 2008. Adiponectin and its receptors are expressed in the chicken testis: influence of sexual maturation on testicular ADIPOR1 and ADIPOR2 mRNA abundance. *Reproduction* 136, 627-638. doi: 10.1530/REP-07-0446.
- Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95, 35–358.
- Oliveira, P.F., Alves, M.G., Rato, L., Silva, J., Sá, R., Barros, A., Sousa, M., Carvalho, R.A., Cavaco, J.E., Socorro, S., 2011. Influence of 5 $\alpha$ -dihydrotestosterone and 17 $\beta$ -estradiol on human Sertoli cells metabolism. *International Journal of Andrology* 34, e612–e620. doi:10.1111/j.1365-2605.2011.01205.x.
- Papaetis, G.S., Papakyriakou, P., Panagiotou, T.N., 2015. Central obesity, type 2 diabetes and insulin: Exploring a pathway full of thorns. *Archives of Medical Science* 11, 463–482. doi:10.5114/aoms.2015.52350
- Phillips K.P., Tanphaichitr N., 2010. Mechanisms of obesity-induced male infertility. *Expert Rev EndocrinolMetab.* 5, 229-251.
- Rak, A., Mellouk, N., Froment, P., Dupont, J., 2017. Adiponectin and resistin: potential metabolic signals affecting hypothalamo-pituitary gonadal axis in females and males of different

species. *Reproduction*. 153, 215-226. doi: 10.1530/REP-17-0002.

Rato, L., Alves, M.G., Socorro, S., Duarte, A.I., Cavaco, J.E., Oliveira, P.F., 2012. Metabolic regulation is important for spermatogenesis. *Nature Reviews Urol.* 9, 330–338. doi:10.1038/nrurol.2012.77.

Rocha, C.S., Martins, A.D., Rato, L., Silva, B.M., Oliveira, P.F., Alves, M.G., 2014. Melatonin alters the glycolytic profile of Sertoli cells: implications for male fertility. *Molecular Human Reproduction*. 20, 1067-76. doi: 10.1093/molehr/gau080.

Rossi, A., Lord, J.M., 2013. Adiponectin inhibits neutrophil apoptosis via activation of AMP kinase, PKB and ERK 1/2 MAP kinase, *Apoptosis*. 18, 1469–1480. doi:10.1007/s10495-013-0893-8.

Scherer, P.E., Williams, S., Fogliano, M., Baldini, G., Lodish, H.F., 1995. A Novel Serum Protein Similar to C1q, Produced Exclusively in Adipocytes. *The Journal of Biological Chemistry* 270, 26746-26749. doi:10.1074/jbc.270.45.26746.

Schoeller, E.L., Albanna, G., Frolova, A.I., Moley, K.H., 2012. Insulin rescues impaired spermatogenesis via the hypothalamic-pituitary- gonadal axis in Akita diabetic mice and restores male fertility. *Diabetes* 61, 1869–1878. doi:10.2337/db11-1527.

Singh, A., Krishna, A., 2012. Effects of adiponectin on ovarian folliculogenesis and steroidogenesis in the vesperilionid bat, *Scotophilus heathi*. *Gen. Comp. Endocrinol.* 178, 502-10. doi: 10.1016/j.ygcen.2012.06.027.

Swan, S.H., Elkin, E.P., 1999. Declining semen quality: Can the past inform the present? *BioEssays* 21, 614–621. doi:10.1002/(SICI)1521-1878(199907)21:7<614::AID-BIES10>3.0.CO;2-B.

- Tomas, E., Tsao, T.-S., Saha, A.K., Murrey, H.E., Zhang, C. c., Itani, S.I., Lodish, H.F., Ruderman, N.B., 2002. Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: Acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proceedings of the National Academy of Sciences* 99, 16309–16313. doi:10.1073/pnas.222657499.
- Verma, R., Krishna, A., 2017. Effect of Letrozole, a selective aromatase inhibitor, on testicular activities in adult mice: Both in vivo and in vitro study. *General and Comparative Endocrinology*. 241, 57–68. doi:10.1016/j.ygcen.2016.02.028.
- Villarroel-Espíndola, F., Maldonado, R., Mancilla, H., Ramírez, A., Vander Stelt, K., Cereceda, K., López, C., Castro, M.A., Angulo, C., Slebe, J.C., Concha, I.I., 2015. The Many Faces of Carbohydrate Metabolism in Male Germ Cells: From Single Molecules to Active Polymers. *Int. J. Med. Surg. Sci.*, 2, 603–619.
- Wu, L., Xu, B., Fan, W., Zhu, X., Wang, G., Zhang, A., 2013. Adiponectin protects Leydig cells against proinflammatory cytokines by suppressing the nuclear factor- $\kappa$ B signaling pathway. *FEBS J* 280, 3920–7. doi:10.1111/febs.12391.
- Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B.B., Kadowaki, T., 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature medicine* 8, 1288–95. doi:10.1038/nm788.
- Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., UchLabela, S., Takekawa, S., Waki,

H., Tsuno, N.H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., Kadowaki, T., 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423, 762–769. doi:10.1038/nature01683.1.

Yang, W.S., Lee, W.J., Funahashi, T., Tanaka, S., Matsuzawa, Y., Chao, C.L., Chen, C.L., Tai, T.Y., Chuang, L.M., 2001. Weight Reduction Increases Plasma Levels of an Adipose-Derived Anti-Inflammatory Protein, Adiponectin. *Journal of Clinical Endocrinology & Metabolism* 86, 3815–3819. doi:10.1210/jc.86.8.3815.

## List of Tables

**Table 1.** Details of antibodies used during the immunological experiments.

**Table 2.** Correlation studies. **(A)** Correlation of pAKT/AKT with IR and GLUT8 expression. **(B)**. Correlation of PCNA and Bcl2 with GLUT8, intra-testicular glucose, SOD, Catalase, and GPx.

## Figures Legends

**Figure 1.** Immunolocalization of adiponectin receptors (AdipoR1 and AdipoR2) in the adult mice testis sections. Representative 6  $\mu\text{m}$  sections, at two different magnifications, are presented. The AdipoR1 **(a & b)** positive immunostaining is mainly localized in the interstitial Leydig cells (Lc) (marked with black arrowhead). The AdipoR2 **(c & d)** positive immunostaining is localized in the seminiferous tubules (ST) mainly in the round spermatids ( $S_R$ ) (marked with red arrowhead). The negative control for AdipoR1 protein was shown **(e)** Scale bars, 50  $\mu\text{m}$  (a, c, e); 10  $\mu\text{m}$  (b & d).

**Figure 2.** Representative western blots and densitometric analysis of **(a)** AdipoR1, **(b)** AdipoR2, **(c)** AMPK, **(d)** IR, and **(e)** pAKT/AKT proteins in the mice testis after *in vitro* adiponectin treatments with low dose (0.1  $\mu\text{g}/\text{mL}$ ) and high dose (1  $\mu\text{g}/\text{mL}$ ) compared to control group. %RIDV, percentage relative integrated density value. Values are represented as mean  $\pm$  SEM. Value (\*) & (#) is significantly different ( $P < 0.05$ ) in both the doses versus control.

**Figure 3.** Representative western blots and densitometric analysis of **(a)** GLUT8, **(b)** MCT2, and **(c)** MCT4 proteins in the mice testis after *in vitro* adiponectin treatments with a low dose (0.1  $\mu\text{g}/\text{mL}$ ) and high dose (1  $\mu\text{g}/\text{mL}$ ) compared to control group. %RIDV, percentage relative integrated density value. **(d)** Changes in the intra-testicular glucose level and **(e)** testicular lactate dehydrogenase

(LDH) activity after *in vitro* adiponectin treatments with a low dose (0.1 µg/mL) and high dose (1 µg/mL) compared to control group. Values are represented as mean ± SEM. Value (\*) & (#) is significantly different ( $P < 0.05$ ) in both the doses versus control.

**Figure 4.** Representative western blots and densitometric analysis of (a) pERK1/2 and PCNA proteins, (b) Bcl2 and Caspase-3 proteins after *in vitro* adiponectin treated testis with a low dose (0.1 µg/mL) and high dose (1 µg/mL) compared to control group. %RIDV, percentage relative integrated density value. Values are represented as mean ± SEM. Value (\*) & (#) is significantly different ( $P < 0.05$ ) in both the doses versus control.

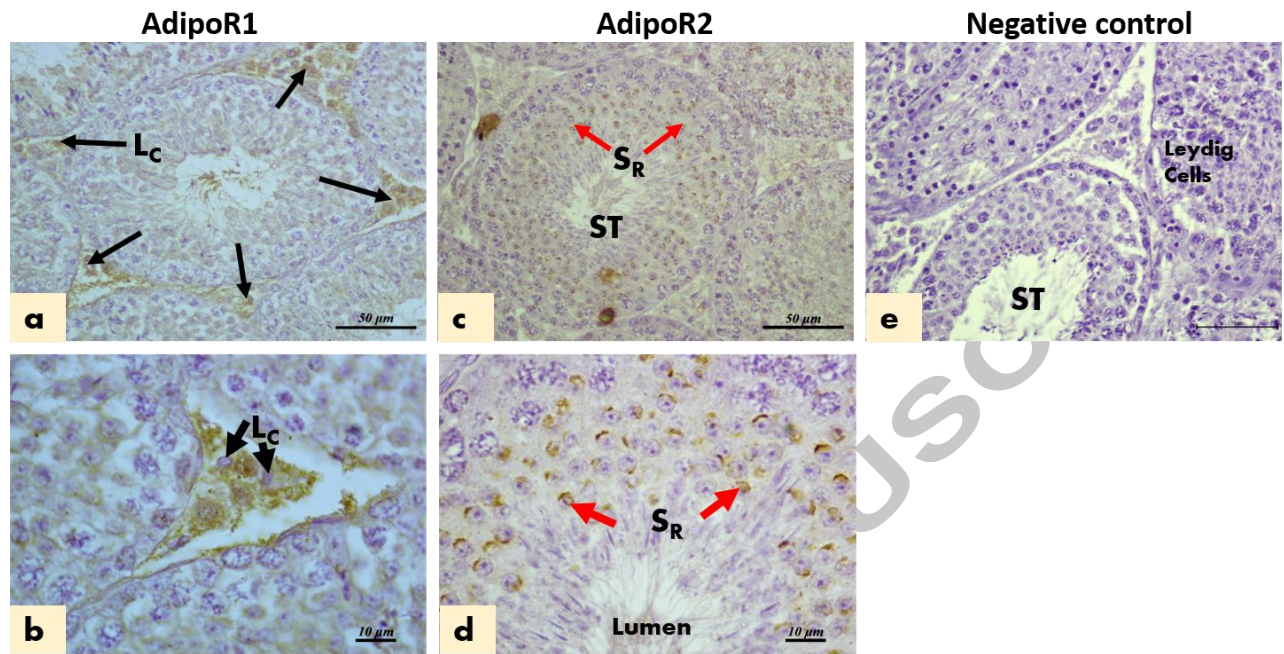
**Figure 5.** Changes in the activities of testicular antioxidative enzymes (a) SOD, (b) Catalase, (c) GPx (d) lipid peroxidation (LPO), and (e) total Nitrate/nitrite after *in vitro* adiponectin treatment with low dose (0.1 µg/mL) and high dose (1 µg/mL) compared to control group. Values are represented as mean ± SEM. Value (\*) & (#) is significantly different ( $P < 0.05$ ) in both the doses versus control.

**Figure 6.** (a) Showed changes in the intra-testicular testosterone (T) and intra-testicular estradiol (E2) level of control; low dose adiponectin (0.1 µg/ml APN) and high dose adiponectin (1 µg/ml APN) (b) Representative western blots and densitometric analysis of LH-R, StAR, Aromatase, and 3β-HSD proteins after *in vitro* adiponectin treated testis with a low dose (0.1 µg/mL) and high dose (1 µg/mL) compared to control testis. %RIDV, percentage relative integrated density value. Values are represented as mean ± SEM. Value (\*) & (#) is significantly different ( $P < 0.05$ ) in both the doses versus control.

**Figure 7.** Schematic illustration of adiponectin signal transduction pathway implicating a crosstalk with the insulin signaling pathway in the regulation of Sertoli cells and germ cells proliferation,

survival, apoptosis, metabolism, and oxidative stress status. Activation of insulin (IR) and adiponectin receptors (AdipoRs) by adiponectin triggers a cascade of signaling events. Most of the metabolic effects of insulin are mediated through phosphorylation of AKT, leading to transport of glucose in the Sertoli cells. Sertoli cells are capable of metabolizing a variety of energetic fuels, including glucose and lactate. Adiponectin-mediated activation of AMPK is the probable mechanism by which adiponectin enhances both glucose uptake and Lactate dehydrogenase (LDH) activity. LDH is the enzyme responsible for the conversion of glucose 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 prevent apoptosis during the normal process of spermatogenesis. Apart from this activation of AdipoRs directly triggers germ cell growth (pERK), proliferation (PCNA), survival (Bcl2), apoptosis (Caspase-3), and antioxidant (SOD, Catalase, GPx), thereby regulating testicular oxidative and nitrosative stress.

Figure 1.





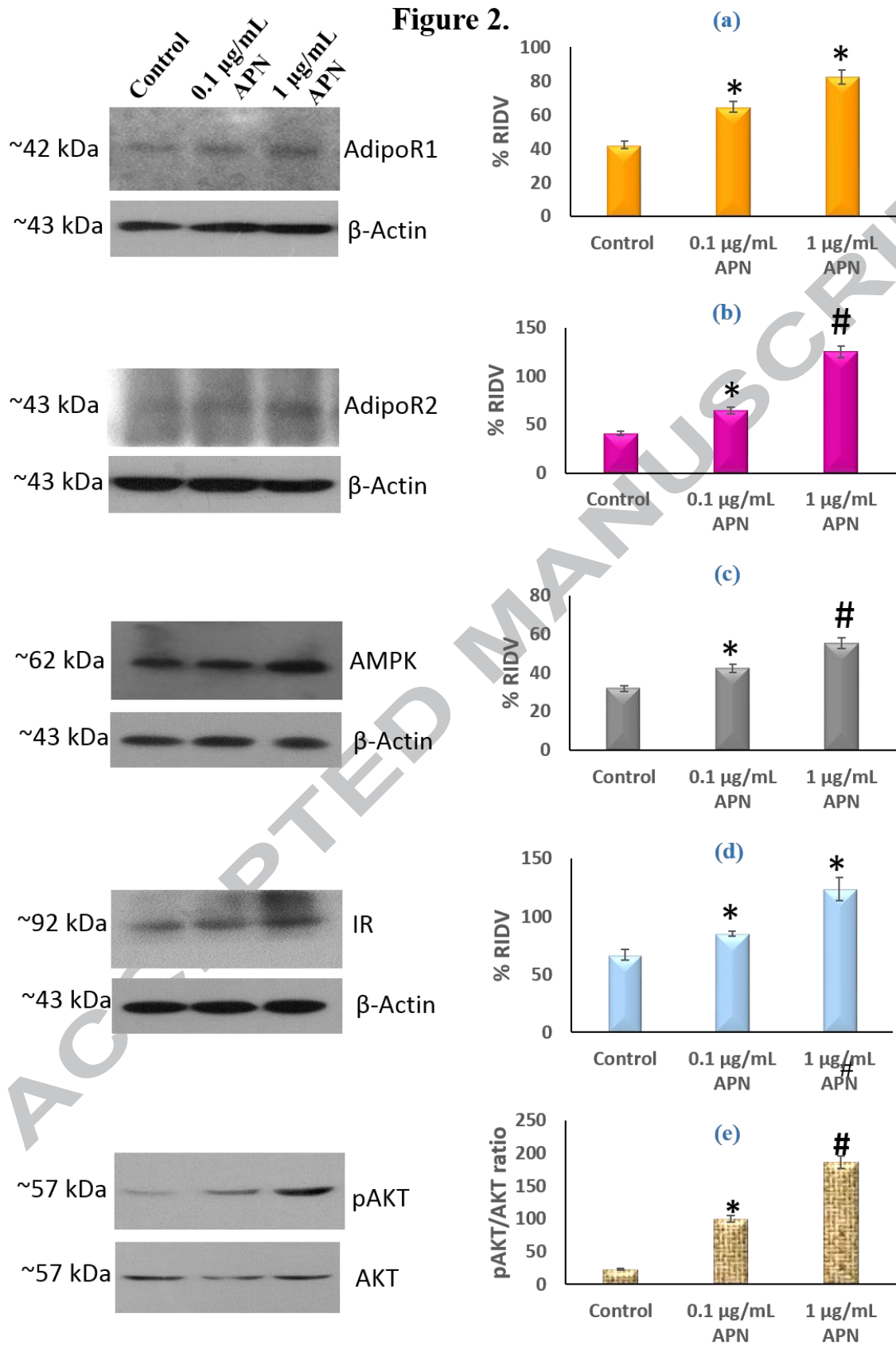


Figure 3.

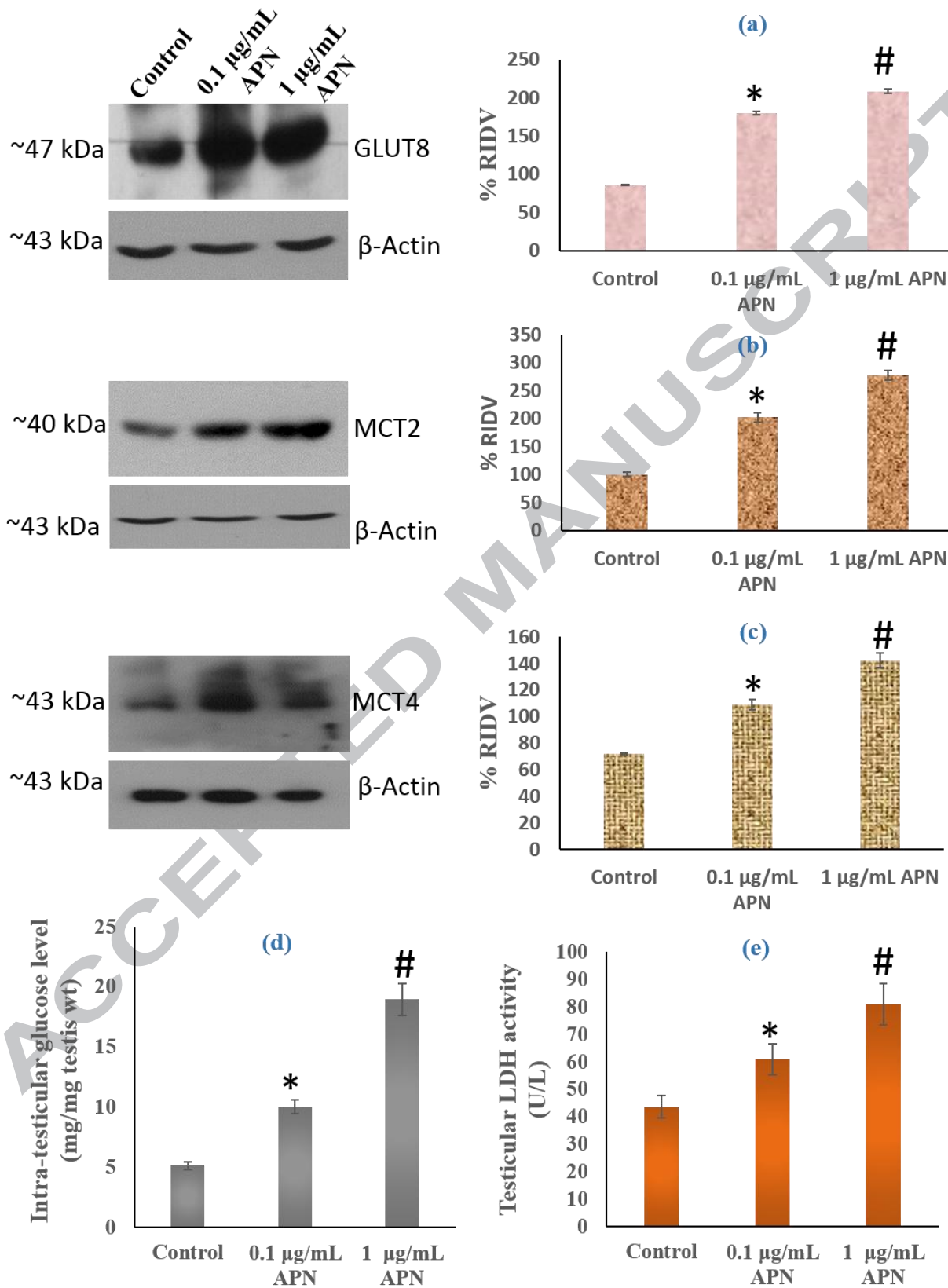


Figure 4.

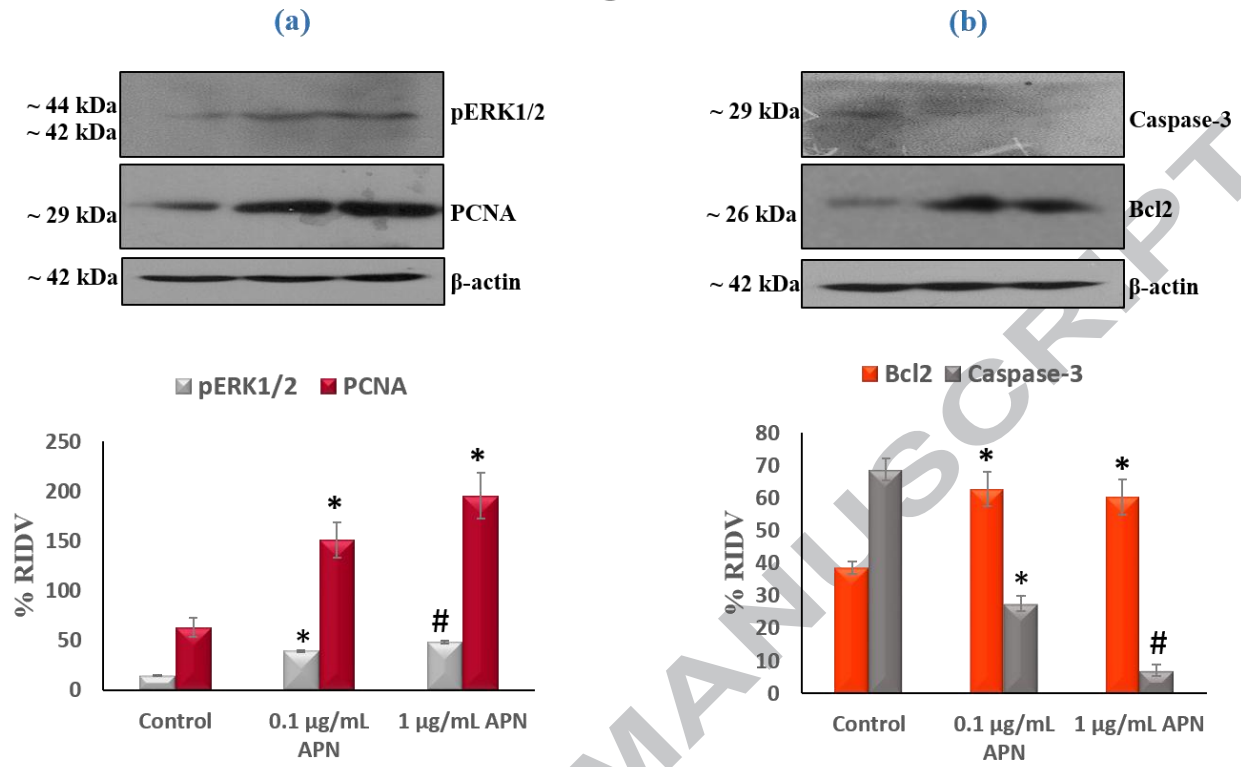


Figure 5.

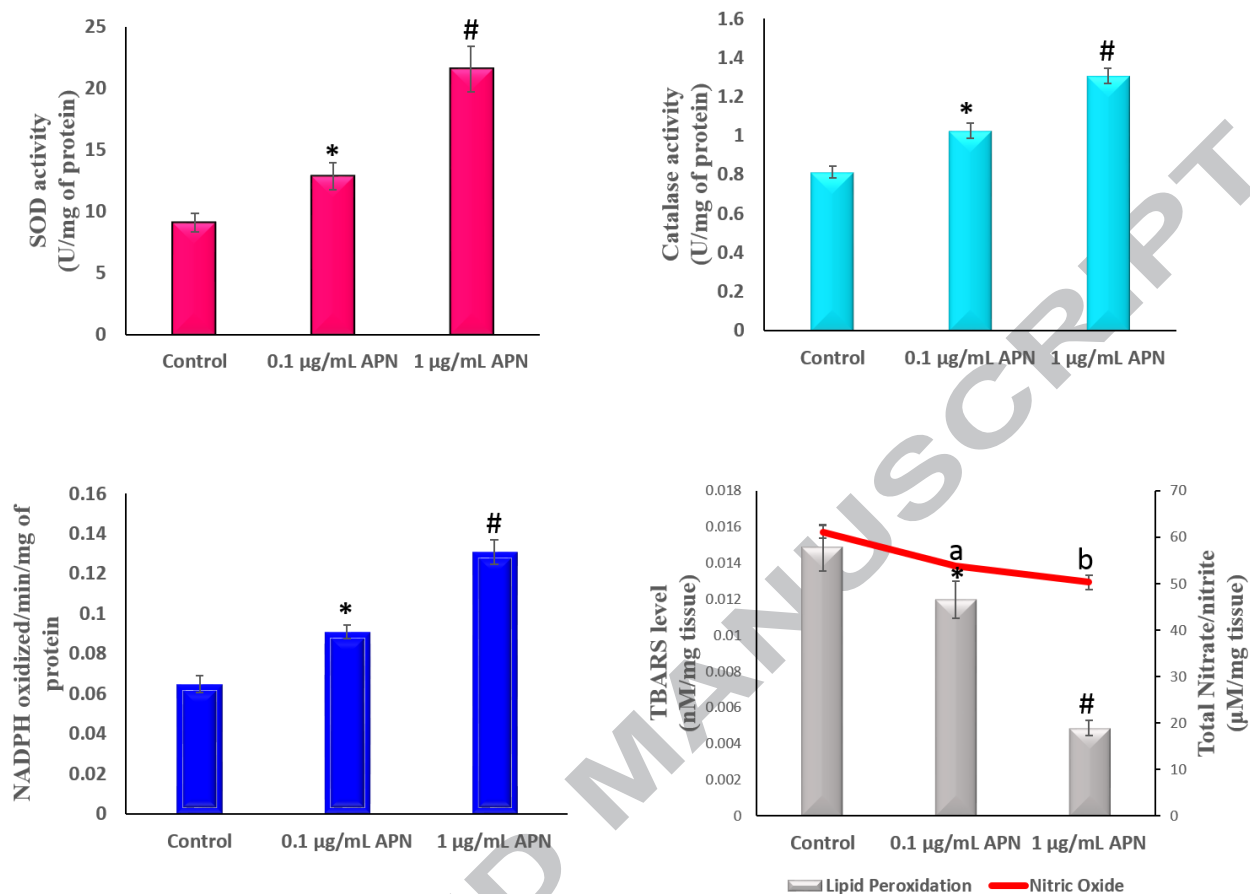


Figure 6.

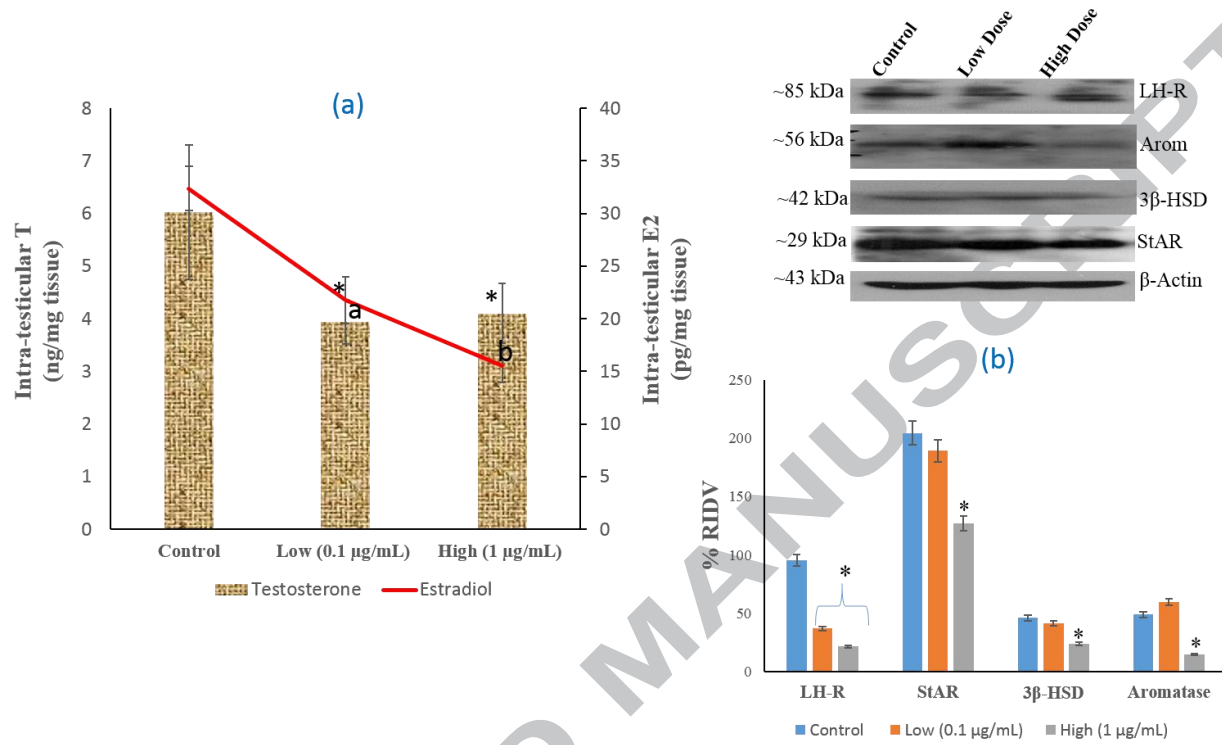
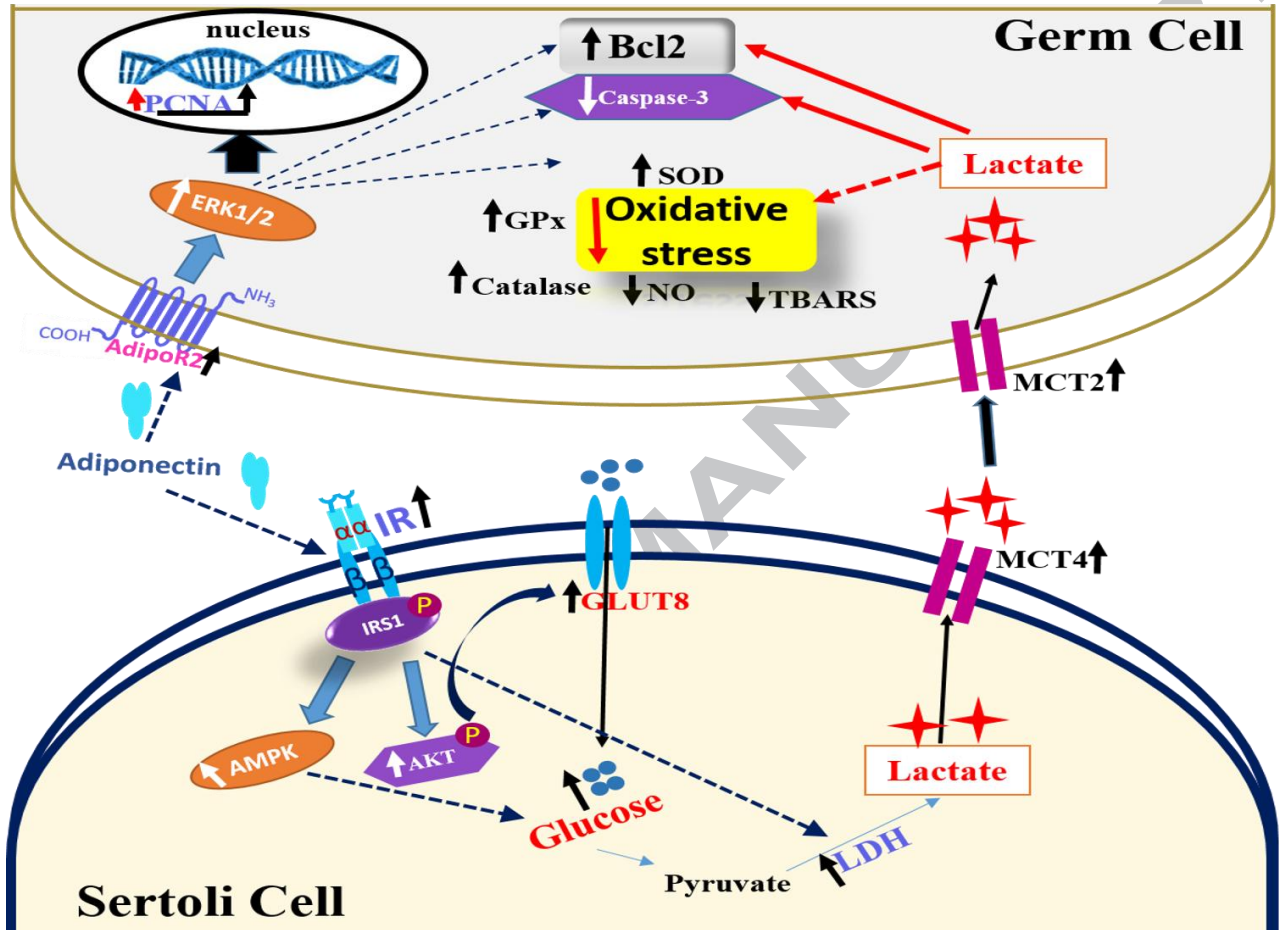


Figure 7.



**Table 1. Details of the antibodies used for Immunohistochemistry and Immunoblotting experiments.**

S.No.	Antibody	Species raised in; Monoclonal/Polyclonal	Source	Dilutions (used for Western blot)
1	AdipoR1	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:300 (WB) 1:50 (IHC)
2	AdipoR2	Rabbit; Polyclonal	Thermo Fisher Scientific Inc.	1:150 (WB) 1:25 (IHC)
3	AMPK	Rabbit; Polyclonal	Genscript	1:1000
4	IR	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:1000
5	AKT	Rabbit; Polyclonal	Genscript	1:500
6	pAKT	Rabbit; Polyclonal	Genscript	1:300
7	GLUT8	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
8	MCT2	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:300
9	MCT4	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
10	pERK1/2	Rabbit; Polyclonal	Genscript	1:500
11	PCNA	Rabbit; Polyclonal	Thermo Fisher Scientific Inc.	1:1500
12	Bcl2	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:1000
13	Caspase-3	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:250
13	LH-R	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
14	STAR	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:1500
15	HSD3b	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:500
16	Aromatase	Rabbit; Polyclonal	Santacruz (Biotechnology Inc., CA, USA)	1:600

**Table 2. Correlation study**

(a) Correlation of pAKT/AKT with IR and GLUT8 expression.

Parameters	pAKT/AKT
<b>IR</b>	0.928*
<b>GLUT8</b>	0.941*

\* Values are significantly different at  $p < 0.05$  level.

(b) Correlation of PCNA and Bcl2 with GLUT8, intra-testicular glucose, SOD, Catalase, and GPx.

Parameters	GLUT8	Testicular glucose level	SOD	Catalase	GPx
<b>PCNA</b>	0.885*	0.889*	0.904*	0.882*	0.78*
<b>Bcl2</b>	-	-	0.83*	0.709*	0.772*

\* Values are significantly different at  $p < 0.05$  level.



**Highlights**

- AdipoR1 is localized in the Leydig cells and AdipoR2 is localized in the seminiferous tubules mainly round spermatids in the testis of adult mice.
- Adiponectin effectively facilitates cell proliferation and survival, and protects from apoptosis.
- Adiponectin treatment showed a significant reduction in oxidative and nitrosative stress.
- Systemic adiponectin treatment may be a promising therapeutic strategy for the improvement of testicular metabolism and hence sperm count.