EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 4
317-329
Year: 2017

KISSPEPTIN ACTS AS A COMMON MESSENGER IN THE PATHWAYS CONTROLLING NUTRITION AND REPRODUCTION IN MALE MOUSE

Shabana Anjum[1] and Amitabh Krishna*[1]

[1]Department of Zoology, Banaras Hindu University, Varanasi 221005, UP, India.

***Corresponding Author: Amitabh Krishna**
Department of Zoology, Banaras Hindu University, Varanasi 221005, UP, India.

Article Received on 01/02/2016 **Article Revised on 21/02/2017** **Article Accepted on 13/03/2017**

ABSTRACT
The aim of the present study was to evaluate the role of Kisspeptin (KP-10) as a modulator of changes in adipose tissue and testicular activity. To achieve this, mice were treated with KP- 10 with different doses (20 pg, 2ng and 2µg/day) for 15 days and evaluated the metabolic and hormonal changes in adipose tissue and testis.The results of this study showed differential effect of KP-10 on changes in metabolic factors of adipose tissue and testis of mice. In adipose tissue, KP-10 promoted uptake of nutrients (triglycerides and glucose) from circulation and resulted in increased accumulation of white adipose tissue. Whereas in the testis, KP-10 caused down-regulation of GLUT8 expression resulting in decreased uptake of glucose, which in turn resulted in decreased synthesis of testosterone. The mice treated with KP-10 showed decreased synthesis of adiponectin in the adipose tissue and decreased expression of AdipoR1 in the testis. The decreased adiponectin synthesis in adipose tissue in turn may be responsible for decreased testicular activity in the mice. The *in vitro* treatment of KP-10 showed increased uptake of nutrients in adipose tissue but inhibitory changes in testis. These findings thus suggest that KP-10 showed direct role in modulating energy balance in adipose tissue, which is reciprocally linked to testicular activities in mice.

KEYWORDS: Kisspeptin, Adiposity, GLUT8, Adiponectin, testis, white adipose tissue.

INTRODUCTION
It is well established that reproductive activity in mammals is associated with nutritional status (Dupont *et al.*, 2014; Wade and Jones, 2004; Wade *et al.*, 1996). Puberty is attained by the animal when appropriate level of body weight or nutrients levels are achieved, whereas nutritional or nutrient deficiency delays the onset of puberty (Kennedy and Mitra, 1963; Foster and Olster, 1985). Any disturbances in nutrients levels such as obesity or under nutrition resulted in fertility impairment (Pasquali *et al.*, 2007). The negative energy balance, when less energy (food) is consumed, inhibit reproductive axis. It has earlier been demonstrated in male rats that food restriction decreased gonadotropin and testosterone synthesis as compared with *ad libitum* fed controls (Compagnucci *et al.*, 2002). Fasting also reduces kisspeptin (KP) mRNA expression (Brown *et al.*, 2008; Castellano *et al.*, 2005; Luque *et al.*, 2007), which precedes the fasting–induced decline of GnRH. Both KP mRNA and gonadotropins are decreased in diabetic or starved animals. The reproductive tissues appears to have a number of "nutrient sensing" mechanism that may link nutrient status to the reproductive system.

Glucose is one of the important nutrients and it is vital for the normal functioning of reproductive processes, including formation of mature germ cells. The passage of glucose across cell membranes is facilitated by a family of integral transport proteins, the glucose transporters (GLUT) (Simpson *et al.*, 2008). GLUTs in cells act as glucose sensors. Metabolic-associated hormones, such as insulin, play crucial roles in the relationship between changes in nutritional levels and reproduction (Crown *et al.*, 2007). The anabolic actions of insulin on peripheral tissues are well established and plasma insulin also serves as a signal of body fat content to the central nervous system (Schwartz *et al.*, 1992). Insulin also amplifies the lipogenesis in adipose tissue (Conway and Jacob, 1993). Glucose is made available in the body by insulin, which helps to lower the level of circulating glucose by promoting its uptake either in adipose tissue or muscle cells through GLUT4. In the gonads, glucose is essential for maintenance of spermatogenesis *in vivo* (Rato *et al.*, 2012; Banerjee *et al.*, 2014). The isoforms of glucose transporters are also expressed in the testis (Kim and Moley, 2007). GLUT8 appears to be one of the main glucose transporters in testis (Doege *et al.*, 2000). Further it has been demonstrated that adequate amount of glucose transporter is required for proper testicular activity (Doege *et al.*, 2000). Insulin and its

receptors (IR) are expressed in both somatic (Leydig, Sertoli and peritubular cells) and germ cells in the testis of various species (Vannelli *et al.*, 1988).

Adipose tissue is an organ involve in passively storing triglycerides/free fatty acid during the time of nutritional affluence and for mobilization during the period of nutrients deprivation. Mature adipocytes synthesize and secrete numerous hormones called adipokines that are involved in overall energy homeostasis and also modulates reproductive activities. Adipose tissue is also known to regulate metabolic processes Including lipid metabolism, glucose homeostasis, insulin sensitivity etc. The adipose tissue is now emerging as an important factor in the complex equation by which the nutritional state regulates reproductive function **(**Dupont *et al.*, 2014**).** Whereas testicular hormone, testosterone, is known to affect adipocytes proliferation, differentiation and fat mass distribution, controlling metabolic function such as food intake,glucose homeostasis, insulin sensitivity etc**.** (Mammi *et al.*, 2012). Several studies have shown negative relationship between adiposity and testicular function (Stewart and Baker, 2008). The mechanism by which nutritional status modulates reproductive processes, there by food restriction inhibits reproduction and increased food intake reactivates it, requires detailed investigation.

The factor that acts as an integrator between adipose tissue and testis is not yet known. Expression of KP/GPR54 has earlier been demonstrated in a number of peripheral tissues, including anterior pituitary, testis, ovary, pancreas, adipose tissue, etc (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001). Role of KP in reproductive processes are well demonstrated (Caraty *et al.*, 2012). KP has also been shown to affect secretion of various metabolic hormones, such as adiponectin, insulin, growth hormone and prolactin (Hauge-Evans *et al.*, 2006; Kadokawa *et al.*, 2008 a, b; Wahab *et al.*, 2010, Wahab *et al.*, 2011). These studies suggest a potential role for KP in connecting nutritional status with reproductive function. Restricted feeding and fasting reduces hypothalamic expression of KP in rodents (Luque *et al.*, 2007), sheep (Beckholer *et al.*, 2010) and primates (Wahab *et al.*, 2011). KP neurons in the hypothalamus are an important component by which the reproductive axis sense nutritional state. Castellano *et al.* (2005) used long-term calorie restriction to inhibit the occurrence of puberty in female rats. Treating these rats with KP rescued gonadotropin secretion and induces puberty; these studies thus clearly suggest. that KP may have a role in integrating the effects of energy balance with the gonadal activities.

Since KP/GPR54 are shown to express both in tissues those governing reproduction such as testis or governing energy homeostasis such as adipose tissue, therefore the aim of this study was to determine the role of KP-10 as modulator of testicular (site of reproduction) and adipose tissue (concerned with energy homeostasis) activities. This was achieved by investigating the effect of KP on changes in metabolic and hormonal factors in the testis and adipose tissue by *in vivo* study and in each of these two tissues separately by *in vitro* study.

MATERIAL AND METHODS

Sample Collection

All experiments were conducted in accordance with principles and procedures of the 2002 Animal act, India, and approved by Animal Ethical Committee, Banaras Hindu University. Adult male laboratory mice (*Mus musculus*) of Parkes strain were obtained from the inbred colony maintained in our animal house. The adult mice (13 weeks old) of nearly equal body weight were used in this study. Mice were housed under optimum conditions of temperature and humidity in a photoperiodically controlled room (12 hr light:12 hr dark) and were provided with commercial food (Pashu Aahar Kendra, Varanasi, India) and tap water *ad libitum*.

Chemicals

Insulin was obtained from Torrent Pharmaceuticals Ltd, Mehsana, Gujrat, India. Antibodies to insulin receptor β-subunit (IR), AdipoR1, GLUT4 and GLUT8 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and adiponectin was purchased from GeneScript USA Inc. (Piscataway, NJ, USA), respectively. All other chemicals were purchased from Merck, New Delhi, India.

The in vivo study

Mice were injected, intraperitoneally, with three different doses of KP-10 (20 pg, 2 ng and 2 µg/day) dissolved in normal saline daily for 15 days (N=10 per group). Mice in the control group received vehicle only. The dose of KP-10 was selected based on previous studies (Thompson *et al.*, 2006; Ramzan and Qureshi, 2011). The animals were sacrificed by decapitation under mild dose of anesthetic ether, 24 hr after the last injection and blood was collected. Body mass of each mouse was recorded before killing. Testes and adipose tissue (accumulated in the abdominal region) of the control and treated mice were excised out, cleaned, and weighed and stored at -40^0C for immunoblot analysis. Serum was collected and stored at -20^0C till used for testosterone, glucose and triglyceride assay.

The in vitro study

Testis culture: Adult testes (n=8 testes) were quickly dissected out and cleaned of any adhered fat tissuein Dulbecco Modified Eagle's Medium (DMEM; Himedia, Mumbai, India) containing 250 i.u./ml penicillin and 250 mg/ml streptomycin sulfate. The testis were cut into equal slices (approximate 10 mg in weight) and cultured by the method as described previously (Anjum *et al.*, 2012). Culture medium was a mixture of DMEM (with sodium pyruvate andL-

glutamine) and Ham's F-12(1:1;v:v) Himedia, Mumbai,India) containing 100 U/ml penicillin, 100 µg/ml streptomycin,and 0.1% BSA (Sigma, USA). After initial incubation for 2 hr at 37^0C, culture medium was discarded and testes slice (one per tube) were finally cultured in 1 ml medium containing 5 pg/ml, 10 pg/ml and 1ng/ml of KP-10 along with 10 ng/ml and 100 ng/ml of LH in a humidified atmospherewith 95% air and 5% CO_2 to maintain pH 7.4 for 24 hr at 37^0C. Each treatment group was run intriplicate. Testes were cultured under these conditions remained healthy and did not show any sign of necrosis. Testes slices were collectedat the end of culture, washed several times with PBS and stored at –40^0C for immunoblot study and media were collected, stored at -40^0C until used for testosterone and glucose uptake assay.

Adipocytes culture: Visceral (Abdominal) white adipose tissue (WAT) was collected from adult mouse (N=6) to determine the *in vitro* effects of insulin on GLUT4, GLUT8 and *Insulin receptor* protein expression in white adipose tissue of male mouse. We assayed these biochemical markers at three doses of insulin. Culture methods for WAT were adopted according to Roy and Krishna. (2011). Following collection, WAT was quickly cut into pieces in delbecco's modified Eagle's medium (DMEM; Himedia, Mumbai, Mahrashtra India) containing 250 i.u./ml penicillin and 250 mg/ml streptomycin sulphate. Pieces of WAT of equal mass were cultured in a mixture of DMEM (with sodium pyruate and L-glutamine) and Ham's F-12 (1/1 v/v) (Himedia) containing 100 i.u./ml penicillin. 100 mg/ml streptomycine and 0.1 % bovine serum albumin (Sigma. St Louise,MO, USA). After initial incubation for 2 hr at 37^0C, the culture medium was discarded and pieces of WAT were cultured in 1 ml medium containing either 1 µg/ml, 5 µg/ml or 10 µg/ml of insulin or 1 ng/ml of KP-10 in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 24 hrs at 37^0C. Each treatment group was run in triplicate. After culture, the WAT was collected, washed several times with phosphate buffer saline (PBS) and kept frozen at -40^0C until immunoblot assay and media were collected, stored at –40°C until used for glucose uptake assay.

IMMUNOBLOTTING

The testes and white adipose tissues collected from the *in vivo* and *in vitro* studies were processed for protein extraction using the method described earlier (Cifuentes *et al.*, 2005, Lowry *et al.*, 1951). Western blot analysis was performed as previously described (Srivastava and Krishna, 2010). In brief, a 20% homogenate (w/v) of adipose tissue was made in suspension buffer containing 0.1 mol/l NaCl, 0.01 mol/l Tris-HCl (pH 7.6), 0.001 mol/l EDTA (pH 8.0) and 10 µ gm/l phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 5000 ***g*** and at 4°C for 15 min; the supernatant was extracted with an equal volume of chloroform and the aqueous phase was recovered. Equal amounts of proteins (40 µg) as determined by Folin's method were used for 10% SDS-PAGE. Thereafter, proteins were transferred electrophoretically to a PVDF membrane (Millipore India Pvt. Ltd, Bangalore, Karnataka, India) overnight at 4°C. Membranes wereblocked for 60 min with Tris-buffered saline [TBS; Tris 50 mmol/l (pH 7.5), NaCl 150 mmol/l 0.02% Tween 20] containing 5% fat-free dry milk. The membranes were further incubated with primery antibody **(see table 1)** for 1 hr in blocking solution. Immunoreactive bands were revealed by incubating the membranes with biotinylated secondary antibody (at a dilution of 1:2000; Vector Laboratories, Burlingame, CA, USA) for 30 min followed by three washings with PBS (0.2mol/l, pH7.4) for 10 min each. After washing, the blots were incubated with avidin–peroxidase conjugate (at a dilution of 1:2000; Vector Laboratories) for 30 min. Finally, the blot was washed three times with PBS and developed with an enhanced chemiluminescence (ECL) detection system (Bio- Rad, Hercules, CA, USA). Similarly, a blot was developed for β-actin (Sigma, Aldritch, India) at a dilution of 1:1000 as a loading control. Immunoreactive bands were later quantified using Image J software (Image J 1.36, NIH, and Bethesda, MD, USA).

Testosterone assay

The ELISA kit for testosterone assay was purchased from Diametra, Folingo (PG) Italy (LOT No: DKO002). To each well of the ELISA plat 25 µl of standard, control or sample was added. Subsequently, 100 µl of the diluted conjugate solution was added to each of these wells. The ELISA plate was then incubated with mild shaking at 37ºC for 1 hr and then aspirated the wells and washed several times with distilled water. Then, 100 µl of the tetra methyl benzidine chromagen substrate was added to each well and the plate was incubated at room temperature for 15 min in the dark. Finally, 100 µl of stop solution was added and absorbance was taken at 450 nm using a microplate reader. The standard curve ranged from 0.2 ng/ml to 16 ng/ml.

Glucose assay

Blood glucose was measured by the glucose oxidase method using a commercially available automated glucose analyzer (Span Diagnostics Ltd, Surat, Gujrat, India) with 10 µl of blood.

Triglycerides levels in the serum and adipose tissue

Triglyceride (TG) in blood was measured using a commercially available automated glucose analyzer (Span Diagnostics Ltd, Surat, Gujrat, India) and TG in WAT was measured with minor modifications (Paglialunga *et al.*, 2007; Lee *et al.*, 1994). A 20% homogenate (w/v) of WAT was prepared in PBS. Then TG was extracted from the homogenate overnight in heptane:isopropanol (3:2) at 4°C. TG content was

measured using a colorimetric kit (GPO-Trinder) from Span Diagnostics Ltd, Surat, Gujrat, India. Results are expressed as mg TG mg^{-1} protein.

Glucose Uptake Assay: *in vitro* study

The media stored at -20 °C was used for glucose uptake assay according to Roy and Krishna. (2011) and Banerjee *et al*. (2014), described earlier to determine the glucose by quantitative colorimetric method. The glucose concentration of the media was measured at the beginning as well as at the end of culture and the difference between initial concentration of media and final concentration of media after the 24 hr culture was taken as the amount of glucose uptake by the WAT or testis. Each group was run in triplicates. The intra-assay coefficient of variation (CV) was <7.5%.

STATISTICAL ANALYSIS

Data were analyzed using one way ANOVA followed by Bonferroni's test using SPSS software 16 for Windows (SPSS Inc, Chicago, IL, USA). Correlation studies were performed to compare data from different groups. The differences were considered significant at the level of $P<0.05$.

RESULTS

Effects of *in vivo* treatment of KP-10 on

Body mass and adipose tissue mass:

Mice treated with KP-10 showed varied effect on body mass **(table 2)**. The mice treated with low dose (20 pg/day) showed a marginal increase in body mass. But body mass increased significantly ($p<0.05$) when treated with moderate dose (2 ng/day) of KP-10, whereas, the body mass decline significantly ($p<0.05$) when treated with high dose (2 µg/day) of KP-10 as compared with the control **(see table 2)**. The WAT mass also changes in response to treatment with different doses of KP-10 similar to that of body mass, but differences are more distinct as compared with the body mass. WAT mass increase significantly in dose-dependent manner in response to low and moderate doses of KP-10 treatment, but declined significantly in response to high dose of KP-10 treatment as compare with the control. **(see table 2)**

Circulating glucose, triglyceride and testosterone levels:

The mice treated with different doses of KP-10 (low dose=20 pg, moderate dose=2 ng and high dose=2 µg/day) showed significant decrease in circulating triglycerides and glucose levels as compared with the control. Both triglycerides and glucose levels dose-dependently decreased significantly ($p<0.05$) in response to low and moderate dose of KP-10 treatment. However, both glucose and triglycerides levels increased significantly ($p<0.05$) when treated with high dose in comparison to moderate dose of KP-10 treatment **(see table 2**). The mice treated with different dose of KP-10 (low dose= 20 pg, moderate dose=2 ng and high dose=2 µg /day) showed dose-dependent significant ($p<0.05$) decline in circulating testosterone levels as compared with the control **(Fig. 1 A).**

***Changes in the level of triglycerides and expression of adiponectin, GLUT 4 and 8 and insulin receptor* proteins in the adipose tissue**

The mice treated with different doses of KP-10 (low dose= 20 pg, moderate dose= 2 ng and high dose= 2 µg/day) showed significant ($p<0.05$) increase in triglycerides level in the adipose tissue as compare with the control mice. But the level of TG declines with the increasing dose of KP-10, whereas high dose of KP-10 treatment caused significant decrease in TG level as compare with the value of TG in moderate dose of KP-10 treated group **(see table 2).**

The mice treated with different doses of KP-10 showed significant ($p<0.05$) decrease in the expression of adiponectin protein in the adipose tissue. Both low and moderate doses showed a dose-dependent decline in the adiponectin expression in adipose tissue. However, high dose of KP-10 treatment showed a significant increase in the expression of adiponectin as compared with moderate dose of KP-10 treated group **(Fig. 2 A).** The mice treated with moderate and high doses of KP-10 showed significant ($p<0.05$) increase in the expression of insulin receptor proteins in the adipose tissue as compare with the control (**Fig. 2 B).** Low dose of KP-10 treatment showed no significant changes in the expression of insulin receptor proteins in the adipose tissue as compare with the control.

The mice treated with moderate dose of KP-10 showed significant ($p<0.05$) increase in the expression of GLUT4 protein in the adipose tissue as compare with the control, whereas high dose of KP-10 treatment showed significant decrease in the expression level of GLUT4 protein in the adipose tissue as compare with the control **(Fig. 3 A).** The mice treated with different doses of KP-10 showed dose-dependent significant increase in the expression level of GLUT 8 proteins in the adipose tissue as compare with the control **(Fig. 3 B).**

***Changes in the level of glucose and expression of AdipoR1, GLUT 8 and insulin receptor* proteins in the testis**

The mice treated with low dose of KP-10 showed significantly ($p<0.05$) increased level of testicular glucose, whereas when treated with moderate and high doses showed decreased level of glucose in the testis as compared with the control **(see table 2)**. The mice treated with different doses of KP-10 showed significant ($p<0.05$) decline in the expression of AdipoR1 protein in the testis. Both low and moderate doses showed a dose- dependent decline in the AdipoR1 expression in testis. However, high dose of KP-10 treatment showed a significant increase in the expression of AdipoR1 as compared with moderate

dose of KP-10 treated group **(Fig. 4)**.

The mice treated with the low dose of KP-10 showed a marginally significant increase in expression of insulin receptor protein in the testis as compared with the control. But the mice treated with moderate and high doses of KP-10 showed significantly sharp decreased in expression of insulin receptor protein in the testis as compared with
the control. **(Fig. 5 A).** The mice treated with different doses of KP-10 showed dose- dependent significant decline in the expression of GLUT8 protein in the testis. The mice treated with low dose of KP-10 showed significant increased expression of GLUT8 protein in the testis as compared to control, whereas treatment with high dose of KP-10 showed significant decreased expression of GLUT 8 protein in the testis as compared with the control **(Fig. 5 B).**

Effects of *in vitro* treatment of KP-10 with or without LH in the testis of mice

Effect on testosterone synthesis

The testis treated in vitro with KP-10 (1 ng/mL) showed significant ($p<0.05$) decrease in testosterone synthesis as compare with the control group. Treatment with both low (10 ng/mL) and high (100 ng/mL) doses of LH alone showed dose-dependent significantly ($p<0.05$) increased level of testosterone synthesis by the testis as compared with the control. Treatment with low dose of LH (10 ng/mL) with KP-10 (1 ng/mL) showed significantly ($p<0.05$) increased, whereas treatment with high dose of LH (100 ng/mL) with KP-10 (1 ng/mL) showed significantly decreased level of testosterone synthesis by the testis as compared with the control (Fig. 6 A)

Effect on Glucose uptake

The testis treated *in vitro* with KP-10 (1 ng/mL) showed significantly ($p<0.05$) complete suppression of glucose uptake **(Fig. 6 B)**. Treatment with both low (10 ng/mL) and high (100 ng/mL) doses of LH alone showed dose-dependent significantly ($p<0.05$) increased in glucose uptake by the testis as compared with the control. Treatment with low dose of LH (10 ng/mL) with KP-10 (1 ng/mL) showed significantly ($p<0.05$) increased, whereas treatment with high dose of LH (100 ng/mL) with KP-10 (1 ng/mL) showed significantly decreased uptake of glucose by the testis as compared with the control **(Fig. 6 B).**

Effect on expression of GLUT 8 protein:

The testis treated *in vitro* with KP-10 (1 ng/mL) showed statistically no significant change in expression of GLUT8 protein **(Fig. 6 C).** However the testis treated *in vitro* with two different doses of LH (low dose=10 ng/mL and high dose=100 ng/mL) showed dose-dependent significant ($p<0.05$) increase in the expression of GLUT8 protein. Treatment with low dose of LH (10 ng/mL) with KP-10 (1 ng/mL) *in vitro* showed a significant increase in expression of GLUT8 protein, whereas treatment with high dose of LH (100 ng/mL) together with KP-10 (1 ng/mL) showed significantly ($p<0.05$) decreased expression of GLUT8 protein in the testis as compared with the testis treated with KP-10 and LH alone **(Fig. 6 C).**

Effects of *in vitro* treatment of kisspeptin-10 with or without insulin to the adipose tissue of mice

***Effect on Glucose uptake*:**

The adipose tissue treated *in vitro* with different (low=5 pg, moderate=10 pg and high=1 ng/mL) doses of KP-10 showed dose-dependent significant ($p<0.05$) variation in glucose uptake (**Fig. 7 A)**. The adipose tissue treated with low and moderate doses of KP-10 showed significantly ($p<0.05$) increased uptake of glucose as compared with the control group, whereas high dose of KP-10 showed significantly ($p<0.05$) decreased level of glucose uptake as compared with the control group. Whereas, treatment with high doses of KP-10 (1 ng/mL) together with insulin (10 µg/mL) showed significantly ($p<0.05$) increased level of glucose uptake by the adipose tissue as compared with the control group (**Fig. 7 A).**

Effect on expression of GLUT 4 protein in adipose tissue

The adipose tissue treated *in vitro* with different (low=5 pg, moderate=10 pg and high=1 ng/mL) doses of KP-10 showed dose-dependent significant variations in expression of GLUT4 protein **(Fig. 7 B)**. The adipose tissue treated with low and moderate doses of KP-10 showed significantly ($p<0.05$) increased expression of GLUT4 protein as compared with the expression of GLUT4 protein in the control group, whereas, high dose. of KP-10 showed significantly ($p<0.05$) decreased expression of GLUT4 protein as compared with the control group. Treatment with high doses of KP-10 (1 ng/mL) together with insulin (10 µg/mL) showed significantly increased level of expression of GLUT 4 protein by the adipose tissue as compared with the control group (**Fig. 7 B).**

Correlation study

The result of correlation studies are summarized in table **3** and **4**. The significant ($p<0.05$) correlation was found between changes in circulating testosterone levels, glucose levels and triglyceride levels with changes in the rate of expression of GLUT4, 8 and insulin receptor in adipose tissue and testis of mice treated with KP-10.

Table.1 Details of antibodies used for Western blot.

Antibody	Target species	Species raised in; Monoclonal/ Polyclonal	Source	Concentration (used for Westernblot)
GLUT4	Human	Rabbit; Polyclonal	Santa cruz Biotechnology Inc. (H-61, SC 7938)	1:500
GLUT8	Human	Rabbit; Polyclonal	Santa cruz Biotechnology Inc. (H-60, SC 30108)	1:500
Insulin Receptor β	Human	Rabbit; Polyclonal	Santa cruz Biotechnology Inc. (C-19, SC 711)	1:500
Adiponectin	Mouse	Rabbit; Polyclonal	Thermo Fisher Scientific Inc./Pierce Biotechnology (PA1-054)	1:500
Adipo R1	Human	Rabbit; Polyclonal	Santa cruz Biotechnology Inc. (N-20, SC 99183)	1:500
Actin	β-Actin	Mouse; Monoclonal	Sigma A2228, 128K4813	1:2000

Table 2. Effect of KP-10 on food intake, body mass, WAT mass , adipose tissue TG, testicular glucose, serum TG and serum glucose mice (*in vivo*)

Treatments	Body mass (g)	White adipose tissue mass (g)	Tissue		Serum	
			Adipose tissue Triglyceride concentration (TG/mg protein)	Testicular glucose concentration (mg/mg testis wt)	Triglyceride level (mg/dL)	Glucose level (mg/dL)
Control	32.14±0.71	1.93±0.412	76.7±2.11	17.26±0.81	107.6±1.22	77.22±1.2
KP-10 20 pg	32.79±0.84	2.27±0.49	127.28±3.12*	20.4±1.14*	98.7±2.15	52.2±1.5*
KP-10 2 ng	33.78±0.48	3.01±0.27*	146.97±3.2*	15.36±0.55*	66.28±1.14*	46.48±1.1*
KP-10 2µg	29.74±0.82*	1.21±0.47*	98.84±2.03*	0.8±0.09*	82.92±2.42*	62.96±2.1*

Values are mean ± SEM for five animals.
*Significantly different from controls ($p<0.05$) by one way analysis of variance (ANOVA) followed by Bonferroni test.

Table 3: Correlation studies of KP-10 treatment between the different parameters in adipose tissue and testis (*in vivo*).

Adipose Tissue / Testis	Effect of KP-10		
	Glut 8	Insulin Receptorβ	Adiponectin
Glut 8	r = -0.98, P<0.05	NS	r=-0.56,p<0.05
Insulin Receptorβ	NS	r= -0.76, P<0.05	NS
Adipo R1	NS	NS	r=0.86, p<0.05

NS- not significant, Values are significantly different at $p<0.05$.

Table 4: Correlation between adipose tissue Glut 8, Insulin receptor and Adiponectin with testicular Glut 8, Insulin receptor and Adipo R1

	Serum TG	Serum Glucose	Testicular Glucose level	Testost-erone level	Adipose tissue			Testis	
					GLUT4	GLUT8	IR	GLUT8	IR
Adipose tissue TG	r= -0.84, p<0.05	r= -0.99, p<0.05	NS	NS	NS	NS	NS	NS	NS
Serum TG		r= 0.72,	NS	r=0.64,	NS	NS	r=-0.61,	NS	r=0.88,
		p<0.05		p<0.05			p<0.05		p<0.05
Serum Glucose			NS	NS	NS	NS	NS	NS	NS

Testicular				r=0.97,				r=0.8,	r= 0.76,
Glucose				p<0.05				p<0.05	p<.0.05
level									
Testoste-								r= 0.67,	r= 0.78,
rone level								p<0.05	p<0.05
GLUT4						r= 0.70,	r= -0.70,	NS	NS
						p<0.05	p<0.05		
GLUT8							r=0.97,	NS	r=0.78,
							p<0.05		p<0.05
	Serum TG	**Serum Glucose**	**Testicular Glucose level**	**Testost-erone level**	**Adipose tissue**			**Testis**	
					GLUT4	**GLUT8**	**IR**	**GLUT8**	**IR**
Adipose tissue TG	r= -0.84, p<0.05	r= -0.99, p<0.05	NS	NS	NS	NS	NS	NS	NS
Serum TG		r= 0.72,	NS	r=0.64,	NS	NS	r=-0.61,	NS	r=0.88,
		p<0.05		p<0.05			p<0.05		p<0.05
Serum Glucose			NS	NS	NS	NS	NS	NS	NS
Testicular				r=0.97,				r=0.8,	r= 0.76,
Glucose				p<0.05				p<0.05	p<.0.05
level									
Testoste-								r= 0.67,	r= 0.78,
rone level								p<0.05	p<0.05
GLUT4								NS	NS
GLUT8								NS	r=0.78, p<0.05

NS- not significant, Values are significantly different at p<0.05

FIGURE LEGENDS

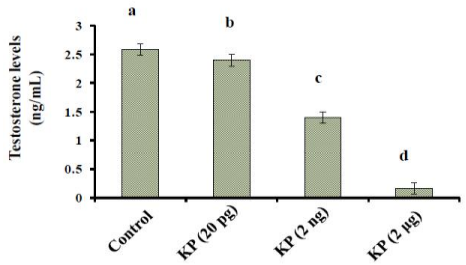
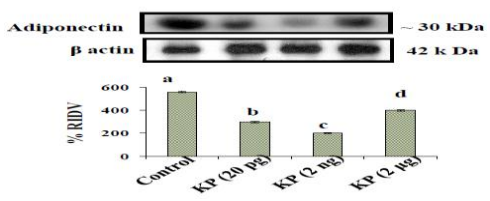


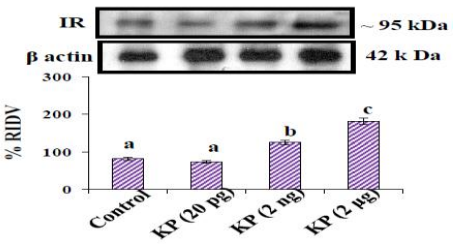
Figure 1. Effect of different doses (20 pg, 2 ng and 2 µg/day) of KP-10 679 treatment *in vivo* on serum testosterone level in mice. All the doses of KP-10 680 showed dose-dependently significant decreased (p<0.05) the circulating levels 681 of testosterone.

(A)



(A). Low and moderate dose of KP-10 showed dose-dependent significant 686 decrease (b and c, p<0.05) expression of adiponectin protein as compare with 687 the control (a, p<0.05) whereas highest dose showed significantly increased (d, 688 p<0.05) in expression of adiponectin as compare with low and moderate doses 689 of KP-10 (b and c, p<0.05) treated groups.

(B)

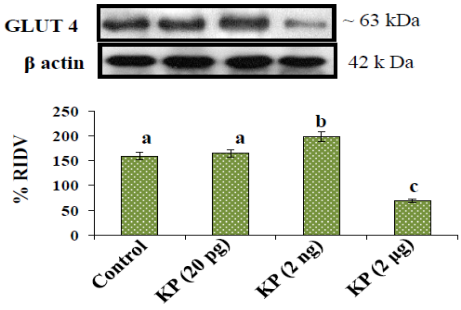


B. Moderate and high doses of KP-10 showed significantly increased (b and c, 691 p<0.05) the expression of IR protein in dose-dependent manner as compare to 692 control and low dose of KP-10 (a, p<0.05) treated groups.

Figure 2. The densitometric analysis of the western

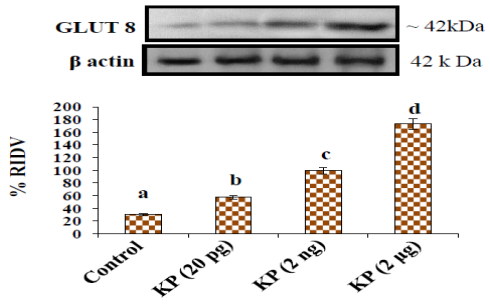
blot showed changes in the 683 effect of different doses (20 pg; 2 ng and 2 µg/day) KP-10 on expression of 684 adiponectin and insulin receptor proteins in adipose tissue of mice.

(A)



(A). Moderate dose of KP-10 showed significant increased (b, p<0.05) in the 697 expression of GLUT 4 protein as compare with control whereas high dose of 698 KP-10 showed significant decreased (c, p<0.05) in the expression of GLUT 4 as compare with compare with control, low and moderate doses of KP-10 (a and b p<0.05) treated groups.

(B)



(B). All the three doses of KP-10 showed dose-dependent significantly 702 increased (p<0.05) expression of GLUT 8 protein as compare to the control (a, 703 p<0.05). Values are significantly different from each other. Values are mean 704 ± s.e.m.

Figure 3. The densitometric analysis of the western blot showed changes in the 694 effect of different doses (20 pg; 2 ng and 2 µg/day) of KP-10 on expression of 695 GLUT4 and GLUT8 proteins in adipose tissue of mice.

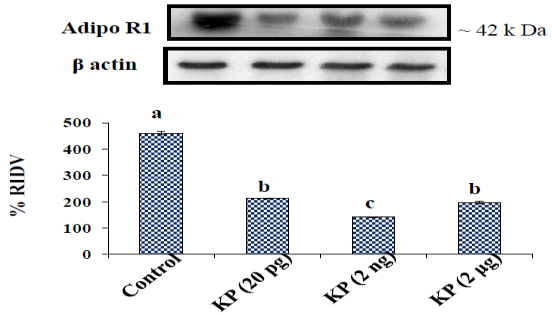
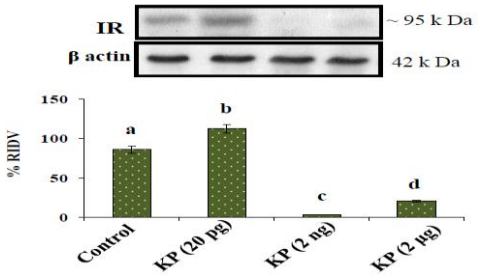


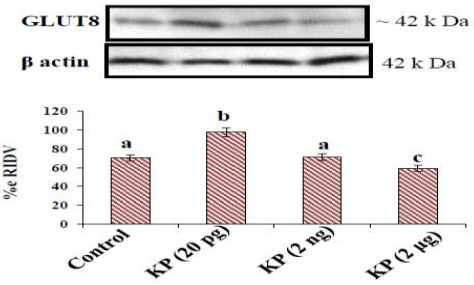
Figure 4.. The densitometric analysis of the western blot showed changes in 706 the effect of different doses (20 pg; 2 ng and 2 µg/day) of KP-10 on the 707 expression of Adipo R1 proteins in testis of mice. All the three doses of KP-10 708 showed dose-dependent significantly decreased (p<0.05) expression of Adipo 709 R1 as compare to the control (a, p<0.05).

(A)



(A). Low dose of KP-10 showed significantly increased (b, p<0.05) the 714 expression of IR protein whereas moderate and high dose of KP-10 showed 715 significantly decreased (c and d, p<0.05) expression of IR protein as compare 716 to the control (a, p<0.05).

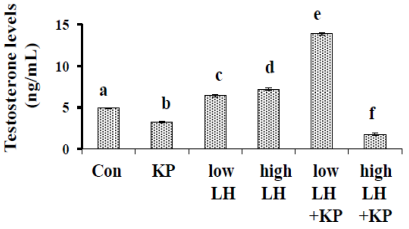
(B)



(B). Low dose of KP-10 showed significantly increased (b, p<0.05) expression 718 of GLUT 8 protein whereas high dose of KP-10 showed significantly 719 decreased (c, p<0.05) expression of GLUT 8 protein as compare to the control 720 (a, p<0.05). Values are significantly different from each other. Values are mean 721 ±s.e.m.

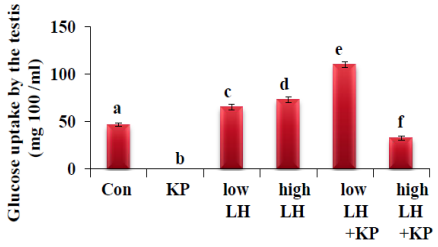
Figure 5. The densitometric analysis of the western blot showed changes in the 711 effect of different doses (20 pg; 2 ng and 2 µg/day) of KP-10 on the expression 712 of IR and GLUT8 proteins in testis of mice

(A)



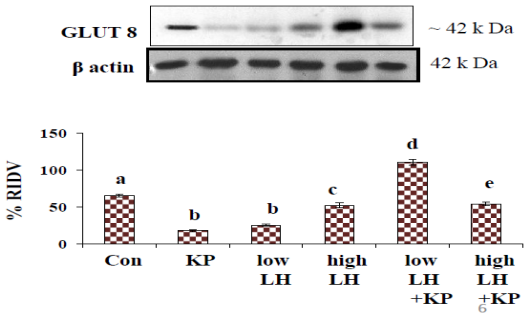
(A). KP-10 showed significantly decreased (b, p<0.05) testosterone synthesis 726 as compare to the control (a, p<0.05). Different doses of LH showed dose- 727 dependent significantly increased (c and d, p<0.05) testosterone synthesis and 728 low dose of LH along with KP-10 showed significantly increased (e, p<0.05) testosterone synthesis whereas high dose of LH along with KP-10 showed 730 significantly decreased (f, p<0.05) testosterone synthesis as compare to the 731control (a, p<0.05).

(B)



(B). KP-10 showed significantly decreased (b, p<0.05) glucose uptake by the 733 testis as determined by the depletion of glucose in culture media. Different 734 doses of LH showed significantly increased (c and d, p<0.05) glucose uptake 735 and low dose of LH along with KP-10 showed significantly increased (e, 736 p<0.05) glucose uptake whereas high dose of LH along with KP-10 showed 737 significantly decreased (f, p<0.05) glucose uptake by the testis as compare to 738 the control (a, p<0.05).

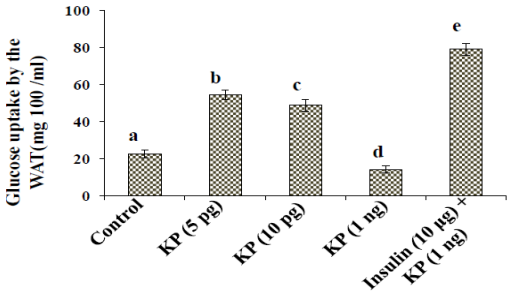
(C)



(C). KP-10 showed significantly decreased (b, p<0.05) expression of GLUT8 740 in the testis. Different doses of LH showed dose-dependent significantly (c and 741 d, p<0.05) increased expression of GLUT 8 protein and low dose of LH along 742 with KP-10 showed significantly (e, P<0.05) increased expression of GLUT 8 743 protein whereas high dose of LH along with KP-10 showed significantly (f, 744 p<0.05) decreased expression of GLUT 8 protein as compare to the control (a, 745 p<0.05). Values are significantly different from each other Values are mean 746 ±s.e.m

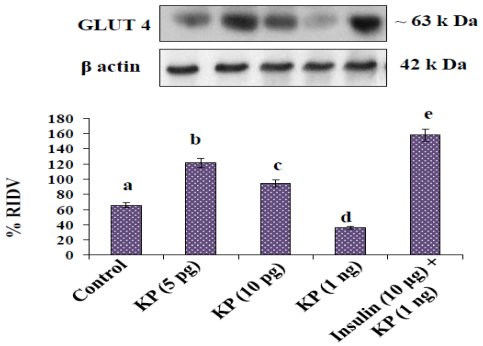
Figure 6. Effect of *in vitro* treatment of KP-10 (1 ng/ml) and different doses 723 of LH (10 ng/ml and 100 ng/ml) with or without KP-10 on testicular synthesis 724 of testosterone, glucose uptake and expression of GLUT 8 protein in the testis.

(A)



(A). Low and moderate dose of KP-10 showed significantly increased (b, c, 752 p<0.05) glucose uptake by the WAT as determined by the depletion of glucose 753 in culture media. High dose of KP-10 showed significantly decreased (d, 754 p<0.05) glucose uptake by the WAT. High dose of KP-10 along with insulin 755 showed significantly increased (e, p<0.05) glucose uptake by the WAT.

(B)



(B). Low and moderate dose of KP-10 showed significantly increased (b, c, 757 p<0.05) expression of GLUT4 protein as compare to the control. High dose of 758 KP-10 showed significantly decreased (d, p<0.05) expression of GLUT4 759 protein. High dose of KP-10 along with insulin showed significantly (e, p<0.05) increased expression of GLUT4 in adipose tissue as compare to the 761 control. Values are significantly different from each other. Values are mean 762 ± s.e.m

Figure 7. . Effect of *in vitro* treatment of different doses of KP-10 (5 pg, 10 pg 748 and 1 ng/ml) with or without insulin (10 µg/ml) on glucose uptake by the white 749 adipose tissue and expression of GLUT 4 protein in the white adipose tissue 750 (WAT).

DISCUSSION

The aim of the present study was to investigate the role of KP as mediator in bridging changes in the nutrients level with the changes in reproduction. This is achieved by evaluating the effect of *in vivo* treatment of KP-10 on changes in nutrients and hormonal levels occurring simultaneously in adipose tissue and testis of mice. The results showed significant changes in the

nutrients and hormonal parameters in both adipose tissue and testis in response to treatment with KP-10. This suggests that KP-10 regulates physiological changes in both testis and adipose tissue and so this neuropeptide may have additional functions beyond reproduction. This study further confirms the earlier report showing the presence of KP-receptor (GPR54) in the adipose tissue (Muir *et al.*, 2001).

The mice treated with low to moderate doses of KP-10 showed significant ($p<0.05$) increase in the levels of triglycerides together with increased expression of GLUT4 protein in the adipose tissue. But high dose of KP-10 caused significant declines in triglycerides and GLUT4 levels in the adipose tissue. This study further showed that KP- 10 treatment caused dose-dependent significant ($p<0.05$) increase in expression of GLUT8 and insulin receptor proteins in the adipose tissue. KP-10 induced increase in concentrations of GLUT4 and 8 and insulin receptor proteins in the adipose tissue suggest increased uptake of glucose by this neuropeptide. This study further suggests that the KP induced increase glucose uptake by adipose tissue may be insulin mediated. The glucose taken up by adipose tissue is converted into triglycerides and free fatty acid, the storage form of nutrients in the adipocytes. In this study the mice treated with low and moderate dose of KP-10 showed significant increase in the weight of accumulated white adipose tissue. This finding thus further confirms the role of KP-10 in promoting triglycerides and glucose uptake in the adipose tissue. The high dose of KP-10 caused decreased uptake of triglycerides as well as down regulation of GLUT4, this could be due to down regulation of GPR54. This observation is supported by decreased level of adipose tissue in the mice treated with high dose of KP-10.

The mice treated *in vivo* with KP-10 showed significant ($p<0.05$) decline in the expression of adiponectin protein in the adipose tissue. The low and moderate doses of KP-10 treatment showed dose-dependent significant decline in the expression of adiponectin protein, whereas high dose of KP-10 showed a significant rise in the expression of adiponectin in the adipose tissue as compared to the moderate dose group. Expression of KISS1 and KISS1R in adipose tissue was earlier demonstrated in the rhesus monkey, *Macaca mulata* (Wahab *et al.*, 2010). This study suggested that KP-10 acted on adipose tissue and stimulated adiponectin release (Huma *et al.*, 2014). The adiponectin secretion from adipose tissue is often diminished during obesity. Adiponectin acts to increase insulin sensitivity, fatty acid oxidation, as well as energy expenditure and reduces the production of glucose by the liver. This study thus suggests that KP-10 acts on adipose tissue and secretes adiponectin, which may play an important role in the regulation of reproductive axis.

The mice treated with low and moderate doses of KP-10 showed significant decline in the circulating triglycerides and glucose levels. The correlation study between serum triglycerides and serum glucose levels ($r=0.72$, $p<0.05$) showed significant relationship, but with triglycerides level of adipose tissue ($r=-0.99$, $p<0.05$) showed significant inverse relationships. Based on present study it may be hypothesized that in response to peripheral administration of KP-10, circulating triglycerides and glucose enters into adipose tissue and accumulated as fat in adipocytes. Recent studies have shown that increased expression of KISS1 gene in adipose tissue was related to the increase in body weight in rodents (Moral *et al.*, 2011; Quennell *et al.*, 2011). This observation suggests that in addition to affect reproduction, KP signaling also influences glucose uptake, accumulation of triglycerides and contribute to increased accumulation of adipose tissue. This implies a possible role for KP-10 in obesity.

The *in vivo* treatment of different doses of KP-10 to mice showed dose-dependent decline in expression of GLUT8 protein and thus decline in glucose concentration in the testis. The study also showed dose-dependent significant decline in the levels of testosterone in the mice treated with different doses of KP-10 as compared with the control. This study further demonstrated a significant correlation between changes in the testicular GLUT8 ($r=0.80$, $p<0.05$) and glucose levels $r= 0.97$, $p<0.05$) with the circulating testosterone level in the mice treated with different doses of KP-10. These observations thus suggest that the mice treated with KP-10 showed reduced availability of glucose to the testis, which in turn is responsible for decreased testosterone synthesis.

This finding further confirms our recent study that the decreased availability of glucose to the testis resulting in decreased synthesis of testosterone (Banerjee *et al.*, 2014). An earlier study has also shown that glucose availability modulates levels of mitochondrial enzymes NADPH (Bajpai *et al.*, 1998) which is required for steroidogenesis.

The mice treated *in vivo* with different doses of KP-10 showed significant ($p<0.05$) decline in the expression of AdipoR1 and insulin receptor proteins in the testis. Low and moderate dose of KP-10 showed dose-dependent decline in both AdipoR1 and insulin receptor protein, but high dose showed a moderate increase in the expression of these proteins. The KP-10 induce changes in the expression of Adipo R1 protein in the testis correlated positively with the synthesis of adiponectin in adipose tissue ($r=0.86$, $p<0.05$), but correlated inversely with the mass of adipose tissue. Inverse correlation between accumulation of adipose tissue in the body and synthesis of adiponectin ($r=-0.78$, $p<0.05$) supported the earlier study showing diminished adiponectin secretion during obesity (Galic

et al., 2010). Most adipokines with pro-inflammatory properties such as leptin are overproduced with increasing adiposity, while adipokines with anti-inflammatory or insulin-sensitizing properties, like adiponectin are decreased during obesity. These observations suggest a potential role for KP in connecting metabolic status with reproductive function.

The present *in vitro* study demonstrated for the first time a direct effect of KP-10 on glucose homeostasis in the adipose tissue of mice. This is supported by presence of KISS1R in the adipose tissue (Wahab *et al.*, 2010). Treatment with low and moderate doses of KP-10 *in vitro* showed significantly ($p>0.05$) increased expression of GLUT4 together with increased uptake of glucose in the adipose tissue, whereas high dose of KP-10 treatment showed decreased expression of GLUT4 and suppression of glucose level. Thus, this *in vitro* study confirms the present *in vivo* findings, where lower and moderate doss of KP-10 treatment showed increased uptake of glucose by adipose tissue resulting in increased accumulation of fat in the adipocytes. The high dose of KP-10 has inhibitory effect on glucose uptake in the adipose tissue. KP-10 together with insulin *in vitro* showed a significant increase in glucose uptake by adipose tissue. This suggests that presence of insulin enhances the effect of KP-induced glucose uptake by adipose tissue. This observation thus suggests that KP-10 may be a direct regulator of metabolic changes in adipose tissue by promoting glucose trafficking.

The testis treated *in vitro* with different doses of KP-10 showed sharp decline in glucose uptake. This observation is further confirmed by study, where the testis treated *in vitro* with KP-10 showed significant ($p<0.05$) decline in the expression of GLUT8 protein. The testis treated with different doses of KP-10 also showed significant decline in testosterone synthesis. These findings clearly demonstrate that KP-induced inhibition of glucose uptake may be directly responsible for inhibition of testicular steroidogenesis. This study confirmed the earlier finding that testis/Leydig cells culture in absence of glucose can synthesis testosterone at very low rate (Rommerts *et al.*, 1973; Banerjee *et al.*, 2014). The testis treated *in vitro* with LH together with KP-10 showed stimulatory effect on glucose uptake together with increased expression of GLUT8 protein and increased synthesis of testosterone. So LH promotes testicular steroidogenesis by enhancing GLUT8 mediated uptake of glucose by the testis.

In brief, the results of this study showed differential effect of KP-10 on uptake of various nutrient factors in adipose tissue and testis of mice. In adipose tissue, KP-10 promoted uptake of triglycerides and glucose from circulation and resulted in increased accumulation of white adipose tissue. At the same time, in testis, KP-10 caused down- regulation of GLUT 8 expression resulting in decreased uptake of glucose, which in turn resulted in decreased synthesis of testosterone. The mice treated with KP-10 showed decreased synthesis of adiponectin in the adipose tissue and decreased expression of AdipoR1 in the testis. The decreased adiponectin synthesis in adipose tissue in turn may be responsible for decreased testicular activity in the mice. The *in vitro* treatment of KP-10 showed increased uptake of nutrients in adipose tissue but inhibitory changes in testis.These findings thus suggest that KP-10 showed direct role in modulating energy balance in adipose tissue, which is reciprocally linked to testicular activities in mice. Based on present study, it may be hypothesized that KP may mediate increase adiposity associated impaired testicular activities.

ACKNOWLEDGEMENT

This work was supported in part by grant-in-aid from UGC, New Delhi to A.K and S.A highly acknowledges the financial assistance from the RFSMS-UGC, New Delhi.

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