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ORIGINAL ARTICLE

Liraglutide modulates adipokine expression during adipogenesis, ameliorating obesity, and polycystic ovary syndrome in mice

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Abstract

Purpose The incidence of obesity is increasing among all age groups throughout the world and it is highly associated with numerous other metabolic disorders, such as insulin resistance, polycystic ovarian syndrome (PCOS) etc.

Methods and Results Using in vitro and in vivo approach, this study investigated the adipokine profile after liraglutide on differentiated murine 3T3-L1 pre-adipocytes. Effect of liraglutide on DHEA-induced PCOS mice were investigated. This study showed Liraglutide treatment resulted in up-regulation of adiponectin and IL-6 along with down-regulation of ICAM 1 in differentiated 3T3-L1 cells. Liraglutide in absence of other differentiating factors, significantly increased glucose, lipid uptake and PPARγ, C/EBPα expression in the adipocytes suggesting its ability to solely promote pre-adipocyte differentiation into mature adipocyte. Liraglutide treatment showed increased adiponectin expression and decreased number of cystic follicles, body weight, circulating glucose, triglyceride and testosterone levels in comparison to the PCOS induced mice.

Conclusion This study suggests that adiponectin may act as a link between metabolic disorders and PCOS and that liraglutide might be a promising therapeutic agent for the treatment of PCOS in addition to obesity and insulin resistance.

Highlights

- The study shows the impact of GLP-1 analogue, liraglutide's impact on adipokine secretion in 3T3L1 adipocytes.
- For the first time, showing that liraglutide in the absence of other differentiating factors (IBMX, insulin, and dexamethasone), significantly increased glucose, lipid uptake and PPARγ, C/EBPα expression in the adipocytes suggesting its ability to solely promote pre-adipocyte differentiation into mature adipocyte.
- Also, for the first time it shows that liraglutide through the up-regulation of adiponectin manages PCOS in mice.

Keywords Liraglutide · Adipocyte · Adiponectin · PCOS

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Introduction

Obesity constitutes a serious health concern worldwide. It is alarming to observe obesity among all age groups in most countries including India [1]. It is a major characteristic of metabolic syndrome and is a risk factor for type II diabetes and cardiovascular diseases [2]. Earlier anti-obesity drugs or anorexins have poor safety records. A better knowledge of the physiology of adipogenesis and fat cell development in obesity is important to identify new biomarkers and therapeutic targets for the development of novel anti-obesity drugs with improved safety profile.

Factors responsible for obesity include those responsible for the expansion of white adipose tissue via the mechanism of hypertrophy of pre-existing adipocytes and hyperplasia resulting from the adipogenesis of pre-adipocytes [3]. However, changes in adipocyte turnover rate, differentiation, and apoptosis could all contribute to changes in fat mass underlying obesity. Studies have suggested that pre-adipocyte differentiation may be impaired in obese humans [4, 5]. One possible avenue to reduce fat mass is to therapeutically regulate pre-adipocyte differentiation, but a better understanding of the pathways controlling adipogenesis is needed. Another important aspect of adipogenesis is the adipokines secretion by the adipose tissue, which is the central factor how fat mass directly influence insulin sensitivity and vascular injury [6]. The success of any new drug to control obesity depends on how therapeutically it might regulate the profile of adipokines secreted from the fat mass.

Glucagon-like peptide 1 (GLP-1), is a proglucagon-derived peptide secreted from endocrine cells in the gut, GLP-1 is used as a therapeutic option for the pharmacotherapy of type II diabetes and obesity. It is responsible for the stimulation of glucose-dependent insulin secretion from pancreatic β cells, inhibition of glucagon secretion, and inhibition of food intake [7–10]. Obese individuals are reported to have reduced GLP-1 levels and improved GLP-1 levels after weight loss [11]. One of the major shortcomings in the implementation of GLP-1 is its rapid inactivation in the body by the enzyme dipeptidyl peptidase IV (DPP-IV) and rapid renal elimination [7]. Studies have shown the effects of GLP-1 on cardiomyocytes involving in the regulation of the PI3K/Akt signaling pathway [12–14]. For the gut–brain axis, GLP-1 and GLP-2 are important for food intake, maintaining glucose homeostasis thereby controlling insulin sensitivity through the PI3K/Akt signaling pathway [15]. To overcome this shortcoming, a long-acting analogue of GLP-1, liraglutide, with a 97% sequence homology to human GLP-1 has been increasingly used as a therapeutic agent in improving insulin resistance, glucose tolerance, and obesity [7].

Both liraglutide and GLP-1 share, some of the glucoregulatory functions including enhancement of glucose-dependent insulin secretion, inhibition of postprandial glucagon secretion, inhibition of gastric emptying, and reduction of food intake [16]. In animal studies, liraglutide maintenance of β-cell mass in diabetes, by inhibiting both cytokine and free fatty acid (FFA)-induced apoptosis [17]. Recently, a study showed that liraglutide prevented hypoadiponectinemia-induced increase in plasma insulin [18]. Liraglutide injection into high fat diet fed mice induced in vivo adipocyte differentiation by regulating cell proliferation and apoptosis of pre-adipocytes [19]. In type II diabetes patients, 14 weeks of liraglutide treatment significantly reduced hemoglobin A1c (HbA1c), body mass index, and total body fat mass, lowered insulin resistance and increased visfatin and resistin levels [20]. A recent study showed that liraglutide facilitates weight loss, improving bleeding pattern, increased SHBG levels, and decreased testosterone levels thereby improving the sex hormone levels and gonadotropin levels further ameliorating other metabolic dysfunction in overweight women with PCOS. This study also assessed the ovarian morphology with three-dimensional ultrasound, however, no effect was observed in both the liraglutide and the placebo groups [21]. The study showed when women with PCOS were treated with liraglutide, the levels of fasting glucose and leptin were reduced, however, measures of insulin resistance, adiponectin, and glucagon strangely did not change. This study further showed liraglutide treatment of 26 weeks in PCOS women resulted in significant reductions of liver fat content, VAT, and the prevalence NAFLD [22]. Another study showed that treatment with liraglutide in combination with metformin resulted in a significant weight loss in overweight and obese women with PCOS [23]. 3T3-L1 cells treated with different doses of liraglutide, showed a significant decrease in the mRNA expression of some adipokines, such as leptin, TNF-α, and IL-6, whereas, the mRNA expression of adiponectin and CTRP3 were increased significantly [24]. Interestingly, liraglutide treatment to type II diabetes patients has also been shown to significantly decrease serum adiponectin level [25] and among Chinese type 2 diabetes (T2DM) patients, liraglutide led to increased adiponectin and decreased resistin levels [26]. However, the direct effect of liraglutide on the adipokine profile of the differentiated pre-adipocyte is not known, knowledge of which will not only help us to better understand the physiology of liraglutide action on adipocytes but also might open new research pertaining liraglutide pharmacotherapy.

The murine 3T3-L1 cells are one of the best in vitro models to study the mechanisms involved in pre-adipocyte proliferation, differentiation, adipokine secretion, and gene/protein expression [2]. Liraglutide previously has been shown to induce differentiation in the presence of differentiation medium consisting of insulin (IN), dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX) [19]. However, whether liraglutide alone has any effect on the differentiation of the 3T3L1 pre-adipocytes has not been studied.

Since 1935, Stein and Leventhal [27] had recognized obesity as a common feature of the PCOS, one of the common metabolic disorders of females, characterized by hyperandrogenism and chronic oligo-anovulation [28]. PCOS has some metabolic characteristics that include impaired insulin action and β-cell function, increased risk for glucose intolerance and type II diabetes [29]. A study also suggests that there is a strong correlation among insulin resistance, low adiponectin level, and PCOS [30]. It is also known that the level of glucose-dependent insulinotropic polypeptide (GIP) in obese women with PCOS is lower in comparison to non-obese PCOS control women and PCOS lean women [31]. It was recently hypothesized that disturbance in incretin hormones, like GLP-1 and GIP, secretion dynamics might contribute to the risk of impaired glucose tolerance and type 2 diabetes in PCOS [32].

Thus far, metformin remains the main choice of drug to manage infertility in PCOS, though studies are in progress investigating the usage of other insulin-sensitizing agents like GLP-1 analogues in managing PCOS [33]. Obese women with PCOS when treated with liraglutide in combination with metformin resulted in significant weight loss [23, 34, 35]. Most of these studies highlighted the weight loss in patients after liraglutide treatment, however, other reproductive parameters were not considered. Hence, investigating the treatment of liraglutide on a PCOS-like condition may improve or manage the reproductive status by regulating the ovarian physiology.

This study was undertaken to investigate the adipokine profile of the mature 3T3-L1 murine adipocytes after liraglutide treatment. The study also determined the effect of liraglutide on 3T3-L1 murine pre-adipocyte differentiation, with and without the differentiation ingredients (IN–IBMX–DEX). Further, the effects of liraglutide on the reproductive parameters in DHEA-induced PCOS mice were investigated, to observe how ovarian physiology is altered after liraglutide treatment.

Materials and methods

Chemicals

Reagents to perform SDS-PAGE and immunoblotting were procured from Bio-Rad, USA. Dehydroepiandrosterone (DHEA) (CAS No. 53-43-0), IBMX (Catalog # I5879), Dexamethasone (Catalog # D4902), and Insulin IN (Catalog # I9278) were procured from Sigma-Aldrich Chemicals Co., USA. Mouse Adipokine antibody array kit was obtained from R&D Systems, USA (Catalog # ARY013). Other chemicals of analytical grade were purchased from Sigma-Aldrich Chemicals Co., USA.

Antibody, catalog number	Host species	Type	Source	Supplier	Dilution IB/IHC
GLUT-4 (H-61):sc-7938	Rabbit	Polyclonal	Human	Santa Cruz Biotechnology Inc., CA, USA	1:500
Adipo R1 (H-40), SC-99183	Rabbit	Polyclonal	Human	Santa Cruz Biotechnology Inc., CA, USA	1:500
Anti-Adiponectin, A6354	Rabbit	Polyclonal	Human	Sigma-Aldrich, MO, USA	1:500
Phospho-PI3K	Mouse	Monoclonal	Human	Santa Cruz Biotechnology Inc., CA, USA	1:100
Phospho-Akt, SC-7985	Rabbit	Polyclonal	Human	Santa Cruz Biotechnology Inc., CA, USA	1:100

Mice

Pre-pubertal female Parkes strain mice ($n = 20$) were used for the present study. Animals were housed under standard laboratory conditions, and were provided with pelleted food and water ad libitum. All animal experimentation and procedures were approved by the Institutional Animal Ethics committee of Birla Institute of Technology and Science (BITS) Pilani Rajasthan, Reference number IAEC/REC/19/21.

Cell culture and treatment

All experiments pertaining to cells were carried out in the Department of Biological Sciences, BITS Pilani KK Birla Goa Campus, Goa, India. The 3T3-L1 pre-adipocytes (CL-173™; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hi Media, India) containing 10% newborn calf serum (NBCS, Invitrogen, Carlsbad, CA, USA) and 10 mg/ml penicillin/streptomycin in an atmosphere of 5% CO_2 at 37 °C. To study the effect of liraglutide on the adipokine profile the following experiment was done. For the 3T3-L1 adipocyte differentiation, the pre-adipocyte cells that reached ~90% confluency after 48 h, were induced with differentiation media containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone (DEX), 1 µg/ml insulin, and 10% NCS in DMEM for 2 days. The cell culture medium was then replaced with insulin media containing DMEM, 1 µg/ml insulin and 10% NCS for the next 2 days, followed by only DMEM and 10% NCS for 3 days. On day 7, maximum number of the cells demonstrated the adipocyte phenotype. Recombinant human glucagon-like peptide, (Liraglutide, Victoza, Novo Nordisk, USA) was added to the culture medium at concentrations of 10 and 100 nM after the pre-adipocytes reached ~90% confluency (after 48 h) for the adipogenic period of 7 days.

To evaluate the effect of liraglutide solely without the differentiating factors IBMX, DEX, and IN, pre-adipocytes after 2 days of confluence were treated with only liraglutide at doses of 10 and 100 nM for 8 days. After 8 days of treatment, cells were subjected to Oil Red O staining. Further, after 8 days of treatment, the media obtained was used to investigate the glucose level.

Glucose level in the media

Glucose was measured from the media after the differentiation of 3T3-L1 cells using the glucose estimation kit obtained from BioLab Diagnostics Ltd., India. In brief, 0.5 ml of working glucose reagent was added and 0.05 ml, mixed well and incubated at 37 °C for 15 min. After incubation, tubes were again thoroughly mixed and the color intensity was read at 500 nm against the blank. All experiments were performed in triplicates. Intra assay variation was less than 3% and inter assay variation was about 5%.

Oil Red O staining and lipid content quantification

The cellular lipid content in the 3T3-L1 differentiated cells was assessed by Oil Red O staining. After 8 days of differentiation, cells were washed, fixed in 4% formalin for 30 min, stained with an Oil Red O working solution, and then incubated for an additional 2 h at room temperature. After washing 3 times with PBS, the cells were photographed with a light microscope at ×40 magnification (Leica microscope, Germany). Next, 125 µl isopropyl alcohol was added to each well, and the cells were maintained at room temperature for 5 min to stain the lipids with Oil Red O. Then, 100 µl of the eluate from each well was transferred to a 96-well plate, and the absorbance values were measured at a 540-nm wavelength using a spectrophotometer (Thermo Fisher Scientific, Inc., USA).

Mouse adipokine antibody array

Modulations of adipogenesis markers in vitro in the liraglutide-treated 3T3-L1 mouse adipocytes was evaluated by using an adipokine proteome profiler array (R&D Systems, USA) as per manufacturer's instructions. Briefly, membranes were treated with 2 ml of blocking buffer and then incubated overnight with 1 ml of cell lysates of liraglutide-treated 3T3-L1 adipocytes containing 500 µg protein at 4 °C. After washing, 1 ml of a mixture of biotin-conjugated antibodies that are specific to the different targets on the array were added for 1 h and then incubated with 2 ml of HRP-conjugated streptavidin at room temperature for 2 h. The membranes were then treated with 500 µl of detection buffer for 2 min and, finally, the images were captured using a ChemiDoc. Densitometric analysis of immunoreaction spots was performed by ImageJ (version 36, NIH, USA). By comparing the signal intensities, relative expression levels of adipokines were determined. Positive controls were used to normalize the results from the different membranes being compared.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol and quantified using spectrophotometer (Nanodrop lite, Thermo Scientific, MA, USA). 3 µg of total RNA was reverse-transcribed into cDNA using Affinity script QPCR cDNA Synthesis Kit (Agilent Technologies, CA, USA. Cat # 600559), and was further amplified using Brilliant III Ultra-Fast SYBR-Green (Agilent Technologies, CA, USA. Cat # 600882) in a fluorescent thermocycler (Agilent Technologies, AriaMx Real Time PCR). The gene sequences of the primers used in the study were PPAR-γ, C/EBPα, and mouse β-actin was used as an internal control (sequence is available upon request). Target gene mRNA levels were normalized to β-actin using the $2^{-\Delta\Delta CT}$ method.

Induction of polycystic ovarian syndrome (PCOS) in mice

To investigate whether liraglutide treatment might improve ovarian physiology in PCOS condition, prepubertal (21–22 days) mice of Parkes strain were injected subcutaneously for 25 days by daily treatment of dehydroepiandrosterone, DHEA to induce PCOS (a dose of 6 mg/100 g body weight per day, dissolved in 0.01 ml 95% ethanol and mixed with 0.09 ml sesame oil) in accordance with Yaba and Damir [36, 37]. The vehicle-treated control group mice were injected with the vehicle, 0.09 ml sesame oil and 0.01 ml 95% ethanol daily for 25 consecutive days. Further, The PCOS mice were treated with two different doses (100 and 200 µg/kg/day) of liraglutide for 14 days twice a day, intraperitoneally. Vaginal smearing was a part of examination during the entire treatment time. After completion of treatments, body weight of all mice was recorded after which mice were sacrificed and ovaries were immediately dissected out. One side of the ovaries was excised, snap frozen and kept at −20 °C until protein extraction for immunoblots, and the contralateral ovary was fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight for histology/immunohistochemistry. The fixed tissues were dehydrated in ethanol, embedded in paraffin wax, and sectioned at 5 µm. Blood samples were collected under light ether anesthesia by retro-orbital plexus. Serum was centrifuged at 4 °C and was kept frozen at −80 °C until further analysis.

Testosterone assay

ELISA kit for testosterone assay was purchased from Dia Metra (catalog number DKO002). To each well of the ELISA plate 50 µl of standard, control, or sample was added. Subsequently, 100 µl of the enzyme conjugate solution and 100 µl of the testosterone antiserum was added to each of these wells. The ELISA plate was then incubated with mild shaking (500–700 rpm) at room temperature for 1 h. The wells were then aspirated and washed several times with wash solution. Then 100 µl of the TMB (tetramethylbenzidine) chromogen solution was added to each well and the plate was incubated at room temperature for 30 min. Finally, 100 µl of stop solution (0.2 M sulfuric acid) was added and absorbance was taken at 450 nm using a microplate reader. The coefficient of intra and inter assay variation were 5.4% and 12%, respectively.

Estrogen assay

ELISA kit for 17β estradiol assay was purchased from Dia Metra (catalog number DKO003). The standard curve ranged from 0.00 to 2000.00 pg/ml. Unknowns were run within the narrow range representing the most linear portion of the standard curve. The coefficient of intraassay variation was <9% and inter-assay variation was <10%.

Glucose and triglyceride (TG) estimation

Serum glucose and serum triglyceride levels were measured by enzymatic procedure in mice serum or from the culture media obtained after 3T3L1 culture under different experimental conditions mentioned above, by commercially available Span Diagnostic Kits.

Histological analysis and immunohistochemistry

After removal, the ovaries were sectioned in blocks, fixed in cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h, dehydrated through a graded series of ethanol and embedded in paraffin. For each specimen, the ovary was entirely cut to serial coronal sections of 5 µm thick and mounted onto cleaned coated slides. Sections were dewaxed in xylene and rehydrated through a decreasing series of ethanol (absolute, 95%, and 70%). One in ten sections was separated to perform classical hematoxylin and eosin staining for comparison with the control and liraglutide-treated PCOS mice ovaries. Further, adjacent sections were used for immunohistochemical assay, the sections were deparaffinized; endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 min. Subsequently, the slides were washed in 0.01 M PBS and further blocked by 5% bovine serum albumin and then incubated overnight at 4 °C with rabbit polyclonal GLUT4 and adiponectin receptor antibodies. Sections were then incubated at RT for 60 min with a biotinylated mouse anti-rabbit IgG (diluted 1:500) streptavidin–biotin horseradish peroxidase tagged complex. The bound complex was made visible by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemicals Co., St. Louis, USA) in 0.05 M Tris, pH 7.6 and 0.1% H_2O_2 for 7–8 min at RT. A minimum of five sections were examined for each sample. Representative tissue sections were viewed, analyzed, and photographed under light microscope with the nucleus counterstained by Elrich's hematoxylin.

Counting of follicles

Counting of different types of follicles in the ovaries was made by examining every fifth serial section of each ovary and then counting the follicles whose plane of section passed through the nucleolus of the oocytes. Early antral: one or more small antral cavities; late antral: one large antrum. In addition to this cystic and atretic follicles were also counted. Cystic follicles were large antral follicle with thin, one or few layers of granulosa cells and large antral fluid [38].

Western blot analysis

Circulating adiponectin level was detected by Western blot [39, 40] followed by densitometric analysis in the four groups of the experimental setup. Serum samples (5 µl) of mice collected from vehicle-treated control, PCOS mice, and low and high dose liraglutide-treated PCOS mice were diluted with 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 50 µl. An equal amount of protein (50 µg) determined by Lowry's method was loaded on to SDS-PAGE (10%) for electrophoresis. Thereafter, proteins were transferred electrophoretically to polyvinylidene difluoride membrane (PVDF) membrane (Millipore India Pvt., Ltd.) overnight at 4 °C. PVDF membranes were blocked for 1 h with phosphate buffered saline (PBS) containing 5% fat-free dry milk and then incubated with primary antibody (adiponectin) 1 h at room temperature. Membranes were then washed with three changes of PBS over 10 min. Immunoreactive bands were detected by incubating the membranes with horseradish peroxidase tagged secondary antibody (at a dilution of 1:4000) for 2 h. Finally, the blot was washed three times with PBS and developed with enhanced chemiluminescence (ECL) detection system (BioRad, USA). All blots were repeated for three times. The densitometric analysis of the blot was performed by scanning and quantifying the bands for density value by using computer-assisted image analysis

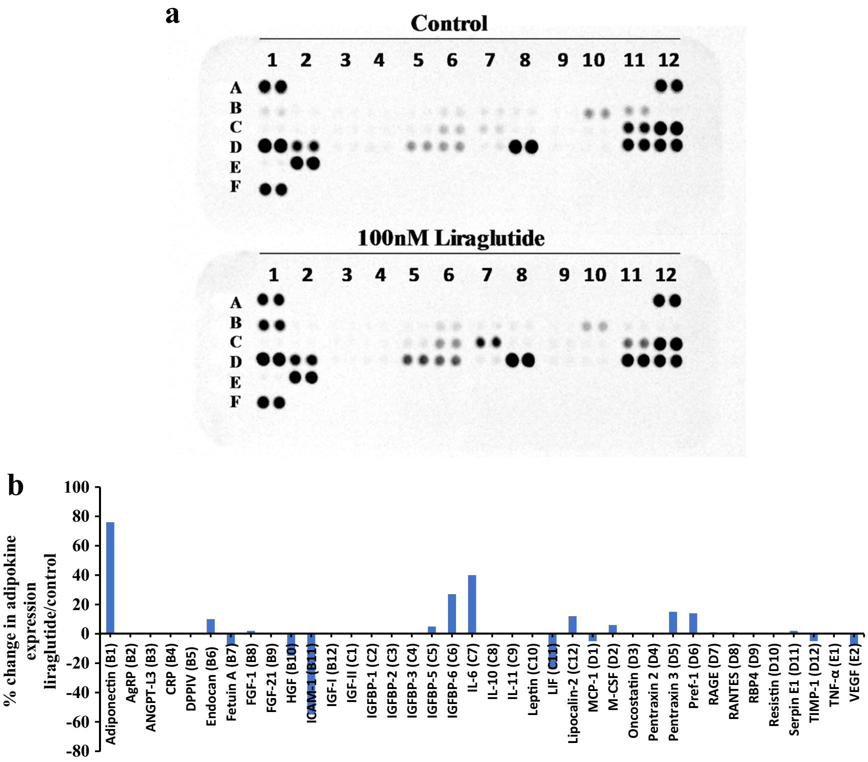


Fig. 1 Effect of the liraglutide on adipokines in adipocytes. Adipokine levels were measured in differentiated 3T3-L1 cells treated with 100 nM liraglutide in comparison to the vehicle control (PBS) using the Proteome Adipokine array. **a** Images of two adipokine profiler membranes showing chemiluminescent reaction spots representing the expression of various adipokines. **b** The bar diagram represents average percentage change in expressions of adipokines obtained from densitometric analysis of the chemiluminescent reaction spots (in duplicates) from the images in panel (**a**)

(Image J 1.38×, NIH, USA). The densitometric data were presented as the mean of the integrated density value ± SEM. Equal loading was confirmed by Ponceau staining of the membrane.

Statistical analyses

Data were expressed as mean ± SEM. The significance of the differences in adiponectin protein levels, body weight, glucose, triglyceride, and testosterone concentration between groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's test. The data were considered significant if $P < 0.05$.

Results

Modulation of adipokines in 3T3-L1 adipocytes after liraglutide treatment

To investigate the effect of liraglutide on adipokines, we used adipokine array to determine adipokines profile in 100 nM liraglutide-treated 3T3-L1 adipocytes. Figure 1 demonstrates the chemiluminescent reaction spots representing expressions of various adipokines in control (PBS-treated) or 100 nM liraglutide-treated adipocytes. Figure 1b shows histogram plotted after densitometric analysis of the chemiluminescent reaction spots showing the percentage up-regulation or down-regulation of adipokines in the liraglutide-treated 3T3-L1 adipocytes in comparison to control. The spots A1, A12, and F1 show the reference controls and rest of the spots represent various adipokines. The adipokines which showed differential expression are adiponectin, intercellular adhesion molecule-1 (ICAM-1), Insulin-like growth factor binding protein 6, IL-6, leukemia inhibitory factor, pentraxin-3, preadipocyte factor 1 (Pref-1). More interestingly, treatment with liraglutide showed 76% up-regulation in adiponectin and 55% down-regulation in ICAM-1. These data revealed that the liraglutide treatment resulted in the modulation of adipokines in differentiated 3T3-L1 cells.

Oil Red O staining

To investigate the effect of liraglutide on 3T3-L1 murine pre-adipocyte, with and without the differentiation ingredients (IBMX-IN-DEX), neutral lipid accumulation and glucose uptake between liraglutide in the presence of IBMX–IN–DEX and liraglutide alone were measured. Adipocytes showed an increase in Oil Red O-stained neutral lipid droplet formation in comparison with that of non-treated control cells (Fig. 2a, b) except for the group where the highest dose of liraglutide (100 nm) was added in the absence of the differentiating agents. The results

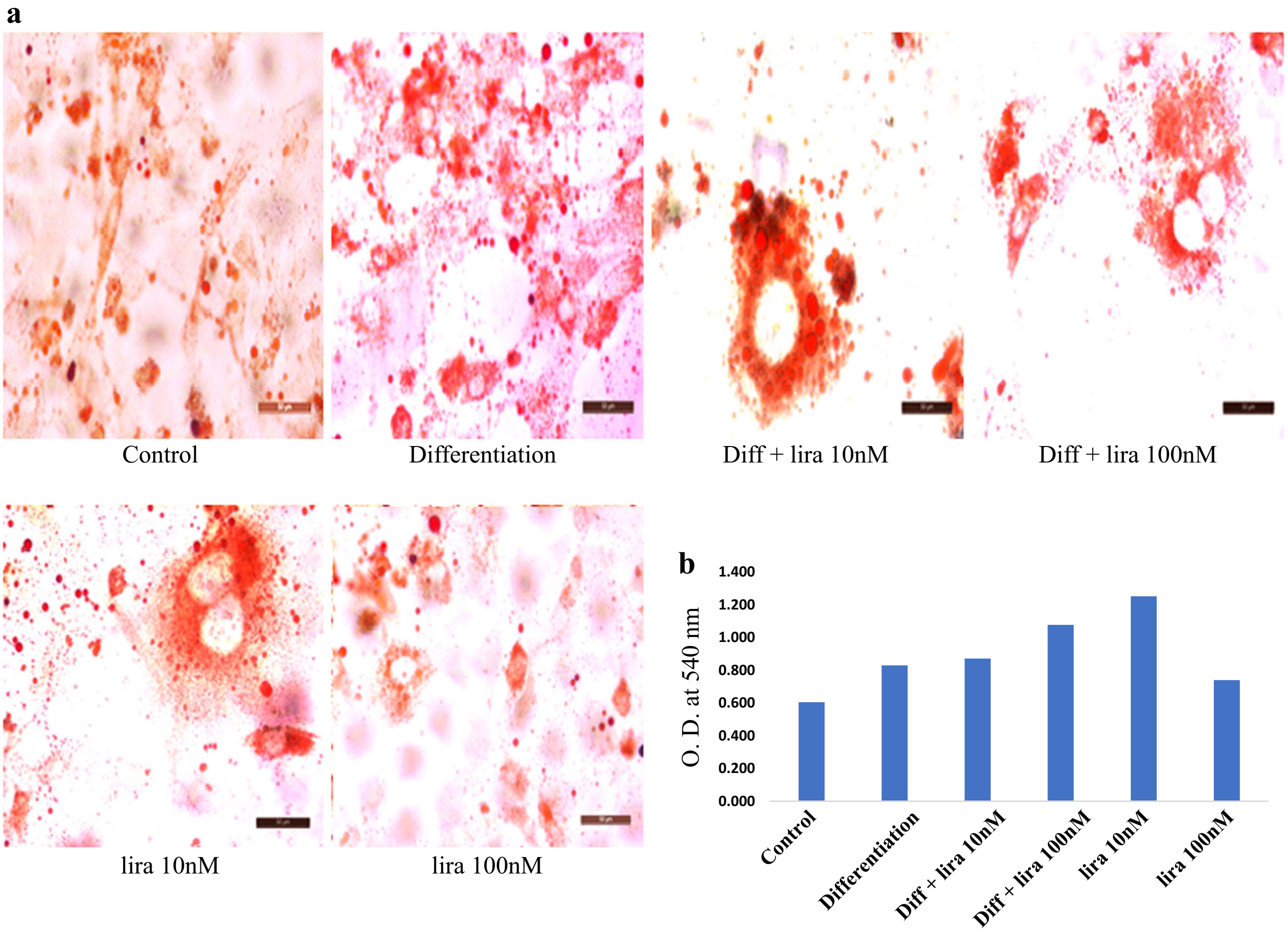


Fig. 2 Effect of liraglutide on lipid accumulation. **a** Murine 3T3-L1 preadipocytes were treated with two concentrations of liraglutide (10 and 100 nM). Liraglutide doses were also added along with or without the differentiation mediators such as IBMX, dexamethasone, and insulin. At day 8 of differentiation, the cells were stained with Oil Red O. **b** Subsequently, 125 µl of isopropyl alcohol was used to elute the Oil Red O, and lipid accumulation was quantified using a microplate reader. The data shown represent the means ± SEM of 3 separate experiments. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. 10 and 100 nM of liraglutide

demonstrate that both the doses of liraglutide when treated in the presence of the differentiating ingredients and the low dose of liraglutide (10 nM) when treated in the absence of the differentiating agents promote adipogenesis in 3T3-L1 preadipocytes, thereby increasing neutral lipid accumulation.

Glucose uptake by cells

To investigate the effect of liraglutide on glucose uptake by the 3T3-L1 murine pre-adipocyte with and without the differentiation ingredients (IBMX–IN–DEX), the indirect assay of glucose estimation in the media was performed (Fig. 3a). There was a significant ($P < 0.001$) decrease in glucose concentration in media in the presence of liraglutide, both with and without the differentiation ingredients (IBMX–IN–DEX), suggesting in the presence of liraglutide there is a significant uptake of glucose by the cells. Moreover, there was a significant ($P < 0.05$) decrease in glucose concentration in media when cells were only treated with low dose of liraglutide in the absence of differentiating agents (IBMX–IN–DEX), thereby suggesting that the low dose of liraglutide in the absence of IBMX, IN, and DEX, more efficiently triggers glucose uptake by the adipocytes and their differentiation.

Effect of liraglutide on adipogenic markers during pre-adipocyte differentiation

To further determine the effect of liraglutide on the differentiation markers like PPAR-γ and C/EBPα, 3T3L1 pre-adipocyte cells were seeded and differentiated with or without liraglutide for 8 days and then qPCR was performed as mentioned above. The expression of PPAR-γ and C/EBPα (Fig. 3b) showed a significant ($P < 0.05$) increase with the 10 nM dose of liraglutide as compared to the

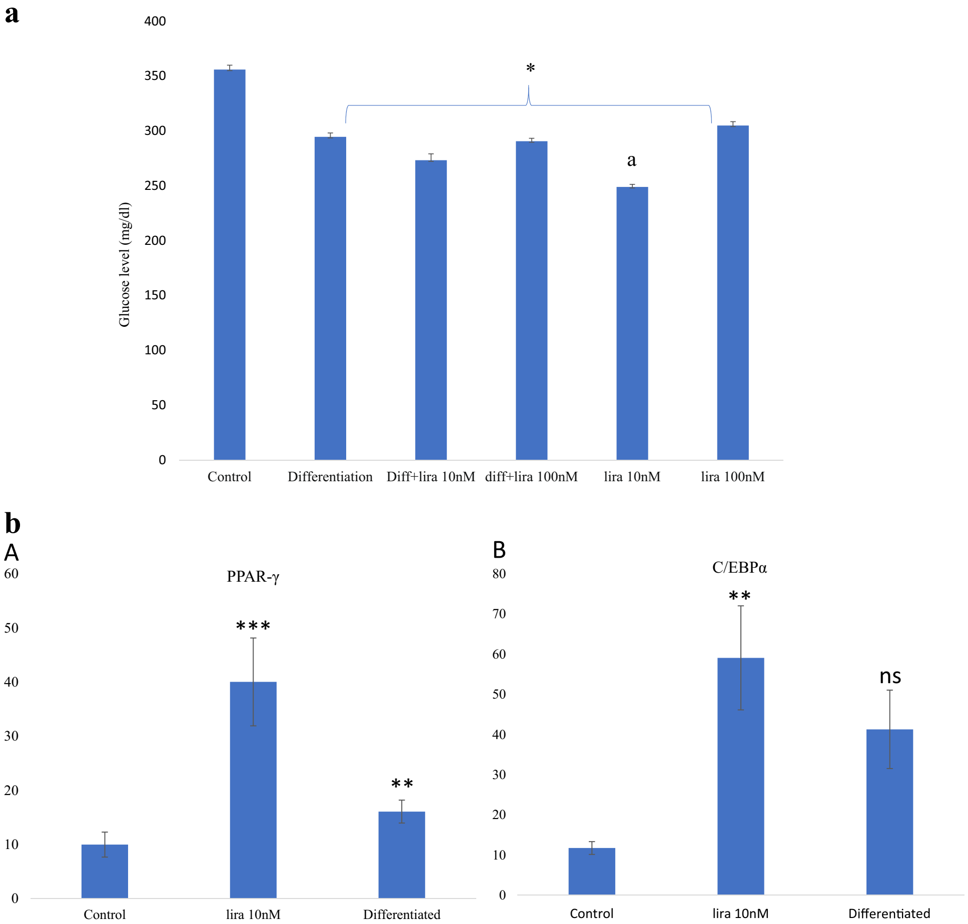


Fig. 3 **a** Effect of liraglutide on glucose uptake by cells. Murine 3T3-L1 preadipocytes were treated with two concentrations of liraglutide (10 and 100 nM). Liraglutide doses were also added along with or without the differentiation mediators such as IBMX, dexamethasone, and insulin. At day 8 of differentiation, glucose was measured in the media. There was a significant (*$P < 0.05$) decrease in glucose concentration in the media of all the groups in comparison to the untreated control group media. Also, there was a significant (**a**, $P < 0.05$) decrease in glucose concentration in the media of the group treated with low dose of liraglutide in comparison to the lira+IN+IBMX+DEX groups (lanes 2 and 3), respectively. **b** Effect of liraglutide on the expression of the adipocyte differentiation markers. Liraglutide (10 nM) was added to the differentiation medium, and the mRNA levels of PPAR-γ and C/EBPα were analyzed by qRT-PCR. **a**, **b** The data shown represent the means ± SEM of 4–5 independent experiments. There was a significant ($P < 0.05$) increase in the expression of PPAR-γ and C/EBPα as compared to the control

control and differentiation ingredients of IBMX, IN, and DEX, thereby strengthening the hypothesis that even liraglutide alone can upregulate adipogenesis.

Effect of liraglutide on body weight, serum levels of glucose, triglycerides, testosterone, and estrogen levels in PCOS mice

There was a significant decrease ($P < 0.05$) in body mass of mice treated with both the doses of liraglutide in comparison to the PCOS mice (Fig. 4a).

Serum glucose level showed a significant ($P < 0.05$) decrease in liraglutide-treated groups in comparison to PCOS mice group (Fig. 4b).

Serum triglyceride showed a significant ($P < 0.05$) decrease in vehicle-treated group and liraglutide-treated groups in comparison to PCOS mice group (Fig. 4c).

Effect of liraglutide on testosterone and estradiol in PCOS mice

Serum testosterone level too followed the pattern of the earlier parameters, decreasing significantly ($P < 0.05$) in the liraglutide-treated groups when compared to PCOS mice group (Fig. 5a).

Serum estradiol level showed a significant ($P < 0.05$) decrease in vehicle-treated group and liraglutide-treated groups in comparison to PCOS mice group (Fig. 5b).

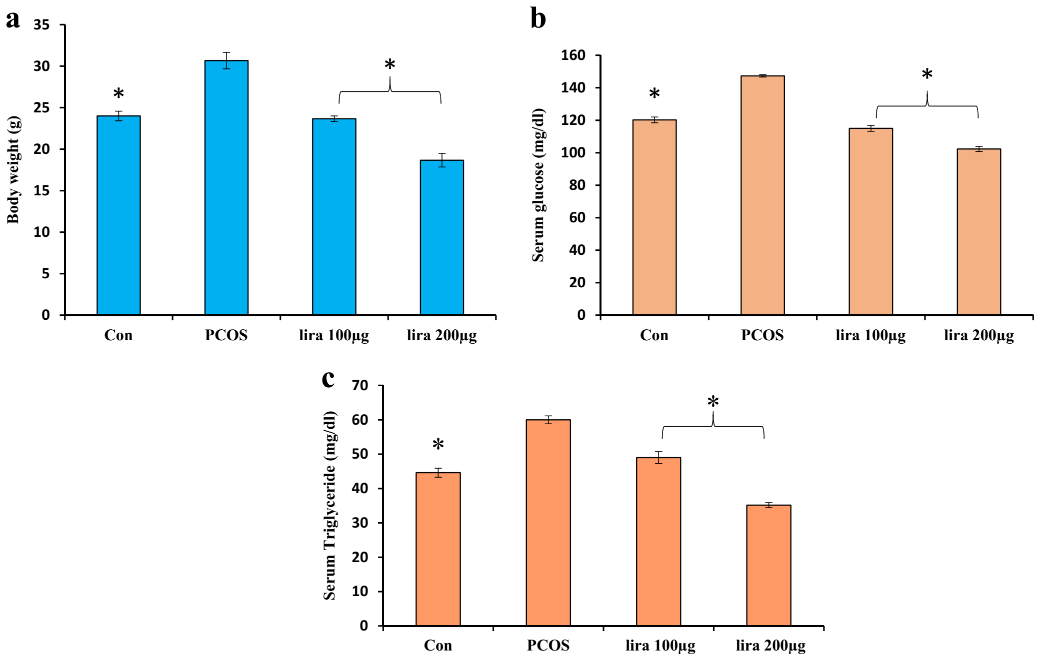


Fig. 4 Effect of liraglutide treatment on **a** body weight, **b** serum glucose, **c** triglyceride (TG) levels. A significant ($P < 0.05$) decrease in body weight after the treatment with both the doses of liraglutide-treated PCO-mice and vehicle-treated control is observed (**a**). Glucose level and TG content showed significant ($P < 0.05$) decline in both the doses of liraglutide-treated PCO-mice and vehicle-treated control (**b**, **c**). Values are mean ± SEM. Values are significant ($P < 0.05$) when compared to PCO-mice

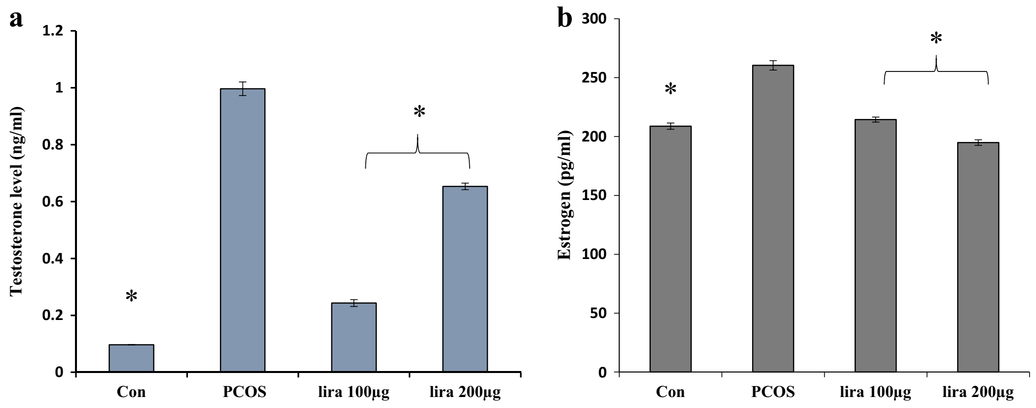


Fig. 5 Effect of liraglutide treatment on **a** circulating testosterone and **b** estradiol levels. Circulating T and estrogen levels were significantly ($P < 0.05$) decreased after the treatment with both the doses of liraglutide as compared to the PCO-mice. Values are mean ± SEM. Values are significant ($P < 0.05$) when compared to PCO-mice

Effect of liraglutide on ovarian histomorphology in PCOS-induced mice

Ovary of DHEA-treated mice showed typical PCOS-like features with increased number of cystic follicles, however, with liraglutide treatment there was an increase in the number of healthy follicles and a decrease in the number of cystic follicles. Oocytes and corpora lutea of liraglutide-treated ovary also featured to be normal and healthy (Fig. 6).

Effect of liraglutide treatment on estrous cycle

Vaginal smearing in the control group showed normal cyclicity as revealed by the vaginal smearing analysis (Fig. 7). PCOS group animals showed constant diestrus condition

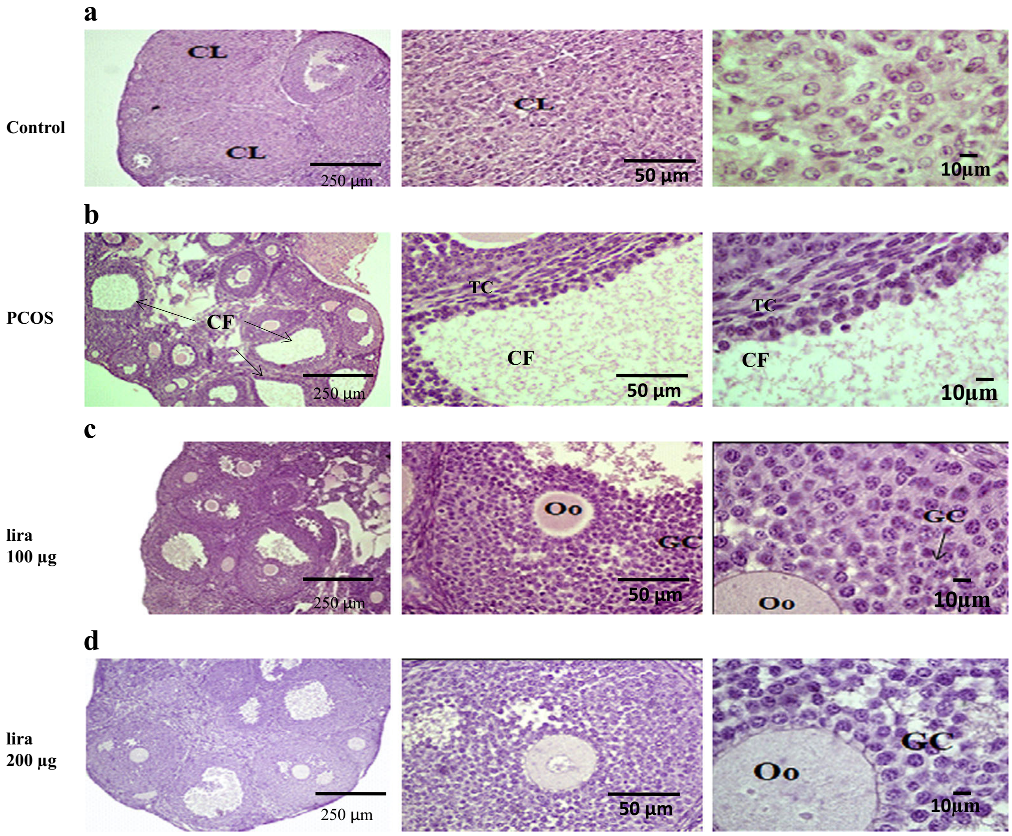


Fig. 6 Histomorphology of ovaries after PCOS induction and liraglutide treatment. Exemplary transverse sections of the ovary of mice ($n = 6$/group) induced with PCOS and further treated with liraglutide. Panel (**a**) shows the ovaries of mice treated with vehicle-treated control and (**b**) induction of PCOS with DHEA for 25 days. Histology of the control ovaries shows normal features compared to increased cystic follicles in PCOS-induced ovaries. **c**, **d** show the ovaries of mice treated with liraglutide for 15 days after induction of PCOS contained mature follicles with healthy oocytes and thick layer of granulosa cells (magnification at ×10, ×40, and ×100). CF cystic follicles, GC granulosa cell layer, TL theca cell layer, O oocyte

indicating the acyclic condition. Low dose of liraglutide-treated animals showed acyclic condition which was long followed by normal cycle. High dose of liraglutide-treated animals showed short acyclic period along with two normal cycles.

Effect of liraglutide on GLUT4 protein in the ovary

Changes in the relative concentration of GLUT4 protein expression in the ovaries of mice were studied in vehicle-treated control, PCOS mice, and PCOS mice treated with liraglutide by using immunohistochemistry (Fig. 8).

The immunostaining of GLUT4 protein showed intense staining in the theca cells of healthy antral follicles in vehicle-treated control mice. In the ovary of PCOS mice GLUT4 is present in follicular fluid. Low dose of liraglutide-treated ovary of PCOS mice showed moderate staining of GLUT4 mainly in theca cells and few granulosa cells of antral follicles. No staining of GLUT4 was found in high dose of liraglutide-treated PCOS mice (Fig. 8).

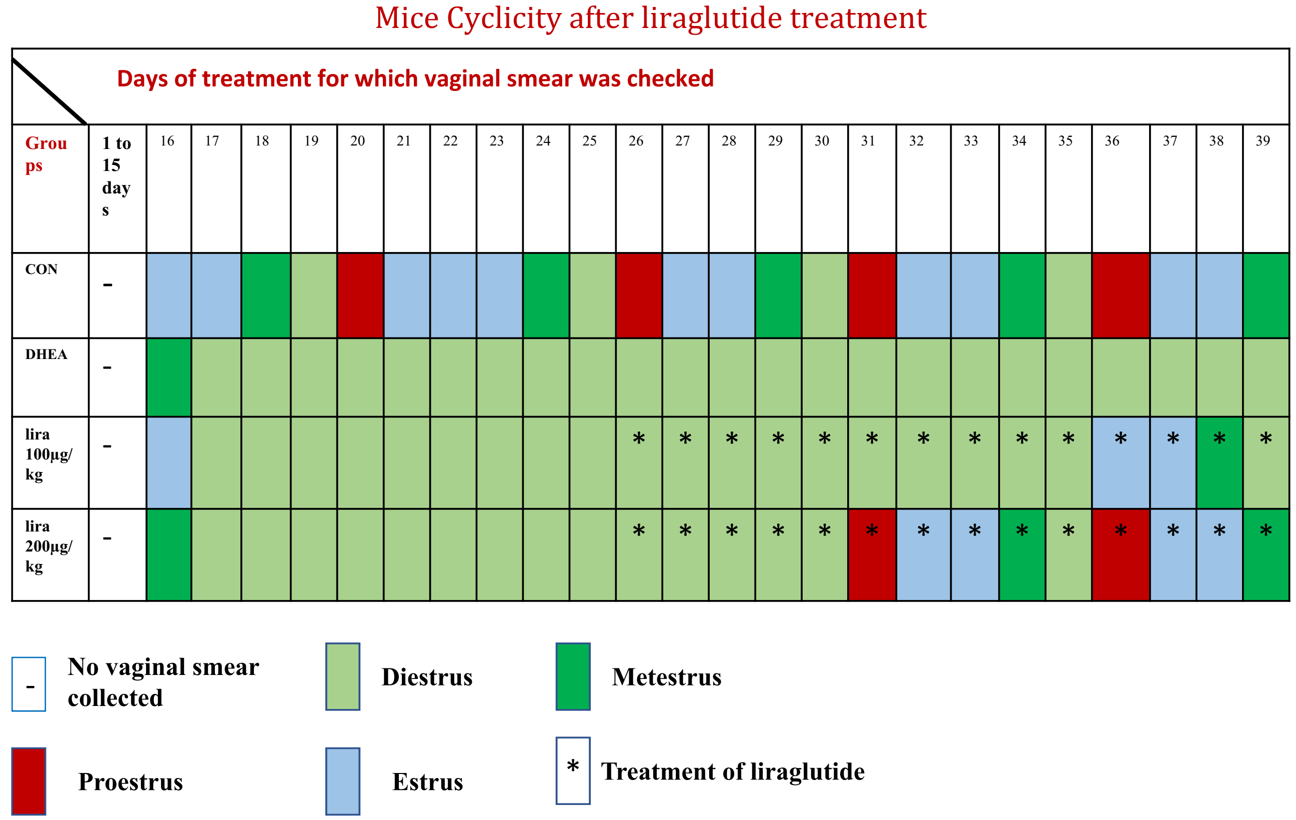
Changes in adiponectin expression/level in serum

The serum adiponectin expression was detected by immunoblot followed by densitometric analysis during PCOS and liraglutide treatment to PCOS mice (Fig. 9a). Densitometric analysis of the immunoblot indicated significant variation in the serum adiponectin level in PCOS study. PCOS-mice showed significant ($P < 0.05$) decrease in the serum adiponectin levels when compared to control whereas there was a significant ($P < 0.05$) increase in the level of adiponectin in those treated with low and high doses of liraglutide.

Immunolocalization of Adipo R1 protein in the ovary

Variation in the immunostaining pattern of Adipo R1 proteins in the ovaries of mice were studied in vehicle-treated control, PCOS mice, and liraglutide-treated PCOS mice using immunohistochemistry as shown in Fig. 9b.

The immunostaining of Adipo R1 protein is intense in the theca cells of healthy antral follicles in vehicle-treated



Mice Cyclicity after liraglutide treatment

	Days of treatment for which vaginal smear was checked																							
Groups	1 to 15 days	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
CON	-	🟦	🟦	🟢	🟩	🟥	🟦	🟦	🟦	🟢	🟩	🟥	🟦	🟦	🟢	🟩	🟥	🟦	🟦	🟢	🟩	🟥	🟦	🟦	🟢
DHEA	-	🟢	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
lira 100µg/kg	-	🟦	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩 *	🟩 *	🟩 *	🟩 *	🟩 *	🟩 *	🟩 *	🟩 *	🟩 *	🟩 *	🟦 *	🟦 *	🟢 *	🟩 *
lira 200µg/kg	-	🟢	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩 *	🟩 *	🟩 *	🟩 *	🟩 *	🟥 *	🟦 *	🟦 *	🟢 *	🟩 *	🟥 *	🟦 *	🟦 *	🟢 *

- No vaginal smear collected

🟩 Diestrus

🟢 Metestrus

🟥 Proestrus

🟦 Estrus

* Treatment of liraglutide

Fig. 7 Estrous cycle after inducing PCOS and after treatment with liraglutide. PCOS group animals showed consistent diestrus condition. Normal cyclicity was attained after treatment with both the doses of liraglutide

control (Fig. 9b). Mild immunostaining was found in the theca cells of cystic follicles. Low dose of liraglutide-treated ovary of PCOS mice showed moderate staining of Adipo R1 in theca cells and oocyte of the antral follicle. Strong immunoreactivity was found in granulosa cells, theca cells, and oocyte of the antral follicle.

Immunolocalization of PI3K and Akt in the ovary

The presence of PI3K and Akt was demonstrated immunohistochemically using phosphospecific antibodies in the ovary of mice. The chromogenic signals were evaluated in vehicle-treated control, PCOS mice, and liraglutide-treated PCOS mice (Fig. 10).

pPI3K showed moderate staining in the vehicle-treated control, immunostaining was mainly observed in the granulosa cells and theca cells. Mild staining was observed in the theca cells of cystic follicles, whereas intense staining was observed after the treatment with liraglutide in the granulosa cells, theca cells, and the stroma. Similar expression pattern was observed with pAkt in the control, mainly in the thecal and granulosa cells, mild staining in the theca cells of cystic follicles in PCOS mice and intense staining was observed after liraglutide treatment mainly in the granulosa cells, theca cells, and the stroma.

Discussion

The present study investigated the expression profile of various adipokines in the differentiated 3T3-L1 cells after liraglutide treatment. The results of this study suggested liraglutide promotes adiponectin, IL-6 synthesis, and inhibits ICAM-1 production in a glucose-dependent mode. The study further showed that liraglutide with or without the potent differentiation causing agents, IBMX–IN–DEX, helps in storing more neutral lipid within the cells thus inducing differentiation. Also, the study showed for the first time that liraglutide treatment to the DHEA-induced PCOS mice improves the ovarian physiology and ameliorates PCOS condition.

The results of the adipokine profile showed that liraglutide upregulates adiponectin and interleukin-6 (IL-6) expression and downregulates intercellular adhesion molecule-1 (ICAM-1) and leptin expression in adipocytes. Currently, few reports exist which discuss about the variation in adipokine profile after liraglutide treatment. In one such prospective study, it was shown that after 14 weeks of treatment of the overweight or obese (BMI, 25 kg/m^2) type II diabetes patients with liraglutide treatment a mild increase in visfatin and resistin was observed however there was no change observed in the circulating level of leptin and

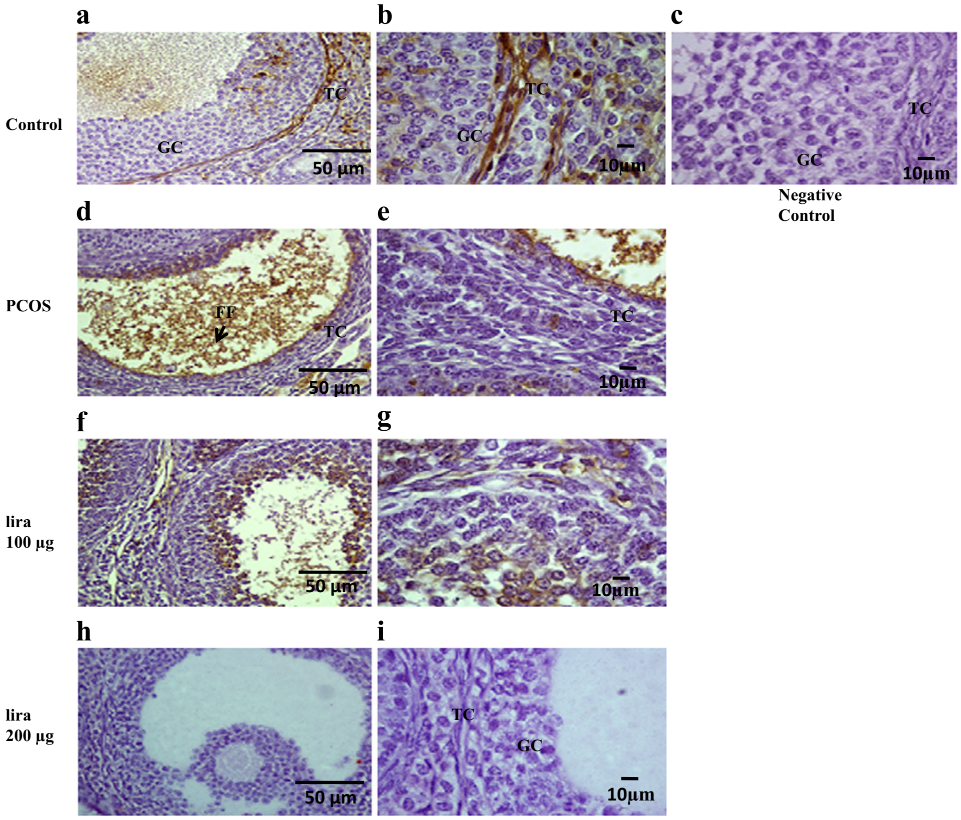


Fig. 8 Immunolocalization of GLUT-4 in the ovary of mice in vehicle-treated control, PCO-mice, and liraglutide-treated group (**a**–**i**). The GLUT-4 positive immunostaining is localized in thecal cells (TC), follicular fluid (FF), and a few granulosa cells (GC). Complete lack of immunostaining for GLUT-4 in the high liraglutide-treated PCO ovary. Follicular fluid showed intense immunostaining in PCO induced cystic follicles. The arrows indicate GLUT-5 immunostaining in the different cell types within ovary. Negative control for GLUT-4 protein was shown in (**b′**). Figures are shown at ×40 and ×100 magnifications

adiponectin, respectively [20]. The present study showed that liraglutide decreased ICAM-1 expression in the adipocytes. Increased expression of ICAM-1 is positively correlated with abdominal fat deposition in obesity and type II diabetes [41, 42]. The present study also shows that liraglutide treatment to the 3T3-L1 cells up-regulates IL-6. Another Glp-1 analogue, exendin 4, had been recently reported to stimulate the expression of IL-6 mRNA in brain and subsequent weight loss of mice [43]. It had been earlier shown that cytokines like IL-1 and IL-6 are involved in regulation of metabolic function in healthy animals as evident from the fact that mice which lack IL-1R or IL-6 develop late-onset obesity and have disturbed glucose metabolism [44]. Increase in adiponectin, IL-6 and subsequent decrease in ICAM-1 levels after liraglutide treatment further suggest anti-obesity potentials of this drug.

Our results suggest that liraglutide promotes alteration in the adipokine profile of 3T3-L1 cells in a glucose-dependent manner. Numerous reports exist suggesting liraglutide improves glucose clearance ability [19, 45, 46]. Further, the Oil Red O staining shows an increase in triglyceride storage within the 3T3-L1 cells during their differentiation after liraglutide treatment which is a prerequisite for proper differentiation. Studies have shown that proper differentiation of adipocyte coincides with increased glucose clearance, lipid storage, and adiponectin secretion [19, 47, 48].

To enhance the differentiation of 3T3-L1 pre-adipocytes into mature adipocytes in vitro, insulin, dexamethasone, and IBMX are commonly used. The present study for the first time shows that the low dose of liraglutide alone in the absence of the classical IN–IBMX–DEX cocktail, promotes the 3T3-L1 cells to differentiate into mature adipocytes, as evident from the increased glucose uptake, the Oil Red O staining, increased expression of PPARγ and C/EBPα. Previously a study suggested that liraglutide promotes adipogenesis in the presence of IBMX in a PPARγ, C/EBP and glucose-dependent pathway [19]. This is the first study showing in the absence of IBMX and other differentiating factors, liraglutide alone might be equally potent to differentiate the 3T3-L1 cells, though the present group is further investigating the molecular pathway to strengthen this hypothesis. Earlier studies have shown that similar insulin-sensitizing agent, rosiglitazone, a member of the thiazolidinediones, acting as an agonist for PPAR gamma, promotes the differentiation of pluripotent stem cells to adipocytes depicting its adipogenic effect [49–51].

Obtaining a surge in adiponectin level secreted by the 3T3-L1 cells after liraglutide treatment, was the rationale to treat PCOS mice with liraglutide. Adiponectin is considered

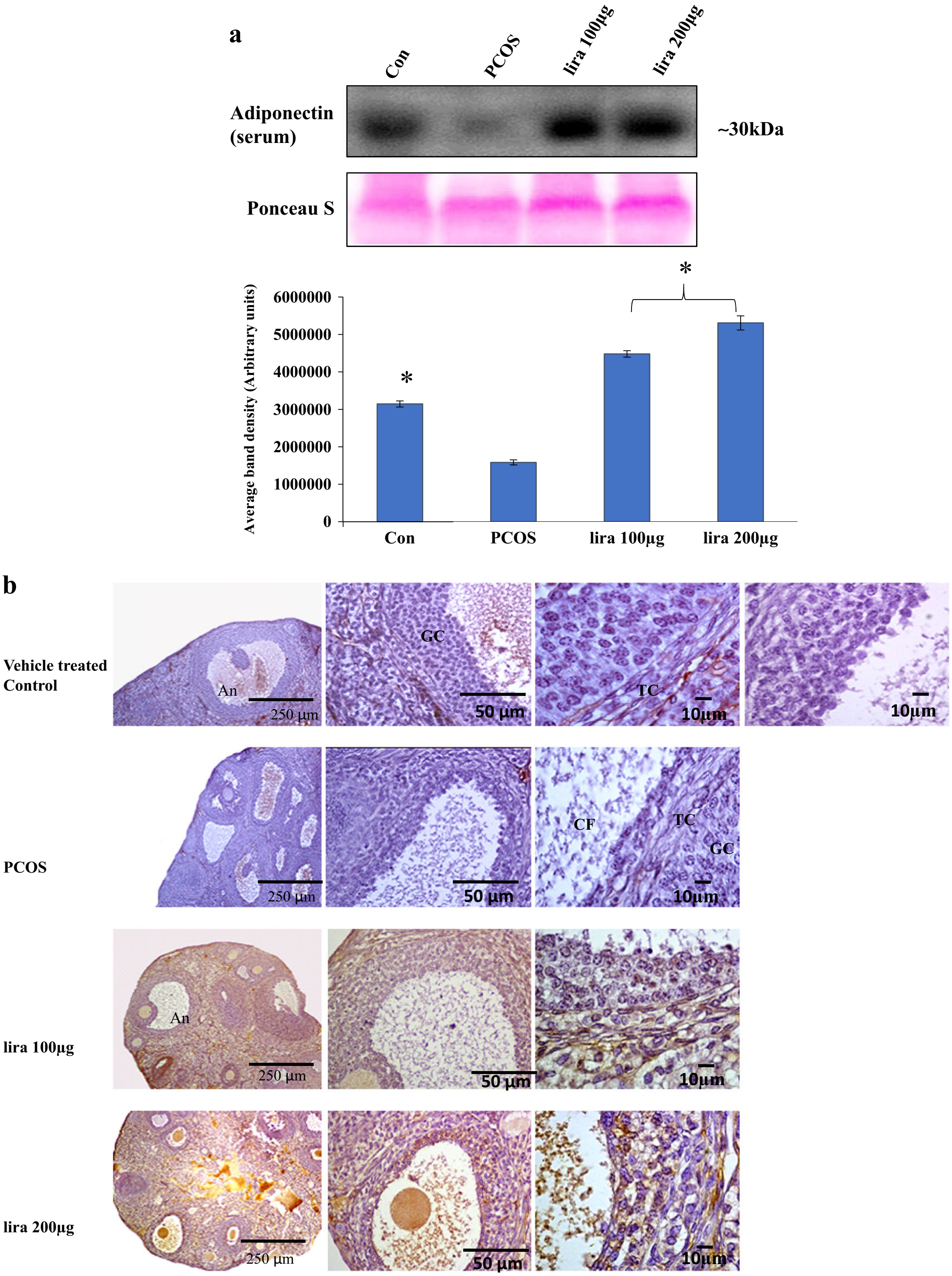


Fig. 9 **a** Western blot analysis of adiponectin. Adiponectin protein in the serum showed increased expression after treatment with both the doses of liraglutide as compared to the PCO-mice. Equal loading was confirmed by Ponceau staining of the membrane. Values are significantly ($P < 0.05$) different from others. Data represent means ± SEM. **b** Immunolocalization of adiponectin receptor1 (Adipo R1) in the ovary of mice. Adipo R1 in vehicle-treated control, PCO-mice, and liraglutide treatment (a–m). Immunostaining of Adipo R1 protein is intense in the theca cells of healthy antral follicles in vehicle-treated control. Mild immunostaining was found in the theca cells of cystic follicles. Low dose of liraglutide-treated ovary showed moderate staining of in theca cells and oocyte of the antral follicle. Strong immunoreactivity was found in granulosa cells, theca cells, and oocyte of the antral follicle. Negative control for Adipo R1 protein was shown in (**d**). Figures are shown at ×10, ×40, and ×100 magnification

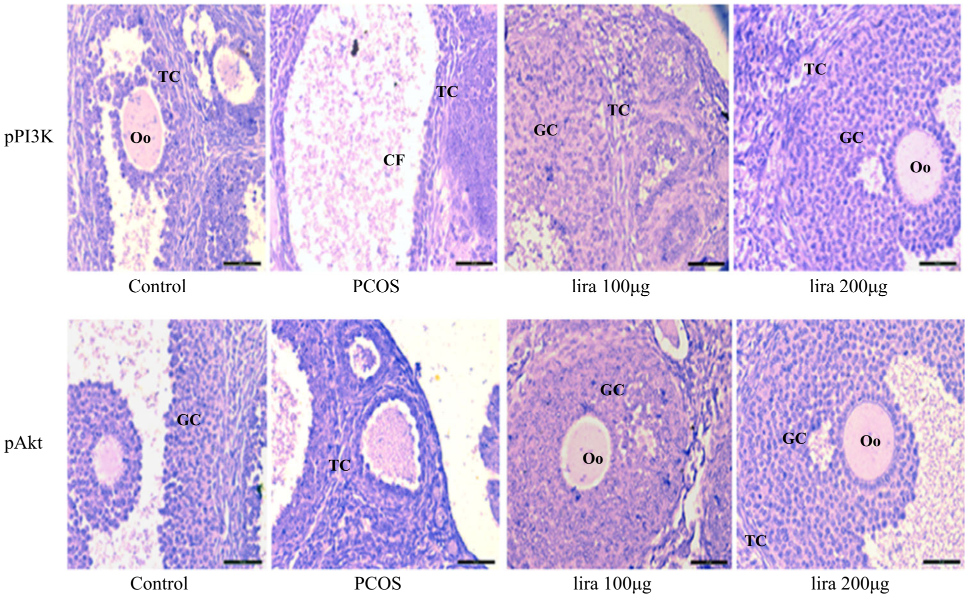


Fig. 10 Immunolocalization of PI3K and Akt in the ovary of mice. Immunostaining of pPI3K and pAkt showed intense staining after liraglutide treatment mainly in the in the granulosa cells, theca cells, and the stroma as compared to the control and PCO-mice. Figures are shown at ×20 magnification

a beneficial adipokine being associated with improved menstrual function and better outcomes in assisted reproductive cycles [52]. PCOS is closely associated with the dysfunction of adipose tissue and altered expression of adipokines [53]. The present study shows pre-pubertal mice treated with DHEA, showed increased number of cystic follicles in the ovaries, increased serum glucose, triglyceride and testosterone levels as features of PCOS-like condition in mice. However, after liraglutide treatment to the PCOS mice there was a decrease in body mass, serum glucose, and testosterone levels. The ovaries also showed normal features of corpus luteum and lacking cystic follicles with increase in both early and late antral follicles. Our result revealed that on injecting liraglutide regulates the disturbed cyclicity with increasing doses as clear from the vaginal smearing. Thus, we positively suggest liraglutide is a crucial candidate drug in improving the ovarian physiology in PCOS-induced mice. Few reports suggest that obese women with PCOS when treated with liraglutide in combination with metformin resulted in significant weight loss [23, 25]. The present study showed that the lower dose of liraglutide decreased serum testosterone level however, the higher dose increased serum testosterone level in comparison to PCOS-induced mice. Liraglutide is capable of managing PCOS condition in PCOS-induced mice. Both the doses of liraglutide decreased body weight, circulating glucose, triglyceride and estradiol levels however, liraglutide in lower dose decreased serum testosterone level and the higher dose increased serum testosterone level in PCOS-induced mice.

It is known that liraglutide increases beta cell proliferation as well as its function and increases serum insulin levels [17]. At higher dose, liraglutide might have increased serum insulin level which in turn has increased serum testosterone level as it is known that insulin triggers testosterone production [54]. Also, a recent study suggests that low dose liraglutide in combination with metformin was more potent to improve androgen profile vs. high liraglutide treatment to obese PCOS patients [55]

The present study also showed that liraglutide treatment to the PCOS mice lowered serum estrogen level. Earlier study demonstrated that enhanced levels of progesterone and estrogen result in follicular cysts formation, thereby preventing the development of dominant follicles [36]. This study is first of its kind showing how liraglutide improves the ovarian physiology in PCOS condition and initiates cyclicity in acyclic PCOS mice.

Also, this study further illustrates the mechanism of the PCOS management which might occur through increased adiponectin secretion in the liraglutide-treated animal model of PCOS. After treatment with liraglutide, there was a significant rise in the circulating adiponectin expression in the PCOS mice followed by an increase in the expression of ovarian adiponectin receptor 1. A recent study had shown that through brown adipose tissue transplantation in DHEA-treated PCOS mice ameliorated PCOS condition and improved ovarian physiology through increased serum adiponectin level [56]. In addition to this, recently direct and systemic action of adiponectin reversing the PCOS condition in mice was reported [37, 40]. Adiponectin levels are lower in obese PCOS patients, individuals suffering from obesity and insulin resistance condition, whereas in non-obese PCOS patient's adiponectin level decreases with increase in body mass index [30, 57]. Recent reports suggest that adiponectin is a biomarker for females to be diagnosed as PCOS risk and provide an opportunity for earlier intervention [30, 58].

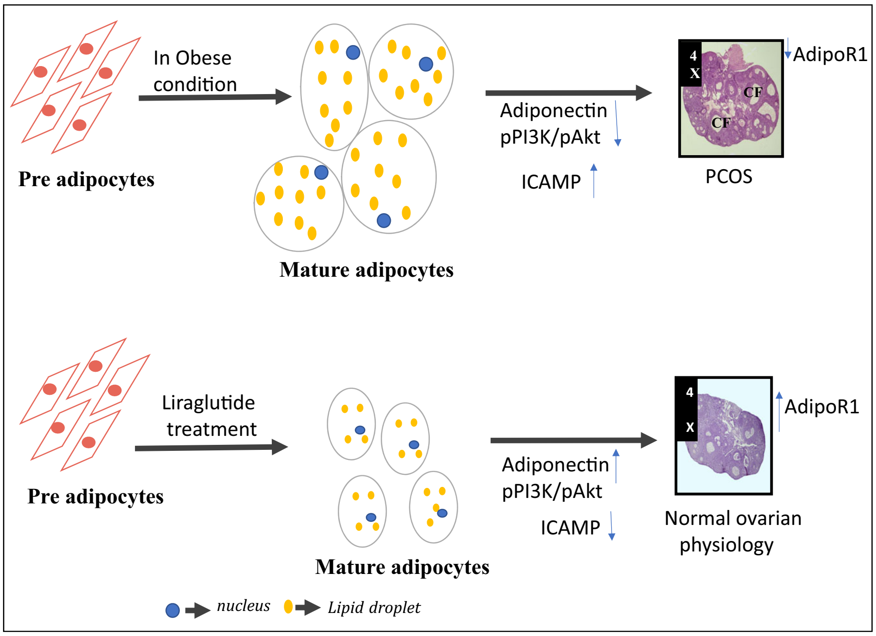


Fig. 11 Liraglutide treatment on adipocytes and PCOS-induced mice. The hypothetical figure summarizing the effect of liraglutide on adipogenesis and PCOS through modulation of adipokines

The present study also showed an increased GLUT4 expression in ovary and a decrease in serum glucose level of PCOS mice after liraglutide treatment coinciding with an increased adiponectin level suggesting an adiponectin-dependent improvement of glucose uptake. Adiponectin has been reported to sensitize insulin action and improve insulin resistance condition [59] and insulin responsiveness of the glucose transport system in adipocytes [60]. A study showed that adiponectin dependent increase of insulin action in glucose uptake by 78% through increased glucose transporter 4 (GLUT4) gene expression and increased GLUT4 recruitment to the plasma membrane of adipocytes [60].

Studies have shown that PI3K is the critical regulator of oogenesis, follicle development, and survival [61–64]. In rodents, Akt1 is expressed in both granulosa and oocytes [65]. In our study, we observed the expression of pI3K and Akt1 in the ovary. Both were mainly localized in the granulosa, theca cells, and mild staining in the oocytes. Moderate staining was observed in the control group, Akt1 mainly localized in the granulosa cells, theca cells. The mice with induced PCOS showed mild staining in the theca cells of the cystic follicles, however, liraglutide-treated mice showed an increased staining in the granulosa cells, theca cells, and the stroma.

Studies in PCOS patients have shown that increased levels of insulin in follicular fluid increases the expression levels of PTEN which further antagonizes the PI3K activity [66]. Numerous studies found PI3K-Akt signaling pathway disorder in patients suffering from PCOS [67–69]. Fukuda et al. showed that PI3K-Akt pathway was related to androgen production and believed that this pathway could be the pathophysiology to PCOS [67]. In this study, we observed mild expression of PI3K and Akt in the theca and granulosa cells in the PCOS-induced mice, however an increased expression of PI3K and Akt was observed after the treatment with liraglutide. Studies have also shown Akt regulates glucose uptake into muscles and fat by stimulating the translocation of GLUT4 to the plasma membrane and defective glucose transport contributes to insulin resistance by inducing serine phosphorylation of IRS-1, followed by reduction of PI3K-Akt activity [70]. Adipo R1 has been reported to be expressed in ovary and with low expression level in the PCOS condition [71], similarly as observed in the present study. Adiponectin receptors have also been shown to be down-regulated in insulin resistance and type II diabetes condition [72] which further strengthens the results obtained in this study.

To summarize (Fig. 11), the present work showed liraglutide enhanced adiponectin and IL-6 synthesis and decreased ICAM 1 in differentiated 3T3-L1 cells. Liraglutide without aid of any differentiating factors, like insulin, IBMX, and dexamethasone, was able to solely differentiate pre-adipocytes as evident from the glucose uptake and Oil Red O staining. Further, liraglutide treatment to the PCOS mice, induced cyclicity in them, improved their ovarian physiology as evident by a decrease in cystic follicles and increase in healthy follicles and the presence of corpus luteum. Moreover, liraglutide treatment decreased serum glucose, triglyceride, and testosterone levels in comparison to the control PCOS mice. The serum adiponectin levels were increased as well as the expression of ovarian Adipo R1, GLUT4, PI3K, and Akt after liraglutide treatment thereby improving ovarian function in PCOS mice through the PI3K/Akt signaling pathway. Hence, we hereby suggest that in addition to managing diabetic and obese condition, liraglutide can also manage obesity-associated PCOS condition by altering the adiponectin levels.

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Author contributions A.S. did the animal-related work and wrote the manuscript. J.R.D.F. did the cell differentiation, partial IHC, and wrote, edited the manuscript. G.C. did the adipokine array work and edited the manuscript. A.K. edited the manuscript. A.B. planned the work and edited the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All animal experimentation and procedures were approved by the Institutional Animal Ethics committee of Birla Institute of Technology and Science (BITS) Pilani Rajasthan, Reference number IAEC/REC/19/21.

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