Oxytocine Counteracts insulin resistance, improves dyslipidemia and acts as anti-inflammation in rats on high fat diet

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Abstract: High fat diet is heavily incriminated in obesity and related insulin resistance, type2 diabetes and metabolic syndrome. Oxytocin is lately reported to decrease body weight and regulate glucose homeostasis. The aim of this study is to investigate the role of oxytocin in metabolic disruption in rats on high fat diet (HFD). Experimental procedures included measurement of body mass index (BMI), blood glucose, serum insulin and basal and insulin-treated glucose uptake by the diaphragm and epididymal fat. Assays of serum triglycerides, and total cholesterol: LDL-C and HDL-C Plasma free fatty acids; as well as serum IL-1β, IL-6 and TNF-α cytokines were performed. Western Blots were done for IRS-1 and IRS-2 in epididymal fat and diaphragm. HFD rats showed significantly higher blood glucose, serum insulin and significantly lower glucose uptake by diaphragm and epididymal fat. There were also significant increase in plasma lipids and pro-inflammatory cytokines from HFD. Moreover, IRS-1 and IRS-2 were significantly decreased. These metabolic disorders however, were reversed in HFD when oxytocin was supplemented. Thus, oxytocin employsa protective mechanismin insulin sensitive tissues which act against metabolic disorders induced by HFD. Further experiments are required to explore these defensive mechanisms.

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1. Introduction

Obesity is a major public health concern with an increasing prevalence worldwide (1). Obesity is associated with insulin resistance (IR) and known as the risk factor for hypertension, dyslipidemia, metabolic syndrome, and type 2 diabetes mellitus (Type 2DM) (2). Chronic inflammation in adipose tissue has been reported to play a crucial role in the development of obesity-induced IR and co-morbidities (3). Adipose tissue macrophage infiltration is considered to contribute to obesity-induced inflammation via secretion of a wide variety of adipose tissue-derived pro-inflammatory cytokines, such as tumornecrosis factor-alpha (TNF-α) and monocyte chemoattractantprotein-1 (MCP-1) (4, 5). It has been shown to have local effects on white adipose tissue as well as potential systemic effects on other organs, including liver or skeletal muscle, to promote the development of IR(6,7).

Coincidently, reactive oxygen species (ROS) generation as a result of oxidative stress has been implicated in the development of insulin resistance and type 2 DM(8, 9). Adipocytes under oxidative stress exhibited increased ROS generation, DNA damage, shortened telomeres and switched to senescent/pro-inflammatory phenotype with impaired glucose uptake8.

Apart from the traditional role of oxytocin in the physiology of reproduction and lactation, earlier reports pointed to the role of oxytocin in the regulation of food intake and control of body weight (10, 11). The role of oxytocin in metabolic regulation started to attract attention with recent reports. Oxytocin deficient mice exhibited decreased insulin sensitivity and impaired glucose tolerance (12). Oxytocin can lead to reversal of obesity as well as related glucose and insulin disorders in mouse models(13,14).

Despite the metabolic advantage of oxytocin in increasing insulin sensitivity and competing insulin resistance, the mechanism of action of oxytocin is not clear. Some scientists referred improvement of obesity-related insulin resistance to body weight reduction and reversal of obesity related metabolic dysregulation. However, this concept was not the same with others (15).

Hence, the aim of this study was to investigate the protective effects of short term administration of oxytocin against metabolic disturbances in high fat fed rats. We additionally aim to investigate the effect of oxytocin on insulin receptor signaling pathways in adipose tissue and skeletal muscle of high fat fed rats.

2. Materials and methods

Ethical approval

This randomized controlled study was conducted in the Physiology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt from June 1st to August 31st 2015. It was approved by the ethics committee of FMSU REC, Cairo, Egypt.

Animals

This work was undertaken on 45 male Wistar rats. The rats were maintained under standard conditions of boarding. The investigation conforms to the guide for the care and use of laboratory animals published by the United States National Institutes of Health. Forty five male Wistar rats were divided into two groups; one group (n=20) was fed normal diet (59% of food intake from carbohydrates, 10% from fat, 21% from protein, and 10% from minerals and ash); and the second group (n=25), was fed high fat diet (43% of food intake from carbohydrate, 26 % from fat, 21% from proteins, and 10 % from minerals and ashes) for 8 consecutive weeks. The dietary formula was constructed in our laboratory according to the normal nutritional dietary requirement. Body weight was measured every week during the entire experimental period.

Each group of rats was then subdivided into another two subgroups. Normal diet group (ND) n=10, normal diet oxytocin-treated group (NDOXY) n=10, and high fat diet group (HFD) n=13, and high fat diet oxytocin-treated group (HFDOXY) n=12. Oxytocin-treated groups received subcutaneous injection of oxytocin in a dose of40 µIU/ kg body weight for 5 days. Control rats were injected with saline as a vehicle. The dose of oxytocin was chosen according to previous studies and pilot study in our lab (14).

Experimental procedures

On the day of the experiments, overnight fasted rats were weighed and were anaesthetized with thiopental sodium 40 mg/kg intra-peritoneally (Sandoz GmbH, Kundl–Austria). Body lengths were measured. Body mass index (BMI) was calculated using the formula: (weight in kg/ body length in m^2) (16).

Blood sugar was tested using glucose strips. The blood samples were collected from the abdominal aorta then centrifuged, and the serum was stored at −20 °C at separate aliquots for biochemical assays. From each rat the diaphragms were collected and divided into two halves one for determination of glucose uptake by the muscles and the other for measurement of IRS-1 and IRS-2. Epididymal pad of fat was removed. One pad was used for glucose uptake, and the other for measurement of IRS-1 and IRS-2 content. The tissue samples for western blot studies were stored at −80 °C.

Glucose uptake by the diaphragm

Experiment 1*:*

Determination of basal glucose uptake in the diaphragm. This was carried out according to the method described by Saleh and Saleh(17).

From each rat, one hemi-diaphragm was used to measure glucose uptake in the skeletal muscle. The diaphragm was dissected, the thick posterior portion was removed, and then the diaphragm was excised into two halves. One hemi-diaphragm was placed in a Warburgflask containing 2 ml ice balanced salt solution (BSS) in which glucose (2 mg/ml) was dissolved. The flask was then aerated for 5 min with a mixture of CO_2 (5%) and O_2 (95%) and then incubated at 37 °C in a Warburg apparatus (shaking rate of 100 cycles/min) for 90 min. At the end of the incubation, the hemi-diaphragm was removed, washed in distilled water, blotted with filter paper and weighed. Half ml aliquots of the incubation medium were used for measurement of glucose concentration. Glucose was measured spectrophotometrically by an enzymatic colorimetric assay (Biodiagnostic, Egypt).

Experiment 2:

Determination of insulin-treated glucose uptake by the skeletal muscle. The other half of each diaphragm was incubated identically to experiment 1 except that the incubation solution was supplemented with insulin in concentration of 20 mU/ml (18).

Calculations:

Glucose concentration in the incubation media was measured. The rate of glucose uptake was determined from the rate of decrease of glucose concentration in the media during incubation. The rates of uptake were expressed as mg/100 ml of glucose taken up per 100 mg tissue/90 min incubation. Rate of glucose uptake =Glucose concentration before incubation - Glucose concentration after incubation / Weight of the tissue in gram divided by 10 (19).

Glucose uptake by the adipose tissues

From each rat, one pad of epididymal fat was used to measure glucose uptake in the adipose tissues. The same steps used for determination of glucose uptake by skeletal muscle were used to determine glucose uptake by adipose tissues where the epididymal fat from one side was dissected and cut into 2 halves one used for determination of basal glucose uptake as done in skeletal muscle (Experiment 1) and the other piece was used to determine insulin-treated glucose uptake (Experiment 2). Again the method for measurement of glucose concentration in the aliquots and glucose uptake calculations mentioned above were followed.

Western blot

To extract protein from the diaphragm and epididymal fat, tissues were homogenized after adding a solution containing 150 mMNaCl, 5 m MEDTA, 50 mM Tri-HCl (pH 8.0), 1 %-NP 40, 1 m Maprotinin, 0.1 mM leupeptin, and 1 mM pepstatin. The solution

was centrifuged for 30 minutes at 13,000 rpm. Supernatants were collected and assayed for protein content. Protein concentration of the supernatant was determined (BCA protein assay; Pierce, Rockford, IL) prior to storage at -70°C. Protein samples were mixed with Laemmli sample buffer (LSB) and placed in a boiling water bath for 5 min. Proteins were resolved by 10,% SDS-polyacrylamide gel electrophoresis (SDS-PAGE; each loaded with same μg of total protein per lane), and transferred to nitrocellulose membranes. Proteins on the membranes were blocked in 5 % skim milk in phosphate – buffered saline (PBS) (NaCl 8 g, KCl 0.2 g, Na2HPO4 1.44 g, KH2PO4 0.24 g, pH 7.4). Thereafter, protein membranes were incubated with the following primary antibodies: IRS-1, 2 number 2382 and 4502, Cell Signaling. for one hour, and washed thrice (15 min each) in a PBS solution containing 0.1 % tween 20. Washed membrane was then treated with secondary antibody (rabbit IgG) conjugated with horseradish peroxidase (HRP). Then, Super Signal West Femto Maximum Sensitivity Substrate was added and immune-reactive bands were developed on Kodak film. The relative strengths of bands were quantitated by densitometry (Sci – Scan, UUSB).

Biochemical analysis

The following analyses were carried out for cholesterol, triglycerides, and high density lipoprotein (HDL) using kits from Biodiagnostic, Egypt: Low density lipoprotein (LDL) was calculated according to the formula described by You et al., (20). Plasma free fatty acids (FFA), were determined with commercial kits (NEFA C: Wako Chemicals, GmbH, Neuss, Germany.

The plasma concentrations of IL-1β, IL-6 and TNF-α cytokines were determined by ELISA (Life Technologies, USA) using DS2 automated ELISA analyzer (Dynex Technologies, USA), according to the manufacturer's instructions. Insulin was measured using a rat insulin enzyme-linked immunosorbent assay kit (ALPCO Diagnostics Salem, NH). HOMA-IR test was used as a marker for insulin resistance using the equation first described by Matthews (21) HOMA-IR = (glucose × insulin)/22.5: Insulin concentrations reported in μU/L and glucose in mmol/L.

Statistical Analysis

Results were statistically analyzed according to Student's "t" test for unpaired data, using GraphPad Prism software version 7.0. Statistical significance was determined at $P< 0.05$.

3. Results

Glucose metabolism parameters

High fat fed rats showed significantly higher blood glucose, serum insulin and HOMA-IR index as compared to normal diet controls**(Table 1).**In normal control rats, no significant changes were found from oxytocin-treated rats as compared to non-treated controls as regard to all the parameters studied. On the other hand, oxytocin-treated high fat fed rats showed significantly lower blood glucose, serum insulin and HOMA-IR index when compared with diet-matched non-treated rats.

Oxytocin promotes the glucose uptake and insulin sensitivity

Indeed, the basal and insulin-treated glucose uptake in both diaphragm and epididymal fat was significantly lowered from high fat group compared to their controls (**Figure I**).

However, basal and insulin-treated glucose uptake by diaphragm and epididymal fat were significantly higher in the high fat fed rats treated with oxytocin as compared to non- treated diet-matched controls and insignificantly different from control normal diet group.

Compared to normal control rats, high fat fed rats showed significantly lower levels of IRS-1 and IRS-2 in both diaphragm and epididymal fat tissue homogenates **(Table II)**. These levels were significantly higher in the high fat group treated with oxytocin treatment and became in significantly different from normal controls.

Oxytocin improves dyslipidemia and inflammation

Compared to normal diet, high fat fed rats showed significantly higher serum levels of triglycerides, total cholesterol, LDL cholesterol whilst significantly lower levels of HDL cholesterol **(Figure 2)**. Moreover, this group of animals showed significantly high levels of, IL-1β, IL-6 and TNF-α pro-inflammatory cytokines **(Figure 3)**. However, all these parameters were significantly lowered except for (HDL-cholesterol which was significantly elevated) after oxytocin administration to high fat fed group and insignificantly different from normally fed animals **(Figures 2 and 3)**.

4. Discussion

The results of the current study demonstrated that high-fat intake induced dyslipidemia, decreased insulin sensitivity and insulin receptor substrates in adipose tissue and skeletal muscle and resulted in hyperglycemia and metabolic syndrome-like model. These biochemical and physiological changes were reversed with oxytocin supplementation. The induction of insulin resistance and dyslipidemia by HFD is in accordance with previous studies (22). A high fat diet in rats has been shown to induce metabolic syndrome similar to type II diabetes and insulin resistance (23).

Table I: Effect of oxytocin (OXY) on the body mass index (BMI), Blood Glucose, Serum insulin, HOMA-IR index in 2 in normal diet (ND) rats and high fat diet (HFD) rats (mean± SEM)

	ND	NDOXY	HFD	HFDOXY
BMI	4.55 ±0.04	4.53±0.04	5.29* 0.03	5.31*** 0.03
Blood glucose (mg/dl)	80.5 0.94	79.7 0.78	88.5* 0.37	80.8## 0.39
Insulin (µU/ml)	7 0.71	7.1 0.77	8.96*** 0.11	7.63# 0.08
HOMA-IR	1.39 0.02	1.4 0.02	1.86*** 0.03	1.52## 0.02

*P<0.05, **P<0.01, and ***P<0.001 significant from ND group, #P<0.05, ##P<0.01, ###P<0.001 significant from HFD group.

Table II: Effect of oxytocin treatment on tissue content of IRS-1 and IRS-2 in normal diet (ND) rats and high fat diet (HFD) rats (mean+ SEM)

	ND	NDOXY	HFD	HFOXY
Diaphragm homogenates				
IRS-1	0.31 0.02	0.29 0.05	0.21*** 0.02	0.28### 0.03
IRS-2	0.57 0.05	0.55 0.07	0.32*** 0.03	0.52### 0.07
Epididymal fat homogenates				
IRS-1	0.27 0.03	0,25 0.03	0.17*** 0.04	0.24# 0,04
IRS-2	0.46 0.05	0.45 0.07	0.32*** 0.03	0.40### 0.07

*P<0.05, ** P<0.01, and *** P<0.001 significant from ND group, # P<0.05, ## P<0.01, ### P<0.001 significant from HFD group.

Figure 1

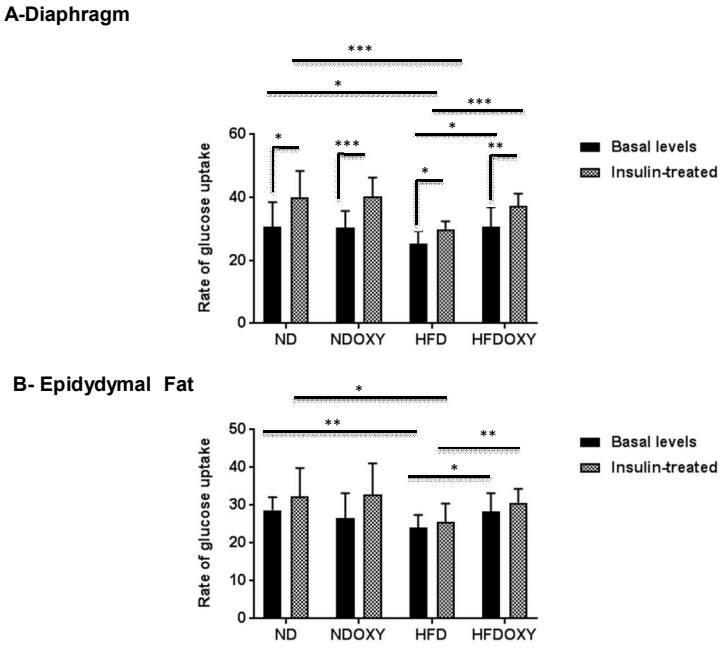

Figure 1: Glucose uptake by A)-diaphragm or B)-epididymal fat at basal levels and insulin-treated.

Tissues were obtained from normal diet (ND) rats, normal diet oxytocin-treated (NDOXY) rats, and high fat diet (HFD) rats, or high fat diet oxytocin-treated (HFDOXY) rats. Comparisons were made between basal control versus insulin-treated in each group; and ND versus HFD, and HFD versus HFDOXY (all either at basal or insulin-treated). Values were illustrated as mean ± SEM. Statistical significance was determined by un-paired student's "t" test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (n=45).

Figure 2

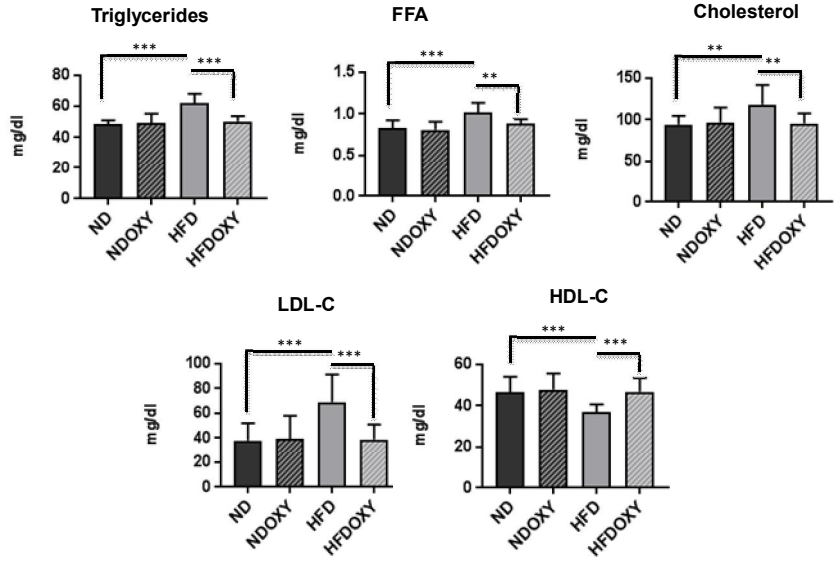

Figure 2: Oxytocin improves dyslipidemia of obese rats.

Serum lipids (triglycerides, free fatty acids, total cholesterol, LDL-C, and HDL-C) were measured in normal diet (ND) rats, normal diet oxytocin-treated (NDOXY) rats, and high fat diet (HFD) rats, or high fat diet oxytocin-treated(HFDOXY) rats. Comparisons were made between ND versus HFD, and HFD versus HFDOXY rats. Values were illustrated as mean ± SEM. Statistical significance was determined by un-paired student's "t" test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (n=45).

Figure 3

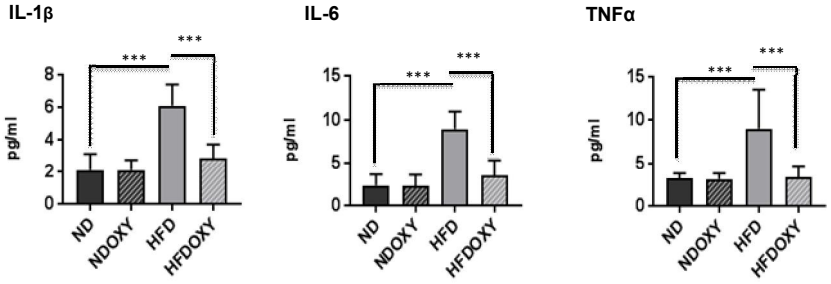

Figure 3: Anti-inflammatory effect of oxytocin on high fat fed rats.

ELISA was used to measure the levels of pro-inflammatory cytokines (IL-Iβ, IL-6 and TNF-α) in serum of normal diet (ND) rats, normal diet oxytocin-treated (NDOXY) rats, and high fat diet (HFD) rats, or high fat diet oxytocin-treated(HFDOXY) rats. Comparisons were made between ND versus HFD groups, and HFD versus HFDOXY groups. Values were illustrated as mean ± SEM. Statistical significance was determined by un-paired student's "t" test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (n=45).

Our data and others (22, 23) showed ahyperlipidemic profile in high fat-fed rats with mainly an increase of triglycerides and plasma free fatty acids. The lipid species may accumulate in insulin sensitive tissues like epididymal fat and skeletal muscle resulting in impairment of insulin signaling in these tissues (24,25). These alterations in peripheral tissues may induce defects in insulin receptor activation (26). Accordingly, our study confirmed the dysfunction of insulin signaling in high fat animals by the significant drop of IRS-1 and IRS-2 in both epididymal fat and muscle tissues. These chemicals are key elements in insulin receptor downstream cascade IRS1/PI3K/PKB/Akt-mediated signaling (27). There is an association of low cellular IRS-1 expression with low Glut4 expression and impaired insulin-stimulated glucose transport in IR (28)due to a marked resistance to the glucose-lowering effects of insulin (29). Conclusively, our results document that high plasma free fatty acids are thought to be associated with decreased levels of IRS-1 and IRS-2 in peripheral tissues and impaired glucose utilization, resulting in subsequent hyperglycemia and hyperinsulinemia. This assumption is supported by the diminished gene expression of IRS-1 in peripheral tissues of animal with IR (30). Findings from other studies have also indicated that dyslipidemia can decrease the expression of insulin receptor IRS-1, and IRS-2 in pancreatic islet cells, thus impairing the insulin signal transduction pathway (31). Impaired insulin signal transduction not only affects signaling in islet β-cells, but can also lead to increased glucagon secretion because insulin negatively regulates glucagon secretion from α- cells through the IRS -1-PI3K pathway (32). These changes, both insulin resistance and increased glucagon secretion, are thought to be associated with the development of type 2 DM(33).

An important finding of our study was that the majority of the aforementioned biochemical and metabolic changes associated with high-fat diet feeding (indicative dyslipidemia and insulin resistance) did not occur when rats fed the high-fat diet were treated with oxytocin. Our results show that oxytocin supplementation in high fat fed rats was associated with significant improvement in insulin sensitivity. These data are in accordance with. (34) and confirmed by the decrease in glucose tolerance and insulin sensitivity in oxytocin deficient mice (12). The improvement of insulin sensitivity of epididymal fat and skeletal muscle upon oxytocin administration indicates involvement of oxytocin in peripheral metabolic functions of skeletal muscle and adipose tissue. It appears that stimulation of glucose uptake in both adipose tissue and skeletal muscle reflects improved insulin sensitivity of oxytocin supplemented HFD rats. One of the main factors suspected of inducing insulin resistance is the increased adiposity (35). Although we did not measure fat mass in our rats, they showed significantly higher body mass index and significantly higher plasma triglycerides. Moreover, we showed a negative correlation between BMI and glucose uptake in both skeletal muscle ($r= -0.6917$, $p<0.05$) and epididymal fat ($r= -0.6338$ $p<0.01$) in high fat fed rats. The latter results indicate that adiposity may play a role in the decline of peripheral tissue sensitivity to insulin in rats used in this experiment. Surprisingly, oxytocin improved insulin action without affecting body weight as there was no significant difference in BMI between control and oxytocin treated rats. These data support the argument that oxytocin related attenuation of insulin resistance may act through a direct role of oxytocin on glucose tolerance and not to its anti-obesity effect(36).

Furthermore, oxytocin at doses similar to ours does not cross the blood brain barrier excluding a central effect of oxytocin on body weight (37). Treatment with oxytocin lowered triglyceride levels, and LDL levels. Oxytocin thus, improves lipid metabolism by decreasing plasma levels of triglycerides and LDL which subsequently decreases the influx of fatty acids into insulin sensitive tissues improving insulin transduction mechanisms. In the present study, we found that the levels of both IRS-1 and IRS-2 in insulin sensitive tissues were significantly decreased in rats fed the high-fat diet compared with rats fed the normal fat diet. Notably, these changes in IRS-1and IR-2 levels were increased upon oxytocin treatment. This finding suggests that oxytocin may have prevented the development of changes consistent with insulin resistance, at least in part, by modulating the level of IRS-1 and IRS-2 in adipocytes and skeletal muscle cells.

Our results also showed an increase in proinflammatory cytokines with high fat diet in accordance to others (38, 39). The adipose tissue macrophages perpetuate inflammation by inducing local and systemic proinflammatory molecules (40). The beneficial effect of oxytocin could be related to the improvement of proinflammatory cytokine profile in HFD rats in our study. This attribution is supported by the findings of Kobayashiand his colleagues (41), They demonstrated that oxytocin infusion in models of myocardial infarction improved function in the injured heart through reduction of inflammation. Furthermore, chronic peripheral oxytocin administration inhibited inflammation and atherosclerotic lesion development where expression of adipokines from visceral adipose tissue was indicative of decreased adipose tissue inflammation (42).

Conclusion

The present study demonstrated the positive role of oxytocin in insulin resistance and hyperlipidemia in high fat fed rats. Oxytocin could improve insulin signaling through enhancement of IRS-1 and IRS-2 in insulin sensitive tissues in high fat-fed rats. Unfortunately, this study does not reach to the exact signaling mechanisms, further experiments are encouraged to explore it. This study is limited by the dose and duration of oxytocin administered. Extrapolation of data from this study is important for human studies. We recommend human studies on the uses of oxytocin in humans suffering from insulin resistance or metabolic syndrome.

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