IMPACT OF CAFETERIA DIET-INDUCED OBESITY ON THE ADRENAL OF ADULT FEMALE RATS

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***Abstract*: Background:** Hormones produced by the adrenal glands and adipose tissues have important roles in normal and physiology and are altered in many disease states. Studies have shown that obesity is associated with changes in adrenal function ,including increase in adrenal medullary catecholamine output ,alterations of the hypothalamic-pituitary adrenal (HPA) axis,elevations in circulating aldosterone together with changes in adipose tissue glucocorticoid metabolism,and enhanced adipocyte mineralocorticoid receptor activity. However ,the impact of obesity on adrenal structure and the mechanism behind the altered adrenal function is not yet studied. **Study Design:** The main objective is to study the effect of cafeteria diet-induced obesity on the adrenal steroidogenic machinery. Sprague-Dawley female rats of 21 days old ,weighing 30-35 grams were taken. The rats were divided into two groups,with 6 animals. Group-1 rats are the controlled rats which were fed with standard chow for 20 weeks and the Group-2 rats are the cafeteria diet fed rats which included standard chow along with random selection of highly energetic and palatable human foods like pork,chicken,cakes ,cookies ,chocolate ,marsh mallow and cheese.During the study period ,food consumption ,body weight and estrous cyclicity were recorded daily.Rats were killed between PND160 and 170 at diestrus phase.Abdominal girth ,and overall fat were weighed,and then adrenals were dissected out and stored at -20 degree. **Results:** StAR ,IR(Insulin receptor) and MCR (Mineralocorticoid receptor) expressions in rat adrenal tissue were assessed by western blotting. The final result is that the StAR protein expression and the Mineralocorticoid receptor protein expression were significantly decreased and the Insulin receptor protein expression was significantly increased in the adrenal of cafeteria diet-induced obese rats compared to control rats.**Conclusion:** The study shows that the rats which were fed cafeteria diet developed hyperphagia and lead to accumulation of fat in their body and also resulted in increased the expression of insulin receptor which might have caused insulin resistance in cafeteria fed rats.

INTRODUCTION

Obesity is a medical condition in which an abnormal accumulation of body fat and these excess fat tissues have a negative effect on health. People are generally considered obese when their body mass index (BMI), a measurement obtained by dividing a person's weight by the square of the person's height. BMI is closely related to both percentage body fat and total body fat (Gray and Fujioka 1991). BMI is a simple and commonly used parameter for classifying various degrees of adiposity.

Table-1. Classification of obesity (Flegal et al., 2010).

BMI (kg/m2)	Classification
< 18.5	Under Weight
18.5 - 25	Normal Weight
25 - 30	Over Weight
> 30	Obese

A BMI greater than 28kg/m^2 in adults is associated with a three to four-fold greater risk of morbidity due to Type-2 diabetes mellitus (T2DM) and cardiovascular diseases (Itallie, 1985). From a large body of evidence, the global epidemic of obesity has resulted mainly from societal factors that promote sedentary lifestyles and the consumption of high-fat, energy-dense diets . While genes are important in the determination of a person's susceptibility to is eaten and drank exceeds energy expenditure over a protracted period.

Obese children are more likely than their non-obese counterparts to grow into obese adults (Goran, 2001). Data from a number of studies also provide robust evidence that children who are growth retarded at birth have an increased risk of becoming obese in later life (Ong *et al.,*2000, Barker *et al.,*1997, Whitaker *et al*.,1998). This phenomenon, which implies foetal programming of adult obesity, is particularly likely to occur when a low body weight at birth from intrauterine growth retardation (IUGR) is over-compensated for by a catch-up growth in later life, and when this adiposity rebound occurs early in childhood (Barker *et al*., 1997).

Obesity is most commonly caused by a combination of excessive food intake, lack of physical activity, and genetic susceptibility (Yazdi ; *et al* 2015). Obesity increases the likelihood of various diseases and conditions, particularly cardiovascular diseases, type 2 diabetes, obstructive sleep apnea, certain types of cancer, osteoporosis, arthritis and depression(Haslam and James (2005), Luppino; *et al* 2010).

In general, obesity is associated with a greater risk of disability or premature death due to type 2 diabetes mellitus and cardiovascular diseases such as hypertension, stroke and coronary heart disease as well as gall bladder disease, certain cancers (endometrial, breast, prostate, colon) and non-fatal conditions including gout, respiratory conditions, gastro-esophageal reflux disease, osteoarthritis and infertility. Obesity also carries serious implications for psychosocial health, mainly due to societal prejudice against fatness.

Leptin, a protein hormone expressed predominantly by adipocytes, is believed to play a major role in this complex mechanism of weight maintenance. Leptin normally acts on receptors in the hypothalamus of the brain to inhibit food intake by counteracting the effects of potent feeding stimulants such as neuropeptide Y, while promoting the synthesis of α-Melanocyte-stimulating hormone (α-MSH), an appetite suppressant (Ong *et al,*1999). Obese individuals, compared with their lean counterparts, have higher leptin levels (Considine *et al.,* 1996), suggesting a 'leptin resistance' rather than a deficiency in obese states. Infrequently, obesity may be a manifestation of other medical conditions such as hypothyroidism, Cushing's syndrome and certain hypothalamic disorders.

Like many other medical conditions, obesity is the result of an interplay between genetic and environmental factors (Albuquerque *et al., 2017).*

Polymorphisms in various genes controlling appetite and metabolism predispose to obesity when sufficient food energy is present. More than 41 of these sites on the human genome have been linked to the development of obesity when a favorable environment is present (Poirier *et al,2006*). People with two copies of the FTO gene (fat mass and obesity associated gene) have been found on average to weigh 3–4 kg more and have a 1.67-fold greater risk of obesity compared with those without the risk allele (Loos and Bouchard). The differences in BMI between people that are due to genetics varies depending on the population examined from 6% to 85% (Yang *et al,2013*).

Studies that have focused on inheritance patterns rather than on specific genes have found that 80% of the offspring of two obese parents were also obese, in contrast to less than 10% of the offspring of two parents who were of normal weight (Kolata and Gina.,2007). Different people exposed to the same environment have different risks of obesity due to their underlying genetics (Walley *et al.,*June 2009).

The fundamental cause of obesity and overweight is an energy imbalance between calories consumed and calories expended. Globally, there has been an increased intake of energy-dense foods that are high in fat; and an increase in physical inactivity due to the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization.

Cafeteria Diet:

Iinvestigations into the aberrant homeostasis responsible for most human obesity were carried out using rodent models of diet-induced obesity (DIO) through the administration of high-fat diets (HFD) (Kennedy., et al., 2010). Traditionally, these diets consist of a simple exchange of carbohydrate-derived calories with fat-derived calories compared to low fat or chow control diets. However, another experimental rodent diet model exists that more accurately reflects the variety of highly palatable, energy dense foods that are prevalent in Western society and associated with the current obesity pandemic. This diet is called as "cafeteria diet" (CAF) (Rothwell et al. 1979; Sclafani et al., 1976). In this model, animals are allowed free access to standard chow and water while concurrently offered highly palatable, energy dense, unhealthy human foods *ad libitum.* This diet promotes voluntary hyperphagia that results in rapid weight gain, increases fat pad mass and prediabetic parameters such as glucose and insulin intolerance (Morris *et al*., 2008; Caimari *et al.,*2010; Heyne *et al.,*2009; Rolls *et al.,*1980). Although both high fat diets resulted in increased adiposity and hepatosteatosis, CAF provided a robust model of human metabolic syndrome compared to traditional lard-based HFD, creating a phenotype of exaggerated obesity with glucose intolerance and inflammation. This model provides a unique platform to study the biochemical, genomic and physiological mechanisms of obesity and obesity-related disease states that are pandemic in western civilization today.

The Adrenal Gland :

The adrenal glands (also known as suprarenal glands) are endocrine glands that produce a variety of hormones including adrenaline and the steroids aldosterone and cortisol (Santulli., 2015) . They are found above the kidneys. Each gland has an outer cortex which produces steroid hormones and an inner medulla. The adrenal cortex itself is divided into three zones: zona glomerulosa, the zona fasciculate and the zona reticularis (Ross and Pawlina., 2011).

The adrenal cortex produces three main types of steroid hormones: mineralocorticoids, glucocorticoids, and androgens. Mineralocorticoids (such as aldosterone) produced in the zona glomerulosa help in the regulation of blood pressure and electrolyte balance. The glucocorticoids cortisol and corticosterone are synthesized in the zona fasciculata; their functions include the regulation of metabolism and immune system suppression. The innermost layer of the cortex, the zona reticularis, produces androgens that are converted to fully functional sex hormones in the gonads and other target organs (Melmed *et al.,*2011). The production of steroid hormones is called steroidogenesis, and involves a number of reactions and processes that take place in cortical cells (Miller and Auchus.,2011). The medulla produces the catecholamines adrenaline and noradrenaline, which function to produce a rapid response throughout the body in stress situations (Melmed *et al,2011.*).

A number of endocrine diseases involve dysfunctions of the adrenal gland. Overproduction of cortisol leads to Cushing's syndrome, whereas insufficient production is associated with Addison's disease. Congenital adrenal hyperplasia is a genetic disease produced by dysregulation of endocrine control mechanisms (Melmed *et al.*,2011, Longo *et al.,2012*).A variety of tumors can arise from adrenal tissue and are commonly found in medical imaging when searching for other diseases (Longo *et al*.,2012).

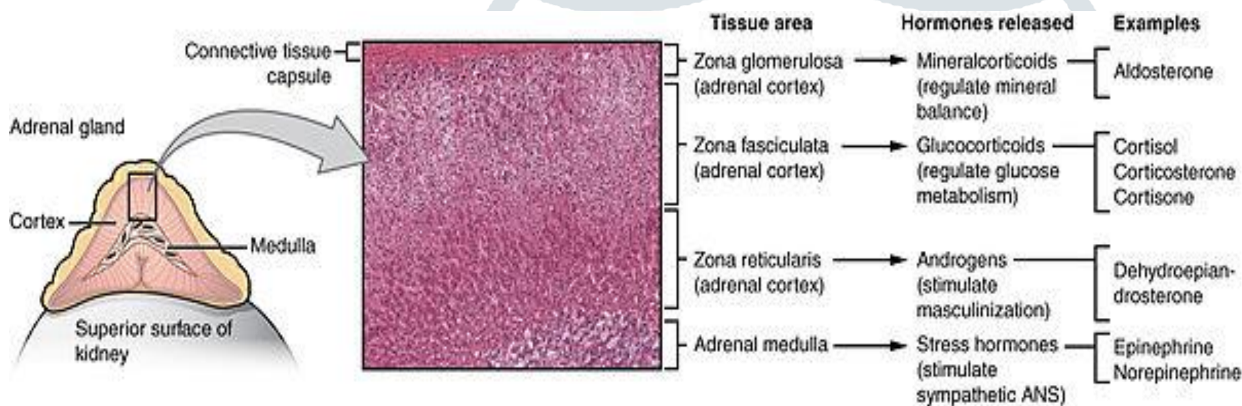
Structure:

The adrenal glands are located on both sides of the body in the retroperitoneum, above and slightly medial to the kidneys. In humans, the right adrenal gland is pyramidal in shape, whereas the left is semilunar and somewhat larger (Nieman.,2010). The adrenal glands measure approximately 3.0 cm in width, 5.0 cm in length, and up to 1.0 cm in thickness (Thomas.,2013). Their combined weight in an adult human ranges from 7 to 10 grams (Antonio Carlos *et al.,2006*). The glands are yellowish in colour (Nieman.,2010).

The adrenal glands are surrounded by a fatty capsule and lie within the renal fascia, which also surrounds the kidneys. A weak wall of connective tissue separates the glands from the kidneys(O'Hare *et al.,1982*). The adrenal glands are directly below the diaphragm, and are attached to the crura of the diaphragm by the renal fascia(O'Hare *et al.,1982*).

Each adrenal gland has two distinct parts, each with a unique function, the outer adrenal cortex and the inner medulla, both of which produce hormones(Moore *et al.,2013*).

Different hormones are produced in different zones of the cortex and medulla of the gland.



Cortex

The adrenal cortex is the outermost layer of the adrenal gland. Within the cortex are three layers, called "zones". When viewed under a microscope each layer has a distinct appearance, and each has a different function(Kay and Saundra.,2015). The adrenal cortex is devoted to production of hormones, namely aldosterone, cortisol, and androgens (Whitehead *et al.,2001*).

Zona glomerulosa

The outermost layer of the adrenal cortex is the zona glomerulosa. It lies immediately under the fibrous capsule of the gland. Cells in this layer form oval groups, separated by thin strands of connective tissue from the fibrous capsule of the gland and carry wide capillaries (Jefferies and William.,2004).

This layer is the main site for production of aldosterone, a mineralocorticoid, by the action of the enzyme aldosterone synthase (Young *et al.*,2013, Curnow *et al.,1991*). Aldosterone plays an important role in the long-term regulation of blood pressure (Zhou and Gomez-Sanchez.,1993).

Zona fasciculata

The zona fasciculata is situated between the zona glomerulosa and zona reticularis. Cells in this layer are responsible for producing glucocorticoids such as cortisol (Marieb and Hoehn.,2010). It is the largest of the three layers, accounting for nearly 80% of the volume of the cortex (Ross and Pawlina.,2011).

In the zona fasciculata, cells are arranged in columns radially oriented towards the medulla. Cells contain numerous lipid droplets, abundant mitochondria and a complex smooth endoplasmic reticulum (Jefferies and William.,2004).

Zona reticularis

The innermost cortical layer, the zona reticularis, lies directly adjacent to the medulla. It produces androgens, mainly dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and androstenedione (the precursor to testosterone) in humans (Dunn *et al,2011*). Its small cells form irregular cords and clusters, separated by capillaries and connective tissue. The cells contain relatively small quantities of cytoplasm and lipid droplets, and sometimes display brown lipofuscin pigment (Jefferies and William.,2004).

Medulla

The adrenal medulla is at the centre of each adrenal gland, and is surrounded by the adrenal cortex. The chromaffin cells of the medulla are the body's main source of the catecholamines adrenaline and noradrenaline, released by the medulla. Approximately 20% noradrenaline (norepinephrine) and 80% adrenaline (epinephrine) are secreted here (Dunn *et al.,2011*).

The adrenal medulla is driven by the sympathetic nervous system via preganglionic fibers originating in the thoracic spinal cord, from vertebrae T5–T11 (Sapru *et al.,2007*). Because it is innervated by preganglionic nerve fibers, the adrenal medulla can be considered as a specialized sympathetic ganglion (Sapru *et al.,2007*). Unlike other sympathetic ganglia, however, the adrenal medulla lacks distinct synapses and releases its secretions directly into the blood.

The adrenal gland secretes a number of different hormones which are metabolised by enzymes either within the gland or in other parts of the body. These hormones are involved in a number of essential biological functions (Nicki *et al.,2010*).

Corticosteroids:

Corticosteroids are a group of steroid hormones produced from the cortex of the adrenal gland, from which they are named. Corticosteroids are named according to their actions. Mineralocorticoids such as aldosterone regulate salt ("mineral") balance and blood volume.Glucocorticoids such as cortisol influence metabolism rates of proteins, fats and sugars.

Adrenaline **and** Nor adrenaline**:**

Adrenaline and noradrenaline are catecholamines, water-soluble compounds that have a structure made of a catechol group and an amine group. The adrenal glands are responsible for most of the adrenaline that circulates in the body, but only for a small amount of circulating noradrenaline (Nicki *et al.,2010*). These hormones are released by the adrenal medulla, which contains a dense network of blood vessels. Adrenaline and noradrenaline act at adrenoreceptors throughout the body, with effects that include an increase in blood pressure and heart rate (Nicki *et al.,2010*). The actions of adrenaline and noradrenaline are responsible for the fight or flight response.

Adrenal Steroidogenesis:

The steroidogenic acute regulatory protein, commonly referred to as StAR (STARD1), is a transport protein that regulates cholesterol transfer within the mitochondria, which is the rate-limiting step in the production of steroid hormones. It is primarily present in steroid-producing cells, including theca cells and luteal cells in the ovary, Leydig cells in the testis and cell types in the adrenal cortex.

Cholesterol needs to be transferred from the outer mitochondrial membrane to the inner membrane where cytochrome P450scc enzyme (CYP11A1) cleaves the cholesterol side chain, which is the first enzymatic step in all steroid synthesis. The aqueous phase between these two membranes cannot be crossed by the lipophilic cholesterol, unless certain proteins assist in this process. A number of proteins have historically been proposed to facilitate this transfer including: sterol carrier protein 2 (SCP2), steroidogenic activator polypeptide (SAP), peripheral benzodiazepine receptor (PBR or translocator protein, TSPO), and StAR. It is now clear that this process is primarily mediated by the action of StAR.

The mechanism by which StAR causes cholesterol movement remains unclear as it appears to act from the outside of the mitochondria and its entry into the mitochondria ends its function. Various hypotheses have been advanced. Some involve StAR transferring cholesterol itself like a shuttle (Kallen *et al.,*1998, Bose *et al.,*1999). While StAR may bind cholesterol itself (Roostaee *et al.,* 2008), the exorbitant number of cholesterol molecules that the protein transfers would indicate that it would have to act as a cholesterol channel instead of a shuttle. Another notion is that it causes cholesterol to be kicked out of the outer membrane to the inner (cholesterol desorption) (Christenson and Strauss.,2001). StAR may also promote the formation of contact sites between the outer and inner mitochondrial membranes to allow cholesterol influx. Another suggests that StAR acts in conjunction with PBR, causing the movement of Cl^- out of the mitochondria to facilitate contact site formation. However, evidence for an interaction between StAR and PBR remains elusive.

StAR is a mitochondrial protein that is rapidly synthesized in response to stimulation of the cell to produce steroid. Hormones that stimulate its production depend on the cell type and include luteinizing hormone (LH), ACTH and angiotensin II. At the cellular level, StAR is synthesized typically in response to activation of the cAMP second messenger system, although other systems can be involved even independently of cAMP (Stocco *et al.,2005*).

StAR has thus far been found in all tissues that can produce steroids, including the adrenal cortex, the gonads, the brain and the nonhuman placenta(Bhangoo *et al.,2006*). One known exception is the human placenta.Alcohol suppresses StAR activity (Srivastava *et al.,2005*).

While loss of functional StAR in the human and the mouse catastrophically reduces steroid production, it does not eliminate all of it, indicating the existence of StAR-independent pathways for steroid generation. Aside from the human placenta, these pathways are considered minor for endocrine production.

It is unclear what factors catalyze StAR-independent steroidogenesis. Candidates include oxysterols which can be freely converted to steroid (Hutson.,2006) and the ubiquitous MLN64.

Mineralocorticoid receptor:

The mineralocorticoid receptor (or MR, MLR, MCR), also known as the aldosterone receptor or nuclear receptor subfamily 3, group C, member 2, (NR3C2) is a protein that in humans is encoded by the NR3C2 gene that is located on chromosome 4q31.1-31.2 MR is a receptor with equal affinity for mineralocorticoids and glucocorticoids. MR is expressed in many tissues, such as the kidney, colon, heart, central nervous system (hippocampus), brown adipose tissue and sweat glands. In epithelial tissues, its activation leads to the expression of proteins regulating ionic and water transports (mainly the epithelial sodium channel or ENaC, Na+/K+ pump, serum and glucocorticoid induced kinase or SGK1) resulting in the reabsorption of sodium, and as a consequence an increase in extracellular volume, increase in blood pressure, and an excretion of potassium to maintain a normal salt concentration in the body.

The receptor is activated by mineralocorticoids such as aldosterone and its precursor deoxycorticosterone as well as glucocorticoids, like cortisol. In intact animals, the mineralocorticoid receptor is "protected" from glucocorticoids by co-localization of an enzyme, Corticosteroid 11-beta-dehydrogenase isozyme 2 (11β-hydroxysteroid dehydrogenase 2; 11β-HSD2), that converts cortisol to inactive cortisone. It also responds to some progestins. Spironolactone and eplerenoneare steroidal MR antagonists of the spirolactone group. Activation of the mineralocorticoid receptor, upon the binding of its ligand aldosterone, results in its translocation to the cell nucleus, homodimerization and binding to hormone response elements present in the promoter of some genes. This results in the complex recruitment of the transcriptional machinery and the transcription into mRNA of the DNA sequence of the activated genes (Fuller and Young) .

Scope of the present study:

Hormones produced by the adrenal glands and adipose tissues have important roles in normal physiology and are altered in many disease states. Studies have shown that obesity is associated with changes in adrenal function, including increase in adrenal medullary catecholamine output, alterations of the hypothalamic-pituitary-adrenal (HPA) axis, elevations in circulating aldosterone together with changes in adipose tissue glucocorticoid metabolism, and enhanced adipocyte mineralocorticoid receptor activity. However, the impact of obesity on adrenal structure and the mechanism behind the altered adrenal function is not yet studied.

Hypothesis:

It is hypothesized that the cafeteria diet - induced obesity may affect the function of adrenal gland in adult female rats.

Objective:

- To study the effect of cafeteria diet - induced obesity on the adrenal steroidogenic machinery.

Materials and Methods:

Experimental Design:

Animals: Sprague-Dawley female rats, 21 days old, weighing 30- 35 grams.

Rats were divided into 2 groups, with 6 animals/group.

Group I (Control) : Rats were fed with standard chow for 20 weeks

Group II (CAF Diet) : Standard chow along with random selection of highly energetic and palatable human foods.

Each day, rats were offered a variety of three human snack foods varied daily (in such a way there were not repeated on simultaneous days to maintain excitement) in addition to *ad libitum* standard chow. The cafeteria diet consisted of different palatable human food items with high energy content (Pork, chicken, cake, cookies, chocolate, Marsh Mallow and cheese).

During the study period, food consumption, body weight and estrous cyclicity were recorded daily. Rats were killed between PND 160 and 170 at diestrus phase. Abdominal girth and overall fat were weighed, and then adrenals were dissected out and stored at -20 degree.

Preparation of tissue lysate:

Adrenal tissue weighing about 50 mg of were homogenized with 495µl of Radio Immuno Precipitation Assay (RIPA) buffer along with 5 µl Protease Inhibitor (PI) and centrifuged at 10,000 g for 10 minutes at 4ºC and then the supernatant was collected and stored at -80ºC.

Quantification of protein

The protein concentration in tissue lysate was estimated by BioRad protein assay kit (Catalog # 223-9950).

Principle

The Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein (Bradford, 1976). The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially

arginine. Thus, Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

Reagents

1) Protein Assay Dye Reagent

Dye reagent was prepared by diluting 1 part of dye reagent concentrate with 4 parts Milli Q water.

2) Protein standard-BSA

Bovine Serum Albumin (BSA), 1 mg was weighed and dissolved in 1ml of Milli Q water. The stock concentration is 1 mg/ml. This can be stored at -20ºC for two weeks.

3) Preparation of Diluent

The diluent was prepared by adding 1 ml RIPA to 4 ml Milli Q water.

Preparation of the diluted BSA standards

Blank/Standards	Volume of the BSA standard solution (µl)	Volume of diluent (µl)	Final BSA concentration (µg/ml)
B	0	50	0
S1	2.5	47.5	50
S2	5	45	100
S3	7.5	42.5	150
S4	12.5	37.5	250
S5	25	25	500

Protocol

The protein standards were prepared ranging from 50-500 µg/ml using a BSA standard. The samples were diluted by adding 45 µl diluent to 5 µl sample. About 5 µl of the protein standards were added to separate wells in the 96 well plate. To the blank wells, 5 µl of buffer was added. To each well being used, 100 µl of the BioRad diluted reagent dye was added and mixed on a shaker for approximately 30 seconds. The samples were incubated at room temperature atleast for 5 minutes and the absorbance was measured at 595 nm. The net absorbance *vs* the protein concentration of each standard was plotted. The protein concentration of the unknown samples was then determined by comparing the Net A595 values against the standard curve.

Calculation

The protein content of the lysate was calculated using the formula.

$$\text{Protein concentration} = \frac{\text{O.D of Unknown}}{\text{O.D of Known}} \times \text{Standard concentration.}$$

Western blot analysis of ER α, ERβ, PPARγ, PR

ER α, ERβ, PPARγ, and PR expressions in rat uterus tissue were assessed by western blotting.

Reagents

1. **Radio Immuno Precipitation Buffer (RIPA):** (150 mM NaCl, 50 mM Tris,1 mM EDTA, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 1 mM β glycerophosphate, 1 mM Sodium orthovanadate and 1 mM Sodium fluoride, pH 7.4).

For 50 ml,

Tris Base	302 mg
NaCl	438 mg
EDTA	186 mg
Na-deoxycholate	25 mg
SDS	50 mg
NP-40	500 µl
β glycerophosphate	10.8 mg
Sodium orthovanadate	9.2 mg
Sodium fluoride	2.0 mg

All these chemicals were dissolved in 40 ml Milli Q water, pH was adjusted to 7.4 with HCl and then the volume was made up to 50 ml. The buffer was covered with aluminum foil and stored in refrigerator.

2. **Protease inhibitor (PI):** Protease inhibitor cocktail was purchased from Pierce Biotechnology Inc, USA. PI was used as per the manufacturer's instruction, 1:100 dilutions (for 1 ml, 10 µl PI).
3. **Running gel buffer** (1.5 M Tris, pH 8.8)**:** For 250 ml, 45.375 g Tris was dissolved in 200 ml Milli Q water and pH was adjusted with HCl and the volume was made up to 250 ml. Stored at room temperature.
4. **Stacking gel buffer** (0.5 M Tris, pH 6.8)**:** For 250 ml, 1 g Tris was dissolved in 200 ml Milli Q water and pH was adjusted with HCl and the volume was made up to 250 ml. Stored at room temperature.
5. **SDS 10%:** For 100 ml solution 10 g SDS was dissolved in Milli Q water and stored at room temperature.
6. **Acrylamide 30%:** 29 g Acrylamide (29%) and 1 g N, N' Bis-acrylamide (1%) were dissolved in 100 ml Milli Q water.
7. **Ammonium Persulfate 10%:** 100 mg Ammonium persulfate was dissolved in 1ml Milli Q water.
8. **10X SDS Electrophoresis buffer** (2.5 M Tris, 1.92 M Glycine and 1 % SDS)**:** For one liter of solution, 30 g Tris, 144 g Glycine and 10 g SDS were dissolved in 800 ml Milli Q water, volume was made up to one liter and stored at room temperature**.**
9. **1X SDS Electrophoresis buffer:** For 500 ml, 50 ml 10X SDS electrophoresis buffer was mixed with 450 ml Milli Q water.
10. **10X Transfer buffer** (250 mM Tris and 1.92 M Glycine)**:** For one liter, 0.3 g Tris and 144 g Glycine were dissolved in 800 ml Milli Q water, volume was made up to one liter and stored at room temperature.
11. **1X Transfer buffer with 20% methanol:** For 1 L, 100 ml 10X transfer buffer was mixed with 800 ml Milli Q water and 200 ml methanol and kept cold until used.
12. **6X Sample buffer with reducing agent** (375 mM Tris-HCl (pH 6.8), 12 % SDS, 40% glycerol, 30 % β-mercapto ethanol , 1 mg/ml bromophenol blue, 300 mM DTT, 120 mM EDTA)**:** For 10 ml, take 2.5 ml 0.5 M Tris-HCl (pH 6.8), 12% SDS, 40% glycerol, 30% β-Mercapto ethanol and 1 mg/ml bromophenol blue, 300 mM DTT, 120 mM EDTA mix well and the volume was made up to 10 ml with Milli Q water. One ml aliquots were stored at -20ºC.
13. **10X Phosphate buffered Saline** (0.1 M Sodium phosphate)**:** For one liter of solution, 13 g NaH_2PO_4 was dissolved in Milli Q water, the pH was adjusted to 7.2 with NaOH, the volume was made up to one litter and stored at room temperature.
14. **Blocking Buffer 5%** (PBS with 5 % glycerol, 5 % milk powder and 0.2 % Tween-20)**:** For 500 ml, 50 ml 10x PBS, 25 ml Glycerol, 4.5 g NaCl, 25 g non-fat dry milk powder, 1 ml Tween-20 and 400 ml Milli Q

water. All these were taken in a beaker and stirred well until dissolved. The volume was made up to 500 ml, with water. Stored in the refrigerator.

15. **Tris Buffered Saline (TBS)** (20 mM, Tris 500 mM NaCl, pH 7.5)**:** For 500 ml, 1.21 g Tris and 14.62 g NaCl were dissolved in 400 ml Milli Q water and pH was adjusted to 7.5 with HCl and made up to 500 ml.
16. **T-TBS** (0.2 % Tween-20 in TBS)**:** For 100 ml, add 0.2 ml Tween-20 to 100 ml of TBS, and stored in refrigerator.
17. **Prestained SDS-PAGE standard:** Protein marker purchased from Bio Rad, Laboratories; USA.

Preparation of gel

Running gel (10 %): The following volumes of solutions were taken for one slab gel.

Acrylamide (30 %)	3.3 ml
Running gel buffer	2.5 ml
DD H_2O	4.0 ml
SDS (10%)	0.1 ml
APS (10%)	0.1 ml
TEMED	0.007 ml

Stacking gel (5%): The following volume of solutions was taken for one slab gel.

Acrylamide (30 %)	0.5 ml
Stacking gel buffer	0.38 ml
DD H_2O	2.1 ml
SDS (10%)	0.03 ml
APS (10%)	0.03 ml
TEMED	0.003 ml

Equal amount of total protein (20 µg) was mixed with 6X sample buffer and boiled for 5 min. The protein was separated on 10 % SDS-PAGE and electrotransfered onto a PVDF membrane (Bio-Rad, USA). The blots were blocked with 5% blocking buffer for 4 hours. After blocking, membranes were incubated with respective rabbit polyclonal antibodies StAR (H-184, cat # sc-7207), Insulin Receptor (C-19, cat # sc-538), MCR(L-20 cat # sc 6822) in 1:1000 dilution for 12 hours at 4ºC. For mouse monoclonal β-actin antibody (sc-8432) in 1: 10,000 dilutions, the membrane was incubated for 12 hours. Then the membranes were washed thrice with T-TBS for 5 minutes each, followed by horseradish peroxidase conjugated secondary antibody (1:10,000 dilutions) incubation for 45 min at room temperature. Finally, signals were visualized using Enhanced Chemiluminescent System (Pierce Biotechnology Inc, USA) and the signals were captured by Chemi Doc XRS system (Bio Rad, USA) and the intensity of the bands were quantified by Quantity One software (Bio Rad, USA).

Statistical analysis

The data were subjcted Student's T test to assess the significance between mean values of control and experimental groups. The data are expressed as mean ± standard error of mean (SEM) and p values ≤ 0.05 were considered as significant. This was carried out using Graphpad prism for windows.

Results:

Fig.1. Effect of Cafeteria diet-induced obesity on StAR protein level in the adrenal of adult female rats.

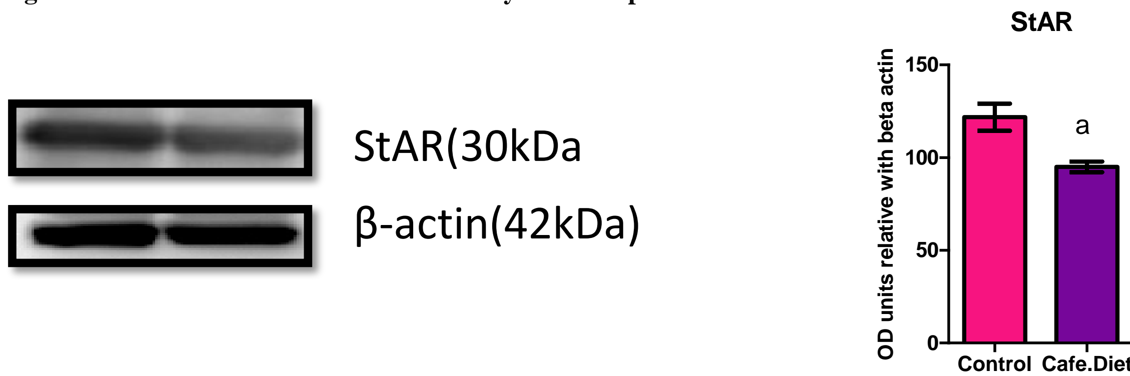


Fig.2. Effect of Cafeteria diet-induced obesity on Insulin Receptor protein level in the adrenal of adult female rats.

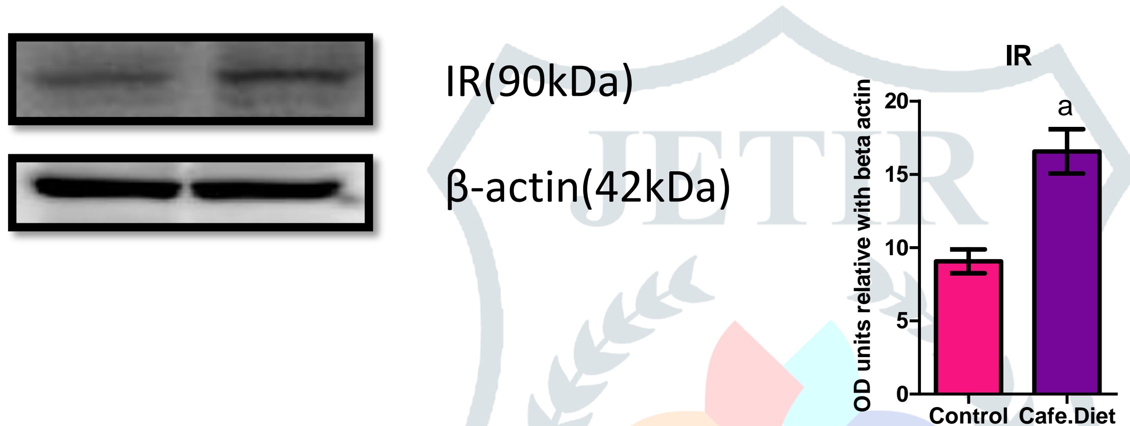
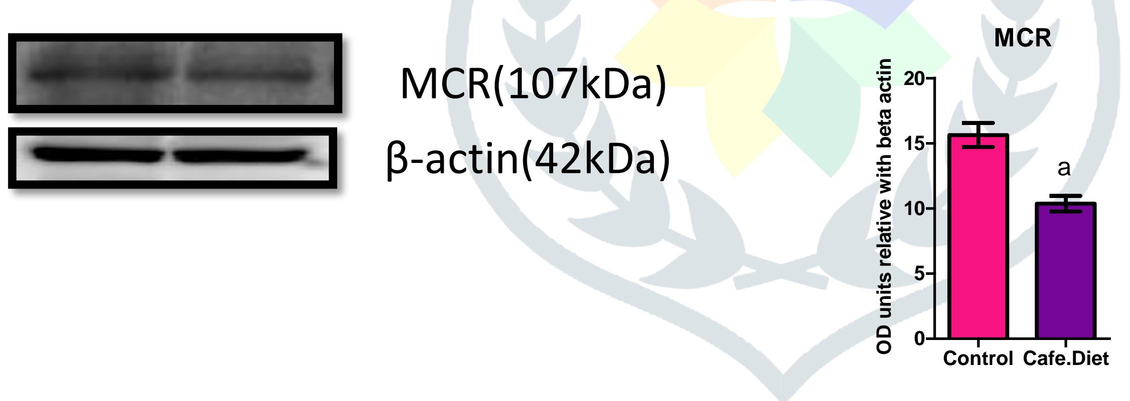


Fig.3. Effect of Cafeteria diet-induced obesity on Mineralocorticoid Receptor protein level in the adrenal of adult female rats.



Each bar represents Mean ± SEM of 3 observations representing 3 animals. Significance at $p<0.05$ a-compared with control

Effect of cafeteria diet on StAR protein level in the adrenal of adult female rats (Fig.1)

The StAR protein expression was significantly decreased in the adrenal of cafeteria diet- induced obese rats compared to control rats.

Effect of cafeteria diet on IR protein level in the adrenal of adult female rats (Fig.2)

The IR protein expression was significantly increased in the adrenal of cafeteria diet-induced obese rats compared to that of control rats.

Effect of cafeteria diet on MCR protein level in the adrenal of adult female rats (Fig.3)

The MCR protein expression was significantly decreased in the adrenal of cafeteria diet-induced obese rats compared to control rats.

Inference:

1) The animals fed with cafeteria diet developed hyperphagia which lead to accumulation of large amount of fat. This increased amount of fat lead to increased body weight in the cafeteria diet fed group compared to that of the control group. Thus, the animals fed with cafeteria diet developed obesity.

2) Insulin receptor has been reported to be involved in adrenal steroidogensis and increased expression of this receptor did not upregulate StAR protein expression. Thus it is inferred that insulin resistance would have occurred in cafeteria diet fed rats.

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