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Systemic Leptin Modulates the Expression of E-cadherin, β-catenin in the Ovary of Dietary-Induced Obese Infertile Rats

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by

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ABSTRACT

Systemic Leptin Modulates the Expression of E-cadherin, β– catenin in the Ovary of Dietary-Induced Obese Infertile Rats

by

Olufunke Abosede Sokan

One of the numerous complications of obesity is infertility. Leptin has been shown to reverse infertility; however, exact mechanism is poorly understood. Recent evidence indicates E-cadherin/β-catenin complex, which is a structural constituent of adherens junction, is expressed in the rat ovary during folliculogenesis. We hypothesized that systemic leptin modulates the expression of E-cadherin and β catenin in dietary- induced obese infertile rats to reverse infertility. Female Sprague-Dawley rats were fed either regular chow diet (RCD) (n=6) or high fat diet (HFD) (n=14). Oestrus cycles were monitored daily until their cycles became irregular. 100 ug/ml of leptin was given intraperitoneally to HFD-fed rats (n=5) with irregular cycles. The control rats HFD (n=9) and RCD received saline. Leptin treatment restored regular estrous cycle and increased the expression of E-cadherin and β-catenin in all the 5 rats (HFD+Leptin). This could represent the mechanism by which leptin reverses infertility in obese infertile rats.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Leptin

Leptin was discovered in 1994 by Zhang and colleagues while they were studying obesity

among mice (1). Leptin is a 16kDa protein hormone. It plays a role in regulating energy intake

and expenditure which includes appetite, hunger, and metabolism (1). The major source of

leptin is from white adipose tissue (2, 3). It is also secreted from the hypothalamus (4) and

other peripheral tissues, such as brain (4), stomach (5), skeletal muscle (6), placenta (7), and

mammary glands (8, 9).

Function of Leptin in Energy Intake and Expenditure

The level of circulating leptin is proportional to the total amount of fat in the body (10).

Fasting or very low calorie diet has been shown to reduce the level of leptin (3). Reduced level

of leptin promotes food intake in excess of energy expenditure in order to restore body fat mass

(3). In the absence of functional leptin, animals fail to restrain their food intake, which leads to

obesity (1). Leptin usually acts as an afferent hormone regulating appetite, weight gain, and fat

deposition via a negative feedback loop involving the hypothalamus (1). Leptin provides the

body with nutritional index (11). Leptin enters the hypothalamus in proportion to its plasma level

and acts on its receptors. Leptin receptors have been found in the mediobasal part of the

hypothalamus, which include the arcuate (ARC), dorsomedial hypothalamic, and ventromedial

hypothalamic nuclei. These receptors are involved in regulation of energy intake, energy

expenditure, thermogenesis, and pituitary hormone levels (12).

There are 2 populations of neurons found in the ARC that are sensitive to leptin. Leptin is

activated when bound to the first population of neurons, the orexigenic peptides, Neuropeptide

Y (NPY), and Agouti-Related Protein (AgRP) (13). This activation leads to increase food intake and reduction in energy expenditure. However, the second population of neurons, proopiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART), give the opposite effect when bound by leptin (10). In the peripheral tissues, leptin modulates adenosine monophosphate-activated protein kinase (AMPK) (14). Leptin uses the ATP-generating pathways (fatty acid oxidation) and avoids ATP-consuming pathways, like fatty acid synthesis, in order to restore energy homeostasis of the cell (14).

Leptin acts as an anorectic hormone to decrease appetite, food intake, and expenditure via various pathways. This is done in both peripheral and central modes of action in order to maintain energy balance (7). It is worthy of note that leptin has also been found to be a crucial hormone for diverse physiological processes such as inflammation, angiogenesis, hematopoiesis, immune function, and most importantly in reproduction (15).

Leptin and Reproduction

The success of reproduction is much dependent on availability of food and body energy stores, with adequate distribution of adipose tissue (16). Because leptin level is related to amount of body fat storage (17), leptin could influence reproductive cycles directly or indirectly. Likewise, lack of biologically active leptin is associated with infertility (18). Leptin is involved in centrally regulated maturation of the reproductive structures (19). This was found in obese female ob/ob mice that were infertile (19). Reversal of their infertility was noted after exogenous leptin treatment (19). Elevation of serum luteinizing hormone, increase in ovarian and uterine weights, and stimulation of ovarian and uterine histology were also documented (20). Some studies have also supported the concept that leptin coordinates the behavioral and endocrine components of reproductive function with adequate amount of energy stores (21).

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<u>Leptin and HPG Axis</u>

Leptin accelerates gonadotropin releasing hormone (GnRH) pulsatility (Figure 1), which plays a major role in fertility (22). However, leptin is not the sole regulator of GnRH secretion but works in supporting other molecules through distinct signaling mechanisms and pathways (23). As much as 20% to 25% of the anterior pituitary cells (predominantly folliculostellate and corticotropes cells) have been found to produce leptin. This may act to regulate pituitary differentiation and cell growth in addition to its effect on luteinizing hormone and follicular stimulating hormone secretion (24). Previous work demonstrated that low levels of leptin have been associated with low levels of luteinizing hormone (LH). This was reversed when leptin was administered (25).
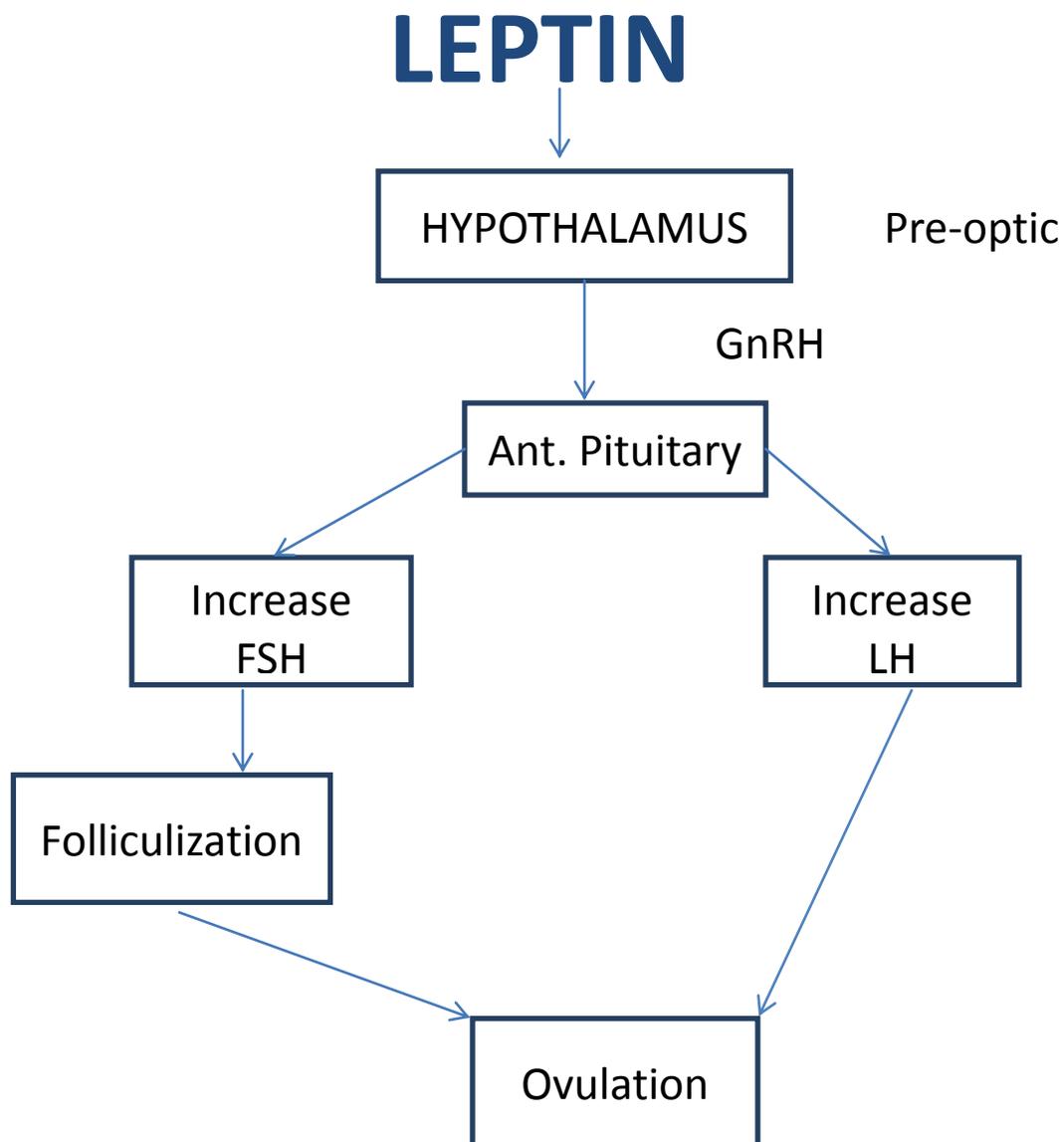
Figure 1: Mechanisms of action of leptin in reproductive system. Leptin acts on the preoptic area of the hypothalamus, increasing GnRH secretion. GnRH stimulates anterior pituitary to release FSH. This causes folliculization in preparedness for ovulation. LH surge initiates ovulation.

E-cadherin and Beta-catenin

E-Cadherin

E-cadherins, also known as CAM 120/80, uvomorulin, or epithelial cadherin, are a subtype in the cadherins family (26). Cadherin family consist of type-1 transmembrane proteins (27). In this family, there are different subtypes. Each of the subtypes is designated with a prefix depending on the tissue type. For example, N-cadherin is found in the neurons; K-cadherin in the kidney; VE-cadherin in the Vascular endothelium; E-cadherin in the epithelia tissues, etc (28). E-cadherin is the most studied member of the cadherin family (27).

E-cadherin is encoded by CH1 gene (26). It was identified in chicken and was originally called L-CAM (29). The mouse counterpart of this protein uvomorulin has about 80% identity in both amino acid nucleotide and sequences to the human counterpart (30). During mammalian development, e-cadherin is first expressed at the 2-cell stage of mammalian development. It then becomes phosphorylated by the 8-cell stage where it causes compaction. E-cadherin's half-life on the cell surface is 5 hours (29). It is found extracellularly at adherens junctions. Adherens junctions are cellular structures near the apical surface of polarized epithelial cells.

For E-cadherin to function, it is dependent on calcium (Ca2+) ions (31). It is composed of 5 extracellular cadherin repeats, (E1–E5) in the extracellular domain, a transmembrane domain, an intracellular domain that binds p120-catenin and beta-catenin, and a highly conserved cytoplasmic tail (32). The intracellular domain of E-cadherin has a highly phosphorylated region vital to β-catenin binding. It is known as a tumor suppressor (33) and plays an important role in cell adhesion (31). E-cadherin ensures that the cells within the tissue are well bound together, hence playing a vital role in normal tissue development (27). The binding of p120 to intracellular domain of E-cadherin regulates cadherin adhesive activity and trafficking. Meanwhile the

binding of β-catenin and γ-catenin play a structural role in junctional complex. E-cadherin-containing cell-to-cell junctions are often adjacent to actin-containing filaments of the cytoskeleton in epithelial cells (31). In addition to the E-cadherin role in normal cells, it can play a major role in malignant cell transformation, tumor development, and progression (31).

β-Catenin

β-catenin is a human protein that was found initially to be a member of Armadillo family of proteins (Homologous protein in drosophila) (34). It is encoded by CTNNB1 gene (34), and the structure has been determined (35). β-catenin family proteins have been found to contain several ARM repeats, sequences of approximately 50 amino acids that are involved in protein-protein interactions. Each of these repeat consists of 3 helices with helix 1 and 3 parallel to each other and helix 3 perpendicular to helix 1 and 3 (7).

β-catenin can be found inside the cell forming complexes with cadherins, axin (a component of the Wnt signaling pathway), galectin-3,transcription factors, and beta-galactoside-binding protein (36). It also associates with α-catenin, ICAT (β-catenin interactive protein 1), and APC (Adenomatous polyposis coli) (37). The ability for β-catenin to bind to other proteins is regulated by tyrosine kinase and serine kinases like GSK-3 (38, 39).   It serves as an anchor to the actin cytoskeleton (38). This may be responsible for transmitting contact inhibiting signal that makes cells stop dividing once the epithelial sheet is complete.

Canonical Wnt /β-catenin Signaling Pathway

   Wnt signaling pathway is a network of several proteins that passes signals from receptors on the cell surface through the cytoplasm to the cell's nucleus (40-42). Compositions of the network are secreted glycoprotein, Wnt family ligand, frizzled and low-density lipoprotein (LDL) receptor-related protein (LRP) families of receptors (40-42). These signals further initiate signaling cascade that lead to expression of target genes (41). Wnt signaling pathway controls cell-cell communication during embryo development, adult cell proliferation, and differentiation during development and healing (43, 44). During development, the Wnt signaling pathway regulates diverse processes, such as cell fate determination, cell polarity, and morphology, structural remodeling and most importantly cell adhesion and growth (40-45). The frizzled receptor component of Wnt signaling pathway is involved in 2 distinct pathways known as canonical or non-canonical pathways (45). The presence or absence of β-catenin is the difference between these two categories of pathways (45).

   The canonical Wnt pathway (Wnt/β-catenin signaling pathway) regulates β-catenin stabilization and gene expression, thereby causing its accumulation in the cytoplasm (46). β-catenin acts as an intracellular signal transducer in the canonical Wnt signaling pathway (47). The non-canonical pathway is the signaling pathway that regulates cell polarization (PCP) (48). This is a process that establishes the uniform alignment of structures in the epithelium (48).

   Normal levels of β-catenin are needed to associate with cadherins to promote cell adhesion and also control cell shape with the microfilament cytoskeletal network (49). Without a normal level, β-catenin will not be able to carry out its numerous functions (49). Normal levels of cytoplasmic β-catenin are maintained by a regulated process that directs degradation of excess β-catenin (49). In the cytoplasm, β- catenin degradation as shown in figure 2a involves phosphorylation by GSK3beta (37, 49, 50). Cyclin-CDK2 primes β-catenin for phosphorylation

by GSK3beta (49, 50). This phosphorylation occurs within the so-called "destruction-complex"

scaffold by the tumor suppressors AXINS (49, 50). Tumor suppressor APC (Adenomatous

polyposis coli) is also required within this destruction complex for an efficient degradation of β-

catenin (37). Phosphorylated beta-catenin is then bound by the beta-TrCP component of

ubiquitin ligase complex and β-catenin is degraded. GSK3beta, which is constitutively active,

must be inhibited to prevent the degradation of β-catenin in order for β-catenin to carry out its

functions (50). This is where canonical Wnt signaling pathway is involved (51).

Canonical Wnt signaling pathway, (figure 2b), through frizzled and LRP receptor (LRP5/6)

pair, activates dishevelled (Dvl) and inhibits GSK3beta. This allows accumulation of β-catenin in

the cytoplasm, hence its ability to migrate to the nucleus. β-catenin enters the nucleus and

interacts with transcription factors and gene regulators, such as T-cell factor (TCF)/lymphoid

enhancer factor (LEF) to mediate gene expression (28, 29). Already bound β-catenins (e.g. E-

cadherin/β-catenin complex) as shown in figure 2c, are not affected by this degradation.

However, the degradation affects the availability of β-catenin to bind to E-cadherin and other

proteins (52).

Canonical Wnt/β-catenin Signaling Pathway and E-cadherin/ß-catenin complex
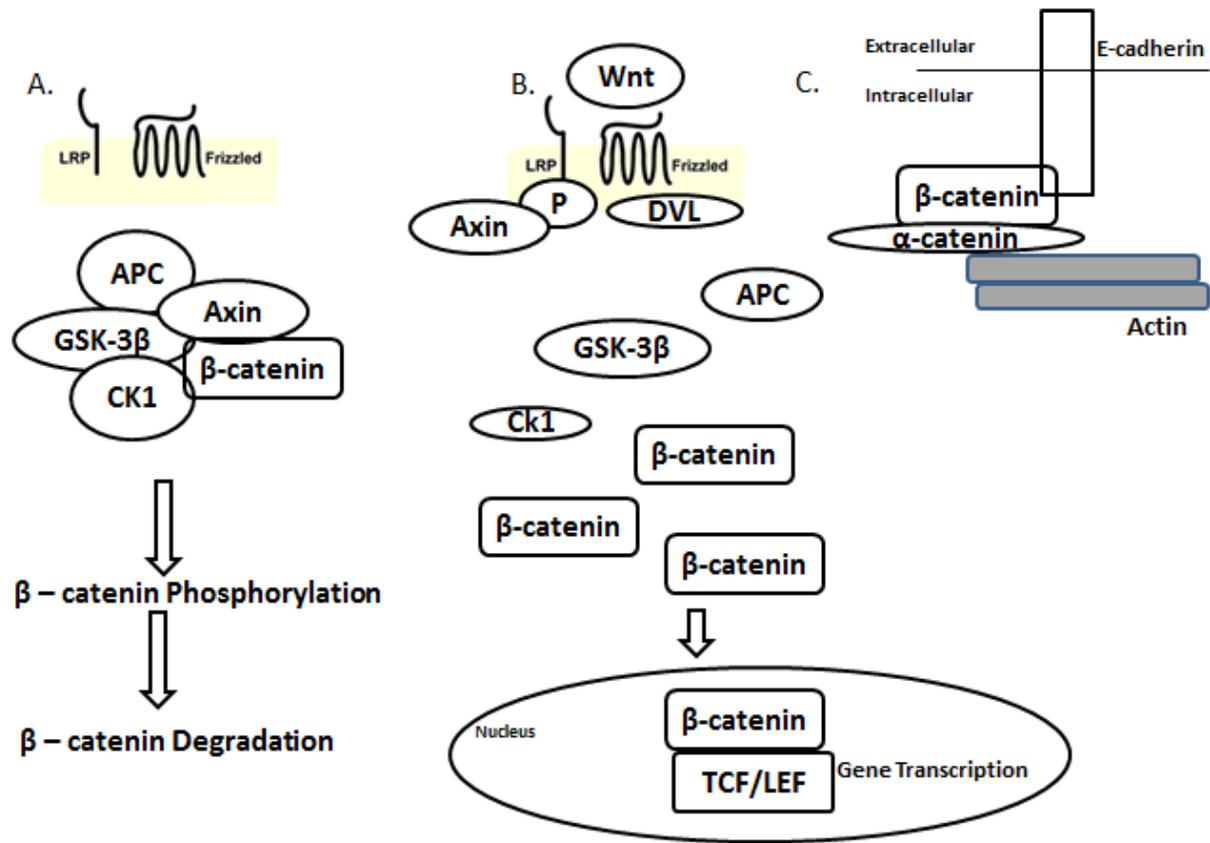
Figure 2: A. The degradation of beta-catenin by GSK3beta complex. B. Wnt/β catenin signaling pathway enhances accumulation of beta-catenin in the cytoplasm. C. E-cadherin/β-catenin complex.

E-cadherin/ß- catenin Complex

β-catenin is part of a complex that constitutes adherens junctions (27, 31). These adherens junctions are needed for the creation and maintenance of epithelial cell layers, through regulation cell growth and adhesion between cells (27).

E-cadherin and β-catenin complex play a crucial role in maintaining epithelial integrity (27, 31). Cell-cell junctions in tissues help to maintain cell and tissue integrity and polarity. In vertebrate animals, there are intercellular junctions systems, namely: Gap junctions (that act as intercellular channels and allow direct cell-cell transfer of small molecules and ions); Tight junctions (the major cellular determinant of epithelial barrier function); Anchoring junctions (these are desmosomes and adheren junctions that interact with the cortical cytoskeleton to mediate cell and tissue behavior) (53). Among the structural molecule constituents that assemble to form the adheren junctions is the E-cadherin/β-catenin complex (53). The extracellular region of E-cadherin spans from the cell surface of a particular cell to other subtypes of cadherins present on adjacent cells. The intracellular region contains binding sites to interact with β-catenins and other regulatory proteins (54).   β-catenin binds to the carboxyl-terminal 100 amino acids of the cadherin cytoplasmic region. Actin cytoskeleton, which has the ability to promote adherens junction protein clustering and stabilization of cell adhesion, is bound to beta-catenin by alpha-catenin (55, 56).

<u>Presence of E-cadherin and β-catenin in Ovary and It's Relation to Ovulation</u>

<u>Folliculogenesis</u>

In mammals there are different dynamic changes that occur during reproduction cycle. The different ovarian compartments are subjected to both differentiation and proliferation events that are regulated by paracrine and endocrine factors (57). Human ovaries normally produce a single dominant follicle that results in a single ovulation during each menstrual cycle (57). The development and maturation of this dominant ovarian follicle involves structural changes that require altered expression and function of the component involved in cell-cell contacts (58).

Intercellular communication through the cell-cell adhesion is crucial in the maintenance of tissue integrity and function (59).

Folliculogenesis is the process of attaining successively higher levels of cell organization by means of cytodifferentiation and cell proliferation of the ovarian follicle (60). This process occurs within the cortex of the ovary (56). The recruitment of a primordial follicle into the pool of growing follicles is the beginning of folliculogenesis (57). This ends with either ovulation or death by atresia. Folliculogenesis includes 4 major developmental events as shown in figure 3 below: 1) primordial follicle recruitment; 2) pre-antral follicle development; 3) selection and growth of antral follicle; and 4) follicle atresia (60-62). The pre-antral phase (gonadotropin-independent phase) is characterized by growth and differentiation of the oocyte and controlled by locally produced growth factors through paracrine/autocrine mechanisms (56-58). A primordial follicle consists of a single layer of flattened or squamous granulosa cells. These granulosa cells are closely juxtaposed to the oocyte and a basal lamina (56). Primordial follicles are formed in the human fetus from the sixth to ninth month of gestation and are arrested in the dictyate stage of meiosis (58). Some of these primordial follicles are recruited to grow soon after their formation in the fetus. This is termed recruitment or primordial follicle activation (56). As the female develops and grows into puberty, the primordial follicle becomes primary follicle. The primary follicle is characterized by a single layer of one or more cuboidal cells arranged around the oocyte (56-58). It is during the conversion of primordial follicles to primary follicles that the importance of cell-cell contacts arises (29). There is a development of intimate intercellular connections between the oocyte and granulosa cells (63). It is believed that the effectiveness of adherens junctions and gap junctions in these regions is important in primary follicle development (59). The importance of oocyte-granulosa intercellular connections makes the E-cadherin/β-catenin complex a potential candidate for a crucial role in folliculogenesis. E-

cadherin/β-catenin is a structural constituent in formation of adherens junction. This support the recent finding of expression of E-cadherin and β-catenin in normal rat ovaries (64, 65).

As the pre-antral folliculogenesis continues, the primary follicle develops into secondary follicle. Secondary follicle development includes the accumulation of multiple layers of granulosa cells around the oocyte and the acquisition of theca cells (theca interna and theca externa) (66). This process is regulated by autocrine/paracrine mechanisms that involve growth factors produced by the oocyte (56-58). Primary-to-secondary transition involves the ability to stimulate granulosa cell proliferation and continual development of inter-cellular junction system between the granulosa cells and oocyte-granulosa intercellular connections (59).

The antral phase (gonadotropin-dependent phase) is associated with tremendous size increase of the follicle (25-30mm) and regulated by follicular stimulating hormone (FSH), luteinizing hormone (LH), and growth factors (57). Determination of how these growth factor pathways negatively or positively regulate folliculogenesis, ovulation, and luteogenesis is not completely understood. The hallmark of the antral phase is the appearance of epitheloid cells in the theca interna and development of a fluid filled antrum (61). The overall size of the antral follicle is determined by the size of the antrum (follicular fluid) and proliferation of the follicle cells (granulosa and theca cells) (57). Cessation of increase in follicular fluid and mitosis under hormonal influence leads to follicular atresia. All the granulosa cells express FSH receptors during antral follicle development. This enables them to respond to FSH in many ways, including induction of luteinizing hormone (LH) receptors (57). The stimulation by gonodotrophins and growth factors causes the growth of the follicle and granulosa cell differentiation, leading to pre-ovulatory follicle. An LH surge causes follicular rupture (ovulation) and formation of corpus luteum (57, 62, 67). Constant communication between the oocyte and its surrounding granulosa cells results in maturation of a fertilizable oocyte (59, 63).

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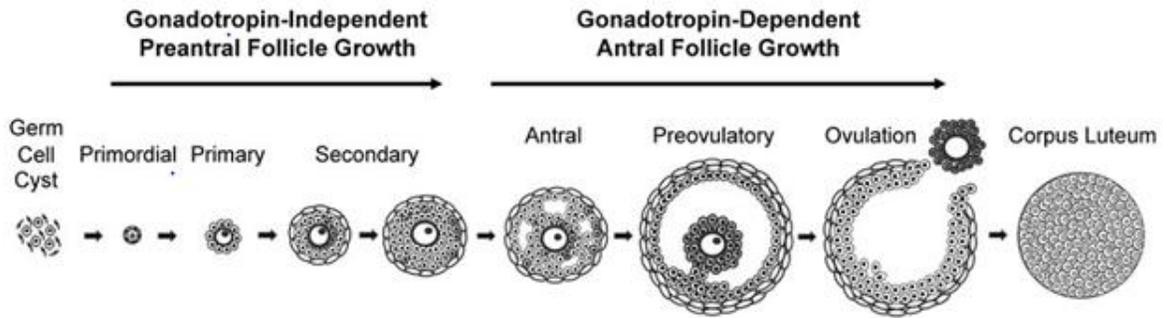
Figure 3: Folliculogenesis. The pre-antral and antral follicular growth phase of folliculogenesis. (adapted from (69))

<u>Expression of E-cadherin/β-catenin in Rats Ovaries</u>

E-cadherin/β-catenin complex plays an important role during tissue development and differentiation of many organs (68). The modulation of E-cadherin-β-catenin expression is an integral component of remodeling processes including corpus luteum formation in the ovary (64). Recent studies have suggested the presence of this complex in the theca-interstitial cells surrounding pre-ovulatory follicles and pre-antral follicles located in the inner region of the ovary (58, 64, 65). E-cadherin/β-catenin complex has been expressed in the rat ovary during folliculogenesis and luteal formation (65). Expressions of E-cadherin and β-catenin were found to be increased in the theca and interstitial cells of the rat ovaries during follicular development. However, granulosa cells had more expression of β-catenin (65).

The concentration of E-cadherin and β-catenin were found to be constant during the follicular phase and present in most ovarian cells at all stages of folliculogenesis (58, 64, 65). This makes them have the potential to participate in the regulation of cytoskeletal structures and intracellular signaling. Studies have shown that modulation of E-cadherin/β-catenin complex expression is an integral component of remodeling processes that includes corpus luteum formation in the ovary and folliculogenesis (61). The complex is under hormonal control during these processes (20, 34). The possibility of E-cadherin having a 'barrier-function' role in limiting

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the accessibility of immune system to the follicular compartment during folliculogenesis has also been postulated (42).

These evidences suggest the involvement of these proteins in reproductive processes. However, the molecule modulating the expression of these proteins (E-cadherin and β-catenin) in these ovarian cells is unknown at the moment. Because leptin is a ploteotropic hormone involved in many physiological processes including reproduction (15), the fundamental questions to be answered are: 1. Is it possible that the direct involvement in modulation of expression of these proteins could be one of leptin actions that has not yet been identified?; and 2. Does leptin work to reverse dietary-induced infertility in an obese rat by modulating E-cadherin and β-catenin expression in ovarian cells? This study was designed to answer these questions.

CHAPTER 2

MATERIALS AND METHODS

Animals Model

Twenty female Sprague-Dawley rats of the same age with the weight range of 220-260g were acquired from Harland and used in this study (Protocol #100501). The animal use protocol was approved by the University committee on animal care (UCAC) of East Tennessee State University. The animals were housed individually and kept in a well-controlled, specific pathogen-free room, located in Brown Hall DLAR facility at ETSU. Food and water were made available ad libitum. Six of the rats were given regular chow diet (RCD), and the remaining 14 were fed with high Fat diet (HFD).

Determination of Oestrous Cycle and Vaginal Cytology

The oestrus cycles were checked daily for each of the rats. A regular 4-day cycle as shown in figure 4, consists of proestrus, estrus, metestrus, and diestrus.

Vaginal Cytology

In determining the different stages of the estrous cycle, a wet mount process was conducted to visualize the vaginal cytology. A small amount (10µl) of saline solution was introduced into the vaginal orifice of each female rat and then aspirated and placed on a clean dry slide. The cells were then examined under a compound microscope at 10X and 40X for analysis. Using a digital camera microscope (manufactured by Nikonci, Japan (Figure 6), pictures were taken.

<u>Oestrus Cycle</u>
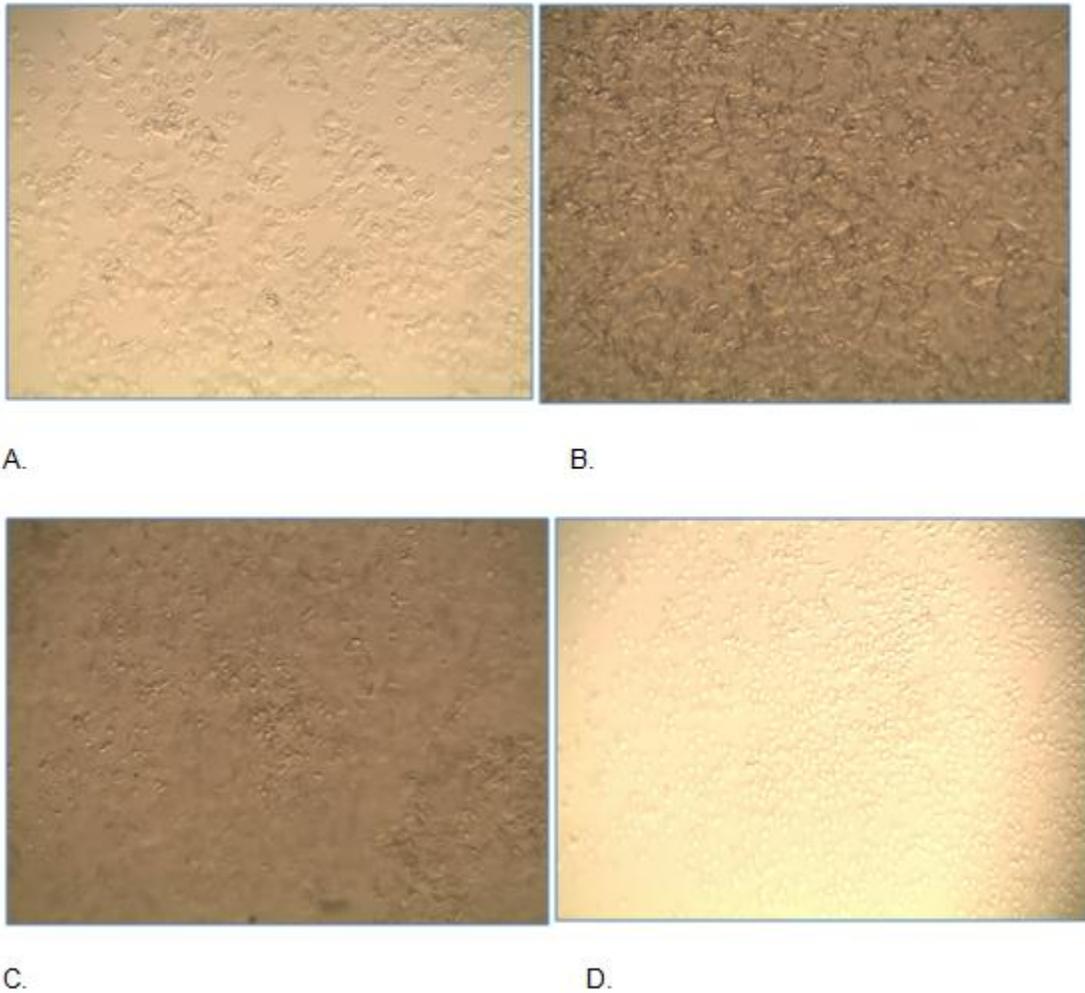
Figure 4: Vaginal smears pictures that were taken at 10X using digital camera microscope. Each of the pictures represents 1 of the 4 cycles of oestrus. (A) Proestrus: predominance of nucleated epithelial cells. (B) Estrus: predominance of anucleated cornified cells. (C) Metestrus: includes nucleated epithelial cells, anucleated cornified cells, and leukocytes. (D) Diestrus: predominance of leukocytes.

Irregular Oestrus Cycle

    The irregularity of the oestrus cycle as shown in figure 5, of those rats given HFD was noticed towards the end of the 12th week of the study, which signified the occurrence of infertility. The regularity of oestrus cycle is determined by a 4-day cycle that consists of: proestrus, estrus, metestrus, and diestrus. Ability of each rat to go through this cycle day by day without skipping any stage signifies a regular oestrus cycle.
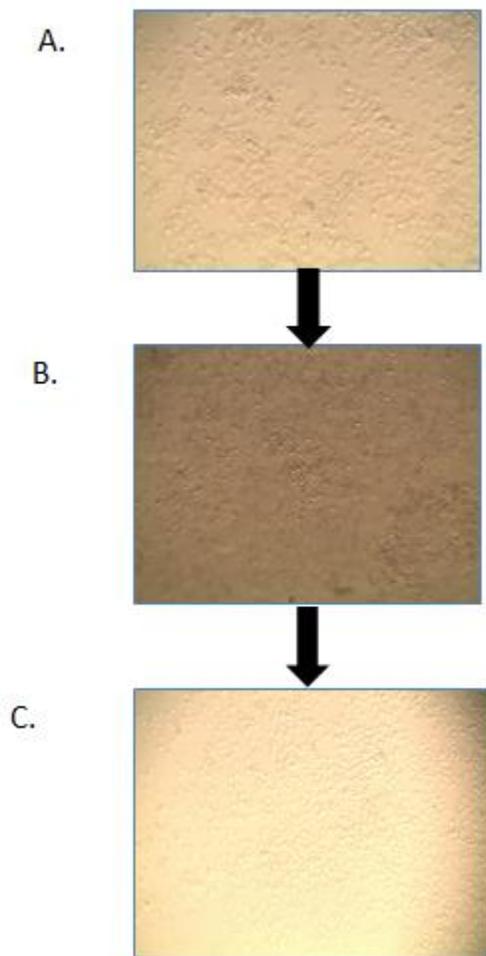
Figure 5: Representation of an irregular oestrus cycle. The cycle moved from (A) Proestrus: predominance of nucleated epithelial cells, to (B) Metestrus: includes nucleated epithelial cells, anucleatedcornified cells, and leukocytes, skipping Estrus and being detained in (C) Diestrus: predominance of leukocytes.

Figure 6: Using a digital camera microscope (manufactured by Nikonci, Japan), pictures were taken at 10X and 40X magnification for analysis.

Overview

Diet-induced obesity in rats has been found to lead to infertility in both female and male rats (15). Leptin is involved in centrally regulated maturation of the reproductive system. The infertility of obese female ob/ob mice can be reversed by leptin treatment (15). In order to study leptin modulation of expression on E-cadherin, β-catenin in dietary- induced obese infertile rats, some of these female rats were given HFD daily. Their weight, food intake, and oestrus cycle were checked daily until the oestrus cycle became irregular.

Six were given RCD and 14 HFD. By the 13th week, 5 out of the 14 female rats that were fed HFD had irregular oestrus cycle earlier than the remaining 9. These were the rats that were treated with leptin. 100ug/ml of leptin was injected to each of the 5 female rats intra-peritoneally (morning and evening) for 2 days. The remaining 9 given HFD and the 6 with RCD were injected with saline. Their body weight and food intake were measured twice weekly and the estrus cycle checked daily.

Statistical Analysis

t-test, one-way ANOVA, two-way ANOVA were used to calculate the significant difference of the average weight change and food intake amongst the rats. The level of significance was set at ($p < 0.05$).

Tissue Harvesting

At the end of the 14th day when the oestrus cycle of those treated with Leptin became regular, the 20 female rats were sacrificed by decapitation. Ovariectomy was done in each rat, and the ovaries were frozen on dry ice and stored at -80 degree until processed for homogenization and Western Blotting.

E-cadherin and β- catenin Identification and Quantification

E-cadherin and β-catenin protein expression was examined using Western Blots. Each sample was homogenized using RIPA lysis buffer (Triton X-100 10%, Sodium Deoxycholate 1%, SDS 0.10%, NaCl 0.15M, NaH2P04 0.01M, NaF 50mM,EDTA 2mM) containing Protease Inhibitor Cocktail (Thermo scientific, #78442) and the cell lysates extracted upon refrigerator centrifugation. The protein concentration of the cell lysates were determined by Bicinchoninic acid Assay (BCA) protein (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated

by electrophoresis on 10% SDS-polyacrylamide gel and electro-transferred onto Hybond-ECL nitrocellulose membranes. Blotted membranes were blocked overnight with 5% skimmed milk and incubated with primary antibodies diluted 1 to 200 in 5% BSA. Antibodies from Santa Cruz Biotechnology (Santa Cruz,CA,USA) included E-cadherin (sc #3195) Beta-catenin (sc #2698). Beta actin was obtained from sigma (F3022). After primary antibody incubation the blots were probed with appropriate secondary antibody conjugated with horseradish peroxidase. The signal was visualized using Super Signal West Dura chemiluminescence substrate (Pierce Biotechnology). Blot images were obtained using the G-box i-chemi XR CCD (Syngene Corporation).

CHAPTER 3

RESULTS

Leptin Effect on Body Weight

The body weight of RCD+ Saline (S), control, and HFD+ Saline (S) were monitored daily

throughout the study with a use of digital scale (Mettler Toledo,OH). The average data (table 1)

and figure (figure 7) are showed below. This parameter was measured in order to see the

difference in body weight change as a result of the type of diet. There was a significant

difference between the weight gain of RCD+S and HFD+S. The HFD+S had more weight gain

than RCD+S.

Table 1: This data shows the difference in the total weight gain at the end of the study between
RCD+S (n=6) vs HFD+S (n=9) rats.

Average Weight Gain of RCD+S and HFD+S At The End Of the Study(g)	
RCD+S	HFD+S
24.5	43.1
28.8	35.5
18.6	42.1
8.3	25.1
17	33.1
30	33.4
	44.6
	28.5
	30.3
Mean	Mean
21.2	35.08
SEM	SEM
3.35	2.29

Average Weight Gain of RCD+S and HFD+S at the End of the Study (g)
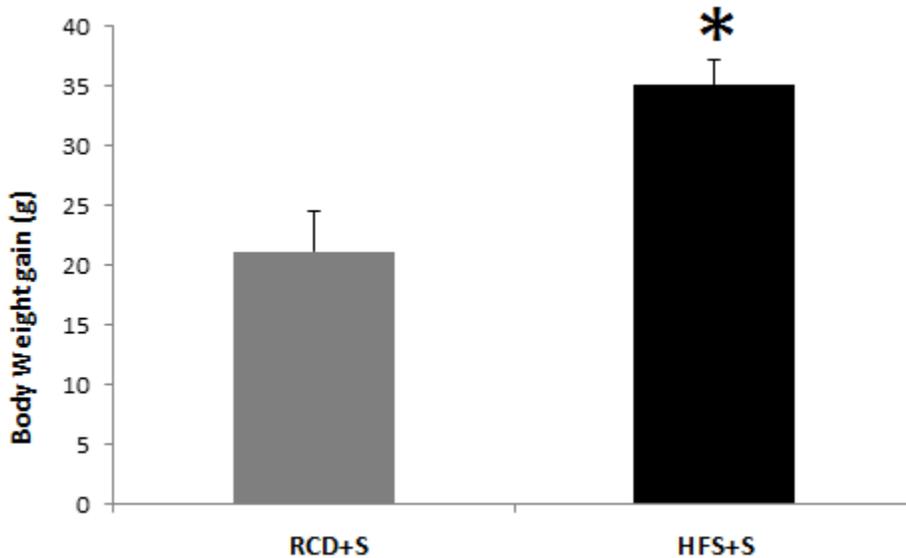
Figure 7: Comparison of average body weight gain of RCD+S and HFD+S at the end of the study. The body weight gain of HFD+S was significantly ($p<0.05$) higher than RCD+S using a Two-sample t-test analysis. (* significant difference)

<u>Leptin Induces Weight Loss</u>

By the 7th day, the oestrus cycle of those treated with leptin (n=5) started showing signs of regularity. By the 13th day, the oestrus cycle of the 5 female rats (HFD+Leptin) became regular, and that of the remaining 9 HFD fed female rats were fully irregular. A significant weight loss occurred in the HFD+Leptin (n=5) rats. The table (table 2) and figure (figure 8) below represent these changes.

Table 2: Data representing the total body weight gain pre-leptin and total body weight loss post leptin treatment (n=5).

Average Weight Gain Pre-leptin And Total weight loss Post Leptin Treatment (g)	
Pre leptin (Weight gain)	Post Leptin (Weight loss)
32	15.3
27.7	7.9
7.4	6.4
28.5	8
24.3	13.9
Mean	Mean
24	13.7
SEM	SEM
4.33	3.64
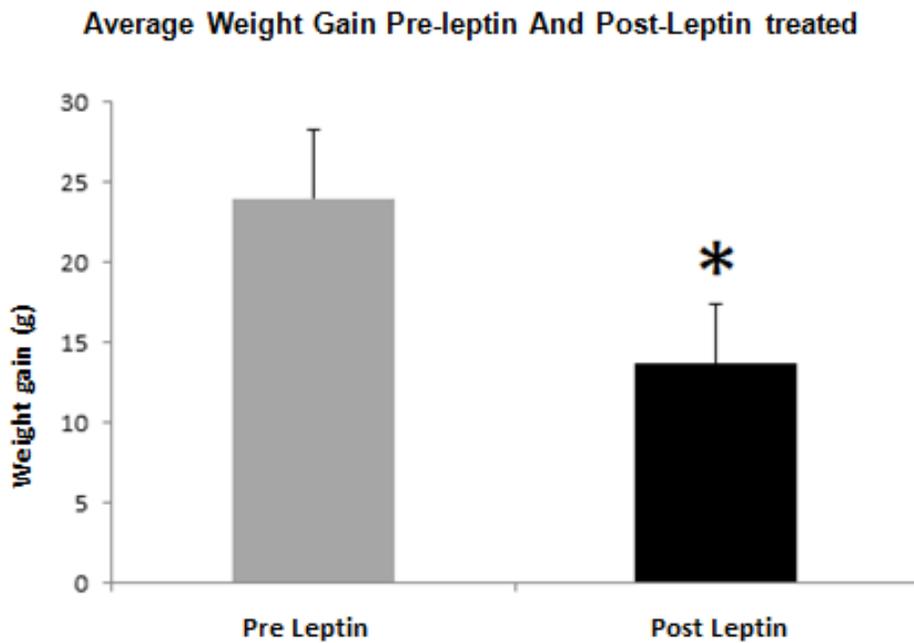
Figure 8: Average weight gain of pre- and post-leptin treatment. There was a significant (p<0.05) weight loss post-leptin treatment (n=5) using a Paired t-test analysis. (* significant difference)

Leptin Effect on Body Weight

Leptin treatment of rats with irregular oestrus cycle resulted in lower weight gain compared to RCD+S and HFD+S as shown in the table (table 3) and figure (figure 9) below.

Table 3: This data represents the differences in the total weight gained of the RCD+S (n=6), HFD+Leptin (n=5), and HFD+S (n=9) at the end of 15 weeks

Average Weight Gain Of RCD+S, HFD+Leptin, and HFD+S At The End Of 15 Weeks (g)		
RCD+S	HFD+Leptin	HFD+S
24.5	16.8	43.1
28.8	19.8	35.5
18.6	1.0	42.1
8.3	20.5	25.1
17	10.4	33.1
30		33.4
		44.6
		28.5
		30.3
Mean	Mean	Mean
21.2	13.7	35.08
SEM	SEM	SEM
3.35	3.64	2.29

Average Weight Gain of RCD+S, HFD+Leptin, and HFD+S at the End of 15 Weeks
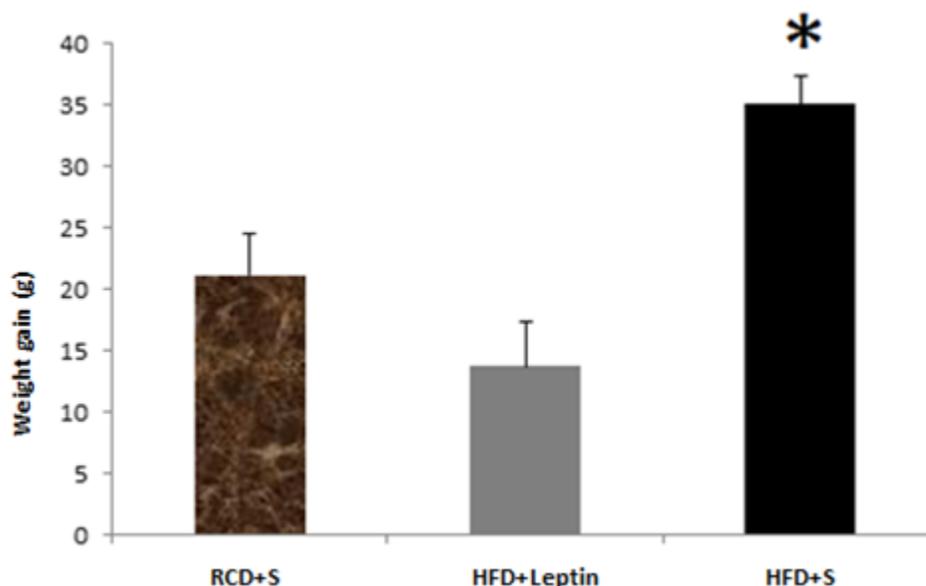
Figure 9: Average weight gain Of RCD+S, HFD+leptin, and HFD+S at the end of 15 weeks. Using a One-way ANOVA analysis test, HFD+S (n=9) was significantly ($p < 0.05$) higher than RCD+S (n=6) and HFD+Leptin (n=5) rats. (* significant difference)

<u>Leptin Reduces Amount of Food Intake</u>

During this study, the amount of food intake by each rat was monitored everyday throughout the study with the use of a digital scale (Mettler Toledo,OH). This parameter was measured to determine if leptin reduces appetite. Leptin caused reduction of the amount of food intake by these rats, which directly had an effect on their body weight change. The HFD+leptin group of rats resulted in a significant reduction in food intake measured in kcal/day when compared to RCD+S and HFD+S as shown in the table (table 4) and figure (figure 10) below. A gram of RCD diet correlates to 3.4 kcal, while a gram of HFD correlates to 4.73 kcal. After the treatment with leptin, there was reduction of food intake of HFD+leptin due to loss of appetite induced by effect of leptin.

Table 4: Average food Intake (kcal/day) of RCD+S, HFD+S, and HFD+Leptin.

Average Food Intake (kcal/day)		
RCD+S	**HFD+S**	**HFD+Leptin**
45.76	52.79	43.04
SEM	SEM	SEM
0.02	0.05	0.03
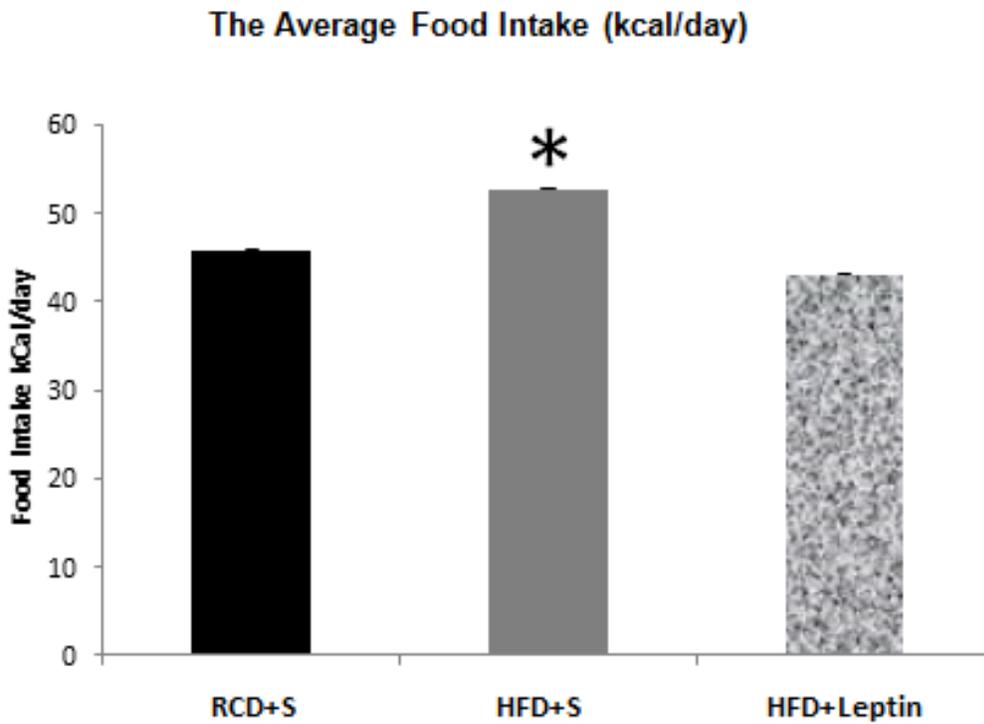
Figure 10: This figure shows the average food intake (kcal/day) of RCD+S, HFD+S, and HFD+Leptin. The calorie intake of the HFD+S was significantly ($p < 0.05$) higher than the RCD+S and HFD+leptin, using a One-way ANOVA analysis test. (* significant difference)

Ovary cell lysate were analyzed for the expression of β-catenin and E-cadherin by Western Blot. It was found that both of these proteins had higher expression levels in rats given RCD+S when compared to HFD+S and HFD+L. There was a significant reduction of β-catenin and E-cadherin in the HFD+S when compared to RCD+S as shown in the figure (figure 11) below. However, the rats fed HFD followed by leptin injection resulted in an increased expression of β-catenin and E-cadherin compared to HFD+S, suggesting leptin induced a recovery from the almost total loss of expression of both β-catenin and E-cadherin due to the effect of obesity on infertility.
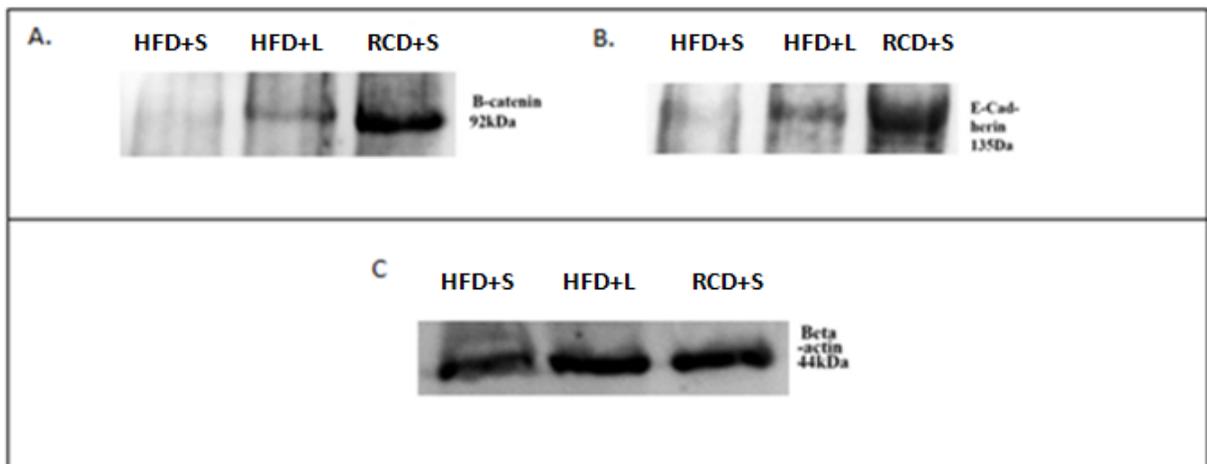
Figure 11: Effects of leptin on expression of (a) β-catenin and (b) E-cadherin in obese infertile ovary cells. Reversal of expression of both proteins in HFD+leptin towards the amount expressed in RCD+S, after almost total loss in HFD+S. (c) Expression of β-actin in the cells, serving as a form of loading control. β-actin, a 44kDa primary antibody, is a house keeping gene that is always in the cell and serves as a form of loading control. This shows that the same amount and concentration of protein was loaded in each well.

CHAPTER 4

DISCUSSION

Leptin is a pleiotropic hormone that has been found to be involved in many physiological functions in the body (9-11). Although leptin has been found to reverse infertility (14), its role in infertility reversal in obese individuals has not yet been examined. The focus of this study is to propose the probable mechanism of leptin in the reversal of infertility in dietary-induced obese infertile rats.

Our first observation was the role of leptin in energy intake and expenditure, which supports previous studies that show leptin regulates appetite, weight gain, and fat deposition via a negative feedback loop involving the hypothalamus (1-4). It was observed that the rats given HFD+leptin had a reduction in their food intake that led to a significant weight loss when compared to rats given HFD+S.

Also, the role of leptin in fertility was observed. HFD feeding for 15 weeks induced irregular oestrus cycle an indicator of infertility in all the rats. The regularity of oestrus cycle was determined by a 4-day cycle that consists of: proestrus, estrus, metestrus, and diestrus. The ability of each rat to go through this cycle day by day without skipping any stage signified a regular oestrus cycle. Two weeks post-leptin injection in the treated group, the irregular oestrus cycle became regular. Leptin is known to reverse infertility in ob/ob infertile rats (14). Exogenous leptin replacement has been found to rescue the infertility of ob/ob females (20). Elevated serum luteinizing hormone, increase in ovarian and uterine weights, and stimulation of ovarian and uterine histology has been documented with exogenous leptin replacement (20). Some studies have supported the concept of leptin coordinating the behavioral and endocrine components of reproductive functions (44-45).

Although recent studies have shown that leptin reverses infertility in ob/ob infertile rats (20), the pathway of reversal is poorly understood. This study will be the first to show the correlation between leptin and a potential pathway in the reversal of fertility in dietaryly induced obese infertile rats. The possibility of calorie restriction/and or weight loss pathway has been excluded (14, 16).

Most importantly, studies have shown that E-cadherin/β-catenin complex is among the structural molecular constituents that assemble to form the adherens-junctions (53). These adherens junction are needed for the creation and maintenance of epithelial cell layers, which is achieved by regulating cell growth and adhesion between cells (53). This complex has also been found to play a crucial role in maintaining epithelial integrity and cell proliferation (35). Expressions of E-cadherin and β-catenin were found to be increased in the theca and interstitial cells of the rat ovaries during follicular development (58, 64, 65). However, granulosa cells had more expression of β-catenin (58, 64, 65). The concentration of E-cadherin and β-catenin were found to be constant during the follicular phase and present in most ovarian cells at all stages of folliculogenesis (58, 64, 65). Folliculogenesis is the process of attaining successively higher levels of cell organization by means of cytodifferentiation and cell proliferation of the ovarian follicle (60). The conversion of primordial follicles to primary follicles involves the development of intimate intercellular connections between the oocyte and granulosa cells (63, 29). The effectiveness of this cell-cell contact is determined by the competency of adherens junction and gap junction at this region (59). The intimate intercellular connections between the oocyte and granulose cells are important in primary follicle development (59). Also, primary-to-secondary transition involves the ability to stimulate granulosa cell proliferation and continual development of inter-cellular junction system between the granulosa cells and oocyte-granulosa intercellular connections (59). The importance of oocyte-granulosa intercellular connections in primordial follicles-primary follicles transition and the stimulation of granulosa cell proliferation and

continual development of intercelluar junctional system between the granulose cell and oocyte-granulosa connections (59), make the E-cadherin/β-catenin complex a potential candidate for a crucial role in folliculogenesis.

In our studies, not did we only demonstrate that E-cadherin and β-catenin are expressed in the rat ovary and that HFD reduced their expression, but surprisingly we also showed that leptin treatment modulates the expression of these proteins in these dietary-induced obese infertile rats. The expression of E-cadherin and β-catenin was markedly reduced in ovaries of rats that were fed with high fat diet (HFD) and reversed expression of these proteins when they were treated with leptin intra-peritoneally. Because E-cadherin/β-catenin complex has been found to be involved in all stages of folliculogenesis (38,41), observations made in this study suggest that leptin has a functional role in the expression of E-cadherin and β-catenin complex in improving fertility in obese infertile rats and could therefore be important in folliculogenesis and ovulation. This could be a novel finding of the potential pathway by which leptin acts in reversing infertility in these rats.

Future Directions

A long-term study including measurement of all the hormones involved in reproduction is needed. The ovarian and uterine weights should also be measured. In respect to the counter effects of E-cadherin and β-catenin that have been recently found to be involved in development of cancer and its metastasis (25,34), it will be of interest to know if these leptin treated rats will develop ovarian cancer or polycystic ovarian disease when they are left for a longer period of time.

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