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Expression of Leptin (Ob Gene Product) in Reproductive System with Special Reference to Polycystic Ovary Syndrome

Abstract

Purpose: To determine serum leptin and its ob mRNA expression both in the PCOS and non-PCOS ovary, endometrium and adipose tissue in normal or polycystic ovary syndrome (PCOS) in South Indian population.

Methods: The regulation of ob gene expression in thin, overweight, obese and morbidly obese PCOS (Polycystic Ovary Syndrome) and non-PCOS subject's endometrium, ovary and adipose tissue were investigated using a reverse transcription-competitive polymerase chain reaction method to quantify the mRNA levels of leptin and compared with normal weight control adipose tissue.

Results: Endometrium, ovary and adipose tissue ob mRNA levels were highly correlated with serum leptin, BMI and body fat distribution of 80 subjects (10 normal weight, 8 thin PCOS, 8 thin non-PCOS, 7 overweight PCOS, 7 overweight non-PCOS, 10 obese PCOS, 10 obese non-PCOS, 10 morbidly obese PCOS and 10 morbidly obese non-PCOS). Ob mRNA levels were positively correlated with serum levels of dehydroepiandrosterone sulphate, free testosterone, luteinizing hormone, and prolactin. Ob mRNA level was inversely correlated with SHBG and androstenedione.

Conclusion: Here, we report for the first time in Indian population, that endometrium, ovary, adipose tissue ob mRNA levels and serum leptin are highly correlated and are different in expression levels in thin, obese, morbidly obese PCOS and non-PCOS, when compared to individuals with normal weight. The leptin could be a novel, independent risk factor for PCOS.

Keywords: Ob mRNA, Serum leptin, body mass index, body fat distribution, infertility, ovary.

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Introduction

Leptin, the product of *ob* gene that is produced by the adipose tissue [1], regulates food intake and energy expenditure [2] and also plays an influential role in reproduction. Injecting leptin into ob/bob mice that are infertile and with low levels of gonadotropin increases the weight of the uterus, ovaries and the number of follicles [3]. Administering leptin to normal female mice accelerates puberty [4], and in humans higher leptin levels are shown to relate to the earlier onset of menarche [5]. Fertility can be restored by treatment with human recombinant leptin [6]. Women undergoing IVF therapy who tend to be obese and show a reduced ovarian response display increased serum leptin concentrations, but unchanged leptin concentration in follicular fluid [7].and for women with, PCOS whether high leptin levels participate in this disturbed gynecological event is still a matter of debate [8]. We have previously, confirmed that the hyper secretion of androgen is a typical biochemical feature of PCOS and they frequently have increased secretion of LH and insulin resistance. Therefore, PCOS subjects are good models for studying serum leptin levels, body fat distribution, measurement of obesity and biochemical analysis in relation to endocrinal parameters [9]. In this study, we investigated the regulation and correlation between ob mRNA and serum leptin levels, BMI, body fat distribution in relation to endocrinal parameters in thin and obese PCOS and non-PCOS subjects and compared it with normal weight controls. However, there is still little information on leptin localization in the intact human ovary, endometrium and adipose tissue and hence the present study.

Our objective is to evaluate serum leptin and its ob mRNA expression both in the PCOS and non-PCOS ovary, endometrium and adipose tissue in normal or polycystic ovary syndrome (PCOS) in South Indian population. Our data confirm the significant correlation between leptin mRNA levels and the BMI as previously observed by others [10]. Interestingly, there is an expression of ob mRNA l in the 18-40y age group subjects in thin, obese and morbidly obese PCOS subject's ovary, endometrium and adipose tissue and compared it with normal weight controls adipose tissue. Inverse correlation is observed between ob mRNA and serum levels of SHBG, androstenedione in thin, obese and morbidly obese PCOS subjects, which is largely, explained by serum leptin and BMI values. A significant note is that, upper body fat distribution was found to be elevated, thus contributing to the increased adiposity in obese and morbidly obese PCOS and non- PCOS subjects, showing a 8 fold increase when compared to normal weight control women, thin PCOS and non-PCOS subjects. One of the striking finding is the pronounced expression of ob mRNA and serum leptin in thin PCOS, irrespective of BMI, and this is indicative of the influence of ovarian hormonal profile, which ultimately leads to defect in leptin expression in female infertility.

Materials and methods

Subjects and sample acquisition

Endometrial, ovary and adipose tissue biopsy specimens and blood samples were obtained from PCOS patients scheduled for laparotomy at the Department of Obstetrics and Gynecology. A total of 35 women of reproductive age underwent Oophorectomy for Polycystic ovary syndrome. Endometrium biopsies were obtained from women diagnosed with PCOS during the mid secretory phase (n=24) of the menstrual cycle. Ovarian cortex with corpus luteum was obtained from PCOS women and adipose tissue as a positive control from the same patients. Classification of the ovarian cycle: (i) Follicular phase (n=3), (ii) Luteal (Secretary) phase (n=8).

The study population consists of the following: 8 thin non-PCOS, 8 thin PCOS, 7 overweight non-PCOS, 7 overweight PCOS, 10 obese non-PCOS, 10 obese PCOS, 10 morbidly obese non-PCOS, 10 morbidly obese PCOS and 10 regularly menstruating control women (Table 1). The subjects were non-alcoholics and non-smokers. Women with PCOS were less than 45 yr old (18–44 yr) with oligo/amenorrhea dating to puberty and polycystic ovarian morphology confirmed by visual inspection of the ovaries by laparotomy,

Table 1: Distribution of subjects

Samples (n=80)	Adipose	Endometrium	Ovary
Thin PCOS [BMI=20.0 kg/m^2]	10	5	3
Thin Non-PCOS [BMI=20.0 kg/m^2]	8		
Overweight PCOS (n) [BMI = 32.2 kg/m^2]	7	5	2
Overweight Non-PCOS [BMI = 32.2 kg/m^2]	7		
Obese PCOS (n) [BMI = 38.0kg/m^2]	10	7	3
Obese Non-PCOS [BMI = 38.0kg/m^2]	10		
Morbidly obese PCOS(n) [BMI= 43.0 kg/m^2]	10	7	3
Morbidly obese Non-PCOS [BMI = 43.0 kg/m^2]	10		
10 Regularly menstruating control women	10		

laparoscopy or by ultrasound examination. There was no evidence of hyperprolactinemia, Cushing's syndrome, congenital or non-classical adrenal hypoplasia, and hormone secreting tumors. Control women were healthy, less than 45 yr old (18–44 yr) with regular menstrual cycle and no evidence of hyperandrogenism, polycystic ovaries, endometriosis or abnormal uterine bleeding. Neither PCOS nor control women had taken medication within 60 days of biopsy specimens and blood sample collection.

This study was approved by the institutional review boards. After obtaining a written consent, biopsy and fasting blood was drawn at the Department of Obstetrics and Gynecology, Padma Hospitals. Measurements of body fat distribution were made during the time convenient for the normal and obese persons. The subjects were allowed to fast for 12 h and then 10 ml of blood was collected from them. The blood was allowed to clot and it was retracted and separated by centrifugation at 2000g for 15 min. The endometrium, ovary, adipose tissue samples were immediately frozen in liquid nitrogen and serum was separated and stored at - 20°C until analysis.

Total RNA preparation

Endometrium, ovary and adipose tissue samples were pulverized in liquid nitrogen and total RNA was prepared from the frozen powder using the RNeasy total RNA kit (QIAGEN, Chatsworth, CA) following the instructions of the manufacturer. The ratio of absorption (260/280 nm) of all preparations was between 1.9 and 2.0.

Quantification of leptin mRNA

Human leptin mRNA was quantified in endometrium, ovary and adipose tissue total RNA preparations by reverse transcription reaction followed by competitive polymerase chain reaction (RT competitive PCR) which consist in the co-amplification of leptin cDNA with known amount of a leptin competitor DNA in the same test tube (11). The competitor DNA molecule was derived from the clone Ob6.1 that contained the complete coding sequence of human leptin cDNA (12). This clone was obtained by screening a human adipose tissue l gt 11 cDNA library with a fragment of mouse ob cDNA clone pmob, and sub cloned into the phagemid pBlue- Script SK (Stratagene). The leptin competitor was constructed by deleting an 80 bp-long fragment (nucleotides +324 to +404 of the coding sequence) by digestion (Eco72I/ Hind III) and ligation. The RT-PCR was performed with 23-nucleotide-long primers that hybridized to the 3' and 5' ends of the leptin coding sequence (sense primer: 5'-ATGCATTGGGGAACCCTGTGCGG - 3', located at position +1 to +23; antisense primer: 5'- GAGGTCCAGCTGCCACAGCATG-3', located at position +465 to +487). Therefore, leptin mRNA yielded a PCR product of 487 bp while the amplification of the competitor gave a product of 407 bp. Working solutions of the leptin competitor DNA at defined concentrations (50–10-3 pmol/liter) were prepared by serial dilution in 10 mM Tris-HCl (pH 8.3)-1 mM EDTA buffer.

Immuno histochemical study

From the above mentioned subjects, the tissue samples were dehydrated in a graded series of ethanol, cleared in Xylene and embedded in paraffin. Serial sections (5 µm thick) were cut on a rotary microtone (Weswicox, Japan) and mounted on egg albumin coated slides. They were then stained with haematoxylin and eosin for histological classification [13].

The sections were deparaffinized by treating with Xylene for 10 min, rehydrated with. isopropanol for 30s and washed with distilled water. Unmarking of tissue antigens was done by treating the sections with sodium citrate buffer (pH 6, 10mM) at 95º C for 15 min. They were then washed with TBS (pH 7.6), incubated in PBS with 1% BSA for 30 min at 37º C for blocking the non-specific protein binding sites and washed with TBS. Later, the sections were incubated with primary antibody (1:500 dilution) rabbit anti-human leptin antibody for 1.5h at 37º C and washed twice with TBS. Immuno-fluorescent labeling was performed with fluorescein isothiocyanate anti rabbit IgG (1: 40 dilution) for 1h at 37º C. The sections were mounted with 90% glycerol and immediately observed under fluorescence microscope at 200X magnification (Nikon model, LABO PHOT –2, Tokyo, Japan). Photographs of all the tissue sections were taken under similar conditions and were given equal exposure time.

Anthropometric variable measurements

Anthropometric measurements, including height, weight, waist to hip ratio, abdominal skin fold thickness were measured. All these measurements were performed twice by certified technicians, and if repeated measurements differed by more than 0.7 cm, they were repeated again.

Determination of biochemical parameters

The circulating leptin concentration was measured using a Human Leptin IRMA Kit (Diagnostic Systems Laboratories, Webster, TX, USA). Radioimmunoassay was adopted in the assay. Serum levels of the major hormones such as testosterone, free testosterone, androstenedione, DHEAS, SHBG, triiodothyronine (T3), thyroxine (T4), thyroid stimulating hormone (TSH), free triiodothyronine (fT3), free thyroxine (fT4), LH, FSH, beta-estradiol and prolactin using kit from Diagnostic Systems Laboratories (Webster, TX, USA) while insulin was assayed using kit from Board of Radiation and Isotope Technology (BARC Vashi Complex, Turbhe, Navi Mumbai). Serum levels of glucose, triglycerides, cholesterol and HDL-cholesterol were estimated by the GOD-POD, GPO-ODESPT, CHOD-POD and direct methods respectively using an autoanalyzer (BAYER RA 50).

Statistical analysis

The data were presented using descriptive statistics (mean±SEM). Multiple linear regression analyses was used to examine the relationship between ob mRNA and serum leptin levels as well as BMI, body fat distribution, percentage of body fat and other biochemical, and endocrinal parameters. Analysis of trends was performed using non-parametric linear regression). Data comparison was carried out using either Student's t-test or one-way ANOVA as appropriate. At 95% confidence interval, 2-tailed p values less than 0.05 were considered significant.

Results and Discussion

Recent data report that serum leptin was correlated significantly with indicators of adiposity. There was a significant relationship between serum leptin and body mass index (BMI) ($r = 0.85$, $P < 0.01$), and between serum leptin and percentage of body fat ($r = 0.92$, $P < 0.01$). In previous studies, a significant relationship was observed between ob mRNA of ovary and adipose tissue (20) in PCOS and compared to normal weight subjects. However, no systematic study had been performed examining the age profile of leptin levels in women with respect to BMI (thin, normal weight vs overweight, obese, morbidly obese) and hormones known to change during the infertility cases. To address this issue, a study was performed in a large group of subjects covering a wide range of age and BMI. We confirmed that BMI is the major determinant

of leptin levels and provided novel evidence showing that hormonal imbalance may affect leptin secretion in non-obese and obese PCOS subjects. Through a cross sectional analysis, treating the data either as groups of ob mRNA levels as continuous variables, we demonstrated that in both PCOS and non- PCOS there was a rise in leptin levels throughout life that is independent from BMI and other hormonal variables. This is the first study demonstrating the influence of sex steroids on ob mRNA and serum leptin concentrations in South Indian Population. With our quantification, we established a BMI-dependent normal range for women. In this study, morbidly obese women have 3-fold higher leptin levels than thin and normal women. The BMI is a good indicator of obesity and total amount of adipose tissue. Therefore, the information on the percentage of body fat in the morbidly obese PCOS and non-PCOS subjects was determined in this study. The ob mRNA levels from our subjects were analyzed with regard to a number of factors. The patients were divided in to thin, overweight, obese and morbidly obese and the results were compared with normal weight control and found endometrium, ovary, adipose tissue ob mRNA and serum leptin levels were significantly higher (Figure 1, r = 0.84, P<0.01, and Figure 2, r = 0.78, P<0.01) in thin PCOS, overweight, obese and morbidly obese PCOS and non-PCOS, when compared to thin non-PCOS and normal weight control adipose tissue. However, endometrium, ovary and adipose ob mRNA levels increased consistently with increased adiposity (r = 0.72, P<0.01) in overweight, obese and morbidly obese PCOS and non-PCOS, when compared with normal weight controls, but in thin PCOS (r = 0.59, P<0.01) ob mRNA levels were expressed irrespective of the BMI. In previous studies, serum leptin levels were not significantly correlated between ob mRNA levels, which were measured in the same subjects [10]. However in our study, serum leptin levels were significantly correlated with endometrium, ovary and adipose tissue ob mRNA levels in thin PCOS, overweight, obese and morbidly obese PCOS and non-PCOS, when compared to thin non-PCOS and normal weight controls (r = 0.92, P<0.01) which were measured in same subjects. Results of ob mRNA from thin and degree of obesity PCOS endometrium, ovarian cortex, adipose tissue and non-PCOS adipose tissue were compared to normal weight control subjects adipose tissue samples using RTcPCR and classical PCR methods. Table 2 showed that absolute leptin mRNA levels were 9 times higher (P < 0.01) in thin and morbidly obese subjects, when compared to thin non-PCOS and normal weight controls. The levels of anthropometric variables and serum bio-chemical

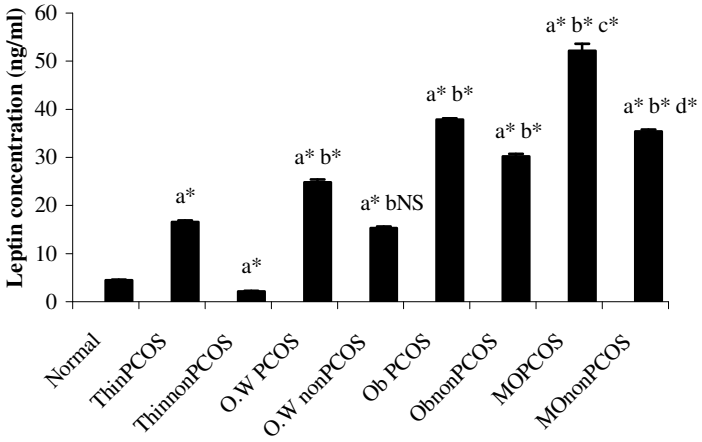

Figure 1: Relationship between serum leptin concentration and BMI

Each bar represents mean ± SEM (Three separate experiment values were calculated). a - as compared with normal weight controls; b - as compared with Thin PCOS; c - as compared with Obese PCOS; d - as compared with Obese non PCOS; Statistical significance are expressed as: * p < 0.01 and NS Non-significant

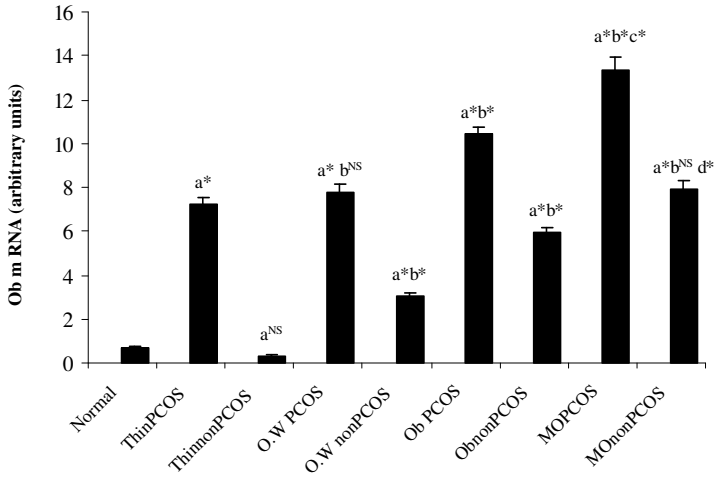

Figure 2: Relationship between ob mRNA expression and BMI
Each bar represents mean ± SEM (Three separate experiment values were calculated). a - as compared with normal weight controls; b - as compared with Thin PCOS; c - as compared with Obese PCOS; d - as compared with Obese non PCOS; Statistical significance are expressed as: * $p < 0.01$ and NS Non-significant

Table 2: Representation of leptin mRNA levels determined by RT-cPCR

Samples	Adipose (amol/µg)	Endometri-um (amol/µg)	Ovary (amol/µg)	BMI (Kg/M2)	Serum Leptin (ng/ml)
Normal weight	15.07±1.20	---	---	27.8±0.10	4.45±0.22
Thin PCOS	78.4±0.32	87.4±0.39	83.5±0.12	19.5±0.12	16.6±0.27 a*
Thin non-PCOS	8.6±0.16	---	---	20.0±0.14	2.1±0.15 aNS
Overweight PCOS	56.5±0.26	63.4±0.48	60.7±0.33	31.5±0.17	24.8±0.66 a* bNS
Overweight non-PCOS	22.3±0.32	---	---	31.8±0.16	15.3±0.33 a* b*
Obese PCOS	71.2±0.32	84.8±0.62	79.6±0.30	37.5±0.21	37.9±0.19 a* b*
Obese non-PCOS	50.5±0.10	---	---	37.6±0.23	32.2±0.47 a* b*
Morbidly obese PCOS	89.7±0.33	96.3±0.36	92.5±0.52	43.6±0.38	52.1±1.5 a* b*c*
Morbidly obese non-PCOS	72.7±0.59	---	---	44.1±0.29	35.4±0.37 a* bNSd*

*ObmRNA, serum leptin and BMI values were represents mean±SEM (Three separate experiment values were calculated); a - as compared with normal weight controls; b - as compared with thin PCOS; c - as compared with obese PCOS; d - as compared with obese non PCOS; * $p < 0.01$; NS - Non-significant*

analysis in thin and degree of obesity PCOS non-PCOS, when compared to thin non-PCOS and normal weight 13 controls are provided in Tables 3 and 4. The measured body fat distribution and bio-chemical analysis was 4 fold higher and 2 to 3 times elevated in obese and morbidly obese PCOS and non-PCOS, when compared to normal weight controls. In simple linear regression analysis, ob mRNA expression was significantly correlated to body fat distribution (abdominal skin-fold thickness ($r = 0.77$, $P < 0.01$), triceps skin-fold thickness ($r = 0.87$, $P < 0.01$), waist circumference ($r = 0.82$, $P < 0.01$), hip circumference ($r = 0.89$, $P < 0.01$), waist to hip ratio ($r = 0.83$, $P < 0.01$), fasting insulin ($r = 0.79$, $P < 0.01$), fasting glucose ($r = 0.44$, $P < 0.01$), fasting cholesterol ($r = 0.92$, $P < 0.01$), fasting triglycerides ($r = 0.94$, $P < 0.01$) and fasting HDL-

Cholesterol (r = 0.37, P <0.01). In Tables 4-6, serum levels of endocrinal parameters were two to three times higher in thin and degree of obesity PCOS, when compared to thin, degree of obesity non-PCOS and normal weight control. Serum levels of DHEAS, free T3, free T4, free testosterone, LH, prolactin, insulin, cholesterol, triglycerides and HDL-cholesterol (Table 7) were positively correlated with ob mRNA, serum leptin and BMI (< 43.0 Kg/m2) in PCOS, when compared to normal weight controls. The fasting serum levels of β-estradiol, FSH, T3, T4, TSH levels had no significant correlation between ob mRNA, serum leptin and BMI (< 43.0 kg/m^2), but the serum levels of SHBG and androstenedione were negatively correlated with ob mRNA, serum leptin and BMI (< 43.0 kg/m^2) in PCOS. The percentage of body fat was 92.8± 0.30 in morbidly obese PCOS, 84.8 ± 0.28 in morbidly obese non-PCOS, 76.2±0.34 in obese PCOS, 72.9±0.32 in obese non-PCOS, 68.4±0.20 in overweight PCOS, 65.8±0.18 in overweight non-PCOS, 22.0±0.12 in thin PCOS, 18.8±0.16 in thin non-PCOS and 44.24±0.24 in normal weight controls. Therefore, a strong positive correlation (r = 0.94, P <0.01) between the ob mRNA and the percentage of body fat was observed in obese, morbidly obese PCOS and non-PCOS subjects. We have previously demonstrated that serum leptin levels were two to three times elevated in thin, obese and morbidly obese PCOS, compared to normal weight controls (16). However, in this study we confirmed that the serum leptin and ob mRNA expression levels were 9 times higher in thin, obese, morbidly obese PCOS, compared to normal weight controls.

Strong positive signal for immunofluorescence was identified for leptin in the endometrial tissue sections. Leptin expression was observed in the luminal and glandular epithelial cells (Figure 4). Leptin immunoreactivity was also evident in the control endometrial sample (Figure 4). Adipose and placenta tissues were taken as positive control, which also immunofluorescence intensely in the adipose cytoplasmic rim and

Table 3: Distribution (mean and SEM) of serum leptin concentration and anthropometric variables in normal-weight and PCOS/non-PCOS of thin, overweight, obese and morbidly obese subjects

Subjects	Skin-fold thickness(mm)		Circumference(cm)		Waist-to-hip ratio
	Abdominal	Triceps	Waist	Hip	
Normal weight (n=10)	21.2±0.18	11.9±0.16	86.5±0.21	93.7±0.25	0.91±0.03
Thin PCOS (n=8)	2.4±0.60^{a*}	3.3±0.15 ^{a*}	55.8±1.0^{a*}	52.1±0.45^{a*}	0.12±0.01 ^{a*}
Thin nonPCOS (n=8)	7.1±0.72 ^{a*}	4.3±0.20 ^{a*}	57.9±1.2 $^{a* bNS}$	46.5± 1.1 ^{a*b*}	0.11±0.01 $^{a* bNS}$
Overweight PCOS (n=7)	24.0±0.19 $^{a* b*}$	14.6±0.12 $^{a* b*}$	87.7±2.9$^{a NS b*}$	106.1±0.45 $^{a* b*}$	1.7±0.13 $^{a* b*}$
Overweight non PCOS (n=7)	25.6±0.44 $^{a* b*}$	14.0±0.23 $^{a* b*}$	79.5±2.8 $^{a* b*}$	101.9±1.0 $^{a* b*}$	1.6±0.14 $^{a* b*}$
Obese PCOS (n=10)	29.0±0.36 $^{a* b*}$	22.1±0.29 $^{a* b*}$	123.2±0.10 ^{a*b*dNS}	126.8±0.17 $^{a* b*}$ dNS	4.0±0.25 $^{a* b*}$
Obese nonPCOS(n=10)	30.2±0.22 $^{a* b*}$	24.1±0.14 $^{a* b*}$	121.6±0.30 ^{a*b*c} NS	125.5±0.19 $^{a* b* c}$ NS	3.8±0.17 $^{a* b*}$
M.obese PCOS(n=10)	33.2±0.14 ^{a*b*c*}	27.0±0.15 $^{a* b*c*}$	133.6±0.18 $^{a* b*c*}$	139.0±0.27 ^{a*b*c*}	5.5±0.11 ^{a*b*c*}
M.Obese nonPCOS (n=10)	33.8±0.23 $^{a* d*}$	26.1±0.13 $^{a* d*}$	132.4±0.32 $^{a* d*}$	138.3±0.24 $^{a* d*}$	4.9±0.11 $^{a* d*}$

*ObmRNA, serum leptin and BMI values were represents mean±SEM (Three separate experiment values were calculated); a - as compared with normal weight controls; b - as compared with thin PCOS; c - as compared with obese PCOS; d - as compared with obese non PCOS; * p < 0.01; NS - Non-significant*

Table 4: Estimation (mean and SEM) of serum levels of bio-chemical analysis in normal-weight and PCOS/non-PCOS of thin, overweight, obese and morbid obese subjects

Subjects	mg/dL	µU/mL	mg/dL	mg/dL	mg/dL
	Fasting Glucose	Fasting Insulin	Fasting Triglycerides	Fasting Cholesterol	Fasting HDL-Cholesterol
Normal weight (n=10)	92.5±0.52	14.8±0.19	96.3±0.36	185.0±0.25	45.3±0.31
Thin PCOS (n=8)	72.7±0.59^{a*}	23.1±0.37 ^{a*}	173.1±0.71^{a*}	177.0±0.65^{aNS}	28.5±0.18 ^{a*}
Thin nonPCOS (n=8)	83.5±0.62 ^{a*}	15.0±0.20 ^{a*}	121.5±0.50 ^{a*}	178.8± 0.22 ^{aNS}	26.1±0.29 ^{a*}
Overweight PCOS (n=7)	56.2±0.40 ^{a*}	30.5±0.21 ^{a*b*}	220.8±0.50$^{a*d NS}$	234.8±0.34 $^{a* d NS}$	18.5±0.20 ^{a*}
Overweight non PCOS(n=7)	63.4±0.48 ^{a*}	22.3±0.31 ^{a*}	197.5±0.36 $^{a* d NS}$	220.7±0.28 $^{a* d NS}$	15.0±0.30 ^{a*}
Obese PCOS (n=10)	35.1±0.33 ^{a*}	39.1±0.25^{a*b*c*}	238.3±0.21 ^{a*}	264.6±0.22 $^{a* d NS}$	12.2±0.24 ^{a*b*c*}
Obese nonPCOS (n=10)	31.4±0.43 ^{a*b*}	31.5±0.21 ^{a*}	211.1±0.50 ^{a*}	254.9±0.34 $^{a* c NS}$	9.4±0.16 ^{a*}
M.obese PCOS(n=10)	44.3±0.53 ^{a*b*c*}	46.3±0.28 ^{a*b*c*}	270.0±0.42 ^{a*b*c*}	316.0±30.0 ^{a*b*c*}	8.6±0.16 ^{a*b*c*}
M.Obese nonPCOS (n=10)	55.4±0.66 ^{a*d*}	41.5±0.26 ^{a*d*}	259.9±0.23 ^{a*d*}	271.2±0.24 $^{a*c NS d NS}$	11.4±0.22 $^{a* b*d*}$

*ObmRNA, serum leptin and BMI values were represents mean±SEM (Three separate experiment values were calculated); a - as compared with normal weight controls; b - as compared with thin PCOS; c - as compared with obese PCOS; d - as compared with obese non PCOS; * p < 0.01; NS - Non-significant*

placental cytosyncyncytiotrophoblast layer at the surface of the chorionic villi (Figure 3). Leptin is known to play a physiological role in early development during the reproductive phase in the fetal stage. Leptin is known to be expressed in the human endometrium throughout the menstrual cycle whereas Ob-Rb is found to be expressed during the mid secretory phase [13]. These findings suggested that endometrial Ob-Rb and the soluble forms of Ob-Rb are present in the endometrial epithelium, and are available at the time of endometrial receptivity, displaying a premenstrual increase. Figure 4 shows the immuno-histochemical staining for endometrium of PCOS. This has revealed higher level of leptin expression in the endometrial epithelium compared to the control. The endometrial samples were obtained during the proliferative phase of the menstrual cycle in PCOS.15 Immuno-histochemical detection of leptin in human ovarian cortex and corpus luteum (CL) Intact antral follicles were absent in polycystic ovaries [18, 19]. Follicular cysts developed with and without hypertrophy of the thecal layer. In addition, we found cysts with a luteinized thecal and granulosa layer. These follicular cysts with utilization appeared to be the largest in diameter and their granulosa cells were distinctly larger in size than the thecal cells. Most follicular cysts displayed a strong leptin expression (Figure 5). It was prominently expressed in the thecal layer (Figure 5). Follicular cysts with luteinization displayed a conspicuous leptin immunoresponse both in the granulosa and thecal layers. In corpus luteum, the structure, amount and distribution of leptin-positive cells depended on the stage of the cycle. In the subsequent stage of secretion, the number of leptin-positive cells was significantly higher in septa (S) containing the larger blood vessels (Figure 6). In the patients suffering from polycystic ovary syndrome of the CL, there is an imbalance between the hormone levels with a

Table 5: Fasting serum levels of hormonal profiles in normal weight controls and PCOS/non-PCOS of thin, overweight, obese and morbid obesity subjects (mg/dL)

Subjects	DHEAS	Testosterone	Free testosterone	β - estradiol	LH
Normal weight (n=10)	5.7±0.19	1.53±0.25	2.5±0.20	107.3±0.29	12.1±0.12
Thin PCOS (n=8)	15.9±0.33 a*	10.5±0.10 a*	11.5±0.21 a*	90.5±0.33 a*c NS	66.9±0.45 a*
Thin nonPCOS (n=8)	3.8±0.62 a*	1.25±0.20 a*	1.85±0.12 a*	105.5± 0.11 a*	10.0±0.20 a*
Overweight PCOS (n=7)	9.4±0.15 a*	3.85±0.23 a*b*	4.5±0.25 a* b*	95.4±0.10 a* b*	36.4±0.36 a* b*
Overweight non PCOS (n=7)	6.8±0.20 a* b*	2.25±0.20 a* b*	3.0±0.10 a* b*	103.5±0.3 a* b*	16.4±0.16 a* b*
Obese PCOS (n=10)	14.0±0.29 a* b*	9.46±0.28 a*b*	8.5±0.23 a* b*	90.5±0.16 a* b NS	56.5±0.26 a*b*
Obese nonPCOS(n=10)	8.4±0.22 a*	3.14±0.11 a* b*	4.0±0.11 a* b*	101.5±0.21 a* b*	23.2±0.41 a* b*
M.obese PCOS(n=10)	19.5±0.17 a*b*c*	12.8±0.12 a*b*c*	13.4±0.23 a*b*c*	85.6±0.22 a*b*c*	71.2±0.32 a*b*c*
M.Obese nonPCOS(n=10)	10.5±0.25 a*d*	6.5±0.11 a*d*	8.6±0.23 a* c NS	99.5±0.16 a*d*	35.7±0.44 a* d*
	Triiodo *THYRONINE*	Thyroxin	Thyroid-stimulating Hormone	Free – T3	Free – T4
Normal weight (n=10)	151.7±0.29	7.6±0.11	3.19±0.21	398.6±0.32	1.64±0.30
Thin PCOS (n=8)	42.2±0.25 a*	3.4±0.25 a*	8.74±0.40 a*	423.8±0.29 a*	1.95±0.33 a*
Thin nonPCOS (n=8)	131.7±0.36 a*	5.7±0.25 a*	5.25±0.25 a*	390.7± 0.31 a*	0.76±0.03 a*
Overweight PCOS (n=7)	98.0±0.30 a* b*	3.8±0.22 a*b*	9.25±0.36 a* b*	414.0±0.43 a* b*	0.97±0.03 a* b*
Overweight non PCOS (n=7)	121.4±0.36 a* b*	6.6±0.13 a* b*	4.26±0.22 a* b*	408.2±0.28 a*	1.34±0.30 a* b*
Obese PCOS (n=10)	63.0±0.68 a* b*	2.5±0.10 a*b*	11.42±0.14 a* b*	415.8±0.44 a* b*	0.71±0.03 a*b*
Obese nonPCOS(n=10)	102.3±0.47 a*	4.8±0.20 a* b*	5.45±0.22 a* b*	402.5±0.61 a*	1.07±0.10 a* b*
M.obese PCOS(n=10)	40.5±0.95 a*b*c*	1.9±0.22 a*b*c*	14.5±0.20 a*b*c*	415.5±0.71 a*b*c NS	0.32±0.02 a*b*c*
M.Obese nonPCOS(n=10)	91.5±0.34 a*d*	3.6±0.22 a*d*	6.84±0.32 a* d*	404.5±0.16 a*d*	1.26±0.28 a* d*

*ObmRNA, serum leptin and BMI values were represents mean±SEM (Three separate experiment values were calculated); a - as compared with normal weight controls; b - as compared with thin PCOS; c - as compared with obese PCOS; d - as compared with obese non PCOS; * p < 0.01; NS - Non-significant*

constant decrease in the estrogen level. The increased expression of leptin in the CL of PCOS might be to the decrease in level of estrogen thus suggesting that leptin expression might be tissue specific and can act in autocrine and endocrinal level.

Leptin levels varied widely between subjects, and large differences were observed even among normal weight control and overweight, obese and morbidly obese PCOS subjects with a similar percentage of body fat. Therefore, fat stores are unlikely to be the only factor that regulates ob mRNA expression and leptin concentration in serum. Even though the correlation between percentage of body fat and leptin level was found to be existing ($r = 0.94$, $P < 0.01$), there was a considerable overlap between obese and morbidly obese PCOS and non-PCOS, as seen in comparison with normal weight controls.

To investigate the regulation of ob gene expression in tissues such as endometrium, ovary and adipose tissue samples in PCOS, we have used RT-competitive PCR based assay [11] which allows to determine the absolute concentration of leptin mRNA. It is well demonstrated that standard RT-PCR should not

Table 6: Fasting serum levels of hormonal profiles in normal weight controls and PCOS/non-PCOS of thin, overweight, obese and morbid obesity subjects

Subjects	FSH	Prolactin	SHBG	Androstenedione
Normal weight (10)	5.27±0.25	16.4±0.12	63.5±0.39	1.63±0.33
Thin PCOS (8)	1.26±0.20^{a*}	29.3±0.19 ^{a*}	14.6±0.22^{a*}	0.92±0.03 ^{a*}
Thin nonPCOS (8)	4.14±0.22 ^{a*}	14.27±0.12 ^{a*}	55.9±0.29 ^{a*}	1.24± 0.20 $^{a*d\ NS}$
Overweight PCOS (7)	2.54±0.17 $^{a*\ b*}$	20.64±0.17 ^{a*b*}	47.8±0.40$^{a*\ b*}$	0.95±0.03 $^{a*\ b*}$
Overweight non PCOS (7)	3.80±0.32 $^{a*\ b*}$	17.3±0.13 $^{a*\ b*}$	58.9±0.20 $^{a*\ b*}$	1.44±0.22 $^{a*\ b*}$
Obese PCOS (10)	1.96±0.13 $^{a*\ b*}$	24.7±0.21 ^{a*b*}	34.8±0.17 $^{a*\ b*}$	0.75±0.03 $^{a*\ b\ NS}$
Obese nonPCOS(10)	3.25±0.30 ^{a*b*}	19.11±0.13 $^{a*\ b*}$	50.5±0.10 $^{a*\ b*}$	1.25±0.2 $^{a*\ b*}$
M.obese PCOS(10)	1.56±0.12 ^{a*b*c*}	32.5±0.16 ^{a*b*c*}	19.4±0.16 ^{a*b*c*}	0.45±0.02 ^{a*b*c*}
M.Obese nonPCOS(10)	2.49±0.11 ^{a*d*}	21.5±0.16 ^{a*d*}	38.2±0.19 $^{a*\ d*}$	0.86±0.02 ^{a*d*}

*ObmRNA, serum leptin and BMI values were represents mean±SEM (Three separate experiment values were calculated); a - as compared with normal weight controls; b - as compared with thin PCOS; c - as compared with obese PCOS; d - as compared with obese non PCOS; * p < 0.01; NS - Non-significant*

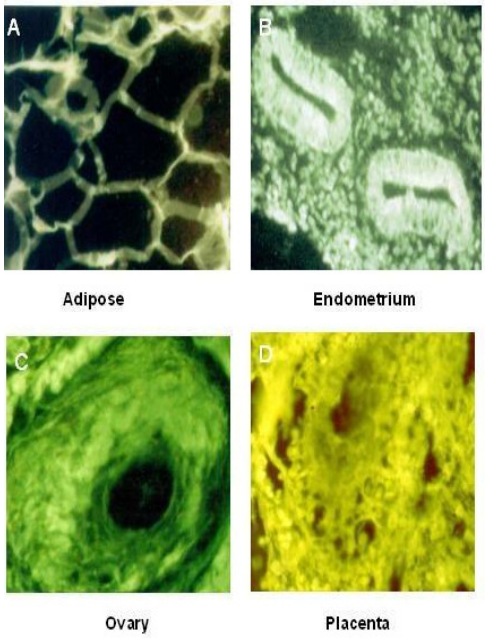

Figure 3: Immunofluorescent detection of leptin in 5 µm section of human adipose endometrium, ovary and placenta

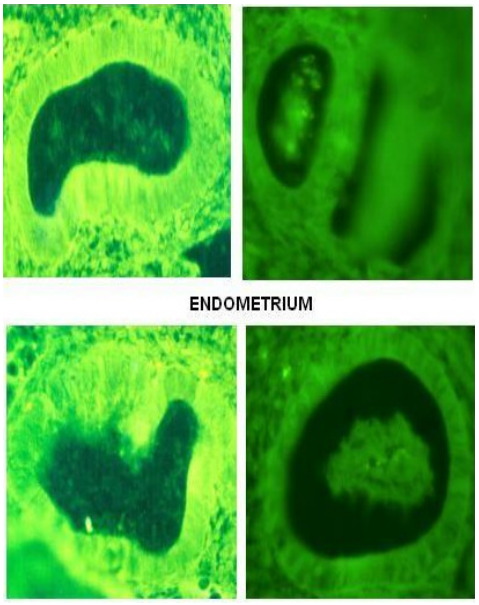

Figure 4: Immunofluorescent detection of leptin in 5 mm section of human PCOS endometrium

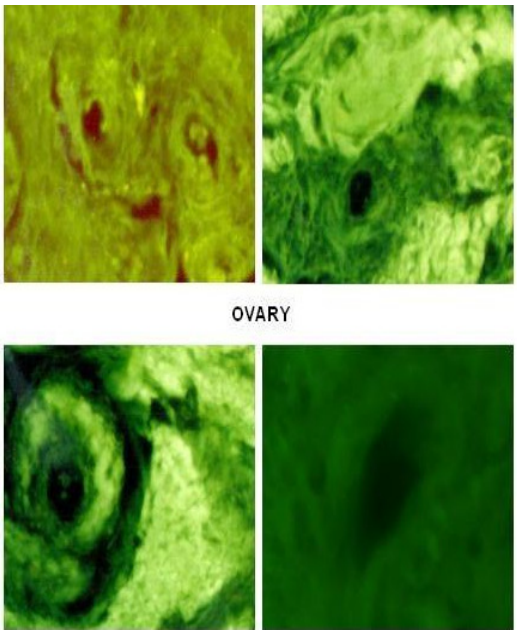

Figure 5: Immunofluorescent detection of leptin in 5 mm section of human PCOS ovary

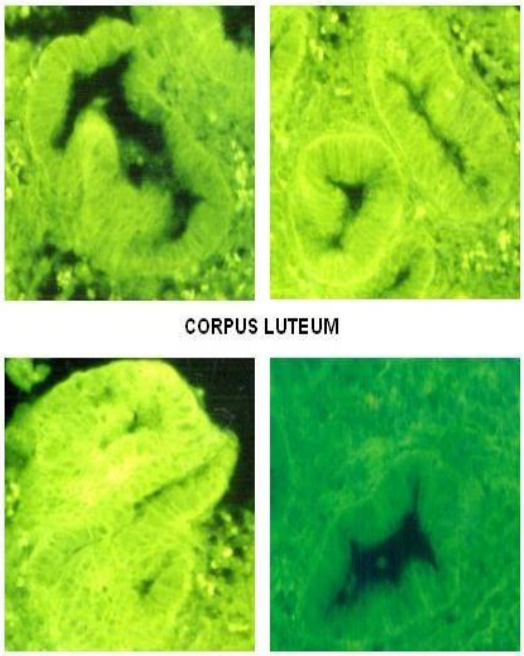

Figure 6: Immunofluorescent detection of leptin in 5 mm section of human corpus luteum

be used as a quantitative assay to measure a given mRNA, mainly because of the uncontrolled efficiency of the PCR. The addition of a competitor DNA molecule which is amplified in the same test tube and with the same primers as the target mRNA overcomes this problem allowing real quantitation [11, 15]. In view of the low yields in total RNA tissue samples and in view of the low levels of leptin mRNA in normal weight subjects, this RT competitive PCR assay, allowing measurement of minute amounts of mRNA, is a powerful method to study variations in leptin mRNA and thus to investigate the regulation of ob gene expression in PCOS. Interestingly, the leptin mRNA levels of obesity with PCOS also correlated positively with BMI, suggested that the expression of ob gene is mainly related to the fat mass rather than to the metabolic and hormonal changes associated with PCOS. But in thin PCOS, ob mRNA and serum leptin levels were highly expressed irrespective of BMI, and this is indicative of the influence of ovarian hormonal profile, which is characteristic of PCOS subjects that might determine the ob mRNA levels. We found that ob mRNA and serum leptin concentration was strongly correlated with overall adiposity as determined by BMI in both normal weight control women and overweight, obese, morbidly obese PCOS and non-PCOS subjects. In addition, leptin concentration was significantly correlated with fat distribution and independent of overall obesity. Specifically, central obesity, as measured by waist to hip ratio and abdominal skin-fold thickness, significantly correlated with leptin levels in both control and overweight, obese and morbidly obese PCOS women [16]. With regard to BMI, there was an increase in leptin levels in overweight, obese and morbid obese PCOS and non- PCOS. However, the observed increase in leptin in thin PCOS is in sharp contrast to the value of normal weight control women, suggesting that leptin could be a detrimental factor in PCOS subjects. From earlier reports [14] it was found that statistically correcting for the body 18 fat distribution eliminated the relation of age (18–45 yr) with ob mRNA and serum leptin concentration, though not available explicitly for control and obese PCOS women's. Leptin levels and anthropometric variables in control and obese PCOS subjects were two to three times higher in

obese PCOS women than in control women. In contrast, there was an independent relation between leptin and fat distribution, as measured by the waist/hip ratio. Though the waist/hip ratio was directly correlated with ob mRNA, the association was statistically significant for body fat. A strong positive correlation was observed between ob mRNA and serum insulin levels in thin, overweight, obese, morbidly obese PCOS and normal weight control women. As expected, fasting serum insulin levels were higher in PCOS than in normal weight control subjects. This is also obviously evident even if BMI is used as a covariate. These results suggest that insulin resistance is not solely dependent on obesity in these patients, but that also contributes to insulin resistance, as reported earlier [16]. However, as ob mRNA levels are not similar in PCOS and in control subjects, the concept that ob mRNA levels would be elevated in insulin resistant states, such as PCOS independent of obesity, has thus been substantiated by the present analysis. s were elevated irrespective of the BMI.

The positive ob mRNA signal observed in the cortex is likely to have been caused by oocytes in primordial and dominant follicles, which was presented in the RTcPCR samples. From this study, the conspicuous occurrence of leptin in the wall of polycystic ovary follicles suggests that the local production of leptin is increased 19 independently of the serum levels, which were probably high in thin and morbidly obese PCOS. The SHBG, androstenedione, LH, β-estrodial, T3 and T4 dysregulation in the ovarian cortex of thin PCOS subscribe to the high local leptin expression. These cause abnormalities in the FSH control. Furthermore, a local expression of ob mRNA production may interfere with the intra ovarian factor, which is dominant follicle. Leptin can bind to its receptor and activate certain protein, which are important for folliculogenesis, such as follistatin a well-known growth factor. The impaired maturation of follicles as seen in PCOS can lead to defective leptin receptor and this can be attributed to the increased levels of leptin resistance [13,14,17]. The suppression of estrogen production is a universal finding in PCOS, it was of interest to determine whether the ovarian endometrium leptin directly antagonize estrogen receptor that could inhibit the estrogen production and lead to LH secretion. The high ob mRNA expression levels play a role in causing the hirsutism that occurs in many obese and thin PCOS women. There is markedly higher expression of ob mRNA in thin-PCOS, indicating that the endometrium is an important source of leptin. The high levels in thin-PCOS are more sufficient to maximally inhibit estrogen production. In contrast, the circulation levels in non-PCOS from normally cycling women do not significantly decrease estrogen production. Taken together, these data strongly suggest that abnormal accumulation of ob mRNA in endometrium of thin-PCOS could play an important role in inhibiting estrogen production in thin-PCOS. In addition, the primary mechanism of leptin could bind to the leptin receptor in vascular endothelial cells and lead to formation of blood vessels or angiogenesis. In the case of thin and obese related PCOS, leptin could not bind to the leptin receptor and it does directly antagonize estrogen receptor, and this might lead to LH secretion and thus the anovulation occurs in the PCOS. This is the first report on the occurrence of transcripts for ob mRNA in various tissue samples as, found in ovarian cortex, endometrium and adipose tissue, of varying intensities.

Conclusion

We conclude that in PCOS, due to the abnormal secretion of LH, ob mRNA expression is associated in the ovarian dysfunction, leading to leptin resistance. It is likely that ob mRNA is involved in steroidogenesis in the intact ovary. The ob mRNA in endometrium, ovarian cortex may indicate a leptin-dependent autocrine and paracrine role, in addition to its endocrinal role in PCOS, and that leptin could be one of the major risk factor for female infertility.

Contribution of Authors

I declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to he content of this article will be borne by the authors. Sample collection and determination of biochemical and molecular

parameters were done by Dr Mani Ravi Shankar Ram. Analysis and interpretation of data and compilation of results were carried out by Dr R Malathi. Sectioning and histochemical analysis was done by Dr P Shanthi

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