Food restriction may not reduce leptin gene expression in obesity condition – A study with reference to an animal model of obesity, WNIN/Ob

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A B S T R A C T

Objective: Leptin an adipokine, deregulated in many cases of obesity is poorly understood under nutrition interventional strategies like food restriction. The present study addresses the above with the help of a rodent animal model of obesity, WNIN/Ob

Methods: Male rats of WNIN/Ob aged 35 days were allotted to three groups (n=4): (1) Rats with lean phenotype and fed *ad libitum*, (2) Rats with obesity phenotype and fed *ad libitum*, and (3) Rats with obesity phenotype and subjected to 60% food restriction. Rats were fed up to 90 days age, after which they were assessed for body weights, fat percentages and leptin.

Results: The higher body weights and fat percentages, in rats with obesity phenotype were reversed by food restriction to that of lean phenotype. Circulating leptin levels though showed a significant reduction (1.7 fold), they were significantly high (43 fold) compared to lean phenotype. The relatively high leptin gene expression in food restricted versus *ad libitum* fed ones (11 fold versus 4 fold with respect to lean) explains the higher circulating leptin levels.

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Conclusions: Results suggest that mere food restriction may not reduce leptin gene expression and hence may not be a safer practice to reduce obesity.

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INTRODUCTION

Obesity or excess body fat was shown to be associated with metabolic syndrome (Metabolic syndrome is a complex of disorders namely dyslipidemia, hypertension, altered hormone signalling, increased pro-inflammatory mediators) and major metabolic diseases like diabetes mellitus, arthritis, cardiovascular disorders, pre-mature ageing, and cancers (1-5). Increase in adipose depots in obesity has been positively correlated with circulating levels of the adipocyte derived satiety signalling hormone 'Leptin', resulting in a hyper-leptinemic condition (6-8). The physiological levels of leptin seems to be in-effective with a consequent development of leptin resistance in many human subjects with obesity (9-12).

Food restriction is a common nutrition interventional strategy to reduce obesity and has been reported to ameliorate various facets of the metabolic syndrome like, dyslipidemia, hypertension, oxidative stress, systemic inflammation, cardiovascular disease risk factors, decrease the risk of age associated diseases, increase longevity and even improve cognitive decline (13-17). Being known to bring the many beneficial effects, its role in leptin gene expression was poorly understood. While few studies have reported decreased circulating leptin levels upon food

restriction (18-20), this reduction was not adequately assessed, particularly with respect to normo-leptinemic / healthy condition. A recent review further highlights many unanswered questions on the regulation of leptin gene expression (21). The present study was aimed to address the role of food restriction on leptin gene expression with the help of a rodent animal model (WNIN/Ob) showing genetic inheritance of obesity.

WNIN/Ob shows Mendelian inheritance of obesity trait with three phenotypes: -/- (rats showing obesity phenotype), +/- (heterozygote / carrier rats with incomplete dominant phenotype), and +/+ (rats with lean or normal phenotype) distinguishable at 35 days age based on body weights and morphological markers. The rats with obesity phenotype of WNIN/Ob are hyper-phagic, hyper-lipaemic, hyper-insulinemic, hyper-leptinemic, and show high frequency of age onset degenerative disorders like cataract and retinal degeneration, impaired immunity, tumors, polycystic ovaries, and early ageing (22-27). WNIN/Ob is a novel rat strain and shows unique features among the known rodent strains with obesity (28-30). Being a valuable resource mimicking human metabolic syndrome, the effect of food restriction on leptin gene expression was studied in this animal model.

MATERIALS AND METHODS

Animals, selection, housing and duration of experiment: Thirty Five days old male rats of lean phenotype (with body weights ranging from 90-120 grams) and rats with obesity phenotype (with body weight ranging from 150-200 grams) of WNIN/Ob rat strain were divided into 3 groups: (1) Rats of lean phenotype and fed *ad libitum*, (2) Rats with obesity phenotype and fed *ad libitum*, (3) Rats with obesity phenotype and subjected to 60%food restriction. The number of animals each group received was 4 (n=4). Rats were housed in clean polypropylene cages with sterilized paddy husk as bedding material. Room temperature was maintained at 22±2°C with 14-16 air changes per hour and 55±5% relative humidity. Rats were maintained with 12 hour light-dark cycles and had free access to water. Rats were fed with standard rat chow (AIN 93 grade) till they have attained 90 days of age after which they were they were assessed for body weights, fat percentages and leptin. Rats were sacrificed following institutional animal ethical guidelines and tissue samples were collected.

Body Weights: Body weights of rats were measured using standard electronic balance with precision of 0.1 gram (Make: Sartorius, Germany).

Estimation of Body fat percentage: Total body fat percentage of animals was assessed using TOBEC (Total Body Electrical Conductivity), a small animal body composition analysis system (Make: EM-SCAN/TOBEC, Model SA-3000 Multi detector, Springfield, III, USA).

Estimation of circulating leptin: Blood samples were collected from 90 days old rats after 12 hours overnight fasting. Plasma was separated from the blood and used for estimation of leptin using the kit, Milliplex Map Rat cytokine / Chemokine Magnetic Bead Panel (Company: EMD Milllipore, MA, USA; Catalog Number: RECYTMAG-65K).

RNA isolation & Gene expression: Total RNA was isolated from Adipose tissue using Trizol (Invitrogen) according to manufacturer's instructions. RNA was converted to cDNA and leptin gene expression was assessed by Real time PCR. Normalization of leptin gene expression was performed against 18S gene expression. Reagents used for Real time PCR were obtained from Takara Bio (PrimeScript™ RT Reagent Kit, cat#RR037A) and primers from Sigma-Aldrich Co. LLC. Primer sequences for leptin are, For 5' ACTTCATTCCCGGGCTTC 3', and Rev: 5' GGTCTCGCAGGTTCTCCA 3'. Primer sequences used for 18s are, For: 5' GCTTAATTTGACTCAACACGGGA 3', and Rev: 5'. AGCTATCAATCTGTCAATCCTGTC 3'. Leptin gene expression between groups was described as Δcq values and fold differences between groups was calculated by $2^{-\Delta\Delta cq}$ method.

Statistical methods

Tests for Normality and Homogeniety of variances: Data was assessed for Normal distribution by Shapiro-Wilk test, skewness and kurtosis. Levene's Test of Homogeniety of variances was performed to determine equality of variances between groups.

Statistical measures and hypothesis testing: Statistical 'Range' as measure of dispersion of the observed data and 'Mean±Standard deviation', as measure of central tendency, and variation was reported for individual groups. Differences between means were assessed for statistical significance by one way ANOVA followed by post hoc comparisons by LSD (Least Significant Difference). Upon violation of the assumption of equality of variances for one way ANOVA, Welch F test was conducted followed by Games-Howel post hoc comparison. One way ANCOVA was further performed to determine differences between groups controlling for a co-variate.

Alpha level was set to 0.05 for all the hypothesis testings. SPSS statistical software (SPSS for Windows, Version 16.0., Chicago, SPSS Inc) was used for the analysis.

RESULTS

Sample characteristics: Normality tests performed on all the data sets suggested that the data are approximately normally distributed (Table 1).

Table 1: Normality test statistics

Dependent variable	Indenpendent variable	Shapiro-Wilk test		Skewness			Kurtosis		
		Statistic	P-value	Statistic	SE	Z-value	Statistic	SE	Z-value
Body Weight(gms)	Lean_Ad	0.824	0.153	1.677	1.014	1.65	3.148	2.619	1.20
	Ob_Ad	0.961	0.785	0.314	1.014	0.31	1.487	2.619	0.57
	Ob_Fr	0.985	0.931	0.367	1.014	0.36	-1.040	2.619	-0.39
Fat percentage	Lean_Ad	0.962	0.790	0.717	1.014	0.71	1.50	2.619	0.57
	Ob_Ad	0.920	0.538	-1.138	1.014	-1.12	0.758	2.619	0.28
	Ob _Fr	0.964	0.804	0.391	1.014	0.38	2.021	2.619	0.77
Circulating leptin levels	Lean_Ad	0.948	0.706	-0.063	1.014	-0.06	1.500	2.619	0.57
	Ob_Ad	0.922	0.546	1.103	1.014	1.08	2.087	2.619	0.79
	Ob _Fr	0.947	0.700	-0.902	1.014	-0.88	0.023	2.619	0.01
Leptin gene expression in Δcq	Lean_Ad	0.991	0.962	0.425	1.014	0.41	-0.076	2.619	-0.02
	Ob_Ad	0.950	0.713	0.768	1.014	0.76	-0.725	2.619	-0.28
	Ob _Fr	0.927	0.580	0.515	1.014	0.51	-2.519	2.619	-0.96

Normal distribution of data as assessed by Shapiro-Wilk test ($p<0.05$), and Skewness & Kurtotic Z values (-1.96 to +1.96).

Sixty percent food restriction from 35 days age reverses body weights and fat percentages in rats with obesity phenotype to that of lean phenotype.

The body weights ranged from 520-624 grams in rats with obesity phenotype fed *ad libitum*, 275-327 grams in rats of lean phenotype fed *ad libitum* and 183-213 grams in rats with obesity phenotype under food restriction. (Figure 1a). Variances of the groups being equal ($F(2, 9) = 0.897$, $P=0.441$) they were tested for one way ANOVA, which suggested statistically significant differences between groups ($F(2, 9) = 179.088$, $p < 0.001$). LSD post hoc comparisons revealed that the body weights of rats with obesity phenotype fed *ad libitum* (569.75±42.5 gms) were

statistically significantly high compared to the body weights of rats with lean phenotype fed *ad libitum* (293.5±22.9 gms, p<0.001) and rats with obesity phenotype under food restriction (197.0±12.9 gms, p<0.001). The body weights of rats with obesity phenotype under food restriction were also statistically significantly lower compared to rats with lean phenotype fed *ad libitum* (p=0.001).

The total body fat percentages ranged from 54.9-55.6 in rats with obesity phenotype fed *ad libitum*, 13.9-16.8 in rats with lean phenotype fed *ad libitum* and 12.8-17.6 in rats with obesity phenotype under food restriction (Figure 1b). Assumption of homogeneity of variances being violated (F (2, 9) = 4.993, P=0.035), Welch F test was performed which suggested statistically significant differences between groups (F (2, 4.324) = 2342, p<0.001). Games-Howell post hoc comparisons revealed that the body fat percentages in rats with obesity phenotype fed *ad libitum* (55.33±0.3) were statistically significantly high compared to the rats with lean phenotype fed *ad libitum* (15.20±1.2, p<0.001) and rats with obesity phenotype under food restriction (15.0±2.1, p<0.001). The body fat percentages in rats with obesity phenotype under food restriction and in rats of lean phenotype were comparable (p=0.985).

Figure 1

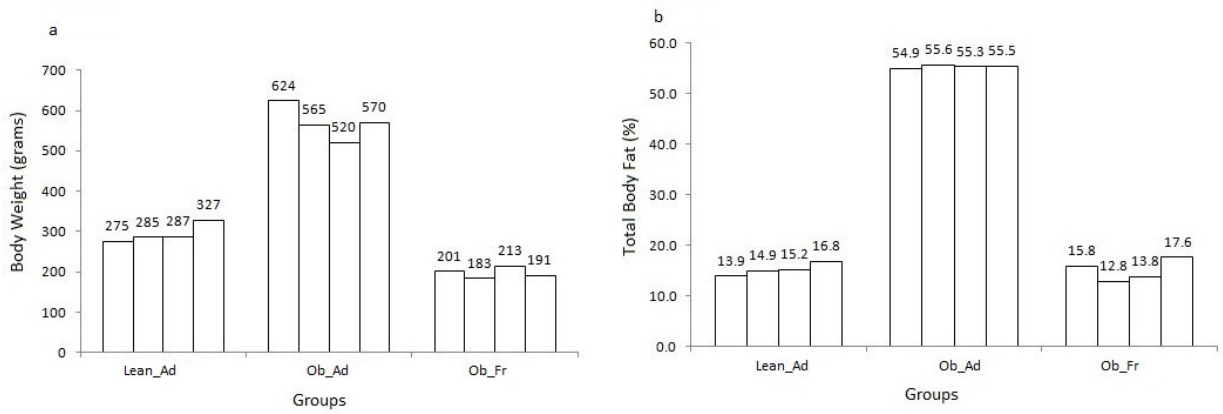

Figure 1. Comparison of (a) Body weights (gms) and (b) fat percentages among the three groups, (1) Rats with lean phenotype and fed *ad libitum* (Lean_Ad), (2) Rats with obesity phenotype and fed *ad libitum* (Ob_Ad), and (3) Rats with obesity phenotype and subjected to 60% food restriction (Ob_Fr).

Impaired leptin gene expression is uncorrected by food restriction in rats with obesity phenotype.

Circulating leptin levels (pg/ml) ranged from 30,381-61,072 in rats with obesity phenotype fed *ad libitum*, 463-695 in rats with lean phenotype fed *ad libitum*, and 19,149-29,794 in rats with obesity phenotype under food restriction. (Figure 2(a)). Variances of the groups being equal ($F(2, 9) = 3.678$, $p=0.068$) they were tested for one way ANOVA, which suggested statistically significant differences between groups ($F(2, 9) = 29.269$, $p<0.001$). LSD post hoc comparisons revealed that circulating leptin levels in rats with obese phenotype fed *ad libitum* (43,200±12,889) were statistically significantly high compared to rats with lean phenotype fed *ad libitum* (580.00±94.7, $p<0.001$) with a fold difference of 74(43,200/580). Food restriction though brought a statistically significant reduction in circulating leptin levels (25,400±4,663.6, $p=0.011$), with a fold difference of 1.7 (43,200/25,400) they were statistically significantly high compared to the rats of lean phenotype fed *ad libitum* ($p=0.002$) with a fold difference of 43.7 (25,400/580).

Fat percentages being comparable between rats of lean phenotype fed *ad libitum* and rats with obesity phenotype under food restriction and the assumption of homogeneity of regression being satisfied (($F(1,4) = 2.15$, $p=0.217$) , one way analysis of co-variance (ANCOVA) controlling fat percentage was performed between the two groups for circulating leptin. ANCOVA results revealed statistically significantly higher leptin levels in rats with obesity phenotype under food restriction ($F(1, 5) =197.2$, $p=0.000$; $\eta^2=0.97$). The adjusted mean values of circulating leptin are 443.5 and 25,500 respectively in rats of lean phenotype fed *ad libitum* and rats with obesity phenotype under food restriction.

The Δcq values for leptin gene expression (Δcq values are inversely proportional to gene expression) ranged from 6.39-7.91 in rats with lean phenotype fed *ad libitum*, 4.72-5.67 in rats with obesity phenotype fed *ad libitum*, and 2.05-5.53 in rats with obesity phenotype under food restriction (Figure 2b). The assumption of homogeneity of variances being violated ($F(2, 9) = 6.788$, $p=0.016$), Welch F test was performed which suggested statistically significant differences between groups ($F(2, 5.226) = 14.763$, $p=0.007$). Games-Howell post hoc comparisons revealed that the Δcq values in rats with lean phenotype (7.09±0.6) were statistically significantly high compared to the rats with obese phenotype fed *ad libitum* (5.12±0.4, $p=0.008$) as well as of rats with obese phenotype under food restriction (3.5±1.6, $p=0.033$). The Δcq values in rats with obese

phenotype fed *ad libitum* versus food restricted were comparable (p=0.273). The fold differences of leptin gene expression in rats of obesity phenotype, fed *ad libitum* and rats of obesity phenotype under food restriction with respect to rats of lean phenotype were, 3.91 and 11.79.

Figure 2

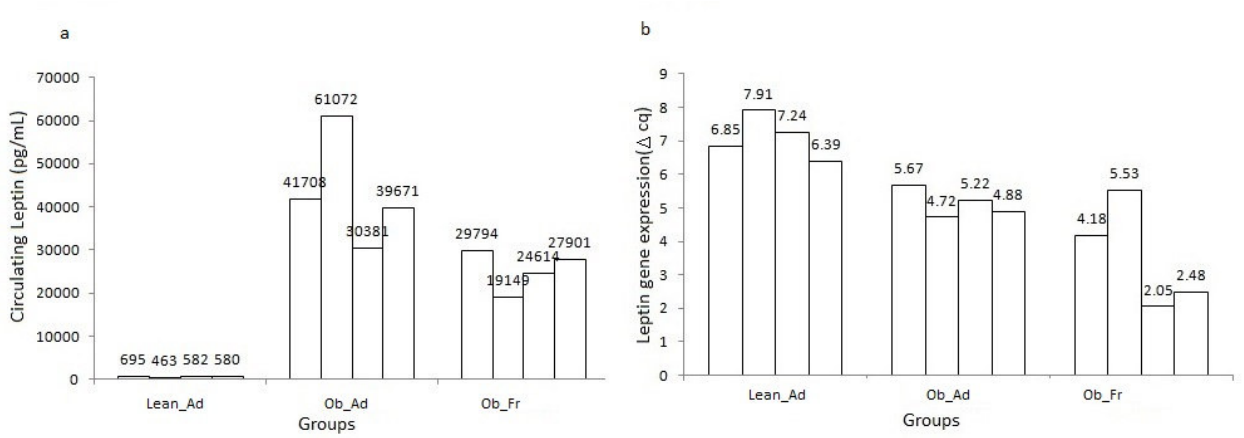

Figure 2. Comparison of (a) circulating Leptin levels and (b) Leptin gene expression (Δcq values) among the three groups, (1) Rats with lean phenotype and fed *ad libitum* (Lean_Ad), (2) Rats with obesity phenotype and fed *ad libitum* (Ob_Ad), and (3) Rats with obesity phenotype and subjected to 60% food restriction(Ob_Fr).

DISCUSSION

The present study shows that food restriction believed to be beneficial to health in a condition like obesity, may in fact add a new facet to the metabolic syndrome. The reduction of leptin levels by food restriction in WNIN/Ob rats appears to be meagre when these were compared with a normo-leptinemic condition. An important aspect the present work highlights is the fact that the body fat percentages of rats of lean phenotype fed *ad libitum* and the rats of obesity phenotype subjected to food restriction despite being similar, their circulating leptin levels were not the same indicating that the circulating leptin levels are not indicative of fat percentages in many cases of metabolic syndrome. The heightened expression of leptin gene in the adipose tissue of rats with obesity phenotype subjected to food restriction suggests for amplification of the molecular signal, increasing the expression of leptin gene. Recent studies have reported weight regain and relapse towards obesity after discontinuing food restriction(31), and hypotheses like; 'activation of anti-starvation mechanisms' which promotes overeating and obesity (32), 'feedback signals from the

depletion of fat mass' modulating energy intake and adaptive thermogenesis and hence increase the risks for fatness (33, 34), 'Signals from adipose tissue after energy-restricted weight loss', a biological drive to regain weight (359), *et cetera* have been suggested by various researchers. Results from the present study and emerging hypotheses on 'food restriction modulated molecular signals' suggest that the excess accumulation of fat in obesity can be a consequence of 'The syndrome X' in many cases and should be addressed rather diligently than mere food restriction, which can be a nutritional stress and may trigger undesirable responses.

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