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RESEARCH ARTICLE

Evaluation of Serum Leptin and Placental Leptin Receptors using (LEP) Marker in Abortion at Second trimester

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Abstract

Objectives: to investigate the relation between serum leptin level and placental leptin receptor in second trimester abortion women.
Methods: A case control study was carried out from November 2011 to April 2012. Sixty pregnant women with a comparable age at second trimester between 14–24 wks of gestation were collected. Blood and placenta tissue samples were taken from them at the time of their admission to hospitals in Baghdad. Control for the leptin receptor was taken from women with premature delivery (28-34 wk). Body mass index was calculated for each woman.
Results: serum leptin concentration were significantly lower in abortion at second trimester compared with leptin of healthy pregnant control (3.5±0.8 pg/ml vs. 24.4±0.7 pg/ml, p=0.0001) and leptin/BMI ratio vs. control (0.1±0.02 vs. 0.9±0.01 ml, p=0.0001. In abortion, structural localization of leptin receptors in placental tissue was mainly confined to the blood lakes and around blood vessels.
Conclusion: Serum leptin level of second trimester abortion women was found normal and not elevated as it is aspect during second trimester of healthy pregnancy. This finding was confirmed by the declines of localization of leptin receptor in syncytiotrophoblast cells where they are responsible for the production of hormones vital to pregnancy maintenance. This finding may suggest a potential for autocrine or paracrine interactions within placental tissue.

INTRODUCTION

Leptin is a hormone-like protein consisting of 167 amino acids was first discovered in 1994.[(1)] It is a hormone secreted mainly by white adipose tissue, but also by the placenta, mammary gland, testes, and ovaries, which is thought to act primarily as a satiety signal to the hypothalamus.[(2)]

Leptin is encoded by the *ob* gene and considered as the first hormone to be released from adipocytes.[(3)] It has important effects in controlling body weight, metabolism and reproductive functions. The function of leptin in resisting obesity and promoting leanness led to the choice of the name "leptin " from the Greek root leptos, meaning thin. [(4)]

During pregnancy, the physiological function of high leptin levels is not clear. Leptin may have effects on thermogenesis, lipid metabolism and mobilization of energy stores rather than on regulation of maternal food intake. [(5)] In maternal circulation, its levels steadily increase during the first and second trimesters and peak in late second or early third trimester. [(6)] These high levels are maintained throughout the remainder of gestation and decline dramatically postpartum.[(7)] Also, it is negatively correlated with serum prolactin level during early lactation. [(8)] Leptin concentrations are dramatically higher in lactating women compared to non-lactating controls and this suggests a role for leptin in mobilizing needed energy reserves [(9)] .

The hyperleptinemia may be part of the insulin resistance syndrome in women with prior pre-eclampsia.[5] Pregnant who subsequently miscarry have significantly lower plasma leptin concentrations during pregnancy, compared to those who subsequently have a term birth. [10] These studies suggest that, leptin may play a role in preventing miscarriage.[11]

Leptin levels were found higher in obese pregnant compared to lean pregnant. Also, its levels were varying depending on the age of the mother. [12]

Placenta-derived leptin may act on the hypothalamus and regulate the maternal energy expenditure and neuroendocrine functions, since leptin receptor is expressed in the hypothalamus. Placenta-derived leptin may also affect glucose metabolism in the liver, pancreas, and muscle, since these organs express functional leptin receptors. In addition, placenta secretes leptin into fetal circulation, although the role of leptin in the fetal growth and development has not been fully elucidated. It is possible that placenta-derived resistin may modulate maternal glucose metabolism since administration of resistin decreases insulin sensitivity in mice.[13]

Since leptin receptor is structurally related to interleukin-6 type cytokine receptors, it is possible that leptin has some other unknown functions as a kind of growth factor during early human development. Leptin is considered as an important new growth factor in intrauterine and neonatal development. It may also function as anti-inflammatory cytokines such as tumor necrosis factor-α (TNFα.). This effect may be important, as successful pregnancy is associated with down-regulation of intrauterine proinflammatory cytokines. [14]

Localization of leptin, OB-Rb and OB-Ra transcripts to the trophoblast may also relate to the placental function as an endocrine organ. In the placenta it could act as a growth hormone (GH) and factor for angiogenesis in an autocrine manner.[15]

The human leptin receptor (OB-R) is a cell surface transmembrane protein, which has sequence homology and functional similarity to mice leptin receptor. The leptin receptors are a member of the class 1 cytocine receptor family, which includes growth hormone receptor (GH-R).[15]

The human leptin receptors are encoded by the diabetes (db) gene and are located on the short arm of chromosome 1 (1p31-p22). [16]

This study was aimed to investigate the relation between serum leptin level and placental leptin receptor in second trimester abortion women.

Materials and Methods:

A case control study was carried out from November 2011 to April 2012. Sixty pregnant women at second trimester between 14–24 wks of gestation were collected from hospitals in Baghdad. They were diagnosed by their physicians to have abortion at second trimester after proper physical and gynecological examination and confirmed by ultrasound findings.

All patients who had hypertension, thyroid disease, diabetes mellitus, smoking, evidence of active infection, fever, chronic inflammatory diseases (including rheumatoid arthritis, joint pain, osteoarthritis, abdominal complain, inflammatory bowel disease); currently taking any medication, Cytomegalovirus (CMV) and toxoplasmosis were excluded from the study.

Blood and placenta tissue samples were taken from patients admitted to the hospitals for abortion at second trimester.

Thirty healthy women with normal pregnancy (at their second trimester of gestation) were used as control. Patients and control were with a comparable age.

Control for the leptin receptor was taken from women with premature delivery (28-34 wk). Placenta and blood samples were obtained from each patient once they admitted to the operation theater.

Five milliliters of venous blood was aspirated from all patients at the time of their abortion.

Serum was collected after blood was transferred into a plain tube, allowed to clot, and then centrifuged for 10 min at 3000 rpm. Then it was used to determine leptin concentration using an Enzyme linked immunosorbant assay (ELISA) technique; Human Leptin (LEP) ELISA Kit (Catalog No. CSB-E04649h, CUSABIO BIOTECH CO., LTD, China). The minimum detectable concentration of human serum leptin is typically less than 1.56 pg/ml. Expected normal concentrations are between (3.5 - 12.5 pg/ml) at 450 nm. The placenta sample was immediately fixed in 10% formalin solution after taken from patient and prepared for Immunohistochemical study for localization of leptin receptor using Leptin receptor (Us biological Company USA).

Ethical approval and patient permission were obtained from the local ethics committee to conduct this study. It was ethically approved by the Local Ethical Committee in College of medicine- Al-Nahrain University.

Statistical analysis:

Data were statistically analyzed by SPSS version 17. All data were presented as a mean ± SE. Statistical differences between value of patients and control groups were determined by student *t*-test. Correlation between the variables was performed by spearman correlation coefficient. *P* value <0.05 was considered as significant.

Results:

Sixty pregnant women in their second trimester between 14–24 wks of gestation were divided into 2 groups: Group (A): includes 30 women at abortion in second trimester and Group (B): 30 normal apparently healthy pregnant women at second trimester.

Anthropometric characteristics such as age, gestational age (GA) and BMI were recorded and calculated for each patient and control in this study. Also, the level of total leptin in patient's sera was determine to be compared with normal healthy pregnant at the same trimester. (table-1)

Table-1: **Anthropometric characteristics and biochemical variables including total leptin level distributed according to BMI in study subjects.**

Variable	*Mean ± SE*		*Pvalue*
	Patients(n=30)	*Control(n=30)*	
Age (years)	26.2±5.8	25.6±6.7	*NS*
GA (weeks)	19.3±0.7	19.9±0.5	*NS*
BMI(kg/m^2)	24.6±3.1	25.9±3.6	*NS*
S.leptin(pg/ml)	3.5±0.8	24.4±0.7	*<0.001*
Leptin/BMI ratio	0.1±0.02	0.9±0.01	*<0.001*

BMI (Kg/m^2)	*Serum Leptin (pg/ml)*				
	Patient(n=30)		*Control(n=30)*		
	No	*Mean±SE*	*No*	*Mean±SE*	
<18.5	-	-	-	-	-
18.5—24.9	19	1.5±0.2	13	20.8±0.5	**0.0001***
25--29.9	9	6.7±1.5	15	26.9±0.5	**0.0001***
≥ 30	2	8.9±5.7	2	28.1±0.2	*NS*
P value	*0.0001**		*0.0001**		

No.= number, SE= standard error, GA= gestational age, BMI= body mass index, S=serum, and NS= not significant.

No significant differences were found between patient's age, GA, and BMI compared with control. While a marked differences (p<0.001) were found between patients serum total leptin and its ratio with BMI compared with its level *vs.* healthy control pregnant (Table-1).

In table-2, no correlation was found between patient's leptin and their age or GA, while a highly significant positive correlation was found between their leptin concentration *vs.* BMI (p= 0.0001, r= 0.682, y=0.880x-18.08) (Figure-1). In other hand, leptin of healthy pregnant was significantly correlated with their age, GA and BMI.

Table-2: Correlations between anthropometric and serum total leptin in study subjects.

Variables	*Serum total leptin(pg/ml)*			
	Patients(n=30)		*Controls(n=30)*	
	P	*r*	*P*	*r*
Age(years)	0.329	-0.184	0.005	0.500**
GA(weeks)	0.583	0.143	0.021	0.156*
BMI(Kg/m^2)	0.0001	0.682**	0.0001	0.897**

GA= gestational age, BMI= body mass index, r= correlation, p= p value, and
*** Correlation was significant at p < 0.05, ** correlation was highly significant at p<0.001.**

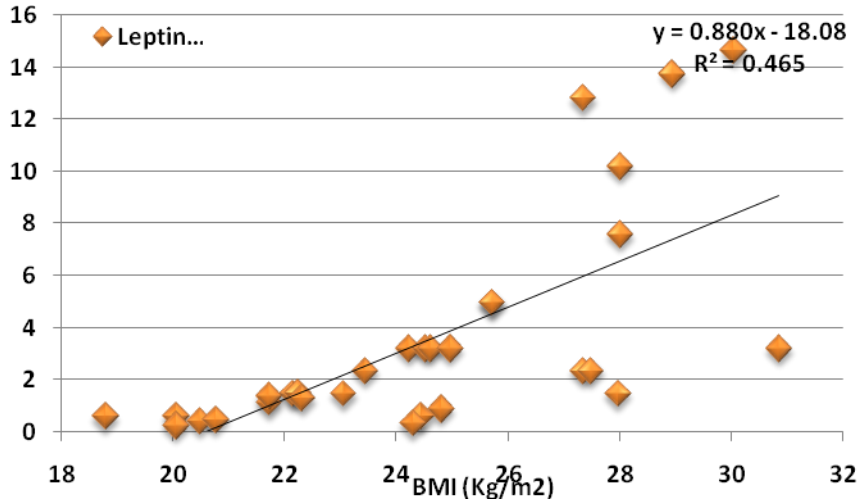

Figure-1: Correlation between serum total leptin BMI in patients group (n=30).

Histological and Immune- histochemical result:

General Morphological features of the placenta using H&E showed that, the placental tissue is arranged as chorionic plate, basal plate and in between these two are villous stem & their branches. Large number of villi was seen during placental tissue examination, these villi have different size & shape, their diameter varied a lot all bathed in maternal blood. Syncytial knots are seen as small darkly basophilic aggregation of degeneration nuclei (Figure2).

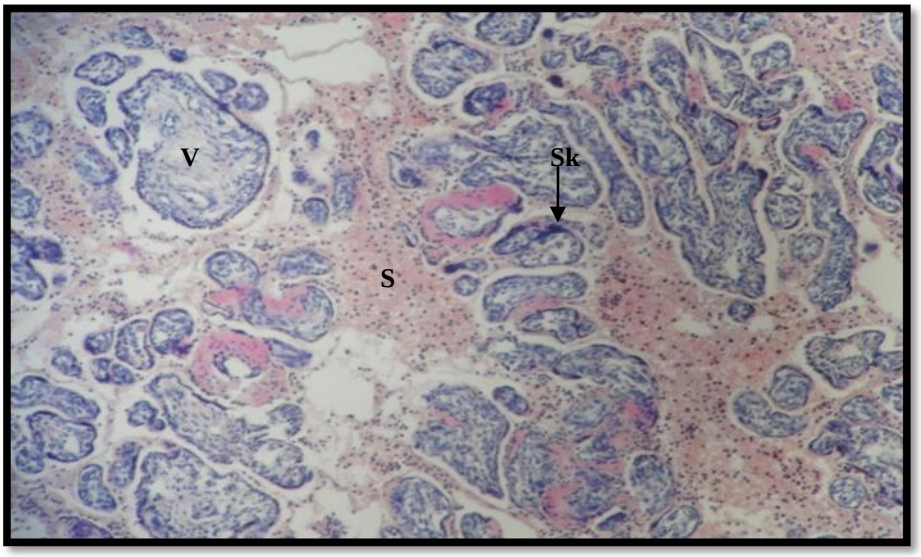

Figure (2): cross section through placental tissue showing chorionic villi (V), inter-villous space (S), syncytial knots (Sk). H&E × 100

Chorionic villi showed number of branches, each villi have a core usually with many blood vessel in it, and some villi are highly vascular, each villus has an outer syncytiotrophoblast layer of basophiles layer cells with short brush border (small microvilli) at their surface. Cytotrophoblast are seen as few cells below the syncytiotrophoblast (Figure 3A and 3B).

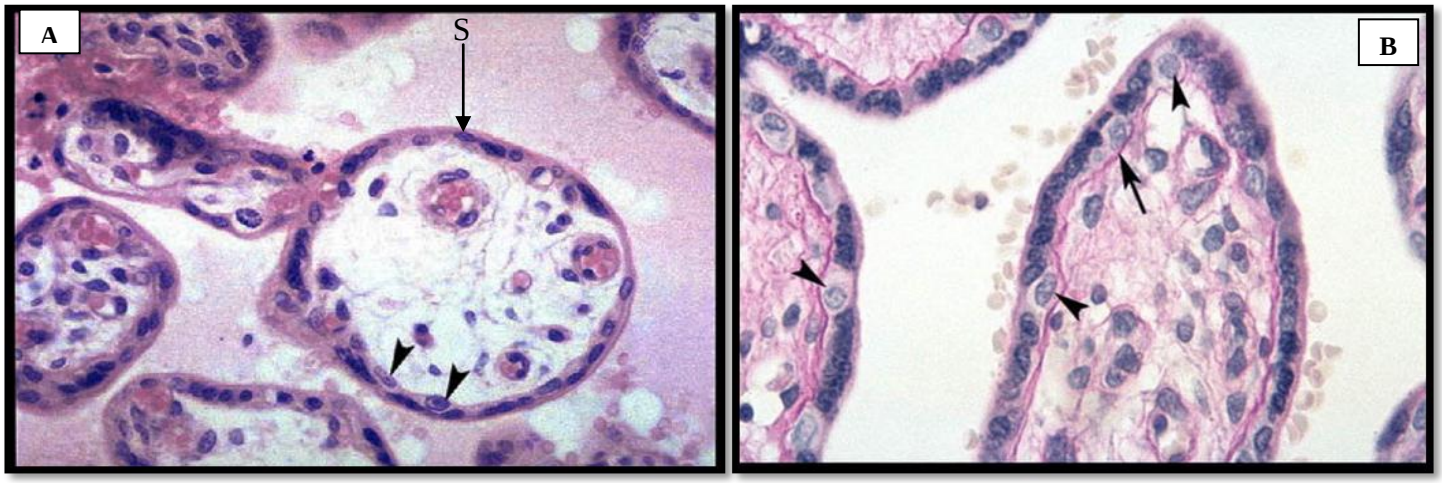

Figure (3):A cross section through placental tissue showing chorionic villi (V), syncytiotroblast(S), cytotrophoblast cell as few large cell below syncytiotrophoblast lightly stain (arrow head). H&E× 400 . (3) B: syncytiotrophoblast. (⟶), the cytotrophoblast (arrow head) & the basal lamina at higher magnification H &E × 1000

For expression of leptin & leptin receptors in human placental tissue (Anti- Leptin Receptor) was used in this study. Immun localization of leptin & leptin receptors in human placental tissue of the control group showed a strongly stained syncytiotrophoblast cells with Anti- human leptin antibody. The most prominent staining was observed in a small apical plasmic rim of the syncytiotrophoblast which provided the strongest signals (Figure 4A, 4B), other cellular components of the placenta tissue (Blood vessel, lakes, and blood) also showed a significant positive staining for the leptin receptor.

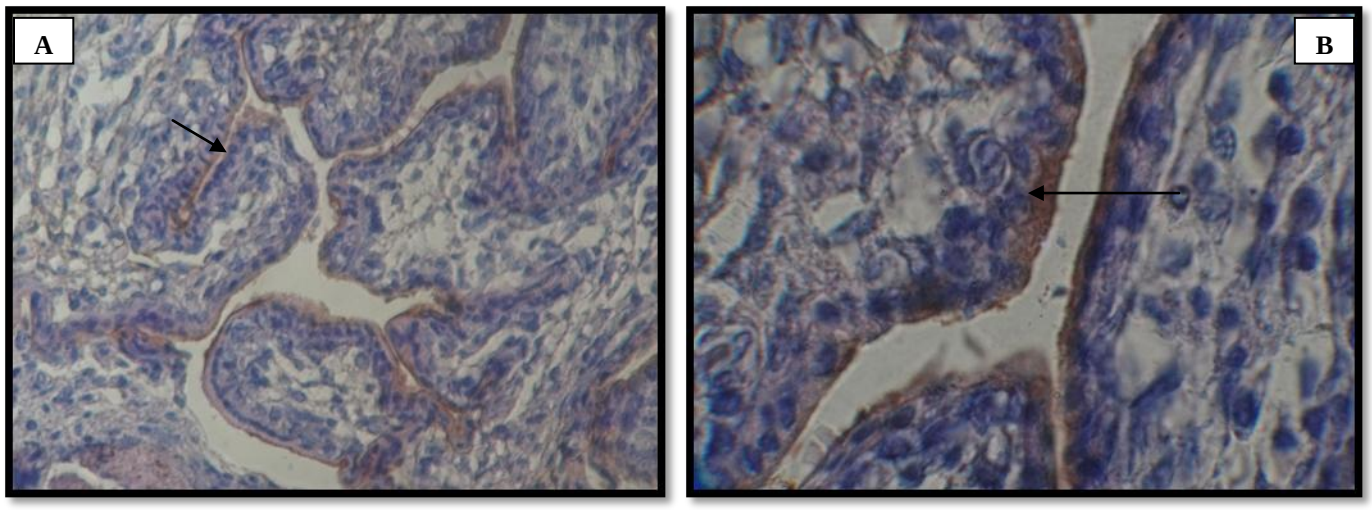

Figure (4): A: cross section through placental tissue showing villi leptin receptor localized in the apex of syncytiotrophoblast (⟶ Anti- Leptin receptor Control group X 100
B: Higher magnification of placental tissue showing chorionic villi (⟶ showing localization leptin receptor in syncytiotrophoblast Anti- leptin receptor control group X400.

Fine small granule was easily seen filling the apical cytoplasm of syncytiotrophoblast leaving the basal cytoplast will no reactivity. (Figure 5)

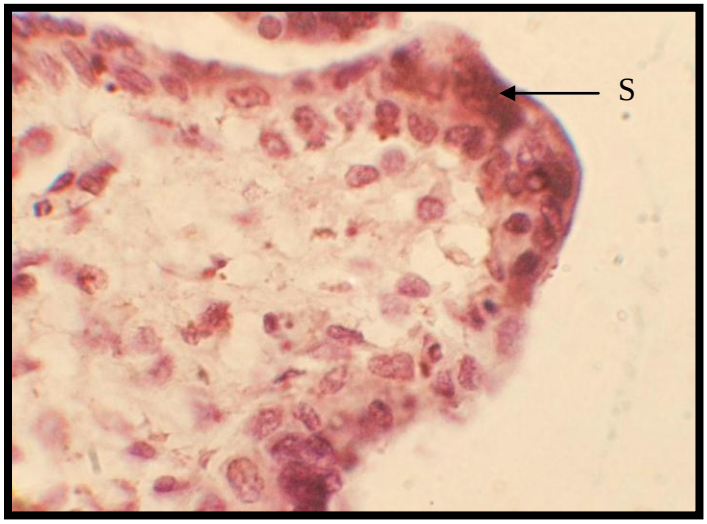

Figure (5): cross section through placental tissue showing chorionic villi with localization of fine granules in the apical of syncytiotrophoblast (⟶) Anti- leptin receptor abortion, Control group X1000.

Detection of leptin & leptin receptors in cytotrophoblast was difficult in females with abortion. No reactivity have been seen in these cell what so ever (Figure6: A and B). In abortion the picture differed a lot from that in control group, structural localization of leptin receptors in placental tissue was mainly confined to the blood lakes and around blood vessels.

No leptin signals was seen inside the placental tissue of women with abortion, syncytiotrophoblast, cytotrophoblast showed no reactivity &loss of the small fine granules. The only positive site for the receptors was localized in syncytial Knots (Figure 6: B).

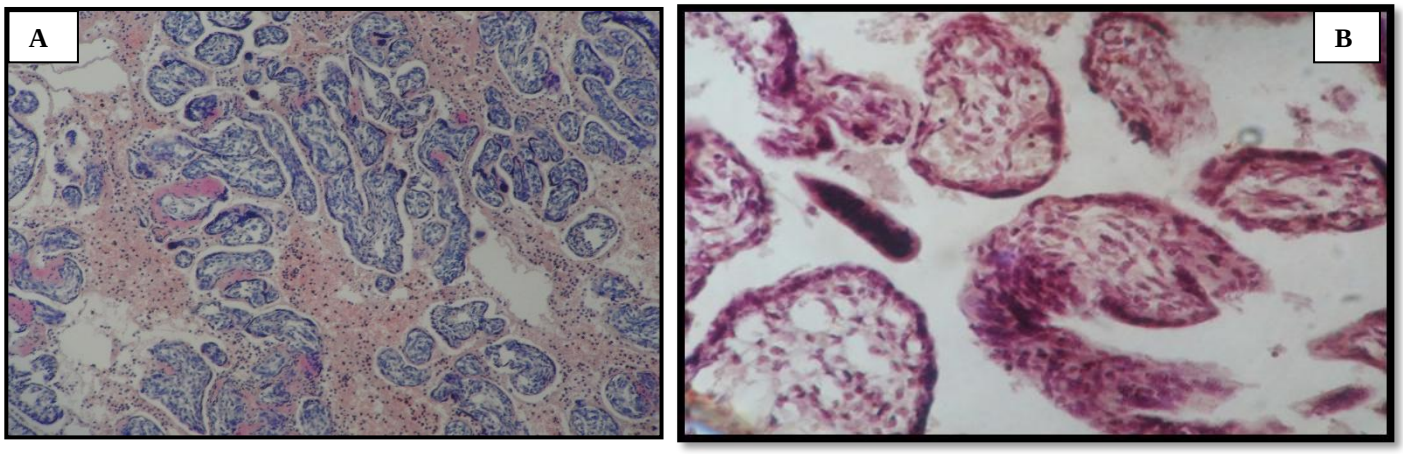

Figure (6): A Cross section through placental tissue showing chorionic villi in abortion. No cellular reactivity. Only blood lakes and vessel reactivity. X 100. B: Higher magnification of placental tissue showing chorionic villi in abortion. No cellular reactivity. Only syncytial knots reactivity. Anti- leptin receptor abortion second trimester group, X 400.

Discussion:

Biochemical study:

In recent years, the adipokines; adiponectin and leptin have been shown to play a role in normal pregnancy, as well as in complications of pregnancy, including GDM and PE. [17]

The aim of the current study was to investigate whether alterations of leptin levels predate the development of abortion in women at second trimester, as assessed by Doppler examination of the uterine arteries during the second trimester of pregnancy with exclusion of the pathological and immunological causes.

We also sought to investigate the relation between circulating level of leptin and the anthropometric parameters (age, gestational age and BMI) and compare it with normal healthy pregnant at same trimester.

Adipokines, predominantly leptin is a hormone produced by the adipose tissue. Fetal adipokines maturation takes place predominantly in second trimester of pregnancy, playing a role in lipid and glucose metabolism. Since leptin is secreted by adipocytes in late stages of differentiation and adiponectin only by fully differentiated adipose cells, they can be used as a marker for adipose tissue development and the amount of adipose tissue. [18]

In this study, patients were classified as having unexplained abortion at second trimester which may be associated with endocrine abnormalities. An abnormality was obvious in their total leptin level which was found significantly lower compared with normal healthy pregnant women at the same trimester.(table-1)

During pregnancy, total leptin levels are substantially elevated [19] and serum leptin levels were significantly higher than levels in non-pregnant women, and increased from the first to the third trimester**.** [20]

The increase in leptin level is due to the additional production of leptin by syncytiotrophoblast in the placenta and thus leptin synthesis is increase with the increase of the placental mass with advancing gestation. [1] He reported that the elevation in serum total leptin level is not due to placenta and adipose tissue only, but also to mammary epithelial cells, fetal tissue, gastric mucosa, and hepatic stellate cells can synthesize leptin, and the production by these organs leads to additional increase in leptin concentration resulted in a significant change in its level related to GA which found in the second trimester**.** [1] Žaneta *et al.* 2004 also have found an elevation in maternal serum total leptin levels during pregnancy with alterations particularly during the second and third trimesters of pregnancy. They mentioned that the occurring physiological hyperleptinemia is not associated with decreased food intake or reduced metabolic activity in pregnant women**.** [21]

Augustine *et al.* 2008 have found that maternal leptin concentrations increase during pregnancy, but the increase seems to occur during the first two trimesters and then leptin levels decrease slightly during the third trimester**.** [22] While Grattan *et al.* 2007 and Ladyman *et al.* 2010 suggested that a lack of increase in leptin level on the 3rd trimester is reflective of late pregnancy being a leptin-resistant stage. [20, 23] In addition, others reported that during the last pregnancy trimester, leptin levels do not rise although body weight increases, indicative of pregnancy induced leptin resistance, and this contributes to reduce insulin sensitivity seen during pregnancy.[20]

Abnormally high leptin level is found in most patients with gestational diabetes mellitus and pre-eclampsia. Increased leptin level in gestational diabetes mellitus might amplify the inflammatory process, whereas elevated leptin concentrations in pre-eclampsia are suggested to be a compensatory response to increase nutrient delivery to the under perfuse placenta.[17]

Regarding relation between total leptin level in serum women with abortion at second trimester and their BMI, in addition to leptin/BMI ratio, compared with same parameters in control, a lower significant was found between patient's leptin and BMI with $p<0.0001$. (Table-1)

The only not significance comparison was observed between patients and control leptin levels was among obese women with BMI $\geq$ 30 Kg/m^2. This result may be due to the small sample size of distributed subjects.

In normal pregnancy, leptin elevation as Kim *et al.* 2008 explained is due to the increase in maternal body weight, as the serum leptin level is dependent on body weight.[24] More explanation was done by Alexe DM *et al.* 2006 whom reported that leptin elevation in maternal serum is due to the gradual increase of the BMI throughout gestation which combines with an increase in estradiol levels that stimulate leptin production from adipocytes.[25]

While Sagawa *et al.* 2002 and Masayo *et al*. 2003 have mentioned that BMI did not necessarily reflect body fat mass in pregnant women, and the remarkable increase of the serum leptin level during pregnancy was not explicable by an increase of fat mass alone. They suggested that the increase of leptin during pregnancy is caused by production in the placenta and that maternal plasma leptin levels are not correlated with BMI. [26,27]

Regarding the ratio of total serum leptin to BMI, a significant decrease was found between abortions L/BMI ratios (0.1 ± 0.02) *vs.* normal pregnant ratio (0.9 ± 0.01) with $p< 0.001$. This ratio was first used in 2001 by Brannian *et al.* as a predictive marker of outcomes in women undergoing IVF (In Vitro Fertilization). They grouped the ratio into three categories, low (0.1-0.3), moderate (0.4-0.6), and high (≥ 0.7). Also, they mentioned, very few patients became pregnant when their leptin was $\geq 3X10^4$ pg/ml, even if their BMI was relatively low. Finally, they concluded

that this relationship may assist clinicians in counseling patients and improving the success of assisted reproduction. [28]

Abortion L/BMI ratio in this study was found ± 0.1 which is like the lowest of low range as Brannian *et al*. suggested. This due to their low leptin levels despite the BMI classifications (normal, overweight and obese). Normal pregnant women L/BMI ratio shows ± 0.9 which is > the high ratio expected by Brannian *et al* 2001. [28]

This study is the first one as our knowledge to determine the relation between serum total leptin levels in abortion women at second trimester and their BMI.

In this study, both maternal age and gestational age were comparable in abortion and normal pregnant group. No significance was found between patient's age and control ($p>0.05$) (table-1). Also, no correlation was found between patient's GA and leptin level (table-2). Yang 2005 [29] found a significant correlation between GA and serum leptin levels among normal pregnant as Tamas *et al.* 1998 [30] found. He concludes that maternal serum leptin concentration throughout the pregnancy course correlates not only to body weight and BMI but also to GA. Because leptin is one of the proteins produced by syncytiotrophoblast in the placenta, theoretically, the amount of leptin synthesized by the placenta should increase due to the increased size of the placental mass with advancing gestation.[29] This was proved by no significant relationship between maternal serum leptin and GA in the first trimester. But serum leptin is correlated to GA in the second trimester and is inversely related to GA in the third trimester. In addition, maternal BMI is related to GA in the second trimester and the whole pregnancy, but not in the first and third trimesters. [29]

The increasing in maternal leptin level with GA increase was not found in women abortion at 2nd trimester in this study unlike the control. Lacroix R *et al*. 2000 [31] have suggested that appetite of most pregnant women increases after the fourth month of gestation when symptoms of nausea and vomiting disappear. Therefore, in addition to uterine content, systemic organs including subcutaneous adipose tissue, omentum, blood volume and breasts increase in size with advancing gestation. However, other than placenta and adipose tissue, leptin can be synthesized by mammary epithelial cells, fetal tissue, gastric mucosa, and hepatic stellate cells. With the additional increase in leptin in these organs, significant changes in serum leptin levels related to GA were found in the second trimester. [31]

Grattan D *et al.* 2007 [20] concluded that leptin is a hormone intimately related to the pregnancy, and its concentrations increases during pregnancy. They also found that leptin concentration in pregnant women was much higher than that of age-matched non-pregnant women. Another study by Žaneta *et al.*2004 [21], found an elevation in maternal serum total leptin levels during pregnancy with alterations particularly during the second and third trimesters of pregnancy. They mentioned that the occurring physiological hyperleptinemia is not associated with decreased food intake or reduced metabolic activity in pregnant women.

Histological Study of the placenta:

The hormone leptin is required for reproducting mammals. Maternal plasma leptin is increased above non pregnant levels is all mammals including human this increased in plasma
leptin appears to be the result in part from upregulation of adipose leptin secretion and/or from production and secretion leptin in placenta.[32]

It has become increasingly clear that leptin is an important reproductive hormone, it is believed to be required for normal pubertal development in rodents.[33] It's also required for fertility in adult rodents.[34] Animal in whom leptin is deficient or unable to act due to mutations in the Ob-R gene , which render the receptor inactive, are infertile.[35]

Leptin receptor seems to be located in syncytiotrophoblast in normal pregnancy. The receptor showed a wide a strong positive staining for anti human leptin antibodies. Apical granulated cytoplasmic reaction was clearly observed. A recent study for leptin and leptin receptor in third trimester in human placenta showed and immunohistochemistry reaction to the syncytiotrophoblast at the maternal interface and to lesser extent a vascular endothelial cells at the fetal interface were localized.[36]

During pregnancy maternal leptin levels increase in plasma in every mammalian and, it have been postulated that increased maternal leptin level may be secondary to the increase in a circulating from of the leptin receptor organized as plasma leptin – binding protein this protein found mostly in plasma of mice and human in secreted placenta in vitro.[37]

Zaho et al. 2004 have identified a soluble leptin- binding protein in plasma of pregnant M. lucifugus this hypothesis is possible in leptin production from adipose tissue in pregnant mice which is recoded is possibly under of circulating steroid hormones.[32]

To our knowledge no study was found to correlate between abortion and leptin receptor localization this study prove that there is a major contribution and relation between circulating leptin during pregnancy and abortion. The diminished localized of leptin receptor in the syncytiotrophoblast layer in women with abortion prove theory.

Human leptin mRNA and co-localized to the syncytiotrophoblast which in direct contact with maternal blood. First trimester cytotrophoblast also contain leptin mRNA and protein.[38] Our study suggest that placenta leptin content increases with procreation of pregnancy this finding is agreement with the gestational increase in leptin measured in the maternal circulation.[39]

In mice leptin and its receptor were similarly localized to the trophoblast giant cells situated in the junction zone at the maternal interface and also in the cytotrophoblasts on the fetal side of the labyrinthine placenta.[40]

In humans the leptin receptor is Co-localized to the syncytiotrophoblast at the maternal interface. Similarly, in the mouse placenta protein and mRNA encoding leptin and its receptor are co-localized to the trophoblast giant cells situated at the maternal interface and to the cytotrophoblast.[40]

So, in conclusion, serum leptin level of second trimester abortion women was found normal and not elevated as it is aspect during second trimester of healthy pregnancy. Also, although patients were not obese, but a significant correlation was found between their leptin and BMI which support our objective that the unexplained abortion at second trimester is due to abnormality in their metabolic hormone action, and the low level of L/BMI ratio in abortion women compared with elevated ratio found in normal second trimester healthy pregnant group confirm the usage of L/BMI ratio as an early predictive marker for second trimester abortion especially when it becomes ≤0.1. The lack of correlation between GA and leptin level of abortion women compared with the presence of positive correlation in healthy pregnant women at the same trimester might due to the lack of leptin production by the placenta. This finding was confirmed by the localization of leptin receptor in syncytiotrophoblast cells where they are responsible for the production of hormones vital to pregnancy maintenance. Their declines suggest a potential for autocrine or paracrine interactions within placental tissue.

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