ORIGINAL ARTICLE

Ghrelin does not modulate angiogenesis in matrigel plug in normal and diet-induced obese mice

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Background: The reciprocal interaction between adipocytes and angiogenesis is considered as an essential component in the development and expansion of adipose tissue. The aim of this study was to evaluate the effect of ghrelin on angiogenic response using *in vivo* angiogenesis assay of matrigel plug and its correlation with serum leptin levels in normal and diet-induced obese mice. **Materials and Methods:** This experimental study has been done on 24 male C57BL/6 mice which were randomly divided into four groups: Normal diet (ND) or control, ND + ghrelin, high-fat-diet (HFD) or obese and HFD + ghrelin (n = 6/group). Obese and control groups received HFD or standard diet for 14 weeks. Then, growth factor reduced matrigel plug (500 µl) containing bFGF (basic fibroblast growth factor; 100 ng) with or without ghrelin (100 µg/kg) was injected subcutaneously in the mid-ventral abdominal region of each mice. After 10 days, blood samples were taken and matrigel plugs were removed under anesthesia and angiogenic response was assessed by immunohisochemical staining. **Results:** HFD significantly increased angiogenesis in matrigel plug as expressed as the number of CD31-positive cells than standard diet (43 ± 5 vs. 13 ± 2.5 $CD31^+$ cells/field). Ghrelin did not alter angiogenesis in matrigel plug in both obese and control groups. There was a strong positive correlation between the number of CD31-positive cells and serum leptin concentration (r = 0.91). **Conclusion:** Leptin as an angiogenic factor has a positive correlation with angiogenesis in matrigel plug model of angiogenesis and ghrelin could not alter angiogenesis.

Key words: Angiogenesis, ghrelin, matrigel plug, obesity

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INTRODUCTION

Obesity that is defined as excess body fat because of alarming rise in the worldwide is recognized as a serious threat for health care.[1] Obesity as a complex metabolic disorder is associated with most common and chronic diseases including: Type 2 diabetes, hypertension, cardiovascular diseases, stroke, osteoarthritis, sleep apnea syndrome, and certain types of cancer.[2]

Adipose tissue because of its ability in rapid and dynamic expansion or shrinkage in excess or demand of energy status is considered as a unique plastic tissue and vasculature can be a causal role in plasticity.[3] Vascular system and angiogenesis (the formation of new blood vessels from existing ones) in adipose tissue with providing of oxygen and nutrients, growth factors and cytokines trigger growth and survival signals for maintenance of physiological function of adipocytes.[4,5] Thus, angiogenesis is critical for adipose tissue expansion.[6] Angiogenesis is regulated by intricate balance between angiogenic factors such as: Vascular endothelia growth factor (VEGF), angiopoietins, leptin, and fibroblast growth factor (FGF2) and angiostatic factors including: Angiostatin, endostatin, and adiponectin.[7]

Ghrelin, a peptide of 28 amino acids, mainly is produced in X/A like cells of the oxyntic mucosa of the stomach.[8] Initially, it was identified as the endogenous ligand of the growth hormone secretagogue receptor (GHS-R);[9] however, it has many other effects including cardiovascular effects such as decreasing of peripheral vascular resistance,[10] vasodilatory effect,[11] increasing in coronary perfusion,[12] decreasing in cardiac injury induced by ischemia/reperfusion.[13] Effect of ghrelin on angiogenesis has been previously documented; however, its effect on angiogenesis has been reported as both proangiogenic or antiangiogenic. The objective of present study was to evaluate the effect of ghrelin on angiogenic response and its correlation with serum leptin levels in normal and diet-induced obese mice. In this study, we used matrigel plug, which is one of the relatively rapid and simple *in vivo* angiogenesis assay.

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MATERIALS AND METHODS

Animals

This experimental study has been carried out on 24 male C57BL/6 mice, (20-30 g, 5 weeks old) which were purchased from Pasteur Institute of Iran. All animals were housed in cages in animal room (22-25°C room temperature and 12-hour daylight cycle). The animals had 7 days to acclimatization to the laboratory conditions and received a standard or HFD chow for 14 weeks. Body weight of the animals was measured weekly. The ethical committee of Isfahan University of Medical Sciences approved all study protocol.

Animal diets

For induction of diet-induced obesity, the obese group consumed HFD (Labratories BioServ, Cat #F3282, USA) included 59% fat, 27% carbohydrate, 14% protein) for 14 weeks.[14] Control group received standard diet (Pasteur Institute, Iran). Body weights of the animals were monitored weekly.

Animal groups, matrigel plug assay for angiogenesis

After 14 weeks, the mice received subcutaneous injection of growth factor-reduced matrigel plug (BD Biosciences; 500 μl) containing bFGF (basic FGF) (Sigma-Aldrich, St. Louis, MO, USA; 100 ng)[15] with or without acylated ghrelin (Tocris Co. Bristol, UK; 100 μg/Kg)[16] in the midventral abdominal region.[17] Thus, the animals were split randomly into four groups: Normal diet (ND) or control, ND + ghrelin, HFD or obese and HFD + ghrelin (n = 6/group). After 10 days, matrigel plugs were removed[17] under anesthesia and used for immunohistochemical staining. At first, the matrigel plugs were fixed in 10% formalin, embedded in paraffin and cut at 4-μm thickness. Then, the slides were incubated with primary antibody (rabbit anti-mouse CD31; 1:50; Abcam Co.) and biotinylated secondary antibody (Novolink polymer; Novocastra Co.). The reaction was developed with DAB substrate (Novolink polymer; Novocastra Co.) and finally the sections were counterstained with hematoxylin.[18] The angiogenic response was expressed as the numbers of CD31-positive cells were counted using an olympus light microscope at ×40 magnification in five different fields for each plug.

Serum leptin measurement

Blood samples were taken at the end of experiment and centrifuged for 30 minutes. The serums were removed and stored at −20°C for subsequent analysis. The serum leptin levels were measured by specific sandwich enzyme immunoassay kit (Invitrogen, Camarillo, CA 93012) and were measured according to the manufacturer's instructions.

Statistical analysis

Data was analyzed with SPSS version 16 and expressed as the mean ± SEM. Statistical comparisons were done between groups with Kruskal-Wallis test using LSD post-hoc test. Correlation analysis was examined using Pearson's correlation coefficient. P-value less than 0.05 was considered statistically significant.

RESULTS

Body weight gain

Figure 1 illustrates the weight gain of the animals in experimental group. As we expected, the obese mice gained more weight during 14 weeks HFD than control animals ($P < 0.05$).

Angiogenic response in matrigel plug: Effect of ghrelin

Quantitative analysis in the matrigel plug demonstrated that the number of CD31-positive cells in obese mice were significantly higher in comparison with control group ($P < 0.05$) [Figure 2e]. There was no significant difference in the number of CD31-positive cells in group that received ghrelin compare to not received and ghrelin could not alter angiogenesis in obese and control groups ($P > 0.05$). Samples of immunohistochemical staining were presented in [Figures 2a-d].

Serum leptin concentration

We found that serum leptin level in obese animals was significantly higher than control group ($P < 0.05$) [Figure 3a].

Correlation analysis

To study the relationship between the number of CD31-positive cells and serum leptin level, we performed correlation analysis and found a strong positive correlation between the number of CD31-positive cells in the matrigel plug and serum leptin levels ($r = 0.91$; $P < 0.05$) [Figure 3b].

DISCUSSION

In this study, was investigated the effect of ghrelin on angiogenesis using an *in vivo* angiogenesis assay of matrigel

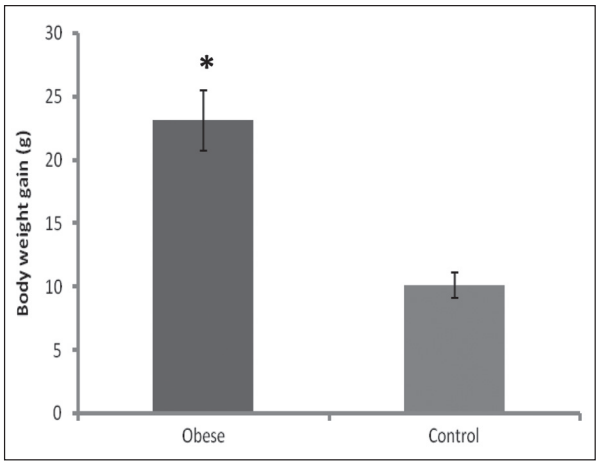

Figure 1: Body weight gain in obese and control groups after 14 weeks receiving diet. Values are expressed as Mean ± SEM, *$P < 0.05$ compare to control

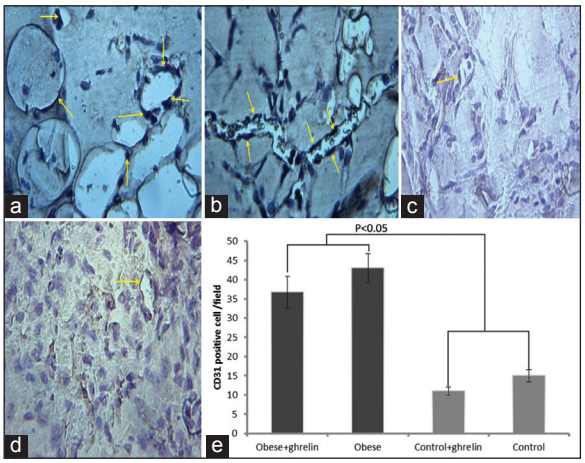

Figure 2: Effect of HFD and ghrelin on the number of CD31-positive cells in the matrigel plug. The histological sections were stained with immunohistochemical technique. (a) Obese + ghrelin, (b) obese, (c) control + ghrelin, (d) control. (e) HFD loading significantly increased the number of CD31-positive cells than standard diet and ghrelin administration did not change the number of CD31-positive cells in obese and control groups. Arrows indicates the CD31-positive cells. Data are shown as mean ± SEM. (n = 6 each group)

plug and its correlation with serum leptin levels in normal and diet-induced obese mice.

Adipose tissue is considered as a unique plastic tissue that angiogenesis and vascular system can be a causal role in plasticity.[2,19] Angiogenesis is controlled by a precise balance between angiogenic factors such as: (VEGF, FGF2, leptin, and angiopoietins) and angiostatic factors including: Angiostatin, endostatin, and adiponectin.[7] In the present study, we demonstrated that HSFD (high saturated fat diet) increased the number of CD31-positive cells in the matrigel plug compare to ND. In a recent study on eNOS-/- knockout and DDAH (overexpression of eNOS) transgenic mice that received HFD for 13 weeks showed that DDAH mice had higher vascularity (number of vessels with lumen, number of vessels without lumen, and number of single CD31-positive cells) in the the matrigel plug in comparison to control or eNOS-/- mice.[20] In the present study, the use of growth factor-reduced matrigel plug decreased the effect of growth factors within matrigel plug that can mask the effects of the test substance or HFD.[21] Thus, possibly, inflammation and/or oxidative stress mechanism through trigger of integrin and metalloproteinases synthesis in HFD and obesity status can be involved in angiogenic response. However, in other study on male mice of wild type and knockout hRXRα (Retinoid X receptor α) that received HFD for 7 weeks, quantitative analysis of CD31-positive structures in the matrigel plug demonstrated that in hRXRα knockout mice, there was a weaker angiogenic response than wild type.[22] Furthermore, in correlation analysis, we found a strong positive correlation between the number of CD31-positive cells and serum leptin levels. Leptin is an adipocyte-derived satiety hormone that mainly contributes in food intake and energy homeostasis.[4] In

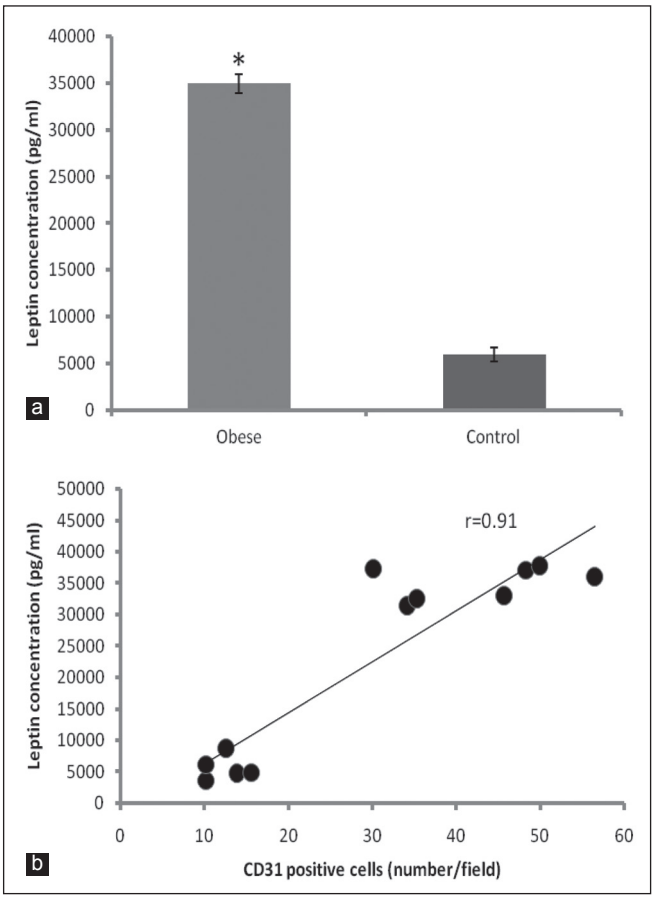

Figure 3: (a) Serum leptin concentration in obese and control groups. (b) Scatter plot shows the correlation between the number of CD31-positive cells and serum leptin concentration (r = 0.91). *P < 0.05 compare to control group

addition, recently it has been demonstrated that leptin has direct angiogenic activity[5] and, thus, can be involved in angiogenic response in this model.

Our results also indicated that ghrelin had no significant effect on the number of CD31-positive cells in matrigel plug in obese and control mice. Ghrelin a peptide of 28 amino acids mainly is produced in the stomach and acts as an endogenous ligand of the GHS-R;[9] however, recently its effect on cardiovascular system especially on angiogenesis has been demonstrated, although its effect on angiogenesis has been reported as proangiogenic or antiangiogenic. A study by Conconi *et al*., indicated that ghrelin can inhibit FGF2-induced proliferation of human umbilical vein endothelial cells ($HUVECS_s$) *in vitro* and also *in vivo* chick chorioallantoic membrane (CAM) model[23] while, Li *et al*., showed that ghrelin increased proliferation, migration, and angiogenesis through ERK2 signaling in human dermal microvascular endothelial cells ($HMVECS_s$).[24] In the present study, ghrelin could not alter angiogenesis in the matrigel plug model. This difference may be related to the model of angiogenesis or local/systemic effect of ghrelin.

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