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Leptin restores markers of female fertility in lipodystrophy

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ABSTRACT

Objectives: Female reproductive dysfunction occurs in patients with pathological loss of adipose tissue, i.e. lipodystrophy (LD). However, mechanisms remain largely unclear and treatment effects of adipocyte-derived leptin have not been assessed in LD animals.

Methods: In the current study, C57Bl/6 LD mice on a low-density lipoprotein receptor knockout background were treated with leptin or saline for 8 weeks and compared to non-LD controls.

Results: The number of pups born was 37% lower in breeding pairs consisting of LD female mice x non-LD male mice ($n = 3.3$) compared to LD male mice x non-LD female mice ($n = 5.2$) ($p < 0.05$). Mean uterus weight was significantly lower in the saline-treated LD group (18.8 mg) compared to non-LD controls (52.9 mg; $p < 0.0001$) and increased significantly upon leptin treatment (46.5 mg; $p < 0.001$). The mean number of corpora lutea per ovary was significantly lower in saline-treated LD animals compared to non-LD controls ($p < 0.01$) and was restored to non-LD control levels by leptin ($p < 0.05$). Mechanistically, mRNA expression of ovarian follicle-stimulating hormone receptor ($p < 0.01$) and estrogen receptor β ($p < 0.05$), as well as of pituitary luteinizing hormone β subunit ($p < 0.001$) and follicle-stimulating hormone β subunit ($p < 0.05$), was significantly up-regulated in LD mice compared to non-LD controls. In addition, mean time to vaginal opening as a marker of puberty onset was delayed by 12.5 days in LD mice (50.9 days) compared to non-LD controls (38.4 days; $p < 0.001$).

Conclusions: Female LD animals show impaired fertility which is restored by leptin. Future studies should assess leptin as a subfertility treatment in human leptin-deficiency disorders.

1. Introduction

Obesity is linked with impaired fertility in both male and female subjects. Thus, reproductive function decreases with increasing body fat stores in both genders [1, 2]. Adipocyte-secreted proteins, i.e. adipokines, might play a significant role in the interplay between body fat stores and fecundity. Among those, leptin is a 16 kDa adipokine which not only suppresses appetite but also is a major factor for reproductive function [3, 4]. Thus, leptin-deficient *ob/ob* mice are infertile [5] and Mounzih and co-workers were the first to demonstrate that leptin administration restores fertility in male *ob/ob* mice [6]. Similarly, leptin treatment significantly improves fertility in obese human subjects with inactivating leptin mutations [7].

Besides obesity, decreased body fat stores in patients with hypothalamic amenorrhea [8, 9], anorexia nervosa [10], and lipodystrophy (LD) [11–13] are also linked to impaired fertility. Among those, LD comprises a cluster of rare acquired or congenital disease states leading to subcutaneous adipose tissue disappearance in a generalized or partial manner [14]. Loss of adipose tissue in LD leads to an impaired ability to store triglycerides, reduced adipokine levels, increased insulin resistance, hypertriglyceridemia, and hepatic steatosis [15]. Small clinical studies suggest that female LD patients show various facets of reproductive dysfunction including menstrual irregularities with oligo- and amenorrhea, polycystic ovary syndrome, as well

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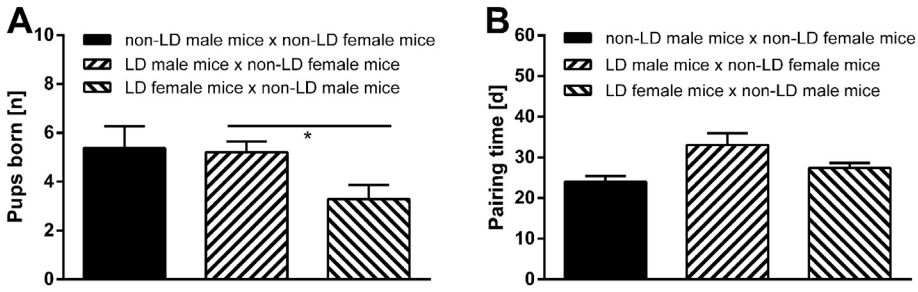

Fig. 1. (A) Number of pups born [n] and (B) pairing time in days [d] of breeding pairs consisting of non-LD male mice x non-LD female mice (Controls), LD male mice x non-LD female mice, and LD female mice x non-LD male mice all on a LDLR$^{-/-}$ background. Data are presented as means ± SEM and represent $N \geq 4$ per group. *indicates $p < 0.05$.

as increased rates for miscarriage and unintended childlessness [11, 13, 16–19]. In contrast, male fertility in LD is not apparently affected [16].

The pathophysiological mechanisms leading to female reproductive dysfunction in LD remain largely unclear. Male and female reproductive function has so far not been characterized in mouse models of LD. Furthermore, treatment effects of physiological leptin doses in LD models have not been assessed. Therefore, aP2-nSREBP1c transgenic mice which are an established mouse model for generalized LD [20], were characterized for the first time with regard to fertility in the current study. Since LD patients are not only at a significantly increased risk for infertility but also for atherosclerotic disease [21], LD mice on an additional proatherogenic LDLR$^{-/-}$ background were used to better resemble the human condition.

Breeding pairs of male or female LD mice were elucidated concerning numbers of pups born and pairing time. Furthermore, female fertility was characterized concerning uterus morphology, ovarian function, gonadotropin expression, and onset of puberty. Male reproductive function was assessed with regard to testis morphology and sperm analysis. We hypothesized that female but not male reproductive function is impaired in LD and that leptin treatment improves fertility in female animals.

2. Materials and methods

2.1. Animal care and treatment

The local ethics committee (Regierungspräsidium Leipzig) of the state of Saxony approved the protocol of all animal experiments (37/12 and 27/16). All treatments were performed in the Medical Experimental Center of the University of Leipzig. All mice were on a C57Bl/6 background. They were maintained in a room under pathogen-free conditions with controlled 21 ± 1 °C on a 12:12 h light/dark cycle (6 AM/6 PM). Eight-weeks-old, generalized aP2-nSREBP1c transgenic, male and female LD mice on a low-density lipoprotein receptor knockout (LDLR$^{-/-}$) background were randomized into two groups and daily treated i.p. with recombinant leptin (3.0 mg/kg body weight [BW]; R&D Systems, Wiesbaden-Nordenstadt, Germany) or saline for 8 weeks. At 3.0 mg/kg BW/d, leptin has physiological effects, i.e. this dose is sufficient to normalize BW in female leptin-deficient *ob/ob* mice (data not shown). Treatment was performed in the morning to imitate the dosing regimen used in LD patients [22]. Non-LD littermates on a LDLR$^{-/-}$ background served as controls. At the end of the treatment period, mice were fasted overnight and the last saline or leptin application was performed 30 min before sacrifice. Plasma samples were obtained. Testes, ovaries, uteri, pituitary glands, hypothalamic, as well as perigonadal and subcutaneous adipose tissues, were removed, weighted, and placed in either formalin or were snap frozen. In male mice, the cauda epididymis and ductus deferens were removed and used for computer-assisted sperm analysis (CASA). Basic characteristics of non-LD control mice, as well as saline- and leptin-treated LD animals, are summarized in Supplemental Table 1.

2.2. Assessment of pairing time, pups born, and reproductive phenotype

To assess fecundity of female LD versus male LD mice, the following three breeding strategies were compared concerning size of first to second litter and pairing time: non-LD male mice x non-LD female mice, LD male mice x non-LD female mice, and LD female mice x non-LD male mice.

In female mice, puberty onset was measured by assessing the age of vaginal opening and first estrus. From day 35 of age, mice were checked daily for vaginal opening based on the criteria described by Champlin and co-worker [23]. After vaginal opening, vaginal cytology was assessed daily until the detection of first estrus. To identify the estrous stage, slides from vaginal smears were stained with May-Grunewald (Carl Roth, Karlsruhe, Germany), Giemsa (Carl Roth, Karlsruhe, Germany), and air-dried. Three different primary cell types were investigated to identify the estrous stage [24, 25]. Vaginal cytology was also assessed immediately before dissection. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were quantified in plasma samples with a commercial multiplex enzyme-linked immunosorbent assay from Merck Millipore (MILLIPLEX Map Mouse Pituitary Kit, Darmstadt, Germany). Plasma leptin (CrystalChem, Zaandam, Netherlands) and testosterone (DRG, Marburg, Germany) were determined according to the manufacturers' instructions. In male mice, CASA was used to assess kinetic and velocity parameters in epididymal semen samples according to Vosoughi and co-workers [26].

2.3. Histological analysis

Uteri and ovaries were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and three slices per animal were stained with hematoxylin/eosin, as well as photographed. Diameter and endometrial area were evaluated in all uteri with Fiji (https://imagej.net/Fiji) [27]. The number of primary and secondary follicles, as well as corpora lutea, in all ovaries was counted by two independent examiners.

2.4. Quantitative real-time RT-PCR analysis

Follicle-stimulating hormone receptor (*Fshr*), luteinizing hormone receptor (*Lhr*), estrogen receptor β (*Erß*), β subunit of luteinizing hormone (*Lhb*), and β subunit of follicle stimulating hormone (*Fshb*) mRNA synthesis was determined relative to *36b4* by quantitative real-time RT-PCR in a fluorescent temperature cycler (Roche, Heidelberg, Germany) as described previously [28, 29]. Primer sequences used are summarized in Supplemental Table 2.

2.5. Data analysis and statistics

Data sets were analyzed using GraphPad Prism 6 (GraphPad Prism Software, San Diego, CA). Values are presented as mean ± standard error of the mean (SEM). Differences were considered significant at $p < 0.05$. To identify significant differences between two groups and more than two groups, unpaired Student's *t*-test and one-way ANOVA followed by post hoc Bonferroni-Holm test were used, respectively.