Effects of diet induced obesity on the gonadal axis in the male rabbit: impact of leptin

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Abstract

It is well established that nutritional status and obesity may disrupt HPG axis function. This work investigates the impact of obesity on changes affecting metabolic parameters, testicular histology and hormones levels of male gonadal axis and the link with leptin and adiponectin levels in two groups of rabbits fed with standard and high calorie diets (HCD) for 15 weeks. HCD increased significantly body, testes and visceral adipose tissue (VAT) weights compared control group and causes lipemia and hyperglycemia. Obese rabbit testis show expanded spaces between seminiferous tubules (ST), while ST diameter, lumen and proportion of sperm were reduced. Lipogenesis in the TS would be a response to the stimulatory effect of hyperinsulinemia (244%) on the production of oestradiol, consecutively to an enhanced aromatization of testosterone. The concomitant increase of plasma leptin, testosterone and FSH seems paradoxical with spermiation impairment and suggests that obese rabbits have developed a leptin resistance whereas adiponectin does not show notable effect. Also, testicular estradiol appears to play a major deleterious role on sperm maturation, without excluding a probable paracrine control by factors such as inhibin B.

Key words: Obesity, spermatogenesis, leptin resistance, testosterone, estradiol

1. Introduction

The reproductive function was closely related to nutritional and energy status of the organism and the control of reproductive capacity in species such as rabbit, since the amount of calories ingested could to disrupt the gonadal axis function. Indeed, it is well established that obesity induces dysfunction of the hypothalamic-pituitary gonadal axis (HPG) and contributes to infertility in
males. In obese humans, blood levels of testosterone and SHBG were decreased (Haffner et al., 1993; Derby et al., 2006) and total sperm count and sperm concentration were inversely related to BMI (Mah and Wittert 2010; Paasch et al., 2010), while rats fed high fat diet (HFD) show a decrease of sperm motility (Fernandez et al 2011) and gonadotropins secretion Olivares et al., 2010). Similarly, insulin resistance resulting in obesity contributes to reduce hepatic production of SHBG (Plymate et al., 1988; Hautanen, 2000). Adipose tissue also is involved in the regulation of energy homeostasis as an endocrine organ secreting adipokines such as leptin, adiponectin, resistin and tumor necrosis factor alpha (TNFα) that regulate satiety, insulin sensitivity and inflammation (Landry et al., 2013). Leptin, encoded by ob gene regulates the activity of HPG axis and acts on target tissues through leptin Ob receptor (Ob-R). In humans, leptin is expressed in TS and sperm (Ishikawa et al., 2007) while in rodents, Ob-R is isolated from Sertoli, Leydig and germ cells (Caprio et al., 2003, El-Hefnawy et al., 2003). Subjects with congenital leptin deficiency developed early-onset obesity, hyperphagia, hypogonadotropic hypogonadism and delayed puberty (Clement et al., 1998, Strobel et al., 1998), whereas overproduction of leptin in obese men contributes to an androgen deficiency (Isidori et al., 1999). The low testosterone level in obese subjects is due to a decrease in the binding capacity of SHBG and/or to a direct action of leptin and adiponectin on testicular function (Caprio et al., 2003; Caminos et al., 2008). Indeed, the ability of leptin produced in other organs to cross the blood-testis barrier suggests an action on the testis by endocrine and/or paracrine/autocrine mechanisms (Banks et al., 1999). Our purpose is to study the impact of a high-calorie diet that may affect reproductive performance such as the morphology of the testis, hormonal secretions of gonadal axis and their relationship with the metabolic hormones: insulin and leptin.

2. Materials and Methods

2.1 Animals

Sixteen male rabbits 3 months-old were purchased from Technical institute des petits élevages (ITELV, Baba Ali, Algeria), housed in individual cages and were given 150g/day of manufactured solid grain (STCO corp, Sig, Algeria) for ten days of acclimation under natural luminosity, room temperature (18-23°C) and hygrometry (70-75%). This diet provided 258 kcal/day, from 8% fat (fat dried fodder + oilseeds), 10% protein (cereals grains) and 15% carbohydrate, with additive vitamins (table I).

2.1.1 Experimental Design

After the adaptive period, rabbits were randomly assigned to two groups: lean (control) and obese (HCD). The experimental design was performed by giving the control group the same diet as in the acclimation phase. The rabbits in obese group consumed a high calorie diet (15% fat, 10% protein, 32% carbohydrate) that provided 454 kcal/day (table I). Body weight changes were estimated weekly. After 15 weeks of feeding, the rabbits were food-deprived for 12h to insure that there was no exogenous food entry. The animals were sacrificed between 9 and 10 am.

Table I: Composition of standard and high calorie diet (HCD).

<table>
<thead>
<tr>
<th>Constituants</th>
<th>Standard diet</th>
<th>HCD</th>
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</thead>
<tbody>
<tr>
<td>Proteins (cereal seeds)</td>
<td>10 g</td>
<td>10 g</td>
</tr>
<tr>
<td>Lipids (Fat dried fodder + oilseeds)</td>
<td>8 g</td>
<td>15 g</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>15 g</td>
<td>32 g</td>
</tr>
<tr>
<td>Energy input/150g</td>
<td>258 Kcal</td>
<td>454,5 Kcal</td>
</tr>
</tbody>
</table>

Additive vitamins

| Vitamin E/kg                      | 11 mg         |
| Vitamin D3/kg                     | 2530 KUI  |
| Vitamin A/kg                      | 11440 KUI  |
| Copper sulfate/kg                 | 11 mg         |
Table 1 shows the amount of ingredients in 150g of both standard and high calorie diets, using McReady (1950), Bradford (1976) and Bligh et Dyer (1959) assay methods to estimate carbohydrates, proteins and total lipids respectively. Considering the equal amounts of additive vitamins in both diets, the estimation of total energy intake in each group is based on the caloric value: 4kcal per g for proteins or carbohydrates and 9Kcal per g for lipids:

2.1.2 Collection of blood and tissues
The blood was removed on EDTA, centrifuged and plasma was frozen under -25°C until assay time. Visceral adipose tissue (VAT) from abdominal cavity, mesenterium and retroperitoneal fat and testis were dissected and weighted. The right testicle was crushed and mixed in 1 ml of distilled water, then frozen in liquid nitrogen and stored at -80°C until hormone assays.

2.1.3 Histology
Structural changes of left testicle were analyzed on 4μm sections, after Bouin-Hollande fixation step and Masson’s trichrome staining. Analysis of spermatogenesis using photomicroscope Motic consists in estimating seminiferous tubules (ST) and lumen diameters and counting within each section the number of ST characterized by the same final stage of spermatogenesis. Estimations were performed on ten selected sections per testis.

2.2 Biochemical and hormonal assays
Plasma levels of glucose, triglycerides (TG), total cholesterol (Tchol), HDL-c. were estimated using kits BioMaghreb, Elitech and Spinreact, while plasma LDL-c was deduced from the formula LDL-c=Tchol–(TG/5+HDL-c). The Homeostasis model assessment index (HOMA index) for insulin resistance diagnosis was calculated using the formula:

\[
\text{HOMA Index} = \frac{\text{Fasting plasma Insulin (mIU/L)}}{\text{Fasting plasma Glucose (mmol/L)}}
\]

Testosterone, estradiol, LH, FSH and insulin levels were determined using RIA Kit protocols (Immunotech SAS, Marseille, France), while leptin and adiponectin were measured by Elisa method (DRG Diagnostics, GmBH, Germany).

(i) The animals were treated according to the precautions stated in the Guide for the Care and Use of Laboratory Animals (http://www.ccac.ca), (ii) The Executive Decrees of the Algerian Ministry of Agriculture and Rural Development No. 88-252 of 31 December 1988 laying down the conditions for the exercise of medical activities, veterinary and animal surgery, and No. 10-90 of 10 March 2010 on the conditions and modalities of health approval and transport of animals and animal products(http://www.minagri.dz/bulletin_officiel.html)

2.3 Statistical analysis
For all parameters, differences between control and obese groups (mean ± sem) were analyzed using Statistica6.0 software for Student t and considered significant if p<0.05.

3. Results and Discussion
3.1 Body and tissues weights.
After 15 weeks of feeding, the mean average body weight increased by 19.4 % (3.69 ±0.112 kg, p = 0.0005) (Fig.1), the average absolute weight of the testis and TAV (PT) are higher by 53.2 % (p = 0.01) and 23.3% (p = 0.03) than controls (Table II).

Table II: Changes in visceral adipose tissue (VAT) and testis absolute and relative weights in control (n=8) and obese rabbit after 15 weeks of feeding (*, p<0.05; ***, p<0.001).
The enriched high-calorie diet (HCD) composed of lipids and carbohydrates led to (develop) the development of a visceral obesity, characterized by a significant increase in body weight, VAT and testis associated to dyslipidemia, hyperglycemia and hyperinsulinemia. Obesity was also induced in the rabbit with HFD or HFD enriched in sucrose (HFSD) (Zhao et al., 2007; Dhungel et al., 2009; Olivares et al., 2010). Conversely, obese rats fed a high fat diet (HFD) for 15, 30 and 45 weeks show no difference in testis and accessory reproductive organs weights, compared to control group (Fernandez et al., 2011).

### 3.2 Biochemical parameters

The obese group shows hyperglycemia (HG) (50%, p < 0.05), associated to dyslipidemia, characterized by significantly higher levels of TG, total cholesterol and LDL-cthan control group, whereas HDL-c shows no significant decrease (table III). The increased VAT weight in obese rabbit and mesenteric and retroperitoneal fat also observed by Dhungel et al., 2009) has been attributed to hypertrophy and hyperplasia of adipocytes (Kitajima et al 2004) consecutively to free fatty acids (FFA) and TG accumulation (Zhang et al., 2008). Free fatty acid (FFA) that support 30 to 50% of basal insulin secretion and potentiate its secretion in response to glucose (Santomauro et al., 1999), could be the cause of hyperinsulinemia and hyperglycemia. In addition, Homa index is 2.28 times higher than that of control group (4.074 vs 1.78), which suggests that the obese group likely developed an insulin resistance. The favorable prognosis of insulin resistance should be confirmed by oral glucose tolerance test (OGTT).

#### Table III: Changes in blood biochemical parameters in control (n=8) and obese rabbit (n=8) after 15 weeks (*, p< 0.05)

<table>
<thead>
<tr>
<th></th>
<th>Glucose (g/L±sem)</th>
<th>T Cholesterol (g/L±sem)</th>
<th>TG (g/L±sem)</th>
<th>HDLc (g/L±sem)</th>
<th>LDLc (g/L±sem)</th>
<th>HOMA Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>1.23 + 0.05</td>
<td>0.48±0.048</td>
<td>0.16±0.008</td>
<td>0.22±0.04</td>
<td>0.22±0.05</td>
<td>1.78</td>
</tr>
<tr>
<td>Obese (n=8)</td>
<td>1.85± 0.17 *</td>
<td>0.99±0.135 *</td>
<td>0.32±0.05</td>
<td>0.16±0.01</td>
<td>0.76±.129 *</td>
<td>4,074</td>
</tr>
</tbody>
</table>

### 3.3 Structural changes in the testis

Testis of the control rabbits shows condensed ST next to the tunica albuginea, separated by very small inter tubular spaces, and an expanded tubular lumen (Fig 2a-c). In obese animals, inter tubular spaces are more abundant, appearing as clear spans (Fig. 2b-d). TS and tubular lumen diameters decreased by 7.6% and 43.3% (p< 0.01) but the height of seminiferous epithelium is increased by10.7% (p>0.05) (Fig.2 B). In obese, 58% of the ST areat the spermatocyte stage, while only 13% of the ST contain spermatozoids (Fig. 2 e-f and, Fig 2 C).
Figure 2: Morphological changes (A) in the testis of control rabbits (a: Gx40, c: x100, F: e: x400) and obese rabbit (b: Gx40; d: Gx100, f: Gx400) using Trichrome Massons’s staining (Al: albuginea; St: seminiferous tubules; IT: interstitial tissue; BL: basal lamina; Sp: spermatogonia; RSp: round spermatids; ESp: elongated spermatids; Sp: sperm; SC: Sertoli cell; ITS: intertubular space; LC: Leydig cells). Height and diameter changes in seminiferous tubules (B) and analysis of germ cells proportions during spermatogenesis (C).

3.4 Hormones of the pituitary-gonadal axis

In the obese group, LH rises slightly (11 %) compared to the control group (0.017 ± 0.3 mIU/ mL, p=0.43), while FSH increases significantly by 102.8 % (45 ± 9 , 67 mIU /mL , p = 0.03) (Fig. 3A-
B). Plasma testosterone was higher in obese by 174% (2.11 ± 0.79 ng/mL, p = 0.09), but its level was lower in the testis by 32.14% (42.24 ± 10.81 ng/g PT, p = 0.22) (Fig. 3 C-D). Plasma estradiol (71.979.7 pg/mL) and testis levels (10.63 ± 1.03 ng/g tw) were higher by 102.7% (p = 0.022) and 34.5% (p = 0.044) than control group (Fig. 3 E-F).

![Figure 3](image)

**Figure 3**: Impact of standard and HCD–induced obesity on plasma gonadotropins (A-B) and on plasma and testis levels of testosterone (C-D) and estradiol (E-F).

### 3.5 Metabolic hormones

In obese group, plasma leptin (2.29 ± 0.89 ng/mL, p = 0.048) and insulin (41.84 ± 8.26 μUI/mL, p = 0.022) were significantly higher by 231% and 244% respectively compared to the control group (Fig. 4 A-B).

![Figure 4](image)

**Figure 4**: Impact of standard and HCD-induced obesity on plasma insulin and leptin in the control (n=8) and obese group (n=8).
On the other hand, the accumulation of lipids in the intertubular spaces could be due to a local edema which caused the increase of the testicular weight and led to the development of a "fat testis". The pressure on the basal lamina may play a major role on decreasing both ST and tubular lumen diameters. The high level of testicular estradiol in obese rabbits seems to regulate this accumulation through the activity of cytochrome P450 aromatase (Hemsell et al., 1974, Simpson et al., 1997). Indeed, hyperinsulinemia may stimulate the production of testosterone (Adashi et al., 1982); a fraction of the testosterone is aromatized to estradiol, while the other fraction is released into the blood circulation leading to a non-significant increase of plasma testosterone and subsequently a reduction of the testicular testosterone level. The testis contributes to 15 % of circulating estrogens (Hemsell et al., 1974) and their physiological action in human adipocytes is exerted mainly via ER\(\alpha\) than ER\(\beta\) receptors (Dieudonne et al., 2004), and /or in combination with G-protein coupled receptors (GPER) (Prossnitz and Barton, 2009). The ER\(\alpha\) receptor expressed in stromal cells causes a cell proliferation unlike the Er\(\beta\) receptor which is abundantly expressed in epithelial cells (Ricke et al., 2007; Korak and Prins, 2008). The initiation of adipogenesis requires the production of the transcription factor peroxisome proliferator-activated receptors gamma (PPAR\(\gamma\)) that induces the expression of specific genes in the adipocyte. During the induction of adipogenesis by troglitazone, estradiol inhibits PPAR\(\gamma\), as well as adipocute protein 2 (aP2) and lipoprotein lipase (LPL) genes expression, involving the coactivator cAMP Responsive Element Binding Protein (CREB-BP) (Jeong Yoon, 2011). Similarly, adipocyte differentiation and maturation are inhibited by genistein and estradiol through an ER-dependent mechanism (Okazaki et al., 2002; Park et al., 2009). However, the concomitant of the high levels of leptin, testosterone and FSH contradict the literature data, knowing that testosterone rise was not significant. Indeed, in obese men, hyperleptinemia has a deleterious effect on the Leydig cells steroidogenesis (Isidori et al., 1999) by decreasing the expression of genes encoding SF-1, StAR, P450scc and Ob-Rb (Tena-Sempere et al., 2001; Caprio et al., 2003). In addition, the low proportion of the ST at the spermatozoids stage in the obese rabbits seems to be paradoxical, considering the mitogenic effect of leptin on germ cells (Bhat et al., 2006) and the synergic action of FSH and testosterone on their complete maturation (Allan et al., 2004; Ruwanpura et al., 2010). Our results also contradict the reported data in rats fed HFD during 90 and 180days that showed a concomitant increase of plasma leptin, insulin and estradiol, plasma FSH decreased after 90days while both LH and testosterone levels were reduced after 180days. In addition, in chromatofocusing of pituitary extracts a trend towards decreased abundance of more basic immunoreactive LH isoforms (pH 9.99–9.0) was apparent in rats fed with the control diet for 180 days but not in those fed the diet enriched in saturated fat (Oliva et al., 2010). Our results also were partially comparable to those reported in rats fed HFD during 15, 30 and 45 weeks, with increased plasma leptin and estradiol, but both control and HFD group show similar levels of testosterone , whereas the sperm quality as well as its motility were altered, and in utero insemination leads to a reduced fertility (Fernandez et al., 2011). The controversial results suggest that obese rabbits have probably developed a leptin resistance, which results in a low sensitivity of the HPG axis. Leptin acts on NPY/AgRP neurons, and POMC in the arcuate nucleus to regulate the GnRH release (Hill et al., 2008), or on the kisspeptin secretion which stimulates the release of LH and FSH probably through an NO synthase activation (Gottsch et al 2004). The altered sensitivity of NPY/AgRP and POMC neurons to leptin has been attributed to pSTAT3-SOCS3activation (Eniori et al., 2007). Therefore, the low proportion of sperm may be mainly related to the increase of testicular estradiol that would play a deleterious role on the spermatogenesis and on the testicular environment (Oliva et al., 2001; Akingbemi 2005). Secondly, as the low level of inhibin B level was observed in obese men (Pauli et al., 2008), the spermiation impairment seems to be linked to a paracrine mechanism, which is due to a change in the secretory activity of Sertoli cells. Thirdly, our investigations on the same rabbit groups have shown that HCD caused a nutritional stress and the stimulation of the adrenal axis in the obese rabbits leading to a significant rise of plasma ACTH and cortisol, thus contributes in the gonadal axis dysfunction.
We conclude that HCD-induced obesity reduced reproductive parameters, and mainly contributes to affect the gonadal axis sensitivity to leptin. Leptin resistance in the rabbit is probably related to the intrinsic physiology of the animal, compared to the reported data on other species such as rats. Therefore, increased synthesis of testicular estradiol appears to play a major "alternative" role in the intracrine regulation, by exerting a deleterious effect on the sperm maturation. The paracrine action is also involved in this regulation through factors secreted by Sertoli cells, such as inhibin B, that contribute to reduce the reproductive capacity in the breeding male rabbit.

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4. References


