Leptin-deficient obese mice: a multi-organ stress model rescued by melatonin

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Obesity is an epidemic health concern in Western countries that strongly involves social and sanitary costs and must be urgently fought. Different animal models of obesity are crucial in basic research to provide information on multifactorial pathogenic mechanisms and pathways useful to develop clinical guidelines. In this chapter we described, through the use of light and transmission electron microscopy, hepatic and renal changes induced in genetically obese (ob/ob) mice characterized by the ablation of leptin gene, a hypothalamic neuropeptide involved in the regulation of food intake and metabolic turnover. In detail, we characterized fibrosis, steatosis, inflammation, and obviously mitochondrial and endoplasmic reticulum stress in ob/ob and B6.V-(lean)/Ola Hsd mice as lean control group. Histopathological (haematoxylin eosin and Azur II-Methylene blue stainings), immunohistochemical (anti-SREBP1, anti-GRP78, anti-CHOP) and ultrastructural analysis greatly indicate that liver, in genetically induced obese mice, is more oriented on macrosteatosis, whereas in the kidney mitochondrial fission is evident. Melatonin intake in drinking water for 8 weeks significantly reduced steatosis, endoplasmic reticulum stress and mitochondrial alterations in ob/ob mice at the level of these two crucial organs for the pathogenic process of obesity. These microscopic findings suggested that melatonin intake may be an effective dietary addition to mitigate obesity.

Keywords: leptin-deficient mice; fatty liver; kidney; mitochondria; TEM

1. Introduction

Metabolic syndrome (MS) is a multifactorial disease involving crucial organs, like brain, heart, liver and kidney, and it is greatly associated to overweight, wrong alimentary habit and sedentary style of life [1-2]. MS is evident in adults and children in Western countries and spreads also in the Eastern Mediterranean area when wrong alimentary habits or lifestyle occurred [3-5], despite the beneficial properties of the traditional cuisine, recognized as an UNESCO intangible cultural heritage [6]. A recent epidemiologic European study demonstrated that, in subjects between 50 and 75 years of age, eliminating smoke and reducing body weight may possible to live 8 years longer and in good health [7]. By contrast, type 1 and 2 diabetes, vascular complications, dementia and cancer are the most important chronic diseases related to MS [8-9].

Despite current limitations on animal experimentation by the European legislation that reduces the use of laboratory animals according to the “Three Rs principle”, i.e. Replace, Reduce, Refine (Directive 2019/63/EU from 1 January 2013), metabolic translational studies still base on in vivo models to best correlate multi-organ changes to the dietary benefit. Obesity animal models that reproduce MS events are heterogeneous and based on diet composition, pharmacological triggers, or on a genetic background [10]. We focused here on leptin-deficient obese (ob/ob) mice obtained by a single autosomal recessive mutation of leptin gene (chromosome 6) and that, due to the complete lacking of leptin signalling, develop hyperphagy, hyperglycemia and hyperinsulinemia, but not circulatory changes, so they are mainly used to study obesity and diabetes. Leptin is a polypeptide synthesized mainly in mature white adipocytes and, in less amount, in brown adipose tissue, skeletal muscle, stomach and placenta, that is then taken into the hypothalamus, where it regulates food intake and energy homeostasis [11-12]. This genetic rodent model has important characteristics similar to human obesity, like overweight and insulin resistance, but it is better considered as an “early-extreme obesity model” [13], because in humans metabolic alterations occur throughout all lifespan with increasing likelihood in advanced age. Obesity, estimated by a body mass index (BMI-obtained by the ratio between the weight in kilograms and the square of the height in meters) > 35 is an index of metabolic dysfunctions in children/adolescents that will become obese when adults, and so exposed to cancer, cardiovascular and neurodegenerative diseases development [14-15].

Besides pharmacological or surgical interventions [16], nutrition, associated to exercise and a healthy life style, are greatly involved in the control of obesity and interesting new reviews and research articles have been produced on this topic [17-19].

Melatonin (5-methoxy-N-acetyltryptamine) (MEL) is an indolamine, synthesized from the essential amino acid tryptophan, in the pineal gland, able to regulate circadian rhythm but actually considered a multi-tasking beneficial
compound with important anti-oxidant, anti-inflammatory and anti-aging effects [20]. Nevertheless, MEL is produced also in extra-pineal sites like the gastrointestinal tract by gut endocrine cells and stimulated by intake of vegetables, caffeine, some vitamins and minerals [21]. Different research teams studied MEL supplementation in obesity-driven dysfunctions in vitro and in vivo [22-24]. Recently, our research group demonstrated that MEL, delivered in rodents in drinking water, had anti-aging, anti-oxidative, anti-apoptotic and anti-inflammatory properties and concurred to reduce atherosclerotic changes and perivascular fat in hypertension [25-26]. Due to its amphipathic nature, MEL easily crosses all biological membranes and settles into the endoplasmic reticulum (ER) and mitochondria where it acts as an effective reactive oxygen species (ROS) scavenger [27]. MEL intake for a long time (at 100 mg/kg/day for 8 weeks) has been previously proven to be devoid of side-effects [28]. In fact, MEL treatment at a dose from 1 to 300 mg per day was considered safe both in humans and rodents [29]. Interestingly, among beneficial MEL actions, there is the modulation of insulin signalling and glucose uptake, that is greatly common in obese humans [30]. An extensive review by Hardeland [31] correlated MEL action to the modulation of metabolic rhythms and sensors in obesity, diabetes and cancer. Remarkably, it must be considered the high nocturnal metabolism of rodents respect the high diurnal activity in humans, and these different activities may be variably influenced by MEL.

Since a long time we used MEL to treat or prevent different pathophysiological conditions like cardiovascular diseases and age-related diseases/alterations [32-33], but in the last five years, we centred our interests on the role of MEL in metabolic diseases. To date many efforts are made to define pathogenetic mechanisms in overweight-related dysfunctions and a strict relationship between adipose tissue and other organs such as gut, liver, heart, kidneys emerged [34]. In a recent study we demonstrated that MEL reduced pro-inflammatory adipokines and stimulated anti-inflammatory signals in ob/ob adipocytes [35]. Indeed, adipocyte hypoxia firstly triggered inflammation and adipokine flux, thereafter involving other organs such as the liver and heart [36-37]. Obesity chronically affected mitochondria size and efficiency, perpetuated ER stress and altered communication sites called mitochondria-associated membranes (MAMs) [38]. In this microscopically oriented chapter, we describe histopathological changes in liver and kidneys, together with evidence of ER stress, in leptin-deficient (ob/ob) mice supplemented or not with MEL. We mainly pointed to analyse steatosis, mitochondria damage and mitochondrial alterations, ER and related stress markers.

2. Liver in leptin-deficient obese mice

The epidemic diffusion of obesity is deeply associated with a common liver alteration called non alcoholic fatty liver disease (NAFLD), without excessive alcohol consumption in humans. To best analyse the pathogenetic changes at the liver level in this dysfunction, a lot of preclinical models have been used, but ob/ob mice are the most studied. It is important to outline that in the last decade, histological analysis of haematoxylin-eosin stained liver sections was the most important diagnostic indicator for NAFLD and that a scale reporting steatosis, hepatocytes apoptosis, ballooning, fibrosis or necrosis was a reliable instrument for any pathologist [39]. Actually NAFLD treatment is directed to weight loss by diet or exercise or to the improvement of insulin resistance, but a pharmacological therapy is still under study [40]. In our study, we fed ob/ob mice with MEL and checked its effects on the liver, starting from the analysis of lipid droplets in semithin sections to best detect macrosteatosis and microsteatosis. We strongly suggested to use this high resolution-technique in every specialized laboratory or University centre where is present a transmission electron microscopy (TEM) facility. Remarkably, we observed panlobular lipid droplets distribution starting from the perivenous zone in ob/ob mice without or with MEL intake at 100 mg/kg/day, as showed in Figure 1.

![Figure 1](http://example.com/figure1.png)

**Fig.1-** Semithin liver section micrographs stained by Azur II-Methylene blue mixture. **A**) ob/ob mice liver with macro and mediovesicular steatosis. **B**) ob/ob mice supplemented with melatonin for 8 weeks. Note the reduced overall steatosis and the residual microsteatosis in the parenchyma. Original magnification 100x

In the liver of genetically obese mice large-size lipid droplets (i.e. macrosteatosis) were scattered in hepatocytes from the pericentral to the perportal zone. Indeed, it is important to consider that liver zonation is linked to circulation flux and it strongly influenced oxidative and glycolitic metabolism and so the “favourite” area for lipid accumulation is
the perivenous zone 3 [41]. The liver lipid droplets of ob/ob mice treated with MEL showed a reduced diameter and were distributed mainly in the pericentral and midzonal area. It is important to outline that large lipid droplets affected oxygen delivery into hepatocytes and mimicked a hypoxic status. Indeed in metabolically-active organs like liver and skeletal muscles, lipid droplets represented the storage of neutral lipids like triacylglycerol or cholesterol esters, but a wrong balance between tissue and circulating lipids induces disorders like insulin-resistance, diabetes, inflammation and hepatosteatosis [42]. The term “lipotoxicity” indicates the dangerous effects of lipid droplets accumulation and of excessive accumulation of fatty acids inside cells that overwhelmed their oxidative ability [43-44]. In steatotic obese liver, large lipid droplets expressed a surface marker called perilipin 1, which is regulated by the most active genes to produce an irreversible damage progression [45]. Moreover abnormal lipid droplets deposition in leptin-deficient mice liver is promoted by genes able to influence metabolic sensors like peroxisome proliferator activated receptor gamma (PPAR gamma), a member of nuclear receptor family that stimulated peroxisome proliferation in response to xenobiotics [46-47]. A fascinating new avenue in the metabolic research is the crosstalk between different key organelles and the active exchanges of proteins and metabolites. Recently, high resolution imaging revealed that peroxisomes are associated with lipid droplets and other organelles like mitochondria and ER in a tissue-specific manner [48]. So we focused on mitochondria and rough ER (RER) as peculiar targets of cellular dysfunctions in leptin-deficient mice. The best tool to show the shape and organization of inner cristae is the ultrastructural analysis of mitochondria in situ [49]. We observed ultrastructural changes in the hepatocytes in the pericentral zone 3 and representative images of mitochondria in different experimental groups are collected in Figure 2.

![Ultrastructural feature of hepatocytes and mitochondria in leptin-deficient ob/ob mice liver](image1.png)

**A)**  
In ob/ob mice fed a normocaloric maintenance diet, the majority of hepatocytes was engulfed by lipid droplets and mitochondria were heterogeneous and located in the cell periphery or around the nucleus (**Fig. 2A**). At higher resolution, the mitochondrial cristae, active sites of oxidative enzymes, appeared poor, the inner mitochondrial membrane irregular and matrix were electron-dense (**Fig. 2C**). In a previous study we demonstrated that the expression of the mitochondrial enzyme ATP-synthase subunit beta decreased in ob/ob mice but was restored by MEL intake [50]. Remarkably, in the liver of ob/ob mice supplemented with MEL, it is evident the reduction of lipid droplets and the prevalence of abundant mitochondria with an elongated shape and well-defined longitudinal cristae (**Fig. 2B-D**), all signs of restored oxidative ability. The liver mitochondria may be of different size and shape and these changes are strictly linked to their activity in pathological conditions [51]. Indeed fission and fusion are two opposite processes that regulate mitochondrial energy demand and cell death [52]. Generally in dietary-induced obese liver the prevalence of small and round mitochondria has been linked to a decrease in respiratory capacity [53]. The liver of “generated” mice
lacking the fission marker dynamin related protein 1 (DRP1) is characterized by reduced steatosis and body weight under an obesogenic diet [54]. However, in the liver, besides peroxisomes and mitochondria, ER is another crucial site involved into metabolic diseases. Indeed ER is the main site of protein folding and sorting, so when it is engulfed by too many proteins or by an altered oxidative pathway, it was unable to perform its function and an unfolded protein response (UPR) occurred. In chronic diseases like obesity, UPR persisted and became inefficient, so an ER stress occurred. Intriguingly, ER stress has been reported in rodent and human obesity and in NAFLD [55-56].

From a morphological point of view a significant ultrastructural change important to measure is the proper distance between ER and mitochondria and their contacts called MAMs [57]. Interestingly, MEL intake was able to change and maintained the regular distance between these two organelles, particularly reduced in ob/ob mice, so confirming its potent hepatoprotective role.

3. Kidney in leptin-deficient obese mice

Actually obesity and related diabetes mellitus have been defined as a unique syndrome called “diabesity” that is a rising epidemic cause of chronic renal diseases [58]. Glomerular damage, proteinuria and proximal tubular alterations have been morphologically described in different models of obesity in rodents [59]. Indeed, systemic lipid overload, defined “lipotoxicity,” can induce inflammation and oxidative stress, crucial mechanisms underlying the MS [60-61]. Similarly to the liver, also in the kidney an optimal way to describe steatosis and its potential reduction after MEL intake may be through the analyses on semithin sections. So we inserted representative images of ob/ob mice kidney without or after MEL intake for 8 weeks (Figure 3).

![Figure 3](image)

**Fig.3-** Light micrographs of renal proximal tubules - Azur II-Methylene blue staining. **A)** The renal proximal tubules of obese mice showed evident lipid droplets and disrupted luminal brush border. **B)** Renal proximal tubules in obese mice orally supplemented with melatonin. Note less lipid droplets and regular nuclei. Semithin (1µm-thick) sections. Original magnification = 100x. **C)** Quantitative analysis of lipid droplets area (expressed as percentage) in proximal tubules in different experimental groups, * significant p<0.05 vs control group # p<0.05 vs ob/ob mice.

Furthermore, we quantified the percentage of area occupied by lipid droplets at proximal tubules level in obese mice and estimated a reduction of about 20% after MEL intake. We also observed proximal tubular alterations because this site in the nephron has been dramatically involved in oxidative and ER stress in lipotoxicity [62]. It is well-known that the proximal tubules are characterized by the presence of elongated mitochondria oriented inside the basal membrane foldings to provide energy and to best reabsorb the glomerular ultrafiltrate. A complex morphological analysis of proximal tubules in mice demonstrated that mitochondrial density was about 44%, but in humans only 16% [63]. This important finding indicated that in small mammals, like mice, cortical renal proximal tubules had a higher metabolic rate then in humans.
In particular in ob/ob mice renal proximal tubules, we reported mitochondrial fission and apoptosis and a significant attenuation after MEL intake [64]. Albuminuria, insulin resistance and hyperglycemia have been documented in leptin-deficient mice and proteinuria triggers ER stress response in renal tubules [65]. Moreover, microscopic analysis of ER stress by immunostaining with specific antibodies anti-glucose regulated protein 78 kDa (GRP78) and anti-transcription factor CCAAT-enhancer binding protein homologous protein (CHOP), indicated a strong altered expression partially recovered by MEL intake.

In Table 1 we resumed the trend of ER stress markers in different experimental groups. Lean group supplemented with MEL showed immunoreactions similar to lean mice and so we refer to both animal groups as “lean control”.

Table 1 – Endoplasmic reticulum (ER) stress markers expression in lean, obese (ob/ob) and obese plus melatonin (MEL) mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lean</th>
<th>ob/ob</th>
<th>ob/ob + MEL</th>
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<tbody>
<tr>
<td>GRP78 Glomeruli</td>
<td>(-)</td>
<td>(+)</td>
<td>(+/−)</td>
</tr>
<tr>
<td>GRP78 Tubules</td>
<td>(+)</td>
<td>(+++)</td>
<td>(+)</td>
</tr>
<tr>
<td>CHOP Glomeruli</td>
<td>(-)</td>
<td>(+)</td>
<td>(+/−)</td>
</tr>
<tr>
<td>CHOP Tubules</td>
<td>(-)</td>
<td>(+)</td>
<td>(+/−)</td>
</tr>
</tbody>
</table>

Legend: (-) absence; (+) increase; (+++) marked increase; (+/−) decrease

In a recent study in diabetic mice receiving tauroursodeoxycholic acid, a drug able to modulate ER stress, proximal tubular injury was attenuated [66]. Similarly, anti-oxidant and anti-apoptotic effects have been demonstrated in different nephrotoxic models after MEL intake [67-68]. In obesity-related renal diseases some proteins able to regulate triglycerides synthesis seemed to act as reliable therapeutic targets [69]. One of these targets is the sterol regulatory element binding protein (SREBP1), involved in the lipid deposition, fibrosis and glucose signalling in obese mice.

According to Jun et al [70], SREBP1 immunostaining enhanced in proximal tubules in diabetic mice due to higher lipid deposition. In contrast, in SREBP1 knockout mice, the effects of obesity are strongly limited [71]. Here, we detected the renal expression of SREBP1 in leptin-deficient mice. As demonstrated in Fig. 4A in the ob/ob mice kidney, glomeruli were poorly stained, but a strong signal was evident in cortical proximal tubules. In contrast, after MEL intake, SREBP1 immunostaining was reduced in intensity and tubular localization, corroborating the beneficial effects of MEL in limiting lipid accumulation (Figure 4B).

Fig. 4 - A) Representative light micrograph of SREBP1 immunostaining in obese mice kidney. Note strong signal in proximal tubules but almost absent in the glomerulus. B) Reduced tubular SREBP1 staining in obese mice kidney after melatonin intake. Original magnification 40x.

4. Conclusions

In our opinion, main considerations on obesity in leptin-deficient mice may be resumed into two points. Firstly, the absence of leptin signalling strongly affected steatosis and mitochondria shape as demonstrated by an integrated microscopic approach, and indicated its fundamental involvement in lipid distribution and energy consumption according to Hsu et al [72]. Second, exogenous MEL intake effectively reduced abnormal mitochondria shape and ER tethering, renal and hepatic steatosis and the expression of ER stress markers in genetically obese mice. We, and others,
strongly believe that this indoleamine may limit the progression of obesity and associated metabolic dysfunctions [73-74].

Despite the emerging imaging of mitochondria dynamic by super-resolution fluorescent microscopy [75], we strongly recommend to consider the ultrastructural techniques to answer complex questions also in the metabolic research.

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