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Melatonin protects female rats against steatosis and liver oxidative stress induced by oestrogen deficiency

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ABSTRACT

Aims: Melatonin has been shown to protect cells against oxidative and inflammatory damage via endocrine, paracrine and autocrine actions. Postmenopausal condition is associated with a high incidence of many features of metabolic syndrome including obesity, steatosis and liver oxidative injuries. The aim of this work was to investigate whether treatment with melatonin improves metabolic disturbances associated with oestrogen deficiency in ovariectomised (OVX) rats.
Main methods: OVX and control (CON) female rats were treated with melatonin (10 mg/kg × day for 3 weeks, p.o.). Body weight gain, adiposity index, plasma biochemical parameters, liver lipid content, hepatic mitochondrial respiration, fatty acid oxidation and mitochondrial H_2O_2 generation and the activity of the most important enzymatic and non-enzymatic reactive oxygen species (ROS) scavenger systems were measured.
Key findings: In OVX rats, melatonin suppressed lipid accumulation and cellular oxidative stress in the liver. There was a reduction in the levels of carbonylated proteins in the mitochondria and cytosol, reduction in the malondialdehyde contents in the liver homogenates, stimulation of cytosolic glutathione peroxidase and glutathione reductase activities and restoration of reduced glutathione contents to normal levels.
Significance: Exogenous melatonin protects the liver of OVX rats against steatosis and cellular oxidative stress, possibly via activation of antioxidant enzymes related to glutathione metabolism and by a direct radical scavenging activity.

1. Introduction

In mammals, melatonin (5-methoxy-*N*-acetyltryptamine) is mainly synthesised in the pineal gland and is secreted during darkness as a hormonal message of the photoperiod [1,2]. Melatonin synthesis also occurs in numerous peripheral organs and tissues such as the retina, liver, kidney, endothelial cells, skin, placenta, endometrium, bone marrow and lymphocytes [3–5]. The broad spectrum of melatonin sources and the presence of melatonin receptors in numerous tissues of different species suggest that melatonin plays an essential role in homeostatic control and cell survival [5,6]. Melatonin has been shown to protect cells against oxidative and inflammatory damage via endocrine, paracrine and autocrine actions [5–8]. It has been reported its involvement in neuronal homeostasis [8], modulation of immunity, cardiovascular responses [7], control of blood pressure [9] and regulation of retinal physiology [10]. Therefore, in addition to its beneficial effect in the treatment of sleep disturbances [4,6], and because of the low toxicity of melatonin in the concentration range of 1 to 200 mg/kg daily [11], melatonin has been studied for various other pharmacological purposes [4,7,12–14].

A hepatoprotective effect of melatonin in suppressing steatosis and oxidative stress has been shown both in human patients with non-alcoholic fatty liver disease (NAFLD) [15] and in animal models of obesity [16,17]. The administration of melatonin at 10 mg/kg day reduces hepatic triglyceride and free-fatty acid (FFA) levels in high-fat diet (HFD)-induced obese mice. Melatonin also induces normalization of the plasmatic levels of triglycerides, FFA, adiponectin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) but without a decrease in the body weight gain [16,17]. At a higher dosage of 30 mg/kg day, melatonin is able to reduce the body weight gain of HFD-fed obese rats and also the plasmatic levels of glucose, leptin, and triglyceride [18].

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In women, the decline in melatonin secretion arises very early in menopause. A close relationship between melatonin levels and oestrogen deficiency is suggested by the observation that the level of overnight melatonin can be restored to normal levels by administration of exogenous oestradiol [19]. Ovariectomised (OVX) rats have been commonly used as an experimental model for studying the consequences of oestrogen deficiency [20–23]. Our previous studies have demonstrated that OVX rats exhibit some features of the metabolic syndrome, such as increased body weight, adiposity associated with liver lipid accumulation (steatosis) and oxidative stress [21–23].

Considering that melatonin appears to exert important systemic and pharmacological effects [1,2,12–15] and that it has been shown to be effective in the treatment of several diseases that are common in the elderly [14,16,19,24], the present study aimed to evaluate whether melatonin protects the liver against metabolic abnormalities and cellular oxidative stress associated with obesity due to oestrogen deficiency in OVX rats. OVX and control rats were treated for three weeks with melatonin (10 mg/kg per day, p.o.) and metabolic parameters, including body weight gain, adiposity index, blood biochemical parameters, liver lipid metabolism and parameters of hepatocyte oxidative stress, were measured.

2. Material and methods

2.1. Material

The following substrates and reagents were purchased from Sigma Chemical Co. (St. Louis, USA): melatonin, adenosine diphosphate (ADP), 2,4-dinitrophenol (2,4-DNP), phenylmethylsulfonylfluoride (PMSF), reduced glutathione (GSH), sodium dodecyl sulfate (SDS), *o*-phthalaldehyde (OPT), 2′,7′-diclorofluorescein diacetate (DCFH-DA), 2′,7′-dichlorofluorescein (DCF), succinate, L-malate, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), β-nicotinamide adenine dinucleotide (phosphate), reduced dipotassium salt (NAD[*P*]H). Kits from Gold Analisa® (Belo Horizonte, Brazil) were used to measure blood lipids and glucose levels. Sodium heparin was obtained from Roche (São Paulo, Brazil). The other reagents used were from Merck (Darmstadt, FRG), Carlo Erba (São Paulo, Brazil) and Reagen (Rio de Janeiro, Brazil).

2.2. Animals

Four groups of 88 female Wistar rats (6 weeks old), weighing 130–160 g, were provided by the Central Biotery of the University of Maringa and were randomly assigned to one of two surgical procedures: sham-operated (CON) or bilateral ovariectomy (OVX). Animals undergoing OVX were anaesthetised with 10 mg xylazine + ketamine 50 mg/kg i.p. The CON rats were subjected to the same procedures, but their ovaries were only exposed without removal.

Throughout the experimental period (16 weeks), rats were housed in polypropylene cages (maximum of four animals per cage), fed a standard diet and water ad libitum, and were kept in a sectorial biotery at a controlled temperature (25 °C) and 12 h light/dark cycle. All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experimentation of the University of Maringá (Certificate no. 056/2011).

2.3. Treatment of the animals and collection of tissues and blood

The rats were divided into four groups: untreated, sham-operated rats (CON); sham-operated rats treated with daily doses of melatonin (CON + MEL); untreated, OVX rats (OVX); and OVX rats treated with daily doses of melatonin (OVX + MEL). Thirteen weeks after surgery, a dose of 10 mg/kg of melatonin dissolved in 0.9% saline solution was administered daily in the morning (at 9:00 h) to CON + MEL and OVX + MEL rats by oesophageal gavage (final volume of 400 μL), over a period of 3 weeks. The rats in the CON and OVX groups received the same volume of 0.9% saline solution. Food intake and body weight (BW) were recorded throughout the trial period. On the day of the experiments, the animals were anaesthetised with thiopental sodium (50 mg/kg i.p.) for blood collection and removal of the liver, adipose tissues and uterus.

The retroperitoneal, uterine, mesenteric and inguinal fat depots were weighed and expressed in g per 100 g of BW. The adiposity index was defined as the ratio of the sum of the weights of these fat depots per 100 g of BW [22]. To confirm the success of OVX, the uterus was also collected, weighed and expressed in g per 100 g of BW. Blood was collected from fasted rats by cardiac puncture to obtain serum and plasma. After blood collection, liver samples were removed, clamped in liquid nitrogen and then stored at −80 °C for subsequent measurements of the lipid content.

2.4. Serum and plasma biochemical analysis

Total cholesterol, high-density lipoprotein (HDL-cholesterol) and triglycerides (TG) were analysed in serum and glucose was analysed in plasma by standard methods using assay kits (Gold Analisa®). Very-low-density lipoprotein (VLDL-cholesterol) levels were calculated using the Friedewald's equation [25], and low-density lipoprotein (LDL-cholesterol) levels were determined by subtracting HDL- and VLDL-cholesterol from total cholesterol.

2.5. Determination of hepatic lipid content

The liver total lipid content was determined using the gravimetric method [26]. Lipids were extracted from homogenized liver samples (approximately 1.0 g) in a chloroform-methanol mixture (2:1). The results were expressed as g of total fat per 100 g of liver wet weight. The cholesterol and TG in the liver were determined after the suspension of fat in 200 μL of 2% Triton, followed by vortexing and heating at 55 °C. Cholesterol and TG contents in the suspension were measured by specific assay kits from Gold Analisa®.

2.6. Isolation of liver fractions for measurements of respiratory activities, parameters of cellular oxidative stress and activities of enzymes of the antioxidant defense system

Liver mitochondria were isolated by differential centrifugation [27]. The livers were homogenized in a medium containing 200 mM mannitol, 76 mM sucrose, 0.2 mM EGTA, 0.1 mM PMSF, 1.0 mM Tris (pH 7.4) and 50 mg/100 mL fatty acid-free bovine serum albumin (BSA, w/v). The homogenate was fractioned via sequential centrifugation at 536 ×*g* and 7080 ×*g* for 10 min each. The sediment of the last centrifugation was washed twice by suspension and centrifugation at 6392 ×*g* and the final mitochondrial pellet was suspended in isolation medium to yield a protein concentration of 60–90 mg/mL. Intact mitochondria were used to measure fatty acid β-oxidation capacity, respiration driven by oxidation of β-hydroxybutyrate and succinate and reactive oxygen species (ROS) generation.

Freeze-thaw disrupted mitochondria were used for reduced glutathione (GSH) content measurements and for respiration driven by oxidation of NADH (NADH oxidase) and succinate (succinate oxidase). Mitochondria disrupted by sonication were used to measure the activities of the mitochondrial antioxidant enzymes, glutathione peroxidase 1 (GPx1) and glutathione reductase (GR): mitochondrial suspensions (50 mg of protein/mL) were diluted 1:10 (v/v) with 0.1 M KCl and 20 mM Tris-HCl (pH 7.4) and sonicated for 60s at maximum power. Aliquots of these fractions were centrifuged at 6000 ×*g* for 10 min, to sediment intact mitochondria, and the supernatants were utilized as enzyme sources.

Total liver homogenates obtained from freeze-clamped liver of overnight fasted rats were used for GSH content, catalase (CAT) activity and