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Histomorphological changes in the pancreas and kidney and histopathological changes in the liver in male Wistar rats on antiretroviral therapy and melatonin treatment

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ABSTRACT

Combination antiretroviral therapy (cART) has shown to cause inflammation, cellular injury and oxidative stress, whereas melatonin has been successful in reducing these effects. The aim of the study was to determine potential morphometric changes caused by cART in combination with melatonin supplementation in human immunodeficiency virus (HIV)-free rats.

Tissue samples (N = 40) of the pancreas, liver and kidney from a control (C/ART-/M-), cART group (C/ART +), melatonin (C/M +) and experimental group (ART + /M +) were collected and stained with haematoxylin and eosin (H&E) and evaluated for histopathology. The pancreata were labelled with anti-insulin and anti-glucagon to determine α- and β-cell regions. Kidneys were stained with periodic acid Schiff (PAS) to measure the area, perimeter, diameter and radius of renal corpuscles, glomeruli and proximal convoluted tubules (PCTs). Blood tests were conducted to determine hepatotoxicity.

No significant changes in histopathology were seen. Melatonin stimulated pancreatic islet abundance, as the number of islets per mm^2 was significantly higher in the C/M+ than in the C/ART-/M- and ART+/M+. Parameters of the renal corpuscle, glomeruli, renal space and PCTs were significantly lower in the C/ART+ compared to the other groups, thus cART may have caused tubular dysfunction or cellular damage. A significant increase in serum haemoglobin was observed in the C/ART+ compared to the C/ART-, which showed cART increases serum haemoglobin in the absence of immune deficiency. Serum lipids were significantly decreased in the C/M+ compared to the C/ART-, possibly due to the effect of melatonin on the decrease of lipolysis, decreasing effect on cholesterol absorption and stimulation of lipoprotein lipase (LPL) activity.

In conclusion, we have demonstrated that melatonin stimulated α-cell production, increased the number of pancreatic islets and caused a decrease in total lipids, whereas cART increased serum haemoglobin and decreased various parameters of the nephron in an HIV-free rat model, suggestive of tubular dysfunction.

1. Introduction

Antiretroviral therapy (ART) has been shown to induce morphological and pathophysiological changes in the pancreas, liver and kidneys (Adjene et al., 2011; Max and Sherer, 2000; Sulkowski, 2004). These changes are often similar to those caused by the HI-virus *per se* (Braithwaite et al., 2008; Huppmann and Orenstein, 2010; Price et al., 2005). In South Africa, a single tablet of 600 mg efavirenz (EFV), 200 mg emtricitabine (FTC) and 300 mg tenofovir disoproxil fumarate (TDF) has been implemented as treatment for HIV since 2013 (Deeks

Abbreviations: α, apha; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate transaminase; β, beta; cART, combination antiretroviral therapy; C, control; DAB, liquid 3'3-diaminobenzidine; DPX, distyrene/plasticizer/xylene; EFV, efavirenz; eGFR, estimated glomerular filtration rate; FFA, free fatty acids; FTC, emtricitabine; H&E, haematoxylin and eosin; HIV, human immunodeficiency virus; IgG, immunoglobulin G; IHC, immunohistochemistry; LDL, low-density lipoprotein; LPL, lipoprotein lipase; MT, melatonin receptor; NGS, normal goat serum; NHS, normal horse serum; NRTI, nucleoside analogue reverse transcriptase inhibitor; NNRTI, non-nucleoside analogue reverse transcriptase inhibitor; PAS, periodic acid Schiff; PBS, phosphate buffered saline; PCTs, proximal convoluted tubules; PFA, paraformaldehyde; ROS, reactive oxygen species; SD, standard deviation; SEM, standard error of the mean; TBS, tris buffered saline; TDF, tenofovir disoproxil fumarate; VLDL, very low-density lipoproteins

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and Perry, 2010). Efavirenz is classified as a non-nucleoside analogue reverse transcriptase inhibitor (NNRTIs), whereas FTC and TDF are nucleoside analogue reverse transcriptase inhibitors (NRTIs) (Brinkman et al., 1998). The latter have been shown to induce pancreatitis, hepatic steatosis and lipodystrophy, whereas NNRTIs may induce nephrotoxicity and an increase in liver enzymes (Adikwu et al., 2013; Esser et al., 2007).

The TDF/FTC/EFV combination may result in weight gain leading to obesity as well as increased insulin secretion and glucose uptake by somatic cells (Barbosa et al., 2013; Guzik et al., 2006). In addition, combination antiretroviral therapy (cART) has been shown to induce pancreatic toxicity in HIV positive patients, which presents as pancreatitis, steatosis, islet hypertrophy, and alpha- (α) and beta- (β) cell dysfunction (Ferreira et al., 2015). In the liver, cART may lead to an elevation of liver enzymes, hepatic fibrosis, hepatic steatosis, hyperlipidaemia and hypercholesterolemia (Fisher et al., 2006; Núñez, 2010; Oliveira et al., 2014; Sulkowski, 2004). Specifically, EFV has shown to induce hypercholesterolemia and increased lipoprotein size (Fisher et al., 2006; Olivero-David et al., 2011). In the kidneys, TDF has been shown to cause a decrease in the estimated glomerular filtration rate (eGFR) and tubular dysfunction due to nephrotoxicity in humans (Jao and Wyatt, 2010; Post et al., 2010; Squillace et al., 2017). Toxicity of the pancreas, liver or kidneys are often linked to inflammatory conditions, caused by increased reactive oxygen species (ROS) (Brown et al., 2010; Jaworek et al., 2005). Inflammation, oxidative stress and cellular injury are increased in patients receiving cART (Barbosa et al., 2013; Brown et al., 2010; Muñoz-Casares et al., 2006). It is unclear whether these pathophysiological changes in patients receiving cART are HIV dependent or due to the specific combination of ART.

Serum melatonin levels have been shown to decrease during HIV progression in patients, due to unclear mechanisms (Carrillo-Vico et al., 2013; Nunnari et al., 2003). Melatonin is mainly produced by the pineal gland and is responsible for the regulation of circadian rhythms in the body. Pinealocytes synthesize melatonin from tryptophan in darkness and secretes it immediately (Acuña-Castroviejo et al., 2014; Jaworek et al., 2010; Radogna et al., 2010). Various studies have proven that melatonin is synthesized by tissues other than the pineal gland, including the retina, liver, kidneys, Harderian gland of the rat, cerebellum, skin, digestive tract and leukocytes. Extrapineal melatonin maintains cell homeostasis and is involved in regulatory mechanisms, whereas pineal melatonin is responsible for biological rhythms, anti-inflammatory and antioxidant properties (Acuña-Castroviejo et al., 2014; Radogna et al., 2010).

Melatonin supplementation have been shown to increase the plasma melatonin levels of humans and middle-aged rats significantly during dark periods (Sharkey and Eastman, 2002; Wolden-Hanson et al., 2000). Treatment with supraphysiological concentrations of melatonin have been successful in decreasing ROS, inflammatory cell recruitment and cellular injury as induced by pancreatic toxicity, hepatotoxicity and nephrotoxicity in rodents and humans (Ersoz et al., 2009; Hu et al., 2015; Jaworek et al., 2012; Radogna et al., 2010). Melatonin is a pleotropic molecule, which acts as an antioxidant, analgesic, free radical scavenger and regulator of circadian rhythm in mammals (Baykara et al., 2009; Hardeland et al., 2010; Maldonado et al., 2010; Nava et al., 2003; Radogna et al., 2010). According to Peschke et al. (2007), β-cells in the pancreas express the melatonin receptor (MT) 1, which opposes insulin secretion by inhibiting forskolin-induced insulin secretion (Peschke et al., 2013; Zibolka et al., 2015). The antioxidant properties of melatonin may also reduce necrosis of beta cells as well as the accumulation of free radicals observed in diabetes and inflammatory conditions (Baydas et al., 2002; Radogna et al., 2010). In addition, Sidhu et al. (2009) found that melatonin can stimulate pancreatic regeneration caused by acute pancreatitis.

Melatonin has been reported to improved carbohydrate and lipid utilization during exercise in rats and humans by regulating plasma glucose and preserving glycogen stores (Maldonado et al., 2012; Mazepa et al., 1999). An elevation in aspartate transaminase (AST), alanine aminotransferase (ALT) and low-density lipoproteins (LDL) caused by hepatic injury was found to be decreased with melatonin treatment (Sigala et al., 2006). Melatonin prevents the accumulation of neutrophils in damaged renal tissue (Sener et al., 2002b) and decreases lipid peroxidation and protein oxidation in the kidneys after drug-induced nephrotoxicity (Hara et al., 2001; Sener et al., 2002a). Furthermore, melatonin protects the kidneys against fibrosis due to its anti-inflammatory properties (Ersoz et al., 2009; Hu et al., 2015).

HIV-infection and ART are known to affect the same organ systems leading to similar pathophysiological and metabolic abnormalities. The present study aimed to use an HIV negative rat model to determine the effects of cART on the pancreas, liver and kidney as determined by histomorphometry and blood parameter tests. In addition, melatonin was evaluated as a complementary therapy to cART. This information is important in order to clarify the putative negative effects of cART and the potential ameliorating effects of melatonin on a tissue level as a possible cost effective therapeutic option in patients receiving cART.

2. Materials and methods

Ethics approval for this study was obtained from the Committee for Experimental Animal Research of Stellenbosch University (SU-ACUM15-00003). Adult male Wistar rats (N = 40) were randomly divided into four experimental groups (n = 10 per group): a control (C/ART-/M-), a control group which received cART (C/ART +), a group which received melatonin administration (C/M +) and an experimental group subjected to both cART and melatonin administration (ART + /M +). The animals were housed in a controlled environment (temperature: 22°C; humidity: 40%; circadian rhythm: 12 h of artificial light per day; 12 h of darkness per day) and fed with normal rat chow (Imbani Nutrition, South Africa) for 16 weeks. The rats were 8 weeks old (180–200 g) when the cART and melatonin treatment was initiated. Antiretroviral therapy (SantaCruz Biotechnology, WhiteHead Scientific, South Africa) was administered by oral gavage (25.8 mg/kg/day TDF, 51.6 mg/kg/day EFV and 17.4 mg/kg/day FTC dissolved in 1 ml distilled water) every morning at the same time for 8 weeks. The cART-naïve groups were gavaged with 1 ml of distilled water. Melatonin (Sigma Aldrich, St Louis, MO, United States of America) (0.01 mg/g/day) (Aydin et al., 2003; Sun et al., 2016) was permanently administered in the drinking water of the melatonin groups for 8 weeks. The amount of water consumed by the rats was measured daily. Melatonin was administered according to the weight of the rats and prepared every second day. The following equation was used to determine the amount of melatonin needed in grams:

$$Melatonin\ needed(mg)for\ 500\ ml = \frac{[(average\ weight\ of\ rats\ in\ cage\ \div\ amount\ of\ rats\ in\ cage)\ \times\ 0.01]}{average\ amount\ of\ water\ drank\ per\ rat\ per\ day\ \div\ 500\ ml}$$

Equation 1 Calculation of melatonin needed for two days of stock

The melatonin was firstly dissolved in 0.2% ethanol (500 µl) before 500 ml distilled water was added.

The body mass of the rats was measured before euthanasia was administered. The animals were deeply anesthetized by an intraperitoneal injection of 160 mg/kg sodium pentobarbitone into the lower right abdomen and euthanized via exsanguination to obtain heart tissue for an unrelated study. For the present study, tissue samples of the pancreas, right median lobe of the liver and the right kidney were harvested and fixed in 4% paraformaldehyde (PFA) for 48 h and embedded in paraffin wax.

2.1. Histopathology

The pancreas, liver and kidney (n = 40 per organ) were sectioned at 3 µm, 5 µm and 5 µm, respectively. One slide of each organ was stained