Original Paper

Combination of Olanzapine Pamoate with Melatonin and Metformin: Quantitative Changes in Rat Adipose Tissue

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ABSTRACT: Olanzapine is one of the atypical antipsychotics widely used in the treatment of schizophrenia and has been associated with metabolic changes as adverse effects, including hyperglycemia, dyslipidemia, and weight gain. In a batch of adult female Wistar rats, we studied the prolonged-release intramuscular olanzapine pamoate induced quantitative changes of visceral and subcutaneous adipose tissue. We also assessed the effects of the combinations of olanzapine pamoate with melatonin, metformin, and melatonin plus metformin, administered by gastric gavage. A higher mean weight of the visceral and subcutaneous adipose tissue per animal was noted in the olanzapine pamoate exposed group compared to controls. The association with melatonin, metformin, or the combination of melatonin with metformin attenuated the olanzapine-induced adipose deposit tissue growth. The effect was more pronounced for the combination of olanzapine with melatonin and metformin. Because most of the results were not statistically significant we can deduce that in the chronic experiment, adaptive type modifications of the receptors on which both olanzapine and melatonin act can occur.

KEYWORDS: *Adipose tissue, rats, olanzapine pamoate, melatonin, metformin*

Introduction

Adipose tissue, which is mainly composed of adipocytes, is essential for maintaining metabolic energy and homeostasis [1]. Complex mechanisms are involved in adipose tissue development and function, regulated by various peptide and steroid hormones. Adipogenesis is assumed to occur in two stages: the involvement of mesenchymal stem cells in a preadipocyte state and terminal differentiation [1,2]. The cellular shape and remodeling of the extracellular matrix have recently been shown to regulate preadipocyte recruitment and competence by modulating GTPase WNT and RHO signaling cascades [1]. The distribution of body fat reflects the central somatotopic organization.

The ability of the CNS to modulate the adipose tissue is based on two separate statements: the balance between sympathetic and parasympathetic innervation, and the selective control loop which depends on the anatomical location of the adipose tissue [3]. The balance between sympathetic and parasympathetic nervous system influences both anabolism and catabolism but also the type of fat distribution-visceral or subcutaneous [4].

Adipocyte expansion can lead to obesity, which is of particular importance in the pathogenesis of metabolic syndrome and insulin resistance. It is well known that during the treatment with atypical antipsychotics, different alterations in the homeostasis of lipid metabolism may occur, with the risk of developing obesity. Therefore, we designed a controlled study in order to evaluate the effect of olanzapine pamoate administered i.m. at two weeks intervals over a period of 57 days on the amount of fatty tissue, in the viscera and subcutaneously, compared with the associated treatment with melatonin, metformin or the combination with the last two substances.

Material and Method

The experiment was performed on a batch of adult female Wistar rats, 4-6 months of age, with an initial weight of 150-200g. Laboratory animals from the animal facility of the University of Medicine and Pharmacy of

10.12865/CHSJ.45.04.05

Craiova were housed in plastic cages in a climate-controlled environment (19-23°C), well ventilated, with a light-dark cycle of 12 hours (light from 8am to 8pm), with water consumption and food *ad libitum* (standard laboratory chow diet). The animals had an acclimatization period of 7 days. The experiment lasted 8 weeks (57 days), and injectable olanzapine pamoate (an atypical antipsychotic) was tested in association with orally administered melatonin (a neurohormone secreted by the pineal gland) and metformin (an oral antidiabetic drug).

Five groups of rats with six animals were tested. The control group of reference animals was marked C1-C6, the group treated with prolonged release injectable olanzapine (olanzapine pamoate) included animals marked O1-O6, the group treated with olanzapine pamoate and oral melatonin consisted of animals marked OMt1-OMt6, the group treated with olanzapine pamoate and metformin used the marking OMf1-OMf6, while the animals in the group treated with olazapine pamoate and melatonin+metformin were marked from OMtMf1 to OmtMf6.

The animals, grouped in the special spaces and individually marked, were fed with standard feed (granulated compound feed, complete feed for rodents used for scientific research, from the National Institute for Medical Research Cantacuzino, Baneasa Station, Bucharest, Romania). Body weight was monitored in the morning, after overnight fasting, between 9am and 10am, for 3 days in the first 2 weeks, and then twice a week until the end of the experiment. Drug doses were administered corresponding to the body weight of each animal. Other parameters not related to the current study were monitored.

Olanzapine pamoate was injected intramuscularly (i.m.) at 14 days interval, using single-use syringes for each animal, while melatonin and metformin was administered daily by gavage (p.o.) using a single-use syringe with a special needle attached to administer the gavage solutions.

The experiment and the number of animals have been approved by the ethical committee of the University of Medicine and Pharmacy of Craiova (73/02.04.2019).

Prolonged-release injectable suspension of olanzapine pamoate was used, commercially available as Zypadhera® (Eli Lilly Nederland BV, Grootslag 1-5, NL-3991 RA Houten, The Netherlands) from vials containing olanzapine pamoate monohydrate equivalent to 210mg olanzapine, after reconstitution each mL of suspension containing 150 mg olanzapine.

Melatonin, chemically identified as N-Acetyl-5-methoxy-tryptamine, was obtained in the form of a solid beige powder (Toronto Research Chemicals, Canada). The melatonin solution was prepared daily by dissolving the melatonin powder corresponding to weight for each animal in a 0.01% hydroalcoholic solution. Metformin hydrochloride, in the form of a white solid powder, was also purchased from the Toronto Research Chemicals.

Drug doses, administered according to body weight (bw), were 100mg/kg bw olanzapine i.m. at 14 day-intervals, 20mg/kg bw melatonin and 300mg/kg bw metformin, daily by gavage, and did not exceed the maximum doses allowed in rats. The animals received gavage at 9-10 am with 0.5mL of the prepared solutions.

Rats were sacrificed according to animal protection standards, and the adipose tissue from the visceral areas and the subcutaneous adipose tissue was harvested separately. Each amount of adipose tissue was weighed and averaged *per* animal/group presented in the descriptive analysis.

Statistical analysis

Statistical analysis was performed using the dedicated program IBM SPSS version 23 (SPSS Inc. Chicago, USA). For the descriptive analysis of the groups, the average, the minimum and maximum values, and the standard deviation were used. For data comparison, the *Z* score with 95% specificity threshold ($p<0.05$) was used. Nonparametric tests were applied, the Mann-Whitney U test, when independent data series were compared, and the Wilcoxon test, for comparisons between measurements in the same subjects. Comparison graphs (with columns) and evolution graphs (with lines) with the average values were performed.

Results

In the table 1, the average quantities ±standard deviation (SD) of adipose tissue in grams *per* female rat and *per* batch for the two forms of adipose tissue (visceral and subcutaneous) are presented: group treated with olanzapine pamoate administered i.m. at 14 day-interval for 57 days, group in which was added melatonin, group in which metformin was added, the group in which melatonin and metformin were added, and control batch.

Table 1. Descriptive analysis of the quantities of adipose tissue in grams/female rat/group isolated from visceral and from subcutaneous levels at the end of the study.

Descriptive Statistics						
Groups		N	Minimum	Maximum	Mean	Std. Deviation
OLANZ	**Visceral adipose tissue**	6	2,38	26,90	17,9367	9,82457
	Subcutaneous adipose tissue	6	,41	17,60	10,0233	6,05074
	Valid N (listwise)	6				
OMt	**Visceral adipose tissue**	6	10,53	33,64	21,9667	8,21233
	Subcutaneous adipose tissue	6	5,00	13,89	9,1017	3,41376
	Valid N (listwise)	6				
OMf	**Visceral adipose tissue**	5	6,35	17,85	12,8160	4,16565
	Subcutaneous adipose tissue	5	1,82	7,87	5,1880	2,44153
	Valid N (listwise)	5				
OMtMf	**Visceral adipose tissue**	6	14,39	26,97	20,0383	4,51022
	Subcutaneous adipose tissue	6	3,49	13,28	8,3017	3,43844
	Valid N (listwise)	6				
CONTROL	**Visceral adipose tissue**	6	7,56	25,28	13,4017	6,90257
	Subcutaneous adipose tissue	6	1,41	8,48	4,3317	2,45004
	Valid N (listwise)	6				

N-number of animals

The difference between the average weight/female rat of visceral and subcutaneous fat tissue deposits in the group treated with olanzapine pamoate, olanzapine pamoate + melatonin, olanzapine pamoate + metformin and olanzapine pamoate + melatonin and metformin *versus* to the control group

In the control group, at visceral level an average of the adipose tissue of 13.40g/female rat was found, and at subcutaneous level 4.33g/female rat was assessed, which represents 32.31% of the amount of adipose tissue at the level of the viscera. In the group treated with olanzapine pamoate the amount of visceral adipose tissue increased compared to the control at 133.84%, and the quantity at subcutaneous level increased to 231.39%. The percentage of cutaneous *versus* visceral adipose tissue in the group treated with olanzapine pamoate was 56.95%. The ratio between visceral and subcutaneous adipose tissue is 3.09 in the control group and 1.79 in the olanzapine pamoate treated group.

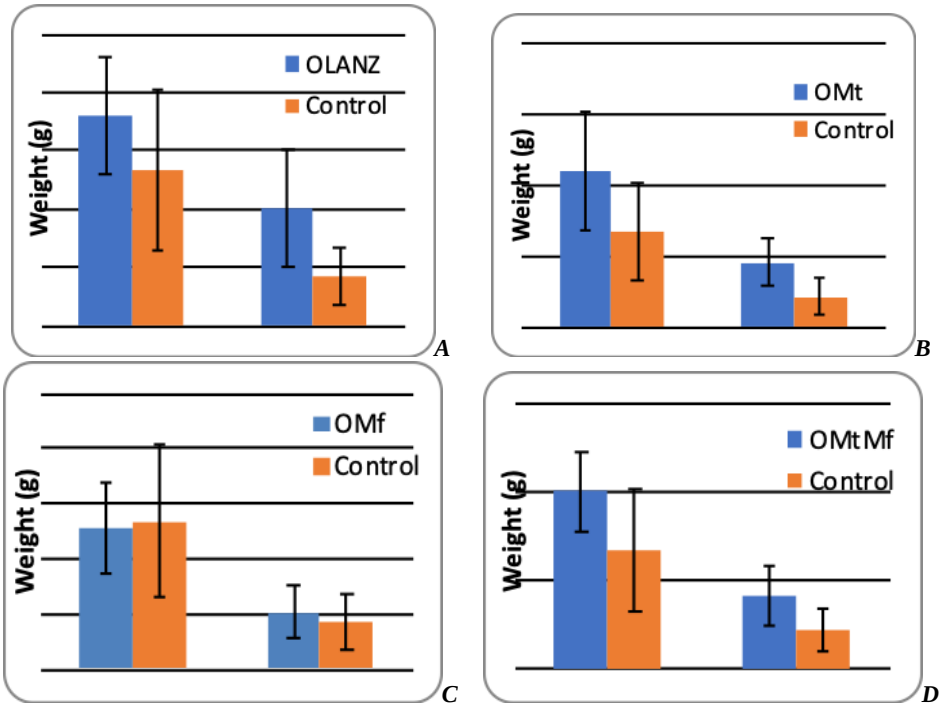

Figure 1. Comparison between the quantitative differences in grams/female rat of visceral and subcutaneous adipose tissue deposits/animal from the control groups and treated with olanzapine pamoate (A), olanzapine pamoate + melatonin (B), olanzapine pamoate + metformin (C) and olanzapine + melatonin and metformin (D).

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Compared with the control group, the amount of adipose tissue in the group with olanzapine pamoate was higher, but at statistically insignificant values for subcutaneous adipose tissue (p=0.093) and visceral adipose tissue (p=0.240). Figure 1A reveals the differences between the weight in grams/rat of visceral adipose tissue and subcutaneous adipose tissue in control and olanzapine pamoate treated groups (Figure 1A).

The difference in adipose tissue/animal between the olanzapine pamoate + melatonin treated group and the control group is statistically significant for subcutaneous tissue (p=0.026) and statistically insignificant for visceral fatty tissue (p=0.093). The amount of visceral adipose tissue increased by 63.91% in the group treated with olanzapine pamoate + melatonin compared to controls, and the amount of subcutaneous fat tissue increased by 110.12% (Table 2). The percentage of cutaneous *versus* visceral adipose tissue in this group is 41.43%, and the ratio between the two locations is 2.41. Figure 1B reveals the differences between the weight in grams of visceral adipose tissue and subcutaneous adipose tissue *per* female rat in the control and treated with olanzapine pamoate + melatonin groups.

In the group treated with olanzapine pamoate and metformin, the visceral adipose tissue /animal decreased statistically insignificantly compared to controls at 95.63% (p=0.792), while the subcutaneous one grows but still insignificant at 119.77% (p=0.662). The ratio between visceral and subcutaneous adipose tissue in this group is 2.31, and the percentage of subcutaneous adipose tissue from visceral adipose tissue is 40.47%. Figure 1C reveals the differences between the gram/animal weight of visceral adipose tissue and subcutaneous adipose tissue in the control groups and treated with olanzapine pamoate+metformin (Figure 1C).

Analyzing the difference between the average quantities of visceral and subcutaneous adipose tissue/animal in the group treated with olanzapine pamoate + melatonin and metformin compared to the control group, we observed a difference in the case of visceral tissue close to significance (p=0.065), and statistically insignificant in the subcutaneous tissue (p=0.093). The percentage visceral adipose tissue/animal compared to the control increased by 2.84% and the subcutaneous level by 6.35%. The ratio between visceral and subcutaneous adipose tissue in this group is 2.41, and the percentage of cutaneous adipose tissue *versus* visceral adipose tissue is 33.52%. Figure 1D reveals the differences between the weight in grams of visceral adipose tissue and subcutaneous adipose tissue *per* animal in control groups and treated with olanzapine pamoate + melatonin and metformin (Figure D).

The difference between the average weight/animal of visceral and subcutaneous fat tissue deposits in the olanzapine pamoate + melatonin, olanzapine pamoat + metformin and olanzapine pamoate + melatonin and metformin treated group compared to the olanzapine pamoate treated group.

The difference between the amount of adipose tissue/animal in the group treated with olanzapine pamoate + melatonin *versus* the group treated only with olanzapine is -11.37% (p=0.589) for visceral localization and -9.19% for subcutaneous localization (p=0.699). The differences are not statistically significant.

Figure 2A reveals the differences between the weight in grams/animal of visceral adipose tissue and subcutaneous adipose tissue in the group treated with olanzapine pamoate + melatonin *versus* the group treated only with the atypical antipsychotic (Figure 2A).

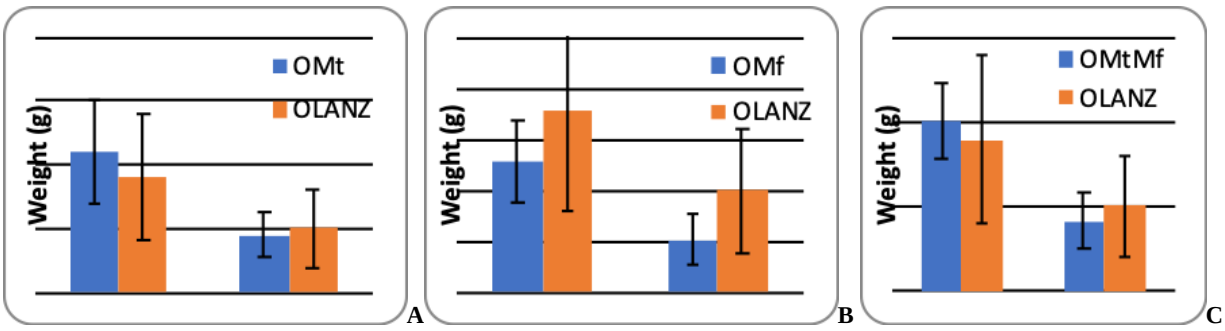

Figure 2. Comparison between the quantitative differences in grams/animal of visceral and subcutaneous fat tissue deposits from the groups treated with olanzapine pamoate + melatonin (A), olanzapine pamoate + metformin (B), olanzapine pamoate + melatonin and metformin (C) versus the treated group only with olanzapine pamoate.

The difference between the amount of adipose tissue/animal in the group treated with olanzapine pamoate + metformin versus the group treated only with olanzapine is -28.55% (p=0.329) for visceral localization and -48.24% (p=0.177) for localization subcutaneous. The differences are not statistically significant. Figure 2B shows the differences between the weight in grams/animal of visceral adipose tissue and subcutaneous adipose tissue in the olanzapine pamoate + metformin-treated group versus the atypical antipsychotic-treated group (Figure 2B).

The difference between the amount of adipose tissue/animal in the group treated with olanzapine pamoate + melatonin and metformin versus the group treated only with olanzapine is -23.36% (p=0.699) for visceral localization and -55.90% (p=0.598) for subcutaneous localization. The differences are not statistically significant. Figure 2C shows the differences between the weight in grams/animal of visceral adipose tissue and subcutaneous adipose tissue in the olanzapine pamoate + melatonin and metformin-treated group versus the atypical antipsychotic-treated group (Figure 2C).

Table 2. Percentage differences between groups treated with olanzapine pamoate, olanzapine pamoate + melatonin, olanzapine pamoate + metformin and olanzapine pamoate + melatonin and metformin vs. control group and vs. olanzapine pamoate group.

Groups of animals	Visceral adipose tissue/animal quantity (g)	Percentages	Subcutaneous adipose tissue/animal quantity (g)	Percentages	Percentage differences to control (U-up) and to olanzapine (D-down)
Control group	13,40g	100%	4.33g	100%	
Olanzapine pamoate G	17,94g	133.84%	10.22g	231.39%	+33.84%/+131.39%
Olanzapine pamoat + melatonin G	21,97g	163.91% 122.47%	9.10g	210.12% 90.81%	U+63.91%/+110.12% D+22,47%/-9.19%
Olanzapine pamoate + metformin G	12,816g	95.63% 71.45%	5.188g	119.77% 51.76%	U-4.37%/+19.77% D-28.55%/-48.24%
Olanzapine pamoate + melatonin and metformin G	13,75g	102.84% 76.64%	4.61g	106.35% 45,10%	U+2.84%/+6.35% D-23.36%/-55.90%

G-group; g-grams; U-Up, D-down; black-visceral adipose tissue; red-subcutaneous adipose tissue;- /+decrease / increase

Discussion

In our study, the amount of adipose tissue in the group with olanzapine pamoate increased, but at statistically insignificant values compared with the control group, both for the subcutaneous adipose tissue (p=0.093) and visceral adipose tissue (p=0.240). In the group treated with olanzapine pamoate the visceral adipose tissue increased by 33.84% and the subcutaneous one by 131.39%. We find a greater amount of visceral adipose tissue compared to subcutaneous level in animals from the control group and those treated with olanzapine pamoate, but the ratio between visceral and subcutaneous adipose tissue decreased in the antipsychotic treated group from 3.09 (control group) to 1.79.

In the group treated with olanzapine pamoate and melatonin, the visceral adipose tissue increased by 63.91%, and the subcutaneous level by 110.12%, compared to controls. There is a marked increase in visceral adipose tissue *versus* subcutaneous tissue for the combination olanzapine pamoate with melatonin. The difference in the amount of visceral tissue compared to the control group is statistically significant (p=0.026). The percentage of cutaneous compared to visceral adipose tissue in this group is 41.43%, and the ratio of visceral and subcutaneous adipose tissue quantity is 2.41.

Regarding the animal group treated with olanzapine pamoate and metformin, the visceral adipose tissue decreased by 4.37% compared to controls, and the amount of cutaneous adipose tissue increases by 19.77%, but these variations were statistically insignificant. Compared with the group treated with olanzapine pamoate, the amount of visceral and subcutaneous adipose tissue was reduced by 28.55% and 48.24%, respectively. The ratio between the amount of visceral and subcutaneous adipose tissue in the group treated with olanzapine pamoate and metformin was 2.31, and the percentage of subcutaneous adipose tissue when compared to visceral adipose tissue was 40.47%.

In the group treated with olanzapine pamoate with melatonin and metformin, the amount of visceral adipose tissue *per* animal increased by 2.84% compared to controls (p=0.065) and that of subcutaneous adipose tissue by 6.35%. Compared with the group treated with olanzapine pamoate, the amount of visceral adipose tissue *per* animal decreased by 23.36%,

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and that of the subcutaneous adipose tissue by 55.90%. without statistical significance. The ratio between the amount of visceral and subcutaneous adipose tissue *per* animal in the group treated with olanzapine pamoate with melatonin and metformin was 2.41, and the percentage of subcutaneous adipose tissue when compared to visceral adipose tissue was 33.52%.

Obesity is not only an excessive accumulation of fat in relation to body weight, but a disease, because it adversely affects the health [5]. It is characterized by a disproportionate increase in adipose tissue relative to body weight.

The treatment with atypical antipsychotics, is regarded nowadays as efficacious antipsychotic pharmacotherapy for the treatment of schizophrenia, but it associates a high risk for obesity, metabolic dysfunctions, increased risk of cardiovascular morbidity and mortality. Although these adverse effects have been widely assessed and discussed in multiple preclinical studies, the underlying mechanisms are not yet completely understood. In recent years, many pathogenic aspects related to adipose tissue metabolism and neurohormonal mediators were assessed [6].

Two atypical antipsychotics, olanzapine and clozapine, have significant dyslipidemic, obesogenic and diabetogenic side effects [7].

Several studies revealed that atypical antipsychotics increase lipid biosynthesis through changes in gene expression. Transcriptional activation of two important genes, the fatty acid synthase gene and the stearoyl-CoA desaturase gene, has been observed in the olanzapine-treated individuals, suggesting a direct lipogenic action, which may be related to adverse metabolic effects [8].

N-desmethyl-olanzapine, a major metabolite of olanzapine which is devoid of its metabolic side effects during obesity, increases uncoupling protein UCP1 (thermogenin) expression and may function to regulate the metabolic responses [9]. Chronic olanzapine treatment may induce pro-inflammatory cytokine expression in peripheral adipose tissue, with elevated plasma levels of IL-1ß, IL-6, IL-8 and TNFα [10].

Cariprazine, a new second-generation antipsychotic, induces a time-dependent decrease in peroxisome proliferator-activated receptor-γ (PPAR-γ) expression in adipocytes derived from murine fibroblasts [11]. There was an increase in IL-6 production and in the number of macrophages (protein expression of F4/80, a phenotypic macrophage biomarker), in the adipose test of rats were administered or long-term administration of olanzapine [12].

Previous studies proposed that olanzapine-induced overexpression of protein tyrosine phosphatase 1B (PTP1B) and G protein-coupled receptor kinase 2 (GRK2), and adipose triglyceride lipase may contribute to the development of metabolic adverse effects, PTP1B being a negative regulator of leptin and insulin signaling pathways, while GRK2 is considered an integrative signaling node in the regulation of cardiovascular function and metabolic homeostasis [13,14]. The Wnt signaling pathway key effector, the TCF7L2 transcription factor, strongly associated with glucose homeostasis, same presents olanzapine-induced expression in liver, or skeletal muscle, and adipose tissues, and it is also involved in its metabolic disturbances [15]. Moreover, distinct metabolic dysregulation induced by olanzapine in obesity may involve the regulation of adipose tissue autophagy [9].

Many studies have reported a relevant role of atypical antipsychotic affinities for the receptors 5HT2A, 5HT6, 5HT7, α1A, and especially H1 and 5HT2C, in their obesogenic effects. It is reported that 5HT2C antagonism or reverse agonism may contribute to olanzapine-induced weight gain [16,17].

5HT2C receptors expressed selectively only in the arcuate pro-opiomelanocortin (POMC) mediate the effects of 5HT2C receptors on energy balance [18].

H_1 receptor blockade has been suggested as a probable mechanism for drug-induced weight gain [17]. H_1 receptor inhibition is directly involved in activating hypothalamic 5'AMP-activated protein kinase/AMPK signaling, which increases appetite and anabolism, and reverses the anorexigenic effect of leptin [17,19]. The risk to gain weight on animal exposure to antipsychotics has been closely linked to the increased affinity for H_1 receptors [17].

Clozapine and olanzapine, with high affinity for H_1 receptors (Ki=1.2nM and Ki=2.0nM, respectively), have shown to have a greater tendency to induce weight gain [17].

Hypothalamic 5-HT2A receptors might have a role in the regulation of feeding and energy homeostasis. In obesity, increased expression of 5-HT2A receptor gene was revealed. Also, 5-HT2A receptor antagonism increases expression of adiponectin and reverses plasminogen activator inhibitor expression [20].

Stimulation of 5-HT2A receptors in certain areas of the brain has anorexigenic effect

because stimulation of 5-HT2A receptors in the paraventricular hypothalamus attenuates neuropeptide Y-induced hyperphagia. Clozapine and olanzapine are potent antagonists of these receptors in antagonizing this effect. Although affinity is lower for alpha1 and beta3 receptors, such antagonism also results in weight gain. Moreover, the brain-derived neuropeptide factor plays a role in weight regulation, and antipsychotics increase the expression of this neuropeptide [21].

Olanzapine administration to mice increases hypothalamic macrophage migration inhibitory factor (MIF) expression, with activation of the appetite-related AMP-activated protein kinase and Agouti-related protein pathway, and upregulates MIF expression in adipose tissue, with increased lipogenic pathways and reduced lipolysis [22].

In a recent study, female rats exposed to olanzapine for 14 days showed weight gain and adiposity, associated with hyperglycemia, hyperinsulinemia, insulin resistance and hyperlipidemia, olanzapine-induced metabolic alterations including a reduction of the AMP-activated and Akt protein kinases [23]. In a rodent model, olanzapine stimulated lipolysis, independent of weight gain, and raised the possibility that endocrine factors may influence gender specificity of metabolic effects [24].

In additional animal experiments, administration of olanzapine increased the accumulation of fat tissue in male rats, uncorrelated with food intake or weight loss, in contrast to female rats, with the involvement of increased uptake of free fatty acids into adipose tissue, increased lipogenesis and decreased lipolysis [25]. In case of chronic exposure to this antipsychotic, other authors revealed hyperglycemia, impaired glucose and insulin resistance, increased adipose tissue, but, in contrast to female rats, without an increase in body weight [26].

Chronic olanzapine treatment with sustained-release intramuscular olanzapine in adult Sprague-Dawley female rats may increase body weight, adipose tissue mass and leptin level [27]. Surprisingly, continuous increase in body weight in response to long-term olanzapine exposure in female rats, for up to 13 months, is accompanied by few concomitant changes in lipogenic gene expression and plasma lipids, suggesting that adaptive mechanisms may be involved to reduce long-term metabolic effects [28]. Other new data indicate that samidorphan, an opioid receptor antagonist, mitigates several metabolic abnormalities associated with olanzapine, regardless of weight variations [29].

In our experiment in adult female rats exposed to olanzapine pamoate, we detected an increase in the amount of visceral and subcutaneous adipose tissue associated with an increase in animal body weight, but not statistically significant, although in the first four weeks this reached statistical significance (unpublished data).

Melatonin is the key mediator for the circadian regulation of physiological and behavioral processes. Also, it to optimizes energy balance and body weight regulation, contributing to a healthy metabolism [30].

Experimental preclinical studies in small mammals revealed that supplementation with this drug, in mature rats with melatonin deficiency, may decrease their increased body weight. Similar findings were detected in experiments on diet-induced obesity. Weight loss was caused by a reduction in visceral fat, in combination with increased plasma levels of insulin and leptin [31,32,33].

Previous studies stated that daily administration of melatonin in middle-aged male rats suppresses body weight gain, intra-abdominal adiposity, leptin, and plasma insulin, unaffected by food intake and independent of total body fat [34].

Although it is currently stated that melatonin plays a role in energy homeostasis regulation, the involvement of this neurohormone in the energy balance is not fully elucidated. In a 13 week-experiment, carried out in male Wistar rats (control batch, melatonin-treated group, pinealectomized group, and pinealectomized group exposed to melatonin 1mg/kg in drinking water, in the dark phase of the day), melatonin treatment reduced dietary intake, body weight and adiposity. Moreover, melatonin restored leptin sensitivity, reduced the expression of Agouti-related peptides and orexin in the group of pinealectomized rats. Such findings reveal the interaction of melatonin and leptin in the hypothalamus to regulate the energy balance [35].

The suprachiasmatic nuclei are one of the main targets of melatonin in the brain, where it plays an inhibitory role. A mechanism mediated by suprachiasmatic nuclei could contribute to the explanation of the metabolic effects induced by atypical antipsychotics and to the positive action of melatonin in mitigating such effects [36].

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Other experiments revealed the role of melatonin as an anti-obesogenic factor. Thus, in young animals, long-term supplementation with this neurohormone decreased the body weight by about 25%, and the visceral fat deposits by about 50% [30]. In mature, already obese animals, melatonin supplementation in drinking water for one year produced a significant reduction in body mass and intra-abdominal visceral fat. Reducing body weight and abdominal visceral fat was neither dependent on reduced food intake, nor by modifying any other hormone that might influence energy metabolism, such as testosterone, thyroxine (T4), T3 or insulin-like growth factor I [30].

Plasma values of basal insulin and leptin decreased in animals treated with melatonin [30].

In our study, there is an increase in the amount of fat, especially visceral fat, the differences being statistical significant, but an insignificant decrease in subcutaneous fat, after the introduction of melatonin in rats exposed to olanzapine, without changes in food consumption (unpublished data).

Melatonin can counteract some metabolic alterations by regulating circadian rhythms. Molecular studies revealed a correlation between biological clock genes and regulation of metabolism, including the control of glucose homeostasis, lipid synthesis with adipogenesis. The physiology of brown adipose tissue is regulated by melatonin, which increases the recruitment of brown adipocytes and amplifies their metabolic activity [37]. Melatonin is efficient in preventing obesity by activating brown adipose tissue and beige cells in the white fat tissue. Rats treated with melatonin revealed an increase in core body temperature, indicating an increase in energy expenditure, rather than a reduction in energy intake. This increase in animal basal body temperature was consistent with an increase in energy expenditure, dependent on melatonin activation of metabolism in brown adipose tissue and brown-white adipose tissue [20,38-41].

There is a link between the activity of brown adipose tissue and melatonin. Brown adipose tissue has high metabolic activity and is responsible for thermogenesis; thus, brown fat tissue burns a large number of calories to produce heat, thus promoting the catabolism of glucose and fatty acids and limiting fat deposits [37,42]. Melatonin can increase brown adipose tissue activity and mass of adipose tissue by different mechanisms, both central and peripheral [43]. Brown adipose tissue is regulated by hypothalamic neurons, especially the suprachiasmatic nuclei [43] by its melatonin receptors. Melatonin acts directly on brown adipose tissue through membrane receptors that are located on adipocytes. It appears that melatonin can also act directly on the mitochondria of adipose tissue, where it causes an increase in the proliferation of brown fat cells, as well as an increase in the thermogenic capacity [43]. Moreover, brown adipose tissue appears to be of crucial importance in regulating blood glucose, lipidemia and insulin sensitivity. Because brown adipose tissue is present in adult humans [44,45], the observed effect of melatonin on weight reduction in rodents may be translated in humans [30].

An interesting study assessed the effect of melatonin on two groups of patients treated with atypical antipsychotics (one with schizophrenia and another with bipolar disorder), and found a marked decrease in fat mass in the group with bipolar disorder, but not in the one with schizophrenia [46]. In patients with psychosis treated with olanzapine, short-term melatonin treatment (3mg/day for eight weeks) attenuated weight gain, abdominal obesity, and hypertriglyceridemia [47].

Another important approach underlines that AMP-dependent protein kinase is a key factor involved in the regulation of adipocyte proliferation. Metformin, which is an AMP analog, suppresses adipogenesis by mechanisms dependent or independent of AMP-activated protein kinase [48]. It appears that the effect of metformin in inhibiting weight gain generated by atypical antipsychotics may be explained by this mechanism of inhibition of adipogenesis. AMPK phosphorylation results in a cascade reaction, including acetyl-CoA carboxylase (ACC) inactivation, inhibition of ACC lipogenesis, and increased fatty acid oxidation [49]. AMPK also decreases the expression of protein 1 binding to sterol regulatory elements and genes such as FAS and S14. All these changes further decrease the lipogenesis and increase the oxidation of fatty acid, which, in turn, decreases the hepatic lipid load and decreases the level of lipoproteins and the serum concentration of triglycerides with very low density [49]. Metformin has also been found to have antifibrotic action in adipose tissue which explains its role in controlling obesity-associated metabolic disorders [50]. Metformin decreases fat mass but does not significantly influence the

insulin-stimulated glucose uptake into the adipose tissue [51]. Moreover, metformin inhibits *in vitro* preadipocyte differentiation and lipogenesis [52].

In a clinical trial conducted in patients with schizophrenia, in which body mass index, the body weight, waist-to-hip ratio levels, and waist circumference, were assessed, metformin was effective and safe in attenuating olanzapine-induced weight gain [53].

The combination of melatonin and metformin had synergistic actions in altering the progression of metabolic dysfunction in rats with diet-induced obesity, circadian activity, pancreatic insufficiency and insulin sensitivity [54].

FDA data recently evaluated the adverse effects of the melatonin and metformin association regarding increased weight, and revealed that 2.78% had weight gain, while 3.7% presented weight loss [55].

In our experimental research, adding metformin to olanzapine treatment for 57 days resulted in a greater reduction in the amount of visceral and especially subcutaneous fat in the female rat group compared to the antipsychotic-treated group to which melatonin was added. To our knowledge, this is an original study of the combination of melatonin with metformin in an animal model with exposure to olanzapine. We did not notice a significant difference between the results obtained in the group with olanzapine treated with metformin compared with the group with olanzapine exposed to metformin and melatonin.

However, a smaller amount of subcutaneous adipose tissue was found in the combination of olanzapine-melatonin-metformin.

Conclusion

In our study, the amount of visceral and subcutaneous adipose tissue *per* laboratory animal was higher in the group treated with olanzapine pamoate at the end of the study compared to the control group, but the increase is statistically insignificant.

In the group treated with olanzapine pamoate, the ratio between visceral and subcutaneous adipose tissue was lower compared to the control group. In the group treated with olanzapine pamoate, the visceral adipose tissue predominated.

Treatment with melatonin, metformin or the melatonin-metformin combination revealed a decrease in subcutaneous adipose tissue mass mainly in the following order: group treated with olanzapine pamoate>group treated with olanzapine pamoate and melatonin>group treated with olanzapine pamoate and metformin>group treated with olanzapine pamoate, melatonin and metformin.

The olanzapine and melatonin-treated animals had a smaller amount of subcutaneous adipose tissue but not of visceral adipose tissue compared to the olanzapine-treated group alone.

The group treated with olanzapine and metformin and the group treated with olanzapine melatonin and metformin had reduced amounts of visceral and especially subcutaneous adipose tissue compared to the group treated only with the typical antipsychotic.

Our experiment emphasizes the potential obesogenic effect of olanzapine in female rats, as well as its reduction by combination with melatonin and metformin.

Because most of the results were not statistically significant we can deduce that in the chronic experiment, adaptive type modifications of the receptors on which both olanzapine and melatonin act can occur.

Conflict of Interest Statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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 10.12865/CHSJ.45.04.05