Int J Clin Exp Med 2015;8(5):8051-8059
www.ijcem.com /ISSN:1940-5901/IJCEM0007244

Original Article

Salivary and serum biomarkers for the study of side effects of aripiprazole coprescribed with mirtazapine in rats

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Received February 21, 2015; Accepted April 22, 2015; Epub May 15, 2015; Published May 30, 2015

Abstract: The aim of this study was to investigate whether the co-administration of aripiprazole and mirtazapine could determine weight gain and lipid metabolism disorders in Wistar rats, compared to the same side effects produced by mirtazapine alone, and the risk of hepatotoxicity due to the combination of the two substances. Tumor necrosis factor alpha (TNF-α), liver fatty acid binding protein (L-FABP/FABP1) and repulsive guidance molecule C/hemojuvelin (RGM-C/HJV) levels were determined in serum and in saliva. Also, serum levels for total cholesterol (TC), low and high-density lipoprotein (LDL, HDL), triglycerides (TG), aspartate aminotransferase (ASAT) and alanine amino transferase (ALAT) were assessed. We found positive and statistically significant correlations between serum and salivary levels of TNF-α, L-FABP/FABP1 and RGM-C/HJV. Mirtazapine determined significantly differences of TNF-α and L-FABP serum levels; final body weight; TC and LDL levels, leading to higher concentrations than its association with aripiprazole. Although not statistically significant, mirtazapine group experienced higher values for salivary levels of TNF-α, TG and ASAT, and lower values for HDL, compared to aripiprazole + mirtazapine group. The results suggest that aripiprazole might improve some of the disturbances caused by mirtazapine, and that the two drugs combination cause no additional alterations in liver function. Also, the findings indicate that TNF-α, L-FABP/FABP1 and RGM-C/HJV levels can be helpful as biomarkers for metabolic disturbances and impaired function of hepatocytes, and that their salivary determination can replace serum determination.

Keywords: Aripiprazole, mirtazapine, weight gain, lipid metabolism disorders, hepatotoxicity, TNF-α, L-FABP/FABP1, RGM-C/HJV

Introduction

Depressive disorders are a major cause of disability and are associated with significant morbidity and mortality [1, 2]. Depressive conditions often complicate the management of other illnesses (cancer, severe trauma, diabetes, myocardial infarction) or can occur secondary to other diseases such as Parkinson's disease, hypothyroidism, and inflammatory conditions [3]. To secure remission of major depressive disorder, the most prevalent of depressive disorders, and treatment-resistant depression there are several pharmacological strategies. Among these alternatives, addition of certain atypical antipsychotics (aripiprazole, quetiapine, and olanzapine) has become a topical choice, and recent scientific data indicate the

efficacy of adjunctive antipsychotic treatment [4-7].

Aripiprazole, a second -generation antipsychotic, is a partial agonist of D_2/D_3 and 5-HT_{1A} receptors with rather weak antagonistic effects at 5-HT_{2A}, 5-HT_7, H_1 receptors, and moderate inhibitory action on serotonin reuptake [8]. Aripiprazole was associated with minimal weight gain and metabolic changes (dyslipidemia, elevated serum triglycerides and impairments in glycemic control) unlike most second -generation antipsychotics [8-12]. Moreover, some researchers showed that adjunctive aripiprazole administered to patients treated with other second -generation antipsychotics might improve some metabolic parameters [12].

Mirtazapine is a second-generation antidepressant which blocks H_1 and α_2 receptors, but also 5-HT_{2A}, 5-HT_{2C} and 5-HT_3 receptors. Sedation, increased appetite and weight gain are the most common adverse affects of mirtazapine [1-3], and studies demonstrated that mirtazapine led to higher weight gains than other antidepressant drugs [13].

Tumor necrosis factor alpha (TNF-α) is a member of the tumour necrosis factor superfamily which is composed of 19 structurally related proteins (ligands) [14]. This potent proinflammatory cytokine also plays a central role in lipid and glucose metabolism [15, 16] and is produced by a wide variety of cell types including adipocytes [17, 18], being a strong autocrine and paracrine regulator of adipose tissue [17].

Liver fatty acid binding protein (L-FABP or FABP1), named after the tissue in which it was first discovered, is one of the low molecular weight proteins (14-15 kDa) belonging to a family of molecules that coordinate lipid responses in cells [19, 20]. The main function of L-FABP is the intracellular transport of lipophilic substrates like long chain fatty acids. Furthermore, antioxidant and hepatoprotective properties of FABP1 have been identified [20, 21].

Repulsive Guidance Molecule C (RGM-C), also known as Hemojuvelin (HJV), a member of the three-gene RGM family in mammals, is a glycoprotein that plays a key role in iron homeostasis [22, 23]. RGM-C/HJV is mainly expressed in hepatocytes, and mutations of RGM-C/HJV cause juvenile hemachromatosis, a hereditary disorder characterized by excessive iron accumulation [22, 23].

The aim of this study was to investigate whether the co-administration of aripiprazole and mirtazapine could determine weight gain and lipid metabolism disorders in Wistar rats, compared to the same side effects produced by mirtazapine alone, and the risk of hepatotoxicity due to the combination of the two substances, by means of TNF-α, L-FABP/FABP1 and RGM-C/HJV serum levels.

Also, the purpose of the present study was to evaluate the salivary levels for TNF-α, L-FABP/FABP1 and RGM-C/HJV and to determine whether they correlate with the serum levels of the three markers. Given that saliva collecting from both laboratory animals and humans is noninvasive, non-traumatic, and the results being easy to interpret, it is a much simpler method than the blood determination and can replace the latter.

Materials and methods

Animals

All animal protocols were approved by The Ethic Commitee from The University of Medicine and Pharmacy of Craiova, Romania (74/29.05.2014). The study was performed in The Experimental Animal Laboratory from The University of Medicine and Pharmacy of Craiova, Romania.

Twenty adult male Wistar rats (255-270 g) were housed in pairs in polycarbonate cages, in a temperature-controlled room (22±1°C) and under 55-60% humidity with a 12 h dark-light cycle (light 07:00-19:00 h). The animals were fed standard laboratory chow (granulated compound feed, a complete provender for laboratory mice, rats or hamsters provided by "Cantacuzino" Institute from Bucharest, Romania and fabricated at Baneasa Station) and water ad libitum, and the wood shaving was changed every two days. For each animal an individual file was opened, registering the weight values measured in the morning between 9 and 10 o'clock, the used drug and the doses administered according to weight. The drugs were administered orally by gavage, daily at the same hour, along a 6 week period.

The rats were randomly assigned to four groups (n = 5), and consulting the literature [24, 25] we

Table 1. Serum levels of TNF-α, L-FABP and RGM-C in the four groups of rats

Variables	Groups			
	C	A	M	AM
TNF α (pg/ml)	13.4832±2.1939	15.7392±0.9700†	17.627±0.9962†,*	16.3976±1.1337†
L-FABP (ng/ml)	0.8708±0.3664	0.5856±0.3246	0.8396±0.0404*	0.6328±0.1682
RGM-C (pg/ml)	857.3840±333.0377	2430.6262±344.2762†	2383.1696±461.3373†	2671.7918±262.8387†

Values are mean±SD. C: control group; A: aripiprazole group; M: mirtazapine group; AM: aripiprazole + mirtazapine group. †$p < 0.05$ compared to corresponding data in C group; *$P < 0.05$ versus AM.

selected the drugs' doses: Group I-Control (C) (saline 10 ml/kg p.o.), Group II-Aripiprazole (A) (4.05 mg/kg p.o.), Group III-Mirtazapine (M) (10 mg/kg p.o.), Group IV-Aripiprazole + Mirtazapine (AM) (4.05 mg/kg p.o. + 10 mg/kg p.o.).

Because the volume of blood, that may be collected from the rats enabling its survival in optimal conditions for the study, is very small, and due to technical limitations, we could not get the baseline values of the subjects included in the study. Thus, we introduced the control group in the experimental protocol, which lived in the same conditions as the rest of the animals, to provide normal values for each determination.

24 hours after the last drug administration, the rats were anesthetized and blood samples were taken. TNF-α, L-FABP/FABP1 and RGM-C/HJV levels were determined both in serum and in saliva. Additionally, serum levels for total cholesterol (TC), low and high-density lipoprotein (LDL, HDL), triglycerides (TG), aspartate aminotransferase (ASAT) and alanine amino transferase (ALAT) were assessed.

Serum

Before blood sampling, the rats were anesthetized using Sevoflurane 5% diluted in a mixture of 70% nitrous oxide and 30% oxygen, given in a special cage for the induction of anesthesia. Following induction of anesthesia, the rats were moved to the surface of a table for infusion, the anesthetic being administered further through a mask for maintenance of anesthesia. After opening the abdominal cavity and severing the abdominal muscles just on the midline, the diaphragm was carefully cut and the chest was open, by cutting the ribs to visualize the heart. The pericardium was removed and a trocar for infusion was inserted into the left ventricle, by means of which 4 ml of intraventricular blood were collected in 2 gel vacutainers. After centrifugation, the supernatant was collected and frozen at -80°C until processing samples.

Saliva

Saliva was collected on filter paper strips (PerioPaper, Oraflow Inc., Smithtown, NY, USA) introduced in the animal's oral cavity for 10 sec, according to the previously described technique, applied in humans, for collecting the gingival crevicular fluid and saliva [26]. Samplings were done prior to beginning of drug administration, as well as 24 hours after the last administration, before animals' sacrifice. The volume was measured with a precalibrated device (Periotron 8000, Oraflow Inc., Smithtown, NY, USA) set for saliva, designed to measure volumes of 10^{-6} l (µl). The absorbed liquid was diluted in 100 µl phosphate-buffered saline (PBS) in polypropylene tubes, the obtained samples being frozen at -20°C until their utilization.

Immunological and Biochemical Investigation

TNF-α, L-FABP/FABP1 and RGM-C/HJV levels were measured (in the total volume of liquid represented by the volume of the collected saliva + 100 µl PBS) by ELISA technique using Quantikine kits from R&D Systems, USA with ASYS Expert Plus (ASYS HITECH GMBH, Austria) according to the manufacturer's instructions.

TC, LDL, HDL, TG, ASAT and ALAT were determined by absorbance photometry using commercial kits obtained from Roche Diagnostics, USA, with an automated analyzer (Cobas Integra 400 Plus, Roche, Switzerland).

Statistical investigation

Statistical analysis was performed by using a dedicated software (SPSS 16.0, Chicago, IL, USA). Differences between groups were calculated using a nonparametric test (Mann Whitney

Table 2. Salivary levels of TNF-α, L-FABP and RGM-C in the three drug treated groups of rats

Variables	Groups					
	A		M		AM	
	Ai	Af	Mi	Mf	AMi	AMf
TNF α (pg/ml)	5.6886±0.4332	6.3338±0.5847#,*	7.7374±2.3340	11.2048±5.0534#	6.1894±0.6918	7.9568±0.7816#
L-FABP (ng/ml)	0.0930±0.0060	0.0858±0.0312	0.0830±0.0200	0.0782±0.0312	0.0838±0.0229	0.0732±0.0282
RGM-C (pg/ml)	18.0026±0.2554	23.3854±4.6623#,*	18.1060±0.5495	18.8392±0.4013#	19.0416±0.5179	18.4418±0.2316#

Values are mean±SD. A: aripiprazole group (Ai: initial value; Af: final value); M: mirtazapine group (Mi: initial value; Mf: final value); AM: aripiprazole + mirtazapine group (AMi: initial value; AMf: final value). #$p < 0.05$ versus initial evaluation in the same group; *P < 0.05 versus AM.

U test). Correlations among groups were calculated with Pearson test. Results are presented as mean±SD (standard deviation) $P < 0.05$ being considered statistically significant.

Results

Serum levels of TNF-α, L-FABP and RGM-C are shown in **Table 1**.

Serum levels of TNF-α were significantly increased ($P < 0.05$) in groups 2, 3 and 4 (A, M and AM groups) compared to group 1 (group C), and were also significantly different between groups 3 and 4 ($P < 0.05$). Regarding L-FABP, only M group displayed a significantly increase ($P < 0.05$) in these values when compared to AM group. The serum RGM-C levels in all groups were significantly different than the C group ($P < 0.01$ for A and M groups; $P < 0.001$ for AM group).

Collected volumes of saliva were within the range 0.518-1.614 µl.

Table 2 presents salivary levels of TNF-α, L-FABP and RGM-C.

Final salivary TNF-α levels were significantly higher in all study groups ($P < 0.05$ for groups 2 and 3; $P < 0.001$ for group 4) compared to the initial evaluation in the same group, and significantly different between groups 2 and 4 ($P < 0.01$). No significantly different results were found in any group for L-FABP. Compared to the initial evaluation in the same group, a highly significant increase ($P < 0.01$) of final RGM-C salivary levels was observed in the M group and significant differences ($P < 0.05$) apeared in A and AM groups; also group A showed significantly different values ($P < 0.05$) versus AM group's values.

We found positive and statistically significant ($P < 0.05$) correlations between serum and salivary levels of all three markers, as shown in **Table 3A-C**. There was a very strong positive correlation for TNF-α in mirtazapine group, for L-FABP in aripiprazole group and for RGM-C in aripiprazole and aripiprazole + mirtazapine group.

In addition to these data, body weight changes and biochemical markers in the four groups of rats (control, aripiprazole, mirtazapine and aripiprazole + mirtazapine) were analyzed (**Table 4**).

A highly significant increase ($P < 0.01$) in final body weight was observed in the M group as compared to the C and AM groups. TC and LDL levels in the A, M and AM groups were significantly greater ($P < 0.01$) than in the C group, and the M group also recorded significant differences compared to AM group ($P < 0.05$). Significantly different results ($P < 0.01$) were found in the three drug treated groups compared to C group for HDL levels. As for TG, M and AM groups showed significantly increased levels ($P <0.01$) compared to C group, and A group compared to AM group ($P < 0.05$).

ASAT experienced significantly different levels ($P < 0.05$) in A, M and AM groups compared to C group, but all three drug treated groups exhibited reduced values as against the ones from C group. Significantly different results were not found for any group in the cases of ALAT, but again A, M and AM groups showed decreased levels than C group.

Discussion

Like J.P. Bastard et al stated, "obesity is associated with a chronic inflammatory response, characterized by abnormal adipokine production, and the activation of some pro-inflammatory signalling pathways, resulting in the induction of several biological markers of inflammation" [18].

Table 3. Correlations between serum and salivary levels of TNF-α (A), L-FABP (B) and RGM-C (C) in the three drug treated groups of rats

A			
Serum levels of TNF-α	Salivary levels of TNF-α		
	A	M	AM
A	0.5932	-	-
M	-	0.9024	-
AM	-	-	0.7194
B			
Serum levels of L-FABP	Salivary levels of L-FABP		
	A	M	AM
A	0.8562	-	-
M	-	0.7274	-
AM	-	-	0.7689
C			
Serum levels of RGM-C	Salivary levels of RGM-C		
	A	M	AM
A	0.8775	-	-
M	-	0.5712	-
AM	-	-	0.8388

A: aripiprazole group; M: mirtazapine group; AM: aripiprazole + mirtazapine group.

TNF-α is a 157-amino acid cytokine, that was first identified in the late-1960s and early-1970s, produced both peripherally and in the central nervous system and which can stimulate its own production [27]. It binds the neuroinflammatory and excitotoxic processes that take place in several neurodegenerative diseases, higher levels of TNF-α being found in traumatic brain injury, Parkinson's disease, multiple sclerosis, ischemia, Alzheimer's disease, and amyotrophic lateral sclerosis [28].

Many studies have evaluated the relationship between plasma levels of proinflammatory cytokines and some psychiatric conditions, including major depression and schizophrenia. Researches have proved that levels of proinflammatory cytokines, including TNF-α, are elevated in depressed patients compared to controls [27, 29].

The "cytokine hypothesis of depression" has been issued, showing the pathway from increased cytokine production to depressive symptoms and describing the important role for pro-inflammatory cytokines. Moreover, it has been suggested that cytokines may serve as biomarkers in individualised treatment of depressive disturbances [29].

In accordance with previous reports [30], in our experiment serum levels of TNF-α were significantly higher in aripiprazole-treated subjects as compared to controls.

Mirtazapine was linked to the highest increase in TNF-α production; this result is consistent with those of an *in vitro* test [29], but different to *in vivo* studies demonstrating that antidepressant treatment does not influence or even decreases TNF-α levels [29, 31].

In terms of body weight changes and biochemical indicators for lipid metabolism disorders, of the three groups of animals treated with drugs, the highest values for final body weight, TC, LDL and TG were recorded in M group, which also had the lowest values for HDL ($P < 0.05$ compared to corresponding data in C group).

L-FABP, that was discovered in 1972 and originally named Z-protein [32], is expressed in elevated levels (7-11% of cytosolic protein) in normal human liver as well as intestine and kidney. Low levels of L-FABP occur in abetalipoproteinemia and Anderson's disease, and L-FABP levels are increased to 20% of cytosolic protein in individuals with Reyes syndrome [33]; its levels being very sensitive to high fat diet, peroxisome proliferators and lipid lowering drugs like fibrates [33].

Regarding the researches upon liver, beside its antioxidant and hepatoprotective properties, L-FABP was found to be helpful in studying the alcoholic liver disease [34], and in the subtyping of hepatocellular adenomas [35].

In the present study, no significant changes were recorded either between initial and final salivary levels of L-FABP, or between its serum levels in the control group and in the three treated groups. These findings support the ideas that aripiprazole and mirtazapine do not cause altered salivary and/or serum levels of L-FABP; and that lipid metabolism disorders that were registered are not related to L-FABP values. Data from similar studies are lacking, and to our knowledge, no researches are available which have analyzed the effect of mirtazapine and aripiprazole on L-FABP in humans or rats.

Table 4. Body weight and biochemical markers in the four groups of rats

Variables	Groups			
	C	A	M	AM
Initial body weight (g)	262.8±2.2803	264±6.5192	258±2.7386	261±4.1833
Final body weight (g)	263.2±2.3874	264.2±5.4954	297 .8±17.1668$^{\#,\dagger,*}$	263.4±5.1768
TC (mg/dL)	32.092±12.1814	66.992±4.6653†	95.174±10.8830†,*	74.942±8.1535†
LDL (mg/dL)	7.814±2.6316	18.138±2.1328†	25.682±2.0435†,*	19.298±3.6294†
HDL (mg/dL)	32.496±9.7133	81.618±10.0974†	62.226±6.7460†	67.462±7.1002†
TG (mg/dL)	41.106±16.0790	51.336±4.5384*	76.13±16.7466†	69.188±16.4595†
ASAT (UI/L)	148.764±16.8484	106.064±11.3571†	115.518±22.9490†	100.812±14.6712†
ALAT (UI/L)	56.654±12.0522	49.91±6.0493	47.76±9.4363	54.782±1.9699

Values are mean±SD. C: control group; A: aripiprazole group; M: mirtazapine group; AM: aripiprazole + mirtazapine group. $^{\#}p < 0.05$ versus initial evaluation in the same group; $^{\dagger}p < 0.05$ compared to corresponding data in C group; *P < 0.05 versus AM.

Regarding hepatotoxicity, drug induced liver injury is a rare incident but many classes of drugs can determine impaired liver function. Genetic, metabolic and immune mechanisms of drug induced hepatotoxicity were investigated [36], using both *in vivo* studies and *in vitro* platforms [37].

In relation to serum biochemical markers associated with the development of hepatotoxicity, in the present study it was found that ALAT concentrations in A, M and AM groups were decreased in comparison to those in the C group and not significant. As for ASAT, in A, M and AM groups significant change was observed, but their results were significantly lower when compared to C group.

Also, neither salivary nor serum levels of L-FABP were significantly influenced by the administration of the two substances. These data are consistent with previous findings which demonstrated that serum L-FABP level is correlated with ASAT and ALAT, and that serum L-FABP is a very sensitive marker of liver injury [19, 38].

It should be noted that ALAT, ASAT and salivary L-FABP levels did not show any significant differences for A and M group compared to AM group; only serum L-FABP levels in M group were significantly elevated as compared to AM group. It has been established that hepatotoxicity is no side effect either of aripiprazole or of mirtazapine, our results confirming this and in addition showing that the combination of the two drugs do not cause additional alterations in liver function.

Identified in humans in 2003 [39], RGM-C/HJV was studied both in genetic and in biochemical tests and it was found to be an upstream regulator of hepcidin, strong hepcidin deficiency leading to onset of juvenile hemachromatosis [39, 40]; until now 43 HJV mutations that cause juvenile hemachromatosis have been described [41]. Although expressed in cardiac and striated muscle, hepatic expression of HJV seems to have the greatest physiologic role in systemic iron homeostasis regulation *in vivo* [41].

Beside the glycophosphatidylinositol-linked membrane form of HJV, endogenous soluble HJV protein was detected in human and rodent serum, but its source, quantity, and physiologic role *in vivo* are still not fully clarified [41, 42].

Inflammatory stimuli, like IL-6 and TNF-α, are linked with severe alterations in iron homeostasis [43, 44]. It was reported the possibility for HJV not to be directly involved in the hepcidin response to inflammatory stimuli whereas it is necessary for hepcidin response to iron [44].

In the current experiment it was observed that administration of both substances still produces a change in the hepatocyte function, thus there were found significantly higher values for serum RGM-C/HJV in A, M and AM groups compared to C group; on the other hand significantly increased levels were registered for serum TNF-α. These results are unlike other findings which showed that TNF-α mediates HJV downregulation [44]. However, there were no significantly differences between A, M and AM group. Future investigations will be needed to elucidate the mechanisms by which RGM-C/HJV levels are modified following the administration of aripiprazol and mirtazapine.

The significantly elevated final values of salivary TNF-α and RGM-C compared to initial val-

ues, observed in the three treated groups, show that both drugs determine salivary detectable changes in these biomarkers' concentration.

Positive correlations, either strong or very strong, between serum and salivary levels of TNF-α, L-FABP and RGM-C allow to say that the analyze of the three biomarkers can be achieved using saliva instead of blood determination.

Comparing mirtazapine group and aripiprazole + mirtazapine group, there was a significant difference of serum levels of TNF-α and L-FABP; final body weight; TC and LDL levels, mirtazapine leading to higher concentrations than its association with aripiprazole. Although not statistically significant, mirtazapine group experienced higher values for salivary levels of TNF-α, TG and ASAT, and lower values for HDL, compared to aripiprazole + mirtazapine group. Taken together, these results suggest that aripiprazole might improve some of the disturbances caused by mirtazapine.

To the best of our knowledge, this is the first study assessing TNF-α, L-FABP/FABP1 and RGM-C/HJV serum levels in rats treated with aripiprazole and/or mirtazapine; furthermore, thisexperiment is the first attempt to evaluate the salivary levels for TNF-α, L-FABP/FABP1 and RGM-C/HJV after administration of aripiprazole and/or mirtazapine, and to determine possible correlations between serum and salivary values of the three markers.

Saliva-based microbial, immunologic, and molecular biomarkers provide unique opportunities to avoid painful invasive procedures, such as repeated blood draws and biopsies, by utilizing oral fluids to assess the condition of both healthy and suffering individuals [45]. Saliva represents a source of indicators for local, systemic, and infectious diseases [45, 46]. An increasing number of specific molecular markers for different disorders, like obesity, inflammation, insulin-resistance [47], cardiovascular diseases, oral and breast cancer and human immunodeficiency virus (HIV) are being identified [46]. Saliva sampling is relatively simple, allows easy storage and transport [48] and constitutes a promising diagnostic alternative, compared to blood sampling, especially among pediatric and geriatric patients, where blood sampling may be difficult [47].

This experiment has several limitations. Side effects of aripiprazole and mirtazapine were tested only after a 6 week period of administration. These drugs' effects upon TNF-α, L-FABP/FABP1 and RGM-C/HJV serum and salivary levels should be evaluated for a longer time period and on a larger number of animals.

Conclusions

In conclusion, data suggest that aripiprazole-augmented treatment might be beneficial for the weight gain and lipid metabolism regulation of patients treated with mirtazapine, and that the combination of the two drugs do not cause additional alterations in liver function.

Insights gained from the present experiment indicate that TNF-α, L-FABP/FABP1 and RGM-C/HJV levels can be helpful as biomarkers for metabolic disturbances and impaired function of hepatocytes, and that their salivary determination can replace serum determination.

Further animal and human studies should investigate the change in the serum levels of these three markers and the correlation with their salivary levels, following different drugs administration.

Acknowledgements

The authors contributed equally to this study. This paper was published under the frame of European Social Found, Human Resources Development Operational Programme 2007-2013, project No. POSDRU/159/1.5/S/13-6893.

Disclosure of conflict of interest

None.

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