Determination of oxidative stress parameters in fluoxetine users

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Abstract

Fluoxetine (FLU), a selective serotonin reuptake inhibitor, is the first line in depression treatment and it is involved in oxidative stress (OE). Thus, this study aimed to analyze the OE parameters in patients diagnosed with depression and treated with FLU. Were evaluated 121 volunteers divided into two groups: 58 fluoxetine users (with major depression) and 63 non-fluoxetine users (control group, without major depression). The OE was evaluated by determining the levels of malondialdehyde (MDA), total antioxidant power (FRAP) and activity of antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD). MDA, FRAP, GPx and SOD were dosed in plasma. The influence of age, smoking, alcoholism, comorbidities, use of another drugs and antioxidants in the OE were evaluated. The results were compared between the groups. In relation to the fluoxetine daily dose, MDA presented higher levels in patients using 20 mg daily FLU when compared to the control group, as well as the activity of the GPx enzyme and the FRAP levels. In this way, the use of fluoxetine may interfere with the OE parameters, causing an increase in OE levels.

Keywords: antioxidants; fluoxetine; reactive oxygen species; oxidative stress; major depression

1. Introduction

Depression is a neuropsychiatric condition with worldwide prevalence reports ranging from 6.5 to 21% [1]. Studies on the causes of depression have shown an increase in the activation of inflammatory immune markers [2], increased production of reactive oxygen species (ROS) [3], and changes in the phospholipids and cholesterol that constitute the cell membrane [4, 5]. In fact, OE has been involved in the occurrence of cognitive disorders. There is evidence that increases in OE levels and/or deficiencies in antioxidant defenses are risk factors for cognitive decline. In this way, the EO seems to play an important role in the pathogenesis of depression [3, 6, 7]. Still, these changes lead to a process of neuroinflammation

and consequent neurodegeneration, which seems to play an important role in the pathogenesis of depression. Several biomarkers have been established in patients with depression, such as cytokines, oxidative stress markers and tryptophan catabolites [8, 9].

The condition known as oxidative stress is a result of the imbalance between the production of reactive oxygen species (ROS) and the antioxidant system. ROS are molecules with an electron spliced and very reactive. This large group of molecules is represented mainly by the radical superoxide ($O_2^{\bullet -}$), peroxyl radical ($ROO^{\bullet -}$), hydroxyl radical ($OH^{\bullet -}$), and nitric oxide ($NO^{\bullet -}$). The enzymatic and non-enzymatic systems that form the antioxidant system, act in a synchronized way to protect the cells from the damage caused by free radicals. The main antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [10, 11]. Non-enzymatic antioxidants include endogenous molecules such as bilirubin, uric acid, and glutathione, as well as exogenous compounds, such as vitamins A, C, and E [10-12]. The action of these compounds along with the antioxidant enzymes result in what we know as total antioxidant status [10, 13].

The oxidative stress is detrimental to cellular metabolism. The process of lipid peroxidation, which also results in the production of free radicals, is the most known damage caused by oxidative stress [14]. Malondialdehyde (MDA) is the main product of lipid peroxidation. This aldehyde is a highly toxic molecule that interacts with proteins and DNA [15]. Studies that address lipid peroxidation in patients with depression describe an increase in the levels of MDA and other lipid peroxidation products [16, 17].

According to some studies, the antioxidant enzymes activities in patients with depression are different from those observed in healthy individuals [18-23]. Indeed, antioxidant enzymes activities are decreased in patients with depression when compared to healthy subjects [22]. Decreased levels of SOD, GPx, and CAT, and increased levels of MDA have been reported in patients with affective disorders [23], and increased urinary excretion of F2 isoprostane in patients with depression [7].

Fluoxetine (FLU) is a first line drug for the treatment of depression. It has emerged as the treatment of choice for depression because of its safer profile, fewer side effects and improved tolerance compared to tricyclic antidepressants and other selective serotonin reuptake inhibitors (SSRIs) [24, 25]. Several studies have reported important effects of fluoxetine on the central nervous system. Novio *et al*. (2011) [26] demonstrated the positive effect of fluoxetine against oxidative damage in stress-induced cells. Zafir and Banu (2007) [27] also showed the antioxidant potential of this drug, stating that this potential could contribute to its therapeutic action. Kolla *et al.* (2014) [28] demonstrated greater survival of neurons and a reduction of oxidative substances with the use of fluoxetine. In addition, Bilici *et al.* (2001) [18] found that ROS may play an important role in major depression and that MDA and antioxidant enzymes may be markers of major depression, as they returned to normal intervals after antidepressant treatment. In this context, this study aimed to analyze the OE parameters in patients diagnosed with depression and treated with FLU.

2. Material and methods

2.1 Study population and sample collection

A cross-sectional study was carried out with 121 individuals, divided into two groups: 58 fluoxetine users (FLU group) and 63 non-fluoxetine users (control group). All volunteers were members of CIES - FEEVALE, Integrity Center of Health Specialties of FEEVALE University, Novo Hamburgo, Brazil and were at least 18 years of age. This study was approved by the Ethics and Research Committee of FEEVALE University (CAAE 44035115.0.0000.5348), and it was carried out according to the resolution 466/2012 of the National Council of Education and Research. All volunteers signed the free and informed consent form.

The fluoxetine user group is designed for patients diagnosed with major depression and taking fluoxetine (doses of 20, 40 or 60 mg/daily) for at least 6 months. Subjects classified as control group were not diagnosed with depression and were not using any antidepressants. Patients that did not present cognitive conditions to respond to the questionnaires, or in use of another antidepressant drug, or were using the fluoxetine less than six months ago were excluded. Both groups had age-related comorbidities (diabetes, hypertension, heart disease, thyroid disorders, and dyslipidemia) and use the same classes of medicines for comorbidities treatment.

All volunteers responded to a structured questionnaire about their lifestyle (use of tobacco, alcohol or antioxidant substance) and socio-demographic profile. The clinical characteristics [age, sex, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (BPD), comorbidities and medications in use] were evaluated from medical records. Blood samples were collected in EDTA and Heparin tubes. Later, they were centrifuged for 10 min at 2500 rpm for plasma separation, which was stored in an ultra-freezer at -80 °C.

2.2 Oxidative stress

The EO was evaluated through the dosages of MDA, FRAP and the enzymatic activities of SOD and GPx.

MDA dosages were initiated with alkaline hydrolysis of plasma in order to release the protein bound fraction, subsequently precipitated by the addition of $HClO_4$. DNPH-derivatized protein was added to the supernatant [29]. The chromatographic run was performed in a Shimadzu Class VP HPLC diode array detector with a Lichrospher RP-18 Merck column (250×4 mm, 5

superoxide radicals which react with 2- (4-iodophenyl) -3- (4-nitrophenol) -5-p-phenyltetrazolium chloride to produce formazan, a compound which absorbs light at 450 nm. Inhibition of chromogen production is proportional to the SOD activity present in the sample. The reading was performed on spectrophotometer microplates and the results expressed as % inhibition of SOD.

The enzymatic activity of GPx was performed by the method described by Pleban; Munyani and Beachum (1982) [31]. First, the working reaction was prepared with 50 mmol / l Tris buffer at pH 7.6, containing 1 mmol Na_2EDTA per liter, 2 mmol reduced glutathione, 0.2 mmol NADPH, 4 mmol sodium azide and 1000 U of glutathione reductase. The mixture was incubated for 5 minutes at 37 ° C. To determine enzymatic activity in plasma, 50 L of plasma was added to 950 L of the working reaction. The activity of GPx was expressed in plasma U/L. After a period of 30 seconds, the decrease in absorbance will be linear with time. At the beginning of the reaction, 10 L of 8.8 mmol/L hydrogen peroxide was added and the spectrophotometer read at 340 nm for 3 minutes.

2.3 Statistically analysis

The clinical characteristics and oxidative stress parameters of fluoxetine users and control group were compared by Student's *t* test, Mann-Whitney *U* test or Pearson Chi-Square, according to the data. The oxidative stress parameters for fluoxetine users, grouped according to the daily fluoxetine doses (20, 40 and 60 mg), and control group were analyzed using the One-way Analysis of Variance (ANOVA), followed by Tukey *post hoc* test for multiple comparisons. Multiple linear regression models were carried out to test the association of oxidative stress parameters (dependent variable) and factors with possible biological relevance (age, use of fluoxetine, anti-inflammatory or antioxidant drugs) or significance at univariate analysis (BMI, SBP, and DBP) were used like independents variables. All independent variables selected were added in a block in a single step. Different oxidative stress parameters were included as dependent variables in each model, one at a time. Data were expressed as medians (percentile 25 and percentile 75) or means ± SD. P values < 0.05 were considered statistically significant. The software SPSS 25.0 (SPSS, Chicago, IL) was used for the statistical analyses.

4. Results

The clinical characteristics of users of fluoxetine and the control group are shown in Table 1. There was no significant difference between the mean ages of both groups. There was a predominance of women among the volunteers, but the proportion of men and women in the groups did not show a statistical difference between them. Both groups contained hypertensive patients, with diastolic and systolic pressures being significantly higher in users of fluoxetine as well as body mass index. Among the drugs used by the volunteers of both groups, we identified anti-inflammatory drugs and antioxidants as possible interferers of the oxidative profile. There was no statistical difference between the groups in relation to the number of non-steroidal anti-inflammatory users (NSAIDs), and only the FLU group presented users of anti-inflammatory drugs of corticosteroids. There was also no statistical difference in the proportion of users of antioxidant drugs in both groups.

Table 1. Clinical characteristics of fluoxetine users and control group

Characteristics	**Control group (n=63)**	**FLU group (n=58)**	
Age (years)	59 ± 11	56 ± 13	0.128
Sex			
Female	81 %	83 %	0.763
Male	19 %	17 %	0.670
BMI (kg/m²)	26.7 ± 4.02	29.58 ± 6.38	0.004
SBP (mmHg)	118.28 ± 18.92	125.94 ± 18.29	0.026
DBP (mmHg)	76.17 ± 13.80	81.20 ± 13.51	0.045
Smoking			
No	94 %	83 %	0.327
Yes	6 %	17 %	0.109
Alcoholism			
No	100 %	100 %	1.000
Uses antioxidante			
No	74 %	84 %	0.838
Yes	26 %	16 %	0.162
Uses anti-inflammatory			
No	78 %	71 %	0.399
NSAI	22 %	22 %	0.847
Corticosteroide	0 %	7 %	<0.001

BMI: body mass index. SBP: systolic blood pressure. DBP: diastolic blood pressure. NSAI: non-steroidal anti- inflammatory. The Student t test (for age, BMI, SBP and DBP variables) and Pearson Chi Square are used in statistical analysis.

Table 2 shows the comparison of the parameters of oxidative stress among control group and users of different doses of fluoxetine. The daily doses of fluoxetine among users of fluoxetine in the study group were 20 mg (n = 49), 40 mg (n = 7) and 60 mg (n = 2). Thus, users of 40 and 60 mg of fluoxetine were grouped for the statistical analyzes related dose of fluoxetine and parameters of oxidative stress. The MDA presented higher levels in patients using 20 mg daily FLU when compared to the control group [1.62 M (1.16 – 2.80) *vs* 1.34 M (1.09 – 1.57) respectively; p <*0.001*], as well as the GPx activity [0.87 U/L (-2.07 – 19.30) *vs* -1.59 U/L (-31.04 – 4.79) respectively; p = 0.004] and FRAP levels [1176 M (980 – 1298) vs 1091 M (738 – 2733) respectively; p =0,017).

Table 2. Oxidative stress parameters of fluoxetine users and control group

OE parameter	Control group (n = 63)	Fluoxetine group		P value	a	b	c
		20 mg (n = 49)	40/60 mg (n = 9)				
FRAP (µM)	1091 (738 – 2733)	1176 (980 – 1298)	1045 (962 – 1121)	0.011	0.017	0.152	0.926
SOD (% inhibition)	85.7 (74.3 – 99.5)	90.4 (84.1 – 93.9)	89.1 (81.7 – 92.9)	0.142	0.338	0.211	0.626
MDA (µM)	1.34 (1.09 – 1.57)	1.62 (1.16 – 2.80)	1.62 (1.45 – 2.34)	<0.001	<0.001	0.125	0.871
GPx (U/L)	-1.59 (-31.04 – 4.79)	0.87 (-2.07 – 19.30)	0.39 (-0.79 – 36.32)	0.006	0.004	0.497	0.812

FRAP: ferric-reducing ability of plasma; SOD: superoxide dismutase; MDA: Malondialdehyde; GPx: glutathione peroxidase. The results are expressed as median (percentile 25 and percentile 75). The One-way Analysis of Variance (ANOVA), followed by Tukey *post hoc* test for multiple comparisons are used in statistical analysis. P value: a) Control Group *vs* 20 mg; b) Control Group *vs* 40/60 mg; c) 20 mg *vs* 40/60 mg.

The statistical differences found in the oxidative stress indicators were adjusted by biological variables (age, BMI, SBP, DBP) and concomitant use of fluoxetine, anti-inflammatories, and antioxidants. The linear regression data for this analysis are presented in Table 3. The use of fluoxetine was shown to be influencing the statistical difference of FRAP, MDA and GPx parameters, while the use of anti-inflammatory drugs seems to influence only the GPx enzyme activity.

Table 3. Multiple linear regression analyses by oxidative stress parameter in FLUOX group (n=58) and Control group (n=63)

Dependent variables	**FRAP**		**MDA**		**GPx**		**SOD**	
(R^2)	**0.360 (0.025)**		**0.416 (0.003)**		**0.364 (0.023)**		**0.181 (0.797)**	
Independent variables	P / beta value		P / beta value		P / beta value		P / beta value	
Use of fluoxetine	0.015	-0.237	<0.001	0.423	<0.001		0.103	0.166
Use of anti-inflammatory	0.236	0.107	0.789	0.023	0.351		0.900	-0.012
	0.562	0.053	0.480	0,063	0.050	-0.178	0.662	-0.042
Use of antioxidant	0.553	-0.055	0.447	0,069	0.590	0.050	0.564	0.056
Age	0.844	-0.019	0.975	-0,003	0.191	0.122	0.660	-0.044
BMI	0.753	-0.040	0.243	-0,145	0.444	-0.074	0.998	0.000
SBP	0.117	-0.192	0.414	0.097	0.642	-0.059	0.983	0.052
DBP					0.547	0.073		

BMI: body mass index; SBP: systolic blood pressure; SBD: diastolic blood pressure. FRAP: ferric-reducing ability of plasma; MDA: malondialdehyde; GPx: glutathione peroxidase; SOD: superoxide dismutase.

5. Discussion

The primary antioxidant defense system involves the coordinated effects of antioxidant enzymes such as SOD, catalase (CAT) and GPx. In this study, we observed differences in OE parameters in users

and non-users of fluoxetine. In fact, the MDA levels, the GPx enzyme activity and the FRAP levels are higher in patients using 20 mg daily FLU when compared to the control group. These findings suggest that the use of fluoxetine may increase oxidative damage (MDA increased), leading to a compensation of antioxidant defenses, both enzymatic (GPx) and non-enzymatic (FRAP) [10]. Another finding of this study concerns the non-difference in levels of SOD enzyme activity between the control and FLU groups.

Studies have shown that patients with depression have increased levels of OE compared to healthy patients [18, 19, 21], as well as levels of MDA, a product of lipid peroxidation. The increased activity of ROS levels, in turn, comes from the activation of immune cells, another feature of major depression [4]. Higher ROS levels trigger lipid peroxidation in polyunsaturated fatty acids (PUFAs) present in cell membranes, destabilizing them and interfering with their functions. In this context, there is information on the influence of fluoxetine on antioxidant enzymes. While some studies suggest that this antidepressant restores the antioxidant capacity in the brain [18, 26], Djordjevic *et al.* (2011) [32] and Zlatkovi *et al.* (2014) [33] suggested that fluoxetine affects the antioxidant system of rat liver, and another study has reported that such therapy does not alter the OE in patients with depression [16].

In the present study, we identified higher MDA levels in fluoxetine users (20 mg daily dose) when compared to the control group. These findings differed from Bilici *et al.* (2001) [18], which showed that after three-month treatment with 20mg daily FLU, MDA levels were significantly reduced. In addition, Belowski *et al.* (2004) [34] showed the ability of fluoxetine to reduce the cytotoxic activity of macrophages, which are sources of ROS. This effect appeared after 2 weeks of treatment with 10 mg fluoxetine. However, the four-week treatment with the same dosage is not shown in the process above. This demonstrates that the effect of fluoxetine may be dependent on the duration and dosage of the treatment. The non-decrease in the levels of MDA in the treated patients could also be attributed to the lack of effectiveness of the treatment in the low adhesion function or without the metabolism of this drug. Further studies in this direction would be necessary.

FRAP (total antioxidant power) measures the antioxidant status, adding the activity of enzymatic and non-enzymatic antioxidant systems. This study found a significantly higher level of this parameter in the FLU group (20 mg daily dose) compared to the control group. Bilici *et al.* (2001) [18], as well as Caiaffo *et al.* (2016) [15], reported that FLU has a significant effect on improving the antioxidant defense system. They suggested an inhibition in the production of pro-inflammatory cytokines, leading to a decrease in the production of free radicals. In this way, they indicated the antioxidant action as a clinical benefit of the administration of this drug. In the multiple linear regression analyses, we found that the concomitant use of anti-inflammatory or antioxidant drugs by volunteers in both groups also did not influence the results of FRAP.

In relation to the GPx enzyme, this study identified a greater activity in the FLU group (20 mg daily dose) in comparison to control group. This increase in GPx activity in the FLU group conflicts with that reported by Bilici *et al. (*2001) [18]. These authors evaluated the antioxidant enzymatic activity in control patients and patients with major depression, before and after three months of treatment with FLU. The latter group significantly reduced the GPx activity. To justify this finding, two mechanisms had been suggested: (i) SSRI treatment has a suppressive effect on cells of the immune system, which can lead to a decrease in ROS levels and consequently levels of antioxidant enzymes; and (ii) Cytochrome P450

enzymes may play a role in ROS production, and these enzymes are inhibited by SSRIs. Thus, according to these authors, it can be speculated that FLU can reduce levels of antioxidant enzymes by inhibiting cytochrome P450 enzymes. Still, this topic requires further investigation.

At the end of this study, we verified whether, in addition to fluoxetine, biological variables (age, BMI, SBP, BPD) and the concomitant use of anti-inflammatory or antioxidant drugs influenced the statistical differences found. The linear regression showed that the use of fluoxetine influenced the results of the FRAP, MDA and GPx parameters. However, the use of anti-inflammatories also showed to influence the GPx parameter.

The main limitations of this study are the small sample size and the non-assessment of fluoxetine users in their baseline status, which is difficult due to the fact that depression requires medical treatment. Therefore, we suggest that one or more factors not considered in this study may be interfering with these results, such as adherence to FLU treatment or alterations in the metabolism of this drug. Complementary studies with these groups are necessary.

6. Conclusion

In this study, we observed differences in OE parameters in users and non-users of fluoxetine. The MDA levels, the activity of the GPx enzyme and the FRAP levels are higher in patients using 20 mg daily FLU dose when compared to the control group, suggesting that the use of fluoxetine may increase oxidative damage (MDA increased), leading to a compensation of antioxidant defenses, both enzymatic (GPx) and non-enzymatic (FRAP).

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7. References

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