EVALUATION OF OXIDATIVE STRESS IN SMOKING AND NON-SMOKING PATIENTS DIAGNOSED WITH ANXIOUS-DEPRESSIVE DISORDER

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Abstract
Oxidative stress is induced by tobacco smoking and is also associated with anxiety and depression, two common psychiatric disorders, frequently associated with tobacco use. The aim of this study was to correlate tobacco use and mixed anxious-depressive disorder (MADD) with oxidative stress markers useful in clinical practice. A study assessing uric acid, vitamin c and malondialdehyde, as oxidative stress markers, was conducted in 31 smokers versus non-smokers with MADD. Other useful parameters assessed were: serum cholesterol, triglycerides and creatinine. Smoking profile was quantified by the number of packs-years (PY) and Fagerström nicotine dependence score (FNDS) with exhaled carbon monoxide (CO) validation, while MADD was certified by the Hamilton Anxiety/Depression Scale (HAM-A/D.) Malondialdehyde (MDA) serum concentration was significantly increased in 73% of the smokers, while vitamin C was lower in 90% of both smoking and no smoking MADD patients. Lower concentrations of uric acid were found in smokers, suggesting a decreased endogenous production. Smoking amplifies oxidative stress described in psychiatric disorders. Monitoring biomarkers of both tobacco exposure and oxidative stress can improve disease management.

Keywords: oxidative stress, smoking, mixed anxious-depressive disorder

Introduction
Tobacco use is the leading cause of precocious disease and death globally. Each year, more than 700,000 Europeans die due to smoking-related disorders [10]. According to the criteria adopted the World Health Organization (WHO) in the international classification of diseases, tobacco/nicotine dependence falls into: “Mental and behavioural disorders due to tobacco consumption” and it is coded with the disease code F.17. There are over 4000 toxic constituents that are formed by burning tobacco, and over 50 of them are proven carcinogens. Although the harmful effects of smoking as well as the passive exposure to tobacco smoke on health have been long demonstrated and sustained, the toxic mechanism with pathophysiological implications in the oxidative stress is not fully understood [6]. Tobacco smoke can induce depression and anxiety due to the resulting free radicals, triggering imbalances between enzymatic and non-enzymatic systems. Moreover, nicotine dependence is
an aggravating factor in the development of depressive and anxiety disorders associated with a poor response to treatment [3]. Nicotine induces the production of oxidative stress markers and reduces the amount of antioxidants, contributing greatly to the development of oxidative stress due to smoking [7, 11, 16]. Epidemiological studies showed that smoking is more frequent in patients with depression and anxiety than in the general population; also, a history of psychiatric illnesses may increase the risk of early initiation of smoking and may contribute to a faster development of nicotine dependence [5]. Predominantly, the effects of oxidative stress arise as a result of the imbalance between oxidants and antioxidants levels. The most studied biomarker of the lipid peroxidation process is malondialdehyde (MDA). MDA can be found in many biological samples (serum, plasma, urine) and has become one of the most used biomarker that can estimate the effects of oxidative stress on lipids. MDA is a biomarker for the evaluation of oxidative stress and it has been found that high values of this parameter coexist with various depressive disorders, proving to be a clinically useful parameter in the diagnosis of depression as well as in the monitoring of the treatment response. More and more studies assess the association between smoking, anxiety symptoms and oxidative stress markers [18]. Oxidative stress can contribute to the pathophysiology of anxiety disorders [12], therefore recent studies have shown a direct association between oxidant/antioxidant levels and anxiety disorders in smokers [18]. There seems to be a decrease in antioxidant capacity in psychiatric disorders such as schizophrenia, bipolar affective disorder, major depression and anxiety [1, 26, 28]. Low concentrations of antioxidants like vitamin C and uric acid (UA) in human serum of patients with depressive disorder have also been reported in other studies [5, 14], and the results have been associated with oxidative stress due to environmental factors and specially to smoking [25].

The primary objective of this study was to evaluate some of the oxidative stress parameters in relation with the nicotine dependence in smokers and non-smokers patients diagnosed with MADD (mixed anxious - depressive disorder). The second objective was to analyse the oxidative stress markers in smokers and non-smokers with MADD, depending on the Hamilton depression rating score (HAM-D) and respectively on the Hamilton anxiety rating score (HAM-A) [13]. This is the first comparative study performed in Romania in order to quantify and to assess the concentrations profile of the oxidative stress markers in smokers and non-smokers diagnosed with MADD.

Materials and Methods

Study population

In the present study there were included 31 smoking and non-smoking patients diagnosed with MADD from the Institute of Psychiatry “Socola” of Iași, Romania. The study was approved by the ethics committee of the “Socola” Institute Iași. All patients have read and signed a written informed consent. All subjects were interviewed at study enrolment on demographics (age, height, gender, area of residence, marital and work status) and on smoking status.

Clinical disease characteristics

Nicotine dependence

Tobacco dependence was assessed by using the Fagerström nicotine dependence test (FNDT), a tool that provides not only yes/no responses, but also a final Fagerström nicotine dependence score (FNDS) that classifies smokers as having low, medium or high levels of nicotine dependence. The higher the score, the higher the nicotine dependence of an individual. The level of nicotine dependence can be used to guide the patient's therapeutic plan. Values of the Fagerström dependence score may range between 0 - 10 and are interpreted as follows, score 0 - 3: lack of dependence or mild dependence, score 4 - 6: moderate dependence, score 7 - 10: severe dependence [4].

Determination of the carbon monoxide (CO)

Carbon monoxide in the exhaled air is the most easily measurable tobacco exposure biomarker; in the absence of CO in the environment, this is the best accepted method for monitoring tobacco exposure. It is easy to determine, it requires the smoker to exhaline in a commercially available portable analyser. CO is expressed in ppm (parts per million), a unit that can be converted as the equivalent of carboxyhaemoglobin [17].

Mixed anxious - depressive disorder

Mixed anxious - depressive disorder is somehow an exclusion diagnosis used when we cannot clearly include the patient only in the anxious disorder or only in the depressive disease. For this diagnosis, the ethology and epidemiology of the aforementioned entities are still valid; also, the evaluation scales and the pharmacological treatment are similar to those applied for anxiety and respectively for depression. The severity of the depressive symptoms was evaluated using the Hamilton Depressive Rating Scale (HAMD) that contains questions about the patient’s mental condition, sleeping, daily activities and cognition, especially attention and concentration. The final score is divided into three levels of severity: mild, moderate and severe [15]. Anxiety was evaluated using the Hamilton Anxiety Rating Scale (HAM-A). The scale contains questions about phobias, fears, the history of panic attacks, if any, and their influence on everyday life. Just like the
scale for depression, there are 3 levels of severity, mild, moderate and severe [21].

**Sample preparation**

The blood samples were collected from all patients. Taking all aseptic precautions, about 5 mL of blood were drawn by venepuncture from a peripheral vein with a disposable syringe, then collected in a clean dry glass tube (lithium-heparin blood test tube in case of vitamin C determination, and clot activator tube in case of MDA) that allowed to stand for 30 min at room temperature for the retraction of the clot. This was centrifuged at 3000 rpm for 10 min to separate the serum. To minimize the oxidation of vitamin C, the blood sample was centrifuged immediately for 15 minutes at 3000 rpm. The serum and plasma samples were stored at -25°C in the refrigerator for analysis.

**Biochemical assay**

Serum parameters were measured using a Cobas Integra 400 plus (Roche) biochemical auto analyser. All tests were performed in the biochemical laboratory, following standard procedures for clinical biochemistry purposes. The biological parameters measured were: uric acid, total cholesterol, triglycerides and creatinine.

**Serum MDA**

Serum MDA concentrations were measured according to the method described by Dobrin I et al. [8] with minor modifications (samples were maintained 30 minutes in a boiling water bath). MDA, the specific product of lipid peroxidation reacts with thiobarbituric acid (TBA) to form a coloured complex that gives maximum absorption at 532 nm. Briefly, 0.2 mL of blood serum was added to 1 mL of 50% trichloroacetic acid, and vortexing 3 minutes. After centrifugation at 14000 rpm for 10 min, the supernatant (1 mL) was decanted and dispersed with 0.9 mL of TRIS-HCL (pH = 7.4) and 1 mL thiobarbituric acid. TBA was carried out by heating in a boiling water bath for 30 min. After cooling with cold water, the absorbance of the supernatant was measured spectrophotometrically (Spectrophotometer Lambda 25, Perkin Elmer) at 532 nm, using 1,1,3,3-tetraethoxy-propane as standard. Thiobarbituric acid reactive substances (TBARS) were expressed as nmol/mL.

**Plasma vitamin C**

In the case of vitamin C, all samples were analysed on a HPLC system (Agilent 1220 Infinity LC) with ultraviolet detector (UV) using a Lichrosphere Amino column from Merck (250 mm x 4.6 mm x 5 μm). The elution conditions and wavelength detection were described in the British Pharmacopeia with a few modifications [4]. The solvents that constituted the mobile phase were water and acetonitrile. The flow rate was maintained at 1.0 mL/min throughout the entire procedure for each sample and standard, and the injection volume was 20 μL. The detection wavelength was 243 nm. Multi-level calibration curves were used for the quantification and good linearity ($r > 0.998$) was achieved for the tested intervals that included the whole concentration range found in the samples. The limit of quantification (LOQ) were calculated as 3x standard deviation of the analysed values for the procedural blanks, LOQ were calculated for a signal-to-noise ratio equal to 10 based on the signal obtained for the standard.

**Statistical analysis**

All statistical analyses were performed using the software package STATISTICA 10 (StatSoft Inc) and Excel program. Normal distribution was tested using the Kolmogorov–Smirnov test. Spearman’s rank correlation coefficient was used to measure the strength of the correlations between biochemical parameters corresponding to the smokers and non-smokers. A p-value less than 0.05 was considered to indicate statistical significance. The characteristics of the two groups of smoking and non-smoking patients were compared using the Mann-Whitney U test. Discriminant function analysis and the Mann-Whitney U test were used to determine whether the two groups of patients differ with regard to MDA, vitamin C and uric acid concentrations. To determine the elements of highest significance in the model, the following parameters were used: partial Wilks’ lambda, the F-test of partial Wilks’ lambda and its p-value.

**Results and Discussion**

*Concentrations profile of oxidant and non-enzymatic antioxidants in smokers and non-smokers with MADD*

**Table I**

Socio-demographic data of the patients with MADD

| Variables                  | Mean |
|----------------------------|------|
| Subjects (n)               | 31   |
| Age (years)                | 57.61|
| Height(cm)                 | 160  |
| **Gender**                 |      |
| Female                     | 68(%)|
| Male                       | 32(%)|
| **Area of residence**      |      |
| Rural                      | (39%)|
| Urban                      | (61%)|
| **Status of smoking**      |      |
| Smoker                     | 19   |
| Non-smoker                 | 12   |
| Cigarettes smoked/day      | 20   |
| **Marital status**         |      |
| Married                    | 75%  |
| Unmarried                  | 10%  |
| Divorced                   | 5%   |
| Widower                    | 10%  |
| **Social status**          |      |
| Not employed               | 10%  |
| Retired                    | 50%  |
| Employed                   | 40%  |
This study analysed the profile of MDA, vitamin C, and uric acid concentrations reported to the Reference Biological Range (RBR) for 31 smoking and non-smoking patients diagnosed with MADD. Information on the demographic characteristics of the studied population are presented in Table I. Descriptive statistic was performed using the EXCEL program and for each biochemical parameter determined in the smokers and non-smokers group the minimum, maximum, average, median and standard deviation were calculated and represented in Table II. The analysis of the data presented in Table II, shows that the mean serum MDA concentrations determined for the smoking patients group (1.51 nmol/mL) are higher than those determined for the group of non-smokers (1.11 nmol/mL). Thus, of the 19 smokers diagnosed with MADD, 77% had the serum MDA concentrations higher than the reference biological range, and 26% had MDA levels in the considered reference biological range. For patients included in this study, it was obtained a good correlation between serum MDA and uric acid concentrations (r = 0.60; p < 0.05). In the case of smokers diagnosed with MADD, the descriptive analysis showed a median concentration value for MDA of 1.73 nmol/mL (with a range of determinations 0.27 - 2.43 nmol/mL) that was much higher than the median concentration in the case of non-smoking patients (0.97 nmol/mL - with a range of 0.27 - 2.38 nmol/mL). (Table II). Similar concentrations for serum MDA were determined by Ashutosh Bajpai, reporting a value of 1.95 ± 1.04 nmol/mL in patients diagnosed with major depression [1].

The prolongation of the level of the stress is an important factor in the major depressive disorder, which can lead to increased serum MDA concentrations, oxidative stress and implicitly to accentuation of depressive symptoms. In the present study, approximately 73% of smokers diagnosed with MADD had the serum MDA concentration higher than the reference interval (0 - 1 nmol/mL). The statistical results showed very good Spearman correlations between the biochemical parameters for smokers and non-smokers with MADD. Positive correlations were observed between: serum concentrations of MDA and uric acid (r = 0.59, p = 0.002), CO and uric acid (r = 0.7, p = 0.002) as well as between uric acid and vitamin C (r = 0.42, p = 0.018) (Table III).

Vitamin C is a non-enzymatic marker for assessing oxidative stress and also a vital antioxidant for the brain. It helps maintaining the integrity and functioning of several processes of the nervous system, including myelin formation, catecholamine and antioxidant proteins synthesis. Vitamin C can neutralize superoxide radicals that are generated in large quantities during neurodegenerative processes.
For vitamin C, very low plasma levels were obtained for both the smokers and the non-smokers group of patients diagnosed with MADD. Thus, for smokers, in 90% of cases, very low plasma vitamin C values were obtained under the considered reference biological range (4.6 - 14.9 mg/L). For non-smokers, levels of vitamin C plasma concentrations between 0.02 and 7.01 mg/L were obtained, with only one patient having the concentration within the considered reference biological range. The very low values of the median concentration for vitamin C determined in human plasma in smokers (median = 1.08 mg/L) and in non-smokers (1 mg/L) are consistent with data reported in the literature and confirm the presence of oxidative stress in depression and in anxiety disorders [13].

Serum triglycerides values of smokers ranged between 76.39 - 186 mg/dL, with an average concentration of 131.60 mg/dL; two of the patients had concentrations above the reference biological range (40 - 165 mg/dL) and it was noticed a dose-response relationship between the number of cigarettes smoked per day and serum triglycerides. For non-smokers, serum triglycerides values ranged from 44.56 to 181.30 mg/dL, with an average concentration of 101.51 mg/dL. The researchers noticed that lipid peroxidation due to exposure to chemical compounds found in tobacco smoke results in changes of antioxidant systems and of plasma lipid profile. Numerous studies have shown that exposure to tobacco has negative effects on lipid metabolism [23]. Smoking is associated with an atherogenic lipid profile, which can also contribute to the production of oxidative stress. Smoking, in its various forms, leads to an increased risk for high total cholesterol serum levels, as well as for high triglycerides levels. Data from the literature reported statistically significant differences (p < 0.001) between the serum concentrations of total cholesterol in smokers (173.44 ± 78.64 mg/dL) compared to non-smokers (115.9 ± 47.67 mg/dL) [15]. In the present study, the total cholesterol serum level was determined in 19 smokers diagnosed with MADD, registering a minimum value of 149 mg/dL and a maximum of 289 mg/dL, with an average value of 199.28 mg/dL. Values above the reference biological range (0.00 - 200 mg/dL) were recorded in 6 smokers (32%). For non-smoking patients, the serum concentrations of total cholesterol were between 111 - 290 mg/dL, with an average value of 197.2 mg/dL, and 4 of the investigated cases had values above the reference biological range. It can therefore be concluded that smoking increases the serum concentration of total cholesterol, especially in individuals with an increased tobacco dependence [27].

Researchers have observed a dose-response relationship between the number of cigarettes smoked per day and the level of serum triglycerides [19]. Thus, mean serum triglyceride concentrations of 176.45 ± 2.19 mg/dL were found in subjects with a consumption of 1 - 10 cigarettes per day and mean serum triglyceride concentrations of 185.15 ± 34.19 in subjects with a consumption of 11 - 20 cigarettes per day compared to non-smoking subjects for whom the mean serum triglycerides levels were 164.1 ± 20.26 mg/dL [19].

Serum creatinine was determined for smokers in the range of 0.49 - 1.35 mg/dL with an average concentration of 0.80 mg/dL; six of the analysed cases had the concentration outside the established reference biological range (0.5 - 1.1 mg/dL). For non-smokers, serum creatinine values were between 0.47 - 0.82 mg/dL, with an average concentration of 0.65 mg/dL; four of the analysed cases had values lower than the reference biological range.

Only a few studies have shown the correlation between smoking and creatinine levels. Dulger et al. compared renal function in active and passive smokers. The study showed that creatinine levels were significantly increased in active smokers (p < 0.01) and it was concluded that the kidneys, and in particular the glomerular function, can be affected even in the case of passive smoking [9]. Few studies in the literature showed that smoking increases the risk of proteinuria and also the risk of mild hyperfiltration, as well as the risk of mild renal impairment, especially in men and in elderly people. Smoking in general has a negative influence on renal function even in subjects apparently not suffering from a kidney disease, but the adverse renal effects due to smoking are present especially in patients with different kidney disorders as well as in hypertensive patients [20].

**Association of nicotine dependence with oxidative stress markers in MADD**

As shown in Table IV, smokers had an average carbon monoxide (CO) concentration in the exhaled air of 14.21 ppm, with values between 10-21 ppm, much higher than the reference biological range (0 - 4 ppm). Also, the number of packs-years (PY) value ranged between 6 and 40 (Table IV). The concentrations of MDA, vitamin C and uric acid of smokers with MADD, according to the Fagerström nicotine dependence score (2 - 8) are shown in (Figure 1).

The statistic processing of the experimental data Statistica 10 followed the biochemical profile of smokers as well as the evaluation of the oxidative stress parameters according to the number of PY. Thus, in the default algorithm of the statistical evaluation method, data were entered on 19 smokers and 10 characteristics.
Table I

| Parameters          | Mean     | SD        | Median | Range |
|---------------------|----------|-----------|--------|-------|
| Fagerström score    | 5.16     | 1.61      | 6      | 2 - 8 |
| CO (ppm)            | 14.21    | 3.60      | 14     | 10 - 21 |
| PY (packs-years)    | 17.72    | 11.15     | 13.50  | 6 - 40 |
| Cigarettes/day      | 11.56    | 5.71      | 10     | 5 - 20 |

Smokers (N = 19)

| Parameters | Mean     | SD        | Median | Range |
|------------|----------|-----------|--------|-------|
| HAM-A      | 29.63    | 5.20      | 29     | 17 - 41 |
| HAM-D      | 17.47    | 4.25      | 16     | 12 - 26 |

Non-smokers (N = 12)

| Parameters | Mean     | SD        | Median | Range |
|------------|----------|-----------|--------|-------|
| HAM-A      | 30.67    | 5.53      | 31     | 17 - 41 |
| HAM-D      | 18.75    | 3.08      | 18.50  | 14 - 25 |

Figure 1.
Concentrations profile of the biochemical parameters against the Fagerström dependence score in patients with MADD

Stepwise forward canonical discriminant analysis was separately performed on data from subjects expressing oxidative stress markers (independent variable) to classify dependence of smoking against PY of smokers (grouping variable): 10 PY; 10 - 20 PY; more than 30 PY.

Figure 2.
2D scatterplot of canonical scores resulting from applying the discriminant function to the data expressing oxidative stress markers in tobacco dependence

Based on the statistical results it can be concluded that total cholesterol (F = 21.24; p = 0.000), MDA (F = 2.83; p = 0.09), Fagerström scores (F = 3.57; p = 0.057), triglycerides (F = 3.30; p = 0.06) where the compounds of highest importance for the discrimination of the three considering dependence levels as low (< 10 PY), medium (10 - 20 PY), severe (more than 30 PY). The scatterplot representing discriminant functions showed a good separation among smokers with medium, low and severe dependence (Figure 2).

Association of oxidative stress markers with the evolution of depression and anxiety in smokers and non-smokers

The evaluation of biochemical parameters associated with depression (MDA, vitamin C and uric acid), in smokers with MADD is shown in Figure 3. Among smokers, 37% had mild depression, 53% had moderate depression and 10% were found with severe depression. Thus, in smokers diagnosed with severe depression, low serum uric acid and plasmatic vitamin C levels were observed compared to patients diagnosed with moderate depression (Figure 2).

Figure 3.
Levels of the oxidative stress parameters against HAM-D score in case of smokers with MADD

Nicotine induces the production of oxidative stress markers and reduces the amount of antioxidants, contributing greatly to the production of oxidative stress due to cigarette smoke [7, 11, 16]. There appears to be a decrease in antioxidant capacity in psychiatric disorders such as schizophrenia, bipolar affective disorder, major depression and anxiety [2, 28]. Two studies have reported low concentrations of vitamin C and uric acid (UA) in human serum of patients with depressive disorders [5, 14]. We found small values of uric acid and vitamin C as well as...
high values of MDA based on the HAM-A anxiety scale (Figure 4) in smokers with severe anxiety.

Moreover, nicotine dependence is an aggravating factor in the development of depressive and anxiety disorders associated with a much slower response to the psychiatric treatment [14]. In order to see if there is a statistical difference between the oxidative stress assessment parameters in smokers and non-smokers diagnosed with severe anxiety (HAM-A > 25), it was applied the Man-Whitney test. Statistical results showed a statistically significant difference between the two groups analysed, namely smokers/non-smokers for uric acid (p = 0.039) in patients diagnosed with severe anxiety. It seems that more and more studies show that this non-enzymatic antioxidant has low serum levels in smokers due to a diminished endogenous production [24]. The HAM-D depression assessment scale had a statistical correlation with serum MDA concentration of r = 0.16, p = 0.51. Similar results have been reported by Rangaswamy R et al., for the evaluation of oxidative stress markers in patients with major depression [22]. The prolongation of physiological stress is considered as a causal factor for major depression, this may lead to an increased serum MDA concentration and to increased oxidative stress and depressive symptoms [16].

Conclusions

Although concentrations of malondialdehyde, vitamin C and uric acid have been determined in various neuro-psychiatric disorders, few studies point to changes in these parameters levels, depending on exposure to tobacco smoke and on disease progression. The discriminatory analysis of smokers showed a direct correlation between malondialdehyde concentrations and the intensity of the tobacco exposure assessed by the number of packs-years. In smoking patients with severe depression, we found low concentrations of serum uric acid and plasmatic vitamin C. Serum malondialdehyde concentrations were higher in smokers diagnosed with severe depression. The concentrations obtained in the quantitative determination of serum uric acid were lower in smokers than in non-smokers diagnosed with mixed anxious-depressive disorders. These results may be explained by the decrease of endogenous production of uric acid and vitamin C that act as antioxidants.

Conflict of interest

The authors declare no conflict of interest.

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