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Blood mononuclear cells as speculum of emotional stress analyzed by synchrotron infrared spectroscopy and a nootropic drug

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ABSTRACT

Chronic psychological stress is an important public health issue which generates behavioral changes, anxiety, immunosuppression and oxidative damage. Piracetam is a cognitive enhancer, at cellular level it protects from oxidative stress. The aim of this study was to evaluate the effect of psychological stress and of piracetam on circulating mononuclear cells by analyzing the biochemical spectrome using Synchrotron Radiation Fourier Transform Infrared Microspectroscopy (SR-μFTIR). Rats were exposed for five days to a stressor (cat odor) under oral administration of piracetam (600 mg/kg). SR-μFTIR analysis showed a decrease in bands associated to the lipids region (2852 cm^{-1}, 2923 cm^{-1} and 2962 cm^{-1}) and an increase absorption of the amide I band (1654 cm^{-1}) under stress conditions. The principal component analysis showed increase oxidation of lipids (decrease of 3010 cm^{-1}, 2923 cm^{-1} and 2852 cm^{-1} bands) as well as proteins denaturation (increase of 1610 cm^{-1} and 1690 cm^{-1} bands) under stress. Piracetam provided protection to polyunsaturated lipids ($p \leq 0.001$) and lipids/proteins ratio ($p \leq 0.001$). Behaviorally, this drug diminished fear and anxiety in stressed animals by the plus maze test ($p \leq 0.002$). However, this drug induced oxidative stress in mononuclear cells from unstressed animals and altered their behavior.

1. Introduction

Psychological stress is a public health issue that generates behavior alteration, fear and anxiety [1] as well as systemic inflammation which overwhelm the neuroimmune axis [2–5]. Using an emotional stress model, we previously showed that neuroinflammation and lymphocytes recruitment into the Central Nervous System can be produced by chronic psychological stress [2]. This neuroinflammation is related to lipid peroxidation in circulating lymphocytes and those changes can be evaluated by the vibrational fingerprints of those cells [3].

To diminish negative effects of stress, psychostimulants and nootropics are widely used without medical prescription in various countries [6]. Piracetam was the first nootropic to be described [7] and is used as cognitive enhancer by healthy people as well as in neuropathologies [6, 8]. It is known that piracetam stabilizes the phospholipids in cell membranes [9] that, in turn, improve the recovering of receptors for glutamate [10] and acetylcholine [11]. This nootropic drug interacts with the polar heads of phospholipids affected by oxidation, restoring the lipid organization and thus, the membrane fluidity [9]. Also, this drug is a metabolic enhancer by increasing the mitochondrial membrane potential and ATP production [12, 13]. Some studies have shown that piracetam treatment does not shift the metabolism behavior, or cognition [14–17]; others report that this nootropic, in high concentrations, has negative effects [18, 19]. Even though many questions remain to be answered about the efficiency and safety, piracetam is one of the most used pharmaceutical products between all 'Performance and image-enhancing drugs'. Thus, piracetam is considered to be a "smart lifestyle drug" being used excessively by healthy individuals to enhance memory and physical performances [6].

Piracetam effect on health and stress were evaluated by behavior and the stress hormone (corticosterone), while the molecular shifts on mononuclear cells were analyzed by using Synchrotron Radiation Fourier Transform Infrared Microspectroscopy (SR-μFTIR), this technique has proved to be a useful research method in Biomedical Sciences [20], it has the power to analyze individual cells in a non-destructive manner [3].

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Our results are consistent with previous works showing that chronic stress is reflected by changes in the behavior and the increase in the stress hormone. This work is adding the evidence on how chronic stress may be related to clinical disorders. Using SR-μFTIR a lipid peroxidation and damage in the backbone structure of proteins in circulating mononuclear cells after chronic stress was observed. Also, to our knowledge, the present study is the first one to use SR-μFTIR to test the effect of piracetam in circulating mononuclear cells from rats exposed to psychological chronic stress giving clues about the effect of this drug in health or stress.

2. Materials and Methods

2.1. Experimental Design

Circulating mononuclear cells were extracted from chronically stressed rats to evaluate the effect of piracetam (600 mg/kg) on biochemical composition in whole cell environment by SR-μFTIR analysis. Also, the effect of stress and piracetam on behavior and plasma corticosterone was studied. The study design is shown in Fig. 1.

2.2. Animals

All experiments were approved by the Bioethics Committee of the Universidad Autónoma de Ciudad Juárez, Ciudad Juárez, Chihuahua, Mexico, following International Guidelines (NIH Publication No. 8023, revised 1978) and the Official Mexican Norms (NOM-062-ZOO-1999).

Female Sprague-Dawley rats of 200–240 g weight at age of 9–11 weeks were used. All rats were maintained at 23–26 °C, 12 × 12 h light/dark schedule, with water and food ad libitum and individually caged for a week before the experiments. Animals were purchased from Charles River (Rismart, Mexico). From birth, up to a month, littermates were maternally nursed; thereafter, female littermates were sorted and kept in groups of two (to avoid stress by isolation).

Rats were separated in four groups: without stress and without piracetam (S−P−); without stress but piracetam (S−P+); with stress but without piracetam (S+P−); with stress and piracetam (S+P+). In the case of the experimental groups without piracetam, water was administered instead of the drug.

2.3. Drug Administration

Piracetam (Nootropil, [1 g/5 mL], UCB Pharma Belgium) or water were orally administrated by using a metal cannula. Piracetam was administrated as previously reported to diminish oxidative stress in brain as in other tissues [21, 22], single dose of 600 mg/kg, at the same hour (8 am), for nine days.

2.4. Stress Exposure

After three days of starting the drug administration, rats were exposed to stress. For five consecutive days, an hour after the medication (9 am), the subjects were individually transferred two times into a test-cage 60 × 27 × 35 cm made of plastic, with one opaque portion and two translucent portions [23]. In the first exposure, the animal was left 20 min in the test-cage for environment habituation, then the rat was returned to the housing-cage for other 20 min to relax. Thereafter, the animal was transferred to the stress-cage for another 20 min, where a piece of cloth (20 × 30 cm) with (stress-cage) or without (control-cage) predator odor (cat) was hinging. The piece of cloth was previously used (or not) as a mattress for a domestic cat for a week, then, it was sectioned in portions of 20 by 30 cm, ziplocked and maintained at −20 °C until used [3]. The rats were videotaped to analyze an ethogram of four behaviors: hiding, exploration, head out and approach. These behaviors were quantitated using JWatcher ™ software (version 0.9) and expressed as the full time spent in each specific behavior on each day [2, 23].

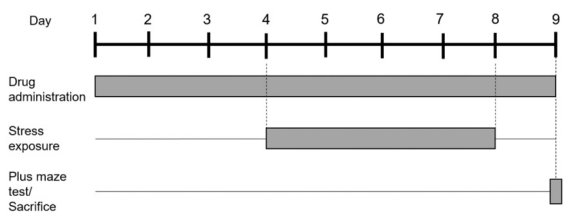

Fig. 1. Study design. For nine consecutive days, rats received piracetam (600 mg/kg), administrated orally using a cannula, in single dose, at 8 am. From 4th day till 8th day, the animals were exposed to stress (at 9 am, after drug administration). In day 9, some rats were tested for anxiety in the elevated plus maze apparatus and another group was sacrificed for circulating mononuclear cells and plasma isolation.

2.5. Elevated-plus Maze Test

In the 9th day, the animals were tested for anxiety behavior in a wood elevated-plus maze with four symmetrical arms (40 cm long and 10 cm wide, walls of 30 cm for closed arms). The center square has 10 cm^2 and all the maze has a high of 50 cm. The experiment took place in a special room with the light and the video camera located in center of the roof [24].

2.6. Blood Sample Collection and Mononuclear Cells Isolation

In the last day of the experiment (the 9th day), 12 animals (3 per group) were anesthetized with a lethal dose of intraperitoneal sodium pentobarbital. When fully anesthetized, the blood was obtained by cardiac puncture using vacutainer EDTA tubes. Whole blood was diluted with PBS-EDTA 1:1 (137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3.2 mM KCl, and 4 mM EDTA, pH 7.4). This dissolution was slowly placed onto a bed of 40% PBS-EDTA, 57.3% Percoll™, 2.7% 10× PBS (in a final relation 9:1). After centrifugation at 1000*g* for 30 min, mononuclear cells were saved and washed three times by centrifugation at 800*g* for 10 min each with 1× PBS containing 4 mM EDTA. Pelleted cells were fixed in 300 μL of 1% paraformaldehyde in PBS for 20 min at room temperature. Finally, fixed cells were washed twice, as before, with 1 mL of deionized water and resuspended with 200 μL of deionized water [3]. Samples were sent in 1.5 mL microtubes, at room temperature, to beamline ID21 at European Synchrotron Radiation Facility (ESRF), Grenoble, France, to be analyzed by SR-μFTIR.

2.7. SR-μFTIR Analysis

SR-μFTIR measurements were done at beamline ID21 at ESRF, Grenoble, France. A Thermo Nicolet Continuum IR microscope (Thermo Scientific, Madison, WT, USA) equipped with a 32× objective coupled to a Thermo Nicolet Nexus FTIR spectrometer (Thermo Scientific, Madison, WT, USA) with a synchrotron IR source was used for this study. The IR microscope was equipped with a liquid nitrogen cooled 50 μm mercury cadmium telluride detector. The spectra were collected in the range 4000–800 cm^{-1}, with a beam size of 8 × 8 μm^2, scanned at 6 cm^{-1} resolution and obtained as an average of 256 scans. 10 μL of sample were placed on a BaF_2 windows with 1 mm height and allowed to dry at room temperature. Two maps for each sample were performed (each map pixel contains the average of the signals from 2 to 3 cells) and, from each map, 21 spectra from aleatory points were obtained.

2.8. Corticosterone Immunoassay

The measurement of corticosterone concentration was performed in blood plasma obtained at the day of sacrifice. An aliquot of 300 μL of freshly extracted blood was centrifuged at 1000*g* for 5 min, plasma was saved and stored at −80 °C until used. For the measurement, a

commercial enzyme immunoassay kit was used following the instructions of the provider (Cat. No: 55-CORMS-E01, Alpco, Salem, NH). The detection range of the kit is 6.1 to 2250 ng/mL. Absorbance were measured with a microplate reader FLUOstar Omega (BMG) at 450 nm.

2.9. Statistical Analysis

FTIR absorbance spectra was normalized using unit vector normalization and was converted in second derivative with Savitzky-Golay algorithm with fifteen points windows and second polynomial order using Unscrambler X 10.3 (Camo). Also, Unscrambler X 10.3 (Camo) was used for principal component analysis (PCA) of the spectra. The robustness of the multivariate model was evaluated using an open code software Orange (ver.3.13) [25]. An Artificial Neural Network (ANN) was applied as a learner on the PCA scores from each lipid and protein region, and then the performance was tested by K-Fold Cross Validation (KFCV) method, both classified model and cross-validation method are very well know and frequently used in the field of vibrational spectroscopy [26]. The parameters of the ANN were 100 neurons per hidden layer, rectified linear unit function (ReLu) as activation function, and Adam method [27] as a solver. The evaluation results after 10 folds KFCV are shown in Table 1.

Excel statistical tools (Office 2016) like average, standard error and *t*-test were used to analyze the normalized second derivative spectra and box plot diagrams with median and inter-quartile range were used to analyze lipid ratios.

For the analysis of the behavior in all five days of stress, average, standard error and *t*-test were applied using MS Excel 2016. p-Values ≤ 0.05 were considered significant. Also, to analyze the behavior from plus maze test and plasma corticosterone level were used box plot diagrams with median and inter-quartile range. Each box shows the value of the 50th percentile, the horizontal line is the median value and the bars indicate the maximum and minimum of the values.

3. Results

3.1. FTIR Spectra Analysis of Circulating Mononuclear Cells

Alone or combined, piracetam and stress changed the biochemical profile of circulating mononuclear cells (Fig. 2).

3.1.1. Lipids Region (3050 cm^{-1}–2800 cm^{-1})

The average and standard errors of SR-μFTIR second derivative spectra in lipids region (3050 cm^{-1}–2800 cm^{-1}) showed three strong bands near 2852 cm^{-1} (assigned to symmetric $\upsilon_s CH_2$ stretching vibration from lipids), 2923 cm^{-1} (assigned to asymmetric $\upsilon_{as} CH_2$ stretching vibrations from lipids) and 2962 cm^{-1} (assigned to asymmetric $\upsilon_{as} CH_3$ stretching vibrations from lipids and proteins). S−P− has increased bands intensity at 2852 cm^{-1} and 2923 cm^{-1} compared with the other experimental groups, specifically with S+P− ($p \le 0.0001$) which has the weakest absorption. S+P+ showed an increase in the absorption of 2852 and 2923 cm^{-1} bands compared with S+P− ($p \le 0.0001$, Fig. 2b). However, S−P+ showed less absorption than S−P− on those bands ($p = 0.002$).

Principal Component Analysis (PCA) was used to study the variance between SR-μFTIR second derivative spectra in the lipids region (Fig. 3). PC1 and PC2 explained together 97% of data variance (Fig. 3a). To test the performance, the model was evaluated using an Artificial Neural Network and 10-Fold Cross Validation methods. From Table 1, the AUC for the lipid region is 0.956 and indicates that the used method produced a strong and robust model. Thus, the data preprocessing and multivariable analysis used method is reliable for effective feature extraction in this study [26]. S−P− and S+P− groups in different clusters. S+P+ has less variation with unstressed groups (S−P− and S−P+) and S−P+ showed less variation with stressed groups (S+P− and S+P+). A relative separation of the experimental groups was obtained, and the differences between them were found mainly by PC2 (8%). Loading plots of PC1 and PC2 (Fig. 3b) indicates that the variation between treatments is ought to several bands such as 3010 cm^{-1} (assigned to *cis* double bounds C═C—H of unsaturated lipids), 2923 cm^{-1} (assigned to $\upsilon_{as} CH_2$ stretching vibrations) and 2852 cm^{-1} (assigned to a symmetric $\upsilon_s CH_2$ stretching vibration from lipids). Because the data is in the second derivate form, the more negative are the values the higher they are (Fig. 3b). Unstressed conditions (S−P− and S−P+) showed the higher absorptions of those three bands where PC2 is positive. On the other hand, stressed groups are characterized by an important decrease of those bands which represent a significant diminution in polyunsaturated lipids quantity [28, 29].

To investigate more in depth the oxidation state of the lipidic components in relation to stress and piracetam treatment analysis of specific absorption band ratios was performed. These ratios offer valuable information about biochemical composition and structure of the cells [28, 29]. The degree of lipids saturation was calculated using 2923 cm^{-1}/2962 cm^{-1} ratio ($\upsilon_{as} CH_2/\upsilon_{as} CH_3$, Fig. 4a) and 2852 cm^{-1}/2962 cm^{-1} ratio ($\upsilon_s CH_2/\upsilon_{as} CH_3$, Fig. 4b). Those two ratios were decreased in S+P− compared with S−P− ($p \le 0.001$), but in S+P+ was found an increase of both ($p \le 0.001$, compared with S+P−), almost to the same level as in unstressed conditions. Also, a weak decrease at these ratios was observed in S−P+ compared with S−P− ($p \le 0.06$) which can be explained by a stress-like condition induced by piracetam in this group. Since $\upsilon_{as} CH_2/\upsilon_{as} CH_3$ ratio is also an indicator of the chain length of fatty acids [29], it is possible that stress cause a shortening of lipids in the mononuclear cells.

Lipid unsaturation (Fig. 4c) was calculated using 3010 cm^{-1}/(2923 cm^{-1} + 2852 cm^{-1}) ratio (*cis* υC═C—H/($\upsilon_{as} CH_2$ + $\upsilon_{as} CH_2$)) where 3010 cm^{-1} represents the unsaturated lipids and 2923 cm^{-1} + 2852 cm^{-1} represent the saturated lipids (also considered as total lipids). This study reveals a significant reduction of this ratio in S+P− compared to S−P− ($p \le 0.001$), which means that the cells under stress are significantly losing unsaturated lipids. Piracetam, in stress, inhibits this damage ($p \le 0.001$, compared with S+P−) and, also, S−P+ showed an important increase in unsaturated lipids ($p \le 0.001$, compared with S−P−).

The changes in the quantity of cell lipids and proteins composition were obtained by calculating the ratio total lipids/total proteins (Fig. 4d). Total lipid content was obtained by summing the intensity absorption bands of $\upsilon_s CH_2$ (near 2852 cm^{-1}) with $\upsilon_{as} CH_2$ (near 2923 cm^{-1}). Total protein represents the sum between Amide I (maximum band near 1654 cm^{-1}) and Amide II (maximum band near 1544 cm^{-1}) intensity absorption bands. S+P− had a lower lipid/protein ratio than S−P− ($p \le 0.001$), which means that in stress, either total lipids are diminished, or total proteins increase. Piracetam in stress alleviates this effect ($p \le 0.001$, S+P+ vs S+P−) but by itself, in unstressed conditions, is a cause for lowering this ratio ($p \le 0.001$, S−P+ vs S−P−).

3.1.2. Proteins Region (1700 cm^{-1}–1500 cm^{-1})

SR-μFTIR second derivative spectra in the proteins region (Fig. 2c; 1700 cm^{-1}–1500 cm^{-1}) showed two strong bands near 1654 cm^{-1} (assigned to amide I caused by υC═O and υCN vibration of protein backbone or β-sheet structures) and near 1544 cm^{-1} (assigned to amide II caused by δNH and υCN vibration of protein backbone or α-helix structures). The Amide II band did not change in stress. However, both stressed groups (S+P− and S+P+) showed higher absorption of

Table 1
Performance metrics results of ANN method using extracted PCA components of the proposed model, where AUC is the area under the curve of the ROC curve [26] and F1 is the weighted harmonic mean of specificity and sensitivity (F-measure or balanced F-score).

Region	AUC	Average F1	Average specificity	Average sensitivity
Lipids	0.956	0.826	0.827	0.826
Proteins	0.972	0.849	0.849	0.849

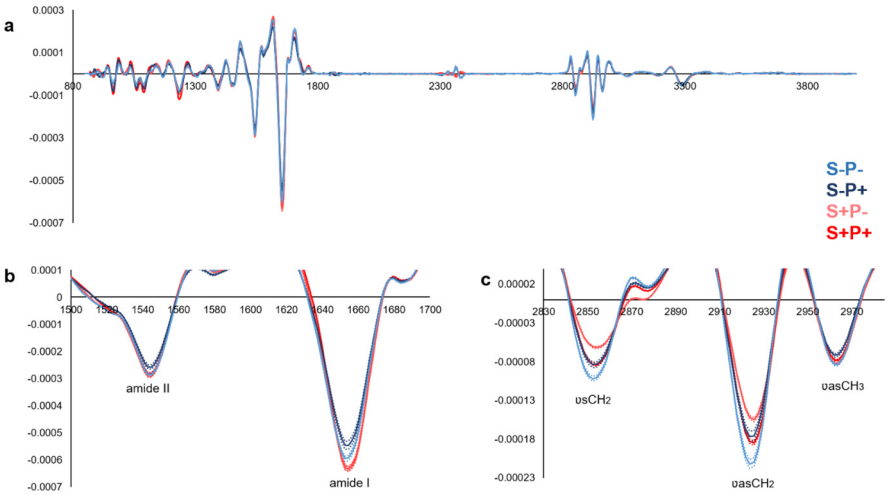

Fig. 2. Stress and piracetam shift the FTIR spectrome of circulating mononuclear cells. a Total SR-μFTIR average second derivative spectra. Red bands: stressed groups; blue bands: unstressed groups. b SR-μFTIR average second derivative spectra in the region 2980–2830 cm^{-1}. c SR-μFTIR average second derivative spectra in the region 1700–1500 cm^{-1}. S−P− mononuclear cells from rats neither exposed to stress nor to piracetam; S−P+ mononuclear cells from rats not exposed to stress but treated with piracetam; S+P− mononuclear cells from rats exposed to stress without piracetam treatment; S+P+ mononuclear cells from rats exposed to stress and treated with piracetam. Solid lines are the mean of 42 spectra from two maps, dotted lines are the ±SEM; n = 3 per group.

amide I band than unstressed groups ($p \leq 0.0001$, Fig. 2c). S−P+ has the weakest absorption of both bands between all groups ($p \leq 0.0001$).

PCA of SR-μFTIR second derivative spectra in the protein region showed that PC1 and PC2 explained together 94% of data variance (Fig. 5a). To test the performance, the model was evaluated using an Artificial Neural Network and 10-Fold Cross Validation methods. From Table 1, the AUC for the protein region is 0.972 and indicates that the used method produced a strong and robust model. Thus, the data preprocessing and multivariable analysis used method is reliable for effective feature extraction in this study [26]. S−P− and S+P− are separate clusters and their scores are distributed on negative and positive part respectively, of the PC2 (14%). S+P+ is distributed in the cluster of S

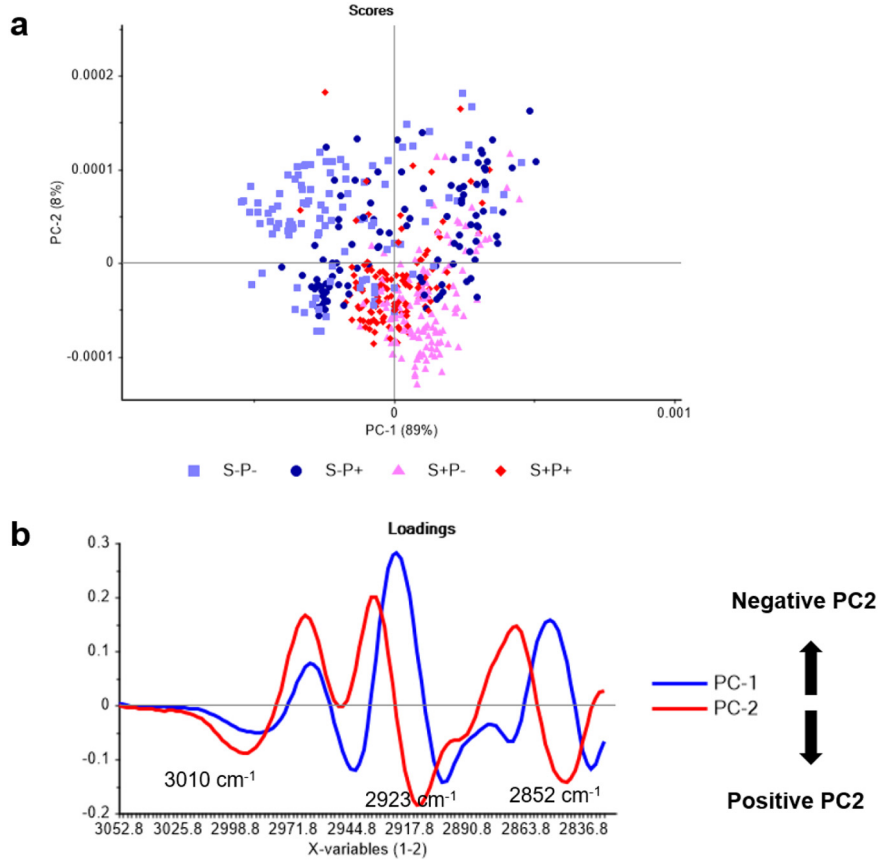

Fig. 3. PCA confirms the effect of stress and piracetam on the bands shifts in lipids region. a Score plots of PC1 and PC2. b Loading plots of PC1 and PC2. S−P− mononuclear cells from rats neither exposed to stress nor to piracetam treatment; S−P+ mononuclear cells from rats not exposed to stress but treated with piracetam; S+P− mononuclear cells from rats exposed to stress without piracetam treatment; S+P+ mononuclear cells from rats exposed to stress and treated with piracetam. Data from 42 spectra coming from two maps per rat; n = 3 per group.

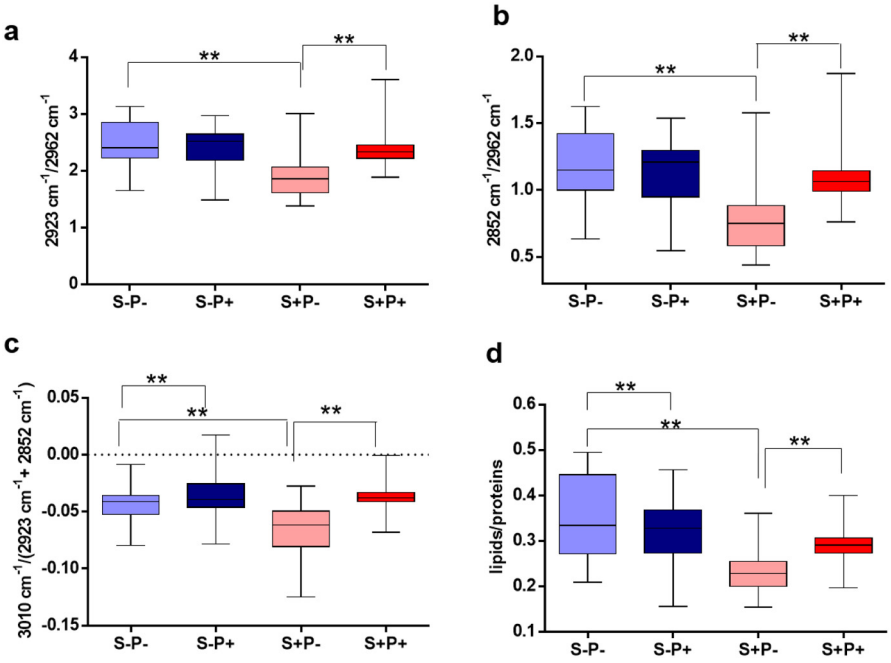

Fig. 4. Stress and piracetam changed the ratio of lipids and proteins in circulating mononuclear cells. a Ratio $\upsilon_{as}CH_2/\upsilon_{as}CH_3$ (2923 cm^{-1}/2962 cm^{-1}). b Ratio $\upsilon_{s}CH_2/\upsilon_{as}CH_3$ (2852 cm^{-1}/2962 cm^{-1}). c Ratio *cis* $\upsilon C{=}C{-}H/(\upsilon_{as}CH_2 + \upsilon_{as}CH_2)$ (3010 cm^{-1}/(2923 cm^{-1} + 2852 cm^{-1})). d Ratio $(\upsilon_{as}CH_2 + \upsilon_{as}CH_2)/(\upsilon C{=}O + \upsilon NH)$ ((2923 cm^{-1} + 2852 cm^{-1})/(1654 cm^{-1} + 1544 cm^{-1}). S−P− mononuclear cells from rats neither exposed to stress nor to piracetam treatment; S−P+ mononuclear cells from rats not exposed to stress but treated with piracetam; S+P− mononuclear cells from rats exposed to stress without piracetam treatment; S+P+ mononuclear cells from rats exposed to stress and treated with piracetam. Data is from 42 spectra coming from two maps per rat; n = 3 per group. Each box shows the value of the 50th percentile, the horizontal line is the median value and the bars indicate the maximum and minimum values. **p ≤ 0.001.

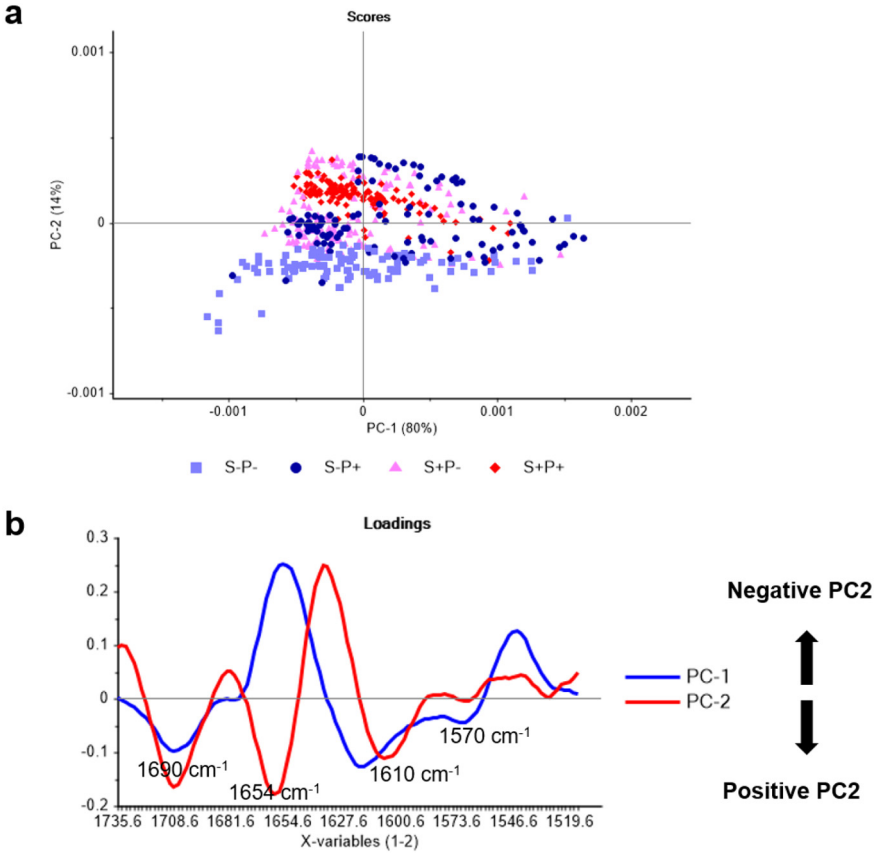

Fig. 5. PCA confirms the effect of stress and piracetam on the bands shifts in proteins region. a Score plots of PC1 and PC2. b Loading plots of PC1 and PC2. S−P− mononuclear cells from rats neither exposed to stress nor to piracetam treatment; S−P+ mononuclear cells from rats not exposed to stress but treated with piracetam; S+P− mononuclear cells from rats exposed to stress without piracetam treatment; S+P+ mononuclear cells from rats exposed to stress and treated with piracetam. Data from 42 spectra coming from two maps per rat; n = 3 per group.

+P−, and S−P+ includes both stress and unstressed groups. The variation between treatments is ought to several bands showed in loading plots of PC2: 1710–1690 cm^{-1} (β-antiparallel sheet structures of proteins), 1654 cm^{-1} (assigned to β-parallel sheet structures) and 1610 cm^{-1}, assigned to misfolding, denatured or aggregated proteins [30, 31]. S−P+, S+P− and S+P+ show increase absorption of those three bands (Fig. 5b).

3.2. Behavior Test Analysis

3.2.1. Behavior Analysis in Cat Odor Exposure Test

In all five days of stress the rats were registered with a camera for previous analysis of their behavior. Fig. 6 shows four of the most significant defensive behavior: time spent in the hide box (hide time), time of exploration in the open part of the experimental box (exploration time), time spent in the entrance of the hide box (head out time) and time of examination of the piece of cotton impregnated (or not) with cat odor (approach time).

Hiding (Fig. 6a) is the behavior to remain in the dark side of the cage and it is an important defensive behavior against predators in nature. Rats exposed to cat odor as stressor (S+P−) spent more time hiding than unstressed ones (S−P−), $p \leq 0.0001$, this last group showed a tendency to increase the hiding time from the third day having a maximum on the fourth day. On the other hand, S−P+ was the group that spent the least time hiding along all days. Group S+P+ had a similar hiding time as S+P−, except on the fifth day where S+P+ showed a tendency to spend less time hiding than S+P− ($p = 0.23$).

Exploration (Fig. 6b) is a non-defensive behavior and is important because it provides information about the surrounding environment, about food, mate and predators, among other clues. S+P− was the group that invested less time in exploration ($p \leq 0.02$, compared with S−P−). Group S−P− explored more in the first two days but on the third day reached a plateau. On the contrary, S−P+ increased the exploration time after the third day and S+P+ tends to rescue the exploration time on the fifth day ($p = 0.3$, compared with S+P−).

Another important behavioral parameter of cognition evaluated in this study is the own rat attention, named as head out, that is when the rat takes out the head from the hiding arena, observing the horizon (Fig. 6c). This strategic position is another defensive behavior when the rat observes the threatened space [23]. Group S+P− showed a significant increase in the time invested in this behavior compared with all groups on the fifth day ($p \leq 0.02$, compared with S−P−). S+P+ showed an increase head out time on fifth day.

Approaching behavior is the motion of the rat to be close enough to perceive the cat odor on the cloth (Fig. 6d). This behavior also confirms that the cloth used to stress the animals does not induce stress by itself. Groups S+P− and S+P+ approached only for a few seconds. Groups S−P− and S−P+ approached to the cloth on each of the five sessions and even played with it ($p \leq 0.0001$, S−P− vs S+P−).

3.2.2. Behavior Analysis in Elevated-plus Maze Test

Elevated-plus maze test was applied the day after the last stress exposure to evaluate the anxiety as a cause of chronic stress (Fig. 7). In this test the paradigm states is that the less anxiety the animal has, the more time it will spent in the open arm exploring and climbing down the head [24]. Thus, the time in the open arm and the number of head deeps were counted. Stressed rats spent less time in the open arms compared with the unstressed ones ($p = 0.007$, Fig. 7a). Piracetam increased significantly this behavior in stressed rats ($p = 0.002$, compared with S+P−). Also, stressed rats climbed down the head less than unstressed ones and piracetam increased significantly this time ($p \leq 0.05$, Fig. 7b).

3.3. Plasma Corticosterone Level

Corticosterone is the main stress hormone, it is produced in the suprarenal gland of mammals and many other vertebrates. Previous studies have shown that under chronic stress the corticosterone in plasma increases [32]. Stressed rats S+P− showed increase plasma corticosterone level compared with S−P− ($p = 0.059$, Fig. 8) which is consistent with the stress axis paradigm [33]. In stressed rats, piracetam showed

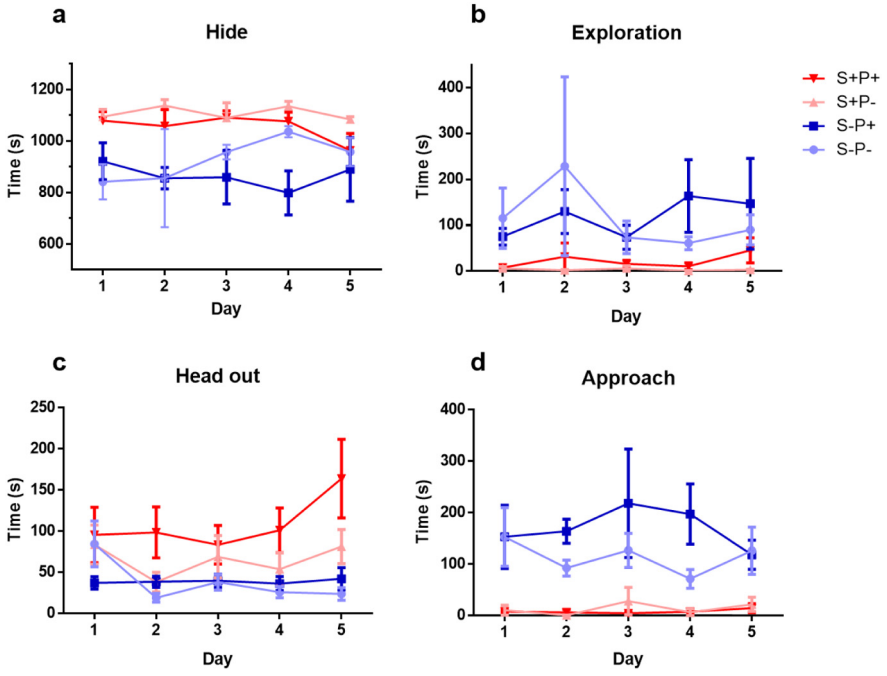

Fig. 6. Chronic stress induces changes in behavior. Rats were exposed to cat odor as stressor for five consecutive days. a Time spent in the hide box (Hiding). b Time of exploration in the opened part of the experimental box (Exploration). c Time spend in the entrance of the hide box (Head out). d Time of examination of the piece of cotton impregnated (or not) with cat odor (Approach). S−P− rats neither exposed to stress nor with piracetam treatment; S−P+ rats not exposed to stress but treated with piracetam; S+P− rats exposed to stress without piracetam treatment; S+P+ rats exposed to stress and treated with piracetam. n = 6–7 per group. Scores are means ± SEM.

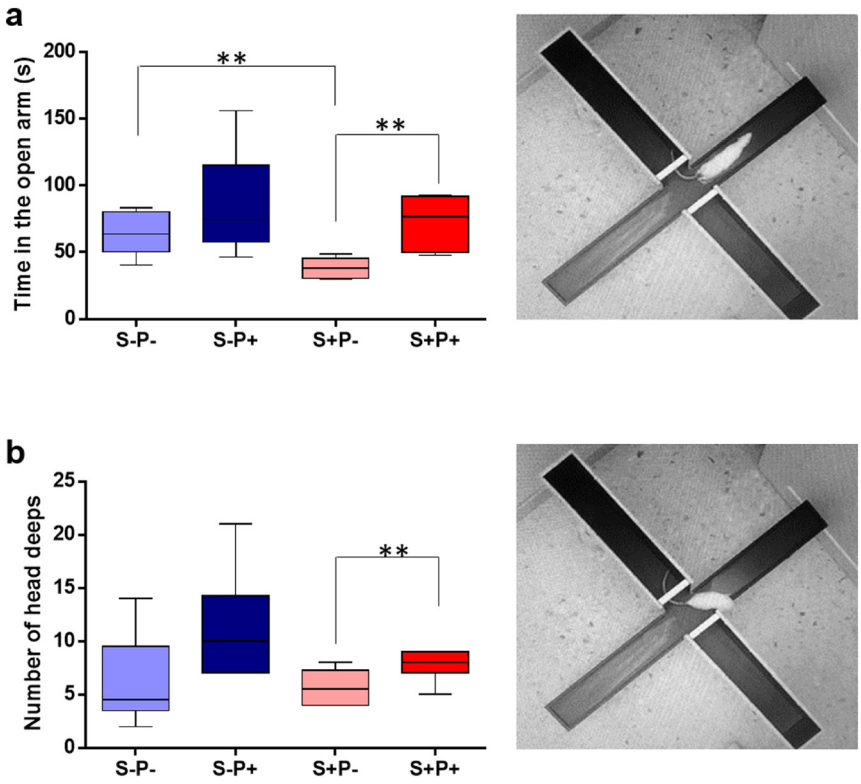

Fig. 7. Stress induces anxiety, piracetam reverts it, but the drug alone induces stress-anxiety like behavior. a Time spent on the open arm. b Number of head deeps. S−P− rats neither exposed to stress nor with piracetam treatment; S−P+ rats not exposed to stress but treated with piracetam; S+P− rats exposed to stress without piracetam treatment; S+P+ rats exposed to stress and treated with piracetam. n = 6–7 per group. Each box shows the value of the 50th percentile, the horizontal line is the median value and the bars indicate the maximum and minimum values. *p ≤ 0.05.

no effect on level of plasma corticosterone but in unstressed condition this drug tends to increase the concentration of this hormone (p = 0.153, S−P− vs S−P+).

4. Discussion

This study provides evidence of the translation mechanism from emotions to the potential immunodepression. Oxidation in living cells is a conserved chemical process on earth since the first living organism [34]. Facing a threat, the whole organism suffers an oxidation process of all molecules and this can be considered an echo of a real, potential or imaginary danger in the environment.

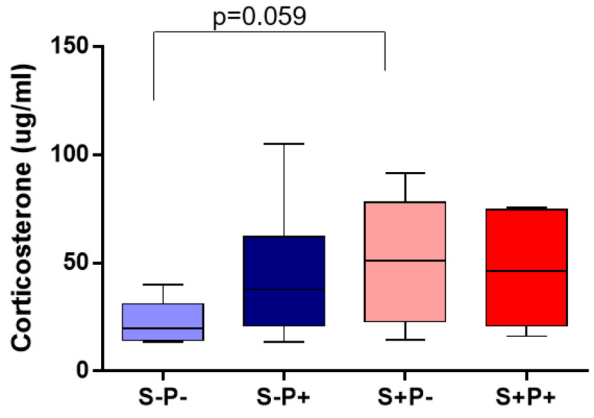

Fig. 8. Chronic stress increases plasma corticosterone, piracetam does not prevent this. Plasma corticosterone concentration was measured in the day 9 of the experiment. S−P− plasma from rats neither exposed to stress nor with piracetam treatment; S−P+ plasma from rats not exposed to stress but treated with piracetam; S+P− plasma from rats exposed to stress without piracetam treatment; S+P+ plasma from rats exposed to stress and treated with piracetam. n = 5–6 per group. Each box shows the value of the 50th percentile, the horizontal line is the median value and the bars indicate the maximum and minimum values.

The present study takes advantage of single cell SR-μFTIR analysis showing global changes in circulating mononuclear cells induced by chronic psychological stress and piracetam. It has been postulated that the principal mechanism of action of this drug is based on its interaction with membrane phospholipids [9]. Also, our previous results showed that chronic stress in rats induces lipid peroxidation in their circulating lymphocytes [3]. SR-μFTIR second derivative spectra from lipids region (3050 cm^{-1}–2800 cm^{-1}) showed three strong peaks near 2852 cm^{-1}, 2923 cm^{-1} and 2962 cm^{-1} which significantly diminished in stress. Piracetam protects lipids membrane from oxidative stress by inhibiting those changes but in non-stressed condition tends to damage them. Free radicals affect cell membranes and determine mitochondrial dysfunction [35]. The binding of piracetam to cell membranes induce restoration of membranes fluidity in stress conditions. In aged mice, this drug improved mitochondrial membrane potential and ATP production [12] and, in lymphocytes of rats with lipopolysaccharide administration, piracetam significantly decreased ROS levels and DNA damage [36]. However, S−P+ showed less absorption than S−P− near 2852 cm^{-1} and 2923 cm^{-1} which means that piracetam, in physiological situations, induce possible alterations. Previous studies showed that piracetam has no effect on unstressed subjects. For example, there were insignificant changes in brain membranes of young rats treated with this drug [37] and no changes were found in control groups in cell viability and level of DNA damage [36]. However, piracetam, being a metabolic enhancer [12, 38], can cause a low increase in oxidative stress in control group (S−P+).

Lipid oxidation is an important parameter in the analysis of cell oxidative damage which can be studied by measuring the loss of unsaturated fatty acids or by quantifying the peroxidation products [39]. Several studies have found an increase in the double bonds HC═CH (at 3012 cm^{-1}) in cells exposed to radiation [29, 40], diabetic platelets

[41], brain tissue from stressed rats [42], or in amyloid plaque spectra from human brain with Alzheimer disease [28]. In those cases, the authors have associated the increase of the absorption of olefinic bonds with the increase of lipid peroxidation products, which are rich in carbon double bonds (malondialdehyde, conjugated dienes, hydroperoxides) [39]. However, the band near 3012 cm^{-1} is characteristic to cis HC═CH from polyunsaturated acyl chains and peroxidation, in erythrocytes showing a significant decrease of this band [43]. Also, a decrease of this peak was obtained in brain tissue from Huntington's disease rat model [44] and in heart tissue from diabetic rats [45]. A diminishing intensity of olefinic HC═CH band indicates a decrease in unsaturated lipid content in cell membranes. In the present study, psychological chronic stress induces a decrease in the quantity of olefinic bonds (cis HC═CH) from polyunsaturated lipids, thus, piracetam inhibits this process.

Proteins fingerprints were also changed by stress and piracetam. SR-μFTIR is a good method to analyze protein secondary structure which is strongly affected by oxidative stress. Spectral alterations of proteins are associated with different diseases like atherosclerosis, epilepsy, Alzheimer disease or Parkinson disease [46]. Increase absorption of the bands near 1610 cm^{-1} and near 1690 cm^{-1} are characteristic for proteins misfolding pathology in samples prepared in aqueous solution [46, 47]. PCA of the proteins region in the present study showed the increase of those bands in stressed groups which means that psychological stress can induce protein denaturation, abnormal aggregation, increase in protein content, or an admixture of these events in circulating mononuclear cells. Piracetam had no effect in stress condition but altered them in non-stress condition. S−P+ showed less proteins expression than all groups and a stress-like PCA. Previous studies showed that this drug seems to be implicated in the metabolism of specific proteins [10, 11, 48] or in total protein expression in liver and brain [49].

The spectra ratios offer valuable information about the structural and compositional changes induced by different conditions, we applied this analysis to chronic stress and piracetam. The decrease of lipids/proteins ratio in stress can be explained by lipids decrease, by proteins increment or by both phenomena at different rates, which can take place in an altered metabolism [40]. Piracetam significantly inhibited the damage at this level but again, in unstressed condition, induced a decrease of this ratio. The diminishing of lipid/protein ratio has also been observed by the toxic effects of herbicides [50] or by ionizing radiations [40] on rat cells in culture.

The neurochemical, neuroanatomic and immune systems are closely linked with behavior. The implication of the knowledge at biomolecular level of the stress on behavior are nowadays being more important since World Health Organization declared that stress is an epidemic illness of this century [51]. The presence of a threat induces defensive behavior like fear and anxiety which can be both an adaptive and pathological emotional states related with psychopathologies. The study of those two conditions brings important information about response of the body to stress [1]. Rats exposed to cat odor recognize the stimulus and show important defensive responses: increase the hiding while decreasing the exploration, augment the alert (head-out position) and spent a minim time in approaching to the cloth to smell it. The initial reaction continues along all days of experiments and culminates with significant anxiety behaviors. Stressed rats treated with piracetam result in a reduction of fear and anxiety behavior like in other studies where this drug improved the transfer latency time of rats [52–54] in plus maze test. However, S−P+ looked more active than S−P−, observation that is not consistent with other studies [53, 54]. The changes in the behavior were reflected in the biochemical profile of the mononuclear cells and, also, in the level of plasma corticosterone. This hormone increased in stress but interestingly, also in the non-stressed rats treated with piracetam.

All cells under chronic emotional stress increase their oxygen consumption and generate high concentrations of ROS [2–5]. Oxidative stress affects the entirely body including the Nervous System, which in the present study was evaluated by changes in behavior. Also, the Immune System, represented by the circulating mononuclear cells, showed loss of unsaturated lipids supported by the fact that lipid/protein ratio decreased and lipid peroxidation increased during emotional stress in consistency with others [55]. Because all of this, we hypothesize that the oxidation ought to emotional stress is the chemical ancestral link between the external danger perception and the internal damage milieu. This observation is plausible because free radicals are chemical chain reactions that spread out of all organelles and tissues without limit barriers [35].

Our findings have important implications in future research about the relationship between psychological stress, behavior, circulating mononuclear cells and pharmacological potential of piracetam. The present study provides clues for the use of circulating mononuclear cells as speculum of the chronic stress damage. Piracetam improves the behavior and shows protective effect against the lipid peroxidation in circulating mononuclear cells from stressed animals, oxidant status being observed trough FT-IR. However, in physiological conditions piracetam induces potential damage on immune system as well as negative behavioral changes.

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Conflict of Interests

The authors declare no competing interests.

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