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Preliminary studies of the effects of psychological stress on circulating lymphocytes analyzed by synchrotron radiation based-Fourier transform infrared microspectroscopy

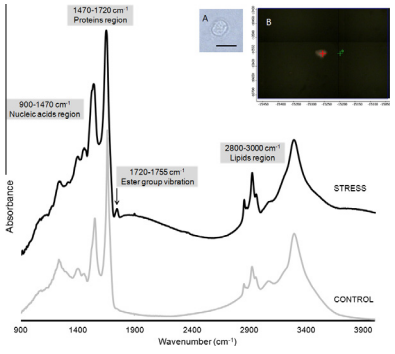
Alejandra Vargas-Caraveo [a,c,1], Hiram Castillo-Michel [b,1], Gloria Erika Mejia-Carmona [a,c], David Guillermo Pérez-Ishiwara [a], Marine Cotte [b], Alejandro Martínez-Martínez [c,*]

[a] Escuela Nacional de Medicina y Homeopatía, Instituto Politécnico Nacional, Guillermo Massieu Helguera, No. 239, Fraccionamiento "La Escalera", Ticomán, C.P. 07320 México DF, Mexico
[b] ID21, European Synchrotron Radiation Facility, 6 Rue Jules Horowitz, 38000 Grenoble, France
[c] Departamento de Ciencias Químico Biológicas, Instituto de Ciencias Biomédicas, Universidad Autónoma de Ciudad Juárez, Anillo envolvente Pronaf y Estocolmo s/n, 32310 Cd. Juárez, Mexico

HIGHLIGHTS

- Biochemical changes by psychological stress in lymphocytes investigated by vibrational spectroscopy at single cell level.
- The results showed an increased absorption at lipid peroxidation region in lymphocytes from stressed rats.
- Changes in peak position and absorbance in nucleic acids were observed suggesting reduction of transcriptional activity.
- Results unravel part of the mechanisms by which psychological stress affects immune system with systemic consequences.

GRAPHICAL ABSTRACT



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ABSTRACT

Psychological stress is a condition that not only generates behavioral disorders but also disrupts homeostasis and immune activity that can exacerbate or lead to inflammatory diseases. The aim of this work was to study biochemical changes in circulating immune cells from rats under psychological stress by using vibrational spectroscopy. A stress model was used, where exposure to a stressor was repeated for 5 days. Subsequently, circulating lymphocytes were examined for their biomolecular vibrational fingerprints with synchrotron radiation based-Fourier transform infrared microspectroscopy. The results showed an increased absorption at the ester lipid region (1720–1755 cm^{-1}) in lymphocytes from stressed rats, suggesting lipid peroxidation. Statistical significant changes in wavenumber peak position and absorbance in the nucleic acid region were also observed (915–950 cm^{-1} Z-DNA, 1090–1150 cm^{-1} symmetric stretching of P—O—C, 1200–1260 cm^{-1} asymmetric PO_2 and 1570–1510 cm^{-1} methylated

* Corresponding author. Tel.: +52 (656) 6881621.
E-mail addresses: alejandra.vargas@uacj.mx (A. Vargas-Caraveo), hiram.castillo_michel@esrf.fr (H. Castillo-Michel), gloria.mejia@uacj.mx (G.E. Mejia-Carmona), dperez@ipn.mx (D.G. Pérez-Ishiwara), marine.cotte@esrf.fr (M. Cotte), alejandro.martinez@uacj.mx (A. Martínez-Martínez).
[1] These authors contributed equally to this work.

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ROS
Z-DNA

nucleotides) which suggest a reduction of transcriptional activity in lymphocytes from stressed rat. These results unravel part of the mechanisms by which psychological stress may affect the immune system leading to systemic consequences.

Introduction

Bidirectional relationships exist between the immune and central nervous systems and occur during situations such as physical and psychological stress. These interactions have been shown to be capable of affecting the activities of both lymphocytes and macrophages [1]. Acute or chronic psychological stress can cause pathophysiological changes that disrupt homeostasis and in turn aggravate disease [2,3]. Previous investigations have studied how different kinds of stressors cause the development of a pro-inflammatory response in the brain and in other systems. The process is characterized by events such as the release of cytokines and prostanoids, transcription factor activation and the production of free radicals [4]. It is known that naturalistic stressors in humans can cause immune suppression [5]. Similarly exposure of rats to predator odor (cat) leads to immunosuppression and reduced infection resistance [6] as psychological stress would do in humans [5].

The relationship between stress and immune function has been demonstrated in many contexts including proliferative response to mitogens and cellular activity [7]. For example, psychosocial stress in humans has been related with cancer development by the increased levels of 8-hydroxydeoxyguanosine, a biomarker of oxidative DNA damage, in peripheral blood leukocytes [8]. On the other hand, lymphocyte function is also important to support learning and memory [9] but maladaptation to psychological stress can be severely increased if the T lymphocyte population is deficient [10].

Fourier Transform Infrared (FTIR) spectroscopy is extensively used in investigations of biological samples. Many important biomolecules, such as nucleic acids, proteins, lipids, and carbohydrates, have well-known vibrational fingerprints and can be identified simultaneously without any staining [11]. The use of a microscope allows the localization of these different species within the sample. A common application of this technique is to distinguish healthy and pathological samples, by measuring differences in the quantity and distribution of specific biomolecules [12]. The objective of this work was to use the Synchrotron Radiation Fourier Transform Infrared microspectroscopy (SR-μFTIR) to study the biochemical changes induced by psychological stress in circulating lymphocytes. Here we provide evidence, for the first time at individual cell level, of the effect that psychological stress can have on lymphocytes, one of the safeguarding cells of the body as a consequence of psychoneuroimmunological interactions.

Methods

Animals

Wistar rats were housed with littermates for the first 30 days after birth. Subsequently, two experimental males were transferred into individual cages, under standard conditions at a temperature of 24 ± 2 °C and with a 12 × 12 h dark/light cycle (lights turned on at 8:00 a.m.). Food and water were available to the animals *ad libitum*. At day 60, the juvenile rats with weights of 233 and 235 g were used as the control and psychological stress subjects, respectively. All experimental procedures were approved by the institutional Bioethical Committee in accordance to international standards.

Stress exposure

Adopting the method described by Dielenberg and McGregor [13], the rats were placed into individual avoidance chambers for two periods of time. The first period consisted of 20 min to allow environment habituation in the avoidance chamber, following which the animal was returned to the housing cage for 20 min to relax. Subsequently, the animals were placed a second time for an additional 20 min in the avoidance chamber containing a 25 × 25 cm piece of cotton fabric, either without odor (control) or impregnated with cat odor (stress). The experimental procedure was repeated for five consecutive days between 8:00 and 9:00 a.m. The cotton fabric was impregnated with cat odor by using it as a carpet for a housed adult cat. Each fabric specimen was stored in airtight plastic bags and maintained at −20 °C until used.

Blood sample collection and lymphocyte isolation

On the 6th day of odor treatment the animals were anesthetized using peritoneal pentobarbital. Blood samples were taken using vacutainer™ EDTA tubes, by cardiac puncture and approximately 4 mL were obtained per rat. Whole blood was diluted with one volume of PBS-EDTA at pH 7.4 (137 mM NaCl; 8.2 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 3.2 mM KCl and 4 mM EDTA). Subsequently 4 mL of this dilution was placed onto 4 mL of a isotonic Percoll solution bed (40% PBS-EDTA, 57.3% Percoll™ and 2.7% 10X PBS) and centrifuged at 1000*g* for 10 min. Lymphocytes appearing in the gradient interface were separated and washed twice by homogenizing with 12 mL PBS-EDTA and re-centrifuged [14]. The final cell pellet was suspended in 1.5 mL of PBS-EDTA and aliquots of 300 μL were then transferred into a microtube containing 4% paraformaldehyde for 20 min at room temperature in order to fix the cells. The fixed cells were precipitated with a 30 s pulse then washed in 1 mL of deionized water and centrifuged for 30 s following which they were resuspended in 200 μL deionized water. Fifteen microlitre of the cell suspension were smeared onto a microscope slide for reflective IR measurements and gently heated to evaporate the liquid. Samples were transported at room temperature to beamline ID21 at European Synchrotron Radiation Facility (ESRF) to be analyzed by SR-μFTIR. Samples were kept inside a desiccator at room temperature until measurements were performed.

White blood cell type determination

In order to confirm which type of white blood cells were isolated [14], four different rat blood lymphocytes isolations were characterized and counted using a Sysmex XT-1800i Hematology Analyzer.

SR-μFTIR

The SR-μFTIR measurements were performed on single cells prepared as previously described, using the FTIR spectromicroscopy end-station of ID21 at the ESRF. The single cell spectra were collected in reflection mode using an IR microscope (Thermo Nicolet Continμum) coupled to a FTIR spectrometer (Thermo Nicolet Nexus). The IR microscope is equipped with a 32× objective, a motorized sample stage and a liquid nitrogen cooled 50 μm mercury cadmium telluride detector. In order to match the beam

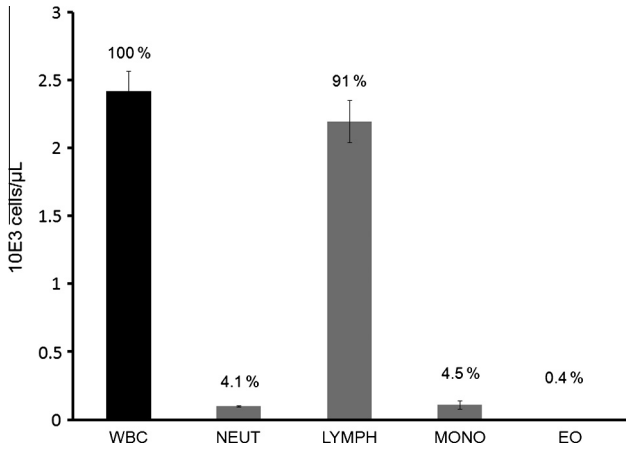

Fig. 1. Pecoll method isolates up to 90% of lymphocytes from the total of white blood cells as determined by hematology analysis. (WBC) white blood cells, (NEU) neutrophils, (LYM) lymphocytes, (MONO) monocytes and (EO) eosinophils. Error bars are indicated, $n = 4$ animals.

size with cell size, a $9 \times 9\ \mu m^2$ aperture was used to acquire spectra from single cells. The spectra were collected from 4000 to 900 cm^{-1}; the spectral resolution was set to 6 cm^{-1} and 128 scans were averaged for each sample. Fourteen and thirty-seven cells from one control and one stressed animal were analyzed respectively.

Spectra analysis

Omnic v8.1 software was used to analyze all spectra. Spectra were converted into second derivatives using the Savitzky/Golay algorithm with a 21 point window and a 2nd polynomial order. The wavenumber shifts and absorbance intensity were obtained using the minimum value from the region of interest (i.e. ester group vibration 1730–1750 cm^{-1} band). Average and standard errors were calculated for spectra and tests for significance differences in wavenumber shifts and absorbances between groups were undertaken using *t*-tests ($p \leqslant 0.05$) in Microsoft Office Excel 2007.

Results

White blood cell type determination

White blood cell typification demonstrated that 90% of the cell isolation were lymphocytes, 4.5% were monocytes, 4.1% were neutrophils and eosinophils 0.4% (Fig. 1).

SR-µFTIR fingerprint region

The characteristic vibrational fingerprint of biological molecules such as nucleic acids, proteins and lipids were observed in all analyzed cells. Representative spectra from control and experimental samples are shown in Fig. 2 and the feature regions of bands and related biochemical components are indicated.

Second derivative analysis

Second derivative spectral analyses were performed in order to detect subtle differences between signals measured from lymphocytes isolated from the control and stressed rats. To provide statistical comparisons between control and stress bands, averages and standard errors were calculated. The nucleic acid related band region provided the most statistically significant difference between groups (Fig. 3A). In lymphocytes from stressed rats a statistically significant decrease in the absorption band at 915–950 cm^{-1} was observed, which corresponded to phosphodiester stretching of DNA, mainly in the Z-DNA conformation [15], (Fig. 3B). In addition, a shift in peak position was also observed in the 1090–1150 cm^{-1} band that originates from the symmetric stretching of P—O—C [16] situated at 1122 cm^{-1} for the control and at 1115 cm^{-1} for the stressed condition (Fig. 3C). A further peak position shift was found

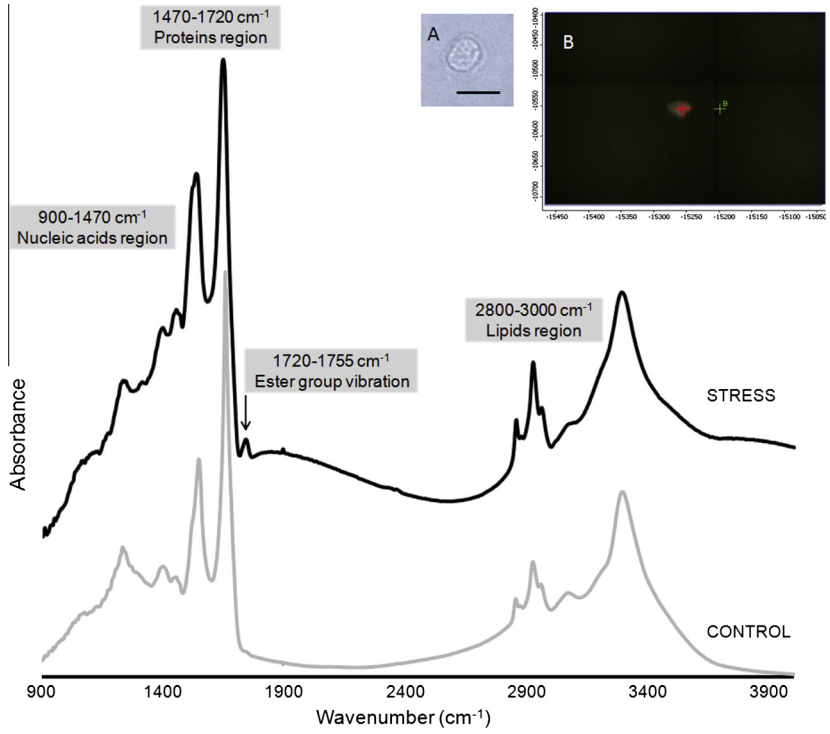

Fig. 2. Single representative spectra of lymphocytes from control and stressed rats. The nucleic acids, protein and lipids regions are indicated as well as ester group vibration. A and B insets are representative images of a lymphocyte cell taken with an optical microscope (scale bar 10 µm) and with the IR microscope 32× objective, respectively.

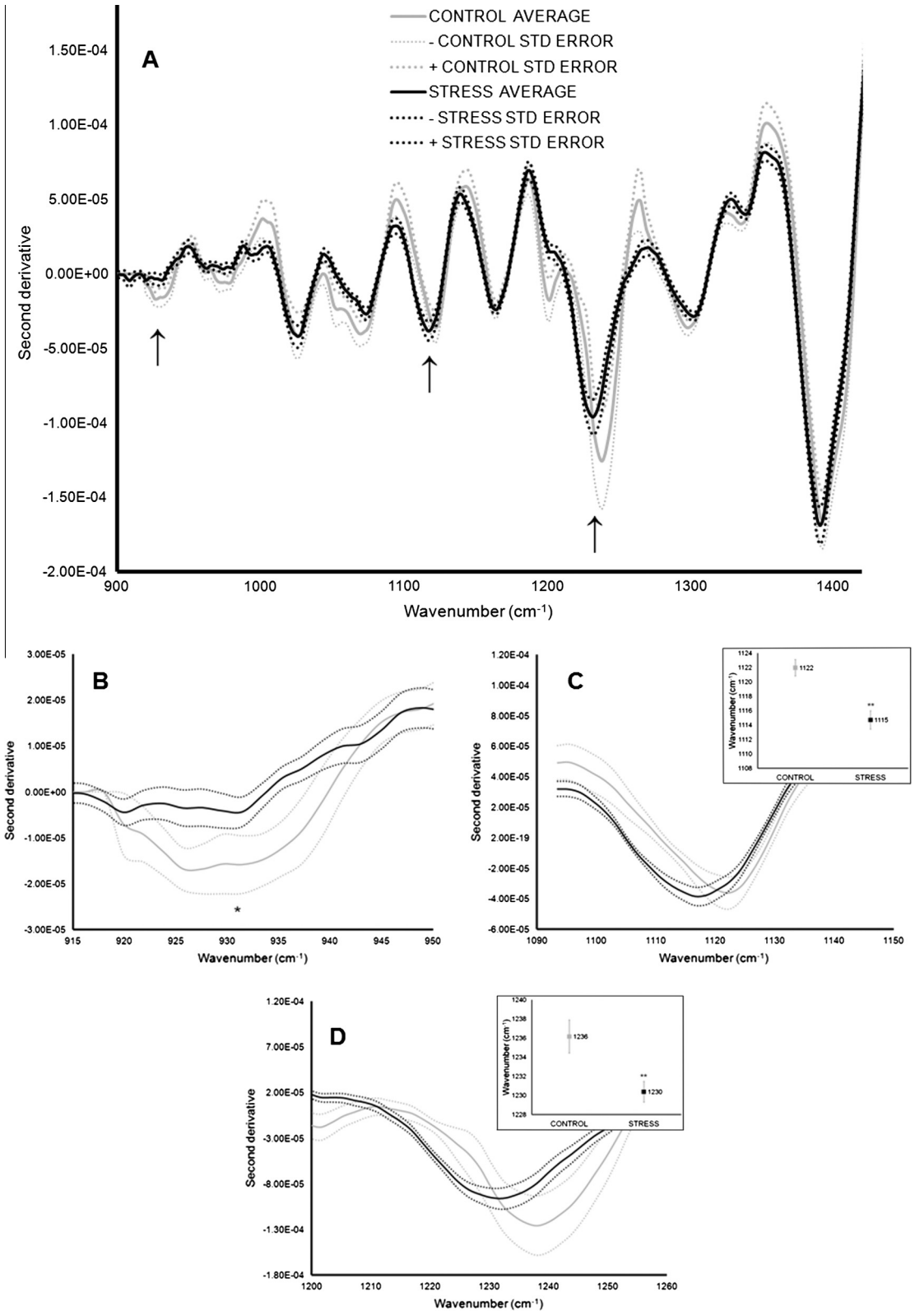

Fig. 3. Second derivate of nucleic acids region for lymphocytes from control and stressed rats. (A) Arrows point out bands with statistically significant changes. (B) Related Z-DNA band shows decrement in peak intensity by stress sample. (C) Related stretching P—O—C band. (D) Related asymmetric PO_2 band. Right on top insets of C and D show average and standard errors for shift peak intensity wavenumber. Values are averages ± error standard, control $n = 14$ and stress group $n = 37$. *Different from the control group, $p < 0.05$. **Different from the control group, $p < 0.01$.

in the 1200–1260 cm^{-1} asymmetric PO_2 band [17] at 1236 cm^{-1} for the control and at 1230 cm^{-1} for the stressed state (Fig. 3D). *t*-Tests confirmed the statistical significance between samples for both band regions corresponding to phosphate ($p < 0.01$). In contrast no statistical differences were observed between groups for absorbance and peak positions in the Amide I and II regions. A shift

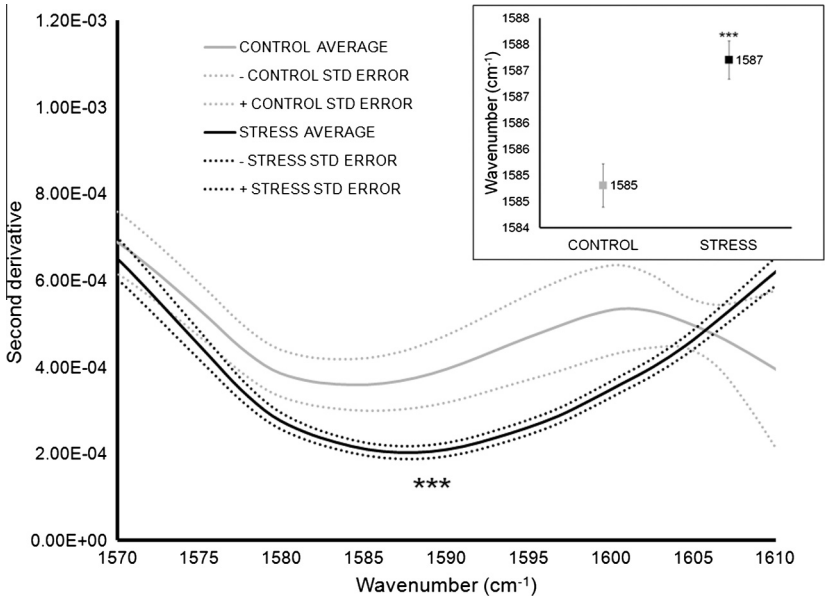

Fig. 4. Methylated DNA band. Increment in peak intensity by lymphocytes from stressed rat. Right on top shift peak intensity wavenumber. Right on top inset shows media and standard errors for shift peak intensity wavenumber. Values are averages ± error standard, control $n = 14$ and stress group $n = 37$. ***Different from the control group, $p < 0.001$.

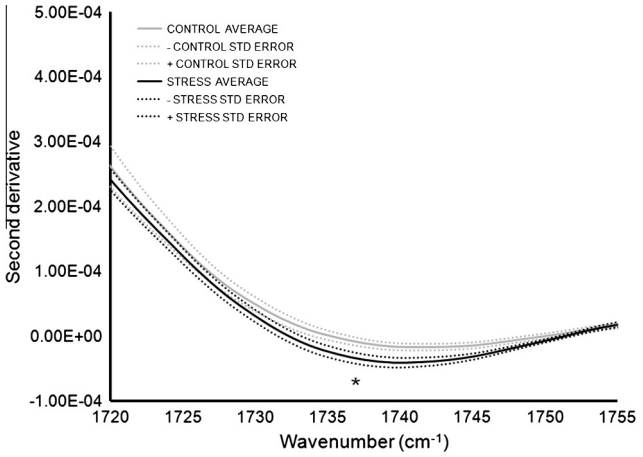

Fig. 5. Ester group vibration band. Increment in peak intensity by lymphocytes from stressed rat. Values are averages ± error standard, control $n = 14$ and stress group $n = 37$. *Different from the control group, $p < 0.05$.

in the peak position and intensity was found in a 1570–1610 cm^{-1} band associated with nitrogenated bases by several functional groups such as C=C stretch, and C=N aromatics: 1585 cm^{-1} for control versus 1587 cm^{-1} for stressed [16,18–20] and is shown in Fig. 4. This band has previously been related to methylated nucleotides [16]. The absorption intensity at 1720–1755 cm^{-1} the ester group vibration band increased significantly in the cells from stressed rats (Fig. 5) and has been linked to lipid peroxidation end products, known as reactive carbonyl compounds [21]. No significant changes in the lipid region of spectra of lymphocytes from control and stressed rats were observed (data not shown).

Discussion

We have demonstrated that SR-μFTIR can be used to study biochemical changes in circulating lymphocytes caused by a psychological stressor. The most significant indicative marker of adverse changes in cell physiology was an increase of the band related to lipid peroxidation end products (1720–1755 cm^{-1}). A similar effect has been observed by other investigators including: in monkey fibroblasts exposed to the reactive oxygen species (ROS) fullerol under white light illumination [21]; in studies examining human lip surfaces to correlate dietary fatty acids with health and diseases [22]; and in biomaterials like ultra-high molecular weight polyethylene used in orthopedic prosthesis exposed to hydroperoxide [23]. Lipid peroxidation in the lymphocytes obtained from the stressed animal may be occurring due to oxidative stress initiated by mental stress. Similar effects have been reported in plasma and in erythrocytes and linked to atherosclerosis disease [24], but inadequately demonstrated in leukocytes. One such study demonstrated that psychological stress in university undergraduates caused by examinations produced lipid oxidation and oxidative damage in DNA which increased significantly in samples taken in two examination periods versus the non-examination period [25].

In contrast in the current study, the analysis of the region associated with nucleic acids (900–1245 cm^{-1}) revealed that the Z-DNA band decreased in the cells taken from the stressed animal. It is known that DNA promoter sites rich in TG or GC repeats allow Z-DNA conformation to occur, with the DNA in a left-handed double helix structure exhibiting a zigzag pattern. This structure forms *in vivo* under physiological conditions in actively transcribed regions of the genome [26,27]. Accordingly the spectral changes in this region observed in the current study can be considered to be associated with regulation of gene expression and genetic instability. These modifications were correlated with psychological stress suggesting the effect on the CNS is translated into physiological changes in the circulating leukocytes. Furthermore the increased absorption observed at 1585–1587 cm^{-1} in the lymphocytes isolated from the stressed rat, is related to DNA methylation [16] that occurs at CpG sites, as well as in the Z-DNA regions [28]. Most likely, the effects observed in both bands are related to DNA methylation which caused a genome silencing action, thereby decreasing the predominance of the Z-DNA conformation. Methylation on CpG sites associated with modification of the epigenetic profile of genes in the brain has been reported in response to psychological stress such as traumatic experiences in early life leading to emotional disorders that may persist in adulthood

[29]. In addition, the detected wavenumber shift of the asymmetric PO_2 band (from1236 cm^{-1} for the control to 1230 cm^{-1} for the stressed cells) could be attributable to the mechanisms reported by Nafisi, who suggested shifting at 1226 to 1231–1233 cm^{-1} was related to the DNA PO_2 coordination to Na_2–SeO_4. Accordingly the presence of potential adducts in DNA such as transcriptional regulators and DNA–protein complexes could also produce this effect [17,27]. This evidence supports how psychological stress could modify gene expression by an epigenetic influence in the circulating lymphocytes, by decreasing the quantity of proteins linked to DNA in the transcription processes under the stress conditions (resulting in the down shifting of asymmetric PO_2 band). Confirmation of such a mechanism requires further experiments to be conducted.

The P—O—C band has been characterized as one of the most representative vibrational groups of RNA [16,30] and in this investigation a down shift in wavenumber from 1122 cm^{-1} in the control cells to 1115 cm^{-1} in cells from stressed animal was observed with, no differences in absorbance. The decrease in Z-DNA alongside an increase in DNA methylation suggests a possible gene silencing in the stressed cell group, however these findings suggest that RNA quantity was unaffected. This may be accounted for by the transcriptional overexpression of pro-inflammatory molecules released by leukocytes from humans exposed to psychological stress [31,32]. The down shifting of the symmetric stretching P—O—C band itself could be explained by changes in the nature of the specific transcripts.

The chronic exposure to psychological stress is accompanied by high O_2 consumption, generating oxidative stress produced by an excess of ROS. This has been shown to contribute to the onset of a large range of diseases, including cancer, diabetes, male infertility, autoimmune diseases, atherosclerosis and cardiovascular disorders [33]. The evidence of lipid peroxidation and nucleic acid composition changes in the lymphocytes obtained from the stressed animal may be due to the over production of systemic ROS that is implicated in adverse effects over the whole organism and immune system, such as causing suppression of immune function and a decrease in leukocyte mobilization to other tissues [34].

Conclusion

The SR-μFTIR data showed statistically significant changes in lipid peroxidation and nucleic acid conformation in lymphocytes from the stressed condition. The advantage of SR-μFTIR over other specific biochemical analyses such as immunohistochemistry, is that it allows the simultaneous observation of the most representative biomolecular changes that immune cells suffer as consequence of an affected psyconeuroimmunological axis.

Acknowledgments

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