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Protein and lipid oxidative damage in healthy students during and after exam stress

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HIGHLIGHTS

- We determined protein oxidative damage in exam stress conditions.
- We measured lipid oxidative damage in exam stress conditions.
- We evaluated relationships between oxidative damage levels and anxiety severity.
- We suggested new mechanisms for anxiety.

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ABSTRACT

Oxidative damage at cellular level is thought to be one of the mechanisms in the pathogenesis of psychological stress (anxiety). The aim of this study was to investigate lipid and protein oxidative damage in exam anxiety conditions. Blood samples were collected in two stages (during the exam period and post vacation) from 51 healthy female students after responding to Beck Anxiety Inventory (BAI) and test anxiety questionnaire. Protein carbonyl, total thiol and malondialdehyde (MDA) levels were determined in serum. Participants reported significantly higher levels of subjective anxiety during the exam period than post vacation. Also the level of total thiol was significantly lower during the exam period compared with post vacation ($p < 0.001$). Meanwhile, protein carbonyl and MDA levels during the exams were significantly higher than those in post-exam period ($p < 0.01$). During the exam period, there was a negative correlation between serum total thiol levels and the severity of anxiety ($r = -0.45$, $p < 0.01$). A significant positive correlation between the changes in serum protein carbonyl and MDA levels, also between those markers and anxiety score was found during the exam period. The high level of protein carbonyl and MDA, also low level of total thiol during the exam period demonstrated an oxidative damage to proteins and lipids in stress conditions. Our results suggest that oxidative damage to cellular compounds may be one of the mechanisms involved in the pathogenesis of anxiety.

1. Introduction

Exam stress (anxiety) is a common physiological condition among students all over the world in which individuals suffer from intense worry and discomfort during exam period [1]. A little anxiety is normal and often helpful keeping individuals mentally and physically alert. However, too much anxiety is usually accompanied with difficulty in concentrating, emotional upset, decrease in normal learning and poor test performance [2,3]. Moreover, there are growing evidences showing that psychological stress is one of the most important reasons in the progression of oxidative stress-related diseases including cardiovascular disorders, diabetes, cancer, and stroke [4–7]. Mechanism(s) that trigger(s) anxiety is/are largely unknown, but there is clear evidence that oxidative stress in neurons is involved in pathogenesis of many neurological disorders including anxiety [8,9].

Oxidative stress is an imbalance between production of reactive oxygen species (ROS) and biological systems that detoxify them. ROSs, such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and peroxides are chemically reactive (oxidant) compounds containing oxygen, which are formed at relatively low level in all cells during normal metabolism and their physiological levels are necessary to maintain normal cell function [10,11]. The additional amounts of ROSs are

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removed by free radical-scavengering enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-PX) [11]. Also, there are nonenzymatic compounds such as reduced glutathione, α-tocopherol, ascorbic acid, uric acid and bilirubin that contribute to antioxidant defenses in the body [12]. When the level of ROSs increases beyond the antioxidant capacity of the body, a condition known as oxidative stress is resulted. In such situation, free radicals interact with proteins, lipids, and nucleic acids and thereby change their function and trigger a number of human diseases [13]. The oxidative damage to proteins can generate protein carbonyl products [14] and decrease free sulfhydryl groups of proteins and other compounds. The reduced sulfhydryl groups of proteins and non-protein compounds are collectively known as total thiol. Lipid peroxidation produces malondialdehyde (MDA) which is measured as one of the most frequently used indicators of lipid oxidative damage [15].

Several studies on animal and human models have demonstrated elevated oxidative stress in psychological stress condition. A relationship between brain oxidative stress and level of anxiety was for the first time described by Hovatta et al. They showed a correlation between anxiety and the expression level of glutathione reductase-1 and glyoxalase-1 genes in mouse brain which protect cells from oxidative damage [16]. A study conducted by Li et al. indicated that psychological stress induces oxidative damage in rat muscles through a decrease in SOD, catalase and GSH-PX activities [17]. The high level of 8-hydroxy-2′-deoxyguanosine, a marker of DNA oxidative damage, was found in the liver of rats exposed to psychological stress. This increase was proportional to the number of times that animals were exposed to stress [18]. Sivonova et al. showed that exam stress in medical students induced lipid peroxidation and nuclear DNA damage [19]. In addition, decreased glutathione and free sulfhydryl levels of seminal plasma were found in healthy medical students undergoing examination stress [20]. Glutathione, in fact, plays an important role in the defense against oxidative damage produced by oxidants and free radicals. In addition, maintenance of free sulfhydryl groups of proteins is important in the proper folding and activity of them [21].

To the best of our knowledge, there is no report regarding protein oxidation damage in psychological stress conditions. Therefore, this study was aimed at evaluating protein and lipid oxidation in healthy students during the exam period as a model of naturally occurring psychological stress.

2. Material and method

2.1. Participants and questionnaires

When aim, anticipated benefits and potential risks in the present study were described in details, fifty nine of first and second-year students from Resalat Teacher Training Institute of Zahedan, Iran volunteered to participate. At this institute, students take 15 weeks of classes followed by 2 week exam periods. All volunteers were female students (age 22.5 ± 2.6) living in dormitory and according to their declaration none of them had a history of chronic physical diseases or psychiatric disorders. Candidates were excluded if they were taking any medication or supplement or if they were in menstrual period. With these criteria, 51 healthy subjects of volunteers were enrolled in the study. The protocol for this study was approved by Deputy for Research of Islamic Azad University, Birjand Branch, Iran. Written informed consent was obtained from participants who were also asked to complete two self-reporting questionnaires. Beck Anxiety Inventory (BAI) [22] was used to evaluate severity of participants' trait anxiety which includes 21 items that are rated on a four-point Likert scale ranging from "not at all" (scale 0) to "extremely" (scale 3) and a total score ranging from 0 to 63 where higher scores indicate greater levels of anxiety. The second questionnaire (Test Anxiety Inventory or TAI) that was used to measure exam anxiety consisted of "25" four-point items with "never", "rarely", "sometimes" and "often" with scores of 0–3, respectively. Total score could range from 0 to 75, where higher scores indicated greater levels of anxiety [23]. Participants were asked to complete TAI only during the exam period. The internal consistency of TAI scale with a Cronbach alpha of 0.95 for female was considered satisfactory.

2.2. Blood sampling

Blood samples were obtained from participants by an experienced phlebotomist one day before the final exam of the trimester, i.e. 12 days after the onset of the course exams (exam or stress period) and 4 weeks later, after term vacation (post vacation or non-stress period). Afterwards, samples were immediately transferred to a biochemistry laboratory and after clotting centrifuged for 20 min at 2500 rpm at 4 °C, and the obtained serum was stored at −70 °C for subsequent analyses.

2.3. Biochemical assay

Serum total thiol levels were determined according to Hu method [24] with modifications performed in our laboratory. Briefly, 30 μL of each sample or of standard was mixed with 255 μL of Tris-EDTA buffer (0.25 mM Tris base, 20 mM EDTA, pH 8.2) and 15 μL of DTNB solution (10 mM in absolute methanol) in each well of a 96-well uncoated microtiter plates. The sample blanks were prepared by mixing 30 μL of serum and 270 μL of Tris-EDTA buffer and the reagent blank contained 285 μL of Tris-EDTA buffer and 15 μL of DTNB solution. The plates were then incubated for 15 min in room temperature and absorbance was measured in an ELISA reader at 405 nm with a reference wavelength of 630 nm. Then, absorbance sample and reagent blank were subtracted from absorbance of corresponding serum. The concentration of sulfhydryl groups was calculated by using reduced glutathione as standard and the result was expressed in μM.

Protein carbonyl assay was performed via ELISA method as were described by Buss et al. [25] using a commercially available kit according to the manufacturer's protocol (BioCell, New Zealand). The results were expressed in nanomole carbonyl/mg protein.

MDA levels were measured spectrophotometrically by the method of Uchiyama and Mihara [26]. Briefly, 3 mL of 1% phosphoric acid and 1 mL of 0.6% w/v aqueous solution of thiobarbituric acid were added to 0.5 mL of serum. The mixture was heated for 45 min in a boiling water bath. After cooling, the mixture was centrifuged at 3000 ×*g* for 10 min and absorbance of upper solution was determined at 535 nm against a blank containing 3 mL of phosphoric acid, 1 mL thiobarbituric acid and 0.5 mL PBS. The concentration of MDA was calculated using 1,1,3,3-tetramethoxypropane standard curve and expressed in μM.

All assays were performed in duplicate and mean score was used in statistical analysis.

2.4. Statistical analysis

All values were expressed as means ± SD and data were analyzed using SPSS 11 software. The normal distribution of the data was evaluated using the Kolmogorov–Smirnov test. A paired samples *t*-test or Wilcoxon signed rank test was used to estimate differences between biochemical analyses and questionnaire evaluations during stress and non-stress period according to the data distribution. Correlations between parameters were examined by Pearson's correlation coefficient test. The minimal level of significance was set at $p < 0.05$.

3. Results

3.1. Self-reporting measures and biochemical analyses

Self-reporting data showed significantly higher Beck anxiety scale during the exam period compared with post vacation ($p < 0.01$) (Table 1). The severity of exam anxiety was more than half of total

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