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Cortisol, β-endorphin and oxidative stress markers in healthy medical students in response to examination stress.

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Abstract

The psychosomatic connection pertaining to the relationship between perceived stress and the milieu intérieur that must be evident during a naturalistic stressor event is not well explored.
Objective: This study therefore examines the interrelationship between perceived stress scores, endocrine levels and oxidant-antioxidant activities under the duress of examination stress, an appropriate example of a naturalistic stressor.
Materials and methods: Apparently healthy year one medical students participated.
Results: Examination stress induced significant increases in perceived stress scores ($p<0.001$), serum cortisol ($p<0.05$) and plasma β-endorphin ($p<0.05$) levels, and erythrocyte lipid peroxidation ($p<0.001$), but a significant ($p<0.001$) decrease in the antioxidant superoxide dismutase activity. In addition, during the examination, the perceived stress scores were found to be correlated positively with lipid peroxidation ($r^2=0.23$; $p<0.01$) but negatively with β-endorphin ($r^2=0.14$; $p<0.05$). After the examination, the perceived stress scores correlated positively only with cortisol ($r^2=0.09$; $p<0.05$).
Conclusion: Sitting for an examination increases cortisol secretion, as well as, β-endorphin levels and induces oxidative stress. The high levels of β- endorphin appear to have an ameliorating effect on cortisol and the perception of stress. This finding awaits further investigation.

Keywords: Examination stress, Perceived stress scores, Cortisol, β endorphin, Oxidative stress markers.

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Introduction

In daily life, all organisms are faced with various types of day-to-day stresses. Some organisms can cope well with the stress stimuli while in others repeated daily stress could lead to derangement of the neuroendocrine coping mechanism and balance between pro- and anti-oxidants, producing a wide range of detrimental effects on the physiological and psychological homeostasis.

It is well-known that the hypothalamo-pituitary-adrenal (HPA) axis is highly sensitive to and easily activated by various stressors. The axis responds by releasing corticotrophin-releasing hormone (CRH) from the hypothalamus and the consequent adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. The circulating ACTH then acts on the adrenal cortex causing the release of glucocorticoids (mainly cortisol), the hormonal end-products of the HPAaxis [1], and it is intricately involved in the adaptation to stress.

In the 1970s, beta-endorphin (β-endorphin), a cleavage product of a precursor hormone for ACTH, was discovered by Li and Chung [2]. This compound is an endogenous opiate and is believed to modulate pain, boost the immune system, promote the feeling of wellbeing, and increase relaxation. However, little is known about the role of β-endorphin in acute naturalistic stress [3-6]. Thus, more studies are called for to determine whether endorphins might be released during times of stress that could have significant roles in preventing wear and tear of the body.

Since the pioneering work of Gerschman in the 1950s on oxygen poisoning [7], the role of free radical reactions in humans has been critically reappraised. In the human body, high levels of constantly formed free radicals and other reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot}-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) are involved in the generation of cascade reactions that attack cell membrane phospholipids and induce membrane lipid peroxidation. In particular, the well-characterised product of lipid peroxidation, malondialdehyde (MDA), can cause damage to proteins and DNA resulting in cellular apoptosis [8]. Hence, protection against and prevention of the consequences

of the deleterious effects of ROS are of critical importance and they could possibly be achieved by non-enzymatic (e.g., glutathione, uric acid, bilirubin, vitamin C and E) and enzymatic antioxidants.

Enzymatic defence against ROS-induced tissue damage in humans includes the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [9]. Superoxide dismutase, present in both mitochondria and cytosol, dismutes $O_2^{\cdot -}$ to form H_2O_2 and oxygen (O_2) [10], while glutathione peroxidase, found partially within cell membranes, helps in removing H_2O_2 from body fluids by converting reduced glutathione to oxidized glutathione [11] as well as being able to terminate the chain reaction of lipid peroxidation by removing lipid hydroperoxides from cell membranes [12]. Catalase, located within peroxisomes and in the cytosol of cells, decomposes H_2O_2 to water and O_2. Therefore, increased H_2O_2 production must be accompanied by increased GPx and/or CAT activity to limit damage. Under normal conditions, a delicate balance between the generation of ROS and heightened antioxidant defence appears to be in place [13]. However, this balance can be readily upset by various factors and this imbalance, the so-called "oxidative stress", appears to play a pivotal in the pathogenesis of several diseases [13-15].

Stress comes in various forms, and for university students, academic stress poses one of the many challenges that they have to contend with during their university years. A review of the literature reveals a paucity of information regarding this aspect of stress, and in particular, that of the effect of academic stress as a brief naturalist stressor on β-endorphin and oxidative markers mediated through the HPA axis. Thus, this study was undertaken to determine the relationship between perceived stress level, endocrine outcomes and antioxidant activities in medical students undergoing examination stress and their responses thereof.

Materials and Methods

This study was approved by the Medical Ethics Committee of the University Malaya Medical Centre (Approval number: 781.12).

Student recruitment

Announcements about the study were made to the first year medical students at the University of Malaya during their routine tutorial and practical classes. Potential subjects were selected based on their responses to a questionnaire designed to gather information on their past and present medical histories. Only those who reported no histories of acute illness, previous known medical conditions or psychological problems were recruited and they were deemed as normal and healthy participants. Further information about the nature and prerequisites of the investigation were provided to each participant in a face-to-face interview prior to the actual day of experiment, during which written consent and personal history, including socioeconomic status and life-style practices, were also obtained. Participants were reimbursed after each visit.

Experimental design

The study was designed such that the subjective as well as objective parameters for all subjects were obtained during the high-stress period identified as 1 or 2 days before the final written academic examination and during the low-stress period, i.e. within 1 week after the examination.

Subjective assessment: stress questionnaire

Perceived self-rated stress levels were measured by a bilingual; English [16] and Bahasa Malaysia [17] version of the validated 42-item questionnaire developed by Lovibond and Lovibond [16]. It produces sets of scales called the depression anxiety stress scales (DASS) that are widely used in clinical and non-clinical settings to elucidate current state or change in state of mind over time on the three dimensions of depression, anxiety and stress.

In the present study, only the stress scale was used, and the final stress scores obtained from stress scale were derived from the summation of scores for relevant items. The stress scale is constructed to measure the sensitivity levels of chronic and acute nervous arousals that pertain to difficulties in relaxation, feelings of being upset/agitated, irritability, over-reactivity and impatience.

Objective assessment: biochemical analysis

Blood samples were taken from the participants on the same day as the subjective assessment, i.e. between 0800 to 0830 h. The samples were collected in non-heparinised and heparinised tubes and kept in ice until centrifugation. For the non-heparinised tubes, the sera were separated after centrifuging and kept at $-80^{\circ}C$ until analysed for neurohormones. The blood samples collected in heparinised tubes meant for oxidative stress marker assays were processed as described below.

Neurohormonal assays

Serum cortisol levels were measured by chemiluminescent immunoassays (ADVIA Centaur, USA) while β-endorphin levels were assessed by enzyme immunoassays (Phoenix Pharmaceuticals Inc, USA).

Oxidative stress marker assays

Blood samples from the heparinised tubes were centrifuged at 3000 rpm for 15 minutes, after which plasma from each tube meant for lipid peroxidation was separated out to be followed by removal of the buffy coat layer. The red cell pellet left behind was hemolysed and used for subsequent analyses of antioxidant enzyme activities. The total hemoglobin content was also measured at the same time by the Drabkin method [18].

Lipid peroxidation assay: The plasma MDA activity was determined according to the method of Akkuş [19] and used as an index of lipid peroxidation [20,21]. This method is based on the reaction of MDA in plasma with 2-thiobarbituric acid (TBA) at boiling-point temperature that would yield a pink-

colour supernatant, the optimal density of which is read spectrophotometrically at 532 and 600 nm, where 532 nm represents the maximum absorbance of the TBA-MDA complex and 600 nm the correction for nonspecific turbidity. The values for absorbance at 600 nm were subtracted from those at 532 nm to give the true MDA values that were expressed as micromoles per gram of hemoglobin (µmol/g Hb).

Antioxidant enzyme activity: Determination of antioxidant status was obtained by the activities of SOD, CAT and GPx that were estimated from the erythrocyte haemolysates. Total SOD activity was determined by the method of Kakkar [22] in which reduction of the substrate, nitroblue tetrazolium (NBT), is used to indicate $O2^{\cdot -}$ production. One unit (U) of the SOD activity inhibits the rate of reduction of NBT by 50%. Catalase activity was measured by measuring the breakdown of H_2O_2 at 240 nm [23]. One unit of CAT decomposes one µmole of H_2O_2 per min with CAT activity being expressed as U/g Hb. The GPx activity was assessed by the oxidation of glutathione by H_2O_2. This reaction is coupled to the reduction of oxidized glutathione by glutathione reductase, which simultaneously oxidizes NADPH to $NADP^+$ [24]. The decrease in absorbance at 340 nm is expressed as units per gram of hemoglobin (U/g Hb).

Statistical analysis

All parameters are reported as means ± SEM. A paired sample t-test was used to compare the scores of the subjective as well as objective parameters during the stress and non-stress periods. Pearson correlations were performed at each time period to ascertain possible associations among all parameters. A probability value of less than 0.05 ($p<0.05$) is taken to be significant.

Results

The participants

Thirty-three students,cmale (n=19) and female (n=14) successfully completed the study. The participants were aged 20.0 ± 0.08 y, single, did not smoke or drink, and were free from medication and financial constraints. They also have no history of previously known medical conditions or psychological problems. No significant difference in these personal data between males and females was found.

Perceived stress scores, cortisol and β-endorphin and oxidative stress markers

The perceived stress scores, serum cortisol and β-endorphin levels and MDA, SOD, CAT, GPx activities during the high-stress and low-stress periods are shown in Table 1. During the high-stress period, the participants showed significantly higher levels of perceived stress ($p<0.001$), serum cortisol ($p<0.05$) and β-endorphin ($p<0.05$) compared with the low-stress period. In addition, the MDA activity, a measure of lipid peroxidation, was significantly ($p<0.001$) reduced upon completion of the examination, while a marked ($p<0.001$) increase in the antioxidant SOD activity was observed at the same time. The CAT and GPx activities remained unchanged during low-stress period.

Table 1: *The perceived stress scores, cortisol and β-endorphin levels and oxidative stress marker activities (mean ± SEM) during high- and low-stress period (n=33).*

Parameters	High-stress period	Low-stress period	p value
Perceived Stress levels			
DASS Stress scores	12.48 ± 0.77	7.27 ± 1.13	***
Neurohormone levels			
Serum cortisol (nmol/L)	465.45 ± 16.29	427.73 ± 18.64	*
Serum β-endorphin (pg/ml)	148.74 ± 22.03	78.52 ± 38.16	*
Lipid Peroxidation			
MDA activity (µmol/g Hb)	0.31 ± 0.01	0.23 ± 0.00	***
Antioxidant activities			
SOD activity (U/g Hb)	1572.93 ± 26.74	1799.70 ± 24.65	***
CAT activity (U/g Hb)	3728.12 ± 94.23	3790.11 ± 67.62	NS
GPx activity (U/g Hb)	24.24 ± 0.68	25.84 ± 0.56	NS

Values are means ± SEM.

*p<0.05, ** p<0.01, *** p<0.001 and NS: Not significant, p>0.05 versus low-stress period

Correlations between perceived stress scores and blood parameters

The correlations between the perceived stress scores and other parameters at both periods are shown in Figure 1. During the high-stress period, perceived stress scores were found to be correlated directly to MDA ($r^2=0.23$; $p<0.01$) but inversely to β-endorphin ($r^2=0.14$; $p<0.05$). With low-stress, only cortisol demonstrated direct correlation with the perceived stress scores ($r^2=0.09$; $p<0.05$). However, no significant correlations were found for β-endorphin ($r^2=0.00$; $p>0.05$) and MDA ($r^2=0.03$; $p>0.05$) during that period. In addition, no significant correlations were found between perceived stress scores and the anti-oxidative enzymes in the high-stress period (activities of SOD ($r^2=0.01$; $p>0.05$), CAT ($r^2=0.00$; $p>0.05$) and GPx ($r^2=0.00$; $p>0.05$) as well as in the low-stressed period (SOD ($r^2=0.00$; $p>0.05$), CAT ($r^2=0.04$; $p>0.05$) and GPx ($r^2=0.05$; $p>0.05$) activities) (graphs not shown).

Discussion

This investigation confirms and extends previous work that clearly demonstrates the effect of examination stress on stress hormones. Taking academic examination as a brief naturalistic stressor, there is evidence that significantly high levels of perceived stress, serum cortisol and β-endorphin have been evoked. Furthermore, convincing evidence of an involvement of the pro- and anti-oxidation system in the physiological

response to stress as well as a novel finding of a role played by β-endorphin are also put forward. It was previously shown that both the nature of the stressor and the state of the respondent are important in determining the endocrine responses to stress. Therefore, in this study, a DASS was used to assess the perceived stress levels of participants as it has been soundly validated in the literature [16,17] as a reliable tool to sensitively reflect the changes in mood, anxiety and distress in individuals or groups.

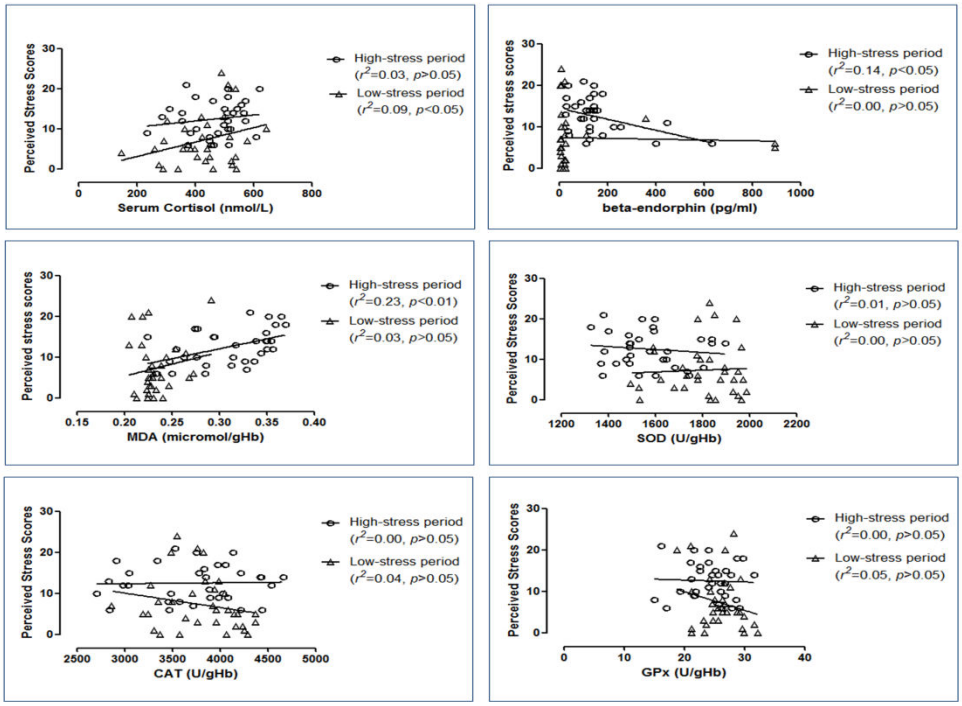

Figure 1: *Correlations between perceived stress scores, neurohormonal and oxidative markers during highand low-stress period.*

Although there are substantial researches done on examination stress and endocrine changes, the results showed inconsistent findings [3,25-30]. Moreover, the studies of cortisol levels by the cortisol awakening response, a new indicator of individual's HPA axis activation, also showed the inconsistent responses of cortisol with the experience of stress itself [31-33]. In this study, significantly high perceived stress levels ($p<0.001$) before the examination indicated that academic examinations are indeed powerful inducers of perceived stress in students. Although serum cortisol levels were significantly increased ($p<0.05$) during the high-stress period, it was not correlated with perceived stress scores suggesting the importance of individual differences in determining the endocrine profile to stress [26]. During the low-stress period, perceived stress scores were found to be positively correlated with cortisol ($p<0.05$). Thus, this clearly demonstrated a heightened HPA involvement during the examination period (under stress) which was quickly and significantly resolved once the examination was over. These data may therefore help to elucidate more definitively the positive response of cortisol to acute stress [3,25,26,30,31,33]. However, there had been few reports of no change and even reductions in cortisol secretion have also occasionally surfaced [27-29,32].

The involvement of the so-called happy hormone, β-endorphin, in acute stress has not been well studied in the literature and may play an important role in the response of the HPA axis and release of cortisol under the duress of stress. In this study, β-endorphin levels were increased ($p<0.05$) during high-stress period when compared to low-stress period, and it was negatively correlated ($r^2=0.14$; $p<0.05$) with perceived stress scores. However, no correlation was found between the two hormones. The particular interplay between cortisol and β-endorphin can be explained by the notion that stress itself has a profound effect on the HPA axis wherein stress per se stimulates the hypothalamic release of CRH, which appears to induce the expression of the proopiomelanocortin (POMC) gene in the anterior pituitary, causing a concurrent secretion of ACTH and β-endorphin from anterior pituitary [1]. It is speculated that the rise in β-endorphin would then attenuate the secretion of cortisol, possibly due partly to the negative feedback of β-endorphin on the HPA itself [34,35], resulting in a lower than expected level of cortisol during the high-stress period. Indeed, such is the power of the benefits of β-endorphin that even the stress scores obtained during the high-stress period appear to be lower, giving rise to the its inverse correlation with perceived stress scores seen in Figure 1. Furthermore, during the high-stress period, due to the lower than expected cortisol levels, a positive correlation with the stress scores could not be discerned. However, during the low-stress period, a significant positive correlation between stress scores and the lingering cortisol levels was obtained (Figure 1). This could be due to the diminishing ameliorating influence of β-endorphin during low-stress period. Thus, it is feasible to

suggest that this increase in β-endorphin could provide a central mechanism for the individual to cope with the stress evoked, and may perhaps also help temper the vulnerabilities of individuals to stress-related psychopathology.

To determine the oxidative stress markers, we used red blood cells as they have high levels of SOD, CAT and GPX activities. Sitting for an academic examination can significantly increase the pro-oxidant MDA while decreasing the anti-oxidant SOD levels. These findings are supported by the positive correlation found between stress and MDA (Figure 1), and are similar to those reported by others [36-38]. As SOD is the first enzyme involved in the metabolism or destruction of superoxide anion radicals, the decreased activity of SOD would cause the formation of high levels of ROS, and subsequently, increase in membrane lipid peroxidation. Although not significant, the other two antioxidant markers, CAT and GPx, showed lower levels during the examination period, and these low levels might contribute the accumulation of H_2O_2 in the body. Thus, examination stress apparently shifts the delicate pro- and anti-oxidation balance to a more pro-oxidative state. While a single episode of exposure to brief naturalistic stressor may not predispose an individual to the damaging effects of elevated ROS, it is possible that repeated activation may subject the susceptible individual to increased allostatic load and risk of chronic ROS-related diseases [10,13,14]. This may also lend support to the anecdotal practice of some individuals consuming antioxidant-rich supplementation during an examination period.

This study has limitations. First, single samples of cortisol level were measured in this study. Although a single morning measurements of cortisol have been reported to be a reliable measure of HPA axis activity, the measures of cortisol secretion with strict reference to the time of awakening [39,40] may have revealed additional results. In addition, as the sample size was small and limited to first year students, these findings may not be generalized to the other year groups of medical students. Further studies are needed to be carried out to address these limitations.

Conclusion

The results of this study confirm and strengthen the hypothesis that brief naturalistic stressors such as an academic examination stimulate both the HPA axis and the endogenous opiate system. Even though cortisol and β-endorphin works independently, the stress-induced release of β-endorphin attenuates the HPA axis and hence cortisol secretion which, in turn, may contribute to lessening of the adverse effects of stress. Sitting for an academic examination shifts the pro- and anti-oxidation balance to a more pro-oxidative state through the impairment of lipid oxidation and enzymatic antioxidant defences. Further studies on the detailed mechanism underlying β-endorphin release and oxidative markers may yield insights into the role of these substances in the allostasis of the stress response.

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