

Salivary Nitric Oxide, a Biomarker for Stress and Anxiety?

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Objective To investigate if salivary nitrate correlates to the daily psychological stress and anxiety in a group of human subjects.

Methods The convenient sample recruitment method was employed; data from seventy three subjects were analyzed. The Perceived Stress Scale (PSS) and Hamilton Anxiety Rating Scale (HAM-A) inventories were used to determine stress and anxiety scores respectively. Salivary nitric oxide was measured through nitrate (NOx) levels using the Griess reaction method.

Results Although stress and anxiety were correlated. No significant correlation exists between salivary nitrate and daily psychological stress and anxiety in the study's participants.

Conclusion While all previous studies focused NOx levels in acute stress models. This is the first study to investigate the correlation between salivary nitrates and daily psychological stress and anxiety. Although stress and anxiety were correlated, there is no correlation between salivary nitrates and daily psychological stress and anxiety. Further studies are required to investigate this correlation using other biological samples such as plasma.

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Key Words Anxiety, Stress, Nitric oxide.

INTRODUCTION

Nitric oxide (NO) is an endogenous gas, produced by different cell types. It plays essential physiological roles, such as in the cardiovascular system and the central nervous system. Nitric oxide is synthesised through the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS).¹ There are three main isoforms, each with a specific distribution profile; neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III).²

Nitric Oxide concentration can be measured through its end metabolite nitrates (NOx). Nitrates can be measured red in central and peripheral tissues.^{3,4} Salivary glands actively take circulating nitrates and release them in saliva.⁵

Stress and anxiety are among the most common psychological disorders that affect the human quality of life. According to epidemiological studies, almost one-third of people will suffer from stress and anxiety disorders.⁶ Studying stress in animal models provided new insights to investigate the underlying mechanisms of anxiety.⁷

It was reported in animal models^{3,8} that stress provokes neurochemical changes in the central nervous system, in particular, the hippocampus and cortex.⁹ The inflammatory status due to NO overproduction in stressed animal models is attributed to iNOS up-regulation.¹⁰ Inflammation due to nitrate accumulation leads to behavioural disorders thus generating a vicious cycle.¹¹ Previous studies focused on the brain nitrate levels in rodent models of stress and anxiety. One study¹² demonstrated that salivary nitrates increase significantly in induced acute stress conditions. To our knowledge, this is the first study to shed light on salivary nitrate in correlation to psychological daily stress and anxiety. Therefore, the aim of this pilot study was to investigate the hypothesis that anxiety and psychological stress in humans correlate with salivary nitrate levels.

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METHODS

Design

A cross-sectional study was conducted to meet the study's aim. The cross-sectional methodology was selected as it was the most appropriate method to explore the correlation between each anxiety and stress with nitric oxide.

Study sample and settings

The study used the convenience sample technique in recruiting the participants. The inclusion criteria were Jordanian, aged 18 years and older, and being willing to participate in the study. The participants were contacted during their visit to the one of the health care centres in Madaba city in April 2015.

Data collection procedure

To ensure homogenous data collection and reduce errors, selected senior pharmacy students from the American University of Madaba were trained for data collection by one of the authors. The study's aim, objectives, data collection procedure, and inclusion criteria were all explained. The students contacted each participant to report to their centres and asked eligible personnel for their willingness to participate in the study, explaining the study's objective and providing them with the study information sheet. The demographical data sheet and the questionnaire were distributed for the participants to be self-completed in Arabic. Ethical approval to conduct the study was obtained from the Latter-day Saint Charities in Jordan. Written informed consent and information sheet were provided, participation was completely voluntary, and participants had the right to withdraw from the study at any time.

Study instruments

The participant demographical sheet, Hamilton Anxiety Rating Scale, and the Perceived Stress Scale were collected from each participant. In addition, saliva was collected and a nitrate assay was performed.

Perceived stress scale

The Arabic version of PSS was used to assess participants' stress levels. The scale consists of 14 items measuring stress in the last month, which was developed by.¹³ Each item has five choices ranged from zero (never) to four (severely frequent), with total scores ranging from zero to 56. The PSS showed an adequate reliability among the different general members of the population with the coefficient alpha reliability ranging from 0.84, to 0.86.¹⁴ The Arabic language translation that was used for this study was available along with other international languages. The Arabic version of PSS showed an adequate reliability and validity with a coefficient alpha of 0.80.¹⁵ Therefore,

the Arabic Perceived Stress Scale is considered a suitable instrument to assess perceived stress in Arabic people. The PSS is not a diagnostic tool, and cut-off point not suggested. The current reliability of the PSS among the current participants shows adequate reliability with a coefficient alpha of 0.81.

HAMA-A

The Arabic version of the Hamilton Anxiety Rating Scale (HAM-A) was used to measure the anxiety levels among participants. It was developed to assess the severity of anxiety symptoms.¹⁶ The scale consists of 14 items measuring both psychic and somatic anxiety. Each item has five choices ranging from zero (not present) to four (severe), with the total scores ranging from zero to 56. The severity of the anxiety scores were categorised into three categories where less than 17 indicates mild severity, between 18 and 24 mild to moderate severity and 25 or higher indicates moderate to severe symptoms.

This scale has shown adequate validity and reliability measures with a coefficient alpha of 0.84 to 0.86. In the current study, the scale shows acceptable reliability with a coefficient alpha of 0.74.

Saliva collection and nitrate assay

Saliva collection and storage were carried out according to standard protocols.¹⁷ In brief, participants were asked to rinse their mouths with distilled water before giving 2 mL of whole saliva. Saliva was collected in a clean cup, transferred to an eppendorf and stored immediately in ice. Afterwards samples were frozen and assayed within 72 hours to prevent nitrate degradation.

The accumulation of nitrate, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with a Greiss reagent.¹⁸ Salivary nitrate was assayed using a Nitric Oxide Assay Kit from Abcam (ab65328) according to the manufacturer's instructions. In brief, saliva samples were allowed to thaw on ice and then centrifuged, then supernatant was used. The nitrate concentration was obtained according to the standard curve generated. All samples and standards were processed in duplicates.

Data analysis

SPSS version 21.0 statistical software was used to analyse the data. Descriptive (including mean and standard) deviations were used to analyse the demographical data. An independent t-test and Mann-Whitney U test were used to examine the differences in anxiety, stress, and NO based on the participants' demographics. An independent t-test and Mann-Whitney U test were used to examine the differences in each NO and stress with anxiety categories.

Spearman's test was used to examine the correlation between each anxiety, stress, and NO concentration. In addition, Pearson's test was used to examine the correlation between anxiety and stress scores. A significant level was set for less than 0.05.

RESULTS

Sample characteristics

Out of the 84 participants asked to participate in the study, 75 participants accepted and signed the informed consent form indicating a response rate of 88.1%. However, 73 were included in the analysis as two participants did not complete most of the data package.

As shown in Table 1 the majority of recruited participants were 30 years old or less ($n=38$, 52.05%), male ($n=41$, 56.16%), married ($n=44$, 60.27%), received secondary school education ($n=48$, 65.75%), do not receive medications for chronic illness ($n=59$, 80.82%) and do not have previous chronic illnesses ($n=57$, 78.08%).

Differences in anxiety, stress and NOx concentration based on participants' demographics

Differences between anxiety, stress and salivary NOx concentration and participants' demographics were aimed to examine the homogeneity of study participants. As each anxiety and stress scores showed to be normally distributed, an Independent t-test was used to assess the differences in each anxiety and stress with each demographics. As shown in Table 1 the independent t-test shows no differences in anxiety and stress based on any of demographical details.

As NOx values were not normally distributed, the Mann-

Whitney U test was used to examine the differences in NOx concentration based on participants' demographics. Mann-Whitney U test showed no significance difference among all demographics.

Mann-Whitney U test was used to examine the differences in NOx concentration based on anxiety categories; less than 17, and 17 or more. The result showed no significant difference. In addition, the independent t-test was used to examine the differences in stress based on anxiety categories. The independent t-test showed significantly higher stress scores ($M=35.33$, $SD=6.91$) among participants with higher anxiety category (more than 17), compared to the lower anxiety category [$M=28.21$, $SD=6.28$; $t(73)=3.54$, $p=0.001$, two tailed].

Correlation of salivary NOx concentration with anxiety and stress

Spearman's rho test was used to examine the correlation of salivary NOx concentration with anxiety and stress scores. No significant correlation was found ($r=-0.117$, $n=73$, $p=0.323$) and stress ($r=-0.084$, $n=73$, $p=0.48$).

Correlation between anxiety and stress scores

Pearson's test was used to examine the correlation between anxiety and stress scores. A positive correlation was demonstrated ($r=0.481$, $n=73$, $p=0.001$).

DISCUSSION

This is the first pilot study to correlate salivary nitrates levels to stress and anxiety in humans. We report a non-significant correlation. Although the vast majority of published data on

Table 1. Demographics and differences based on demographics

Demographics (total participants included in the analysis)	Categories	Number (percentage)	Anxiety score mean (standard deviation)	Stress score mean (standard deviation)	Salivary NOx mean (standard deviation)
Age (73)	30 years or less	38 (52.05)	17.68 (10.17)	29.11 (5.98)	1.56 (3.12)
	More than 30 years	35 (47.95)	18.11 (9.89)	29.8 (7.81)	1.41 (1.15)
Gender (73)	Male	41 (56.16)	20.53 (10.56)	29.90 (7.27)	1.12 (0.97)
	Female	32 (43.84)	14.5 (8.11)	28.84 (6.38)	1.95 (3.39)
Marital status (73)	Single	29 (29.73)	19.00 (10.04)	29.79 (7.87)	1.62 (2.77)
	Married	44 (60.27)	16.20 (9.79)	28.89 (5.18)	1.28 (1.63)
Education level (73)	Secondary school	48 (65.75)	17.77 (9.20)	30.52 (8.35)	1.56 (2.66)
	University degree	25 (34.25)	18.12 (11.15)	23.00 (7.81)	1.34 (1.75)
Chronic illness (73)	Yes	16 (21.92)	16.06 (8.74)	28.93 (7.61)	1.31 (0.79)
	No	57 (78.08)	17.84 (10.36)	29.57 (6.72)	1.52 (2.66)
Receiving medication(s) for chronic illness (73)	Yes	14 (19.98)	15.78 (8.32)	27.28 (5.82)	1.23 (0.89)
	No	59 (80.02)	18.38 (10.32)	29.94 (7.05)	1.55 (2.61)

animals confirmed this correlation, however our results are contradictory. This controversy may be attributed to many factors. Firstly, the difference in the model employed i.e., this is a human-based study, while previous studies were conducted on stressed animals. Secondly, the type of stress and anxiety measured herein is chronic daily life stress and anxiety compared to an acute induced stress in previous works. Thirdly, nitrate levels were measured in saliva not in hippocampal or cortical tissue. Fourthly, the sample size used in this study is considered relatively low.

Psychological and physical stress contributes to anxiety.¹⁹ Stress triggers the Hypothalamus-Pituitary-Adrenal (HPA) axis to release stress hormones such as glucocorticoids. This mechanism seems to be mediated by NOS. Previous reports studied the brain expression of NOS in stressed animal models; all reports indicated an increase in nitrates levels due to NOS over expression. Pre-treating mice with L-NAME, a widely used NOS inhibitor attenuated the anxious behaviour in animals.²⁰ Moreover; NOS knockout mice demonstrated anxiolytic behaviour, further confirming the previous results.²⁰

A possible explanation for the different results is that the stress type, duration and intensity in animals may not be representative to humans. The PSS and HAMA-A inventories were aimed at asking participants their daily stressful conditions during the last month. However, referred animal models were stressed by immobilisation for hours. Similarly, the only study on human salivary nitrate used an acute stress method.¹² Indeed, healthy volunteers were asked to jump from a platform of 70 metres, and the salivary nitrates increased one hour later after the jump. It can be suggested that nitrates may not be elevated during chronic every day stress and anxiety conditions.

Another explanation for the results may be the salivary samples used. Human studies in this field are scarce; one similar study pointed out the increase in plasma nitrates in depressed patients.⁴ The use of saliva samples in stressed patients has substantial evidence.²² Moreover, saliva is rich in nitrates.²² The fact that saliva is rich in potential proteins and biomarkers, its accessibility, and ease of collection makes it an attractive subject to investigate. For example, salivary cortisol and testosterone were studied in correlation with mood and behavioural changes.²⁴ It is suggested that the expression of salivary NOS and nitrates has to be studied in the light of the hippocampal and cortical levels in stressed animal models. Therefore, this may provide new insights for studying anxiety and stress objectively via peripheral and accessible biological samples that may be the “window” for the brain tissues.

Although this is the first study that employs human salivary nitrates, however it remains a pilot study. The study did not measure basal salivary nitrates in control population. Future investigation with a larger sample size can provide more con-

firmatory results and drive more substantial conclusions.

In conclusion, the authors report no significant correlation between stress, anxiety and salivary nitrates in this pilot investigation. The results may be attributed to a difference in methods, models and sample size. Further studies have to be performed to explore and compare salivary with hippocampal and cortical NOS and nitrate expression in models of stress and anxiety.

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