Original Research Article

ESTIMATION AND COMPARISON OF LEVELS OF SALIVARY CORTISOL AND NITRIC OXIDE IN PATIENTS WITH RECURRENT APHTHOUS STOMATITIS IN ACTIVE PHASE AND REMISSION PHASE

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Abstract

Problem Considered- Recurrent Aphthous Stomatitis (RAS) is one of the most common and poorly understood mucosal disorders. Psychological anxiety is considered one of the causative factors which alters cortisol levels in the body. Nitric Oxide (NO) is a cytotoxic molecule and is produced from neural tissue, blood vessels, macrophages and T-lymphocytes. The aim of the present study was to estimate and compare the levels of salivary cortisol and NO in patients with RAS in active phase and remission phase and in normal healthy individuals. Also to correlate the salivary cortisol and NO levels with anxiety levels of the patients.
Methods- the present prospective study comprised of 25 RAS patients and 25 sex and age matched normal healthy individuals. Saliva samples were collected from RAS patients once in active ulcer phase and once in remission phase. A questionnaire called 'State-Trait anxiety test' was filled by the patients. One part of collected saliva was analysed for NO by Griess reagent and spectrophotometer and the other part for cortisol by ELISA method.
Results- the study showed that both salivary cortisol and NO were significantly more in RAS patients when compared to the control group. Within the study group there was a significant difference in both the salivary cortisol levels and NO levels between the stage of active ulcer and stage of remission. The anxiety levels were more in RAS patients than the control group but this was not statistically significant. Poor correlation was seen among the levels of anxiety, salivary cortisol and salivary NO.
Conclusion- salivary cortisol and NO can be used as a diagnostic feature and possible treatment of RAS.

Keywords- Recurrent Aphthous Ulcer, Salivary Cortisol, Salivary Nitric Oxide, State Anxiety, Trait Anxiety.

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1. Introduction

Recurrent Aphthous Stomatitis (RAS) is the most common type of ulcerative disease of the oral mucosa affecting 5-25 % of the general population.[1,2] The lesions of RAS are self-limiting, persisting for 1-2 weeks, resolving with or without scarring, and recurring after periods of remission.[3] Many factors have been implicated in the promotion and/or exacerbation of RAS; these include positive family history, local trauma, nutrition deficiency, food hypersensitivity, immune disturbance, smoking cessation and most commonly psychological stress/anxiety.[4,5]

Numerous studies have shown association of anxiety and RAS, nevertheless, many patients make an association between RAS and what they term anxiety.[6] Anxiety can be of two types: (i) state anxiety is a psychological entity which manifests as an exaggerated response to a given situation, whereas, (ii) trait anxiety is present all the time as a behavioural characteristic.[7]

Cortisol is a steroid hormone secreted by adrenal gland. Almost any kind of stress/anxiety, whether physical and neurogenic, causes an immediate and marked increased in ACTH secretion by the anterior pituitary gland, followed within minutes by greatly increased adrenocortical secretion of cortisol.[8]

Nitric oxide (NO) is a highly reactive free radical present in freshly secreted in human saliva and plays an important role in modifying processes of oral mucous membrane in health and disease.[9] The current concept states that all the factors implicated in the aetiology of RAS essentially disturb the oxidant-antioxidant balance of the organism by increasing the oxidative stress which triggers free radical formation including NO.[10,11]

Thus the aim of the study was to measure the salivary cortisol and NO levels and to correlate it with anxiety in RAS patients.

2. Materials and Methods

A total of 25 patients of RAS were enrolled in the study. 25 age and gender matched patients with no history of RAS were kept in control group. Unstimulated saliva samples were collected in the morning between 9am to 10 am to avoid diurnal variation. In study group saliva was collected twice from each patient, once in phase of active lesion and once in remission phase, 2 weeks after healing of ulcer.

Each saliva sample was divided into two parts. One part was analysed for NO by Griess reagent and spectrophotometer.[12] The second part was analysed for cortisol by Enzyme-linked Immunosorbent Assay (ELISA) method using Salivary Cortisol Kit, DIAMETRA, Italy.

A questionnaire named 'State-Trait Anxiety Test' from National Psychological Corporation was used to assess the anxiety in all patients at the time of collection of saliva samples. It consisted of a total of 40 questions out of which, 20 questions assessed the level of state anxiety and 20 questions assessed trait anxiety of all subjects. The score was calculated and accordingly patients were classified under the grading system of the questionnaire.[7]

The results were analysed and tabulated. Frequency and percentage was calculated. Statistical analysis was done by using Chi square test and ANOVA test. Correlation of cortisol and NO with anxiety was done by using Pearson's correlation test.

3. Results

This study included 50 patients out of which 25 were RAS patients and 25 were normal healthy individuals as control group.

There was no significant difference in age distribution and gender of the subjects between the study group and control group. Hence, the age and gender of both the groups was matched. Majority of the patients of RAS said psychological stress/anxiety was the probable cause of recurrent ulcerations in their mouth. (Table 1)

The mean salivary cortisol level in study group was more than the control group. Salivary cortisol levels in active stage were more than that in the remission stage which was more than the control group. There was statistically significant ($p<0.001$) difference among the 3 groups (Table 2).

The mean salivary nitric oxide level in study group was more than the control group. Salivary NO levels in active stage were more than in the remission stage which was more than the control group. There was statistically significant ($p<0.001$) difference among the 3 groups for salivary NO levels (Table 2).

The anxiety level in study group was more than the control group but the difference was not statistically significant ($p=0.130$). Individually state anxiety and trait anxiety in study group were more than the control group but that difference also was not statistically significant ($p=0.169$ and $p=0.242$ respectively) (Table 3).

There was a weak correlation (0.226) between cortisol levels in active stage and state anxiety levels ($p=0.278$) in the study group (Graph 1).

There was a weak correlation (-0.122) between cortisol level in remission stage and trait anxiety levels ($p=0.278$) in study group (Graph 2).

There was a weak correlation (0.227) between NO level in active stage and state anxiety levels (0.274) in study group (Graph 3).

There was a weak correlation (0.078) between NO levels in remission stage and trait anxiety (0.964) in study group (Graph 4).

There was a weak correlation (0.10) between cortisol level and NO levels (p=0.964) in active stage in study group (Graph 5).
There was a weak correlation (-0.063) between NO levels and cortisol levels (p=0.767) in remission stage in study group (Graph 6).

4. Discussion

In the present study, the study group included patients with ages between 19-56 years. Mean age of the study group was 26 years which was similar to the findings that show that the peak age of RAS is in second and third decade.[13,14] Also, there were a greater number of females (64%) as compared to males, which is in accordance to several studies in literature which have described a female predominance.[14-16]
In the detailed case history which was taken for every patient, a question was asked regarding the probable cause which the patient presumed could be activating ulcers in their oral cavity. Out of 25 patients 68% attributed the ulcers in their mouth to what they called was stress/anxiety. 24% patients said ulcers were due to reasons other than stress/anxiety namely stomach upset, constipation, diet, chocolate and dehydration, whereas, 8% patients could not link RAS with any particular factor. Numerous studies have been documented linking RAS to psychological stress/anxiety.[15-18]
Salivary cortisol measurement is a reliable indicator of free cortisol or biologically active cortisol, whereas serum cortisol assess total cortisol (bound and free).[7] It has been shown that salivary cortisol is not influenced by plaque and gingival status of the patient,[19] making saliva an ideal medium for estimating cortisol levels. An insight into patient's psychological status can be estimated from both serum and salivary cortisol levels.[20] Morning collection of saliva is necessary to avoid diurnal changes on cortisol levels.
In the present study, patients with RAS had significantly higher cortisol levels in the active stage (11.47 ng/ml) when an ulcer was present in the patient's mouth than in the remission stage (9.27 ng/ml). [Table 2] The cortisol levels in the control group (5.81ng/ml) were much lower and this difference also was statistically significant.
Cortisol is a stress hormone. Psychological stress like anxiety, depression affects the HPA axis and cause increase in cortisol levels. Hence, increased levels of persistent stress/anxiety have been associated with changes in the HPA axis functioning and increased cortisol levels.[8,21] The present study revealed a positive correlation between RAS and salivary cortisol levels. Salivary cortisol was not just increased in active phase of RAS but also in the remission stage showing a generalised increase of cortisol in RAS patients all the time.
Among various factors thought to be involved in the aetiology of RAS, recent research has indicated that they all contribute to the pathogenesis of RAS by a direct or indirect impact on the oxidant-antioxidant balance of the body.[22-24] NO is a highly reactive free radical and in the present study was measured by Greiss reagent in patient's saliva in active stage and remission stage. (Table 2) Statistically significant difference was seen in the study group and the control group (16.03μM) and also, with in the study group, significantly higher levels of salivary NO was observed in active stage (39.56μM) than in the stage of remission (30.76 μM). These results were similar to results in a study by Ohashi et al.[12]
Both T lymphocytes and macrophages implicated in the aetiology of RAS serve to point a possible role of NO in the mediation of disease process and cell injury in RAS. Inducible NO synthase enzyme is capable of producing NO over a longer period of time by various immunological mediators and a wide variety of cells including macrophages, T cells and natural killer cells.[25,26]
NO is synthesized from L-arginine by a family of enzymes called nitric oxide synthase (NOS).[27]
There are 3 forms:

a. Type I NOS- brain enzyme (bNOS)
b. Type II NOS- inducible enzyme (iNOS)
c. Type III NOS- endothelial cell enzyme (eNOS)

Free radicals, including NO, play an important role in ulceration induced by several kinds of stress/anxiety.[28] NO can also modulate stress induced activation of HPA axis and the sympatho-adrenal system. It has been reported that psychological stress causes NO release in correlation with increase in neural NOS activity.[28]
3 isoforms of NOS have been shown and the inducible NOS is known to be synthesised by macrophages, mast cells and T lymphocytes.[29] The inducible NOS once induced continues to produce NO for a much longer period of time than either neural or endothelial NOS.[30] NO when in excess is known to produce cell damage, tissue injury and cause severe damage to fibroblasts and keratinocytes.[28] Thus, it is proposed that free radicals including NO may play a role in RAS.
In the present study significantly increased levels of NO were seen in active stage of RAU patients. When compared to controls significantly higher levels of NO were also noted in remission stage though they were less than that in active stage. This gradual decrease in NO levels in RAS patients may be because inducible NOS enzyme is capable of producing NO over a longer period of time.[30]

Thus, in the present study both salivary cortisol and NO were significantly increased in RAS patients when compared to non-RAS patients. Moreover, within the study group significantly higher levels of salivary cortisol and NO were seen in active stage when compared to remission stage.

Anxiety is a psychological entity which is manifested as an exaggerated response to a given situation (state anxiety) or present all the time as a behavioural characteristic (trait anxiety).[19] As elevated level of anxiety has been commonly associated with exacerbation in RAS, a questionnaire named 'State-Trait Anxiety Test' was filled by the patients at the time of active aphthous ulcer period. The questionnaire included 20 questions which analysed state anxiety and 20 questions which analysed trait anxiety.

It was seen that state (5.24) and trait (4.88) anxiety levels were more in study group than the state (4.44) and trait (4.28) anxiety levels in control group (Table 4). However, the difference was not statistically significant which may be because questionnaires are not an objective method of analysing anxiety. The most important factor that influences stress is the individual's perception of the challenge posed by the specific stimulus. The extent to which recurrent ulcers are themselves stressful to patients who suffer from life-long RAS must also be considered. More over expressing that perception to an examiner may be biased.

Since anxiety levels in patients influence both cortisol and NO levels, Pearson's correlation test was also done for the 3 variables to see if any correlation was present.

In the present study, in the study group the salivary cortisol levels measured in the active stage in RAS patients were considered to be influenced by the anxiety levels present at the moment, and so they were correlated with state anxiety levels of the patient (Graph 1). Similarly, the cortisol levels measured in the remission stage were considered to be influenced by the anxiety which is present all the time as a behavioural characteristic thus they were correlated with trait anxiety levels of the patients (Graph 2). A weak correlation was seen between cortisol and anxiety in both active stage and remission stage. Thus, the patient's psychological response to anxiety was not correlating with the psychological response as expressed by the patient through questionnaire. This result was in correlation with results observed by Farmaki et al.[7]

Anxiety is one of the factors known to be associated with increase in NO release. Thus, in the present study NO levels were also correlated with anxiety levels. Here also, the NO levels measured in the active stage in RAS patients were considered to be influenced by anxiety levels present at the moment, and so they were correlated with state anxiety levels of the patient (Graph 3). Similarly, the NO levels measured in remission stage were considered to be influenced by the anxiety which is present all the time as a behavioural characteristic thus they were correlated with trait anxiety levels of the patient (Graph 4). A weak correlation was seen between NO and anxiety in both active stage and remission stage. NO levels were also correlated with the salivary cortisol level. The NO levels measured in the active stage in RAS patients were correlated with cortisol levels in active stage of the patient (Graph 5). Similarly, the NO levels measured in remission stage were correlated with cortisol levels in remission stage of the patients (Graph 6). A weak correlation was seen between NO and cortisol in both active stage and remission stage. Cortisol is a hormone secreted by the adrenal glands, whereas NO is cytotoxic chemical released by various cells like inflammatory cells. As they have different physiologic pathways in the body, their role in the pathogenesis of RAS may be different which requires further studies to be established.

5. Conclusion

In the present study, RAS patients showed an increased level of salivary cortisol and NO not only in the stage of active ulceration but also during the stage of remission when compared with the normal healthy individuals suggesting their role in the pathogenesis of RAS. The state and trait anxiety levels measured by the questionnaire were increased in RAS patients than in control group but this difference was not statistically significant. Salivary cortisol and NO levels were correlated with anxiety levels and it was seen that both cortisol and NO in active stage and remission stage were poorly correlated with state and trait anxiety respectively.

6. References

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	Age (in years)	Gender (in %)		Probable reason for RAS (in %)		
		Male	Female	Stress/anxiety	Other than stress/anxiety	Did not know
Study group	26.32 ± 8.562	36%	64%	68%	24%	8%
Control group	27.52 ± 5.832	36%	64%	-	-	-

Table 1: table showing the mean age and gender distribution in study group and control group, and also the probable reason for RAS as told by the patients themselves.

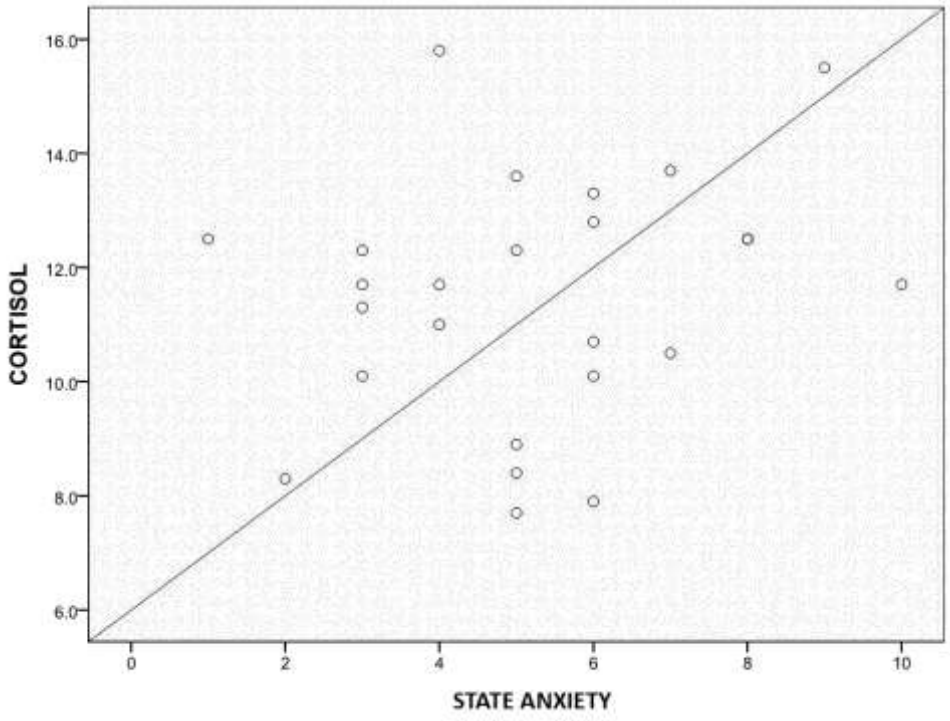
	Study group		Control group
	active stage	remission stage	
Cortisol (ng/ml)	11.47 ± 2.16	9.27 ± 2.93	5.81 ± 3.12
Nitric oxide (μM)	39.56 ± 7.59	30.76 ± 5.80	16.03 ± 5.64

Table 2: table showing mean values of salivary cortisol and NO levels in active stage and remission stage of RAS in study group and in the control group.

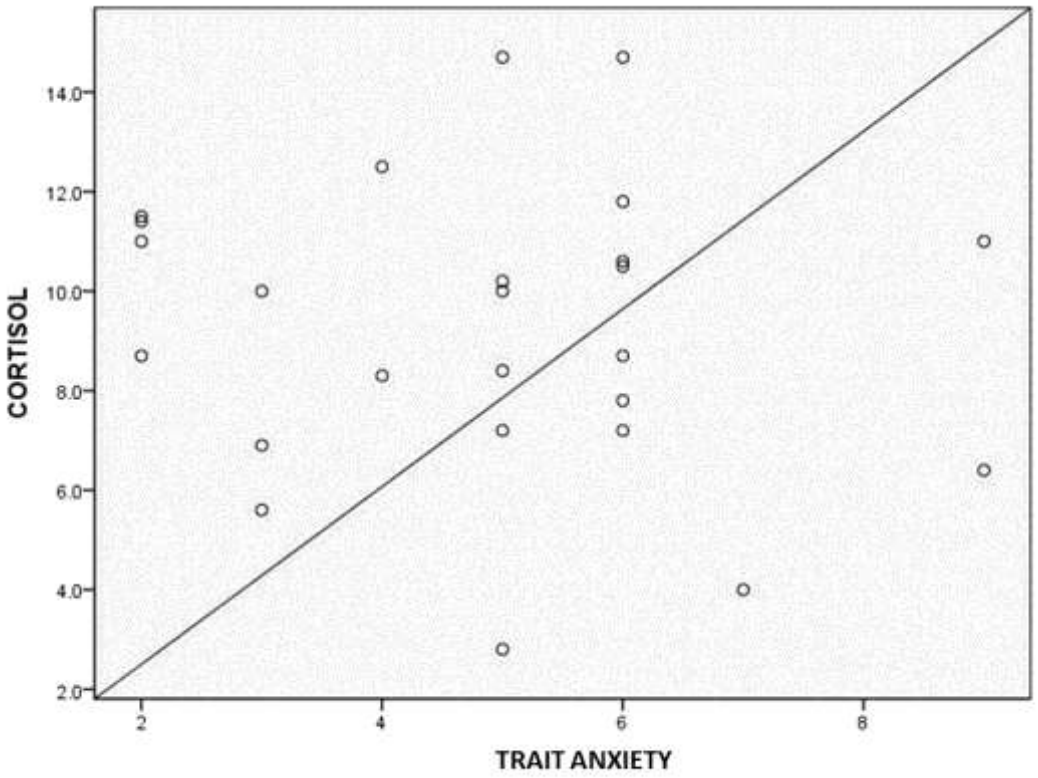
	Study group	Control group
Anxiety	5.20 ± 1.803	4.44 ± 1.685
State anxiety	5.24 ± 2.185	4.44 ± 1.850
Trait anxiety	4.88 ± 1.965	4.44 ± 1.595

Table 3: table showing mean level of state and trait anxiety in study and control group

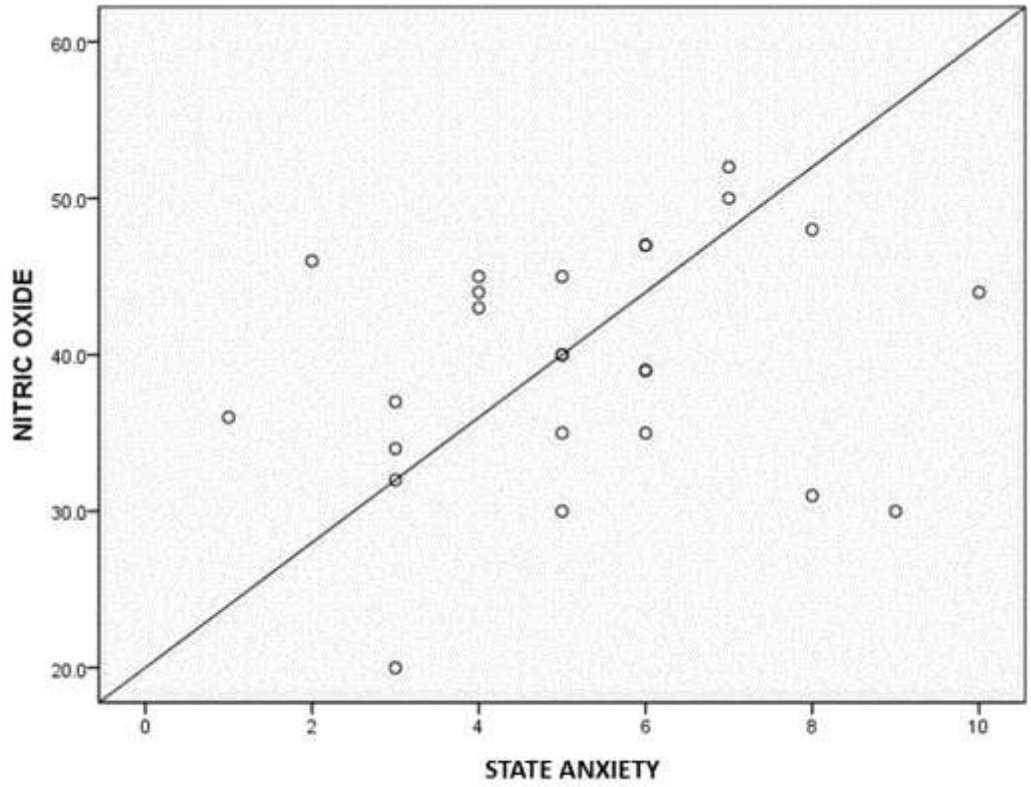
Graph Legends



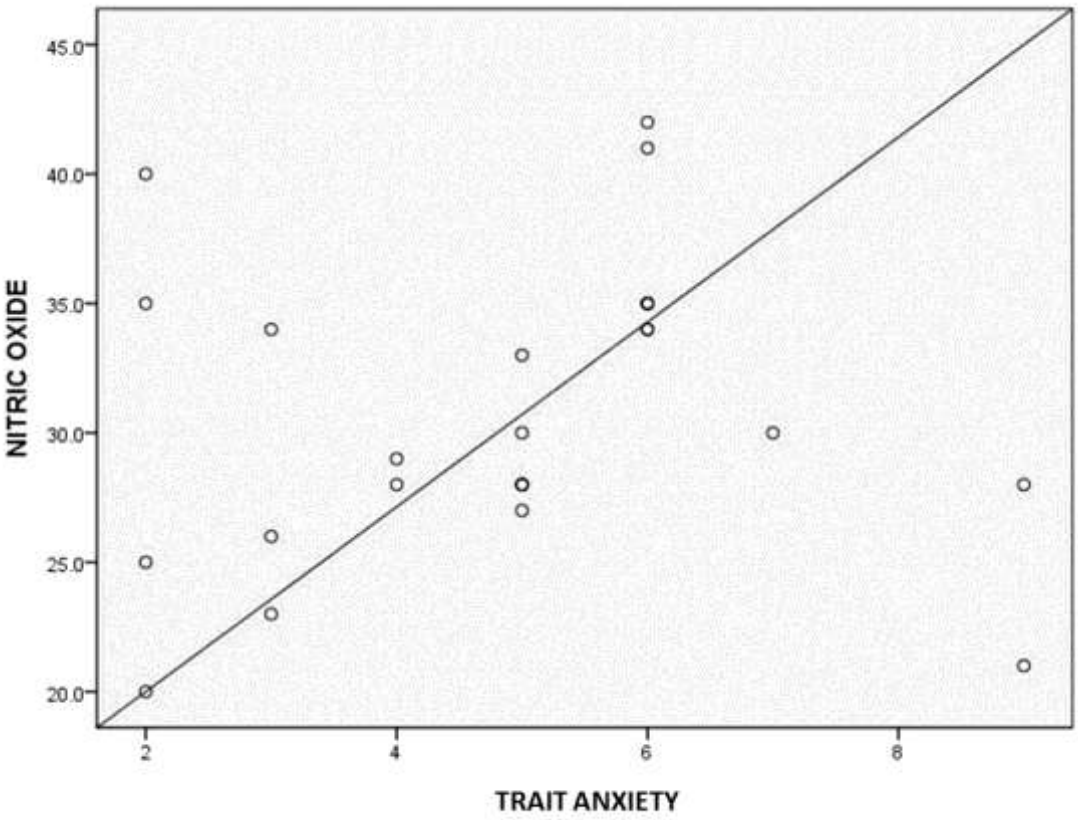
Graph 1: Correlation between cortisol levels in active stage and state anxiety levels in study group.



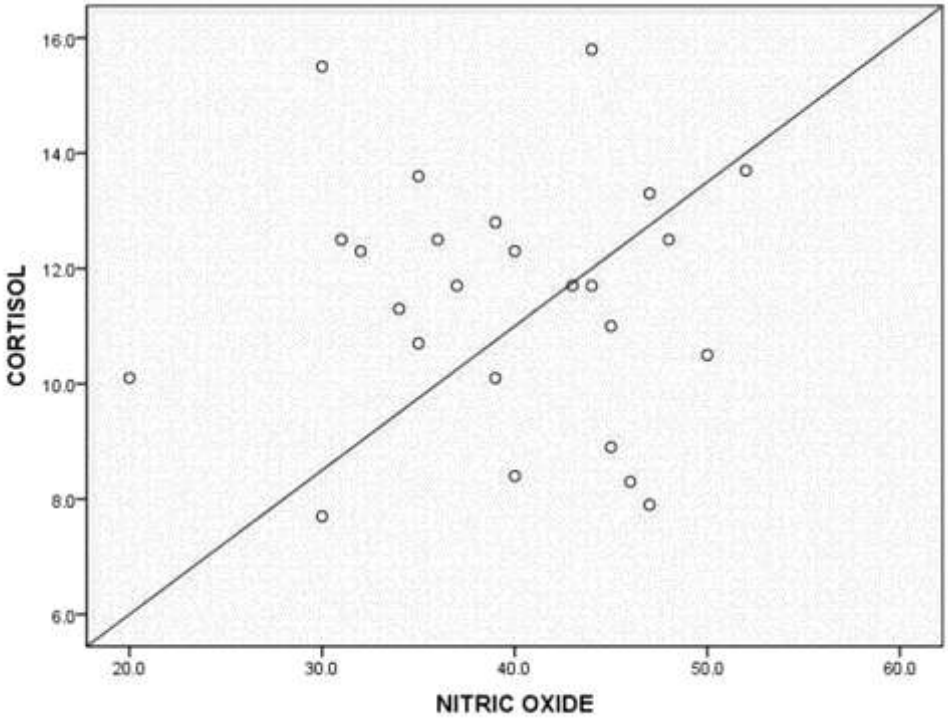
Graph 2: Correlation between cortisol levels in remission stage and trait anxiety levels in study group.



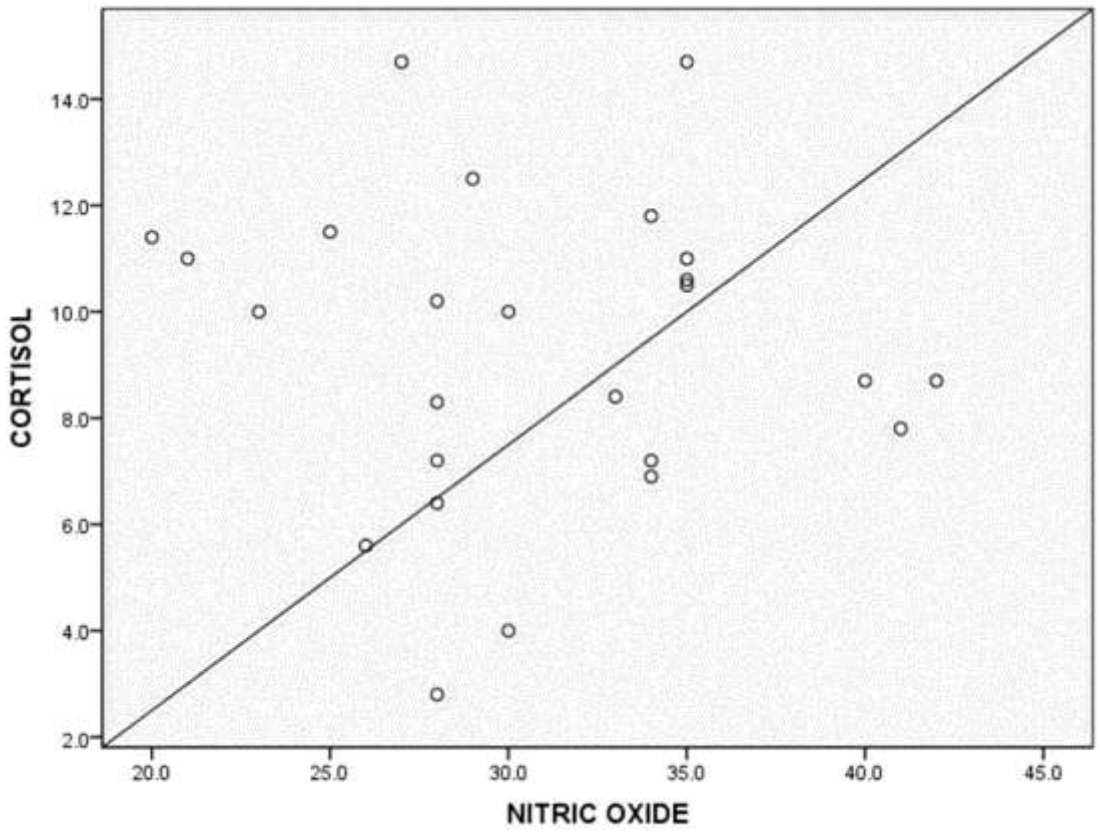
Graph 3: Correlation between nitric oxide levels in active stage and state anxiety levels in study group



Graph 4: Correlation between nitric oxide levels in remission stage and trait anxiety in study group.



Graph 5: Correlation between cortisol levels and nitric oxide levels in active stage in study group.



Graph 6: Correlation between nitric oxide levels and cortisol levels in remission stage in study group