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EVALUATION OF BIOCHEMICAL MARKERS OF OXIDATIVE AND NITROSATIVE STRESS PATHWAYS IN MAJOR DEPRESSIVE DISORDERS (MDD)

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Abstract

In major depressive disorders (MDD) the oxidative stress and alterations to nitric acid pathways play an important role. The two pathways interact quite closely but are never studied simultaneously in major depressive disorders.The aim of the study is to assess and compare the levels of oxidative and nitrosative stress in the neutrophils (PMNs) of drug naïve MDD patients and their first degree relatives. To assess the levels of reactive oxygen species (ROS), nitrites, neuronal NO synthase (nNOS),myeloperoxidase in PMNs and cortisols in serum of drug naïve patients. To study the correlation of the levels these markers in the MDD patients and their first degree relatives. A case control study was carried out where in 30 drug naive major depressive disorder patients and 25 healthy first degree relatives and healthy controls aged 18-45 years were included in the study. The levels of reactive oxygen species (ROS), nitrites, neuronal NO synthase (nNOS), and myeloperoxidase in PMNs, and cortisol in serum was assessed. As compared to healthy controls the generation of free radicals, myeloperoxidase activity and nNOS mRNA expression in PMNs, and cortisol level in serum were significantly higher in the drug naive patients. Whereas the increased levels were seen in first degree relatives. The total nitrite content in the PMNs and the plasma have significantly lower value in both patients and first degree relatives. A positive correlation was seen in ROS levels in the PMNs, plasma and neutrophil nitrite and the serum cortisol level between major depressive disorder patients and their first degree relatives.The results of this study gives a better understanding of the familial association of the major depressive disorder, and demonstrates that neutrophil ROS/RNS, plasma nitrite and serum cortisol levels are positively correlated between the major depressive disorder patients and their first degree relatives. But the studies more diverse samples are required to extend these pathways as the potential biomarkers for the identification of persons at high risk psychopathology at early stages.

Keywords: Major depressive disorders, Depression, Oxidative stress, Nitrosative stress

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INTRODUCTION

Depression is common mental disorder that has affected people all over the world. According to World Health Organization, it currently affects closed to 264 million people all across the globe affecting almost all the age groups[1] and has been projected to become the second leading cause of worldwide disease and disability by 2030[2]. There are strong evidence that severe depressive disorders can lead to suicide[1]. The etio-pathogenesis of depression states that it's a multifactorial and it also involves closed interplay of biological and psychosocial factors[3].Its many years, the theories and research in relation to biological factors leading to depression were mainly focused around the monoamine neurotransmitters i.e. norepinephrine, dopamine, serotonin, and histamine. Lately there have been researches on other biological factors for depression and important information has been generated regarding the role of other neurotransmitters, second messengers, hormonal regulation, immunological disturbances and inflammation [3]. Amongst the biological factors responsible for the depression, oxidative stress is a key factor responsible for depression.[4,5,6,7]

The human brain is more vulnerable to oxidative stress as compared to other organs and tissue in the body[8]. It can be because of high consumption of oxygen in the brain, which is around 20% of the total amount of oxygen in the body. This vulnerability is further increased by high amounts polyunsaturated fatty acids, iron and low concentrations of antioxidant enzymes[8]. Several studies have shown the positive correlation between the severity of depression and the oxidative stress.[4,9,10,11,12]Depression is related to increased levels of reactive oxygen species (ROS) levels and also depleting levels of antioxidants like vitamin E, zinc, coenzyme Q10 and glutathione and exhaustion of anti-oxidative enzyme activities such as xanthine oxidase and superoxide dismutase.[4,5,9,10]In post-mortem studies also it has shown that brain has increased level of ROS including peroxide and superoxide and altered levels of antioxidant defensesin cases with depression[13,14]. The oxidative vulnerability in cases with depression leads to damage of DNA and RNA and telomere shortening. Thus antioxidant treatment has been shown to improve psychiatric symptoms in clinical trials[10].

People suffering from unipolar i.e. major depressive disorder or bipolar depression show deregulated redox signaling. Nitric oxide (NO), a redox active small molecule, is an important signaling molecule in many neural processes and it exerts both pro-and antioxidant responses. At lower concentrations, it helps in generation of free radicals, while at higher concentration of NO protects oxygen free radicals.[15,16] NO also helps in release of neurotransmitters including dopamine, neural development, regeneration, the regulation of gene expression and synaptic plasticity which is believed to be related to learning and memory[17]. The levels of nitric oxide in the body is carried out by assessing the levels of its metabolites, nitrites and nitrates. There are studies conducted that demonstrate the role of nitrates and its metabolites in depressive disorder.[18,19,20,21,22]

Nitric oxide s synthesized L-arginine in the presence of nitric oxide synthase (NOS) enzymevia the L-arginine pathway-NO pathway[23]. Three isoforms of the enzyme NOS have been known i.e. endothelial NOS or eNOS, neuronal NOS or nNOS and inducible NOS (iNOS). Amongst these, the nNOS is found primarily in the nervous tissue and is linked with neuro-psychiactric disorders including depression[7,24,25,26]. Previous studies have also shown the probable role of nNOS in the anti-depressant action of selective serotonin reuptake inhibitors[27,28,29]. Although nNOS is majorly

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expressed by the neuronal cells and its localization in the human brain, the decreased amount of para-ventricular nNOS positive neurons in depressed cases makes its assessment difficult in most of the studies. Many studies also state that, just like neurons, human neutrophils i.e. PMNs also express neuronal nitric oxide synthase[30]. The penetration of neutrophils in the CNS results in development of numerous neurodegenerative disorders including major depressive disorders[31]. Several clinical studies have shown that an alteration in the nNOS - NO pathway in the human brain is also reflected in circulating neutrophils[32,33]. Thus nNOS expression in circulating PMNs of depression patients is being studied as a proxy marker in our current study. The activity of nNOS is dependent on many factors like age, gender, diet, diurnal variation, glucocorticoids, antidepressant drugs, nicotine and other substances of abuse, high blood glucose, high cholesterol, deranged liver functions, and major physical illnesses especially autoimmune disorders.[4,9,34,35,36,37,38,39] Any study that's assesses nNOS should note these factors especially glucocorticoids. The level of serum cortisol is elevated in patients under depression and it is resolved with the treatment. The patients under depression have also shown elevated levels of inflammatory markers.[3,40,41]Myeloperoxidase i.e. MPO is a marker that has shown positive correlation with the severity of depression. In-fact lower levels of myeloperoxidase are associated with reduced cardiovascular diseases, cytokine production, and inflammation that usually exist with depression. To confirm the genetic association of these parameters the assessment of non-symptomatic blood relatives of the patients can bring valuable insights. With these criteria in mind the current study was done to evaluate oxidative and nitrosative stress markers in patients with depression. The primary aim of this study being to assess and compare the levels of oxidative and nitrosative stress in drug naive patients with MDD. The study also assessed the association between the oxidative or nitrosative stress and the clinical profile of the patients under depression and its relation with the first degree relatives.

METHODOLOGY

Convenience sampling was the method used for the selection of the cases. Total number of 30 cases who were diagnosed with major depressive disorder as per the Diagnostic and Statistical Manual of Mental disorders, 4th edition, text revision (DSM-IV-TR)[42] were included in the study who were aged between 18-45 years and had not received psychotropic medications or electroconvulsive therapy during the last three months. Those who were suffering from any other comorbid psychiatric disorder were excluded from the study. 25 first degree relatives were randomly selected if he/she were aged between 18 to 45 years and scored less than three on the General Health Questionnaire, 12 item version (GHQ-12) which had positive and negative descriptions of mood states. The first degree relatives were not included in the study if they had any psychiatric disorder. 25 healthy controls were included if they were aged between 18 and 45 years and they scored less than 3 on GHQ-12[43]. Healthy controls were also excluded from the study if they suffered from any psychiatric disorder. The cases included in the study were included in the study after taking written voluntary consent. The cases and subjects who were found suffering from or were taking treatment for any major medical illness, had deranged blood cholesterol or sugar levels or liver function tests, used substances like nicotine or were unable to give informed consent were not included in the study.

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Procedure

A qualified psychiatrist assessed the patients, their relatives and the healthy controls prior to their inclusion in the study. Hence the diagnosis and suitability for inclusion in the study were confirmed by the psychiatrist. Their clinical details were noted and the severity of the depression was assessed on the Global Assessment of Functioning (GAF) scale [44]. The blood samples were collected from all the participants in the study who were kept in overnight fasting between 8 am and 10 am. The samples were assessed for blood sugar, liver function tests and cholesterol levels.

Separation of Neutrophils and Plasma

Neutrophils were isolated from the venous blood samples which were collected in the sodium citrate which had specifications of 0.129M, pH 6.5, 9:1 –v/v). Platelet rich plasma was removed by centrifugation at 250Xg for 2 mins at 20 degrees Celsius and the buffy coat was subjected to Precoll density gradient at 700g for 30 min at 25 degrees Celsius. The neutrophils from healthy volunteers, patients and their first degree relatives were also isolated using the same method[45] and the purity was confirmed by using a Flow cytometer using a CD15 antibody and it was always more than 95%. The viability of isolated neutrophils was more than 95% as confirmed by Trypan blue exclusion test [45].

Assessment of ROS generation

The levels of intercellular ROS were measured using the redox sensitive probes dichlorohydroflourescein diacetate (DCF-DA). The neutrophils (1X105) were incubated with DCF-DA (10 micrometers) for 15 minutes at 37 degree Celsius and were assessed for ROS generation by acquiring 10,000 events on FAC Calibur and followed by analysing on Cell Quest program.

Estimation of Neutrophil and Plasma Nitrites

NO production in the plasma or Neutrophils was determined by calculating the nitrite content which is the metabolic end product of formation of nitric oxide using the Griess reagent. To estimate the total nitrite content in plasma the cadmium pellets were added to convert the nitrate to nitrite as mentioned earlier[46] and the equal volume of Griess reagent which is 0.1% aqueous – N- (l-naphthyl)-ethylenediamine dihyrochloride, 1% sulphanilamide, 2.5% phosphoric acid was added to the respective samples and incubated for 30 mins at 37 degrees Celsius. The nitric concentration was calculated by measuring the absorbance at 545 nm and 630 nm against sodium nitrite as a standard using an ELISA palate reader and it was represented as microMoles per ml [47].

To calculate the estimated value of the total nitrite in the neutrophils, the cells were sonicated by placing on ice after adding hypotonic TKM solution i.e. 25 mM Tris HCl, ph-7.4, 5mM KCl, 1 mM MgCl2, and 1% NP-40. The protein was removed from the cell lysates by adding 96% cold ethanol (1/2 v/v) for 30 minutes at 4 degrees Celsius. To the supernatant the cadmium pellets were added to convert nitate into nitrite and were similarly treated with an equal volume of Griess reagent. The total nitrite content in the neutrophils was noted as µM/107 cells[47]

Estimation of Neutrophil nNOS Expression

Total RNA from human neutrophils were isolated using the Tri reagent and cDNA was synthesized by RevertAid ^{TM} H Minus first strand cDNA synthesis kit using an oligo (dT) primer. Quantification of nROS mRNA was performed by a real time PCR using the Light Cycler instrument with a 2X

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Maxima SYBR green RT-PCR Master mix and nNOS (forward 50 - TCTAACAGGCTGGCAATGAAG-30, reverse 50 - TCTCTAAGGAAGTGATGGTTGAC-30) and β-Actin (forward 5'-AACTGGAACGGTGAA GGTG-3', reverse 5'-CTGTGTGGACTTGGGAGAGG-30) primers. A three step cycling protocol was used which protocol was used where in initial denaturation at 95 degrees Celsius for 10 minutes, followed by 45 cycles of 15 seconds denaturation 19 95 degrees Celsius , 20 seconds annealing at 57 degrees Celsius and 20 seconds extension at 72 degrees celsius was used to amplify the genes.The specificity of the PCR products was determined by a melting curve analysis consisting of 1 cycle: 95 ◦C for 0 s, 65 ◦C for 10 s, 95 ◦C for 0 s, and 1 cooling cycle: 40 ◦C for 3 min. The relative fold differences between the groups were calculated by using the comparative cycle threshold (2−ΔΔCp) method. β-Actin was used as an internal standard for the calculation of relative mRNA expression[45,47].

Estimation of Neutrophil MPO Activiity

Neutrophils ($5X10^6$ cells/ml) were freeze – thawed consecutively three times and then sonicated in three times and then sonicated in three cycles of 10 seconds each at 95 watts in ice.Hexadexyl trimethyl ammonium bromide was added to the cell lysate , incubated at 37 degrees Celsius for 30 mins, and centrifuged at 3000 Xg for 20 min at 20 degress Celsius. Twenty microliters sample was mixed with a phosphate buffer which Na2HPO4 – 50 mM; NaH2PO4 -50 mM, pH-6.0), o-dianisidine (7.09 nM) and hydrogen peroxide (4.4mM). Optical density was recorded at 462 nm using ELISA plate reader and MPO activity has been expressed as µmole/1X106 cells/3 min for neutrophils using the molar extinction coefficient of oxidized o-dianisidine Ɛ= 10,062 (Mxcm) -1[48]

Estimation of Cortisol level

Serum cortisol was calculated by using Cortisol ELISA kit. 20µl od standard or serum samples were added to wall pre-coated with anti-cortisol monoclonal antibiodies followed by the addition of 200 µL enzyme conjugate, mixed for 10 seconds and incubated for 60 min at room temperature. The enzymatic action was stopped by addition of 100 µL of stop solution which is 0.5M H2SO4 and subsequently the plate was read at 450+/- 10 nm using a =n ELISA reader. Standards provided in the kit were used for drawing the standard curve and for calculating the absolute cortisol concentration in the sample. The cortisol level was reported as ng?ml of serum.

Statistical Analysis

Data has been represented as mean +/-SEM and the statistical significance was analysed by one way analysis of variance or ANOVA which was followed by post hoc analysis using Tukey's multiple comparison's test. Pearson's correlation coefficients or r were performed to check whether there was any correlation between the patient's profiles and the investigated parameters or between the investigated parameters of the patients and their first degree relatives. All the statistical analysis were calculated with GraphPad Prism 5.0 program and p value less than 0.0 was considered as statistically significant.

RESULTS

Thus 30 cases under depression, 25 first degree relatives and 25 healthy controls were included in the study. The demographic profile of the study samples is shown in the Table 1. There was not any major difference in the age and gender of the depressed patients, their first degree relatives and the

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healthy controls. The majority of the cases i.e. 23 out of 30 patients (76.67%) included in the study were diagnosed MDD in their first episode, while the remaining 7 cases i.e.23.33% had experienced recurrent MDD episode. The mean duration of the current episode of depression was 3.36+/-6345 months. A family history of mood disorder was there in only 6 i.e. 24 percent of the cases. The average score of the cases on HAM-D and GAF was 18.23+/-7324 and32.23 +/-1867 respectively. The clinical profile of the patient is shown in the Table 2.

Oxidative stress and MPO in the Neutrophils of Depressed patients and their first degree relatives

Free radical generation is assessed by DCF DA fluorescent probe and it was seen that free radicals generation was significantly more in neutrophils of drug naive patients under depression as compared to that of the healthy volunteers. To calculate the risk estimates in the family, the next assessment was done for ROS generation in the first degree relatives of the patients under depression. No statistically significant change in the ROS levels was observed between Neutrophils of healthy controls and the first degree relatives, an elevated trend was observed. A positive direct correlation was seen the ROS levels between the cases and the first degree relatives were seen where $r = 0.7543$, $p < 0.0001$), Table 3. No significant relation was found between ROS generation and the disease severity HAM-D and GAF score, age, gender, number of episodes, and duration of illness or family history. **Table 1.** Demographic profile of depressive patients, their first-degree relatives, and healthy controls.

Table 1: Demographic profile of cases under depression, their first degree relatives and healthy controls

Details	Control	Case	Relative
Number	25	30	25
Age, years (mean+/- SEM)	27.15 +/- 0.80	29.52+/- 1.76	30.12 +/-1.74
Gender (M/F), n(%)	16 (64%)/9(36%)	14(46.67%) / 16(53.33%)	14(56%) /11 (44%)
FBS, mg/dl (mean +/- SEM)	75.53 +/- 1.9	81.07 +/-1.56	75.34 +/-2.1
Cholesterol, mg/dl (mean +/- SEM)	107.32 +/- 2.1	113.8 +/-2.6	116.3 +/- 4.2
Total Billirubin mg/dl (mean +/- SEM)	0.5690 +/- 0.022	0.5156 +/- 0.0158	0.5331 +/- 0.0209
ALP U/L (mean +/- SEM)	119.9 +/- 7.2	125.1 +/- 9.3	113.4 +/-
SGPT U/L (mean +/- SEM)	29.56 +/- 1.47	29.45 +/- 1.89	33.12 +/- 1.91
TLC (mean +/- SEM)	6552 +/-211.1	6715 +/- 208.2	6631 +/- 221.3

FBS – Fasting blood sugar, ALP – Alkaline Phosphatase, SGPT – Serum glutamic pyruvic transaminase, TLC – Total leukocyte count.

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Table 2: Clinical profile of cases under depression

Variable	
Diagnosis	
Major depressive disorder (1st episode), n(%)	23 , 76.67%
Major depressive disorder, recurrent, n (%)	7, 23.33%
Duration of current episode, (mean +/- SEM)	3.36+/-6345 months
Family of mood disorder present, n(%)	6, 24%
Family of depression, score on HAM-D, (mean +/- SEM)	18.23+/-7324
Functioning, score on GAF (mean +/- SEM)	32.23 +/-1867

HAM-D: 17 item Hamilton Depression Rating scale, GAF – Global Assessment of Functioning scale

MPO is a critical component of oxidative activity of the neutrophils, so MPO activity was measured in these cases. MPO activity was comparatively higher in the neutrophils of cases under depression (˜1.21 fold, $p<0.05$, 95% CI: 1.764 to 0.02365) and their first degree relatives (˜1.27 fold, $p<0.05$, 95% CI:- 1.845 to 0.07252), than their healthy volunteers. MPO activity was independent of disease severity, HAM-D and GAF score, age, gender, number of episodes, duration of illness and blood relation.

Nitrite level and NOS Isoform expression in the Neutrophils of cases under depression and their first degree relatives.

It was observed that the total nitrite content in the plasma was lower in both cases under depression, the value being ˜0.53 fold, $p<0.05$, 95% CI: 1.163 to 10.87 and their first relatives, whole values were ˜0.51 fold, $p<0.05$, 95 %CI : 0.1087 to 10.46 as compared to ealthy volunteers. A statistically significant positive correlation in the plasma nitrite content was seen in the cases and their first relatives where $r=0.5165$, $p=0.0105$). To investigate the regulation of NO, nitrite content and nNOS, mRNA expression were assessed in the neutrophils of cases under depression and their first degree relatives and healthy controls. The cases under depression had shown decrease in the total nitrite content in neutrophils i.e. ˜0.57 fold, $p<0.05$, 95% CI: 0.05418 to 2.398, the expression of the nNOS transcript was considerable more ˜ 2.45 fold, $p <0.05$, 95% CI : 0.006557 to 0.0003329) in the neutrophils as compared to their first degree relatives and the healthy controls. A positive correlation was seen in the total nitrites in the neutrophilsbetween cases under depression and their first degree relatives where $r = 0.6437$, $p=0.0021$, **Table 3**. nNOS isonorm mRNA expression was also not dependent of disease severity, HAM-D and GAF score, age, gender, number of episodes, duration of illness and family history.

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Table 3: Correlations between the investigated parameters of case under depression and their first degree relatives

	Neutrophil's ROS	Plasma Nitrite	Neutrophils'Nitrite	nNOS mRNA Expression	Neutrophils' MPO	Cortisol
R	0.7543	0.5165	0.6437	0.2450	0.01273	0.3984
P	0.0001*	0.0101*	0.0019**	0.2472	0.9534	0.0356*
N	19	22	17	20	14	25
95 % CI	0.5182-0.9165	0.1365-0.7589	0.3054-0.8654	0.1829-0.6167	0.4756-0.5045	0.03357-0.6815

Serum Cortisol level in cases under depression and the first degree relatives

As the chronic stress raises the incidence of depression and its majorly correlated with cortisol hypersecretion [47]. Thus the serum cortisol levels were analysed for the association between cases under depression and their first degree relatives. The serum cortisol level was elevated in cases i.e. ˜1.87 fold, p<0.001, 95 % CI : -254.3 to -142) and in their first degree relatives which was ˜ 1.45 fold, p<0.001 95% CI : -165.7 to -52.65 as compared to their healthy controls. It was further seen that there was a positive correlation in serum cortisol levels between patients and their first degree relatives where in the r = 0.3984, p =0.0351) and the serum cortisol level in the cases under depression was considerably higher than their first degree relatives.

DISCUSSION

Oxidative/nitrosative stress (ROS/RNS) and inflammation are main pathological processes of MDD. This study contributes towards a better understanding of the familial connection of the depression and positive correlation in neutrophil ROS/RNS, plasma nitrite and the serum cortisol levels between the cases under depression and their first degree relatives. The study sample had 30 cases under depression, 25 first degree relatives and 2 healthy controls. As shown in the Table 1 the majority of the and the study was almost equally distributed as far as gender was concerned. Even the leukocytes count and other biochemical parameters were equally distributed in the sample. Oxidative stress levels are elevated in the MDD cases and it is well established through studies,[4,6,7,8,10,13,14,36] however he familial connection is not well defined. Parental depression predisposes a risk factor in the offspring[49], Zalar *et al*. have shown that genetic makeup of Major depressive disorder patients with positive family history of depression is different from sporadic cases[50].

A study by Frank *et al*. has shown that elevated ROS generation by the monocytes of depression cases and their association with inflammation[51]. Majority of the studies are focussed on analysing oxidative stress in depression cases and the positive correlation is seen between oxidative stress and severity of depression[10,12]. Our study has established a positive correlation in the ROS/RNS level between major depressive disorder patientsand their first degree relatives and it also highlighted as the potential biomarker to study risk factors in the family. MPO, the inflammatory enzyme plays an important role in oxidant production by neutrophils and is linked as a specific marker of microglial immune activation in MDD[41]. The current study had shown the findings of elevated MPO activity in

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the neutrophils of both depression and their first degree relatives, although no correlation between the blood relatives were observed. These findings were similar as Talarowska *et al* and Galecki *et al*, who had shown the findings of elevated leukocytes MPO transcript and serum MPO protein in depression patients[52,53] Nitric acid modulates the major neurotransmitters like norepinephrine, serotonin, dopamine and glutamate which are majorly involved in the neurobiology of the major depression. The current study shows that there is decrease in the level of total nitrite content in the plasma of both depressed patients and their first degree relatives. This study also shows the positive correlation in the plasma nitrite between the patients of MDD and their relatives, which suggests and elevated risk of depressive disorders in blood relatives. A study by Ali –Sisto *et al* which also evaluated NO metabolism in the MDD cases also demonstrated lower levels of arginine as well as a lower global arginine bioavailability ratio (GABR) in the serum of MDD patients[54]

LIMITATIONS

The major limitation of this study was its small sample size. The diet of the subjects in the study could not be matched hence it affected the nitrite level. The case using nicotine could not be excluded as this could have limited the size of the sample. The comparison between the groups of first episode depression and recurrent depression could not be made because of fewer subjects in the latter group. Similarly a comparison could not be made in the positive and negative history of mood disorders in blood relatives as there was very small number of cases in the former group.

CONCLUSION

The findings of the study backs the findings of familial association of depressive disorders and have shown that ROS/RNS, plasma nitrite and serum cortisol levels are positively correlated between the patients of MDD and their first degree relatives and can be used as potential biomarkers to calculate the risk factor in the family. Obtaining a family history of depression and its severity and impairment in old generations might help to identify persons at high risk for psychopathology at young age. But to extend these observations further studies on more diverse samples of depression cases are required.

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