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Identification of metabolomic changes before and after exercise regimen in stress induced rats

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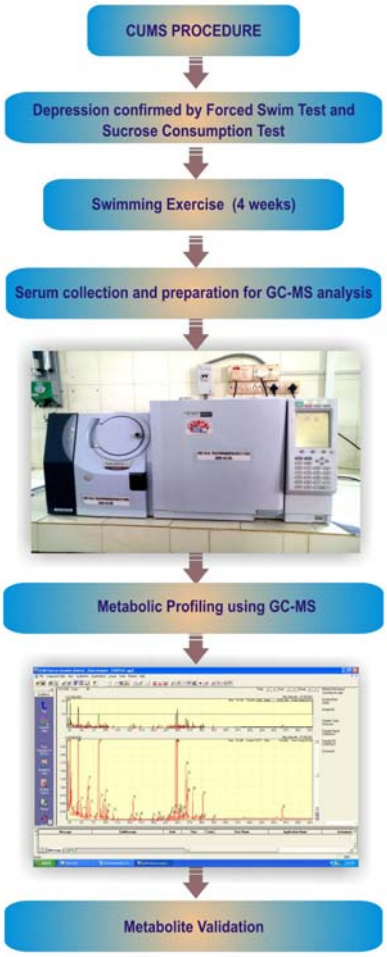
Abstract

Aim : To identify the metabolomic changes before and after exercise regimen in depression induced animal models.

Methodology : Severity of depression was measured by forced swim test (FST) and sucrose consumption test (SCT) and their statistical significance was obtained by ANOVA followed by post hoc test. Swimming protocol was followed for 4 weeks of exercise treatment. Serum obtained from depressed and exercise treated rats were used for the metabolite analysis by GCMS. Subsequent statistical analysis (ANOVA followed by post hoc test) revealed significant metabolic changes.

Results : About 20 metabolites were found to be differentially expressed in control, depressed and exercise treated groups. Serum levels of glycine and serine were significantly increased in depressed groups, whereas the levels of leucine, proline, valine (BCAs), glucose, fructose, galactose, mannose, xylitol myo-inositol, lactic acid, oleic acid, palmitic acid, stearic acid were significantly decreased when compared to control group (Group I). After 4 weeks of swimming exercise regimen procedure, lower levels of glycine, serine and higher levels of sugars, myo-inositol, oleic acid, palmitic acid, stearic acid and BCAs were found in the exercised groups (Group III) when compared to the depressed groups (Group II).

Interpretation : These observations suggested that the depressed state may be associated with the changes in the level of few metabolites involved in amino acid, fatty acid, glucose and energy metabolisms, which may get reverted after chronic exercise.



Introduction

Depression is a serious psychiatric disorder often associated with stressful events that can lead to emotional and physical problems (Shenl *et al.*, 2003). The global burden percentage of depression is about 12.3 % and is predicted to rise by 15 % in 2020 (Reynolds, 2003). Cognitive and emotional biases play an important role in the development of depression (Anisman and Matheson, 2005; Coles and Heimberg, 2002). Chronic unpredictable mild stress (CUMS) is a well-validated animal model that mimics several human symptoms of depression (Willner, 1997) and had been used widely for studying clinical depression and effect diverse antidepressant drugs (Redrobe *et al*., 1998).

Metabolomics enables the investigation of the metabolic response of the living system by measuring the variations in the metabolic profiles in biological fluids (Kell, 2006). Metabolomics is a non-targeted approach used in the study of metabolic networks for identifying disease-specific metabolic profiles and biomarkers of CNS disorders (Schwarz and Bahn, 2008; Kadddurah – Daouk *et al.*, 2008). Gas chromatography – mass spectrometry (GC-MS) is one of the most efficient, sensitive and reliable metabololite detection tool due to its high resolution and selectivity. It produces reproducible molecular fragmentation patterns making it an integral tool for metabolite identification (Pasikanti *et al.*, 2008).

Metabolomics was used to study the pathogenesis of major depressive disorder (Wingenfeld and Wolf, 2011). There is a promising evidence that exercise can increase synaptic plasticity, enhance neuronal survival, while the stress shows contradictory effects, which indicates a potential mechanism for exercise to reduce stress (Cotman *et al*., 2007). A previous study has reported the antidepressant like effect of exercise which promotes neurogenesis and inhibits neurodegeneration (Eyre and Baune, 2012).

In the present study, Chronic Unpredictable Mild Stress was induced to produce gradual development of depression. The central objective of this study was to investigate the metabolic changes in the serum of control, depressed and exercised rats. It was hypothesized that depressed rats, subjected to moderate swimming exercise for 28 days would show up some changes in their metabolic profile that may bring out some reduction in the stress level. To our knowledge, this is the first analysis comparing metabolomic changes in the serum of depressed and exercised animal models.

Materials and Methods

Animals : Female Wistar rats weighing about 150 – 200g were purchased from Kovai Medical Center and Hospital, Tamilnadu, India. Animals were maintained at constant temperature (37°C), humidity (45 ± 15%) controlled room with 12 hrs light/dark cycle and free access to food and water. The ethical clearance for handling of experimental animals was obtained from the Institutional Animal Ethics Committee (IAEC) of PSG Institute of Medical Sciences and Research.

Chronic Unpredictable Mild Stress (CUMS) procedure : Rats were randomly divided into three groups (n=6 per group): control group, depression-induced group and exercise treated group. The control rats were housed together without any disturbance and the model rats were exposed to the following CUMS procedure following the method of Willner, (1997) : cage tilting (tilt at 45°C angle) for 24 hrs, crowded housing (5 rats per cage), noises for 1 hr (alternative periods of noise 10 min and silence 10 min), 24 hrs food, 24 hrs water deprivation, soiled bedding (wetting cage with water), removal of bedding materials for 12 hrs, reversed light-dark cycle respectively. One stressor was applied per day and the whole stress process was done in a random order for 4 weeks and at the end of it, depressive behaviour was confirmed by forced swim test and sucrose consumption test.

Behaviour test

Forced swim test : The forced swim test was carried out according to Porsolt *et al*. (1977). Here, the experimental rats were made to swim individually in a cylindrical container of 35 cm in height and 15 cm in diameter, such that the rats could not touch the bottom of the cylinder with its limb or tail or climb over the edge of the chamber.

Trial sessions were conducted before the start of actual 6 min test. The initial 2 min in the total 6 min were not considered, because during that time period, animals treid to escape. A rat was considered immobile when it remained floating in the water without struggling, making only minimum movements to keep its head above water. The total duration of immobility during final 4 min were recorded. The rats were then allowed to dry in a pre-washed enclosure (~32°C) before being returned to their cages.

Sucrose consumption test : This test was performed following the method of to Willner *et al.* (1987). After 24 hr period of food and water deprivation, each rat was rested to be in individual cage and was given two bottles containing 1% sucrose solution and water. The preference for sucrose was calculated as a percentage of sucrose solution consumed in the total amount of liquid intake.

Exercise protocol : Rats were trained in a moderate swimming program according to the method of Liu *et al.* (2010). Daily swimming exercise was performed in a large plastic barrels (45 cm in diameter and 60 cm in height) filled with fresh water (32°C ± 2°C) to the depth of 50 cm. Exercise was performed at the same time daily (between 10.00 a.m. to 12.00 p.m.). After swimming, the rats were towel dried and kept warm by electric drier.

The swimming program included two phases: adaptation and training. During first week (adaptation), the training begun for 15 min on the first day and it was gradually increased for 60 min on the last day. The adaptation was performed for reducing the water induced stress without promoting physiological alterations in

relation to physical training (Contarteze *et al*., 2008).

Analysis of metabolites : Rats were sacrificed and the blood collected was centrifuged at 4000 rpm for 20 min at 4°C to separate the serum. The serum was stored at -20°C and used for the analysis of amino acids, fatty acids, sugars, sugar alcohol, organic acids, cholesterol, glycerol and urea.

Sample preparation for GC-MS : The sample preparation for GC-MS analysis was performed according to previous studies with few modifications (Gao *et al*., 2014). Serum was thawed at 4°C and mixed well before use. About 100 µl of serum was subjected to protein precipitation using 250 µl of acetonitrile and centrifuged at 12,000g for 10 minutes at 4°C. Then, a volume of 200 µl supernatant was transferred to a tube and evaporated to dryness under the stream of nitrogen prior to derivatization. All the dried samples were derivatized with 30 µl methoxylamine hydrochloride (20 mg/ml in pyridine) at 70°C for 60 minutes, followed by 50 µl MSTFA (N-Methyl-N (trimethylsilyl) trifluoracetamide) at 40 °C for 90 minutes. After derivatization samples were cooled at room temperature and 1 µl was injected into the GC-MS.

The samples were analysed using a Shimadzu GC-2010 Plus gas chromatography instrument coupled to a Shimadzu QP2010 mass spectrometer (Shimadzu, Japan). The column used for analyses was a DB-5MS fused – silica capillary column (30m x 0.25mm I.D, 0.25 µM film thicknesses) chemically bonded with a 5% phenyl 95% methylpolysiloxane cross-linked stationary phase. Helium was used as the carrier gas with a flow rate of 1.0 ml min-1. The initial temperature was held at 100°C for 4 min, increased to 270°C at the rate of 5°C/ minute for 15 minutes. The temperatures of injection, interface and ion source were set at 280°C, 250°C and 200°C respectively. One microlitre of the sample was injected in the 10:1 split mode, and the solvent delay time was set to 9 min. The mass spectrometer was operated in electron ionisation mode (electron energy of 70eV) and was implemented with the range of m/z of 35 to 800. The identification of the metabolites was based on NIST & WILEY mass library.

Statistical analysis : Quantitative data were expressed as mean ± SD (standard deviation). The levels of significance between the groups were determined by performing ANOVA (Analysis of Variance) and subsequent post hoc (Scheffe's test) analysis using SPSS (version 16.0).

Results and Discussion

Forced swim test is an established test for depressive animal model. The longer the immobility time, more the depression. The immobility (floating) time was measured as an estimate of phenotypes of depression. Zhoa *et al*., (2015) reported higher immobility time in case of depressed rats and lower levels in antidepressant (imipramine) - treated rats. In the present study, in FST, significant ($p<0.05$, $p<0.01$) increase (114.06 ± 8.22 seconds) was found in the immobility time of group II (depressed) rats which after subjecting to swimming regimen for four weeks showed a significant reduction (76.69 ± 4.20 seconds).This indicates that exercise ameliorates depression like behaviour induced by chronic stress (Table 1).

Table 1 : Effect of exercise on behavioural changes in depression induced animal models

Groups	FST Duration of mobility in seconds	SCT Sucrose preference in %
Normal control	47.96 ± 1.76	92.04 ± 1.45
Depression induced	114.06 ± 8.22**	66.91 ± 0.67**
Depression + exercise treated	76.69 ± 4.20**	82.10 ± 0.82**

FST – Forced Swim Test; SCT – Sucrose Consumption Test. Data are expressed in mean ± SD (n=6), **p<0.01 – significant

In Sucrose Consumption Test, stressed animal models of depression showed less preference to sucrose consumption 'anhedonia' which was reversed by antidepressant treatment (Willner 1997). In a previous study decreased sucrose consumption found in CUMS induced rats reverted after the swimming exercise which alleviated depression-like behaviour (Lie *et al*., 2013). Likewise in the present study also, the preference for sucrose consumption was found to decrease (66.91 ± 0.67 %) significantly ($p<0.05$) in group II rats when compared to control group (group I). After 4 weeks of exercise regimen sucrose consumption had increased significantly (82.10 ± 0.82 %) in group III stressed rats, showing that the exercise reduces the Chronic Unpredictable Mild Stress induced depression - like behaviour.

Having seen that exercise produced improvements in the above two behavioural tests, serum metabolic profiling of control, CUMS and exercise groups were carried out by GC-MS analysis. Twenty nine metabolites were identified and they were subjected to one way ANOVA followed by post hoc (Scheffe's test) analysis, which revealed differences in 20 serum metabolites of 3 groups viz., normal, depressed and exercised rats in aminoacids, fatty acids, sugars, sugar alcohol and organic acid metabolite levels (Table 2).

Aminoacids play an important role in human metabolome as they are the precursors of brain neurotransmitters and hence, have been studied as biomarkers for several disorders including schizophrenia (Do *et al*., 1995), Major depressive disorder Altamura *et al*., 1995; Xu *et al.* (2012), Alzhemier's disease Fonteh *et al.* (2007), cancer Leichtle *et al.* (2012) and metabolic syndrome Cheng *et al.* (2012). In a recent study (Batch *et al*., 2014) reported the significance of Branched chain aminoacids (BCAs) as specific biomarker for certain diseases and observed decreased levels of BCAs and reduced activation of Mammalian

Table 2 : Serum metabolites detected by GCMS in the depressed and exercised rats

Compounds	Concentration (mmol ml^{-1})				
	Control ¥	Depression ¥	Exercise ¥	P value	% change after exercise
Aminoacids					
Alanine	1.49 ± 0.15	1.32 ± 0.157	1.43 ± 0.105	0.378 ns	7.69
Glycine	0.12 ± 0.03	0.47 ± 0.09**	0.19 ± 0.06	0.001**	-147.37
Isoleucine	0.93 ± 0.07	0.75 ± 0.08	0.77 ± 0.8	0.062 ns	2.59
Leucine	2.44 ± 0.18	1.38 ± 0.15	1.70 ± 0.04	0.000**	18.82
Proline	1.01 ± 0.10	0.073 ± 0.02**	0.92 ± 0.09	0.000**	92.07
Serine	0.39 ± 0.18	5.01 ± 0.16**	0.94 ± 0.02**	0.000**	-432.98
Threonine	0.21 ± 0.105	0.97 ± 0.18	0.32 ± 0.14	0.020*	-203.13
Valine	1.53 ± 0.11	0.66 ± 0.05	1.03 ± 0.40	0.000**	35.92
Fatty Acids					
Arachidonic acid	1.43 ± 0.15	1.073 ± 0.01**	1.479 ± 0.19 ns	0.060 ns	27.45
Linoleic acid	0.25 ± 0..4	0.18 ± 0.026*	0.40 ± 0.032	0.001**	55.00
Oleic acid	0.26 ± 0.04	0.08 ± 0.13**	0.23 ± 0.07	0.003**	65.22
Palmitic acid	4.13 ± 0.14	3.16 ± 0.25*	3.77 ± 0.16	0.044*	16.18
Stearic acid	1.21 ± 0.05	1.02 ± 0.07	1.20 ± 0.08	0.027*	15.00
Tetradecanoic acid	0.04 ± 0.22	0.06 ± 0.01	0.07 ± 0.10	0.722 ns	14.28
Sugars					
Fructose	1.27 ± 0.2	0.85 ± 0.04**	1.13 ± 0.115	0.020**	24.78
Galactose	13.97 ± 0.11	7.35 ± 0.24	11.23 ± 0.15	0.000**	34.55
Glucose	12.6 ± 0.45	8.53 ± 0.115**	9.72 ± 0.16**	0.000**	12.24
Mannose	0.186 ± 0.02	0.09 ± 0.01	0.34 ± 0.04	0.000**	73.53
Xylitol	1.44 ± 0.23	0.36 ± 0.19	0.93 ± 0.71	0.010*	61.29
Sugar Alcohol					
Myo-inositol	3.38 ± 0.08	0.70 ± 0.10**	2.32 ± 0.03**	0.000**	69.83
Organic Acids					
3-hydroxy butyric acid	3.64 ± 0.04	1.66 ± 0.03**	2.26 ± 0.06**	0.000**	26.54
Lactic acid	12.51 ± 0.21	7.63 ± 0.22**	12.14 ± 0.19	0.000**	37.15
Malic acid	0.09 ± 0.02	0.08 ± 0.05	0.07 ± 0.05	0.932 ns	-14.28
Oxalic acid	0.91 ± 0.02	1.23 ± 0.107**	1.06 ± 0.15	0.004**	-16.04
Propanoic acid	0.56 ± 019	0.42 ± 0.14	0.75 ± 0.04	0.069 ns	44.00
Succinic acid	0.05 ± 0.03	0.71 ± 0.06	0.29 ± 0.08	0.000**	-144.83
Others					
Cholesterol	1.33 ± 0.16	1.22 ± 0.05	1.25 ± 0.04	0.060 ns	2.40
Glycerol	1.8 ± 0.19	1.52 ± 0.16	1.35 ± 0.20	0.064 ns	-12.59
Urea	11.20 ± 0.16	11.72 ±0.11	12.09 ± 0.70	0.104 ns	3.06

↑, ↓ indicate significant increase and decrease in depressed rats when compared to healthy rats. Data are expressed as mean ± SD (n=6), *p<0.05, **p<0.01 – significant, ns– not significant, ¥ n=6 for each group

target of rapamycin (mTor) ubiquitous serine/threonine protein kinase. This shows that BCAs play a crucial unrecognized role in the etiology of depression (Baranyi *et al*., 2016). Likewise in the present study, BCAs such as leucine (1.38 mmolml^{-1}) and valine (0.66 mmolml^{-1}) were found to be decreased in stress induced group. After the exercise, the BCAs level was found to be increased significantly, which may due to the increased activation of mTor.

Excitatory neurotransmitters such as serine (5.01 mmolml^{-1}) and glycine (0.47 mmolml^{-1}) are found to be higher in depressed animals when compared to control. Serine and glycine, one of the excitatory amino acids in brain are implicated in neurotransmission acting either as neurotransmitters or as modulators of NMDA receptors for excitation. Previous studies have reported increase levels of excitatory amino acids such as serine and glycine in major depressive disorder (Mauri *et al*., 1998; Hashimoto *et al*., 2016) and after the exercise regimen, there was a significant decrease in their levels when compared to that of the depressed rats. Exercise, as found in this study, had brought down the increased levels of these excitatory aminoacids (serine and glycine) almost to normal levels in depressed rats.

Stearic acid seems to possess neuroprotective effects (Wang *et al*., 2006). In this study, decreased concentrations of

fatty acids such as palmitic acid (3.16 mmol ml^{-1}), stearic acid (1.02 mmol ml^{-1}), linoleic acid (0.18 mmol ml^{-1}) and oleic acid (0.08 $mmolml^{-1}$) was observed in depressed rats when compared to control rats. These results suggest of alterations in fatty acid metabolism in depressed rats as they are associated with metabolic pathways related to TCA cycle (Serretti *et al*., 2004). Previous metabolic studies have reported that fatty acid metabolism is significantly lower and shifted from β – oxidation to ω–oxidation in depressed patients, in comparison to non-depressed controls (Maes *et al*., 1996; Paige *et al*.,2007; Steffens *et al*., 2010). Thus, decreased serum fatty acids may reduced result in oxidation of fatty acids, which in turn reduced the production of acetyl CoA that leads to dysfunction of TCA cycle, a possible cause for fatigue, which is most frequently representated symptom in major depressive disorder (Serretti *et al*., 2004). After 28 days of swimming, the fatty acid levels were found to increase significantly which in turn resulted in increased β – oxidation leading to increased TCA cycle pathway with subsequent relief from fatique.

Myo-inositol, a key metabolic precursor to phosphoinositide pathway was reported to be decreased in major depression (Coupland *et al*., 2005). Decreased levels of myo-Inositol have been reported in CSF and post-mortem frontal cortex of patients with mood disorders (Barkai *et al*., 1978; Shimon *et al*., 1997). Glial cells contain a high concentration of myo-Inositol (Brand *et al*., 1993; Griffin *et al*., 2002) and it contributes to glial osmoregulatory functioning (Strange, 1992; Fisher *et al*., 2002), as well as a wide range of other structural and signalling functions. Stress induced disruption of glial-neuronal interaction lead to glial deficits in mood regulation (Edgar and Sibille, 2012), may be due to reduced myo-inositol levels in the brain. this in turn might result in reduced osmoregulatory and cell signalling functions of myo-inositol. Accordingly, in the present study decreased level (0.70 $mmolml^{-1}$) of myo-inositol was found in depressed rats possibly due to glial dysfunction and the same improved (2.32$mmolml^{-1}$) significantly after exercise regimen, with concurrent improvement in depressive behaviour (Table 2).

A previous study reported decreased levels of plasma glucose and lactate in major depressive disorder that results in putative imbalance in glycolysis and gluconeogenesis (Zheng et al., 2012). Likewise, in this study, levels of other metabolites viz., sugars such as glucose (8.53$mmolml^{-1}$), fructose (0.85$mmolml^{-1}$), Galactose (7.35$mmolml^{-1}$), Mannose (0.09 $mmolml^{-1}$), Xylitol (0.36 $mmolml^{-1}$) and the organic acids lactate (7.63 $mmolml^{-1}$), 3-hydroxy butyric acid (1.66 $mmolml^{-1}$) decreased significantly in depressed rats and their levels increased after swimming. Neverthless, in the present study no significant levels in the baseline levels of metabolites (glycerol, malic acid, propanoic acid, arachidonic acid, Cholesterol, Tetradecanoic acid, alanine, isoleucine and urea) was found among the healthy control, depressed and exercised groups of rats.

GCMS metabolomic analysis carried out in this study found significant differences in few unique metabolites among aminoacids, sugars, fatty acids, sugar alcohols and organic acids between the groups viz normal control, depressed and exercised rats. Identifying the biochemical mechanisms of these both in depression and after exercised would enhance our understanding of the etiology of depression and if validated, may become useful diagnostic bio-markers for improving the treatment of depression, as well. This presents new opportunities for translational research in depression and schizophrenia.

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