



Received on 28 January 2019; received in revised form, 29 April 2019; accepted, 14 June 2019; published 01 October 2019

## NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY BASED URINARY METABOLIC PROFILING IN POST TRAUMATIC STRESS DISORDER RATS

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### Keywords:

Post traumatic stress disorder,  
Animal model, Underwater trauma,  
Metabonomics, Nuclear magnetic  
resonance

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**ABSTRACT:** Though at present, Post-Traumatic Stress Disorder (PTSD) stands to be one of the major mental debilitating psychiatric disorders, however, its clinical diagnosis remains unachieved due to the absence of any biological marker. Hence, this study is aimed at the identification of putative biological underpinnings of PTSD through the metabonomic approach. For this purpose, the animal model based NMR spectroscopy approach was undertaken for profiling of urine samples, for classification of metabolic changes brought about in body due to PTSD. Multivariate statistical analysis techniques, namely Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA) were performed on <sup>1</sup>H NMR spectral data, which was obtained from both rat urine sets that are, before and after exposing the rats to Underwater Trauma (UT) for PTSD. The behavioral changes were measured and recorded by conducting the Open Field Test (OFT). PCA showed partial separation with PC1 = 49.8% and PC2 = 21.7%, however PLS-DA completely separated both urine sets with R<sup>2</sup> = 0.9 and Q<sup>2</sup> = 0.8. Eight endogenous metabolites were tracked whose concentration attenuated majorly post PTSD, about the changes recorded. The metabolites included citrate, hippurate, aspartate, n-methyl nicotinamide, betaine, creatine, creatinine, and β-hydroxybutyrate. OFT indicated heightened anxiety behavioral changes brought about in post-trauma rats. The metabolites explored were primarily involved with energy metabolism, lipid metabolism, and intestinal microflora metabolism. These observations enable to assess the changes in the physiology of PTSD by evaluating the concentration of these metabolites in urine. This study would have an imperative role in assessing the biological underpinnings of PTSD and can further be taken into account for developing, the clinical screening parameters for PTSD.

**INTRODUCTION:** Increasing stress and unhealthy lifestyles have led people to be more prone to various psychiatric disorders.

These issues are currently being addressed enormously worldwide. Post-Traumatic Stress Disorder (PTSD) is an anxiety disorder <sup>1</sup>, experienced ensuing exposure to a traumatic event or a situation in which an individual faces acute stress <sup>2</sup>. Traumatic event herein refers to any life-threatening event, *e.g.*: military combat, physical or sexual assault, natural disasters, or loss of a loved one in some cases. Symptoms include re-experiencing trauma, avoidance, negative thoughts, or mood <sup>3</sup>.

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|   | <b>QUICK RESPONSE CODE</b><br><b>DOI:</b><br>10.13040/IJPSR.0975-8232.10(10).4636-43       |
|   | This article can be accessed online on<br><a href="http://www.ijpsr.com">www.ijpsr.com</a> |
| DOI link: <a href="http://dx.doi.org/10.13040/IJPSR.0975-8232.10(10).4636-43">http://dx.doi.org/10.13040/IJPSR.0975-8232.10(10).4636-43</a> |  |

Since PTSD studies in humans are subjected to diverse variations, like nature of trauma, impact on the individual, period of induction, type of stressor confronted, *etc.* the knowledge in this area remains restricted. Therefore, animal models have key importance in PTSD related research. Animal models have played, an elemental role in providing insight into the diverse bio-physiological phenomenon taking place during a psychiatric disorder<sup>4,5</sup>. Rats are widely used and accepted as a model organism for PTSD<sup>6</sup>. Understandings developed in rat model can be used, to expand insight into the human system, as the emotional behavior generating brain systems, are highly conserved throughout various levels of evolution<sup>6</sup>. Diagnosis in rats depends upon various behavioral and physiological markers, which can be easily evaluated. Model development for PTSD is based upon, exposure of animals to trauma like events, wherein they encounter near death/severe traumatic experiences<sup>7</sup>. Several methods have been devised for developing knowledge in PTSD, based on exposing the animal to acute stress conditions such as Single Prolonged Stress (SPS)<sup>8</sup>, Restraint stress<sup>9</sup>, Forced Swim Test<sup>10</sup>, Foot Shock<sup>11</sup>, Predator exposure<sup>12</sup>, *etc.* Although several limitations are encountered while studying the above models such as Footshock and Social Defeat models, are primarily used as models for depression<sup>13</sup> and are not specific to PTSD; SPS does not contribute to the effect of each stressor involved in procedure<sup>14</sup>.

The Underwater trauma (UT)<sup>15</sup> is considered to be a superior method for inducing PTSD trauma since it is more ethologically significant than other methods like electrical shocks<sup>15</sup>. Additionally, after being subjected to UT, animals showed impaired learning in the Morris water maze even after 3 weeks. This finding is also in line with 'negative alterations in cognition' criterion according to the "Diagnostic and Statistical Model" for mental disorders (DSM-5)<sup>16</sup>. Hence, the UT model was carried out in this study for inducing PTSD in the rats.

Studies conducted in PTSD using different techniques until now reveals, psychophysiological<sup>17</sup>, structural<sup>18</sup>, functional<sup>19, 20</sup>, neuroendocrinological<sup>21</sup> and genetic<sup>22, 23</sup> information. Although some studies like microdialysates<sup>24</sup> and peptide assay<sup>25</sup> have been conducted on PTSD rat model,

the metabolomic information remains restricted. For studying changes in metabolites within in vivo systems, metabonomics is systematic method<sup>26</sup>, which is defined as "the quantitative measurement of the dynamic multi-parametric metabolic responses of living systems to pathophysiological stimuli or generic modifications." Biofluids like urine, blood, *etc.* contains metabolites circulating in the body as a result of various metabolic cycles. Longitudinal studies conducted on these biofluids from an animal before and after exposure to stress, could reveal the various pathways getting perturbed due to stress. Urine is a complex biofluid, encompassing information about metabolites secreted by the body. Attenuations in the homeostatic cycles of an individual are directly reflected by altered metabolite pool in the urine. Metabolic assessment of urine helps in tracking of metabolites non-invasively<sup>26</sup>. Furthermore, no additional stress is caused to the animal in the process of collection, *e.g.* serum/blood. These changes can be assessed using various modalities like NMR, LC, and GC. NMR based metabolomics is a non-invasive, non-destructive<sup>27</sup> and quantitative technique<sup>28</sup> used for detection of a wide spectrum of metabolites, providing a snapshot of metabolites, at a particular moment.

In this study, the metabolic changes caused by PTSD was assessed through NMR metabolic approach. The underwater trauma model was used for analyzing the changes brought about (PTSD symptoms) in the body due to the induction of trauma and to indicate possible metabolic biomarkers for PTSD. Behavioral analysis, along with metabolic profiling, was performed before and after the sensitization of animals to PTSD underwater trauma. The NMR metabolic information was subsequently processed using Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA). Further analysis was conducted for the development of a better understanding of the alterations in biological pathways brought about in PTSD.

## MATERIAL AND METHODS:

**Animal Handling:** The study was carried out with 10 healthy male Sprague-Dawley (SD) rats, weighing 200-300 g (about 2-3 months of age), obtained from the animal facility of the institute.

Female rats were not included, to negate the hormonal effects from the resulting spectra. They were housed in polypropylene cages at an average room temperature of 23 °C and humidity of 50-60% in 12 h (h) light-dark cycle. Food and water were provided *ad libitum*. All rats were acclimatized to the room conditions for 2 weeks, before the beginning of experiments. The study was approved by the animal ethics committee of our institute.

**Behavioural Assessment through Open-Field Test (OFT):** All rats were tested one by one in an open field apparatus, which consisted of a black square base (100 cm × 100 cm) with black walls (40 cm). The base was divided into 25 squares by white stripes and organized into peripheral and central sectors. The peripheral sector consisted of squares, close to the wall, and the center sector consisted of the nine central squares. Testing was conducted under dim light during the morning in an undisturbed room. A rat was placed in the center of the apparatus and then allowed to behave and move freely for 5 min. In between each test, the arena was thoroughly cleaned with a 5% ethanol solution.

Tests were conducted between 10:00 and 12:00 h. While in the arena, the rat's behavior was taped and given to personnel, who were blinded whether the animals were in before trauma (pre-PTSD) or after trauma (post-PTSD) group. Rats were subjected to this test before and after their exposure to the trauma, and behavioral aspects, including climbing, central activity, peripheral activity, grooming, and rearing, were studied and marked.

**Underwater Trauma (UT):** The procedure followed in this study was similar to that described by Richter Levin, 1998<sup>29</sup>. Rats were given 4 days of training, wherein they were left in the water-filled the cylinder to freely swim for 45 sec and on the 5<sup>th</sup> day, following the swim session they were forcefully drowned to the bottom of cylinder for 30 sec (using metal mesh). Swim sessions were conducted by placing the rats in a glass cylinder (50 cm tall × 25 cm in diameter) containing 23-25 °C water and was 30 cm deep. At the 30 cm water depth, rats could not support themselves by touching the bottom with their feet. This gave them a drowning experience. Between each test, the glass cylinder was thoroughly cleaned using 5% ethanol solution.

**1-H NMR Spectroscopic Urine Analysis:** Urine samples were collected from each rat twice, namely pre-trauma and post-trauma respectively, by placing them in metabolic cages prewashed with sodium azide. Urine samples were stored at -80 °C for NMR spectroscopy analysis. Urine samples were thawed and brought to room temperature at the time of analysis. Each sample was centrifuged at 10,000 rpm. 400 µL supernatant was taken and mixed with 200 µL of deuterated buffer solution [0.2 M Na<sub>2</sub>HPO<sub>4</sub> + 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4 containing 1 Mm Trimethylsilylpropanoic acid (TSP) prepared in D<sub>2</sub>O] to remove particulate contaminants. The supernatant was poured into 5 mm NMR tubes, for The TSP acts as a chemical shift reference (d = 0) and D<sub>2</sub>O as locking agent. Urine and buffer were thoroughly mixed and again centrifuged at 5000 rpm for 5 min to remove further analysis.

NMR spectrums were acquired on a Bruker Avance III spectrometer (Bruker, Germany), operating at a frequency of 600.1 MHz at 298 K using standard one-dimensional water pre-saturation pulse sequence Nuclear Overhauser Enhancement Spectroscopy - 1 dimensional (NOESYPR1D) was used to suppress water signal. A total of 128 scans (acquisition time 1.7 s/scan) were collected into 64 k data points over the spectral width of 9615.4 Hz with a relaxation delay of 2 s. The raw free induction decay (FID) signal was multiplied by an exponential weighing function corresponding to line broadening (0.3 Hz) before Fourier transform. The metabolites were assigned based on their chemical shifts and signal multiplicity as described in earlier literature<sup>23</sup>.

**Data Reduction and Pattern Recognition (PR) Analysis:** Individual <sup>1</sup>H NMR raw spectra were manually phased and baseline corrected for distortions, on Topspin software (Version 3.5, 2014) Bruker, Germany. After that peaks were referenced to the chemical shift of TSP (δ 0.0 ppm). Further the spectra were binned using AMIX software (Version 3.9.15) Bruker, Germany, to 0.01 ppm segments starting from 0.5 to 9.5 ppm; however regions of spectra containing water (4.6-5.3 ppm) and urea (5.3-6.4 ppm) peaks were excluded to overcome the spurious effect of water suppression and urea. The binned data was exported to MATLAB (Mathworks, 2016) and then

normalized to the total area. Normalized spectral data were further subjected to multivariate analysis. Statistical analysis methods, including principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were performed on both data groups pre-trauma and post-trauma PTSD using MetaboAnalyst (4.0). The output from these techniques helped in visualizing the separation between the groups. Furthermore, the VIP score plot and the Loadings plot helped in identifying the metabolites responsible for bringing about the difference in the post-trauma and pre-trauma PTSD groups.

## RESULTS:

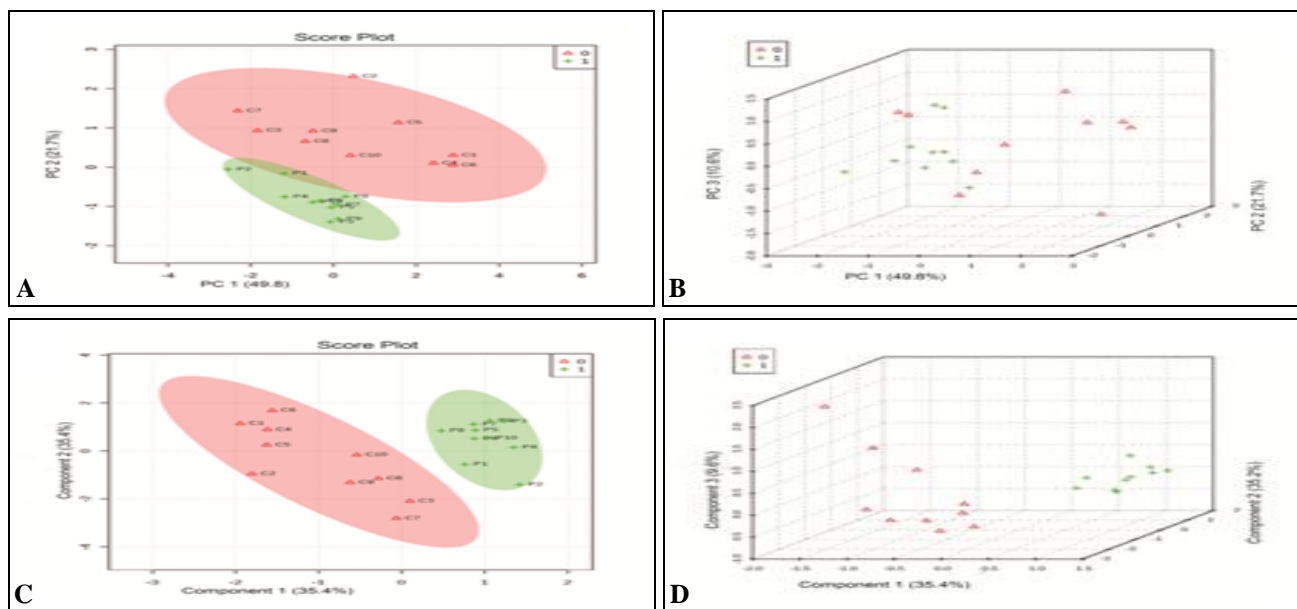
**<sup>1</sup>H NMR Spectral Analysis of Urine Samples:** 600 MHz <sup>1</sup>H NMR revealed a lot of valuable information regarding metabolic changes in the body taking place due to PTSD, which was directly reflected in the urinary metabolic pool. Based upon the chemical shifts published in the literature<sup>23</sup>, eight endogenous metabolites accounting for the variation amongst the two groups (pre and post-trauma) majorly were identified. They included hippurate, aspartate, betaine, N-methylnicotinamide, creatine, creatinine, citrate, and  $\beta$ -hydroxybutyrate, as represented in **Table 1**.

**TABLE 1: RELATIVE CONCENTRATION OF THE METABOLITES IN URINE WHICH ALTERED MAJORLY, AFTER THE RATS WERE SUBJECTED TO THE PTSD TRAUMA, INCLUDING NAMELY CITRATE, CREATINE, CREATININE, BETA-HYDROXYBUTYRATE (BHB), BETAINE, N-METHYLNICOTINAMIDE (NMNA), HIPPURATE AND ASPARTATE**

| S. no. | Metabolite | Urinary levels post trauma PTSD | PPM                          |
|--------|------------|---------------------------------|------------------------------|
| 1      | Citrate    | ↓                               | 2.67 (ABX)                   |
| 2      | Creatine   | ↑                               | 3.04 (s)                     |
| 3      | Creatinine | ↑                               | 4.05 (s)                     |
| 4      | BHB        | ↓                               | 1.24 (d)                     |
| 5      | Betaine    | ↓                               | 3.27 (s)                     |
| 6      | NMNA       | ↓                               | 4.45 (s), 8.84 (s)           |
| 7      | Hippurate  | ↑                               | 3.97 (s), 7.55 (s), 7.84 (s) |
| 8      | Aspartate  | ↑                               | 2.8 (s)                      |

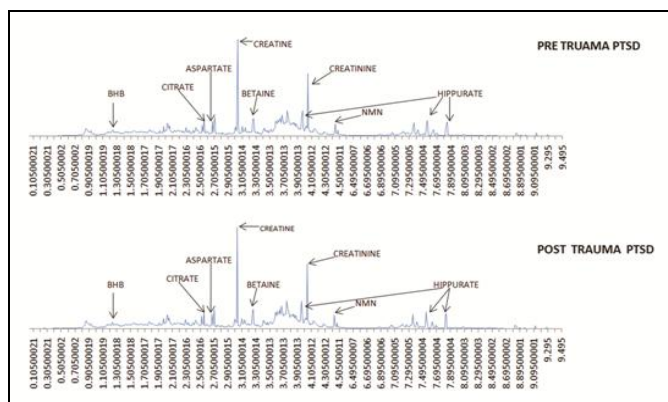
**Pattern Recognition (PR) Analysis of Urine Samples:** <sup>1</sup>H NMR spectral data of urine samples obtained from the rats before (pre) and after (post) subjecting them to PTSD trauma, was investigated and compared using multivariate statistical analysis

technique including Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA). Both the techniques used showed a clear separation between the two groups.



**FIG. 1: PATTERN RECOGNITION (PR) ANALYSIS OF URINE SAMPLES OF RATS FROM PRE AND POST TRAUMA GROUPS OF PTSD. PCA SCORE PLOTS; 2D (A) AND 3D (B) SEGREGATING THE PRE AND POST TRAUMA GROUPS PARTIALLY. PLS-DA SCORE PLOTS; 2D (C) AND 3D (D) SEGREGATING THE PRE AND POST TRAUMA GROUPS COMPLETELY**

PCA showed partial separation with values of 49.8% in Principal Component (PC) 1 and 21.7% in PC 2, whereas PLS-DA showed complete separation between the pre and post group with values of 35.4% in both PC 1 and PC 2, in the 2D score plot as represented in **Fig. 1**. The 3D score plots of both PCA and PLS-DA represented distinction from each other, segregating the two groups. The  $R^2$  and  $Q^2$  constant values are used for representing the goodness of fit and predictive ability, respectively. The third component in PLS-DA was shown to be the best classifier with an  $R^2$  value of 0.9 and  $Q^2$  value of 0.8. VIP score plot indicated a difference in the concentration of metabolites in post and pre-group along with the significance of the metabolite in a particular group. The spectral overlay graph, as represented in **Fig. 2**, illustrates the difference in both the pre and post PTSD spectra, indicating variation in peak intensities at several points.

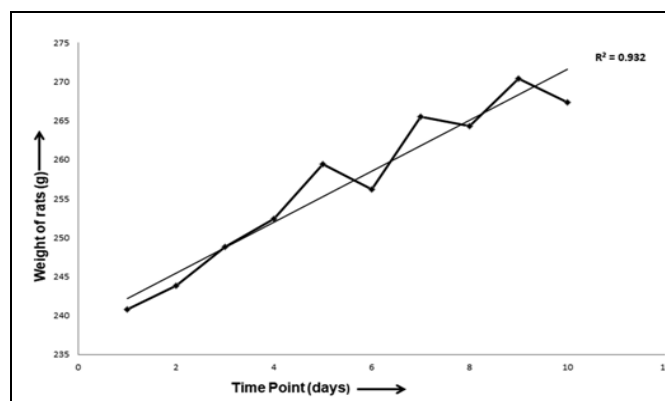


**FIG. 2: SPECTRAL OVERLAY GRAPH SHOWING DIFFERENCE IN URINARY LEVELS OF EIGHT ENDOGENOUS METABOLITES OF RATS, NAMELY CITRATE, CREATINE, CREATININE, BETA-HYDROXYBUTYRATE (BHB), BETAINE, N-METHYLNICOTINEAMIDE (NMN), HIPPURATE AND ASPARTATE, BEFORE (PRE TRAUMA PTSD) AND AFTER (POST TRAUMA PTSD) EXPOSURE TO PTSD TRAUMA**

### Behavioral and body weight analysis:

Measurement of body weight was done at regular intervals, starting from 1 week after acclimation period to 1 week after the exposure to the trauma. Weights of rats in the acclimation period increased from  $240.8 \pm 22.42$  g to  $252.40 \pm 76.80$  g, linearly with a regular feeding pattern. During the training period gain in weight showed a zigzag pattern, from  $259.4 \pm 18.88$  g to  $265.5 \pm 20.21$  g. After trauma also it followed the same erratic pattern of feeding ( $264.3 \pm 20$  g to  $267.4 \pm 20.4$  g) and

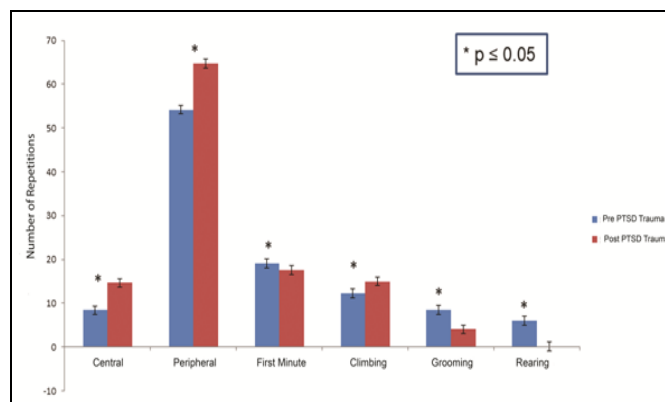
continued in both, training as well as trauma period. Mean gain in weight was 11.5 g in pre PTSD rat, during training period it was 6.1 g and 3.4 g in post PTSD rats, as represented in **Fig. 3**.



**FIG. 3: GRAPH REPRESENTING BODY WEIGHT GAIN PATTERN OF RATS THROUGHOUT THE EXPERIMENT. BODY WEIGHT IS REPRESENTED ON X AXIS (G) VERSUS TIME POINT ON Y-AXIS (DAYS)**

Behavioral analysis was conducted through an open field test (OFT). In OFT, certain activities were marked to assess the anxiety and stress levels of the animal. These activities included central square crossing, peripheral square crossing, first-minute activity, climbing, rearing, and grooming.

These activities were marked before and after subjecting the rats to trauma, respectively. Results depicted that central square crossing, peripheral square crossing, and climbing were increased after the rats were subjected to trauma. Whereas the first min activity, grooming activity, and rearing activity were found to be more in pre-trauma rats as compared to the post-trauma one's **Fig. 4**. Paired t-tests for all set of activities were conducted, for which p-value for each set came out to be  $\leq 0.05$ .



**FIG. 4: BAR GRAPH REPRESENTING BEHAVIOURAL ACTIVITIES PERFORMED BY RATS IN OPEN FIELD TEST (OFT), PRE TRAUMA AND POST TRAUMA**

**DISCUSSION:** In the study conducted 1H-NMR based metabolomics approach was undertaken to detect the alterations brought about in the metabolic pool of urinary samples of rats due to PTSD trauma. Multi-parametric statistical methods, including PCA and PLS-DA, lead to the inferences indicating that the two groups that were; pre PTSD and post PTSD were different from each other. Although, PCA showed only partial separation between the two groups, PLS-DA indicated a further diversification between these two groups. The loadings plot revealed major metabolites causing the variation between the two groups, which aided in the identification of the biochemical cycles, which are getting affected due to the induction of underwater trauma in PTSD.

Citrate level was found to be lowered in rat urine samples after PTSD trauma was imparted (post-PTSD). Citrate plays the central role in tricarboxylic acid cycle (TCA). TCA cycle is one of the most vital metabolic pathways in the body and is associated with energy metabolism. TCA is responsible for pyruvate metabolism for aerobic respiration, and also assists in fat and amino acid metabolism pathways. The lowered concentration of citrate indicates TCA cycle disruption caused due to PTSD trauma. Literature also reports the lowering of TCA cycle metabolites in case of shocked mice<sup>31</sup>, which is by our study.

Hippurate is the major metabolite associated with gut microflora. This metabolite is resultant of the metabolic activity of the bacterial population present exclusively in small intestine<sup>32</sup>. A significantly high urinary hippurate concentration was observed in post PTSD rats. Therefore PTSD may be associated with the fluctuations in intestinal microflora.

In related studies, it has been reported, animals showing depressed behavior also exhibit changes in gut microflora<sup>33</sup>. This linkage of depressed behavior with gut microbiome has already been well established in literature<sup>34</sup>. Thus, elevated levels of hippurate in post-trauma PTSD animal urine samples signifies that mood disorder/stress is associated with variations in intestinal microflora. This finding also explains the erratic pattern of feeding and zigzag pattern of weight gain during trauma.

N-methlnicotineamide (NMNA) is the end product of nicotinamide metabolism. NMNA is the key metabolite in the tryptophan-nicotinic acid pathway<sup>35</sup>. The lower concentration of urinary NMNA in the post-trauma PTSD rats indicates the down-regulation of the tryptophan-nicotinic acid pathway in PTSD. Also, the reduction of NMNA probably indicates a deficiency of nicotinamide in PTSD cases, which possess an elemental role in oxidation-reduction reactions.

Very high levels of urinary creatine and creatinine were detected post-PTSD. Creatine is the primary regulator of energy metabolism in the brain and skeletal muscles. Creatine phosphate dephosphorylates to give rise to creatinine. Thus, both creatine and creatinine fulfill the energy demand of the body by acting as energy reserves, especially in the brain and muscles. Higher levels of both creatine and creatinine in post PTSD rats suggests, disrupted metabolism of both the metabolites in PTSD. Increase in concentration of these metabolites could be a consequence of heightened anxiety and fear, after exposure to the trauma. Creatinuria may be due to attenuated muscle metabolism or skeletal muscle degeneration as observed in high altitude stress<sup>36</sup>. This was also reflected in the behavioral activity wherein the tendency to run, that is the central and peripheral activity, were found to be high in the post PTSD rats.

Reduction in beta hydroxybutyrate (BHB) levels was observed post trauma. The elevation in ketone bodies such as BHB causes a fundamental shift in metabolic physiology and brain neuro-pharmacology that is associated with brain homeostasis<sup>35</sup>. Numerous studies have demonstrated the role of BHB in treating of anxiety related symptoms<sup>36</sup>.

BHB can be used as an energy resource by the brain when the blood glucose level is low. Lowering of BHB levels in urinary samples in post-trauma PTSD rats has a role in the anxiety of the rats reflected after trauma, both behaviourally and metabolically. This evidence matches with our study of  $\beta$ -hydroxybutyrate in urine are significantly lowered after exposure to trauma. Use of  $\beta$ -hydroxybutyrate to treat anxiety is well established in literature<sup>37</sup>.

Betaine concentration was also found to be lowered in the urine of post-trauma rats. Betaine serves as organic osmolytes and is taken up by cells in situations of environmental stress such as high temp, salinity, osmotic stress, *etc.* Role of betaine has been well established in treating depression<sup>38</sup>. The lowered concentration of betaine signifies cellular stress in PTSD. Urinary aspartate levels were found to be high post-trauma signifying aberration in the amino acid pathway of the uric acid cycle along with liver dysfunction<sup>39</sup>. Aspartate was also found to be high in studies including depression<sup>39</sup> as well in shocked mice<sup>25</sup>, which is by our findings.

The pattern in weight gain and the behavioral study shows a close correlation to that of the metabolic data acquired through NMR metabolomics. Weight of the rats increased almost linearly, in a stable manner during the acclimation period. Proceeding to which, during the training period, the linear trend of weight gain was lost, and it showed a zigzag pattern. The similar zigzag pattern in weight gain continued during the post-PTSD underwater trauma. Maximum mean gain in weight was found during acclimation period that is (11.5 g), following which was the training period (6.1 g) and minimum mean weight gain was during post-PTSD underwater trauma period (3.4 g). Reason of this fluctuating weight can be explained by, various hormonal and metabolic changes occurring in the rat at the onset of the training period. These changes lead to behavioral changes and disrupted feeding patterns in rats. The climbing, central, and peripheral activity was found to be high in post-trauma rats. This signifies the behavioral anxiety and tendency to escape inculcated in rats after exposure to underwater trauma. Whereas 1<sup>st</sup> min activity, grooming, and rearing were considerably decreased post trauma, indicating the onset of anhedonia in the rats.

**CONCLUSION:** A metabolomic approach based on NMR has been used to demonstrate the effects of PTSD on the various metabolic pathways. The findings herein demonstrate changes in urinary levels of eight endogenous metabolites in rats after being exposed to trauma, which is namely, citrate, hippurate, aspartate, betaine, N-methylnicotinamide, creatine, creatinine, and  $\beta$ -hydroxybutyrate. These metabolites are participants of major

metabolic pathways such as energy metabolism, lipid metabolism, and intestinal microfloral metabolism. These outcomes aid in providing the essential biological underpinnings for PTSD and bring an insight into the biochemical pathways getting affected in PTSD. Consequently, this would lead to the discovery of new therapeutic breaks to supplement PTSD stress resiliency aids. Overall, these findings may lay the groundwork for the future development of a clinical urine-based diagnostic test for PTSD.

**ACKNOWLEDGEMENT:** The author would like to express her thanks to Dr. Ajay Kumar Singh, Director General Life Sciences, DRDO, Delhi, for providing necessary research facilities. The author is also thankful to Prof. Rina Chakrabarti, Department of Zoology, University of Delhi, for her support and guidance.

**AUTHOR DISCLOSURE STATEMENT:** The author reports no conflict of interest to declare.

**ETHICAL STATEMENT:** All experimental procedures were carried out by the animal ethical committee of the Institute of Nuclear Medicine and Allied Sciences, Defence Research and Development Organisation.

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**How to cite this article:**

Singh P: Nuclear magnetic resonance spectroscopy based urinary metabolic profiling in post traumatic stress disorder rats. *Int J Pharm Sci & Res* 2019; 10(10): 4636-43. doi: 10.13040/IJPSR.0975-8232.10(10).4636-43.