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Effect of paroxetine exposure on the proteome expression of mice liver

Sandeep Kumar Thakur [1]**, Manish Nivsarkar[1]

1 Department of Pharmacology and Toxicology, Ahmedabad, India

**Registered at Institute of Pharmacy, Nirma University, Ahmedabad

Corresponding author: Sandeep Kumar Thakur

Abstract

Paroxetine is a Selective Serotonin Reuptake Inhibitor (SSRI) utilized for the treatment of Major Depressive and Stress issues. One of the significant drawbacks in Paroxetine treatment is the pharmaceutical impelled hepatic damage. In this study impact of paroxetine presentation on the liver were described utilizing *in vivo* mouse model. Swiss albino mice received the medication for distinctive time periods and the alterations in the levels of protein explored by proteomic examination. There were 44 down-controlled proteins, some of which are differentially conveyed at diverse treatment stages.

Keywords: Paroxetine, Proteomics, Stress, Hepatic

Introduction

Paroxetine is a drug belonging to the selective serotonin reuptake inhibitors (SSRIs) class which are first line antidepressants used in the primary care and psychiatric practices due to their fewer side-effects in comparison to old generation antidepressants (1,2). It is mainly used to treat depression, obsessive-compulsive disorder, anxiety disorders, post-traumatic stress disorders (PTSD), and premenstrual dysphoric disorder (PMDD)(3,4). Paroxetine (PXT) is associated with side effects such as nausea, vomiting, diarrhoea, loss of appetite, weight loss, yellowing of the skin or whites of the eyes, dark urine, and pale stools (1,5,6). There are reported in paroxetine administration is associated with reports of an increase in AST, ALT levels, and acute liver failure. The mechanism of the PXT induced liver toxicity is still very less studied (6–8). It is considered that it causes the idiosyncratic toxicity. Drugs that have the potential to cause idiosyncratic drug toxicity may regulate common physiological or biochemical processes(9,10). Understanding the response to drugs/chemicals at the molecular level has potential both to elucidate the mechanisms of toxicity and to predict idiosyncratic toxicity(11,12). Recently many studies have been conducted to study and analyze gene expression, protein expressions altered by toxicants so that the mechanism behind their toxicity could be studied(13–16). However, very few studies have been conducted to understand the toxicity mechanisms of paroxetine.

Our objective was to study the changes in protein profile of the liver when animals were exposed to paroxetine. This type of studies will help in understanding the pathways by which the paroxetine causes liver damage and can help in the development of markers for early identification of toxicity so that preventive steps can be taken to reduce the damage and continue the therapy with alternative drugs. Commonly used markers such as AST, ALT intend to show the cell damage which is not limited to hepatic cells.

The purpose of the current study was to measure and identify changes in the liver proteomic profile after exposing animals with a drug with different time duration. Identifying changes in global protein expression will not only strengthen our understanding of paroxetine toxicity, but it will also help us in the identification of biomarkers which will help in improving the diagnosis and identification of toxicity at early stages.

Methodology

Chemicals and Reagents

Urea, thiourea, glycine, acrylamide, bromophenol blue, Chaps, dithiothreitol and iodoacetamide were purchased from Himedia (Mumbai). Complete protease inhibitor cocktail tablets were purchased from Sigma-Aldrich (USA). Sequencinggrade trypsin was purchased from Promega (USA). Acetone and ethanol were purchased from Fischer Scientific. (Shanghai, China). All of the water used in this study was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

Animals

Swiss albino mice (age 3-4 month) used in this study were obtained from the animal house of the B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, Ahmedabad, India. The animals were housed in polypropylene cages and placed in the experimental room where they were allowed to acclimatize for a week before the experiment. A 10% air exhaust conditioning unit was maintained along with a relative humidity of 60 ± 5% and a temperature of 25 ± 3°C was stabilized. A 14:10 h light: dark cycle was also regulated for the experimental animals. VRK Laboratory Animal Feed (VRK Nutrition Solution) and RO water were provided *ad libitum* to the experimental animals. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee (IAEC) prior to initiation of the experiment. Techniques with Good Laboratory Practice (GLP) were followed.

Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India, vide registration no.1661/PO/a/12/CPCSEA, dated 21/11/2012.

For this study animals were divided into 4 groups of 3 animals each

Control group: No treatment was given in this group

Treatment group 1: This group was treated for 5 days with the Paroxetine (123.33mg/kg)

Treatment group 2: This group was treated for 10 days with the Paroxetine (123.33mg/kg)

Treatment group 3: This group was treated for 15 days with the Paroxetine (123.33mg/kg)

Blood collection was done from retro-orbital on day six, eleven and sixteen days post PXT administration for serum biochemistry analysis and the animals were euthanized by CO_2 asphyxiation. The liver samples were frozen and stored at -80°C until the protein isolation.

Sample preparation and Gel Electrophoresis

Liver tissue was homogenized in sample buffer (7 M urea, 2 M thiourea, 4%w/v CHAPS, 20 mM DTT containing protease inhibitor cocktail) and then centrifuged at 15,000 rpm for 1 h at 4°C. All samples were stored at -80°C prior to electrophoresis. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad Laboratories, USA). Isolated protein were mixed with rehydration buffer (5 M urea, 2M Thiourea, 2% CHAPS, 100 mM DTT, 0.5% v/v pH 3-10 IPG buffer, 40 mM Tris Base, 2% SB 3-10, and trace bromophenol blue) to a final volume of 270µg/200µl for each strip, and applied to immobilized 7 cm pH 3-10 nonlinear gradient strips by over-night re-hydration. isoelectric focusing (IEF) was performed with an Ettan IPGphor apparatus (GE Healthcare) as follow steps: 0-200 V (STEP for 1Hr), 500 V (STEP for 4Hr), 500—1000 V (STEP for 1Hr), 1000-5000 V (GRADIENT for 4Hr), 5000 V (STEP for 30min), 500 V (STEP for 8Hr). All IEF steps were carried out at 20°C. After the first dimensional IEF, IPG gel strips were placed in an equilibration solution (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCI, pH 8.8) containing 1% DTT for 10 min. The gels were then transferred to the equilibration solution containing 2.5% iodoacetamide and kept for a further 10 min before placing them on 12% polyacrylamide gel slab(17–19).

Visualization and image analysis: Gels were stained with coomassie brilliant blue and visualized in Bio-Rad Gel Doc X1R.Spot detection and Quantification were carried out using FLICKER (20).

Spot intensity was quantified automatically by calculation of spot volume after normalization of the image by taking the ratio of intensity of one spot to the total spots

In-Gel Digestion and MS analysis

In Gel digestion was performed according to He Ping *et al* (2001). Excised gel spots were minced into $1mm^3$ pieces and transferred to a sterile microcentrifuge tube. Pieces were washed using 500 µl 50mM ammonium bicarbonate in 50% acetonitrile (ACN) at room temperature for 30 min with mile shaking/agitation. Washing was repeated until the complete dye got removed. The gels were dehydrated using 100% acetonitrile for 5 min. After discarding the acetonitrile, the gels were dried at room temperature. Dried gel pieces were reduced with 10mM dithiothreitol (DTT) (56°C; 30 min) and alkylated with 50mM iodoacetamide in 100mM NH_4HCO_3 (dark conditions, room temperature, 30 min).After discarding the alkylation solution, the gel were dried using 100% acetonitrile and then incubated in the digestion solution (40mM NH_4HCO_3, 9% ACN, and 20 µg/mL trypsin; overnight, 37°C). The tryptic peptides were extracted with 50% ACN/2.5% TFA and then dried using a gentle nitrogen stream. The sample was analyzed as per method of He Ping for mass analysis (21–23).

Protein identification and data analysis

Raw spectral data files were converted to suitable format and the list of masses containing all the fragment information was submitted to Mascot (Matrix Science version 2.1) in order to identify the proteins using the International Protein Index (IPI) database for mouse proteins, plus common contaminants such as trypsin and BSA. The search was performed using the following parameters: maximum of three missed trypsin cleavages, carbamidomethylation (Cys) as fixed variation, oxidation (Met) and acetylation (N-terminal of the protein) as variable modifications, and mass accuracy of 0.2 Da. Peptides with a minimum Mascot score of 38 indicate identifications with an error of less than 5% ($p < 0.05$). Proteins matching, at least, two peptides by Mascot were accepted automatically while identifications on the basis of only one peptide were accepted if the score was at least twice the threshold value for acceptance of MS/MS sequenced peptides and using an MS/MS fragment of at least 7 amino acids, and after manual validation. Spectra and protein validation were performed using open source software called MS Quant, extensively used for MS data analysis (20) .

Statistical analysis

Statistical analysis was made by means of Student's t-test and values with $p<0.05$ were considered to be statistically significant.

Results

The representative 2 DE gels of control and treatment groups are displayed in Figure 1

Quantitative comparison of protein spots was conducted by Flicker software. We compared the 2-DE gels from control groups and paroxetine-treated groups (5 Day, 10 Day and 15 Day).

A total of 33, 30 and 28 protein spots were down-regulated significantly respectively in 5 days, 10 day and 15-day treatment groups in comparison to control group 24 were present in more than one treatment duration. According to KEGG pathway and Protein Database in Mascot, these proteins were found to involve in signal transduction, homeostasis and protein synthesis, energy and primary metabolism. Figure 2 summarize the pathways related to the proteomic response of the liver to paroxetine exposure.

In five day treatment group of Paroxetine, 14 proteins Arginase-1, Carbamoyl-phosphate synthase, Glutamate dehydrogenase 1, Serum paraoxonase/arylesterase 1, 3-ketoacyl-CoA thiolase mitochondrial, Ketohexokinase, Aconitate hydratase, mitochondrial precursor,26S protease regulatory subunit S10B, Methylcrotonoyl-CoA carboxylase alpha chain, Dimethylglycine dehydrogenase, 60 kDa heat shock protein, Actin, Sulfite oxidase were significantly altered.

In the ten-day treatment group, 8 proteins Xanthine dehydrogenase/oxidase, Alpha-2-HS-glycoprotein, Programmed cell death protein 8, Ubiquinol-cytochrome-c reductase complex core, Adenosylhomocysteinase, Superoxide dismutase, Serotransferrin precursor, Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP, were significantly altered in comparison to control group. Total 10 proteins were significantly altered in 15-day group which include Dimethylaniline monooxygenase, Murinoglobulin-1, Hemopexin, Senescence marker protein-30 (Regucalcin), Glutathione S-transferase, N-Myc downstream-regulated gene 2 (NDRG2), Cathepsin L1, Ceruloplasmin, Vitamin D-binding protein, Alpha-1-antitrypsin 1-4.

Discussion

In recent research findings the -omic approaches have demonstrated their capability to elucidate toxicological effects of chemicals and drugs. In this Work, animals were exposed to high dose of paroxetine for different time durations followed by the proteomic analysis of liver.

In the 5 days, exposure of Paroxetine total 14 proteins was significantly altered. Arginase-1 is the final enzyme of the urea cycle when released from hepatocytes it increases the arginine breakdown (24). Carbamoyl-phosphate synthase is also a metabolic protein which is involved mainly in ammonia metabolism, Recently WG Carter et al found that the level of Carbamoyl-phosphate synthase can be useful in establishing new biomarkers for ethanol-induced liver damage. Also, the level of Carbamoyl-phosphate synthase is decreased in acetaminophen-induced hepatotoxicity (25). Schomaker and colleagues evaluated the diagnostic utility of glutamate dehydrogenase (GLDH) and Serum paraoxonase (PON), for liver injury in over 800 patients. They found that GLDH shows best predictive power for prediction of liver damage whereas serum paraoxonase levels were unpredictable even in thecontrol population(26). 3-ketoacyl-CoA thiolase mitochondrial

which is a terminal enzyme in fatty oxidation system is involved oxidative stress mediated hepatotoxicity. Newton BL et al demonstrated that level of ketohexokinase is significantly decreased in alcohol induced steatosis(27). Dimethylglycine which is an end product of homocysteine pathway is an indirect biomarker for the fatty liver condition(28). 5-day exposure of paroxetine leads to decrease in various metabolic and regulatory proteins. This directly indicated the system was under stress and tries to compensate the overload via utilizing metabolic machinery.

Total eight proteins were significantly down-regulated in the10-day treatment group. Oxidative stress-induced cell death involved various proteins which include the Xanthine dehydrogenase/oxidase, Superoxide dismutase, Ubiquinol-cytochrome-c reductase, TNF. It results in the triggering of apoptosis and programmed cell death which is various proteins such as Programmed cell death protein 8. Decrease in the level of Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP is an indicator of increased energy demand as it is involved in Glyceraldehyde and FAD metabolism. Alpha-2-HS-glycoprotein which is also known as fetuin-A has been associated with various diseases such as cancer, psoriasis, osteoporosis and non-alcoholic fatty liver syndromes, although earlier studies shows an increase in the level of Alpha-2-HS-glycoprotein while in this study we found that its expression is decreased, whereas the results for decrease in the level of the Serotransferrin are in accordance with the earlier studies (29). In third treatment group which received the therapy for 15 days, ten proteins were significantly altered. The Ceruloplasmin which plays an important role in storage and transport of ions such as Copper and iron is synthesized in the liver. Since these ions are important for energy production, connective tissue formation along with other functions. Although the level of this protein is found to be elevated in obsessive compulsive disorder, but is used in a lab test for the identification of a rare Wilson's disease(30). Similarly, Hemopexin which is also majorly present in the liver is responsible for the heme binding and iron metabolism. It plays a protective role against the heme-mediated oxidative stress. A reduction in the level of Hemopexin in indicator of increased oxidative stress. Reduction in the levels of Glutathione S- transferase also an indicator of increased oxidative stress in the system, Clarke et al found the GST as more sensitive and accurate indicator to carbon tetrachloride-induced hepatotoxicity in comparison to AST. Cathepsin L1 which is a protease in mainly responsible for the catabolism of collagen and elastin. Decrease in the level of Cathepsin may be due to its activation and subsequent catabolism of collagen and elastin which are key factors in liver fibrosis. Murinoglobulin has been studied as an indicator for pancreatitis, but its role in the liver is still unexplored. The increase in the levels of the murinoglobulin has been found in metastatic cancer in theliver.NDRG2 modulated the HSC activation and liver fibrosis, a decline in the levels of this may indicate the negative impact on liver architecture which may be in a compromised state due to catabolism of collagen and elastin.

Conclusions

In summary, on the administration of Paroxetine for the different duration, we found that initially it alters the normal metabolic process of the liver, which is a common process in the all the xenobiotic exposure. But at latter stages, the levels of stress protector molecules declined followed by a decrease in the molecule which is essential for maintaining the normal structure and function of the liver. These results were in agreement to studies for other hepatotoxins, we can say that this molecule should be further investigated with other hepatotoxicity models for establishing better predictive power.

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Figure 1 Representative 2D-gel image of comparative liver proteome of control and treatment groups

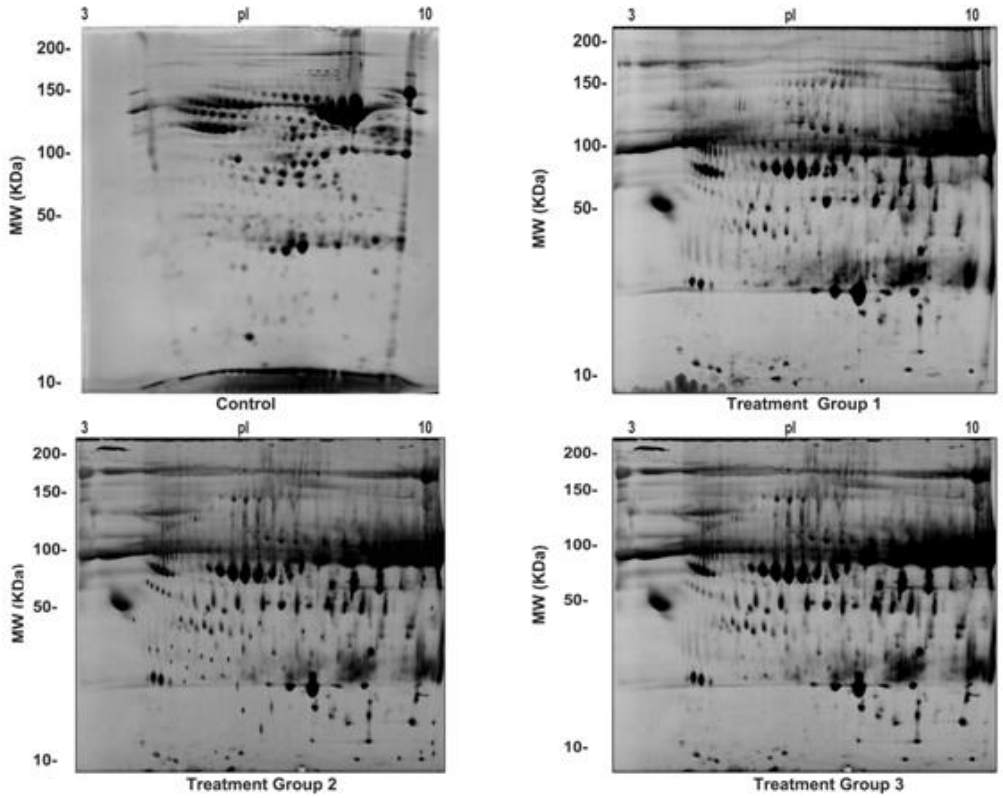

Figure 2 Altered signaling pathways in PXT induced liver damage. This interaction map was created in the String 9.0 program (http://string-db.org) and summarizes the affected signaling pathways.