Toxicity of Bisphenol-A and Probiotic Treatment on Total Proteins in Hypothalamus and Liver of Rattus norvegicus.

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Abstract In the present study, total proteins were analyzed in *Rattus norvergicus* (SD rats) of healthy, Bisphenol A treated and probiotic treated along with Bisphenol A for 30days. 18 rats weighing between 230-270g were used and divided into 3 groups of six each. 1st Group received soyabean pellet diet only and served as control, 2nd group were exposed with Bisphenol-A (32.2mg/kg /bw) and 3rd group were exposed with probiotic *Lactobacillus salivarus* (one capsule weight 300mg) along with Bisphenol-A and the total protein contents were estimated in hypothalamus and liver tissues. To determine the best method for protein quantification, we followed three different methods for quantification, Lowry's method, Bradford method and BCA kit method. BCA kit method was more appropriate method when compared to other methods for conducting further analysis of proteomic studies. The protein levels were significantly decreased in hypothalamus and liver after Bisphenol-A treatment when compared to control. While, the animals supplemented with probiotics along with Bisphenol A were showed significantly increased protein level in hypothalamus and liver when compared to Bisphenol A treated animals. These results indicated that probiotic *Lactobacillus* species work as an antidote against Bisphenol A toxicity in Sprague dawley rats.

Keywords: Bisphenol A, Hypothalamus, *Lactobacillus,* Liver, SD rats, Total Proteins.

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INTRODUCTION

BPA (2,2-bis(4-hydroxyphenyl propane) an endocrine disruptor compound is a small (228 Da) molecule polycarbonate plastics, epoxy resins and other polymer Materials for manufacturing plastic utensils and is among the highest-prod

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uction volume chemicals in the world, with an annual production of over 2 million tonnes [1]. The manufacturing of Bisphenol A involves the acid catalyzed condensation of phenol and acetone. These estrogen-like chemicals are becoming ubiquitous in the environment, and humans are being exposed to these chemicals through the food chain [2]. Many of the physiological effects of BPA have been described in the context of its ability to interact with classic ER (nuclear hormone receptor activity). The estrogenic activity of BPA was reviewed in detail by the NTP-CERHR Expert Panel on Bisphenol A. Exposure to high doses of BPA causes toxicity in multiple organ systems such as the kidney, liver, spleen and pancreas [3&4]. The liver is often a target for these compounds due to the function of the organ in cleansing the body of contaminants. For the next few decades, BPA use continued to grow as it was used in more and more applications, such as, CDs, DVDs, and water bottles [5].Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, including the gastrointestinal tract. [6]. [7] found that *L. salivarius* can survive in acidic conditions and it mimics the variable changes in the stomach because bacterium can grow at low pH.

Tissue homogenization is a process used to prepare tissue samples for certain types of studies. It involves encouraging the cells to lyse, or break apart to release their contents. This method is used to collect DNA samples, proteins, enzymes, specific organelles, and other things that may be present inside a cell. It is performed under controlled environment and analysis was carried out. The homogenized tissue can be spin in a centrifuge to separate it into layers, the sample of interest is obtained. (http://www.wisegeek.com)

Proteins are the building blocks of amino acids which are assembled on needed basis. They regulate body functions. Protein quantification is often important to know the concentration of protein in a sample. The most common situations where such information is necessary fall into three categories, characterization and purification of proteins and enzymes, physiological studies of protein expression, and clinical diagnosis of altered protein levels in body fluids, indicative of a variety of diseases. [8]

Trizol Reagent is a ready to use mixture of phenol, guanidine isothiocyanate, red dye and other proprietary components that can be used to isolate total RNA, DNA and

proteins can be recovered with sequential precipitation from the organic phase. Trizol was developed by Chomczynski .The red dye allows easy detection of the organic phase and is non-interactive with nucleic acids. In Lowry's method combines the reactions of copper ions with the peptide bonds under alkaline conditions and the reduction of the Folin-Ciocalteu acid to hetero polymolybdenum blue by the copper catalysed oxidation of aromatic acids. The Lowry method is sensitive to pH changes [10]. In Bradford reaction, peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. In BCA Protein assay combines the well-known reduction of Cu_2+ to Cu_1+ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu_1+) by bicinchoninic acid. It is the extinction method of the Bradford method.

The objective of this paper is to quantify the protein concentration in hypothalamus and liver tissues using different methods like Lowry's method, Bradford method, and BCA kit based method and analyzed the accuracy of protein levels. It is a preliminary study and also evidenced the further research work, 1D-PAGE, 2D-PAGE, MS analysis, protein expression, and clinical diagnosis of altered protein levels in body fluids, indicative of a variety of toxicants/diseases.

MATERIAL AND METHODS

Tissue sampling and preparation of hypothalamus and liver homogenate

The male Sprague dawley rats were sacrificed on 4 th week of experiment. The hippocampal regions of the brain were dissected and then stored at -80˚C until use. The tissue was homogenized on ice with a cold Tris/EDTA (ethylene diamine tetra acetic acid) buffer (50 mmTris, 1 mm EDTA, p^H 7.5), and centrifuged at 10,000 g for 20 min at 48˚C. Supernatant was collected and processed for protein analysis. [11]

The abdominal cavities of the anesthesitized rats were immediately opened and the livers were excised, perfused with normal saline to remove blood, blotted between filter papers and used for the preparation of tissue homogenate. About 0.5g of each liver was homogenized in 4.5 ml of phosphate buffered saline (PBS p^H 7.0, containing 1µm EDTA).The crude tissue was centrifuged at 8000 g for 30 min and the supernatant was collected and stored at 4˚C for further analysis. [12]

Protein extraction

Proteins were extracted by the method of Trizol protein extraction method. [9]. Trizol reagent consisting of guanidine thiocyanide, phenol and chloroform to the supernatant obtained after homogenization, mixed the contents thoroughly by vortex mixture. Added chloroform to this solution, mixed the contents and placed the tube on ice for few min, centrifuged the tube at 2000rpm for 5 min. Three distinct layers

were obtained at this stage the top most is the aqueous layer containing RNA, Inter phase is the protein and the bottom layer contains DNA. Discarded the transparent top layer having the RNA, then added absolute alcohol to the remaining layers, and mixed the solution as well centrifuged the contents at 2000 rpm for 5min. the DNA forms a white precipitate at the bottom of the tube, while the protein remain clear in the supernatant. Supernatant was collected in a fresh tube, and then added chilled acetone to this tube and mixed well by vortex mixture. The solution was stored at – 20˚C at least one hour before centrifugation.

Protein pellet was collected, discarded the supernatant and dried the protein pellet at room temperature. Re-constitutes the dried pellet with rehydration buffer and stored over night at-20˚C before carry out protein quantification.

Quantification

Lowry's method

In this method, prepared BSA working standards in test tubes and make up to 1 ml by using distilled water. To this solution, 4.5 ml of reagent –I (Na_2CO_3, NaOH, Na-K Tartrate, and $CuSO_4$.5 H_2O) was added and allowed to incubation for 10 min. After incubation 0.5 ml of reagent –II (Folin-Phenol) was added and allowed to incubation for 30 min. Blank was also maintained and prepared for unknown solutions as mentioned above. The absorbance was measured at 660nm and standard graph was plotted. The amounts of proteins present in the unknown samples were estimated by using standard graph.

Bradford method

It is a colorimetric analysis procedure used to measure the concentration of protein. Standard protein samples were prepared with BSA ranges from 0.1-0.8 mg/ml, to this test tube, added 5 ml of Bradford reagent (Coomassie-Brilliant blue G250+phosphoric acid+ glycerol) allowed for incubation (10-30 min) and read each of the standard and unknown samples at 595 nm.

BCA (Bicinchoninic acid) method:

It is a simple method to quantify the protein concentration. Standard series of protein samples and unknown samples were prepared, mixed with BCA reagents A and B (50:1 (v/v)). Then 25µl of each standard and unknown sample were pipetted and transferred to micro titer plate. 200µl of BCA working reagent was added to each well and allowed for incubation at 37˚C for 30 min, absorbance at 562nm by using Elisa reader.

Statistical analysis

Statistical analysis was performed with SPSS (version 11), using one way ANOVA followed by Dennett multiple comparisons. Significance of the samples obtained *$p<0.05$ when compared with control.

RESULTS

The present results indicating that the protein concentration in the Bisphenol A treated sample was decreased than the control, indicating the protein synthesis has been arrested. The protein concentrations in Bisphenol A with probiotic treated samples were higher than the Bisphenol A influenced samples indicting that probiotc has been act as detoxicant against Bisphenol A. These results were supported by wessam mohmmad Abdel- wahab(2014) under toxicity of BPA on liver tissue. This report covers Lowry's method, Bradford method and BCA kit method for protein extraction methods getting a better understanding about which methods give better exact quantity and higher amount of proteins and also perform one-way ANOVA for comparison of three method results. Our results indicated that, BCA kit method is fast and easy way to quantify the concentration of proteins and it is more appropriate method for further analysis of proteomic studies.

Table: 1 Total protein in hypothalamus tissue of SD rats.

S.NO	Lowry method	Bradford method	BCA method
Control	123.35 ±2.23	121.38 ±2.21	120.35 ±2.20
Bisphenol treated	110.21 ±1.19	109.35 ±1.14	108.45 ±1.16
Bisphenol+probiotic treated	119.25 ±1.17	115.3± 1.45	112.45 ±1.52

Total Proteins (Lowry, Bradford and BCA methods) in hypothalamus tissue of (1) healthy (2) BPA treated and (3) probiotic treated along with BPA of rats

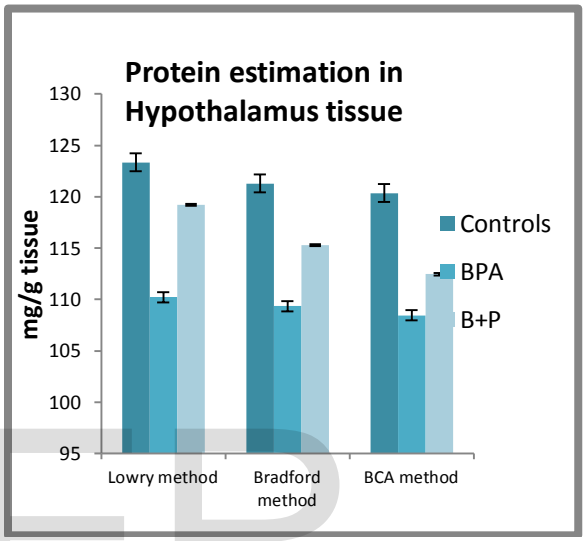

Fig 1: Effect of bisphenol A on protein content in SD rat hypothalamus and its possible alleviation by lactobacillus. The values were expressed as the mean ± SE, n = 6. The protein content was expressed as mg/100 mg tissue weight. In all the groups the significance was observed as $p < 0.05$.

Table: 2 Total protein in liver tissue of SD rats

S.NO	Lowry method	Bradford method	BCA method
control	98.62± 1.23	96.62± 1.47	94.62± 1.32

Bisphenol treated	68.72± 1.04	64.22± 1.06	66.22± 1.08
Bisphenol+probiotic treated	80.76± 1.14	77.65± 1.19	74.62± 1.18

Total Proteins (Lowry, Bradford and BCA methods) in liver tissue of (1) control (2) BPA treated and (3) probiotic treated along with BPA of rats

DISCUSSION

The present results showed that Bisphenol A is more toxic on liver when compared to hypothalamus tissue. It is confirmed liver undergo for toxicity by Bisphenol A because liver is often a target for these compounds due to the function of the organ in cleansing the body of contaminants

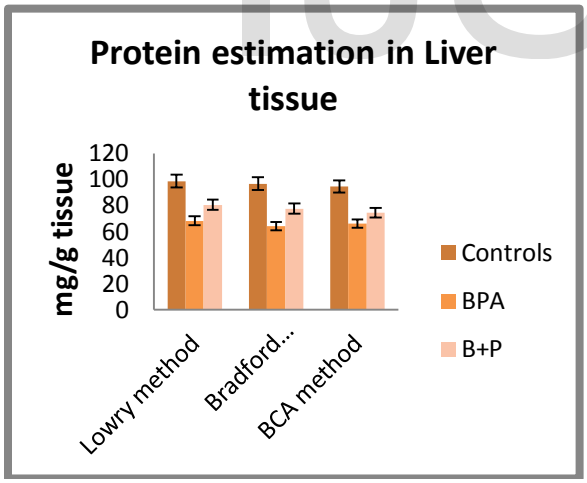

Fig 2: Effect of bisphenol A on protein content in SD rat hypothalamus and its possible alleviation by lactobacillus. The values were expressed as the mean ± SE, n = 6. The protein content was expressed as mg/100 g tissue weight. In all the groups the significance was observed as $p < 0.05$.

The results of NEHA P *et al.*, shown that the protein content bisphenol effected rats shown 7mg /100mg in liver tissue weight (i.e. 210mg/3gms tissue wt) and the hypothalamus is also effected by bisphenol A.

CONCLUSION

Based on the current study, it was concluded that BCA method was accurate method for protein analysis. BPA has detrimental effect on protein synthesis as protein contents decrease in case of hypothalamus and liver tissue of bisphenol A treated animals. Also, this study clarifies that the damage caused by Bisphenol A in rat can be recovered by treatment of probiotics. Further research analysis is needed to be carried out to study complex protein mixture extracted from both tissues followed by proteomic analysis of bisphenol induced proteins with the help of 2-DE and 1-DE electrophoresis.[19]

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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