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Research Article

Docosahexenoic acid prevents aluminum induced metabonomic changes in rat urine: A Proton nuclear magnetic resonance study

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Abstract: High resolution proton nuclear magnetic resonance (NMR) spectroscopy has proved to be one of the most powerful technologies for producing a comprehensive profile of metabolite signals. Metabonomics is successful because disease, drugs or toxins cause perturbations of the concentrations and fluxes of endogenous metabolites involved in cellular pathways. The metabolic profile of urine, in particular, often shows changes in response to toxic or disease-induced stress, because of the system's attempt to maintain homeostasis. In the present study, we aimed to explore the protective effect of DHA against aluminum (Al) induced nephrotoxicity and metabonomic changes in rats using histopathology and proton Nuclear Magnetic Resonance. The study highlights significant decrease the concentration of citrate, creatinine, allantoin, trans-aconitate succinate and increased acetate level in urine profile of experimental rats when compared with control rats. The docosahexaenoic acid (DHA) found to be reverses these metabonomic and cellular changes. These modulations may lead to identification of possible therapeutic intervention for Al induced nephrotoxicity, dialysis dementia and Alzheimer disease (AD).
Keywords: NMR, Aluminum, DHA, Urine.

INTRODUCTION

In the recent years, much attention has been focused on the potential of a wide range of xenobiotic chemicals to interact with and disrupt the internal system of animal and human population. Among these chemicals, metal especially aluminum (Al) belong to a group of widespread toxicant. Among these toxins, Al is extensively used in dialysis, antacids, drug aspirin, as food contamination and added in drinking water for purification purpose [1]. Al is excreted by the kidneys and therefore toxic amounts can impair kidney function. Al can also accumulate in the brain causing Alzheimer's disease [2,3].

Aluminium magnesium silicate and aluminium hydroxide are extensively used as antacid and aluminium gel as phosphate binding agent to lower plasma phosphorous level in patients suffering from acute renal failure [4]. However, the National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines for the care of patients with end-stage renal disease still include screening for Al toxicity with plasma aluminum concentrations [5]. Moreover, the wide use of Al cookware and storage vessels, the intake of Al by Indian population is much higher than what has been reported for the West. One of the main organ affected by Al ingestion is brain and kidney [6, 7].

Docosahexaenoic acid (DHA) is a major component of fish oil. It is a long chain polyunsaturated fatty acid. DHA is vital component of the phospholipids of human cellular membranes, especially those in the brain and retina, necessary for optimal neural development and visual acuity. Earlier some of the studies reported that DHA may have a protective role against oxidative stress as demonstrated by decreased lipid per oxidation, both ex vivo and in vivo [8,9]. Moreover, it plays an important role as critical modulator of neuronal function and regulation of oxidative stress mechanisms in brain health and disease [10].

High resolution 1H nuclear magnetic resonance (NMR) spectroscopy has proved to be one of the most powerful technologies for bio fluids and essentially the only one capable of studying intact tissues, producing a comprehensive profile of metabolite signals without the need for pre-selection of measurement parameters or selection of separation or

derivatisation procedures. Metabonomics is successful because disease, drugs or toxins cause perturbations of the concentrations and fluxes of endogenous metabolites involved in cellular pathways. The response of cells to stressors generally results in an adjustment of their extra-cellular environment in order to maintain homeostasis. The metabolic profile of urine, in particular, often shows changes in response to toxic or disease-induced stress, because of the system's attempt to maintain homeostasis. Hence, even when cellular homeostasis is maintained, subtle responses to toxicity or disease are expressed in alterations in bio fluid composition. In the present study, we aimed to explore the protective effect of DHA against Al-induced nephrotoxicity and metabonomic changes in rats using histopathology and proton Nuclear Magnetic Resonance.

MATERIAL AND METHOD

Animals:

Forty male albino rats (162.8 ± 4.3 gram) were taken from university animal house. The animals were housed separately in polypropylene cages in a room, which was maintained at a temperature of 22 ±2 ^{0}C, relative humidity of 50 ±10 % and 12h light dark cycles. They were fed a commercial pellet diet (Dayal Industries, Barabanki, UP, India) and allowed access to water ad libitum. The Institutional Animal Ethics Committee approved the study prior to the initiation of the experiment and also approved all experimental protocols. Animals were randomly divided into four groups (n = 10) viz. Group 1 served as control treated with normal saline, Group 2 treated with 100mg / kg body weight of DHA, Group three treated with 100mg /kg body weight of $AlCl_3$ and Group four treated with 100mg $AlCl_3$ + 100mg DHA for 90 days. Dose was directly introduced into the rat pharynx via a feeding cannula to rats for 90 days.

Urine collection:

The urine samples were collected at every morning using metabolic cages on 45 and 90 days of treatment and centrifuged at 3000 rpm for 5 min at 4◦C to remove the particulate contaminants. Samples were stored at −80◦C until NMR spectroscopic analysis.

Nuclear Magnetic Resonance Experiments:

1H NMR spectra for all urine samples were obtained on a Bruker Biospin Avance 400 MHz spectrometer (Faellanden, Switzerland) using 5-mm broad band inverse probehead at 300 K. 500 µL urine samples (each) were taken in 5-mm NMR tubes, a sealed coaxial capillary tube containing 0.375% trimethyl silyl propionic acid sodium salt-*d*4 (TSP) in 35 µL deuterium oxide and was inserted into the NMR tube before obtaining the NMR spectra. TSP served as a chemical shift reference as well as the standard signal for absolute quantitative estimation of the metabolites, whereas deuterium oxide served as solvent for "field-frequency-locking." One-dimensional 1H NMR spectra were obtained for the samples using one-pulse sequence with suppression of water resonance by presaturation. For all the serum samples, additional one dimensional 1HNMRspectra were also obtained using Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with suppression of water resonance by presaturation to remove the broad resonances arising from macromolecules. The typical parameters used were spectral width: 8000 Hz; time domain points: 32K; relaxation delay: 5 s; pulse angle: 90◦; number of scans: 128; spectrum size: 32 K and line broadening: 0.3 Hz. For CPMG experiment total echo time of 0.64 ms with 420 echoes was used. The concentrations of metabolites were obtained using the integral area of respective metabolite marker signal with reference to the integral area of TSP in CPMG spectra [11].

Histopathology:

After 90 days, control and experimental rats were sacrificed by cervical dislocation. Kidneys were removed surgically and rinsed with physiological saline. A portion of kidney cortex was fixed with neutral formalin, dehydrated with different concentrations of ethyl alcohol and embedded in paraffin. Tissue sections were cut (4 µm) and stained with hematoxylin and eosin for histological grading of renal and hepatic injury.

RESULTS

Proton NMR spectral analysis of urine samples of control and experimental rats: Typical ^{1}H NMR spectra of urine at 45 and 90 days of control and experimental rats, shown in Figure-1 with the labeling of the signals. Six metabolites viz., acetate, succinate, citrate, creatinine, allantoin and trans-aconitate were identified and quantified (table-1). The concentrations of these urine metabolites on 45 and 90 days of experimental rats, and of same age group control rats were shown in Table 1 along with statistical evaluation.

The concentration of citrate were found to be significantly ($p<0.001$) reduced by 43% and 60% in Al treated rats when compared with the controls respectively. While DHA + Al treated rats shows significant ($p<0.001$) recovery by 53% and 84% when compared with the Al treated rats on 45 and 90 days of treatment respectively. The creatinin were found to be markedly reduced by 41% and 38% in Al treated rats as compared with the controls on 45 and 90 days respectively. While, DHA +Al treated groups indicated that significant increment by 32% and 70% when compared with the Al treated rats on 45 and 90 days respectively.

The levels of allantoin were found to be markedly reduced by 66% and 64% in Al treated rats as compared with the controls on 45 and 90 days of treatment respectively. While the concentration of allantoin were found to be increased by 59% and 120% in DHA+ Al treated rats as comparison with Al treated

rats on 45 and 90 days of treatment respectively.Acetate levels were significantly increased in Al treated rats when compared with the controls on 45 and 90 days of treatment respectively. While the concentration was significantly elevated by 80% in Al + DHA treated rats on 90 days as compared with the Al treated rats.

Trans-aconitate levels were found to be markedly decreased by 64% and 70% in Al treated rats when compared with the controls on 45 and 90 days of treatment respectively. While it was increased in Al + DHA treated rats on 90 days when compared with Al treated rats. The concentrations of succinate in urine were found to be reduced by 68% and 85% in Al treated rats as compared with the controls on 45 and 90 days of treatment respectively. While it was significantly elevated by 368% and 678% in Al + DHA treated rats as compared with the Al treated rats on 45 and 90 days of treatment respectively.

Histopathology: Among all experimental groups the specific changes were seen as mild congestion in the glumeroli, focal congestion and vacuolar (hydropic) degeneration of tubular cells in Al treated rats as compared with the control rats. No histopathology changes were seen among control group. The kidneys from the co- administrated with Al and DHA showed no abnormality in the cellular changes when compared with the Al treated rats (figure-2).

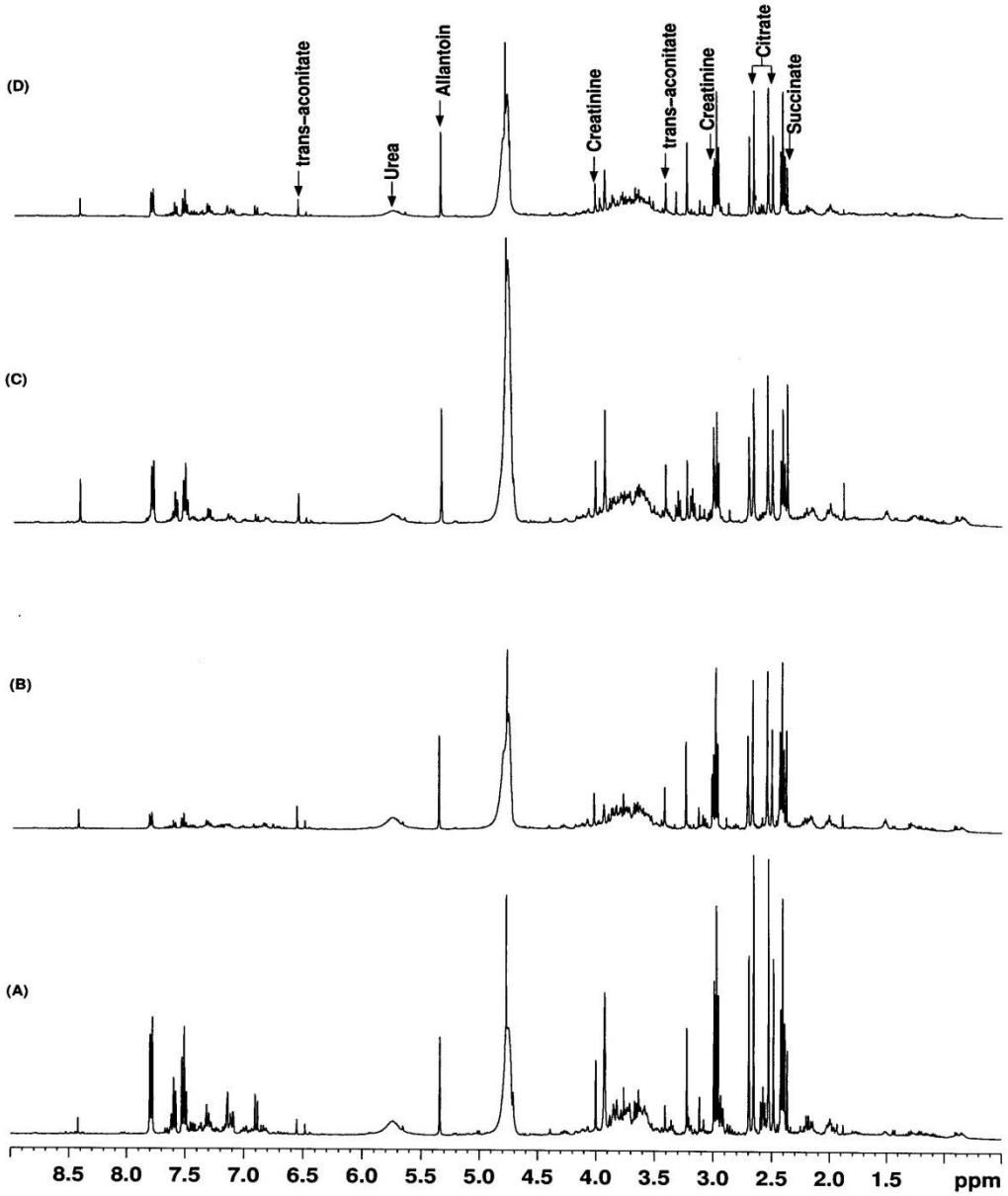

Fig-1: ^{1}H NMR spectra of urine of (a) a control (b) DHA treated (C) Aluminium treated and (D) DHA + Aluminium treated rats on 90 days.

Table-1. Semi Quantitative Concentration of Urine Metabolites in mg/ dL of control and experimental rats

	45 Days					90 Days			
	CT	**DHA**	**Al**	**Al + DHA**		**CT**	**DHA**	**Al**	**Al + DHA**
Citrate	345.5 ± 87.5	369.5 ± 93.6^{ns}	198.2 ± 66.7^{a}	302.6 ± 41.4^{c}		394.1 ±109.3	408.3 ±106.3	156.4 ± 80.7^{a*}	288.2 ± 102.3^{c}
Creatinine	84.4 ± 36.6	79.4 ± 24.3	41.1 ±15.6^{a}	54.1 ± 10.3^{ns}		96.9 ± 33.7	101.6 ± 3.8a	37.7 ± 12.5^{a}	63.9 ±12.1^{c}
Allantoin	451.9 ±104.9	470.6 ±126.2	152.3 ±104.8^{a}	242.6 ±74.2^{c}		419.4 ±115.5	405.14 ± 135.1	149.19 ±45.7^{a}	328.2 ±89.7^{c}
Acetate	ND	ND	3.44 ± 0.9^{a}	2.99 ± 0.49^{ns}		ND	ND	5.0 ±3.5^{a*}	1.01 ±0.5^{c}
trans-Aconitate	130.2 ± 68.3	135.01 ± 76.4	46.8 ±3.8^{a}	35.6 ± 3.5^{ns}		123.00 ±73.8	116.8 ± 50.2	43.78 ± 10.4^{a}	74.6 ± 16.3^{c}
Succinate	22.2 ± 3.8	28.03 ± 5.2	7.22 ± 2.16^{a}	33.8 ± 14.1^{c}		18.97 ± 10.5	19.0 ± 5.2	2.75 ± 1.7^{a*}	21.4 ±8.7^{c}

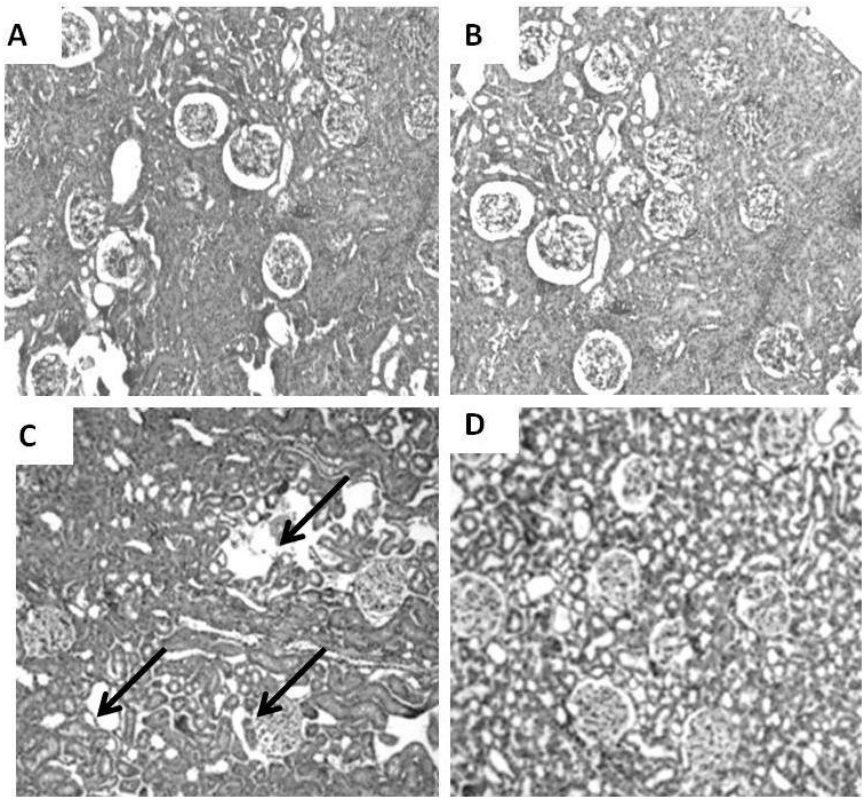

Fig-2: Light photomicrograph (H&E) of kidney (cortical part) of control (A), DHA treated(b), Aluminium treated (C) and DHA + Aluminium treated (D) rats on 90 days (40x). In control and DHA treated rat renal glomeruli showed normal structure, lobular organization of the glomeruli, and flat epithelium lining of the glomerular capsule. However, in Al treated rat enlargement of vascular glomeruli, vaculate and degenerated epithelia of glomeruli observed while the the coadministrated with Al + DHA treated rats showed significant recovery.

DISCUSSION

The present study was carried out mainly to investigate the protective effects of docosahexaenoic acid on Al induced endogenous metabolic changes in the urine. Previous study shown numerous physiological changes take place and they are reflected by the physical and biochemical perturbations in the animal, resulting in a difference in proportion of endogenous intermediate metabolic products excreted in the urine [11].

In the present study, concentration of citrate, creatinine, allantoin, trans-aconitate and succinate were found to be gradually decreased in Al treated rats when compared with the controls on both 45 and 90 day of treatment (table-1). On the other hand, treatment of DHA were recovered these metabolites near to controls on both 45 and 90 days of treatment respectively (figure-1).

The reduced concentration of citrate in urine reflects the urinary pH. Moreover, intracellular pH and the intracellular bicarbonate concentration control the rates of transport of citrate across the inner mitochondrial membrane with the help of aconitase enzyme in the TCA cycle and the degree of utilization of citrate by the renal tubular cells [12.13]. In addition, reduced citrate level was recorded during renal tubular acidosis [14], it may be due to reduced conversion of oxaloacetate to citrate in the TCA cycle. Moreover oxaloacetate is the marker of kidney stone of oxalate. Following DHA supplemented rats exhibited reversal changes of citrate in Al treated rats. This results agreement with the finding of Siener et al. [15], they proposed that omega fatty acid supplementation increases the concentration of citrate in urine.

The elevated creatinine concentration in urine is an important indicator of renal functioning because and high concentration in blood and easily measured byproduct of muscle metabolism that is excreted by the kidneys [16.17]. Additionally, the creatinine level has also been associated with decreased renal blood flow in diabetes and cardiovascular disease [18, 19]. In the present study, DHA supplementation recovered the concentration of creatinine which reflects the ameliorating property of DHA. Our result similar with the study of Rizwan et al. [20] and Palaniswamy et al. [21], they reported that omega-3- fatty acid reduces the risk of nephrotoxicity.

In the present study, the concentration of allantoin was found to be significantly recovered by DHA supplementation. Allantoin is the marker of oxidative stress. Uric acid is the final product of purine metabolism in human and may be oxidized to allantoin by various ROS that are the hallmark of oxidative stress and can damage proteins, lipids and DNA [22]. Our result is the concomitant with the finding of Fukuhara et al [23], they reported that high level of allantoin in oxidative stress conditions in neurodegenerative animal model. On the contrary, several studies have been divisive over the ultimate effects of DHA on lipid peroxidation. The LPO products stimulate DNA damage and depletion of ATP and further lead to cell death [24].

A significant decrease in *trans*-aconitate in Al treated rat urine in the present study may be due to mitochondrial dysfunction, it may be due to Al induced mitochondrial oxidative stress in kidney [25,26]. The DHA treated rats exhibited significant improvement near to control rats. The result indicated that DHA is the most potent n-3 fatty acid that suppresses Al induced oxidative stress at mitochondrial level [27]. The present study demonstrated that significantly decrement of succinate levels (which is the important excretion product of the Krebs cycle) in Al treated rat urine further it is evidenced that the pathophysiological changes in liver and kidney [28].

The present metabolic profiling study using 1H NMR showed that DHA caused a systemic recovery from the AL induced metabolic perturbation in rat kidney, an animal model for the kidney disfunctioning. This study also demonstrates that metabolic profiling is a useful method to study therapeutic effects of DHA. Interestingly, DHA was found to act as a kidney protector. Indeed, in the Al treated group, DHA succeeded to counterbalance the cellular changes (figure-2). Moreover, it has significantly improved the metabonomic alterations and histological parameters.

CONCLUSION

In this study, the Al induced kidney damage was illustrated not only by a significant alteration in serum metabolites, but also an altered histological feature in the kidney tissue reminiscent of some known diseases. In this rat model, Al was also able to cause neurotoxicity and improved histological alterations by DHA. In conclusion, the overall results have clearly shown the ability of DHA to offer protection against some aspects of Al ingestion in the serum and kidney, probably due to a synergic effect of many compounds. Thus, DHA can be used as a regular nutrient to alleviate the side effects of Al ingestion during Al exposure, especially for dialysis patients who are more susceptible to developing Al induced nephrotoxicity and dialysis dementia.

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